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Investigation of the antifouling constituents from the brown alga *Sargassum muticum* (Yendo) Fensholt

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Abstract One of the most promising alternatives to toxic heavy metal-based paints is offered by the development of antifouling coatings in which the active ingredients are compounds naturally occurring in marine organisms and operating as natural antisettlement agents. Sessile marine macroalgae are remarkably free from settlement by fouling organisms. They produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against other settling organisms. In this study, a dichloromethane extract from the brown seaweed *Sargassum muticum* was tested in situ and, after 2 months of immersion, showed less fouling organisms on paints in which the extract was included, compared to paints containing only copper after 2 months of immersion. No barnacles or mussels have been observed on the test rack. Identification by NMR and GC/MS of the effective compound revealed the abundance of palmitic acid, a commonly found fatty acid. Pure palmitic acid showed antibacterial activity at 44 μg mL^{-1}, and also inhibited the growth of the diatom *Cylindrotheca closterium* at low concentration (EC_{50}=45.5 μg mL^{-1}), and the germination of *Ulva lactuca* spores at 3 μg mL^{-1}. No cytotoxicity was highlighted, which is promising in the aim of the development of an environmentally friendly antifouling paint.

Keywords Antifouling · Brown algae · Dichloromethane extract · Dioctyl phthalate · Palmitic acid · *Sargassum muticum*

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Introduction

Engineered structures such as ships and marine platforms, as well as offshore rigs and jetties, are under constant attack from the marine environment. These structures need to be protected from the influences of the key elements of the marine environment such as saltwater, biological attack and temperature fluctuations. The settlement and accumulation of marine organisms on an inanimate substrate can cause large penalties to engineered structures. In heat exchangers, biofouling can clog systems, and on ship hulls it can

increase the hydrodynamic drag, lower the manoeuvrability of the vessel and increase the fuel consumption. This leads to increased costs within the shipping industry through the increased use of manpower, fuel, material and dry docking time (Abarzua and Jakubowski 1995; Lambert et al. 2006; Chambers et al. 2006).

The process of biological fouling is often grouped in the literature into key growth stages, which include an initial accumulation of adsorbed organics, the settlement and growth of pioneering bacteria creating a biofilm matrix and the subsequent succession of micro and macrofoulers (Wahl 1989; Abarzua and Jakubowski 1995; Yebra et al. 2004). Methods for inhibiting both organic and inorganic growth on wetted substrates are varied, but most antifouling systems take the form of protective coatings. Unfortunately, operational profiles vary; hence the application of one universal coating to ship hulls is unlikely and specific coatings designed for the particular needs of certain exposure and operational profiles may need to be targeted individually. Heavy metals and booster biocides such as Irgarol 1051 and Diuron, are not environmentally acceptable alternatives due to increased concerns over their toxicity, but do offer cost benefits (Chambers et al. 2006). The International Maritime Organisation (IMO) legislation and the increased legislation of local and regional pesticide control authorities are the largest driving forces for the design and implementation of non-toxic antifouling coatings (Chambers et al. 2006).

The biomimetics approach implies the use of the natural world as a model on which to base an engineering development. Marine organisms have been shown to use both physical and chemical methods to protect themselves from biofouling (Bakus et al. 1986; Davis et al. 1989; Wahl 1989; Steinberg et al. 1998; Fusetani 2004; Bazes et al. 2006).

The introduced macroalga *Sargassum muticum* (Heterokonta, Fucales) is found along the coasts of South Brittany (Critchley et al. 1990; Plouguerne et al. 2006). Similar to many other macroalgae, *S. muticum* may accumulate quantities of secondary metabolites (Hay and Fenical 1988; Steinberg 1992; Hay 1996) generally assumed to be a chemical defence against grazers and bacterial colonisation (Sieburth and Conover 1965; Hay and Fenical 1988; Harlin 1996; Plouguerne et al. 2006). The chemical composition of *Sargassum* has been studied extensively, and phlorotannins (Kubo et al. 1992), phlorethols (Banaimoon 1992), sterols (Harvey and Kennicutt 1992) and dicotylpthalate (Sastry and Rao 1995) have been isolated. Various extracts from this alga have shown biological activities, including bactericidal and fungicidal (Sastry and Rao 1994, 1995; Hellio et al. 2001; Bazes 2006).

In this paper, we report on the isolation and identification of a potential natural antifouling compound from a dichloromethane extract of *S. muticum*.

Materials and methods

The brown alga *Sargassum muticum* (Yendo) Fensholt was harvested in Locmariaquer (47.55°N, 2.90°W, Brittany, France) in March 2004. After collection, the material was rinsed in sterile seawater and 5% ethanol in order to remove any associated microflora. Algae were then dried at room temperature under shade, and stored in the dark before use.

Extraction was performed as previously described by Hellio et al. (2001). The dried algae were suspended by stirring in ethanol (2,000 g/12 L). After decantation, the resultant pellet was re-extracted five times in the same way. The alcoholic extracts were combined and evaporated under vacuum at low temperature (35°C). Distilled water (4 L) was then added and partitioned with dichloromethane (4×4 L). The organic phases were collected, left dry in presence of Na_2SO_4 for 24 h, filtered and concentrated under vacuum at low temperature (dichloromethane extract). The resulting dichloromethane extract (A) was stored at 4°C before use. The extraction yield was 0.85%.

Biocides commonly used in commercial antifouling paints were also evaluated on marine bacteria, microalgae and macroalgae spores. Diuron, Irgarol 1051, Tolylfluanid and Dichlofluanid were provided by Nautix, France.

Binders and paints

The binder used was purchased from ZENECA. It is a mixture of an acrylic copolymer (polybutylmethacrylate-co-polymethylmethacrylate) with rosin. The relative amount of rosin influences the erosion properties of the final paints. Paints were formulated with this polymer (cf. Table 1). All the ingredients were dispersed under vigorous agitation (2,000*g*) for 1 h. Then the paints were filtered through a 100-μm sieve.

Table 1 Composition of paints (wt.%)

Composition	Paint
Polymer	16.3
Solvent (xylene)	40
Extract	9.5
Copper	10
Fillers	18.7
Additives	5.5

Immersion and test procedures

Test panels were coated by using an automatic film applicator (Sheen 1137). The wet films were 200 μm thick. After drying, plates were immersed in the harbour of Lorient (Britanny, France) for 2 months, July and August, when the fouling pressure is the highest. The plates were immersed under the surface, where most of the fouling organisms live.

Purification of the active extract

For each step of purification, the different fractions were tested against three agents of microfouling. The most active fraction was retained for a further purification step.

1.5 g of the dichloromethane extract (A) was added to a Solid Phase Extraction (SPE) column (Chromabond SiOH, 150 ml/50 g, Macherey-Nagel) previously conditioned with hexane. Elution was carried out using a gradient of CH_2Cl_2/MeOH from 99:1 to 0:100 (v/v). The resulting fractions were collected, evaporated and stored at 4°C before use.

The most active fraction (20 mg) was then laid on a preparative pre-coated TLC plate (SIL G-200, Macherey-Nagel) and eluted with CH_2Cl_2/MeOH 85:15 (v/v). After drying, part of the plate was revealed with sulphuric vanillin (1 g of vanillin in 100 mL of MeOH and 1 mL of concentrated sulphuric acid). Each spot was then scratched, dissolved in CH_2Cl_2/MeOH 85:15 (v/v), centrifuged and the supernatants were evaporated and stored at 4°C before use.

The active spot was investigated on a HPLC system (Dionex) with a 600E pump and an ASI-100 autosampler injector and UV detection at 215 nm. Separation was performed on an Econosil C18 (Alltech) column (10 mm ID×250 mm l) heated at 30°C. A multi-step eluting gradient (MeOH/H_2O 85:15 0–15 min, MeOH/H_2O 100:0 15–30 min, MeOH/H_2O 85:15 30–40 min) was used at a flow rate of 3 mL min^{-1}. The volume used for each injection was 500 μl. Each peak was collected, evaporated and stored at 4°C before use.

Identification of the active compound

Mass spectrometry experiments An Agilent Technologies 1100 Series vacuum degasser, LC pump and autosampler (Hewlett-Packard, Germany) were used to analyse the fraction isolated after C_{18} HPLC. Twenty microliters of sample solutions were applied onto an analytical C_{18} reversed-phase column (Hypersil ODS, 250×4.6 mm, particle size 5 μm). The elution procedure consisted of an isocratic profile of methanol–water (15:85, v/v) for 5 min, followed by a linear gradient from 85 to 100% methanol over 15 min, and an isocratic profile with 100% methanol over 20 min. The LC flow (0.4 mL min^{-1}) was split (1/12) using a micro-splitter valve (Upchurch Scientific, USA). The post-column additive, a mixture of 5 mM ammonium acetate and 0.05% trifluoroacetic acid (TFA) (Analysis grade, Carlo Erba) in methanol–water (50:50, v/v), was added using a Cole-Parmer (USA) syringe pump and a 2.5-mL SGE syringe at a flow-rate 150 μL h^{-1}. The LC-separated compounds were detected by electrospray ionisation ion trap mass spectrometry (ESI-MS) using a Bruker Esquire-LC spectrometer (Bruker Daltonic, Germany) under positive-ion conditions. For each compound, two ions were formed: the $[M+H]^+$ and the $[M+Na]^+$ ions. The $[M+H]^+$ ions were isolated for MS–MS fragmentation. The MS–MS chromatographic analysis is segmented for the isolation and fragmentation of the eluted $[M+H]^+$ ion. The electrospray used nitrogen as a nebulising gas (pressure set to 15 p.s.i.) and a drying gas (flow set to 7 mL min^{-1}). The drying temperature was 300°C. The helium pressure in the ion trap was 6×10^{-6} mbar. Full-scan mode detection was used with a scan range from m/z 50 to 700. The software used was Bruker Esquire-LC NT version 6.08 and Agilent Technologies ChemStation May 1998.

NMR experiments NMR experiments were performed at 25°C in a Bruker Avance DRX 500 spectrometer equipped with an indirect 5 mm triple TBI 1H/{BB}/13C probehead using standard pulse sequences available in the Bruker software. The samples were dissolved in 700 μL of 99.8% MeOD. 1D 1H spectra were recorded at 500.13 Mhz with a 30° pulse, a delay D1 of 2s and 64 scans. Chemical shifts were expressed in ppm relative to TMS (Tetrametylsilane) as external standard. Double-quantum filtered ^1H-^1H correlated spectroscopy (DQF COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC) with a 60-ms mixing time were performed according to standard pulse sequences to assign ^1H and ^{13}C resonances.

Gas chromatography experiments Active fraction was evaporated under nitrogen and methylated by contact with methanol/sulphuric acid (98:2, v/v) in excess for one night at 50°C. After cooling, 2 mL of pentane and 1 mL of water were added and vortexed. The upper organic phase was assayed using GC-MS on a Hewlett-Packard model 6890 series II gas chromatograph attached to an Agilent model 5973N selective quadripole mass detector. GC-MS was connected to a computer with Hewlett-Packard chemstation and the ionisation voltage used was 70 eV at 250°C. The temperature of injector and interface were maintained at

250°C and Helium was used as a carrier gas under constant flow (1 mL min^{-1}). Separation was realised on a CP-Sil 5 CB low bleed MS (Chrompack; 60 m×0.25 mm i.d., 0.25-μm film thickness). The oven temperature was programmed from 80 to 170°C at a rate of 30°C min^{-1}, then from 170 at 295°C with a rate of 3°C min^{-1}.

Bioassays

Antibacterial activities The marine bacterial strain was obtained from the Culture Collection of the IUT of Quimper (LUMAQ, UBO, France) and identified by the CIMB (Institut Pasteur, Paris, France) as Rhodobacteraceae bacterium *R11* A. This bacterium was associated with immersed surfaces and isolated from decomposing seaweeds (Hellio et al. 2004). Antibacterial evaluation of the extracts and fractions was performed in 96-well plates as previously described in Bazes et al. (2006). Samples of cultures grown overnight (2×10^8 cells mL^{-1}) were incubated with extracts and biocides (at the concentration of 25, 50, 100, 200 and 300 μg mL^{-1}) for 48 h at 20°C (Maréchal et al. 2004). All inhibition assays were carried out in triplicate. Growth was monitored by measuring OD_{600} with a Packard Spectracount microplate spectrophotometer and the percentage of inhibition was calculated for each concentration:

$$\%\ \text{inhibition} = (OD_c - OD_c)/OD_c \times 100$$

where OD_c is the mean optical density of the bacterial controls and OD_t is the mean optical density of the test samples. Control testing with the solvents and *N*-decane 1% was performed for every assay and showed no inhibition of the microbial growth. Seawater was used as a negative control.

Inhibition of phytoplankton growth Cylindrotheca closterium (Diatomophyceae, AC515) was obtained from the Culture Collection of Algae of the University of Caen (France). It was used as a common fouling species (Jackson 1991; Hellio et al. 2004). Screening for bioactivity was performed as described by Sawant et al. (1995) and modified in Bazes et al. (2006). The effect of algal extracts and fractions (at the concentration of 25, 50, 100, 200 and 300 μg mL^{-1}) was assessed after 72 h by estimating the chlorophyll-a (Aminot 1983). All the screening experiments were carried out in triplicate. The percentage of growth inhibition was calculated:

$$\%\ \text{inhibition} = (\text{Chla}_c - \text{Chla}_t)/\text{Chla}_c \times 100$$

where $Chla_c$ is the mean concentration of chlorophyll-a of the algal controls and $Chla_t$ is the mean concentration of chlorophyll-a of the test samples. Control tests with the solvents and *N*-decane 1% were performed for every assay and showed no inhibition of the microalgal growth. Seawater was used as a negative control.

Inhibition of germination of Ulva spores Ulva lactuca (Ulvales, Chlorophyta) samples were collected in July and September 2004 at Locmariaquer, South Brittany, France (47°33′N, 02°56′W). Spores were obtained using the osmotic method (Fletcher 1989). Tests of algal extracts and fractions on spores were performed as described by Hattori and Shizuri (1996) and Bazes et al. (2006), by determining the percentage of inhibition of germination of spores (600 mL^{-1}) in plastic Petri dishes after incubation for 5 days at 20°C under 24 h light. All the screening experiments were carried out in triplicate. The percentage of growth inhibition was calculated:

$$\%\ \text{inhibition} = (gs_c - gs_t)/gs_c \times 100$$

where gs_c is the mean number of germinated spores for the controls and gs_t is the mean number of germinated spores for the test samples. Seawater was used as a negative control.

Toxicity

Cytotoxicity evaluation by cell viability was performed by the neutral red dye method (McLaren et al. 1983) on 3T3 as described in Bazes et al. (2006). Cellular suspensions ($3{,}5\times10^5$ 3T3 cells mL^{-1} purchased from Eurobio) were incubated with various concentrations of algae extracts and biocides (10–300 μg mL^{-1}, 4 wells per concentration) in 96-well plates (72 h, 37°C, 5%CO_2) in Eagle's MEM 10% FCS. The same experiment has been conducted with Vero cells. All the cytotoxicity experiments were carried out in triplicate. The cytotoxic concentration (CC_{50}) was expressed by a percentage of destruction:

$$\%\ \text{destruction} = (OD_c - OD_t)/OD_c \times 100$$

where OD_c is the mean optical density of the cell controls at 540 nm and OD_t is the mean optical density of the test samples at 540 nm.

Statistical analysis

Percentage of growth inhibition was calculated for each microfouling organisms as described previously. The 50% effective concentrations (EC_{50}) and cytotoxic concentrations (CC_{50}) were estimated by regression analysis with Prism software, Version 4 (GraphPad Software). All calculations were based on measured concentrations of extracts, and CC_{50} and EC_{50} were given when it was in the range of concentrations.

Results

In situ testing

Results of the in situ tests are shown on Fig. 1. No barnacles or mussels were observed on the test rack. The paint including only copper is less efficient than the paints including the crude extract. When copper and crude extract are included together in the paint, there is much less fouling than on the other coatings: 37% of the plate painted with crude extract of *Sargassum muticum* and copper are covered with young thalli of *Ulva sp*., while 92% of the plate painted only with *S. muticum* extract, and 100% of the plate painted only with copper, are covered with different green algae (mostly from the genus *Ulva*), and red algae (mostly from the genus *Polysiphonia*). Well-developed thalli of *Ulva sp*. were observed on those last two plates.

Identification of the active compound

Solid phase extraction of the crude extract (A) allowed for the isolation of an active fraction in the CH_2Cl_2/MeOH 70:30 part (B) (91.5 mg). The preparative TLC of that sample gave 8 fractions. Fraction no.5 (C) (22.8 mg) was active and it was analysed by HPLC on a C18 column using a MeOH/H_2O gradient as the eluent. After HPLC, ten fractions were isolated. The eighth one (D) (2.7 mg of a pale green oil) was inhibitory to the growth of the three organisms tested. This last fraction was isolated in large quantities for the identification of bioactive molecules.

MS on the D fraction showed a peak corresponding to two ions. The m/z 413.4 ion was found in a majority while the m/z 391.4 ion was in a minority, corresponding to the [M+H]+ and [M+Na]+ ions of the octyl phthalate. MS-MS detection was then used for identification. The m/z 413.4 ion gave no signal in those conditions, while the m/z 391.4 ion produced a m/z 149 fragment, typically representative of phthalates.

NMR on the D fraction showed that protons from terminal methyl groups show one badly defined triplet at 0.88 ppm (mainly from 16:0 acyl chains). A multiplet at 1.2 ppm is assigned to the methylene protons. The multiplet at 2.36 ppm corresponds to the C_aH_2 group and the signal at 1.58 ppm to the C_bH_2 group (where a and b positions are relative to the carbonyl group). Those signals reveal that this fraction is mostly constituted of lipid chains. The integration and the lack of unsaturated signals at about 5.5 ppm confirm that most of the lipid chains are 16:0 (palmitic acid). The HMQC spectrum allowed the specific assignment of ^1J carbons and HMBC sequencing determined the shift of the carbonyl group. To confirm the results obtained by NMR and to determine the abundance of fatty acids, GC-MS analyses were performed on every sample.

The fatty acid content of every sample is shown in Table 2. The crude extract contains 99% fatty acids and 1% contaminant. This table highlights that the different steps used during the purification process led to the loss of most of the fatty acids present in the crude extract, the only main one remaining in the D fraction. The analysis of fraction D confirmed the results obtained with NMR. As it was not possible to separate the two main compounds from the D fraction, palmitic acid and dioctyl phthalate were purchased from Sigma and were investigated separately for their antifouling activity in order to determine the role of each in the activity of the fraction

Antifouling activities of the different isolated fractions

The antifouling activities of the crude extract, the HPLC purified fraction, the palmitic acid and dioctyl phthalate

Fig. 1 A test rack, which was exposed to a marine environment for 2 months, showing less fouling on the samples containing extract and copper than on the samples only containing extract or copper. 1: Paint with crude extract from *Sargassum muticum* without copper. 2: Paint with crude extract from *Sargassum muticum* and copper. 3: Paint only with copper

Table 2 GC-MS analysis of the methylated isolated fraction

Crude extract (A)	Solid phase extraction (B)	TLC (C)	HPLC (D)
16:0 (31.5%)	16:0 (62.1%)	16:0 (49.8%)	16:0 (71%)
14:0 (3.14%)	14:0 (6.2%)	14:0 (7.1%)	14:0 (1%)
15:0 (0.36%)	15:0 (0.8%)	18:0 (8.1%)	18:0 (7%)
16:1ω7 (7.60%)	16:1ω7 (1.3%)	Dioctyl phthalate (6.3%)	22:0 (3%)
16:1ω9 (0.91%)	16:1ω9 (1.5%)		Dioctyl phthalate (16%)
18:4ω3 (2.28%)	18:1ω9 (3.9%)		
18:2ω6 (5.39%)	18:0 (1.8%)		
18:1ω9 (15.68%)	20:1ω7 (0.9%)		
18:1ω7 (1.57%)	Dioctyl phthalate (4%)		
18:0 (0.70%)			
20:4ω6 (9.74%)			
20:5ω3 (5.66%)			
20:3ω6 (0.68%)			
20:2ω6 (2.14%)			
20:1ω9 (2.29%)			
22:1ω7 (1.67%)			
Dioctyl phthalate (0.95%)			

Dioctyl phthalate = 1,2 benzenedicarboxylic acid, bis(2-ethylhexyl)ester

(Sigma) and commercial biocides were evaluated and are presented in Table 3. The remaining active fraction after the HPLC step (D) contains at least 70% palmitic acid and shows a better activity on every fouling organism tested than the four chemical biocides. Moreover, this fraction showed no toxicity on 3T3 cells. Palmitic acid purchased from Sigma was also tested for its antifouling activities and showed good antibacterial activity against Rhodobacteraceae bacterium R11A with an EC_{50} at 44 μg mL^{-1}. Besides this antibacterial activity, palmitic acid also inhibits the development of a microalgal strain (*C. closterium*) at 45.5 μg mL^{-1} and the germination of spores from *U. lactuca* at 3 μg mL^{-1}. Myristic (14:0) or stearic (18:0) acids were also tested on fouling organisms and none of them showed antifouling activity (data not shown).

Those results also show that palmitic acid has a better antibacterial activity than dioctyl phthalate while the dioctyl phthalate is inactive at concentrations up to 300 μg mL^{-1}. However, the comparison of the activity of those two products on *C. closterium* shows that the dioctyl phthalate is more efficient than the palmitic acid with an EC_{50} lower than 25 μg mL^{-1}. This EC_{50} is the same as for the crude extract. This suggests a synergetic effect between the dioctyl phthalate and the palmitic acid on the growth of *C. closterium*.

The biocides tested here showed a low antibacterial activity. Diuron and Dichlofluanid showed an inhibition of Rhodobacteraceae bacterium *R11 A* at less than 200 μg mL^{-1}, while Tolylfluanid showed no bacterial inhibition under 300 μg mL^{-1}. Conversely, they all showed a good microalgal inhibition under 25 μg mL^{-1}. Irgarol, Tolylfluanid and dichlofluanid showed less inhibition of the germination of the spores than the extracts and purified fractions, while Diuron was active at 25 μg mL^{-1}. The four biocides tested here showed high toxicity against 3T3 cells with a CC_{50} under 32 μg mL^{-1}, while no cytotoxicity was observed at concentration lower than 300 μg mL^{-1} for the crude extract and purified fractions.

Discussion

In 2000, a study on the antifouling activity of extracts from 30 marine algae has been conduced (Hellio 2000). Statistical analysis of this study allowed the isolation promising potential antifouling of extracts from the brown seaweed *Sargassum muticum*. This study was confirmed and completed by the work of Hellio et al. (2004), while the study by Bazes (2006) showed that the most efficient

Table 3 Antifouling activity (EC_{50} in μg mL^{-1}) and toxicity (CC_{50} in μg mL^{-1}) of every most active fraction of the purification process and of palmitic acid and dioctyl phthalate

Fraction	Rhodobacteraceae bacterium *R11 A*	*C. closterium*	Germination of *Ulva lactuca*	CC_{50} on 3T3 cells
Crude extract (A)	206.0	<25.0	<25.0	>300.0
SPE fraction (B)	150.0	<25.0	<25.0	>300.0
TLC fraction (C)	100.0	<25.0	<25.0	>300.0
Purified fraction (D)	50.0	<25.0	18.0	>300.0
Dioctyl phthalate (Sigma)	>300.0	<25.0	>300.0	>300.0
Palmitic acid (Sigma)	44.0	45.5	<3.0	>300.0
Diuron	80.7	<25.0	25.0	32.0
Irgarol	180.0	<25.0	69.0	10.0
Tolyfluanide	>300.0	<25.0	64.0	12.0
Dichlofluanide	150.6	<25.0	71.0	18.0

All assays were carried out in triplicate.

extract was produced from *S. muticum* harvested in March. The chemical composition of *Sargassum* has been studied extensively, but the present investigation was undertaken because there is no report regarding antifouling activity of purified compounds derived from *S. muticum*.

Palmitic acid is a common fatty acid in brown algae and, in the genus *Sargassum*, it can represent 20–40% of the total fatty acids (Vaskovsky et al. 1996; Li et al. 2002; Hossain et al. 2003; Kornprobst 2005). In *S. muticum*, it was shown to constitute 21.5% of the total fatty acids (Vaskovsky et al. 1996). Fatty acids can be produced from triglycerides by action of lipases and have to be included in chemical ecology studies (Noguchi et al. 1979). The fats and fatty acids from marine organisms can play an important role due to the wide diversity of their biological characteristics and their oxidative enzymes leading to the formation of many other bioactive secondary metabolites (Ganti et al. 2006). Indeed, some fatty acids have shown antibacterial or bacteriostatic activities (C8-C12), while C4-C12 fatty acids have antifungal activities and C7-C12 fatty acids inhibit the growth of *Chlorella* sp. (Noguchi et al. 1979). Active antibacterial extracts from different brown algae (*Alaria marginata, Desmarestia ligulata, Dictyota pfaffii, Egregia menziesii, Eisenia arborea, Fucus distichus, Laminaria saccharina, Macrocystis integrifolia, Nereocystis luetkeana* and *Pleurophycus gardneri*) have been found to be made up of saturated and unsaturated fatty acids, with a predominance of myristic, palmitic, oleic, arachidonic and eicosapentaenoic acids (Rosell and Srivastava 1987; Barbosa et al. 2007). A mixture of fatty acids from a lipophilic fraction from *Skeletonema costatum* has also shown an interesting level of inhibition on the growth of *Vibrio anguillarum* and other pathogens associated with the aquaculture industry (Naviner et al. 1999). Hexadecyl palmitate isolated from the alcyonacean soft coral *Sinularia polydactyla* was also shown to be active against *Vibrio harveyi* (Risk et al. 1997). The sulphoglycerolipid 1-*O*-palmitoyl-3-*O*(6′-sulpho-α-quinovopyranosyl)-glycerol isolated from the methanolic extract of the brown seaweed *Sargassum wightii* is active against *Xanthomonas oryzae*. This compound is mainly formed from palmitic acid (Arunkumar et al. 2005). A method developed to control biofouling using polyglycol fatty ester has shown that pure palmitic acid may be used to inhibit bacteria from adhering to a submergible surface (Glover et al. 1997). Those studies confirm the biological activity of palmitic acid observed in our work. The type and amount of free fatty acids can then have a role in the overall defence against microbial colonisation (Benkendorff et al. 2005), but we have shown here that a fatty acid can have an effect on other microfouling organisms.

However, we have also highlighted the presence of the 1,2 benzenedicarboxylic acid, bis(2-ethylhexyl) ester (or dioctyl phthalate; di-(2-ethylhexyl)-phthalate or DEHP), which is a plasticiser and constitutes 16% of the active fraction of *S. muticum*. Pthalate esters are likely contaminants from plastics in the laboratory encountered during the extraction or isolation process and are commonly found during natural products isolation. However, phthalate ester may also come from the coastal environment and/or reflect a phenomenon of bioaccumulation (Peakall 1975). Phthalate esters have been found in soils, plants, and aquatic organisms (Morris 1970; Peakall 1975; Melancon and Lech 1976; Noguchi et al. 1979; Wofford et al. 1981; Stales et al. 1997; Chen 2004; Mackintosh et al. 2004; Cho et al. 2005). Because of their lipophilicity, they can be potentially bioaccumulated by organisms (Mackintosh et al. 2004). The biosynthesis of di-(2-ethylhexyl)-phthalate has been studied by Chen (2004), who has shown that the red alga *Bangia atropurpurea* was synthesising this compound de novo. In algae, di-(2-ethylhexyl)-phthalate has been isolated from *Ceramium rubrum*, but the origin of this phthalate had not been elucidated (Noguchi et al. 1979). Dioctyl phthalate has also been isolated from the brown algae *S. wightii* (Sastry and Rao 1995), *Ishige okamurae* (Cho et al. 2005) and *S. confusum* (Ganti et al. 2006). The dioctyl phthalate isolated from *S. wightii* has shown antibacterial activity against *Staphylococcus aureus, Proteus vulgaris, E. coli, Salmonella typhi, S. paratyphi A, S. typhiridium* and *Pseudomonas aeruginosa* (Sastry and Rao 1995). This phthalate was isolated in a lipid fraction, not unlike in our study. The other extracts of algae studied by those authors and harvested at the same place did not show any antibacterial activity (Sastry and Rao 1994), which suggested that the antibacterial compound of interest did not come from the environment. Di-n-octylphthalate (DNOP) has been isolated from *Ishige okamurae* and tested against the mussel *Mytilus edulis* and the green alga *U. prolifera* (Cho et al. 2005). Total repulsion of the mussel feet was induced by 0.3 mM of DNOP, and 1 mM of DNOP showed a reduction of 7.5% in spore fixation compared to a seawater control. Dioctyl phthalate also has been isolated from *S. confusum* and tested on spore attachment of *U. pertusa* (Ganti et al. 2006). Concentrations of 1 to 100 μg mL^{-1} inhibited 53–86% of spore attachment.

The role of the phthalate ester in the active fraction of *S. muticum* does not seem to be important, except against the growth of the phytoplanktonic strain, but it would be interesting to determine its origin.

In conclusion, fatty acid esters can play an important role in the defence and protection against bacterial development, providing they can be present in sufficient quantities on the considered surface (Benkendorff et al. 2005). The relevant literature leads us to believe that palmitic acid could be responsible for the antifouling activity observed in the active fraction isolated from *S. muticum*. Two patents

(Glover et al. 1997; Risk et al. 1997) already show the potential of pure palmitic acid as an antibacterial compound which could be used in wood protection (Risk et al. 1997), or of one of its derivates, hexadecyl palmitate, which could inhibit bacterial colonisation of a submersed surface (Glover et al. 1997). The tests conducted with commercial palmitic acid on representative organisms of primary colonisation show good activity at low concentrations. Moreover, no toxicity was observed on the cell models used. This compound has potential for the development of an environmentally friendly antifouling product. Furthermore, it can be easily purchased, so there is no need to carry out expensive and time-consuming extractions from *S. muticum*. New assays in paints for commercial palmitic acid and D fraction are currently under development to compare their activity in vivo.

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