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Sargassum polyceratium (Phaeophyceae, Fucaceae) surface molecule activity towards fouling organisms and embryonic development of benthic species

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Abstract

Coral reefs have undergone profound ecological changes over recent decades. Areas formerly covered by scleractinian coral species are now often overgrown by macroalgae. In Martinique (West Indies), this phenomenon has lead to the colonisation of numerous coral reefs by algae, amongst which Sargassum is one of the most prominent. This study focuses on potential defence molecules produced by Sargassum polyceratium. The hexane dipping method was employed to extract surface molecules on fresh material, and their bioactivities were assessed against bacteria (marine and estuarine), and marine tropical invertebrates. The hexane dipping method was employed to extract surface molecules on fresh material, and their activities have increased over the past 20 years. Particular attention has been given to the production of secondary metabolites by macroalgae, microalgae, invertebrates, Cyanobacteria, octocorals, sponges and ascidians (Hay and Fenical 1996, Paul and Puglisi 2004, Paul et al. 2007, Hellio et al. 2009). Algae produce secondary metabolites with a wide range of biological activities including antifungal, antibacterial, antibiotic, anti-fouling (AF), UV radiation protection, feeding deterrence, inhibition of competitors, gamete attraction and inhibition of larval settlement and development (Hay and Fenical 1988, Paul et al. 1988, Hay and Fenical 1996, Hellio et al. 2001, 2002, 2005, Steinberg and de Nys 2002, Birrell 2003, Paul and Puglisi 2004, Paul et al. 2007, Plougüerné et al. 2010a).

Several examples demonstrate that specific structures localised on the surfaces of algae release active compounds into the environment (Dworjanyn et al. 1999, De Nys and Steinberg 2002); Vesicular physodes located on the surfaces of phaeophyceans contain phlorotannins (Ragan and Glombitza 1986). The role of phlorotannins as macroalgal defence mechanism has been extensively described in the literature, but yet remains not fully understood. These compounds have been described as UV screens, herbivore deterrents and antimicrobial agents, but are also known to play a role in primary metabolism (Paul and Puglisi 2004). Moreover, fluorescence microscopy, combined with chemical analyses, has demonstrated that Delisea pulchra (Grev.) Mont. (Rhodophyceae) releases AF compounds (halogenated furanones) from gland cells located on its surface (Dworjanyn et al. 1999, De Nys and Steinberg 2002).

The ‘‘corps en cerise’’ found on cortical cells of Laurencia snyderiae E.Y. Dawon (Rhodophyceae) may be a primary location of halogenated MNPs (Young et al. 1980). However, even though it has been stated that the ‘‘corps en cerise’’ are main reserves for halogenated compounds in the alga Laurencia obtusa Lamour and store high concentrations of bromine and chlorine (Salgado et al. 2008), they are not found at the surface of the algae and no structure linking them to the algal surface have been found (De Nys et al. 1998).

Sargassum species have been extensively analysed for their allelochemical activities for several reasons: 1) some of them are lightly fouled in the field, 2) the genus is one of the most conspicuous algae in numerous areas, especially the tropics (Ang 1986, De Ruyter van Stevenick and Breeman 1987, Littler et al. 1993, Lapointe 1997, Engelen et al. 2001), 3) some species have invaded many parts of the world (Plougüerné et al. 2006). Antibacterial, anti-algal, anti-fungal and anti-invertebrate activities have been demonstrated in S. muticum (Yendo) Fensholt samples (Hellio et al. 2002, Plougüerné et al. 2008, 2010b, Maréchal and Hellio 2011), S. wightii Greville ex J. Agardh and S. johnshonii V.J. Chapman (Sastry and Rao 1994).

Keywords: bacteria; embryonic development; hexane dipping; Sargassum polyceratium; toxicity.

Introduction


Several examples demonstrate that specific structures localised on the surfaces of algae release active compounds into the environment (Dworjanyn et al. 1999, De Nys and Steinberg 2002); Vesicular physodes located on the surfaces of phaeophyceans contain phlorotannins (Ragan and Glombitza 1986). The role of phlorotannins as macroalgal defence mechanism has been extensively described in the literature, but yet remains not fully understood. These compounds have been described as UV screens, herbivore deterrents and antimicrobial agents, but are also known to play a role in primary metabolism (Paul and Puglisi 2004). Moreover, fluorescence microscopy, combined with chemical analyses, has demonstrated that Delisea pulchra (Grev.) Mont. (Rhodophyceae) releases AF compounds (halogenated furanones) from gland cells located on its surface (Dworjanyn et al. 1999, De Nys and Steinberg 2002).
Sieburth and Conover (1965) found antibiotic activity in phlorotannins extracted from *S. vestitum* (R. Brown ex Turner) C. Agardh and *S. natans* (Linnaeus) Gaillon, and Tanaka and Asakawa (1988) found antialgal activity in extracts from *S. horneri* (Turner) C. Agardh. *S. vulgar* C. Agardh extracts from Brazil had AF activity against microalgae and mussel settlement (Plougrenz et al. 2010a). *S. muricatum* and *S. tenerillum* J. Agardh induce modifications of swimming activity in two-day-old larvae of *Platygrya daedalea* Ellis et Solander (coral) and decrease their proportional settlement, indicating chemical defences (Diaz-Pulido et al. 2010).

*Sargassum polyceratium* W.R. Taylor, one of the most abundant macroalgae of Martinique tropical reefs (M. Thabard, unpublished data), has, however, never been investigated. *Sargassum* species have been recorded since the 1970s on the Atlantic coast of the island (Battistini 1978). Coral reef colonisation by macroalgae has induced significant changes in the structure and diversity of communities. As an example, *S. polyceratium* and *S. hystrix* J. Agardh have supplant ed hermatypic coral species on the Jamaican north coast (Lapointe 1997, Lapointe and Thacker 2002). Algal canopies are known to affect understory species (Eckman and Duggins 1991) and may interfere with invertebrate larval recruitment processes (Pawlak 1992, Birrell 2003, Titlyanov et al. 2005). To survive the environmental pressures they are subjected to, and to successfully colonise new areas, macroalgae have developed several strategic mechanisms, including both specific morphological characteristics and production of deterrents to avoid epibiont overgrowth (Littler and Littler 1980, Littler et al. 1983a,b, Paul and Puglisi 2004).

In a survey conducted in Martinique in 2007 (M. Thabard unpublished data), the sea urchin *Diadema antillarum* Philippi was found to be almost absent from the *Sargassum* area, but a sea urchin population had developed nearby, forming distinct belts. *D. antillarum*, which is considered as a key-stone herbivore species on Caribbean coral reefs (Knowlton 2001), suffered mass mortality in 1983 in this region (Lessios et al. 1983, 1984). This induced profound changes on coral reefs, and is thought to have played a major role in the community composition shift observed nowadays in the Caribbean due to reduced grazing pressure on macroalgae (Hughes 1994). *Diadema* recovery is proving to be a very slow process in the Caribbean although its reproductive biology seems to indicate that the species should recover rapidly (Lessios 1995). There are several hypotheses (too few adults, pathogens remaining in water, etc.) for this phenomenon (Lessios 2005). It may be that fast colonisation by *Sargassum* sp. on coral reefs in the 1980s (Littler et al. 1993) is in part responsible for this slow recovery process as it is possible that the seaweed prevents the larval recruitment process.

The present study focuses on potential antibacterial and deterrent activities of *Sargassum polyceratium* and their possible interactions with other tropical species. In order to perform a broad spectrum analysis of the toxic activity of MNPs extracted from *S. polyceratium* surface, three marine tropical invertebrates were tested, one sea urchin, one bivalve and one worm, representing organisms from different phyla. *In vitro* effects of *S. polyceratium* extracts towards bacteria and embryos of marine invertebrates were investigated.

### Materials and methods

#### Algal collection site

*Martinique* is a volcanic island located in the eastern Caribbean Sea (Lesser Antilles) between latitudes 14°50′N and 14°23′N and at mean longitude of 62°12′W. It has a land area of 1128 km² and is bordered by the Caribbean Sea to the west and the Atlantic Ocean to the east. The climate is defined by distinct dry and wet periods, the dry season lasting from December to July and the wet season from August to early December.

The site chosen for this study is a fringing coral reef to the south of Martinique, close to the mouth of Trois Rivières river. This site was chosen for the dominant presence of *Sargassum polyceratium*.

#### Study organism

*Sargassum polyceratium* is commonly found in moderately turbulent habitats from the lower intertidal zone to depths over 50 m (Littler and Littler 2000, Engelen 2004). It has a tough crowded thallus and dense branches reaching to 100 cm. The main axes roughened with small spines may be numerous. The blades measure 3–8 mm in width and 1.5–2.0 cm in length. The holdfast is strong and disc like. The importance of recruitment vs. regeneration was modeled and demonstrated to vary with population, year and disturbance (Engelen et al. 2005).

#### Algal extractions

*Sargassum polyceratium* samples were collected in October 2008 and January 2009 (wet and dry seasons). Thalli were removed with their holdfasts by breaking away pieces of substratum, thus reducing stress on the algae; stress may be responsible for secondary metabolite production. The samples were collected by SCUBA diving at 18 m depth. The algae were cleaned of epiphytes, rinsed with seawater (SW) and transported to the laboratory in a container filled with clean SW. The fresh samples were soaked in hexane (Fisher, Loughborough, UK) in the ratio 1 l hexane kg⁻¹ thalli were dipped for 30 s in hexane for preliminary tests.

Two protocols were used:

- **Protocol A** (October 2008 samples, rainy season), *Sargassum polyceratium* thalli were dipped for 30 s in hexane for preliminary tests.
- **Protocol B** (January 2009 samples, dry season) was developed based on results of both tests conducted on 30 s extracts (October) and the observations of algal surfaces for breaks (see results). This amended protocol was used to test for the effect of dipping time on extract efficiency.
This was done by dipping algae in hexane for two different periods (10 s and 30 s), with the algal surface remaining intact.

Observation of algal surface

The objective of our experiments was to select the molecules present at the surface of Sargassum polyceratium only. The hexane dipping method was shown to break some algal surface cells when thalli were dipped for more than 30 s (De Nys et al. 1998). The algal surface was therefore checked for breaks that would result in leaks of cell contents in thalli dipped for 30 s, 10 min and 30 min to determine the best dipping time. As UV excitation of plant leaves is known to induce two distinct types of fluorescence, damage caused to the surface cells of S. polyceratium by hexane dipping was investigated by epifluorescent microscopy (Nikon, Tokyo, Japan, Eclipse 80i microscope; Filter FITC 494 nm excitation). Observation of algal surface was done by screening the surface molecules that were present at the surface of Sargassum polyceratium through the use of filters, namely 514 nm emission. Nikon Dxm1200F camera was used in the protocol adapted from Cerocic et al. (1999).

Bioassays

Bacteria  Culture of bacteria: Four marine bacterial strains were used: Halomonas marina (Cobet et al.), Dobson et Franzmann (ATCC 25374), Pseudalteromonas elyakovii (Ivanova et al. Sawabe et al. ATCC 700519), Polaribacter igensii Gorsk et al. (ATCC 700398) and Vibrio aestuarianus Tilson et Seidler (ATCC 35048). These bacteria were chosen because they are typical marine fouling bacteria (Plouguerné et al. 2010b). Pseudalteromonas elyakovii and Vibrio aestuarianus are also known to cause infections in marine organisms, such as molluscs, crustaceans, fishes or algae, the latter being thought responsible for the summer mortality of Crassostrea gigas Thunberg (Labreuche et al. 2005). Marine bacteria were cultivated with marine broth (5% tryptone, Oxoid, Basingstoke, UK, diluted in SW) and incubated at 30°C to allow development (Plouguerné et al. 2008).

Five terrestrial bacterial strains known to be present in estuaries and coastal environment (Mokrini et al. 2008) were used: Bacillus subtilis (Ehrenberg) Cohn (NCIMB 1026), Enterobacter aerogenes Hormaeche et Edwards (ATCC 13048), Escherichia coli (Migula) Castellani et Chalmers (B 81), Pseudomonas aeruginosa (Schroeter) Migula (NCIMB 10390) and Staphylococcus aureus Rosenbach (NCIMB 8625). B. subtilis and S. aureus are Gram-positive bacteria. The remainder are Gram-negative. Terrestrial bacteria were cultivated on a nutrient broth (CM0067. No. 2, Nutrient media Powder Oxoid 25 g l⁻¹) and incubated at 30°C. Biological activities of extracts were evaluated following the method of Amsterdam (1996).

Antibacterial assays: Aliquots of 100 µl of each hexane extract were poured in six wells of 96 well plates (Fisher) for each bacterial assay following the protocols of Plouguerné et al. (2010b). The solutions were tested at three concentrations: 15, 150 and 300 µg ml⁻¹. In addition, six wells free of extracts and six wells containing hexane were used as controls. The plates were first dried in a flow cabinet to evaporate the solvent and then left for 15 min in a UV cabinet for sterilization.

The optical densities (OD) of bacterial stock cultures were measured at 630 nm for every sample to determine the quantity of solution required to obtain 1 mOD (mili optical density). Then, 100 µl of bacterial solutions were added under aseptic conditions and the plates were incubated for 48 h at 30°C for bacterial growth. Activity was obtained comparing the controls and the wells containing the extracts. Solutions were considered to be active when bacteria did not grow in four, five or six wells. Bacterial growth was noted by the presence of a cloudy solution. One plate was used for each strain to limit the cross-contamination risk.

Invertebrates  Organisms: The toxicity of the extracts was tested against larvae of Codakia orbicularis L., Diadema antillarum and Pseudonereis sp. These organisms are tropical and represent typical species from three marine ecosystems (seagrass bed, reef and mangrove). Their spawning and early larval development has been described previously (Gros et al. 1997, Eckert 1998). For both C. orbicularis and D. antillarum, spawns were induced in the laboratory under controlled conditions, while Pseudonereis sp. embryos were collected from the wild.

Codakia orbicularis is a tropical bivalve mollusc distributed from Florida to Brazil (Abbott 1974). Adult C. orbicularis (between 40 and 60 mm shell length) were collected by hand from seagrass beds in Ilet Cochon (Guadeloupe, Figure 1B) in July 2009. Fertilization was induced following the method described by Gros et al. (1997). Adults were cleaned with a brush and spawning was induced by injection of 0.3 ml of a 4 mM serotonin solution in 0.22 µm filtered SW into the visceral mass. Sperm and oocytes were mixed in a 1 l cylinder until the appearance of two-cell embryos. Fertilization occurred under constant aeration to hold eggs in suspension as they are slightly negatively buoyant. C. orbicularis embryonic development follows the general development of bivalves (Gros et al. 1997). Appearance of the first polar body (indicating fertilization) is not always visible under a dissecting microscope, thus the two cells embryos were chosen to ensure the fertilization had occurred, and these were used in toxicity experiments.

Diadema antillarum: The black spined sea urchin D. antillarum was selected for experiments. Adults were collected on the shore at Port-Louis (Guadeloupe, Figure 1B) during summer 2009. Urchins were acclimated in the laboratory (25°C) for a week and fed on agar pellets containing a mixture of algae (including Ulva lactuca L. and Sargassum sp.) following the protocol of Pereira et al. (2003). After a week, the urchins were transferred to another tank containing 29°C filtered SW. Thermal shocks (3–5°C) that induce spawning of D. antillarum (M. Moe, personal communication) were applied over a few minutes. Both male and female gametes were pipetted from this tank and diluted in 10 l of 0.22 µm filtered SW (25°C) to induce the fertilization. The eggs are slightly negatively buoyant, thus aeration was used to keep them suspended. Embryos at the two cells stage (fertilization (To)+1h) were chosen for experiments.
Pseudonereis sp.: Egg balloons were collected from the mangrove Manche à Eau (Guadeloupe, Figure 1B) in summer 2009. Egg balloons containing young embryos of Pseudonereis sp. (i.e., at the blastula-early gastrula stage) were chosen to conduct the experiment.

Embryo toxicity tests: Dimethyl sulphoxide (DMSO) (Fisher) was used as a solvent carrier in order to dilute molecules extracted with hexane in 0.22 μm filtered SW. Preliminary tests demonstrated that this solvent was not toxic for embryos until the trochophore stage for both Codakia orbicularis and Pseudonereis sp. and prism stage for Diadema antillarum when used at a concentration of 0.5 μg ml⁻¹ (data not shown).

Tests were conducted in 96-well plates (Fisher). Experiments were tested at seven concentrations: 1, 5, 10, 15, 50, 100 and 200 μg ml⁻¹. Two hundred microlitres of each extract were added to each well (six replicates). In addition, six wells filled with SW, six with 1% CuSO₄ (known to kill larvae, Bielmeyer et al. 2005) and six with 0.5% DMSO were used as controls. Four larvae were added to each well and allowed to develop for 24 h at 25°C to reduce bacterial development. Percentage mortality and embryonic development (stage reached) were recorded. All the assays were performed on two independent batches of embryos (Hellio et al. 2004).

Statistical analyses

Embryo toxicity assays results were analysed using non-parametric tests (Kruskal-Wallis) and comparison between treatments was performed using a multiple comparisons test with the software R and the package npmc. The results of the two larval batches were pooled as no significant differences were apparent. These statistical tests were selected as the data (percentages) did not fit a normal distribution (Kolmogorov-Smirnov) even after transformation.

Results

Algal surface observation

Epifluorescence microscopy images showed cells to be well formed and not broken (Figure 2A, B) in the control and after 30 s of dipping in solvent, while there were leaks, suggesting lyses of the cell surfaces, after longer dipping times (Figure 2C, D). The assays tests were thus conducted only with the extracts prepared for the shortest times (10 and 30 s), protocols A and B.

Bacterial bioassay

Sargassum polyceratium extracts at a concentration of 15 μg ml⁻¹ did not inhibit bacterial strains (Table 1). The January 10 s extraction sample was more more active against bacteria than the January 30 s sample (except in the case of Vibrio aestuarianus). The 30 s extraction samples prepared in October were the second most active. Marine bacteria were most sensitive to extracts; growth of terrestrial bacterial strains was inhibited by only the 30 s October and 10 s January extracts.

Activity towards larvae

Diadema antillarum  Algal extracts affected Diadema antillarum embryo development (Kruskal-Wallis: p<0.0001). One hundred percent of embryos died in the highest concentrations (from 10 to 200 μg ml⁻¹) and in the copper solution (positive control) (Figure 3). Less than 70% of embryos survived when exposed to 5 μg ml⁻¹. Only solutions at concentrations of 1 μg ml⁻¹ and the 10 s January extract concentration of 5 μg ml⁻¹ permitted good survival of embryos (not significantly different from the control; p=0.9 for comparison between DMSO and the January 10 s at a concentration of 5 μg ml⁻¹ and p=1.0 for the others; multiple comparisons tests).

Apart from the 10 s January extract at a concentration of 5 μg ml⁻¹, there were no significant differences between the October and the January extracts.

Embryonic development stopped at different stages depending on the solution tested (Table 2, Figure 5). Only embryos developing in the controls (DMSO and SW) or the extract at 1 μg ml⁻¹ survived to the prism stage. The development of the other embryos was stopped progressively with decreasing concentrations of algal extracts, i.e., 2–4 cell stage at concentrations 100–200 μg ml⁻¹, morula at 15 and
Figure 2  Epifluorescent microscopic images of *Sargassum polyceratium* “leaf” surfaces: (A) control, (B) 30 s, (C) 10 min and (D) 30 min after being dipped in hexane. (magnification=×100). L, cellular content leaks; SF, surface cells.

50 μg ml⁻¹, late blastula early gastrula at 10 μg ml⁻¹, gastrula at 5 μg ml⁻¹ and prism at 1 μg ml⁻¹.

**Codakia orbicularis** The results for *Codakia orbicularis* embryos were similar to those for *Diadema antillarum* (Figure 4) (Kruskal-Wallis: p<0.0001). All embryos died in the highest concentrations assayed (50–200 μg ml⁻¹) and in the copper control. Percentage mortality decreased at 15 μg ml⁻¹ but the embryos did not develop to the trochophore stage. Only embryos cultured in the 1 μg ml⁻¹ solution devel-

Table 1  Antibacterial activity of hexane dipping extracts expressed as minimum inhibitory concentrations (μg ml⁻¹).

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>October 2008</th>
<th>January 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 s</td>
<td>10 s</td>
</tr>
<tr>
<td>Terrestrial</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>N</td>
<td>300</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>N</td>
<td>300</td>
</tr>
<tr>
<td>Marine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Halomonas marina</em></td>
<td>300</td>
<td>150</td>
</tr>
<tr>
<td><em>Polaribacter irgensii</em></td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td><em>Pseudoalteromonas elyakovii</em></td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td><em>Vibrio aestuarianus</em></td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

N, no active extracts.

Months of algal collection and dipping times are indicated.
Figure 3  Mean percentage mortality of *Diadema antillarum* embryos after 30 h exposure to molecules extracted from *Sargassum polyceratium*. Dates of algal collection and extraction times are indicated in the key. Values are means±SE, n=12. *Significant differences (p<0.05, multiple comparisons test) between control (DMSO) and one of the solutions; **significant differences (p<0.05) observed between control (DMSO) and a group of extract solutions.

Table 2  Stages of development in *Codakia orbicularis* and *Diadema antillarum* embryos after 30 h exposure to *Sargassum polyceratium* hexane surface extracts.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Stage of development</th>
<th></th>
<th>Stage of development</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Codakia orbicularis</em></td>
<td></td>
<td><em>Diadema antillarum</em></td>
<td></td>
</tr>
<tr>
<td>1 µg ml⁻¹</td>
<td>Trochophore</td>
<td></td>
<td>Prism</td>
<td></td>
</tr>
<tr>
<td>5 µg ml⁻¹</td>
<td>Late blastula-early gastrula</td>
<td></td>
<td>Gastrula</td>
<td></td>
</tr>
<tr>
<td>10 µg ml⁻¹</td>
<td>Late blastula-early gastrula</td>
<td></td>
<td>Late blastula-early gastrula</td>
<td></td>
</tr>
<tr>
<td>15 µg ml⁻¹</td>
<td>Morula</td>
<td></td>
<td>Morula</td>
<td></td>
</tr>
<tr>
<td>50 µg ml⁻¹</td>
<td>Morula</td>
<td></td>
<td>Morula</td>
<td></td>
</tr>
<tr>
<td>100 µg ml⁻¹</td>
<td>2–4 cells</td>
<td></td>
<td>2–4 cellules</td>
<td></td>
</tr>
<tr>
<td>200 µg ml⁻¹</td>
<td>2–4 cells</td>
<td></td>
<td>2–4 cellules</td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>Trochophore</td>
<td></td>
<td>Prism</td>
<td></td>
</tr>
<tr>
<td>DMSO (0.5%)</td>
<td>Trochophore</td>
<td></td>
<td>Prism</td>
<td></td>
</tr>
<tr>
<td>Seawater</td>
<td>Trochophore</td>
<td></td>
<td>Prism</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4  (A–D) SEM images (magnification=×500) of early stage of *Codakia orbicularis* development: (A) 4 cell stage, (B) morula (t+6 h), (C) gastrula (t+21 h), and (D) trochophore (27 h). (E–H) Light microscopy images of *Diadema antillarum* early stage of development: (E) 4 cell stage, (F) morula, (G) gastrula, (H) prism.
Figure 5 Mean percentage mortality of *Codakia orbicularis* embryos after 24 h exposure to molecules extracted from *Sargassum polyceratium*. Dates of algal collection and extraction times are indicated in the key. Values are means±SE, n=12. *Significant differences (p<0.05, multiple comparisons test) between control (DMSO) and one of the solutions; **significant differences (p<0.05) observed between control (DMSO) and a group of extract solutions.

opened to the last stage and had percentage mortalities similar to the control (p=1.00 for the January extracts and p=0.55 for the October extracts).

Embryonic development was a function of the concentration of the solution tested, and stopped progressively with decreasing extract concentrations (Table 2, Figure 4), i.e., 2–4 cell stage at concentrations 100–200 µg ml⁻¹, morula at 15 and 50 µg ml⁻¹, late blastula early gastrula at 5 and 10 µg ml⁻¹ and trochophore at 1 µg ml⁻¹.

*Pseudonereis* sp. *Sargassum* extracts were less effective against *Pseudonereis* embryos than against other species (Figure 6). Only the highest concentrations of extracts tested (100 and 200 µg ml⁻¹) and the copper solution completely inhibited larval development. The *Sargassum polyceratium* solution extracted for 30 s (1 µg ml⁻¹) in January had some activity against the larvae (15% mortality), although it was not significantly different from the control. Even though all larvae survived the treatments, their swimming behaviour differed between the control and the lowest concentrations (except 1 µg ml⁻¹); affected larvae were almost still or swam very slowly.

**Discussion**

Molecules extracted at the algal surface with hexane are non-polar. Sargassum species produce polyphenols (polar metabolites), but some non-polar extracts, such as S. vulgare hexane extracts, are highly active towards the development of microalgae, suggesting production of active non-polar secondary metabolites by this alga (Plougenné et al. 2010b). So far, no methods allowing the extraction of polar molecules located at the surface of the algae have been developed and the activity observed here thus represents only a portion of the possible MNPs present at the surface of S. polyceratium.

There were no differences in toxic activity towards embryos between the 30 s extracts in October and January. However, antibiotic activity was different between these two extracts, suggesting a possible seasonality in surface molecule activities. Temperate algal extracts have a seasonal variation in molecular composition, antimicrobial activity, and AF activity (Steinberg and Van Altena 1992, Hellio et al. 2004, Maréchal et al. 2004, Plougenné 2006). The October extracts were from the rainy season, while those from January were collected in the dry season. Hellio et al. (2004) demonstrated that S. muticum secondary metabolite activity varies with seasons and is higher during the summer months when fouling pressure (including fouling by bacteria) is most intense. It is thus possible that macroalgae develop specific mechanisms for protection in the rainy season when bacterial concentrations are high in coastal environments (Futch et al. 2010). In the present study, the levels at which extracts were active towards bacteria were high (150–300 μg ml⁻¹) in comparison with other algal crude extracts. Crude extracts obtained from seaweeds collected in Brittany were active with concentrations ranging between 24 and 96 μg ml⁻¹ (Hellio et al. 2001) and between 0.1 and 100 μg ml⁻¹ (Plougenné et al. 2008). Marine bacteria were the most sensitive strains, suggesting that defence strategies of S. polyceratium are specific. Such targeted defence strategies have been described for other algal species (Paul and Puglisi 2004).

Bioactivity of Sargassum polyceratium extracts was marked; however, we do not know whether the active molecules were produced by Sargassum polyceratium or by its associated biofilm. Secondary metabolite isolation from algae can be confounded by associated microorganisms. As this study focused on surface molecules only, it was impossible to clean the macroalgal surface from microepiphytes using existing methods, such as ethanol, without breaking the surface cells (De Nys et al. 1998). The extract obtained therefore corresponds to Sargassum and/or its associated biofilm. Numerous bacteria living in SW produce active secondary metabolites (Jensen and Fenical 1994). Moreover, there are host-specific associations between algae and bacteria, and algae may control associated bacteria (Lachnit et al. 2009). The bacterial biofilm may in turn confer a protection to the host alga through production of secondary metabolites.

Sargassum polyceratium surface extracts inhibited bacterial growth and embryo development of three tropical marine invertebrates one of which was Diadema antillarum, a tropical herbivorous keystone species that controls macroalgal...
populations. Testing surface molecules was a first investigation step and further work will be carried out to relate the natural compounds to their possible ecological role. These results require more work focused on 1) concentrations produced on the algal surface, 2) the source of production (alga or biofilm, or both) and 3) the release of compounds into the water column, a process that might interact with embryonic development of organisms surrounding Sargassum. We are currently performing tests on molecules present in the waters in which algae are immersed (conditioned water) in order to assess the activity of these cues against tropical invertebrate marine larvae in the same environment.

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References


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