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REGULAR ARTICLE

Xavier Turon · Mikel A. Becerro · María J. Uriz

Distribution of brominated compounds within the sponge *Aplysina aerophoba*: coupling of X-ray microanalysis with cryofixation techniques

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Abstract The major secondary metabolites of the sponge *Aplysina aerophoba* are brominated compounds. X-ray energy dispersive microanalysis was therefore used to locate secondary metabolites via the Br signal in energy emission spectra from sponge sections. To test the reliability of this method in the face of the loss or redistribution of metabolites during processing, we compared the results obtained by conventional aldehyde fixation with those obtained by cryofixation and cryosubstitution with and without cryoembedding. Bromine appeared to be concentrated in two sponge structures, viz. fibres and spherulous cells, when cryofixed material was examined. However, X-ray microanalysis failed to demonstrate the presence of bromine in spherulous cells in chemically fixed samples, showing the need for cryotechniques to avoid the loss of compounds. Cryofixation plus cryosubstitution methods performed best regarding structural preservation and the immobilization of metabolites. The presence of bromine in the spherulous cells suggests that this cell type is the producer of the secondary metabolites, as described for other sponge species. Nevertheless, the presence of bromine in sponge fibres indicates that they can accumulate metabolic substances, although we have been unable to assess whether the chemicals are in their original form or in a modified state within the fibres. *A. aerophoba* has both bacterial and cyanobacterial symbionts in its mesohyl; the absence of brominated compounds in them contrasts with previous findings in other sponges with prokaryote symbionts.

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X. Turon (✉)
Department of Animal Biology (Invertebrates),
Faculty of Biology, University of Barcelona, 645 Diagonal Ave.,
E-08028 Barcelona, Spain
e-mail: xaviert@bio.ub.es
Fax: +34 93 4035740

M.A. Becerro · M.J. Uriz
Centre for Advanced Studies (CSIC), Camí de Sta. Bàrbara,
s/n, E-17300 Blanes (Girona), Spain

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Introduction

The study of the within-specimen distribution of bioactive compounds in marine organisms has provided information regarding the ecological roles of these compounds (Hay et al. 1988; Paul and Van Alstyne 1988; Fontana et al. 1993; Di Marzo et al. 1993; Uriz et al. 1996a, 1996b) and their biosynthetic origin, i.e. whether diet-derived, symbiont-derived or produced "de novo" (e.g. Paul and Pennings 1991; Cimino and Sodano 1993; Fontana et al. 1994; Faulkner et al. 1994). Knowledge of the origin of a metabolite can also be crucial for potential biotechnological applications, e.g. cell selection and culturing for the production of chemicals (Garson 1994; Munro et al. 1994).

Sponges are a vast source of new bioactive compounds (Sarma et al. 1993; Faulkner 1999); however, the within-specimen distribution and cellular location of these compounds 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. Wilkinson 1978; Rützler 1990) further complicates the issue, sometimes leading to contrasting conclusions as to which organism produces the bioactive metabolites (e.g. results obtained by Elyakov et al. 1991 and Unson et al. 1994 for the genus *Dysidea*). Another problem that hinders comparison between studies is the diverse array of methods used for the cellular location of metabolites in sponges. In most cases, the diverse cell types and symbionts are dissociated and then separated on density gradients (Bretting and Konigsmann 1979; Müller et al. 1986; Garson et al. 1992; Uriz et al. 1996a, 1996b; Flowers et al. 1998), flow cytometry (Unson and Faulkner 1993; Unson et al. 1994) or centrifugation (Bewley et al. 1996). The fractions are then subjected to chemical analyses or toxicity tests. Another technique

used to locate the active compounds is symbiont isolation and culture, followed by chemical analyses of the strains (Stierle et al. 1988; Voinov et al. 1991; Elyakov et al. 1991). In some cases, X-ray energy dispersive microanalytical techniques have been used to locate active compounds (Thompson et al. 1983; Unson et al. 1994).

All these techniques have some drawbacks for fine location studies. The preparation of sufficient amounts of cellular fractions (which are never pure) for chemical analysis or toxicity tests is often a limiting factor (Flowers et al. 1998). Moreover, symbionts are difficult to culture and may function quite differently in isolation and in association with the sponge (e.g. they may not produce the metabolites of interest; Unson and Faulkner 1993). In theory, the best possible approach to establishing the cellular location is direct coupling between ultrastructural observations and chemical analysis. This coupling can be achieved by X-ray energy dispersive spectrometry provided that the metabolites under study have a good atomic marker that allows easy identification in spectra. Some halogenated organochemicals have such markers and, in particular, brominated compounds are widespread secondary metabolites in marine organisms (Gribble 1992; Fielman et al. 1999). Indeed, Br^- is one of the major anions present in seawater (at more than 50 µmol/kg; Kennish 1994), so there is an unlimited source of this element for covalent bonds with organic compounds. Microanalytical techniques allow a fine level of morphological resolution, as the diverse symbionts, cells, cell organelles and non-cellular components can be analysed 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 (Roomans 1988a; Edelman 1991; Quintana 1994) and provide reliable data. To our knowledge, previous studies of the location of secondary metabolites with this method have used only chemically fixed material.

We have studied the fine location of secondary metabolites in the sponge *Aplysina aerophoba* Schmidt, 1862 (O. Verongida) by means of energy dispersive X-ray microanalysis. Previous studies (Thompson et al. 1983) of the congeneric species *Aplysina fistularis* (Pallas, 1776) have been used for interspecies comparison. Verongida sponges contain a variety of brominated metabolites (Thompson et al. 1983; Teeyapant and Proksch 1993; Aiello et al. 1995; Fendert et al. 1999). Over 100 halotyrosine-derived compounds have been reported for sponges of this order (Carney and Rinehart 1995). We have taken advantage of the well-known chemistry of brominated compounds in *Aplysina aerophoba* (Cimino et al. 1983; Teeyapant et al. 1993a, 1993b; Ciminiello et al. 1997), which has, as major brominated secondary metabolites, aerophobin-2, aplysinamisin-1 and isofistularin-3 (Ebel et al. 1997). Bromine-containing metabolites can account for 7%–12% of the sponge dry weight (Teeyapant et al. 1993a). *A. aerophoba* has received special attention since Teeyapant and Proksch (1993) first demonstrated the bioconversion of the major brominated compounds into aeroplysinin-1 and a dienone. The latter are much more bioactive (with antibiotic, cytotoxic, growth inhibitory and repellent properties) than their precursor compounds (Teeyapant et al. 1993b; Koulman et al. 1996; Weiss et al. 1996; Ebel et al. 1997). Moreover, this activation is wound-induced, fast (less than 1 min) and enzymatically triggered (Weiss et al. 1996; Ebel et al. 1997). This case constitutes the first instance of a wound-induced defence mechanism in a marine invertebrate, paralleling the finding of Paul and Van Alstyne (1992) for marine algae of the genus *Halimeda*.

The goals of our work were two-fold. First, we wished to study secondary metabolite production and storage in *A. aerophoba*, which has both bacterial and cyanobacterial symbionts. Second, we wanted to assess the reliability of the X-ray method in secondary metabolite location by applying techniques of cryofixation, cryosubstitution and cryoembedding to this issue for the first time and by comparing the results with those obtained following conventional chemical fixation.

Materials and methods

Specimens of *A. aerophoba* were collected in spring 1998 in the northwestern Mediterranean, in the locality of Cadaqués (NE of Spain, 42° 16.9' N 3° 17.8' E). At this locality, this species is fairly abundant on sublittoral rocky substrata to a depth of 15 m. *A. aerophoba* forms thick masses over the substratum from which chimney-like structures, each ending in an osculum, protrude. The sponge is intense yellow but turns dark upon exposure to air, hence the species' name. Six specimens were collected by scuba divers, placed in plastic jars and transported to the laboratory, where they were either chemically fixed or cryofixed.

Chemical fixation

Small fragments (about 1 mm^3) from the sponge ectosome and choanosome were taken separately. They were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for about 6 h at 4°C. They were then postfixed with OsO_4 (1% in the same buffer) for 2 h at 4°C, dehydrated in a graded acetone series and embedded in Spurr resin at room temperature. Ultra-thin sections (ca. 60 nm) were cut on a Reichert Ultracut microtome and stained with uranyl acetate and lead citrate for ultrastructural observation (Reynolds 1963). For microanalysis, semi-thin sections (0.3 µm) were either left unstained or contrast was enhanced with uranyl acetate alone. Sections for microanalysis were mounted on Cu grids and carbon-coated to reduce specimen charging under the beam.

Cryotechniques

Two cryotechniques were used for the preparation of the samples: cryofixation, cryosubstitution and then heating to room temperature for inclusion in Spurr (hereafter called the CRYO-2 method, because it included two low temperature processes) and cryofixation, cryosubstitution and cryoembedding in Lowicryl HM23 resin (three low temperature processes, hence the proposed name as the CRYO-3 method).

For fast-freeze fixation (cryofixation), small sponge fragments (about 1 mm^3) from both the ectosome and the choanosome of the

sponge were cut from live specimens and positioned on a small sheet of paper coloured for later identification. The paper was glued with a drop of agar onto a slightly larger thin plate of mica attached, with bi-adhesive tape, to a thick layer of rubber foam that acted as a shock absorber. The specimen was then placed upside down on the mobile shaft of a Cryoblock device (Leica, Vienna) and projected against a cooler copper plate. The specimen holder was then quickly transferred into a bath of liquid nitrogen.

After cryofixation, the samples were cryosubstituted with acetone at –90°C for 3 days in a home-made cryosystem (Quintana 1994). The samples were then transferred to a vessel that had previously been cooled to –90°C and that contained different solvents according to the two protocols followed. (1) For the CRYO-2 method, the vessel contained acetone with 0.1% uranyl acetate plus 2% osmium tetroxide (Porta and López-Iglesias 1998). The temperature was increased to –20°C by 5°C per hour. The samples were then transferred to a fridge, allowed to reach 4°C and finally exposed to room temperature. They were rinsed several times for 3–4 h in acetone without chemical fixatives and embedded in Spurr resin. The resin was allowed to polymerize at 60°C for 2 days. (2) For the CRYO-3 method, acetone with no chemical fixative was used. The temperature was increased to –80°C by 5°C per hour. At this point, the samples were embedded in Lowicryl HM23 resin:acetone 1:1 for 24 h, 2:1 for a further 24 h and, finally, pure Lowicryl HM23 for 24 h. The resin was polymerized in UV light at –80°C for 6 days.

The samples processed by cryofixation were then treated and stained in the same way as those chemically fixed for both ultrastructural observation and microanalysis.

Energy dispersive X-ray microanalysis

Under the impact of the beam in an electron microscope, electrons from the atoms in a sample are displaced from the inner orbital shells and replaced by electrons from outer shells. This last transition produces X-rays that can be detected. The orbital shells are designated K, L and M (from inner to outer) and the energy released by a replacement depends on the element involved and each possible electron jump, resulting in a series of signals that are characteristic for each element. These signals can be examined in a spectrum of X-ray energies obtained when the beam is focussed on a particular structure.

Microanalysis and ultrastructural examination were performed, respectively, on a Hitachi H800 MT and a Hitachi H600 microscope (Hitachi, Tokyo), both belonging to the Microscopy Unit of the Scientific Services (University of Barcelona). For microanalysis, an acceleration of 100 kV was used in scanning transmission electron microscopic mode. Electron beam excitation was detected in a thin-window (10 mm^2) Kevex detector (Kevex, California) connected to a Kevex 8000 analytical system with Quantex 6.13 software. The gain rate was adjusted to 1500–2000 counts s^{-1} and an acquisition time of 100 s was used.

The presence of signals corresponding to bromine was examined on the spectra obtained. The two main energy peaks emanating from bromine corresponded to the signals Lα (peak at 1.5 keV) and Kα (peak at 11.9 keV). A third smaller peak, corresponding to the Kβ signal, appeared at 13.3 keV. The Br Lα peak was the highest but it partially overlapped the Mα peak of osmium (1.9 keV), an element present in some samples because of the fixation procedure. The Br Kβ peak overlapped the U Lα peak (13.5 keV). The Br Kα peak provided the clearest indication of the presence of bromine, since it did not overlap other peaks (lead citrate was not used in the staining procedure to avoid the close Pb Lβ peak at 12.5 keV). Therefore, the presence of this peak was preferentially used as a qualitative marker of the presence of bromine. A second quantitative measure used was the height ratio of the Br Kα peak to the Cl Kα peak at 2.6 keV, as the Cl signal came from the embedding Spurr resin and could be used as an internal standard to control for the differences in the area scanned, magnification and count rate (Thompson et al. 1983). The use of the Br/Cl ratio allowed formal statistical comparison (ANOVA) between the compartments under study. This quantification could not be carried out in the sections embedded in Lowycril, as this resin contains no Cl.

X-ray analyses were performed at high magnification, producing spectra for small windows that covered a single cell or organelle. The electron beam covered the area studied with a raster pattern. At least five readings were made 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. Cyanobacteria and bacteria (n>20 each) were also scanned for the presence of bromine.

Results

Overview of general structure and cellular types

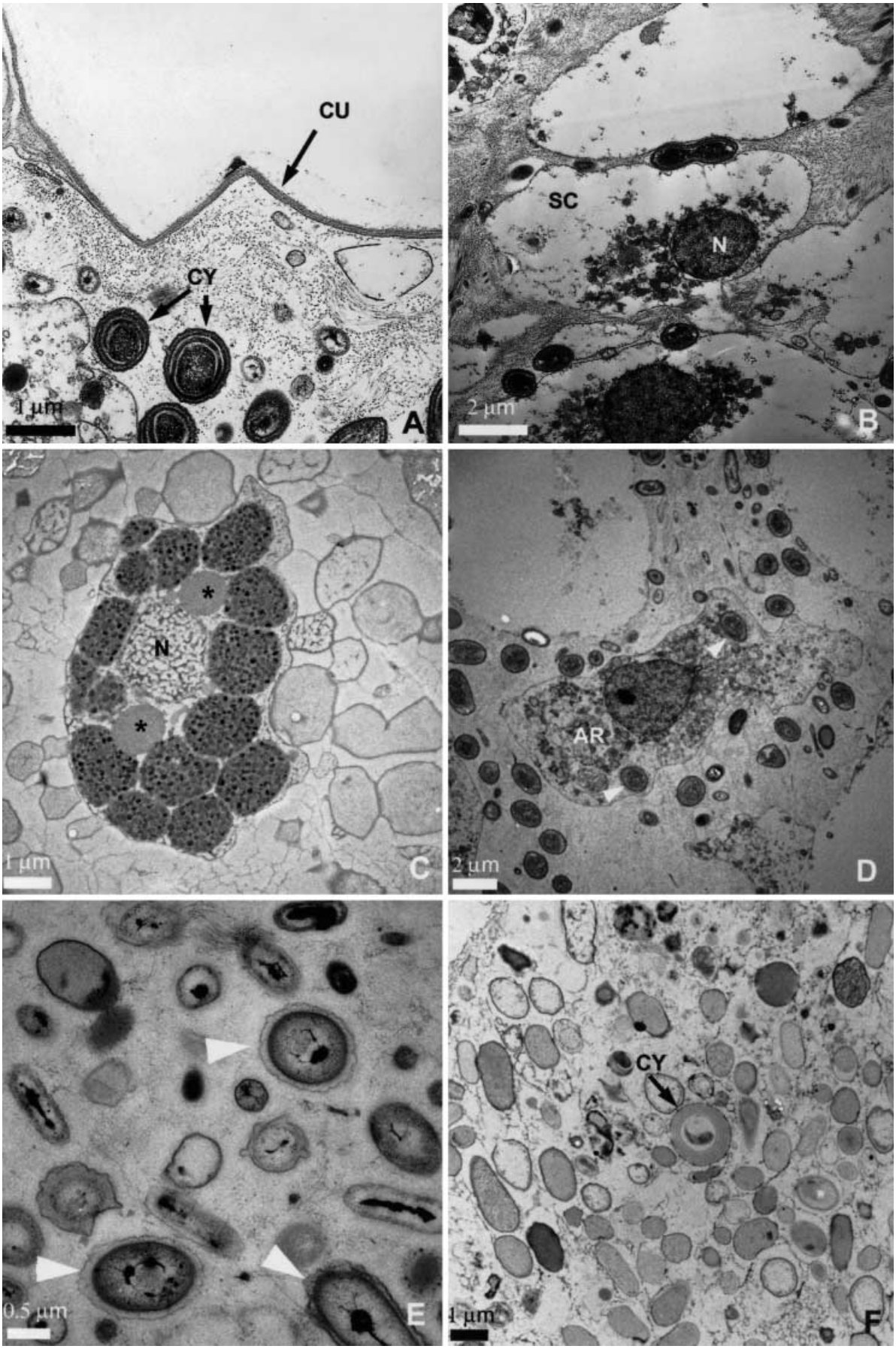
The ectosome of *A. aerophoba* is a 1-mm- to 2-mm-thick layer characterized by the absence of choanocyte chambers and the abundant presence of cyanobacteria and bacteria. The choanosome accounts for most of the sponge thickness, contains the choanocyte chambers and harbours several types of heterotrophic bacteria. Thick spongin fibres cross the sponge mesohyl.

The pictures obtained by conventional chemical fixation and cryofixation (CRYO-2 method) provided good morphological resolution but the appearance of some sponge components changed depending on the method used, so we decided to combine both types of images in the presentation of the results. We did not obtain good quality images of the sponge ultrastructure with the CRYO-3 method.

We did not find a developed exopinacorderm. Instead, a fibrilar cuticle lined the external sponge surface (Fig. 1A). Numerous collagen fibrils, bacteria and cyanobacteria were accumulated under this layer. In many areas, the cell components appeared swollen and disintegrating, giving a vacuolar appearance to the ectosome (Fig. 1B).

Spherulous cells and archeocytes were common in both the ectosome and the choanosome. The spherulous cells were elongated cells, up to 10–12 µm in length, with a large (2 µm) anucleolate nucleus (Fig. 1C). The cytoplasm contained numerous large inclusions ("spherules": Vacelet 1967) that were 0.5–2 µm in diameter and membrane-bound. The contents of the spherules consisted of granules of an amorphous material whose density varied from electron-dense to electron-clear depending on the cell. Intermediate forms were also encountered and all these cells were otherwise indistinguishable. Occasionally, some crystalline material could be seen among the granules of the spherules of the more electron-dense type. A second type of inclusion found in spherulous cells took the form of smaller vesicules (about 1 µm in diameter) with finely granulated material (Fig. 1C). Some images of apparently degenerating spherulous cells could be seen, especially in the ectosome, with the the spherules' contents being released to the surrounding mesohyl.

The archeocytes (Fig. 1D) were 10–15 µm in length, with a large nucleolate nucleus and the cytoplasm filled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with phagosomes in which prokaryote symbionts that had been phagocytosed were sometimes discernible. We did not see typical collencytes. Cells that could potentially be ascribed to collencytes always had phagosomes within them.

The prokaryote symbionts consisted of cyanobacteria in the ectosome (Fig. 1A) and bacteria in both the ectosome and choanosome (Fig. 1E). These symbionts live extracellularly within the mesohyl. The cyanobacteria were spherical (0.5–1.5 µm in diameter) or ovoid (up to 2 µm, major axis), with an external layer of tylakoids surrounding a central core of granular material (Fig. 1A, F). Some images of cyanobacteria could be seen undergoing fission. The bacteria had circular or rod-shaped profiles (Fig. 1E). The circular outlines were usually about 0.5 µm in diameter in chemically fixed samples. The bacteria inhabiting *Verongia* species have been described in detail by Vacelet (1975) and Friedrich et al. (1999). In our chemically fixed samples, Vacelet's type D (with slime layers) and E (with enlarged periplasmic material) bacteria were abundant. Condensed nucleic material was often seen at the centre of the bacteria. Following cryofixation, the appearance of bacteria changed (Fig. 1F). They appeared swollen and were larger and two types could be discerned: one with a uniform low-density granular material and simple coatings, and the other bearing denser reticulate material (probably nucleic). In the latter, the cell coatings usually conformed to that of Vacelet's type B bacteria: a unit membrane and a wall composed of two dense layers separated by a lighter zone. In some areas of the choanosome, bacteria occupied more than 50% of the ground substance of the mesohyl.

The spongin fibres had two clearly distinct zones (Fig. 2A): the outer zone made up of compact spongin material and the inner zone in which the density of spongin was much lower, leaving many empty spaces that gave the fibre an alveolar aspect. No foreign material was embedded in the spongin fibres. The canals of the aquiferous system were lined by endopinacocytes (Fig. 2B) that were triangular in shape, with a flattened surface that lined the canals.

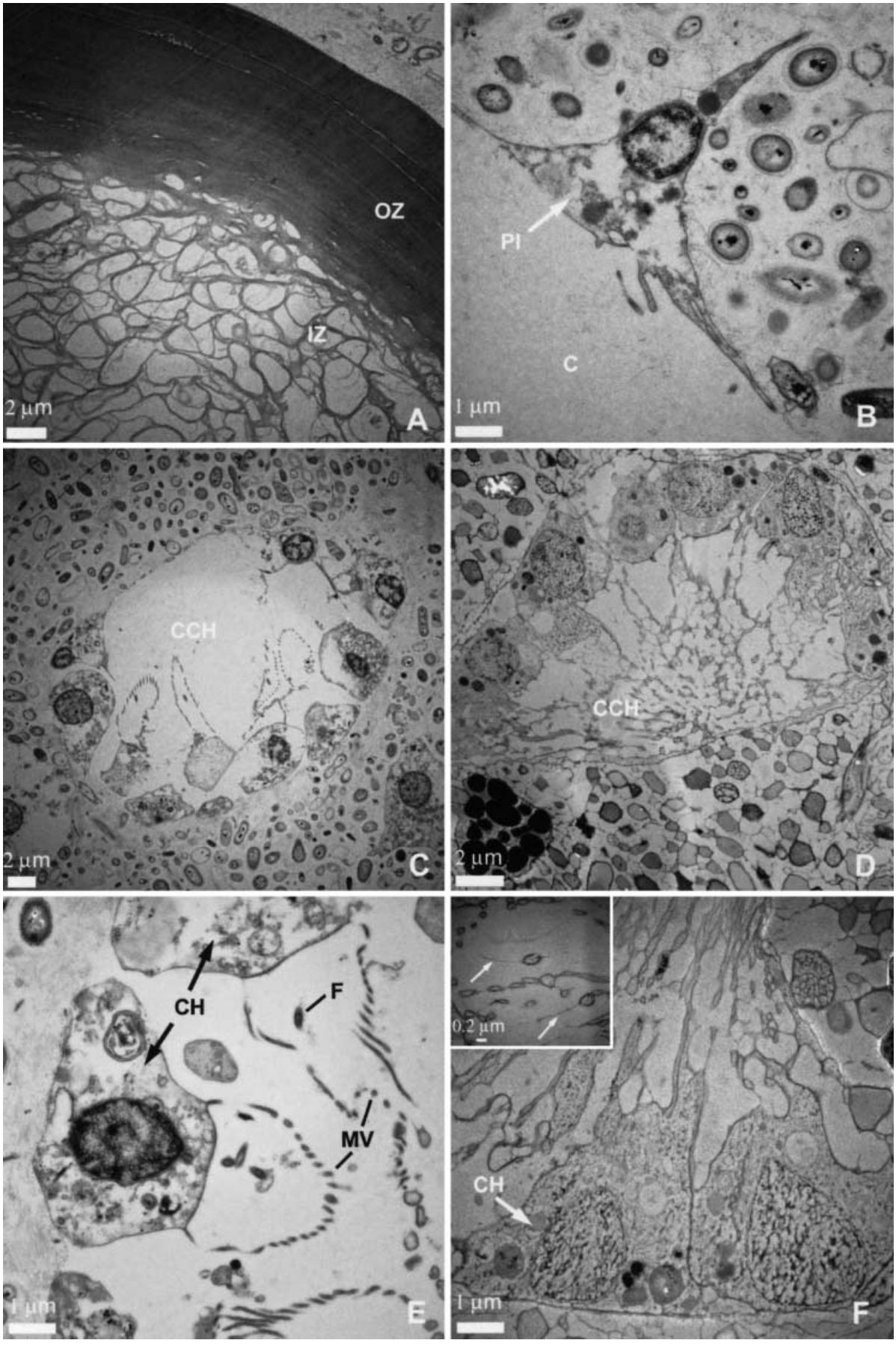
The choanocyte chambers were small and circular or elongated in section, with a diameter up to 50 µm (Fig. 2C, D). Choanocyte cells did not carpet the entire perimeter of the chamber. They were small ovoid cells, 2–4 µm in diameter, featuring a winged flagellum surrounded by microvilli and bearing diverse types of vesicules in their cytoplasm (Fig. 2E, F). The appearance of the choanocytes and the chambers was different under chemical fixation and cryofixation (Fig. 2, compare C with D and E with F). Following cryofixation, the nuclei contained reticulate chromatin and the microvilli and glycocalyx elements in the lumen of the chamber were better preserved. Some features, such as the flagellar "wings" of the glycocalyx (Fig. 2F), could only be seen in cryofixed material. Another difference was the better preservation of membranes and cell contacts between choanocytes with cryofixation (Fig. 2D, F).

◀ **Fig. 1** **A** Image of the cuticular layer (*CU*) surrounding the sponges. Cyanobacteria (*CY*) are visible underneath it; chemical fixation. **B** Vacuolar appearance of some zones of the ectosome because of the abundance of swollen cells (*SC*); chemical fixation (*N* nucleus). **C** Spherulous cell with large spherules and smaller, less dense vesicules (*asterisks*); cryofixation (*N* nucleus). **D** An archeocyte (*AR*) with engulfed cyanobacteria (*arrowheads*); chemical fixation. **E** Bacteria in sponge mesohyl; chemical fixation (*arrowheads* type D bacteria). **F** Bacteria and a cyanobacterium (*CY*); cryofixation

Microanalysis

To illustrate the kind of data obtained from the micronalysis, three spectra from sections processed by several fixation and preparation methods are shown in Fig. 3. All correspond to fibres and show the presence of bromine. The bromine signal, especially that corresponding to the Kα shell at 11.9 keV, is clearly distinct irrespective of the method. Figure 3A corresponds to chemically fixed material without contrasting. Figure 3B corresponds to cryofixed (CRYO-2) material with uranyl acetate for enhanced contrast, which results in the presence of U peaks that add some noise but this only interferes with the smallest Br peak (Kβ). On the other hand, this contrast permits a much better definition in the observation of the material. Some of the remaining noise peaks correspond to the Cu in the grids, Fe from the microscope and the osmium used in the processing. The spectra obtained by CRYO-3 methods (Fig. 3C) are the least noisy but the preservation of the material is poor and, hence, histological observation is hindered.

With regard to the location of bromine in the sections, we used the criterion proposed by Roomans (1988a): we scored bromine as being present in a structure whenever the pressumptive Br peak exceeded the background level by twice the standard deviation of the background intensity. In samples prepared by conventional chemical methods, we detected a clear Br signal only in the fibres. In samples prepared by cryofixation and cryosubstitution (CRYO-2 method), we obtained clear Br peaks not only in the fibres, but also in the spherulous cells. In samples cryofixed, cryosubstituted and cryoembedded (CRYO-3 method), the results were the same as for CRYO-2 but identification of the cell types was more difficult and less reliable. Figure 4 summarizes the results of the analyses performed on the main components of the sponge and in the embedding plastic, in terms of the height ratio between the peaks of Br and of Cl (see Materials and methods). In order to make them comparable, all results presented in this figure were obtained in samples prepared by CRYO-2 methods, except the ones from spherulous cells, for which results obtained by conventional chemical fixation are also shown for comparison. For fibres, we separately analysed the outer compact part and the inner loose material. An ANOVA performed on the data in Fig. 4 (chemical fixation values excluded) re-


OZ
IZ
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1 µm
F

vealed significant differences between the compartments analysed; a pairwise Student-Newman-Keuls test indicated that this significant effect was the result of the higher values in the outer part of the fibres and in the spherulous cells. The inner part of the fibres featured a weak Br signal but it was not significantly different from that obtained in the other components analysed. Bromine levels in the sponge components other than fibres (outer) and spherulous cells were not significantly different from the background level (as assessed by the signal obtained for the plastic material).

In order to illustrate the coupling between ultrastructural observation and microanalysis, Figs. 5 and 6 show images of spherulous cells and fibres and spectra obtained from selected windows of these images. The section in Fig. 5 was prepared by CRYO-2 techniques and contrasted with uranyl acetate; the one in Fig. 6 was prepared by conventional fixation methods with no contrast added. The quality of the images was affected by the thickness (0.3 µm) of the sections for microanalysis. Figure 5 shows the two types of spherulous cells observed: cells with clear inclusions and cells with dense inclusions. Both types feature bromine in their vesicules but the Br peaks are slightly higher in the spherules from "dense" spherulous cells. Figure 5 also depicts spectra corresponding to one bacterium close to the spherulous cells and to the mesohyl in their vicinity, featuring no Br peaks. Regarding the fibres (Fig. 6), the Br signal in the compact outer layer of the fibres was strong, whereas the height of the Br peak was much lower in the inner part of the fibres, probably because of the much lower density of fibre material in this alveolar area. Bromine was absent in windows analysed just outside the fibres (Fig. 6).

Discussion

Our data show that bromine in *A. aerophoba* accumulates in the spherulous cells and the fibres. As the secondary metabolites of the sponge contain most of the organic bromine, detection of this element can be used for the localization of these compounds in the sponge (Thompson et al. 1983). The data also show that the choice of fixation and processing techniques is crucial when using energy dispersive X-ray microanalysis. Conventional chemical methods have failed to demonstrate the presence of bromine in the spherulous cells; this has only been substantiated in cryofixed material.

◀ **Fig. 2** **A** Image of the periphery of a fibre, showing the compact outer zone (*OZ*) and the alveolar inner (*IZ*) zone; chemical fixation. **B** A pinacocyte (*PI*) lining an internal canal (*C*); chemical fixation. **C** A choanocyte chamber (*CCH*); chemical fixation. **D** A choanocyte chamber (*CCH*); cryofixation. **E** Close up of a choanocyte chamber showing choanocytes (*CH*), flagella (*F*) and microvilli (*MV*); chemical fixation. **F** Enlarged view of some choanocytes (*CH*); cryofixation. *Inset* Magnification of a zone of a choanocyte chamber showing transversely cut flagella with well-preserved wing-like expansions (*arrows*) presumably made of mucopolysaccharide material

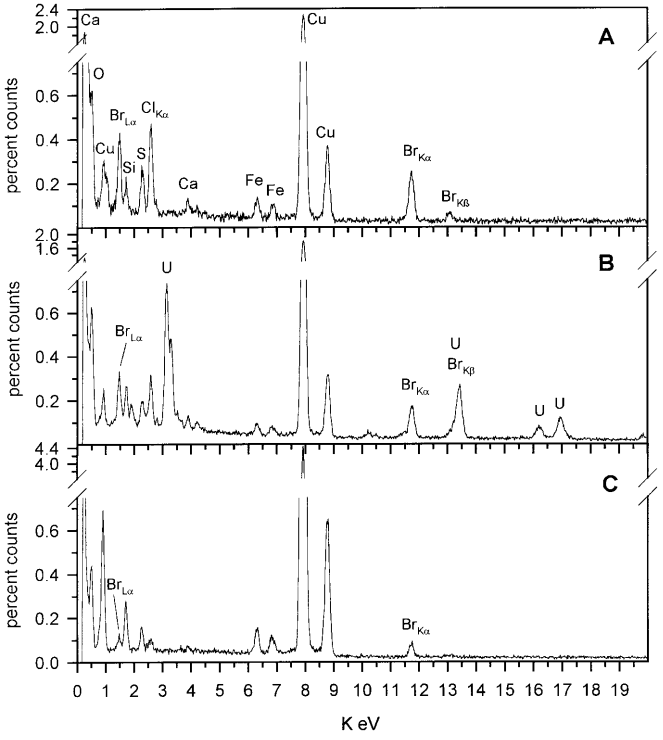

Fig. 3A–C XMRA spectra obtained from 2×2 µm windows placed on the outer compact layer of fibres of *A. aerophoba* in sections obtained by diferent methods. **A** Chemical fixation, no contrast. **B** CRYO-2 fixation with uranyl acetate contrast. **C** CRYO-3 method, no contrast. The main peaks are labeled in **A**, uranyl peaks are labeled in **B**, and bromine peaks are indicated in the three spectra

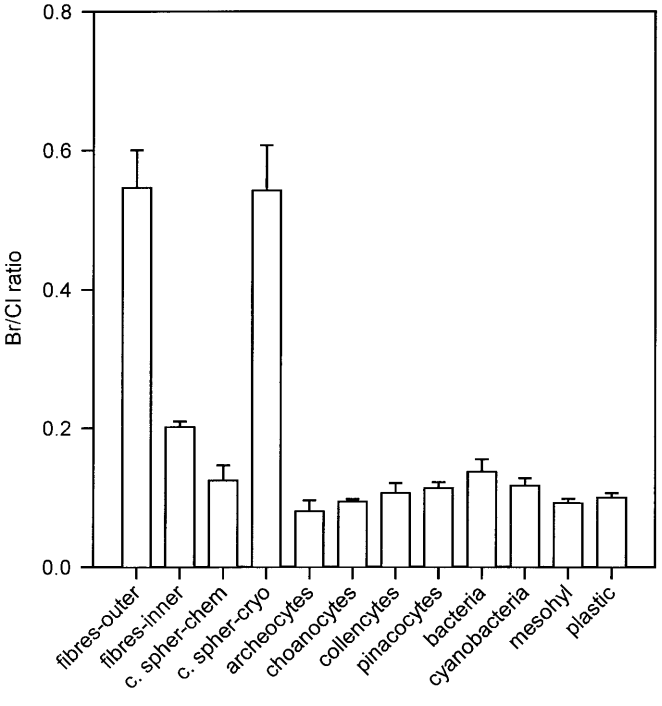

Fig. 4 Mean and standard error of the Br/Cl ratio, measured as the ratio between the height of the Br Kα peak and that of the Cl Kα peak in the diverse sponge components

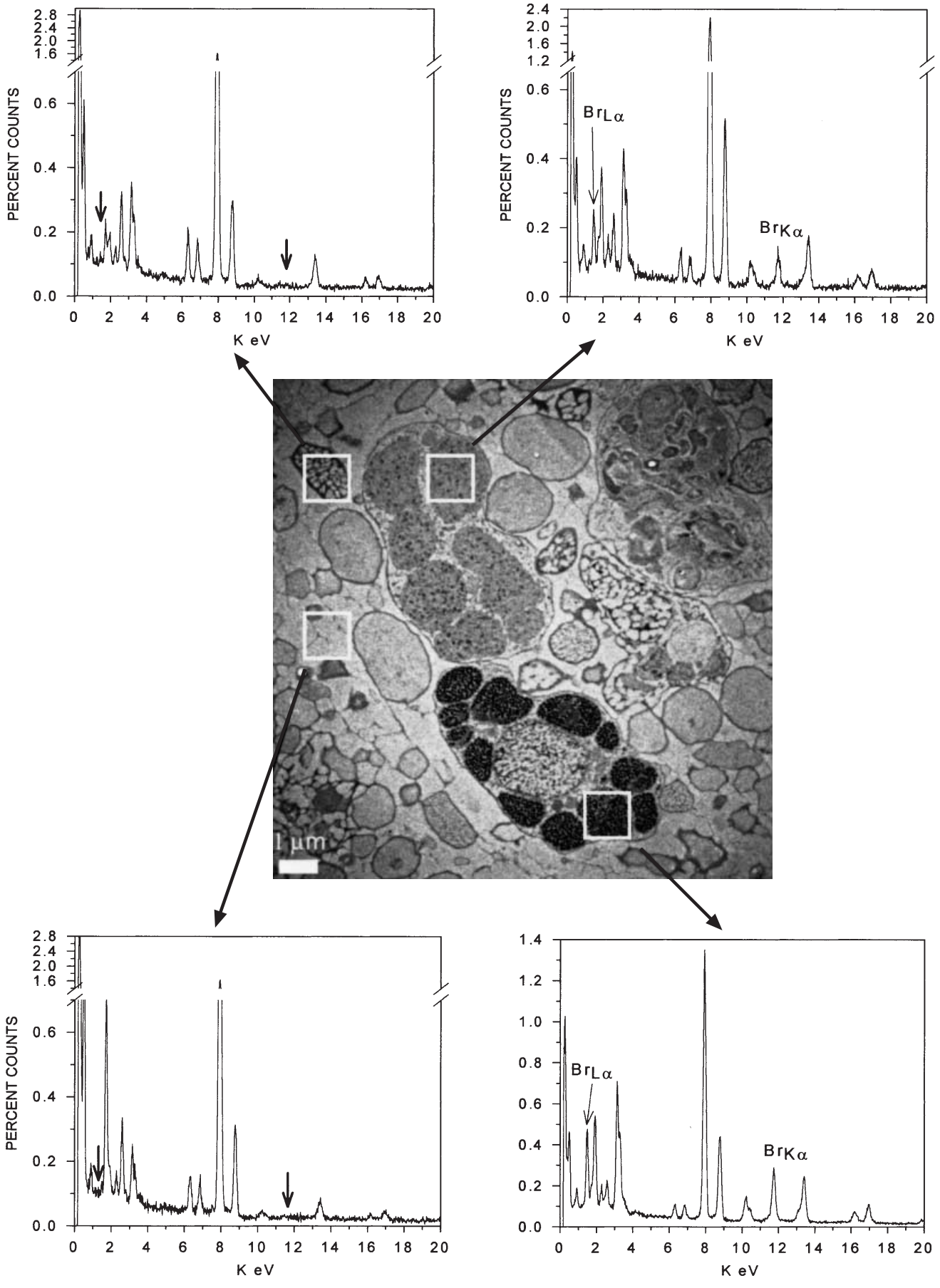

Fig. 5 Semi-thin section (0.3 µm) of two spherulous cells (CRYO-2 fixation with uranyl acetate contrast) and the corresponding XMRA spectra from selected windows of the section. Bromine peaks are labeled; the lack of them is indicated by *bold arrows*

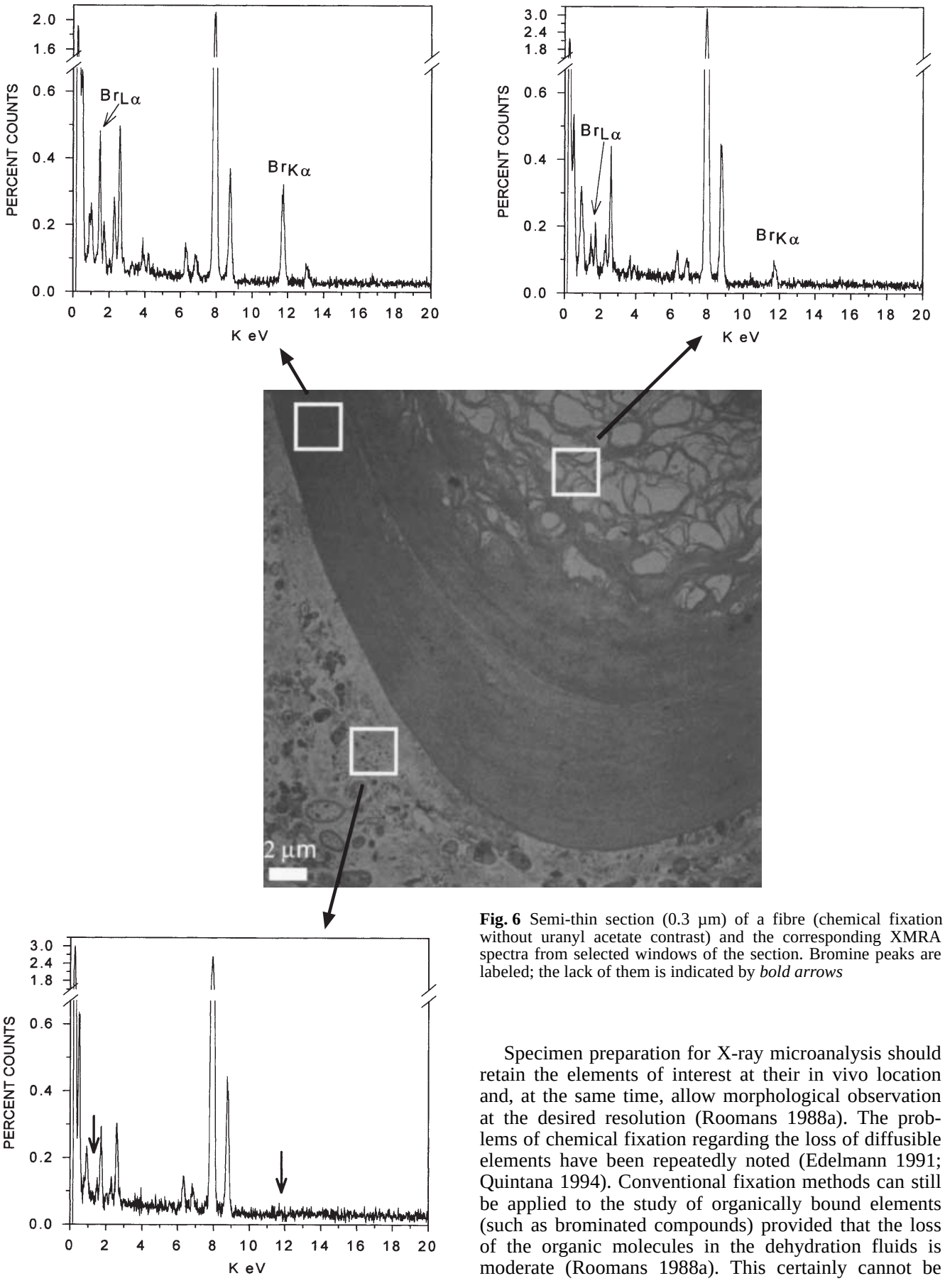

Fig. 6 Semi-thin section (0.3 µm) of a fibre (chemical fixation without uranyl acetate contrast) and the corresponding XMRA spectra from selected windows of the section. Bromine peaks are labeled; the lack of them is indicated by *bold arrows*

Specimen preparation for X-ray microanalysis should retain the elements of interest at their in vivo location and, at the same time, allow morphological observation at the desired resolution (Roomans 1988a). The problems of chemical fixation regarding the loss of diffusible elements have been repeatedly noted (Edelmann 1991; Quintana 1994). Conventional fixation methods can still be applied to the study of organically bound elements (such as brominated compounds) provided that the loss of the organic molecules in the dehydration fluids is moderate (Roomans 1988a). 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. The Br retention in the fibres observed by chemical methods may be the result of the better immobilization of these organic molecules or their derivatives within the spongin matrix of the fibres. Cryomethods should therefore to be used for the reliable location of brominated metabolites in our samples.

A properly cryofixed sample can be observed in a hydrated state in a cryomicroscope, although this type of sample does not withstand the high doses of electrons necessary for microanalysis and so dehydrated samples should be used. Cryodehydration can be performed by freeze-drying or cryosubstitution. The former causes problems with ultrastructure preservation and, thus, cryosubstitution may provide the best preservation (Quintana 1994; Porta and Lopez-Iglesias 1998), while allowing the satisfactory retention of diffusible elements (Edelmann 1991). Methods including cryoembedding (CRYO-3 method) are believed to perform best in element retention, as tested with highly diffusible ions such as K^+ (Quintana 1993, 1994) but, in our case, the CRYO-2 method has yielded the best balance in terms of both bromine detection and ultrastructure preservation. No new site of bromine accumulation has been detected with the CRYO-3 method and morphological observation is much poorer. In addition, the use of Spurr resin in the CRYO-2 method provides a good standard (the Cl peak) for comparison.

The quantitative analysis of elements by X-ray microanalysis is fraught with methodological problems (reviewed in Roomans 1988b) but comparative measures with reference peaks (such as the Cl peak used in this work) are nonetheless possible and allow for numerical comparison among structures. Moreover, the most suitable method for the preservation of brominated compounds in situ may vary according to the species being studied. For instance, Thompson et al. (1983) have detected bromine in the spherulous cells of *A. fistularis* by X-ray analysis after chemical fixation. The only difference from our procedure is that these authors use ethanol, instead of acetone, as the dehydrating fluid. It seems advisable to compare the results obtained by several fixation techniques for each case under study. We have also found that, for this element, the clearer signal is the one from the Kα line, which does not interfere with fixation or contrasting elements (such as Os and U).

The ultrastructure observed in our samples is similar to that reported for *A. fistularis* by Thompson et al. (1983). The spherulous cells are very similar to those described for *A. cavernicola* by Vacelet (1967) and the finding of different bacterial morphotypes within the mesohyl of the sponge is in agreement with Vacelet's (1975) observations in *Aplysina* species. We suggest that the spherulous cells are the site of production of the brominated secondary metabolites in *A. aerophoba*. Cells of this kind have been shown to produce defence metabolites in other species (Thompson et al. 1983; Bretting et al. 1983; Uriz et al. 1996a). We have observed numerous instances of apparent degeneration of spherulous cells that have released their contents to the mesohyl; this may be the usual way of metabolite release. However, we have not seen any image of spherulous cells leaving the sponge mesohyl through canals or external surfaces, a route that has been suggested as a means of chemical release to the environment (Uriz et al. 1996a), although the release of chemicals after wounding is of course compatible with our observations. Indeed, Kreuter et al. (1992) have demonstrated the release of metabolites from incubated sponge cubes of this species.

On the other hand, the fibres may play a role in the sequestering and accumulation of brominated compounds, perhaps as inactive residues. The ability of sponges to accumulate trace metals and halogens is well known (Patel et al. 1985) and fibres of keratose sponges have been demostrated to be sites of immobilization and accumulation of heavy metals (Vernedal et al. 1990). Fibres are the longest-lasting structures in the dynamic system formed by the living parts of the sponge and seem to contribute an excretory function by accumulation. To some extent, the fibre compartment, surrounded by pynacocyte-like cells, can be considered as an external medium for the sponge. Some of the mechanisms for the extrusion of toxic substances, such as the MXR pump described by Müller et al. (1996), may concentrate these substances in the fibres. It is not surprising, therefore, to find an accumulation of bromine in the fibres of sponges, a large number of their secondary metabolites containing this element (up to 12% dry weight of brominated metabolites; Teeyapant et al. 1993a). It would be interesting to test whether the chemicals are in their original or in a modified residual form within the fibres. Another possible role for halogenated derivatives in structural proteins may be to provide stability by non-specific incorporation of halogen atoms into aromatic amino acids; this may enhance molecular cohesion (Hunt 1984).

In the two best known instances of brominated secondary metabolite production by sponges, different sources for these compounds have been demonstrated: symbiotic prokaryotes (whether cyanobacteria or bacteria: Elyakov et al. 1991; Unson et al. 1994) in Dysideidae and sponge cells (spherulous cells) in members of the Verongida (Thompson et al. 1983; this work). The same pattern seems to apply to other sponge groups: several studies suggest or demonstrate secondary metabolite production by sponge symbionts (e.g. Stierle et al. 1988; Molinski 1993; Oclarit et al. 1994; Bewley et al. 1996), whereas others indicate the production by sponge cells (e.g. Bretting et al. 1983; Uriz et al. 1996a, 1996b). Even in the same species, some metabolites may be produced by sponge cells and others by symbionts (Flowers et al. 1998) or different metabolites may be produced by different kinds of prokaryote (Bewley et al. 1996). There is also the very interesting possibility that, in *Aplysina*, the sponge cells produce the inactive precursors and the symbionts produce the enzymes that activate them (Ebel et al. 1997). The finding by Kreuter et al. (1992) that the

various 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; this makes any generalization or prediction difficult. Considering only brominated compounds, those of more than 25 sponge species had been chemically identified by the early nineties (Gribble 1992). It would be interesting to ascertain whether all sponges with brominated compounds contain prokaryote symbionts; this would point to these symbionts as being producers and/or to a role of these antimicrobial compounds in the regulation of symbiont populations. On the other hand, the presence of the same or similar brominated metabolites in distant groups (Gribble 1992; Molinski 1993; Faulkner et al. 1994; Unson et al. 1994; Bewley et al. 1996), suggests the convergent evolution of successful kinds of active molecules and introduces the hitherto unexplored topic of chemical convergence and its evolutionary implications.

Aside from the particular case under study, it seems that X-ray microanalysis, coupled with cryofixation techniques, allows the accurate detection of metabolites within invertebrates. The potential applications of this method are important given the number of halogenated compounds that are present in marine organisms. These techniques may allow us to increase our knowledge about secondary metabolite biosynthetic pathways; this has far-reaching ecological, evolutionary and biotechnological implications.

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