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A new antifouling assay method: results from field experiments using extracts of four marine organisms

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Abstract

Antifouling is one possible defensive function of marine natural products isolated from sessile benthic organisms, but there is little experimental evidence to support this claim. We developed a technique in which crude organic extracts of marine organisms are incorporated into hard, stable gels that serve as substrata for larval settlement in the field. These gels contain extracts at concentrations that are volumetrically equivalent to those in living tissues, and compounds diffuse from assay gels in a manner that may mimic their natural release from some organisms. After 21 days in flowing seawater, a mean of 56% of the mass of crude extract from the sponge *Hymeniacidon heliophila* (Parker) remained in gels. Extracts from two sponges, *Aplysilla longispina* (George & Wilson) and *Hymeniacidon heliophila*, an ascidian, *Eudistoma hepaticum* (VanName), and an alga, *Codium decorticatum* (Woodward & Howe), were incorporated into gels and deployed in the field over a period of 28 days; extracts from *A. longispina* deterred settlement of invertebrates and algae relative to control gels, while extracts from *C. decorticatum* enhanced settlement. Mean settlement on control gels was similar to that on plexiglas plates of the same size. This technique represents a more ecologically relevant method for assaying the antifouling properties of extracts of marine organisms because (1) assay gels are exposed to a natural population of settling propagules, (2) extracts are incorporated into gels at natural volumetric concentrations, and (3) extracts within the gel matrix do not alter the physical characteristics of the settlement surface.

Keywords: Antifouling; Biofouling; Natural products; Secondary metabolites; Settlement

1. Introduction

Although epibiosis is ubiquitous in benthic marine environments, many con-

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spicuous organisms that lack apparent physical defenses remain unfouled. Colonization by fouling organisms can be inhibited or reduced by one or any combination of mechanical, physical and chemical processes. For example, Barthel & Wolfrath (1989) found that the sponge *Halichondria panicea* sloughs its outer tissue layer at regular intervals. Surface tension and energy (Becker & Wahl, 1991), wettability (Brewer, 1984), pH (Baker & Orr, 1986), and secondary metabolites (Bakus et al., 1983) may also play a role, allowing host organisms to inhibit fouling. Wahl et al. (1994) found that metabolites of the ascidian *Cystodytes lobatus* deterred bacterial settlement and suggested that secondary metabolites in some invertebrates may control bacterial colonization. Because many fouling invertebrates require the presence of a surface film of microorganisms as a prerequisite for settlement (ZoBell & Allen, 1935; Scheltema, 1974), chemical control of bacterial epibiosis might reduce subsequent fouling by macrofauna. In addition, some metabolites of invertebrates and algae may have a direct role in preventing fouling and overgrowth (Pawlik, 1993). Deterrent compounds may provide a mechanism for slow-growing sessile organisms to persist and maintain living space in competitive, substrata-limited communities. Some marine natural products have been demonstrated to induce or inhibit settlement of larvae, and, as a result, secondary metabolites may play an important role in larval ecology and recruitment processes (reviewed by Davis et al., 1989; Bakus et al., 1991; Pawlik, 1992, 1993).

Although the biological roles of marine natural products have been of interest for several decades, only recently have there been ecologically relevant investigations of their functions (Paul, 1992; Pawlik, 1992). Antifouling research has typically focused on toxicity and settlement inhibition assays (e.g. Rittschof et al., 1985; Davis & Wright 1990). Most of these studies have been conducted in the laboratory, and it is unclear whether their results can be reliably extrapolated to the field because they have been performed with homogenates or extracts of organisms placed in small volumes of static seawater (e.g. Stoecker, 1978; Standing et al., 1984; Thompson et al., 1985; Rittschof et al., 1986; Sears et al., 1990; Martin & Uriz, 1993), and it is doubtful that larvae experience such conditions in nature. Antifouling properties observed in the laboratory may simply be artifacts of excessive metabolite concentration, static water, or extract preparation (Pawlik, 1992).

The few examples of field studies of the antifouling properties of natural products involved "painting" extracts or compounds onto surfaces. For example, Bakus et al. (1991) coated plywood panels with extracts from various sponges, but did not observe any reduction of settlement. Moreover, this painting method may not accurately assess antifouling potential, because oily extracts remain on the surface and alter wettability. Wettability is the capacity of a substratum to induce spreading of a liquid on its surface, and may greatly alter settlement of propagules solely for physical reasons (Mihm et al., 1981; Brewer, 1984).

It is clear that a more ecologically relevant technique is needed to test marine natural products for antifouling properties. Herein we present a method in which crude organic extracts of marine organisms are incorporated into stable gels and

deployed in the field, where the gels are subject to settlement by a natural population of propagules. This technique has three advantages over previous antifouling assay methods: (1) organic extracts are present at natural concentrations in a tissue-like matrix from which they can diffuse into seawater, rather than being dissolved in a small volume of static seawater or present at high concentrations on the surface of an impermeable panel, (2) because they are incorporated into gels, extracts do not affect the physical properties of the settlement surface, and (3) settlement on the gels occurs under realistic flow conditions and from a natural supply of larvae and algal spores. In this study, we determined the rate at which sponge crude extract was lost from the gel matrix when gels were placed in flowing seawater. We investigated the use of phosphatidyl-choline chloride as an agent for creating micelles (spherical clusters of phospholipids that spontaneously form in aqueous solutions; Darnell et al., 1990), which were expected to form around lipid-soluble metabolites and increase extract retention in gels. Finally, the new method was used to assay crude extracts from two sponges, an ascidian, and an alga for antifouling activity in the field.

2. Methods

Four species of sessile marine organisms were collected from floating docks in Banks Channel, Wrightsville Beach, North Carolina and taken to the lab for immediate processing: two sponges, *Aplysilla longispina* (George & Wilson) and *Hymeniacidon heliophila* (Parker) an ascidian, *Eudistoma hepaticum* (Van Name), and an alga, *Codium decorticatum* (Woodward & Howe). These species were chosen to represent a taxonomic range of both infrequently fouled (*A. longispina, H. heliophila*, and *E. hepaticum*) and frequently fouled (*C. decorticatum*) organisms.

Six 50-ml aliquots of fresh tissue of each species were chopped into 1 cm^2 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 lyophilized and extracted for 24 h in 1:1 dichloromethane: methanol, then the tissue was extracted a second time in methanol for 24 h. The extracts were combined and the solvent was removed by a rotary evaporator, leaving a lipid soluble crude extract for each species.

Gels were made by adding 2.17 g of Phytagel™ (Sigma Chemical) to 50 ml distilled water and mixing for 5 s with a hand-held electric blender. 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 (top of plastic drinking cup). Strips of fiberglass window screen were embedded in each gel before it cooled and hardened to provide support for securing cable tie hangers.

There were six replicates of each extract treatment, as well as six gel controls that contained 3 ml of methanol but no extract, and six Plexiglas plates of the same size. The gels were hung randomly from floating docks in a secure facility at

the La Que Center for Corrosion Technology, Inc. Wrightsville Beach, North Carolina in March, 1994 (water temperatures = 15–20°C). 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 tidal flow. Gels were visually monitored for hardness and resistance to degradation by microorganisms. 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 days.

To determine the diffusion rate of extracts from the gel matrix, a time series experiment was set up in a 1000 l mesocosm tank continuously supplied with unfiltered seawater and covered with an opaque lid. Gels were prepared as previously described with extracts from *Hymeniacidon heliophila*, but with one additional step: extracts were dried completely and the mass recorded before resolublizing in methanol for addition to the gel. Fifteen gels were suspended in the tank from monofilament lines attached to dowels and three replicate gels were removed after 0, 3, 7, 14, and 21 days. For comparison, another set of gels containing 100 mg of phosphatidyl-choline chloride per gram of extract was treated the same way. Phosphatidyl-choline chloride was expected to form micelles around the lipid-soluble metabolites to increase retention time.

Upon removal from the mesocosm tank individual gels were macerated and twice extracted for a minimum of 10 min in methanol, then extracted in 1:1 dichloromethane: methanol for the same amount of time. The combined extracts were dried completely and weighed. Because gels placed in seawater acquire salts, the mass of salt per gel was determined by extracting control gels placed in seawater for 24 h. The amount of extract remaining in each gel was calculated by subtracting the final mass of extract from the mean salt mass and the initial mass of extract.

3. Results

Gels slowly leached an extract of *Hymeniacidon heliophila* in flowing seawater, with the mean mass of extract gradually decreasing from 94 to 56% of the initial concentration after 21 days (Fig. 1). There was no significant difference in the mean retention of extracts of *H. heliophila* in gels that contained phosphatidyl-choline chloride than in gels that did not contain choline after 21 days (*t*-test, $p > 0.05$).

Settlement became obvious on both gels and plexiglas plates after 14 days in the field. Gels remained resilient, and showed no evidence of degradation, for periods

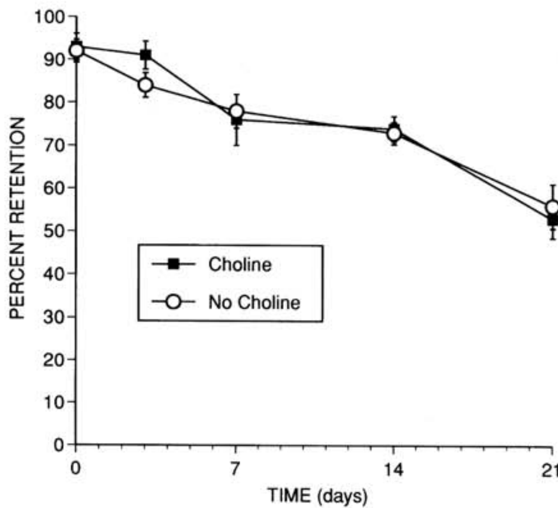

Fig. 1. Percentage retention (mean ±SD, $N = 3$ replicates per point) of an extract of the sponge *Hymeniacidon heliophila* in gels over time. Choline (phosphatidyl-choline chloride) was added to a separate set of gels to assess its effect on extract retention. There was no difference in mean retention between the two treatments after 21 days.

up to 6 wk. Percentage cover of fouling organisms steadily increased over 28 days, with a mean maximum of 73% coverage on gels containing an extract of *Codium decorticatum* (Fig. 2). There were significant differences in mean percentage cover among treatments (ANOVA, $p < 0.05$, $F = 67.8$, $df = 5$). A pairwise comparison (Tukey-HSD) revealed that gels containing extracts of *A. longispina* had less settlement, while gels containing *C. decorticatum* had more settlement than control gels. Mean settlement on the other treatments, including the Plexiglas plates, was not different from settlement on control gels. The most common invertebrate settlers on gels were barnacles, followed by spirorbid worms, hydroids and bryozoans (Table 1).

4. Discussion

There is a large body of literature describing unusual natural products from marine invertebrates and algae (e.g. Faulkner, 1991), yet a lack of experimental evidence of any biological function for these compounds (Pawlik, 1992). The new assay method described in this study may provide more ecologically relevant answers to questions regarding the antifouling properties of secondary metabolites. Incorporation of compounds into a gel matrix offers an improved alternative to painting them onto panels because compounds are present in gels at natural volumetric concentrations and are not pooled on the settlement surface, where they may alter surface characteristics. Gels in the antifouling assays remained

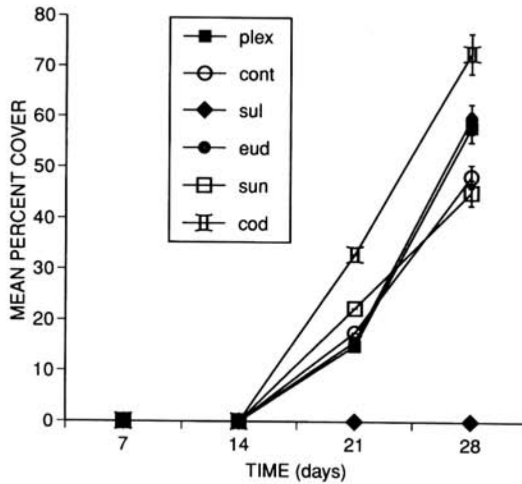

Fig. 2. Percentage cover of algae and invertebrates on gels over time. All points are mean values of six replicates, vertical bars indicate standard deviations. Treatments are as follows: plex = Plexiglas control, cont = gel control, then treatment gels containing extracts of: sul = *Aplysilla longispina*, eud = *Eudistoma hepaticum*, sun = *Hymeniacidon heliophila*, cod = *Codium decorticatum*. Relative to control gels, settlement was significantly diminished on gels containing extracts of *A. longispina*, and enhanced on gels containing extracts of *C. decorticatum*.

solid and proved to be a suitable substratum for larval and algal propagules, with no differences in settlement on the gels vs. plexiglas plates. Because it is performed in the field, the technique described herein eliminates problems associated with laboratory assays such as unrealistic water flow and compound concentrations, and exposure to only one or a few species of larvae. The gel technique can be applied to test pure compounds as well as crude extracts, and can be adjusted to address other possible functions such as defense against overgrowth. It remains to be demonstrated that any of the organisms tested in this study elaborate secondary metabolites on their surfaces, or into the water column. Thompson (1985) determined that the sponge *Aplysina fistularis* produces exudates containing the dibromotyrosine-derived metabolites aerothionin and homoaerothionin, which were toxic to invertebrate larvae in small volumes of water (Thompson et al., 1985). Similar compounds may also be produced by the sponge *Aplysilla longispina*, extracts of which inhibited the natural settlement of propagules in the present study. Because assay gels contained a natural concentration of metabolites at the onset of the experiment, and the concentration decreased with time, the technique presented herein should give conservative evidence of antifouling activity. Moreover, antifouling compounds may be concentrated near the surface of the living organism, but they are homogeneously distributed in the assay gels.

Several other types of gels were experimented with in the course of this study, including acrylamide, alginic acid, and various types of carrageenan. Some of

Table 1
Mean (±SD) percentage cover estimate and mean number of individuals or colonies of invertebrates on Plexiglas plates and experimental gels after 28 days in the field

Treatment	% cover	Mean number on gels					
		B. eburneus	*C. fragilis*	*S. unicornis*	*B. neritina*	*E. carneum*	*Spirorbis*
Plexiglas	58.0 ± 11.9^B	5.3 ± 2.5	7.5 ± 3.0	0.3 ± 0.5	0.2 ± 0.4	0.5 ± 0.8	1.7 ± 2.1
Control gels	48.3 ± 5.6^B	7.8 ± 3.3	4.7 ± 1.9	0.2 ± 0.4	0	0.3 ± 0.5	1.8 ± 2.8
A. longispina	0^A	0.2 ± 0.4	0	0	0	0	0
E. hepaticum	59.7 ± 9.1^B	5.2 ± 1.5	3.5 ± 2.6	0	0	0.2 ± 0.4	0.7 ± 1.2
H. heliophila	45.2 ± 5.3^B	6.7 ± 2.7	5.2 ± 2.4	0	0	0	0.2 ± 0.4
C. decorticatum	72.7 ± 7.2^C	8.5 ± 2.4	6.2 ± 2.1	0.2 ± 0.4	0	0.3 ± 0.5	1.6 ± 1.6

N = six replicates per treatment. Significant differences in mean percentage cover of algae and invertebrates are indicated by superscripts; means with the same superscript are not significantly different at $p \leqslant 0.05$ (see Fig. 1). The most commonly observed invertebrate settlers were barnacles: *Balanus eburneus* Gould and *Chthamalus fragilis* Darwin; bryozoans: *Schizoporella unicornis* (Johnston) and *Bugula neritina* (Linné); a hydroid: *Eudendrium carneum* Clarke; and polychaetes of the *genus Spiorbis*.

these would not solidify when extracts were added to them (acrylamide, carrageenan), while others disintegrated after a few days in the field (alginic acid, carrageenan). In contrast, gels made of Phytagel™ were stable under all field conditions. One surprising characteristic of gels made from this material was noted, however. We attempted to recover crude extracts from freeze-dried gels, but less than 50% of the initial concentration was liberated by organic solvents, while recovery was close to 100% when wet gels were extracted. It may be that the sugar residues of the Phytagel™ matrix strongly bind organic compounds in the absence of water, which may also explain the slow rates of extract diffusion from hydrated gels exposed to seawater.

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