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Antifouling activity of macroalgal extracts on *Fragilaria pinnata* (Bacillariophyceae): A comparison with Diuron

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ABSTRACT

The tributyltin-based products and organic biocides which are incorporated into antifouling paints have had a negative impact on the marine environment, and the ban on tributyltin-based antifouling products has urged the industry to find substitutes to prevent the development of fouling on ship hulls. Natural antifouling agents could be isolated from marine resources, providing an alternative option for the industry. The effects of different marine seaweed extracts from *Sargassum muticum* and *Ceramium botryocarpum* on the growth, pigment content and photosynthetic apparatus of the marine diatom *Fragilaria pinnata* were compared with those of Diuron, a biocide widely used in antifouling paints. The addition of the macroalgal extracts in the culture medium resulted in an inhibition of the growth of *F. pinnata*, but this inhibition was lower than that obtained with Diuron. After transfer to a biocide-free medium, *F. pinnata* cells previously exposed to the macroalgal extracts exhibited normal growth, in contrast to Diuron-treated cells, which died, demonstrating that the effects of the natural antifouling agents were reversible. Macroalgal extracts and Diuron-induced modifications in *F. pinnata* cellular pigment content. Chlorophyll *a*, fucoxanthin, and the xanthophyll pool, diadinoxanthin and diatoxanthin, were the most affected. Changes in the structure and function of the photosynthetic apparatus were studied by microspectrofluorimetry, and provided a comprehensive evaluation of the inhibition of the diatom Photosystem II (PSII) by the biocides. This study confirms that natural extracts from the macroalgae studied have the potential to be used as a substitute to commercial biocides in antifouling paints.

1. Introduction

Biofouling, the undesirable accumulation of organisms on submerged surfaces, is estimated to cause great economic losses of more than $5.7 billion US dollars per year worldwide (Rouhi, 1998; Jacobson and Willingham, 2000). It is generally agreed that the prevention of marine fouling can be achieved by the use of coatings, from which a controlled release of toxic molecules (biocides) prevents the growth of adhered organisms (bacteria, algae, molluscs) by killing them (Yebra et al., 2004; Faÿ et al., 2007). Due to the international ban on tributyltin (TBT) as an antifouling agent for ship hulls on 1 January 2008, paint manufacturers now often use organic compounds. The herbicide Diuron (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea) is one of the most representative of "organic booster biocides", replacing organotin compounds in antifouling paints (Callow and Willingham, 1996; Konstantinou and Albanis, 2004). The use of Diuron has been reported in numbers of European countries such as the United Kingdom, Sweden, Spain, the Netherlands, Portugal, as well as outside Europe, e.g., in Japan (Dahl and Blanck, 1996; Ferrer et al., 1997; Thomas, 1998; Ferrer and Barcelo, 1999; Azevedo et al., 2000; Boxall et al., 2000; Martinez et al., 2000; Thomas et al., 2000, 2002; Martinez and Barcelo, 2001; Lamoree et al., 2002; Okamura, 2002; Okamura et al., 2003). There is

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increasing evidence that Diuron concentrations in seawater can have a potentially deleterious impact on seagrasses (Scarlett et al., 1999), and limit the photosynthetic activity of micro- and macroalgae (Kobayashi and Okamura, 2002; Nystrom et al., 2002; Chesworth et al., 2004; Gatidou and Thomaidis, 2007), thus affecting primary producers in aquatic ecosystems. Due to a growing awareness of the environmental issues associated with antifouling paints, the UK Health and Safety Executive (HSE) imposed a ban on the use of Diuron as antifouling agent in November 2002. In fact, the ban is total for Diuron and other booster biocides on boats over 25 m in length. Unfortunately, the ban is not Europe-wide and the persistence of Diuron suggests that herbicides may still pose a threat to the marine environment (Advisory Committee on Pesticides, 2000; Chesworth et al., 2004). Due to effective or imminent restrictions on the use of traditional toxic antifouling paints, the biotechnological field is currently searching for alternative, pollution-free compounds (Yebra et al., 2004; Perez et al., 2006). One of the most promising alternatives to heavy metal- and organic compound-based paints, is offered by the development of antifouling coatings in which the active ingredients are compounds found in marine organisms, acting as natural anti-settlement agents (Bazes et al., 2009).

Remarkably, sessile marine algae are usually not colonized by fouling organisms. It has been shown that these organisms secrete chemicals which prevent the larvae of other organisms from settling and growing on them (Hellio et al., 2000, 2001, 2004; Bazes et al., 2006; Dubber and Harder, 2008). Ethanolic extracts from *Sargassum muticum* (Heterokonta, Sargassaceae) and *Ceramium botryocarpum* (Rhodophyta, Ceramiaceae) present antifouling activities against representative marine organisms such as bacteria, phytoplankton, and spores of macroalgae. Compared with synthetic biocides commonly used in antifouling paints, the natural extracts of these algae present no cytotoxicity (Bazes et al., 2006, 2009). The aim of the present study is to evaluate the impact of the organic extracts of two macroalgae, *S. muticum* and *C. botryocarpum*, on the growth, pigment composition and photosynthetic apparatus of the diatom *F. pinnata*, and to compare their efficiency with those of Diuron by HPLC analysis and microspectrofluorimetry. Among organisms responsible for the development of microbial biofilms on man-made surfaces placed in aquatic environments, diatoms are a major component (Molino and Wetherbee, 2008). The marine diatom *F. pinnata* is commonly involved in biofouling (Jackson, 1991), and has been used as a bioindicator in ecotoxicological studies to assess the physico-chemical quality of aquatic environments (Fisher, 1977; Jurgensen and Hoagland, 1990; Rao, 1994). Furthermore, it has been recently shown that *F. pinnata* is a sensitive microalgal species for antifouling biocide tests (Bazes et al., 2006; Khatoon et al., 2007).

2. Materials and methods

2.1. Test organism

The phytoplanktonic pennate marine diatom *F. pinnata* (Fragilariophyceae) was obtained from the Algae Culture Collection of Caen University (France). It was grown at 18 °C in sterile conditions in a simplified seawater-based culture medium with Guillard F/2 (Sigma) (Guillard and Ryther, 1962). The simplified seawater was made with NaCl (30 g L^{-1}), $MgCl_2$ (10.2 g L^{-1}) and KCl (0.74 g L^{-1}), and sterilised before use. Guillard F/2 was added after sterilisation and the culture medium was stored at 4 °C until use. Cultures were grown in 100 mL Erlenmeyer flasks under controlled illumination (100 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps) with a 16 h:8 h light:dark cycle. Regular dilutions with fresh medium ensured cells were maintained in an exponential growth phase prior to testing.

2.2. Natural antifouling extracts

The natural extracts were prepared from two species of macroalgae, *S. muticum* (Heterokonta, Fucales) collected in Locmariaquer (48°44′N, 3°59′W, France) and *C. botryocarpum* (Rhodophyta, Ceramiales), cultivated in raceways by the Innovalg Company (Bouin, France). For this study, two types of extracts with different polarities were tested, namely A extract (ethanol/water) and B extract (ethanol/dichloromethane). The fresh algae were cleaned in ethanol 5% in order to remove associated microflora before extraction in ethanol 95° (50 g/300 mL). After centrifugation (1.5 h, 10,000 × *g*, 4 °C), the pellet was re-extracted four times using the same procedure. The 5 alcoholic extracts were combined and evaporated in a vacuum at low temperature (35 °C). Distilled water (100 mL) was then added and partitioned with dichloromethane (4 × 100 mL). The aqueous phase was collected, lyophilised, re-suspended in absolute ethanol (100 mL), filtered and concentrated in a vacuum at a low temperature (extract A). The organic phases were collected, dried for 24 h under Na_2SO_4, filtered and concentrated in a vacuum at a low temperature (extract B). The resulting ethanolic (A) and dichloromethane (B) extracts were stored at 4 °C before use (Hellio et al., 2001; Bazes et al., 2006).

2.3. Organic booster biocide and solvent

Diuron (N-[3,4-dichlorophenyl]-N,N-dimethylurea, purity 97.6%) (Nautix, France) demonstrating a low solubility in water, an organic solvent was used for the preparation of stock and working solutions. Among the different water-miscible solvents proposed in standardized protocols (OECD, 1981; ASTM, 1996), DMSO was the least toxic for diatoms (Okumura et al., 2001), and was used as the carrier solvent for Diuron in the bioassays. Stock and working solutions were prepared in acetone-rinsed glassware. Diuron was first dissolved in dimethyl sulphoxide (DMSO; 99%, BDH Ltd., England), then further diluted with autoclaved, deionised water to give a stock solution of 10 mg mL^{-1} and kept in the dark at room temperature to prevent photodegradation. The stock solution was further diluted with the culture medium to achieve the target concentrations for toxicity tests. The final DMSO concentration in experimental vessels never exceeded 0.1% (v/v), a concentration lower by one order of magnitude than the NOEC (no observable effect concentration) values (11,000 ppm) observed for DMSO in two diatom species, *Skeletonema costatum* and *Chaetoceros calcitrans* (Okumura et al., 2001). Furthermore, the toxic effect of DMSO alone towards *F. pinnata* was examined in a preliminary experiment at 200 and 1000 ppm, to estimate the optimum solvent volume which did not affect the growth of this diatom. Experiments were run in triplicate, and none of the tested concentrations were toxic towards *F. pinnata.*

2.4. Growth inhibition test of biocide toxicity

For the growth inhibition test, cultures of *F. pinnata* were grown for 72 h at 18 °C under 16 h:8 h (light:dark cycle) (100 μmol photons m^{-2} s^{-1}). Cultures of *F. pinnata* (initial cell concentration, 67 × 10^5 cells mL^{-1}) were exposed to different concentrations of Diuron and of macroalgal extracts ranging from 0.01 to 100 μg mL^{-1}, along with a control with DMSO (1000 ppm) and a control without DMSO. Cultures were swirled manually twice a day. For each treatment, cell counts were performed daily with a Malassez haemocytometer, and growth rates calculated according to Rioboo et al. (2002). Experiments were run in triplicate, growth being expressed as cells mL^{-1}.

For the toxicity tests, a concentration–response relationship was constructed. The purpose of this 72-h test was to determine the effects of Diuron and natural macroalgae extracts on actively

growing *F. pinnata* cultures. In spite of the relatively brief test duration, effects could be assessed over several generations. The growth of the cultures exposed to Diuron and macroalgal extracts was expressed as relative growth rates (% μ), the ratio of growth rate between that in the presence of the biocide and that in the control, measured at the end of the 72-h toxicity test period. Experiments were run in triplicate. From the average relative growth rates recorded in a series of test solutions, the concentration causing 50% inhibition of growth rate was determined and expressed as the EC_{50} (OECD, 2006).

2.5. Irreversibility of biocide toxicity

Aliquots of an actively growing culture of *F. pinnata* were grown for 72 h in a medium containing effective concentrations (25 μg mL^{-1}) of the different biocides, either Diuron or macroalgal extracts. The algal cells were then centrifuged (2000 × *g*, 10 min, 20 °C, Beckman Avanti J30I) to separate the cells from the medium containing the Diuron and the macroalgal extracts. The pellets containing the algal cells were then resuspended in a fresh plain medium (without biocides) for another 96 h. The algal growth was assessed daily (Sawant et al., 1995) and all the experiments were carried out in triplicate (Bazes et al., 2006).

2.6. HPLC pigment analysis

The HPLC device consisted of a dual-piston pump (Waters 600 E), an automatic injector (Waters 715 Ultra Wisp) connected to a separating column in inverse phase C18 (Waters NovoPack C18 150 × 3.9), preceded by a precolumn (Waters NovoPack C18), and a photodiode array detector (Waters 996). HPLC data were collected using Chromeleon software (Dionex). Pigment extraction was carried out on glass microfibre filters using 90% acetone (24 h at 4 °C in the dark) prior to the injection of sample (100 μL) as recommended by Brotas et al. (2007). Acetone extracts were then filtered (size of filter pores 0.45 μm) and diluted volume-to-volume (30:70) in 1 M ammonium acetate. The HPLC protocol used was adapted from Arsalane et al. (1994). This method can separate about 30 chlorophyll and carotenoid pigments and their breakdown products. Solvent A consisted of Milli-Q water (13%):methanol (51%):acetonitrile (36%):ammonium acetate (23%); solvent B was acetonitrile (30%):ethyl acetate (70%). A linear gradient (solvent A 100% 0–27 min, solvent B 100% 27–42 min) was used at a flow rate of 1.4 mL min^{-1} to separate the pigments (Kraay et al., 1992; Arsalane et al., 1994; Lavaud, 2002; Brotas et al., 2007).

Pigment composition was measured after addition of Diuron or natural algal extracts (A extracts of *S. muticum* and *C. botryocarpum*) at a 1 μg mL^{-1} concentration, after a 144-h growth period. Experiments were run in triplicate. Ten mL aliquots of *F. pinnata* culture were filtered (GF/F Whatman) for pigment extraction. After extraction by acetone, pigments were detected and characterized by diode array measurements of their absorption spectra recorded between 300 and 600 nm. Chromatograms were done at a wavelength of 440 nm, and standards of chlorophyll *a* and β-carotene (Sigma–Aldrich) were used for pigment identification and quantification. Pigment concentrations were expressed by surface area (mg m^{-2}). For some pigments, concentrations were summed: chlorophyll *a* (Chl *a*) and its allomer, chlorophyll $c_1 + c_2$, fucoxanthin and its by-products (*cis*-fucoxanthin and neofucoxanthin), diadinoxanthin and diatoxanthin. The extinction coefficient for Chl *a* determination was used as recommended by Berkaloff et al. (1990).

2.7. Microspectrofluorescence

During photosynthesis, light energy absorbed by the antennae is transferred to the reaction centres of photosystem II (PSII) and photosystem I (PSI). The excitation energy absorbed by chlorophyll *a* molecules can drive photochemistry (delocalisation of electrons at the reaction centres), or be dissipated as heat, or re-emitted as fluorescence (Lazár and Jablonský, 2009). By measuring microspectrofluorescence, the impact on photosynthetic apparatus was estimated by changes in the relative contribution of PSI and PSII. The measurements of the emitted fluorescence from *F. pinnata*, exposed to the macroalgal extracts and to Diuron, with comparison to the control, provided data about the activity of the photosynthetic apparatus. All fluorescence measurements (in triplicate for each concentration of extracts, Diuron treatments and control) were made with the microspectrofluorometer belonging to the Laboratory of Biophysics and Dynamics of Integrated Systems of Perpignan University. Fluorescence digital imaging microscopy (Vigo et al., 1991) consisted of an inverted fluorescence microscope IMT2 (Olympus, Rungis, France) equipped with an epi-illuminator, a 40× objective (Leitz, Rueil-Malmaison) and a Silicon Intensified Target (SIT) camera (Ihesa, Saint Quentin, France), coupled to a Matrox MVP-AT digitizing card (Matrox, Dorval, Canada). For the fluorescence excitation, cells were lit by a Xenon flash lamp (FX-4400, PerkinElmer Optoelectronics, Cambridge, UK) illuminating from a distance of 0.5 m a circular area with a diameter of ca. 0.3 m. The lamp operated with an input energy of 0.5 J per flash, and produced a flash with a 2.5 μs main peak and a 16 μs afterglow. The frequency was set to 50 Hz. A filter wheel placed in front of the lamp housing allowed a choice in the spectral range of excitation. The excitation wavelength was set to 430 nm. The slit of the excitation monochromator was set to 15 nm, the slit of the polychromator to 10 nm for the detection. Fluorescence emission spectra of algal cells were measured between 530 and 770 nm, with an acquisition time of less than 5 s, to be compatible with the follow-up of the intracellular biological phenomena.

2.8. Statistical analyses

For the toxicity tests, concentration–response relationships were constructed and analyzed using a Probit analysis (Finney, 1971). Differences in cell growth between the control and treatment groups were also compared using one-way analysis of variance (ANOVA). Assumptions of normality and homocedasticity were tested using the Shapiro–Wilk and Brown–Forsyth tests, respectively. The lowest observable effect concentrations (LOEC) and the no observable effect concentrations (NOEC) were determined using Dunnett's post hoc multiple comparison tests (Worboys et al., 2002). The parametric inverse prediction (discrimination) method was applied to determine the 95% confidence intervals (95% CI) of the 50% effective concentrations (EC_{50}) (Seber and Lee, 2003). Statistics computations were performed using standard software packages (SPSS version 13.0, Chigago, IL, USA and GraphPad Prism, version 4.02, San Diego, CA, USA).

3. Results

3.1. Growth toxicity test

All the macroalgal extracts, and Diuron inhibited the growth of the diatom *F. pinnata* in a clear concentration-dependent effect (Fig. 1). For each treatment, results were expressed as relative growth rates, in comparison with the negative control. The results of EC_{50} (the concentration causing 50% growth inhibition), the LOEC (the lowest observed effect concentration) and NOEC are presented in Table 1. When compared to Diuron (Fig. 1A), all tests of growth inhibition with the macroalgal extracts (Fig. 1B–E) resulted in significantly higher values, indicating a lower growth inhibition for the natural extracts. The A extracts of *S. muticum* and *C. botry-*

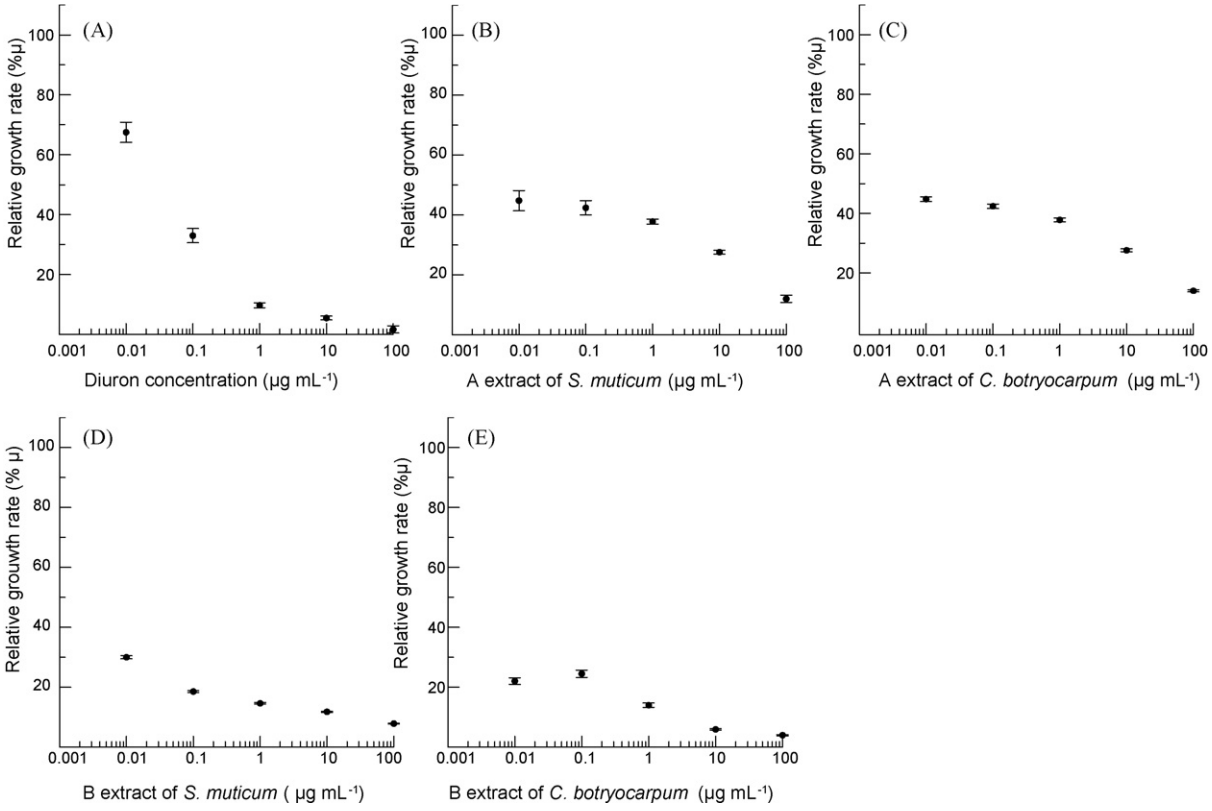

Fig. 1. Relative growth rates of *F. pinnata* exposed to Diuron (A); A extract of *S. muticum* (B); A extract of *C. botryocarpum* (C); B extract of *S. muticum* (D) and B extract of *C. botryocarpum* (E) for 72 h at five concentrations. Values are mean ± SE ($n = 3$).

ocarpum resulted in significantly lower EC_{50} and LOEC values than B extracts. For each type of extracts (A or B), there were no significant differences between macroalgae, as indicated by the largely overlapping standard errors (SE) of their 72-h EC_{50} values (Table 1). Values of EC_{50} and LOEC for the B extract of *S. muticum* were 2/3 of those of *C. botryocarpum*, reflecting a higher growth inhibition. Regarding the NOEC, the A extract of *S. muticum* resulted in value significantly lower than that of *C. botryocarpum*, in contrast to the B extracts.

3.2. Irreversibility of biocide toxicity

The irreversibility of growth inhibition by biocides was tested by transferring algae exposed for 72 h either to Diuron or to the different macroalgal extracts, to a fresh, biocide-free medium for another 96-h period of growth (Fig. 2). For this series of experiments, all biocides were used at 25 μg mL^{-1}, a concentration demonstrated to be effective as previously tested in the growth inhibition test. Diuron completely inhibited the growth of *F. pinnata*, and this inhibition persisted after the transfer to a medium free of biocide, most likely due to the death of the cells. All the macroalgal extracts did inhibit the growth of the diatom, but to a lower extent than Diuron. Furthermore, the cells exhibited an almost normal growth after the transfer to an extract-free medium. A extracts of *S. muticum* and *C. botryocarpum* inhibited the growth of *F. pinnata* more than their B extracts.

3.3. Changes in pigment composition with biocide exposure

The influence of biocides on the pigment content of *F. pinnata* was tested by comparing the effects of Diuron with those of A extracts of *S. muticum* and *C. botryocarpum*. Biocide concentration was reduced to 1 μg mL^{-1}, in order to allow the algal cultures to grow for a 72-h period and for possible pigment alterations to develop. Controls consisted of cultures without biocides. HPLC chromatograms for the 3 experimental conditions and the control revealed 12 peaks, corresponding to chlorophylls *a* and *c*, major and minor carotenoids, on the basis of their *in vitro* absorption spectra and retention times (not shown). When compared to the control, a 72-h exposure to the different biocides (1 μg mL^{-1}) caused a decrease in the pigment content in *F. pinnata* (Fig. 3). For all pigments, the decrease was significantly higher with Diuron, ranging from ca. 50% for β-carotene to ca. 90% for the pool of xanthophylls DD + DT. Both macroalgal A extracts showed a similar influence on the pigment composition of *F. pinnata*, the decrease ranging from ca. 10% for Chl *c* and β-carotene, to 40–60%, for Chl *a*, fucoxanthin

Table 1
Results of the 72 h toxicity tests for Diuron and macroalgal A and B extracts of *S. muticum* and *C. botryocarpum* with cultures of *F. pinnata*. Values are mean ± SE ($n = 3$).

	EC_{50} (μg mL^{-1})	LOEC (μg mL^{-1})	NOEC (μg mL^{-1})
Diuron	0.090 ± 0.005	0.070 ± 0.017	0.050 ± 0.010
A extract of *S. muticum*	4.890 ± 0.225	2.110 ± 0.680	1.710 ± 0.541
A extract of *C. botryocarpum*	5.100 ± 0.236	3.420 ± 0.692	2.930 ± 0.421
B extract of *S. muticum*	15.120 ± 0.803	10.300 ± 1.023	8.000 ± 0.792
B extract of *C. botryocarpum*	15.150 ± 0.815	14.410 ± 1.583	9.740 ± 1.291

EC_{50}: half maximal effective concentration; LOEC: lowest observed effect concentration; NOEC: no observe effect concentration.

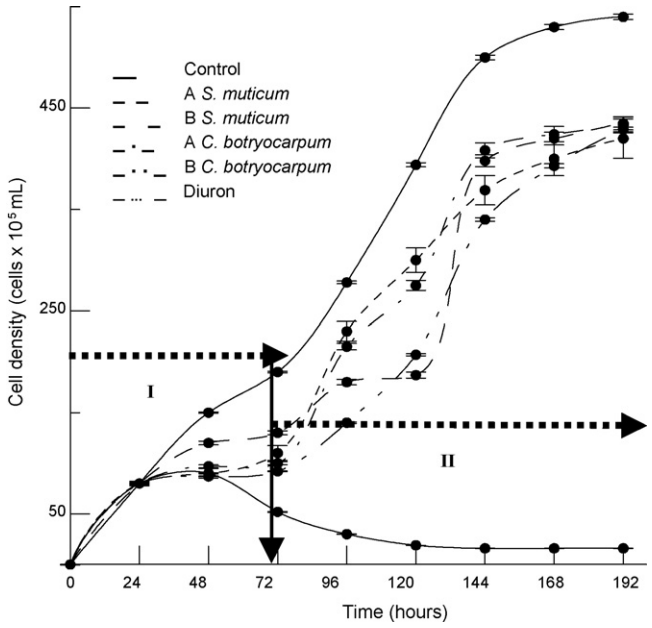

Fig. 2. Growth (cellular concentration) of *F. pinnata* cells exposed to 25 μg mL^{-1} of the A and B macroalgal extracts or Diuron (I) before and (II) after transfer to an extract or biocide-free medium. Values are mean ± SE (n = 3).

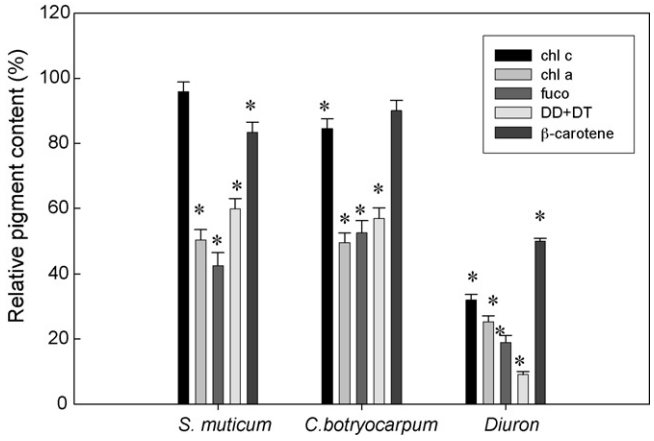

Fig. 3. Pigment content in *F. pinnata* exposed for 72 h to the macroalgal A extracts or Diuron (all antifouling compounds at 1 μg mL^{-1}), expressed as percent of cellular pigment content of the control (mean ± SE, n = 3). For all pigments, the cellular content was significantly higher in presence of macroalgal extracts than in the presence of Diuron (p < 0.05, ANOVA). *Values significantly different from the control (p < 0.05, ANOVA) for algal extracts and Diuron.

and the pool of xanthophylls. Relative changes in the pigment content for Chl *c*, fucoxanthin, the xanthophyll pool, and β-carotene, expressed in function of the molar Chl *a* content, are presented in Table 2. Apart from the drastic decrease of xanthophylls, the greatest change in presence of Diuron occurred for Chl *c* and fucoxanthin.

3.4. Microspectrofluorescence

Microspectrofluorometry was used to investigate the effects of biocides on the photosynthetic apparatus of *F. pinnata*. Concentrations for Diuron ranged from 0 to 1 μg mL^{-1}, and for macroalgal extracts from 0 to 10 μg mL^{-1}. The choice of these concentrations was based on previous results (growth inhibition and pigment alteration). The fluorescence emission spectra of *F. pinnata* cells were recorded daily on cells collected from cultures exposed to different concentrations of biocides over a 72-h period. The fluorescence emission spectra from 630 to 770 nm were deconvoluted into 3 Gaussian bands (Fig. 4). Band I and band II originate from PSII antenna, band III, from the PSI antenna. From the deconvoluted spectra, three parameters can be used to characterize the different bands at their maximal emission: the wave number, the width halfway, and the peak wavelength. Moreover, the ratio of the band areas, $(A_I + A_{II})/A_{III}$ can be used as an index reflecting changes in the structure of the photosynthetic apparatus due to the presence of biocides. For the control (cells not exposed to biocides), the values of these parameters were almost constant over the 72-h period of growth (Table 3). Whatever the concentration of Diuron added to the medium, and whatever the growth phase, the wave number

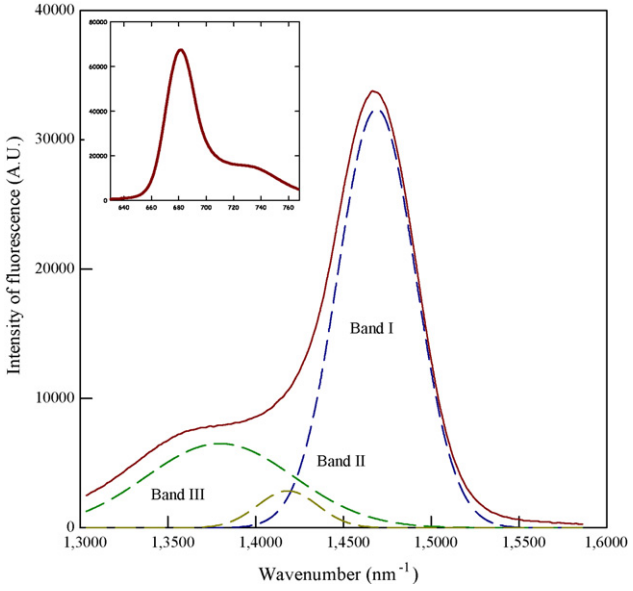

Fig. 4. *In vivo* emission spectrum (insert) and deconvolution spectrum of chlorophyll fluorescence of *F. pinnata* for λ_{exc} = 430 nm. Band I and band II present PSII emission of fluorescence, band III, the PSI emission of fluorescence.

and the halfway width for each band were not significantly different from the control (not shown). Nevertheless, the addition of Diuron to the culture medium altered the physiological state of *F pinnata*, as illustrated by changes in the ratio $(A_I + A_{II})/A_{III}$. The higher the concentration of Diuron, the higher the ratio $(A_I + A_{II})/A_{III}$, whatever the exposure time (Fig. 5). A concentration of 0.002 μg mL^{-1} of Diuron was sufficient to induce a 50% increase of the ratio $(A_I + A_{II})/A_{III}$.

Table 2
Pigment molar ratio/Chl *a* of *F. pinnata* after natural extract treatments and Diuron exposure. Values are mean ± SE (n = 3).

Biocide treatments	Pigment ratios			
	Chl *c*/Chl *a*	Fucoxanthin/Chl *a*	(DD + DT)/Chl *a*	β-Carotene/Chl *a*
Control	1.900 ± 0.004	0.926 ± 0.002	0.913 ± 0.017	0.917 ± 0.026
A extract of *S. muticum*	1.899 ± 0.010	0.833 ± 0.026	1.170 ± 0.006	1.759* ± 0.047
A extract of *C. botryocarpum*	1.716 ± 0.050	1.068 ± 0.028	1.155 ± 0.056	1.887* ± 0.186
Diuron	1.258* ± 0.005	0.748* ± 0.003	0.363* ± 0.017	1.982* ± 0.168

* Values are significantly different (p < 0.05) from control (ANOVA).

Table 3
Mean values of the wavenumber, the width halfway and the wavelength of maximal emission for three bands of the emission spectrum for control. Values are mean ± SE ($n = 3$).

	Wavenumber of maximal emission (nm^{-1})	Width halfway (nm^{-1})	Wavelength of maximal emission (nm)
Band I	$1.471 \times 10^{-3} \pm 0.042$	$5.250 \times 10^{-5} \pm 0.001$	680
Band II	$1.421 \times 10^{-3} \pm 0.043$	$3.796 \times 10^{-5} \pm 0.002$	703
Band III	$1.377 \times 10^{-3} \pm 0.001$	$1.002 \times 10^{-4} \pm 0.006$	726
$(A_I + A_{II})/A_{III}$		2.460 ± 0.180	

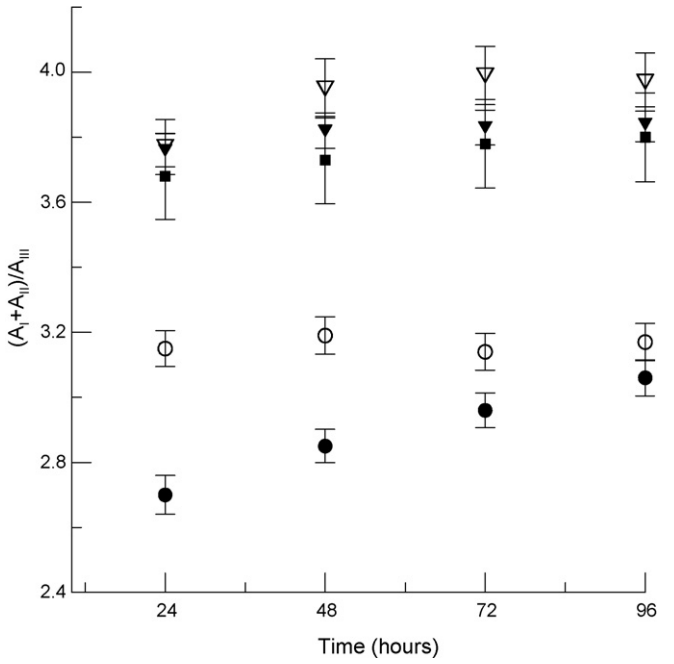

Fig. 5. Changes with time of the ratio $(A_I + A_{II})/A_{III}$ in *F. pinnata* cells exposed to different concentrations of Diuron (●, 0 μg mL^{-1}; ○, 0.001 μg mL^{-1}; ▽, 0.01 μg mL^{-1}; ■, 0.1 μg mL^{-1}; ▼, 1 μg mL^{-1}). Values are mean ± SE ($n = 3$).

Similarly, natural biocides did not change the general characteristics of the fluorescence emission spectra of *F. pinnata* cells (same wave number and halfway width), whatever the concentration or the growth phase (data not shown), but they altered their photosynthetic apparatus, as demonstrated by changes in the ratio $(A_I + A_{II})/A_{III}$ with macroalgal extracts (Fig. 6B and C). The ratio increased with the concentration of the A extracts, but remained constant with the B extracts. The A extract of *S. muticum* was more active than A extract of *C. botryocarpum*. The concentration needed to induce a 50% increase of the ratio $(A_I + A_{II})/A_{III}$ was of 0.02 μg mL^{-1} for *S. muticum* (Fig. 6 B), and 0.08 μg mL^{-1} for *C. botryocarpum* (Fig. 6C). These concentrations were higher by one order of magnitude than those of Diuron.

4. Discussion

4.1. A demand for environment-friendly antifouling coatings

By default, antifouling paints are used to hinder the colonisation of marine organisms, which increase the drag and fuel consumption of ships. The aim of antifouling paints is to kill or repel the organisms before they can attach to the hull (Yebra et al., 2004; Kristensen et al., 2008). In recent years, antifouling products incorporated in boat paints have often included organic "booster" biocides to enhance performance without using tributyltin compounds, which have been restricted in most countries due to their demonstrated toxicity to non-target organisms at very low concentrations (Guitart et al., 2007). Furthermore, the International Maritime Organization (IMO), through the Marine Environmental Protection Committee, approved a resolution that globally prohibited the application of TBT as biocide in antifouling systems on ships by 1 January 2003, and completely prohibited its presence by 1 January 2008 (Champ, 2003). However, some of the booster biocides used to replace TBT were later also banned, and their use regulated in Europe, because of their remanence and toxicity to non-target organisms (Voulvoulis et al., 1999; Ranke and Jastorff, 2000; Thomas et al., 2002; Konstantinou and Albanis, 2004). Among these biocides, Diuron showed an impact on aquatic ecosystems by exerting selective pressure and altering phototrophic species assemblages (Thompson et al., 1993; Seguin et al., 2001; Kish, 2006; Muller et al., 2008). Hence, the control of fouling and environmental constraints requires not only adequate risk assessment procedures for antifouling biocides (Bellas, 2006), but also alternatives to the toxic coatings currently used on ship hulls. Thus, the incorporation in matrix paints of bioactive compounds derived from marine organisms has become a promising strategy for the development of environment-friendly antifouling coatings (Holmstrom and Kjelleberg, 1994; Kjelleberg and Steinberg, 1994; Hellio et al., 2001; Bazes et al., 2006; Culioli et al., 2008).

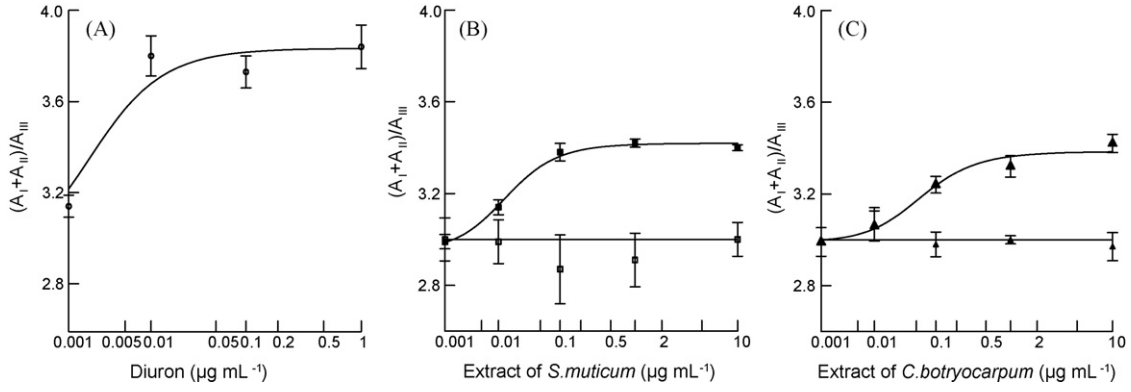

Fig. 6. Changes with time of the ratio $(A_I + A_{II})/A_{III}$ in *F. pinnata* cells exposed to different concentrations of Diuron (A), or natural extracts from *S. muticum* extracts (B), or *C. botryocarpum* (C). A extracts (■, ▲), B extracts (□, △). Values are mean ± SE ($n = 3$).

Marine macroalgae represent a high biomass in the marine environment, and they often carry significantly less macro and microepibionts on their thalli than co-occurring biofilms on inanimate substrata (Hellio et al., 2001; Lam and Harder, 2007; Dubber and Harder, 2008). Indeed, they can produce a wide variety of metabolites, e.g. fatty acids, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles or steroids, some of them with antibacterial, antialgal, or antifungal properties, which has justified large-scale screenings of crude algal extracts in the last decades (Hornsey and Hide, 1976; Caccamese and Azzolina, 1979; Reichelt and Borowitzka, 1984; Febles et al., 1995; Amade and Lemee, 1998; Maximilien et al., 1998; Hellio et al., 2000, 2001, 2004; Gonzalez del Val et al., 2001; Liao et al., 2003; Sandsdalen et al., 2003; Marechal et al., 2004; Bansemir et al., 2006; Bazes et al., 2006, 2009; Dubber and Harder, 2008). Hence, some of these bioactive compounds, which seem to specifically protect macroalgae from settling organisms, should be effective in the prevention of biofouling on submerged structures. The isolation of these biogenic compounds, the determination of their structure, and their commercial production are key steps in the development of environment-friendly antifouling paints (Dubber and Harder, 2008; Xiong et al., 2009).

Ceramium and *Sargassum* are two genera which are considered to be good sources of bioactive compounds (Hellio et al., 2000, 2001; Stiger et al., 2004; Bazes et al., 2006, 2009). For instance, it has been shown that *C. botryocarpum* is a rich source of allelochemicals and secondary metabolites, exhibiting strong antifouling activity of marine bacteria, phytoplankton and spores of *Ulva* sp. (Bazes et al., 2006). Temperate brown seaweeds are known to produce secondary metabolites like phenolic acid sulphate esters (Steinberg, 1992), generally considered as a chemical defence against fouling colonisation (Sieburth and Conover, 1965; Norris and Fenical, 1982; Hay and Fenical, 1988; Harlin, 1987; Hay, 1996; Amade and Lemee, 1998; Targett and Arnold, 1998; Stiger and Payri, 1999; Connan et al., 2004; Plouguerne et al., 2006). In particular, it has been shown that *Sargassum* is a genus grouping species producing compounds with a broad spectrum of action against fouling organisms. These compounds are harmless to the environment (Hellio et al., 2000, 2001; Stiger et al., 2004; Bazes et al., 2009).

4.2. Potential interest of macroalgal extracts to prevent fouling

The present study confirmed the presence of active substances in both extracts of *C. botryocarpum* and *S. muticum*, and demonstrated their inhibitory potential on the growth of *F. pinnata* at concentrations ranging from 8 to 30 $\mu g\,mL^{-1}$. Whatever the response of *F. pinnata*, A extracts (ethanol/water) were more effective than B extracts (ethanol/dichloromethane). Natural extracts were less toxic than Diuron, as they presented a comparable inhibitory effect on the growth of *F. pinnata* but at concentrations one/two orders of magnitude higher. The macroalgal extracts are not purified compounds, like Diuron, and the lower efficiency observed in the present work does not preclude the use of their bioactive substances as antifouling agents. Furthermore, the antifouling effects of the two macroalgal extracts were reversible, in contrast to the organic booster biocide. *F. pinnata* cells recovered after their transfer to a medium not containing any bioactive substances, and displayed almost a normal growth rate. This confirms the potential of compounds extracted from the two macroalgae as environmentally acceptable antifouling agents.

4.3. The impacts of antifouling agents on the changes in the pigment content

In unicellular algae like diatoms, the pigment content usually changes with the photoacclimation state (Falkowski and Raven, 1997), and is an indicator of cell physiology. Thus, pigments have been used as biomarkers to estimate the influence of biocides (Teisseire and Vernet, 2001; Geoffroy et al., 2002). Regarding the impacts of the macroalgal extracts on the pigment composition of *F. pinnata*, the most obvious effect observed was the decrease in Chl *a*, fucoxanthin and xanthophylls (DD + DT). In contrast, Chl *c* and β-carotene decreased to a lower extent, reflecting that this was not a consequence of an inhibition of growth, and that the natural biocides have a direct impact on the pigment content, like Diuron, a common reference for the assessment of antifouling activities. In particular, our results show that the xanthophyll pool, which is essential in photoprotection and which, in diatoms, consists of the reversible light-induced de-epoxidation from diadinoxanthin to diatoxanthin (Falkowski and Raven, 1997; Lavaud, 2002), was strongly affected by Diuron, as it is by other chemicals like heavy metals (Bertrand et al., 2001). This organic compound is a phenyl urea herbicide used to control broadleaf weeds, and it has been commonly incorporated as booster biocide in antifouling paints (Antizar-Ladislao, 2008). Diuron is considered to be one of the most potent photosynthetic inhibitors, which act by blocking electron transport between PSII and PSI (Hall et al., 1999; Thomas et al., 2003). More precisely, Diuron binds with a high affinity to the Q_B binding site in the D1 protein of PSII, displacing Q_B and preventing electron transfer from Q_A to Q_B (Cremlyn, 1991; Jansen et al., 1993; Dahl and Blanck, 1996; Hall et al., 1999; Thomas et al., 2003). This inhibition in the electron transport from PSII to PSI ultimately leads to a reduced carbon dioxide uptake, a decrease in carbohydrate production, alteration in the biosynthesis of pigments (carotenoids and chlorophylls) and proteins, and the eventual starvation of the organism (e.g., Frank and Cogdell, 1996; Teisseire and Vernet, 2001; Geoffroy et al., 2002).

4.4. Microspectrofluorometry can evidence the antifouling action of biocides

The photosynthetic processes in algae were especially affected by the A extract of *S. muticum*, *C. botryocarpum* and by Diuron, as demonstrated by the decrease of chlorophyll fluorescence emission spectra. The structure of the photosynthetic apparatus was also altered, since some significant changes in chlorophyll fluorescence parameters were observed at concentrations as low as 0.01 $\mu g\,mL^{-1}$ for macroalgal extracts and 0.001 $\mu g\,mL^{-1}$ for Diuron. This is in line with observations on the sensitivity of PSII in aquatic plants exposed to pollutants reported earlier (Juneau et al., 2002; Geoffroy et al., 2003, 2004; Olette et al., 2008). In particular, changes in the ratio $(A_I + A_{II})/A_{III}$ give information on how the photosynthetic apparatus is affected by Diuron and by A extracts of both macroalgae. Fluorescence emission can provide general information on the functioning of photosystems, such as modifications in the antenna and reaction centres, in the electron transport chain, non-photochemical quenching processes or photoinhibition (Horton and Hague, 1988; Ruban and Horton, 1995; Muller et al., 2001). At room temperature, an increase in the ratio $(A_I + A_{II})/A_{III}$ reflects an increase in the fluorescence emission at the PSII RC level, hence a decrease in the linear electron transport rate from PSII to PSI, possibly due to changes in the redox environment of RC PSII. Thus the changes in $(A_I + A_{II})/A_{III}$ ratio evidenced in our study, confirm the damage to the *F. pinnata* photosynthetic apparatus due to A extracts of *S. muticum*, *C. botryocarpum*, and Diuron. This is in line with previous studies (e.g., Agati et al., 1995; Eullaffroy and Vernet, 2003), which reported that the F684/F735 or F680/F730 chlorophyll fluorescence ratios, similar in essence to $(A_I + A_{II})/A_{III}$, were reliable and early indicators of changes in the functioning of the photosynthetic apparatus under herbicide or metal stress.

4.5. .The environmental toxicity of Diuron

Many studies have reported water contamination by the Diuron contained in antifouling paint (e.g., Boxall et al., 2000; Okamura, 2002), or used as herbicide (Magnusson et al., 2008). For instance, in the marine environment, Diuron concentrations as high as 6.7 $\mu g\,L^{-1}$ (Thomas et al., 2001) and 8 $\mu g\,L^{-1}$ (Mitchell et al., 2005) were detected. In the coastal waters of western Japan, 86% of 142 samples studied contained Diuron up to 3.05 $\mu g\,L^{-1}$ (Okamura et al., 2003). In Dutch coastal waters and marinas, levels higher than the maximum permitted (430 $ng\,L^{-1}$) were observed (Lamoree et al., 2002). Although Diuron and its degradation products were found mainly in water, it has been shown that they can also be detected in sediments (Thomas et al., 2002), that an increase in suspended particulate matters enhanced the transfer of Diuron to sediments, and that this phenomenon was not easily reversible (Voulvoulis et al., 2002).

The present study shows that Diuron concentrations lower than 10 $\mu g\,L^{-1}$ affect algal biomass, by altering the electron transport rate and decreasing growth, a result in agreement with Magnusson et al. (2008). Hence, inhibition of the photosynthetic apparatus by Diuron, even at low concentrations, is likely to change pigment content, reduce growth and alter biomass of critical primary producers in estuarine habitats, which in turn could induce changes in the composition of the microorganism community, thus altering food availability and quality for benthic feeders. Taking into consideration the important ecological role of microalgae in aquatic food webs, the results of the present study suggest a possible environmental risk due to the direct release of Diuron into the marine environment, or due to its products of transformation, either from biotic or abiotic degradation (Giacomazzi and Cochet, 2004). Single-species bioassays give only a first estimate of the ecological impact of pollutants on phytoplankton. More research is needed concerning their combined action, especially in more realistic conditions like microcosms, but certain aspects (the mechanisms of pollutant removal and co-existence with other toxic compounds) complicate the estimation of the ecological risk (Gatidou and Thomaidis, 2007).

5. Conclusion

It can be concluded from this study that crude extracts of *S. muticum* and *C. botryocarpum* displayed clear antifouling properties against *F. pinnata*. These natural compounds affected the growth and the pigment content of the diatom. The mechanism by which the extracts act as antifouling agents relies on the alteration of the photosynthetic apparatus, as evidenced by pigments changes and PSII inhibition. The efficiency of the macroalgal extracts was lower than that of Diuron, but as their influence is reversible, they represent an environmentally acceptable alternative to toxic products for the control of fouling organisms. Hence these extracts could be used as non-toxic coatings in marine aquaculture, for which a demand exists (Da Gama et al., 2002; Bazes et al., 2006, 2009). Since the crude extracts are not yet characterized, further research is needed to purify and identify the active molecules responsible for this activity, before assessing definitely their potential as alternative antifouling agents. Provided a natural efficient pathway of bioactive algal metabolites is found in *C. botryocarpum* and *S. muticum*, these macroalgae may be suitable candidates for the production of antifouling compounds. Experiments aiming at identifying the naturally released active molecules and quantifying production rates are currently underway.

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