Seasonal and spatial variation of species toxicity in Mediterranean seaweed communities: correlation to biotic and abiotic factors

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ABSTRACT: The toxicity of crude extracts of 32 seaweed species from the western Mediterranean was analysed by Microtox® assay in spring and autumn of 1996 and 1997. The species analysed represented more than 76% of seaweed coverage in the 3 algal communities studied: photophilic and sciaphilic communities from the Cabrera Archipelago (Balearic Islands), and a hemisciaphilic community from the Medes Archipelago (northeastern Iberian Peninsula). Most species showed seasonal variation of toxicity, which was greater in species from Cabrera than in those from Medes. Both, intra and interspecies variation of toxicity were found. Moreover, comparison of mean toxicity of these communities showed that toxicity was higher in November than in June in all cases, and that the photophilic community had both the highest number and the most toxic species. To make an ecologically relevant interpretation of the toxicity detected by Microtox®, we compared the toxicity of extracts analysed by the Microtox® test and those analysed by the commonly used sea urchin embryo assay. In addition to seaweeds, some species of invertebrates (sponges and ascidians) were compared to ascertain whether the relationship between the 2 tests was applicable to species belonging to different phyla. These comparisons allowed us to establish that 0.5 gamma units in Microtox® assay is the threshold value between toxic and non-toxic species. Following a light gradient from the photophilic to the sciaphilic communities, the seaweed species that were occasionally toxic increased while the always-toxic seaweeds decreased. Rhodophyta and Phaeophyta had a higher percentage of toxic species than Chlorophyta. Non-encrusting seaweed forms were more toxic than the encrusting ones, and in contrast to most other seaweeds, the non-encrusting calcareous species that increased coverage from June to November simultaneously displayed a marked decrease in toxicity. We conclude that the temporal variation of toxicity observed in the seaweeds studied may be partially explained by intrinsic factors of the species (growth rates and growth shapes).

KEY WORDS: Natural toxicity · Seaweed · Seasonal variation · Spatial variation · Microtox® assay · Sea urchin assay · Mediterranean Sea · Growth shapes

INTRODUCTION

Toxicity screenings of seaweed species from various geographic regions (Caccamese & Azzolina 1979, Henriquez et al. 1979, Caccamese et al. 1980, Chénieux et al. 1980, Naqvi et al. 1980, Ballesteros et al. 1992) have shown repeatedly that seaweeds are an important source of bioactive products. Consequently, seaweeds have often been targeted in the search for compounds with pharmacological properties. The most common property of seaweed extracts tested is their effectiveness against pathogenic micro-organisms such as bacteria, fungi and viruses.

However, studies to clarify the ecological functions of toxic algal secondary metabolites are scarce and usually focus on just 1 or a few species. Seaweeds and, in general, benthic invertebrates are exposed to variable environmental factors such as light intensity, food...
availability, salinity and pollution concentrations, as well as to biotic pressures such as predation and spatial and trophic competition. All these factors affect the organisms’ physiology and have direct effects on the production of seaweed secondary metabolites that can have several functions (Schmitt et al. 1995). Among the different ecological functions reported, the best known (possibly because it is more amenable to experimental manipulation) is the anti-predatory role (Hay et al. 1987, 1988, Meyer & Paul 1992, Cronin & Hay 1996, Stachowicz & Lindquist 1997, Schnitzler et al. 1998, 2001). The anti-fouling activity of algae is less well known (de Nys et al. 1995, Schmitt et al. 1995, Kjelleberg et al. 1997). However, the localization of some bioactive compounds in cells and their release to the seaweeds surface suggest an anti-fouling role (Wolk 1968, Ragan 1976, Ragan & Glombitza 1986, de Nys et al. 1996, Dworjanyn et al. 1999). The allelochemical function of secondary metabolites from seaweeds has also been postulated (Fletcher 1975, Helleburst 1975, Vadás 1979, Harlin 1987), but evidence of negative effects of these compounds on neighbouring organisms is scarce (de Nys et al. 1991).

The aims of this study were to investigate the relationships of several structural and dynamic descriptors of algal-benthic communities with the seasonal and spatial variation of toxicity in the most abundant species of 3 algal communities from the Mediterranean Sea. These descriptors were species shape (Martí 2002) and seasonal changes in coverage (i.e. as an estimation of growth).

The novelty of the study is the analysis of a high number of species (as in pharmacological-oriented screenings), which are representative of whole communities, thus allowing an interpretation of the results at a community level. The high number of samples analysed made it necessary to use a standardized bioassay such as the Microtox® assay (Becerro et al. 1995, Martí et al. 2003). This assay is highly repeatable and precise, allowing analysis and quantification of the toxicity of a large number of taxonomically diverse species to detect intra and interspecies variation and community-level patterns. Furthermore, it correlates well with many other biological assays (e.g. Botsford 2002). Of course, this method, as with any general test, cannot detect all kind of species toxicity. That is why we are using our data only for comparative purposes to analyse variation in toxicity among individuals, species, communities or seasons.

The relationship between growth shape and survival strategies of algae has been studied by Littler & Littler (1980) and Littler et al. (1983), who proposed a functional-form hypothesis for benthic marine macroalgae. Here, we have studied whether different growth shapes relate to species toxicity.

It has been suggested that the production of chemical defences is costly in most cases (Cronin 2001). Therefore, organisms have to allocate resources and energy to different activities (i.e. growth, reproduction, defence, e.g. Uriz et al. 1995) for their well-being, which often implies trade-offs in resource allocation from one process to another. In particular, investment in chemical defence may be at odds with investment in growth (Cronin 2001). Thus, in order to examine this trade-off, seasonal changes in coverage were compared to seasonal changes in toxicity for each seaweed species.

Our purpose was to obtain new insights into the role that species toxicity may play in the field, and on the factors that influence the high variation of toxicity found, by using a a previously studied system of 3 communities (Martí et al. 2004) as a case study.

MATERIALS AND METHODS

Sampling. Samples of 3 algal communities were collected by SCUBA divers from 2 Mediterranean archipelagos in June and November 1996 and 1997. Sampling was performed according to previous inventories of these communities (Martí et al. 2004). The communities studied followed a gradient of light as they were located at the entrance of submarine caves. We selected a photophilic (from 70 to 15% of superficial light) and a sciaphilic (5 to 1.5%) community from the Cabrera Archipelago (Balearic Islands) and a hemiscaphilic (20 to 5%) community from the Medes Archipelago (Catalan coast). A more detailed description of these communities and the coverage of every species can be found in Martí et al. (2004). Our communities correspond to what Martí et al. (2004) called Zone 1 and Zone 2 of the Cabrera cave (the photophilic and sciaphilic communities, respectively), and Zone 1 of the Medes cave (the hemiscaphilic community).

Whenever the number of specimens available allowed, 3 replicates of each species were collected, extracted and analysed separately.

Due to the clonal nature of seaweeds, we considered the thalli that were physically contiguous, forming a single patch, as an individual (i.e. same clone) and considered as replicates those thalli collected from patches, which were at least 1 m apart.

Chemical extraction. Once in the laboratory, samples were cleaned of epibionts, frozen, lyophilised and stored for subsequent chemical extraction. Samples were extracted and analysed directly after collection, in both seasons.

A known weight of each lyophilised sample was extracted 3 successive times in 10 ml of methanol (MeOH) for 5, 10 and 15 min, respectively, using an ultrasonic bath. The solvent from the 3 extractions was filtered,
pooled and completely evaporated under reduced pressure and a stream of nitrogen. The dry crude extracts were then weighed and kept at –20 C° until dilution in artificial seawater for the Microtox® assay.

**Microtox® assay.** The Microtox® assay analyses the toxicity of the extracts to a marine abyssal bacterium, *Vibrio fischeri*. Previous studies have shown that the Microtox® assay has higher precision and sensitivity than other commonly used tests (Becerro et al. 1995, Del Valls et al. 1997, Pedersen et al. 1997, Burridge et al. 1999, Chiaridini et al. 1999, Sabaliunas et al. 2000) and that the toxicity measured with this test correlates well with both chemical quantification (Martí et al. 2003) and many other biological assays (Botsford 2002). These reasons made this assay the preferred choice for our study.

Just before the Microtox® analyses, crude extracts were dissolved in artificial seawater (prepared following Margalef 1977) to obtain a final concentration of 1000 µg crude extract ml⁻¹. We used a dilution factor of 2, and tested concentrations of 500, 250, 125 and 62.5 µg ml⁻¹ (i.e. 50, 25, 12.5 and 6.25% of the initial 1000 µg ml⁻¹).

The toxicity index provided by Microtox is measured in Gamma units, which are calculated as:

\[
\text{Gamma} = \left(\frac{I_0 \times \text{correction factor}}{I_t} \right) - 1
\]

where \(I_0\) and \(I_t\) are the bioluminescence measured before and after 5 min of exposure of bacteria to the crude extract solution or suspension, respectively. The correction factor is the ratio between the initial bioluminescence and that measured after 5 min in the control wells.

To compare results across samples we used the gamma value corresponding to the concentration of 1 mg sample DW (dry weight) ml⁻¹. The toxicity of this concentration was obtained by using the resulting regression equation of the Microtox® assay (Martí et al. 2003). We chose to use a concentration based upon sample DW (instead of crude extract DW) to make the results comparable in spite of different weights of sample extracted or different proportions of crude extract g⁻¹ DW across samples. We believe that the ecologically relevant information is the amount of toxicity per sample unit, not per extract unit.

**Sea urchin assay.** To determine whether species toxicity measured by Microtox® can also extend to marine invertebrates, we compared the Microtox® results with those from the sea urchin assay, which assesses cytotoxic (i.e. number of divided but dead or aberrant embryos) and antimiotic (number of non divided but fertilized embryos) effects of extracts on *Paracentrotus lividus* embryos (see Martín & Uriz 1993 for a detailed description of this method). We used the same crude extracts and tested the same concentrations (500, 250, 125 and 62.5 µg ml⁻¹) of species belonging to different phyla with both tests. The target species were previously known to have some kind of active metabolites (Martín & Uriz 1993 and unpubl. data). The seaweed *Flabellia petiolaris* (Turra) Nizamuddin, the sponges *Dendroxea lenis* (Topsent, 1892) and *Axinella damicornis* (Esper, 1794) and the tunicate *Pseudodistoma cyrmusense* Péres, 1952 were analysed from the Cabrera site. The Medes’ species analysed were the seaweeds *F. petiolaris* and *Peyssonnelia squamaria* (Gmelin) Decaisne, and the sponges *Dysidea avara* (Schmidt, 1862) and *Aplysina aerophoba* Schmidt, 1862. The toxicity index (Ti) used for the sea urchin test was \(Ti = 1 - \left(\frac{A_0}{A_t} - 1\right)\), where \(A_t\) and \(A_0\) are the average percent of aberrant (i.e. irregularly divided and resulting in non-viable embryos) plus non-divided embryos in 3 replicates from treatment (t) and control (0), respectively. This index ranges from 0 (non-toxic) to 1 (no viable embryo).

**Qualitative classification of seaweeds depending on their toxicity.** The previous step gave the calibration of a threshold for toxicity measurements with the Microtox® assay. This value allowed us to classify the species as toxic or non-toxic. Moreover, we were able to detect shifts from one condition to the other depending on season or on community. The abundance of species that were always toxic, in all communities and seasons (hereafter always-toxic species), those which were never toxic in any community or season (hereafter never-toxic species) and the ones which were toxic only in some community or season (hereafter occasionally toxic species) were scored and compared. We also counted the number of toxic seaweeds in the main taxonomic groups, Chlorophyta, Rhodophyta and Phaeophyta.

**Relationship between species toxicity and growth shapes.** As for seaweeds, we followed the functional-form groups established by Littler et al. (1983), i.e. crustose group (e.g. *Mesophyllum alternans*), thick, leathery group (e.g. *Cystoseira balearica, Padina pavonica, Flabellia petiolaris*), sheet group (e.g. *Dictiopteris membranacea*) and coarsely branched group (e.g. *Halopteris* sp.). Afterwards we compared the mean toxicity between encrusting and non-encrusting species (Fig. 7).

**Seasonal changes in coverage and toxicity.** Twenty photographs of 310 cm² were randomly taken in each community in June and November. The photographs were projected on an inverse slide projector and the patches of each species were outlined. These outline drawings allowed us to record the contacts among the species, but overgrowth processes might not be clearly discernible. By digitalising these photographs and using image analysis we obtained the coverage of each species. The seasonal changes in coverage and toxicity...
were estimated from the formula: Seasonal change of coverage = (coverage in November – coverage in June)/coverage in June.

A similar formula was applied for toxicity by using toxicity values instead of coverage values. This change indicates whether coverage or toxicity increased or decreased (indicated by a plus/minus sign) in November compared to June.

**Statistical analyses.** Seasonal changes in toxicity were statistically analysed for each species. Student's t-test analyses for differences between seasons were performed for species present in only 1 community. Species present in more than 1 community from a single archipelago were analysed by 2-way ANOVA with season and community as factors. To make a comparison between communities, the mean toxicity for species present in both seasons was calculated and compared by 2-way ANOVA.

Unless otherwise stated, Tukey post-hoc tests were used for *a posteriori* comparisons. In all cases, when data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlet test), rank transformation was carried out and parametric analyses were performed on ranked data (Conover & Iman 1981, Potvin & Roff 1993). All analyses used the Systat v5.0 and Statistica v4.0 packages.

The toxicity indices obtained with the sea urchin assay for all concentrations and samples were related by regression analyses to the gamma units obtained for the corresponding concentrations with the Microtox® test.

Seasonal changes in coverage were plotted against seasonal changes in toxicity. In this plot the species appear divided into 4 groups following graphical representation in quadrants: those that increased or decreased concomitantly coverage and toxicity from June to November (Quadrants II and III, respectively) and those that increased coverage with decreased toxicity or vice-versa (Quadrants I and IV). We tabulated our observations as a 2-way contingency table and used a chi-square goodness of fit analysis with Yates correction for continuity (Zar 1984) to test whether the distribution of the species among quadrants deviated from a random pattern.

**RESULTS**

**Quantitative approach to seaweed toxicity**

Photophilic and sciaphilic communities from the Cabrera Archipelago

A total of 25 seaweed species from the 2 Cabrera communities were analysed: 21 species from the photophilic seaweed community and 5 species from the sciaphilic seaweed community (1 species was common to both communities). Some of these seaweeds were not present in both seasons due to seasonal dynamics, which implied changes in species coverages (Martí 2002).

In the photophilic community, the analysed species (Table 1) represented 97 and 94% of seaweed coverage of the zone in June and November, respectively. In the sciaphilic seaweed community (Table 1), these percentages varied from 77% in June to 84% in November. Therefore, the samples collected were representative of the phytobenthic communities studied in both sampling seasons.

For the species for which there was replicate samples in both seasons and/or in both communities, the variation in toxicity is shown in Figs. 1 & 2. Summarizing the results of the statistical tests, we found significantly higher toxicity in *Pseudochlorodesmis furcellata* during November than in June, whereas for *Lobophora variegata* we found the opposite pattern. For *Corallina elongata* and *Haloptilon virgatum* there was no significant variation.

Among the 12 species without replicates from the photophilic community of Cabrera analysed in both seasons, 11 species (79%) showed higher toxicity in November than in June with important seasonal differences. Six of these 11 species (55%) were toxic in both seasons (*Dicytota dichotoma* var. intricata, *Anadyomene stellata*, *Dicytota* *polypodioides*, *Halopteris scoparia*, *Amphiroa rigida* and *Valonia utricularis*), and 5 species (45%) (*Mesophyllum alternans*, *Polysiphonia fruticulosa*, *Cystoseira compressa*, *Padina pavonica* and *Neogoniolithon notarisii*) were toxic in November but not in June. *C. balearica* and *Falkenbergia rufolanosa* were highly toxic in both seasons and seasonal variation was very low. *F. rufolanosa* was the most toxic seaweed from Cabrera, whilst *Halimeda tuna* was never toxic.

In the sciaphilic community, 2 species were not toxic, *Peyssonnelia squamaria* and *Palmpophyllum crassum*. The latter was the least toxic seaweed species analysed. *P. rosamaria* was toxic in November but not in June, following the same seasonal pattern as most species from the photophilic community.

Hemisciaphilic community from the Medes Archipelago

A total of 15 seaweed species from the hemisciaphilic seaweed community of Medes were analysed (Table 1). These represented 97 and 94% of algal coverage in June and November, respectively.

Species from Medes from which we collected replicates showed higher intraspecies variation of toxicity than those from Cabrera (Fig. 1), and thus standard
errors were higher. No species showed significant seasonal variation of toxicity.

As at Cabrera, *Falkenbergia rufolanosa* was the most toxic seaweed analysed. In contrast, *Flabellia petiolata* was non-toxic in all seasons (Fig. 2). Again as at Cabrera, *Corallina elongata* (Fig. 2) was toxic in both seasons and did not show seasonal variation, but its toxicity was lower in Medes. *Peyssonnelia squamaria* and *Halopteris filicina* showed mean values of toxicity slightly over 0.5 gamma only in 1 of the 2 seasons: November and June, respectively.

### Table 1. Toxicity of seaweeds from different communities of the Cabrera and Medes Archipelagos in June and November

<table>
<thead>
<tr>
<th>Species and authorities</th>
<th>Toxicity (gamma units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>June</td>
</tr>
<tr>
<td><strong>Photophilic community (Cabrera Archipelago)</strong></td>
<td></td>
</tr>
<tr>
<td><em>Amphiroa rigida</em> Lamouroux</td>
<td>0.569</td>
</tr>
<tr>
<td><em>Anadyomene stellata</em> (Wulfen) C. Agardh</td>
<td>1.359</td>
</tr>
<tr>
<td><em>Codium bursa</em> J. Agardh</td>
<td>0.294</td>
</tr>
<tr>
<td><em>Corallina elongata</em> Ellis &amp; Solander*</td>
<td>1.537</td>
</tr>
<tr>
<td><em>Cystoseira compressa</em> (Esper) Gerloff &amp; Nizamuddin, Ercegovici</td>
<td>2.033</td>
</tr>
<tr>
<td><em>Dictyota dichotoma</em> (Hudson) Lamouroux <em>v. intricata</em> (C. Agardh) Greville</td>
<td>0.000</td>
</tr>
<tr>
<td><em>Dictyopteris polyiodoides</em> (Stackhouse) Batters</td>
<td>0.647</td>
</tr>
<tr>
<td><em>Falkenbergia rufolanosa</em> (Harvey)</td>
<td>5.170</td>
</tr>
<tr>
<td><em>Flabellia petiolata</em> (Turra) Nizamuddin*</td>
<td>1.077</td>
</tr>
<tr>
<td><em>Halimeda tuna</em> (Ellis &amp; Solander) Lamouroux</td>
<td>0.067</td>
</tr>
<tr>
<td><em>Haliphtilon virgatum</em> Ellis &amp; Solander*</td>
<td>0.947</td>
</tr>
<tr>
<td><em>Halopteris scoparia</em> (Linnaeus) Sauvageau</td>
<td>0.695</td>
</tr>
<tr>
<td><em>Lobophora variegata</em> (Lamouroux) Womisleya</td>
<td>0.603</td>
</tr>
<tr>
<td><em>Mesophyllum alternans</em> (Foslie) Cabioch &amp; Mendoza</td>
<td>0.226</td>
</tr>
<tr>
<td><em>Neogoniolithon brassica-florida</em> (Dufour) Athanasiadis</td>
<td>0.447</td>
</tr>
<tr>
<td><em>Padina pavonica</em> (Linnaeus) Thivy</td>
<td>0.399</td>
</tr>
<tr>
<td><em>Polysiphonia fruticulosus</em> (Wulfen) Sprengel</td>
<td>0.381</td>
</tr>
<tr>
<td><em>Tricleocarpa</em> sp.</td>
<td></td>
</tr>
<tr>
<td><em>Valonia utricularis</em> (Roth) C. Agardh</td>
<td>0.622</td>
</tr>
<tr>
<td><em>Wurdermannia miniata</em> (Sprengel) J. Feldmann &amp; Hamel</td>
<td></td>
</tr>
<tr>
<td><strong>Hemisciaphilic community (Medes Archipelago)</strong></td>
<td></td>
</tr>
<tr>
<td><em>Asparagopsis armata</em> (Harvey, 1855)</td>
<td>1.143</td>
</tr>
<tr>
<td><em>Colpomenia sinuosa</em> (Mertens ex Roth) Derbès &amp; Solier in Castagne</td>
<td>0.849</td>
</tr>
<tr>
<td><em>Corallina elongata</em> Ellis &amp; Solander*</td>
<td>0.728</td>
</tr>
<tr>
<td><em>Dictyota dichotoma</em> (Hudson) Lamouroux</td>
<td>0.601</td>
</tr>
<tr>
<td><em>Dictyota dichotoma</em> (Hudson) Lamouroux <em>v. intricata</em> (C. Agardh) Greville</td>
<td>0.772</td>
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<tr>
<td><em>Dictyota fasciata</em> (Roth) Howe</td>
<td>0.359</td>
</tr>
<tr>
<td><em>Falkenbergia rufolanosa</em> (Harvey)*</td>
<td>5.204</td>
</tr>
<tr>
<td><em>Flabellia petiolata</em> (Turra) Nizamuddin*</td>
<td>0.157</td>
</tr>
<tr>
<td><em>Halimeda tuna</em> (Ellis &amp; Solander) Lamouroux</td>
<td>0.102</td>
</tr>
<tr>
<td><em>Halopteris filicina</em> (Grateloup) Kützing*</td>
<td>0.570</td>
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<tr>
<td><em>Litophyllum incrustans</em> Philipsi</td>
<td>0.325</td>
</tr>
<tr>
<td><em>Mesophyllum alternans</em> (Foslie) Cabioch &amp; Mendoza</td>
<td>0.154</td>
</tr>
<tr>
<td><em>Padina pavonica</em> (Linnaeus) Thivy</td>
<td>0.374</td>
</tr>
<tr>
<td><em>Peyssonnelia squamaria</em> (Gmelin) Decaisne*</td>
<td>0.345</td>
</tr>
<tr>
<td><em>Taonia atomaria</em> (Woodward) J. Agardh</td>
<td>0.611</td>
</tr>
<tr>
<td><strong>Sciaphilic community (Cabrera Archipelago)</strong></td>
<td></td>
</tr>
<tr>
<td><em>Flabellia petiolata</em> (Turra) Nizamuddin*</td>
<td>0.555</td>
</tr>
<tr>
<td><em>Palmophyllum crassum</em> (Naccari) Rabenhorst</td>
<td>0.041</td>
</tr>
<tr>
<td><em>Peyssonnelia rosa-marina</em> Boudouresque &amp; Denizot</td>
<td>0.244</td>
</tr>
<tr>
<td><em>Peyssonnelia squamaria</em> (Gmelin) Decaisne</td>
<td>0.240</td>
</tr>
<tr>
<td><em>Pseudochlorodesmis furcellata</em> (Zanardini) Borgesen*</td>
<td>0.580</td>
</tr>
</tbody>
</table>

*Species for which replicates were obtained: toxicity values are means

Comparison of species present in communities at the 2 sites

*Corallina elongata* and *Flabellia petiolata* (Fig. 2) were the only species common to both archipelagos and abundant enough to allow us to obtain replicates. The differences found in the ANOVA for *C. elongata* (Table 2) were due to the community factor, with the specimens from the photophilic community of Cabrera more toxic than those from the hemisciaphilic community of Medes.
Toxicity of *Flabellia petiolata* varied differently with the season, depending on the cave community (significant interaction term, Table 2). Separate analyses in each community indicated that the species only had a significant seasonal variation of toxicity (p < 0.01) in the photophilic community: higher in June than in November. When we analysed seasons separately, this species was significantly more toxic in June in the photophilic community of Cabrera than in the sciaphilic community of Cabrera and the hemisciaphilic community of Medes (ANOVA p = 0.007 and Duncan post-hoc test). However, in November the species was significantly more toxic in the sciaphilic community of Cabrera than in the photophilic community of Cabrera and the hemisciaphilic community of Medes (ANOVA p = 0.034 and Duncan post-hoc test). It should be noted that the toxicity of the species from the sciaphilic community of Cabrera and the hemisciaphilic one of Medes showed the same seasonal pattern namely toxicity was higher in November than in June.

Comparison between communities for the species present in both seasons

The number of seaweed species present in both seasons was 17 in the photophilic community and 5 in the sciaphilic community of Cabrera, and 7 in the hemisciaphilic community of Medes (Fig. 3). A 2-way ANOVA (Table 2) detected significant differences in toxicity between zones and seasons. The post-hoc Duncan test comparison indicated that the photophilic community of Cabrera was significantly more toxic than the hemisciaphilic community from Medes, and that the sciaphilic community of Cabrera did not differ significantly from either of the other 2 communities. The 3 communities showed significantly higher toxicity in November than in June.
Correlations between sea urchin and Microtox® assays

Fig. 4 shows the results of the regressions between the gamma units (Microtox® test) and the toxicity index from the sea urchin assay. The regression was positive ($r > 0.9$) and significant ($p < 0.05$) for all the samples analysed. Therefore, the Microtox® assay correlated well with the sea urchin test for these samples.

On the basis of all these regressions, we selected a threshold to separate toxic from non-toxic species. This threshold was 0.5 gamma units, because all the samples showed activity against sea urchin embryos at this gamma value.

Qualitative approach to seaweed toxicity

To make a qualitative interpretation of the toxicity data, all the seaweed species analysed from both archipelagos in both seasons were divided into 3 categories: those which were never-toxic in any community, archipelago or season, always-toxic ones, and those which shifted from toxic to non-toxic or vice versa in any community, archipelago or season (species occasionally toxic).

The never-toxic species category (Table 3) contained only 2 species, which represented 8% of the species analysed. The always-toxic category (Table 3) contained 11 species, 46% of the 24 species analysed. The occasionally toxic category also had 11 species (Table 3), 46% of the species analysed.

There was an increase (Fig. 5) in the percentage of always-toxic species and a decrease in non-toxic species from the darkest zone (the sciaphilic seaweed community) to the most illuminated zones (the photophilic seaweed community). The percentage of occasionally toxic species was higher in the sciaphilic and hemisciaphilic communities than in the photophilic community.

Chlorophyta was the group with the fewest toxic species (60%), whereas 100% of the Phaeophyta and Rhodophyta analysed were toxic.

Table 2. Toxicity of species and communities in June and November (2-way ANOVA). C: community, S: season

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corallina elongata (in photophilic and hemisciaphilic communities)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community</td>
<td>108.0</td>
<td>1</td>
<td>108.0</td>
<td>24.923</td>
<td>0.001</td>
</tr>
<tr>
<td>Season</td>
<td>0.333</td>
<td>1</td>
<td>0.333</td>
<td>0.077</td>
<td>0.789</td>
</tr>
<tr>
<td>C × S</td>
<td>0.000</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Error</td>
<td>34.667</td>
<td>8</td>
<td>4.333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flabellia petiolata (in photophilic, hemisciaphilic and sciaphilic communities)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community</td>
<td>0.675</td>
<td>2</td>
<td>0.338</td>
<td>9.190</td>
<td>0.004</td>
</tr>
<tr>
<td>Season</td>
<td>0.017</td>
<td>1</td>
<td>0.017</td>
<td>0.453</td>
<td>0.514</td>
</tr>
<tr>
<td>C × S</td>
<td>0.883</td>
<td>2</td>
<td>0.442</td>
<td>12.020</td>
<td>0.001</td>
</tr>
<tr>
<td>Error</td>
<td>0.441</td>
<td>12</td>
<td>0.037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean toxicity (all species in the 3 communities)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community</td>
<td>2433.9</td>
<td>2</td>
<td>1216.9</td>
<td>5.113</td>
<td>0.009</td>
</tr>
<tr>
<td>Season</td>
<td>1171.2</td>
<td>1</td>
<td>1171.2</td>
<td>4.920</td>
<td>0.031</td>
</tr>
<tr>
<td>C × S</td>
<td>62.5</td>
<td>2</td>
<td>31.2</td>
<td>0.131</td>
<td>0.877</td>
</tr>
<tr>
<td>Error</td>
<td>12377.2</td>
<td>52</td>
<td>238.0</td>
<td></td>
<td></td>
</tr>
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</table>

The never-toxic species category (Table 3) contained only 2 species, which represented 8% of the species analysed. The always-toxic category (Table 3) contained 11 species, 46% of the 24 species analysed. The occasionally toxic category also had 11 species (Table 3), 46% of the species analysed.

There was an increase (Fig. 5) in the percentage of always-toxic species and a decrease in non-toxic species from the darkest zone (the sciaphilic seaweed community) to the most illuminated zones (the photophilic seaweed community). The percentage of occasionally toxic species was higher in the sciaphilic and hemisciaphilic communities than in the photophilic community.

Chlorophyta was the group with the fewest toxic species (60%), whereas 100% of the Phaeophyta and Rhodophyta analysed were toxic.

Relationship between species toxicity and growth shapes

We observed a similar pattern of toxicity with respect to growth shape in both archipelagos (Fig. 6, Table 4): in general sheet forms tended to be more toxic than the other morphotypes. When we pooled non-encrusting morphotypes (thick leathery, sheet and coarsely branched groups) and compared them to crustose forms, a significant difference ($t$-test) was detected only in Cabrera: encrusting forms were less toxic than non-encrusting forms.

Significant seasonal differences ($t$-test, $p < 0.05$) of toxicity were detected only for sheet seaweeds from Cabrera Archipelago, which were more toxic in November than in June.
Seasonal changes in coverage and toxicity

The spatial representation of the relationship between change in toxicity and change in coverage of seaweed species in June and November is represented in Fig. 7A. Fig. 7B represents the same relationship but excludes the species *Lobophora variegata* since its high change in coverage compared to the other species made the spatial representation of the remaining species cluttered.

In Quadrant I there were 6 species, 11 in Quadrant II, only 2 in Quadrant III and 4 in Quadrant IV. The pattern deviated significantly from an expected random distribution across quadrants (p < 0.05). It is interesting to note that many species of Quadrant II, which increased both coverage and toxicity in November compared to June, were encrusting forms: *Mesophyllum alternans* (9, 12), *Neogoniolithon brassica-floride* (11), *Peyssonnelia rosa-marina* (8) and *P. squamaria* (13). The higher coverage of these species in November (especially *N. brassica-floride* and *M. alternans*) may be due to the disappearance of the canopy layer that covered them in June, so that the increase in coverage observed may not be due to true growth. Thus, these results should be taken with caution.

![Table 3. Classification of seaweed species as a function of their toxic behaviour](image)
The 6 species depicted in Quadrant I increased slightly in toxicity while decreasing in area from June to November. The only 2 species in Quadrant III, i.e. Falkenbergia rufolanosa (18) and Cystoseira balearica (19) from Cabrera, also decreased slightly in both coverage and toxicity in November.

Finally, 2 of the 4 species from Quadrant IV, i.e. Falkenbergia rufolanosa (21) and Halopteris filicina (20), both from Medes, showed a discrete increase in coverage from June to November as compared to Flabellia petiolata (22) and Lobophora variegata (Fig. 7A). The 2 latter species have tropical affinities (Ballesteros 1993) and are characterised by maximum growth at the end of summer, which can explain their higher coverage in November.

**DISCUSSION**

Many studies have demonstrated that seaweeds are an important source of bioactive secondary metabolites (Burkholder et al. 1960, Hornsey & Hide 1974, 1976, Naqvi et al. 1980, Reichelt & Borowitzka 1983, Hodgson 1984, Paul & Fenical 1986, 1991, Van Alstyne & Paul 1988, Munro et al. 1991, Hay 1996). Our results showed that 87% of the seaweed species analysed were toxic (mean toxicity higher than 0.5 gamma value) in at least 1 season, archipelago or community.

At Cabrera 64% of the seaweed species analysed were more toxic in November than in June, and only 3 species (7.3%) showed the opposite pattern (Cystoseira balearica, Flabellia petiolata and Lobophora variegata, all 3 from the photophilic community). Moreover, 10 species of those that were toxic in November were non-toxic or at the limit of the 0.5 gamma value in June.

In summary, most algal species from Cabrera had marked seasonal changes in toxicity, which increased from spring to autumn. In contrast, the species from Medes showed greater intraspecies variability in toxicity but had no significant seasonal variation of toxicity.

The good correlations between the results of Microtox® and the sea-urchin tests indicate that these toxicities may have an effect on both prokaryotic and phage populations.
eukaryotic cells and may have an ecological role as deterrent, antifouling or space competition mechanisms due to their antimitotic and cytotoxic properties. An ecological role can be proposed for seaweeds toxicity since several toxic species (according to Microtox results) were also deterrent when given to fish and sea urchins (Hay et al. 1998) while others deter amphipods from grazing (Hay et al. 1998, Schnitzler et al. 1998).

However, from these laboratory tests, we can only speculate about their possible effects in the field. Field experiments, which were not the aim of the present investigation, would be necessary to confirm the ecological trends here reported, and would represent a further step of the study.

On comparison of the percentage of species belonging to each of the established toxic categories (always-toxic, never-toxic and occasionally toxic) in the 3 seaweed communities, differences became clear. The decrease in light received from photophilic to sciaphilic communities coincided with a marked decrease in the always-toxic species and an increase in both never-toxic and occasionally toxic species. This positive correlation between light and toxicity might be due to an optimization of the species fitness under high irradiance. However, nitrogen can be limiting in the Cabrera waters (Vives 1993). The carbon-nutrient balance hypothesis (Cronin & Hay 1996) suggests that part of the differences in toxicity detected within species between Cabrera and Medes could be due to nitrogen limitation in Cabrera, which would direct excess of photosynthates toward the production of chemical defences. If this hypothesis is right, then these defences should be C-based rather than N-based.

A higher allocation of the available resources to defence in environments where seaweeds are limited by nutrients is consistent with the higher mean toxicity found in species from Cabrera. In our design, the community factor cannot be separated from the geographic area factor, as our 3 communities belong to 2 different areas. However, in the 2 Cabrera communities, the pattern of a decrease in toxicity from the photophilic to the sciaphilic community still holds true and cannot be explained on the basis of resource allocation hypotheses because there were no significant differences in nitrogen concentration between the 2 communities (Martí et al. 2004). As in the comparison between archipelagos, the species from a less lighted community of Cabrera (sciaphilic community) are less toxic than those of the photophilic community. This seems to be a consistent pattern in Mediterranean communities, because Uriz et al. (1991) also found lower antibacterial activity in species from sciaphilic seaweed communities than in those from photophilic ones.
In our study, Phaeophyta and Rhodophyta contained the most toxic species and the highest percentage of toxic species, followed by Chlorophyta. These percentages contrast with the results from a previous screening against pathogenic microbes in which Chlorophyta contained the highest number of active species (Ballesteros et al. 1992). However, in most previous studies, Rhodophyta and Phaeophyta were the 2 most bioactive groups (Barbagallo et al. 1979, Caccamese et al. 1981, 1985, Padmakumar & Ayyakkannu 1997). Furthermore, Harper et al. (2001) listed the known secondary metabolites from the 3 groups of seaweed and showed that 51% of the compounds described up to now belonged to Rhodophyta, 41% to Phaeophyta, and only 8% to Chlorophyta. Therefore, the activity measured by Microtox® coincided quite well with most previous studies and reflects the patterns of abundance of bioactive compounds in seaweeds.

Some of the species that did not show toxicity in our study were reported as toxic in previous screenings, or vice versa (Porzi & Minelli 1975, Caccamese & Azzolina 1979, Caccamese et al. 1980, 1981, 1985, Pesando & Caram 1984, Ballesteros et al. 1992). Differences in extraction procedures and bioassay methods may account for these differences. However, the geographic, seasonal or spatial variation in toxicity of seaweeds found in this study and reported previously (Hornsey & Hide 1976, Caccamese et al. 1980, Meyer & Paul 1992, Pavia & Aberg 1996, Padmakumar & Ayyakkannu 1997, Van Alstyne et al. 1999, Wright et al. 2000) may also contribute to different outcomes in toxicity assessment.

When we studied the relationship between toxicity and growth shapes, we observed that the non-encrusting (thick, leathery, sheet and coarsely branched groups) forms tended to be more toxic than the crustose forms. This pattern may reflect a higher investment in chemical defences in those algae (sheets) more susceptible to grazing. Of course, other defence mechanisms (e.g. physical defences such as calcification or leathery consistency) can act to prevent grazing by herbivores in seaweeds. This can explain why Littler et al. (1983) reported that functional-form groups of stony, tough or leathery-rubbery texture were the forms most resistant to herbivores. Although seasonal differences of toxicity were general in the various forms of seaweeds analysed, these differences were only significant in non-encrusting forms from Cabrera, where seaweeds toxicity was higher in November than in June.

At a community level as well as at species level, there was an increase in toxicity for most species in November compared to June (i.e. those species in Quadrants I and II in Fig. 7B), that may be attributed to a physiological process of ageing, but also to a lower requirement of photosynthates for maintenance or resource acquisition, which would produce a shift in allocation of resources from growth or reproduction to production of toxic compounds. Following the cycles reported for photophilic communities in the Mediterranean (Ballesteros 1991), the photophilic seaweed community of Cabrera was in a diversification phase in November (Martí 2002). After a phase of high growth rates and reproduction in summer, many species had smaller coverage and lower growth rates in November, and may allocate more resources to synthesis of secondary metabolites than in June, when the energy should be devoted to somatic growth and reproduction. This behaviour matches Grime’s plant strategy model (Grime 1979), slightly modified by Cronin (2001), which states that, when growth and reproduction occurred, a lower portion of the acquired resources can be allocated to chemical defences.

Conversely, the species Lobophora variegata from the photophilic community of Cabrera and, to a lesser extent, Flabellia petiolata, increased in coverage from June to November (warmest months in the Mediterranean) possibly because they have tropical affinities (Ballesteros 1993) and, as it would be expected according to Grime’s theory, they experienced a decrease of toxicity during the same period. Consequently, our results suggest that Grime’s model could explain the seasonal variation of toxicity found in non-encrusting algae.

Increased levels in production of chemical defences associated with decreases in growth, as those reported here, survival or reproduction processes have already been described in previous studies for terrestrial plants (Berenbau et al. 1986, Coley 1986) and for the seaweed Fucus vesiculosus (Yates & Peckol 1993).

As for the encrusting algae with a weak seasonal variation of toxicity, it is difficult to ascribe toxicity variation to changes in coverage because quantifying growth from the pictures may be inaccurate, as the change in coverage recorded may have been partially due to the canopy layer that covered them in June but not in November and, besides, they usually have very slow growth rates (Garrabou & Ballesteros 2000).

This study clearly shows a spatial and temporal variation of toxicity in several Mediterranean seaweeds. It also illustrates that toxicity varies intra-species as well as inter-species as a function of community or season, which points to the role of abiotic and biotic factors in the production of chemical defences by seaweeds. However, this variability should be addressed experimentally in the future by means of transplantation experiments, to verify whether there is a cause/effect relationship and to find the concrete factors that affect toxic metabolites production.
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