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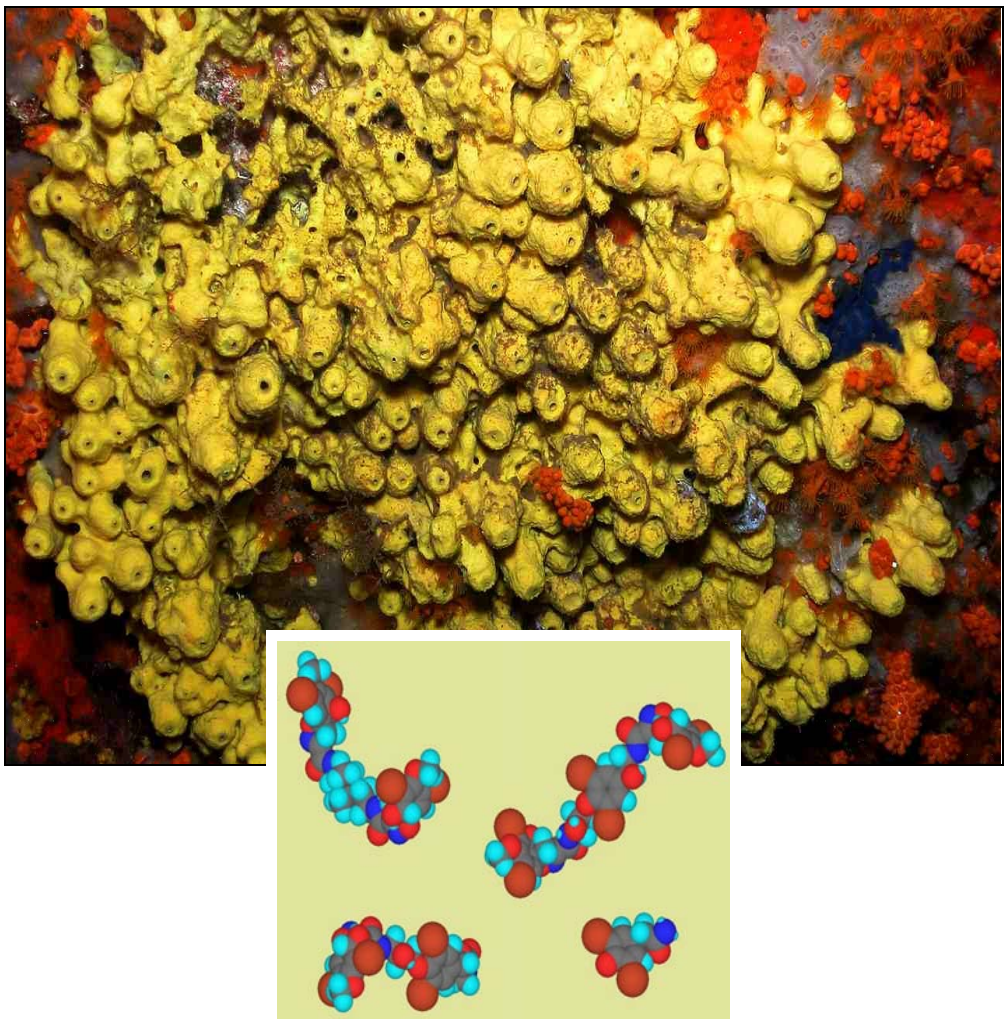

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Expression of secondary metabolites by the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*

Mémoire présenté par Nabil MAJDI

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Contents

1. INTRODUCTION

Sponges (phylum Porifera) are very ancient marine metazoans: the first fossil records from sponges date from the ediacarian period (about 600 million years ago) [1]. Some studies [2,3] seem to attribute to sponges the status of early metazoan ancestors, that is to say that sponges appeared before 600 million years. These organisms show primitive attributes: only 2 cell layers, absence of digestive or nervous system, regenerating and burgeoning [4]. They are essentially filter feeders, and they live all their adult life as sessile organisms fixed to a benthic substrate. Despite this relative simplicity, sponges are important marine actors for several hundreds of million years, and nowadays the phylum Porifera has about 9000 living species with a world-wide distribution, from infra-littoral zones to abyssal plains [5].

Moreover, the success of sponges depends on the accumulation of toxic or repellent compounds [6] (also called secondary metabolites). This chemical shield repulse predators and protect the sponges from invasion by microorganisms and biofouling [7].

It seems that the chemical properties of sponges were well known by our ancestors: already from late Greek antiquity, sponges were used by men for his personal hygiene (*Spongia officinalis*), pain relief and treatment of diseases [8,9]. Nowadays, with the development of pharmacotherapies, more and more scientists attempt to isolate active compounds from marine organisms [10]. And it is assessed that sponges have the most valuable potential for development of new pharmaceuticals [7].

38 species of sponges belong to the genus *Aplysina* Nardo, 1834 (Class Demospongiae, Order Verongida, Family Aplysinidae). *Aplysina* are great producers of a highly toxic family of isoxazolic alkaloids, that are derived from amino-acid tyrosine [11]. This genus is also characterized by a typical bright yellow pigment called uranidine which warns predators of the toxic content of *Aplysina* sponges [12]. The genus *Aplysina* is more present at tropical latitudes, but 2 identified species are living in the Mediterranean sea.

The first Mediterranean *Aplysina* was described by Schmidt in 1862, and was named *aerophoba* because of its color change (from bright yellow to dark violet) when exposed to air. *A. aerophoba* can be found on all well-exposed rocky substrate in shallow waters (0 to 30 m deep) from the Mediterranean sea to the near Atlantic: around Canary Islands [13] .

In 1959, Vacelet [14] described another Mediterranean *Aplysina* naming it *cavernicola* because of its preferred habitat: contrary to the photophile *A. aerophoba*, *Aplysina cavernicola* is a sciaphile species that can be found in semi-obscure entry of submerged caves, or on deeper substrates (40 to 70 m deep).

External and internal morphology is almost identical in the two species. So, according to a lack of traditional taxonomic character (spicule comparison), taxonomists have long debated if these 2

Aplysina species, showing so different ecological requirements but identical morphology, should be regarded as a single or distinct species [15]. Now, molecular phylogeny has brought response to this question: *A. aerophoba* and *A. cavernicola* are lately distinct species [16,17].

These 2 species can contain high amounts of brominated alkaloids derived from tyrosine (up to 13% dry weight) [18]. These alkaloids are found in a particular sponge cell type named "spherulous cell" [18,19]. These large vacuolar cells stock Bromotyrosine Alkaloids (BA) that are transformed into simple but more toxic forms when a wound of plasmatic membrane occurs [20] (Fig.1).

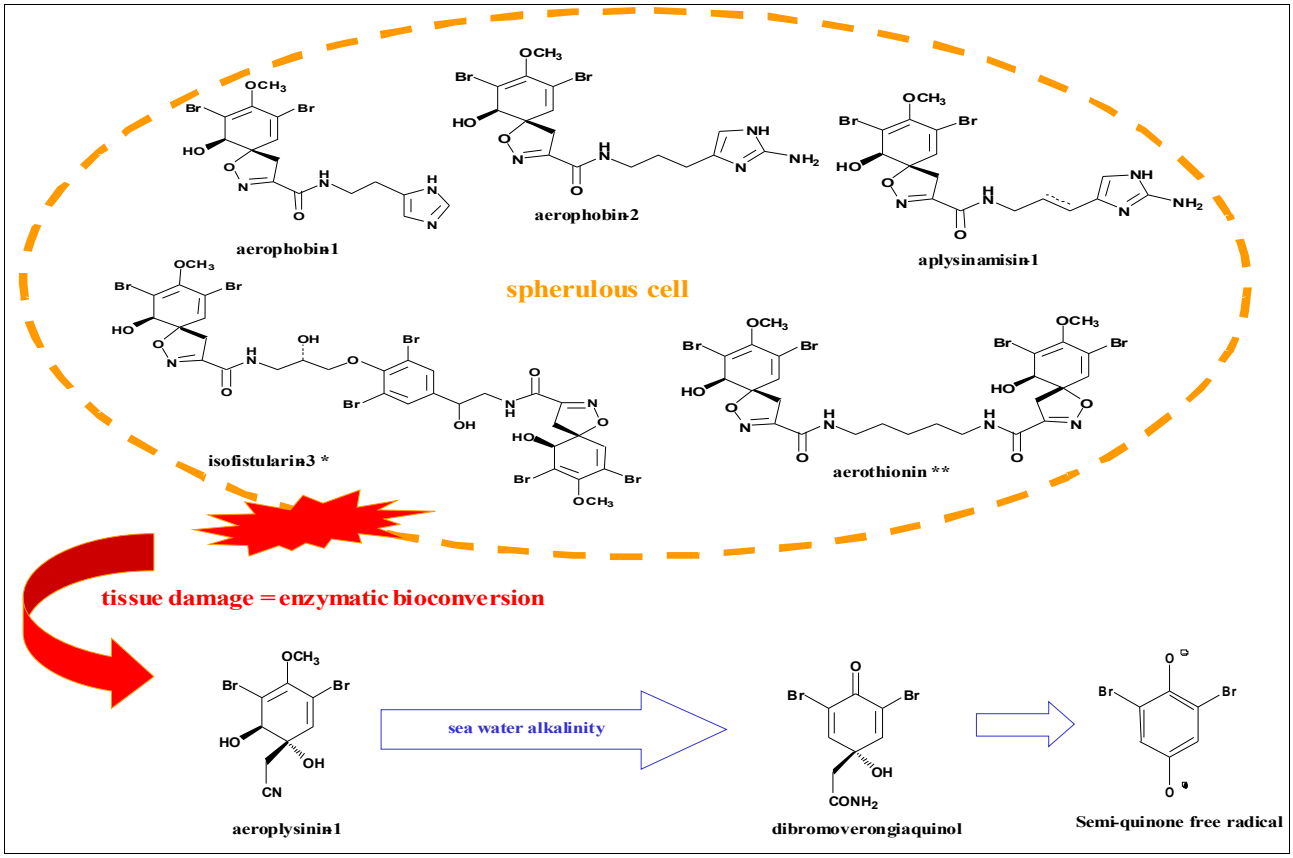


Fig.1. Scheme of Mediterranean *Aplysina* chemical defense. Bromotyrosine Alkaloids (BA) are located in spherulous cells. A lesion of these cells, causes enzymatic bio-conversion of BA into aeroplysinin-1 [20]. Then with sea-water alkalinity, aeroplysinin-1 gives dibromoverongiaquinol and semi-quinone free radicals. Both aeroplysinin-1 and dibromoverongiaquinol have strong antibacterial and cytotoxic activity [20-23]. This toxic release in seawater repels predators and protects sponge from the invasion of microorganisms [21,23,24].

(* isofistularin-3 is only present in *A. aerophoba*. ** aerothionin is only present in *A. cavernicola*).

Some taxonomist called *Aplysina* sponges « bacteriosponge » because of their dense community of symbiotic bacteria, that can amount up to 40 % of their dry weight [25]. These so-called endobionts [26] can help their hosts in nutrient uptake, stabilization of skeleton, and protection against UV radiation [21,25,27]. Despite the fact that Bromotyrosine Alkaloids (BA) are

concentrated in sponge cells (Fig.1), endobionts are suspected to play a critical role in BA production [20,28].

Aplysina aerophoba and *Aplysina cavernicola* are two target-species in the ANR program ECIMAR [29], a program developed in marine chemical ecology to evaluate the potentialities of marine biodiversity in terms of chemodiversity. The aim of this program is to better understand the processes controlling the chemical diversity. As part of this program, specific goals of this study are fourfold:

- Finalizing the methodology to quantify the major secondary metabolites produced by the two Mediterranean *Aplysina* species.
- Identifying chemotaxonomic markers in order to clearly discriminate the two sibling species.
- Assessing whether variation, in quality and quantity, of secondary metabolism occurs at different biogeographic scales.
- Investigating the role of microbial symbionts in BA biosynthesis.

2. MATERIALS AND METHODS

2.1. Sample collection and storage

Specimens of *Aplysina* sponges were collected by scuba divers (Pérez T., Becerro M., Banaigs B.) at different locations (see Table 1). Samples were then freeze-dried and stored in the dark at – 25°C. Samples of *A. aerophoba* were "nested sampled" (see Annexes) in Tenerife and Cap Creus.

Species	Region	Samples	Date	Divers
Aplysina aerophoba	Tenerife (Canary Islands)*	82	03/2003	M. Becerro (CEAB)
	Cap Creus (Spain)*	70	03/2003	M. Becerro (CEAB)
	Lebanon	1	08/2007	T. Pérez (DIMAR)
	Marseille (France)	1	03/2006	B. Banaigs (LCBE)
	Banyuls (France)**	15	03/2008	B. Banaigs (LCBE)
Aplysina cavernicola	Costa brava (Spain)	3	09/2003	M. Becerro (CEAB)
	Marseille (France)	30	2007-2008	T. Pérez (DIMAR)
	Ceuta (Spain)	3	07/2007	T. Pérez (DIMAR)

Table 1. **Sponge sampling** thanks to divers from "Centre d' Estudis Avançats de Blanes" (CEAB), "Laboratoire de Chimie des Biomolécules et de l'Environnement EA 4215" (LCBE), and "Diversité, évolution et écologie fonctionelle marine UMR 6540" (DIMAR).

* See Annexes for more information on "nested sampling sites". ** Fresh *A. aerophoba*

Fresh *A. aerophoba* were taken at the Oullestrell's cap (near Banyuls s/ mer) by B. Banaigs. These fresh sponges were kept alive in a water tank and quickly brought to the "Observatoire Océanologique de Banyuls s/mer" (OOB). It is important to strictly avoid any contact with air

during this operation, because tissues of *A. aerophoba* are very sensitive to air oxydation. Then, the sponges were transplanted in OOB's water tanks which are supplied with non-filtered sea water.

2.2. From a standardized to an optimized protocol for the HPLC analysis and quantification of Aplysina's BA

At the beginning of my work, I used the standardized ECIMAR protocol for the extraction and analysis of the samples (available at [30]). This protocol is well adapted for the preliminary analysis and the comparisons of marine organism chemical extracts. But in our case, this protocol is time-consuming and needs too much material (2 g of dry sponge). So, with the little material we have at our disposal, it was crucial to develop an optimized protocol for the identification and quantification of *Aplysina*'s BA. All analyses, comparisons, and results are based on this optimized protocol.

2.2.1. Chemical procedures

Freeze-dried sponge samples were ground to powder. Approximatively 50 mg of each freeze-dried sample was weighted, extracted 3 times with 1.5 mL of HPLC-grade methanol (MeOH) in an ultrasonic tank, and passed through a 20 µm PolyTetraFluoriEthylene (PTFE) filter. The 4.5 mL resultant Crude Extract (CE) was then ajusted to 5 mL with MeOH. An aliquot of 1.5 mL was passed through a 13 mm, 0.2 µm PTFE syringe-filter before HPLC injection.

2.2.2. High Pressure Liquid Chromatography (HPLC) analysis

The analyses were performed with a Waters Alliance 2695 separation module and a Waters 996 photodiode array detector. The HPLC conditions consisted in:

Column: Phenomenex Synergy (250x3 mm/4 µm ∅)

Flow rate: 0.4 mL.min^{-1}

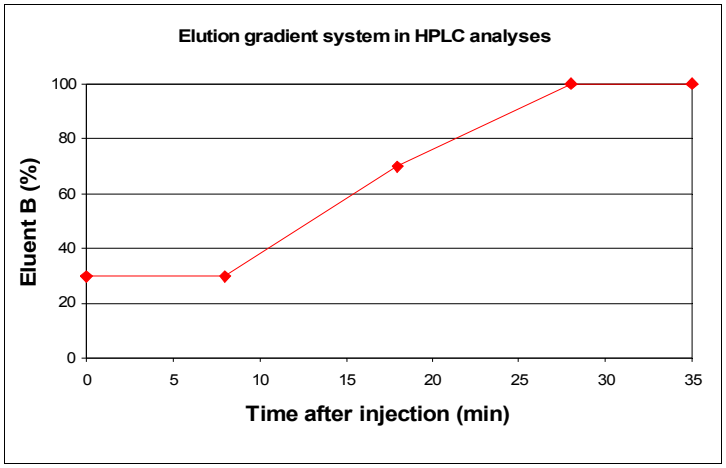
Fixed temperature: 30°C

Injection volume: 20 µL

Eluant A: MilliQ water with 1‰ TriFluoroAcetic acid (TFA)

Eluant B: HPLC-grade Acetonitrile

UV detection: λ = 245 nm



2.2.3. Secondary metabolites identification

The major compounds observed in the HPLC chromatograms have already been characterized in the laboratory by classic spectrometric techniques (LC/MS, NMR, UV). Five compounds (aerophobin-1, aerophobin-2, aplysinamisin-1, aerothionin and isofistularin-3) were chosen for quantification, as they are representative of the sponge BA content [20]. The identification of these compounds was made thanks to their Retention Time (RT) and their UV profiles (Fig.2).

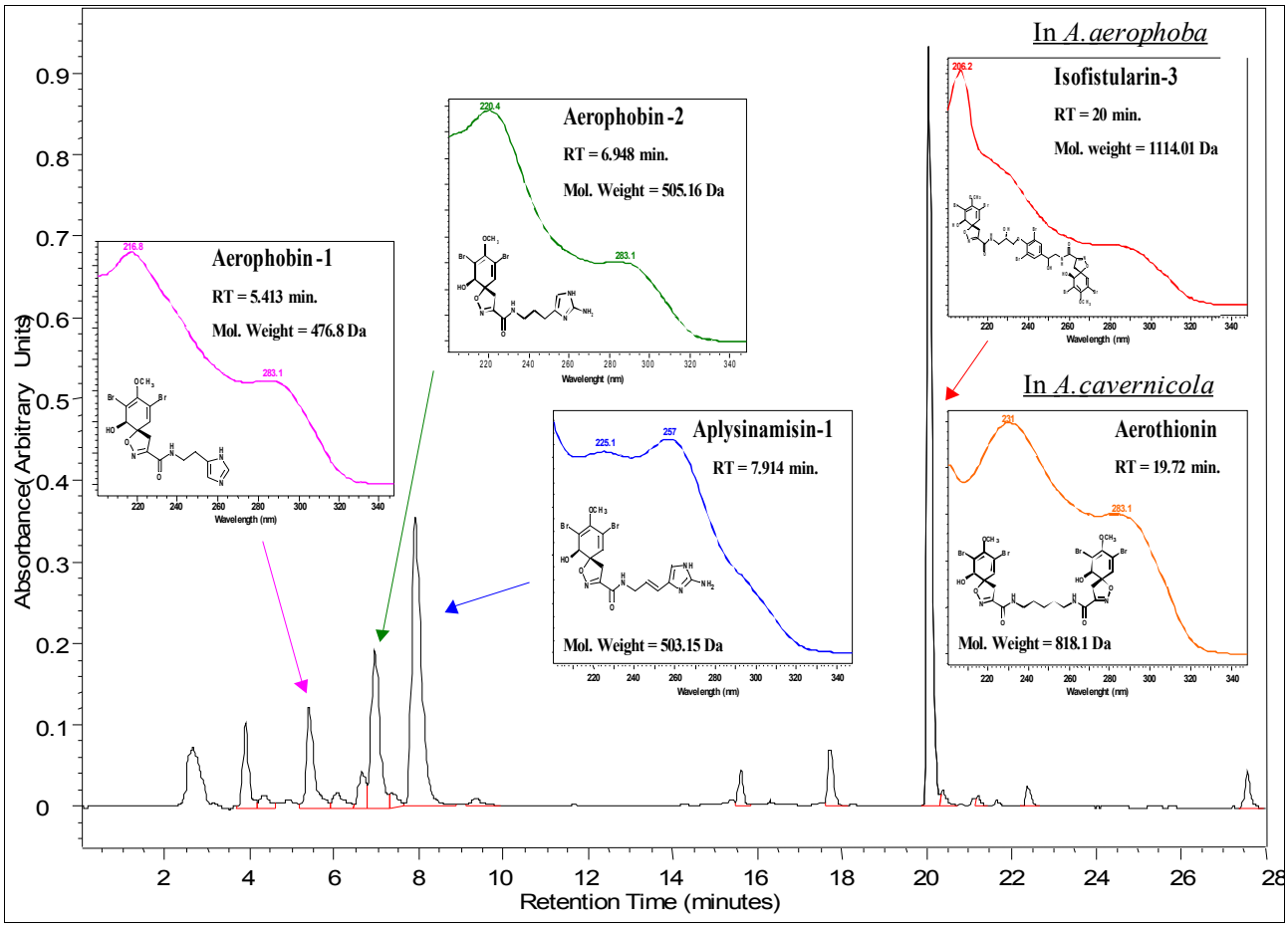


Fig. 2. Example of a typical chromatogram of *A. aerophoba* recorded at 245 nm with the optimized protocol. Each peak on chromatogram represents the absorbance at λ=245 nm of a single compound. Here, BA are identified with their RT and their absorbance profile between 200 and 350 nm. Peak areas can be measured and related to concentration. It is to be noted, that *A. cavernicola* shows almost the same chromatographic pattern except that isofistularin-3 is replaced by aerothionin (the 2 compounds have, in this chromatographic conditions, very close RT).

2.2.4. HPLC quantification

A Crude Extract (CE) was prepared with 50 g of freeze-dried sponge. Extraction was carried out as stated in (2.2.1.), except that MeOH volumes were 100 times higher. So, 500 mL of CE was first fractionated by Flash-Chromatography. Then, CE Fractions were manually injected with a syringe into a Waters 1525 binary HPLC pump coupled with a Waters 2487 dual λ absorbance detector. HPLC conditions consisted in:

Column: Gemini RP-18 (250x10 mm/5 µm ∅) / Flow rate: 3 mL.min^{-1} / Injection volume: 100 µL

Isocratic conditions: 70% of MeOH, 30% MilliQ water / UV detection: λ = 320 nm

Each BA compound detected was identified and carefully isolated. After evaporation on a rotary evaporator, dry pure BA recovered were weighed. Then, a series of dilution on the pure compounds coupled to peak area calculation in HPLC (at 245 nm) allowed to trace calibration-curves (Fig. 3). Thanks to calibration curves, BA amount was quantified in all HPLC analyses: for each BA

compound, peak area obtained at 245 nm was calculated and compared to correspondent calibration-curve. For more reliable results, the final amount of each compound in the optimized protocol was calculated by averaging 2 replicate HPLC injections. The Bromotyrosine Alkaloid (BA) amount was expressed as percentage of dry mass of sponge.

2.3. *Aplysina aerophoba and its associated cyanobacteria*

2.3.1. Concentration of Chlorophyll A (ChlA): an indication on the density of autotrophic symbionts

100 mg of freeze-dried *A. aerophoba* powder was approximately weighed. 5 mL of PestiPur-grade Acetone was added and extraction occurred for 12 hours in the dark at +1°C. Then, 2 mL were passed through 0.2 µm PTFE syringe filter before placing it in a quartz cuvette. A Hewlett-Packard 8452 diode array spectrophotometer was used to measure absorbance at 630, 647, 664 and 750 nm. The trichromatic equation of Hemphrey and Jeffrey [31] was used to deduce ChlA concentration:

$$\mathbf{C_{ChlA}(mg.L^{-1}) = 11.85\,(Abs664 - Abs750) - 1.54\,(Abs647 - Abs750) - 0{,}08\,(Abs630 - Abs750)}$$

Concentration was linked to weight, therefore ChlA was expressed in percentage of dry weight.

2.3.2. Microorganisms extraction

To avoid contamination by other bacteria, it was very important to work under sterile conditions (laminar flow chamber, gloves and sterile material). Approximately 100 g of fresh, living *A. aerophoba* was carefully cut with a sterile scalpel in small cubes of about 1 cm^3. Then, cubes were manually ground in a beaker containing 1 L of sea-water passed through a 0.2 µm Whatman's filter. By grinding this way, living symbiotic microorganisms are expelled from the sponge tissues. Then, this suspension of living symbionts and sponge tissues was twice-filtered through 100 µm (to retain sponge tissues) and 11µm (to retain sponge cells [32]). So, 1L of "Endobiont Suspension" (ES) with the associated bacteria of *A. aerophoba* was obtained.

2.3.3. Fluorescence-Activated Cell Sorting (FACS) – experiments performed at the OOB

FACS is a powerful instrument to analyze mixed populations of single cells [33]. FACS allows counting and sorting of these cells according to their fluorescence and size characteristics.

1 mL of ES was analysed by a Becton-Dickinson FACSaria. SYBRgreen-II dye was done [34] to visualize the total content of living cells in ES. FACS will also be used to sort cyanobacteria cells from *A. aerophoba.*

2.3.4. Biliproteins pigment analyses of associated cyanobacteria – exp. performed at the OOB

The characteristic pigments of cyanobacteria have a proteic part (phycobiliproteins), and are hydrosoluble [35]. So, to extract them, we ground fresh *A. aerophoba* in a simple phosphate buffer (NaH_2PO_4; pH = 6.7). Then, this extract was centrifugated at 3000g for 10 minutes. Supernatant was transfered in a glass cuvette, which was introduced into a Perkin-Elmer LS55 spectrofluorimeter for fluorimetric analyses.

2.4. Data analysis and statistical tests

HPLC data were treated with EMPower software. FACS results were treated with BDFACS Diva software. According to M. Becerro, statistical analyses based on multivariate methods available in the PRIMER software [36] were used to analyze differences in the BA content of our samples. Log-transformed data were used to calculate the Brain-Curtis similarity and the analysis of similarities (ANOSIM). Non-metric multidimensional scaling (MDS) was also used to analyze our data more deeply. Then, we used SIMPER procedure in the PRIMER software to quantify the relative contribution of each BA to dissimilarities. An analysis of variance (ANOVA) was also carried out on the average amount of each BA.

3. RESULTS

3.1. Calibration curves (made with HPLC's peak area at 245 nm)

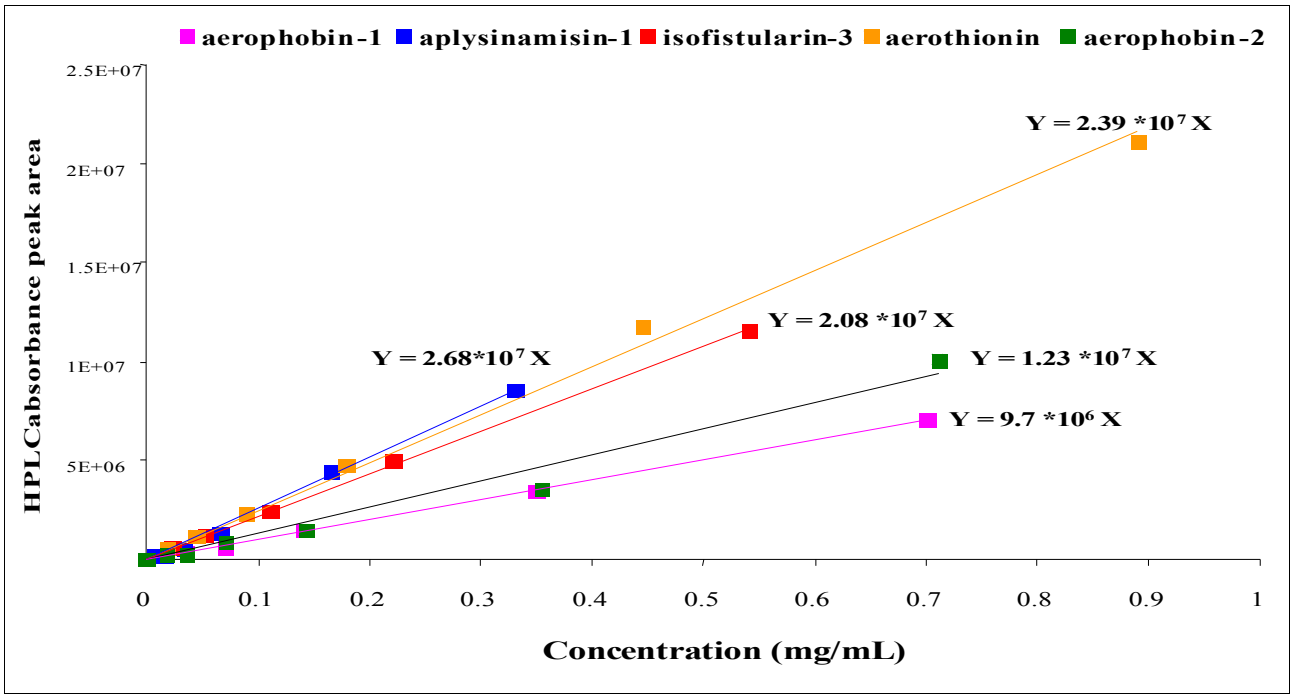


Fig. 3. Calibration curves for each BA. For all curves, $R^2 > 0.99$

3.2. Chemical fingerprints: a tool for Aplysina species determination

According to molecular phylogeny, *A. aerophoba* and *A. cavernicola* are early distinct species [16,17]. Their identification in the field could be problematic, therefore chemotaxonomic markers without resorting to molecular techniques could be interesting for Mediterranean *Aplysina* identification. Chemical fingerprints obtained from 155 samples of *A. aerophoba* and 36 samples of *A. cavernicola* are very close (Fig.4): the two species have a complex secondary metabolism, with numerous compounds that are present in both. Nevertheless, a more detailed analysis showed that there is a notable difference in chemical fingerprints between both species: about 20 minutes after HPLC injection, an intense peak occurred in all chromatograms. However, UV spectrum of this

peak differs according to species. In fact, in all *A. aerophoba*, this peak corresponds to isofistularin-3, when in all *A. cavernicola* this peak corresponds to aerothionin. These 2 compounds are species specific. So, occurrence of aerothionin or isofistularin-3 is a good chemotaxonomic marker for Mediterranean *Aplysina* identification.

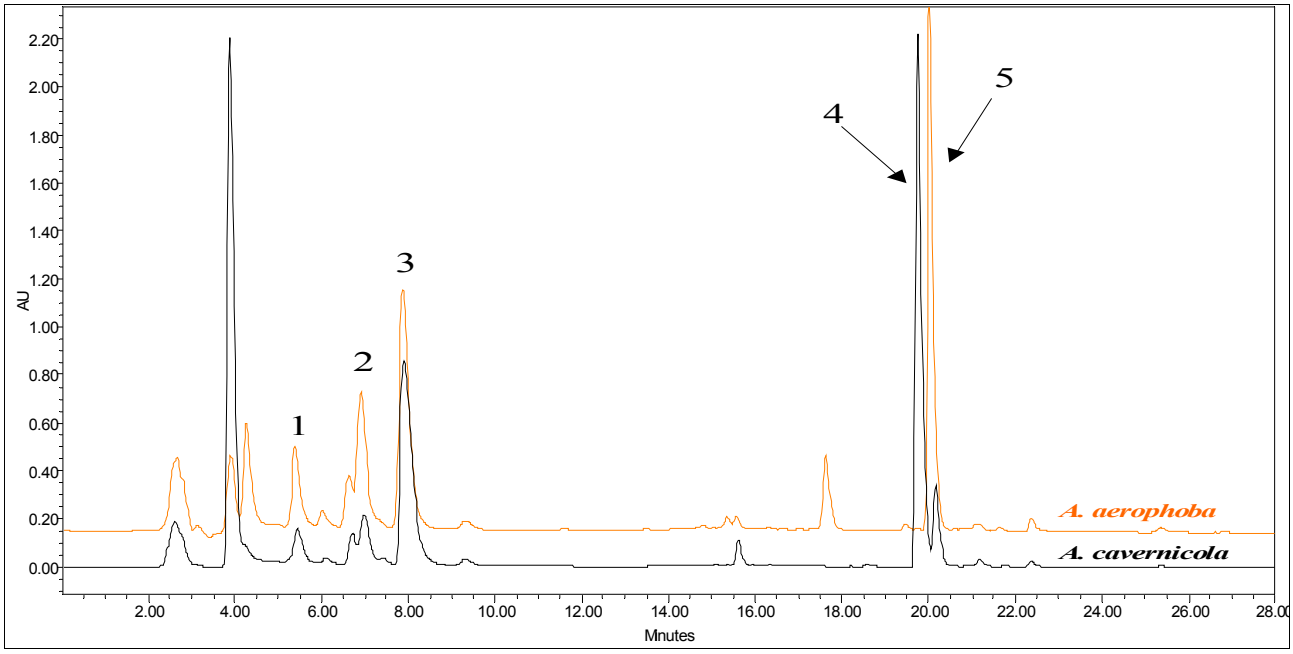


Fig 4. Comparison of chromatograms showing the typical chemical fingerprint from *A. aerophoba* and *A. cavernicola.* Aerophobin-1 (**1**), aerophobin-2 (**2**), aplysinamisin-1 (**3**) are found in the both species, whereas aerothionin (**4**) and isofistularin-3 (**5**) are specific to respectively *A. cavernicola* and *A. aerophoba*.

3.3. The intra-specific variations of secondary metabolites in A. aerophoba

We have quantified the four major BA: aerophobin-1, aerophobin-2, aplysinamisin-1, and isofistularin-3, in more than 150 samples of A. aerophoba (Table 1). The amount of each of these compounds is expressed in percentage of sponge dry weight. We analyzed the variability of chemical patterns of these samples as function of their geographical origin with multivariate statistical tests.

3.3.1. Variability in secondary metabolism of *A. aerophoba* at different geographical scales

The 82 samples from Tenerife and the 70 samples from Cap Creus (Table 1) allowed us to compare the amount of BA in geographically close or distant populations of *A. aerophoba*. Data for aerophobin-1, aerophobin-2, aplysinamisin-1 and isofistularin-3 were log-transformed for Brain-Curtis similarity. Then, they were compared between geographical distances (in the declining order: Region > Zone > Location > Site; see Annexes) using ANOSIM. The Global-R reflects differences in variability between groups. Results were listed in Table 2:

ANOSIM using Site groups as samples	Test for differences between	p-value	Global-R
Site within Location	Site group	0.001*	17.3 %
	Location group	0.204	15.8 %
Site within Zone	Site group	0.001*	15 %
	Zone group	0.07	18.9 %
Site within Region	Site group	0.001*	17.7 %
	Region group	0.001*	43.2 %

Table 2. ANOSIM test, * represents significant difference

So, Bromotyrosine Alkaloid (BA) amount differs significantly between Sites and between Regions in *A. aerophoba*. That is to say that BA amount vary at very small scales (between bays), but also at much higher geographical scales: here, between Tenerife and Cap Creus. Moreover, with a Global-R of 43.2 %, variability between Regions is more important than between Sites.

Then, a MDS analysis was carried out to represent Sites and Regions variability (Fig. 5). On MDS we observe that Sites repartition is more extend in Tenerife than in Cap Creus. So, small-scale variability is more important in Tenerife than in Cap Creus.

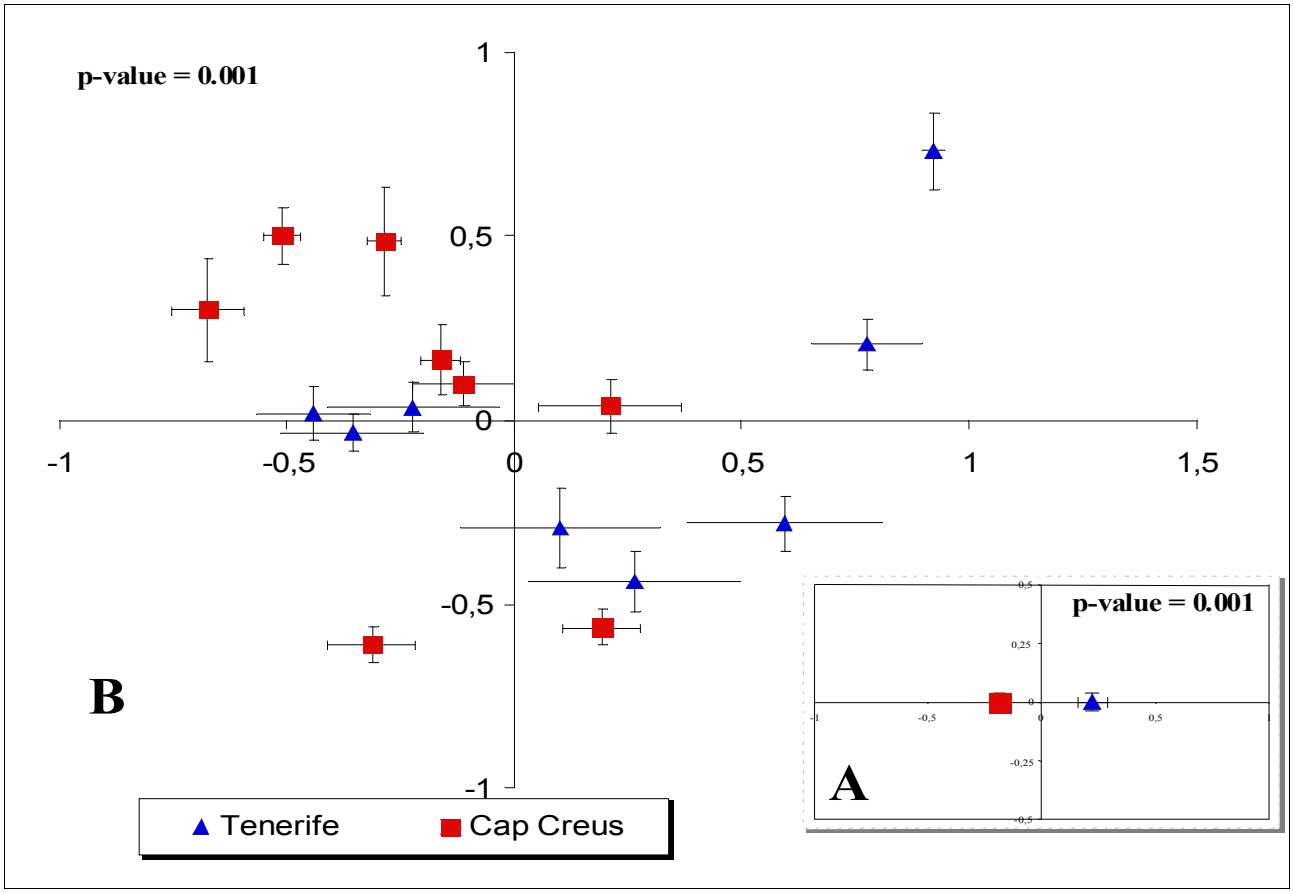


Fig. 5. non-metric MDS performed separately for (A) Regions and (B) Sites. MDS generates plots in which the distance between points is proportional to their degree of similarity. Averaging matrix similarity of samples was made before to better visualize Sites and Regions plots.

3.3.2. Relative contribution of each BA compound to dissimilarities between Regions

To point out variability in secondary metabolism between *A. aerophoba* from Tenerife and Cap Creus, we have made a SIMPER procedure (Table 3).

Region	Average Dissimilarity	Relative contribution of			
		aerophobin-1	aerophobin-2	aplysinamisin-1	isofistularin-3
Tenerife	17.09 %	12.04 %	26.9 %	40.28 %	18.54 %
Cap Creus					

Table 3. Average dissimilarity between Regions. Relative contribution of each BA compound to Region dissimilarities is deduced by performing a SIMPER procedure in PRIMER software.

With this test, we can see, that aplysinamisin-1 is responsible for 40 % of dissimilarities in BA content between regions.

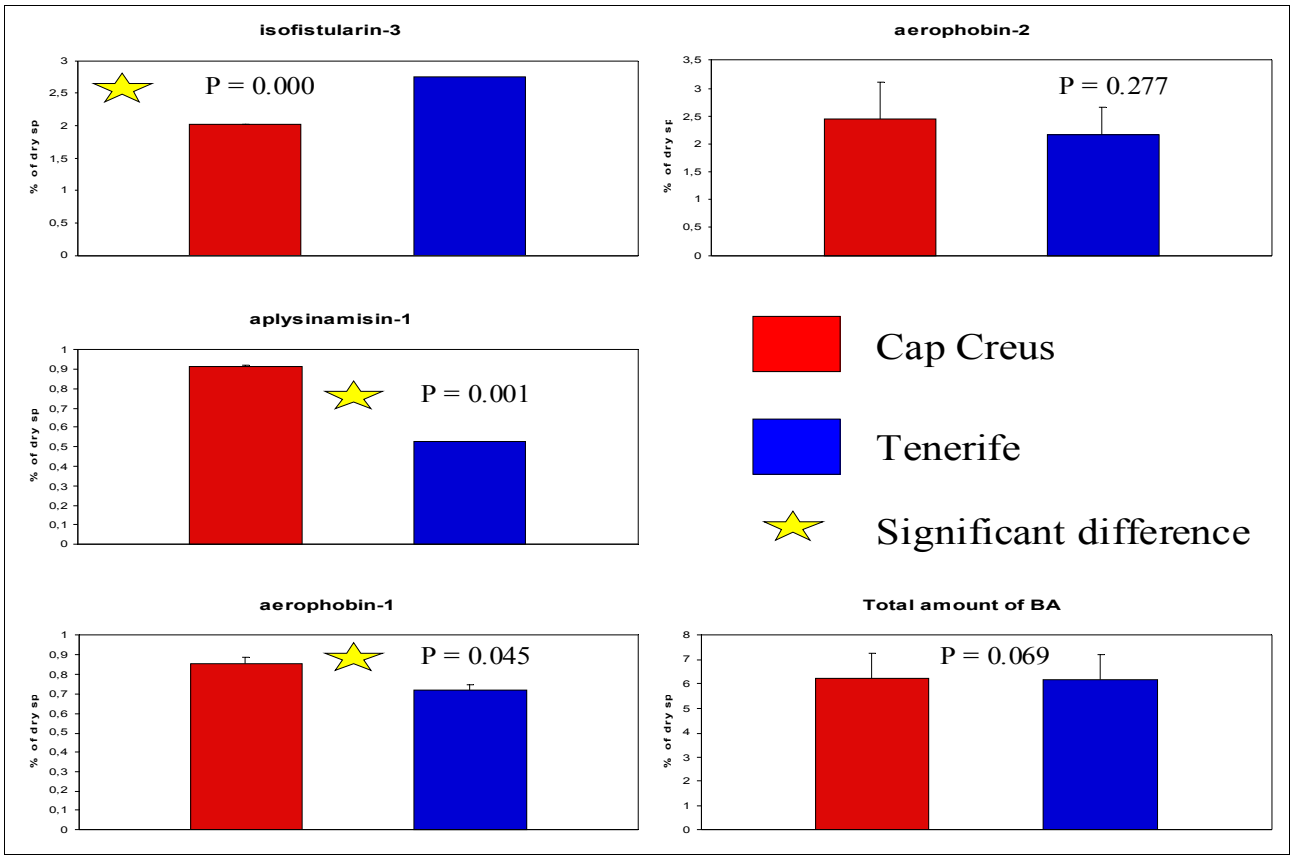


Fig. 6. ANOVA on each BA and total amount of BA, p-values < 0.05 were counted as significant differences in amount of BA between Regions.

Aplysinamisin-1 and aerophobin-1 were significantly less produced by the *A. aerophoba* from Tenerife. Contrary to isofistularin-3 which was less produced by the *A. aerophoba* from Cap Creus. Aerophobin-2 do not show significant difference. And surprisingly the average total amount of BA between Cap Creus and Tenerife is almost identical. So, some BA compound can vary with location, but global amount of BA seems to be constant in all *A. aerophoba* sampled.

3.4 Role of symbiotic microorganisms in Bromotyrosine Alkaloids (BA) production

3.4.1 Quantification of ChlA amount

A. cavernicola did not show any ChlA in its tissues. It was predictable, because this species lives in dark places. On the contrary, all *A. aerophoba* samples had ChlA. This means that, autotrophic symbionts occur in *A. aerophoba*, but are absent in *A. cavernicola*. Moreover, it seems that ChlA amount (as well as autotrophic symbiont density) varies with geographical location and decreases with depth (fig. 7).

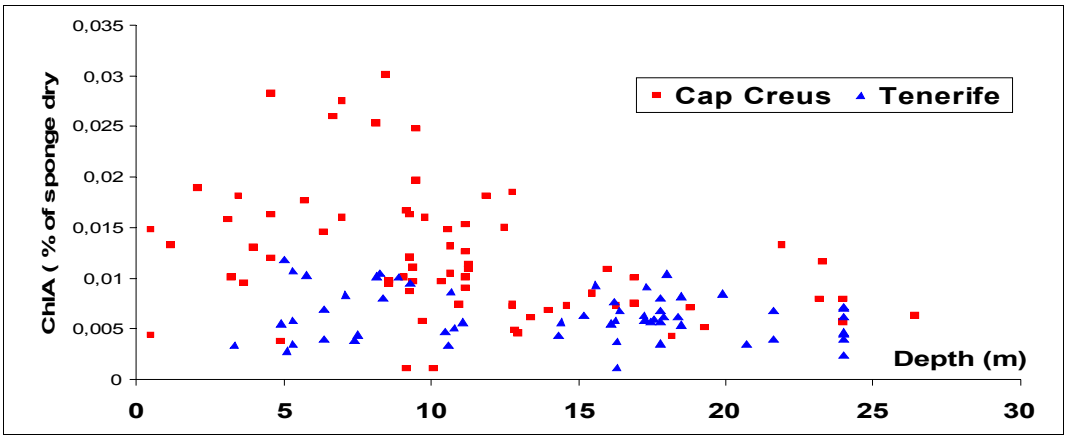


Fig. 7. ChlA amounts by *A. aerophoba* samples from Cap Creus and Tenerife. ChlA is of autotrophic origin. So, the ChlA concentration gives an indication on density of autotrophic symbiont. Here, ChlA concentration logically decreases with depth. This decrease is more pronounced in Cap Creus than in Tenerife.

3.4.2. Spectrofluorimetric analysis of cyanobacterial pigments

ChlA could be found in various autotrophic organisms. To precise nature of the autotrophic symbionts from *A. aerophoba*, the analysis of characteristical pigments was performed.

The spectrofluorimetric analyses led to the identification of a phycoerythrin with 2 chromophores: phycourobilin and phycoerythrobilin (Fig.8A). A phycocyanin characteristical response was also recorded (Fig.8B). Phycoerythrins (red) and phycocyanins (blue) are phycobiliproteins: proteins to which chromophores are covalently bound. The occurrence of these 2 phycobiliproteins is a characteristic clue for the presence of cyanobacteria.

However, phycoerythrin is more abundant than phycocyanin, and such occurrence of phycourobilin and phycoerythrobilin as main chromophores of phycoerythrin could be regarded as a characteristical marker for the presence of *Synecococcus* [37,38]. *Synecococcus* is a cyanobacterial genus abundant in marine planktonic communities. They are also known as symbiotic partners for several sponge, especially *Aplysina* [39].

Nevertheless, fluorescence intensity decreased rapidly with time (Fig. 8C), and a progressive darkening was observed. This darkening is probably due to the air oxydation of the sponge pigment uranidin [12].

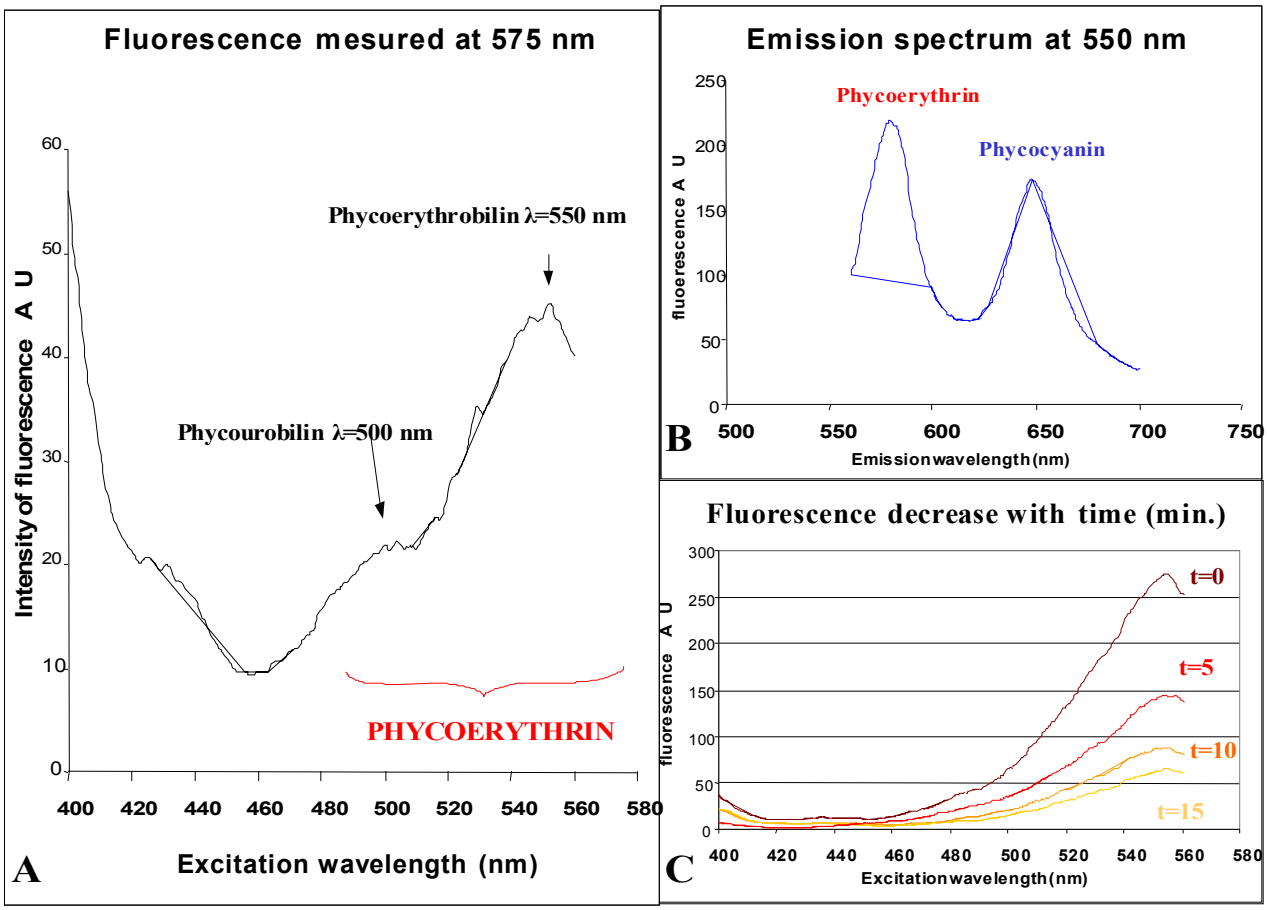


Fig. 8. Fluorescence spectra of *A. aerophoba* pigment content. A. Characteristical excitation spectrum of phycoerythrin. **B.** When excited at 550 nm, a typical emission spectrum for phycoerythrin and phycocyanin was recorded. **C.** The same sample analyzed for fluorescence at 575 nm at different times after extraction from sponge shows an important decrease in intensity with time. After 15 min, the sample has lost 80% of its fluorescence intensity.

3.4.3. FACS results: a tool to count and sort associated micro-organisms

SYBRgreen is an asymmetrical cyanine dye which binds to double-stranded DNA. The resulting DNA-dye-complex absorbs blue light (λmax = 488 nm) and emits green light (λmax = 522 nm). It was shown [34], that SYBR-II is the most appropriate dye for bacterial enumeration of unfixed and fixed seawater samples.

A. aerophoba contains a very dense and complex community of associated microorganisms. In a first experiment, we used SYBR-II to visualize living cells (Fig.9A). Approximately 1.5×10^6 cells/mL were counted in the "Endobiont's Suspension" (ES). This concentration is several times higher than normal concentrations of microorganisms found in sea-water.

Without SYBR-II dye, only autotrophic microorganisms are detected due to fluorescence of their photosynthetic pigments (Fig.9B). Parameters of size and fluorescence showed that *Synecococcus* are the most important autotrophic symbionts. Consequently, 1.34×10^6 *Synecococcus* cells were

sorted with FACS and further analyzed for their chemical content (3.4.4.).

A small population of eukaryots from Cryptophyta phylum also seems to live with *A. aerophoba*.

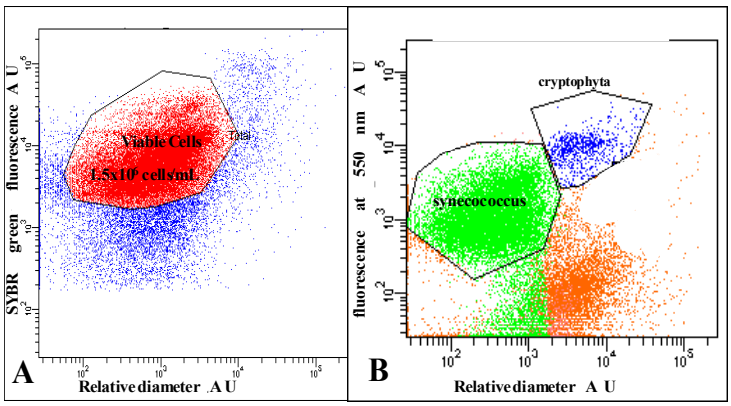


Fig. 9. Cytograms obtained from ES. A. SYBRgreen fluorescence permits distinction between viable cells and debris. Viable cells in 1 mL of ES were also counted. **B.** Fluorescence at 550 nm without SYBRgreen dye shows autotrophic cells: density of *Synecococcus* is important.

3.4.4. Chemical profile of *Synecococcus* from *A. aerophoba*

Symbionts from *A. aerophoba* could contribute to BA production. So, occurrence of a BA compound in a symbiont could constitute an interesting finding to understand sponge-symbiont relashionships in secondary metabolites production. *Synecococcus* sorted by FACS were extracted with MeOH and analyzed in HPLC. The Comparison of chemical fingerprints between *Synecococcus* and *A. aerophoba* showed that isofistularin-3 occurred in both (Fig.10).

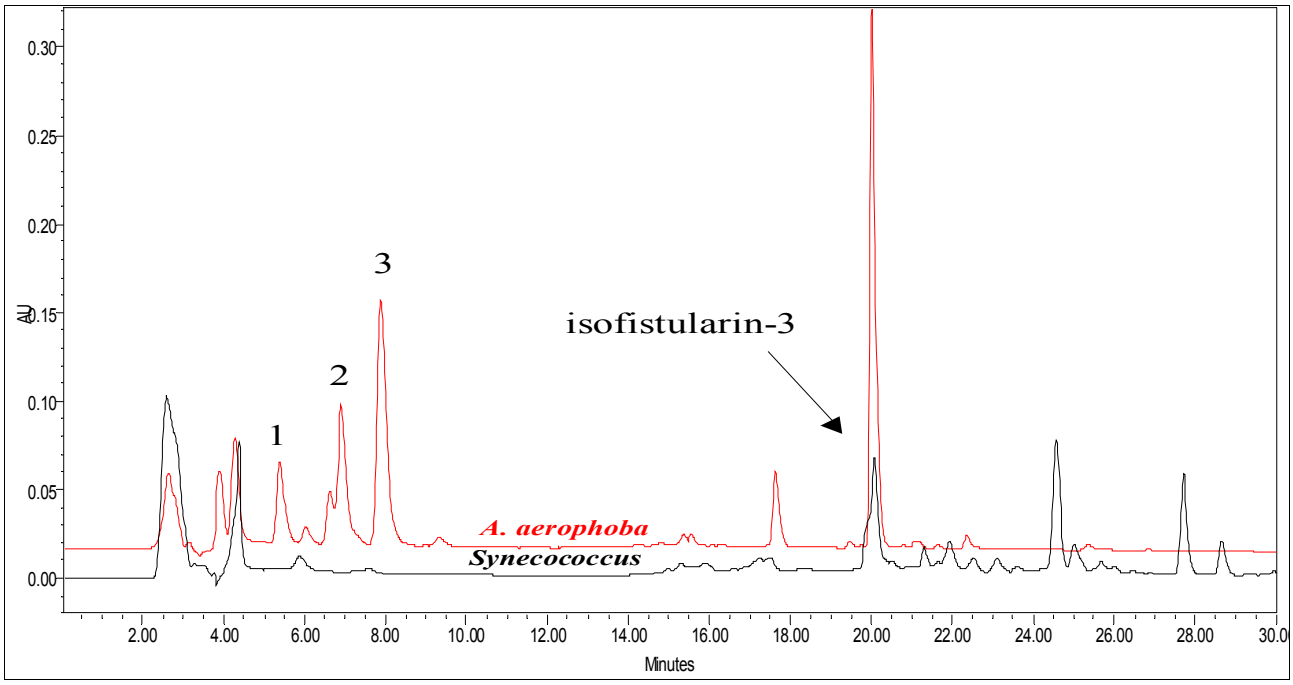


Fig. 10. Chromatograms of *A. aerophoba* and its associated *Synecococcus*. Both chromatograms show occurrence of isofistularin-3. However other BA (**1**,**2**,**3**) do not appear in *Synecococcus*.

4. DISCUSSION

The first specific goal of this study was to finalize a methodology to quantify the major secondary metabolites produced by the two Mediterranean sibling species *A. aerophoba* and *A. cavernicola.* So, We have developed an optimized protocol from which we were able to obtain a well-resolved HPLC chromatogram from a minimum of biological material. This protocol brought us chemical fingerprints from 155 *A. aerophoba* and 36 *A. cavernicola* samples. From these fingerprints, the 4 major BA biosynthesized by *A. aerophoba* have been quantified. This protocol places at our disposal a very powerful analytical tool for further studies in chemical ecology.

A. aerophoba and *A. cavernicola* have an almost identical morphology, and can be easily confounded. They can be identified with their preferred habitat, however, in some cases, identification could be problematic. For example, 2 samples from Ceuta were reported as *Aplysina sp.* (field observations were not sufficient to discriminate between *A. aerophoba* or *A. cavernicola*). In making their chemical fingerprints, we were able to set these unidentified samples as *A. cavernicola*. So, isofistularin-3 and aerothionin could be used as useful chemotaxonomic markers to help for Mediterranean *Aplysina* identification. This result confirms the preliminary result obtained by Ciminiello et al. [40] on a comparison of 2 samples both collected in Sardinia (Italy). Now, our study with numerous specimen taken in a wide geographic range strenghtens this previous finding without doubt.

Secondary metabolites content of Mediterranean *Aplysina* is quite homogen compared to those of other marine invertebrates where we can find different chemotypes associated with different morphotypes [41]. We studied the intra-specific variability in secondary metabolites production in more than 150 samples of *A. aerophoba* collected during the same period at different geographical scales (see Annexes) that can be distant (Regions = 2800 Km), medium (Zones = 10 Km), small (Locations = 1 to 5 Km) or very close (Sites= 100 to 500 m). We have noted, that expression of secondary metabolites varied at very close scale (between Sites), and vary the most between Regions. The variations recorded is principally due to the compounds isofistularin-3, aerophobin-1, and aplysinamisin-1. In particular, aplysinamisin-1 was responsible for 40 % of variability recorded. Another investigation work on *A. aerophoba* chemical content could be from great interest to identify causes of such variability.

Fortunately, *A. aerophoba* and *A. cavernicola* are model organisms for the program ANR-ECIMAR. So, there is no doubt, that future ECIMAR diving missions (Crete, Corsica, Spain and Tunisia) will bring other specimens of *Aplysina*. With a view to further studies on Mediterranean *Aplysina*

chemical content, our work constitute a first step for secondary metabolites survey in these sponges.

Another aim of this work was to determine the role of symbiotic microorganisms of *Aplysina* sponges in secondary metabolites production. We focus our work on *A. aerophoba.*
According to previous studies [25,27] we found with FACS that associated microorganisms from *A. aerophoba* were more abundant in the sponge than in seawater. So, the protocol we developed for symbiotic microorganisms extraction proved to be efficient.

ChlA measurements showed that contrary to *A. cavernicola*, *A. aerophoba* possess autotrophic microorganisms. Detailed pigment analysis and FACS showed that these autotrophic microorganisms were principally cyanobacteria from *Synecococcus* genus.
We choose to concentrate our study on these cyanobacterial symbionts, because FACS permits to sort them specifically. After sorting and extracting these *Synecococcus*, isofistularin-3 was detected. Nevertheless, we cannot exclude that isofistularin-3 was released in "Endobiont Suspension" during sponge grinding, and was then detected as an artifact due to the protocol. But if it was the case, why other BA did not appear ? It is also striking, that *A. cavernicola* which lacks *Synecococcus,* lacks isofistularin-3 too. This preliminary result shows that we are able to obtain an HPLC chemical fingerprint with a very small amount of precise cells sorted from a very complex and mixed population of cells. Our work constitutes another first step to be continuated as follows: further work has to be done to point out the role of *Synecococcus* in isofistularin-3 production.

Molecular phylogeny based on 16s rDNA was done on *A. aerophoba* [42], the results were very useful to identify and classify the huge symbiotic bacteria diversity of these sponges. With FACS experiments, we observed these bacteria, and we obtained information on density of certain symbiotic bacterial populations. Moreover, we could see, that *A. aerophoba* harboured Cryptophyta eukaryots cells as symbionts. This type of symbiont does not appear in 16S rDNA based molecular phylogeny. So, our FACS-based protocol can be useful to estimate importance of bacterial and micro-eukaryot populations associated with sponges.

So, to sum up, in this study we have developed an optimized protocol for Mediterranean *Aplysina* chemical content survey. This protocol permits us to observe the geographical variability of chemical content in *A. aerophoba*. We also demonstrated that identification between sibling species *A. cavernicola* and *A. aerophoba* could be done by comparing Bromotyrosine Alkaloid patterns. Lastly we developed a protocol for symbionts separation from host sponge. These symbionts (in particular *Synecococcus*) might be implicated in isofistularin-3 production (which is a precursor to antibacterial and cytotoxic compound Aeroplysinin-1).

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6. ANNEXES

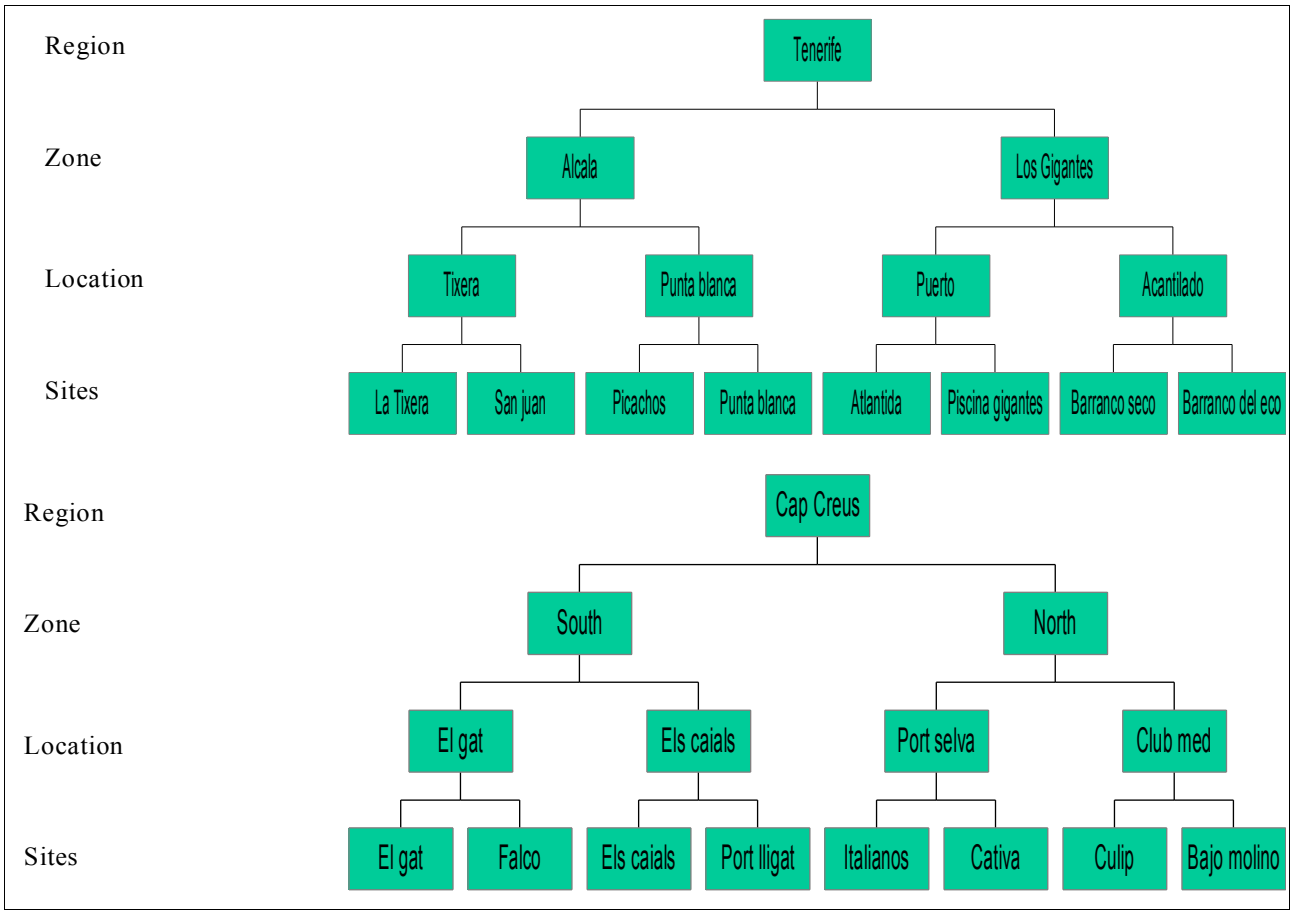

Fig. 11 Scheme of nested sampling

The 2 regions nested sampled were Tenerife (Canary Islands) and Cap Creus (Spain). The distance between the 2 region is about 2800 Km.

Each Region was divided in 2 Zones. Distance between 2 Zones from the same Region is about 6 to10 Km.

Each Zone was divided in 2 Locations. Distance between 2 locations from the same Zone is about 1 to 5 Km.

Each Location was divided in 2 Sampling Sites. Distance between Sites from the same location, is about 100 to 500 m

In each Site, about 10 specimens of A. aerophoba were sampled.

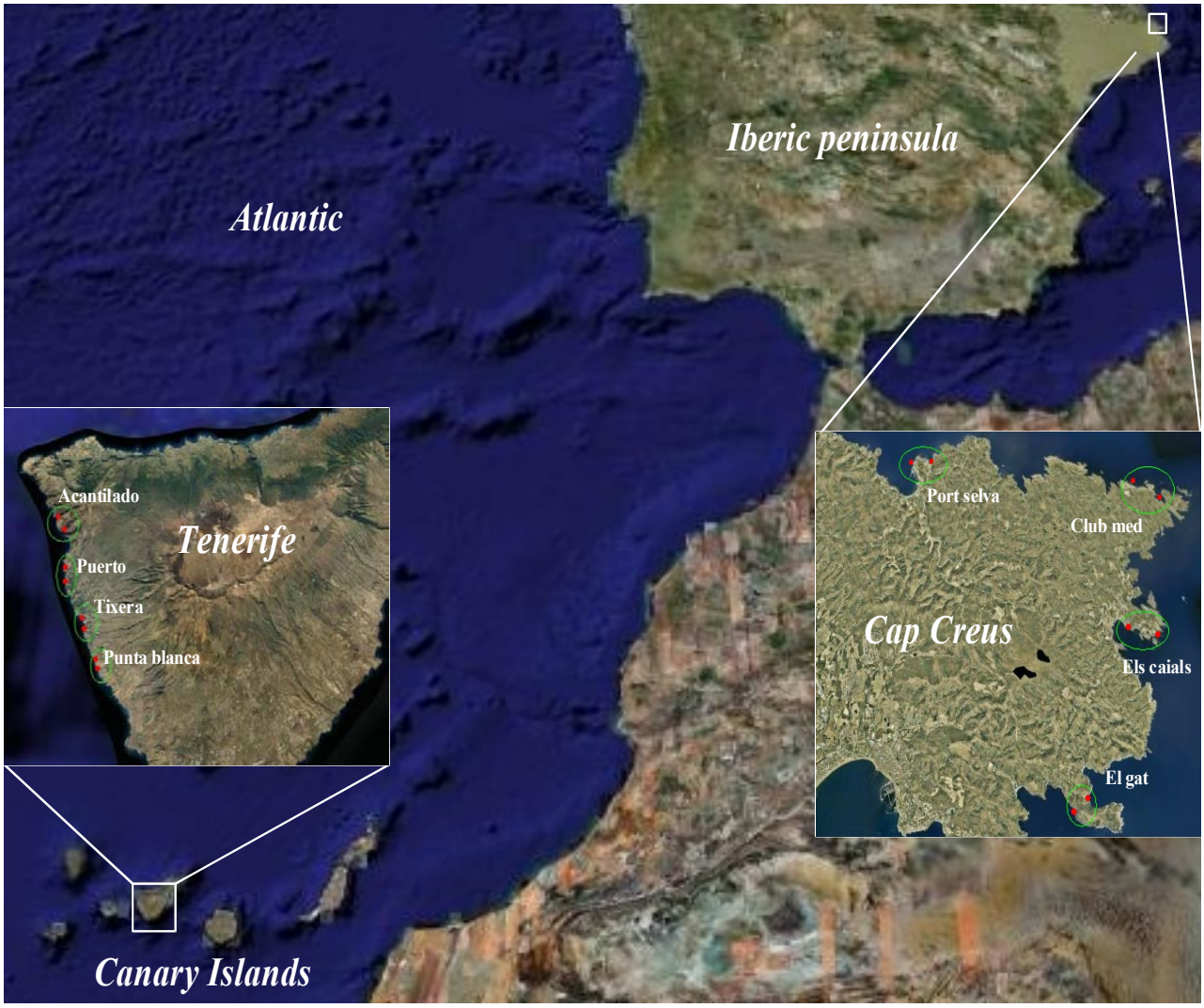

Fig. 12. Simplified map of western Mediterranean sea and near Atlantic. Sites are represented by red dots. Locations are named and are represented by green circles.

7. ABSTRACT

Aplysina aerophoba and *Aplysina cavernicola* are two sibling sponge species common in Mediterranean sea. External and internal morphology is almost identical in the two species. So, according to a lack of traditional taxonomic character (spicule comparison), taxonomists have longly debated if the two taxa, showing different ecological requirements but identical morphology, should be regarded as ecotypes or distinct species. Now, molecular phylogeny has brought response to this question: *A. aerophoba* and *A. cavernicola* are lately distinct species. The two species, also called bacteriosponges because of their dense community of symbiotic bacteria that can amount up to 40 % of their dry weight, can contain high amounts of secondary metabolites: brominated alkaloids derived from tyrosine (up to 13% of dry weight).
As part of a program in chemical ecology where the main goal is to better understand the processes controlling the chemical diversity and its variation in marine invertebrates, we finalized the methodology to quantify the major secondary metabolites produced by the two Mediterranean *Aplysina* species, we identified chemotaxonomic markers in order to clearly discriminate the two sibling species, we explored whether variation, in quality and quantity, of secondary metabolites expression occurs at different biogeographic scales, and we investigated the role of microbial symbionts in bromotyrosine alkaloids biosynthesis.

8. RÉSUMÉ

Aplysina aerophoba et *Aplysina cavernicola* sont deux espèces d'éponges Méditerranéennes. Leur morphologie interne et externe est trés semblable (on les dit “espèces voisines”). Les taxonomistes ont longtemps débattu sur la position spécifique de ces deux espèces, en raison de leurs forte ressemblance malgrés des caractéristiques écologiques trés différentes. De nos jours, les techniques de phylogénie moléculaire ont permis de trancher: *A. aerophoba* et *A. cavernicola* sont deux espèces distinctes depuis peu. Ces deux espèces aussi appellées “bacteriosponges”, car elles peuvent habriter une communautée de bactéries symbiotiques représentant 40 % de leur poids sec, contiennent également de grandes concentrations de métabolites secondaires: alcaloides bromés dérivant de la tyrosine (jusqu'à 13 % de leur poids sec).
En tant que membres d'un programme européen d'écologie chimique dont le but principal est de mieux comprendre les processus controlant la diversité chimique chez les invertébrés marins, nous avons dans cette étude: Finalisé une méthodologie pour quantifier les métabolites secondaires majoritaires produits par les deux espèces Méditerranéennes d' *Aplysina*. Nous avons identifié des marqueurs chemotaxonomiques permettant de clairement discriminer ces deux espèces voisines. Nous avons observé la variation qualitative et quantitative du métabolisme secondaire a différentes échelles biogéographiques. Et, enfin, nous avons étudié le role des microorganismes symbiotiques dans la biosynthèse des alcaloides dérivés de la bromotyrosine.

9. KEYWORDS

Chemical ecology; Secondary metabolites; Aplysina sponges; Chemotaxonomy; Symbiotic microorganisms