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MULTIPLE FUNCTIONS FOR SECONDARY METABOLITES IN ENCRUSTING MARINE INVERTEBRATES

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Abstract—We used three chemical fractions (spanning a wide range of polarities) from the extracts of four marine invertebrates, the sponges *Crambe crambe* and *Hemimycale columella* and the ascidians *Cystodytes dellechiajei* and *Polysyncraton lacazei*, to test inhibition of cell division, photosynthesis, and settlement. We used assay organisms from the same habitat, seeking to determine whether a species may display diverse, ecologically relevant bioactivities and, if so, whether the same types of compound may be responsible for such activities. Cell division was strongly inhibited by the sponge *C. crambe*. A dichloromethane fraction from *C. crambe* prevented development of sea urchin *Paracentrotus lividus* eggs at a concentration of 10 μg/ml, as did the butanolic fraction, but at higher concentrations (50 and 100 μg/ml). At 50 μg/ml, the aqueous fraction of *C. crambe* allowed cell division but prevented eggs from developing beyond the gastrula stage. Similar results were recorded with the dichloromethane fraction of *P. lacazei* and from the aqueous fraction of *H. columella*. Photosynthesis was unaffected by any of the species at 50 μg/ml. Larval settlement was inhibited by one or another fraction from the four species surveyed at a concentration of 50 μg/ml, although *C. crambe* exhibited the greatest amount of activity. We therefore found that various fractions displayed the same type of bioactivity, while compounds from the same fraction were responsible for multiple activities, suggesting that secondary metabolites are multiple-purpose tools in nature, which is relevant to our understanding of species ecology and evolution. Moreover, results showed that the assessment of the role of chemical compounds is significantly influenced by the assay organism, fractionation procedure, concentration, and

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duration of experiments. All these factors should be carefully considered when testing ecological hypotheses of the roles of chemically-mediated bioactivities.

Key Words—Secondary metabolites, chemical defense, evolution, ascidians, sponges.

INTRODUCTION

The field of natural products research has uncovered a vast source of new compounds in marine organisms (Faulkner, 1996, and previous reviews by the same author), many of which show antimitotic, cytotoxic, antibacterial, and antiviral properties that may play important ecological roles (Bhakuni and Jain, 1990). Despite the number of compounds reported, comparatively few of them have been properly tested for an ecological role, and so the biological significance of most secondary metabolites remains largely unknown. Indeed, the bioactivity of a metabolite has frequently been described much later than its chemical characteristics. For instance, avarol, a sesquiterpenoid hydroquinone, was first isolated from the sponge *Dysidea avara* (Schmidt) in 1974 (Minale et al., 1974), but it was not until several years later that avarol was reported to produce aberrations in the development of sea urchins (Cariello et al., 1980) and to display antibacterial, antifungal, and antiviral activities (Seibert et al., 1985, Uriz et al., 1992). Additional ecological functions of avarol have been suggested (Martin and Uriz, 1993; Uriz et al., 1992a), although only the antipredatory role has been experimentally tested (Uriz et al., 1996a). Similar stories can be told for a variety of bioactive marine natural products.

The importance, however, of predator–prey interactions in the production of chemical defenses has been highlighted both in terrestrial and benthic organisms. Chemical defenses may have evolved to avoid predation (Rosenthal and Berenbaum, 1992), resulting, in many instances, in a chemically mediated predator–prey coevolution with ecological advantages for both parties (Paul et al., 1990). However, the importance of antipredation in the evolution of chemical defenses in marine environments has been, in our opinion, overemphasized, perhaps because many predictions stem from works on chemical adaptations to herbivory in terrestrial plants. The idea that the antipredatory function is the sole or the most important selective process in the evolution of chemical defenses underlies most of the literature on seaweeds (Hay and Steinberg, 1992; Steinberg and van Altena, 1992; Yates and Peckol, 1993; Hay et al., 1994, to cite some recent examples) and also benthic animals (Van Alstyne et al., 1994; Pawlik et al., 1987, 1995; Paul et al., 1990; Uriz et al., 1996a). Historically, comparatively less attention has been given to other possible roles of chemical substances, such as competition for space or antifouling mechanisms, although more recently they have been the subject of a growing number of studies, especially

those considering benthic animal groups (Coll et al., 1987; Sammarco and Coll, 1988; Porter and Targett, 1988; Davis et al., 1989; Coll, 1992; Maida et al., 1993; Becerro et al., 1995; Clare, 1996; Turon et al., 1996a). It would seem that the most realistic scenario is that secondary metabolites have evolved in response to a panoply of selective pressures and that they fulfill a variety of ecological roles in nature.

Whether a species may display multiple chemically mediated types of interaction is a suggestive hypothesis to test. Such multiple functions could provide a range of ecological advantages in addition to that of the well-known antipredator role. For instance, space is usually a limiting resource in rocky littoral communities; and for encrusting filter-feeding organisms certain basic physiological parameters, such as feeding, reproduction, and mortality, are a function of their area (Jackson, 1979; Ryland and Warner, 1986; Sebens, 1987). Thus, the need for mechanisms for space acquisition and maintenance would seem paramount, and the use of "chemical warfare" can be expected. Furthermore, fouling organisms may interfere with the filtering activity of the basibionts and diminish their efficiency, especially in those filter-feeding organisms in which water is pumped through the free surfaces, as in sponges and some compound ascidians. In organisms of this kind, mechanisms that fight against fouling could also be expected. If secondary metabolites have multiple ecological functions, then changes in chemical defenses could not be modeled as a response to changes in a particular competitor, predator, or fouler, since the producer may be constrained by the other functions of the metabolites (Schmitt et al., 1995). The possibility that particular secondary metabolites have multiple functions has recently been considered, but as yet only occasionally tested in marine environments (Paul, 1992; Schmitt et al., 1995).

This study seeks to determine whether a species may display diverse chemically mediated functions, focusing on functions related to antifouling and space competition. Four common encrusting, filter-feeding species on hard substrata were extracted and their chemical compounds fractionated according to their polarity. Each group of compounds was tested for the inhibition of cell division, photosynthesis, and settlement using ecologically relevant organisms. These activities were selected since they may be involved in growth inhibition, competition with algae, and antifouling processes.

METHODS AND MATERIALS

Previously gathered chemical and ecological data allowed the following species to be selected from among a pool of Mediterranean species whose bioactivity was already known (Uriz et al., 1992a; Martin and Uriz, 1993; and authors' unpublished research):

Crambe crambe (Schmidt) is a red encrusting sponge (Poecilosclerida). It can reach surface areas of 0.5 m^2 in the study zone and is always found free of macroepibionts. This species has been reported to have an array of bioactivities (Berlinck et al., 1990, 1992a,b; Jares-Erijman et al., 1991; Becerro, 1994; Becerro et al., 1994). *C. crambe* is one of the most abundant species in Mediterranean littoral waters (Uriz et al., 1992b). It inhabits both well-illuminated and dark habitats at depths ranging from 1 to 60 m and can be found on hard substrata as well as seagrass (*Posidonia oceanica* Delile) beds; it is one of the few species able to grow among the "new colonizing alga" in the western Mediterranean, *Caulerpa taxifolia* (Vahl) C. Agardh (authors' personal observation).

Hemimycale columella (Bowerbank) is a pinkish encrusting sponge (Halichondrida). Maximum size in the study area is about 500 cm^2. It is also a common species in the Mediterranean, where it inhabits *P. oceanica* beds and hard substrata at depths ranging from 1 to 300 m (Uriz, 1978).

Cystodytes dellechiajei (Della Valle) is a polycitorid ascidian, with a blue tunic (occasionally white, green, or brown) free of epibionts and sand. It has a maximum size in the study area of about 400 cm^2. Although *C. dellechiajei* is preferentially a sciaphilous species, it can also be observed in well-lit habitats (Turon, 1988, 1990). Specimens collected in Japanese waters have been reported to have alkaloids with antineoplastic activity (Kobayashi et al., 1988). Mediterranean specimens featured antimitotic and cytotoxic activities (authors' unpublished research).

Polysyncraton lacazei (Giard) is a red encrusting didemnid ascidian that can reach a surface area of 400 cm^2 in the zone studied. It is usually clean of macroepibionts (Wahl and Lafargue, 1990). It is one of the most abundant colonial ascidians in the zone of study between 0 and 20 m of depth (Turon, 1990). *P. lacazei* has been reported to have antimitotic and cytotoxic activities, and its antifouling mechanisms have been extensively studied (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991).

All specimens were collected in the locality of Tossa de Mar (Northwestern Mediterranean; 41°43.2′N, 2°56.0′E) from a vertical wall at a depth ranging from 1 to 20 m, by scuba diving.

Extraction Procedure. Several specimens of each of the four species selected were freeze-dried and ground together. This was necessary to obtain sufficient amounts of extract. Interspecimen variation was, therefore, not addressed in this work. The samples were extracted three times [1/2 hr, 1/2 hr, and overnight (13 hr)] with a mixture of dichloromethane (DCM) and methanol (MeOH) (1 : 1) in the proportion of 20 ml of solvents per gram (dry weight) of sample. The solvent was evaporated to dryness under reduced pressure and the residue was dissolved in 20 ml of a mixture of DCM and distilled water (1 : 1). These solvents yielded two layers (DCM and aqueous phases) in which the extracted compounds

were separated according to their polarity. The DCM phase was then isolated. Addition to the water phase of 10 ml of DCM yielded new DCM/aqueous layers. The new DCM phase was isolated and a further 10 ml of DCM was added to the aqueous phase, obtaining a third pair of DCM/aqueous layers, from which the DCM layer was isolated again. The three DCM phases were then pooled, evaporated under reduced pressure and weighed. This residue contained the lowest polarity compounds of the crude extract, and it will hereafter be referred to as the DCM fraction. The remaining aqueous phase was extracted with 10 ml of *n*-butanol (BuOH). Two new layers (aqueous and butanolic phases) were obtained. The butanolic layer was isolated and 10 ml BuOH was again added to the aqueous phase. The formation of this layer was performed for a third time. The three butanolic phases were then pooled and evaporated under reduced pressure. This residue contained the mid-range polarity compounds, and it will hereafter be referred to as the BuOH fraction. The remaining aqueous fraction was also evaporated, and the residue, containing the highest polarity compounds of the initial crude extract, will hereafter be referred to as the aqueous fraction. Consequently, this separation procedure gave three fractions of the crude extract—DCM, aqueous, and BuOH fractions—which were used to test the inhibition of cell division, photosynthesis, and settlement in three bioassays using organisms that share the habitat with the four species being investigated.

Inhibition of Cell Division. The bioassay was run using the eggs and embryos of the sea urchin *Paracentrotus lividus* (Lamarck), commonly found in the same habitats as the species analyzed, to detect both antimitotic and cytotoxic properties in the samples. These properties may be useful in competing for space, as antifouling mechanisms or in those processes in which the inhibition of another's growth is a determining factor. A general description of this method can be found in Reish and Oshida (1987), Dinnel et al. (1987), and Martin and Uriz (1993). Adult specimens of *P. lividus* were collected in the field and carried to the laboratory. Spawning was induced by peristomic injection of 1 ml of 0.5 M potassium chloride and both sperm and eggs were released into filtered (0.45-μm pore diameter) seawater (FSW). Cross-fertilization was carried out by adding a small quantity of sperm to the eggs. Tests were run with eggs and sperm from the same couple of sea urchins, since they deliver sufficient numbers of gametes for the simultaneous testing of several samples. Fertilization was usually successful—over 80%. Otherwise the eggs were rejected and the cross-fertilization process was repeated with other specimens. The eggs were then placed in a vessel containing the necessary FSW to a concentration of ca. 200 eggs/ml. Each of the fractions (DCM, BuOH, and aqueous) for each species was tested at three concentrations: (1) 100 μg of extract per ml, (2) 50 μg of extract per milliliter, (3) 10 μg of extract per milliliter. These concentrations were obtained by diluting 100, 50, and 10 μg of extract per 100 μl of ethanol (EtOH). The final solution was obtained by adding 0.2 ml of this ethanolic

solution to 1.8 ml of FSW with eggs. These steps were followed to ensure a good solution of the solid residue in the FSW, i.e., to ensure that the active compounds, if any, diffuse adequately in the aqueous solution. Three replicates were used for each treatment and control. The control was performed by adding 0.2 ml of EtOH to 1.8 ml of FSW. Experiments were stopped after 80 min by adding a drop of formaldehyde (40%). The number of eggs [non-fertilized (NF)] and embryos [fertilized without division (ND), in two-cell stage (TC), and in four-cell stage (FC)] were counted and the proportion of each category was calculated. A three-way analysis of variance of the proportion of embryos at the first cellular division (TC) was performed using species, fraction (nested within species), and concentration (orthogonal to fraction) as factors. A treatment (i.e., any combination of the factors investigated) was referred to as bioactive whenever it differed significantly from the control, as assessed by one-way ANOVA. The proportion of TC was the variable selected on which to run the analyses since this category was the most abundant in the controls. Data were arcsine transformed in order to meet the assumptions of normality and homoscedasticity (as tested by Kolmogorov-Smirnov and Bartlett tests, respectively).

A second experiment was performed as previously described but with a final concentration of only 50 μg of fraction per milliliter. The aim of this second bioassay was to follow the embryonic development of the eggs to the pluteus stage, thus allowing the compounds to act at different stages of the larval development. This may also help to clarify the mechanisms of action of the compounds tested. The number of NF, ND, nonpluteus (NP), and pluteus (P) stages were recorded after 48 hr, and their relative proportions were calculated. To test the effect of time in the detection of fraction activity, data from the two experiments described (experiment 1: concentration = 50 μg/ml and time = 80 min; experiment 2: concentration = 50 μg/ml and time = 48 hr) were combined. Since the most abundant stage in the controls changed between the two experiments (two-cell embryos after 80 min, and pluteus after 48 hr), it was necessary to formulate a new standardized variable that would allow for comparison. This standardized variable was calculated by dividing the proportion of TC and P in the treatments after 80 min and 48 hr, respectively, by the mean proportion of TC or P in their respective controls. Thus, at both times, controls have a mean value of 1, while the treatments may have values higher than 1 (indicating a higher proportion of TC or P than in controls, 1 (no effect), or less than 1 (lower proportion of TC and P). In practice, however, values ranged from about 1 to 0, indicating inhibition of development. A three-way ANOVA was performed on this variable using species, fraction (nested within species), and time (orthogonal to fraction) as factors. As in the shorter-term experiment, and in order to detect bioactivity, data from all combinations of species and fractions were compared with the respective controls with one-way ANOVAs (one per observation time). No data transformation was necessary since the standardized variable met the assumptions on which the ANOVAs were run.

Photosynthesis Inhibition. The test was conducted with the green alga *Ulva rigida* C. Agardh. This test shows whether photosynthesis is affected by the samples. Generally speaking, algae are fast-growing organisms when compared to most zoobenthic species. Thus, inhibition of the photosynthetic ratio may favor zoobenthic organisms competing with algae for space. *U. rigida* was collected in the field and taken to the laboratory to measure oxygen production. This alga was selected because of its morphology, which allowed a clean thallus to be cut in uniform pieces of approximately 1–2 cm^2. These pieces were individually introduced in 280-ml flasks. Oxygen content was measured with an oxygen indicator (Orbisphere Laboratories) after 90 min. Incubation was performed in a Mk X Incubator Shaker (LH Fermentation) at 100 rpm, 20°C, and 500 $\mu mol/m^2/s$ of light intensity. The extraction procedure was as described above. Treatments were tested at the concentration of 50 μg of extract per milliliter. Three replicates were used for each treatment and control. Oxygen content was adjusted to grams dry weight of algae (four days at 80°C, constant weight). No data transformation was necessary since normality and homoscedasticity assumptions were met by the data. Therefore, two-way ANOVA of the oxygen content was performed with species and fraction (nested within species) as factors. As before, bioactivity was assessed by one-way ANOVA using all combinations of species and fractions, together with the controls, as levels of the main factor.

Settlement Inhibition. The settlement inhibition bioassay was run with larvae of the bryozoan *Bugula neritina* (Linné). This bioassay records larval settlement success and posterior survival under the effect of the extracts. Bryozoans are significant components of fouling communities, so this test can be considered as an indicator of general antifouling properties. Colonies of *B. neritina* were collected in the sea, taken to the laboratory, and placed in a completely dark, areated aquarium for one day. After this period, release of larvae was achieved in response to exposure to natural light (Keough, 1984). The colonies were collectively subjected to the photic shock, and consequently larvae from all the colonies were sufficiently mixed to ensure that no colony-dependent differences in the settlement behavior were present among treatments. Treatments were obtained as previously described and consisted of 50 μg of extracts per milliliter. Between 12 and 34 larvae were pipetted into 5-ml Petri dishes where the experiment was performed. Three replicates were used for each treatment and control. The number of larvae settled [settled (ST) and ancestrula (AN)] and not settled (NS), and their movements, were recorded at 1 hr, 30 min; 5 hr, 30 min; 9 hr, 30 min; and 20 hr (times 1, 2, 3, and 4, respectively) and the proportion of each category was calculated. A three-way ANOVA was performed using species, fraction (nested within species), and time (orthogonal to time) as factors. Bioactivity was assessed by one-way ANOVA (one for each observation time) comparing all levels of species and fractions with the controls. As in the experiment of cell division inhibition, the most abundant variable in the controls

changed as a function of time, so it was necessary to use a standardized variable. Here, the standardized variable was calculated by dividing the proportion of ST (time 1 and 2) and AN (time 3 and 4) in the treatments by the mean proportion of ST or AN in their respective controls. As before, at any time, controls have a mean value of 1 while in the treatments it ranged between about 1 (no effect) and 0 (strongest inhibition). No data transformation was necessary since the assumptions of normality and homoscedasticity were met by the standardized variable.

RESULTS

Inhibition of Cell Division. Results from the inhibition of cell division after 80 min are given in Figure 1, while the corresponding ANOVA output is shown in Table 1. Bioactive treatments (significantly different from the controls—$P = 0.05$) are indicated by asterisks in the figure (note that they correspond to the three DCM concentrations and the two highest BuOH concentrations of *C. crambe*). At the conclusion of the experiment, most embryos in the controls (over 80%) had accomplished the first or second cellular division (mainly the former). The low number of unfertilized eggs (<5%) and undivided embryos (<15%) approximated that expected under normal experimentation conditions (Reish and Oshida, 1987). All factors showed a significant effect in the three-way ANOVA (Table 1). The effect of species was due to the inhibition of the sponge *C. crambe* (Tukey multiple comparison $P < 0.001$ with the other species). The effect of fraction did not change significantly with concentration (nonsignificant interaction, Table 1), although this might be due to our sample size since the BuOH fraction was inactive at a concentration of 10 μg/ml but active at higher levels (Figure 2). The effect of fraction was due to the aqueous fraction (Tukey, $P < 0.001$ with the other fractions), which was the only fraction inactive in *C. crambe* (Figure 2). The effect of concentration was due to the significant differences found between 10 and 100 μg/ml (Tukey, $P = 0.002$).

When the eggs of the sea urchin were allowed to reach the pluteus stage, and the data for both experiments combined, new activities were detected (Figure 2, ANOVA output in Table 2). The aqueous fraction of *C. crambe* and *Hemimycale columella* as well as the DCM fraction of *Polysyncraton lacazei*, inactive in the first experiment, became active in the second (bioactive treatments indicated by asterisks in the figure). Accordingly, a significant fraction × time term was detected in the three-way ANOVA (Table 2). In the presence of a significant interaction term, no conclusion can be drawn for the main factors, and data should be reanalyzed for one variable within fixed values of the other variables (Underwood, 1981). We used two-way ANOVAs to look for effects

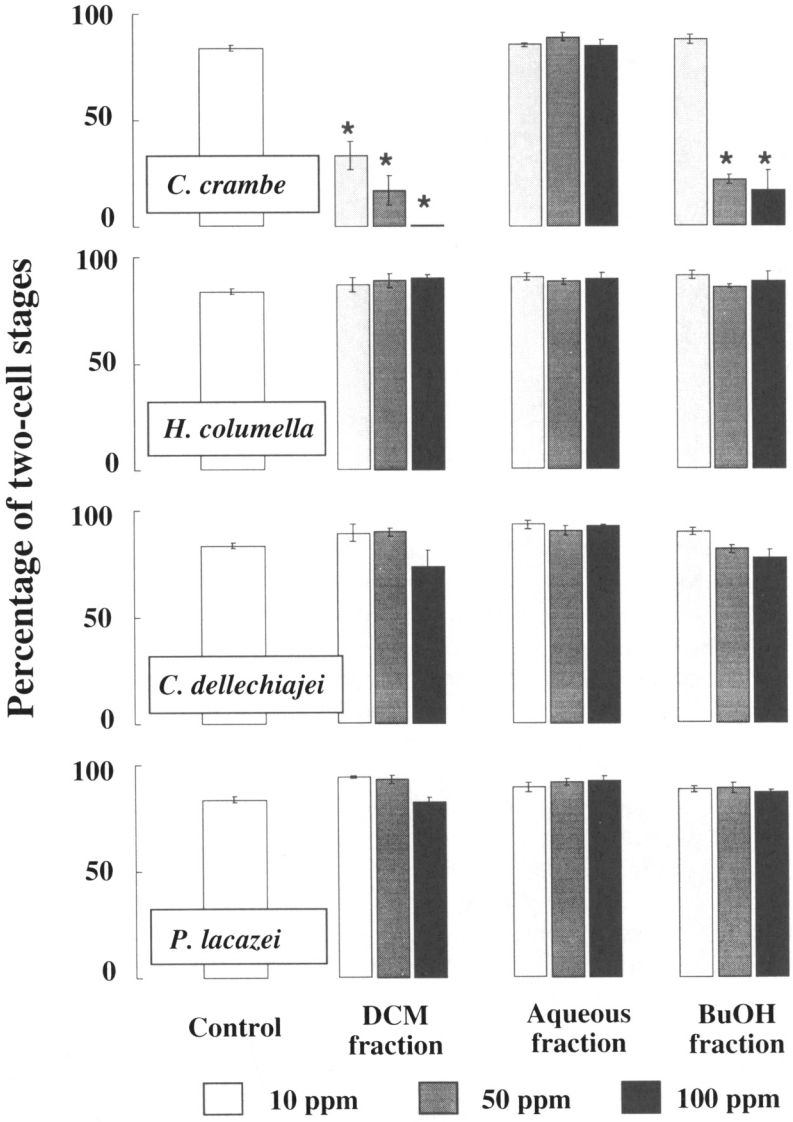

FIG. 1. Percentage of embryos at the two-cell stage 80 min after fertilization when incubated with extracts at different concentrations. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

TABLE 1. RESULTS OF THREE-WAY ANOVA OF EFFECT OF SPECIES, FRACTION, AND CONCENTRATION ON PROPORTION OF SEA URCHIN EMBRYOS AT TWO-CELL STAGE (ARCSINE TRANSFORMED) AFTER 80 MINUTES OF INCUBATION

Source	SS	DF	MS	F	P
Species	4.791	3	1.597	39.013	<0.001
Fraction	1.181	2	0.590	14.425	<0.001
Concentration	0.472	2	0.236	5.773	0.004
Fraction × concentration	0.387	4	0.096	2.366	0.058
Error	3.930	96	0.040		

of species and fraction within the two experiments. In the first experiment, both species and fraction had a significant effect (Table 3). The species effect was due to the sponge *C. crambe* (Tukey, $P < 001$ with the rest of species), while the fraction effect was due to the aqueous fraction (Tukey, $P = 0.028$ with the other fractions), which remained inactive in this experiment (Figure 3). In the second experiment, the species factor had the same results (Table 3, *C. crambe* responsible for significant effect; Tukey, $P < 0.01$ with the remaining species). Fraction had no effect in the longer experiment (Table 3). Time comparisons were made between each combination of species and fraction by t tests. Significant differences between the activity reported for the two times were found in the aqueous and BuOH fractions of *C. crambe* ($P < 0.001$ and $P = 0.014$, respectively), the aqueous fraction of *H. columella* ($P = 0.002$), and the DCM fraction of *P. lacazei* ($P < 0.001$).

Photosynthesis Inhibition. Results of the oxygen production of *Ulva rigida* are summarized in Figure 3. Neither species nor fraction had a significant effect (Table 4) on the oxygen content (milligrams O_2 per liter per gram) at the concentration tested in this experiment (50 μg of extract per milliliter) and all treatments remained inactive when compared with the controls (one-way ANOVA, $P = 0.716$).

Settlement Inhibition. All factors investigated affected the larval settlement of the bryozoan *Bugula neritina* (results summarized in Figure 4 and ANOVA output in Table 5). The effect of species was due to the strong inhibitory effects of the sponge *C. crambe* (Tukey, $P < 0.001$ with the rest of species) and the ascidian *Polysyncraton lacazei* (Tukey, $P < 0.001$ with the rest of species). The aqueous fraction showed the weakest inhibitory effect and accounted for the significant differences detected in the fraction factor (Tukey, $P < 0.001$ with the other two fractions). Time also showed a significant effect. Multiple comparisons showed no significant differences between times 1 and 2 (Tukey,

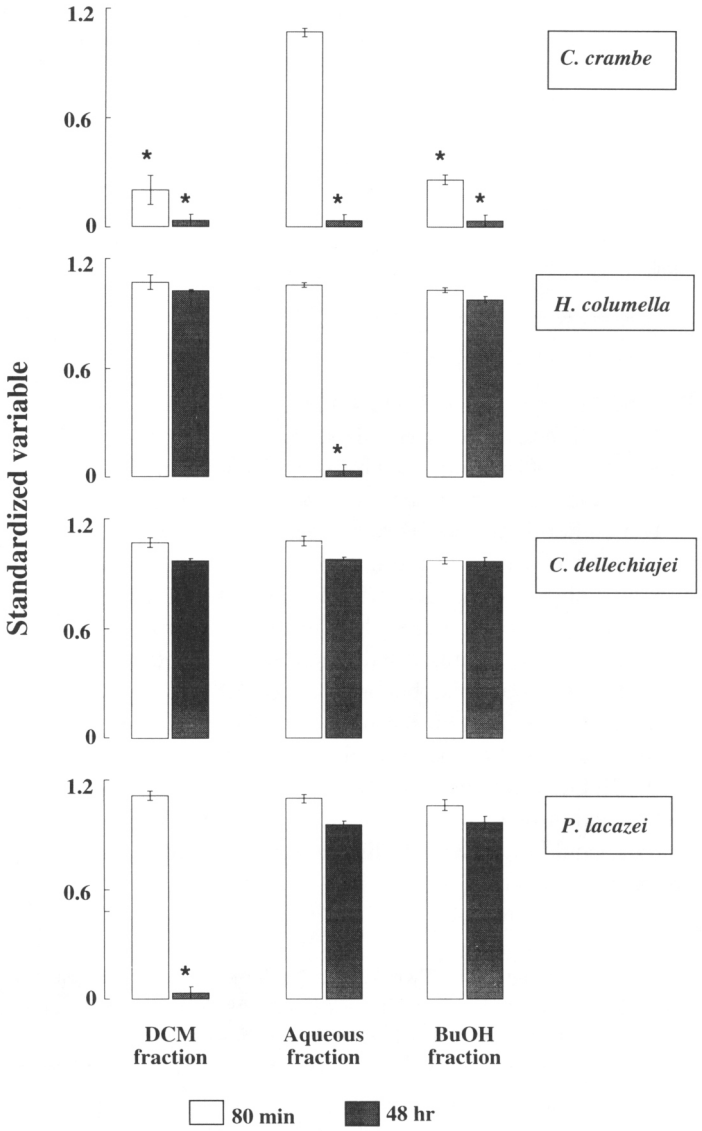

FIG. 2. Values of the standardized variable (see Methods and Materials) measuring the effect of the treatments with respect to the controls in the *Paracentrotus lividus* experiment 80 min and 48 hr after fertilization. The concentration of extracts was 50 μm/ml. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

TABLE 2. RESULTS OF THREE-WAY ANOVA ON STANDARDIZED VARIABLE MEASURING EFFECT OF SPECIES, FRACTION, AND TIME ON DEVELOPMENT OF SEA URCHIN EMBRYOS

Source	SS	DF	MS	*F*	*P*
Species	5.871	3	1.957	25.712	<0.001
Fraction	0.149	2	0.074	0.983	0.379
Time	2.067	1	2.067	27.154	<0.001
Fraction × time	0.693	2	0.346	4.558	0.014
Error	4.795	63	0.076		

$P = 0.998$) and between times 3 and 4 (Tukey, $P = 0.877$). The effect was due to significant differences between these two groups ($P < 0.001$).

Bioactive treatments are indicated by asterisks in Figure 4. It is worth noting that some of the fractions that did not prevent settlement at the beginning of the experiment (i.e., after 1 hr and 30 min) became active after a longer period. This trend could be observed in the aqueous and BuOH fractions of *H. columella*. It was also observed that some fractions produced detachment of settled larvae, a trend that was particularly clear in the DCM fraction of *P. lacazei*. After 5 hr and 30 min no significant differences were detected in this fraction when compared to the controls, but at 9 hr and 30 min larvae had become detached and were lying dead on the bottom of the Petri dish, giving a highly significant effect compared to the controls (Figure 4). This trend could also be observed in the BuOH fractions of *P. lacazei*, the three fractions of *C. crambe*, and the DCM fraction of *Cystodytes dellechiajei*. In the remaining treatments, nonsettled larvae could be observed alive and moving around during the whole duration of the experiment.

TABLE 3. RESULTS OF SEPARATE TWO-WAY ANOVAS OF EFFECT OF SPECIES AND FRACTION ON DEVELOPMENT OF SEA URCHIN EMBRYOS (STANDARDIZED VARIABLE) 80 MINUTES AND 48 HOURS AFTER FERTILIZATION

Time	Source	SS	DF	MS	*F*	*P*
1 hr, 20 min	Species	2.096	3	0.698	19.040	<0.001
	Fraction	0.426	2	0.213	5.815	0.007
	Error	1.100	30	0.036		
48 hr	Species	4.237	3	1.412	13.106	<0.001
	Fraction	0.416	2	0.208	1.933	0.162
	Error	3.233	30	0.107		

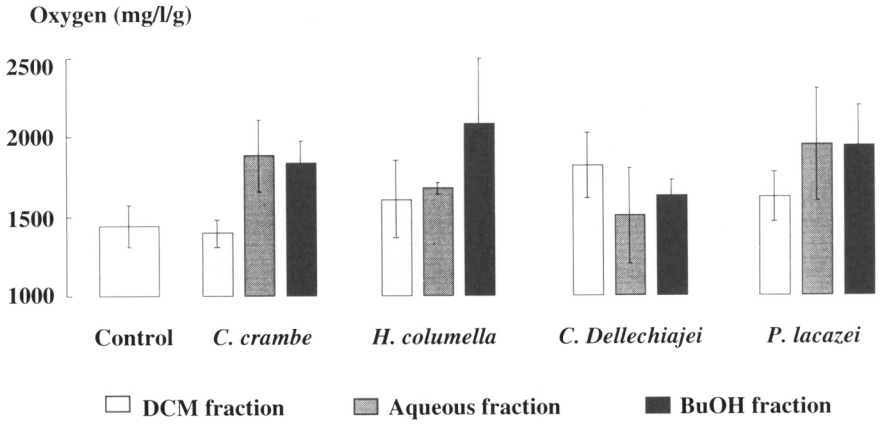

FIG. 3. Oxygen production of *Ulva rigida* when incubated for 90 min with extracts (50 μm/ml) of the different species.

TABLE 4. TWO-WAY ANOVA TABLE OF EFFECT OF SPECIES AND FRACTION ON OXYGEN PRODUCTION BY ALGA *Ulva rigida*

Source	SS	DF	MS	*F*	*P*
Species	0.194×10^6	3	0.648×10^5	0.341	0.795
Fraction	0.420×10^6	2	0.210×10^6	1.107	0.343
Error	0.570×10^7	30	0.190×10^6		

TABLE 5. RESULTS OF THREE-WAY ANOVA ON STANDARDIZED VARIABLE MEASURING EFFECT OF SPECIES, FRACTION, AND TIME ON SETTLEMENT OF BRYOZOAN LARVAE

Source	SS	DF	MS	*F*	*P*
Species	7.922	3	2.640	45.912	<0.001
Fraction	1.204	2	0.602	10.467	<0.001
Time	2.311	3	0.770	13.392	<0.001
Fraction × time	0.240	6	0.040	0.697	0.652
Error	7.420	129	0.057		

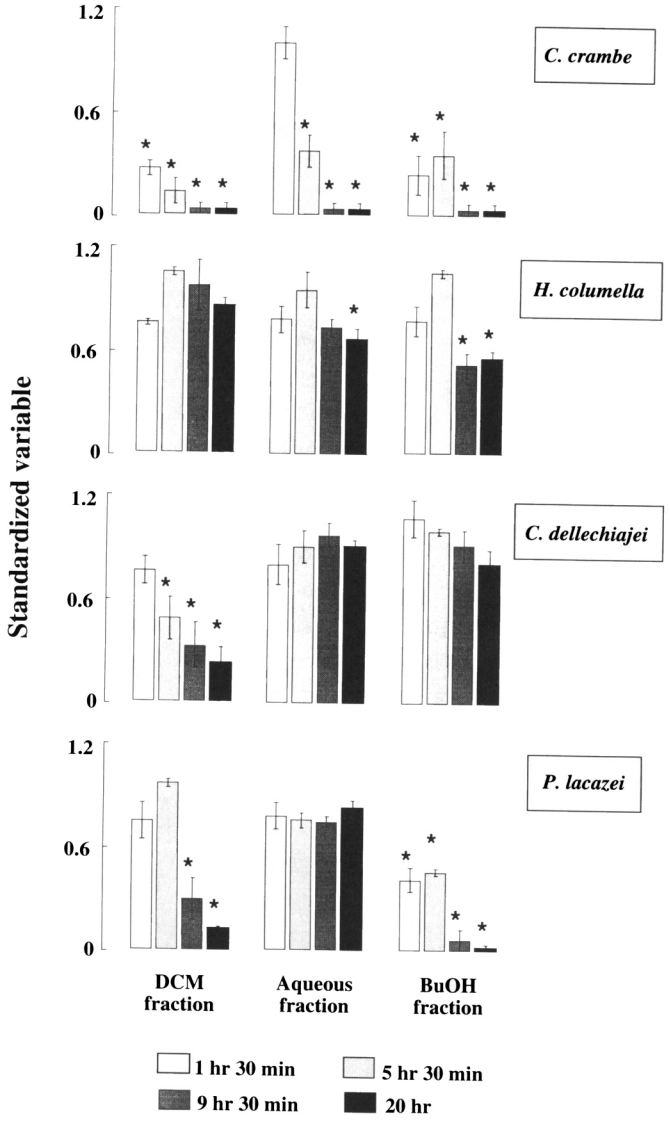

FIG. 4. Values of the standardized variable (see Methods and Materials) measuring settlement inhibition with respect to the controls after different times of incubation with extracts (50 μm/ml) of the species under study. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

DISCUSSION

The results obtained showed that: (1) different fractions of the same species displayed similar ecologically relevant activities in certain cases, (2) different fractions displayed different activities in other instances, and (3) the same fraction could also display diverse activities. "Fraction" is not the same as "compound," of course, but compounds belonging to diverse fractions can be assumed to be different (given the range of polarities covered), and the fact that one single fraction was able to display diverse activities strongly suggests that the same compound, or a group of related compounds, can have multiple ecological roles. To the extent that the active chemicals in our fractions corresponded to single (or groups of related) compounds, which are unrelated to those of the other fractions, our results indicate that the relationship between secondary metabolites and ecological functions is indeed complex. This is consistent with the idea that secondary metabolites have evolved as a response to diverse selective pressures from a variety of competitors.

The sponge *Crambe crambe* was overall the most active species. It showed a strong inhibition of cell division in its DCM and BuOH fractions. These fractions even caused cell destruction in the eggs of the sea urchin *Paracentrotus lividus*. Egg destruction could be due to cytotoxic rather than antimitotic activity of the fractions, as cytotoxic activity has previously been described for this sponge (Berlink et al., 1992b; Jares-Erijman et al., 1991; Uriz et al., 1992a). The development of pluteus larvae was inhibited by the three fractions, which prevented the formation of the first skeletal rods. Settlement in *Bugula neritina* was also inhibited by all fractions from *C. crambe*; the DCM and BuOH fractions did so at the first observation time, while the aqueous fraction had a significant effect after 5 hr and 30 min of incubation.

Apparently *C. crambe* has low-, middle-, and high-polarity compounds that are able both to inhibit cell division and to prevent settlement. Over 15 compounds have already been described for this sponge (Berlinck et al., 1990, 1992a,b; Jares-Erijman et al., 1991). Response to chemical defenses has been shown to be usually species-dependent (Schupp and Paul, 1994). A high number of compounds sharing the same biological activity could be interpreted as an attempt to increase the number of species targeted by chemical defenses. They could have additive or even synergistic effect (Van Alstyne et al., 1994), making them more effective in their role(s). Compounds from *C. crambe* have been reported to reduce predation, fouling, and competitors' growth effectively (Becerro et al., 1994; Turon et al., 1996a; Uriz et al., 1996a). *C. crambe* is one of the most widespread benthic invertebrates in Mediterranean littoral waters (Uriz et al., 1992b), where it successfully inhabits a great variety of contrasting habitats. Thus, *C. crambe* encounters more diverse competitors than any other species dwelling in a narrower range of habitats. Particular responses (coevo-

lution) to one of the competitors would be constrained by this fact (Schmitt et al., 1995). The diverse biological activities that this species displays may be responsible for such a widespread distribution in nature. In this sense, the evolution of "chemical warfare" in *C. crambe* may have favored the acquisition of generalist, multiple-purpose compounds to effectively protect the sponge against a wide array of competitors.

Information on the other species investigated in this work is more limited. Antifouling properties in the ascidian *Polysyncraton lacazei* have been reported (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991). Our findings are consistent with the idea of an ascidian that is chemically defended against fouling, since the BuOH fraction prevents settlement and successful development of the larvae of *B. neritina*. Even if larvae are able to contact the *P. lacazei* surface and settle, they still have to avoid being detached, a trend that was particularly clear in the DCM fraction of this ascidian. Sloughing off the external layer of the tunic has been reported in this species (Wahl and Lafargue, 1990; Turon, 1992). This process and the settlement inhibition activities found here may act together and contribute to the maintenance of a epibiont-free surface. Whether the inhibition of cell division found in the DCM fraction in the *P. lacazei* experiment strengthens the antifouling effect or serves other functions remains unknown.

Results with the sponge *Hemimycale columella* are similar to those from the ascidian *P. lacazei*, although the active compounds of the sponge are more polar than those in the ascidian. High polar compounds (aqueous fraction) of *H. columella* inhibited cell division in *P. lividus*, while the formation of ancestrula in *B. neritina* was retarded by less polar compounds (BuOH compounds, although the aqueous fraction also had an effect at the end of the experiment). As in *P. lacazei*, the activities found in the sponge *H. columella* may also play a role as an antifouling mechanism of this (macroscopically) epibiont-free species.

Active compounds from the ascidian *Cystodytes dellechiajei* are restricted to the lowest polar fraction (DCM fraction). Several toxic alkaloids with antineoplastic activity have been described from this species (Kobayashi et al., 1988). These compounds may be responsible for the antimitotic and cytotoxic activities featured by Mediterranean specimens (authors' unpublished research). Our data exclusively show an inhibitory effect on the settlement of *B. neritina* that may suggest a chemically mediated antifouling mechanism in this ascidian, which also features macroscopically clean surfaces.

None of the four species investigated inhibited photosynthesis of the alga *Ulva rigida*. Whether this is due to a true lack of activity against photosynthesis or is due to an inappropriate concentration or another methodological problem in the experiments remains unclear. An equivalent ecological function could be achieved by increasing respiration rates (Porter and Targett, 1988), but unfortunately no data are available to support this possibility.

Overall, the aqueous fractions tended to have lower activity than the less polar compounds. This may indicate energetic constraints related to the cost of production of substances readily diffusible in water. There would be a continuous waste of these compounds unless they are retained or encapsulated in some way, while apolar compounds, even if released, would remain on the animals' surfaces for longer times.

The assays run in this work were chosen in an attempt to select biological tests with ecological relevance. The activities were tested against marine organisms that are abundant in the communities where the selected species live, which allows for an ecological interpretation. However, it is difficult to determine the concentrations at which the compounds in our fractions may operate in nature. The metabolites were extracted and placed in the water to check their activity. Whether these compounds are naturally released to the water and contact other organisms is at present unknown (but see Uriz et al., 1996b). Furthermore, while intraspecific and intraspecimen variations in the production of secondary metabolites have been described for a variety of benthic organisms, they were not considered here (Paul and Van Alstyne, 1988; Harvell and Fenical, 1989; Wylie and Paul, 1989; Maida et al., 1993; Turon et al., 1996b). In spite of these cautionary remarks, our experiments showed variability in the activities of the fractions within the range of concentrations tested, which may indicate that we were not far from the ecologically relevant concentrations.

The results obtained in this study suggested that *C. crambe* is a highly active species that uses its activities against fouling and in competition for space, while *P. lacazei*. *H. columella*, and *C. dellechiajei* may preferentially use toxicity as an antifouling mechanism. For *C. crambe* and *P. lacazei*, studies carried out previously provide firm support for the proposed ecological roles and also for an antipredation role in *C. crambe* (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991; Becerro, 1994; Becerro et al., 1994; Uriz et al., 1996a; Turon et al., 1996a). In the case of *H. columella* and *C. dellechiajei*, the extrapolation of our laboratory results to ecological functions is at present an assumption that should be tested with more specific work.

Methodological procedures may play a major role in evaluating both compound levels and ecological functions. Cronin et al. (1995) showed how storage and extraction procedures determine the levels of secondary metabolites found in two *Dictyota* species. Selection of an ecologically relevant organism does not guarantee a correct interpretation of the ecological role. Responses to chemical defenses vary depending on test organisms. Schupp and Paul (1994) reported that the response to *Halimeda macroloba*'s secondary metabolites differed among fish species. Pawlik et al. (1986) concluded that diterpenes defended the limpet *Collisella limatula* against natural predators, while Manker and Faulkner (1996) found that the diterpenes did not prevent predation by the starfish *Astrometis* sp., a well-known limpet predator. The test organism used should vary for each life-history stage of the species whose activity is being studied (Uriz et al.,

1996a). Our results confirm that final activity (either in ecologically relevant experiments or in more general screenings) is significantly affected by assay organism, fractionation procedure, concentration, and duration of experiments. Therefore, all these factors should be carefully selected to test ecological roles. Selection should be based on: observational work in the field that leads to ecologically relevant hypotheses and, accordingly, set up extraction procedures, ecologically relevant concentrations and appropriate time lengths, and a range of laboratory or field experiments with a variety of ecologically relevant organisms on which to test these hypotheses.

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