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SEASONAL VARIATION IN BIOFOULING OF GELS CONTAINING EXTRACTS OF MARINE ORGANISMS

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Many benthic marine organisms have no obvious physical defenses against propagule settlement, and yet remain free of epibionts, suggesting that they are chemically defended against fouling. Crude organic extracts of four marine organisms were assayed for antifouling properties by incorporating them into hard, stable gels that served as substrata for propagule settlement in the field. Half of the gel replicates were maintained in darkness and half were subjected to natural lighting while suspended from floating docks at Wrightsville Beach, North Carolina, for 28 d. The assay was repeated 7 times over 2 years to examine seasonal variation in settlement. Crude organic extracts from the sponge *Aplysilla longispina* deterred settlement of a variety of invertebrates and algae relative to control gels, while extracts from the sponge *Hymenacidon heliophila*, the ascidian *Eudistoma hepaticum*, and the alga *Codium decorticatum* were not deterrent. In one assay (March 1994), gels containing an extract of *C. decorticatum* had enhanced settlement relative to controls. In contrast, extracts of *A. longispina* were effective against fouling organisms in all assays year-round. There was not a significant difference in mean percentage cover of fouling organisms between dark and natural light treatments. Algae did not settle on gels maintained in the dark, therefore a greater abundance of invertebrates settled on gels kept in the dark compared to those in natural light, although the diversity of invertebrates did not change. This gel assay technique represents the most ecologically relevant system for assessing the antifouling effects of secondary metabolites from marine organisms described to date.

Keywords: antifouling; settlement; natural products; secondary metabolites; biofouling

INTRODUCTION

Benthic marine communities are characterized by diverse assemblages of invertebrates, most of which include a free-swimming larval stage as part of

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their life cycle (Thorson, 1964). This vast array of meroplankton requires substrata for attachment as a prerequisite to metamorphosis for completion of their life cycle. Free space becomes an important limiting resource in marine hard-bottom communities. In many parts of the world, any substratum that is not ephemeral, regularly disturbed, or defended, will become fouled by space-limited organisms. Biofouling and epibiosis (settlement on living substrata) therefore play an important role in the ecology of sessile organisms.

The presence of a variety of unfouled organisms in benthic communities indicates that these organisms elaborate some form of defense against fouling. In addition to physical antifouling defenses, such as mucus sloughing (Barthel & Wolfrath, 1989), chemical compounds have been implicated in the inhibition of propagule settlement. Chemical deterrence against settlement may be one mechanism that allows physically undefended sessile organisms to resist colonization and overgrowth. Past studies suggest antifouling chemical defenses against a variety of taxa, including bacteria, fungi, algae and invertebrate larvae (Pawlik, 1993). Many studies have shown various compounds extracted from marine organisms are effective in preventing bacterial film formation (*e.g.* Sieburth & Conover, 1965; Bell *et al.*, 1974; Stoecker, 1978; Lynch *et al.*, 1979; Harrison, 1982; Wahl, 1989). A film of microorganisms can be a prerequisite for settlement of macrophytes and epizooites (*e.g.* Crisp & Ryland, 1960; Mihm *et al.*, 1981). Therefore an antimicrobial defense may also prevent settlement of macroflora and macrofauna. Many studies have indicated that secondary metabolites are effective against marine fungi (Caccamese & Azzolini 1979; Pesando *et al.*, 1979; Caccamese *et al.*, 1980; Accoriniti, 1983; Pesando & Caram, 1984). Compounds capable of inhibiting algal propagules also have been investigated (*e.g.* Sand-Jensen, 1977; Targett *et al.*, 1983; Harrison & Durance, 1985). Marine invertebrate larvae are highly discriminatory with regard to choosing a substratum on which to settle (*e.g.* McDougall, 1943; Knight-Jones & Crisp, 1953; Ryland, 1959; Osman, 1977). Larvae possess photo-, tactile- and chemosensory organs and are capable of responding to numerous environmental stimuli (*e.g.* Thorson, 1964; Crisp, 1974; Young & Chia, 1987; Boudreau *et al.*, 1990; Pawlik, 1992). Marine natural products have been demonstrated to induce or inhibit settlement of larvae (reviewed by Davis *et al.*, 1989; Pawlik, 1992). Chemically-mediated processes may partially determine which species can invade in spatially limited communities, thereby altering distributions and abundances.

Marine organisms, sponges in particular, produce a diversity of natural products (*e.g.* Faulkner, 1994). Only in the last decade has research

focused on identifying the biological function of these unique metabolites (Pawlik, 1993). The commonly held view is that most of these compounds function in a defensive capacity. However, most studies which indicate an antifouling role for secondary metabolites have been conducted in the laboratory with compounds suspended in seawater or adsorbed onto assay surfaces. Such experiments often subject marine invertebrate larvae to conditions not encountered in the field, for example, placing larvae in small quantities of static water. Also, laboratory studies typically assay antifouling compounds against only one or two species of larvae. In addition laboratory studies cannot take into account seasonal variation in the abundance of propagules.

A new field assay method for testing the antifouling activity of crude organic extracts of marine organisms has recently been devised (Henrikson & Pawlik, 1995). This technique, in which organic extracts are incorporated into a gel matrix, has several advantages, including (1) assay gels are exposed to a natural population of settling propagules, (2) extracts are incorporated into gels at the same volumetric concentration that they occur in the organism, (3) extracts slowly diffuse from the gels, at a rate of 1–2% per day, and (4) extracts within the gel matrix do not alter the physical characteristics of the settlement surface. In the process of testing this assay method, it was discovered that extracts of the sponge *Aplysilla longispina* inhibited settlement of invertebrate and algal propagules (Henrikson & Pawlik, 1995). In the study reported herein, variation in settlement on assay gels was examined throughout the year, thereby determining whether certain extracts were only effective during part of the year, or only effective against certain species of epibionts and not others. Finally, an investigation was carried out to determine whether there was a difference in settlement on gels maintained in natural light *vs* gels maintained in darkness. It was hypothesized that greater numbers and greater diversity of invertebrates would settle on gels if algal settlement was limited by darkness.

MATERIALS AND METHODS

Four species of sessile marine organisms were collected from floating docks and bridge pilings in Banks Channel, Wrightsville Beach, North Carolina and taken to the laboratory for immediate processing. They were the two sponges, *Aplysilla longispina* and *Hymeniacidon heliophila*, an ascidian, *Eudistoma hepaticum*, and an alga, *Codium decorticatum*. Organisms were generally collected and processed within one month of each assay.

Six 50 ml aliquots of fresh tissue of each species were chopped into 1 cm^3 pieces and frozen overnight. The amount of tissue used was volumetrically equivalent to the amount of gel made, so that each gel would have a natural concentration of metabolites. Frozen tissue was Iyophilized and extracted for 24 h in 1 : 1 dichloromethane : methanol. The tissue was then extracted for a second time in methanol for 24 h. The extracts were combined and solvent was removed by a rotary evaporator, leaving a lipid soluble crude extract for each species.

Gels were made by adding 1.63 g of Phytagel™ (Sigma Chemical Company) to 50 ml distilled water and mixing for 5 s with a hand-held electric blender (note that the recipe was incorrectly reported as 2.17 g 50 ml^{-1} in Henrikson & Pawlik, 1995). The gel mixture was heated in a microwave oven until boiling and allowed to cool slightly before an aliquot of extract dissolved in 3 ml of methanol was added and stirred. The mixture was then poured into a 10 cm diameter circular mold. Strips of fiberglass window screen were embedded in each gel before it cooled and hardened to provide support for securing cable tie hangers.

For each assay, there were 6 replicates of each extract treatment, as well as 6 gel controls that contained 3 ml of methanol but no extract, and 6 plexiglas plates of the same size. The gels were hung randomly from floating docks at a secure facility at the La Que Center for Corrosion Technology Incorporated, Wrightsville Beach, North Carolina. One set of replicates was maintained in darkness under an opaque canvas tarp, while another set of replicate gels and plates was kept in natural lighting, which included direct sunlight during daylight hours. Each gel was hung 30 cm below the water surface on monofilament line, with a lead weight and swivel attached 4 cm above the gel to allow it to orient parallel to the tidal flow. Assays were conducted in March 1994 and repeated in June 1994, August 1994, September 1994, January 1995, March 1995, and June 1995.

Settlement of invertebrates and algae was measured as percent cover on the front and back sides of each gel using a dot-grid estimate method (Foster *et al.*, 1991; Meese & Tomich, 1992). The outline of a gel was traced onto a clear acetate sheet and the area within was marked in a dot grid with all points 1 cm apart. The acetate sheet was then fitted over both sides of each gel and the number of points with organisms underneath was recorded. Percentage cover was then calculated by dividing recorded points by total number of points. In addition to percentage cover, the total number of individuals and colonies of sessile invertebrates on gels was recorded after 28 d. All data were arcsine transformed prior to analysis (Zar, 1984).

RESULTS

Settlement was obvious on both gels and plexiglas plates between 7 and 14 d in the field. Percentage cover of fouling organisms steadily increased over 28 d for all assays, with a mean maximum of 73% coverage on gels containing extract of *C. decorticatum*. There was not a significant difference in mean percentage cover between control gels and plexiglas plates (ANOVA, $p < 0.05$, $F = 0.68$, $df = 1$, Figure 1). There was a significant difference in mean percentage cover on control gels between months (ANOVA, $p < 0.05$, $F = 6.89$, $df = 6$, Figure 1). A pairwise comparison (Tukey–HSD) revealed that settlement during the months of August 1994 and January 1995 was significantly less than settlement in June 1994.

There was a significant difference in mean percentage cover between treatments for all months (Two-way ANOVA, $p < 0.05$, $F = 367.5$, $df = 5$, Figure 2). A pairwise comparison (Tukey–HSD) revealed that gels containing extract of *A. longispina* had less settlement than control gels. Mean settlement on the other treatments, including the plexiglas plates, was not different from settlement on control gels except for March 1994, when

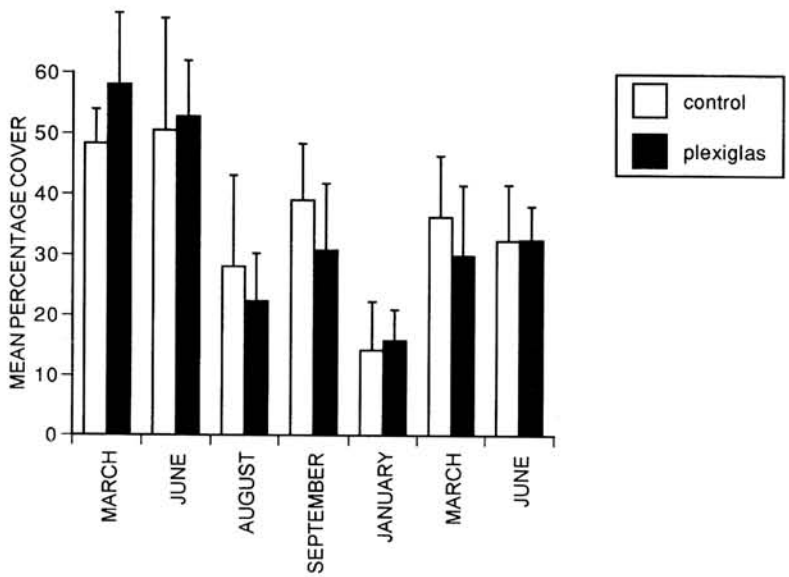

FIGURE 1 Percentage cover of algae and invertebrates on control gels and plexiglas plates after 28 d during March, June, August and September 1994, and during January, March and June 1995. All points are mean values of six replicates; vertical bars indicate standard deviations.

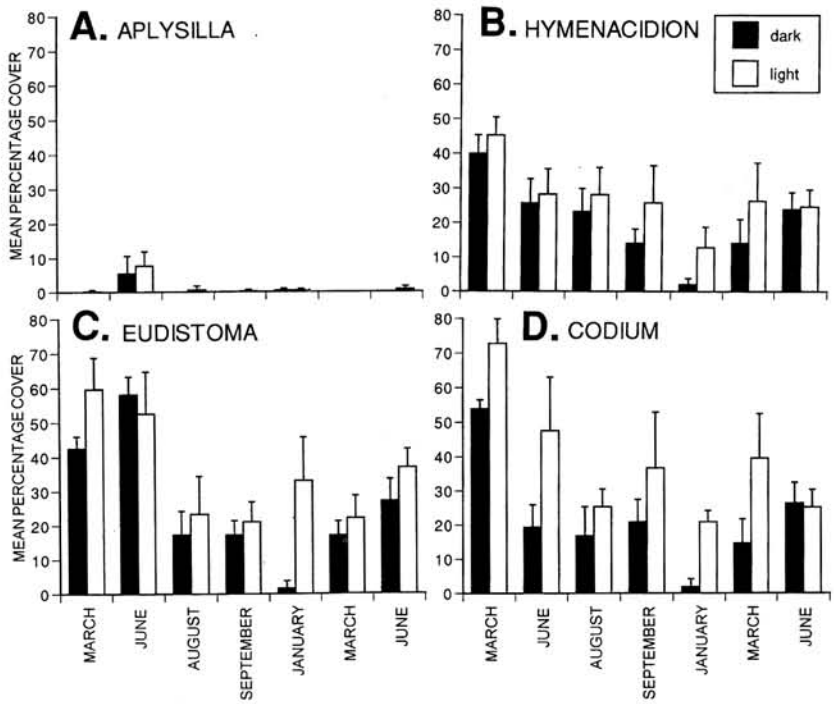

FIGURE 2 Percentage cover of algae and invertebrates on gels containing extract of A. *A. longispina*, B. *H. heliophila*, C. *E. hepaticum*, and D. *C. decorticatum* after 28 d during March, June, August and September 1994, and during January, March and June 1995. All points are mean values of six replicates; vertical bars indicate standard deviations.

there was enhanced settlement on gels containing extracts of *C. decorticatum* relative to controls.

There was a significant difference in mean percentage cover between light and dark treatments (Two-way ANOVA, $p < 0.05$, $F = 209.3$, $df = 1$, Figure 2), but a pairwise comparison (Tukey–HSD) revealed that this difference existed only in the month of January 1995 and only for gels containing extracts of *H. heliophila*, *E. hepaticum*, and *C. decorticatum*.

DISCUSSION

Secondary metabolites have long been thought to have defensive functions such as feeding or fouling deterrency. This field study confirms that secondary metabolites from the tissues of some organisms may defend against

biofouling. Mean percentage settlement was not different between control gels and plexiglas plates, indicating that assay gels are as suitable a substratum for propagule settlement as a hard plastic surface. Gels with extracts of the sponge *Aplysilla longispina* inhibited settlement of fouling organisms, as found in a previous 1-month study (Henrikson & Pawlik, 1995). A variety of organisms, algae, barnacles, bryozoans, spirorbids and hydroids, settled on all other treatment and control gels (Table I), indicating that *A. longispina* has a chemical antifouling defense that is effective against a broad range of fouling taxa. Like the sponge *A. longispina*, the sponge *Hymenacidon heliophila* and the tunicate *Eudistoma hepaticum* were never observed with epibionts in the field, but extracts of these two species did not deter fouling. These organisms may have a mechanical or physical, rather than a chemical, defense against fouling, or several non-chemical mechanisms may act in concert to provide defense. It is also possible that these organisms may have chemical defenses against fouling that are not apparent using the assay technique described herein, either because the compounds are too unstable or diffuse too quickly from the gel matrix to influence settlement over a 2 week period.

The antifouling properties of extracts of the sponge *A. longispina* did not change seasonally, either on account of changes in the activity of the sponge extract, or on account of changes in the composition of propagules that were available for settlement. Chemical defense against fouling was effective year-round. A few barnacles settled on the *A. longispina* treatment in June 1994, but this was minor compared to the abundance and diversity of settlers on control gels and other treatments. Settlement on treatments other than *A. longispina* did not differ from the controls, except in March 1994, when gels containing an extract of *C. decorticatum* experienced enhanced settlement relative to controls. The seasonal aspect of this survey is important because a seasonally effective chemical defense might be missed if the survey was conducted for a single month. A comparison of mean percentage cover of fouling organisms on control gels shows there was a significant difference in settlement between months. Gels containing an extract of *A. longispina* experienced settlement for only one month out of seven. Likewise, gels containing extracts of *C. decorticatum* enhanced settlement for only one month over the duration of the study. Although extracts of *A. longispina* were deterrent year-round, seasonally effective chemical defenses may be discovered as more species are assayed for antifouling properties. Chemical defenses against fouling that are metabolically expensive may be more prevalent during times of peak larval production.

TABLE I Mean (±SD) percentage cover and mean number of individuals or colonies of invertebrates on control gels after 28 d in the field. N = 6 replicates. The invertebrate settlers were hydroids: *Eudendrium carneum*; *Tubularia crocea*; barnacles: *Balanus* sp.; bryozoans: *Schizoporella unicornis*; *Bugula neritina*; *Membranipora tenuis*; polychaetes: *Hydroides dianthus*; *Spirorbis* sp.; molluscs: *Pteria colymbus*

Month/Year	*% Cover*	*Mean number on gels*								
		E. carneum	*T. crocea*	*Balanus* sp.	*S. unicornis*	*B. neritina*	*M. tenuis*	*H. dianthus*	*Spirorbis* sp.	*P. colymbus*
Mar 94	48.3 ± 5.7	0.4 ± 0.5	0.0	11.7 ± 2.2	0.2 ± 0.4	0.2 ± 0.4	0.0	0.0	1.2 ± 0.8	0.0
Jun 94	31.3 ± 9.9	0.2 ± 0.4	0.0	6.8 ± 3.4	6.8 ± 3.4	0.4 ± 0.5	0.0	0.0	0.0	0.0
Aug 94	28.0 ± 15.1	0.0	0.0	9.3 ± 1.5	9.3 ± 1.5	0.0	0.0	0.2 ± 0.4	0.2 ± 0.4	0.0
Sept 94	39.0 ± 9.3	0.2 ± 0.4	0.0	14.5 ± 2.7	14.5 ± 2.7	0.0	0.5 ± 0.5	2.8 ± 2.3	1.5 ± 1.9	0.2 ± 0.4
Jan 95	14.2 ± 8.1	0.0	0.2 ± 0.4	3.8 ± 1.9	3.8 ± 1.9	0.0	0.0	0.0	0.0	0.0
Mar 95	36.2 ± 10.1	0.0	0.2 ± 0.4	9.8 ± 3.9	9.8 ± 3.9	0.8 ± 0.8	0.2 ± 0.4	2.7 ± 1.0	0.0	0.0
Jun 95	32.3 ± 9.2	2.5 ± 1.8	0.0	9.7 ± 2.4	9.7 ± 2.4	0.4 ± 0.5	0.0	0.2 ± 0.4	0.0	0.0

A greater diversity of invertebrate settlers was expected on gels that were kept in darkness to inhibit algae, but this was not the case. There was not a difference in mean percentage settlement between gels maintained in natural light *vs* darkness; however, because algae were absent, or nearly so, on gels maintained in the dark, there were greater numbers of invertebrates on these gels. Although invertebrate abundance was greater on gels maintained in the dark, there was not an increase in diversity. Organisms found on gels maintained in darkness were the same as those found on control gels kept in sunlight (Table I). Month-to-month changes in the diversity of settlers on control gels and on gels containing extracts other than *A. longispina* are likely to be due to differences in the availability of propagules in the water column, rather than any species-specific defense. For example, the hydroid species replace each other seasonally; *E. carneum* is present in warm months and *T. crocea* is present in cold months (Table I).

This study, along with that of Henrikson and Pawlik (1995), presents a novel approach for assaying secondary metabolites for antifouling properties in the field. Extracts of *A. longispina* were a deterrent against settlement in both studies, indicating the presence of a compound whose biological function is a defense against fouling. The seasonal aspect of the present study enables detection of variation in defensive capacity against fouling by using a seasonally changing natural pool of larval and algal propagules. This assay system could also be used to detect variation in chemical antifouling defenses seasonally, or over the lifespan of an organism.

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References

Accorinti J (1983) Antifungal products of algal origin. *Rev Int Oceanogr Med* **67**: 45–53

Barthel D, Wolfrath B (1989) Tissue sloughing in the sponge *Halichondria panicea*: a fouling organism prevents being fouled. *Oecologia* **78**: 357–360

Bell W H, Lang J M, Mitchell R (1974) Selective stimulation of marine bacteria by algal extracellular products. *Limnol Oceanogr* **19**: 833–839

Boudreau B, Bourget E, Simard Y (1990) Benthic invertebrate larval reponse to substrate characteristics at settlement: shelter preferences of the American lobster *Homarus americanus*. *Mar Biol* **106**: 191–198

Caccamese S, Azzolini R (1979) Screening for antimicrobial activities in marine algae from Eastern Sicily. *Planta Med* **37**: 333–339

Caccamese S, Azzolini R, Fumari G, Cormaci M, Grasso S (1980) Antimicrobial and antiviral activities of extracts from Mediterranean algae. *Bot Mar* **23**: 285–288

Crisp D J, Ryland J S (1960) The influence of filming and surface texture on the settlement of marine organisms. *Nature (Lond)* **185**: 119

Crisp D J (1974) Factors influencing the settlement of marine invertebrate larvae. In: Grant P T, Mackie A M (eds) *Chemoreception in Marine Organisms*. Academic Press, New York, pp 77–265

Davis A R, Targett N M, McConnell O J, Young C M (1989) Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer P J (ed) *Bioorganic Marine Chemistry, Vol 3*. Springer-Verlag, Berlin, pp 85–114

Faulkner D J (1994) Marine natural products. *Nat Prod Rep* **11**: 355–394

Foster M S, Harrold C, Hardin D D (1991) Point versus photo quadrat estimates of the cover of sessile marine organisms. *J Exp Mar Biol Ecol* **146**: 193–203

Harrison P G (1982) Control of microbial growth and of amphipod grazing by water-soluble compounds from leaves of *Zostera marina*. *Mar Biol* **67**: 225–230

Harrison P G, Durance C D (1985) Reductions in photosynthetic-carbon uptake in epiphytic diatoms by water-soluble extracts of leaves of *Zostera marina*. *Mar Biol* **90**: 117–119

Henrikson A H, Pawlik J R (1995) A new antifouling assay method: results from field experiments using extracts of four marine organisms. *J Exp Mar Biol Ecol* **194**: 157–165

Knight-Jones E W, Crisp D J (1953) Gregariousness in barnacles in relation to the fouling of ships and to antifouling research. *Nature (Lond)* **171**: 1109

Lynch J M, Fletcher M, Latham M J (1979) Biological interactions. In: Lynch J M, Poole N J (eds) *Microbial Ecology: a Conceptual Approach*. Blackwell Scientific Publishing, Oxford, pp 171–187

Meese R J, Tomich P A (1992) Dots on rocks: a comparison of percent cover estimation methods. *J Exp Mar Biol Ecol* **165**: 59–73

McDougall K D (1943) Sessile marine invertebrates at Beaufort, North Carolina. *Ecol Monogr* **13**: 321–374

Mihm J W, Banta W C, Loeb G I (1981) Effects of adsorbed organic and primary fouling films on bryozoan settlement. *J Exp Mar Biol Ecol* **54**: 167–179

Osman R W (1977) The establishment and development of a marine epifaunal community. *Ecol Monogr* **47**: 37–63

Pawlik J R (1992) Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr Mar Biol Annu Rev* **30**: 273–335

Pawlik J R (1993) Marine invertebrate chemical defenses. *Chem Rev* **93**: 1911–1922

Pesando D, Caram B (1984) Screening of marine algae from the French Mediterranean coast for antimicrobial and antifungal activity. *Bot Mar* **27**: 381–386

Pesando D, Gnassia M, Gueho E (1979) Antifungal properties of some marine planktonic algae. In: Hoppe H A, Levring T, Tanaka Y (eds) *Marine Algae in Pharmaceutical Science*. Walter de Gruyer, Berlin, pp 461–472

Ryland J S (1959) Experiments on the selection of algal substrates by polyzoan larvae. *J Exp Biol* **36**: 613–631

Sand-Jensen K (1977) Effect of epiphytes on eelgrass photosynthesis. *Aquat Bot* **3**: 55–63

Sieburth J McN, Conover J T (1965) *Sargassum* tannin, an antibiotic which retards fouling. *Nature (Lond)* **208**: 52–53

Stoecker D (1978) Resistance of a tunicate to fouling. *Biol Bull* **155**: 615–626

Targett N M, Bishop S S, McConnell O J, Yoder J A (1983) Antifouling agents against the benthic marine diatom, *Navicula salinicola*: homarine from the gorgonians *Leptogorgia virgulata* and *L. setacea* and analogs. *J Chem Ecol* **9**: 817–829

Thorson G (1964) Light as an ecological factor in the dispersal and settlement of larvae of marine bottom invertebrates. *Ophelia* **1**: 167–208

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Mar Ecol Prog Ser* **58**: 175–189

Young C M, Chia F S (1987) Abundance and distribution of pelagic larvae as influenced by predation, behavior, and hydrographic factors. In: Giese A C, Pearse J S, Pearse V B (eds) *Reproduction of Marine Invertebrates*, Vol 9. Blackwell Scientific Press, Palo Alto, CA, pp 385–463

Zar J H (1984) *Biostatistical Analysis*. 2nd edition. Prentice-Hall, Englewood Cliffs, New Jersey