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# Inhibition of biofouling by marine microorganisms and their metabolites

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**Abstract**
Development of microbial biofilms and the recruitment of propagules on the surfaces of man-made structures in the marine environment cause serious problems for the navies and for marine industries around the world. Current antifouling technology is based on the application of toxic substances that can be harmful to the natural environment. For this reason and the global ban of tributyl tin (TBT), there is a need for the development of ''environmentally-friendly'' antifoulants. Marine microbes are promising potential sources of non-toxic or less-toxic antifouling compounds as they can produce substances that inhibit not only the attachment and/or growth of microorganisms but also the settlement of invertebrate larvae and macroalgal spores. However, so far only few antilarval settlement compounds have been isolated and identified from bacteria. In this review knowledge about antifouling compounds produced by marine bacteria and diatoms are summarised and evaluated and future research directions are highlighted.

**Keywords:** *Marine microbes, bacteria, diatoms, fouling, invertebrate larvae, settlement inhibition, antifouling, defence*

## Introduction

The colonisation of a substratum in the aquatic realm has been viewed as going through a four-step process, viz. biochemically conditioning the surfaces, bacterial colonisation, diatom and protozoan colonisation and settlement of invertebrate larvae and algal spores (Wahl, 1997; Maki, 2002). This multistage process involves i) adsorption of dissolved organic molecules to a newly submerged surface, ii) colonisation of the surface by bacteria, iii) colonisation by microscopic eukaryotes (e.g. diatoms, fungi, and other heterotrophic eukaryotes) and iv) settlement and subsequent growth of invertebrate larvae and algal spores. This successional scenario is disputable with respect to the fact that ''uncolonised'' surfaces will rarely be available in natural situations. Instead, disturbances may provide opportunities for colonisers at any successional stage in the development of mosaic colonisation patterns (i.e. patch dynamics, see Wright et al. 2004).

A biofilm itself is distinguished from other types of microbial aggregations by its formation at interfaces (Costerton et al. 1995; Wahl, 1997; Maki, 2002). In marine environments biofilms mainly consist of bacteria and diatoms (Marshall et al. 1971). Microbial cells in biofilms are enmeshed in a matrix of extracellular polymers (EPS) that are mainly composed of high-molecular weight polysaccharides (Decho, 2000). The structure of biofilms is complex and three-dimensional (Stoodley et al. 2002). It had been proven that Gram-negative bacteria in biofilms produce cell-to-cell communication signals (*quorum sensing* signals) that accumulate to threshold concentrations and activate target genes, as well as having effects on bacterial biofilm formation (Davies et al. 1998; Parsek & Greenberg, 2000; Sauer et al. 2002).

A broad range of marine invertebrate larvae utilize biofilms as indicators of substratum suitability for prospective settlement (see reviews by Scheltema, 1974; Wieczorek & Todd, 1998; Railkin, 2004). Since the formation of biofilms on newly submerged substrata as a rule precedes colonisation by invertebrates, the establishment of microbial biofilms is regarded as a general prerequisite for the colonisation of macroorganisms such as invertebrate larvae and algal spores (Scheltema, 1974; Rodriguez et al. 1993; Burgess et al. 2003; Patel et al. 2004). The dynamics of biofilms mediate not only subsequent microbial colonisation but also macrofoulers. This also leads to

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a highly variable effect of biofilms on the colonisation of macroorganisms (Keough & Raimondi, 1996; Wieczorek & Todd, 1998; Maki, 1999). The physical properties of biofilms, biotic composition of biofilms, and accumulation of chemical compounds, as well as the dynamics of these parameters provide a discriminative mechanism in shaping biofouling communities including algal and invertebrate colonisation (Hodgson, 1990; James & Underwood, 1994; Wahl, 1997; Qian, 1999; Qian et al. 2000).

Natural and artificial substrata in the marine environment are quickly colonised by marine micro- and macroorganisms in a process known as ''biofouling''. Biofouling causes serious problems for marine industries and navies around the world (Yebra et al. 2004). So far, the most effective methods of biofouling control are based on the application of toxic substances like tributyl tin (TBT) (Rouhi, 1998), copper or organic compounds (e.g. Sea-Nine, Isothiazolone) (Thomas, 2001; Yebra et al. 2004). All these chemicals are toxic and are pollutants in aquatic environment (see reviews of Evans, 1999; Yebra et al. 2004). The International Maritime Organisation (IMO) and Marine Environmental Protection Committee (MEPC) decided to ban the usage of TBT or other substances containing tin as biocides in antifouling paints effective in January 2008. Therefore, there is a need for the development of ''environmentally-friendly'' non-toxic antifoulants.

Biofilms can enhance (Kirchman et al. 1982; Lau & Qian, 1997; 2001; Qian et al. 2003) or inhibit larval settlement of marine invertebrates and attachment of algal spores (Rodriguez et al. 1993; Egan et al. 2000; 2001; Dobretsov & Qian, 2002; Huang & Hadfield, 2003). Chemical compounds produced by bacteria and diatoms, as well as biofilms of live microorganisms can lead to the disruption of biofilm formation and/or prevent the epibiosis, and, therefore, they may be useful for the biotechnological development of an ''environmentally-friendly'' protection against marine biofouling (Clare et al. 1992; Holmstrøm & Kjelleberg, 1999; Armstrong et al. 2000a).

The application of natural products from marine organisms (algae, invertebrates, and microorganisms) as active ingredients in coatings that deter the growth and settlement of fouling organisms has been proposed since the 1980s (Mitchell & Maki, 1988). In laboratory bioassays, a wide variety of marine natural products from macroorganisms, such as macroalgae and invertebrates, that show activity against fouling organisms have been identified (e.g. Clare, 1998; Rittschof, 2001). The information about ''antifoulants'' from marine microorganisms, however, is rather limited in comparison with that from macroorganisms (Fusetani, 2004). In order to develop an effective ''environmentally-friendly defence'' against fouling it is necessary to understand how existing marine biofilms affect subsequent micro- and macrofouling and why some microorganisms are ''more effective'' in terms of antifouling protection than others.

This overview will focus on antifouling compounds produced by bacteria and diatoms, as these are the main components of biofilms in a marine environment and possibly show important effects on biofouling (Maki, 2002). Antifouling compounds from cyanobacteria were reviewed earlier (Abarzua et al. 1999), therefore, they have been excluded from this review. As this review covers only marine applications, microbial compounds against medical pathogens (Demain, 1999; Bush et al. 2004) are not included. The following objectives are particularly pursued here: i) to summarise the available information about the process of biofilm formation and production of antifouling compounds by marine bacteria and diatoms, ii) to evaluate the ecological relevance of antifouling compounds that are produced by marine bacteria and diatoms, and iii) to highlight future directions in the search of novel antifouling compounds and the development of new bioassay techniques.

## Antifouling activity expressed by bacteria and diatoms

### *Antimicrobial activity of bacteria*

In spite of much success in antibiotic drug discovery from non-marine microorganisms, marine microorganisms have received comparatively little attention in this regard (Faulkner, 2000). Several studies show independently that marine bacteria are capable of producing bioactive compounds that have not been isolated from terrestrial species (Burkholder et al. 1966; Fenical, 1993; Fenical & Jensen, 1993). Since the discovery of the first antibiotic compound produced by the marine bacterium *Alteromonas* sp. that was isolated from the Caribbean seagrass *Thalassia* sp. in 1966 (Fenical, 1993), the number of new compounds isolated from marine bacteria has increased substantially. Some of these compounds are already in pharmacological clinical trials and as biocontrol agents in aquaculture (Faulkner, 2000). Most metabolites with antibiotic effects were isolated from species that belong to the *Streptomyces*, *Alteromonas*, *Pseudoalteromonas* and *Roseobacter* clades (Fenical, 1993; Wagner-Döbler et al. 2002). For example, the actinomycete *Chainia purpurogena* SS-228 produces antibiotic benzanthraquinone (Okazaki et al. 1975). The bacterium *Streptomyces griseus* SS-20 produces antibiotic aplasmomycin that inhibits Gram-positive bacteria (Hotta et al. 1980). Antibiotic bonactin that inhibits the growth of

Gram-positive and Gram-negative bacterial strains, as well as yeasts was isolated from liquid cultures of another *Streptomyces* sp. (Schumacher, 2003).

Only a limited number of phylogenetic clades of marine bacteria have been screened so far with respect to their antifouling activity. The difficulty in the search of metabolites from marine bacteria is mainly due to the non-cultivability of bacterial strains. It has been estimated that 95% of marine bacterial strains are non-cultivable (Haglund et al. 2002). Extreme environments, such as deep-sea, hot spring environments and the external and internal surface of marine organisms are a less explored source for discovering new bacterial strains and new metabolites.

The following strains provide examples of marine bacteria associated with marine organisms that produce antibiotic substances. The bacteria *Pseudoalteromonas luteoviolacea*, *P. tunicata* and *P. aurantia* isolated from sponges and algae inhibited the growth of other bacteria from the marine environment (Holmstrøm et al. 2002). The antibacterial compounds produced by *P. aurantia* (Gauthier & Breittmayer, 1979), *P. rubra* (Gauthier, 1976), and *P. luteoviolacea* (Gauthier & Flatau, 1976) are of high molecular weight and inhibit bacterial cell respiration. Furthermore, the antibacterial activity displayed by *P. luteoviolacea* has been suggested to be attributable to two classes of compounds, viz. cell-bound acidic polysaccharides that are partly diffusible in a culture medium (Gauthier & Flatau, 1976) and high molecular proteinaceous compounds (McCarthy et al. 1994). Antibiotic activity being mediated by polyanionic molecules has also been demonstrated for the bacterium *P. citrea* (Gauthier, 1977). The antibacterial compound produced by *P. tunicata* isolated from the ascidian *Ciona intestinalis* is a high molecular mass protein (>190 kDa) consisting of 2 subunits (James et al. 1996). The mode of action of this protein has not been investigated, but the investigators proposed that it has bacteriostatic effects. The bacteria *Bacillus pumilus*, *B. licheniformis*, *B. subtilis* and *Pseudomonas* sp. isolated from algae and from a nudibranch produced antibiotic compounds that inhibited bacterial attachment (Burgess et al. 2003). Phenazine-1-carboxylic acid isolated from the epibiotic strain *Pseudomonas* sp. showed the highest activity with a minimal inhibitive concentration of 1–10 $\mu$g against different bacterial strains (Burgess et al. 2003). Several studies also showed antibacterial effects of streptomycetes associated with corals and jellyfishes (Fenical, 1993).

However, the true source of bioactive compounds from sponges, corals and other marine organisms remains unclear. For instance, 6-bromindole-3-carbaldehyde previously isolated from the ascidian *Stomozoa murrayi* can be produced by the epibiotic bacterium, *Acinetobacter* sp. associated with this ascidian (Olguin-Uribe et al. 1997). An anti-bacterial peptide that was originally isolated from the sponge *Hyatella* sp. is synthesised by an associated *Vibrio* sp. (Oclarit et al. 1994). In addition, bioactive metabolites isolated from the sponge *Theonella swinhoei* by Bewley et al. (1996) could be produced by microbial symbionts rather than by the sponge itself (Thakur & Anil, 2000).

A diverse array of antibiotic compounds can be produced by even phylogenetically closely related microorganisms and the production of bioactive compounds is often dependent on the medium. For instance, the bacteria *P. rubra* and *P. luteoviolacea* do not express any antibacterial activity when grown on blood-containing media. The expression of active compounds is very low in bacteria grown on nutrient agar and tryptic soy agar media (Gauthier, 1976). Similarly, the actinomycete strain number 18 isolated from sediments, produces antibiotics against Gram-positive bacteria only in the presence of salt at concentrations ranging from 60–110% (v/v) (Imada, 2005). Since the production of most, if not all, antifouling compounds takes place only under laboratory conditions, it remains unknown if bioactive compounds will be produced and be effective under natural conditions.

Bacteria in a biofilm can also affect the growth of other bacteria in the same biofilm (Burgess et al. 1999). For example, the presence of ''resident'' bacterial strains on particles either increases or decreases the colonisation rate of ''newcomer'' strains (Grossart et al. 2003). The marine bacterium *Alteromonas* sp. produces antibiotic 2-*n*-phenyl-4-quinolinol that alters the composition of the bacterial community developed on particles (Long et al. 2003). A comparison between strains producing antibiotics and their antibiotic free mutants showed no inhibitory effect of the newcomers due to the production of antibiotics. In the mixed-species biofilms, the antibiotics produced by the bacterium *P. tunicata* removed the competing bacterial strain unless its competitor was relatively insensitive to an antibacterial protein or produces a strong inhibitory activity against the bacterium *P. tunicata* (Rao et al. 2005). On the contrary, hydrolytic activity of the antibiotic producing strain enhances the colonisation rate of newcomers. The growth of a number of diatoms and other unicellular algae is also inhibited by marine bacteria in laboratory studies (see antialgal activity of bacteria, Holmstrøm et al. 1996).

Many marine organisms, such as the red alga *Delisea pulchra*, use *quorum sensing* inhibition targeting acetyl homoserine lactone (AHL) dependent signals to control epibiotic biofilm formation (Rice et al. 1999). This red alga produces furanones that interfere with bacterial *quorum sensing* signals and therefore inhibit

bacterial growth and biofilm formation. Similarly, marine bacteria may produce *quorum sensing* inhibitors that interfere with the formation of biofilms (McClean et al. 1997; Bauer & Robinson, 2002). For example, the bacterium *Aeromonas veronii* inhibits *quorum sensing* through competition for AHL production (McLean et al. 2004).

It must be pointed out that in most previous antibacterial bioassays pathogenic strains were used. In order to investigate the antifouling activity of bacterial isolates, it is necessary to use bacterial isolates from the same environmental situation in order to target ecologically relevant bacterial taxa. This issue is discussed in more detail in the section "A search for antifouling compounds".

## Antilarval activity of bacteria

Inhibitive bacterial strains for larval settlement are commonly found in seawater (Wieczorek & Todd, 1998). The relative proportion of inhibitive and inductive strains in biofilms varies with changes in environmental condition, species and the physical conditions of larvae used in bioassays (Hadfield & Paul, 2001; Lau et al. 2002; Lee & Qian, 2003). For instance, the marine bacteria *Halomonas* (*Deleya) marina* and *Vibrio campbelli* from natural biofilms suppress cyprid attachment of the barnacle *B. amphitrite* but the inhibitive effect of the bacteria varies with the age of the biofilm and the age of the cyprid larvae (see Table I, Maki et al. 1988; 2000). It was also reported that biofilms could remain inhibitive even in the presence of strong promoters for cyprid settlement (conspecific factor) of *B. amphitrite* (Rittschof et al. 1984; Lau et al. 2003). Although inhibitive compounds have not been identified, Maki and co-authors (1988) proposed that extracellular polysaccharides were involved; bacterial polysaccharides attached to lectin receptors of the larvae and thus, block larval settlement (Oclarit et al. 1994). In the natural environment, large variation in biofilm composition may lead to differential larval settlement (Keough & Raimondi, 1996; Qian et al. 2003).

Mary et al. (1993) studied the effects of bacterial strains isolated from biofilms associated with adult shells of the barnacle *B. amphitrite* on cyprid settlement and found that 12 out of 16 isolates were inhibitory to larval settlement. The Gram-negative and motile isolates were identified as belonging to the genera *Vibrio, Alteromonas, Alcaligenes, Flavobacterium* and *Pseudomonas*. The most inhibitory isolate was identified as a *Vibrio* sp. (Mary et al. 1993). Similarly, inhibitive strains were obtained from natural biofilms by Lau and Qian (2001) and Lau et al. (2003) (Table I).

Epibiotic bacteria associated with soft-bodied organisms could be a particularly useful source of antifouling compounds since they may protect their hosts from biofouling (Holmstrøm & Kjelleberg, 1999). Holmstrøm and co-authors (2002) investigated antifouling activity of 10 bacterial strains from the genus *Pseudoalteromonas* which were isolated from marine sponges and algae and found that four of them inhibited cyprid attachment of the barnacle *B. amphitrite*. The bacteria *P. citrea* and *P. ulvae* partially inhibited the larval settlement of *H. elegans,* while *P. tunicata* prevented larvae from attachment to

Table I. Antilarval compounds from marine bacteria.

| Bacterium | Inhibits | Inhibitive compound | Reference |
|---|---|---|---|
| *Alteromonas* sp. | Attachment of *B. amphitrite* | Ubiquinone | Kon-ya et al. 1995 |
| *Acinetobacter* sp. | Attachment of *B. amphitrite* | 6-bromoindole-3-carbaldehyde | Olguin-Uribe et al. 1997 |
| *Pseudoalteromonas marina* | Attachement of *B. amphitrite* and *Enteromorpha* sp. | Polar low molecular weight (>500 Da) heat stable compound | Holmstrøm et al. 1992; 1996; Egan et al. 2001 |
| *Pseudomonas citrea* and *P. ulvae* | Settlement of *H. elegans* and *B. amphitrite* | Unknown | Holmstrøm et al. 2002 |
| *Pseudomonas* sp. | Microbial fouling, attachment of algal spores and *B. amphitrite* | Phenazine-carboxylic acid; hydroxyphenazine; heptylquinol-one; nonylquinol-one; pyolipic | Burgess et al. 2003 |
| *Halomonas (Deleya) marina*, *Vibrio campbelli* | Attachment of *B. amphitrite* | Unknown exopolysaccharides | Maki et al. 1988; 2000 |
| *Micrococcus* sp., *Rhodovulum* sp. and *Vibrio* sp. | Attachment of *B. amphitrite* | Unknown extracellular polymers | Lau et al. 2003 |
| *Vibrio* sp. | Attachment of *B. amphitrite* | Unknown | Mary et al. 1993 |
| *Vibrio alginolyticus* | Attachment of *B. amphitrite*, *H. elegans*, and *B. neritina* | Heat stable, polar, high molecular weight (>200 kDa) carbohydrate | Dobretsov & Qian, 2002; Harder et al. 2004a |
| *Vibrio* sp. and an unidentified α-Proteobacterium | Settlement of *H. elegans* and *B. neritina* | Large (>100 kDa), polar, heat stable polysaccharides | Dobretsov & Qian, 2004 |

the surface (Holmstrøm et al. 2002). The antifouling effect of *P. tunicata* was attributed to its toxicity.

More recent studies confirm the antifouling activity of epibiotic bacteria from sponges, soft corals and green algae (Dobretsov & Qian, 2002; 2004; Dobretsov et al. 2004; Harder et al. 2004a; Lee & Qian, 2004). For instance, most of the bacterial isolates from the alga *Ulva reticulata* are either non-inductive or inhibitive to larval settlement of *H. elegans* (Dobretsov & Qian, 2002). The bacterial strains *Pseudoalteromonas* sp. and *Vibrio alginolyticus* isolated from this alga produce either non-soluble or waterborne metabolites that inhibit larval settlement (Dobretsov & Qian, 2002; Harder et al. 2004a). The most active antifouling compounds from *V. alginolyticus* are >200 kDa polysaccharides consisting of glucose, mannose, galactose and glucosamine (Table I). In a separate study, two strains out of 11 bacterial isolates from the soft coral *Dendronephthya* sp. were found to be ''inhibitive'', four strains ''inductive'', and the remaining five ''non-inductive'' for larval settlement of *H. elegans* (Dobretsov & Qian, 2004). There is no correlation between the antifouling activities of bacterial isolates and their phylogenetic allocation, i.e. closely related bacterial strains showing different effects on the larval settlement of *H. elegans* and *Bugula neritina*. In addition, inhibitive compounds from *Vibrio* sp. and an unidentified α-proteobacterium for larval settlement of *H. elegans* and *Bugula neritina* are waterborne, heat-stable polysaccharides with a molecular weight > 100 kD (Dobretsov & Qian, 2004).

One bacterium can produce more than one type of antifouling compound. For example, the pigmented bacterium *Pseudoalteromonas tunicata* (Holmstrøm et al. 1992) originally isolated from the surface of the ascidian *Ciona intestinalis* produces a polar, heat stable, low molecular weight compound (<500 Da) that is inhibitory to larvae of both barnacles and tunicates but acts in a toxic mode of action (Holmstrøm et al. 1992; Holmstrøm & Kjelleberg, 1999). This bacterium also produces an antibacterial compound (James et al. 1996) and compounds inhibitory to algae and fungi (Holmstrøm et al. 1996; Egan et al. 2001). Surprisingly, the antifouling activity of this bacterium is correlated with its colour (Egan et al. 2002). Usually, the bacterium is dark-green in colour. The yellow-pigmented mutant retains antifouling activity whereas the purple mutant loses some or all of its ability to inhibit larval settlement. Further analysis of these mutants identifies the genes that may be involved in the synthesis and regulation of synthesis of pigment with or without the antifouling bioactivity. One of these mutants was disrupted in a particular gene (WmpR), which has a similar sequence to the ToxR gene from *Vibrio cholerae* and the CadC gene from *Escherichia coli*. Analysis of protein expression using two-dimensional gel electrophoresis showed that WmpR is essential for the expression of at least 15 proteins important for the synthesis of antifouling compounds.

With respect to antifouling compounds from epibiotic bacteria of animate surfaces, it is not yet understood how bacteria protect their hosts and what kind of compounds they may produce under natural conditions. For instance, Harder et al. (2004a) did not detect bioactive bacterial metabolites that epibiotic bacteria produced under laboratory conditions in the *Ulva reticulata* habitat. This may suggest that either that the compounds released by bacteria under natural conditions are different from those produced under laboratory conditions, or the number of bacteria on the surface of the alga responsible for the production of antifouling compounds is low. This argument is partially supported by the work of Skovhus et al. (2004), who found that the relative abundance of antifouling bacterium *Pseudoalteromonas* spp. was the highest on the surface of the alga *Ulva lactuca* and lowest on the tunicate *Ciona intestinalis*.

So far, only a few anti-larval settlement compounds have been isolated and identified from bacteria. One example is provided by the marine bacterium *Alteromonas* sp. isolated from the marine sponge *Halichondria okadali* that produces the antifouling compound ubiquinone inhibiting larval settlement of the barnacle *B. amphitrite* at concentrations of 12.5–25.0 ppm and becoming toxic to larvae at concentrations >25 ppm (Kon-ya et al. 1995) (Table I). Similarly, an epibiotic bacterium, *Acinetobacter* sp., isolated from the surface of the ascidian *Stomozoa murrayi* produces 6-bromindole-3-carbaldehyde that inhibits cyprid settlement of the barnacle *B. amphitrite* at concentrations of 10 $\mu$g ml$^{-1}$ (Olguin-Uribe et al. 1997). Recently, Burgess and co-authors (2003) isolated 650 marine bacteria from different surfaces and screened them for their antifouling activity. They found that paints incorporated with supernatants of the inhibitive bacterium *Pseudomonas* sp. contained several antifouling compounds (Table I) that inhibited settlement of the barnacle *B. amphitrite* in laboratory experiments and the settlement of algal spores in field experiments.

Based on the above information, it would be argued that bacteria growing on ships' hulls may protect the hulls from biofouling (Wahl, 1997). To test this hypothesis, Holmstrøm et al. (2002) produced inhibitory ''living paints'' by incorporating live cells of the inhibitory bacterium *P. tunicata* into hydrogels and showed that ''living paints'' inhibited the settlement of barnacles for 14 d. Their results suggest that, given the right bacterium and the right matrix, inhibitory ''living paints'' that persist for

months or years may provide a reasonable antifouling biotechnology.

## Antialgal activity of bacteria

Only few studies report the antialgal activity of marine bacteria. For instance, the marine bacterium *P. tunicata* produces antialgal compounds that inhibit settlement of spores of *Ulva lactuca* (Egan et al. 2002). The antialgal compound is extracellular, heat sensitive, polar, with a molecular size between 3 and 10 kDa (see Table II), and consists of an aromatic domain bound to a fatty-acid side chain. The bacteria *Zobellia galactanovora*, *Pseudoalteromonas citrea*, *P. elyakovii* and *P. halopanktis* isolated from 1.5 h, 3 h and 48 h biofilms also inhibit spore attachment of the alga *Ulva (Enteromorpha)* sp. (Patel et al. 2004). 2-*n*-Pentyl-4-quinolinol produced by the marine bacterium *Alteromonas* sp. inhibited the growth of plankton diatoms at concentrations >10 nM (Long et al. 2003).

The strain Y of *Pseudoalteromonas* sp. produces water-borne algicidal compounds against the planktonic algae *Chatonella, Gymnodium,* and *Heterosigma* (Lovejoy et al. 1998). The antidiatom effects of the bacterium HYK0203-SK02 *Pseudomonas putida* were also shown to be effective against the pelagic diatom *Stephanodiscus hantzschii* and the cyanobacterium *Microcystis aeruginosa* (Kang et al. 2005). Lectins binding to D-galactose and mannan produced by the bacterium *Pseudoalteromonas* sp. were shown to reduce the ability of the diatoms *Amphora coffeaeformis* and *Navicula* sp. to adhere and to move (Wigglesworth-Cooksey & Cooksey, 2005). Most bacterial isolates from the alga *Ulva reticulata* inhibited the growth of the diatom *Nitzschia paleacea* but did not cause its mortality in a study by Dobretsov and Qian (2002). On the other hand, spores of the alga *Ulva (Enteromorpha)* sp. settle on biofilms of the bacterium *Vibrio anguillarum* that produces acetyl homoserine lactones (AHLs) (Joint et al. 2002). When Joint and his co-workers used mutant strains that cannot produce AHLs, bacterial films inhibited the attachment of algal spores. They concluded that the alga can detect concentration gradients of homoserine lactones, and argue that some bacterial strains can suppress the growth of Gram-negative bacteria that produce AHLs, which in turn, suppress the settlement of the macroalga *Enteromorpha* sp. Certainly bacterial antialgal activity in general deserves further studies.

## Antifouling activity of diatoms

In contrast to bacteria-derived cues, diatom-derived cues are less explored. Evidence could not be found that pennate diatoms produce antibacterial compounds, despite the fact that several diatom toxins and other forms of bioactivity have been discovered (Pohnert & Boland, 2002; Adolph et al. 2004; Wichard et al. 2005). A recent investigation shows that high abundance of the diatom *Asterionellopsis glacialis*, correlates with a low abundance of bacteria compared to nearby estuarine waters (Abreu et al. 2003). Decanal, produced by the diatom *Thalassiosira rotula*, inhibits the growth of the bacterial strains *Vibrio splendidus* and *Bacillus megaterium* (Table III, Adolph et al. 2004). Conversely, most of the investigations show that bacteria inhibit the growth of diatoms (see above).

On the other hand, there is only limited information about the effect of diatoms on larval development and settlement. For instance, aldehydes produced by diatoms are responsible for a suite of physiological dysfunctions during egg development, hatching and morphogenesis in copepod, sea urchin and oyster larvae (Romano et al. 2003; Adolph et al. 2004). The

Table II. Antialgal activity of marine bacteria.

| Bacterium | Inhibits | Inhibitive compound | Reference |
|---|---|---|---|
| *Pseudoalteromonas tunicate* | Settlement of *Ulva lactuca* | Heat sensitive water-borne (protein) 3 and 10 kDa | Egan et al. 2001; 2002 |
| *Alteromonas* sp. | Growth of *Thalassiosira weissflogii*, *Chaetoceros simplex*, *Cylindrotheca fusiformis* | 2-n-pentyl-4-quinolinol | Long et al. 2003 |
| *Zobellia galactanovora*, *Pseudoalteromonas citrea*, *P. elyakovii, P. haloplanktis* | Settlement of *Ulva* (*Enteromorpha*) sp. | Unknown | Patel et al. 2004 |
| HYK0203-SK02 *Pseudomonas putida* | Growth of *Stephanodiscus hantzschii* | Unknown, surface attached | Kang et al. 2005 |
| *Pseudoalteromonas sp.* | Growth of *Chatonella* sp., *Gymnodium* sp. and *Heterosigma* sp. | Unknown water-borne | Lovejoy et al. 1998 |
| *Vibrio sp.*, *Pseudoalteromonas* sp. | Growth of *Nitzschia paleacea* | Unknown | Dobretsov & Qian, 2002 |
| *Pseudoalteromonas* sp. | Adhesion of *Amphora coffeaeformis* and *Navicula* sp. | Unknown lectin binds to D-galactose and mannan | Wigglesworth-Cooksey & Cooksey, 2005 |

Table III. Antifouling compounds from marine diatoms.

| Diatom | Inhibits | Inhibitive compound | Reference |
|---|---|---|---|
| *Thalassiosira rotula* | Growth of *Vibrio splendidus* and *Bacillus megaterium* | Unsaturated aldehydes (decanal) | Adolph et al. 2004 |
| *Thalassiosira rotula* | Disruption of egg development and morphogenesis of *Sphaerechinus granularis*, *Crassostrea gigas* and *Calanus helgolandicus* | Unsaturated aldehydes (2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, 5*E*,7*E*-9-oxononadienoic acid, 4*Z*-decenal; 2*E*-decenal, decanal) | Romano et al. 2003; Adolph et al. 2004 |
| *Achnantes parvula* | Settlement of *Semibalanus balanoides* | Unknown | Le Tourneux & Bourget, 1988 |
| *Amphora tenerrima*, *Nitzschia frustulum* | Settlement of *H. elegans* | Unknown, non-water soluble | Harder et al. 2002; Lam et al. 2003 |
| *Nitzschia frustulum* | Attachment of *B. neritina* | Unknown | Dahms et al. 2004 |
| Monospecies biofilms | Settlement of *Haliotis rubra* | Unknown | Daume et al. 2000 |

larvae of *Semibalanus balanoides* tend to attach at high density to rough substrata bare of diatoms and detritus in field experiments (Table III, Le Tourneux & Bourget, 1988). Out of 32 diatom species isolated from Hong Kong waters, only three were highly inductive, 10 were weakly inductive, and 19 had no inductive effect at all on the larval settlement of the tubeworm *Hydroides elegans* (Harder et al. 2002). There is a clear negative correlation between the diatom cell- and extracellular polysaccharide-free space available and larval settlement (Lam et al. 2003). The diatoms *Amphora tenerrima* and *N. frustulum* cause low settlement of the polychaete *H. elegans* (Table III: Harder et al. 2002; Lam et al. 2003). Large carbohydrates ($>100$ kDa) in the biofilm matrix have a decisive role in the process of larval settlement of the tubeworm *H. elegans* (Lam et al. 2005). The absence of mannose, galactose and glucose in the extracellular polymers (EPS) matrix of diatoms causes low larval settlement of *H. elegans*. In a separate study, it was found that the diatom effect on larval settlement of the bryozoan *Bugula neritina* was lower in the presence of the diatom *Nitzschia frustulum* than in the control (seawater) and in the presence of other diatom species (Dahms et al. 2004) (Table III). Larval settlement of the mollusc *Haliotis rubra* is also lower on monospecies diatom biofilms than that on mixed biofilms of *Amphora* sp. and *Navicula* sp. (Daume et al. 2000). On the contrary, larvae of the abalone *H. laevigata* prefer to settle on diatom biofilms of *Navicula ramosissima* (Daume et al. 1999). In conclusion, the available information suggests that larval responses to diatom films depend on the diatom species present in biofilms, their ability to produce EPS, and the uncovered space available on a given substratum.

## A search for antifouling compounds

To gain a better understanding of chemical antifouling mechanisms of marine organisms in general, it is necessary i) to identify the settling preferences of common fouling species, ii) to be able to determine the concentrations of secondary metabolites that settlers would experience in the field and iii) to develop assay methodologies that deploy compounds in ecologically realistic ways (see Hay, 1996). However, from a biotechnological point of view, conclusive bioassay-derived evidence would be required that antifouling compounds are not active against non-target species, harmless to humans, non-polluting, and biodegradable.

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 (Rittschof, 2001). Natural products generally will not be available in sufficient quantity to be commercially harvested from marine macroorganisms. This holds particularly for natural resources that are rare or endangered. Moreover, most potent natural product compounds are structurally too complex to be commercially synthesised. Alternative compounds must have a potency that makes them applicable and the potential for being effectively synthesised. Possible ways to obtain adequate quantities of useful natural products include fermentation technologies and genetic manipulation (Clare, 1998). Incorporation of compounds that will individually be most effective against a particular group of fouling organisms in broad spectrum antifouling coatings may have some potential. From the perspective of potency for theoretical use in an antifouling coating, a vast majority of compounds exceeding the potency criterion should be active at $<25$ $\mu$g ml$^{-1}$ in static bioassays and have a therapeutic ratio of the effective median concentration to the 50% lethal concentration ($EC_{50}/LC_{50}$) larger than 10 (Rittschoff, 2001).

Many active compounds cannot easily be detected because they degrade during collection, storage, and extraction. Compounds that are stable in the cells of microorganisms can become labile following extraction (Fenical & Pawlik, 1991) and volatile

compounds may be lost during freeze-drying (Hay & Fenical, 1988). Extraction procedures that have not removed some of the bioactive compounds may also be responsible for screening differences. Unfortunately, little has been published regarding the effects of various extraction or storage procedures on individual compounds.

Concentration is a confounding factor in biofouling assays. At some exposure level, the same substance may be attractive, repellent, and even toxic. Hence, it is crucial to relate the response to a biologically relevant concentration (Steinberg et al. 2002). For bioassays in the field, an attempt must be made to reproduce the concentrations potentially experienced by biota in nature. However, when an organism is being tested, it is difficult to estimate ecologically relevant concentrations or to determine whether and to what extend these substances are released into the surrounding seawater, if the microscale localisation of metabolites is not known.

Considering the possibility of multifunctional effects of bioactive substances interacting with fouling processes, ideally, the compounds should be tested against several different naturally co-occurring organisms. This is of particular importance for antimicrobial bioassays if compounds are tested against cultivable pathogenic bacteria (Fenical & Pawlik, 1991). Also, the compounds will likely interact with the chemical-producing organisms under consideration in the natural environment. Therefore, the use of field assays becomes indispensable if it is intended to determine how specific or broadly active bioactive substances are, and where they are produced or applied later on (Hay, 1996).

In contrast to the rapid progress made in documenting the ecological effects of environmentally relevant bioactive substances, ecologically realistic demonstrations of semiochemical microbial activities are hampered by a lack of data on where microorganisms produce antifouling compounds and how the compounds are deployed under natural conditions (Thompson, 1985).

## Looking ahead

The studies reviewed here demonstrate that marine microorganisms, such as bacteria and diatoms, provide a significant source of new antifouling compounds. Although there are numerous examples of compounds being extracted from marine microbes which have antimicrobial or antitumor properties (Demain, 1999), there is little information on antifouling compounds from marine microorganisms. Furthermore, bioactive compounds from marine bacteria are far better investigated than from diatoms and other marine microalgae in general. Therefore, marine diatoms and other marine microalgae may hide a high potential for antifouling compound production.

There are a number of benefits associated with using microorganisms as sources of antifouling compounds. The first advantage is the relief of problems relating to compound supply. For the extraction of an antifouling compound from a marine organism, large numbers of animals or algae would have to be collected. In contrast, bacteria described in this review can be easily cultured. Another advantage of using microbes as a source of antifouling compounds is that microorganisms can produce compounds much more rapidly and in large amounts compared to invertebrates and algae. By analogy to biomedical applications, antifouling molecules from microorganisms may be easily genetically and chemically modified (Demain, 1999). In addition, bacterial strains of the same species can produce different bioactive compounds under different culture conditions, therefore, increasing the potential number of useful compounds (Armstrong et al. 2000a). Many microbes do not produce compounds in single culture; therefore co-culture of microbial strains with target marine biofilms may also increase antifouling production (Mearns-Spragg et al. 1998). Since only a small proportion of microorganisms have been screened for the production of antifouling compounds, much research has to be done in order to isolate and culture these organisms to fully utilise their potential.

The surfaces and internal spaces of marine organisms, such as sponges, are unique microhabitats where bacteria are regularly detected. These environments contain more nutrients than the ambient seawater and sediments and thus provide a rich source for the isolation of diverse bacteria with diverse metabolic capabilities and potentials. As more evidence is obtained, it is also becoming clearer that bacteria associated with macroorganisms may have highly specific, symbiotic relationships with their ''hosts''. In many cases, they may be considered as an important source of antifouling compounds. Since most of them are not cultivable under ''standard'' conditions, it becomes necessary to develop new methods, such as using special media for cultivation or by the transfer of target genes into cultivable species that allow the production of bioactive compounds from such ''uncultivable'' species. For example, the uncultivable symbiotic bacterium *Endobugula sertula* has been shown to be the true source of bryostatin – a potent antitumor compound – by cloning and expressing the target genes responsible for the production of this compound by the epibiotic bacterium on the bryozoan *Bugula neritina* (Hildebrand et al. 2004). An alternative to this approach is to demonstrate that certain biosynthetic machinery resides in symbiotic

microorganisms. For example, the application of nucleotide probes for biosynthethic genes may confirm their presence in the uncultiviable symbiont. Real time PCR (RT-PCR – see Heid et al. 1996) can be used for the analysis of bacterial species abundance associated with hosts (Skovhus et al. 2004), as well as for the investigation of the expression of functional biosynthetic genes *in situ*. It will particularly be the source allocation of a compound under natural conditions that will prove the obligatory association of a host with such microorganisms.

It has been demonstrated that some bacteria can produce a set of different antifouling compounds targeting different groups of microorganisms and macroorganisms (Holmstrøm & Kjelleberg, 1999). Bacteria that are capable of producing a broad spectrum of antifouling compounds are promising sources of biotechnologically interesting substances for ''environmentally-friendly'' antifouling applications. Searching for bacteria that interfere with other microorganisms *via* the production of antibiotics or inhibitors of *quorum sensing* signals shall be an important future research direction. Those promising bacterial candidates will not only inhibit the formation of biofilms and the growth of microfoulers directly, but also affect the settlement of invertebrate larvae and algal spores indirectly in terms of mediating properties of microbial communities (Jin & Qian, 2005).

In most studies, antifouling activity of microorganisms is tested for several target fouling organisms while in antibacterial bioassays the organisms tested are predominantly pathogenic strains. Practically, prospective antifouling compounds should prevent fouling of all fouling taxa, not just of pathogens and dominant foulers. More importantly, it is necessary to test antibacterial properties of compounds against environmentally relevant microorganisms under natural conditions because most of them are uncultivable. In the authors' laboratory, compounds are incorporated into Phytagel$^{TM}$ (Sigma, USA) matrices and then such gels are exposed for microbial settlement under natural conditions (Harder et al. 2004b). This method allows not only consideration of the abundance of bacteria on gels but also characterisation of differences in bacterial community composition by utilising molecular finger printing methods, such as terminal restriction fragment polymorphism (T-RFLP) (Liu et al. 1997).

Most antifouling compounds tested in laboratory assays are adsorbed to glass or simply added to a dish containing test organisms. Therefore, it is also necessary to investigate the performance and effectiveness of antifouling compounds included in a potential paint coating under natural conditions. It will be important to consider publications where investigators have used ship paint matrices for their studies (i.e. Willemsen & Ferrari, 1993; Railkin & Dobretsov, 1994; Armstrong et al. 2000b; Burgess et al. 2003). Because the composition of paint matrices is important for the effectiveness of antifouling compounds, cooperation between academic institutions and the industry should be fruitful (Rittschof, 2000).

Previously, it was suggested that in contrast to inducers, deterrents appear to be primarily nonpolar secondary metabolites with low solubility, being effective only at low concentrations (Steinberg et al. 2001; 2002). Recent findings demonstrate that some bacteria can produce large (>100 kDa) waterborne, polar compounds that act as antifoulants (Dobretsov & Qian, 2002; 2004; Harder et al. 2004a). Antifouling compounds could be particularly large carbohydrates from bacteria that mainly accumulate in the bacterial film matrix, and are released to boundary layers only at low concentrations. Therefore, the dispersal kinetics of water-borne biofouling microbial metabolites will be another important future research direction.

Overall, marine bacteria could provide a source of biologically active metabolites for the antifouling industry and other biotechnological applications. With the improvement of isolation and cultivation techniques and the employment of molecular tools for uncultivable strains, it may be possible to detect more isolates, to identify more novel antifouling compounds and to engineer ''environmentally-friendly'' biotechnologies against biofouling.

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