Screening of biological activities of extracts of *Ralfsia verrucosa*, *Petalonia fascia* and *Scytosiphon lomentaria* (Phaeophyceae, Scytosiphonales) for potential antifouling application.

Marie Thabard[1], Ghezlane Id Daoud[1], Benoît Véron[2], Robert L. Fletcher[1], Claire Hellio[1*].

[1]*School of Biological Sciences, King Henry Building, University of Portsmouth, Portsmouth PO1 2DY, UK.*
[2] *Algobank-Caen, Université de Ca en Basse-Normandie, 14032 Caen cedex, France.*
* *Corresponding author: phone: +44 2392 84 20 73; fax: +44 2392 84 20 70; email: claire.hellio@port.ac.uk*

Abstract:
Fouling process is often detrimental to the efficiency of man made structure, and preventative measures are required. With increasing concern about the polluting impact of the biocides used as antifouling, non-toxic compounds are being sough after. Pertinent to this requirement is the increased interest being given to the potential use of natural biogenic compounds.
The present study involves an investigation of the potential antifouling and bactericidal properties of extracts of three species *Ralfsia verrucosa*, *Petalonia fascia* and *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae). Methanol and ethanol extracts of these algae were tested for their potential activities against bacteria, microalgae and spores of macroalgae.
Bioassays were performed using strains of marine bacteria, human pathogenic bacteria, marine microalgae, freshwater microalgae and marine macroalgae.
No antifouling and antibacterial activity was recorded when testing the methanol extracts. Activity was, however, found when using the ethanol extracts. The best activity was obtained with *R. verrucosa* ethanol extract against *Shewanella putrefaciens* at a MIC (minimum inhibitory concentration) of 10 µg/mL.

Keywords: Antifouling, crustose, erected shoot, natural products, Scytosiphonales.

Introduction:

Biofouling is a major economic problem concerning man made structures such as, ships, boys, pontoons etc.. It costs the U.S Navy approximately 1 billion $ per annum (Callow and Callow 2002). This phenomenon, which results from the settlement of organisms on any surface submersed in the aquatic environment can be responsible for the corrosion of steel made structures (such as the ship hulls), the decrease of the ships speed due to increased drag, technical problems in aquaculture systems, fish nets and power plant cooling system (Hellio and Yebra, 2009).
Biofouling can be described in a 4 main stages process (Wahl 1989): the biochemical conditioning, the microfouling (involving bacteria), the unicellular eukaryote colonisation (yeasts, protozoa and diatoms) and finally the multicellular eukaryote adhesion and growth (larvae and algae spores). However, all these phases are not always represented in the environment; these stages can overlap or occur in parallel and one or two phases can also be missing, depending on the environmental conditions (Maréchal and Hellio, 2009). Therefore, it appears necessary to focus on the different layers of the colonisation to prevent the fouling of a substratum.
Several antifouling (AF) technologies were developed in the past to avoid fouling on man made structures. Paints containing organotin such as tributylin (TBT) were first applied in the 1960's to prevent biofouling. These paints were worldwide used until recently. However, various studies have demonstrated TBT to cause many adverse ecotoxicological effects (Coelho et al. 2006; Abarzua and Jakubowski 1995; Grondin et al. 2007). The International Maritime Organisation (IMO) has reported accumulation of TBT in mammals, and deficiency of the immunological defences in fishes

(Yebra et al. 2004). As a result, its use has progressively been restricted in numerous countries and is prohibited worldwide on any surface since January 2008 (Van Wezel and Van Vlaardingen 2004). New paints containing organic biocides were developed, but it has already been proven that some of them pollute the environment and are very harmful to many organisms (De Sousa et al. 1998; Marechal and Hellio, 2009).

As man made structures need to be protected from biofouling, new environmentally products must be developed to replace the toxic paints. Numerous studies focused on physical, chemical, mechanical and biological methods to prevent marine biofouling (Abarzua et al. 1999). Recent studies point out the fact that biogenic compounds are a promising field of research regarding fouling problem (Abarzua et al. 1999). Marine organisms produce a large range of bioactive compounds (Bhadury and Wright 2004) that could be added in paints as AF agents. Studying marine algae as producers of AF molecules is thus promising because they synthesise biogenic compounds and can be cultivated (Hellio et al. 2002; Maréchal et al. 2004; Barbosa et al. 2007; Mokrini et al. 2008). Many seaweed species were reported to inhibit the growth of both gram positive and negative bacteria (Caccamese et al. 1979, 1980, 1981, 1985; Rao and Parekh 1981; Pesando et al. 1984; Sastry et al. 1994; Devi et al. 1997; Gonzales del val et al. 2001; Bhosale et al. 2002; Hellio et al. 2001, 2004). There are evidences that algae also produce toxic compounds preventing the settlement and development of unicellular and multicellular eukaryote (Harada et al. 1984; Vanelle and Le Gal 1995; Cho et al. 2001; Hellio et al. 2002, 2004, 2009).

In the following experiments, a study was carried out on the natural products of some selected brown algae (Phaeophyceae) members of the Scytosiphoniales, a group of algae which has received very little attention up to date. Three species were studied here: *Scytosiphon lomentaria* (Lyngbye) Link 1983, *Petalonia fascia* (O.F. Müller) Kentze 1898 and *Ralfsia verrucosa* (Areschoug) Areschoug 1845. Extracts of these algae were prepared and tested for potential AF activities towards bacteria, micro- and macroalgae. The purpose of these experiments was both to study AF activities in the Scytosiphonales group, and to highlight any possible differences between the production of inhibitory compounds between crustose and erect thalli growth forms.

Material and Methods:

Biological material:

Test algae:

S. lomentaria and *P. fascia* were collected from Swanage (Dorset, UK, 50º36'N; 1º 56'W) in January 2007. Also, *P. fascia* was collected on the buoys of Langstone harbour (Hampshire, UK, 50º 47'N; 1º 01W). *R. verrucosa* was collected at Whiteness Gap (Kent, UK, 51°21'N; 1°22'E) in December 2006. Both *S. lomentaria* and *P. fascia* are erect thalli growth forms while *R. verrucosa* is a crustose alga. The latest was collected by scrapping the rock surface. After collection, the algae were cleaned from epiphytes and washed with a solution made of pasteurised seawater and ethanol (3%) to remove the microflora present on the surface of algae. After being weighted, they were stored in a freezer at -80°C for a few days and then freeze-dried for 3 Days.

Two extractions were performed on the algae collected using both absolute ethanol and absolute methanol (Fisher, UK). For both samples of *P. fascia* and *S. lomentaria*, 100 mL of solvent were added to 1.50 g of algae, for *R. verrucosa* 6.6 mL of solvent were added to 0.22g of algae. The extraction (maceration) lasted for three days in a dark place at a temperature of 20°C. The samples were then dried under reduced pressure.

Dilutions of the extracts were performed to make 6 concentrations: 0.5, 5, 10, 30 and 100 µg/mL.

Bacteria:

The 5 marine bacteria strains (*Pseudoalteromonas elyakovii* ATCC 700519, *Shewanella putrefaciens* ATCC BAA-453, *Polaribacter irgensii* ATCC 700398, *Vibrio aestuarianus* ATCC 35048 and *Cobetia marina* ATCC 25374) used in these experiments were obtained from the Culture Collection of the University of Portsmouth. One of these strains is Gram-positive (*S. putrefaciens)*. These bacteria were chosen because they are involved in marine fouling. *S. putrefaciens* is known to cause organoleptic alteration of sea products, it can settle easily on different types of substrata and is responsible for the corrosion of metals due to its ability to reduce a variety of compounds such as Fe (III) and Mn (IV) by anaerobic respiration (Dichrisitina and Delong 1993; Nealson and Myers 1992). Marine bacteria were cultivated with marine broth (5% Tryptone) and incubated for five days at 30° C to allow for their development.

The 5 terrestrial bacteria strains (*Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 6896, *Klebsiella pneumonia* ATCC 13883, *Salmonella typhirium* ATCC 14028 and *Bacillus subtilis* ATCC 21332) used in these experiments were provided by the University of Portsmouth. One of these strains (*B. subtilis*) is a Gram-positive bacteria, the others being Gram-negative. Terrestrial bacteria can be found in the coastal waters and are thus interesting species to study. Terrestrial bacteria were cultivated in a nutrient broth (CM0067, Nº2, 25g/L) and incubated for five days at 30° C to allow for their development.

Microalgae:

The 5 marine microalgae strains (*Navicula jeffreyi* Hallegraef and Burford AC181, *Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann 1964 AC170, *Chlorarachnion globosum* K. Ishida and Y. Hara 1994 AC132, *Pleurochrysis roscoffensis* (P.

Dangeard) J. Fresnel and C. Billard 1991 *AC1* and *Exanthemachrysis gayraliae* H. Lepailleur 1970 *AC15*) used in these experiments were obtained from Algobank-Caen, Universite de Caen Basse-Normandie, France. These microalgae are found in coastal and/or estuarine waters and can thus form biofilms on ship hulls and other man made structures. Marine microalgae were cultivated with an f/2 culture medium (Guillard and Ryther 1962) and incubated for 5 days in a growth room (constant light, 20˚C) to allow for their development.

The 3 freshwater microalgae strains *Scenedesmus armatus* CCAP 276 (R. Chodat) R. Chodat 1913, *Cosmarium* sp. CCAP 612 and *Fragilaria crotonensis* CCAP 1029 (Kitton 1869) used in these experiments were obtained from the culture collection of CCAP (Scotland). Freshwater microalgae were cultivated with a Bold Basal Medium (Stein, 1973) and incubated for a few weeks in a growth room (constant light, 20˚C) to allow for their development. These freshwater microalgae are very common in Europe and are responsible for important blooms.

Microalgae produce extracellular polymeric substances (EPS) that are involved in the formation of biofilms and are thus major organisms to study in the research for AF compounds.

Macroalgae:

Undaria pinnatifida (Harvey) Suringar 1873, a brown invasive species, was used in these experiments. The assays (settlement and germination) were performed using the zoospores.

Assays:

Antibacterial assays (Marechal et al. 2004):

The Optical Density (O.D) of stock cultures was measured at 630nm for every sample to calculate the quantity of solution required to obtain 1 mDO. This was determined using the table of Amsterdam (1996), to ensure that the assay started with the inoculation of the same number of bacteria (2.10^8 cells/mL).

50 µL of each test solution (extracts) were poured in 6 wells of 96 wells' plates (Fisher, UK) for each bacterial assay. In addition, 6 wells free from extracts were used as a control. These plates were dried under a U.V cabinet for 2 hours to evaporate the solvent under sterile conditions. 50µl of bacteria solutions were then added under aseptic conditions and the plates incubated for 48 hours at 30°C to allow for the development of bacteria. (Algal extracts are considered to be active if bacteria do not grow in 4, 5 or 6 wells over 6 wells.) One plate was used for each strain to decrease the contamination risk.

Microalgae assays:

The quantity of chlorophyll a was determined in every sample by an indirect method using the equation of Lorenzen (1967): Ca [mg/m^{-3}] = ($11.6\ D_{665} - 1.31\ D_{645} – 0.14\ D_{630}$) $v\ l^{-1}\ V^{-1}$

With v = volume of acetone (ml), l = cell length (cm) V = volume of filtered microalgae solution (L), and D_{665}, D_{645}, D_{630}, the O.D of the solution measured at 3 different wavelength 665, 645 and 630 nm. (Trichromatic method). To do this, microalgae solutions were filtered using a pump and Whatman filters (GF/F 0.7µm, 47mm diameter).

After measuring the quantity of chlorophyll a present in the microalgae solutions, dilutions were made to obtain solutions containing 1 mg/L of chlorophyll a. These solutions were poured in 96 wells' plates (already containing evaporated extracts to test) following the method described above for the bacteria.

Germination and settlement assay:

96 wells' plates were filled with the Scytosiphonales extracts following the same method as described in the antibacterial assay. In addition, 6 wells free from extract, 6 wells filled with methanol and 6 wells filled with ethanol were used as controls. The plates, once dried under a UV cabinet were filled with the spore solutions made of spores and Von Stosch (1964) culture medium (50 µL per well). The number of spores in each well (10500 spores/50 µL) was determined using a hemocytometer cell.

The plates were incubated for 2 hours in the dark to allow for spore settlement. The wells were then carefully washed with a Von Stosch solution to eliminate the non settled material. 50 µL of new fresh media were added and the plates were incubated in a growth room (temperature of 15°C, photon irradiance of 45 $\mu Molsm^{-2}s^{-1}$, 16/8 hour light to dark ratio) for 5 days to allow for the spore germination. The material was then fixed using a 10% formalin solution.

The total number of spores (germinated + non germinated spores) and the number of germinated spores were counted in 10 random fields in 3 wells for each treatment using a microscope (x20 x10 magnification). The relative number of spores was then compared with the controls (no treatment, ethanol and methanol) to determine the amount of settlement and the percentage of germination was calculated (Fletcher 1989).

The zoospores which had produced a germ tube followed by a transverse division into two cells (with evacuation of the original spore cell) were considered as germinated spores.

Statistical treatment:

To ensure the results were showing significant differences, a kruskal-wallis test was performed and the quantification of the difference was observed on a graph (Box-plot) using the software MINITAB. The extracts possessing a percentage of germination differing from both the control and the solvent (methanol or ethanol depending on the solvent used for the extraction) were considered as active

Results:

Table 1: Minimum Inhibitory Concentration (MIC in µg/mL) of Scytosiphonales extracts showing an activity against Shewanella putrefaciens.

Algae	Location	Solvent	MIC (µg/mL)
P. fascia	Langstone	Ethanol	100
P. fascia	Swanage	Ethanol	100
R. verrucosa		Ethanol	10

Antibacterial assay:

The terrestrial bacteria growth was not inhibited by the extracts tested here (results not showed). Some activity has been observed against the marine bacterium *S. putrefaciens*. Ethanol extracts of *P. fascia* collected from both Swanage and Langstone harbour, showed an activity at a concentration of 100 µg/mL (table 1). *R. verrucosa* ethanol extract showed an activity at a concentration of 10 µg/mL. *S .putrefaciens* is, therefore, more sensitive to *R. verrucosa* extracts than to the other Scytosiphonales extracts tested. It can be noticed that no inhibition was observed with *S. lomentaria* extracts.

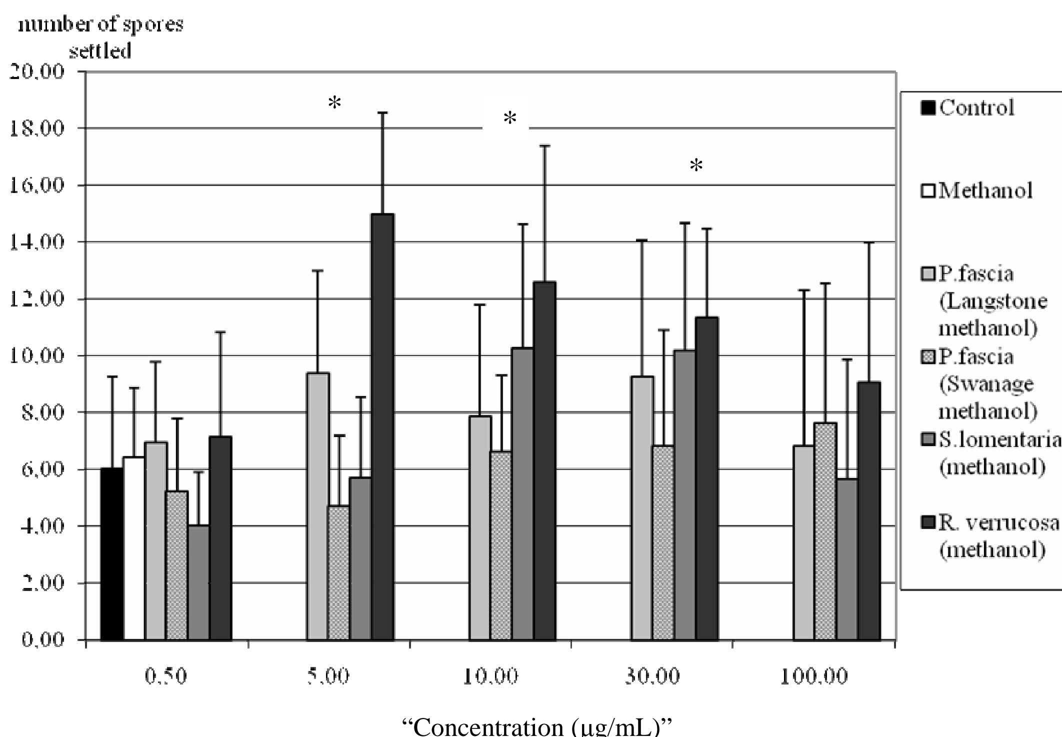
Antimicroalgal assay:

No activity was observed against all the strains of microalgae studied.

Macroalgae assay:

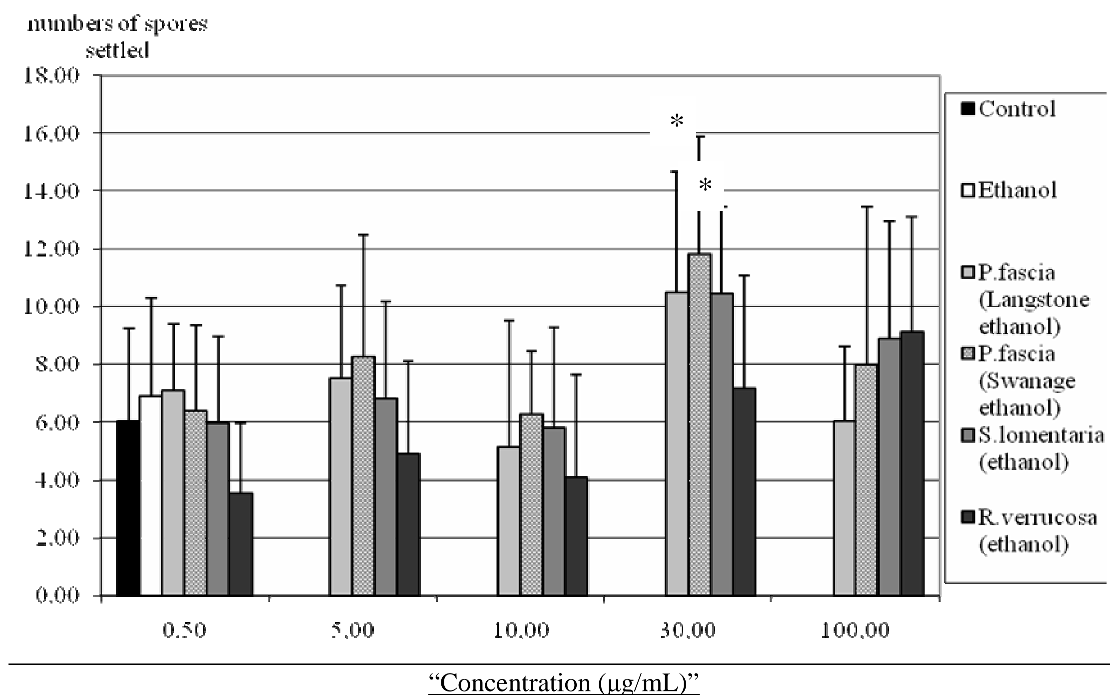
Undaria pinnatifida spore settlement:

It can be noticed from the results that the number of spores settled in the wells is almost constant. The statistical treatment (Kruskall-wallis) showed a significant difference between 5 treatments and the control, the number of spores being higher in the treatments (Figure 1 and 2).



"Concentration (µg/mL)"

Figure1 : number of *U. pinnatifida* spores settled under the different treatments (methanol extracts). The * codes for a significant difference between the treatment and the control.

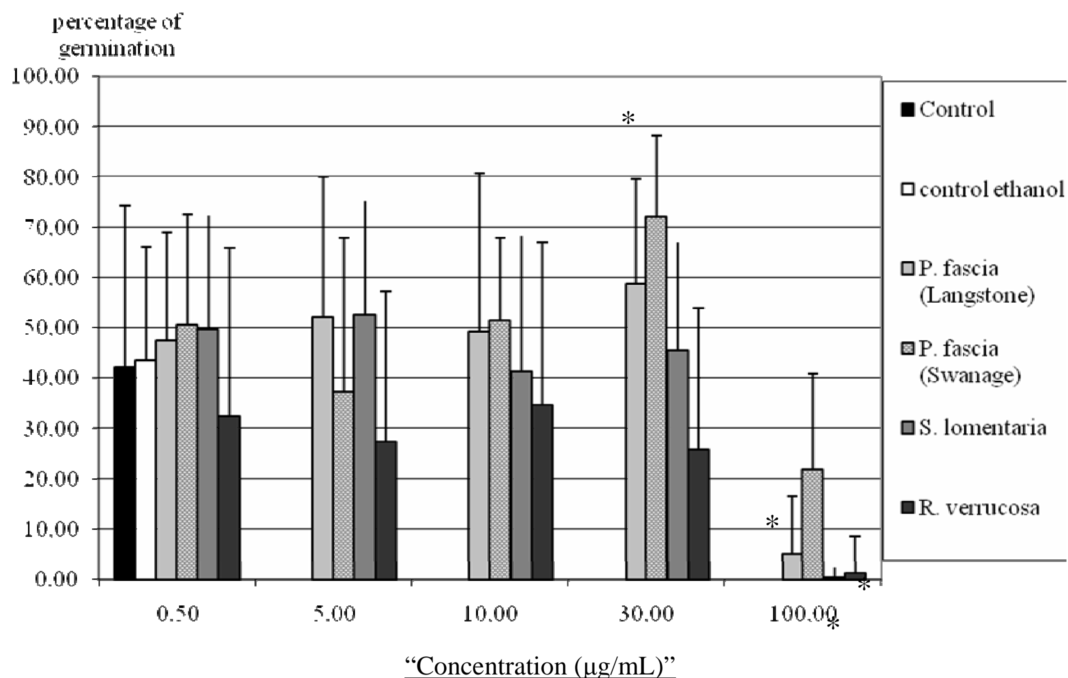


"Concentration (µg/mL)"

Figure 2: number of *U. pinnatifida* spores settled under the different treatments (ethanol extracts). The * codes for a significant difference between the treatment and the control.

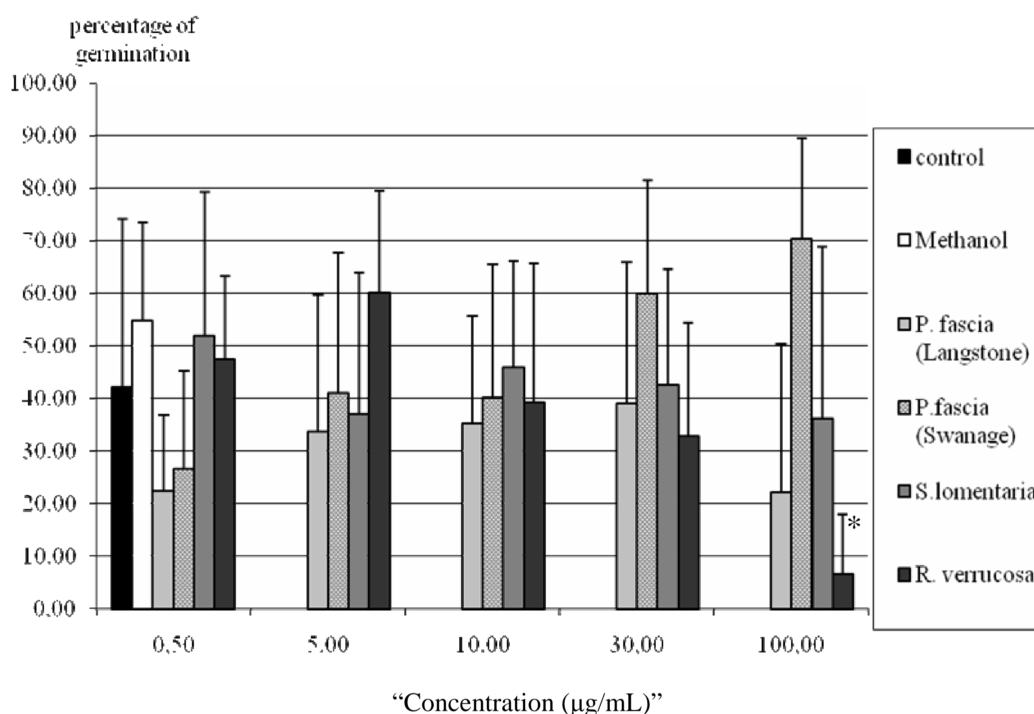
<u>*U. pinnatifida* germination assay:</u>

The statistical treatment (Kruskal-wallis) showed a significant difference between the percentages of germination depending on the solution tested ($p<0.001$). The results for *U. pinnatifida* are presented in Figure 3 and 4. No differences can be seen between the 3 controls (methanol, ethanol and control). It can be seen that 5 treatments (*P. fascia* Swanage methanol extract at 30 µg/mL, *P. fascia* Langstone ethanol extract at 100 µg/mL, *S. lomentaria* ethanol extract at 100 µg/mL, *R. verrucosa* ethanol and methanol extracts at 100 µg/mL) differ from both the control and the solvent control (either methanol or ethanol depending on the solvent used for the extraction). Some of the treatments, such as *R. verrucosa* ethanol extract at 30 µg/mL, differ from the solvent control, but not from the blank control.



"Concentration (µg/mL)"

*Figure 3: percentage germination of U. pinatifida spores under the different treatments (ethanol extracts). The * codes for a significant difference between the treatment and the control.*



"Concentration (µg/mL)"

*Figure 4: percentage germination of U. pinatifida spores under the different treatments (methanol extracts). The * codes for a significant difference between the treatment and the control.*

Discussion:

Biofouling of man made structures results in major economic costs. Different approaches were developed in the past to reduce both macro and micro fouling; however most of them appeared to be toxic for the environment (Gibbs 1993; Gibbs et al. 1987, 1988; Iwata et al. 1995). It is evident that non toxic strategies must be developed to prevent biofouling. Numerous studies showed algae to produce molecules that could act as repellent against both micro and macro organisms involved in the fouling process (Da Gama et al. 2002). The AF compounds found in marine organisms incorporated to paints could be less toxic for the environment, especially against the non target species (Hellio et al. 2000b).

Some species, have received little attention in the past, and more experiments are required to determine their potential as producers of AF agents. The current study aimed at determining if the alga members of the Scytosiphonale group had some activity against both micro and macro fouling.

None of the algae tested here had any activity against the five terrestrial bacteria strains studied, *P.fascia* has already been demonstrated not to have any activity against terrestrial bacteria such as *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Salmonella paratyphi, Aeromonas hydrophila, Vibrio vulnificus* and *Vibrio parahaemolyticus* (Caccamese et al. 1985; Magallanese et al. 2003), while it was shown by Magallanes et al. (2003) to have antibacterial activity against two terrestrial strains of bacteria: *Staphylococcus aureus* and *Enterococcus faecalis*. *S. lomentaria* was shown not to have activity against two strains of terrestrial bacteria: *E. coli* and *B. subtilis* (Caccamese et al. 1985). The activity is thus specific and depends on the strains studied. None of the extracts had activity against the 8 strains of microalgae tested.

Among all the species tested, *R. verrucosa* (extracted with ethanol) is the most active against the marine bacteria *S. putrefaciens*. Indeed this extract inhibited *S. putrefaciens* growth at a minimum concentration of 10 µg/mL while the other active extracts. *fascia* collected from both Swanage and Langstone Harbour, extracted with ethanol), showed activity at a minimum concentration of 100 µg/mL.

The macroalgae assays showed some significant differences depending on the treatment (extract tested). Previous studies have shown numerous algal extracts to have activity against macroalgae (Hellio et al. 2002). No differences were observed in the spore settlement assay apart from a few wells which contained significantly more spores than the control (Figure 1 and 2). The heterogeneity of the spore solution could explain such observation (even if the solution was carefully mixed during the experiment). However, the percentage of germination (Figure 3 and 4) showed some interesting results (some of the highest concentrations tested had activity). The extracts tested here do not seem to prevent the fouling of macroalgae (same number of spore settled) but their development (percentage of germination was different). It can be concluded from these experiments that the Scytosiphonales extracts tested are toxic against *U. pinnatifida* spores development.

The percentages of germination of *U. pinnatifida* seem to be low even in all the wells, including the controls (around 50%) while nothing should prevent its growth. This may be due to the technique used in these experiments. Indeed, the 96 wells' plates, which are narrow and "deep", might prevent a good oxygenation

of the medium. Previous experiments carried out by Hellio et al. (2002), were carried out using Petri dishes as a substratum. However, the amount of extract we had in these experiments did not allow us to use such a method.

Despite this low percentage of germination, some significant results were observed. The 3 controls tested were not significantly different from one to another.

The percentage of germination of the ethanol extract of *P. fascia* collected from Swanage at 30 µg/mL was significantly higher than the one observed with the control (p= 0.096). It has been demonstrated that algae (both encrusting and erected thalli) can produce secondary metabolites facilitating the recruitment of invertebrates' larvae (Pawlick, 1992; Heyward and Negri 1999; Soares et al. 2008). It is also possible that marine algae produce molecules capable of enhancing the algae recruitment (in order to out compete other species). This property is important regarding marine aquaculture and could be used to facilitate the recruitment of larvae or algae spores of cultured species (Soares et al. 2008).

P.fascia and *S. lomentaria* ethanol extracts also showed some activity at 100 µg/mL against spore germination. Both methanol and ethanol extracts of *R. verrucosa* showed some activity against spore development at a concentration of 100 µg/mL. This agrees with previous experiments suggesting *Ralfsia* species to be resistant against grazing and fouling activities in both field and laboratory conditions. For example, Conover and Sieburth (1966) noticed that the pools containing *R. verrucosa* were not colonised by *Balanus balanoides* Linnaeus, 1767 and *Mytilus edulis* Linnaeus, 1758 while other pools located at the same level on the shore without *R. verrucosa* were colonised. They also found the seawater taken from these pools to affect organisms such as *Acaria tonsa*, trochophores, veligers and Nereid worms. The *Ralfsia* species are thought to produce tannins that would act as herbivory deterrents. Moreover, Fletcher (1975) reported the brown alga *R Spongiocarpa* Batters, 1888 to prevent the development of the crustose marine red alga *Porphyrodiscus simulans* Batters, 1897 in laboratory culture.

Interestingly, in this respect, observations made by Lubchenco (1978) and Lubchenco and Cubit (1980), suggested that the crustose life stages are more resistant against grazers and fouling. Several hypotheses were drawn to explain such results, among them are often stated the morphology of the crustose stage and the production of active molecules. Observations made here tend to show that the production of natural compounds by crustose algae could act for their protection. However, it is hard to conclude about the role of these molecules in the environment and their ecological effect. The significance of such results should be taken with caution as we do not know whether these substances are released in the environment by the algae.

Fluorescence microscopy, combined to other studies (such as chemical analyses) demonstrated the red alga *Delisea pulchra* to release AF compounds (halogenated furanones) by gland cells located on the surface of the alga (de Nys and Steinberg 2002; Dworjanyn et al. 2004). Cortical cells of *Laurencia snyderae* exhibit a particular structure named "corps en cerise" that may be a primary location of halogenated natural products (Young et al. 1980). Salgado et al. (2008) showed corps en cerise to be the main reserve for halogenated compounds in *Laurencia Obtusa* and to store high concentrations of bromine and chlorine. Further studies, analysing the composition of the algae should be undertaken to see if crustose algae (*Ralfsia*) can release active molecules by particular cells in the environment. De Nys et al. (1998) described a new technique: "Hexane dipping" to determine the concentration of non polar molecules at the surface of the algae, this method could be easily performed on *Ralfsia* species to determine if they release any AF molecules at their surface.

The results discussed here are part of our ongoing research for new non-toxic AF compounds. It would be very interesting to purify the active compounds, to test them against other organisms such as fungi or barnacles (*Semibalanus balanoides*). If activity is found in these experiments it could be possible to carry out further studies to determine the composition of the fractions and the structure of the active compounds.

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