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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