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# Sponge chemical ecology: Considering cytology into the equation

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## ABSTRACT

Chemically mediated interactions are complex. The field of chemical ecology requires scientific disciplines other than just chemistry and ecology to achieve a throughout understanding of the ecological and evolutionary role that chemistry plays in natural systems. In marine environments, sponges are a vast source of natural products and there is ample evidence that these compounds play ecological roles such as relief from competition or predation. Sponges are relatively simple metazoans that lack true tissues and therefore accomplish their functions mostly at a cellular level. Ultrastructural techniques may provide key information on the chemical mechanisms behind sponge biology and ecology. Localization of bioactive substances within sponges (whether sponge cells, heterotrophic bacteria, or cyanobacteria) is receiving increasing interest by the scientific community. In this chapter, we review our work on this challenging area. As in other aspects of ecology, there is great variety of methods and results that hinder extrapolation. Our two-fold goal with this review is i) to highlight the importance of ultrastructural and cytological studies to better understand sponge biology and ecology and ii) to strengthen the need for cytological approaches in

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sponges given the scarcity of studies available and the relevance of their results. Incorporating cytological knowledge into sponge chemical ecology will significantly improve our understanding of the role that chemistry plays in the biology and ecology of sponges.

## INTRODUCTION

Ecology is a multidisciplinary science that integrates knowledge from a variety of fields such as chemistry, physic, genetics, or taxonomy to cite just a few examples. Ecology puts together the knowledge obtained from these "unrelated" disciplines to better under-stand interactions of species among them and with the environment. This is particularly true in the field of chemical ecology, where it has been necessary a technological development to actually demonstrate the chemical mechanisms behind many of the biological patterns suggested even centuries ago [1].

Cytology is one of the fields that have benefited from recent advances in technology and are providing relevant information from a chemical ecology perspective. Microscopic techniques are nowadays more powerful than a few years ago and allow investigation of features other than just ultrastructural microscopic observation. For example, we can nowadays obtain information on the chemical composition of cellular components with X-ray microanalysis, take advantage of the physical and chemical properties of the cell components to better isolate and quantify specific cell types with flow-cytometers, or accurately quantify many biological features such as investment in cellular, structural, and reproductive material at an individual scale with image analysis tools, to cite just a few examples.

Studies at a cellular scale are crucial to increase our understanding of biological and ecological processes, including chemically mediated interactions. Cell is the most basic unit at which biological functions occur and even the most complex organisms accomplish their functions mostly at a cellular level. This is especially evident in sponges since these simple Metazoans lack true tissues [2]. Thus, because of the lack of specialized organs accomplishing specific tasks, biological and ecological functions are direct consequences of sponge cytology and morphology. This brings up a world of possibilities in ecology since we can reach biological and ecological goals based upon proper investigation of relevant cytological, structural, and morphological features.

The study of the within-individual distri- b ution of bioactive compounds in marine organisms has been a source of information about the ecological roles of these compounds [3, 4, 5, 6, 7, 8] and their biosynthetic origin: whether diet-derived, symbiont-derived or produced "de novo" (e.g.: [9, 10, 11, 12]). Knowledge of the origin of a metabolite can also be crucial for potential bio-technological applications (e.g. cell selection and culturing for the production of chemicals) [13, 14, 15].

In this chapter, we present data from several studies on which a cytological approach has provided significant information to understand the biology and ecology of sponge species. We also review the most relevant references available in this area and provide critical assessment of the most commonly used techniques. We will point to the pros and cons of these techniques and will discuss the suitability of linking chemical and structural studies to address specific ecological goals.

## GROWTH FORM AND LIFE STRATEGY

Growth form has been suggested as a major correlate of biological characteristics and space occupation strategy in benthic modular animals [16, 17, 18]. Sponges are surface-dependent organisms since they perform water exchange through small orifices spread through their surface (i.e., incurrent and excurrent orifices). In encrusting forms, sponge surface depends on the amount of substrate occupied and, therefore, their fitness relies on some efficient mechanism for space acquisition and maintenance. However, as reported for other benthic groups such as ascidians, comparable

growth shapes may include contrasting ecological strategies [19, 20].

In this section, we report the cytology and microstructure of two co-occurring encrusting sponges: the poecilosclerid *Crambe crambe* (Schmidt, 1862) and the dictyoceratid *Dysidea avara* (Schmidt, 1862). These two sympatric species share growth form but have contrasting ecological features, such as differences in growth or investment in defensive mechanisms. We set forth the hypothesis that these contrasting strategies correlate with cytological and morphological characteristics other than growth form and investigate the possibility to use structural and cytological parameters as descriptors of their biological strategies.

**First level: light microscopy approach**

We can obtain relevant cytological information with light microscopy. For instance, a more developed aquiferous system can imply higher food capture efficiency and hence higher potential growth rates; a higher amount of structural material (collagen, spicules) may render the species less susceptible to predation, and so on. These features are easily observed under light microscopy with adequate staining (Figure 1), which may allow the use of image analysis techniques for accurate and unbiased quantification of relevant parameters. These parameters are presented as ratios between the component quantified and the total area of the

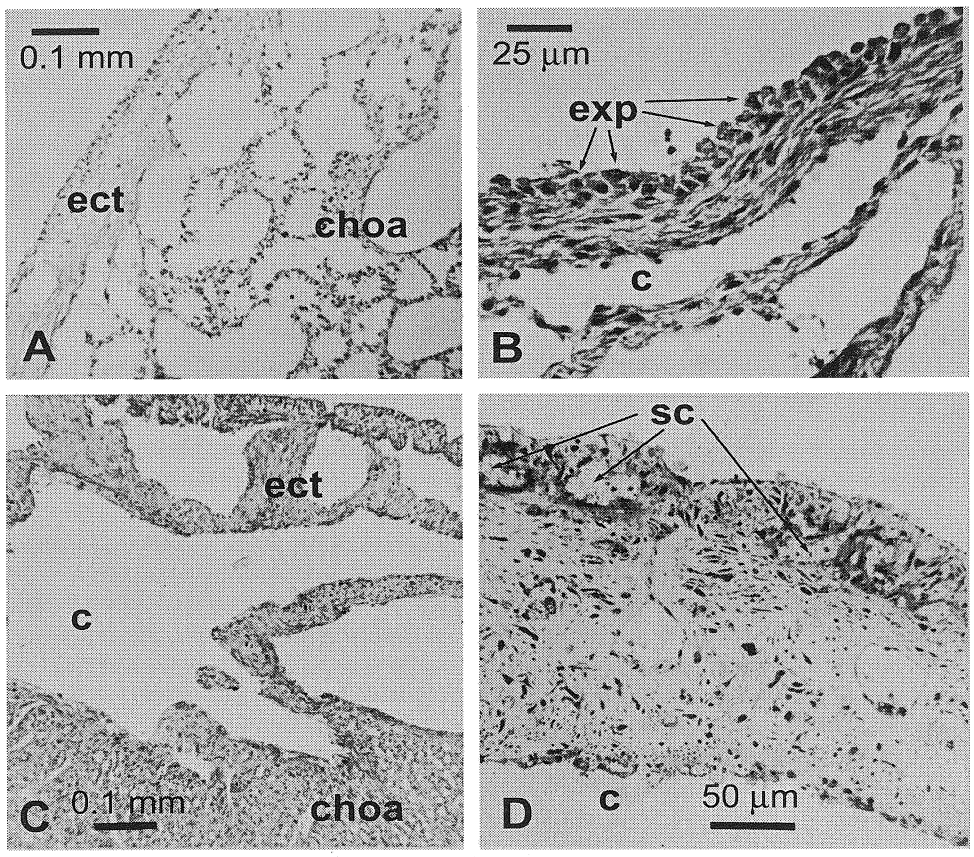


**Figure 1.**- Light microscope images of *Dysidea avara* (A, B) and *Crambe crambe* (C, D). A and C show general organization of ectosome (ect) and choanosome (choa). B and D show enlarged views of the ectosome of both species. c, canals, exp, exopinacocytes, sc spherulous cells. Modified from [21].

sponge section (or total area except the aquiferous system). For further information on the methods used in comparing *Crambe crambe* and *Dysidea avara* see study by Galera and co-workers [21].

We fixed materials in 4% formalin, decalcified them with a 19:1 solution of 4% formalin and pure nitric acid for 2h and desilificied them with 5% hydrofluoric acid (HF) for 2h. We then dehydrated (ethanol series), embedded (paraffin), and sectioned (5-8μm thick) our samples prior to staining them with haematoxilineosin (general observation), Mallory's stain (component quantification for *Crambre crambe*) and Diff-Quick (parameter quantification for *Dysidea avara*). These staining procedures differentially stain cellular and structural components with colors, which allow easy recognition of the relative abundance of both components and their quantification by image analysis techniques.

For parameter quantification we digitized histological sections and studied them with IMAT software (developed by the University of Barcelona). Due to the differential staining, we were able to quantify under light microscopy porosity (canals and chambers of the aquiferous system), sponging fibers, cells, intercellular collagen, and matrix in both choanosome and ectosome.

**Second level: fine structural approach**

Gathering information about cell types and other ultrastructural sponge traits needs transmission and scanning electron microscopy techniques (TEM and SEM respectively). For TEM, we fixed materials for 5h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed them for 90 min with $OsO_4$ (1%) in the same buffer. We dehydrated samples in an acetone series and embedded them in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate. For SEM, we fixed materials in a 6:1 mixture of 2% $OsO_4$ in seawater and saturated $HgCl_2$ in distilled water for 90 min. Sponges were then frozen in liquid nitrogen, freeze-fractured, critical-point dried, mounted, and sputter-coated with gold.

**Are structural parameters good descriptors of life strategy?**

Structural parameters may be good descriptors of the general biological strategy of the sponges. Among these parameters are the degree of development of the aquiferous system and the relative investment in supportive (spicules, spongin, intercellular collagen), cellular and matrix material. These parameters have the advantage that they are not only qualitative, but can be quantified and analyzed statistically, allowing formal comparisons among species, as well as intraspecific studies of relative allocation of the energy expenditure [22]. To our knowledge, the study by Galera and co-workers [21] is the first instance of a quantitative comparison of microstructural pattern in sponges.

Although the two species studied are encrusting, their architecture is clearly distinct. The most conspicuous differences are the higher thickness and ectosome/choanosome rate, the higher porosity of *Dysidea avara*, and the higher amount of matrix (ground substance) in *Crambe crambe* (see Table I for a comparison of the main structural parameters in both sponges).

*Crambe crambe* is a sponge with abundant matrix substance, its aquiferous system occupies less than 25% of the sponge section, and it produces a mineral skeleton and an array of chemical defenses. Overall, it invests more in structural (fiber + collagen) than in cellular components (Structure/Cell ratio of 1.188). These features point to a slow growing species that is able to maintain the space acquired in the face of other competitors. The known biological parameters of *Crambe crambe* are in agreement with these microstructural features: *Crambe crambe* grows very slowly (average monthly growth rate of small sponges during a two-year period of $0.03 \pm 0.01$, [23]). It is well defended chemically and is a good space competitor [24].

On the other hand, *Dysidea avara* has most of its sponge section occupied by the canals and chambers of the aquiferous system with little matrix material and, among the cellular

**Table I.-** Summary of the main structural components quantified in *Crambe crambe* and *Dysidea avara* by means of image analysis techniques. Data are mean ± se. P values correspond to statistical tests performed to compare each parameter in both species

| Variable | *Dysidea avara* | *Crambe crambe* | P value |
|---|---|---|---|
| Ectosome thickness (μm) | 295 ± 25 | 376 ± 38 | 0.175 |
| Choanosome thickness (μm) | 3335 ± 257 | 689 ± 71 | <0.001 |
| Choanosome/Ectosome ratio | 12.05 ± 1.29 | 2.09 ± 0.23 | <0.001 |
| Porosity (%) | 67.2 ± 0.03 | 22.5 ± 0.01 | <0.001 |
| Ostia diameter (μm) | 30.8 ± 2.2 | 11.4 ± 1.2 | <0.001 |
| Chamber surface ($\mu m^2$) | 3404 ± 473 | 261 ± 21 | <0.001 |
| Fibers (section ratio vs. whole section) | 0.065 ± 0.02 | 0.004 ± 0.01 | <0.001 |
| Collagen (section ratio vs. whole section) | 0.067 ± 0.006 | 0.167 ± 0.02 | <0.001 |
| Cells (section ratio vs. whole section) | 0.261 ± 0.006 | 0.442 ± 0.024 | <0.001 |
| Fibers (section ratio excluding aquif. system) | 0.217 ± 0.017 | 0.006 ± 0.001 | <0.001 |
| Collagen (section ratio excluding aquif. system) | 0.204 ± 0.019 | 0.218 ± 0.024 | 0.361 |
| Cells (section ratio excluding aquif. system) | 0.796 ± 0.019 | 0.561 ± 0.030 | <0.001 |
| Structure (fiber + collagen)/Cell ratio | 0.455 ± 0.059 | 1.188 ± 0.111 | <0.001 |

types, those able to ingest food particles (choanocytes and pinacocytes) largely predominate. *Dysidea avara* produces twice as much cellular material as structural material (S/C ratio of 0.455). These features suggest that this species can grow at high rates and has a more opportunistic strategy of space acquisition and maintenance. Again, the existing evidence is coherent with the structural results: *Dysidea avara* has a strong filtering activity [25], it is susceptible to predation [26], and it features a dynamic growth pattern with alternating phases of active growth and retraction (average monthly growth rate for small sponges during a two-year period of 0.1 ± 0.02, [27]).

## DISTRIBUTION OF BIOACTIVE COMPOUNDS WITHIN SPONGES

Sponges are a vast source of new bioactive compounds [28, 29 and references therein], yet the within-specimen distribution and cellular location of these have been studied in only a few species. The common presence of prokaryote symbionts (bacteria and cyanobacteria) in the mesohyl of many sponge species (e.g.: [30, 31]) further complicates the issue, sometimes leading to contrasting conclusions as to which organism produces the bioactive metabolites (e.g. results by Elyakov and co-workers [32] and Unson and co-workers [33] for the genus *Dysidea*).

There are three basic methodologies to localize cell components containing secondary chemistry in sponges: i) separation of cell types and microsymbionts by flow-cytometry [33, 34], centrifugation [35], or density gradients techniques [7, 8, 36, 37, 38, 39], ii) symbiont isolation and culture techniques [32, 40, 41], and iii) X-ray microanalytical techniques [33, 42, 43].

All these techniques have some drawbacks for fine location studies. The preparation of sufficient amount of cellular fractions (which are never pure) for chemical analysis or toxicity tests is often a limiting factor [39]. Cells and symbionts are difficult to culture and they may function quite differently in isolation and in association with the sponge (e.g. they may not produce the metabolites of interest: [34]). Microanalysis techniques require

halogen elements that are rare or absent from the primary metabolites [42].

In this section, we detail the X-ray microanalysis and density gradients techniques we used to determine the cell localization of secondary metabolites in the sponges *Aplysina aerophoba, Crambe crambe,* and *Dysidea avara.*

**Cell separation in sponges**

Cell separation by centrifugation, density gradients, or flow cytometers followed by chemical analyses of the cell fractions obtained in the process has been successfully used to identify the location of secondary metabolites in several sponge species [7, 8, 33, 34, 35, 36, 37, 38, 39]. Cell and metabolite characteristics are crucial to selecting the best method. Flow cytometers can provide excellent results when cells or symbionts show some fluorescence [34]. However, when secondary metabolites do not have any halogen atom or cells do not show any fluorescence, centrifugation or density gradients are most suitable. We have applied density gradient methods to locate secondary metabolites in the sponges *Crambe crambe* [7] and *Dysidea avara* [8].

Methods followed for each species are similar. We characterized cell types on fresh and fixed material through light (Nomarsky and phase-contrast optics) and electron microscopy. We dissociated cells by cutting fresh sponge into small pieces (up to 3 mm in diameter) up to a volume of 2 ml. We rinsed them with artificial seawater free of Calcium and Magnesium (CMF-ASW), transferred them to 100ml CMF-ASW with pH adjusted (ClH, 7.3) and dissociated the cells by slow stirring (200 rpm) for 3 hours at 21°C. The resulting hyaline cell suspension was first filtered through a nylon-mesh (30 µm in diameter) to eliminate most spicules and cell aggregates [44] and then pelleted by centrifugation at 1000 rpm for 2 minutes 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. We used no pronase and collagenase enzymes [42] to improve sponge dissociation.

Two to three discrete Ficoll gradients for each sponge sample 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% for *Crambe crambe* and 12%, 9%, 6%, and 3% for *Dysidea avara*. These particular densities were chosen after preliminary studies on the cell fractionation of the two species on a continuous gradient from 1 to 10% [45] and on various discrete gradients. After 3 hours of 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 haemato-cytometer and observed through light microscope (Nomarsky optics) to quantify the relative number of the different cellular types present. We conducted 2 readings per sample and repeated the whole procedure on twelve sponge specimens to obtain sufficient cells of each fraction to allow toxicity (*Crambe crambe*) or chemical analysis (*Dysidea avara*) of the fractions.

For *Crambe crambe*, we found high toxicity levels (as measured by the Microtox method [46]) in the ectosome of the sponge, where only pinacocytes, collencytes, archeo-cytes (very few) and two types of spherulous cells (very abundant) are present. Cellular types in the much less toxic choanosome include pinacocytes, choanocytes, collencytes, sclerocytes, archeocytes, spongocytes, with spherulous cells in much lower numbers. 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, see Figure 2A).

Avarol, the main secondary metabolite in *Dysidea avara*, is found in the choanocytes as shown by the chromatographic study of the cell fractions resulting from the density gradients (Figure 2B). Contrary to that reported by other authors [37], no true spherulous cells have been found in this species. Although the authors correctly isolated avarol-containing cells, they probably misinterpreted their results because neither choana nor flagellum are detectable in fixed cells such as those used by Muller and co-workers [37] to perform cell fractionation, and choanocytes show an unusual spherulous appearance due to the presence of numerous phagosomes. This misinterpretation of cell types has persisted in the literature [39].

Both *Dysidea avara* and *Crambe crambe* lack associated microsymbionts, and show that the use of gradient methods can successfully locate sponge cells with secondary chemistry. The location of toxic metabolites in the spherulous cells of *Crambe crambe* reinforces the role of its secondary chemistry against fouling [47], predation [26], and competition [48] since spherulous cells are concentrated in the ectosome of the sponge and are released into the environment where they reach the sponge competitors (in a wide sense). The location of avarol within cells inside *Dysidea avara* seems to constrain the possible ecological roles of this compound. The efficiency of avarol as feeding deterrent depends on the quality of food [49]. *Dysidea avara* can be considered as energy-rich as some littoral algae and, in fact, *Dysidea avara* did not prevent sea urchins from consuming the brown alga *Cystoseira mediterranea* [26]. This sponge species may also harbor endobiont organisms such as the cnidarian *Nausitöe punctata* [50] and the rodophycean *R. membranaceum* [8] so avarol seems to play no role in preventing colonization of the sponge inner spaces. The interpretation of the role that avarol plays in nature, if any, is difficult since none of the functions traditionally proposed for chemical defense appears to be likely. Other roles suggested include enhancement of filter-

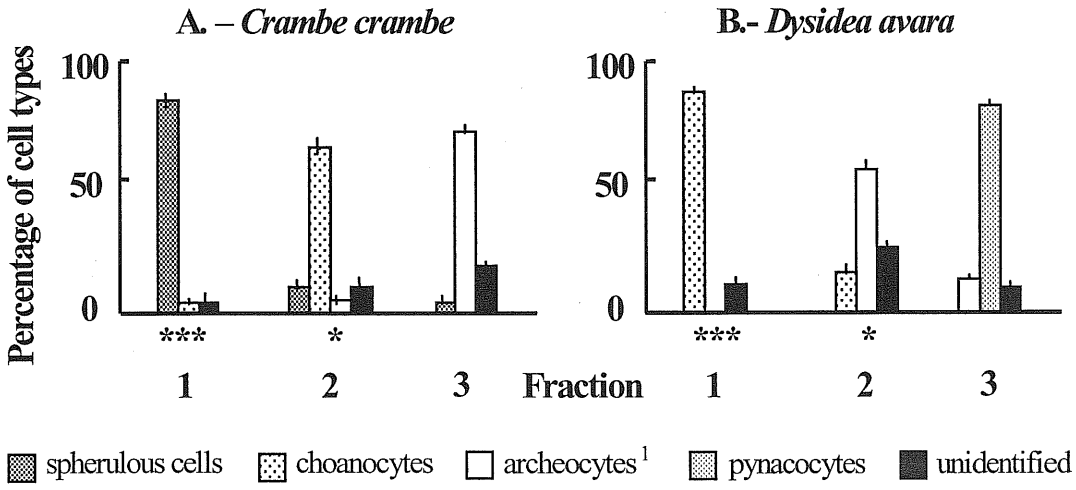


**Figure 2.**- Relative percentage of sponge cells in the 3 fractions of the ficoll gradient in *Crambe crambe* (A) and *Dysidea avara* (B). Asterisks represent a semi quantitative estimation of toxicity (A) or abundance of avarol (B) in each fraction. 1: Mixture of archeocytes and collencytes in *Dysidea avara*.

feeding efficiency by killing planktonic bacteria and facilitating their phagocytosis [51]. Avarol might play this role in *Dysidea avara* given the antimicrobial activities reported for this compound [52].

**X-ray microanalysis techniques**

In theory, the best possible approach to cellular location is the direct coupling between ultrastructural observation and chemical analysis. X-ray energy dispersive spectrometry can achieve this coupling provided the metabolites under study have a good atomic marker that allows easy identification in the spectra. Some halogenated organochemicals have such markers and, particularly, brominated compounds are widespread secondary metabolites in marine organisms [53, 54]. Microanalytical techniques allow a fine level of morphological resolution, as the diverse symbionts, cells, cell organelles and non-cellular components can be analyzed individually. These techniques, however, are not free of potential shortcomings. One of them is the possible loss or redistribution of diffusible elements and metabolites during processing for chemical fixation, which includes dehydration through solvents. The use of cryofixation and/or cryoembedding techniques may help to overcome this problem [43, 55, 56, 57] and provide reliable data. To our knowledge, most studies on the location of metabolites using this method have used only chemically fixed material. We have investigated the localization of secondary metabolites in *Aplysina aerophoba* using energy dispersive X-ray microanalysis (ED-XRMA) and cryofixation and/or cryoembedding techniques and compared the results with those obtained using conventional chemical fixation [43].

A general description of the chemical fixation procedure is described in the "Growth form and life strategy" section. Detailed information on the several methods can be obtained from Turon and co-workers [43]. Briefly, we used two cryotechniques for preparation of the samples: i) cryofixation and cryosubstitution followed by heating to room temperature for inclusion in Spurr (CRYO-2 method) and ii) cryofixation, cryosubstitution, and cryo-embedding in Lowicryl HM23 resin (CRYO-3 method). After cryofixation, samples were cryosubstituted with acetone at -90°C for 3 days. For the CRYO-2 protocol we transferred the samples to a vessel containing acetone with 0.1% uranyl acetate plus 2% osmium tetroxide and increased the temperature to -20°C (5°C/hr), then to 4°C, and finally exposed to room temperature. For the CRYO-3 protocol we transferred the samples to a vessel with acetone (with no chemical fixative added) and increased the temperature at a rate of 5°C/hr until -80°C. At this point we embedded the samples in Lowicryl HM23 resin:acetone 1:1 for 24 hrs., 2:1 for 24 more hrs., and pure Lowicryl HM23 for another 24 hrs. The resin was allowed to polymerize under UV light at -80°C for 6 days.

We perform microanalysis and ultrastructural examination on a Hitachi H800 MT and a Hitachi H600 microscopes (Hitachi Ltd., Tokyo). For microanalysis, we used acceleration of 100 Kv in STEM mode. Electron beam excitation was detected in a thin window (10 $mm^2$) Kevex detector (Kevex Corporation, California) connected to a Kevex 8000 analytical system with software Quantex 6.13. We adjusted gain rate to 1500-2000 counts $s^{-1}$ and acquisition time to 100 s.

We examined the obtained spectra for the presence of signals corresponding to bromine. The two bromine corresponded to the signals Lα (peak at 1.5 keV) and Kα (peak at 11.9 keV). The Br Kα peak provided the clearest indication of the presence of bromine since it did not overlap with other peaks (lead citrate was not used in the staining to avoid the close Pb Lβ peak at 12.5 keV). We used as a quantitative measure the height ratio of the Br Kα peak to the Cl Kα peak at 2.6 keV (the Cl signal came from the embedding Spurr resin and could be used as internal standard to control for the differences in the area scanned, magnification and count rate [42]). The use of the Br/Cl ratio allowed formal statistical comparison (ANOVA) between the compartments

under study. This quantification could not be done in the sections embedded in Lowycril, as this resin has no Cl in its composition.

We perform X-ray analyses at high magnification, producing spectra for small windows that covered one single cell or organelle (see Figure 3 for examples of such windows, and their associated spectra, covering several spherulous cells, a bacterium, and an area in the mesohyl of Aplysina aerophoba). The

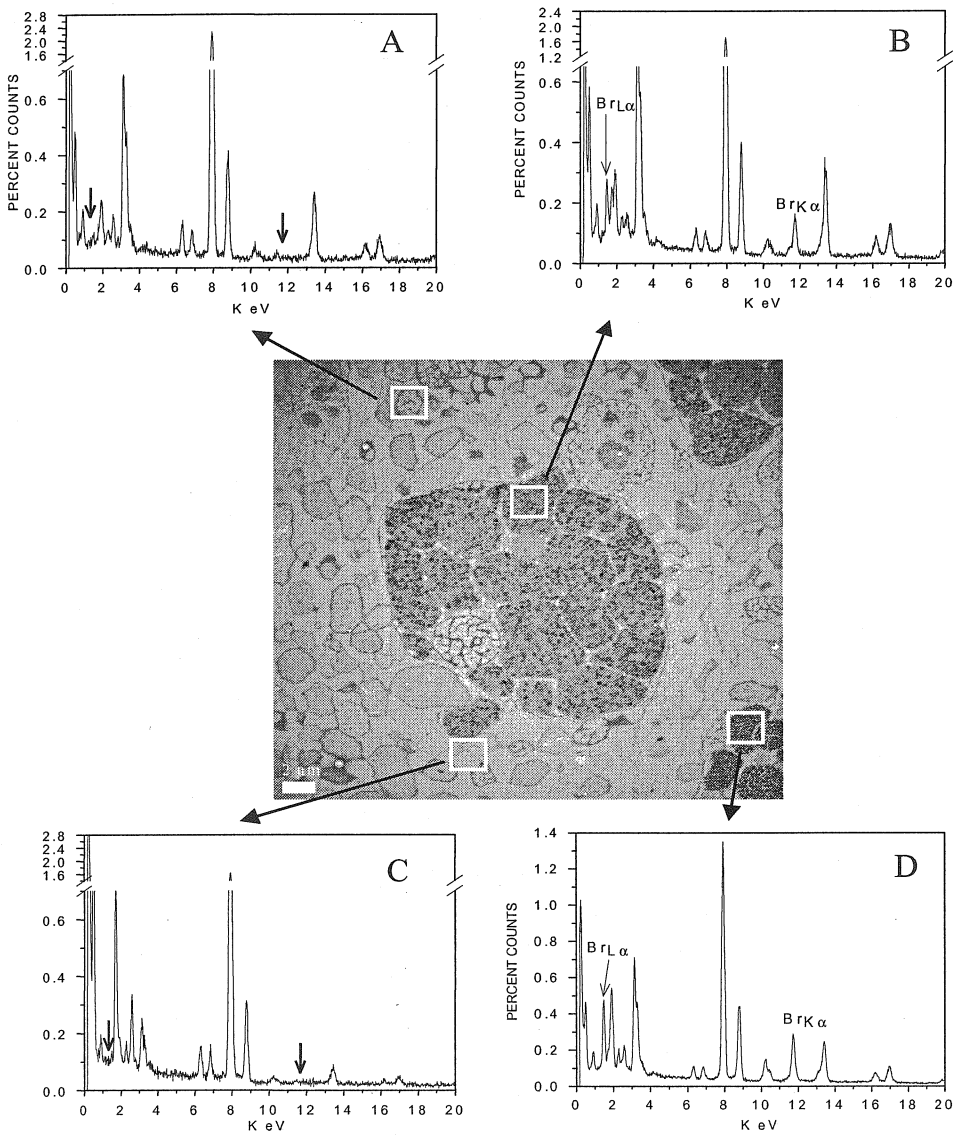


**Figure 3.**- Semi-thin section showing spherulous cells (CRYO-2 fixation with uranyl acetate contrast) and the corresponding XMRA spectra from selected windows of the section. Bromine peaks are labeled, and lack of them indicated by arrows. A: bacterium. B: spherulous cell vesicle with heterogeneous contents. C: mesohyl. D: electron dense vesicle from another spherulous cell.

electron beam covered the area studied with a raster pattern. We made a minimum of five readings for each fixation method on each of the main cell types: choanocytes, collencytes, pinacocytes, spherulous cells and archeocytes, on the mesohyl and on sponge fibres. We also scanned cyanobacteria and bacteria (n>20 each) for the presence of bromine.

Our data show that bromine in *A. aerophoba* accumulate in the spherulous cells and the fibers (Figure 4). As the secondary metabolites of the sponge contain most of the organic bromine, detection of this element can be used for localization of these compounds in the sponge [42]. The data also show that the choice of fixation and processing techniques is crucial when using ED-XRMA (Figure 4). Conventional chemical methods failed to demonstrate the presence of Br in the spherulous cells, which could only be substantiated in cryofixed material.

Specimen preparation for X-ray micro-analysis should retain the elements of interest at their *in vivo* location and, at the same time, allow morphological observation at the desired resolution level [55]. The problems of chemical fixation as regards the loss of diffusible elements have been repeatedly noted [56, 57]. Conventional fixation methods can still be applied for the study of organically-bound elements (such as brominated compounds) provided the loss of the organic molecules in the dehydration fluids is moderate [55]. This certainly cannot be taken for granted in the case of secondary metabolites, as shown by the lack of Br signal in the spherulous cells in our chemically preserved samples. Cryomethods have therefore to be used for reliable location of brominated metabolites in sponges and other invertebrates. The Br retention in the fibers observed with chemical methods may be due to the immo-

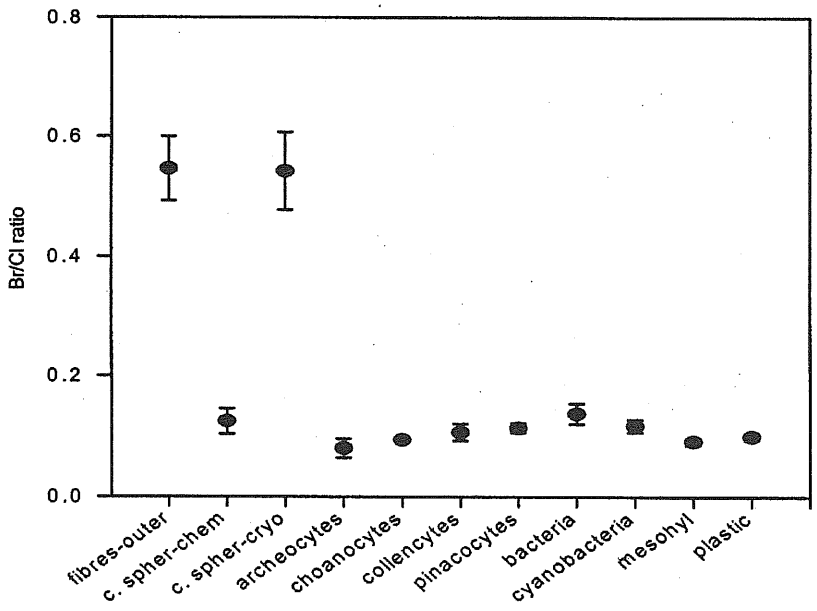


**Figure 4**.- Mean and standard error of the Br/Cl ratio, measured as the ratio between the height of the Br Kα peak, in several sponge components and in the embedding plastic material. The notation –chem and –cryo for the spherulous cells refers to the type of fixation performed (see text). Modified from [43].

bilization of bromine and other metals within the spongin matrix of the fibers reported for other sponges [58] or even to brominated aminoacids taking part of the spongin [59].

A properly cryofixed sample can be observed in a hydrated state through a cryomicroscope, but this type of sample does not withstand the high doses of electrons necessary for microanalysis, so dehydrated samples should be used. Cryodehydration can be performed through freeze-drying or cryosubstitution. The former has problems with ultrastructure preservation, and cryo-substitution may provide the best preservation [57, 60], while allowing satisfactory retention of diffusible elements [56]. Methods including cryoembedding (CRYO-3 methods) are believed to perform best in element retention, as tested with highly diffusible ions such as $K^+$ [57, 61], but our data show that CRYO-2 method yielded the best balance in terms of both Br detection and ultrastructure preservation. No new site of Br accumulation was detected through the CRYO-3 method, and the morphological observation was much poorer. In addition, the use of Spurr resin in CRYO-2 methods provided a good standard (the Cl peak) for comparison.

We suggest that the spherulous cells are the sites of production of the brominated secondary metabolites in *A. aerophoba*. Cells of this kind have been shown to produce defense metabolites in other species [7, 42, 62]. We have observed numerous instances of apparent degeneration of spherulous cells, releasing their contents to the mesohyl, which may be the usual way of metabolite release. However, we did not see any image of spherulous cells leaving the sponge mesohyl through canals or external surfaces in *A. aerophoba,* which has been suggested as a way of chemical release to the environment [7], although release of chemicals after wounding is of course compatible with our observations. In fact, Kreuter and co-workers [63] demonstrated the release of metabolites from incubated sponge cubes of this species.

## IS THERE ANY GENERALIZATION POSSIBLE?

Sponge secondary chemistry has been localized in sponge cells and associated symbiotic bacteria and cyanobacteria. When sponges have been identified as the source organism, the few studies performed seemed to suggest that spherulous cells contain, and most likely produce, secondary chemistry [7, 37, 42, 62]. Our study shows that choanocytes may also contain and, most likely, produce secondary metabolites in some sponges.

In the two best known instances of brominated secondary metabolite production by sponges, different sources for these compounds have been demonstrated: symbiotic prokaryote in Dysideidae (although source organisms vary with authors [32, 33, 64] and sponge cells (spherulous cells) in members of the Verongida [42, this work]. The same pattern seems to apply to other sponge groups: a number of studies suggest or demonstrate secondary metabolite production by sponge symbionts (e.g.: [35, 40, 65, 66]), while others indicate the production by sponge cells (e.g.: [7, 8, 62]. Even in the same species, sponge cells may produce some metabolites and symbionts others [39], or different metabolites may be produced by different kinds of prokaryote [35]. There is also the very interesting possibility that in *Aplysina* spp the sponge cells produce the inactive precursors, and the symbionts produce the enzymes that activate them [67]. Interestingly, the finding by Kreuter and co-workers [63] that the different biotransformation products of *A. aerophoba* are found in different sponge zones raises the possibility that distinct prokaryote symbionts produce enzymes involved at different levels of the biotransformation pathway. What emerges is a complex pattern of sponge secondary metabolite production related to symbiosis, which makes any generalization or prediction difficult. Considering the large amount of brominated compounds isolated from numerous sponges [53], it would be interesting to ascertain whether all sponges with

brominated compounds contain prokaryote symbionts, which would point to these symbionts as producers and/or to a role of these antimicrobial compounds in regulation of symbiont populations. On the other hand, the presence of the same or similar brominated metabolites in distant groups [12, 33, 35, 53, 65] suggests the convergent evolution of successful kinds of active molecules and introduces the hitherto unexplored topic of chemical convergence and its evolutionary implications.

## CHEMISTRY-CYTOLOGY INTERFACE: BENEFITS AND RISKS

The study of cell localization of secondary metabolites is widening our ecological vision since it provides information of great biological, ecological, and evolutionary relevance at a microscopic scale. We are just scratching the surface of the role that symbionts play in the production of secondary compounds and, therefore, in the acquisition of important defensive mechanisms by their hosts. Moreover, the incorporation of cytology into chemical ecology is expanding the possibility of investigating chemically mediated interactions at an ultrastructural level, since it is bringing to our attention a world of possible direct and indirect interactions among symbionts and their host sponge. Genetic studies aimed at characterizing the variability of prokaryote symbionts seem promising to unravel the causes of the variability in chemical defense in sponges. Experimental approaches similar to those followed with other groups (i.e., soft corals [68]) would also prove beneficial for understanding the role of symbionts in the production of secondary metabolites in sponges and will adequately complement current research on this area.

We have summarized in this review the ultrastructural studies carried out by our research group on sponges and show how these studies provide valuable ecological information. Thus, the microarchitectural patterns of some sponges correlate well with their life history strategy. The presence of the bioactive compounds of *Crambe crambe* and *Aplysina aerophoba* in the spherulous cells helps explain their ecological role against foulers, predators, and competitors, providing further indirect evidence of these roles and their mechanism of action. The localization of avarol in the choanocytes of *Dysidea avara* opens new interpretations as to its relevance as a chemical defense for this species.

Finally, we want to emphasize the need for a multidisciplinary approach in this field since, despite the benefits, research on the chemistry-ecology-cytology interface can lead to some mishaps. Some of the methods traditionally used for chemists may not be the best way to preserve material for cytological observation. The opposite is true and some common methods for ultrastructural observation should be avoided for chemical analysis (e.g., dehydration procedures may wash out compounds as detailed above). Also, lack of expertise when working in the interface can lead chemists to inaccurate conclusions on biological information and viceversa (e.g., misinterpretation of choanocytes by spherulous cells in *Dysidea avara* [37], and of choanocytes by archeocytes - and thus choanocyte chambers by archeocyte clusters - by wrongly assuming that only archeocytes have nucleolus in *Dysidea herbacea* and that archeocytes can be flagellated [39]). Truly multidisciplinary cooperation and integration at both research design and analysis of results will prove a satisfactory step in overcoming limitations and biases associated with our own area of expertise. Last but not least, editors may help reduce the shortcomings of working in a multidisciplinary field by carefully considering appropriate reviewers for manuscripts in the interface between chemistry, ecology, and cytology. The need for valid reviewers within each of the disciplines seems advisable to help prevail benefits over risks. "Multidisciplinary" seems the keyword in this research area. If we apply this keyword from research design to manuscript evaluation we will apply our best knowledge and best tools to understanding the

role of secondary chemistry in sponge biology, ecology, and evolution.

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