
Bacterial Sensors in Microfouling Assays

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1. Introduction

1.1. Fouling, the undesirable load

A lot of the marine invertebrates have a planktonic larval stage, in this period the larvae are dispersed and transported by currents. When larvae mature and attain the ability to metamorphose, they start looking for suitable substrates, swimming toward the bottom and exploring the surfaces. When larvae encounter suitable substrate, they settle and metamorphose into juveniles; the survival of them is heavily dependent on where they settle. On the other hand, larval settlement and metamorphosis are influenced by local factors as salinity, temperature, light, kind of substrates, larval age, and nutritional conditions of larvae. However, one of the most important factors for settlement is the presence of chemical tracks originated from nonspecific adults and prey organisms. Microbial films are included also in those kinds of tracks and induce differentially larval settlement and metamorphosis in many invertebrate species; unfortunately, these bacterial biofilm factors have not been fully characterized [1]. The first biofilm formed on a surface, the settlement and the following steps of biological colonization are known as fouling, which could be defined (since an industrial point of view) as the undesirable accumulation of dissolved chemical compounds, microorganisms, algae and animals on submerged substrates leading to subsequent bio-deterioration of the colonized surface.

The fouling process is an ecologically complex of interactions between basibionts, surface-colonizing microbes, and fouling larvae, all mediated by chemical signaling. The assessment of fouling organism over basibionts can have severely deleterious effects on them such as inhibition of photosynthesis, blockage of filter feeding, and elevated risk of mechanical dis-

lodgement or predation. In this scenario, competition for space represents ecological forces comparable to predation, because the space is a limited resource in the ocean; therefore, marine invertebrates have to compete for their place on a surface. Consequently, sessile invertebrates establish evolutionary weapons to colonize: when they are larvae, they must locate and colonize a surface in order to colonize and metamorphose; but when they are adults, they have to keep their own surfaces clean and ward off settlement by larvae [2].

Nowadays, the biofouling has been understood as a four-step sequential process. The initial step consists in the adsorption of organic macromolecules, it occurs almost immediately after submersion of any surface, and is characterized by the formation of a film composed by proteins, glycoproteins and polysaccharides, this film is colonized subsequently by bacteria. The second step, which occurs within an hour of surface immersion in water, is characterized by the assessment of prokaryotes and the subsequent development of a bacterial biofilm. Once the bacterial attachment to the surface has occurred, bacterial cells begin producing a matrix of extracellular polymeric substances (EPS) that is critical for maintaining adhesion and subsequent biofilm development and consolidation. Those biofilm are bacterial communities assembled coordinating different phenotypes that change with the time and the environment, and depend of different factors that influence, as well, further colonization of a surface. Recently, Quorum Sensing (QS) has been recognized as one of the main factors that determinate biofilm maturation, and this is perhaps the strongest determinant for the establishment of a proper biofilm. This phenomenon is defined as the regulation of gene expression depending on the bacterial population density and allows the synchronization of phenotypes by bacterial communication. It is important to explain briefly, how this QS regulation works (Figure 1). Basically, it works through a genetic circuit compose by a transcriptional factor (LuxR) and an acyl homoserin lactones (AHL's) synthetase (LuxI). The accumulation of AHLs in the media, due to the amount of bacterial cells preset in a culture, leads to the expression of the genes regulated by LuxR; some of them involved in biofilm maturation (for review see: [3]).

The third step in fouling formation is the colonization by unicellular eukaryotes; these include photosynthetic taxa such as diatoms, and heterotrophic suspension feeders and predators. The final step is the attachment of propagules of multicellular organisms, invertebrate larvae and algal spores, the predominant organisms differ in temperate zones and the tropics, season and other local conditions. It is now recognized that the nature of biofilms varies widely, and can present a range of positive and negative stimuli to settling larvae. Larvae may respond in the water column to chemical cues emanating from the substratum, and/or upon contact to physicochemical and biological characteristics of the substratum and elect to settle or reject the surface. In this context, is not surprising that the host-specific bacterial communities are maintained by many invertebrates, and may inhibit fouling by chemical deterrence of larvae, or by preventing biofilm formation by inductive strains. Finally, the larval settlement naturally occurs in a turbulent environment, yet the effects of waterborne versus surface-adsorbed chemical inductors and/or defences have not been completely understood [1].

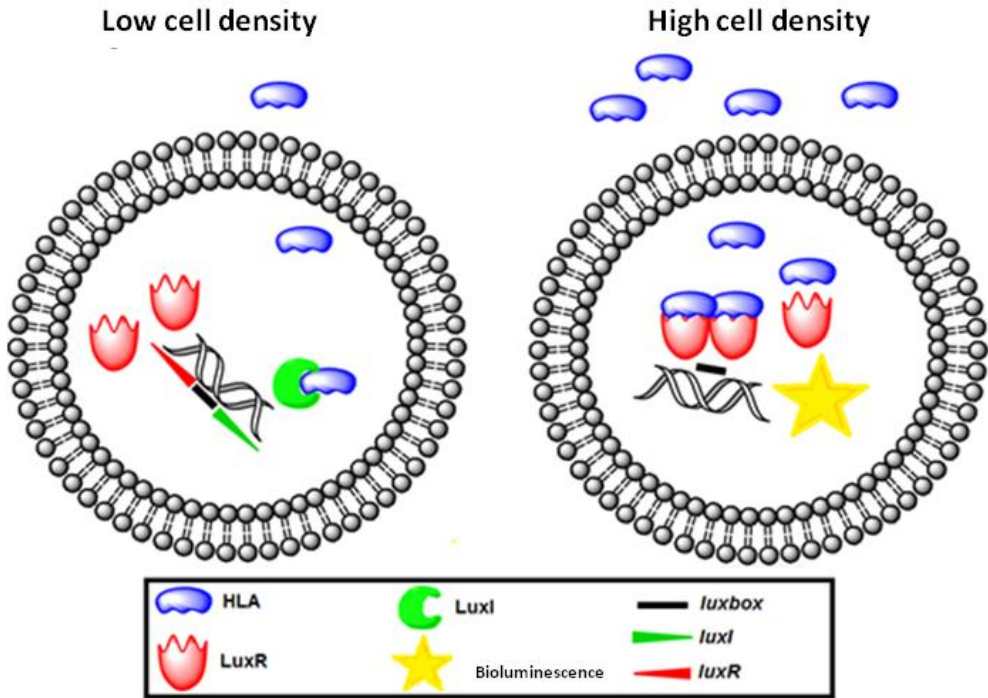


Figure 1. LuxR/I system in *V. fischeri*. QS is defined as the gene expression regulated by the population density. The *luxI* gene encodes the AHL's synthetase. The AHL's molecules works as an "auto-inductor" that can diffuse freely through the cell membrane, and when it is accumulated up to a specific threshold it binds to the LuxR protein, a transcriptional factor. The complex LuxR-AHL activates the transcription of genes regulated under the *luxbox* promoter, in this case those responsible of bioluminescence.

To sum up, we can distinguish within the fouling structure two levels of organization, the microfouling and the macrofouling. The first one rules the second, and nowadays the efforts to control the fouling phenomena are concentrated in biofilm control, because its inhibition, by the use of quorum sensing inhibitors, could avoid the macrofouling assessment.

The fouling has been identified as a cause of severe problems in different scenarios, e.g., it is estimated that fuel consumption of ships increases 6% for every 100 of hull roughness caused by fouling organisms. Another example is the higher frequency of dry-docking operations required or the invasive species that can be spread inadvertently by fouled ships. Therefore, inadequate protection against fouling is a consequent threat to marine ecosystems with incalculable damage, and just for the shipping industry the cost could be estimated in several billion of dollars [4]. Other industries could be affected by this phenomenon, i.e. marine industries as gas and petroleum exploitation, aquaculture, cooling towers, drinking water distribution systems, building materials, etc; and hull fouling is also a major vector for marine invasive species. The fouling problem is quite important for sensor devices,

particularly in marine and riverine sensors [5]; even more, not only the bacterial and marine invertebrates are challenging for antifouling technologies the blood cells are too [6]. In order to deal with this problem the use of antifouling (AF) paints has emerged as the most useful solution; antifouling paints contain biocides that are released during the lifetime of the coating; these biocides are present within a surface micro-layer of water adjacent to the paint surface, avoiding the settlement of juvenile fouling organisms. Due the great number of organisms involved on marine fouling the biocides used in antifouling paints must have a wide spectrum of activity to be able to deter the colonizer organisms on the ship's surface. So, the antifouling products play an important role in the shipping industry and are of significant economic importance [4].

The use of antifoulants to protect the boats is not a new concept; furthermore, the Romans and Greeks coated their boats with lead sheathing. In the discovery and colonization of America the vessels were coated with pitch and tallow, and the British Empire used as antifouling paints grease, sulphur pitch and brimstone, and later copper sheathings were used. Finally, in the mid-1800s the antifouling paints were developed, because with the introduction of iron ships the copper sheathing caused corrosion of the iron, and new formulation were necessary. These antifouling paints included a lot of toxic compounds as copper oxide, arsenic, and mercury oxide to resin binders. After Second World War, the synthetic copper based paints became most popular until the tributyltin (TBT) proved to be excellent in the prevention of fouling [7]. A lot of advantages were attributed to TBT antifoulant, e.g. it exhibits broad-spectrum biocidal properties, and is effective against most of the colonizing organisms. It could be incorporated into coloured paints because it does not have color, and does not promote galvanic corrosion on iron ships; furthermore, it can also be used on aluminium surfaces. Due it is an organic compound it can be co-polymerised into resin-based paints, and incorporated into self-polishing coatings that remain effective for long periods of time [4]. However, nowadays it is known that organotins, such as tributyltin (TBT) and tributyltin oxide (TBTO), are the most toxic biocides ever introduced to the marine environment [8] because they have important deleterious biological effects over a great number of marine organisms, a lot of them non-target marine organisms, e.g. tissue's analyses of sea mammals, fish and some birds have revealed detectable concentrations of TBT. In this way, the International Maritime Organization (IMO) adopted an international treaty entitled "*The International Convention on the Control of Harmful Anti-Fouling Systems on Ships* in 2001", which banned the presence of organotin compounds in antifouling paints by 1 January 2008 [4].

The prohibition of TBT-based paints forced to develop alternatives that includes booster biocides, like irgarol 1051, sea-nine 211, dichlofluanid, chlorothalonil, zinc pyrithione, diuron, TCMS pyridine, TCMTB, zineb, etc. These biocides are being used in many countries, but they have been found to accumulate in coastal waters and have become a threat to the marine environment as well. These alternatives to TBT are also toxic and their putative impact on non-target organisms is poorly known in some cases, for that reason their contamination in the aquatic environment has been a topic of increasing importance over the last few years, interesting review could be consulted in Antifouling Paint Biocides [7] or Ecotoxicology of

Antifouling Biocides [4]. Nowadays, some countries have signed an agreement to restrict the use of Irgarol 1051 and diuron biocides [7,8].

In this context, the developing of “environmentally friendly” antifoulants is an urgent necessity, which include fouling-release coating and electrical antifouling systems; however, many researchers are trying to employ chemical defense systems from sessile marine organisms for this purpose [8]. These natural compounds incorporated into a painting would mimic the marine organisms, which keep their body surfaces clean due to the natural production of antifouling substances with high anesthetic, repellent, settlement deterrent, and settlement inhibitory properties, but without having biocidal effects. Compounds with different structures have been identified as antifoulants, and include terpenes, nitrogen-containing compounds, phenols, steroids and others. Additionally, mixtures of natural products could be useful taking advantages of the synergistic properties observed; it is expected this mixture to be much better antifoulants than the organotin compounds, see [9]. Dr. Fussetani has presented several reviews about the use of natural products as antifoulants, where different compounds have been identified as the most promising natural product for the antifouling paints. To mention some examples: the sesquiterpene elatol isolated from red alga *Laurencia elata*, the furanones isolated from the red alga *Delisea pulchra* with, some isocyanoterpenes isolated from the sponge *Acanthella cavernosa* and from the nudibranchs *Phyllidia pustulosa*, *P. ocelata*, *P. varicosa* y *Phyllidiopsis krempfi*, and the 5,6-Dichloro-1-methylgramine (DCMG) inspired in the natural product 2,5,6-tribromo-1-methylgramine.

There is, however, one drawback; the known supply problem for Marine Natural Products (MNP), that has to be overcome in order to apply these products in antifouling technologies. In this context, bacteria and fungi are promising sources, and more efforts towards the development of antifouling compounds from marine microorganisms should be made [1,8,10].

On the other hand, current antifouling paints are not effective against microfouling colonization (bacterial and diatom species) because the microorganisms have the ability to colonize entire surfaces previously treated with common antifouling paints. Therefore, the development of new compounds to regulate the density of microbes on antifouling coatings is urgently needed. In this context, the Quorum Sensing Inhibitors (QSI) are a new alternative, and these compounds can be used too for antimicrobial protection in aquaculture, and even more in the control of medical caterer biofilm development. Dobretsov *et al.* [11]. established the ability to prevent microfouling by the kojic acid in a controlled mesocosm experiment. This acid inhibited formation of microbial communities on glass slides, decreasing the densities of bacteria and diatoms. The study suggested that natural products with QS inhibitory properties can be used for controlling biofouling communities [11-15].

2. Common bioassays used in the evaluation of chemical compounds as candidates to combat the microfouling

As it was mentioned before the fouling is a natural process of colonization of submerged surfaces, involving a wide range of organisms from bacteria until invertebrates. Due to the

diverse range of organisms involved, there are not specific assays that may show the antifouling potential of a compound, moreover the latest publications agree that no single substance could inhibited the settlement and growth of all the organisms implicated in the marine fouling. However, several bioassays have been developed to determine whether natural products inhibit specific organisms known to be involved in the microfouling process, mainly directed to understand the influence of the initial colonization by bacteria (microfouling) on the subsequent settlement and growth of macrofouling.

Because it is necessary to screen plenty of candidates in order to select the most promising among them, the identification of effective biocides and coatings requires laboratory tools development. The direct evaluation of the candidates in field conditions demands great amounts of each compound, and these assays are affected in an uncontrolled way by numerous factors, including the season, weather etc, so a previous selection is strongly recommended, and the most recommended way is the use of laboratory test. As a consequence, a number of laboratory-based AF assays have been developed in recent years; however few compounds have been tested in field assays or in moving water, which is needed to evaluate the ecological role of a putative antifouling compound. The test could be grouped in three main groups according with the target involved, microfoulers, macrofoulers and enzymes [16].

Because the wide diversity of organisms involved in the fouling process, several different AF targets (organisms) are required for proving the antifouling activity of a particular compound. So, a wide range of test organisms has been used in AF bioassays; however the selection of the target most be done according with the answer to be solved. E.g. For understanding the ecological process must be selected as target organisms those that reflect the potential micro- or macrofoulers of the studied species; for the discovery of new biocides, to be used in AF paints, the AF assays are conducted using the dominant fouling species in the area (marine bacteria, diatoms, algae, mussels and barnacles). However, the difficulty and cost of culturing higher benthic organisms has influenced the final choice of test organism. Some bioassays use a single species of micro- or macrofouler, but for microfouling assays, mixed consortia are sometimes used. In this sense, the most used microorganisms are strains of bacteria, isolated from marine biofilms, especially these pioneer strains since marine bacteria are relatively easy to isolate and maintain. Some diatoms have been also widely used for bioassays, and it is important because many AF coatings fail against microalgal slimes dominated by diatoms. Marine fungi have been used in a few studies, because they are not in the main group of fouler organisms. On the other hand, the most important macroorganisms involved in ship shield colonization are the barnacles; so on the most popular test is involved cypris of the subtropical barnacle *Balanus amphirite*. The second group of macrofouling organisms of importance in terms of the number of publications is the mussels, *Mytilus* spp are the most used for bioassays [16].

Growth inhibition test as microfouling assays could be done both disc diffusion and liquid media; however these assays are not so relevant because the fouling involves biofilm, and these assays do not take account this fact, furthermore, it is well known that the sensitivity of microorganisms growing in a biofilm is lower than those in planktonic culture. On the

other hand, these tests could be automated and the compounds evaluated rapidly in multi-well plates by measuring the change in turbidity. E.g. For diatoms, the inhibition of growth can be evaluated in liquid flasks by measuring absorbance or chlorophyll a concentration. The attachment assays (Bacteria and Microalgae) are another test to evaluate microfouling activity; however, most of them generate an *in vitro* biofilm under static water conditions, with a few exceptions using flow chambers, and those conditions do not reflect the real fouling conditions. Additionally, a bacterial multispecies biofilms reflect in the best way the natural conditions because the role of synergy in the resistance to antimicrobial agents. Finally, nowadays it is recognized the fundamental role of Quorum Sensing in the biofilm consolidation and in the fouling process, due this fact the QS inhibition assays are being conducted to determinate the antifouling potential of pure compounds [8,16].

3. Asking for antifouling molecules with the use of bacterial sensors

QS inhibition has been a strategy of algae, animals, plants, bacteria and other microorganism to control its own population and to synchronize the expression of different phenotypes in a community. QS can be inhibited in several points of the communication circuits. The most used inhibition mechanisms are degrading the signaling molecules or competing with the signaling molecule for the binding site in the regulatory protein. Some bacteria have useful phenotypes regulated by QS, for example bioluminescence, or different pigments as violacein, these traits can be exceptional reporters for QS inhibition. Some of these genes have been used in synthetic biosensors to study with a molecular sight the mechanisms of QS inhibition. Those systems are the common tool to evaluate the activity of new molecules using them in simple Petri dishes assays. In this section we introduce our current classification of QS inhibition biosensors and the next generation of biosensors made to order.

3.1. Searching molecules into a complex ecosystem

In a complex social-competitive environment, organisms have developed several mechanisms to control their own populations; one of them is the inhibition of QS, a phenomenon called quorum-quenching (QQ) [30]. Many quorum-quenching molecules have been identified since the 2000 when Rasmussen and Co-workers reported the halogenated furanone produced by the seaweed *Delisea pulchra* (see [17,18]). They show how this molecule and some derivatives could affect the QS and the swarming motility in *Serratia liquefaciens*. Some of the examples are previously mentioned in this chapter, however it is important to mention that moreover that secondary metabolites there are other examples of QS inhibitors including AHL-lactonases, AHL-acylases and paraoxonases (PONs), which degrade AHL signals [19]. *Bacillus sp.* strain 240B1 produces an AHL-lactonase metalloprotein, encoded by the *aiiA* gene, able to attenuate the virulence of *Erwinia carotovora* [20], even many species of *Streptomyces* have been reported to encode AHL-acylases able to degradate AHL signals, decreasing the production of virulence factors such as elastases, proteases and LasA in *P. aeruginosa* [21]. Currently, the use of quorum-quenching molecules could be applied to the

control of AHL-mediated pathogenicity and biofilm formation as has been proposed by Park and Co-workers [21].

Because of its own wide-diversity, searching of quorum-quenching molecules in the environment is a complex procedure. Different aspects must be considered, for example the complexity of the holobiont and the conditions that should be carefully selected depending on the organism that may be producing the molecules. In fact, the biggest problem is to be sure of the origin of such a molecule in a complex sample where bacteria, microalgae, fungi, invertebrates among other organism could be included and responsible of the activity. Consequently, the current challenge is to design a wide-ranging method that allows sensing these molecules avoiding the conflicts caused by the origin. The main problem after defining the chemical extraction required and the amount of sample according to the producer organism is it to find a wide detection method for the bioactivity.

Since years, biosensors have been used for the detection of QS activity, induction or inhibition, and recent studies suggest that the biosensor assays in the simple Petri dishes methodology are the best way, up to now, to detect such molecules. Some of the biosensors used as tool for quorum-quenching detection are wild type strains with reporter phenotypes that can be inhibited by the selected substance or could be mutant biosensors with the AHLs synthetase disrupted and a reporter gene induced by synthetic AHL's; this signalization is then impaired by the molecule being tested [22,23]. The main goal now is to find a wide range of detection because usually the biosensor strains have a specific LuxR type protein that can detect a specific range of AHL's.

3.2. Kind of quorum-quenching biosensors:

We define two main approaches to identify quorum-quenching molecules using the biosensors mentioned above: **(1)** Inhibiting a certain phenotype under the regulation of QS. **(2)** Disrupting the induction of QS regulated reporter gene in a biosensor strain stimulated with foreign AHL's. Both approaches are illustrated in the Figure 2.

3.2.1. Inhibiting a certain phenotype under the regulation of QS

The best example for the first approach is the inhibition assay using the *Chromobacterium violaceum* wild type as a biosensor strain. This strain is able to produce the pigment Violacein under the regulation of QS when a quorum-quenching molecule is present in the medium. *C. violaceum* is not able to produce the pigment (Figure 3).

This approach use wild-type strains able to express phenotypes regulated by QS, some of the most used phenotypes are bioluminescence from *Vibrio* sp, pigments from *Serratia marcescens* or *Cromobacterium violaceum* or antibiotics from *Erwinia carotovora* among others. These phenotypes are multi-factorial traits, which mean there are many genetic and metabolic determinants involved. Consequently special condition should be provided in order to test the quorum-quenching activity of a selected molecule (Figure 3, left).

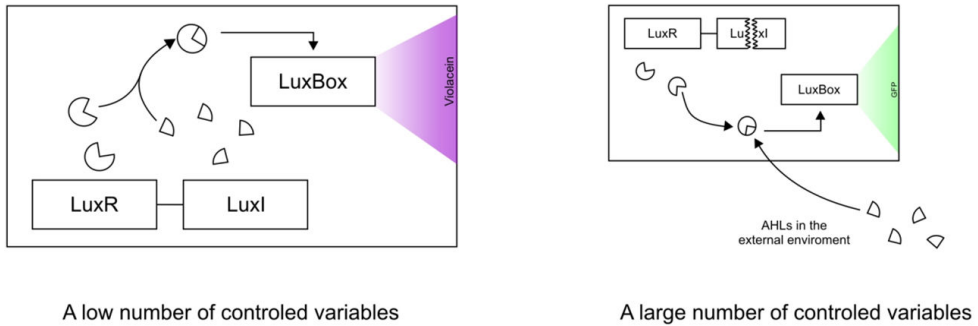


Figure 2. Approaches to identify quorum-quenching molecules. Left: Inhibiting a certain phenotype under the regulation of QS. This approach use wild-type strains able to express phenotypes regulated by QS. Right: Disrupting expression of a reporter gene regulated by QS in a biosensor mutant. This approach use biosensors strains designed by transformation with synthetic plasmids. The arrows represent potential targets for quorum-quenching molecules: Left: their own wild-type regulation circuit. Right: the communication between strains, the reporter system and the stimulatory system.



Figure 3. Inhibition of Violacein in *Chromobacterium violaceum*: Right: control disk with no QS inhibition halo. Left: Disk impregnated with a QSI compound (results under publication)

3.2.2. Disrupting the induction of QS regulated reporter gene in a biosensor strain stimulated with foreign AHL's

The second approach uses biosensors made with synthetic plasmids; those are more stable and can be easily manipulated. The biosensors mutants in the Quorum Sensing systems (mainly from Gram-negative bacteria) have been a helpful tool to analyze the communication system present in a complex environment sample. Therefore, a large number of strains, genetically transformed with truncated QS circuits, have been developed (for review see [22]). These mutants require AHLs in the media to induce the reporter phenotype regulated by QS, consequently the media to test the quorum-quenching activity must be supplemented with the respective AHL's. However, it should be mention that the QS mutated strains have lots of problems with the laboratory maintenance and the growth conditions.

These transformed biosensors can be classified in two kinds concerning the response level of the reporter gene:

- a. The first one is the plasmid-based biosensors of overall population response, the best example are the plasmids pSB403 and pHV200I designed by Winson *et al.* 1998 [23]. Both used the system LuxR/I able to sense the molecule 3-oxo-C6HL. The strains transformed with this plasmid are able to sense the 3-oxo-C6AHL molecule in the growth media and induce the expression of the reporter system LuxCDABE. This reporter system induces the expression of bioluminescence over all the population and the bioluminescence is the summatory of the entire bacterial culture.
- b. The second type is the plasmid-based biosensors of single cell response, the best example is the plasmid pJBA-132 designed by Andersen *et al.* 2011. This plasmid used also the system LuxR/I but with the reporter gen *gfp* (green fluorescent protein). Certain bacterial strain transformed with this plasmid is able to sense, as well the 3-oxo-C6AHL (because it has the same LuxR protein), in the growth media and induce the expression of GFP. The fluorescence of GFP can be determined in a single cell and therefore it can be quantified individually into a population or community.

Additionally, plasmid-based biosensors can be classified regarding the kind of promoter used. These can be constitutive promoters (for example, the plasmid pSB403) or inducible promoters (pHV200I). The use of inducible promoters allows the directly manipulation by activating certain biosensor as desired. The most common used promoter is the PlacO1 promoter. Despite the role of PlacO1 promoter in lactose catabolism has been widely described, we propose this promoter as a mechanism to switch between an activated or inactive state at the genetic level of expression of certain reporter system. PlacO1 promoter regulation mechanism works as a response of environmental lactose levels. In an environment without lactose the protein LacI repressor binds to operator region of lac operon inhibiting the transcription of genes under its control (inactive biosensor). On the other hand, in an environment with lactose or analogous (IPTG), this can bind to LacI to assemble a complex Lactose-LacI and it is possible to induce the expression of genes (active biosensor). We used this mechanism to switch our biosensor strain whenever we want it. These biosensors are used in the screening of quorum-quenching molecules adding in the media the respective AHL molecule to produce the specific stimulus. The use of plasmids with designed circuits improves the knowledge of the variables that should be controlled in the experiment, in contrasts with the wild type-strains biosensors mentioned above (Figure 3, right).

Nowadays, it is possible to select the biosensor following the questions we have for the environmental sample more over a Synthetic Biology approach through successive steps of *in silico* design, *in vitro* construction and *in vivo* expression is a very useful tool to allow scanning and testing quorum-quenching molecules in an artificial communicational environment.

3.3. The next generation, biosensors made to order:

As an interesting perspective, we could select our desired biosensor-stimulatory environment from parts assembly by Synthetic Biology approaches to test quorum-quenching molecules.

Dubrin *et al.* 2007 [24] define Synthetic Biology (SB) as a variety of experimental approaches with the aim to mimic or modify a biological system. This recent field of research has been widely studied [25-27]. A model to test quorum-quenching molecules through SB may be constructed following two main steps: (1) design and (2) manufacturing. Heinemann *et al.* 2006 [28] reported a deep description of each step.

For the particular case of systems of QS, a large number of studies have established a pattern of interactions, levels of regulation, transcription rates and other parameters relating to components of these networks [3,29,30]. This allows us to meet one of the main requirements in SB, in which you have sufficient data to perform a mathematical and computational modelling of various QS systems based on the parameters reported in the literature in order to predict the behaviour of the entire network under certain events that can be simulated (e.g. lactonase activity, AHL acylases concentrations, a mutation in the gene, response to external signals, receptor competition, etc).

The assumptions and standard parameters to be used when performing a computational or mathematical modeling of a QS system have been clearly described in the work of Garcia-Ojalvo, *et al.*, 2004 [31], McMillen *et al.*, 2002 [32] and Dockery & Keener 2001 [33].

The goal of synthetic biology is not only perform a mathematical modelling *in silico*, but to perform an assembly *in vitro* and monitoring *in vivo* of a particular genetic network. In that sense, it is necessary to establish requirements to design a model and that it can be viable at all levels (*in silico*, *in vitro* and *in vivo*).

Friesen *et al.*, 1993 [34] and Kaznessis 2007 [35] have defined four criteria to design a model in SB, these are: (1) there must be a network topology, where some biomolecules control the concentration of others. (2) Any unit of excitation must activate the system. (3) An oscillating system must include a restoration process that returns the oscillating system to steady state (negative feedback) and (4) there must be a process leading to overcoming the steady state values before the inhibition will be fully effective. A diagram illustrating the basic structure which must have a model designed in SB studies are presented in Figure 4.

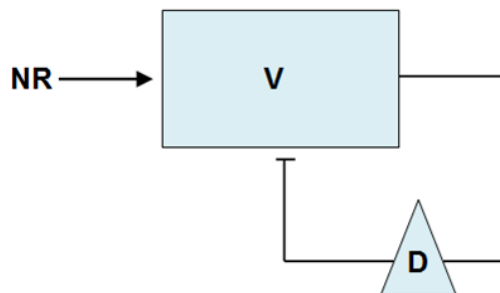


Figure 4. Adapted from Friesen *et al.* (1993) [34]. Basic structure of a model built in Synthetic Biology. NR represents the excitatory input may or may not be rhythmic. V represents the variables and D delays. Arrows indicate induction and perpendicular indicates inhibition.

Many components of QS systems have been employed in the construction of artificial modules and these meet the requirements presented in Figure 4.

Following these criteria it is possible to simulate a QS- communication environment when we can test our samples with the aim to search Quorum-Quenching activity and establishing the presence of quorum quenching molecules.

Finally, we can use bacterial as bio-factories to produce inhibitors molecules in higher amounts than classical approaches under regulation of inducible promoters by using easily manipulated strains.

4. Working model (combined strategy) to measure the potential of inhibitors in marine microfouling process

According to the current knowledge of the fouling process mentioned in the preceding sections, the selection of compounds capable of inhibiting it is not an easy task. For the search of good antifoulants most researchers in this area use the results of bioassays mainly against larvae or spores of macro-organisms. Once selected these compounds, they are applied as additives in antifouling paints. However, there is increasing evidence that microorganisms, in particular bacteria, re-colonize entire surfaces treated with commercial antifoulants [11]. Consequently, there is a real need to find new and more potent antifouling compounds effective against the microorganisms that are deposited in the first stage of the process of colonization.

Therefore, we have developed a strategy using natural compounds or their analogues to interfere with the microfouling considering that the process of biofouling consists of three main stages developed in a sequential manner (Figure 5). The first, usually known as microfouling (attachment of microorganisms mainly bacteria and algal cells), involves the adsorption of dissolved organic molecules; those molecules provide nutrients for attaching the primary colonizers. The subsequent stages involve the recruitment of invertebrate larvae and algal spores (macrofouling) [36,37]. However, it is important to have in mind that these stages can overlap, could be developed in a succession or occurs in parallel [8].

The Figure 5 which illustrates schematically the process of fouling. One can observe several key points within the microfouling step: firstly the reversible attachment of microorganisms (A) (mainly bacteria and algal cells, assay a, blue arrow), at this point they could be detached from the surface just by washing with water; secondly the irreversible attachment of those microorganisms to the solid surface (B) followed by cell division (assay b, red arrow) (C), growth (D), biofilm formation (assay c, yellow arrow) (E) and continuing recruitment (F). At this point Quorum Sensing and biofilm formation of bacteria which are found in all submerged structures in the marine environment frequently in association with algae, protozoa and fungi [38] are key points. Although it is likely that for the majority of organisms a biofilm surface is not a pre-requisite for settlement, in practice colonization by spores and larvae of fouling organisms almost takes place via a biofilmed surface (G). Therefore, we

think that in the way to prevent fouling in marine submerged surfaces we could use natural compounds or their analogs selected through their evaluation as potential antifouling agents using antibacterial activity test against marine bacteria associated with heavy fouled surfaces, quorum sensing inhibition QSI and biofilm inhibition assays.

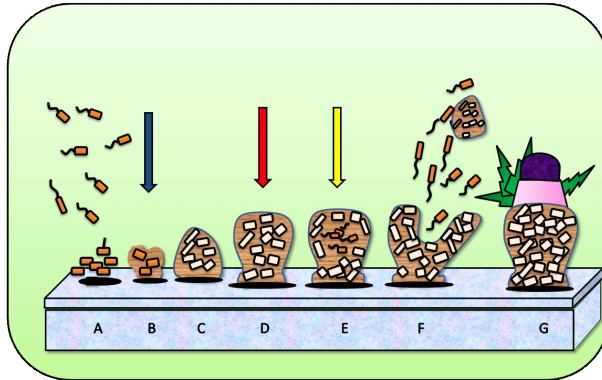


Figure 5. Scheme of the process of fouling. In the Figure 5 which illustrates schematically the process of fouling one can observe several key points within the microfouling step: firstly the reversible attachment of microorganisms (A) (mainly bacteria and algal cells, **assay a**, blue arrow), at this point they could be detached from the surface just by washing with water; secondly the irreversible attachment of those microorganisms to the solid surface (B) followed by cell division (**assay b**, red arrow) (C), growth (D), biofilm formation (**assay c**, yellow arrow) (E) and continuing recruitment (F). At this point Quorum Sensing and biofilm formation of bacteria which are found in all submerged structures in the marine environment frequently in association with algae, protozoa and fungi [38] are key points. Although it is likely that for the majority of organisms a biofilm surface is not a pre-requisite for settlement, in practice colonization by spores and larvae of fouling organisms almost takes place via a biofilmed surface (G). Therefore, we think that in the way to prevent fouling in marine submerged surfaces we could use natural compounds or their analogs selected through their evaluation as potential antifouling agents using antibacterial activity tested against marine bacteria associated with heavy fouled surfaces, quorum sensing inhibition QSI and biofilm inhibition assays.

Although, no laboratory bioassay could replicate the complex process of fouling since it involves a wide range of physical, chemical, and biological interactions [16]; recently, some technical approximations have been reported as indicators of the antifouling potential of chemical compounds. Those strategies use as a model the growth inhibition bioassay and the disruption of biofilm formation in bacteria associated with heavily fouled marine surfaces [8]. As we mention, it has been established that bacteria are the first organisms to colonize immersed surfaces [2] and they are usually associate with soft-bodied organisms or with inert surfaces. The formation of bacterial biofilms is regulated by QS systems, and it has been recognized as fundamental track for the attachment and growth of other organisms such as other bacteria, invertebrate larvae or spores of algae [15]. Thus, the conformation of the bacterial community is involved in the process of fouling by regulating the settlement of organisms and by promoting or inhibiting colonization [15,16,41,47].

Therefore, in our recent research on marine metabolites we have been focused on the search of compounds that exhibit antifouling properties mainly from octocorals that keep their sur-

faces free of fouling organisms [39-45] using their own metabolites as chemical defenses against the settlement and metamorphosis of invading species. After a quick *in vitro* antifouling test using the bacterial strains associated with fouled surface *Ochrobactrum pseudoringnonense*, *Alteromonas macleodii*; *Kocuria* sp., and *Oceanobacillus iheyensis* (described below in section a) [41] to evaluate 39 extracts of marine organisms, the *Eunicea knighti* and *Pseudoplexaura flagellosa* extracts showed the strongest antifouling properties [41,43-45]. Consequently, these octocorals *E. knighti* and *P. flagellosa* were collected in Santa Marta bay, Colombian Caribbean Sea by scuba diving. The animals were identified by Prof. Dr. S. Zea, and Prof. Dr. M. Puyana. The fresh coral colonies were immediately frozen after collection and remained frozen until extraction. The organisms *E. knighti* (650 g) and *P. flagellosa* (360 g) were cut in small pieces and separately extracted with a MeOH-CH₂Cl₂ (1:1 v/v) mixture, concentrated by rotary evaporation and the extracts obtained subjected to reversed-phase HPLC for final purification, to afford pure compounds 1-8 from *E. knighti* and 9-16 from *P. flagellosa* (Figure 6) as was reported by Tello *et al*, 2009 [43], 2011 [44] and 2012 [45].

The structures of compounds 1-16 (Figure 6) were established by the analysis of their spectroscopic features (MS, one- and two- dimensional NMR) and their absolute configurations were determined by a combination of chemical and NMR methods (multiple correlations observed in a ROESY and NOESY experiments, and by the modified Mosher method and the values of their optical rotations). Additionally, the stereostructures of compounds 9-16 (Figure 6) were confirmed by single-crystal X-ray diffraction.

The cembranoid type compounds isolated from *E. knighti* and *P. flagellosa* were subjected to Quorum Sensing and Bacterial Biofilm inhibition assays (Tables 1 and 2) finding interesting values for microfouling activity particularly for compounds 2-5, 7-9, 11, 12, 14, and 15. These results and their structure similarity, indicated an interesting structure-activity relationship, in this specific case of antifouling activity. For this reason, we envisaged a strategy to obtain diverse analogues with a high functional diversity, in order to enhance their activity making possible their use in antifouling technologies. Thus, we selected among the most active natural compounds, six lead compounds (2-5, 9, 11, and 12), based on their antifouling properties, on the easy access to substantial amounts of those compounds and on the presence of highly reactive functional groups in their structure. The latter made them suitable templates for the synthesis of analogues possessing uncommon structural features which enhanced its antifouling properties and leaves the possibility of assessing new interesting biological activities. Thus, we selected a group of regioselective, straightforward, fast, reproducible and high yield reactions to afford the synthetic analogues of cembranoids 17-52 (Figure 7) i.e. epoxide ring opening, oxidation reactions, treatment with iodine, photochemical reactions, methylation and acetylation, and cyclizations [46]. The analogues thus synthesized were subsequently subjected to antimicrofouling assays as explained below to evaluate their properties i.e. if they were capable of inhibiting marine bacteria involve in marine fouling, disrupt Quorum Sensing systems and avoid or inhibit biofilm formation and the subsequent steps of fouling as described in section 1.

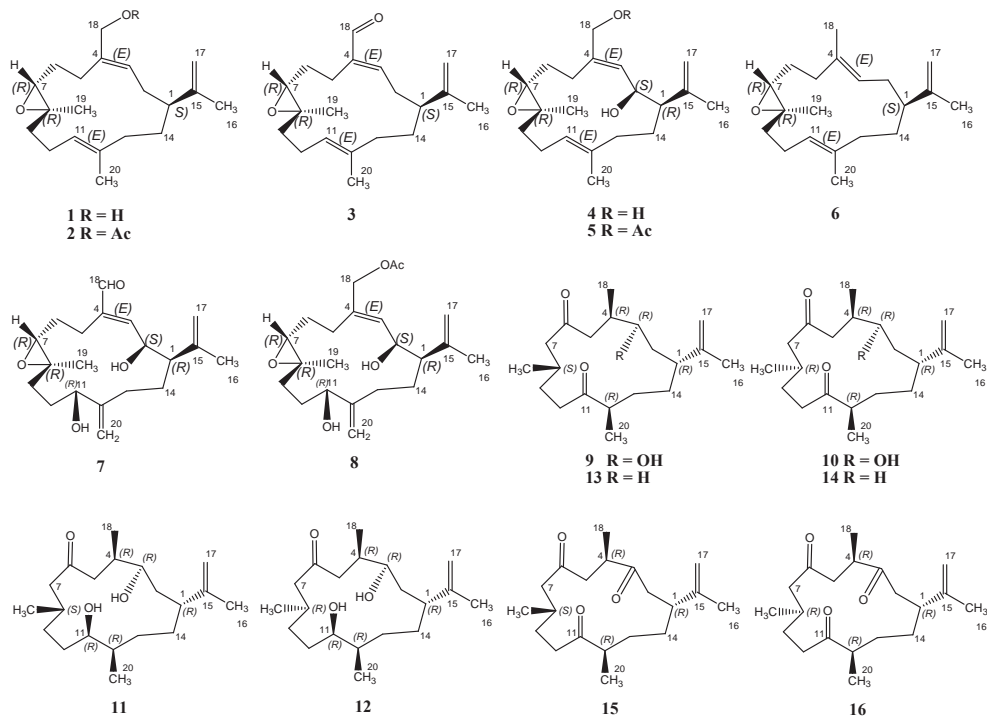


Figure 6. Natural products isolated from *E. knighti* (1-8) and *P. flagellosa* (9-16).

4.1. Marine bacteria involved in fouling process and their use as preliminar sensors to evaluate the antimicrofouling activity of compounds 1-16

As no laboratory bioassay could replicate the complex process of fouling as mentioned before, some technical approximations have been reported as indicators of the antifouling potential of chemical compounds [16]. These approximations use as one of the models, marine bacteria involved in fouling process.

Our experiments used bacterial strains associated with the fouled surface of the sponge *Aplysina lacunosa* and from the calcareous surface of a shell of the bivalve *Donax* sp. Those bacteria were collected at Santa Martha Bay in the Colombian Caribbean, and identified by the 16S rRNA sequencing as *Ochrobactrum pseudogrignonense*, *Alteromonas macleodii*; *Kocuria* sp., and *Oceanobacillus iheyensis* [41]. The natural compounds were tested in a growth inhibition assay against these marine bacterial strains, using the common antimicrobial test (disk-diffusion technique) [41,48,49]. The results showed in general that most of the assayed cembranoids exhibited high activity against most of the isolated bacteria, mainly *O. pseudogrignonense*. Specifically, compound 1 has a wide antibacterial activity and the best potency (halo >10 mm) of all compounds tested, although it was more specific against Gram (+) bacteria. Compounds 4 and 5 exhibited activity against isolates of *O. pseudogrignonense*. The

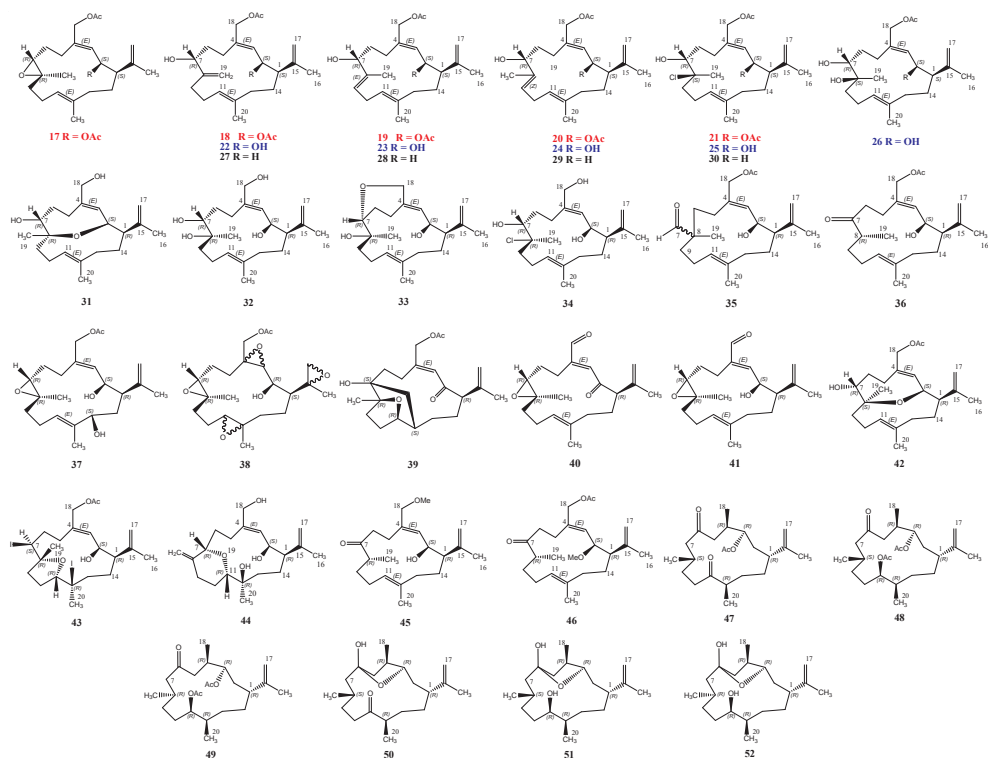


Figure 7. Analogues of cembranoids (17-52).

compound 3 showed a wide activity, but it was more powerful against Gram (+) bacteria as was reported previously by us in Tello *et al.*, 2009 [43].

4.2. Quorum sensing inhibition assay and its role as a tool to decide which of the natural and or the analogues synthesized exhibited the best potency as antimicrofoulants

The Quorum Sensing involves the cell control of bacterial population by communication using chemical signals (molecules) and a complex network of genetic circuits with a positive feedback regulation. Sensing these chemical signals bacteria can respond as groups and detect the “quorum” of a population in order to regulate different phenotypes [11,36], i.e. the biofilm formation and maturation [50]. Thus, QS inhibitors can avoid the maturation of bacteria biofilms, and affect the attachment larval indirectly [15]. We decided to evaluate all 52 compounds (naturals and synthetics) in this bioassay, in order to establish whether these cembranoid compounds can interfere with the QS systems signals and at the same time if the chemical transformations to the natural cembranoids led to obtain synthetic analogues with a QS inhibitory activity increased. Thus, to evaluate the QSI activity of the natural and synthetic cembranoid analogues the *Chromobacterium violaceum* ATCC 31532 biosensor strain

was used in a standard disk–diffusion assay [49]. The disks were loaded with µg amounts of every compound (1-52) as was previously reported by us in Tello, *et al*, 2011 [44], 2012a [45], and 2012b [46]. The known QS inhibitor kojic acid [11] was used as a positive control together with the common antifouling agent Cu₂O.

Assay		Quorum Sensing Inhibition ^a (µg/disk) ^b	
Compounds	<i>Chromobacterium violaceum</i>	Compounds	<i>Chromobacterium violaceum</i>
1	-	27	-
2	-	28	5.0
3	7.5	29	-
4	-	30	-
5	-	31	30.0
6	-	32	-
7	7.5	33	-
8	15.0	34	30.0
9	-	35	7.5
10	-	36	30.0
11	7.5	37	30.0
12	-	38	-
13	-	39	15.0
14	7.5	40	-
15	30.0	41	-
16	-	42	-
17	-	43	-
18	7.5	44	-
19	2.5	45	-
20	7.5	46	-
21	-	47	7.5
22	7.5	48	30.0
23	2.5	49	15.0
24	-	50	-
25	7.5	51	7.5
26	7.5	52	-
Kojic acid	90.0	Solvent	-

^aActivity was measured by the inhibition of violacein pigment. ^bMinimum quantity in µg per disk of compound required to inhibit violacein pigment. – No zone of inhibition was observed, even at 30 µg/disk.

Table 1. Quorum Sensing Inhibition activity of compounds 1-52.

All compounds listed in the Table 1 were tested in the QS inhibition assay and were evaluated. The results showed mainly, that only six of the natural compounds showed QSI activity, being the most active compounds 3, 7, 11, and 14 (all 7.5 µg/disk). The synthetic compounds that showed the best QSI activity were 19 (2.5 µg/disk), 23 (2.5 µg/disk), and 28 (5.0 µg/disk) which present a similar structure and functional groups. All in all, the carried out reaction resulting in an enhanced QS Inhibitors compounds, because, eighteen active analogue compounds were obtained and is noteworthy to say that the most active cembranoid analogues (19, 23, and 28) were most potent than the most active natural products 3, 7, 11, and 14, achieving the main proposal of the work [46].

4.3. Disruption of Bacterial biofilm formation and maturation as indicator of antifouling potency of natural and semisynthetic cembranoid analogues

Bacterial biofilms have long been recognized as fundamental settlement cues for many invertebrate larvae that colonize hard substrata, such as sponges, cnidarians, mollusks, barnacles, bryozoans and ascidians [8]. This intrinsically complex process is the result of a network of interactions both in the pioneering biofilm and in the community of colonizers [14]. These interactions in a biofilm determine the composition of different populations and the establishment in a specific environment, all these together create a chemical pattern that gives specific signals for the subsequent colonization of other organisms.

Biofilm inhibition assay ^a			
Compounds	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Vibrio harveyi</i>
1	4,1	2,0	0,3
2	12,8	1,3	9,8
3	9,3	3,2	1,0
4	14,7	0,01	>100,0
5	5,0	0,3	>100,0
6	8,3	0,5	>100,0
7	10,1	0,01	80,2
8	17,8	0,8	>100,0
9	4,0	10,1	17,1
10	4,5	10,0	69,7
11	11,5	">100,0	11,0
12	17,2	>100,0	>100,0
13	9,2	20,9	0,3
14	6,4	1,0	>100,0
15	12,2	5,7	9,5
16	6,8	1,4	53,8
Kojic acid	17,2	24,7	>100,0
Solvent	NI	NI	NI

^aConcentration that inhibits 50% of the biofilm and expressed in ppm. NI Non biofilm inhibition.

Table 2. Biofilm inhibition activity of compounds 1-16.

For biofilm inhibition assays, *Pseudomonas aeruginosa* ATCC 27853 (Gram negative), *Staphylococcus aureus* ATCC 25923 (Gram positive) and *Vibrio harveyi* PHY-2A (Gram negative) were used as model bacteria. The pure compounds 1-52 were evaluated in this bioassay to test if they are capable of inhibit the bacterial biofilm formation [44-46]. The bioassay was performed in a 96-well polystyrene microtiter dishes as was previously described in Tello, *et al*, 2011 [44]. For the natural compounds the IC₅₀ values were calculated (Table 2).

The results of the disruption of Bacterial biofilm formation showed that approximately 60% of compounds inhibited the biofilm formation in a 50% extension, in at least one strain of the three used for this bioassay. Most of the cembranoid compounds showed outstanding biofilm inhibition activity against all the strains used in this bioassay, mainly against the Gram positive bacterium *S. aureus*. Thereby, fifteen compounds inhibited the bacterium *S. aureus* at lower concentrations than 1.0 ppm, being the compounds 4, 7, 18, 19, 25, 35, and 36 the most active even better than the known QS inhibitor kojic acid (24.7 ppm). Against *P. aeruginosa* nine compounds showed IC₅₀ values lower than 10.0 ppm and the most active compounds were 1, 5, 9, and 10. The control kojic acid showed an IC₅₀ of 17.2 ppm. Finally, against *V. harveyi* just three natural compounds (1, 3, and 13) presented lower IC₅₀ values than 1.0 ppm,, the kojic acid presented an IC₅₀ value upper than 100 ppm against this strain (data being published). The kojic acid has been proved by Dobretsov *et al*. [11] to have the ability to prevent microfouling in a controlled mesocosm experiment by inhibition of microbial communities on glass slides, decreasing the densities of bacteria and diatoms. Additionally, the biofilm inhibition in all cases was achieved without interference in the bacterial growth.

Then, based on the results obtained in the above assays for all compounds (1-52), it was possible establish the natural compounds 3 and 7, and the synthetic compounds 18, 19, 25, 35, and 36 as the most active cembranoids in the anti-microfouling bioassays, which could be used as additive in antifouling coating.

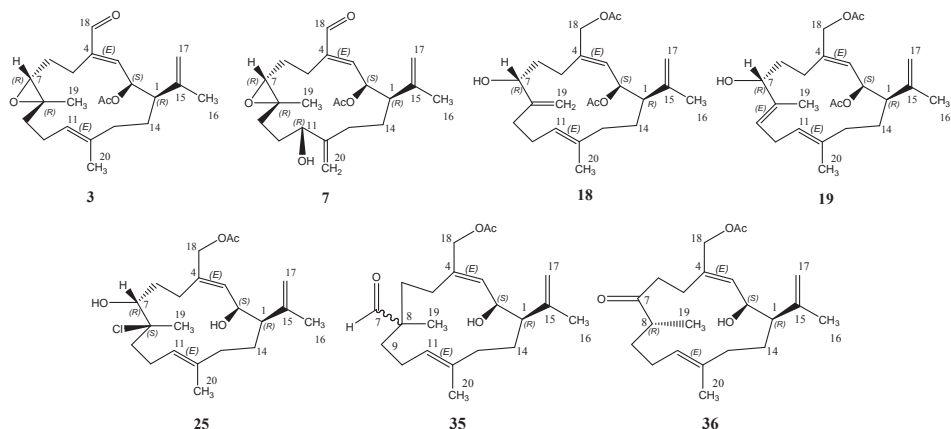


Figure 8. Most active compounds in the anti-microfouling bioassays.

4.4. Field test

Based on the previous results we chose compound (19), one of the most active compounds in the antimicrofouling bioassays to evaluate its activity in a field experiment with natural conditions (the sea) as additive in an industrial coating (Table 3). Thus, white ceramic panels (12 cm × 12 cm × 5 mm) used as surface were polished and then coated with the test paints (code I and II), a copper-based paint (30% of Cu²O), and a kojic acid-based paint (0.5% of kojic acid).

Code	Paint	Compound 19	epoxymastic (marine paint)	catalyst	Acetone
I	1	2.0%	47.0%	47.0%	4.0%
II	1	0.5%	48.0%	48.0%	3.5%

Table 3. Formulation of the paints used in field tests.

Field experiments were conducted at Rosario Islands, Caribbean coast of Colombia, between 25 April and 9 July of 2012. The test panels were placed at random at a depth of 2 to 6 meters and vertically using SCUBA diving.

The results showed that the copper-based paint was cover with microfouling during the test period, otherwise showed good antifouling activity against macroorganisms after 75 days, the panel control was completely fouled mainly by algae, the test panels treated with the compound 19 completely prevented settlement of macrofouling during the tested period, further, the panels were slightly fouled with microalgae but remained most free of fouling and showed excellent antifouling performance after 75 days of exposure. Finally, barnacles and bivalves were observed alongside the pier wall, but were not found on the test panels. The results of biofilm inhibition activity and the field test will be published in near future.

As summary, QSI and Bacterial biofilm inhibitors compounds were more effective than the known antifoulants kojic acid and Cu₂O, and is noteworthy that the most of the analogues of cembranoids were significantly less toxic against the tested bacteria (*C. violaceum*, *V. harveyi*, *S. aureus* and *P. aereginosa*). Thus, marine cembranoids are recognized to be promising environmentally friendly candidates to be included in industrial coatings as antimicrofouling agents. The results showed that the analogue 19 completely prevented settlement of macrofouling and inhibited most of the microfouling during the period of testing. The above support the use of cembranoids (particularly compound 19) as excellent candidates to be used as antifoulant agents in a commercial antifouling paint, but because of its structural complexity, the synthesis of more simple compounds together with QSAR studies should be the next step in the search for potential non-toxic antimicrofoulant agents.

5. Concluding remarks

Although it is well known that in the marine environment all natural and artificial substrata are quickly colonized by micro- and macroorganisms in a complex physical, chemical and biological process, only recently the studies on microfouling have emerged as a key area of research, in an effort to develop more efficient and environmental friendly antifoulants. Since, the formation of a biofilm is considered an initial step in the development of fouling and taking into consideration that QS controls bacterial biofilm differentiation and maturation, the use of chemical compounds (natural or synthetic analogs) as interferences in these processes has been proposed as one potential approach for controlling microfouling. Furthermore, since larvae of many marine invertebrates preferentially settle on bacterial biofilm, disruption of bacterial biofilm could lead to the reduction of macrofouling of submerged surfaces as well. In this sense, this chapter, that includes our own working model (combined strategy) to measure using bacterial sensors, the potential of marine isolated compounds and their synthetic analogs as inhibitors in marine microfouling, should surely contribute to the significant expansion of this area of research.

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