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Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida)

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Abstract

We studied the chemically-mediated interactions of the encrusting sponge *Crambe crambe*, one of the most toxic and widespread species in rocky sublittoral habitats in the Northwestern Mediterranean. Guanidine alkaloids accounted for *C. crambe*'s toxicity, which seems to have multiple functions in nature, as evidence has been found for antifouling, antipredation, and space competition roles.

We investigated the factors underlying the chemical defence strategy of this species by assessing variation in the production of toxic substances as a function of different biological and environmental variables. The working hypothesis was that the production of these metabolites should be optimized according to the biological features (morphogenesis, reproduction, growth, life history) and ecological conditions (biotic pressures and abiotic factors) of the particular specimens.

One cell type, the spherulous cell, which was concentrated near the sponge's surface, accumulated the toxic substances. Within-specimen analyses showed that toxicity was higher in the ectosome than in the choanosome of the sponges. There was a seasonal pattern of change in the toxicity of the species. Life-history stage also proved significant in the production of toxic substances: larvae were non-toxic, and feeding-deterrence experiments showed that larvae and newly metamorphosed individuals were not protected from predation, while two-week-old recruits already showed strong feeding deterrence. Overall, toxicity increased from small to medium-sized adult sponges, and decreased again in larger individuals. Variation in toxicity was also found at an ecological level: the values at a highly competitive site dominated by slow-growing animal species were higher than those at an adjacent, well-lit site with algal dominance. The relative investment in structural material (collagen, fibres, spicules...) was also higher in the shaded habitat, thus a positive relationship was found between investment in chemical and physical defences. In the two habitats compared, allocation to defence correlated negatively with reproduction and growth, and positively with survival.

The results showed that *C. crambe* can adjust, at organismal and population levels, the production of bioactive substances to different environmental and physiological situations. Space competition emerged as a key factor explaining the variation found in the production of bioactive substances.

Introduction

Marine organisms have been a rich source of novel chemical compounds (see Faulkner, 1996, and previous reviews by the same author), which have boosted the development of marine natural products chemistry for about 3 decades. Pharmacologists soon joined this field because of the applied interest of many compounds. Only over the last fifteen years, however, has the incorporation of marine biologists and ecologists resulted in the development of the interdisciplinary field of marine chemical ecology (Faulkner, 1993; Pawlik, 1993; Hay, 1996), which focuses on the ecological functions of marine organisms' secondary

metabolites and on the selective forces modeling their evolution (for reviews on different aspects, see Paul, 1992; Coll, 1992; Hay & Steinberg, 1992; Pawlik, 1992, 1993; Clare, 1996).

In seaweeds, models explaining evolution and selection for different types of chemical defence originate largely from models developed for terrestrial plants. Plant apparency models, resource availability models and spatial variation-in-herbivory models have been analysed as potential descriptors of algal chemical defence evolution (e.g. Hay & Steinberg, 1992; Yates & Peckol, 1992). The concepts of quantitative vs. qualitative, or those of constitutive, inducible, and activated defences have been applied to marine plants (Van Alstyne, 1988; Paul & Van Alstyne, 1992; Hay & Steinberg, 1992; Steinberg, 1994). Underlying all these models and concepts is the notion of a cost associated with the production of bioactive metabolites and a need for optimization of this cost (the optimal defence theory, Fagerström et al., 1987). Clear demonstration and quantification of costs, however, remains as elusive for marine organisms as it has been for terrestrial ones (Hay & Steinberg, 1992; Adler & Karban, 1994).

Possibly because of the roots of marine chemical ecology in terrestrial plant studies, most developments in this field encompass the idea of adaptation to herbivory (or, more broadly, to predator-prey) interactions as the main factor explaining the evolution of chemical defences. Thus, predation-oriented studies are common in the literature on seaweeds (Steinberg & Van Altena, 1992; Hay et al., 1994; Pennings et al., 1996, to cite some examples) and also on benthic animals (*e.g.* Paul et al., 1990; Pennings et al., 1994; Van Alstyne et al., 1994; Pawlik et al., 1995). Other factors have received less attention. Competition for space or antifouling mechanisms may also be chemically-mediated (Coll, 1992) and, therefore, may be important factors affecting both production and evolution of chemical defence, particularly in sessile forms. It is generally acknowledged that many herbivore-susceptible marine species contain potentially defensive compounds, and that there are a large number of metabolites which appear not to have a role in antipredation (Hay, 1984; Steinberg & Van Altena, 1992; Pawlik, 1993). Notwithstanding, studies considering functions other than antipredation as the role of chemical defences or as the underlying selective theme are scarce and usually focused on benthic invertebrates (e.g. Sullivan et al., 1983; Coll et al., 1987; Porter & Targett, 1988; Davis et al., 1991; Sammarco & Coll, 1988; Maida et al., 1993, 1995; Wahl et al., 1994; Teo & Ryland, 1995). As there are diverse selective pressures, chemical defences may have evolved as a multiple-purpose response. Such a possibility is relevant for evolutionary scenarios as it hinders co-evolution of particular pairs of interacting species (Schmitt et al., 1995).

We are not able at present to make generalizations about the applicability of the proposed models of chemical defence evolution. Likely, there is no generalization that can be made, different factors being important for different types of organisms. We need more data on the ecological roles of bioactive metabolites and, crucial to this issue, on variation of bioactivity at several levels (intra- and inter-specimen, spatial, temporal, etc.) and its relationship with other biological parameters of the organisms. Without estimates of this variation, we can do little to increase our understanding of processes behind the patterns observed (Cronin et al., 1995). The present contribution is intended to provide such a framework of variation of bioactivity and its correlates in a model organism.

The aim of this work is to study the ecological functions and variability of the chemical bioactivity of the encrusting sponge *Crambe crambe* (Schmidt). This variability is addressed from several points of view and at several scales, and combined with variability in other biological parameters. This allowed us to correlate differences in chemical bioactivity with other traits of the life-history. It is the first instance, to our knowledge, in which the bioactivity of a species is studied in a comprehensive manner from the cell level to the population level. The particular questions we wanted to answer were: what function or functions does toxicity fulfil in the biology of this species? are there any patterns of toxicity variation at the intra- and inter-individual levels? what information on the underlying selective pressures can be gleaned from the patterns observed? how does investment in chemical defence correlate with investment in other biological functions? As we will review studies performed during the last few years, we will draw partially on data published by the authors since 1994, but we will present them within a common framework and add new evidence to provide a general picture of the chemical ecology of this species.

Material and methods

The poecilosclerid sponge *Crambe crambe* (Schmidt) is a red encrusting form, which can attain surface areas of 0.5 m^2 in the study zone (Northwestern

Mediterranean). This species was selected because it is one of the most abundant sponges in Mediterranean littoral communities (Uriz et al., 1992b), where it is found in a wide range of habitats. At the same time, *C. crambe* featured strong bioactivities in previous, pharmacologically-oriented, screenings (Jares-Erijman et al., 1991; Berlinck et al., 1992; Uriz et al., 1992a). *C. crambe* also possesses an array of potentially active metabolites, grouped into two types of guanidine alkaloid: crambines and crambescidins (Berlinck et al., 1990, 1992; Jares-Erijman et al., 1991). Besides, this species is a thinly encrusting form and therefore highly surface-dependent, which implies strong space competition with neighbours. All these features make *C. crambe* a suitable species for the purposes of assessing ecological roles and the variation of its chemical bioactivity.

All specimens studied were collected by SCUBA-diving at Blanes (NE Spain, Western Mediterranean). For chemical analyses, sponge pieces were blotted on paper towels and freeze-dried. Unless stated otherwise, secondary metabolites were obtained by three successive extractions (for 5, 15, and 30 min, respectively) with dichloromethane (DCM). Preliminary studies showed that this method extracts all compounds of interest (Becerro, 1994).

In some of the experiments we quantified the bioactive properties of the sponges. We chose to use a toxicity test instead of chemically analysing the active compounds, and this point requires some justification. First, we were unable to quantify (by chromatographic methods or by magnetic nuclear resonance) all the potentially active compounds (up to four crambines, plus homologues, and four crambescidins have been described). Second, the relative activity of these compounds is poorly known, and the potential synergistic effects between them are unknown, so trying to quantify one or several of them may not provide the information sought. We looked for a quick and precise tool for measuring bioactivity (i.e. the end-product of all these compounds and their interactions), and so we resorted to a standard toxicity test, the Microtox bioassay (Ribo & Kayser, 1987). This method is based on measurements of bioluminiscence of the deep sea bacterium *Photobacterium phosphoreum*. Although of no ecological meaning in themselves, the results of this test correlate well with those of other, more ecologically relevant analyses, and Microtox performed best in terms of repeatability and accuracy (Becerro, 1994; Becerro et al., 1995b). Pastorok & Becker (1990) also found this method to be the most sensitive in a comparison of marine species used in bioassays. Therefore, toxicity analyses were carried out using the Microtox device and crude DCM extracts, homogeneously resuspended in water (through sonication), and at an initial concentration of 250 ppm relative to initial sponge dry weight. We present the results in Toxicity Units (TU), which equal $100/EC_{50}$ (EC_{50} = concentration which resulted in 50% decrease in light production). For details see Becerro et al. (1995b).

The methods employed in data collection and analysis not reported elsewhere are explained in full in the following sections, while we will only outline the methods used in previously published experiments; readers may refer to the references given for more details on the techniques and the statistical tests used.

Study of ecological roles

Antifouling

For the study of an antifouling effect, we analysed the ability of *C. crambe* to prevent the formation of microbial film on its surface. We quantified the amount of epibiotic bacteria in five living sponges by swabbing surfaces with sterile cotton, seeding culture media with the swabs and counting the bacteria that developed in the cultures. We also tested the antimicrobial effect of crude acetone extracts on seven bacterial strains, isolated from the field in the vicinity of *C. crambe* specimens, by the paper disk diffusion method. For details see Becerro et al. (1994).

A direct test of the effect of *C. crambe* extracts on naturally developing microbial films was performed by adding crude acetone extracts of five sponge individuals (20 cm^2 pieces were cut from each) to five marine agar (2216, Difco, 5% w/v) plates 20 cm^2 in area (5 ml of acetone with the extract was added when the agar temperature fell below 50 °C). Five further plates served as controls, and only solvent was added to them. The plates were left for three days in individual 30 l flow-through cages submersed in large aquaria supplied with running seawater. After this period, the plates were rinsed with filtered seawater and gently swabbed with sterile cottons. These swabs were used to seed agar plates which were incubated in an oven at 21 °C for 21 days; the area occupied by bacteria colonies was then measured.

We also analysed the inhibition of settlement of larvae of *Bugula neritina* (Linné), a common bryozoan in the study zone. This was studied by placing from 12 to 34 larvae in each of three Petri dishes contain-

ing 50 ppm of three subfractions (aqueous, DCM, and butanolic) of a DCM:methanol (1:1) extract of *C. crambe*. Three more Petri dishes served as controls. The number of larvae that settled relative to the controls was measured after 1 h 30 min and 5 h 30 min. The number of ancestrula relative to the controls was measured after 9 h 30 min and 20 h.

Competition for space

For the study of space competition mechanisms, we used both an observational and an experimental approach (Turon et al., 1996b). We studied the small-scale interspecific associations of *C. crambe* with its neighbours in a sublittoral community, and tested the significance of the associations found and the strength of the associations at increasing distances from the contact borders. The experimental approach evaluated regeneration rates of one of the main space colonizers in the community studied, the encrusting sponge *Scopalina lophyropoda* Schmidt. We scraped to rock replicate circular holes (about 6 cm^2) in specimens of this sponge, and the holes were then rubbed with either *C. crambe* fragments, *S. lophyropoda* fragments (rubbing controls), or not rubbed at all (absolute controls). We then surveyed the regeneration of the sponge (i.e. sealing off of the holes) in the different treatments.

We also performed a test of photosynthesis inhibition using as an assay organism the alga *Ulva rigida* Agardh and the same three subfractions of crude DCM/methanol (1:1) extracts of *C. crambe* as in the *B. neritina* experiment. Oxygen production by algal pieces in the presence of extracts (at 50 ppm) was measured in replicate ($n = 3$) samples after 90 min in an incubator and compared to controls.

We assessed whether the active substances of *C. crambe* were found on the sponge surfaces: we gently swabbed an area of 20 cm^2 on each of five large sponge specimens with ca 1 g of glass wool fibre (Sigma) for 1 min, while another five wool pieces (controls), were taken underwater and taken out of their containers at the same time, but not used for swabbing. Control and treated wool was extracted twice with DCM (10 min in 13 ml of DCM each time), the two extracts were pooled, the solvent was allowed to evaporate, and the extracts were tested with Microtox.

Antipredation

We also investigated the predator-deterrent properties of larvae, juvenile and adult sponges. We selected the benthic fish *Parablennius incognitus* (Bath) for predation tests on larvae and rhagons (functional sponge recruits), and the sea urchin *Paracentrotus lividus* (Lamarck) for tests of consumption of artificial food with chemicals and materials from adult sponge specimens. Details of these tests are given in Uriz et al. (1996b).

Study of variation in chemical bioactivity

Within-individual variation

Within-individual location of toxicity was also investigated to provide clues to the function of the active substances. This was done (Uriz et al., 1996a) by separately analysing (Microtox) in five specimens the two layers that comprise the sponge structure: the basal choanosome and the distal ectosome. Further work addressed the identification of the cell type(s) responsible for the toxic properties of the sponges. Cells were separated by depositing cell suspensions (obtained through stirring of small sponge pieces in calcium- and magnesium-free artificial sea water) in a gradient of four densities made with decreasing concentrations of Ficoll (Merck). The cells accumulated in one of the three density interfaces, and in this way we obtained three fractions, each enriched in a different cell type (quantified with a haematocytometer), whose toxicity was analysed by the Microtox procedure.

Intra-individual variation of toxicity was also studied during the seasonal cycle (Turon et al., 1996a). To this end, we selected five large specimens and took small samples from the center and the periphery of the sponges over 15 months (January 1993–March 1994). Samples were extracted and analysed with the Microtox.

Between-individual variation

Inter-individual variation was studied as a function of size and habitat (Becerro et al., 1995a). To this end, we chose a sampling site with two parallel vertical walls between 6 and 12 m in depth, which were only 3 m from each other. They were identical in all respects (including trophic and physical conditions) except in the orientation: one wall faced North, the other faced South. As a result, the former received much less irradiance (relatively shaded wall) than the latter (well-illuminated wall). Communities in these walls showed remarkable differences in species composition: a space-limited community (which will be hereafter called the sciaphilous assemblage) mainly dominated by encrusting animal species was found

on the shaded wall; whereas the well-lit habitat was dominated by erect algae interspersed with patches of bare substrate (photophilic assemblage). On the latter wall, *C. crambe* was the only conspicuous macroinvertebrate (at the landscape level). Our design included these two sites and three size classes of sponges: small (<1000 mm^2 in area), medium (1000 to 10 000 mm^2) and large ($>10\,000$ mm^2) specimens. Ten specimens for each category of size and habitat were selected at random, samples were taken and extracted, and their toxicity was quantified.

Biological parameters

We characterised the pattern of resource allocation following the same design of habitat and size by studying the following parameters in individuals chosen at random on the two walls: thickness, biomass cm^{-2}, organic matter cm^{-2}, spicule content cm^{-2}, porosity, relative amount of spongin fibres, collagen, cells and matrix, and investment in reproduction (number of larvae incubated cm^{-2}). Several techniques were used to analyse these parameters, and they are explained in detail in Uriz et al. (1995).

We also studied the growth rates and mortality on both walls. To this end, in November 1994, we selected small specimens (average area less than 100 mm^2) on each wall. Every month we drew their outlines underwater on acetate sheets. The outlines were then digitized and their surface areas were calculated. Since a high mortality was found on the well-illuminated wall from the beginning of the study, new individuals from this wall were included in the monitoring during the first 4 months of study. Final numbers of sponges monitored were 24 on the shaded wall, 51 on the well-illuminated wall. The survey lasted until January 1997.

Results

Results reported here for the first time are explained in full, while we summarize the results already reported in previous papers by the authors. The reader may refer to them for full details and ANOVA and statistical tables, which will not be presented here.

Study of ecological roles

Antifouling

When the surfaces of individuals of *Crambe crambe* were swabbed with a sterile cotton, we found (Figure 1) that the estimated number of bacteria was about 60 ± 40 cm^{-2} (all results are mean $\pm$ SE), which was of the same order as the number found by swabbing sterile Petri dishes used as controls (30 ± 25 bacteria cm^{-2}), and much lower than the figures obtained by swabbing glass slides immersed in water for three weeks (mean $18\,940 \pm 4303$ bacteria cm^{-2}). The surfaces of *C. crambe*, therefore, were almost axenic. To test for a possible antimicrobial effect of the secondary metabolites of the sponge, cultures of seven bacterial strains (four Gram + and three Gram $-$) isolated from the field were assayed with paper disks (6 mm in diameter) soaked in 25 mm of crude *C. crambe* extract. A significant inhibition effect was found on the seven strains. The diameter of the inhibition zone that developed after 24 h varied from 7.7 ± 0.25 mm to 24.2 ± 0.45 mm (including the disk) depending on the bacterial strain, in contrast to the non-inhibition found in control disks (Figure 1). In the experiment of inhibition of natural bacterial films by extracts of *C. crambe*, bacteria had occupied 5.37 ± 2.51 cm^2 of the culture plates seeded with cottons swabbed in control plates, while only 0.37 ± 0.19 cm^2 of those seeded with swabs from the treatment plates, and this difference was significant ($p = 0.028$, Mann-Whitney U test).

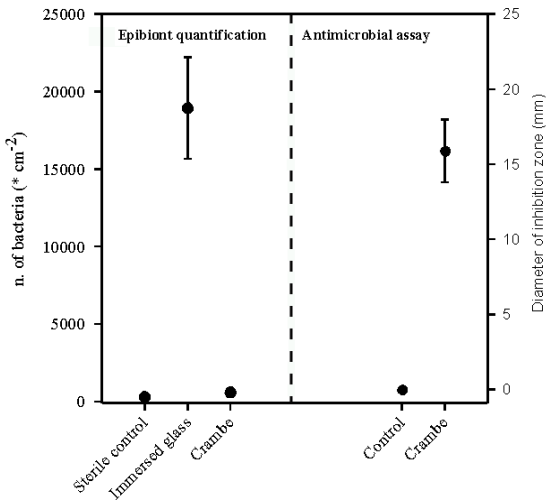

Figure 1. Summary of results from the microepibiont quantification experience and the bacterial inhibition assay. Bars are standard errors.

All subfractions studied showed an inhibitory effect on larval settlement of *Bugula neritina* larvae (Figure 2), although we present only the results for the DCM subfraction (which is the most comparable with the DCM extracts used in the other parts of this study).

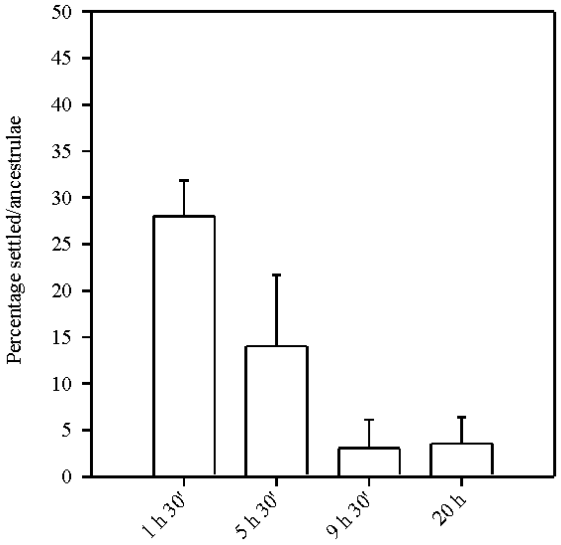

Figure 2. Percentage (relative to controls) of settlers (first and second observation times) or ancestrulae (third and fourth observation times) of *Bugula neritina* in the presence of extract of *C. crambe*. Bars are standard errors.

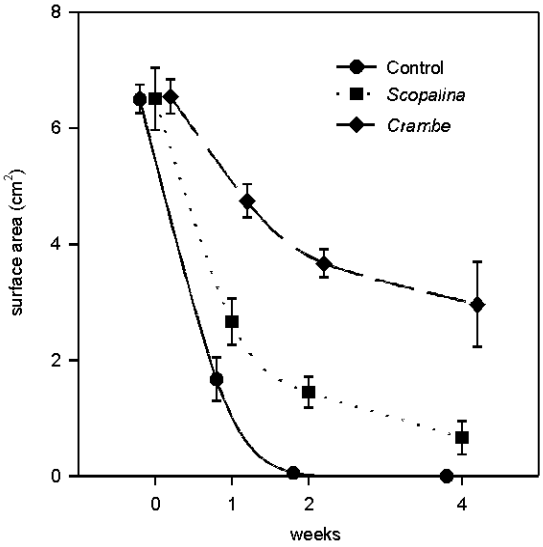

Figure 3. Time course of the area of holes scraped in colonies of *Scopalina lophyropoda*. Treatment holes were rubbed at the end of one and two weeks with either *C. crambe* or *S. lophyropoda*. Bars are standard errors.

There was a significant inhibitory effect at all observation times.

Competition for space

Small-scale association measurements of *C. crambe* were made in a community in which this species was abundant. This community was located on the shaded wall of the ecological variation study. The results showed that, out of ten main states identified (bare rock, nine different species and a miscellaneous group including all species with low abundances), *C. crambe* had negative associations with the five zoobenthic species recorded (3 sponges, 1 ascidian, and 1 bryozoan) and a crustose alga. Monte Carlo analyses revealed that these associations were significant in the case of the interactions with the three sponge species present. In contrast, *C. crambe* was positively and significantly associated with bare rock. Interestingly, when the associations were studied at increasing distances from the contact borders, their intensity fell drastically over the first few centimeters. On the other hand, when holes scraped in *S. lophyropoda* were rubbed with *C. crambe*, the regeneration rates were significantly lower than those of holes rubbed with *S. lophyropoda* (Figure 3). A rubbing effect was also apparent, as at week four non-rubbed holes were sealed off, while the rubbed ones were not. On the other hand, none of three subfractions (aqueous, DCM, and butanolic) of a DCM:MeOH extract of *C. crambe* exerted, at 50 ppm of concentration, a significant effect on *U. rigida* oxygen production rates, so no interference with photosynthesis was detected.

As for the results of the swabbing of *C. crambe* surfaces with glass wool fibre, very small amounts of substances (from 300 to 600 μg, extract dry weight) were recovered from the swabs, and a toxicity value could not be calculated with the Microtox device, as the EC_{50} was greater than the highest concentrations tested in all cases. We were, however, able to compare bioluminescence readings between controls and treatments to identify the presence of a toxic substance, even if in very small amounts. We compared the luminescence decrease (after 5 min of incubation) at the highest concentration possible (60 ppm with respect to extract weight) of the replicate for which we had least material. The variable analysed was Gamma Units, which measured the ratio of light expected from a non-toxic sample to that observed, minus 1. The Gamma Units were significantly higher (*t*-test, $p = 0.0112$) in the swabs from *C. crambe* (0.370 ± 0.051) than in the control swabs (0.129 ± 0.053). Another evidence of the presence of bioactive substances on sponge surfaces came from the histological observations of spherulous cells accumulating and being released through the surfaces of the sponges (see below).

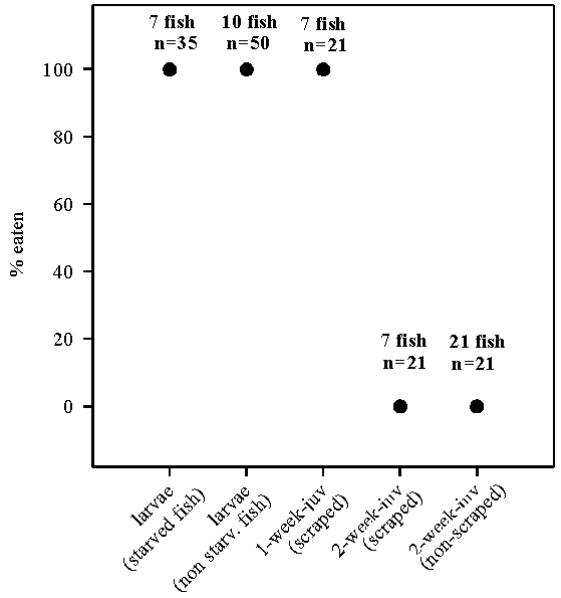

Figure 4. Summary of results from the feeding experiment with *Parablennius incognitus*. The number of fish used and the total number of larvae (n) offered to them is indicated. For the non-scraped 2-week-old juveniles, 7 fish were added to each of 3 Petri dishes with 7 sponge juveniles.

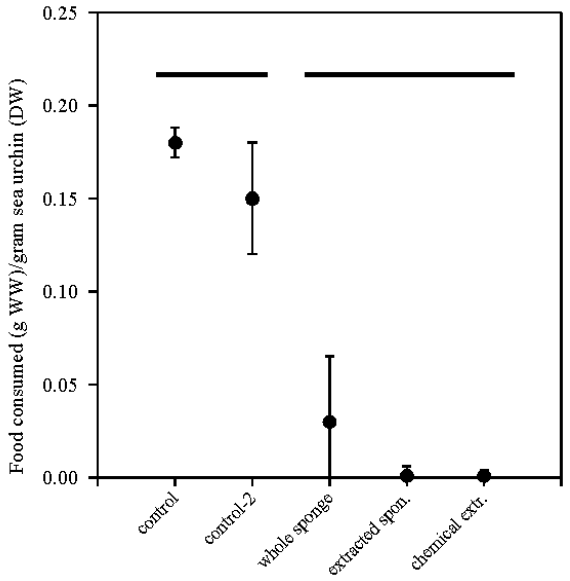

Figure 5. Artificial food consumption by *Paracentrotus lividus*. Horizontal lines join treatments non-significantly different (Tukey test). Bars are standard errors.

Antipredation

The results of fish predation on larvae and juvenile sponges were clearcut (Figure 4): all larvae offered were immediately eaten by *P. incognitus*, irrespective of whether the fish were starved or not. One-week-old juveniles (scraped from the substrate) were also readily eaten in all trials, whereas no two-week-old juvenile was eaten in any case, whether scraped from the substratum or still attached to it. The consumption of larvae is consistent with the finding that the DCM extract of 300 larvae showed hardly any toxicity in the Microtox test (0.24 TU). Furthermore, the experiment with the sea urchin *P. lividus* also showed a distinct effect of all treatments with respect to controls (Figure 5): untreated sponge material, extract from sponge, and the sponge material remaining after extraction (i.e. with all physical structures but without toxic metabolites) all significantly deterred the sea urchins from feeding on one of their preferred algae. We used sponge fragments of the same size as the food plates to keep realistic concentrations of chemicals. Control plates made with fresh (control-1) or extracted algae (control-2) were eaten at similar rates (Figure 5), so no effect due to extraction *per se* could be substantiated.

Study of variation in chemical bioactivity

Within-individual variation

The toxicity was significantly higher in the distal part of the sponge (ectosome, 12.54 ± 1.4 TU) than in the basal part (choanosome, 2.58 ± 0.92 TU) (Figure 6). There are many spherulous cells in the ectosome, which were frequently observed in histological sections clustered beneath the exopinacoderm, releasing their vacuole contents, or being shed themselves to the outside.

The Ficoll procedure gave three cell fractions. Fraction 1 (interface between 2% and 5% Ficoll) contained $90 \pm 0.9\%$ spherulous cells, the 10% of other cell types consisted of choanocytes and non-identified sponge cells or debris. Fraction 2 (interface 5–8% Ficoll) was enriched in choanocytes ($70 \pm 0.95\%$), and also had spherulous cells ($12 \pm 0.74\%$), archeocytes ($6.2 \pm 0.74\%$) and unidentified cells. Fraction 3 (interface 8–11% Ficoll) mainly contained archeocytes ($75 \pm 0.66\%$), a few spherulous cells ($7 \pm 0.74\%$) and cell aggregates ($18 \pm 0.41\%$). Fraction 1 was the most toxic (mean 9.08 TU), while Fraction 2 was mildly toxic (mean 0.48 TU) and Fraction 3 did not show any toxicity (Figure 6). There was, therefore, a good correlation between presence of spherulous cells and toxicity, both at the cellular level and between sponge

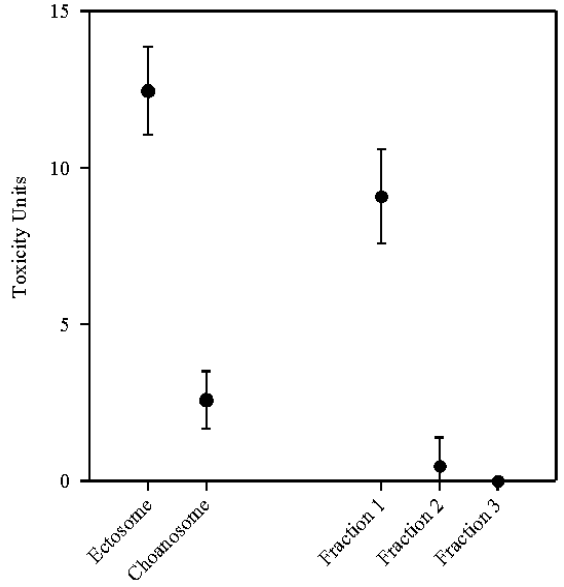

Figure 6. Toxicity readings from the ectosome and choanosome of sponges, as well as from the three cellular fractions obtained by a Ficoll gradient. Bars are standard errors.

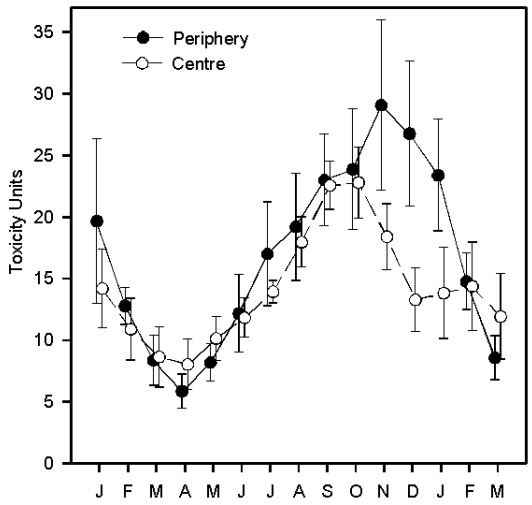

Figure 7. Time course of the toxicity values in the centre and at the periphery of the colonies monitored. Bars are standard errors.

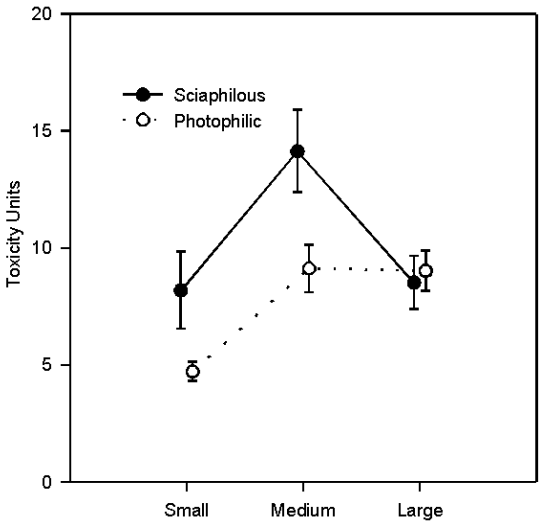

Figure 8. Toxicity values as a function of habitat (sciaphilous or photophilic assemblages) and size class. Bars are standard errors.

layers. The spherulous cells were identified as those that contained the toxic metabolites in this species.

The seasonal variation in toxicity is presented in Figure 7. Two aspects are noteworthy: firstly, there was a clear seasonal pattern, with a minimum in April and maxima at the end of summer-autumn (centre of sponges) and autumn-winter (periphery of sponges). Secondly, toxicity was significantly higher at the periphery than in the centre during the period of high toxicity.

Between-individual variation

When toxicity was analysed as a function of habitat (shaded versus well-lit community) and size class (Figure 8), both factors proved significant (two-way ANOVA), while the interaction was not. Overall, toxicity was higher in the shaded (sciaphilous) community, and in both habitats toxicity was higher in medium-sized specimens (although in the photophilic assemblage, medium- and large-sized sponges showed similar values).

Biological parameters

Table 1 summarizes the results of the analyses performed for the biological and morphological parameters studied following the habitat-size design. Many patterns found proved significant in two- and three-factor (with specimen as a nested factor) analyses of variance. Multiple comparisons were made by Ryan's *Q* method (Day & Quinn, 1989), and the results are also reported in Table 1. Sponges were thicker in the photophilic assemblage. In general, variables associated with structural materials (collagen, fibres, spicules) were higher on the shaded wall, at least (spicules) for large sponges. In contrast, more matrix material was found in photophilic specimens, and also more organic matter (in large sponges) and more cells (in medium-sized sponges). There was a higher production of larvae

Table 1. Summary of the significant effects found in ANOVA analyses of the morphological and biological parameters studied (∗∗=significant at 0.05 probability level; ns=non significant; –=main effect not tested because of significant interactions). Whenever an effect was found significant, *post hoc* tests were made by Ryan's *Q* method. Only significant comparisons are reported. If the interaction size-habitat was not significant, levels of a significant main factor were compared pooling across levels of the other factor. If the interaction was significant, levels of each factor were compared within levels of the other factor (ph, photophilic specimens; sc, sciaphilous specimens).

Variable	Effect			Comparisons
	Habitat	Size	Habitat-Size	
Thickness	∗∗	ns	ns	photophilic>sciaphilic
Porosity	ns	ns	ns	
Biomass	ns	∗∗	ns	large>small & medium
Silica content	–	–	∗∗	ph small>sc small
				sc large>ph large
				sc large>sc medium>sc small
Organic matter	–	–	∗∗	ph large>sc large
				ph large>ph small & medium
Amount of cells	–	–	∗∗	ph medium>sc medium
				sc small>ph small
				sc medium>sc small
Amount of collagen	∗∗	ns	ns	sciaphilous>photophilic
Amount of fibres	∗∗	∗∗	ns	sciaphilous>photophiic
				small>medium
Matrix	∗∗	ns	ns	photophilic>sciaphilous
Larvae	∗∗	∗∗	ns	photophilic>sciaphilous
				large>medium
				large>small

in sponges from the well-illuminated wall, and there was a trend towards increased investment in reproduction with size in both habitats.

As for the growth rates, sponges grew more in the photophilous assemblage. Figure 9 shows the cumulative growth rates (final area minus initial area divided by initial area) at the end of the first and the second years of monitoring. The values in the photophilic habitat were higher, especially during the second year, resulting in a final mean cumulative growth rate of about 2 (i.e. initial areas had been, on average, trebled), while in the shaded wall the mean cumulative growth rate was ca 1 (i.e. initial areas had been, on average, doubled). However, high variances resulting from high inter-individual variability prevented these final cumulative growth rates from being statistically different in the two habitats (Mann-Whitney U test). When growth rates were considered on a monthly basis and compared between habitats, higher growth rates were found in the photophilic habitat in 21 out of 26 months surveyed, and the differences were significant (Mann-Whitney U-test, with Bonferroni correction for the number of comparisons) in May, June, and July 1996. Mortality, on the other hand, was higher in the well-illuminated habitat, in which only 31% of sponges survived by the end of the study, against 62% survival on the shaded wall.

Discussion

The surfaces of *Crambe crambe* were almost axenic, and the antimicrobial properties of its toxic metabolites could explain why development of the microbial film (and hence subsequent steps in the fouling sequence) was prevented. Moreover, antilarval effects of *C. crambe* extracts were also demonstrated. The pattern of small-scale associations of this sponge was consistent with the presence of a short-range inhibition mechanism which may serve for space-competition. Moreover, the rubbing experiment showed that some substance from *C. crambe*, which remained in the substrate for some time, prevented growth of one of the main space competitors of *C. crambe*.

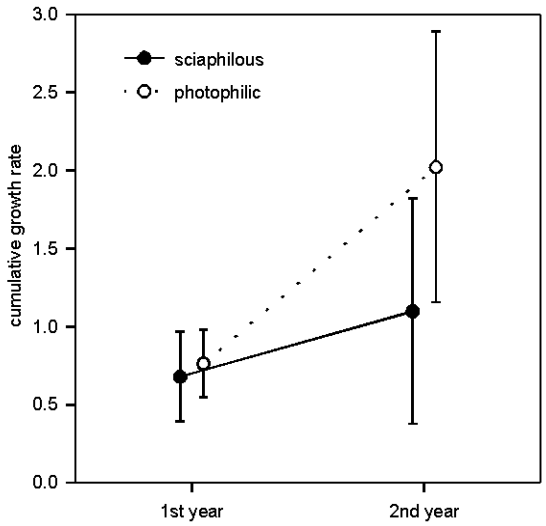

Figure 9. Cumulative growth rates of sponges at the end of the first (Jan 96) and second (Jan 97) years of the growth monitoring in the two habitats considered. Bars are standard errors.

The commonest grazer in the zone (*P. lividus*) was prevented from feeding by all sponge treatments. Feeding deterrence experiments should consider all the potential defences, not only the effects of single defences in isolation (Van Alstyne et al., 1994). We separated the effect of chemicals (extracts) from that of structural materials (extracted sponge), and found that the sponge material deterred feeding by the sea urchin, but that either the chemicals (at an ecologically relevant dose) or the physical structures, considered separately were enough to account for this deterrence. The metabolites of *C. crambe*, however, did not affect photosynthesis rates of the alga assayed. This suggests that these compounds do not constitute an antialgal competition mechanism.

Our results, therefore, showed evidence of a range of ecological functions for the bioactive metabolites of *C. crambe*. Van de Vyver et al. (1990) and Becerro (1994) also found a broad spectrum of bioactivities in sponges and other benthic invertebrates, suggesting that their toxic substances may be multiple-purpose defensive weapons. This may be the rule in benthic, encrusting (i.e. surface dependent) organisms which are subject to high fouling and space competition pressures, as well as to predation.

A key aspect of the ecological roles of bioactive chemicals is that they must be released to the medium if they are to work in antifouling or space competition. Conversely, release is not necessary if their function is solely to prevent predation. The continuous release of a highly diffusible (i.e. polar) compound to the sea would be wasteful, so active compounds that are released are most likely non-polar molecules that may remain on the surfaces and diffuse very slowly. This is consistent with the structure of crambines and crambescidins, which have a mixture of lipophilic (due to the non-polar chain) and hydrophilic (due to the polar head) properties. Although the results of our swabbing experiment were not clearcut, since we recovered very small amounts of material and obtained low activities, our *C. crambe* swabs were significantly more active than the controls, indicating the presence of bioactive substances. A second line of evidence came from the histological studies, in which the spherulous cells, those that accumulate the toxic substances, were shown to be concentrated near the exopinacoderm and to be released through the sponge surface (Uriz et al., 1996a). The available evidence, therefore, points to the release of toxic substances, possibly at small doses, to the medium.

Insight into the processes modeling the defence strategy in this sponge may come from the study of variation at several scales. There are differences between cell types, as the spherulous cells were responsible for the accumulation (and probably production) of the active substances. The same pattern has been found in other sponge species (Bretting et al., 1983; Thompson et al., 1983, but see Uriz et al., 1996c, for contrasting results in another species). The high abundance of spherulous cells in the ectosome of the sponge explains the high toxicity of the distal layer.

We also detected ontogenetic variation in chemical defences. The larvae were not toxic (Microtox), and neither the larvae nor the one-week-old recruits had predation-deterrent properties. Deterrency developed somewhere between one and two weeks after settlement. This may correlate with the time of differentiation of the first spherulous cells in the juveniles. This result is consistent with the hypothesis that rapidly developing juvenile tissues cannot produce bioactive metabolites (growth-differentiation balance hypothesis, Cronin & Hay, 1996), although many exceptions have been reported (e.g. in ascidians: Lindquist et al., 1992). Larvae of another sponge species, *Dysidea avara* (Schmidt), were found to be defended against predation (Uriz et al., 1996b). Seasonality imposes strong periodicity on the biological parameters of marine organisms in temperate seas. Investment in toxin production was also found to fluctuate seasonally in *C. crambe*. Differences between centre and periphery

of the sponges were also substantiated in this study. These differences were evident during the season of high toxicity. The time course of toxicity in the centre may be modulated by internal parameters (e.g. investment in reproduction); while at the periphery external interactions (e.g. space competition) may be the dominant factor (Turon et al., 1996a). In this sense, it is indicative that the season of high peripheral bioactivity coincides with the season in which many invertebrates reactivate growth after the aestivation period. Fouling pressure varies seasonally, but its maxima are in spring in the study zone, which cannot explain our autumn peak. Mature larvae are present within *C. crambe* at the end of spring and summer, so gametogenesis and associated changes occur in spring. It seems, therefore, that the timing of allocation to reproduction and to toxin production is reversed, thus suggesting a trade-off between reproduction and chemical defence production. A combination of internal and external pressures probably determined the cycles found.

Habitat-related variation in toxicity was found, with sponges from a space-saturated (sciaphilous) community dominated by slow-growing animal species featuring more toxicity than sponges from an adjacent habitat (photophilic community) dominated by algae and with patches of bare space being continuously produced. It seems likely that space competition pressures explain the differences. This does not imply, however, that algae do not compete for space. More specifically, the key factor may be the different turnover and growth rates of the interacting species. A slow growing form such as *C. crambe* would hardly outcompete fast-growing algal species which appear and disappear seasonally, so there would be no selective advantage in investing in costly defences (physical and chemical) that would not prevent mortality in this habitat anyway. On the other hand, allocation to defensive chemicals would be more advantageous in a community dominated by perennial, surface-dependent organisms.

C. crambe was very plastic in most biological parameters. Many of the features studied varied significantly among size classes and habitats, indicating an ability to adjust its relative energy allocation in response to physiological and environmental changes. We did not find any trade-off between allocation to chemical and physical defences, as reported in other studies (between species: Sammarco et al., 1987; Coll, 1992; at the intra-individual level: Harvell & Fenical, 1989; Pennings et al., 1996). Our results on an intraspecific scale agree with those of Chanas & Pawlik (1995) in an interspecific comparison among Caribbean sponges. In our study, sponges that invested more in chemical defences (sciaphilous sponges) also allocated more resources to tough, structural materials (collagen, spongin fibres, spicules) able to serve as physical defences. It may also be that the combined effect of both types of defence is greater than their separate sums (as found by Hay et al., 1994).

The question arises as to the allocation of the energy expenditure spared by specimens (photophilic) that invest less in defence; obvious responses are growth and reproduction (Paul & Van Alstyne, 1988; Chanas & Pawlik, 1995). Reproductive output, as measured by the number of larvae incubated, is indeed higher on the photophilic habitat (Table 1). Growth rates are also higher in this habitat, although large variances prevented this effect from being statistically significant in some of the analyses. Preferential allocation to growth in small specimens, and to reproduction in larger ones, may also explain why toxicity, overall, is higher in medium-sized individuals (Figure 8). Mortality, on the other hand, was higher in the well-illuminated wall, where sponges invest less in physical and chemical defences.

In summary, the results are consistent with the idea that chemical defence is costly in *C. crambe*, and that it is optimized with respect to within-individual, ontogenetic, seasonal, and ecological constraints. Clonal construction is an energetically favourable situation for the production of costly defences (Adler & Harvell, 1990; Harvell, 1990), which may explain the lack of a dichotomy between allocation to physical and chemical defences in our between-habitat comparison. We found, however, a trade-off between allocation to defences and to reproduction and growth. What can be said about the factors determining the variation found? Predation can apparently be avoided with just physical defences, and no indication of an effect of variable fouling pressures was apparent (peaks of toxicity did not correspond to recruitment peaks of foulers, and we did not expect much difference, at least at the microfouling level, between our two adjacent walls). Differences in food availability are also unlikely between the walls studied, so resource limitations cannot explain the between-habitat pattern observed. The modulation of defence levels seems, therefore, best explained by differences in space competition pressures.

In conclusion, the optimal defence theory seems to apply in the case of *C. crambe*, and a model of variation-in-space competition pressures is consistent with most patterns observed. This may prove to be a

general feature of indeterminate-growing organisms, especially those that are more dependent on free space (e.g. those of encrusting morphology), and which feature a strategy of slow growth and perennial life span.

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