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Location of toxicity within the Mediterranean sponge *Crambe crambe* (Demospongiae: Poecilosclerida)

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Abstract Within-specimen location of toxicity in *Crambe crambe* (Schmidt) has been addressed by complementary procedures on specimens collected in north-east Spain (Western Mediterranean) in winter of 1993. The toxicity of the distal (ectosome) and basal (choanosome) sponge parts have been analysed and the main cellular types present in these two layers have been studied by light and electron microscopy. The toxicity of the three main cell types, separated by the gradient-density method, has also been analysed. Three main fractions, each of them enriched in a different cellular type, were obtained: Fraction 1 (interface between 2 and 5% Ficoll) contained 90 ± 0.9% (mean ± SE) of spherulous cells and 10% of different cell types consisting of choanocytes (5 ± 0.54%), and unidentified cells or cell debris (5 ± 0.84%); Fraction 2 (interface between 5 and 8% Ficoll) was enriched in choanocytes (70 ± 0.95%), and also contained spherulous cells (11.8 ± 0.73%), archeocytes (6.2 ± 0.74%) and unidentified sponge cells (12 ± 0.74%); Fraction 3 (interface between 8 and 11% Ficoll) mainly consisted of archeocytes and archeocyte-like cells (75 ± 0.66%), together with spherulous cells (7 ± 0.74%) and other unidentified sponge cells and cell aggregates mainly formed by choanocytes (18 ± 0.41%). Toxicity [measured in toxicity units, TU, using the Microtox® procedure] was significantly higher in the sponge ectosome (12.45 ± 1.4 TU) than in the choanosome (2.58 ± 0.92 UT). Only the abundance of spherulous cells in the sponge tissues correlated well with the pattern of toxicity observed, and this was corroborated by the toxic behaviour of the three cellular fractions obtained: the one enriched in spherulous cells was highly toxic (9.08 UT), whereas those enriched in choanocytes and in archeocytes were almost inactive (0.48 UT) or totally innocuous, respectively. All these results point to the spherulous cells being responsible for the storage (and possibly production) of the toxic compounds in *C. crambe*. Toxicity is concentrated in the sponge periphery. Spherulous cells are also concentrated in this area and can also be observed outside the sponge exopinacoderm. These results correlate well with the assumption of a defensive role of toxicity, since encounters with potential epibionts, predators and competitive neighbours take place through this peripheral zone. However, we found two types of spherulous cells (orange and colourless, respectively) coexisting in the same sponge zones as well as in Cell Fraction 1. Thus, we cannot at present determine whether one or both types are responsible for the toxicity encountered, although it is likely that the two correspond to different states of the same cell type.

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Introduction

Within-specimen location of bioactive compounds in a species provides clues to the biological and ecological roles that these substances may play in nature. The location of bioactive metabolites in the mantle, cerata, or mucus glands of opisthobranchs indicates that these bioactive substances may have an anti-predatory function (e.g. Avila et al. 1991; Di Marzo et al. 1993; Fontana et al. 1993). Their location in the digestive glands provides information as to whether the metabolites were diet-derived compared to being biosynthesised "de novo", (e.g. Paul and Pennings 1991;

Cimino and Sodano 1994; Fontana et al. 1994). There is evidence of intracolony or intraplant variation of toxicity. Hay et al. (1988) and Paul and Van Alstyne (1988) found that the most toxic and deterrent compounds of *Halimeda macroloba* were allocated to newly grown areas which are more vulnerable to herbivores, where they would play a deterrent role relevant for survival. Harvell and Fenical (1989) provided evidence of intracolony variation of chemical defences in two gorgonian species. Van Alstyne et al. (1994) found that concentration and types of antipredator defences vary among tissues within colonies of *Sinularia* spp. Determining the precise location of bioactive substances within seaweeds or primitive marine invertebrates is a complex task, due to the lack of discrete organs on which to perform separate chemical or toxicological analyses. The complexity of locating a certain metabolite or bioactivity is still greater in sponges, since the biological processes in these organisms take place at a cellular level (Simpson 1984) and separation and study of the different cellular elements is the only known way to locate biological activity in a sponge.

Crambe crambe (Schmidt) is a highly toxic sponge featuring an axenic surface free of epibionts (Becerro et al. 1994a). The antifouling role of the metabolites displaying toxicity in this species has already been shown for microfoulers (Becerro et al. 1994a) and invertebrate larvae (Martín and Uriz 1993). The hypothesis of the present study was that if a defensive role against external organisms is played by the toxicity of *C. crambe*, toxic compounds would be produced and/or stored near the sponge surface and, thus, toxicity would be higher in the distal part of the sponge (ectosome) than in the inner choanosome. Consequently, some cells abundant in the former zone and absent or present in lower numbers in the choanosome, would be responsible for the toxicity.

Location of bioactive metabolites in sponge cells has rarely been accomplished (Thompson et al. 1983; Garson et al. 1992) due to the difficulty of obtaining sufficiently pure cell populations (De Sutter and Tulp 1981). When the active metabolite possesses an halogen element that is rare or absent from the primary metabolites, X-ray microanalysis on thin sponge sections provides an accurate and relatively easy solution (Thompson et al. 1983). In the same way, when some fluorescence is shown by any of the cell types, flow-cytometric methods of separation can provide good results (Unson and Faulkner 1993). However, the crambines and crambescidines accounting for the toxicity of *Crambe crambe* (Berlinck et al. 1990; Jares-Erijman et al. 1991) do not possess any halogen atom nor do sponge cells fluoresce. Therefore, cell dissociation is necessary in this case, and traditional techniques such as density gradients are the most suitable to perform it.

Materials and methods

Sampling

Sampling was carried out in the locality of Blanes, north-east Iberian Peninsula (Western Mediterranean) at depths of 6 to 13 m (Becerro et al. 1994b). The sponge *Crambe crambe* (Schmidt) is an encrusting species ranging in size from a few mm^2 to 1 m^2. It is one of the landscape-dominant forms of the semi-dark, shallow, sublittoral habitats in the study area.

Specimens for cellular dissociation were collected in winter (December to February), before the period of reproduction and the associated cellular changes, transported to the laboratory in seawater, and immediately fractionated. Thick specimens were preferred, since these contained comparatively less collagenous material that preliminary assays had shown to interfere with cell dissociation by forming aggregates.

For evaluation of differences in toxicity between basal (choanosomal) and apical (ectosomal) sponge zones, a total of five relatively thick specimens was collected from the same site as the previous specimens. Each sample was carefully examined under a stereomicroscope and cleaned of any foreign body present. The basal and the apical zones of the five specimens were then separated by dissecting under a steromicroscope, and lyophilised. Their separation was feasible due to the large aquiferous canals running between the two zones. Amounts of 0.01 g (dry weight) of each sponge zone were then set aside for toxicological quantification.

Cell characterisation

Cytological studies on fresh and fixed sponge material through light (Nomarsky and phase-contrast optics) and transmission electron (TEM) microscopes were carried out to characterise the main cellular types of *Crambe crambe* and their features, and to allow easy identification after dissociation in a liquid medium. For cytological characterisation of the sponge layers, the specimens were fixed in formaldehyde, embedded in paraffin, and sectioned (5 μm thickness) perpendicularly to the sponge surface. Sections were then stained by Mallory's technique (Martoja and Martoja 1970) and observed with light microscopy. For dissociated cell identification, fresh specimens were squeezed in seawater and the cells leaving the sponge tissue were observed alive by light microscopy (Nomarsky and phase-contrast optics). Samples for the electron microscopy were treated according to the method of Vacelet et al. (1989) and were embedded in Spurr's resin. Silica dissolution (with hydrofluoric acid, HF) was not performed before sectioning to prevent possible artifacts in the cells due to HF. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963), and observed in a Hitachi H-600 electron microscope belonging to the Microscopy Service of the University of Barcelona.

Cell dissociation

Cell dissociation was performed on Ficoll gradients, according to the following procedure (modified from De Sutter and Van der Vyver 1977 and from Thompson et al. 1983): a fresh sponge was cut into small pieces ($\simeq$ 3 mm diam) up to a total volume of 2 ml, rinsed several times in artificial seawater free of calcium and magnesium to eliminate the aggregation factor released by the cells (Burkart et al. 1979), transferred to 100 ml vol seawater [calcium/magnesium-free artificial seawater, (CMF–ASW)] adjusted to pH 7.3 with HCl, and dissociated by slow stirring ($\sim$ 200 rpm) for 3 h at room temperature ($\sim$ 21 °C). The resulting hyaline cell suspension was first filtered through a nylon-mesh (30 μm diam) to eliminate most spicules and cell aggregates (De Sutter and Tulp 1981), then pelleted

by centrifugation at 1000 rpm for 2 min in a refrigerated centrifuge (temperature = 10 °C). The wet pellet was then dispersed into single cells by repeated pipetting before carefully layering onto the top of the Ficoll gradients. In a preliminary assay we added pronase and collagenase enzymes (Thompson et al. 1983) to improve sponge dissociation, but this procedure failed to fractionate the cells of *Crambe crambe* adequately.

For each sponge sample, 2 to 3 discrete Ficoll gradients were simultaneously prepared in 30 ml tubes by adding, from bottom to top, 6 ml of the following solutions of Ficoll in CMF–ASW: 11, 8, 5 and 2%. These particular densities were chosen after preliminary studies on the cell fractionation of the species on a continuous gradient from 1 to 10% (De Sutter and Van der Vyver 1977) and on various discrete gradients. After 3 h sedimentation, cell fractions accumulated at interfaces between successive densities along the gradients. Each fraction was isolated individually by aspiration into a pipette and placed in a graduated tube. Filtered seawater was added up to a final volume of 2 ml. An aliquot of each fraction consisting of 5 µl of the cell suspension was placed in a haematocytometer and observed through light microscope (Nomarsky optics) to quantify the relative number of the different cellular types present. Two readings (each corresponding to a volume of 2.5 µl) of each sample were obtained, and the mean value of these two readings was calculated. Cell fractions of the same density from different gradients were pooled together and frozen (− 40 °C). The whole procedure was conducted on 12 sponge specimens on successive days to obtain sufficient cells of each fraction to allow the toxicity assays to be performed. By averaging the values of the 12 sponges we obtained the final values for the mean and standard error of the percent of each cell type in each fraction.

Chemical extraction

The extraction procedure used has been described in detail by Becerro (1994). Since dichloromethane has been shown to extract all the toxic compounds of *Crambe crambe*, sponge fragments (0.01 g dry wt) were extracted successively three times with 5 ml of dichloromethane (DCM), for 5, 15, and 30 min, respectively. The three extracts were pooled, the DCM was allowed to evaporate, and the residue was weighed and resuspended in 20 ml of distilled water (in an ultrasonic bath to ensure homogeneity) to a final concentration relative to the initial sponge dry weight: 500 ppm. Cell fractions were also extracted as above, but their amount did not reach the dry weight used for the sponge layers. Thus, the absolute values of toxicity encountered are only comparable between sponge layers and among cell fractions.

Toxicity analyses

Toxicity of sponge fragments and cell fractions was quantified by the Microtox® bioassay (Ribo and Kaiser 1987; Kaiser and Ribo 1988; Becerro et al. 1995). This method evaluates toxicity against a marine bioluminescent bacteria by measuring loss of bioluminescence in living cell suspensions of the deep-sea bacterium *Photobacterium phosphoreum*. This procedure has proved to be more sensitive and precise than other commonly used methods such as the disk diffusion and the sea-urchin assays (Becerro et al. 1995). Moreover, Becerro et al.'s results correlate well with those obtained in assays on bacteria from *Crambe crambe*'s habitat (Becerro et al. 1994a) and embryos and larvae of benthic sublittoral organisms (Martín and Uriz 1993; Becerro 1994). It is therefore used here as a measure of the general toxicity of the samples. Freeze-dried bacteria were rehydrated in a reconstituent suspension at 4 °C. Four different concentrations and one control per sample were analysed. Experiments were run for 5 min at 15 °C. The light produced by the bacteria was recorded before and after the experiments and transformed to gamma units (GU) (Ribo and Kaiser 1987): $GU = (R_t\, I_0/I_t) - 1$, where R_t = correction factor, I_0 = light at time 0, and I_t = light at time t.

Finally, the concentration at which GU is equal to 1 (50% of light reduction or estimated median effective concentration, EC_{50}) was calculated by fitting the data in logarithmic scale to a linear regression. The toxicity units (TU) used in this study were defined as $100/EC_{50}$.

Results

Main cell types

Two clearly differentiated structures can be observed in a section of *Crambe crambe* (Fig. 1A): the ectosome, at the sponge periphery, with a thickness ranging from 200 to 600 µm, and the choanosome, in the inner part 400 to 5000 µm in thickness, separated from the ectosome by wide canals and delimited at the sponge base by a thin spongin layer in contact with the substratum. From this basal layer, spicule bundles, partially surrounded by spongin, arise perpendicularly to the substratum without reaching the sponge surface.

The ectosome is traversed by oscula, ostia, inhalant and exhalant canals, and abundant subectosomal spaces (Fig. 1A, B). It is externally delimited by an unicellular layer of pinacocytes (exopinacocytes) which also underlie the canal system (endopinacocytes). Besides the pinacocytes, the ectosome of *Crambe crambe* contains large amounts of diffuse collagen (Becerro 1994) and few cellular elements, mainly consisting of collencytes (cells producing collagen) and large cells packed with large spherical uniform inclusions (hereafter termed spherulous cells) of two morphological types. These types are orange and colourless under the light microscope, and feature high- and low-density to electrons (Fig. 1C, D) in TEM, respectively. They both lie close to subectosomal spaces and to the sponge surface where they form clusters beneath the pinacoderm (Fig. 1B). They can be frequently observed leaving the sponge tissue (Figs. 1B and 2A).

The choanosome is basically formed by the choanocyte chambers and the whole system of canals entering and leaving these chambers. Although it mainly consists of choanocytes (the most abundant cell category of this sponge), its mesohyl also contains collagen fibrils, abundant endopinacocytes lining the surface of canals, archeocytes, sclerocytes and collencytes/spongocytes (Becerro 1994), with spherulous cells in much lower numbers than in the ectosome. Some spherulous-like cells, in a more or less degraded state, are often visible beneath the endopinacoderm or within the aquiferous canals (Fig. 2B, C).

The cells and mesohyl of both layers were free of microsymbionts (bacteria or cyanobacteria) in all specimens studied. This observation contrasts with Sarà's (1966) report on the sporadic occurrence of symbiotic cyanobacteria and microalgae in specimens of this species from Italian shores.

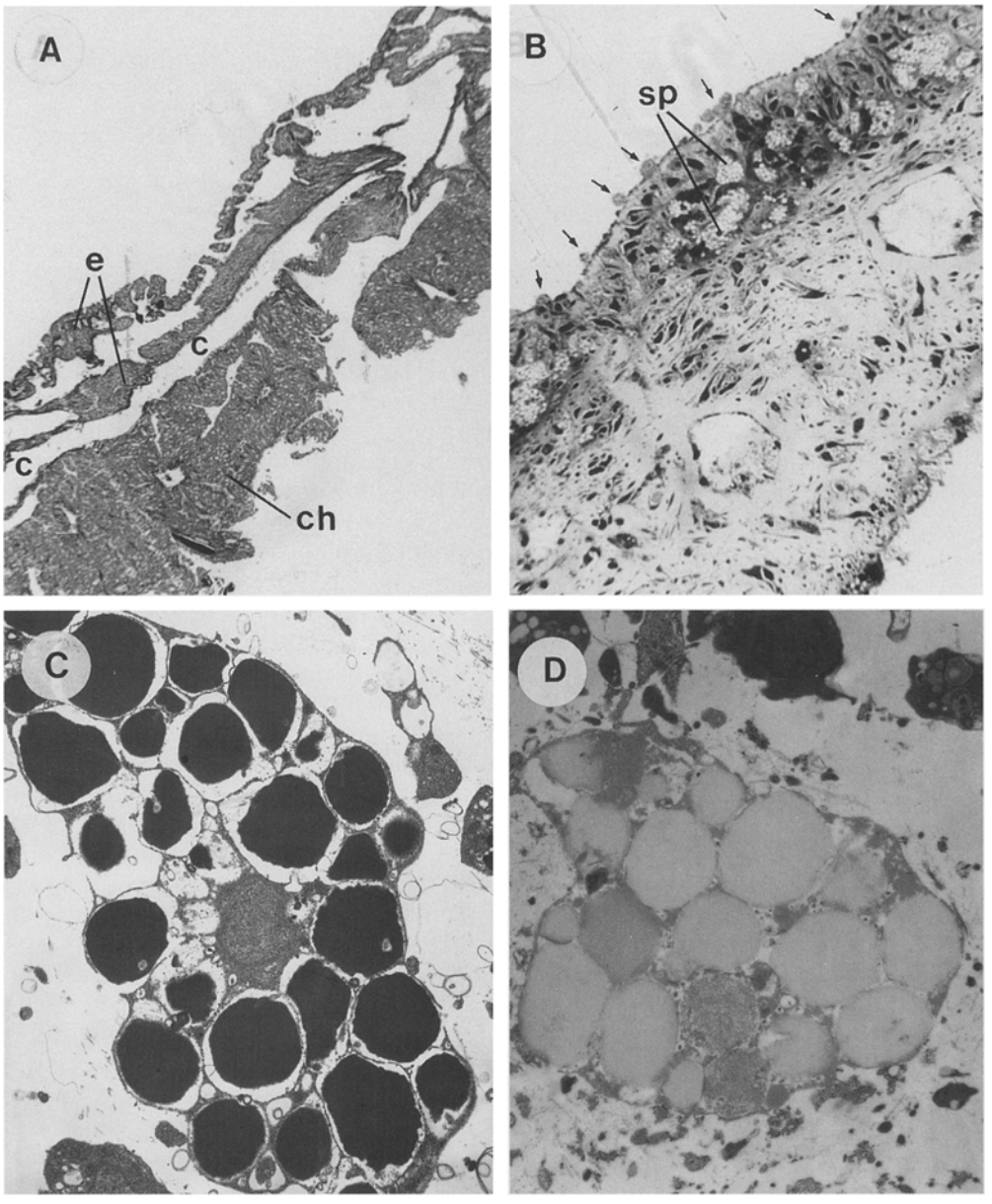

Fig. 1 *Crambe crambe*. **A** Light microscope image of transverse section (×31.25). **B** Light microscope image of ectosome (*arrows* indicate spherulous cells leaving sponge) (×300). **C** TEM image of a spherulous cell featuring dense inclusions (×6600). **D** TEM image of a spherulous cell with clear inclusions (×6600) (*c* subectosomal canals; *ch* choanosome; *e* ectosome, *sp* clusters of colourless spherulous cells)

Cell fractions

Three main fractions, each enriched in a different cellular type, were obtained from the Ficoll gradients. Fraction 1 (interface between 2 and 5% Ficoll) contained 90 ± 0.9% (mean ± SE) spherulous cells, which were characterised by a mulberry-like appearance with dense spherules, and a diameter of 10 to 15 µm (Fig. 2D). The two types of spherulous cells described in the previous subsection were distinguishable by their colour (orange and colourless, respectively) but their similar size and density rendered them impossible to separate by the density-gradient method. The orange cells resemble the chromocytes of *Cyamon neon* de Laubenfels and *Trikentrion helium* Dickinson (Smith 1968) and the so-called spherulous cells of *Axinella polypoides* Smidt (Simpson 1984). They have dense membrane-bound inclusions. There was also 10% of other cell types consisting of choanocytes (5 ± 0.54%) and non-identified sponge cells or cells debris (5 ± 0.84%). Fraction 2 (interface between 5 and 8% Ficoll) was enriched in choanocytes (70 ± 0.95%), identified by their size (4 to 6 µm diam), shape (somewhat narrower at the collar zone), transparency, and the

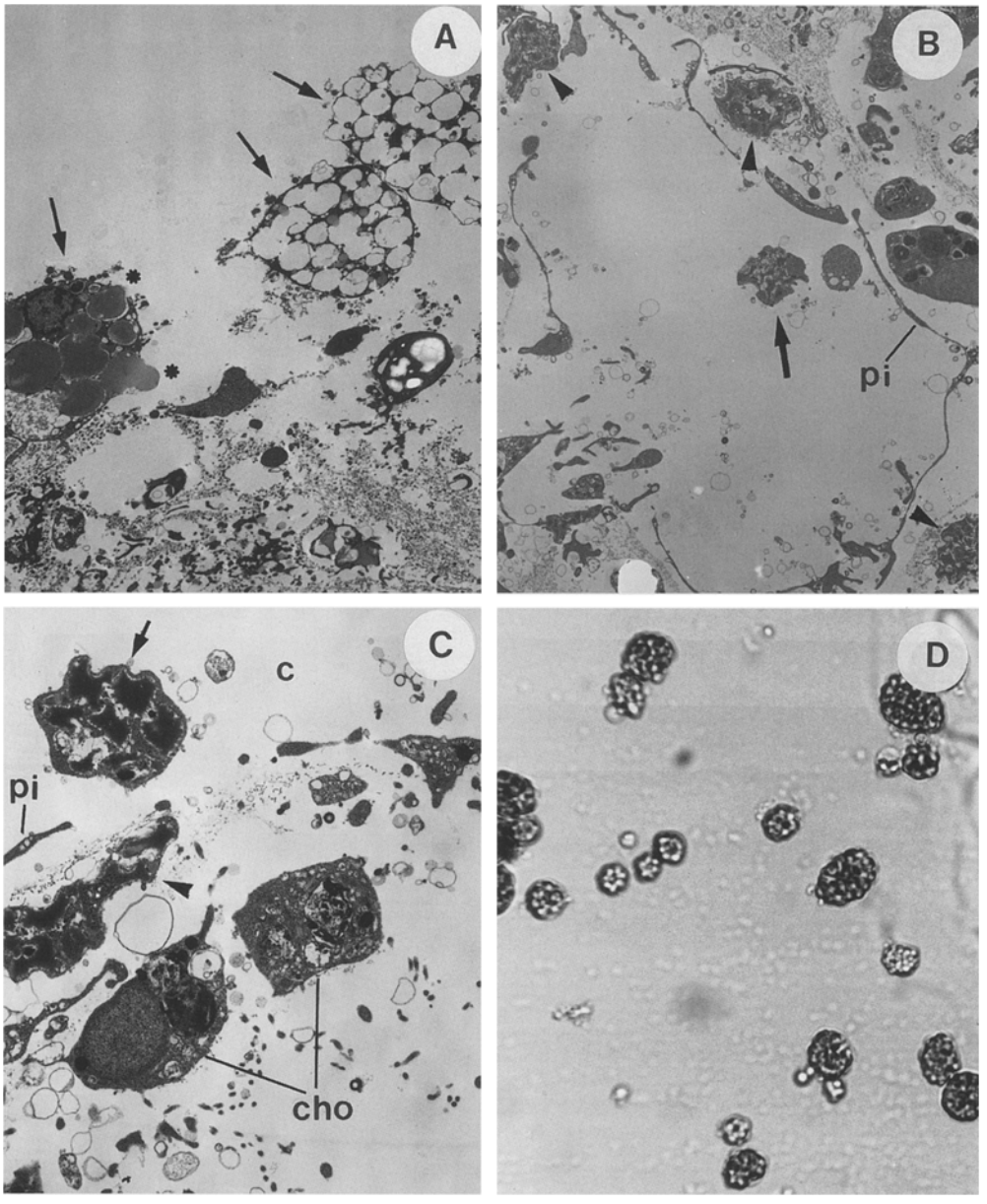

Fig. 2 *Crambe crambe*. **A** TEM image of spherulous cells (*arrowed*) leaving sponge surface; note that vesicles of two of them appear empty, while in the third they appear to be discharging their contents to surrounding medium (*) (× 4000). **B** Choanosomal aquiferous canal with spherulous-like cell in lumen (*arrowed*); other cells of same type appear just beneath pinacoderm (*pi*) layer (*arrowheads*) (× 3300). **C** Enlarged image of spherulous-like cell (*arrowed*) entering an exhalant choanosomal canal (*c*); a second cell (*arrowhead*) lies just beneath picacocytes (*pi*) (*cho* choanocytes) (× 6500). **D** Light image of spherulous cells fraction (Fraction 1) obtained by density-gradient method (× 600)

presence of the flagellum, besides spherulous cells (12 ± 0.74%), archeocytes (6.2 ± 0.74%) and unidentified sponge cells (11.8 ± 0.73%). Fraction 3 (interface between 8 and 11% Ficoll) mainly contained archeocytes and archeocyte-like cells (75 ± 0.66%), characterised by a diameter of 8 to 10 μm, a large nucleus and inclusions of variable refringence, size, and number (mostly phagosomes), together with spherulous cells (7 ± 0.74%) and other unidentified sponge cells and cell aggregates mainly formed by choanocytes (18 ± 0.41%).

Toxicity

Toxicity was significantly higher in the distal part of the sponge (ectosome) than in the basal part (choanosome) (Student *t*-test, $p = 0.001$). The corresponding mean values and standard errors were: 12.45 ± 1.4 TU

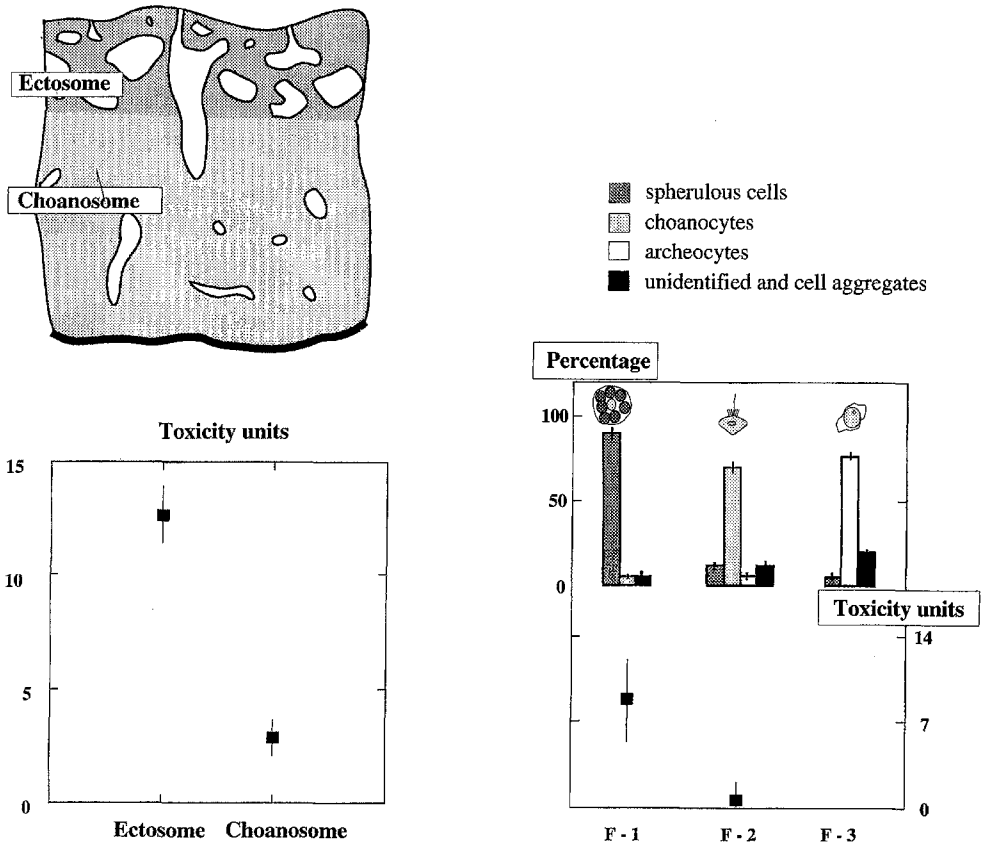

Fig. 3 *Crambe crambe*. Schematic drawing of sponge section, showing toxicity in different parts and cell fractions of sponge. Percentage of main cell types in the three fractions of density gradient is also indicated (*vertical bars* standar errors)

($n = 5$) for the distal zone, and 2.58 $\pm$ 0.92 TU ($n = 5$) for the basal zone (Fig. 3).

The corresponding toxicity of the different cell fractions were: Fraction 1 (mainly formed by spherulous cells) 9.08 TU (6.38 to 12.92, confidence level = 95%); Fraction 2 (mainly consisting of choanocytes) 0.48 TU (0.09 to 2.39, confidence level = 95%); Fraction 3 (mainly formed by archeocytes) did not show any toxicity (Fig. 3).

Discussion

The possible ecological roles of the toxicity of *Crambe crambe* have been addressed by specific tests against marine sublittoral bacteria (Becerro et al. 1994a), invertebrates, and fishes (Martín and Uriz 1993; Becerro 1994). For precise quantification purposes, however, we chose the standardized Microtox method because it has been shown to correlate well with the previous tests (Becerro 1994; Becerro et al. 1995). We assume, therefore, that the measure of toxicity of *C. crambe* against this deep-sea bacteria is a good estimate of its general toxicity against marine organisms. Noticeable differences in toxicity between the ectosome and choanosome zones were found in the present study, the ectosome being highly toxic and the choanosome showing toxicity below the lowest values found in a study on between-colony variation of toxicity in which ectosome and choanosome were not separated (Becerro 1994). The concentration of the toxicity in the sponge ectosome correlates well with the assumption of a defensive role, since encounters with potential epibionts, predators and competing neighbours take place through the peripheral layer of the sponge. Since no symbionts were found, a symbiotic origin of the toxic substances can be ruled out. Only pinacocytes, collencytes, archeocytes (very few) and two types of spherulous cells could account for the high toxicity found in the sponge ectosome since they are the only cells present there. Conversely, the much less toxic choanosome mainly contains choanocytes, pinacocytes, archeocytes, sclerocytes, collencytes, spongocytes and some spherulous-like cells, with "true" spherulous cells in much lower numbers. Moreover, there is a higher relative abundance of collagen in the ectosome (Becerro 1994) and, consequently, a lower proportion of cells there, which highlights the considerably higher toxicity of the cell components of the ectosome with respect to those of the choanosome. Only the abundance of spherulous cells in the sponge tissues correlates well with the pattern of toxicity observed and, thus, these cells seem to

be responsible for the storage of the toxic compounds. This result is corroborated by the toxic behaviour of the three cellular fractions obtained: the fraction enriched in spherulous cells was highly toxic whereas those enriched in choanocytes and in archeocytes were almost inactive (the contamination of Fraction 2 with 12% spherulous cells may account for the presence of slight toxicity in this fraction).

It is not possible to ascertain from the results obtained here which one of the two types of spherulous cells found is responsible for the toxicity. It may even be that both types correspond to different states of the same cell type. The orange cells are morphologically similar to those described as chromocytes in several sponge species (Smith 1968), and their contents may be related to carotenoid pigments. The location of these coloured cells beneath the pinacoderm agrees well with a possible protective function against light for these cells. The colourless spherulous cells, which exhibit a spatial distribution similar to the orange cells, are accumulated in clusters within the ectosome and can also be seen leaving the sponge through the ectopinacoderm. They would be more likely to be responsible for toxicity, since the known secondary metabolites (crambines and crambescidines) accounting for the toxicity found in the crude extract are colourless compounds (Berlinck et al. 1990, 1992; Jares-Erikman et al. 1991). However, both types might also correspond to different developmental states of the same cells. The less electron-dense inclusions may be the result of modifications of the chemical compounds contained in the spherules. Separate chemical analyses on these two cell types are necessary to definitely settle this point. However, the chemical complexity and instability of the toxic metabolites of *Crambe crambe* (Berlinck et al. 1990 1992; Jares-Erikman et al. 1991) make larger quantities of pure cells necessary for these chemical analyses than can be obtained by density gradients. Accurate methods based on specific markers and cell separation by flow–cytometric techniques (Pomponi and Willoughby 1994) must be developed in order to obtain sufficient pure populations of these two cell types for chemical analytical procedures.

The separation of the spherulous cells in the less dense fraction of the Ficoll gradient was unexpected, since these large cells were thought to be more dense than the choanocytes and archeocytes, as found in previous studies of other sponge species such as *Aplysina fistularis* de Laubenfels (Thompson et al. 1983). The different microstructure of the spherules of the spherulous cells of *A. fistularis* and *Crambe crambe*, and the chemical nature and, consequently, density of the substances contained in the spherules could account for their different behaviour in the density gradients. In fact, the crambescidins, a family of toxic compounds recently discovered in *C. crambe* (Jares-Erijman et al. 1991) which cause most of the sponge toxicity, are oily compounds whose low density may account for the behaviour of the spherulous cells in the density gradient.

Two previous studies described the spherulous cells as those harbouring secondary metabolites in sponges. Aerothionin and homoaerothionin were located in spherulous cells of the species *Aplysina fistularis* (Thompson et al. 1983). In the same way, the sesquiterpene avarol was said to be present in the spherulous cells of *Dysidea avara* (Müller et al. 1986), although, according to the TEM images provided by these authors, there is some doubt as to the correct identification of the cell types in their study. The presence of bioactive metabolites in the spherulous cells that are usually concentrated just beneath the sponge pinacoderm and leave the sponge surface fits well with the interpretation of a defensive role for toxicity in sponges, and can also explain how the toxic compounds are encapsulated within the cells as spherules without resulting in self-toxicity. Spherulous cells have also been observed in a collagenous envelope around spawned oocytes of *Aplysina* spp. (Gallisian and Vacelet 1976), and have been interpreted as having a defensive function for the sexual products (Thompson et al. 1983). However, other roles such as an excretory function (Vacelet 1967) or secretion of substances associated with collagen or spongin (De Vos et al. 1991) have also been attributed to the spherulous cells. The presence of two categories of these cells in *Crambe crambe*, along with other spherulous-like cells within or close to choanosomal canals (which may have an excretory function), is an additional indication of the frequent misinterpretation of functionally different spherulous cells (UV protection, chemical defence, excretion) lumped together under the same name merely on the basis of morphological (ultrastructural) similarities.

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