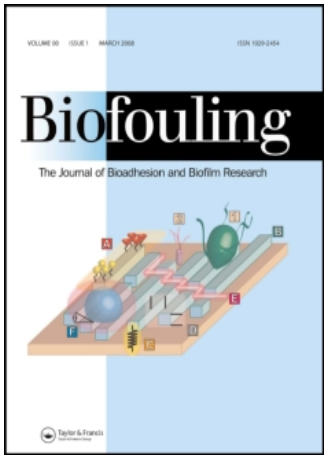

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Tannin and tannate from the quebracho tree: an eco-friendly alternative for controlling marine biofouling

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Abstract
Current antifouling coatings are based on toxic compounds that can be harmful to the natural environment. A promising alternative to these compounds is the use of natural products that are non-toxic, but have antifouling properties. Tannins are natural, water-soluble, complex polyphenolic substances, which precipitate proteins and have anticorrosive and antimicrobial properties. In this study, the effect of quebracho tannin as a probable antifouling pigment in both laboratory and field trials is evaluated. As tannins have high solubility in aqueous media and consequently would leach rapidly, they were precipitated as aluminium tannate, which has an adequate solubility for use as a component in marine paints. *In vitro* exposure of *Balanus amphitrite* and *Polydora ligni* larvae to low concentrations of both quebracho tannin and saturated aluminium tannate solutions produced complete appendage immobilisation. In 28-d field trials of test gels, a significant decrease in micro- and macrofouling density and diversity in relation to the control gel was detected ($p < 0.05$). This study suggests that natural tannins could be employed as bioactive pigment for new antifouling technologies.

Keywords: *Quebracho tannin, aluminium tannate, non-toxic antifouling*

Introduction

Marine biological fouling can be defined as the undesirable accumulation of micro- and macroorganisms on artificial surfaces immersed in seawater. Biofouling is a severe problem for the shipping industry. Biological fouling on vessels leads to an increase in weight, subsequent speed reduction and loss of manoeuvrability and as a consequence, higher fuel consumption is needed (Lewis, 1994; Callow & Callow, 2002).

Traditionally, biofouling can be prevented by means of antifouling (AF) paints containing one or more toxic compounds, such as copper and organotin derivatives in a paint matrix (Fusetani, 2004; Konstantinou & Albanis, 2004; Yebra et al. 2004). When submerged, AF paints release compounds at levels that can cause adverse environmental effects, such as imposex in oysters, and death of dolphins, porpoises and whales (Simmonds, 1986; Ponasik et al. 1998; Brady, 2000). Environmental and human health problems are associated with metal complexes that are used as bioactive pigments (Gibbs, 1993; Voulvoulis et al. 1999). The ban on harmful substances in AF paints requires the development of new AF strategies and it is clear that alternatives should be as effective as conventional paints, but of lower toxicity and friendly to the environment.

In nature, some organisms may be heavily fouled on their surfaces while others can be totally fouling-free. The surfaces of sessile benthic marine algae are particularly susceptible to fouling because they are restricted to the photic zone where conditions for fouling growth are optimal (de Nys et al. 1995). However, some algae are rarely epiphytised, indicating potential AF mechanisms (Hellio et al. 2000; 2001; Dobretsov & Qian, 2002). It has been observed that natural compounds from marine organisms and terrestrial plants could be very promising AF agents (Fusetani et al. 1996; Dworjanyn et al. 1999; Rittschof, 2000; Hellio et al. 2004; Yang et al. 2006). This has generated interest in identifying the mechanisms by which fouling organisms are repelled or inhibited. These mechanisms may involve dissolution of adhesives by enzymatic action, interference in metabolic processes of the organisms, inhibition of attachment, growth or metamorphosis, or modification of organism surfaces with repellents or biocides

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(Abarzua & Jakubowski, 1995; Steinberg et al. 1998; Rittschof, 2000; Yebra et al. 2004).

Tannins are natural compounds common in higher plants and brown algae. They are usually defined as water-soluble polyphenolic substances that have high molecular mass (>500) and possess the ability to precipitate proteins, such as gelatin, from solution (astringency). Tannins are also important in industry and environmental sciences (Ho, 1992; Chung et al. 1998; Hagerman, 2002). Tannins also have anticarcinogenic properties and protect cells from oxidative damage (Hagerman et al. 1998; De Bruyne et al. 1999; Oszmianski et al. 2007). In the last 50 years, anticorrosive properties of tannins have been observed and, subsequently, a number of tannin-based products appeared in the market and found certain success as pre-treatment primers for rusted steel without requiring complete removal of the corrosion product (Knowles & White, 1958; Matamala et al. 2000). Tannins are widely found in foods, including beverages (red wine, tea, cider, coffee, cocoa and beer), legumes (fava beans, cowpeas and common beans), fruits (bananas, persimmon and apples), cereals (sorghum and barley), and berries (strawberries, blueberries and raspberries) (Bennick, 2002).

Some inhibitory properties of condensed tannins are well known. They are used as antifungal agents, against yeasts and bacteria (Zucker, 1983; Scalbert, 1991; Digrak et al. 1999) and as wood preservatives when they are copper-complexed (Laks & McKaig, 1988). Despite the antimicrobial properties of tannins, some microbes are resistant and have developed various mechanisms and pathways for tannin degradation in their natural milieu (Deschamps, 1989; Saxena et al. 1995; Bhat et al. 1998). Some moulds develop easily on the surface of tannin-rich woods such as quebracho or European oak. Moulds such as *Aspergillus niger* or *Penicillium glaucum* grow on the surface of the liquid of tannery pits (Rajakumar & Nandy, 1983; Scalbert, 1991). Also, tannins are degraded by white rot fungi (*Ceriporiopsis subvermispora* and *Cyathus stercoreus*) (Gamble et al. 1996).

Little information is available on the activity of condensed tannins on fouling macroorganisms (Ayoub, 1982). In a previous study (Pérez et al. 2006), it was established that quebracho tannin (condensed tannin) combined with a low copper content has an AF performance as good as a conventional paint. AF agents of natural origin incorporated into paints may be less damaging to the environment and may have less activity on non-target organisms (Hellio et al. 2001).

The aim of this study was to evaluate the potential AF properties of a natural compound, quebracho tannin (*Schinopsis* sp. tannin), on fouling organisms in the laboratory and in the field. As quebracho tannin is highly soluble, a less soluble compound, aluminium tannate, also was evaluated. In the laboratory, tests were carried out in order to estimate the effect of quebracho tannin on larvae of *Balanus amphitrite* (Cirripedia, Balanidae) and *Polydora ligni* (Polychaeta, Spionidae). Fifty-inactivity time (It_{50}) or the time required to inhibit 50% of the larval population for a given tannin concentration was determined. In addition, fouling coverage percentage was estimated by exposure of plates of inert gels (Phytagel^{TM}) containing quebracho tannin and aluminium tannate in Mar del Plata harbour (Argentine).

Material and methods

Preparation and characterisation of the bioactive pigment

In the laboratory, stock solution of quebracho tannin (commercial product; Quimica Oeste) was prepared at a concentration of 1 g l^{-1}. Since quebracho tannin is highly soluble, a less soluble compound, aluminium tannate, was also prepared.

Aluminium tannate was precipitated from quebracho tannin solution with aluminium nitrate solution. For this, both solutions were slowly dropped at the same time into a glass beaker with continuously stirring at 60°C. The pH of the resulting pigment suspension was finally adjusted to 4.5–4.7 in order to avoid aluminium oxide precipitation. After that, the precipitated pigment was filtrated using a Büchner funnel, washed three times with distilled water and dried in air at room temperature. The composition of lab-prepared bioactive pigment and its physicochemical features are shown in Table I. The composition of bioactive pigment is presented as percentage by weight due to the non-stoichiometric nature of this compound. The precipitated pigment is a product from the coagulation of natural tannin, which is a complex mixture of phenolic compounds with trivalent aluminium salt. Its composition is highly reproducible, however, if the conditions of preparation are maintained constant. The soluble aluminium tannate concentration (solubility) in artificial seawater was determined by colorimetric

Table I. Pigment characterisation.

Pigment features	Tannate	Aluminium (Al^{3+})
Composition (as % by weight)	94.5	5.5
Aqueous extract* composition (ppm)	0.5	4.0
Aqueous extract pH*	7.28	

*in seawater.

techniques (Snell & Snell, 1941; Pérez et al. 2006). A saturated solution of aluminium tannate was prepared for the laboratory assays described below.

Laboratory assays

Bioassays were carried out using *Balanus amphitrite* and *Polydora ligni* larvae. *B. amphitrite* adults were collected from Club de Motonáutica piers in the harbour of Mar del Plata (38°08′ 17″S, 57°31′ 18″W). *P. ligni* larvae were obtained with a 25 μm zooplankton net at the site, isolated under a stereomicroscope, and fed with cultures of the diatom *Skeletonema costatum*. In the laboratory, all organisms were conditioned in artificial seawater (ASTM, D1141, pH 8.2) at 20 ± 1°C with suitable aeration and natural light. Adult barnacles were fed a daily diet of *Artemia salina* nauplii. Newly released *Balanus* larvae (nauplii I) were transferred to a beaker containing filtered seawater; they molted and became nauplii II approximately 1 h after release. Some nauplii II actively swimming toward a light source were selected for each bioassay, while the remainder were put into a beaker containing seawater and fed with *S. costatum*. In these conditions, 30–35% of the larvae metamorphosed to the cyprid stage and were kept at 4°C.

Thirty nauplii II and twenty cyprids of *B. amphitrite*, and thirty larvae of *P. ligni* (15–16 setigerous, i.e. close to the tube-forming stage) were used for the toxicity assays. Larvae were added using a Pasteur pipette to small crystallising dishes containing a 30 ml of each solution. The two compounds, quebracho tannin (1 g l^{-1}) and aluminium tannate (saturated solution), were assayed at 100, 50, 25, 12.5, and 6.25 v/v% dilutions. Observations were made under a stereomicroscope for 90 min. The inability of *B. amphitrite* nauplii II to stay in the water column and the loss of phototactic reaction were scored as toxic responses. Cyprids were scored as dead if they did not swim or move or close their valves, or if their appendages were extended and they did not respond when touched lightly with a metal probe. The response of *P. ligni* larvae to compounds was estimated by the same parameters as used for nauplii II, i.e. inability to stay in the water column and loss of phototactic reaction.

In all cases, experiments were compared with controls (filtered artificial seawater only). All bioassays were carried out with four replicates of each treatment and a control, and repeated twice with separate batches of larvae.

To study the 'refreshing effect', larvae were removed from the test solutions after the 90-min observation period and placed in vessels with artificial seawater. The refreshing effect was determined by observations of the organisms' recovery of swimming movements and ability to continue their development.

Field trials

In order to establish the AF properties of quebracho tannin and aluminium tannate in seawater, tests were carried out at Club de Motonáutica (Mar del Plata).

Two samples of gel were made by adding 3.2 g of Phytagel^{TM} (Sigma Chemicals) to 100 ml of distilled water and mixing for 5 s. Gel mixtures were heated until boiling and allowed to cool to 45°C. Then, 5 ml of a suspension of 100 g l^{-1} of aluminium tannate (i.e. 0.05 g tannate) were added to one gel sample, and 5 ml of 0.5 g l^{-1} of quebracho tannin (i.e. 0.0025 g tannin) to a second gel sample; both were easily stirred. Each mixture was poured into 10 cm circular plastic moulds. After the gels solidified (1 cm thick, 10 cm diameter), they were removed from the moulds and hung from the marina in the sea. Gels without adding any test compound were used as controls. Gels were hung from the marina at 50 cm below water line for 28 d. Settlement of fouling organisms was measured as percentage cover on each gel using a dot-grid estimate method (Foster et al. 1991). All field tests were carried out in quadruplicate.

Statistical analysis

All statistical analyses were performed with Statistica 6.0. The normality assumption was verified with the Shapiro-Wilk's test (Shapiro & Wilk, 1965). The differences between treatment and control were determined by one-way analysis of variance (ANOVA) followed by LSD *post hoc* test. Differences were considered to be significant at $p < 0.05$.

Results

The present study revealed that quebracho tannin and aluminium tannate, a less soluble compound obtained from quebracho tannin, inhibited *B. amphitrite* and *P. ligni* larval activity in laboratory experiments and reduced fouling coverage in the sea.

Laboratory experiments

When healthy larvae (nauplii II and cyprids) of *B. amphitrite* and *P. ligni* were exposed to either quebracho tannin or saturated aluminium tannate solutions, they fell to the bottom of the vessels so that the number could readily be counted. In this way, the total number of animals that had lost all signs of movement (including that of their appendages) was estimated at suitable time intervals. The percentage values plotted against time gave sigmoid curves, from which the time required for 50% of the test animals

to be quiescent was calculated (Figures 1 and 2). The toxicity of quebracho tannin and aluminium tannate to the larvae covered a broad range, and all concentrations studied showed a larval inhibitory effect, except for cyprids at 6.25 v/v% for both solutions. Loss of activity was not an indication of the death of organisms, because when inactive larvae were transferred to fresh, non-toxic artificial seawater, they were able to recover immediately and continue their development. This observation confirmed that the effect of tannin was not permanent. In contrast, no changes in larval behaviour in control vessels were observed, and cyprids attached to the vessels in 4–7 d.

For all concentrations, the pH of quebracho tannin solutions were similar to normal seawater, i.e. ranging between 7.8 and 8.2. In contrast, the pH for the saturated solution of aluminium tannate was 7.28 (Table I), suggesting that the larvae in the saturated solution were affected not only by tannate anion, but also by the decrease in pH (Pérez et al. 2001). The pH of the diluted aluminum tannate solutions ranged between 7.9 and 8.2.

Field trials

An *in situ* observation at 15 d showed that both the quebracho tannin and aluminium tannate gels had a clear AF effect. At the 15-d observation, the quebracho tannin gels still had their brown-reddish colour. After 28 d, the fouling coverage of the experimental gels initially containing 0.5 g aluminium tannate was significantly lower for both micro- and macrofouling species than those on the control gels ($p < 0.05$). In contrast, some settlement was recorded for gels initially containing 0.0025 g quebracho tannin, probably due to the lower original compound content in the gel matrix and exhaustion due to its extremely high solubility. Almost total loss of the quebracho tannin from the gel was supported by the observation that the originally brown-reddish coloured quebracho tannin gels became colourless after 28 d in the seawater.

Relative to the control gel, there were significant differences in settlement of the diatoms *Amphora*, *Grammatophora*, *Melosira*, *Navicula* and *Pleurosigma* and in the settlement of the macrofoulers *Enteromorpha*, *Polydora*, *Hydroides* and *Ciona* on the gels containing aluminium tannate ($p < 0.05$) (Figures 3 and 4). The results showed that aluminium tannate diffused from gels deterred settlement of the main fouling organisms at the Mar del Plata harbour (Figure 5).

Discussion

The group of vegetable tannins or plant polyphenols are promising natural AF compounds. The present study combined laboratory and field experiments to test whether quebracho tannin and a less soluble salt, aluminium tannate, could suppress marine fouling at Mar del Plata harbour.

Many marine organisms protect the surfaces of their bodies with AF substances without causing

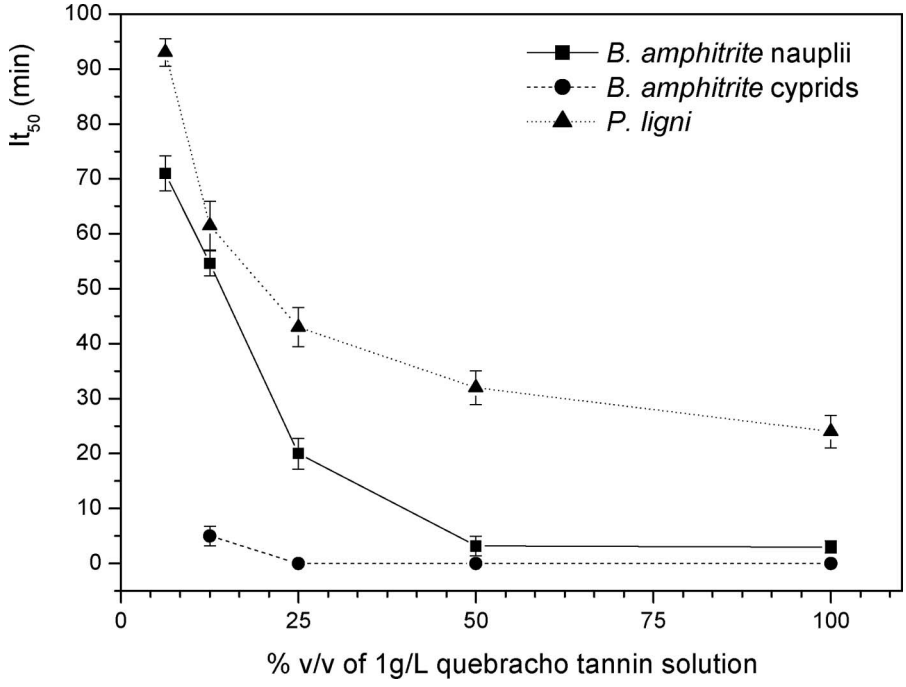

Figure 1. It_{50} curves for *B. amphitrite* and *P. ligni* larvae in dilutions from 1 g l^{-1} quebracho tannin solution. (It_{50} is the time required for 50% of the test organisms to become inactive). Error bars = SE.

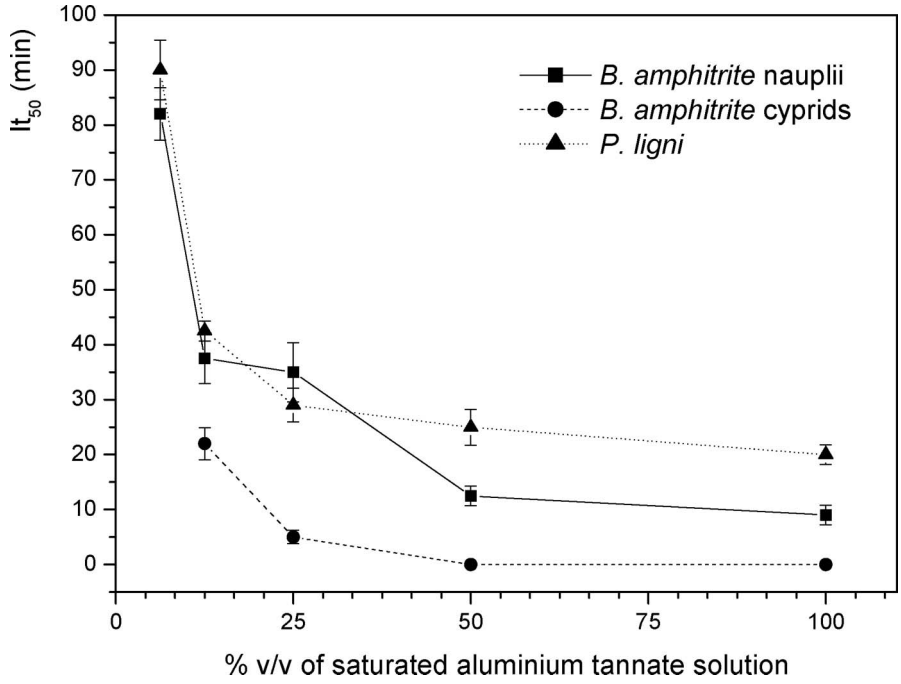

Figure 2. It_{50} curves for *B. amphitrite* and *P. ligni* larvae in dilutions from saturated aluminium tannate solution. Error bars = SE.

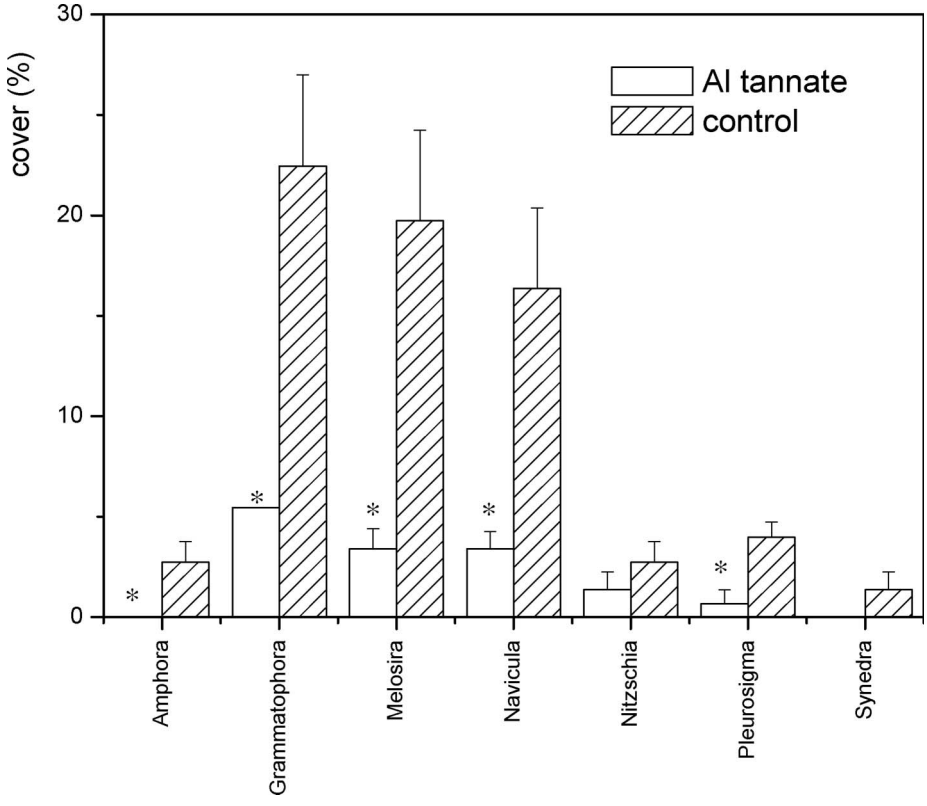

Figure 3. Coverage percentage for microfouling organisms on aluminium tannate gel and control gel. Bars = mean ± SE. *significant difference from controls.

serious environmental problems. Some terrestrial plants also have the similar AF substances. Therefore, these substances may be expected to be utilised as new, environmentally friendly AF agents, especially those having high anesthetic, repellent, settlement deterrent, or settlement inhibitory properties

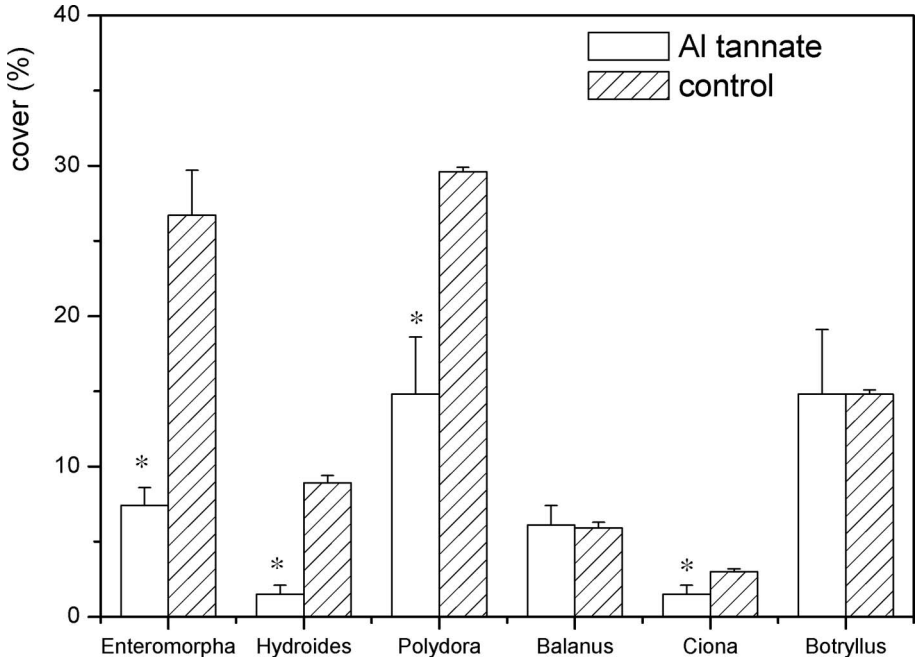

Figure 4. Percentage cover for macrofouling organisms on aluminium tannate gel and control gel. Bars = mean ± SE. *significant difference from controls.

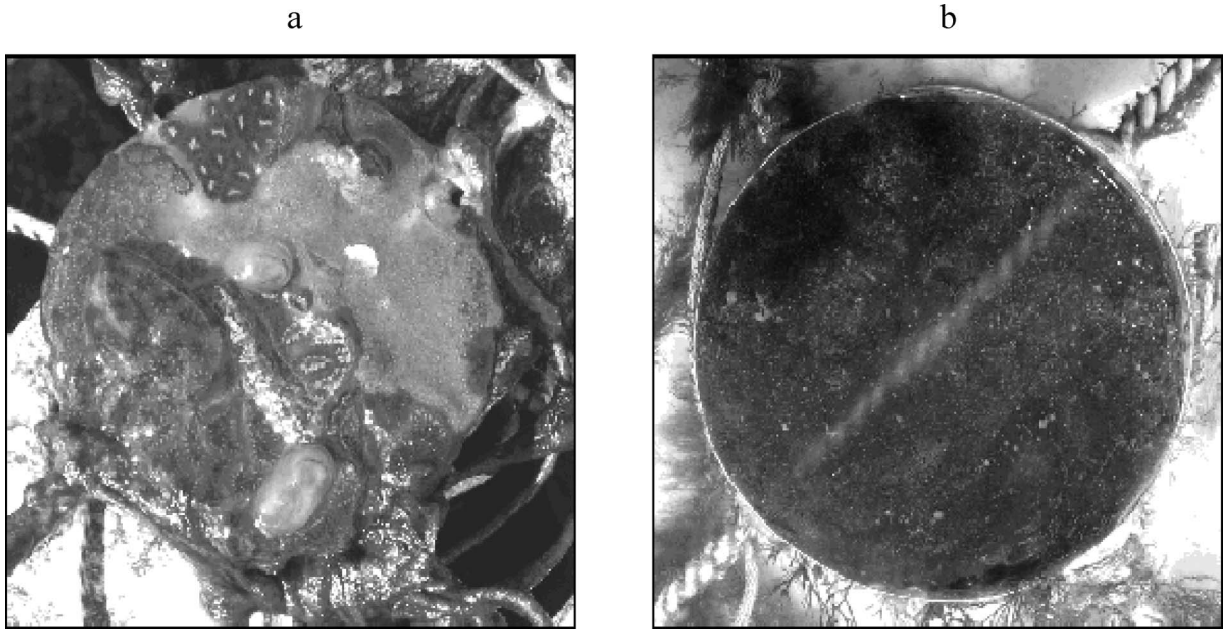

Figure 5. Gels after 28-d exposure in seawater; a = control gel; b = aluminium tannate gel.

without having biocidal properties (Omae, 2006). As a result of the need for environmentally safe AF systems, in the last 20 years, a wide variety of natural compounds from seaweeds and sessile marine organisms have been isolated and identified as settlement inhibitors for *Balanus amphitrite*. For instance, diterpenes from the sea pansy *Renilla reniformis* (Keifer et al. 1986), pukalide from the whip coral *Leptogorgia virgulata* (Gerhart et al. 1988), compounds from sponge species (Sarma et al. 1991; Goto et al. 1993; Thirionet et al. 1998; Hellio et al. 2005; Dobretsov et al. 2005; Nogata & Kitano, 2006), cinnamic acid from *Zostera marina* (Todd et al. 1993), juncellin from the octocoral *Juncella juncea* (Avelin et al. 1993), furanones, isethionic acid and floridoside from red algae (de Nys et al. 1995; 2006; Hellio et al. 2004), phenolic compounds (Lau & Qian, 2000), extracts from bryozoan (Kawamata et al. 2006) and extracts from nudibranchs (Nogata & Kitano, 2006).

The use of phlorotannins to inhibit the growth of a variety of marine organisms is well documented. Phlorotannins exist exclusively in brown algae and are present in considerable amounts (Ragan & Glombitza, 1986). They are polymerised phloroglucinol (1,3,5-trihidroxybenzene), extremely water soluble and enclosed in subcellular structures. Phlorotannins are released continuously from the algae under normal conditions, but an increased rate is observed under stress. It has been shown that phlorotannins released into the surrounding environment are inhibitory to microorganisms and reduce the survival of barnacle, mussel larvae and other algal species (Sieburth & Conover, 1965; Ryland, 1974; Targett & Stochaj, 1994; Lau & Qian, 1997). In contrast, little information is available on inhibitory effect of condensed tannins on settlement of benthic macroorganisms.

This study focused on the antisettlement activity of *Schinopsis* tannin on nauplii and cyprids of *B. amphitrite* and *P. ligni* larvae. In laboratory experiments, it was confirmed that *B. amphitrite* larval activity was strongly affected by exposure to each dilution, while cyprids responded immediately when they were put into solutions and were more sensitive than nauplii for each concentration (Figures 1 and 2). However, after 28 d in the sea, gels treated with aluminium tannate were colonised by a few small barnacles and no significant differences in relation to control were observed (Figure 4). It is hypothesised that the tannate concentration at the gel/seawater interface was insufficient to prevent cyprid settlement, but did affect other foulers such as *Amphora*, *Grammatophora*, *Melosira*, *Navicula* and *Pleurosigma*, *Enteromorpha*, *Polydora*, *Hydroides* and *Ciona* (Figures 3 and 4).

The power of laboratory-based bioassays is the rapid screening of potential compounds for AF toxicity and effectiveness (Rittschof et al. 1992). However, there are some aspects to compare between laboratory and field trials; for instance, organism response in the static conditions of water *vs* flow conditions (Hay et al. 1998) and the use of one or two species for evaluating compound activity in the laboratory *vs* all fouling organisms in the sea (Rittschof, 2001).

Undoubtedly, the identification of an active compound and its properties is just one of the steps before the compound can be incorporated in an AF formulation. A good AF coating probably will need to contain a number of different compounds to ensure that all biofouling organisms are repelled from the surface. The use of quebracho tannin is a very promising and environmentally benign option for AF technology; it also is a cheap natural product. It is recognised that compounds with high solubility, such as quebracho tannin, are a disadvantage for a marine paint. However, tannin precipitation as aluminium tannate solved this problem in the current study because the tannate was less soluble and was as effective as quebracho tannin.

These results suggest that tannins, which are found in many plants, are potentially very important pigments for AF coatings.

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