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Antifouling activity against barnacle cypris larvae: Do target species matter (*Amphibalanus amphitrite* versus *Semibalanus balanoides*)?

Jean-Philippe Maréchal [a], Claire Hellio [b,*]

[a] *Observatoire du Milieu Marin Martiniquais, 3 Avenue Condorcet, 97200 Fort de France, Martinique, French West Indies*
[b] *School of Biological Sciences, University of Portsmouth, King Henry Building, Portsmouth PO1 2DY, UK*

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ABSTRACT

Larvae of many benthic invertebrates settle on surfaces where they metamorphose into juveniles if suitable substrata are available, and are responsible for the major costs of biofouling. When assessing new formulations or compounds for potential antifouling (AF) application, constraints such as seasonal availability may restrict most bioassays to relatively few taxa and species. For example, amongst barnacles, *Amphibalanus amphitrite* is popular as a test organism but is it really representative of other barnacle species? In order to test this hypothesis, we have chosen to work with marine natural extracts as a probe. Indeed, one substitution technology to toxic metal-based coatings to control fouling is the development of AF coatings with active compounds derived from marine organisms or analogues of the lead compounds. In this study, the AF activity and toxicity of extracts from 30 algae from the North East Atlantic coast were investigated for their potential anti-settlement activities against larvae of two species of barnacle, *A. amphitrite* and *Semibalanus balanoides*. As a trend, most of the active extracts displayed activity towards *S. balanoides*, only few displayed targeted activity against *A. amphitrite*, or against both species. In order to better understand if this tendency could be linked to chemical ecology, surface extracts were prepared on a selection of species. The results highlight that surface extracts of algae all displayed highest levels of activity than total extracts when tested on *S. balanoides*. This difference illustrates that specific compounds in their ecological context can have potentially a better efficacy on target species.

1. Introduction

The control of marine biofouling on artificial structures is essential to maintain operational effectiveness and to minimise associated costs (Armstrong et al. 2000; Rittschof 2000; Yebra et al. 2004). Toxicant-based coating systems can provide effective fouling control, but those based on organotins are now banned from being applied to submarine structures due to environmental concerns and regulations (Maguire 2000; Appel 2004; Giacomazzi and Cochet 2004; Yebra et al. 2004). Thus, there are now opportunities to introduce new, efficient and non-toxic substitutes to limit the most severe fouling organisms such as algae, barnacles, tubeworms, mussels and bryozoans (Dahlström et al. 2000). Even if field experiments are the best assays to assess the performance of marine paints formulations in real conditions (Henrikson and Pawlik 1995), they have the disadvantage to require larger quantity of the test compound(s) and to be conducted over a longer time scale (Da Gama et al. 2008; Dhams and Hellio 2009). However, a recent study has demonstrated that results from laboratory assays did not fully concur with the AF activity of the paints in the field trial (Bressy et al. 2010). In most cases, because of the lack of field data, the reliability and the validity of in-vitro bioassays cannot be critically discussed (Bressy et al. 2010). Thus, the screening process always starts with lab-based experiments, which has the advantage of requiring low amounts of compounds but with the limitation that the complexity of the natural environment is not replicated. An ideal bioassay should be ecologically relevant, quick, independent of season, reliable, require a small amount of the test compound and able to be performed in any laboratory (Dhams and Hellio 2009). As such, barnacles represent an excellent biological model to assess AF efficacy of marine natural products (MNPs). Barnacles are among the most successful fouling animals (Koryakova and Korn 1993) and have a unique degree of adaptation to the sessile life. They are found attached to all kind of hard surfaces and are better equipped than other animals to colonise immersed artificial structures. Barnacles are problematic as fouling organisms because their firm attachment and heavy calcification can make them difficult and expensive to remove. The prolific settlement of

* Corresponding author. Tel.: +44 2392 84 2073.
E-mail address: claire.hellio@port.ac.uk (C. Hellio).

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barnacle larvae and their global distribution has made them the most common fouling marine invertebrate in the world (Clare and Alred 2009; Hayward et al. 1995; Hills et al. 1999; Knight-Jones and Crisp 1953). Traditionally, the warm-water barnacle species, *Amphibalanus amphitrite* (previously named *Balanus amphitrite*, Clare and Hoeg 2008), has been used extensively for AF activity screening. This success can be explained by the fact that this species is easy to culture under laboratory conditions and reproduction can be manipulated so that larvae are available throughout the year for toxicity and settlement tests. *A. amphitrite* is a common, broadly distributed coastal and estuarine biofouling organism and is now a dominant fouling organism found in warm and temperate waters worldwide (Clare and Alred 2009). Individuals can survive water temperatures as low as 12 °C, but will not breed in water colder than 15–18 °C (Anil and Kurian 1996). Bishop (1950) reported that low temperature reproductive limits defined the northern most extent of *A. amphitrite* distribution in England, while Vaas (1978) noted that it survives in colder waters in Britain and the Netherlands at sites bathed in heated power plant effluent. It was demonstrated that embryonic development of *A. amphitrite* is accelerated by temperature increase (Anil et al. 1995; Anil and Kurian 1996). Our study aimed at exploring the relevance of species choices for the screening of MNPs as potential AF agents. We focused on two species of barnacle: *A. amphitrite* and *Semibalanus balanoides* (a keystone species, which is widespread in the Northern hemisphere but has not found favour as an AF test species in large part due to its short reproductive season). *S. balanoides* is borea-arctic species with its northern limits being defined by the extent of summer peak ice, whilst it is not found south of northwest Spain due to increasing water temperatures preventing final maturation of gametes (Fish and Fish 1996). It occurs on shores at all levels of wave exposure and is typically found from the upper to the lower eulittoral zones. Larval release is synchronised with the spring diatom bloom to ensure larvae grow and develop under optimum conditions (Salman 1982).

One approach for the research on new non-toxic AF is to exploit the inherent capabilities of particular marine organisms to synthesize chemical defence compounds to maintain an epibiont-free surface (Armstrong et al. 2000). Indeed, many fixed and soft-bodied marine organisms produce molecules involved in deterring potential predators, warding off pathogens, keeping their living space free from competitors and/or reducing the impact of exposure to environmental stresses (Clare 1996; Fusetani 1997; Fusetani 2004; Wahl 2008; Hellio et al. 2009). So far, numerous compounds and extracts, obtained from cold-water organisms have been assessed for their AF activities using mostly *A. amphitrite* as target organisms, but most of the studies highlighted low or absence of bioactivity. Within this work, we aimed at testing the potential difference in the susceptibility of the two barnacle species to AF substances, using algal extracts as a probe. Our hypothesis was that the lack of potency of some MNPs and extracts could be partially explained by the fact that *A. amphitrite* is not ecologically relevant to cold waters. In order to test this hypothesis, extracts of 30 marine algae from the North East Atlantic coast (France) were tested for their *in vitro* anti-settlement activity against the cyprids of these two barnacle species while toxicity was assayed using their naupliar stages.

2. Material and methods

2.1. Preparation of the algal extracts

Specimens of thirty species of marine algae were collected in spring from the North Atlantic coast of France (Concarneau Bay, Brittany, 47°52 N–3°55 W): (1) *Ulva intestinalis* (Linnaeus) (Ulvophyceae, Ulvales, Ulvaceae), (2) *U. Lactuca* (Linnaeus) (Ulvophyceae, Ulvales, Ulvaceae), (3) *Cladophora rupestris* (Linnaeus) Kützing (Ulvophyceae, Cladophorales, Cladophoraceae), (4) *Ascophyllum nodosum* (Linnaeus) Le Jolis (Phaeophyceae, Fucales, Fucaceae), (5) *Fucus serratus* (Linnaeus) (Phaeophyceae, Fucales, Fucaceae), (6) *F. Spiralis* (Linnaeus) (Phaeophyceae, Fucales, Fucaceae), (7) *F. Vesiculosus* (Linnaeus) (Phaeophyceae, Fucales, Fucaceae), (8) *Himanthalia elongata* (Linnaeus) Gray (Phaeophyceae, Fucales, Himanthaliaceae), (9) *Pelvetia canaliculata* (Linnaeus) Decaisne & Thuret (Phaeophyceae, Fucales, Fucaceae), (10) *Sargassum muticum* (Yendo) Fensholt (Phaeophyceae, Fucales, Sargassaceae), (11) *Ectocarpus siliculosus* (Dillwyn) Lyngbye (Phaeophyceae, Ectocarpales, Ectocarpaceae), (12) *Alaria esculenta* (Linnaeus) Greville (Phaeophyceae, Laminariales, Alariaceae), (13) *Chorda filum* (Linnaeus) Stackhouse (Phaeophyceae, Laminariales, Chordaceae), (14) *Laminaria digitata* (Hudson) Lamouroux (Phaeophyceae, Laminariales, Laminariaceae), (15) *L. Ochroleuca* Bachelot de la Pylaie (Phaeophyceae, Laminariales, Laminariaceae), (16) *Saccorhiza polyschides* (Lightfoot) Batters (Phaeophyceae, Tilopteridales, Phyllariaceae), (17) *Chrondus crispus* Stackhouse (Florideophyceae, Gigartinales, Gigartinaceae), (18) *Gigartina stellata* (Stackhouse) Batters (Florideophyceae, Gigartinales, Gigartinaceae), (19) *Gelidium latifolium* Bornet ex Hauck (Florideophyceae, Gelidiales, Gelidiaceae), (20) *Palmaria palmata* (Linnaeus) Weber & Mohr (Florideophyceae, Palmariales, Palmariaceae), (21) *Dilsea carnosa* (Schmidel) Kuntze (Florideophyceae, Gigartinales, Dumontiaceae), (22) *Bornetia secundiflora* (J. Agardh) Thuret (Florideophyceae, Ceramiales, Ceramiaceae), (23) *Ceramium virgatum* Roth (Florideophyceae, Ceramiales, Ceramiaceae), (24) *Cryptopleura ramosa* (Hudson) Newton (Florideophyceae, Ceramiales, Delesseriaceae), (25) *Delesseria sanguinea* (Hudson) Lamouroux (Florideophyceae, Ceramiales, Delesseriaceae), (26) *Dasya hutchinsia* Harvey (Florideophyceae, Ceramiales, Dasyaceae), (27) *Halurus equisetifolius* (Lightfoot) Kützing (Florideophyceae, Ceramiales, Wrangeliaceae), (28) *Osmundea pinnatifida* (Hudson) Stackhouse (Florideophyceae, Ceramiales, Rhodomelaceae), (29) *Plumaria plumosa* (Hudson) Kuntz (Florideophyceae, Ceramiales, Wrangeliaceae), and (30) *Polysiphonia lanosa* (Linnaeus) Tandy (Florideophyceae, Ceramiales, Rhodomelaceae).

After collection, the samples were washed in sterile filtered seawater (22 μm) to remove associated debris and large epiphytes. A 10 min 5% ethanol wash was performed to clean the surface from microflora (Hellio et al. 2000). The cleaned material was then air dried in the shade at 30 °C for 24 h. Aqueous (A), ethanol (B) and dichloromethane (C) extracts were prepared as previously described (Hellio et al. 2000). All three phases were stored at −80 °C before their use in settlement and toxicity assays.

A selection of ten species was submitted to surface extraction using the hexane dipping method following the protocol previously published by De Nys et al. (1998). Thus specimens of *U. intestinalis* (1), *A. nodosum* (4), *F. serratus* (5), *S. muticum* (10), *L. ochroleuca* (15), *P. palmata* (20), *B. secundiflora* (22), *D. sanguinea* (25), *P. elegans* (29) and *P. lanosa* (30) were soaked into hexane following the ratio 1 L hexane/1 kg wet weight algae.

2.2. Preparation of multi-wells plates for bioassays

Extracts were assayed at concentrations of 0 (control), 0.5, 1, 5, 10, 25, 50 and 100 μg ml^{-1} (Hellio et al. 2005), with 6 replicates of each concentration. Methanol was used as a carrier solvent for the aqueous, ethanol and dichloromethane. Extracts in methanol were then added to the wells of 24-well plates (Iwaki), and then evaporated to dryness at room temperature. Two ml of filtered seawater (0.45 μm) were the added to each well. Controls consisted of well containing dried methanol and filtered seawater.