BRAZILIAN JOURNAL OF OCEANOGRAPHY, 55(4):257-264, 2007

ANTIFOULING ACTIVITY OF SEAWEED EXTRACTS FROM GUARUJÁ, SÃO PAULO, BRAZIL

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ABSTRACT

Marine biofouling historically constitutes one of the major constraints faced by mankind in its oceanic activities. The search for alternatives to TBT-based antifouling paints has led several researchers to focus efforts in the development of environmentally friendly natural compounds. This work has contributed with this search, testing the antifouling potential of crude organic extracts from four seaweed species collected at Praia Branca, Guarujá district, São Paulo, Brazil. Throughout laboratory antifouling assays in which the attachment of a common fouling organism, the brown mussel *Perna perna*, was employed, antifouling activity ($p < 0.05$) was detected in natural concentrations of the extracts of *Jania rubens* (Rhodophyta, Cryptonemiales) and *Bryothamnion seaforthii* (Rhodophyta, Ceramiales), while *Dictyopteris delicatula* (Phaeophyta, Dictyotales) and *Heterosiphonia gibbesii* (Rhodophyta, Ceramiales) did not exhibit fouling inhibition. From the algae that exhibited antifouling activity, *J. rubens* presented best performance when compared to that of *B. seaforthii*. Future field studies would be necessary to obtain results that can better reflect natural conditions, as well as to assess the activity spectrum of the antifouling activity presently recorded. Further bioassay-guided purification of the active extracts can lead to new alternatives to the metal-based antifouling paints currently in use.

RESUMO

A incrustação biológica constitui, historicamente, um dos maiores problemas encontrados pelo homem em suas atividades no mar. A busca por alternativas a tintas antiincrustantes contendo tributilestanho (TBT) tem levado diversos pesquisadores a concentrar esforços no desenvolvimento de substâncias naturais menos danosas à biota marinha. Este trabalho procurou contribuir com essa busca, testando o potencial antiincrustante de quatro diferentes espécies de macroalgas da Praia Branca, município de Guarujá, SP. Através de testes antiincrustantes em laboratório utilizando a fixação de um organismo incrustante comum, o mexilhão *Perna perna*, foi constatado que os extratos de *Jania rubens* (Rhodophyta, Cryptonemiales) e *Bryothamnion seaforthii* (Rhodophyta, Ceramiales), à concentração natural, apresentaram atividade antiincrustante significativa ($p < 0,05$), enquanto *Dictyopteris delicatula* (Phaeophyta, Dictyotales) e *Heterosiphonia gibbesii* (Rhodophyta, Ceramiales) não demonstraram eficiência na inibição da fixação de bissos do molusco. Das algas que indicaram potencial atividade contra a incrustação, *J. rubens* apresentou melhor desempenho em relação a *B. seaforthii*. Futuras investigações em campo serão necessárias para a obtenção de resultados que possam refletir melhor as condições naturais, bem como avaliar o espectro de ação da atividade antiincrustante observada. A purificação guiada por bioensaios dos extratos ativos poderá levar a novas alternativas para os antiincrustantes metálicos atualmente em uso.

Descriptors: Antifouling activity, Seaweeds, Secondary metabolites, Marine natural products, Biofouling, Marine chemical ecology, Epibiosis.
Descritores: Atividade antiincrustante, Algas marinhas, Metabólitos secundários, Produtos naturais marinhos, Bioincrustação, Ecologia química marinha, Epibiose.

INTRODUCTION

Fouling organisms play an important role in marine ecosystems, since besides being efficient filter feeders, they are also an important source of food for several marine animals, as well as for man. However, when interacting with manmade structures, they incur in severe costs to navigation and other maritime activities (DAVIS et al., 1989; FULTON-HOWARD; FORT, 2004).

Biofouling makes ship hulls irregular and rough, increasing drag, and decreasing maneuverability and cruising speed (SAFRIEL et al., 1993; DAVIS et al., 1989). An increase of 10 μ (1/1000 cm) in ship hull mean roughness results in an increase of 0.3 to 1.0% of fuel consumption (CHAMP; LOWESTEIN, 1987). In temperate waters, estimates suggest that fouling causes an increase of 35 to 50% in fuel consumption of a ship that has spent 6 months at sea (W.H.O.I., 1967). However, this percentage may be fairly larger in tropical environments where, in general, fouling development is much faster, reaching extremely high biomass values (DA GAMA et al., 1999; RAILKIN, 2003). In structures such as oil platforms, fouling promotes corrosion and increases the mass of their underwater structures, distorting their original configuration (SAFRIEL et al*.,* 1993; YAN; YAN, 2003); in floating structures such as navigation buoys, fouling overwhelmingly increases weight, reducing buoyancy and sometimes sinking structures; interfering with mobile mechanisms such as swivels and mooring chains, increasing the breakage likelihood (COUTINHO, 1992); in maritime piping, such as those used to cool down nuclear power plants, fouling reduces the internal diameter and maximizes material fatigue and corrosion (DA GAMA; PEREIRA, 1995b).

Other detrimental consequences of fouling comprise the effects on sound transmission, making echo soundings more difficult; the role in the transport of non-indigenous species around the world, attached to the hulls of ships or inside ballast tanks (GODWIN, 2003; MINCHIN; GOLLASCH, 2003), and the clotting of net cages and protection nets in aquaculture (W.H.O.I., 1967). In Australia, 80% of the total pearl cost production is allocated to combat or prevent biofouling (FULTON-HOWARD; FORT, 2004).

As an efficient form of prevent marine fouling, the use of TBT (tributyl tin) based antifouling paints has been spread worldwide in the last decades, but evidence of harmful effects to many non-target organisms followed soon after (CHAMP; LOWESTEIN, 1987). However, effects of TBT paints have only recently been documented in Brazilian waters (CAMILLO et al., 2004; FERNANDEZ et al., 2005; LIMAVERDE et al., 2007).

These papers reported high indices of imposex in the Brazilian whelk *Stramonita haemastoma* as a possible consequence of severe TBT contamination. Such evidence is particularly noteworthy when taking into account that *S. haemastoma,* as well as its main prey, the brown mussel *Perna perna*, are edible species largely consumed along the Brazilian coast (LIMAVERDE et al., 2007).

In face of this environmental issue, environmentally friendly alternatives to toxic biocides in antifouling paints are urgently needed, and have been investigated in a number of countries and research groups (FUSETANI, 2004). Among the compounds under investigation, marine natural products have been highlighted as promising new antifoulants in a number of studies (DA GAMA; PEREIRA, 1995a; DEVI et al., 1998; DA GAMA, 2001; DA GAMA et al., 2002; HELLIO et al., 2002; PEREIRA et al., 2002; BURGESS et al., 2003; NOGATA et al., 2003; NYLUND; PAVIA, 2003; STUPAK et al., 2003; PEREIRA et al., 2003; BHADURY; WRIGHT, 2004; HELLIO et al., 2004; MARÉCHAL et al., 2004; VILLAS-BÔAS; FIGUEIREDO, 2004).

Marine organisms, including seaweeds, sponges, corals, ascidians, and many other invertebrates are frequently found devoid of epibionts in nature, thus becoming natural targets of investigation seeking the discovery of the antifouling compounds responsible for epibiosis prevention (DA GAMA; PEREIRA, 1995b).

Seaweeds are among the more prolific natural product synthesizers, from which ca. 2000 secondary metabolites have been isolated to date (HARPER et al., 2001), most of which displaying interesting biological activities (SENNETT, 2001). However, little is known about the chemical ecology of Brazilian algae (PEREIRA, 2002). This study was aimed at investigating whether the macroalgae *Heterosiphonia gibbesii* (Harvey) Falkenberg, *Dictyopteris delicatula* Lamouroux., *Bryothamnion seaforthii* (Turner) Kützing and *Jania rubens* (Linnaeus) Lamouroux exhibit antifouling activity toward the fouling mussel *Perna perna*.

MATERIAL AND METHODS

Sampling Procedure

Samples of *Heterosiphonia gibbesii*, *Bryothamnion seaforthii* (Rhodophyta, Ceramiales), *Jania rubens* (Rodophyta, Corallinales), and *Dictyopteris delicatula* (Phaeophyta, Dictyotales) were collected by hand during low tide at Praia Branca (23º51'59"S and 46º10'22"W, Guarujá district, São Paulo State, southeastern Brazil) in 2 October, 2005.

Sampled material was transferred to dark recipients and stored in isothermal boxes to prevent photo- and thermal degradation during the transport to the laboratory.

Wet algae were then cleaned from sediments and associated organisms (but no special treatment was employed to remove microorganisms) and divided into two aliquots: one for taxonomic identification (preserved in 4% formalin in seawater) and other to be used to extraction after weighing and dryness. This portion was dried at a room temperature of 17°C and in the dark until a steady weight was obtained (dry weight).

Extraction Procedure

Dried seaweeds of the 4 species were cut into small pieces to increase extraction effectiveness in aluminum foil-covered Pyrex beakers (to prevent extract photodegradation) to which pure methylene chloride (100% dichloromethane, CH_2Cl_2, analytical grade, Merck) was added until samples were totally immersed. A total of 5 successive extractions were performed at room temperature (ca. 25°C), at 24 h intervals. After extraction, the solvent was filtered and vacuum-evaporated to yield the crude organic extracts, which were then weighed. Pure compounds were not obtained from active crude extracts in the present work.

Antifouling Activity Bioassays

Juvenile mussels (*Perna perna*) were collected during low tide from the rocky coastal area of Itaipu (22º58'26"S, 43º02'47"W; Niterói City , Rio de Janeiro, Brazil) and kept in a 400 l recirculating laboratory aquarium (equipped with biological filtering, protein skimming and activated carbon) at a constant temperature (20°C), salinity (ca. 35) and aeration for 12 h. Individuals were then disaggregated by carefully cutting the byssus threads, and divided into size groups according to total shell length, ranging from 0.8 to 4.1 cm, in plastic trays with seawater. Individuals exhibiting substrate exploring behavior (actively exposing their foot and crawling) were selected for experiments.

Antifouling activity was measured by the following procedure, modified by Da Gama et al. (2003) from the original methods described by INA *et al*. (1989) and Goto et al. (1992). Water-resistant filter paper was cut into 9 cm diameter circles and soaked in solvent (control filter). Another 9 cm diameter set of filter papers (treatment filters) was cut in a chess board pattern (1 cm squares) and soaked in a natural concentration of each of the four algal extracts employed (determined as the extract equivalent to the DW of alga = DW of filter paper) or in solvent only (control experiment). All filter paper circles were allowed to air dry. Entire filter circles were then placed in the bottom of sterile polystyrene Petri dishes, over which treated chess board filters were placed. Dishes were filled with 80 ml of seawater and three mussel specimens (1.5 – 2.5 cm length) were added. In this way, mussels would have the same area of treated (superior, squared) and control (inferior, entire) filter paper to which to attach. Ten replicates of each treatment (control, *H. gibbesii*, *B. seaforthii*, *J. rubens* and *D. delicatula* extracts) were used. Experimental dishes were kept in total darkness, as mussels have been shown to produce more byssal threads when held in the dark (DAVIS and MORENO, 1995). Experiments were allowed to run for 12 h. Mussel activities were recorded in the 30 min immediately after the start of the experiment, and then after 12 h. The activities recorded were substratum exploring behavior, eventual gamete release (as an indicator of possible stress or positive cues; although juvenile mussels were not supposed to release gametes, this behavior has been described in controlled conditions by Da Gama et al., 2003) and number of byssal threads attached to each substratum (control or treated filter paper, shell of another mussel or border of Petri dish). After the 12 h period, all records of attachment were checked, mussels were placed in plastic mesh bags tagged according to treatment, and suspended in a sea aquarium for 24 h to check for possible mortality due to exposure to the test substances.

After the trials, filter papers treated with algal extracts were taken from dishes and allowed to air dry. The filter papers were then reextracted, the solvents evaporated and the residue remaining applied to a TLC plate for comparison with the original crude extracts.

Statistical Analysis

The number of attached byssal threads was analysed by a one-way analysis of variance (ANOVA). Normality of distribution and variance homogeneity were tested using Shapiro-Wilk's W and Cochran's tests, respectively, and both assumptions were satisfactorily met. Significant differences (α = 5%) were located a posteriori using Dunnett's test, which compares several treatments with a single control group, thus having a lower type I error rate than other unplanned multiple comparison tests (ZAR, 1999).

RESULTS

The natural concentrations of crude organic extracts employed in antifouling assays were somewhat variable among macroalgae species. The brown seaweed *Dictyopteris delicatula* presented the

highest concentration (0.6% of algae dry mass - DM), followed by the red algae *Bryothamnion seaforthii* (0.3% DM), *Heterosiphonia gibbesii* and *Jania rubens* (both with 0.2% DM).

Juvenile mussels could possibly attach to three different types of substrata within Petri dishes: filter paper (control or treated), inner border of the dish or the shell of another mussel (generally the preferred substrate in this gregarious organism). Differences in attachment preferences within treatments were not considered for the purpose of this work, but differences among treatments were taken into account. The mean numbers (± 95% confidence intervals) of attached byssal threads per treatment are presented in Figure 1. A total of 875 byssal threads were counted in all 5 treatments (control + 4 algal extracts). Mussels attached a mean of 27.6 byssal threads in controls, with a slight, non-significant reduction of attachment (Dunnett's test, $p > 0.05$) in *H. gibbesii* (18.4), and *D. delicatula* (17.8). The extracts of *B. seaforthii* and *J. rubens* significantly inhibited mussel adhesion (12.4 and 11.3, respectively) relative to controls (ANOVA $F_{4,45}$ = 3,6077, p = 0,01; Dunnett's test, $p < 0.01$ in both cases). As a general trend, all extracts inhibited mussel attachment to some degree.

The substrate exploring behavior of the mussels, in which they expose the feet, sense and crawl through the substrata, was recorded immediately after the experiment set up. A mean of 2 individuals per dish (from a total of 3 per dish) exhibited exploring behavior (data not shown) in all treatments.

As shown, among the 4 macroalgal extracts tested as antifoulants, only *B. seaforthii* and *J. rubens* exhibited antifouling activity at the natural concentrations tested.

Extracts were not acutely toxic to mussels, since no mortality was recorded across all treatments during the experiments or in the 24 h following exposure to test compounds (data not shown). Compounds from the extracts were still present after the end of the trials, as shown by TLC comparative analyses.

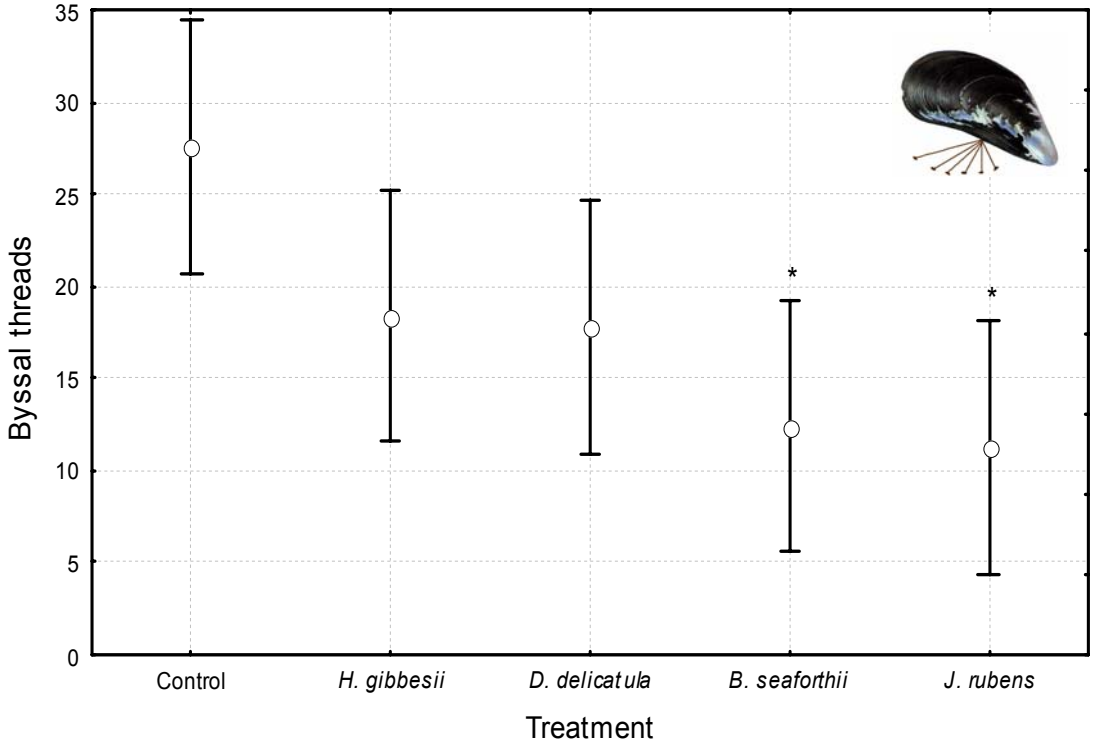

Fig. 1. Mean number of attached byssal threads (± 95% confidence interval) produced by the mussel *Perna perna* in response to four algal extracts and compared to controls. Number of replicates is 10 per treatment. * denotes significant differences compared to the control (Dunnett's test following ANOVA, $p < 0.01$).

DISCUSSION

Through the experiments, it was possible to observe that natural concentrations of the organic extracts from the red algae *Bryothamnion seaforthii* and *Jania rubens* exhibited strong antifouling activity when compared to the control. Such results, expressed by a lower byssal attachment by *Perna perna* juvenile mussels, suggest the existence of a chemical defense mechanism against epibionts in these algae.

Some calcareous red algae (family Corallinaceae) are known to defend themselves against epibionts throughout a physical antifouling mechanism of epithallus sloughing (e.g., LITTLER; LITTLER, 1999; VILLAS-BÔAS; FIGUEIREDO, 2004), however, without the release of chemical compounds with antifouling activity.

The results of the present study confirm, in part, previous observations from other researchers for calcareous algae. The arborescent red alga *Jania rubens* is described as an alga strongly impregnated with calcium carbonate (JOLY, 1967), and its extract has shown strong antifouling activity toward mussels, reinforcing the idea of a chemical defense mechanism in this alga already physically defended. However, it is not possible to infer about a simultaneously physical and chemical antifouling line of defense, since the former mechanism was never tested in this organism.

Such combined defense mechanism could also be found in *Bryothamnion seaforthii*. Also according to the classical observations reported by Joly (1967), *B. seaforthii* has the ability to dissociate its dense external epithallus in the older branches. Although this behavior suggests a physical antifouling mechanism, our study points out to a chemical mechanism against epibionts. During the field work, this alga has shown to be considerably more covered by epibionts than the other species sampled, although the laboratory results show an undeniable antifouling activity. Epibionts were found along the thallus and at the basal portions, where thalli are thicker. More external branching portions, however, were devoid of epibiosis. It seems possible, if not likely, that these portions are younger, more active producers of defensive secondary metabolites than older portions (HAY; STEINBERG, 1992), or even that they allocate defenses only to the external branches as those structures can shelter reproductive traits (JOLY, 1967; PEREIRA, 2002). Studies on secondary metabolite localization within algae are still scarce (HAY; STEINBERG, 1992) and were not the focus of this paper. However, it seems important to emphasize that there are known examples of differences in natural product concentrations within thallus and also when comparing within thallus and surface concentrations (STEINBERG et al., 2001). Compounds found at the thallus surface would be more prone to act as antifoulants (STEINBERG et al., 2001).

The fact that *B. seaforthii* thalli exhibit epibiosis, even though it clearly has antifouling chemical defenses active against *P. perna,* may occur due to the specific activity of chemical defenses in relation to target species, i.e., may be due to a narrow spectrum antifouling activity. This means that some algae seem to possess secondary metabolites that are efficient antifoulants only against some epibiont species: biofilm bacteria, microalgae, macroalgae, or larvae of some specific invertebrate (HAY; FENICAL, 1992; DA GAMA et al., 2002). The mentioned alga seems to lack defenses active against epibiont bryozoans (unidentified species) or other epibiont macroalgae (*Heterosiphonia gibbesii*, *Dasya* sp.) found associated in the field.

Another possible explanation could be that epibiosis induced the production of antifouling defenses in *B. seaforthii* in order to prevent further overgrowth by epibionts. Some algae are known to produce constitutive defenses, i.e., defenses whose concentrations do not vary in function of ecological variables such as herbivory pressure (CRONIN, 2001). On the other hand, other algae produce inducible defenses only when they are needed, e.g., under herbivore attack (e.g., WEIDNER et al., 2004; MACAYA et al., 2005) or, in the context of this paper, under epibiosis pressure. Defense production possibly incurs in metabolic costs (HAY; FENICAL, 1992; VAN ALSTYNE *et al.*, 2001), but these costs can be supplanted by the benefits gained with protection when under attack. The costs of epibiosis for algae are huge and relatively well-documented, and include reduction of photosynthetic rate by overshadow, increasing breakage and even increased consumption (e.g., KAREZ et al*.,* 2000). Thus, it seems reasonable that inducible defenses could be produced in response to epibiosis, but such a mechanism has not yet been described in macroalgae, although similar responses to herbivory are now extensively studied (e.g., HAY; FENICAL, 1992; PAUL et al., 2001; VAN ALSTYNE et al., 2001; PAUL et al., 2006). Further investigation is needed to determine whether the production of chemical defenses in *B. seaforthii* is a consequence of the intensity of epibiosis or a response to other environmental or biotic factors.

The brown alga *Dictyopteris delicatula* did not show a significant fouling inhibition. Some papers have demonstrated the presence, in this alga, of some C_{11} metabolites active as defenses against some herbivores (predatory reef fishes) but not against others (low mobility herbivores or mesoherbivores, such as amphipods) (HAY et al., 1988). The *Dictyopteris* group of brown algae is known to produce sesquiterpenes (TEIXEIRA, 2002), a class of natural products that has sometimes been described as

having antifouling activity (MASUDA et al., 1997; DA GAMA, 2001; RITTSCHOF, 2001). However, in our study this activity was not observed for individuals of this genus.

Extracts of the red alga *Heterosiphonia gibbesii* also did not reveal a significant antifouling activity. The thalli of this alga are extremely soft, what may provide an inappropriate substrate for fouling settlement, thus acting as a physical defense and then making the use of chemical defenses not useful. Such fact reinforces the hypothesis that species that do not suffer environmental pressure from stressful factors - such as the presence of biofouling or epibiosis – would not be selected for the production of antifouling chemical defenses. Other explanations, however, cannot be excluded without further investigation.

The absence of mortality after exposure to all algal extracts reinforces the idea that seaweed natural products may, in the future, really provide an effective (in some cases) and more environmentally friendly alternative to currently used metal-based antifouling agents (DA GAMA et al., 2002). Further studies must deal with the purification of the active extracts in order to obtain the antifouling compounds, and then test them in a more ecologically realistic situation, *i.e*., field experiments.

ACKNOWLEDGEMENTS

This work was part of a larger research effort seeking to identify algae with antifouling activity, supported by grants or fellowships from CNPq, CAPES and FAPERJ. The authors wish to thank E.A. Xavier, T.F. Porto and C.L. Teixeira for their help during laboratory work and R. Villaça and Y.Yoneshigue-Valentin for algal identification. B.A.P. da Gama thanks CNPq for his Research Productivity fellowship.

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(Manuscript received 09 June 2006; revised 16 April 2007; accepted 30 August 2007)