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Chemical bioactivity of sponges along an environmental gradient in a Mediterranean cave

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SUMMARY: The bioactivity of the most abundant sponges from three communities in a Mediterranean cave was assessed by Microtox® assay in two seasons, spring (June) and autumn (November). We quantified bioactivity as a proxy for the investment in production of biologically active substances, and we related sponge bioactivity to growth form, growth rates, and physical contacts of each species with other species. We established a threshold for classifying a species as bioactive based on a comparison between the results of the Microtox® and the sea urchin embryo toxicity bioassay. A total of 30 species were included in the study, of which 50% were bioactive in some community or season. Significant ecological (between communities) and seasonal variation in mean bioactivity was found. When sponge bioactivity was related to sponge growth shape, it was found that the encrusting species tended to be more toxic than the non-encrusting ones. There was a negative relationship between bioactivity and sponge growth, suggesting a trade-off in energy allocation to defence and to other biological functions. Furthermore, a negative correlation was found between bioactivity and positive associations with other species. These results highlight the important role of chemically-mediated interactions in cave communities.

Keywords: sponges, bioactivity, natural toxicity, Microtox® assay, temporal variation, ecological variation, caves, western Mediterranean.

RESUMEN: BIOACTIVIDAD QUÍMICA DE LAS ESPONJAS A LO LARGO DE UN GRADIENTE AMBIENTAL EN UNA CUEVA MEDITERRÁNEA. – La bioactividad de las esponjas más abundantes en tres comunidades a lo largo de una cueva mediterránea fue cuantificada por medio del bioensayo Microtox® en dos estaciones, primavera (junio) y otoño (noviembre). La bioactividad medida se usó como una aproximación a la inversión en producción de sustancias bioactivas, y se relacionó la bioactividad de las esponjas con su morfología, tasas de crecimiento, y contactos de cada especie con otras. Se estableció un umbral para determinar si una especie es bioactiva mediante una comparación entre el test Microtox® y el test de biotoxicidad en embriones de erizo. En total se estudiaron 30 especies, de las que un 50% fue bioactivo en alguna comunidad o estación del año. Se encontraron importantes diferencias ecológicas (entre comunidades) y estacionales en la bioactividad media. Cuando se relacionó la bioactividad con la morfología de las esponjas se encontró que las esponjas incrustantes tendían a ser más tóxicas que las de otras morfologías. Se detectó una correlación negativa entre bioactividad y crecimiento, lo que sugiere un balance entre inversión de energía en defensa y en otras funciones. Por otro lado, se encontró una correlación negativa entre bioactividad y las asociaciones positivas con otras especies. Estos resultados ponen de manifiesto el importante papel de las interacciones mediadas por sustancias químicas en comunidades de cuevas.

Palabras clave: bioactividad, toxicidad natural, bioensayo Microtox®, variación temporal, variación ecológica, cuevas, Mediterráneo occidental.

INTRODUCTION

Studies of the chemical ecology of marine organisms have consistently shown that sponges are among the main producers of biologically active substances (Amade *et al.*, 1987; Uriz *et al.*, 1992; Newbold *et al.*, 1999). Bioactive secondary metabolites have been found in all orders of demosponges (Garson, 2001), and the number of new compounds isolated from sponges is the highest found for marine invertebrates (Blunt *et al.*, 2008, and references therein).

The ecological role of sponge secondary metabolites in nature has been analysed in a number of studies. It has been shown that many of the bioactive compounds have an antipredatory function (e.g. Chanas and Pawlik, 1995; Pawlik *et al.*, 1995; Becerro *et al.*, 2003; Burns *et al.*, 2003). Occasionally, although rarely, an allelopathic function has been documented (Porter and Targett, 1988; Turon *et al.*, 1996a; Thacker *et al.*, 1998; Engel and Pawlik, 2000). This has been analysed in more depth in sponges in which exudation of active metabolites has been proven (Thompson, 1985; Walker *et al.*, 1985). Moreover, the multifunctional nature of some secondary metabolites of sponges has been reported (Becerro *et al.*, 1997a; Thacker *et al.*, 1998; Newbold *et al.*, 1999), which gives an idea of the multiple roles that they may play in nature by mediating relationships among marine organisms.

The variability in the production of secondary metabolites in sponges has been documented at inter- and intra-specific levels (e.g. Turon *et al.*, 1996b; Betancourt-Lozano *et al.*, 1998), and more rarely within specimens (Turon *et al.*, 1996b; Becerro *et al.*, 1998; Schupp *et al.*, 1999). Genetic, biological, and/or environmental factors (both biotic and abiotic) can account for this variation. The genetic component of this variability has not been explored yet, and only the environmental factors (i.e. light, nutrient availability, pollutants) and biotic pressures (i.e. space competition, fouling or predation) to which sponges are exposed have been considered to date (Uriz *et al.*, 1995; Becerro *et al.*, 1997b; Agell *et al.*, 2001).

In this study we analysed the bioactivity and how it varies in the most abundant sponges from 3 different communities of a cave in the western Mediterranean, in two seasons of the year (spring and autumn). Although there are a few studies on the community structure and dynamics of Mediterranean caves (e.g. Gili *et al.*, 1986; Bibiloni *et al.*, 1989; Zabala *et al.*, 1989), to our knowledge chemically mediated interactions in invertebrate assemblages of caves have never been investigated (but see Martí *et al.*, 2004a, for a study on seaweeds).

We used a standardised bioactivity assay as a proxy for production of bioactive substances since the high number of species and samples to be analysed precluded individual identification and quantification of the bioactive secondary metabolites (Martí *et al.*, 2003). We selected the Microtox® method, which quantifies toxicity against a marine photobacterium (Ribo and Kaiser, 1987; Ribo and Rogers, 1990). It is highly repeatable and precise, and allows toxicity in taxonomically diverse species to be quantified. We admit that this method, like other general assays, cannot detect all kinds of ecologically relevant bioactivities. However, it correlates well with a panoply of other biological assays (e.g. Botsford, 2002) and, particularly for sponges, other ecologically relevant tests (Becerro *et al.*, 1995, Martí *et al.*, 2003). Given these good correlations, we used this method here for comparative purposes to detect intra- and interspecies variation and community-level patterns.

To interpret the possible biological role of the bioactivity detected and its seasonal and ecological variation, we assessed the relationship of several biotic parameters (i.e. growth shape, seasonal changes in coverage, and contacts with other species) with sponge bioactivity. Sponge growth form may influence intra-species interactions and competition, since vertical growth makes colonial invertebrates less dependent on the available free substratum (e.g., mound-, tree-, and vine-like organisms) than encrusting, two-dimensional forms (sheet-like organisms) (Jackson, 1979), which rely heavily on the acquisition and maintenance of free substratum. However, the production of chemical defences is costly in most cases (Cronin, 2001). Therefore, organisms may partition the available resources to different activities for their well-being (i.e. growth, reproduction, defence). In most cases this implies trade-offs in resource allocation from one process to another (López-Legentil *et al.*, 2007). We compared the allocation of resources to growth or defence by relating seasonal changes in coverage with the bioactivity levels of the species.

Since it has been demonstrated that space competition is strong in benthic species inhabiting hard substrata, and that allelochemical production is one of the mechanisms involved (e.g. Jackson and Buss, 1975; Jackson, 1977; Engel and Pawlik, 2005a,b), we focused on the possible effect of allelochemical (toxic metabolites) production on competition for the substrata. Thus, we related sponge bioactivity to the number of contacts with other species, as an indirect measure of competition for space.

Therefore, we investigated: (1) the abundance of bioactive sponge species in the cave studied, (2) intra- and interspecies variation in bioactivity, (3) ecological (between communities) and temporal (between seasons) variation in bioactivity and, (4) we related the patterns of variation found to the biological and ecological characteristics of the species.

We aimed to gain a new perspective on the abundance of bioactive species of sponges in a little known community type (caves) and to assess the extent of the seasonal and ecological variation in bioactivity, as well as the potential implications of this variation at both the species and the community levels.

MATERIAL AND METHODS

Sampling

Sampling was performed by Scuba diving in Cova Blava cave in Cabrera archipelago (Balearic Islands, western Mediterranean, 39°09'37.93"N; 2°56'47.30"E). A description of the topography of this cave can be found in Martí *et al.* (2004b). The three communities sampled followed a horizontal gradient. These communities were: a sciaphilic seaweed community (SSC) dominated by seaweeds close to the cave's entrance, and two contiguous semi-dark cave communities, dominated by sponges, one located at a more external position (external semi-dark community, ESC), and the other located in the innermost part of the cave (internal semi-dark community, ISC). The rationale for choosing these categories, and a detailed description of the communities, is given in Martí *et al.* (2004b,c). Our communities corresponded to zone 2, zone 3, and zone 4 of the Cabrera cave in the terminology by Martí *et al.* (2004b).

According to previously recorded quantitative inventories (Martí *et al.,* 2004b) we collected the most abundant species from each community in order to assess bioactivity. Whenever the abundance and available biomass allowed it, we collected replicates for each species. Replicates (n=3 to 6) consisted of different individuals or colonies of a species located at least 1 m apart from each other.

Chemical extraction

Once in the laboratory, samples were cleaned of epibionts and separated from the substratum and from any foreign bodies when necessary. Afterwards, they were frozen, lyophilised, and stored at -20°C for later chemical extraction.

A known weight of each lyophilised sample (ca. 0.5 g) was ground in a mortar, and extracted three successive times in 10 ml of methanol for 5, 10, and 15 minutes respectively using an ultrasonic bath. We used methanol as a solvent because we specifically targeted the range of polar compounds that are diffusible in water and thus are the most likely to be involved in competitive interactions. Non-polar compounds are usually bound inside or at the surface of organisms (De Nys *et al.,* 1998).

The solvent from the three extractions was filtered, pooled, and completely evaporated under reduced pressure and a nitrogen stream. The dry crude extracts were then weighed and kept at –20°C until analysis.

Bioassays and statistical analyses

Dried crude extracts were resuspended in artificial sea water to obtain a final concentration of 1000 µg crude extract ml-1 for analysis by Microtox®. Since the dilution factor was 2, the concentrations tested were 500, 250, 125, and 62.5 µg ml^{-1} (i.e. 50%, 25%, 12.5%, and 6.25% of the initial concentration).

The Microtox® assay measures the decrease in light emitted by a marine bioluminescent bacterium (*Vibrio fischeri*) in gamma units, and was used here as a measure of toxicity. In order to compare results across samples we used the gamma value corresponding to the concentration of a 1 mg sample of DW ml^{-1}. This value was obtained by using the regression equation in gamma units on the concentration resulting from the Microtox® assay (Martí *et al.,* 2003) and the known relationship between the dry weight and extract weight. By referring the results to the same concentration of the sample weight, we could compare all the samples even if the proportions of crude extract per sponge weight varied across species.

We compared mean bioactivity using 2-way ANOVA with community and season as factors, pooling all species to obtain an overall picture. For the species for which we had replicate samples (ca. 85% of the total) we performed statistical comparisons of bioactivity at species level. For species only present in one community, t-tests were used to check differences between seasons. For species present in more than one community we performed two-way ANOVAs with community and season as factors.

Qualitative classification of the sponges depending on their toxicity

The bioactivity measure used was a continuous variable and we did not know at which level the sponges had potentially noxious effects on other

species; therefore, we established a criterion based on the relationship between the Microtox® assay and the sea-urchin test (Martín and Uriz, 1993; Martí *et al.*, 2004a) to classify species into either a bioactive (toxic) or non-bioactive category. A threshold of 0.5 gamma units/mg sample was found to be appropriate to separate non-toxic from toxic species, because all the samples that reached this value had detrimental effects on sea urchin embryos (see Martí *et al.*, 2004a, for a more detailed explanation).

We determined the abundance of species which were always toxic (gamma values higher than 0.5) in all communities and seasons (hereafter always-bioactive species), those which were not toxic in any community or season (hereafter never-bioactive species), and the ones which were occasionally toxic (hereafter occasionally-bioactive species). The percentages of these categories were compared between communities. We also measured and compared the mean bioactivity of the species in each category.

Relationships between bioactivity, taxonomic group and the biological parameters

In order to determine potential phylogenetic patterns, we classified the species found in the different sponge orders and compared the bioactivity of those that were well represented in our samples by ANOVA. We also categorised the sponge species into the main growth forms found (sheet-like, mounds, and tree-like) and analysed differences in bioactivity between them using ANOVA.

The growth rates of the species were approximated by measuring changes in coverage of species between the two sampling dates. The coverage data was obtained from a previous work (Martí *et al.*, 2004b), in which 20 pictures (each covering ca. 310 cm^2) were taken at random in each community and season. The cover area of all benthic species identifiable in the pictures was calculated. This dataset included most sponges considered in this work. From this information we calculated the seasonal changes in coverage using the formula:

$$SCC=(C_N-C_J)/C_J$$

where SCC is the seasonal change in coverage, C_N the coverage in November and C_J the coverage in June. These changes in coverage were compared with the mean bioactivity value of the species by correlation analysis.

To assess possible implications of bioactivity levels to spatial competition abilities, we recorded the contacts among all species (algae and invertebrates) present in the same 20 pictures per community and season taken to measure species coverage, using a modification of the method described in Turon *et al.* (1996a). These contacts were tabulated in triangular matrices. The diagonal was set to 0 as we were only interested in interspecific contacts. Using a log-linear model of quasi-independence (Knoke and Burke, 1991, implemented in Systat v11), we calculated the expected matrix of contacts under the assumption of independence between rows and columns (the first and the second species of any contact respectively), setting the diagonals to 0 as in the observed matrices. We then determined the species that were positively or negatively associated (i.e. more or less in contact than expected on the basis of species abundance alone) with others. We estimated the significance of these associations by randomly resampling by replacing the expected matrix of contacts (obtained under the assumption of independence). The procedure was repeated 1000 times to produce a distribution that mimics the variation in the number of contacts that can be found by chance alone in the absence of interspecific associations.

We used these generated distributions to test the significance of the observed associations by comparing the number of contacts observed for each species-pair with the distribution generated. When the value observed fell within the distribution generated, we concluded that these two species did not have significantly more or less contacts than expected due to their respective abundances. However, when the value fell outside the distribution generated or in 2.5% of the most extreme values at the two tails of the distribution, we concluded that the two species showed a significant positive or negative (according to the sign or the difference) association (0.05 significance level). The matrices included all relevant species in the communities; however, we have only focused here on the results for sponges. We computed the correlation between the mean bioactivity levels of each sponge species and the number of significant positive associations with other species, as a measure of the amount of physical interactions of these species.

In all parametric analyses, when data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett test), rank transformation was carried out and analyses

TABLE 1. – Values of bioactivity (gamma units) of sponges from Cabrera cave in the different communities and seasons. For species with replicate samples, the SE of toxicity values is indicated between parentheses. Codes after the species name refer to bioactivity category/order/growth shape. Codes for bioactivity category: A, always bioactive; N, never bioactive; O, occasionally bioactive. Codes for orders: Ag, Agelasida; As, Astrophorida; Ch, Chondrosida; D, Dictyoceratida; Hc, Halichondrida; Hd, Hadromerida; Ho, Homoscleromorpha; Hp, Haplosclerida; Pe, Petrosida; Po, Poecilosclerida. Codes for growth form: S, sheet-like; M, mound; T, tree-like.

Species	Bioactivity (gamma units)					
	SSC		ESC		ISC	
	June	Nov.	June	Nov.	June	Nov.
Acanthella acuta (O/Hc/T)	0.483 (0.005)	0.669 (0.077)	0.424 (0.195)	0.200 (0.105)	0.488 (0.059)	0.221 (0.108)
Agelas oroides (N/Ag/M)			0.375 (0.065)	0.308 (0.061)	0.289 (0.052)	0.497 (0.071)
Axinella damicornis (A/Hc/T)	1.999 (0.700)	2.188 (0.192)	1.010 (0.875)	1.221 (0.414)	2.370 (0.948)	1.969 (0.820)
Crambe crambe (A/Po/S)	*	*	*	*	*	*
Chondrosia reniformis (N/Ch/M)			0.214 (0.067)	0.335 (0.036)	0.190 (0.083)	0.174 (0.025)
Dendroxea lenis (O/Hp/S)			1.211 (0.560)	8.555 (2.561)	4.462 (4.039)	0.077 (0.023)
Dictyonella sp. (O/Hc/M)			0.349 (0.159)	0.702 (0.093)	0.452 (0.115)	0.493 (0.165)
Diplastrella bistellata (O/Hd/S)					0.743 (0.204)	0.472 (0.138)
Dysidea avara v. *pallescens* (A/D/S)					12.618 (1.946)	
Erylus euastrum (N/As/M)			0.018 (0.011)	0.004 (0.002)	0.000 (0.000)	0.000 (0.000)
Eurypon sp. (N/Po/S)			0.032	0.002	0.098 (0.044)	0.004 (0.001)
Hexadella pruvoti (N/Po/S)					0.082	0.007
Hymedesmia sp. 1 (O/Po/S)			0.03 (0.014)	2.568 (2.168)	0.016 (0.008)	9.447 (6.184)
Hymedesmia sp. 2 (N/Po/S)					0.808 (0.100)	0.033 (0.030)
Hymedesmia sp. 3 (A/Po/S)						0.777 (0.059)
Hippospongia communis (N/D/M)					0.324 (0.012)	0.244
Ircinia oros (A/D/M)			1.287 (0.370)	0.556 (0.177)		
Ircinia variabilis (O/D/M)	0.145 (0.145)	1.267 (0.092)	0.129 (0.048)	2.087 (0.862)	1.474 (0.469)	0.336 (0.162)
Merlia lipoclavidisca (N/Po/S)					0.074	
Microciona sp. (N/Po/S)					0.049 (0.000)	0.015
Myceliospongia sp. (A/?/S)					17.483 (6.544)	1.254 (0.721)
Oscarella tuberculata (A/Ho/M)			0.665 (0.014)	2.510 (0.802)		
Petrosia ficiformis (N/Pe/M)					0.000	
Phorbas tenacior (N/Po/S)			0.181 (0.043)	0.096 (0.029)	0.310 (0.059)	0.273 (0.046)
Pleraplysilla spinifera (A/D/S)			1.263 (0.192)	1.399 (0.334)	0.914	
Raspaciona aculeata (O/Po/S)			0.314	0.574	0.165	
Sarcotragus spinosula (N/D/M)			0.000 (0.000)	0.000 (0.000)		
Spirastrella cunctatrix (A/Hd/S)			1.010 (0.268)	1.310 (0.122)	1.079 (0.127)	0.896 (0.131)
Terpios fugax (N/Hd/S)					0.202 (0.037)	0.064 (0.046)
Topsentia garciae (N/Hp/S)					0.000 (0.000)	0.018 (0.009)

* Toxicity was too high to calculate a regression line with the concentrations used, and therefore no gamma value could be established for this species.

were performed on ranked data (Conover and Iman, 1981; Potvin and Roff, 1993). Student-Newmann-Keuls post-hoc tests were used for *a posteriori* comparisons. All the analyses were carried out with the packages Systat v11 and Sigmastat v.1. A Turbo Pascal routine was written to perform the randomisation procedure and is available on request.

RESULTS

Sponge toxicity

A total of 30 sponge species were collected in Cova Blava cave. More than half (56.6%) of them were present in more than one community (Table 1). We analysed 4 species from SSC, which represented 63% and 56% of the sponge cover in the community in June and November respectively (coverage data obtained from Martí *et al.,* 2004b). 18 species were studied from ESC, which represented 95% of sponge coverage in both seasons; and 26 species from ISC, which represented 75% and 97% of the sponge cover in the community in June and November respectively. The sponges analysed were therefore the most representative of the phylum in the communities studied.

Table 1 lists the sponge species with their bioactivity (in gamma units) measured in the two seasons. We could obtain replicate samples within communities for most species (94%). When all species were pooled, bioactivity levels showed different patterns of variation among the communities in spring (June) and autumn (November) (Fig. 1). In spring, bioactivity was higher than in autumn in the innermost community (ISC) and the reverse occurred in the other two communities (SSC and ESC). Accordingly, a 2-way ANOVA revealed a significant interaction term between community and season (Table 2). The highest mean toxicity was obtained in the inner com-

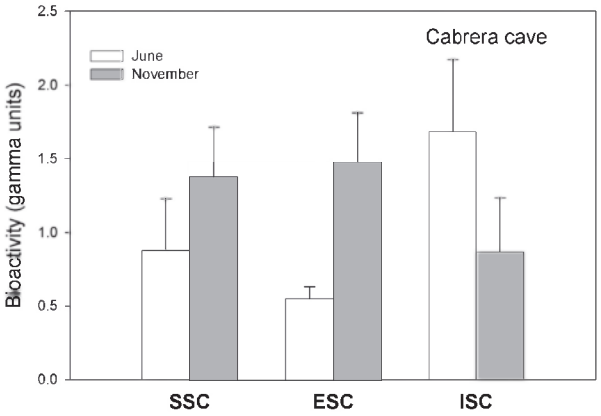

FIG. 1. – Mean values of bioactivity of the samples analysed in the different communities and seasons. Bars are standard errors. SSC, sciaphilic seaweed community; ESC, external semi-dark community; ISC, internal semi-dark community.

TABLE 2. – Two-way ANOVA of the bioactivity of sponges from Cabrera cave according to community and season, all species pooled (rank-transformed data)

Source	SS	DF	MS	F	P
Community	51673.4	2	25836.7	4.86	0.009
Season	14338.5	1	14338.5	2.70	0.102
C*S	51417.4	2	25708.7	4.83	0.009
Error	1345483.7	253	5607.7		

munity (ISC) in June, followed by the intermediate community (ESC) in November (Fig. 1).

A total of 3 species with replicates from SSC, 16 from ESC and 26 from ISC were analysed statistically. The species *Crambe crambe* could not be used in these analyses because of its high toxicity, which did not allow a regression line to be calculated as all bacteria were killed at the lowest concentrations tested. Among the species present in only one community for which we had replicates (n=10), significant seasonal differences in bioactivity were found in: *Oscarella tuberculata* in ESC, which was more toxic in autumn than in spring (Student *t*-test, $p<0.001$); and *Hymedesmia* sp.2 ($p<0.005$) and *Myceliospongia* sp. ($p<0.05$) in ISC, which were more toxic in spring than in autumn, and had much lower standard errors (lower intra-species variation) in autumn than in spring.

Among the species present in two communities with replicates (n=8), only 2 had significant outcomes in 2-way ANOVAs: *Dendroxea lenis* showed a significant interaction term (Table 3), which indicates that its seasonal variation in bioactivity was different in each community. The species is more

TABLE 3. – Two-way ANOVAs of the bioactivity of sponges from Cabrera cave present in more than one community in June and November. Only species with significant outcomes are shown.

Source	SS	DF	MS	F	P
Dendroxea lenis					
Community	62.83	1	62.83	7.06	0.021
Season	2.83	1	2.83	0.32	0.583
C*S	127.44	1	127.40	14.33	0.003
Error	106.75	12	8.90		
Hymedesmia sp. 1					
Community	1.33	1	1.33	0.24	0.636
Season	96.33	1	96.33	17.52	0.003
C*S	1.33	1	1.33	0.24	0.636
Error	44.00	8	5.50		
Ircinia variabilis					
Community	13.00	2	6.50	0.75	0.494
Season	60.50	1	60.50	6.97	0.022
C*S	306.33	2	153.17	17.65	0.000
Error	104.17	12	8.68		

bioactive in autumn than in spring in ESC ($p<0.05$), while in ISC it was significantly more bioactive ($p<0.05$) in spring than in autumn. *Hymedesmia* sp. 1 from ISC was significantly more bioactive in spring than in autumn ($p<0.005$) (Table 3). The three species present in the three communities (*Acanthella acuta*, *Axinella damicornis,* and *Ircinia variabilis*) showed different trends. Significant differences in bioactivity were only found for *I. variabilis*, which showed a significant interaction between season and community (Table 3), and was more bioactive in November than in June both in the SSC ($p<0.005$) and ESC ($p<0.05$) communities, while in ISC, seasonal differences were not detected.

Qualitative classification of the sponges depending on their bioactivity

The percentage of species belonging to the three categories previously established were: 46.6% of never-bioactive species (14 species), 30% of always-bioactive species (9 species), and 23.3% of occasionally-bioactive species (7 species) (Table 1). The mean bioactivity values of the species comprising each category (averaged across seasons and communities, if applicable) were clearly different (Fig. 2A), and were 0.132 ± 0.039 gamma units (mean ± SE) for the never-bioactive species, 1.339 ± 0.514 for the occasionally-bioactive, and 3.666 ± 1.632 for the always-bioactive. Note the increase in dispersion (higher SE) for the last two categories. The ANOVA analysis of these values (rank-transformed) showed significant differences between categories ($p<0.001$),

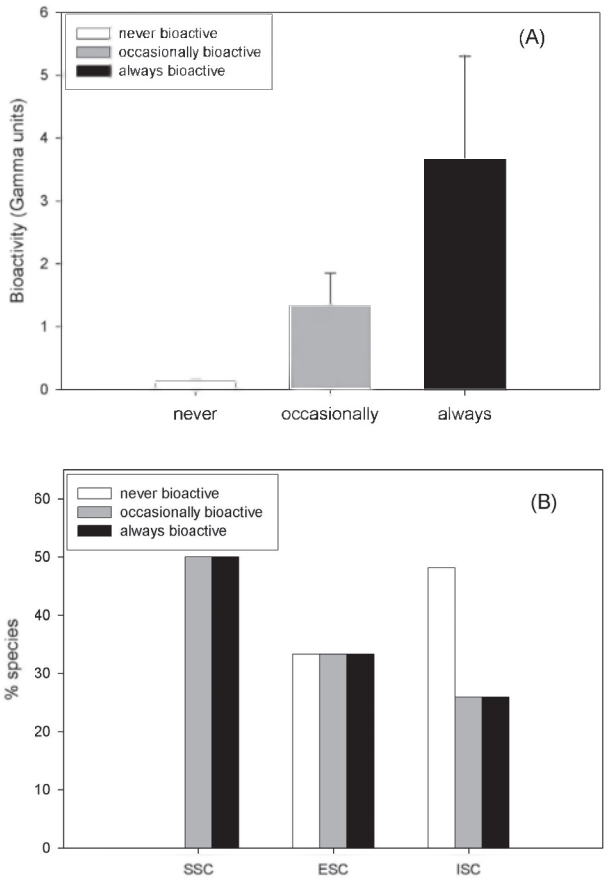

FIG. 2. – (A) Values of bioactivity of the species for each bioactivity category. The mean measure for each species (averaged across season and community, if applicable) was used. Bars are standard errors. (B) Percentage of sponge species belonging to each bioactivity category in the different communities of the cave. SSC, sciaphilic seaweed community; ESC, external semi-dark community; ISC, internal semi-dark community.

and the post-hoc test revealed that only the comparisons between never-bioactive species and the other two categories were significant.

There was a clear increasing trend in never-bioactive species from the external communities to the most internal ones, while the always-bioactive and occasionally-bioactive species decreased along this same gradient in the cave (Fig 2B).

Relationships between bioactivity, taxonomic group and the biological parameters

Ten orders of sponges were represented in our samples (Table 1), but only five of them had more than one species. Of these, Dictyoceratida and Haplosclerida showed the highest bioactivity and Hadromerida and Poecilosclerida the lowest (Fig. 3), although the results for the latter may be misleading as the highly bioactive poecilosclerid Crambe crambe could not be evaluated, as explained above. However, bioactivity varied greatly within orders and an ANOVA showed no significant differences between taxonomic groups (rank-transformed gamma values, p=0.659). Myceliospongia sp. could not be assigned to any order, as this genus is at present incertae sedis (Vacelet et al., 2002).

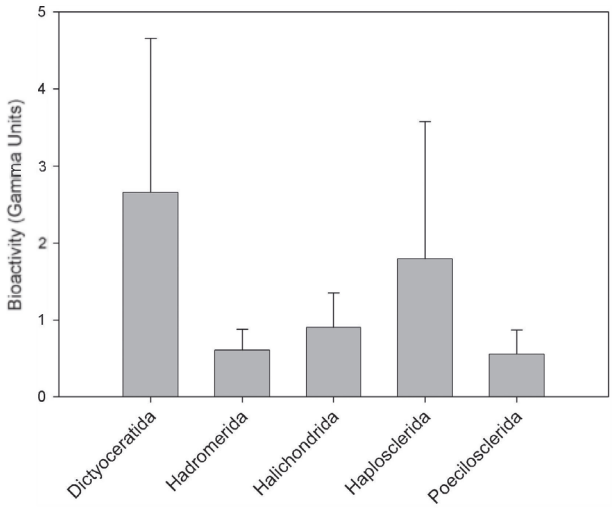

FIG. 3. – Mean values of natural bioactivity of sponges per order. Orders represented by a single species are not shown. Bars are standard errors.

Sheet-like (encrusting) sponge forms tended to be the most bioactive (Fig. 4). The differences among growth forms, however, were not significant (ANOVA on rank-transformed values, p=0.566), due to high interspecies variability.

We obtained information on coverage in both seasons for 27 species (from Martí *et al.*, 2004b) and we could therefore compare the mean bioactivity found in a particular community (averaging across seasons)

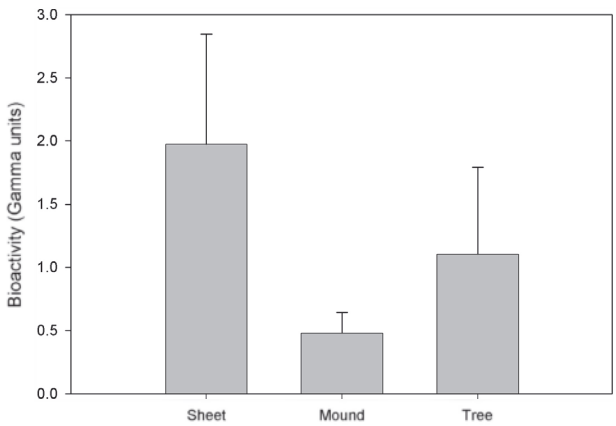

FIG. 4. – Mean values of natural bioactivity in sponges according to their growth form and season. Bars are standard errors.

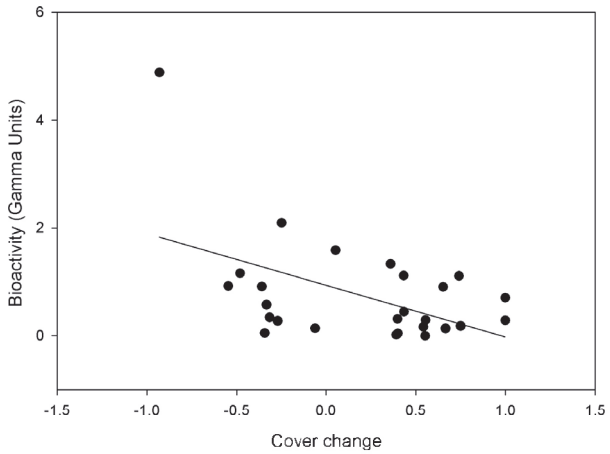

FIG. 5. – Relationship between species bioactivity and growth rates estimated as changes in coverage between observation times.

with the monthly growth rate estimated from the changes in coverage of that species in that community from spring to autumn (Fig. 5). We found a significant negative relationship (r=-0.505, p=0.008), which indicates a lower growth trend in species with high bioactivity. The relationship was still significant if we removed the extreme point on the left, in Figure 5.

Not all sponge species appeared in sufficient abundance in the pictures to allow contacts to be quantified. Among those that were sufficiently represented, all the species showed some significant associations (either positive or negative) with other species. The bioactivity of each sponge species in the cave was plotted against the percentage of species that were positively associated with it (significant relationships detected by the randomisation routine) to assess the relationship between bioactivity and associations (Fig. 6), and a significant negative trend was found ($r = -0.36$, $p<0.05$).

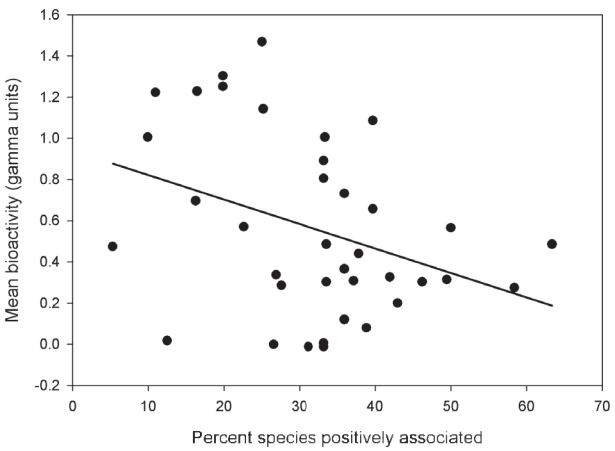

FIG. 6. – Relationship between species toxicity and percentage of significant positive associations.

DISCUSSION

A total of 50% of the sponge species analysed was bioactive (mean bioactivity higher than 0.5 gamma units) at least in one season or community. The percentage of bioactive species found is similar to that reported for Mediterranean sponges in a previous study using different bioassays (Uriz *et al.*, 1991), and agrees with the high percentage of bioactive sponge species detected in other studies from different geographical regions (e.g. Amade *et al.*, 1987; Uriz *et al.*, 1992; Newbold *et al.*, 1999).

Large interspecies variation was found in all communities and seasons, but when species were pooled to analyse the overall trends, the pattern of change in bioactivity among communities varied according to the season, with the highest mean bioactivity recorded in the innermost community in spring.

However, at species level, large variation in bioactivity was detected within species as well as between communities (ecological variation) and between seasons (temporal variation). Variation of bioactivity in sponges has already been described and has been attributed to different causes. Ecological variation in production of bioactive molecules has been addressed by comparing the concentration of secondary metabolites or bioactivity of specimens of the same species living in different habitats. The results of these types of studies on sponges are often contradictory. Thompson *et al.* (1987) found higher concentrations of diterpenes in specimens of *Rhopaloeides odorabile* living in illuminated habitats than in those living in shadow habitats, and speculated that light activates the biosynthesis of diterpenes or some intermediate metabolite. Becerro *et al.* (1997b) found the opposite trend for *Crambe crambe*. In this case, differences in bioactivity were related to different competition pressures for the available space.

There was significant seasonal variation of sponge bioactivity (the mean percentage of change in the three communities was 64.3%). In SSC and ESC, most species tended to be more bioactive in autumn than in spring, while in ISC the opposite trend was found. As expected, the always-bioactive species have less seasonal variation in bioactivity than the occasionally-bioactive species. There have been few reports of seasonal variation in the production of sponge secondary metabolites (Turon *et al.*, 1996b). Antimicrobial bioactivity varied seasonally in sponges from shallow Mexican waters, which were more bioactive during the warmest parts of the

year (Green *et al.*, 1985). The proliferation of bacteria and possible pathogens in the warmest months was pointed out as the cause for the increase in the production of the sponge defensive compounds.

The number of non-bioactive species increased towards the inner part of the cave, while the reverse was true for the occasionally or always bioactive species. The seasonal pattern of bioactivity in the innermost community was also the opposite of that found in the external and intermediate communities (see above). All this suggests a particular dynamic in this community where inter-species contacts are less frequent since the space is not saturated, but where food depletion could strongly affect the biology of the species present.

We did not find clear evidence of a phylogenetic pattern in the bioactivity of sponges. The different orders analysed had bioactive species, although in varying percentages. More species should be studied before any phylogenetic pattern can be formally outlined. We did not find significant differences between growth forms, although encrusting sponges tended to be more bioactive than non-encrusting forms (mounds and tree-like morphologies). We should mention again that we couldn't include the most toxic sponge found, *Crambe crambe*, in the calculations because its toxicity was too high for the concentrations tested. This species is encrusting and its inclusion would undoubtedly have resulted in a significant increase in mean bioactivity of the encrusting category. Encrusting forms are characterised by a higher proportion of surface area per volume unit than non-encrusting forms; hence encrusting sponges have more potential for interactions and may need more powerful chemical warfare. Furthermore, it has been reported that the spherulous cells of sponges, which are concentrated at the periphery of the sponge (Uriz *et al.*, 1996; Willenz and Pomponi, 1996) represent the storage compartment of the bioactive compounds (Uriz *et al.*, 1996; Turon *et al.*, 2000). The high area to volume ratio of encrusting forms would allow them to accumulate more spherulous cells per biomass unit at their surfaces and thus become more bioactive than massive forms or non-encrusting forms in general.

In the western Mediterranean area, most sponges grow at very low rates (Turon *et al.*, 1998, Garrabou and Zabala, 2001; De Caralt *et al.*, 2008). In our case, there was a similar number of species that increased or decreased coverage between spring and autumn, and Martí *et al.* (2004b) did not find a clear growth pattern. We did find a trade-off between growth and production of toxic substances, as there was a significant negative relationship between cover change (here used as an estimation of growth rates) and bioactivity levels. Trade-offs of toxicity with other parameters have rarely been described for sponges (Uriz *et al.*, 1995), but they are consistent with a negative relationship with reproduction rather than with growth. Likewise, no negative relationship was found between production of bioactive metabolites and growth in other invertebrates (colonial ascidians) (López-Legentil *et al.*, 2007), while a trade-off with reproductive activity seems to shape the seasonal patterns of metabolite production. In our study we detected a negative relation to growth, but we did not analyse reproduction specifically, and more focused research is necessary to ascertain whether there is also a pattern of metabolite production in relation to reproductive activity.

The less bioactive sponge species tended to be in contact with more species than those with a higher toxicity. This significant negative correlation between sponge bioactivity and the number of positively associated species indicates that bioactivity may play an important role in the micro-structuring of animal-dominated benthic communities, as was found for algal-dominated communities from the same cave (Martí *et al.*, 2004a). It has also been reported that allelochemical production plays a role in interference interaction between sponges in Caribbean communities (Engel and Pawlik, 2000, 2005a,b).

The results of this extensive correlational study showed that a significant number (50%) of sponge species in the cave communities studied were bioactive above the threshold established. We also found a high degree of variation in bioactivity among individuals, species, and communities. Furthermore, the seasonal component of variation in sponge bioactivity in a temperate sea became manifest. Finally, we have shown that encrusting sponges tend to be more toxic than non-encrusting ones, and that in general bioactive sponges grow at lower rates and have fewer contacts with other species. Taken together, these results indicate that bioactivity plays an important role in structuring the sponge-dominated communities of caves, and provide the preliminary knowledge necessary to design formal experimental studies addressing the causes of the variations in bioactivity found.

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