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Chemical bioactivity of Mediterranean benthic organisms against embryos and larvae of marine invertebrates

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Abstract: Thirty-two species of Mediterranean benthic organisms were assayed for antimitotic-cytotoxic bioactivity against the embryos of the sea urchin *Paracentrotus lividus*. A further 19 of these species were tested for anti-fouling properties (biocide and anti-fixation) against the larvae of the bryozoa *Bugula neritina*. Nineteen of the 32 species were non-active against the sea urchin embryos at the initial concentration of 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ of species crude extract. Highly significant differences ($p > 0.001$) in antimitotic-cytotoxic response were found for the remaining species except *Acanthella acuta*, *Dyctionella obtusa*, *Alcyonium acaule* and *Bugula neritina*. The most active species were the sponges *Crambe crambe* and *Ircinia oros* which displayed antimitotic-cytotoxic activity down to a concentration of 8 $\mu\text{g}\cdot\text{ml}^{-1}$, to be followed by *Reniera mucosa* (down to 30 $\mu\text{g}\cdot\text{ml}^{-1}$) and by *Ircinia fasciculata* and *Pseudodistoma crucigaster*, grey form (down to 125 $\mu\text{g}\cdot\text{ml}^{-1}$).

A bioassay using larvae of *Bugula neritina* showed highly significant ($p > 0.001$) biocide activity of tissue crude extracts for all the species except *Corallina elongata*, *Agelas oroides* and *Microcosmus sabatieri* (tunic). *Acanthella acuta*, *Crambe crambe* and *Pseudodistoma crucigaster* were the most active species (down to a concentration of 60 $\mu\text{g}\cdot\text{ml}^{-1}$). All the assayed species displayed anti-fixation activity. Sublethal toxicity is believed to be responsible for this anti-fixation behaviour at lower concentrations. This may imply that benthic organisms could avoid becoming fouled without the necessity killing larvae, by using small amounts of toxic substances.

Key words: Antifouling; Benthic invertebrate; Chemical bioactivity; Mediterranean Sea

INTRODUCTION

The observed space-temporal variability of bioactivity by benthic organisms (e.g. sponges) has been related both to environmental factors (Thompson et al., 1987) or to the presence and/or abundance of microsymbionts (Bergquist & Wells, 1983) without rigorous demonstration of either hypotheses. Only in the case of some seaweeds (Paul & Hay, 1986) and gorgonians (Harwell & Fenical, 1989), has correlation been found between bioactivity and some biological parameters. In both cases, the variability of the bioactive responses was ascribed to quantitative changes of the toxic metabolites. Nevertheless, bioactivity by chemicals in marine organisms has usually been assessed by testing a single concentration, either of the active metabolites (Thompson et al., 1985; Amade et al., 1987), the different chemical fractions (Wahl & Banaigs, 1991) or the crude extract (Uriz et al., 1991, 1992).

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Most interpretations of the ecological role played by these bioactive substances presuppose their release out of the organism's boundaries (e.g. Jackson & Buss, 1975). Nevertheless, only small scale releases of toxin have been proved (Coll et al., 1982; Walker et al., 1985). Consequently, it would be interesting to know the amount of substance(s) necessary to display a given activity.

The list of problems encountered when trying to quantify toxicity or any other bio-activity by chemicals is extensive. Frequently, several active substances are contained in different chemical fractions of a sole species (i.e. *Crambe crambe*, Berlinek et al., 1992) in such a way that the total active response can be a simple addition of the individual toxicities or the result of a synergistic effect. Consequently, testing the crude extract has been considered an acceptable way to take into account the species activity as a whole, even though it also contains substances other than bioactive metabolites (e.g. salts, fatty acids, proteins).

Antimitotic and cytotoxic activities are closely associated in many Mediterranean benthic invertebrates (Uriz et al., 1991, 1992). Both activities have been chosen to be tested in this study, together with biocide and anti-fixation activities. These activities have been considered because they may represent a suitable combination to act against larvae, embryos, propagules or spores of invertebrates or algae in their attempt to colonize the surface of sessile invertebrates. Consequently, they may serve a function in the species survival affecting its capacity to compete for the substratum and contributing to define the structure of the community.

Among the organisms studied, only six marine sponge species had previously been screened for cytotoxic activity against aquatic organisms, freshwater sponge cells, but no information on the concentration at which the assays were performed was supplied (Van der Vyver et al., 1990). Moreover, none of them had been tested against marine larvae.

MATERIAL AND METHODS

MATERIAL

Thirty-two species of benthic organisms were collected by SCUBA diving along the sub-littoral zone of the Costa Brava, North East of the Iberian Peninsula (North Western Mediterranean). These species included 4 algae, 15 sponges, 1 cnidarian, 1 polychaete, 1 nudibranch, 1 crinoid, 6 tunicates (plus 1 morphotype) and 3 bryozoans, each of which was assayed for activity against embryos of the sea urchin *Paracentrotus lividus* Lamarck. Nineteen species of which 12 sponges, 1 cnidarian, 1 polychaete, 3 bryozoans, and 2 ascidians were tested for anti-fouling properties (biocide and anti-fixation) against larvae of the bryozoan *Bugula neritina* (Linné).

In the case of the solitary tunicate *Microcosmus sabatieri*, the tunic and the soft part were tested separately.

All specimens were transported live to the laboratory, frozen and preserved at -32°C before being extracted.

Reproductive specimens of *P. lividus* and colonies *B. neritina* were collected off the shallow sub-littoral zone (from 0 to 1 m in depth) of the Blanes Bay, the former from hard-bottoms covered by seaweeds and the latter from the submerged hulls of boats.

PREPARATION OF TEST MATERIAL

Since the aim of this study was to detect species (not individuals) bioactivity, pieces of tissues from 4 to 6 specimens of each species up to a total wet weight of 20 g were drained for 1 min after thawing for 60 min, homogenized together with a mortar and pestle, extracted three successive times with 30 ml of methanol/acetone (1:1) at ambient temperature, and centrifuged at 3000 rpm. The supernatant was evaporated at reduced pressure to constant dry weight of the solid residue.

PREPARATION OF TEST SOLUTIONS

Each weighed crude extract was re-dissolved in adequate volumes of acetone to obtain an initial concentration of $10 \text{ mg}\cdot\text{ml}^{-1}$ in every case. Aliquotes of the initial solutions were diluted to obtain solutions of decreasing concentrations for the bioassays. A volume of $250 \mu\text{l}$ of each of these solutions was added to the wells to supply the amount of tissue extract necessary to reach the required test concentrations for the anti-embryo bioassays. In the case of the anti-larvae assays, a volume of 4 ml was added to each Petri dish.

The acetone was totally evaporated prior to the assays, since the presence of small quantities of acetone or other organic solvents (i.e. ethanol), specially at the low concentration such as that needed to avoid toxic effects, enhances embryo division and metamorphosis (Pennington & Hadfield, 1989; authors pers. obs.).

Previous tests to assess the total evaporation time of the acetone were performed by using series of two acetone dosages with three replicates of the treatments and seawater controls. The series were kept at 4°C during periods of 1, 2, 4, 12, 24 and 48 h, respectively, before adding the test embryos and larvae. The dosages of acetone used were those representing the maximum solvent required in the most concentrated assays, that is 0.05 ml and 1 ml for embryos and larvae, respectively. These tests clearly showed that a period of 1 h, in the case of the embryos, and 48 h for the larval assays, was sufficient to ensure the total absence of solvent, that is to say the absence of any effect on the embryos with regard to those of the seawater control.

BIOASSAYS

Antimitotic-cytotoxic bioassays

Antimitotic-cytotoxic assays were conducted on embryos of the sea urchin *P. lividus* placed in multi-well plates (24 wells of 2.5 ml each one). Sea urchin oocytes and

spermatozoids were obtained by injection of 5 ml of a KCl solution (0.1 N) through the aboral membrane of adult specimens (Kobayashi, 1971), and collected in glasses with 100 ml of filtered seawater at ambient temperature (22 °C). External fertilization was carried out at the same temperature by adding 5 ml of seawater with spermatozoids to the 100 ml of seawater containing the oocytes. The fertilization was detected under a stereomicroscope by the presence of the fertilization membrane surrounding the embryos.

The volumes of the tissue extract/acetone solutions required to obtain the successive test dilutions were placed into the assay wells and maintained at 4 °C till the total evaporation of the acetone (cf. above). A volume of 2.5 ml of seawater containing more than 100 recently fertilized eggs (less than 15-min old) was added to each well, and then stirred for a few seconds.

The assays were stopped by adding three drops of 4% formaldehyde when all the fertilized embryos in the control were, at least, at the two-cell stage (\approx 1 h and a half after fertilization).

Four replicates of each concentration were tested in every bioassay, and four of the seawater control.

Antifouling assays

Anti-fouling assays were carried out on larvae of the bryozoan *B. neritina*, a typical fouling species. Mature colonies were maintained in the dark during 24 h in closed-system aquariums at ambient temperature (22 °C). Larvae were obtained from these colonies by light shock (Keough, 1984). Adequate quantities of crude extract/acetone solution were placed in Petri dishes to obtain a uniform bottom layer of crude extract. After the total evaporation of the solvent, 40 ml of filtered seawater containing 100 larvae of *B. neritina* were added to each Petri dish. Fixed (ancestrulae), mobile and dead larvae were counted 24 h after the beginning of the experiment.

Four replicates of each concentration and four of the seawater control were performed in every bioassay.

General procedures

A concentration of 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ of the crude extract was arbitrarily considered high enough to separate active from non-active species. Subsequently, concentrations ranging from 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ to 4 $\mu\text{g}\cdot\text{ml}^{-1}$ for the embryos tests, and from 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ to 60 $\mu\text{g}\cdot\text{ml}^{-1}$ for the larval tests, were simultaneously assayed for the resulting active species. Concentrations lower than 60 $\mu\text{g}\cdot\text{ml}^{-1}$ were not assayed in the anti-larval tests due to the impossibility of obtaining enough larvae for all the replicates from the first light shock, and because a second shock usually produced a high rate of non-viable (immature) larvae.

TREATMENT OF RESULTS

Student-*t* test (Sokal & Rohlf, 1979) was used to detect significant activity ($p < 0.01$) at an initial concentration of $1000 \mu\text{g}\cdot\text{ml}^{-1}$, allowing active species to be separated from non-active.

The effects of the concentration of the crude extract of the resulting active species were tested for significance on arc-sin transformed percent data, using one-way ANOVA analysis. Multiple comparisons of means followed the Tukey's test (Tukey, 1953).

An activity index (*I*) was calculated for the antimitotic-cytotoxic, biocide, and anti-fixation activities, by dividing the mean percentage of non-divided embryos, dead or non-fixed larvae respectively, in the seawater control (% *C*), by that in the treatment (% *T*).

$$I = 1 - \frac{\%C}{\%T}$$

This activity index ranged from 1 (maximal activity) to 0 (no activity). Nevertheless, as mean percentages were used, the statistical tests must be taken into account to know the significance of the activity level indicated by the index.

RESULTS

ANTI-EMBRYO ACTIVITIES

Only 12 (2 algae, 4 sponges, 1 crinoid, and 1 colonial and 4 solitary ascidians) of the 32 tested species were non-active at the initial concentration of $1000 \mu\text{g}\cdot\text{ml}^{-1}$ (Table I), whilst the remaining 20 were active at some of the crude extract concentrations tested (Table II). One-way ANOVA analysis showed highly significant differences ($p < 0.001$) in the active response among the different concentrations assayed for the seaweeds *Corallina elongata* and *Flabellia petiolata*, the sponges *Agelas oroides*, *Aplysina aerophoba*, *Axinella* sp., *Crambe crambe*, *Hemimycale columella*, *Ircinia fasciculata*, *Ircinia oros*, *Phorbas tenacior* and *Reniera mucosa*, the polychaete *Hermione histrix*, the gastropod *Platydoris argo*, the bryozoans *Myriapora truncata* and *Sertella beaniana*, and the ascidians *Microcosmus sabatieri* (tunic) and *Pseudodistoma crucigaster* (red and grey forms) (Table III). On the contrary, the differences were not significant in four species, the sponges *Acanthella acuta* and *Dictyonella obtusa*, the cnidarian *Acyonium acaule* and the bryozoan *B. neritina* (Table III). Multiple comparison tests of mean percentages of divided embryos showed the concentrations responsible for the concentration effects detected in the ANOVA to vary, according to the species considered. Only the concentration of $500 \mu\text{g}\cdot\text{ml}^{-1}$ was significantly antimitotic-cytotoxic ($p < 0.001$) in the case of *C. elongata*, *Axinella* sp., *H. histrix*, *P. argo*, *M. truncata*, *S. beaniana*, and *M. sabatieri* (tunic). *F. petiolata*, *A. aerophoba*, *A. oroides*, *H. columella*, *P. crucigaster* (red form),

TABLE I

Results of bioassays on embryos of *Paracentrotus lividus*: tested species which non-significantly differed from the controls following Student-*t* test at a concentration of 1000 $\mu\text{g}\cdot\text{ml}^{-1}$. %ND = percentage of non-divided embryos; AI = activity Index; PHY = phylum; AVG = average (ALG = algae; SPO = sponges; CRI = crinoids; ASC = ascidians).

Species	%ND			AI	
	PHY	AVG	SD	AVG	SD
Control		3.5	1.8	—	—
<i>Halopteris scoparia</i> (L.) Sauvag.	ALG	4.5	1.5	0.22	0.07
<i>Halimeda tuna</i> (Ellis & Sol.)	ALG	3.5	2.1	0.00	0.00
<i>Aplysina cavernicola</i> (Vacelet)	SPO	4.5	2.5	0.22	0.12
<i>Axinella damicornis</i> (Esper)	SPO	5.5	3.2	0.36	0.21
<i>Petrosia ficiformis</i> (Poiret)	SPO	3.8	3.3	0.07	0.06
<i>Chondrosia reniformis</i> Nardo	SPO	5.5	2.7	0.36	0.18
<i>Antedon mediterranea</i> (Lam.)	CRI	10.8	1.7	0.68	0.11
<i>Diazona violacea</i> Savigny	ASC	11.8	3.5	0.70	0.21
<i>Halocynthia papillosa</i> (L.)	ASC	8.5	4.0	0.59	0.28
<i>Microcosmus sabatieri</i> Roule (WT)	ASC	10.5	7.4	0.67	0.47
<i>Microcosmus vulgaris</i> Heller	ASC	3.5	1.7	0.00	0.00
<i>Phallusia mammillata</i> (Cuvier)	ASC	4.3	2.3	0.18	0.09

($p < 0.001$) and *P. tenacior* ($p = 0.001$) displayed highly significant antimitotic/cytotoxic activities down to a concentration of 250 $\mu\text{g}\cdot\text{ml}^{-1}$. *I. fasciculata* and *P. crucigaster* (grey form) were significantly active ($p < 0.001$) to a concentration of 125 $\mu\text{g}\cdot\text{ml}^{-1}$. *R. mucosa* was significantly active ($p < 0.001$) down to a concentration of 30 $\mu\text{g}\cdot\text{ml}^{-1}$. Finally, *C. crambe* and *I. oros* were the most toxic species assayed, displaying highly significant activity ($p < 0.001$) down to a concentration of 8 $\mu\text{g}\cdot\text{ml}^{-1}$. The values of the activity index (*I*) at the different concentrations of crude extract are represented in Figs. 1 and 2.

ANTILARVAL ACTIVITIES

Biocide effect

All the assayed species except *A. oroides* and *M. sabatieri* (tunic) were active at some of the concentrations tested (Table IV). One-way ANOVA analysis showed highly significant differences ($p < 0.001$) among the mean number of dead larvae between the different concentrations tested for all the species except *C. elongata*, *A. oroides* and *M. sabatieri* (tunic) (Table III). Multiple comparison tests showed: (1) All the concentrations tested were responsible for the concentration effects detected in the ANOVA in the case of *C. crambe* and *P. crucigaster* (grey form) ($p < 0.001$). (2) Concentrations ranging from 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ to 125 $\mu\text{g}\cdot\text{ml}^{-1}$ significantly affect ($p < 0.001$) larval survival in the case of *Axinella* sp., *H. columella*, *P. ficiformis*, *H. histrix*, *B. neritina*, and

TABLE II

Percentages of non-divided embryos of *Paracentrotus lividus* for the active species, up to the concentration at which anti-embryo activities were not significant following Tukey's test ($p \geq 0.1$). CTRL = control; PHY = phylum; AVG = average (ALG = algae; SPO = sponges; CNI = cnidarians; POL = polychaetes; MOL = moluscs, BRY = bryozoans; ASC = ascidians). Concentrations in $\mu\text{g}\cdot\text{ml}^{-1}$.

Species	PHY		CTRL	1000	500	250	125	60	30	15	8	4
<i>Corallina elongata</i>	ALG	AVG	7	100	69	7	-	-	-	-	-	-
Ellis & Sol.		SD	0.6	0.0	10.3	1.9	-	-	-	-	-	-
<i>Flabellia petiolata</i>	ALG	AVG	3	82	99	34	6	-	-	-	-	-
(Turra) Nizamuddin		SD	0.6	6.5	1.5	4.2	1.3	-	-	-	-	-
<i>Acanthella acuta</i>	SPO	AVG	8	100	16	-	-	-	-	-	-	-
Schmidt		SD	3.9	0.0	2.9	-	-	-	-	-	-	-
<i>Agelas oroides</i>	SPO	AVG	7	100	100	100	25	19	-	-	-	-
(Schmidt)		SD	4.7	0.0	0.0	0.0	3.3	19.4	-	-	-	-
<i>Aplysina aerophoba</i>	SPO	AVG	5	91	74	91	7	-	-	-	-	-
Schmidt		SD	0.3	5.1	18.7	1.9	1.1	-	-	-	-	-
<i>Axinella</i> sp.	SPO	AVG	2	100	96	12	-	-	-	-	-	-
		SD	0.7	0.0	3.0	6.7	-	-	-	-	-	-
<i>Crambe crambe</i>	SPO	AVG	7	100	99	100	100	100	89	70	64	14
(Schmidt)		SD	1.8	0.9	1.1	0.0	0.0	0.4	7.3	4.3	4.6	1.1
<i>Dyctionella obtusa</i>	SPO	AVG	6	80	24	-	-	-	-	-	-	-
(Schmidt)		SD	1.5	5.9	8.4	-	-	-	-	-	-	-
<i>Hemimyscale columella</i>	SPO	AVG	5	100	100	39	7	-	-	-	-	-
(Bowerbank)		SD	0.6	0.0	0.0	8.2	2.1	-	-	-	-	-
<i>Ircinia fasciculata</i>	SPO	AVG	4	100	100	100	56	7	-	-	-	-
(Pallas)		SD	0.5	0.0	0.0	0.0	13.2	1.3	-	-	-	-
<i>Ircinia oros</i>	SPO	AVG	6	100	100	100	100	100	96	86	40	13
(Schmidt)		SD	1.2	0.2	0.0	0.0	0.0	0.0	4.5	5.7	4.6	2.2
<i>Phorbos tenacior</i>	SPO	AVG	8	100	100	19	9	-	-	-	-	-
(Topsent)		SD	3.6	0.4	0.0	1.1	2.2	-	-	-	-	-
<i>Reniera mucosa</i>	SPO	AVG	4	92	100	75	60	42	31	5	-	-
Griessinger		SD	0.5	9.0	0.0	12.0	8.5	5.7	6.6	1.5	-	-
<i>Alcyonium acaule</i>	CNI	AVG	6	80	13	-	-	-	-	-	-	-
(Kükenthal)		SD	5.9	10.7	4.9	-	-	-	-	-	-	-
<i>Hermione histrix</i>	POL	AVG	8	100	61	9	-	-	-	-	-	-
Savigny		SD	0.6	0.0	13.8	1.1	-	-	-	-	-	-
<i>Platydoris argo</i> L.	MOL	AVG	4	100	78	8	-	-	-	-	-	-
		SD	0.6	0.0	14.2	0.8	-	-	-	-	-	-
<i>Bugula neritina</i> (L.)	BRY	AVG	3	45	12	-	-	-	-	-	-	-
		SD	0.9	6.1	6.0	-	-	-	-	-	-	-
<i>Myriapora truncata</i>	BRY	AVG	4	100	88	7	-	-	-	-	-	-
(Pallas)		SD	0.6	0.0	8.6	2.9	-	-	-	-	-	-
<i>Sertiella beaniana</i>	BRY	AVG	492	14	7	-	-	-	-	-	-	-
(King)		SD	0.6	5.0	2.4	2.1	-	-	-	-	-	-
<i>Microcosmus sabatieri</i>	ASC	AVG	6	42	22	7	-	-	-	-	-	-
Roule (T)		SD	1.9	1.9	5.4	2.1	-	-	-	-	-	-
<i>Pseudodistoma crucigaster</i>	ASC	AVG	3	100	100	32	23	8	-	-	-	-
Gaill (G)		SD	1.6	0.0	0.0	10.3	0.7	2.4	-	-	-	-
<i>Pseudodistoma crucigaster</i>	ASC	AVG	7	100	99	59	7	-	-	-	-	-
Gaill (R)		SD	2.3	0.0	1.1	8.0	2.2	-	-	-	-	-

TABLE III

Summary of one-way analysis of variance in the different assays. ANTI-EMBRYOS = for means of divided embryos of *Paracentrotus lividus*; Biocide = for means of death larvae of *Bugula neritina*; Anti-fixation = for means of non-fixed larvae of *Bugula neritina*; *F* = value of Fischer distribution; *p* = probability.

Species	Anti-embryos		Biocide		Anti-fixation	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
<i>C. elongata</i>	203.27	<0.001	3.23	<0.05	3.43	<0.05
<i>F. petiolata</i>	260.98	<0.001	—	—	—	—
<i>A. acuta</i>	3.66	<0.05	246.01	<0.001	95.43	<0.001
<i>A. aerophoba</i>	52.85	<0.001	129.48	<0.001	129.48	<0.001
<i>A. oroides</i>	61.67	<0.001	—	—	52.15	<0.001
<i>C. crambe</i>	259.00	<0.001	244.88	<0.001	156.62	<0.001
<i>D. obtusa</i>	2.63	<0.1	13.47	<0.001	6.12	<0.01
<i>H. columella</i>	256.14	<0.001	11.25	<0.001	7.74	<0.01
<i>I. fasciculata</i>	203.74	<0.001	1486.61	<0.001	90.01	<0.001
<i>I. oros</i>	617.13	<0.001	1348.65	<0.001	269.17	<0.001
<i>P. ficiformis</i>	—	—	16.32	<0.001	6.81	<0.01
<i>P. tenacior</i>	857.70	<0.001	296.68	<0.001	134.02	<0.001
<i>R. mucosa</i>	27.23	<0.001	137.74	<0.001	72.32	<0.001
<i>Axinella</i> sp.	109.86	<0.001	23.46	<0.001	5.55	<0.01
<i>A. acaule</i>	0.98	<0.462	94.45	<0.001	41.43	<0.001
<i>H. histrix</i>	27.23	<0.001	24.79	<0.001	33.26	<0.001
<i>P. argo</i>	40.61	<0.001	—	—	—	—
<i>S. beaniana</i>	13.77	<0.001	22.66	<0.001	6.18	<0.01
<i>B. neritina</i>	1.98	<0.174	16.61	<0.001	4.59	<0.05
<i>M. truncata</i>	153.43	<0.001	25.77	<0.001	14.13	<0.001
<i>M. sabatieri</i> (T)	8.61	<0.003	—	—	36.73	<0.001
<i>M. sabatieri</i> (WT)	—	—	27.62	<0.001	13.38	<0.001
<i>P. crucigaster</i> (G)	423.77	<0.001	—	—	79.02	<0.001
<i>P. crucigaster</i> (R)	597.71	<0.001	124.96	<0.001	—	—

M. truncata. (3) Significant biocide activity ($p < 0.001$) was found against larvae at concentrations of 1000 and 500 $\mu\text{g}\cdot\text{ml}^{-1}$ in *A. aerophoba*, *I. fasciculata*, *R. mucosa*, *A. acaule* and *M. sabatieri* (without tunic), whereas *D. obtusa* and *P. tenacior* were only significantly active ($p < 0.001$) at a concentration of 1000 $\mu\text{g}\cdot\text{ml}^{-1}$. The index of biocide activity is represented in Fig. 3.

Anti-fixation activity

All the assayed species were active at some of the concentrations tested (Table IV). One-way ANOVA analysis showed highly significant differences ($p < 0.001$) among the mean number of fixed larvae (ancestrulae) between the concentrations tested for the nineteen species assayed (Table III). Multiple comparison tests showed all the concentrations assayed as responsible for the concentration effects detected in the ANOVA ($p < 0.001$), for *A. acuta*, *C. crambe*, *I. oros* and *P. crucigaster* (grey form). *A. acaule*

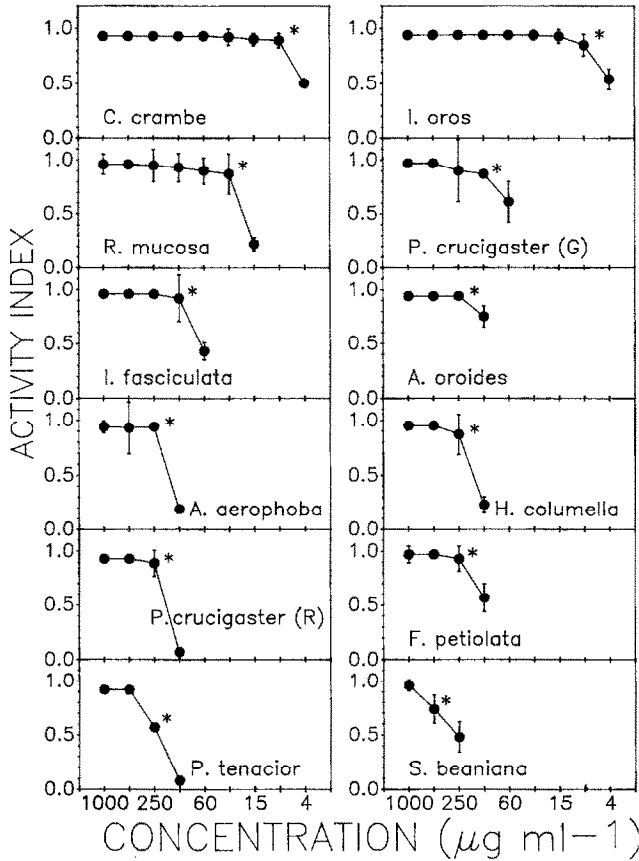


Fig. 1. Bioassays on embryos of *Paracentrotus lividus*: relationships between test concentration and index of activity in the most active species (* = minimum concentration at which the species were significantly active; vertical bars represent \pm SD).

was significantly active ($p < 0.001$) down to a concentration of $125 \mu\text{g}\cdot\text{ml}^{-1}$. Highly significant anti-fixation effects ($p < 0.001$) were displayed by *A. aerophoba*, *I. fasciculata*, *P. tenacior* and *R. mucosa* at concentrations of down to $250 \mu\text{g}\cdot\text{ml}^{-1}$. Finally, *A. oroides*, *D. obtusa* and *M. sabatieri* showed significant anti-fixation activity ($p < 0.001$) only at a concentration of $1000 \mu\text{g}\cdot\text{ml}^{-1}$. The index of anti-fixation activity is represented in Fig. 4.

Comparative behaviour

When comparing the activity displayed against embryos and larvae by the same crude extracts, different behaviour can be shown, depending on the species under study

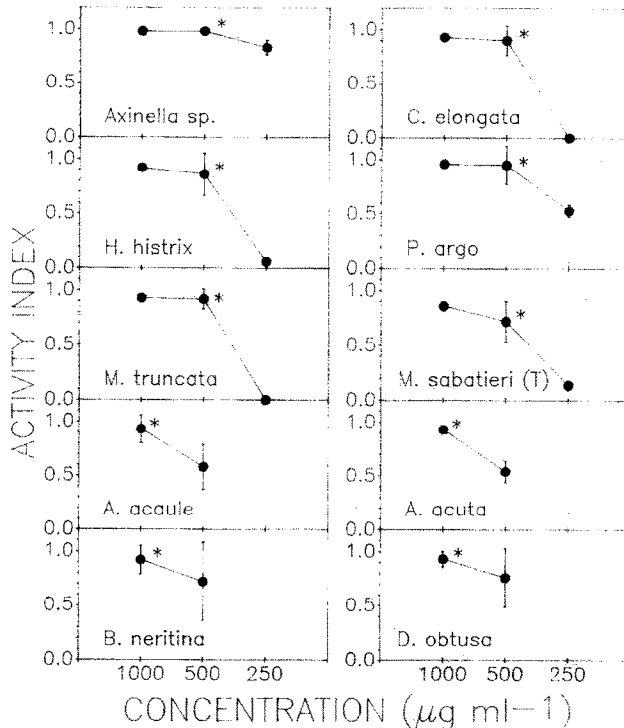


Fig. 2. Activity on embryos of *Paracentrotus lividus*: relationships between test concentration and index of activity in the less active species (* = minimum concentration at which the species were significantly active; vertical bars represent \pm SD).

(Fig. 5). Only the sponges *C. crambe* and *D. obtusa* were active against both organisms down to the same concentration of sponge's crude extract. In contrast, other species such as the sponges *A. aerophoba*, *A. oroides*, *I. fasciculata*, *I. oros*, *P. tenacior*, and *R. mucosa* and the ascidian *M. sabatieri* (tunic), displayed higher cytotoxic-antimitotic activity than anti-larval effects. On the contrary, the sponges *Axinella* sp., *H. columella*, *P. ficiformis*, the cnidarian *A. acaule*, the polychaete *H. histrix*, the ascidians *P. crucigaster* (grey form) and *M. sabatieri* (without tunic), and the bryozoans *B. neritina*, *M. truncata* and *S. beaniana*, were much more active against larvae of *B. neritina* than against embryos of *P. lividus* (Fig. 5).

With regard to the anti-larval activity, in most cases, the crude extracts of the biocide species, showed no biocide effects when diluted to lower concentrations, since all the larvae remained alive after 24 h, but inhibition of the settlement and fixation processes was clearly evident ($p < 0.001$). The sponge *P. tenacior* and the ascidian *M. sabatieri* (tunic) did not kill larvae at the concentration of $1000 \mu\text{g}\cdot\text{ml}^{-1}$ but inhibited larval fixation ($p < 0.001$) down to a concentration of $250 \mu\text{g}\cdot\text{ml}^{-1}$ and $500 \mu\text{g}\cdot\text{ml}^{-1}$,

TABLE IV

Results of the assays on larvae of *Bugula neritina* for the active species, down to the concentration at which biocide and anti-fixation activities were not significant following Tukey's test ($p \geq 0.1$).

Activities	Species	Biocide						Anti-fixation					
		Control	1000	500	250	125	60	Control	1000	500	250	125	60
<i>Axinella</i> sp.	AVG	12	94	100	85	95	24	65	100	100	94	98	51
	SD	3.2	4.5	0.0	8.6	3.4	26.1	7.1	0.0	0.0	4.5	1.7	18.6
<i>A. acuta</i>	AVG	0	100	100	100	85	25	32	100	100	100	87	60
	SD	0.0	0.0	0.0	0.0	2.7	9.3	8.9	0.0	0.0	0.0	1.6	4.0
<i>A. aerophoba</i>	AVG	0	96	54	0	-	-	20	100	100	34	-	-
	SD	0.0	5.2	18.3	0.0	-	-	7.9	0.0	0.0	6.6	-	-
<i>A. oroides</i>	AVG	0	-	-	-	-	-	18	100	21	-	-	-
	SD	0.0	-	-	-	-	-	6.9	0.0	7.8	-	-	-
<i>C. crambe</i>	AVG	0	100	100	100	82	48	30	100	100	100	100	82
	SD	0.0	0.0	0.0	0.0	8.1	3.7	5.9	0.0	0.0	0.0	0.0	4.9
<i>D. obtusa</i>	AVG	0	46	0	-	-	-	36	97	57	-	-	-
	SD	0.0	17.7	0.0	-	-	-	8.4	4.7	26.5	-	-	-
<i>H. columella</i>	AVG	12	74	91	67	100	49	65	87	96	83	100	90
	SD	3.2	11.7	12.3	14.4	0.0	23.8	7.1	5.8	6.1	7.2	0.0	7.4
<i>I. fasciculata</i>	AVG	0	99	87	2	-	-	20	99	91	29	-	-
	SD	0.0	1.8	1.9	3.1	-	-	7.9	1.8	1.3	10.5	-	-
<i>I. oros</i>	AVG	0	100	100	100	92	13	30	100	100	100	100	100
	SD	0.0	0.0	0.0	0.0	2.9	3.3	5.9	0.0	0.0	0.0	0.0	0.0
<i>P. ficiformis</i>	AVG	12	75	100	95	100	41	65	87	100	97	100	84
	SD	3.2	3.7	0.0	4.2	0.0	30.4	7.1	1.8	0.0	2.1	0.0	16.0
<i>P. tenacior</i>	AVG	0	79	6	-	-	-	9	95	70	31	20	-
	SD	0.0	6.3	1.0	-	-	-	4.7	0.3	7.1	5.4	2.2	-
<i>R. mucosa</i>	AVG	0	88	60	0	-	-	8	88	60	11	-	-
	SD	0.0	9.3	6.7	0.0	-	-	1.5	9.3	6.7	8.5	-	-
<i>A. acaule</i>	AVG	0	80	85	0	-	-	8	100	85	73	40	9
	SD	0.0	8.8	12.2	0.0	-	-	1.5	0.0	12.2	13.8	9.2	4.0
<i>H. histrix</i>	AVG	12	97	100	100	100	33	65	98	100	100	100	95
	SD	3.2	4.3	0.0	0.0	0.0	27.2	7.1	2.1	0.0	0.0	0.0	3.6
<i>B. neritina</i>	AVG	12	92	92	85	79	27	65	96	96	93	90	-
	SD	3.2	6.0	11.8	4.6	14.7	22.0	7.1	3.0	5.9	2.3	7.4	-
<i>M. truncata</i>	AVG	12	99	100	98	100	47	65	100	100	99	100	74
	SD	3.2	1.0	0.0	2.6	0.0	25.2	7.1	0.0	0.0	1.3	0.0	12.6
<i>S. beaniana</i>	AVG	12	85	93	91	81	64	65	95	96	95	90	92
	SD	3.2	8.0	10.5	6.9	14.2	7.8	7.1	3.7	5.2	3.4	7.1	11.1
<i>M. sabatieri</i> (T)	AVG	0	-	-	-	-	-	22	87	40	-	-	-
	SD	0.0	-	-	-	-	-	4.8	3.9	11.4	-	-	-
<i>M. sabatieri</i> (WT)	AVG	0	82	41	0	-	-	40	97	63	-	-	-
	SD	0.0	15.8	16.1	0.0	-	-	3.7	2.1	11.3	-	-	-
<i>P. crucigaster</i> (G)	AVG	0	100	100	89	73	32	15	100	100	100	86	81
	SD	0.0	0.0	0.0	5.3	6.9	9.1	6.4	0.0	0.0	0.0	9.7	5.4

respectively. In the same way, the sponges *H. columella* and *I. oros*, the cnidarian *A. acaule*, the polychaete *H. histrix*, and the bryozoan *S. beaniana*, display anti-fixation activity down to one concentration level lower to that exhibiting biocide activity.

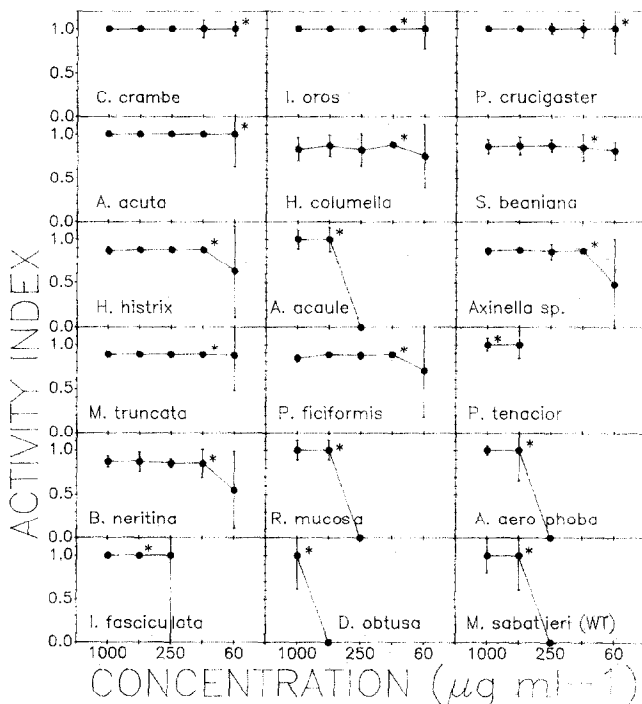


Fig. 3. Biocide activity in larvae of *Bugula neritina*. Relationships between test concentration and index of activity (* = minimum concentration at which the species were significantly active; vertical bars represent \pm SD).

DISCUSSION

Most of the assayed species exhibited antimitotic/cytotoxic activities. These activities were specially remarkable in the sponges *C. crambe*, *I. oros*, and *R. mucosa* which displayed similar activities when tested against kidney cells of monkey and leukemic cells of mice (Uriz et al., 1992). A comparable behaviour against marine and terrestrial organisms has been also assumed by Thompson et al. (1985), in contrast to the results found by Bakus et al. (1990). Thus, generalizations seems to be precipitate and must be done with due regard to the limitations of the present knowledge on this subject.

All the assayed species but two were able to kill larvae at some of the highest concentrations tested whereas anti-fixation effects were usually present down to lower concentrations.

The inhibition of the larval settlement exhibited by some biocide tissue extracts when diluted to lower concentrations, can be an indication of a sublethal toxicity that may result efficacious for the organisms to prevent being fouled.

Sublethal toxicity resulting in various effects such as incapacitation of ciliary mechanisms leading to disorientation (Dyrynda, 1986), anesthetic properties, and delay in

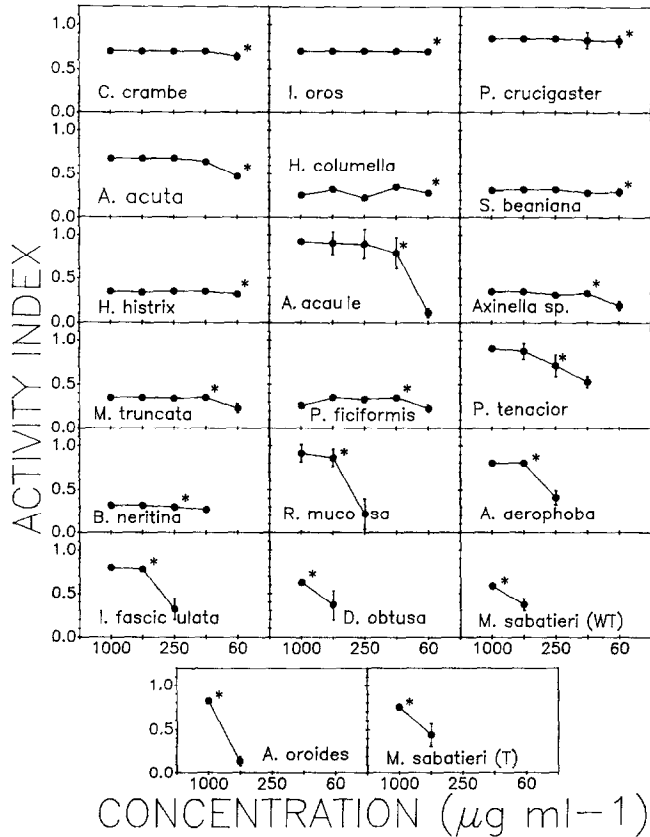


Fig. 4. Anti-fixation activity on larvae of *Bugula neritina*. Relationships between test concentration and index of activity. (* = minimum concentration at which the species were significantly active; vertical bars represent \pm SD).

larval metamorphosis (Davis et al., 1989), have been suggested as the possible ways that toxicants may act at low concentrations. This may be the case in some of the assayed species, although biochemical studies would be required to verify these hypotheses and to pinpoint the site and mode of action. Anyway, as it has been shown for organisms from other latitudes (see Davies et al., 1989 for a review), it seems that most of the assayed species would not need to release the required amounts of toxic substances to kill larvae to prevent being fouled, but only the necessary amounts to inhibit settlement or fixation. Nevertheless, it seems that some exceptions to this rule have been found among the assayed species, as in the case of some sponges (*A. acuta*, *A. aerophoba*, *Axinella* sp., *C. crambe*, *I. fasciculata* and *P. ficiformis*), bryozoans (*M. truncata*) and ascidians (*P. crucigaster*, grey form). These species showed biocide and anti-fixation effects down to the same concentration. Nevertheless, the "in situ" effectiveness of these anti-larvae substances tested in the laboratory remains unknown. The

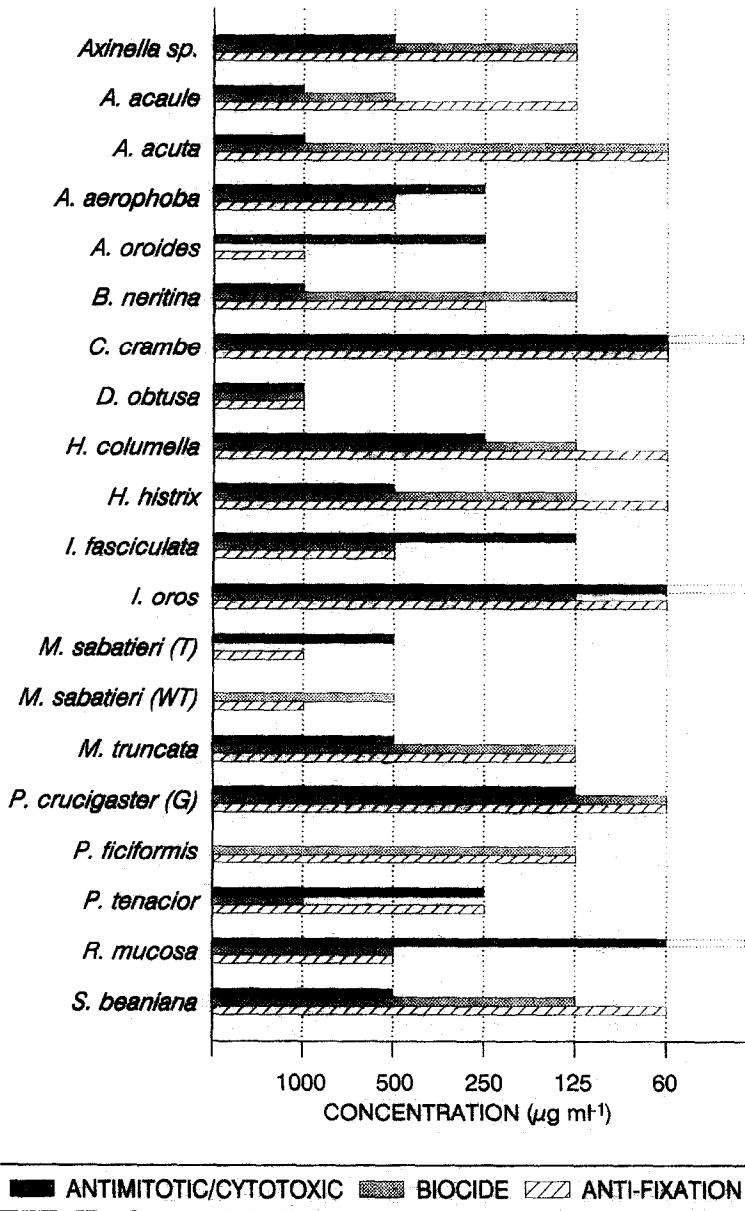


Fig. 5. Comparative activities against embryos of *Paracentrotus lividus* (antimitotic/cytotoxic activities) and larvae of *Bugula neritina* (biocide and anti-fixation activities). Bars extend to the last concentration at which the species were significantly active in each assay.

overall anti-fouling mechanisms displayed by each particular organism must be taken into account (Davies et al., 1989), and complementary experimental research must be addressed to know whether these substances are actually produced in nature.

Some unavoidable restrictions of the anti-fouling assays must be pointed out although they do not significantly affect the interpretation of our results. The consistency and physical properties of the crude extract layer may vary according to the species thus influencing the proportion of larvae inclined to settle. In any case, the results are comparable among the different concentrations assayed.

Intraspecific variability in the chemically mediated activities has been evidenced (i.e. Green et al., 1990). The different varieties of a species can display different rates of bioactivity as has been shown in the grey and red forms of *P. crucigaster*, the former being much more active than the latter. If this is due to the presence of different metabolites, and not to a greater production of the same metabolites, these differences could have taxonomic relevance since qualitative variations in secondary metabolites are considered to indicate subspecific, specific or generic differences (Bergquist et al., 1983).

Taxonomic implications may also be inferred from the very different responses displayed in the tests by the two taxonomically closed sponge species *A. aerophoba* and *A. cavernicola*. The latter was not active at any concentration assayed whereas *A. aerophoba* was active down to a concentration of $250 \mu\text{g}\cdot\text{ml}^{-1}$ (both against embryos or larvae). This could be related to the symbiotic cyanophyceae of *A. aerophoba*, and its associated cytotoxicity (Moore, 1977) but could also confirm the specific differences between both species (Vacelet, 1959; Wilkinson & Vacelet, 1979), contrary to Voultziadou (1987).

Finally, although some extracted substances other than secondary metabolites, may contribute to the toxicity of the crude extract (particularly fatty acids, which may display some degree of toxicity in their own right once extracted), the finding of a reasonable estimate of the crude extract concentration ranges between which the species bioactivities vary, appears to be a convenient previous step in the design of experimental studies dealing with bioactivity of natural toxins.

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REFERENCES

- Amade, P., G. Charroin, C. Baby & J. Vacelet, 1987. Antimicrobial activities of marine sponges from the Mediterranean Sea. *Mar. Biol.*, Vol. 94, pp. 271–274.
- Bakus, G.H., B. Schulte, S. Jhu, M. Wright, G. Green & P. Gomez, 1990. Antibiosis and antifouling in marine sponges: laboratory versus field studies. In, *New perspectives in sponge biology*, edited by K. Rützler, Smithsonian Institution Press, Washington D.C., pp. 102–114.
- Bergquist, P.R. & R.J. Wells, 1983. Chemotaxonomy of the Porifera: the development and current status of the field. In, *Marine natural products*, edited by P.J. Scheuer, Academic Press, New York, Vol. 5, pp. 1–50.
- Berlink, R.G.S., J.C. Braekman & D. Daloz, 1992. Crambines C1 and C2: two further ichthyotoxic guanidine alkaloids from the sponge *Crambe crambe*. *J. Natl. Prod.*, Vol. 55, pp. 528–532.
- Coll, J.C., B.F. Bowden & D.M. Tapiolas, 1982. In situ isolation of allelochemicals released from soft corals (Cocenterata: Octocorallia): a totally submersible sampling apparatus. *J. Exp. Mar. Biol. Ecol.*, Vol. 60, pp. 293–299.
- Davis, A.R., N.M. Targett, O.J. McConnell & C.M. Young, 1989. Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In, *Biorganic Marine Chemistry*, edited by P.J. Scheuer, Springer-Verlag, Berlin, Vol. 3, pp. 85–114.
- Dyrinda, P.E.J., 1986. Defensive strategies of modular organisms. *Phil. Trans. R. Soc. Lond.*, Vol. B 313, pp. 227–243.
- Green, G., P. Gomez & G.J. Bakus, 1990. Antimicrobial and ichthyotoxic properties of marine sponges from Mexican waters. In, *New perspectives in sponge biology*, edited by K. Rützler, Smithsonian Institution Press, Washington D.C., pp. 102–114.
- Harwell C.D. & W. Fenical, 1989. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.): intracolony localization of defense. *Limnol. Oceanogr.*, Vol. 34, pp. 382–389.
- Jackson, J.B.C. & L. Buss, 1975. Allelopathy and spatial competition among coral reef invertebrates. *Proc. Natl. Acad. Sci. USA*, Vol. 72, pp. 5160–5163.
- Keough, M.J., 1984. Kin-recognition and the spatial distribution of larvae of the bryozoan *Bugula neritina* (L.). *Evolution*, Vol. 38, pp. 142–147.
- Kobayashi, N., 1971. Fertilized sea urchin eggs as an indicatory material for marine pollution bioassay: preliminary experiments. *Publ. Seto Mar. Biol. Lab. Jpn.*, Vol. 18, pp. 379–406.
- Moore, R.E., 1977. Toxins from blue-green algae. *Bioscience*, Vol. 27, pp. 797–802.
- Munro, M.H.G., J.W. Blunt, G. Barns, C.N. Battershill, R.S. Lake & N.B. Perry, 1989. Biological activity in New Zealand marine organisms. *Pure Appl. Chem.*, Vol. 61, pp. 529–534.
- Pennington, J.T. & M.G. Hadfield, 1989. Larvae of a nudibranch mollusc (*Phestilla sibogae*) metamorphose when exposed to common organic solvents. *Biol. Bull.*, Vol. 177, pp. 350–355.
- Paul, V.J. & M.E. Hay, 1986. Seaweed susceptibility to herbivory: chemical and morphological correlated. *Mar. Ecol. Progr. Ser.*, Vol. 33, pp. 255–264.
- Sokal, R.R. & F.J. Rohlf, 1979. *Biometria. Principios y métodos estadísticos en la investigación biológica*. H. Blume Eds., Madrid.
- Thompson, J., R.P. Walker & J. Faulkner, 1985. Screening and bioassays for biologically-active substances from forty marine sponge species from San Diego, California, USA. *Mar. Biol.*, Vol. 88, pp. 11–21.
- Thompson, J.E., P.T. Murphy, P.R. Bergquist & E.A. Evans, 1987. Environmentally induced variation in diterpene composition of the marine sponge *Rhopaloeides odorabile*. *Biochem. Syst. Ecol.*, Vol. 15, pp. 595–606.
- Tukey, J.W., 1953. *The problem of multiple comparisons*. Department of Statistics, Princeton University.
- Uriz, M.J., D. Martín, X. Turón, E. Ballesteros, R. Hughes & C. Acebal, 1991. An approach to the ecological significance of chemically mediated bioactivity in Mediterranean benthic communities. *Mar. Ecol. Progr. Ser.*, Vol. 70, pp. 175–188.
- Uriz, M.J., D. Martín & D. Rosell, 1992. Relationships between chemically mediated bioactivity and

- taxonomical and biological characteristics of Mediterranean sponges. *Mar. Biol.*, Vol. 113, pp. 287–297.
- Vacelet, J., 1959. Répartition générale des éponges et systématique des éponges cornées de la région de Marseille et de quelques stations Méditerranéennes. *Recl. Trav. Stn. Mar. Endoume*, Vol. 16, pp. 39–101.
- Van der Vyver, G., J. Huysencomb, J.C. Braekman & D. Daloze, 1990. Screening and bioassays for toxic substances in sponges from Western Mediterranean Sea and North Brittany. *Vie et Milieu*, Vol. 40, pp. 285–292.
- Voultsiadou, E., 1987. Some remarks on the Mediterranean species of the genus *Aplysina* (Demospongiae, Verongida). In, *Taxonomy of Porifera*, edited by J. Vacelet & N. Boury-Esnault, NATO Series, Springer-Verlag, Berlin, Vol. G 13, pp. 275–279.
- Wahl, M. & B. Banaigs, 1991. Marine epibiosis. III Possible antifouling defence adaptations in *Polysincraton lacazei* (Giard) (Didemnidae, Ascidiacea). *J. Exp. Mar. Biol. Ecol.*, Vol. 45, pp. 49–63.
- Walker, R.P., J.E. Thompson & D.J. Faulkner, 1985. Exudation of biologically-active metabolites in the sponge *Aplysina fistularis*. II. Chemical evidence. *Mar. Biol.*, Vol. 88, pp. 27–32.
- Wilkinson, C. & J. Vacelet, 1979. Transplantation of marine sponges to different conditions of light and current. *J. Exp. Mar. Biol. Ecol.*, Vol. 17, pp. 91–104.