

# *Sargassum polyceratum* (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 led 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 polyceratum*. 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 [an annelid (*Pseudonereis* sp.), a bivalve (*Codakia orbicularis*) and a sea urchin (*Diadema antillarum*)]. Extracts were active against all microorganisms tested (MIC=150 or 300  $\mu\text{g ml}^{-1}$ ), early stages of development in *Pseudonereis* sp. (MIC=100  $\mu\text{g ml}^{-1}$ ) and embryos of *C. orbicularis* and *D. antillarum* (MIC=5  $\mu\text{g ml}^{-1}$ ), suggesting the production of defence compounds by *S. polyceratum*.

**Keywords:** bacteria; embryonic development; hexane dipping; *Sargassum polyceratum*; toxicity.

## Introduction

Studies on production of marine natural products (MNPs) 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, antifouling (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, Plouguerné 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 snyderae* 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 alga 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 (Plouguerné 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, Plouguerné et al. 2008, 2010b, Maréchal and Hellio 2011), *S. wightii* Greville ex J. Agardh and *S. johnshonii* V.J. Chapman (Sastri and Rao 1994).

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 anti-algal activity in extracts from *S. horneri* (Turner) C. Agardh. *S. vulgare* C. Agardh extracts from Brazil had AF activity against microalgae and mussel settlement (Plouguerné et al. 2010a). *S. muricatum* and *S. tenerimum* J. Agardh induce modifications of swimming activity in two-day-old larvae of *Platygyra daedalea* Ellis et Solander (coral) and decrease their proportional settlement, indicating chemical defences (Diaz-Pulido et al. 2010).

*Sargassum polyceratum* 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. polyceratum* and *S. hystrix* J. Agardh have supplanted 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 (Pawlik 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 keystone 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 polyceratum* 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. polyceratum* 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. polyceratum* 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 km<sup>2</sup> 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 polyceratum*.

### Study organism

*Sargassum polyceratum* 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 polyceratum* 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<sup>-1</sup> wet weight *S. polyceratum* (De Nys et al. 1998). All the extractions were performed in the dark as some secondary metabolites, such as the polyphenolics are known to react to light. The goal of using the hexane dipping method was to extract molecules produced both by the alga at its surface and by its associated biofilm in order to obtain the whole range of MNPs that might interact with eukaryotic and/or prokaryotic organisms present in the algal environment.

Two protocols were used:

- Protocol A (October 2008 samples, rainy season), *Sargassum polyceratum* 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 polyceratum* 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. polyceratum* by hexane dipping was investigated by epifluorescent microscopy (Nikon, Tokyo, Japan, Eclipse 80i microscope; Filter FITC 494 nm excitation/514 nm emission; Nikon Dxm1200F camera) using a 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), *Pseudoalteromonas elyakovii* (Ivanova et al.) Sawabe et al. (ATCC 700519), *Polaribacter irgensii* Gosink 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). *Pseudoalteromonas elyakovii* and *Vibrio aestuarianus* are also known to cause infections in marine organisms, such as molluscs, crustacean, 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<sup>-1</sup>) 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<sup>-1</sup>. 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

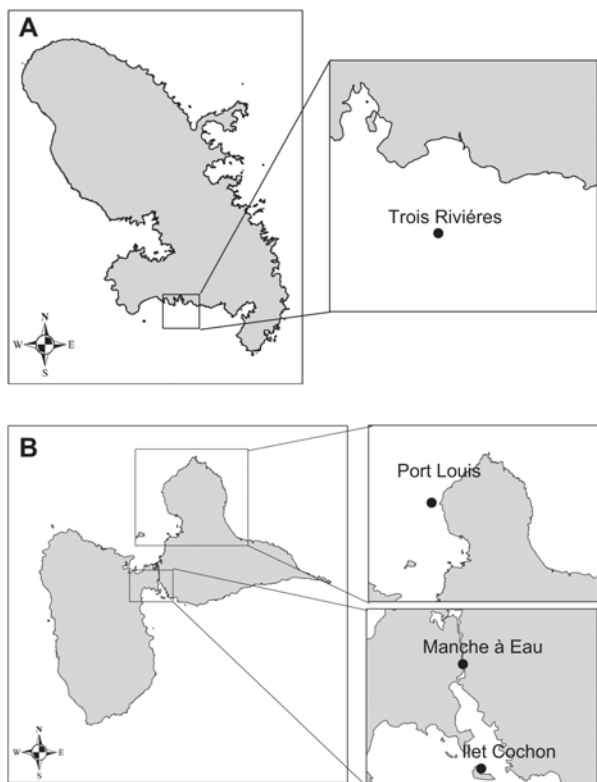
evaporate the solvent and then left for 15 min in a UV cabinet for sterilization.

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.



**Figure 1** (A) Map showing the algal collection site (Martinique) at *Trois Rivières* (14°27'22"N, 60°58'03"W). (B) Map showing the invertebrates collection sites (Guadeloupe) at *Ilet Cochon* (16°12'56"N, 61°32'20"W), *Manche à Eau* (16°16'36"N, 61°31'24"W) and *Port Louis* (16°25'18"N, 61°32'02"W).

*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  $\mu\text{m}$  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 0.5  $\mu\text{g ml}^{-1}$  (data not shown).

Tests were conducted in 96-well plates (Fisher). Extracts were tested at seven concentrations: 1, 5, 10, 15, 50, 100 and 200  $\mu\text{g ml}^{-1}$ . Two hundred microlitres of each extract were added to each well (six replicates). In addition, six wells filled with SW, six with 1%  $\text{CuSO}_4$  (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

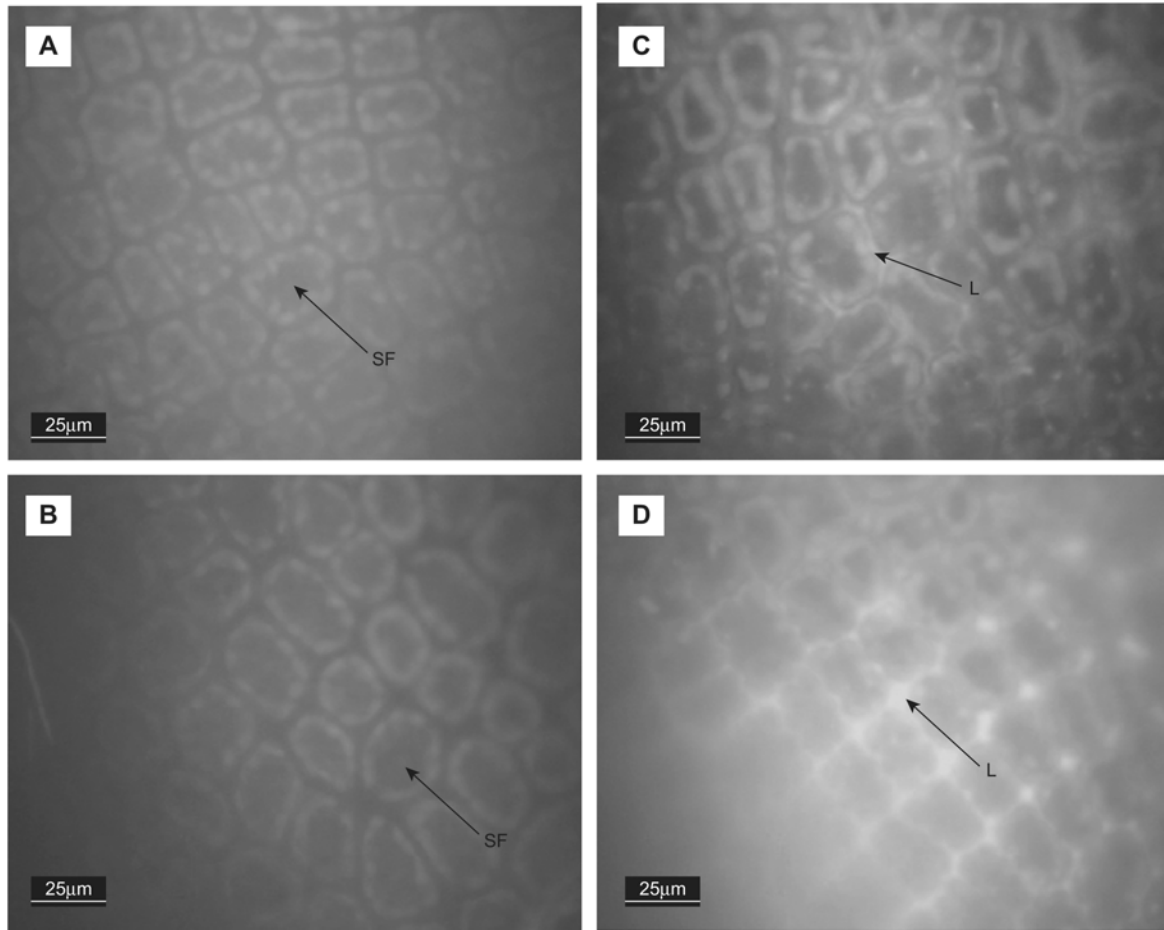
*Sargassum polyceratum* extracts at a concentration of 15  $\mu\text{g ml}^{-1}$  did not inhibit bacterial strains (Table 1). The January 10 s extraction sample was more more active against bacteria than the January 30 s sample (except in the case of *Vibrio aestuarianus*). The 30 s extraction samples 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  $\mu\text{g ml}^{-1}$ ) and in the copper solution (positive control) (Figure 3). Less than 70% of embryos survived when exposed to 5  $\mu\text{g ml}^{-1}$ . Only solutions at concentrations of 1  $\mu\text{g ml}^{-1}$  and the 10 s January extract concentration of 5  $\mu\text{g ml}^{-1}$  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  $\mu\text{g ml}^{-1}$  and  $p = 1.0$  for the others; multiple comparisons tests).

Apart from the 10 s January extract at a concentration of 5  $\mu\text{g ml}^{-1}$ , 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  $\mu\text{g ml}^{-1}$  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  $\mu\text{g ml}^{-1}$ , morula at 15 and



**Figure 2** Epifluorescent microscopic images of *Sargassum polyceratum* ‘leaf’ surfaces: (A) control, (B) 30 s, (C) 10 min and (D) 30 min after being dipped in hexane. (magnification= $\times 100$ ). L, cellular content leaks; SF, surface cells.

50  $\mu\text{g ml}^{-1}$ , late blastula early gastrula at 10  $\mu\text{g ml}^{-1}$ , gastrula at 5  $\mu\text{g ml}^{-1}$  and prism at 1  $\mu\text{g ml}^{-1}$ .

**Codakia orbicularis** The results for *Codakia orbicularis* embryos were similar to those for *Diadema antillarum*

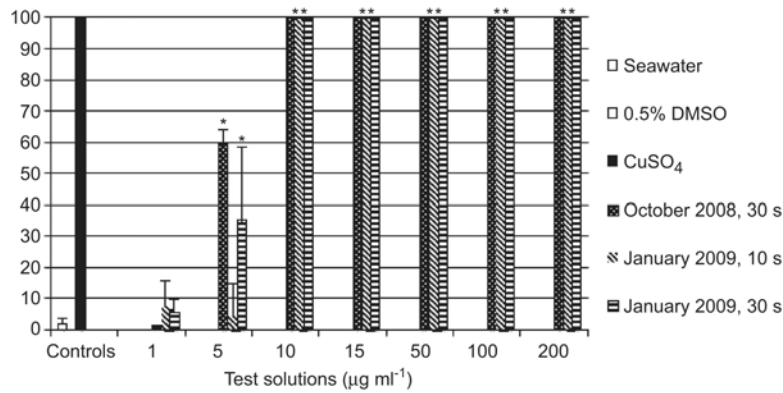
(Figure 4) (Kruskal-Wallis:  $p < 0.0001$ ). All embryos died in the highest concentrations assayed (50–200  $\mu\text{g ml}^{-1}$ ) and in the copper control. Percentage mortality decreased at 15  $\mu\text{g ml}^{-1}$  but the embryos did not develop to the trochophore stage. Only embryos cultured in the 1  $\mu\text{g ml}^{-1}$  solution devel-

**Table 1** Antibacterial activity of hexane dipping extracts expressed as minimum inhibitory concentrations ( $\mu\text{g ml}^{-1}$ ).

Bacterial species	October 2008		January 2009	
	30 s		10 s	30 s
<b>Terrestrial</b>				
<i>Bacillus subtilis</i>	150		300	N
<i>Enterobacter aerogenes</i>	N		300	N
<i>Escherichia coli</i>	300		300	N
<i>Pseudomonas aeruginosa</i>	150		300	N
<i>Staphylococcus aureus</i>	N		300	N
<b>Marine</b>				
<i>Halomonas marina</i>	300		150	300
<i>Polaribacter irgensii</i>	150		150	300
<i>Pseudoalteromonas elyakovii</i>	150		150	300
<i>Vibrio aestuarianus</i>	150		150	150

N, no active extracts.

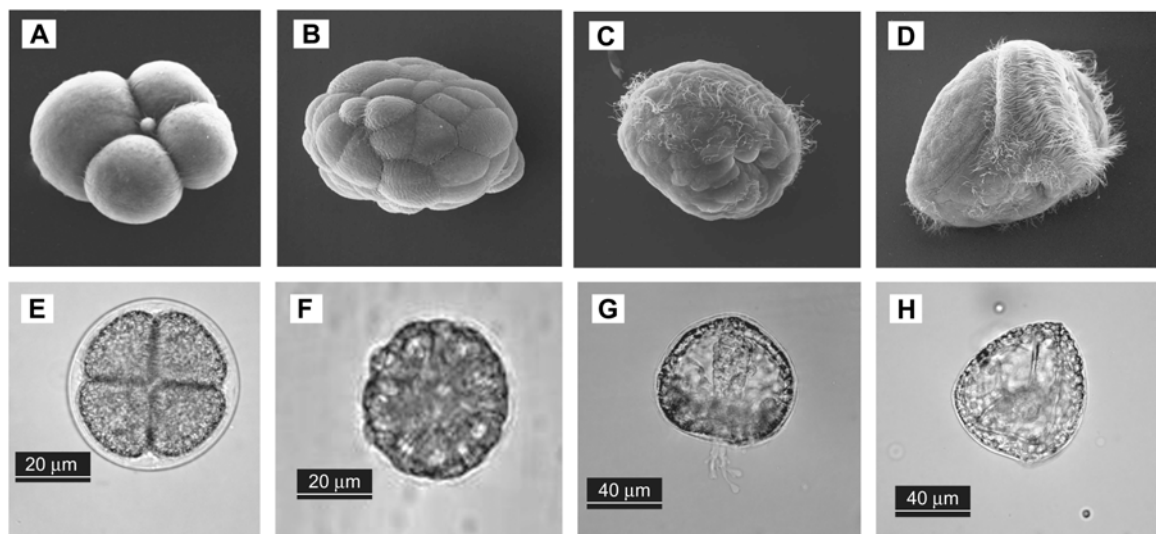
Months of algal collection and dipping times are indicated.



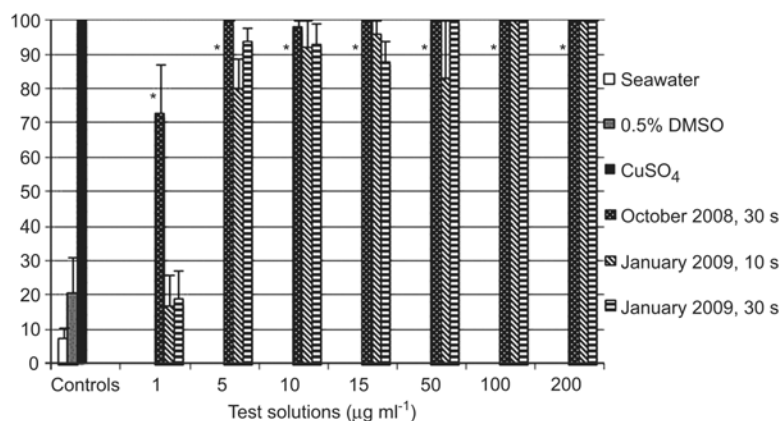
**Figure 3** Mean percentage mortality of *Diadema antillarum* embryos after 30 h exposure to molecules extracted from *Sargassum polyceratum*. Dates of algal collection and extraction times are indicated in the key. Values are means+SE, n=12. \*Significant differences ( $p < 0.05$ , multiple comparisons 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 polyceratum* hexane surface extracts.

Concentration	Stage of development	
	<i>Codakia orbicularis</i>	<i>Diadema antillarum</i>
1 $\mu\text{g ml}^{-1}$	Trochophore	Prism
5 $\mu\text{g ml}^{-1}$	Late blastula-early gastrula	Gastrula
10 $\mu\text{g ml}^{-1}$	Late blastula-early gastrula	Late blastula-early gastrula
15 $\mu\text{g ml}^{-1}$	Morula	Morula
50 $\mu\text{g ml}^{-1}$	Morula	Morula
100 $\mu\text{g ml}^{-1}$	2-4 cells	2-4 cellules
200 $\mu\text{g ml}^{-1}$	2-4 cells	2-4 cellules
CuSO <sub>4</sub>	2-4 cells	2-4 cellules
DMSO (0.5%)	Trochophore	Prism
Seawater	Trochophore	Prism



**Figure 4** (A–D) SEM images (magnification= $\times 500$ ) 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 polyceratum*.

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.

oped 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).

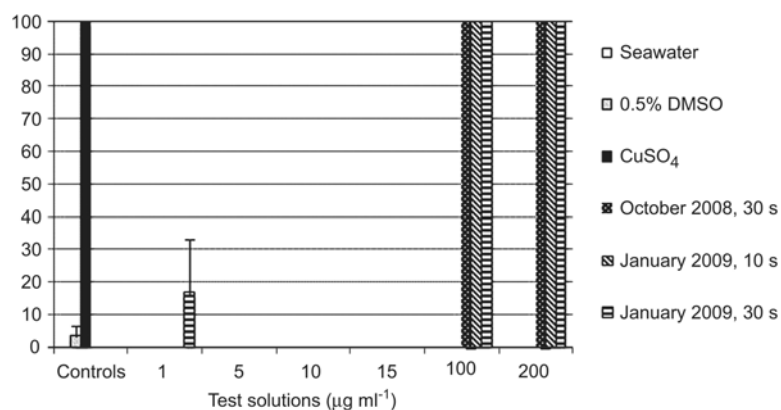
Embryonic development was a function of the concentration of the solution tested, and stopped progressively with decreasing extract concentrations (Table 2, Figure 4), i.e., 2–4 cell stage at concentrations 100–200  $\mu\text{g ml}^{-1}$ , morula at 15 and 50  $\mu\text{g ml}^{-1}$ , late blastula early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

***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  $\mu\text{g ml}^{-1}$ ) and the copper solution completely inhibited larval development. The *Sargassum polyceratum* solution extracted for 30 s (1  $\mu\text{g ml}^{-1}$ ) 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  $\mu\text{g ml}^{-1}$ ); affected larvae were almost still or swam very slowly.

## Discussion

Secondary metabolites produced by algae affect settlement, recruitment and development of numerous organisms (Pawlik 1992, Steinberg and de Nys 2002, Paul and Puglisi 2004, Paul et al. 2007). Compounds isolated from *Dictyota menstrualis* (Hoyt) Schnetter, Hörning and Weber-Peukert and *D. ciliolata* Sonder ex Kützing caused significant mortality [*Bugula neritina* Linnaeus (bryozoan)], abnormal development (*B. neritina*) and reduced settlement rates [*Amathia convoluta* Lamarck, (bryozoan), *B. neritina*, *Eudendrium carneum* Clarke, (hydroid)] (Schmitt et al. 1998). Algal waterborne cues affect larval recruitment in *Acropora millepora* Ehrenberg, (coral) (Birrell 2003) and are toxic towards early developmental stages of the fouling polychaete *Hydroides elegans* Haswell (Dobretsov et al. 2006). Algal



**Figure 6** Mean percentage mortality of *Pseudonereis* sp. embryos after 48 h exposure to molecules extracted from *Sargassum polyceratum*. Dates of algal collection and extraction times are indicated in the key. Values are means+SE, n=12.

MNPs are often species-specific and can have an effect on different larval development stages. *Dictyota* spp. and *Lau-rencia* sp. are toxic against larvae of *H. elegans* and *B. neritina*; *Padina* sp. and *Halimeda* sp. inhibit their larval settlement; *Hypnea* sp. and *Ulva* sp. stimulate larval settle-ment and *Sargassum* spp. have no effect (Walters et al. 1996).

Our results demonstrate that *Sargassum polyceratium* sur-face molecules significantly affect embryonic development of three tropical invertebrates and bacterial growth. These organisms are not all in direct contact with *S. polyceratium* (except *Diadema antillarum*), but they represent species from three invertebrate phyla and thus give some indication of potential toxic activity of *Sargassum* towards several types of organisms. In the embryo toxicity assays, the active extracts concentrations varied by species tested. Both *Coda-kia orbicularis* and *D. antillarum* had similar responses although *C. orbicularis* was expected to be more resistant because of the large envelope surrounding its embryos (Gros et al. 1997). *Pseudonereis* embryos were the most resistant to the extracts. In the control, *C. orbicularis* embryos devel-oped in 24 h to the trochophore stage and *D. antillarum* embryos developed in 30 h to the prism stage, consistent with previously reported values (Gros et al. 1997, Eckert 1998). However, *S. polyceratium* surface compound treat-ments had progressive effects (according to increasing con-centration) on embryonic development, suggesting the extracts block egg cleavage. The highest concentrations blocked development at the 2–4 cells stage, the medium con-centrations allowed development until the morula stage and the lowest concentrations until the blastula, gastrula and finally trochophore or prism stages (respectively for *C. orbi-cularis* and *D. antillarum*). Similarly, Paul and Fenical (1986), showed that caulerpyne blocks the cleavage of devel-oping sea urchin eggs. The division of *Paracentrotus lividus* Lamarck (urchin) embryos was inhibited by algal extracts (Martin and Uriz 1993). Dihydrorhocephalin, aldehyde, udoteal, petiodal, dihydroudoteal, rhocephalin, halimeda-trial, halimeda tetraacetate (4,9-diacetoxy-udoteal) isolated from siphonous green algal species (*Halimeda* spp., *Penicil-lus* spp., *Rhizocephalus phoenix* Ellis et Solander, *Udotea* spp.) are toxic against developing sea urchin eggs, sperm and larvae (Paul and Fenical 1986), with ED<sub>100</sub> levels (lowest concentration leading to 100% inhibition of cell division) ranging from 0.2 to 16 µg ml<sup>-1</sup>. Concentrations of MNPs extracted from siphonous algae have higher activity against both *Codakia* and *Diadema* than the solutions tested in this study (ED<sub>100</sub> ranging between 50 and 100 µg ml<sup>-1</sup>). How-ever, the tests performed by Paul and Fenical (1986) were performed with isolated secondary metabolites and not crude extracts. It is possible that the active compounds tested in this study were present in the crude extract at very low con-centration (µg ml<sup>-1</sup>). On the other hand, some molecules act synergistically such that a pool of molecules is required to induce a biological effect (Hay 1996). Further analytical chemistry tests involving molecule purification and analyti-cal chemistry analyses (GC-MS and NMR) will give further insights into the chemical nature of the bioactive compounds and their activity levels.

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 sec-ondary metabolites by this alga (Plouguerné et al. 2010b). So far, no methods allowing the extraction of polar mole-cules 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 embri-os between the 30 s extracts in October and January. How-ever, antibacterial activity was different between these two extracts, suggesting a possible seasonality in surface mole-cule activities. Temperate algal extracts have a seasonal var-iation in molecular composition, antimicrobial activity, and AF activity (Steinberg and Van Altena 1992, Hellio et al. 2004, Maréchal et al. 2004, Plouguerné 2006). The October extracts were from the rainy season, while those from Jan-uary 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<sup>-1</sup>) in comparison with other algal crude extracts. Crude extracts obtained from seaweeds collected in Brittany were active when concentrations ranged between 24 and 96 µg ml<sup>-1</sup> (Hellio et al. 2001) and between 0.1 and 100 µg ml<sup>-1</sup> (Plou-guerné 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 mole-cules 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 impos-sible 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 bio-film. Numerous bacteria living in SW produce active sec-ondary metabolites (Jensen and Fenical 1994). Moreover, there are host-specific associations between algae and bac-teria, and algae may control associated bacteria (Lachnit et al. 2009). The bacterial biofilm may in turn confer a protec-tion to the host alga through production of secondary metabolites.

*Sargassum polyceratium* surface extracts inhibited bacte-rial growth and embryo development of three tropical marine invertebrates one of which was *Diadema antillarum*, a trop-ical 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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