International Biodeterioration & Biodegradation xxx (2010) 1–10

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

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

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ARTICLE INFO

Article history:
Received 30 July 2010
Received in revised form
3 October 2010
Accepted 3 October 2010
Available online xxx

Keywords:
Algae
Antifouling
Amphibalanus amphitrite
Chemical ecology
Extracts
Semibalanus balanoides
Settlement
Surface
Toxicity

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

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doi:10.1016/j.ibiod.2010.10.002

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 north-west 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.

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A slightly different protocol was used for the hexane extracts. They were added in the wells of glass 96-well plates and tested for anti-settlement activities (only) at the same concentration than above (from 0.5 to 100 μg ml^{-1}). Controls consisted of dried hexane and filtered seawater.

2.3. Organisms used for anti-settlement assays

2.3.1. A. amphitrite

Adult barnacles, settled on PVC pipes, were collected at the Duke University Marine Laboratory, North Carolina (courtesy of Dr D Rittschof) and were shipped to Newcastle University (UK). Adults were maintained at 22 °C and fed on a daily diet of Artemia sp. nauplii (7 nauplii ml^{-1}) (Hellio et al. 2004a). Release and culture of nauplii were performed as previously described (Hellio et al. 2004b). After 4 days, when most of the nauplii had metamorphosed, cyprids were collected by filtration (250 μm). *A. amphitrite* cyprids were allowed to age at 6 °C for 1 to 5 days, before being used in settlement and discriminatory assays (Maréchal et al. 2004b). Low temperature (6 °C) storage is a method used routinely to age cyprids (Rittschof et al. 1984) as they become inactive and do not settle at this temperature.

2.3.2. S. balanoides

Cyprids were collected in spring (May) from the Menai Strait (North Wales, UK) using planktonic mesh. After collection, cyprids settlement was prevented by containing them within Nitex plankton netting (150 μm) which is an unfavourable surface for settlement. Cyprids were kept at 6 °C and then sampled everyday from day 1 to 5 for settlement and discriminatory experiments.

For all the settlement experiments, the temperature of incubation was respectively 14 °C for *S. balanoides* and 28 °C for *A. amphitrite* in order to mimic the environmental conditions.

2.4. Validation of the method for bioassays

2.4.1. Size of the cyprids

Cyprids of both species were measured and the average size was calculated.

2.4.2. Settlement abilities of the cyprids

A. amphitrite: Ten cyprids were added to each well of Iwaki microplates (24 wells). Settlement was enumerated after incubation for 24 h in the dark at 28 °C. The physical state of each larva was examined under a dissecting microscope. Cyprids with extended thoracopods that did not move and did not respond after a light touch by a metal probe were regarded as dead (Rittschof et al. 1992; Lau and Qian 2000). Permanently attached and metamorphosed individuals were counted as settled. All others were counted as swimmers.

S. balanoides: the assays and data analysis were carried out as described for *A. amphitrite*, except that five cyprids were added per well in order to have a biomass comparable to the one used for the bioassays with *A. amphitrite* and that the temperature of incubation was 14 °C.

2.4.3. Discriminatory abilities of the cyprids

A nitrocellulose membrane (9 × 13 cm) (M-tech Diagnostics, Ltd, UK) was freshly prepared by soaking in 50 mM tris–HCl, pH 7.5 for 5 min and sandwiched in a 24-well dot-blot apparatus following the method described by Matsumura et al. (1998). Diluted *A. amphitrite* (0.18 mg protein ml^{-1}) and *S. balanoides* (0.18 mg protein ml^{-1}) adult extracts and buffer (used as a non-treated control) were added in a random design to the wells of the vacuum manifold and aspirated. This resulted in 24 concave depressions (10 mm diameter) in the membrane; 8 with *A. amphitrite* adult extract adsorbed, 8 with *S. balanoides* adult extract and 8 non-treated buffer controls. The membrane was partially dried at 4 °C and fixed to the bottom of a new polypropylene container.

A. amphitrite: Two hundred *A. amphitrite* cyprids were then added to the container in 150 ml artificial seawater (ASW) and incubated for 24 h in the dark. Settlement was recorded following the incubation. A 'Selection Index' based on settlement-area preference was calculated as follows: Conspecific index = (settlement on *A. amphitrite* extracts/total settlement) × 100; Allospecific index = (settlement on *S. balanoides* extracts/total settlement) × 100.

S. balanoides: One hundred *S. balanoides* cyprids were then added to the container in 150 ml ASW and incubated for 24 h in the dark. Settlement was recorded following the incubation. A 'Selection Index' based on settlement-area preference was calculated as follows: Conspecific index = (settlement on *S. balanoides* extracts/total settlement) × 100; Allospecific index = (settlement on *A. amphitrite* extracts/total settlement) × 100.

2.5. Anti-settlement assays with the extracts to test

2.5.1. A. amphitrite

Cyprids were allowed to age at 6 °C for 3 days, before being used in settlement assays. Ten cyprids were added to each well of the prepared Iwaki microplates (24 wells) and two cyprids were added when 96 well plates were used. Settlement (at 28 °C) was enumerated as explained above. For each extract, an EC_{50} value (concentration of extract which results in a 50% inhibition of settlement compared with the seawater control) was calculated using SigmaPlot (Tsoukatou et al. 2007).

2.5.2. S. balanoides

The assays and data analysis were carried out as described for *A. amphitrite* except that 5 cyprids were added per well for 24 wells-plates and 1 cyprid per well for 96 well-plates and that the plates were incubated at 14 °C.

2.6. Toxicity tests

2.6.1. A. amphitrite

Toxicity tests were conducted on nauplii of *A. amphitrite* according to Wu et al. (1997). Extracts were tested at the same concentrations used for the settlement assays, with 6 replicates of each treatment and control (Hellio et al. 2004b, Maréchal et al. 2004a). Fifteen stage-II nauplii were added to each well of a 24-well (Iwaki) plate containg 2 ml FSW and extracts. The number of swimming and dead nauplii was recorded after 24 h incubation at 28 °C. Non-swimming larvae were regarded as dead (Rittschof et al. 1992). Toxicity results are presented as 24 h LC_{50} with 95% confidence intervals (Hellio et al. 2005).

2.6.2. S. balanoides

Nauplii were collected in summer in Concarneau Bay (France). The assays were carried out as described for *A. amphitrite* except that seven nauplii were added per well, the plates were incubated at 14 °C and mortality was enumerated after 24 h. The LC_{50} was determined as above and the data are expressed as a 24 h LC_{50} with a 95% confidence interval.

2.7. Statistical analysis

Percentage settlement values were arcsine transformed prior to statistical analyses (Maréchal et al. 2004a). As the assay data were not normally distributed (Bartlett's test), a non-parametric test (Kruskall–Wallis) was used for all data. Post hoc comparison of

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treatment means employed Dunn's test. The level of significance was set at $\alpha < 0.05$. The analysis was performed using Instat.

Data (EC_{50} and LC_{50}) were re-coded for hierarchical analysis and clustering (Table 1). Cluster analysis methods were applied to investigate the relationships between the species of algae sampled and their activities towards settlement and toxicity of *A. amphitrite* and *S. balanoides* larvae. In this study, Euclidean distances with Ward's clustering method were used: average, complete, single, Ward's and weighted linkage type (Jain et al. 1999; Xu and Hagler 2002). Then, the linkage type similarity within each cluster was calculated and plotted. The analysis was performed using © Minitab.

3. Results

3.1. Validation of the experimental methods

3.1.1. Size of the cyprids

Measurements of cypris of *A. amphitrite* and *S. balanoides* were conducted on several batches of larvae, as a result we found that usually *S. balanoides* cyprids were on average body length about 1 mm and *A. amphitrite* about 0.5 mm (data not shown). Based on these data, for all the subsequent experiments, we used twice more *A. amphitrite* larvae than *S. balanoides*.

3.1.2. Settlement and discrimination abilities of the cyprids

Settlement and discrimination abilities of both species of barnacles were compared and results are presented in Fig. 1. Cypris of *A. amphitrite* showed no settlement at day 1. Settlement started from day 2 only with 8.9% of the larvae attached after 24 h. Settlement rate then increased concomitantly with age and reached value of 25.3% for day 3 larvae and 31.1% for day 5 larvae. When measuring conspecific index, values were very high for day 2 and day 3 cypris (with values >95%) and then decreased significantly for day 4 and day 5 cyprids. At the opposite, the allospecific index did increase with the age of the cyprids, with very low values for day 2 and day 3 cypris (<2%) reaching 20.1 and 29.8% respectively for day 4 and day 5 cypris.

Cypris of *S. balanoides* showed high level of settlement at all the ages tested, with 49.8% settlement at day 1, 75.1% at day 2 and 100% for day 3 to day 5. Conspecific index was high (>95%) for day 1 larvae, then decreased to 49% for day 2 larvae and 38% for day 3%, to then stabilise at 33% for day 4 and day 5 cypris.

From these results, we decided to run the screening of algal extracts using day 3 *A. amphitrite* and day 1 *S. balanoides* larvae as they both are competent with high level of discrimination towards substrate.

3.2. Hierarchical analysis of the screening data

3.2.1. Anti-settlement activities of the aqueous, ethanol and dichloromethane algal extracts towards A. amphitrite and S. balanoides

The anti-settlement activities of the 90 algal extracts against *A. amphitrite* and *S. balanoides* are presented in Tables 2a and 2b respectively. After re-coding (Tables 2a and 2b), data were analysed through multivariate statistical tools. A cluster analysis was used using ward linkage and Euclidian distance and is presented Fig. 2. It was possible to classify the extracts into 4 groups:

Table 1
Data code used for hierarchical analysis and clustering.

Anti-settlement activity − EC_{50}	Data code	Toxicity − LC_{50}	Data code
0–20 μg ml^{-1}	1	>100 μg ml^{-1}	1
20–40 μg ml^{-1}	2	80–100 μg ml^{-1}	2
40–60 μg ml^{-1}	3	60–80 μg ml^{-1}	3
60–80 μg ml^{-1}	4	40–60 μg ml^{-1}	4
80–100 μg ml^{-1}	5	20–40 μg ml^{-1}	5
>100 μg ml^{-1}	6	0–20 μg ml^{-1}	6

- Group A comprised 34 extracts, which were active against settlement of *S. balanoides* but not *A. amphitrite*; Extracts classified within this group were *U. intestinalis* (1A,B [1, 2]), *C. rupestris* (3B, [8]), *A. nodosum* (4A, [10]), *F. serratus* (5A,B, [13, 14]), *F. vesiculosus* (7B,C [20, 21]), *H. elongata* (8A [22]), *E. siliculosus* (11A [31]), *C. filum* (13A,B,C [37, 38, 39]), *S. polyschides* (16A,B,C [46, 47, 48]), *G. latifolium* (19A,C [55, 57]), *P. palmata* (20A [58]), *B. secundiflora* (22A,B,C [64, 65, 66]), *C. virgatum* (23B,C [68, 69]), *C. ramosa* (24A,C [70, 72]), *D. sanguinea* (25A,B,C [73, 74, 75]), *D. hutchinsia* (26A,C, [76, 78], *P. elegans* (29B,C, [86, 87]) and *P. lanosa* (30A, [88]).
- Group B comprised 4 extracts with anti-settlement activity towards *A. amphitrite* but not *S. balanoides*; these extracts were fractions of *A. nodosum* (4B [11]), *S. muticum* (10B [29]), *L. ochroleuca* (15B, C [44, 45]).
- Group C comprised 6 extracts which were active against the settlement of both species; Extracts classified within this group were *A. nodosum* (4C [12]), *C. crispus* (17B [50]), *P. palmata* (20C [60]), *O. pinnatifida* (28B [83]), *P. lanosa* (30B, C [89-90]).
- Group D comprised 46 extracts, which were inactive. Extracts classified within this group were *U. intestinalis* (1C [3]), *U. lactuca* (2A,B,C [4, 5, 6]), *C. rupestris* (3A,C [7, 9]), *F. serratus* (5C [15]), *F. spiralis* (6A,B,C [16, 17, 18]), *F. vesiculosus* (7A, [19]), *H. elongata* (8B,C [23, 24]), *P. canaliculata* (9A,B,C [25, 26, 27]), *S. muticum* (10A,C [28, 30]), *E. siliculosus* (11B,C [32, 33]), *A. esculenta* (12A,B,C [34, 35, 36]), *L. digitata* (14A,B,C [40, 41, 42]), *L. ochroleuca* (15A [43]), *C. crispus* (17A,C [49, 51]), *G. stellata* (18A,B,C [52, 53, 54]), *G. latifolium* (19B [56]), *P. palmata* (20B [59]), *D. carnosa* (21A,B,C [61, 62, 63]), *C. virgatum* (23A [67]), *C. ramosa* [24B (71)], *D. hutchinsia* (26B [77]), *H. equisetifolius* (27A,B,C [79, 80, 81]), *O. pinnatifida* (28A,C [82, 84]), *P. elegans* (29A [85]).

Figs. 3 and 4 summarise this analysis. Among the 90 extracts investigated for their potential anti-settlement activities, 37.7% belonged to group A; 4.5% to group B; 6.7% to group C; and 51.1% to group D (Fig. 3). It is of interest to note that aqueous extracts are not present in groups B and C (Fig. 4) and that none of them were active against *A. amphitrite* settlement.

3.2.2. Anti-settlement activities and toxicity of the extracts from groups A, B and C

For the second part of the multivariate analysis, we omitted inactive extracts and included the toxicity data (the toxicity data are presented in Tables 3a and 3b respectively for *A. amphitrite* and *S. balanoides*). Detailed clustering was produced using ward linkage and Euclidian distance and is presented in Figs. 5 and 6.

- Fig. 5 represents clusters obtained from the 34 extracts of Group A (active specifically towards *S. balanoides*). From the analysis, Group A can be divided into five sub-groups:
  - Sub-group A1: 10 extracts, namely *U. intestinalis* (1A,B [1, 2]), *F. vesiculosus* (7C [8]), *E. siliculosus* (11A [10]), *C. filum* (13B [12]), *B. secundiflora* (22A [20], *D. sanguinea* (25A,B [27, 28]), *D. hutchinsia* (26C [31]) and *P. lanosa* (30A [34]). These extracts are active against *S. balanoides* but could not be used industrially as they displayed EC_{50s} and LC_{50s} values, which are in the same range (40–60 μg ml^{-1}).
  - Sub-group A2: 2 extracts, *S. polyschides* (16B [15]) and *D. sanguinea* (25C [29]). These extracts cannot be used for AF

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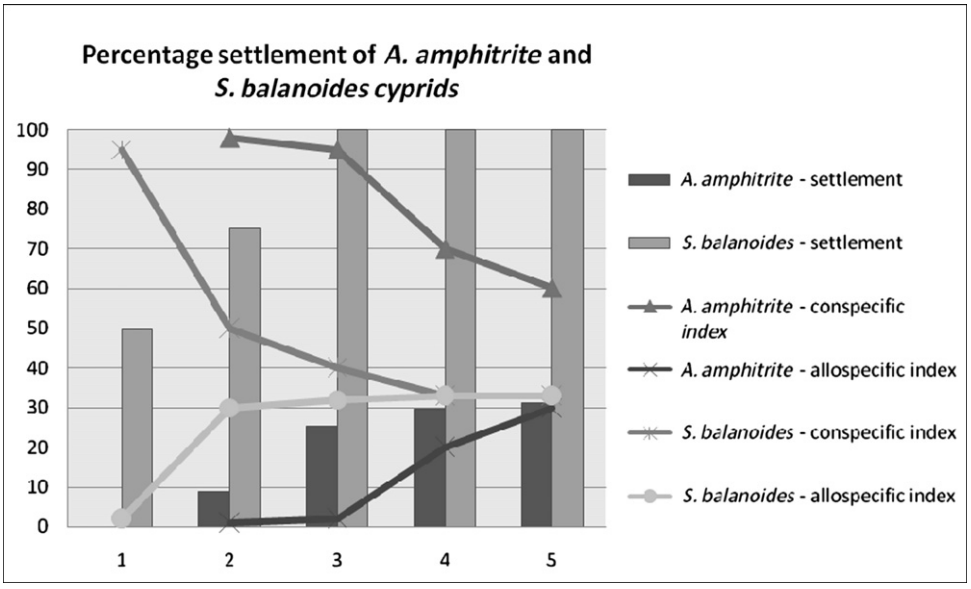

Fig. 1. Settlement and discriminatory ability of day 1 to day 5 cyprids of *A. amphitrite* and *S. balanoides.*

formulations as they display $EC_{50s} > LC_{50s}$ and the levels of their AF activities are not in the highest range (40–60 μg ml^{-1}).

- ○ Sub-group A3: 5 extracts *H. elongata* (8A [9]), *S. polyschides* (16C [16]), *G. latifolium* (19C [18], *B. secundiflora* (22B [21]) and *P. elegans* (29C [33]). They all displayed $EC_{50s} < LC_{50s}$, but with a quite low range of anti-settlement activities (40–60 μg ml^{-1}).
- ○ Sub-group A4: 9 extracts *C. rupestris* (3B [3]), *F. serratus* (5A, B [5, 6]), *C. filum* (13A [11]), *G. latifolium* (19A [17]), *P. palmata* (20A [19]), *B. secundiflora* (22C [22]), *C. virgatum* (23B,C [23, 24]). All the extracts from this cluster showed $EC_{50s} < LC_{50s}$ with very high levels of anti-settlement activities (in the range 0–20 μg ml^{-1}) concomitant with very low levels of toxicity (80–100 μg ml^{-1}). These extracts are the most interesting for a potential industrial use.
- ○ Sub-group A5: 8 extracts are grouped within this cluster, *A. nodosum* (4A [4]), *F. vesiculosus* (7B [7]), *C. filum* (13C [13]), *S. polyschides* (16A [14[), *C. ramosa* (24A,C [25, 26[), *D. hutchinsia* (26A [30]) and *P. elegans* (29B [32]). All these extracts displayed $EC_{50s} < LC_{50s}$ with very high levels of anti-settlement activities (within the range 0–20 μg ml^{-1}) but with slightly levels of toxicity than the extracts from Sub-group A6 (within the range 60–80 μg ml^{-1}).

From this analysis, we can conclude that the best extracts for inhibition of *S. balanoides* settlement are those from sub-groups A4 and A5.

- ✔ All the extracts from Group B displayed $EC_{50s} < LC_{50s}$ towards *A. amphitrite* larvae. The most active extracts from this group are

Table 2a
Effects of the algal extracts on the settlement of cypris larvae of *A. amphitrite*. Results are expressed as EC_{50} values (in μg ml^{-1}). Data code used for hierarchical analysis and clustering is provided.

	Aqueous extract		Ethanolic extract		Dichloromethane extract	
	EC_{50}	Data code	EC_{50}	Data code	EC_{50}	Data code
(3) *C. rupestris*	>100	6	>100	6	98.1	5
(4) *A. nodosum*	>100	6	25.2	2	9.2	1
(9) *P. canaliculata*	>100	6	>100	6	96.2	5
(10) *S. muticum*	>100	6	25.8	2	97.1	5
(15) *L. ochroleuca*	>100	6	24.2	2	49.3	3
(17) *C. crispus*	>100	6	49.0	3	98.3	5
(20) *P. palmata*	>100	6	99.8	5	49.1	3
(21) *D. carnosa*	>100	6	97.1	5	99.0	5
(24) *C. ramosa*	>100	6	>100	6	98.7	5
(28) *O. pinnatifida*	>100	6	49.2	3	>100	6
(30) *P. lanosa*	>100	6	24.3	2	49.1	3

Extracts of (1) *U. intestinalis*, (2) *U. lactuca*, (6) *F. spiralis*, (5) *F. serratus*, (7) *F. vesiculosus*, (8) *H. elongata*, (11) *E. siliculosus*, (12) *A. esculenta*, (13) *C. filum*, (14) *L. digitata*, (16) *S. polyschides*, (18) *G. stellata*, (19) *G. latifolium*, (22) *B. secundiflora*, (23) *C. virgatum*, (25) *D. sanguinea.* (26) *D. hutchinsia*, (27) *H. equisetifolius*, (29) *P. plumosa* were not active ($EC_{50} > 100$ μg ml^{-1}, data code: 6) (data not shown).

Table 2b
Effects of the algal extracts on the settlement of cypris larvae of *S. balanoides*. Results are expressed as EC_{50} values (in μg ml^{-1}).

	Aqueous extract		Ethanolic extract		Dichloromethane extract	
	EC_{50}	Data code	EC_{50}	Data code	EC_{50}	Data code
(1) *U. intestinalis*	48.3	3	48.1	3	>100	6
(3) *C. rupestris*	>100	6	8.8	1	>100	6
(4) *A. nodosum*	8.1	1	>100	6	7.1	1
(5) *F. serratus*	9.3	1	8.7	1	>100	6
(7) *F. vesiculosus*	>100	6	9.2	1	49.3	3
(8) *H. elongata*	49.1	3	>100	6	95.5	5
(10) *S. muticum*	>100	6	95.5	5	>100	6
(11) *E. siliculosus*	48.8	3	96.3	5	97.4	5
(13) *C. filum*	10.0	1	48.0	3	8.3	1
(16) *S. polyschides*	9.4	1	49.2	3	47.8	3
(17) *C. crispus*	>100	6	49.7	3	>100	6
(19) *G. latifolium*	8.1	1	>100	6	49.0	3
(20) *P. palmata*	9.47	1	>100	6	49.4	3
(22) *B. secundiflora*	48.1	3	48.2	3	8.2	1
(23) *C. virgatumum*	>100	6	9.3	1	9.4	1
(24) *C. ramosa*	8.8	1	>100	6	8.7	1
(25) *D. sanguinea*	47.5	3	48.1	3	47.3	3
(26) *D. hutchinsia*	9.1	1	96.6	5	48.5	3
(28) *O. pinnatifida*	>100	6	49.7	3	>100	6
(29) *P. plumosa*	>100	6	8.8	1	49.1	3
(30) *P. lanosa*	49.3	3	49.2	3	8.7	1

(2) *U. lactuca*, (6) *F. spiralis*, (9) *P. canaliculata*, (12) *A. esculenta*, (14) *L. digitata*, (15) *L. ochroleuca*, (18) *G. stellata*, (21) *D. carnosa*, (27) *H. equisetifolius* were not active ($EC_{50} > 100$ μg ml^{-1}, data code: 6) (data not shown).

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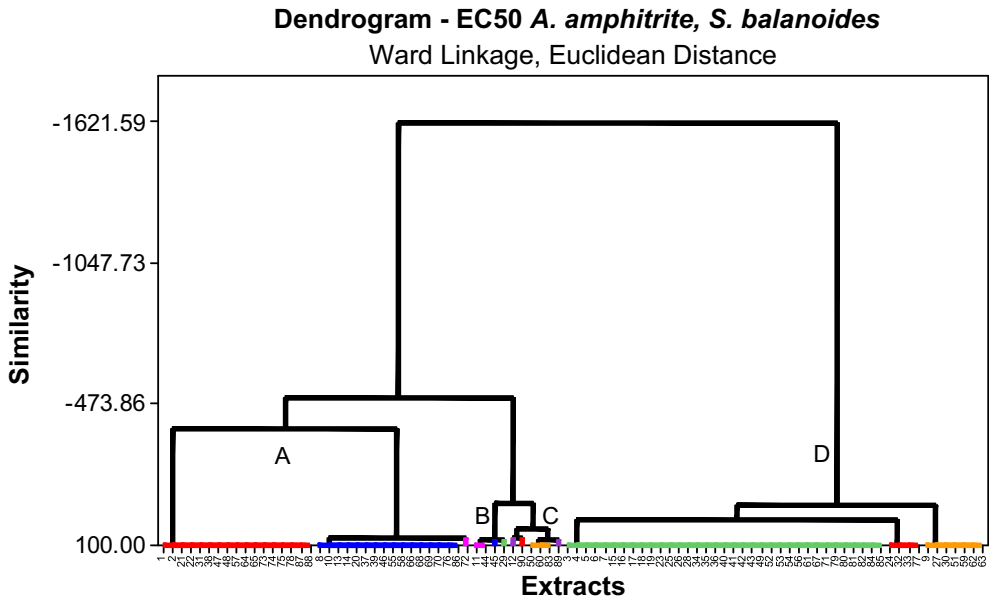

Fig. 2. Clusterisation of algal extracts regarding their anti-settlement activities towards *A. amphitrite* and *S. balanoides*. Extracts were divided in four groups A [34 extracts-1A,B (1, 2), 3B (8), 4A (10), 5A,B (13, 14), 7B,C (20, 21), 8A (22), 11A (31), 13A,B,C (37, 38, 39), 16A,B,C (46, 47, 48), 19A,C (55, 57), 20A (58), 22A,B,C (64, 65, 66), 23B,C (68, 69), 24A,C (70, 72), 25A,B,C (73, 74, 75), 26A,C (76, 78), 29B,C (86, 87), 30A (88)], B [4 exrtacts-4B (11), 10B (29), 15B (44), 15C (45)], C [6 extracts-4C (12), 17B (50), 20C (60), 28B (83), 30B (89), 30C (90)] and D [46 extracts-1C (3), 2A,B,C (4, 5, 6), 3A,C (7, 9), 5C (15), 6A,B,C (16, 17, 18), 7A (19), 8B,C (23, 24), 9A,B,C (25, 26, 27), 10A,C (28, 30), 11B,C (32, 33), 12A,B,C (34, 35, 36), 14A,B,C (40, 41, 42), 15A (43), 17A,C (49, 51), 18A,B,C (52, 53, 54), 19B (56), 20B (59), 21A,B,C (61, 62, 63), 23A (67), 24B (71), 26B (77), 27A,B,C (79, 80, 81), 28A,C (82, 84), 29A (85)].

A. nodosum (4B), *S. muticum* (10B) and *L. ochroleuca* (15B) with EC_{50s} values within the range 20–40 μg ml^{-1} with significantly lower LC_{50s}. Extract 15C was less active than the three others but still has some potential as it was non-toxic (45).

Fig. 6 represents the cluster results for the six extracts from Group C. All these extracts showed antifouling activities towards the two species of barnacles studied. From the analysis, Group C can be divided into three sub-groups:

- Sub-group C1: 2 extracts, *A. nodosum* (4C [1]) and *P. lanosa* (30C [5]), very active on both species (EC_{50s} 0–20 μg ml^{-1}) with very low level of toxicity.
- Sub-group C2: 2 extracts, *C. crispus* (17B [2]) and *P. palmata* (20C [3]), less active than extracts from C1 with EC_{50s} within the range 40–60 μg ml^{-1} and low levels of toxicity.
- Sub-group C3: 2 extracts, *O. pinnatifida* (28B [4]) and *P. lanosa* (30B [6]). They displayed the same levels of AF potency than extracts from Sub-group C2 with slightly highest levels of toxicity. However, they are non toxic with $EC_{50s} < LC_{50s}$.

3.2.3. Anti-settlement activities and toxicity of the hexane extracts

Additional experiments were run on a limited numbers of algal species to test the anti-settlement potency of hexane fractions to address surface compounds potential activity.

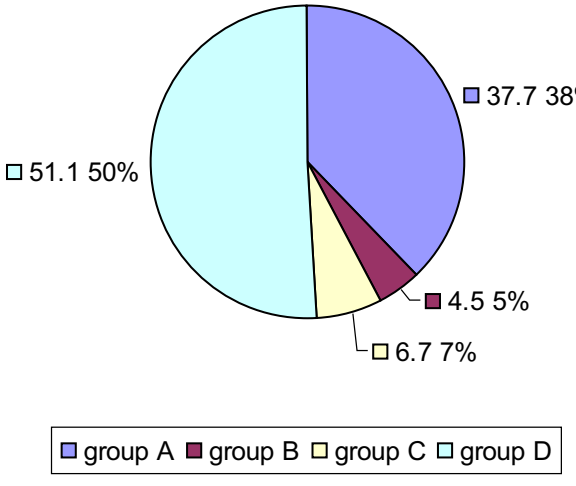

Fig. 3. Distribution of algal extracts within groups A (active specifically towards *S. balanoides*), B (active specifically towards *A. amphitrite*), C (active against both *S. balanoides* and *A. amphitrite*), and D (inactive).

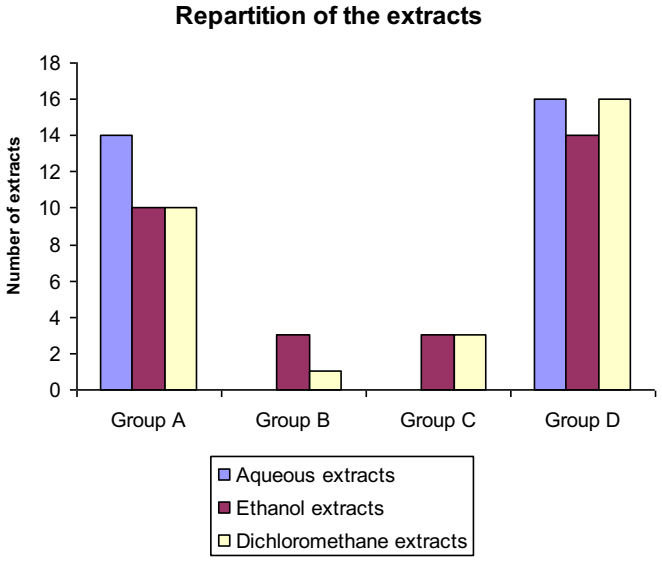

Fig. 4. Distribution of aqueous, ethanol and dichloromethane withing groups A (active specifically towards *S. balanoides*), B (active specifically towards *A. amphitrite*), C (active against both *S. balanoides* and *A. amphitrite*), and D (inactive).

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Table 3a
Effects of the algal extracts on the viability of nauplii of *A. amphitrite*. Results are expressed as LC_{50} values (in $\mu g\,ml^{-1}$).

	Aqueous extract		Ethanolic extract		Dichloromethane extract	
	LC_{50}	Data code	LC_{50}	Data code	LC_{50}	Data code
(3) *C. rupestris*	98.5	2	83.1	2	85.3	2
(4) *A. nodosum*	>100	1	91.0	2	74.1	3
(8) *H. elongata*	>100	1	83.2	2	93.5	2
(9) *P. canaliculata*	89.6	2	91.2	2	93.1	2
(10) *S. muticum*	95.5	2	58.4	4	83.3	2
(15) *L. ochroleuca*	61.9	3	63.9	3	78.1	3
(17) *C. crispus*	>100	1	91.1	2	>100	1
(22) *B. secundiflora*	73.5	3	68.4	3	52.0	4
(23) *C. virgatum*	93.8	2	>100	1	91.4	2
(24) *C. ramosa*	89.3	2	83.5	2	93.6	2
(25) *D. sanguinea*	75.7	3	78.6	3	83.5	2
(28) *O. pinnatifida*	61.8	3	69.5	3	59.2	4
(30) *P. lanosa*	58.1	4	78.5	3	98.1	2

(1) *U. intestinalis*, (2) *U. lactuca*, (5) *F. serratus*, (6) *F. spiralis*, (7) *F. vesiculosus*, (11) *E. siliculosus*, (12) *A. esculenta*, (13) *C. filum*, (14) *L. digitata*, (16) *S. polyschides*, (18) *G. stellata*, (19) *G. latifolium*, (20) *P. palmata*, (21) *D. carnosa*, (26) *D. hutchinsia*, (27) *H. equisetifolius* and (29) *P. plumosa* were non-toxic (LC_{50}>100 $\mu g.ml^{-1}$, data code: 6) (data not shown).

Among the extracts tested towards *S. balanoides*, only the hexane fraction of *L. ochroleuca* showed no bioactivity. However, all remaining extracts were active and it is of interest to note that they all displayed EC_{50} values lower compared to aqueous, ethanol and dichloromethane extracts. *A. nodosum*, *F. serratus*, *P. palmata*, *B. secundiflora*, *D.elegans* and *P. lanosa* were very active with $EC_{50s} < 10\ \mu g\,ml^{-1}$. *U. intestinalis* and *S. muticum* exhibited fair activity levels with $EC_{50s} < 60\ \mu g\,ml^{-1}$ (Table 4).

Among the extracts tested towards *A. amphitrite*, 6 hexane fractions were not active (*F. serratus*, *L. ochroleuca*, *P. palmata*, *D. sanguinea*, *D. elegans* and *P. lanosa*). The active extracts all showed moderate activity with $EC_{50s} = 80\ \mu g\,ml^{-1}$ (Table 4).

Table 3b
Effects of the algal extracts on the viability of nauplii larvae of *S. balanoides*. Results are expressed as LC_{50} values (in $\mu g\,ml^{-1}$).

	Aqueous extract		Ethanolic extract		Dichloromethane extract	
	LC_{50}	Data code	LC_{50}	Data code	LC_{50}	Data code
(1) *E. intestinalis*	56.3	4	50.1	4	>100	1
(3) *C. rupestris*	11.1	6	88.7	2	>100	1
(4) *A. nodosum*	70.4	3	>100	1	82.6	2
(5) *F. serratus*	98.1	2	91.5	2	>100	1
(7) *F. vesiculosus*	>100	1	73.8	3	52.1	4
(8) *H. elongata*	68.3	3	18.4	6	25.3	5
(10) *S. muticum*	17.4	6	28.1	5	12.8	6
(11) *E. siliculosus*	45.3	4	27.4	5	23.5	5
(13) *C. filum*	85.3	2	51.3	4	79.2	3
(16) *S. polyschides*	76.5	3	35.1	5	63.2	3
(17) *C. crispus*	7.3	6	52.1	4	15.7	6
(19) *G. latifolium*	82.4	2	7.5	6	67.8	3
(20) *P. palmata*	92.4	2	13.9	6	51.4	4
(22) *B. secundiflora*	58.1	4	60.2	3	93.5	2
(23) *C. virgatum*	>100	1	>100	1	95.2	2
(24) *C. ramosa*	77.8	3	>100	1	74.6	3
(25) *D. sanguinea*	48.6	4	41.3	4	33.5	5
(26) *D. hutchinsia*	79.0	3	21.1	5	45.4	4
(28) *O. pinnatifida*	21.3	5	52.6	4	15.7	6
(29) *P. plumosa*	>100	1	73.1	3	60.5	3
(30) *P. lanosa*	65.2	3	63.1	3	94.0	2

(2) *U. lactuca*, (6) *F. spiralis*, (9) *P. canaliculata*, (12) *A. esculenta*, (14) *L. digitata*, (15) *L. ochroleuca*, (18) *G. stellata*, (21) *D. carnosa* and (27) *H. equisetifolius* were non toxic (LC_{50}>100 $\mu g\,ml^{-1}$, data code: 6) (data not shown).

4. Discussion

The significance of the physiological condition of cyprids on recruitment in barnacles has been previously demonstrated (Hadfield 1984; Jarrett and Pechenik 1997; Miron et al. 2000; Harder et al. 2001) and it was shown that the metamorphic success increased in the following order of seasonal conditions: winter < spring < summer for *S. balanoides* collected in the field (unpublished data). Similar results were obtained by Thiyagarajan et al. (2002) on *A. amphitrite* and it was stated that, at elevated temperatures, cyprids had an increased metamorphic rate (Anil et al. 2001; Pineda et al. 2002). Physiological age of the larva used for bioassay is very important as it can influence the rate of settlement and the discrimination ability, which are the two key factors influencing assays for MNPs (Dhams and Hellio 2009). Within this study, *A. amphitrite* larvae were produced under controlled laboratory-conditions, at the opposite of *S. balanoides* which were collected from the field. In order to be able to compare the effects of algal extracts on both species, we had to work on larvae of similar physiological ages, so we decided to set up the bioassays using competent larvae with high level of discrimination and for this purposes, from our experimental data, we did select day 3 cyprids of *A. amphitrite* and day 1 wild cyprids of *S. balanoides* (collected in spring) which show these characteristics. Another important factor, when working on bioassays with two different species, is to make sure that all the tests are performed with the same biomass as this could affect the dose–response pattern. As *S. balanoides* larvae are on average twice larger than *A. amphitrite* ones, all the assays were set up with double numbers of *A. amphitrite* compared to *S. balanoides*.

When screening for new AF compounds, the choice of the bioassays usually is based on key species used. Although, the selection of candidate organisms for MNP discovery is usually made on the basis of one or more of the following strategies: a) screening a broad and diverse range of organisms which may produce compounds of interest; b) choosing specific taxonomic groups that are known to be a rich source of marine natural products or c) selecting organisms for which an ecological role of marine natural products is suspected. However, while studies can clearly demonstrate that for instance macroalgae commonly contain metabolites with AF activities; it is far from clear whether they are involved in ecological interactions with their natural competitors. The halogenated furanones are a notable exception (Steinberg et al. 2001). For most of these compounds, however, it is not known whether they have evolved in another context (e.g. in anti-herbivory) and whether their AF activity may only be an artefact generated by breaking-up cell structures (Jormalainen and Honkanen 2008). An indication of a broader ecological role for AF compounds was suggested by the seasonal variations in the activity of extracts of three macroalgae *Bifurcaria bifurcata*, *Sargassum muticum* and *Ascophyllum nodusum*. A clear trend in their biological activity was observed (Hellio et al. 2004a; Maréchal et al. 2004a), however, the link between major chemical components, their ecological role and their AF activities was not demonstrated. This inability to establish a link may have been related to the fact that a warm-waters barnacle was used to assay the potency of algal extracts of North Atlantic origin.

The present study sampled macroalgae from a single location on a NE temperate Atlantic shore, to determine if the species selected showed any AF activity, which could be explained by ecological interactions. Accordingly, the bioactivity of the macroalgal extracts was assayed against *A. amphitrite* (warm-waters species) and *S. balanoides* (temperate/cold species), the later being found in the same environment as the macroalgae. Our main objectives were to determine: (1) whether the results obtained using *A. amphitrite*

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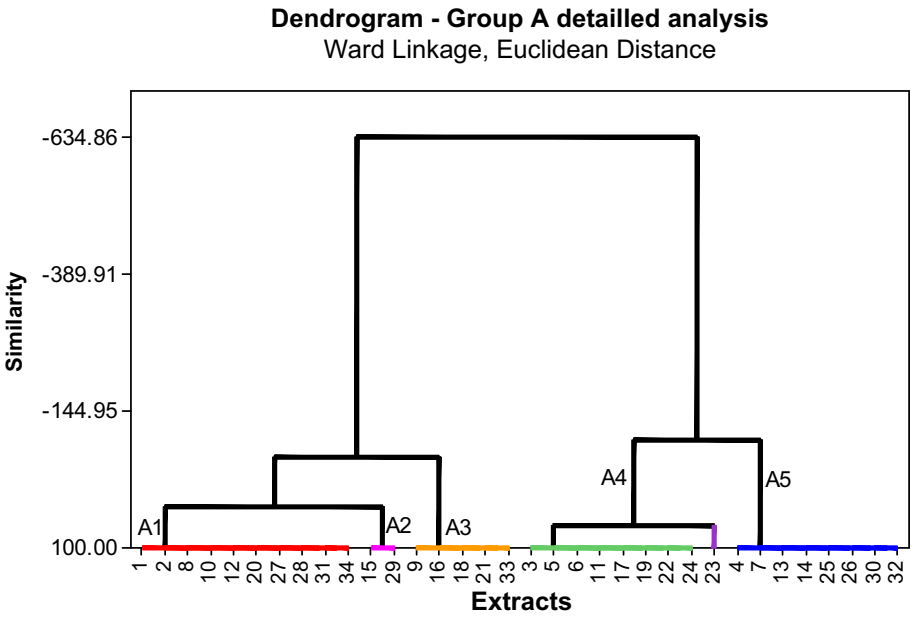

Fig. 5. Detailed clusterisation of algal extracts from group A (active specifically towards *S. balanoides*) regarding their anti-settlement activities and toxicities levels.

could be extrapolated to *S. balanoides* and *vice versa* and (2) the appropriateness of using warm water species for AF tests when screening for marine natural products of marine organisms from temperate waters.

From the first set of experiments using aqueous, ethanol and dichloromethane, it is clear that, with some exceptions (46 inactive extracts and 6 extracts active against the two species of barnacles), the two species of barnacle reacted differently to identical algal extracts. This result may have a significant impact on the protocol used for AF testing as so far *A. amphitrite* is the only species of barnacles used for the assessment of most AF formulations. We proved that *S. balanoides* larvae were significantly more sensitive to the algal extracts tested than *A. amphitrite* larvae. Of the 90 extracts tested, 34 inhibited specifically the settlement of *S. balanoides* while only 4 inhibited specifically the settlement of *A. amphitrite*. The variability in anti-settlement results between these two species suggests that the active ingredients in the extracts could be target-specific. Previous results obtained with the same algal extracts support this hypothesis. Among the 17 promising extracts that were active at non-toxic concentrations specifically towards *S. balanoides*, 7 (3B, 4A, 7B, 23B, 23C and 24A) inhibited other fouling species from the same environment as the algae (diatoms, bacteria, macroalgae and mussels) (Hellio et al. 2001, 2002).

The observation that 8 extracts (4B, 15B, 17B, 20C, 28B, 30B, 30C)) showed activity against the two species of barnacle could be explained by the fact that the sample site is located at the southern limit of the cold waters and the northern limit of the warm waters. So in terms of chemical ecology, it is reasonable to expect that some species present in transitional geographical regions may produce compounds with activity towards both warm waters and cold

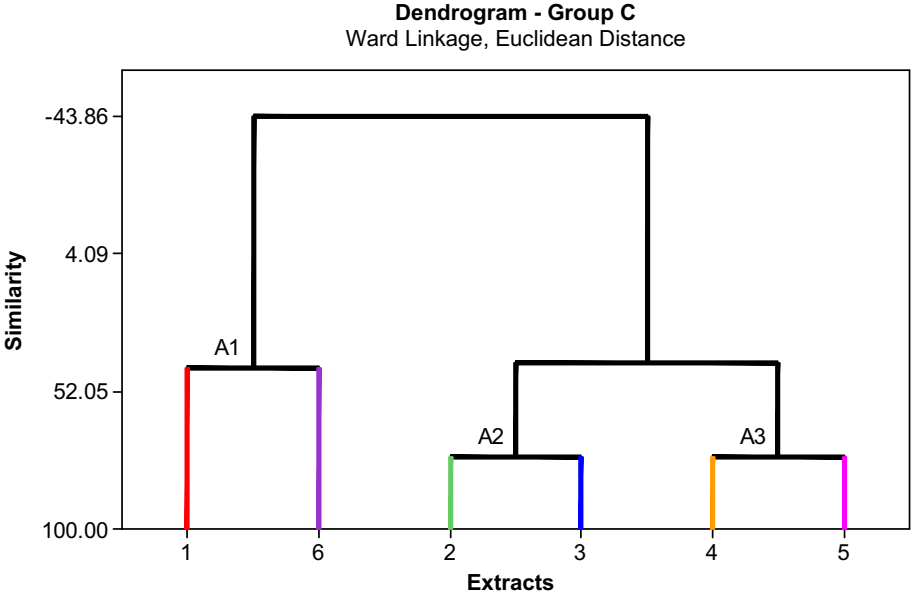

Fig. 6. Detailed clusterisation of algal extracts from group C (active against both *S. balanoides* and *A. amphitrite*) regarding their anti-settlement activities and toxicities levels.

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Table 4
Effects of the algal hexane extracts on the settlement of cypris larvae of *A. amphitrite* and *S. balanoides*. Results are expressed as EC_{50} values (in $\mu g\,ml^{-1}$).

	A. amphitrite	*S. balanoides*
	EC_{50}	EC_{50}
(1) *U. intestinalis*	80.2	45.5
(4) *A. nodosum*	80.2	6.1
(5) *F. serratus*	>100	8.3
(10) *S. muticum*	>100	58.2
(15) *L. ochroleuca*	>100	>100
(20) *P. palmata*	>100	9.9
(22) *B. secundiflora*	80.3	9.6
(25) *D. sanguinea*	>100	33.3
(29) *D. plumosa*	>100	8.3
(30) *P. lanosa*	>100	8.2

waters species. Similarly, Plouguerné et al. (in press) demonstrated that *Sargassum vulgare* when collected at 5 different locations of the Rio de Janeiro State (Brazil) exhibited variable chemical defence against bacteria and mussels. The distribution of species is limited by their ability to adapt to local environments. For adaptation by selection, genetic variability is crucial (Schmeller et al. 2005) and incorporating locally adapted genes might extend the range limit. This can be linked to the biosynthesis of a more active chemical defence.

Another interesting fact to consider is that the most active extract which acts specifically on *A. amphitrite*, namely the ethanolic extract of *S. muticum* (10B), inhibited two alien macroalgae with world-wide distribution (*S. muticum* and *U. intestinalis*) (Hellio et al. 2002) and the tropical mussel *Perna perna* (Plouguerné et al. in press), but was inactive towards the blue mussel *Mytilus edulis* and the cold water macroalga *Polysiphonia lanosa* (Hellio et al. 2000, 2002). In this case, it appears that the bioactivity of the ethanolic extract of *S. muticum* is specifically targeted towards warm-waters species. *S. muticum* is classified as an alien species at the geographic location the sample was collected for this study. This species originates from North-eastern Asia and was introduced to the Pacific coast of North America and to Western Europe where it has spread widely during the last 40 years (Plouguerné et al. 2008). Recent studies have highlighted the link between success of marine bioinvasion and the establishment of chemical defences by the introduced organism (Cipollini 2005; Lages et al. 2006; Wikstrom et al. 2006; Simoncini and Miller 2007; Cassano et al. 2008; Plouguerné et al. 2008). The bioactivity results obtained for the extracts of *S. muticum* corroborate this hypothesis; this alga is particularly well equipped for biological competition, with its aqueous fraction showing high levels of inhibition of cold/temperate species' growth and settlement, its ethanolic fraction being active towards tropical species and its dichloromethane fraction showing modest activity towards a large range of organisms (Bazes et al. 2009; Plouguerné et al. 2008, 2010). One hypothesis is that alien species produce a full range of bioactive compounds or compounds with a wide range of activity that could be expressed in a context of colonisation processes (and could modulate their biosynthetic pathways to produce the specific compounds they need depending on the environment they develop in).

In order to better understand if the AF activities of the extracts may be linked to chemical ecology, surface extracts of 10 algal species were produced and their potency were assessed towards the 2 species of barnacles. 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. Thereof it could be argued that active AF compounds located at the surface of the algae can have a role in the chemical communication between species. This hypothesis is corroborated by the fact that the same hexane extracts showed no activity against *A. amphitrite*, which is not present in *S. balanoides*' environment were the seaweeds were sampled. Four extracts displayed activity towards the two barnacle species, interestingly from the algae that are classified as alien species (*S. muticum* and *U. intestinalis*) and the 2 species that has the broadest geographical distribution (*A. nodosum* and *B. secundiflora*). In this context, the chance of finding new compounds displaying targeted activity might be higher when sampling and testing organisms from the same environment are examined, and the chance of finding compounds with broad range of activity is better when focusing on alien species or species displaying a large geographic distribution. These thoughts have an obvious impact on the choice of species used to screen for biological activity in AF research and the choice of the species for the bioassays. Most research projects have focused on MNPs from tropical and sub-tropical zones (Hellio et al. 2009) and most of the bioassays have been targeted on fouling organisms from warm waters (Dhams and Hellio 2009). So far the choice of bioassays has been driven by both the industry requirements and their feasibility. As the main maritime route goes through tropical zones, paint manufacturers are looking for new compounds with activity towards tropical organisms. Moreover, from an experimental point of view, developing bioassays using tropical species rather than temperate/cold water species has the great advantage of eliminating the time constraints, as they reproduce all year round. However, with the recent and growing development of the Arctic maritime transport, it may be necessary to develop new specific paint formulations that are active in cold and temperate environments. In this regard, when working on MNPs, it would be more judicious to sample organisms from temperate climate and to test them primarily on fouling species from the same area. Such targeted formulations could be marketed for the leisure sailing and motor yachts, as well as for any permanent structures (such as pontoon, buoys, aquaculture equipments) (Maréchal and Hellio 2009). This argues for the development of new AF bioassays targeting cold and/or temperate species, which have been so far neglected.

Acknowledgements

Our special thanks to Prof. D. Rittschof (Duke University Marine Laboratory, Beaufort, North Carolina, USA) for providing *A. amphitrite* adults and to Prof. A.S. Clare (Newcastle University, UK) for his comments to improve this manuscript.

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Please cite this article in press as: Maréchal, J.-P., Hellio, C., Antifouling activity against barnacle cypris larvae: Do target species matter (*Amphibalanus amphitrite* versus *Semibalanus*..., International Biodeterioration & Biodegradation (2010), doi:10.1016/j.ibiod.2010.10.002