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Antifouling paints based on marine natural products from Colombian Caribbean

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ABSTRACT

Biofouling control involves the application of paints containing toxic substances to the marine ecosystem. One of the most promising alternative technologies to antifouling paints based on heavy metals is the development of coatings whose active ingredients are compounds naturally occurring in marine organisms. This study investigated the antifouling activity of organic extracts from some epibiont-free Colombian Caribbean Sea sponges (*Agelas tabulata*, *Myrmekioderma gyroderma*, *Oceanapia peltata*, *Aplysina lacunosa*, *Neopetrosia* sp.) and a sea-cucumber (*Holoturia glaberrima*). Extracts were incorporated into hard stable gels and into soluble matrix antifouling paints and exposed in the sea (Colombia and Argentina). After 45 and 90 days, significant differences in fouling cover percentages between painted panels and controls were found ($p < 0.05$). It was demonstrated that the greatest antifouling activity was contained in extracts of *A. tabulata* and *Holoturia glaberrima* in both study sites. This study successfully identified potential new sources of natural antifouling compounds.

1. Introduction

Marine biofouling is an extensive phenomenon causing large problems to engineered structures such as ships and offshore platforms by way of increased use of manpower, fuel, material and dry-docking time. Shipping accounts for approximately 90% of world trade and seaborne trade has quadrupled over the past three decades (ICS and ISF, 2009). The economic costs of hull fouling have been a driving force behind the development of antifouling technologies, a global industry that is now worth approximately US$ 4 billion annually (Wright, 2009).

Antifouling paints have traditionally incorporated toxicants including copper and tributyltin into a matrix that gradually leaches the biocide from the surface layer to prevent settlement. However, these compounds, particularly TBT, were reported to be highly toxic and persistent in the marine environment, and proved to have adverse effects on non-target organisms (Alzieu et al., 1989; Claisse and Alzieu, 1993; Yebra et al., 2004; Limna Mol et al., 2009; Pérez et al., 2009). Because of its toxicity, a worldwide ban on TBT went into effect in 2008.

Increased awareness of the impacts resulting from the use of toxic antifouling paints has prompted investment in the research and development of non-toxic alternatives such as coatings that incorporate natural origin compounds as derivates from algae and other marine organisms (Holmstrom and Kjelleberg, 1994; Kjelleberg and Steinberg, 1994; De Nys et al., 1995; Rittschof, 2000; Hellio et al., 2002, 2009).

In the marine environment, where all surfaces are constantly exposed to colonization, many sessile organisms remain relatively free of biofouling. Marine invertebrates are a potential source of natural, bioactive products that act against external threats (Sipkema et al., 2005; Paul et al., 2006). They are involved in a great variety of interactions, many of which are chemically mediated (Paul et al., 2006; Egan et al., 2008). These compounds often play multiple ecological roles, primarily protection against predators (Pawlik et al., 1995; Waddell and Pawlik, 2000; Burns et al., 2003; Ruzicka and Gleason, 2009), competitors for space (Engel and Pawlik, 2000; Luter and Duckworth, 2010), biofoulers (Becerro et al., 1994) and opportunistic pathogenic microorganisms

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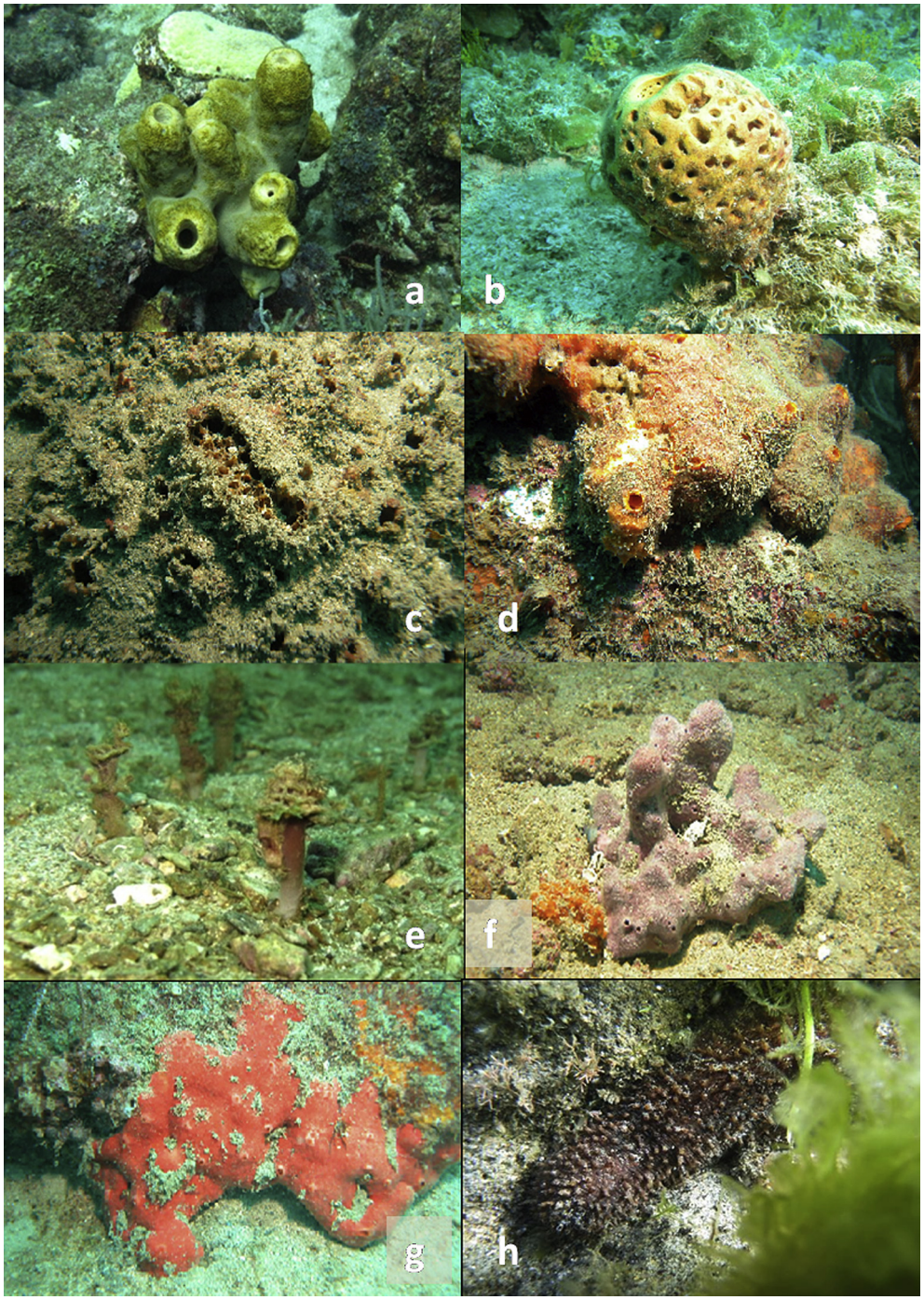

Fig. 1. Species studied. (a) *Agelas tubulata*, (b) *Aplysina lacunosa*, (c) *Biemna cribaria*, (d) *Myrmekioderma gyroderma*, (e) *Oceanapia peltata*, (f) *Neopetrosia proxima*, (g) *Spirastrella coccínea*, (h) *Holothuria glaberrima*.

(Newbold et al., 1999; Kelman et al., 2001). Particularly, secondary metabolites produced by marine sessile organisms represent a new perspective on preventing overgrowth by epibionts and could potentially be used as commercial antifoulants (Willemsen and Ferrari, 1993).

Sponges, with their rich chemical defense mechanisms, are among the most studied organisms for the isolation of natural products antifoulants (NPAs) (Thakur and Anil, 2000). Marine sponges have received much attention because they represent a source of unique and diverse secondary metabolites with novel structures and potential biological activities (Becerro et al., 1997; Thacker et al., 1998; Sera et al., 1999; Schupp et al., 1999; Faulkner, 2000, 2002; Matsunaga et al., 2000; Harper et al., 2001; Blunt et al., 2003; Dobretsov et al., 2005; Stowe et al., 2011). Previous studies have shown that sponges are rich in terpenoids and steroids which can function in antipredation, competition for space and control of epibionts (Becerro et al., 2003; Hellio et al., 2005; Clavico et al., 2006; Tsoukatou et al., 2007).

In contrast, echinoderms are one of the groups less explored in relation with NPAs. Only a few investigations of antifouling compounds have been examined with starfish, basket stars and sea urchins (Bryan et al., 1996; De Marino et al., 1997; Haug et al., 2002).

However, sea cucumbers are a large and diverse group of organisms from which a wide range of secondary metabolites have been isolated. A number of these compounds possess biological activity such as toxicity, antibacterial, antifungal, antiviral, anti-tumor and other specific activities (Bryan et al., 1992; Villasin and Pomory, 2000; Haug et al., 2002; Han et al., 2009).

The properties established for both, sponges and sea cucumbers, and particularly their antimicrobial activity, support the hypothesis that these groups could be potential candidates to obtain antifouling compounds. The inhibition of the organism settlement process could be achieved by breaking one or more of the steps implicated in biofouling development (Hellio et al., 2001). Additionally, bacterial biofilms can play an important role in mediating settlement and metamorphosis of larvae, e.g. they can enhance or inhibit larval and algal spore attachment.

Most of the experiments conducted to evaluate antifouling activity of a vast range of substances have been laboratory-based using different larval stages, and also field tests employing gels containing extracts (Henrikson and Pawlik, 1995, 1998). However, few of these substances have incorporated into marine paints for testing.

In this paper, the antifouling properties of seven sponge extracts *Agelas tubulata* (Schmidt, 1870), *Aplysina lacunosa* (Lamarck, 1814), *Biemna cribaria* (Alcolado and Gotera, 1986), *Myrmekioderma gyroderma* (Alcolado, 1984), *Neopetrosia proxima* (Duchassaing and Michelotti, 1864), *Oceanapia peltata* (Schmidt, 1870) and *Spirastrella coccinea*, and a sea cucumber, *Holothuria glaberrima* (Selenka, 1867), from the Caribbean Colombian Sea included into an inert gel (Phytagel™) and into a paint matrix were examined. Effectiveness of extracts was evaluated in two different harbous, Santa Marta (Colombia) and Mar del Plata (Argentina).

2. Material and methods

2.1. Extraction procedure

Extracts were obtained from hand-collected marine organisms by Scuba diving in the Colombian coast from Santa Marta to Neguanje (Tayrona National Park, 11°19′52″N–74°05′28″W). Seven marine sponge species (*A. tubulata*, *Aplysina lacunosa*, *Biemna cribaria*, *Mymekioderma gyroderma*, *Neopetrosia proxima*, *Oceanapia peltata* and *Spirastrella coccinea*), and a sea cucumber (*H. glaberrima*) were selected for the experiments (Fig. 1).

Organism surfaces were free of macroepibionts and had no evidence of depredation. For the experiments, tissues were rinsed to remove debris and cut in small pieces, and wet weights were determined. Sponges and holothurians were stored at −15 °C until processed. Then, samples were lyophilized (36–48 h/−45 °C/0.120 mbar) in order to eliminate water from tissues and dry weights recorded.

Dry tissues were macerated in a mixture methanol/dichloromethane (1:1) at 20 °C during 24 h and continuous stirring (120 rpm) to obtain a broad polarity range of metabolites (Castellanos, 2007). After filtration, tissues were extracted twice more. Solutions obtained from each filtration were concentrated using a rotary evaporator with a heating bath at 38 °C and controlled pressure to remove remaining solvents. Extracts were once more lyophilized up to complete dryness and were labeled as 'total extract'. In addition, natural concentration was determined as total mass of metabolites after extraction process per unit volume of organism (Chaves, 2003; Newmark et al., 2005).

2.2. Phytagel assays

The method employed for immobilization of extracts into gels was adapted from that described by Henrikson and Pawlik (1998) and Newmark et al. (2005). Gels were prepared by adding Phytagel® (Sigma Chemical) into a beaker containing deionized water up to complete dissolution; then, they were placed on a hot plate with a magnetic stirrer. When gels reached 75 °C, extracts were added at twice natural concentration to detect the activity of all metabolites including those at low quantities.

The gel/extract mixture was poured into polystyrene Petri dishes (9 cm diameter) containing a nylon mesh to reinforce structural integrity and to prevent detachment of gel. Likewise, gels without adding any compound were used as controls. Petri dishes were attached to acrylic plates which were placed on PVC rectangular structures (80 × 160 cm) and submerged at Punta de Betín station, Santa Marta (Colombia) for 28 days. Results from gels were used as a screening test in order to detect those extracts with antifouling activity.

Tests were carried out in triplicate. Settlement of fouling organisms was estimated as percentage cover on each gel using a dot-grid estimate method. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was applied to determine significant differences between treatments and control; the significant level was set at $p < 0.05$.

2.3. Soluble matrix paints

Soluble matrix antifouling paint was prepared by dissolution of colophony (resin) and oleic acid (plasticizer) in the solvent using a high-speed disperser. Then, a laboratory scale ball mill was loaded with this mixture ('vehicle') and pigments, and dispersed for 24 h. The composition of this paint, expressed as volume percentage, was as follow: 27.0% colophony, 6.0% oleic acid, 20.0% xylene, 20% white spirit, 16.2% zinc oxide and 10.8% calcium carbonate.

For each organism, the total extract was fractionated and two phases were obtained, aqueous and organic one. Due to compatibility criteria between solvents used in paint formulation and solvent mixture used to fractionate the extracts, only the organic phase was incorporated into the paint. In contrast, metabolites from aqueous phase could not be dispersed into base paint and were not included in the experiments. In particular, only extracts for which antifouling activity was observed in gels were employed to prepare antifouling paints.

Then, paint was filtered and fractionated in seven portions, one of which was used as a negative control (P1). For the remaining ones each organic extract (1% w/w) was added (P2: *A. tubulata*; P3: *Myrmekioderma gyroderma*; P4: *H. glaberrima*; P5: *Oceanapia peltata*; P6: *Aplysina lacunose*; P7: *Neopetrosia proxima*). Finally, paints were dispersed during 1 h.

Sandblasted acrylic tiles (4 × 12 cm) were used for field trials. Paints were applied by brush on tiles previously degreased with toluene. Four coats of paint were applied and allowed to dry for 24 h between each application, resulting in a final dry thickness of 75±5 μm. Coated panels were hung in a marina in Mar del Plata harbor (Argentina) (38°02′30″S–57°32′00″W) and in Santa Marta (Colombia) (11°14′50″N–74°12′06″W). Additionally, unpainted acrylic tiles were simultaneously submerged.

All tests were performed in triplicate. Abundance percentages for each species of fouling settled on panels were estimated with a grid after 45 and 90 days exposure in the sea. Coverage data refers to percentage of surface colonized by fouling organisms on the side of the panels exposed to light.

2.4. Statistical analysis

Differences in organism settlement between the experimental treatments and controls were determined by one-way ANOVA followed by a post-hoc Tukey test. Data for the fouling cover on the control and Phytagels/antifouling paints containing extracts were

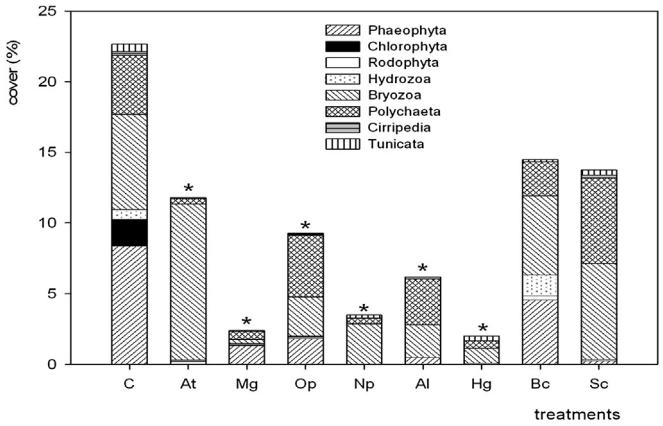

Fig. 2. Fouling percentage cover on gel discs containing organic extracts. *At: Agelas tubulata*; *Mg: Myrmekioderma gyroderma*; *Op: Oceanapia peltata*; *Np: Neopetrosia próxima*; *Al: Aplysina lacunosa*; *Hg: Holothuria glaberrima*; *Bc: Biemna cribaria*; *Sc: Spirastrella coccinea*; C: control. (*) significant differences, $p < 0.05$.

checked for normality using Shapiro–Wilk's test and for homogeneity of variance using Levene test. Differences were considered to be significant at $p < 0.05$.

Percentage cover data from Santa Marta were normalized by using arcsin-square-root transformation.

3. Results

3.1. Phytagel assays

After 28 days in the sea, there were significant differences in mean percentage cover among treatments ($p < 0.05$). A pairwise comparison revealed that gels containing organic extracts of *Myrmekioderma gyroderma*, *Oceanapia peltata*, *Neopetrosia proxima*, *Aplysina lacunosa*, *A. tubulata* and *H. glaberrima* had less fouling settlement than controls. In contrast, gels containing extract of the sponges *Biemna cribaria* and *Spirastrella coccinea* showed similar amount of fouling to that of control. The most common settlers on gels were phaeophyte algae and some invertebrates like barnacles, tube worms and bryozoans (Fig. 2).

Results obtained from gels allowed selecting organic extracts with antifouling properties in order to be included in paints.

3.2. Field trials

Fouling communities on hard substrates in Santa Marta and Mar del Plata are different in relation to density and diversity of organisms. In Santa Marta harbor the community is dominated by serpulid polychaetes, barnacles and sponges, and in lower abundance encrusting bryozoans and colonial ascidians species all year round (García and Salzwedel, 1995). The groups of fouling organisms recorded in Mar del Plata on control panels were characterized by algae (principally chlorophyta, phaeophyta and rodophyta), tube worms (mainly *Hydroides elegans*), bryozoans (mainly *Bugula* sp.), barnacles (*Balanus amphitrite*), solitary and colonial ascidians (*Ciona intestinalis* and *Botryllus* sp., respectively) with seasonal or annual cycles of recruitment depending on species (Pezzani et al., 1996). During the experiments seawater temperature ranged between 21.2 °C (February) and 28.5 °C (December) in Santa Marta and 14.3 °C (December) and 24.7 (January) in Mar del Plata.

Panels submerged in the sea showed significant differences in both study sites.

3.3. Field studies after 45 days exposure

In Santa Marta, results from statistical analysis between control paints vs. treatments revealed that extracts of sponges *Agelas* and *Aplysina*, and the extract of the sea cucumber *Holothuria* inhibited settlement of barnacles ($p < 0.05$) (Fig. 3a).

Similarly, these extracts showed antifouling activity in Mar del Plata harbor due to settlement inhibition of *Enteromorpha intestinalis*, *Ectocarpus* sp., *Bugula*, *Corophium* and *Hydroides elegans* (Fig. 3b). Unfortunately, antifouling activity on barnacle species could not be confirmed because larvae were not available in the plankton during the experiment.

In contrast, different performances were detected in paints containing *Oceanapia* and *Neopetrosia* extracts in relation to latitude. These extracts were effective in inhibiting the settlement of *E. intestinalis*, *Ectocarpus* sp., *Bugula*, *Corophium* and *H. elegans* in Mar del Plata harbor but had no antifouling properties in Santa Marta. On the other hand, similar performance was obtained for paints containing *Myrmekioderma* extract, they had no antifouling properties in both study sites.

3.4. Field studies after 90 days exposure

In Santa Marta harbor, paints containing extracts of *Agelas* and *Holothuria* maintained antifouling properties during the experiment. In contrast, paints formulated with *Aplysina* extract reduced effectiveness and panels were invaded by barnacles and tube worms, mainly (Fig. 4a and Fig. 5a).

On the other hand, all of paints that showed antifouling properties after 45 days exposure in Mar del Plata harbor maintained their performance after 90 days. In spite of this, it is important to

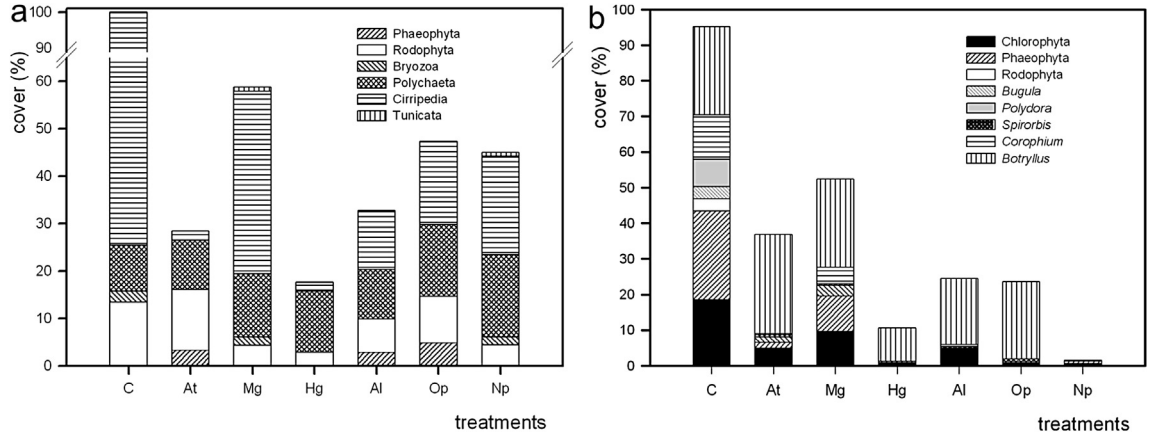

Fig. 3. Fouling percentage cover on painted panels vs. control, 45 days exposure (a) Santa Marta, (b) Mar del Plata. References as in Fig. 2.

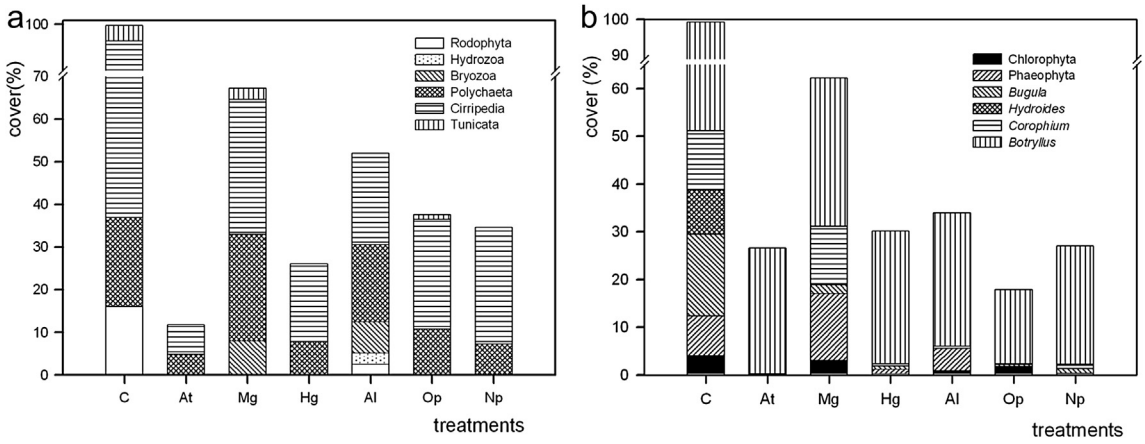

Fig. 4. Fouling percentage cover on painted panels vs. control, 90 days exposure (a) Santa Marta, (b) Mar del Plata. References as in Fig. 2.

remark that neither of paints could inhibit colonial ascidians attachment (Fig. 4b and Fig. 5b).

A particular case was observed for paints containing *Agelas* extract in both study sites. These series of paints showed lesser amount of fouling cover for 90 days than 45 days. It was considered that *Agelas* extract interfered in some way in organism cementation to substrate.

It is important to note that after 90 days in the sea, the paint films kept their integrity and had no signs of deterioration (i.e. adhesion failure, cracking or wrinkling).

4. Discussion

The widespread use of toxic biocides in antifouling paints has introduced high levels of contamination into the environment and raised concerns about their toxic effects on marine communities.

Natural product antifoulants are one of the most promising alternatives to the toxic and non-biodegradable used antifouling agents. Secondary metabolites from marine invertebrates have diverse ecological roles and may be used in antipredation, antifouling and spatial competition. It was established that secondary metabolites may deter predators (Pawlik, 1993; Chanas and Pawlik, 1995; Pawlik et al., 1995), prevent fouling organisms and pathogens (Uriz et al., 1992; Becerro et al., 1994), and may aid organisms in competition for space via allelopathic effects (Becerro et al., 1994; Engel and Pawlik, 2000). Additionally, a single secondary metabolite may also act in a range of roles, and hence serve multiple ecological functions (Uriz et al., 1992; Becerro et al., 1994).

The present research focused in the antifouling activity of five sponges and one holothurian extracts included in marine paints.

Sponge extracts account for almost 50% of the reported natural products (Harper et al., 2001). Particularly, some studies have shown that sponge extracts that display high antimicrobial activities are also efficient antifouling agents (McCaffrey and Endean, 1985; Uriz et al., 1992). For example, the guanidine alkaloids responsible for the toxicity of the sponge *Crambe crambe* had antimicrobial, antifouling, antipredation and spatial competition properties (Becerro et al., 1997). Other marine sponges such as *Oceanapia fistulosa* and some species of *Haliclona* (*Haliclona exigua* and *H. cribricutis*) exhibited considerable activity against both bacterial strains as well as barnacle larvae (Limna Mol et al., 2010). In contrast, these experiments demonstrated that crude extract of *Oceanapia peltata* had no effect on barnacle settlement in Santa Marta.

It is well known that some species of *Aplysina* produce a diverse array of secondary metabolites exhibiting cytotoxic and antimicrobial activities and a brominated alkaloid located in ectosome may be responsible of antifouling effect (Gopichand and Schmitz, 1979; Goo, 1980; McMillan et al., 1981; Thompson et al., 1985; Muricy et al., 1993; Teeyapant and Proksch, 1993; Ziefer et al., 1995; Ebel et al., 1997; Fendert et al., 1999; Newbold et al., 1999; Sacristán-Soriano et al., 2012). The present experiments showed the potentiality of crude extract of *Aplysina lacunosa* as antifoulant depending on geographic location. In Santa Marta the extract did not inhibit the establishment of fouling and this is in accordance with results obtained for Brazilian coasts (Pereira et al., 2002). However, *Aplysina* extract inhibited the attachment of most of fouling community at Mar del Plata harbor.

The performance of the extract of the sponge *A. tubulata* showed promising antisettlement activity in both localities. This is in accordance with earlier studies which demonstrated that some secondary metabolites such as brominated alkaloids and bromopyrrol derivatives of certain species from the genus *Agelas* affected biofilm and barnacle attachment (Tsukamoto et al., 1996; Sjögren et al., 2008; Hertiani et al., 2010).

Bioactive compounds with antimicrobial activity have been identified in *Neopetrosia proxima*, principally halogenated fatty acids and sterols (Minh et al., 2007). Additionally, polyacetylenes compounds were found in extracts of sponges of this genus; these metabolites showed a range of biological activities which include antimicrobial, cytotoxic, antitumor, antiviral, immunosuppressant, and enzyme inhibition (Ankisetty and Slattery, 2012). Ecological significance of these compounds includes preventing fouling by barnacle larvae (Tsukamoto et al., 1997). In these experiments the action of this extract was dependent (as *Aplysina lacunosa*) on geographic location, i.e., it showed antifouling effect only for Mar del Plata; in this harbor *N. proxima* extract inhibited the settlement of tube-worm *Hydroides elegans*.

The extract of the sponge *Myrmekioderma gyroderma* had no antifouling effect and panels were colonized abundantly from the beginning of the experiments in both sites. In spite of this result, metabolites isolated from the sponge *M. dendy* has been reported with antifouling activity at laboratory scale (Tsukamoto et al., 1997).

Echinoderms in general produce secondary cerebroside and saponin metabolites to deter many infections, parasites and predators. Sea cucumbers saponins are triterpenoid glycosides mostly based on the 'holostane' skeleton with cytotoxic, antimicrobial and antifungal activity (Jayasree et al., 1991; Minh et al., 2007; Muniain et al., 2008; Careaga et al., 2011; Mokhlesi et al., 2012; Omran and Allam, 2012). In the literature, a controversy has been found in relation to the antifouling activity of sea cucumber extracts. According to Dobretsov et al. (2009) *Holothuria atra* and *H. edulis*

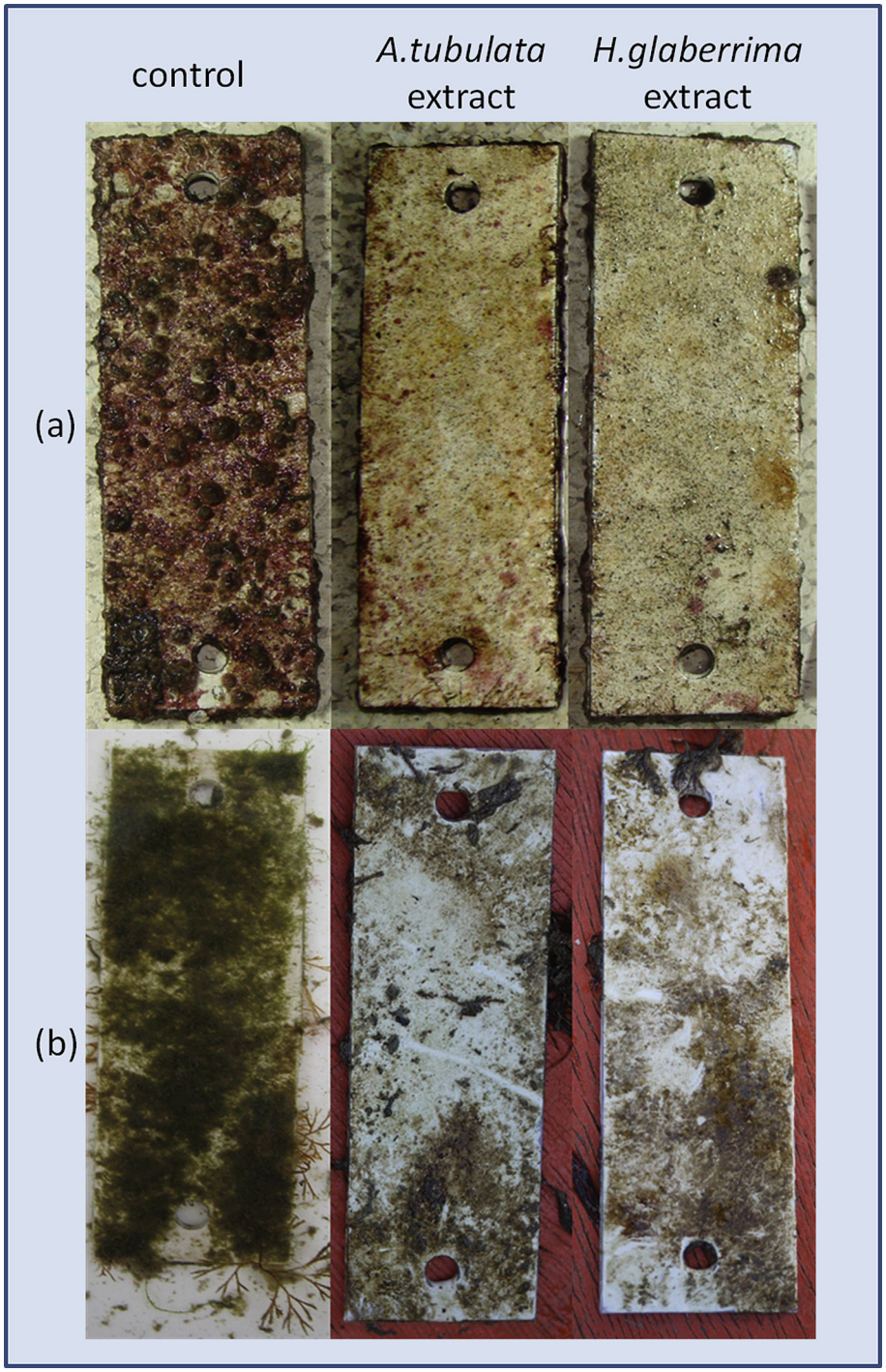

Fig. 5. Painted panels after 90 days exposure. (a) Santa Marta, (b) Mar del Plata.

extracts had no effect on microfouling settlement. However, some authors reported that methanol extract of *Holothuria leucospilota* effectively prevented the growth of biofilm forming marine diatoms (Mokashe et al., 1994; Gonsalves, 1997; Selvin and Lipton, 2004). In the present experiments the extract of *H. glaberrima* significantly reduced the attachment of fouling species including barnacle larvae. This extract was an effective deterrent for mostly fouling organism in both harbors and might form a rich source for developing potent novel antifoulants. In addition, complementary field assays confirmed that the extract of *H. glaberrima* was effective for twelve months.

Microorganisms associated with their invertebrate hosts have been described as a source for bioactive metabolites. Particularly, it was shown that some bacteria which are associated with the organism's surface are able to produce diverse compounds and the determination of ecological roles of bioactive metabolites is complicated by the nature of the symbiotic relationships (Kalinovaskaya et al., 1995; Thakur and Anil, 2000). In the present study, the evaluation of antifouling activity was based on the organic fraction of the crude extract having complex mixtures of primary and secondary metabolites. Consequently, the settlement inhibitory effect could be produced either by metabolites exuded

by associated bacteria, compounds from host tissues itself or a combination of all these compounds. As a consequence, further bioassay-guided research is needed to identify those molecules responsible of antifouling activity.

5. Conclusions

After immersion in Santa Marta (Colombia) and Mar del Plata (Argentina) all extracts included in paints showed antifouling activity except for *M. gyroderma*. The organic fraction of the extracts of *A. tubulata* and *H. glaberrima* were the most effective in both study sites and inhibited settlement of relevant species of local fouling community. For marine antifouling research, bioactive substances of particular interest should be ones that show deterrence properties and can be used for the development of antifouling coatings. In spite of the great number of antifouling compounds identified in laboratory research, only a few of these substances have incorporated into marine paints for testing. A combined approach of laboratory and field assays would be useful in understanding how natural substances affect fouling attachment. In this sense, this work allowed to obtain efficient paint formulations at laboratory scale and evaluate and evaluate their antifouling activity under natural conditions. Natural products rarely are available in sufficient quantity to be commercially harvested from marine macroorganisms. However, it is expected that the identification of new antifouling products from marine invertebrates provide new insights into potential structural modifications of other abundant substances that could produce efficient bioactive compounds in a sustainable way.

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