COPPER TANNATE COMPLEX AS A POTENTIAL MARINE ANTIFOULING AGENT

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COPPER TANNATE COMPLEX AS A POTENTIAL MARINE ANTIFOULING AGENT

by

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LIST OF ABBREVIATIONS

AHL	Acyl-homoserine lactone
Al	Aluminum
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSLT	Brine Shrimp Lethality Test
$CaCo_3$	Calcium carbonate
CDP	Controlled Depletion System
CFU	Colony Forming Unit
$CHCL_3$	Chloroform
CLSI	Clinical and Laboratory Standard Institute
CO	Copper omadine
CP	Chloramphenicol
CPT	Copper pyrithione
CT	Copper-tannate
CTAB	Cetyltrimethylammonium bromide
Cu	Copper
$CuSO_4.5H_20$	Copper sulphate pentahydrate
DF buffer	Detergent-free reaction buffer
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular Polymeric Substances
FB	Fouling Bacteria
Fe	Iron
H_20_2	Hydrogen peroxide
HCIO	Hypochlorous acid
HMDS	Hexamethyldisilazane
HSL	Homoserine-lactone
IAA	Isoamyl alcohol
IBRL	Industrial Biotechnology Research Laboratory
IMO	International Maritime Organization
INT	p-iodonitrotetrazolium violet salts
KH_2PO_4	Dipotassium orthophosphate
KOH	Potassium hydroxide
F	Fermentative
LC_{50}	50% Lethal Concentration
LPS	Lipopolysaccharide
LuxI	Autoinducer synthase
LuxICDABE	Luciferase operon
LuxR	Binding transcriptional activator
MA	Marine agar

MBC	Minimum Bactericidal Concentrations
MIC	Minimum Inhibitory Concentrations
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTBA	Mangrove Tannin-Based Absorbent
N	Negative
O_2	Oxygen
OECD	Organization of Economic Cooperation and Development
OX	Oxidative
PCR	Polymerase Chain Reaction
PDMSE	Polydimethylsiloxane
PTFE	Polytetrafluoroethylene
QS	Quorum sensing
RNase A	Ribonuclease A
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
SiO_2	Silicon dioxide
SPC	Self-Polishing Copolymer
SYEP	Seawater Yeast Extract Peptone agar
TAE	Tris-acetate-EDTA buffer
TBT	Tributyltin

TBTO	Tributyltin oxide
TEEB	The Economic of Ecosystems and Biodiversity
TEM	Transmission Electron Microscope
TiO_2	Titanium dioxide
WHOI	Woods Hole Oceanographic Institute
Zn	Zinc
ZnO	Zinc oxide

KOMPLEKS KUPRUM-TANNAT SEBAGAI AGEN ANTI-TEMPEL MARIN BERPOTENSI

ABSTRAK

Ekoran daripada penguatkuasaaan undang-undang yang mengharamkan penggunaan agen anti-tempel seperti tributyltin dan diuron yang mengancam hidupan laut, wujud satu keperluan mendesak untuk menghasilkan agen anti-tempel baharu yang lebih mesra alam. Oleh itu, antara salah satu alternatif untuk mengatasi masalah penempelan mikrooganisma adalah dengan menggunakan teknologi hijau. Dalam penyelidikan ini, ekstrak tannin daripada pokok bakau minyak atau nama saintifiknya *Rhizophora apiculata* telah dipilih sebagai sumber semulajadi yang memiliki keupayaan antimikrob dan berkebolehan untuk bergabung dengan ion logam seperti kuprum untuk menghasilkan kompleks logam-tannat yang akan dicampurkan ke dalam formula cat anti-tempel. Empat jenis kompleks logam-tannat yang di hasilkan kemudiannya di saring untuk mengesan aktiviti antimikrob dengan menggunakan asai agar plug ke atas beberapa pencilan bakteria marin. Kompleks kuprum-tannat menunjukkan aktiviti antimikrob yang paling kuat berbanding kompleks logam-tannat yang lain dengan diameter zon perencatan antara 10-22 mm. Nilai MIC yang diperolehi pula ialah antara 0.25-1.00 mg/ml dan nilai MBC pula ialah antara 0.50-2.00 mg/ml. Daripada nilai nisbah MBC/MIC, kompleks kuprum-tannat menunjukkan kesan bakterisid terhadap tujuh pencilan bakteria marin dan kesan bakteriostatik terhadap pencilan *Vibrio alginolyticus* IBRL FB6 dan pencilan *Bacillus aquimaris* IBRL FB13. Aktiviti antibakteria kompleks kuprum-tannat

adalah bergantung kepada kepekatan ekstrak. Di antara perubahan pada bentuk dan struktur sel yang di kesan melalui Mikroskop Elektron Imbasan dan Mikroskop Elektron Transmisi ialah, kompleks kuprum-tannat menyebabkan sel-sel bakteria berubah daripada bentuk asal normal rod kepada bentuk yang tidak teratur, pembentukan kaviti atau lubang, permukaan sel yang berkedut serta kebocoran kandungan sitoplasma sel yang menyebabkan sel-sel akhirnya musnah. Ketoksikan kompleks kuprum-tannat juga di uji ke atas anak udang brin. Nilai LC_{50} menunjukkan ketoksikan kompleks kuprum-tannat di sumbang oleh ion-ion kuprum. Ketoksikan ekstrak berkadaran terus dengan kepekatan ekstrak dan lama masa. Untuk ujian lapangan, kompleks kuprum-tannat yang berbeza kepekatan di cat pada panel jaring ikan sebelum direndam di dalam laut selama dua bulan. Panel dengan kepekatan yang lebih tinggi iaitu 19.35 mg/ml, mempunyai aktiviti anti-tempel yang lebih kuat berbanding panel dengan kepekatan ekstrak yang lebih rendah iaitu 12.9 mg/ml. Secara kolektif, kajian awal ini membuktikan keupayaan kompleks kuprum-tannat untuk memperlahankan dan mengurangkan penempelan mikroorganisma pada jaring ikan.

COPPER TANNATE COMPLEX AS A POTENTIAL MARINE ANTIFOULING AGENT

ABSTRACT

Due to the banned of many antifouling biocides such as tributyltin and diuron because of their toxic impacts on the marine environment, there is an urgent need for a novel antifouling agent. Therefore, one of the alternatives to overcome this biofouling problem is by shifting to the green technology. In this present study, tannin extracted from *Rhizophora apiculata* was selected as the natural source as it was proven to posses antimicrobial property and can be easily combined with metal ions (i.e: copper) to form a metal-tannate complexes that later can be incorporated in the antifouling paint. Four different metal-tannate complexes were tested for antimicrobial properties via disc-diffusion assay against a series of marine bacterial isolates. Copper-tannate complex showed the strongest antimicrobial activity with diameter zone of inhibition ranged from 10- 22 mm. The MIC and MBC values obtained ranged from 0.25 mg/ml to 1.00 mg/ml and from 0.50 mg/ml to 2.00 mg/ml, respectively. From the ratio of MBC/MIC, copper-tannate complex showed bactericidal effect on seven marine bacteria and bacteriostatic effect on *Vibrio alginolyticus* IBRL FB6 and *Bacillus aquimaris* IBRL FB13. Time kill assay revealed that the antibacterial activity of copper-tannate complex was a concentration-dependent. The main abnormalities observed via SEM and TEM study after treatment with copper-tannate complex were the alterations in morphology and cytology of the bacterial cells where bacterial cells changed from normal rod-shaped

bacillus to having an irregular appearance, showing formation of pits and cavities, wrinkle surface and lost in rigidity of the cells due to the leakage of cell cytoplasm. The toxicity of copper-tannate complex was determined on *Artemia salina*. By comparing the LC_{50} values for acute (6 h) and chronic (24 h) toxicity of copper-tannate complex, mixed-tannin and copper sulphate pentahydrate, it can be concluded that the copper ions contributed to the toxicity of copper-tannate complexes and the increase of mortality is proportional to increase of extract concentration and exposure time. For the field test, copper- tannate formulated antifouling paint was applied on fishnet panels. After two months of exposure in the seawater, panels with higher concentration (19.35 mg/ml) of copper tannate complex were less affected with biofoulers compared to panels painted with lower concentration of copper-tannate (12.9 mg/ml). In conclusion, this preliminary study on the effects of copper-tannate complex formulated paint on the fishnets revealed the potential use of this complex in slowing the attachment of the biofoulers on the substract (the fish net).

CHAPTER 1 INTRODUCTION

1.1 Biofouling and antifouling technologies

As happens to all the great majority of solid surfaces immersed in seawater, after a relatively short immersion time they will become fouled with numerous marine organisms, which are more than 4000 species. This biofouling phenomenon is also defined as the accumulation of biotic deposits on a submerged surface that caused major technical and economical problems worldwide (Eguia & Trueba, 2007; Iyapparaj *et al*., 2012). The development of biofouling involves both physical and biochemical reactions. The first step occurs within the first minutes of the biological settlement. It involves physical reaction, where a layer of 'conditioning' film builds from organic materials mostly (protein, proteoglycans and polysaccharides) provides a sticky surface that aid in microorganism adherence (Loeb & Neihof, 1975; Dexter *et. al*., 1978; Baier, 1984; Lewin, 1984). Next step is the microorganism colonization where biofilm develops as bacteria and microalgae adhere to the surface.

Microorganism colonization involves two distinct steps: reversible adsorption and irreversible adhesion. Physical forces such as Brownian motion, electrostatic interaction, gravity and van-der-Waal forces essentially govern the former step (Fletcher & Loeb 1979; Walt *et al.,* 1985). The latter occurs mainly through biochemical effects such as secretion of extracellular polymeric substances (EPS). The biochemical reactions are effectively irreversible. Therefore, it would be easier to prevent biofouling at the physical reaction as efficient inhibition of the physical reaction would prevent the later biochemical reactions (Cao *et al*., 2010).

Biofouling has been recognized for causing many problems for more than 2000 years (Callow 1990). In shipping industry, microfouling alone can increase the fuel consumption by up to 18% and reduce the ship speed by at least 20%. This is due to the increased of frictional resistance that makes the hull rougher and the ship heavier. The fish farming industry and aquaculture in general suffer significantly from the effects of biofouling. For example the heavy fouling of fish cages and netting, which is costly to remove, is detrimental to fish health and yield and can cause equipments failure (Hellio *et al*., 2000; Ross *et al.*, 2004; Braithwaite & McEvoy, 2005; Bazes *et al*., 2006).

Natural product such as wax and tar were used as antifouling product. The first antifouling paints was reported in the mid-19th century, which contained copper and arsenic as toxicants agent. Among the currently available biocide (co-biocides) are, Irganol 1051 (2-methylthio-4-*tert*-butylamine-6-cyclopropylamine-*s*-triazine), Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), copper pyrithione, zinc pyrithione, Sea-nineTM 211 (member of 3(2H)-isothiazolone), and Zineb. However, their effects have not been fully studied.

Since the end of 1990s, antifouling paints have been made with combination of polyacrylic resins with biocides to prevent biofouling formation. Organotin compounds including tributyltin (TBT) and tributyltin oxide (TBTO) were widely used for controlling these sessile organisms (Suzuki *et al.,* 1992). In general, the TBT based paint was widely applied as an antifouling coating in the shipping industry before it was banned. The use of TBT has been restricted as of the International Maritime Organization (IMO) conference in 1998, and these coatings have been banned completely starting from 1st January 2008 (Clare, 1998; Champ 2000; Anna, 2009).

Due to the ban, there is a growing need for other methods of prevention of the biofouling. It is reported that the prevention of marine fouling can be achieved by coatings from which a controlled release of toxic molecules prevents the growth of adhered organisms (bacteria, algae and mollusks) by killing them (Fay *et al*., 2007). The ideal replacement for TBT is an environmentally neutral coating with both antifouling and fouling-release properties (Magin *et al.,* 2010). In addition, the response to this ban has been the use of copper, zinc and a variety of organic compounds as the active, antifouling components.

The antifouling properties of tannins were claimed as early as 1881 (Jones, 1881). In recent studies, tannin from *Rhizophora apiculata* barks has been reported to possess antibacterial and anticandidal properties (Suraya *et al.,* 2011). In Malaysia, *R. apiculata* is a plant widely used in charcoal industry, where the barks of the *R. apiculata* are normally scraped out from the log and left to rot in the fields (as agrowaste). The barks of these plants are able to produce high yields of tannins.

1.2 Problem statements

Because non-toxic antifouling paints cannot as yet be produced on an industrial scale, there is an urgent need for the development of alternative formulations that promotes good antifouling performance without polluting the marine environment.

Therefore, in this present study, the mixed-tannin extracted from *R. apiculata* mangrove barks, which is a polar substance that is easily dissolved in water, will be combined with non-polar substance such as metal ions (copper) to form metal-tannate complexes, in order to reach an adequate leaching rate and ensures antifouling control when incorporated in antifouling paint formulation. The antimicrobial activities of all

metal-tannates complexes were studied. The selected metal-tannate complex with the strongest antimicrobial activity was further studied for the effect of exposure time on the growth profile of marine fouling bacteria, the toxicity and also antifouling activity of the metal-tannte on field test.

1.3 Research Objectives

1. To identify the marine bacteria isolates.
2. To extract mixed-tannin from *Rhizophora apiculata* barks using 70% acetone-water mixture and to prepare metal-tannate complexes by chelating process.
3. To screen the antimicrobial activity of the four metal-tannate complexes (copper-, zinc-, aluminium- and ferum-tannate) against identified marine fouling bacteria.
4. To determine the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) and time kill study of the copper-tannate complex against marine fouling bacteria, as well as its structural degeneration and toxicity tests.
5. To evaluate the potential application of the new antifouling paint containing copper-tannate complex on fishnet panels in submerged seawater.

CHAPTER 2 LITERATURE REVIEW

2.1 Biofouling

Commonly in the marine environment, any natural and artificial surfaces immersed in seawater are colonized by biofoulers. This biofouling event is generally defined as the undesirable phenomenon of adherence and accumulation of biotic deposits on a submerged artificial surface or in contact with seawater (Iyapparaj *et al*., 2012). Ever since 2000 years ago, biofouling has been recognized for causing various problems worldwide (Callow, 1990). Biofouling involves a series of discrete physical, chemical and biological events, which later results in the formation of a complex layer of attached organisms known as biological fouling. Marine bacteria, fungi and yeast are major organisms involved in the formation of the microlayer, which is the first step in the process of biofouling formation (Holmstrom & Kjellberg, 1994). In general, there are two major categories of marine adhesion organisms. The first category includes 'micro fouling' or biofilm organisms, which consist of marine bacteria, micro-algae, protozoa and diatoms. The next category includes 'macro fouling' organisms such as macro-algae, barnacles, bryozoans and tubeworms (Dobretsov *et al.,* 2006). The five most important macro-fouling species that have been reported are barnacles, mussels, polychaete worms, bryozoans and seaweeds (Stefan, 2009).

2.1.1 Biofouling process

The development of biofouling involves both physical and biochemical reactions as summarized in Table 2.1 (Almeida *et al.,* 2007). The first step of biofouling is

Table 2.1: Stages of attachment of marine organisms on surfaces immersed in seawater

Processes involved	Attached organisms	Nature of film formed	Initiation time
Stage 1: Essentially physical forces, such as electrostatic interactions, Brownian movement and Van der Walls forces.	Adhesion of organic molecules, such as proteins, polysaccharides and proteoglycans and, possibly, some inorganic molecules.	Conditioner	1 min
Stage 2: Reversible "adsorption" of species, especially by physical forces and their subsequent adhesion interacting together with protozoans and rotifers.	Bacteria, such as *Pseudomonas putrefaciens* and *Vibrio alginolyticus* and diatoms (single-cell algae) such as *Achnantes brevipes, Amphora coffeaformis, Amphiprora paludosa, Nifzschia pusilla* and *Licmophora abbreviata*.	Microbial biofilm	1-24 hour (s)
Stage 3: Arrangement of microorganisms with greater protection from predators, toxicants and environmental alterations, making it easier to obtain the nutrients necessary for the attachment of other microorganisms.	Spores of microalgae, such as *Ulothrix zonata* and *Enteromorpha intestinalis* and protozoans, including *Vaginicola* sp., *Zoolhamnium* sp. and *Vorticella* sp.	Biofilm	1 week
Stage 4: Increase in the capture of more particles and organisms, such as larvae of marine macroorganisms, as a consequence of the pre-existence of the biofilm and the roughness created by the irregular microbial colonies that comprise it.	Larvae of macroorganisms, such as *Balanus amphitrite* (Crustacea), *Laomedia flexuosa* (Coelenterata), *Electra crustulenta* (Briozoa), *Spirorbis borealis* (Polychaeta), *Mytilus edulis* (Mollusca) and *Styela coriacea* (Tunicata)	Film consisting of the attachment and development of marine invertebrates and growth of macroalgae.	2-3 weeks

[Source: Almeida *et al.,* 2007]

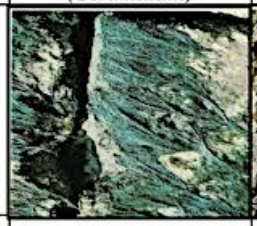
conditioning that occurs within the first minutes of the biological settlement. This stage involves physical reaction, where organic materials mostly protein, proteoglycans and polysaccharides form a layer of 'conditioning' film that provides a sticky surface that aid in microorganism adherence (Loeb & Neihof 1975; Dexter, 1978; Baier, 1984). Next step is the microorganism colonization where bacteria and microalgae adhere to the submerged surface and biofilm start to develop. Microorganism colonization involves two distinct steps: the reversible adsorption and irreversible adhesion. Physical forces such as Brownian motion, electrostatic interaction, gravity and van-der-Waal forces essentially administer the former step (Fletcher & Loeb 1979, Walt *et al.*, 1985). The latter step occurs mainly through biochemical effects such as secretion of extracellular polymeric substances (EPS). The biochemical reactions are effectively irreversible. Theoretically, it would be effective to prevent biofouling at the physical reaction stage as efficient inhibition of the physical reaction would prevent the later biochemical reactions (Cao *et al*., 2011).

2.2 Marine organisms

With a diversity of life forms that cover more than 70% of the earth's surface, the oceans represent the largest ecosystem on earth. Whereas, the largest fraction of biomass in the oceans is represented by microbes which comprise of both prokaryotes (bacteria and archae) and eukaryotes (algae, protists and fungi). Among the microbes, bacterial populations ranks as one of the dominant communities with a cell count in the pelagic water, is typically about 10^6 cells/ml (Ducklow, 2000). The total number of bacteria in oceanic waters has been estimated to 10^{29} cells (Whitman *et al.*, 1998). This number proves the existence of bacteria almost everywhere in the oceans. Their habitats are

diverse, including the open water, sediment, bodies of marine macro and microorganisms, estuaries, and hydrothermal vents (Goecke *et al.*, 2010). Bacteria play an important role in controlling the life of many marine organisms including plants and animal. For example, biofilm-forming bacteria are known to transmit signals (quorum sensing) that affect the settlement of invertebrate larvae on those biofilms (Hadfield, 2011). Established associations between bacteria and animals are widely distributed in both marine and terrestrial ecosystems. Conserve associations with a more diverse microbial assemblage have also been reported where bacteria can induce permanent attachment in variety of taxa including the alga *Ulva* sp. (Joint *et al.*, 2000), the coral *Acropora microphthalma* (Webster *et al.*, 2004) and the polychaete *Hydroides elegans* (Hentschel *et al.*, 2002; Lau *et al.*, 2003; Bourne *et al.*, 2008). Recently, marine organisms have attracted much attention for scientific researches due to their importance in various domains of sciences. Biofouling is one of the reasons that trigger the vast study of marine biofoulants and antifouling technologies. As in 1960s, the Organization of Economic Cooperation and Development (OECD) in many countries, including Portugal, has performed studies on the identification of the main marine macroorganisms that fix themselves to ship hulls and caused biofouling. Table 2.2 is showing the characteristics of main macroorganisms species that fouled on the ship hulls. Each of the groups and subgroups possess different different characteristics is more or less prevented from becoming fixed based on the toxicant level that have been incorporated in the different antifouling products used over the years.

Table 2.2: Characteristics of main marine macroorganism species

Groups	Algae (plants)	Invertebrates (animals)							
Subgroups	(a) green, (b) brown and (c) red	Hard shell organisms				Grass type organisms	Small bush organisms	Spineless organisms	
Designation	*(a) Enteromorpha, Ulva and Cladophora), (b) (Ectocarpus and Fucus, and (c) (Ceraminum)*	*Balanus*	*Barnacles*	*Molluscs*	*Fouling bryozoans*	*Hydroids or bryozoans*	*Hydroids or bryozoans*	*Ascidians*	*Sponges and sea anemones*
Example of typical aspect									
Designation	*Green algae*	*Balanus*	*Calcareous polychaetes*	*Molluscs*	*Fouling bryozoans*		*Bryozoans*	*Ascidians*	
Example of typical aspect									
Short description	Only plants that become attached to immersed surface: a) close to surface; b) at mid depth; and c) at depth	Attached trunco-conical or cylindrical crustaceans	Barnacles are Balanus that are fixed to surfaces via a stem	Bivalves containing a spineless animal in their interior	Calcareous incrustations that multiply from a central individual	Organisms that cover surfaces with an open grass or fur	Like bushes of several centimetres and with branches	Constituted by a spineless bag with two tubular openings or starry plates	Spineless and spongy aspect (sponges) and sea anemones

[Source: Almeida *et al.*, 2007]

2.3 Quorum sensing and biofilm development

Quorum sensing (QS) and biofilm development are closely interconnected processes (Solano *et al.,* 2014). Quorum sensing is chemical communication in bacteria that involves producing, releasing, detecting and responding to small hormone-like molecule called auto-inducers. Biofilm formation is a cooperative group behavior that involves bacterial populations living embedded in a self-produced extracellular matrix (Solano *et al.,* 2014). Bacteria use the chemical signal molecules that contain crucial information to communicate and coordinate the activities of large group of bacterial cells (Waters & Bassler, 2005). Quorum sensing might coordinate the switch to a biofilm lifestyle when the bacterial population density reached a threshold level (Solano *et al.,* 2014). Thus, bacteria are able to monitor the environment for other bacteria and to alter behavior on a population-wide scale in response to changes in the number and/or species present in a community (Waters & Bassler, 2005). Most quorum-sensing-controlled processes are ineffective when single bacterium act alone, however it becomes effective when carried out simultaneously by a large number of bacterial cells. Quorum sensing enables bacteria to act as multicellular organisms by manipulating the distinction between prokaryotes and eukaryotes (Waters & Bassler, 2005). It is crucial to understand the quorum sensing systems in order to prevent the biofouling formation at early physical-reaction stage.

2.3.1 Quorum sensing in Gram-negative bacteria: HSL mediated system

The first described quorum-sensing system is that of the bioluminescent marine bacterium *Vibrio fischeri* that colonize the light organ of the Hawaiian squid *Eupryma scolopes* or bobtail squid (Figure 2.1) (Nealson & Hastings, 1979). It is a mutual

relationship where the bacteria benefit from the nutrient-rich light organ which encourage cells proliferation in numbers that are unachievable in seawater, whereas the squid (Figure 2.1a) uses the light provided by the bacteria (Figure 2.1b) for counter illumination to mask its shadow and prevent threat from the predators (Visick *et al.*, 2000). As shows in Figure 2.2, two proteins LuxI, autoinducer synthase that produce acyl-homoserine lactone (AHL) and LuxR, binding transcriptional activator, control the expression of the luciferase operon (luxICDABE), which is required for the light production. When the AHL reaches a critical threshold concentration, LuxR binds to the AHL and form complex of (LuxR-AHL) that later activates the transcription of the operon encoding luciferase. The complex also induces expression of *luxI* because it is encoded in the luciferase operon. This regulatory configuration floods the environment with the signal that creates positive feedback loop and causes the entire population to switch into "quorum-sensing mode" and produce light. A large number of other Gram-negative proteobacteria possess LuxIR-type proteins and communicate with AHL signals (Manefield & Turner, 2002). Rather than rely exclusively on one LuxIR quorum sensing system, normally bacteria use one or more LuxIR systems, often in conjunction with other types of quorum-sensing circuits.

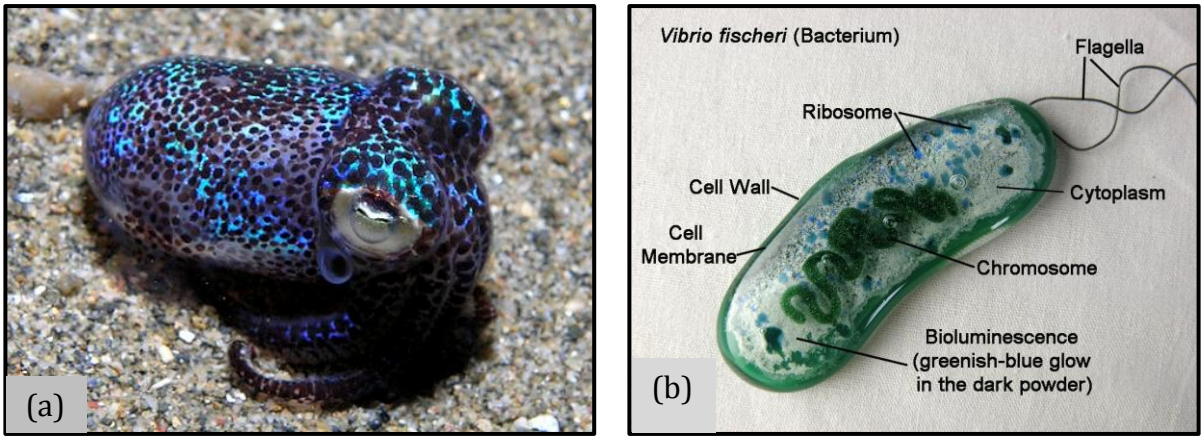


Figure 2.1: Quorum sensing system of two mutual organisms. (a) Bobtail squid from East Timor with visible blue glow that results from the presence of (b) *Vibrio fischeri* luminescence bacteria in the light organ.
[Source:http://germzoo.blogspot.com/2012/01/living-machines-and-flashing-lights.html]

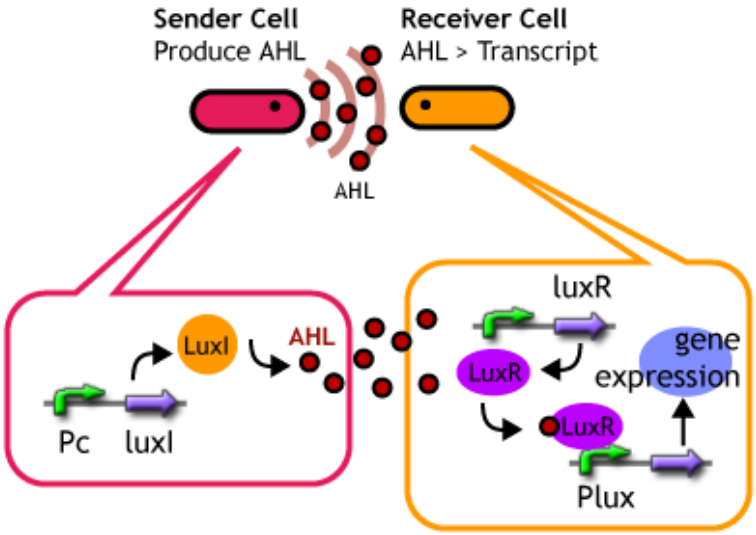


Figure 2.2: Diagrammatic representations for Homoserine-lactone (HSL) mediated quorum-sensing system in Gram-negative bacteria *Vibrio fischeri*.
[Source:https://bli-research-in-syntheticbiologyandbiotechnology.wikispaces.com/Anna]

2.3.2 Quorum sensing in Gram-positive bacteria: Small peptide mediated systems

Differ from Gram-negative bacteria, Gram-positive bacteria use modified oligopeptides as signals to communicate and also "two component"-type membrane-bound sensor histidine-kinases as receptors. Figure 2.3 is showing the diagrammatic representation of the quorum sensing system in Gram-positive bacteria. A phosphorylation cascade that influences the activity of a DNA-binding transcriptional regulatory protein termed as response regulator mediates the signaling. Each Gram-positive bacterium uses a signal different from that used by other bacteria and the cognate receptors are exquisitely sensitive to the signals' structures. Peptide signals are not diffusible across the membrane; hence dedicated oligopeptide exporters mediate the signal release. Many gram-positive bacteria communicate with multiple peptides in combination with other types of quorum-sensing signals.

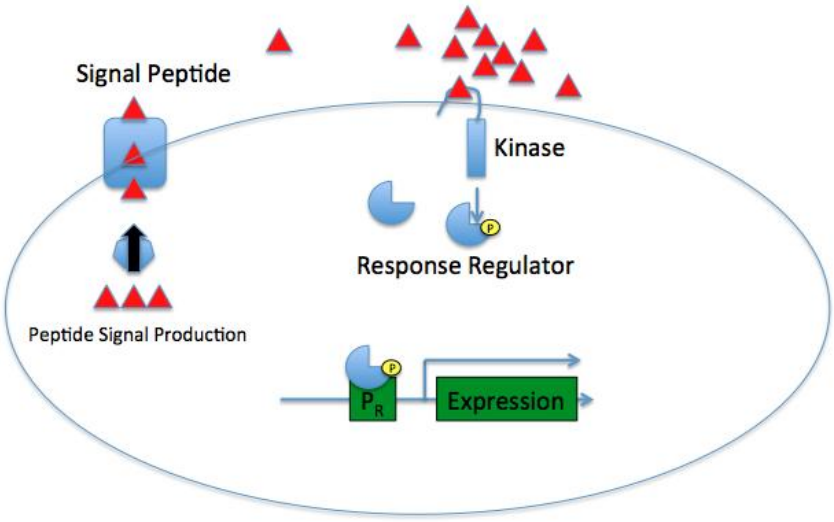


Figure 2.3: Diagrammatic representation for small peptide quorum sensing mediated systems in Gram-positive bacteria of *Staphylococcus aureus.* [Source: http://openwetware.org/wiki/CH391L/S12/Quorum_Sensing]

Figure 2.4 is showing an example of peptide quorum sensing exists in *Staphylococcus aureus* (Tenover & Gaynes, 2000). *Staphylococcus aureus* uses a strategy to cause disease to the host. When at low cell density, the bacteria express their protein factors that promote attachment and colonization, whereas at high cell density, the bacteria repress these traits and initiate secretion of toxins, hemolysins and proteases that are presumably required for diffusion (Lyon & Novick, 2004).

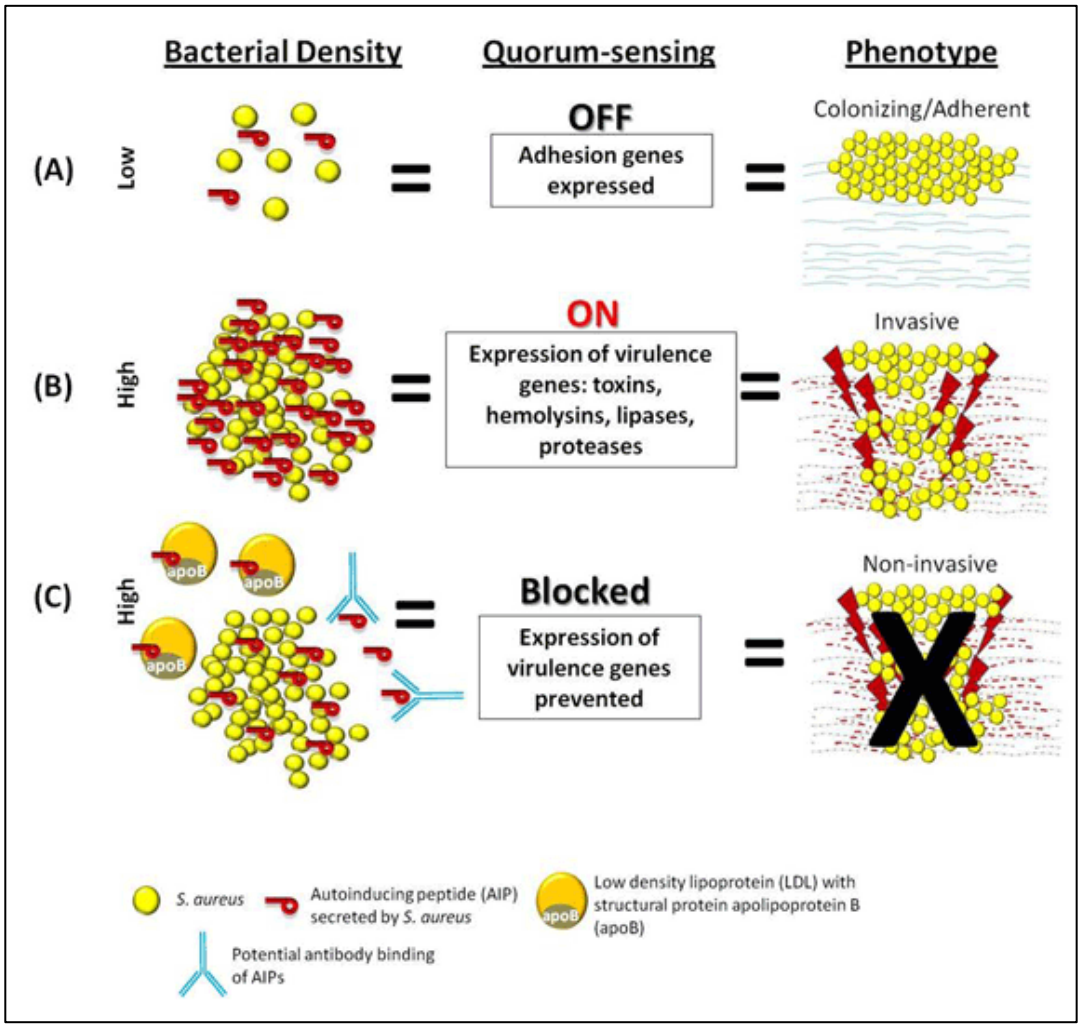


Figure 2.4: Diagrammatic representation of quorum sensing strategy used by *Staphylococcus aureus* to cause disease in the host. [Source: Lyon & Novick, 2004]

2.4 Anti-fouling timeline

2.4.1 First technologies used prior to mid-19th century

Since ancient age, natural products such as wax, tar and asphalt were used as antifouling product. In the history of antifouling product, the Phoenicians and Carthaginians were the pioneer to introduce copper for antifouling purpose. The Greeks and Romans on the other hand started the use of lead sheathing. In the 18th century, the use of wooden sheathing covered with mixtures of tar, fat and pitch and studded with numerous metal nails was common as antifouling approach. Non-metallic sheathings were also used, such as rubber, vulcanite, cork and others. The first antifouling paints appeared only in the mid-19th century, containing copper, arsenic or mercury oxide as toxicants dispersed in linseed oil, shellac or rosin (WHOI, 1952; Lunn, 1974; Callow, 1990). The main antifouling products used prior to the mid-19th century is summarize in Table 2.3.

2.4.2 First antifouling paints used on steel hulls prior to 1960

The development of antifouling paints continues with the emergence of paints with binders based on different bituminous products and natural resins. However, it was reported to cause corrosion on the steel hulls. New products were then applied including “hot plastic paints, “rust preventive compounds” and “cold plastic paints”. The first organometallic paints (with tin, arsenic, mercury and others) appeared in 1950 and gave rise to tributyltin (TBT)-based antifouling paints after numerous successive developments. Table 2.4 summarizes the main antifouling paints used on steel hulls prior to 1960.

Table 2.3: Antifouling products used in the past, prior to mid-19th century

Civilization / navigator	Approximate period	Antifouling product	Reference
Oldest	Oldest	Wax, tar and asphalt	WHOI,1952; Callow, 1990
Phoenicians, Carthaginians	700 B.C.	Pitch and possibly copper sheathing	WHOI,1952; Callow, 1990
Phoenicians	700 B.C.	Lead sheathing and tallow	Lunn, 1974
	500 B.C.	Coating of arsenic and sulphur mixed with oil	Callow, 1990
Greeks	300 B.C.	Wax, tar and lead sheathing	Callow, 1990
Romans, Greeks	200 B.C. to 45 A.D.	Lead sheathing with copper nails	WHOI, 1952
Vikings	10 A.D.	Seal tar	WHOI, 1952
Plutarch	45-125 A.D.	Scraping of algae, slime and pitch	WHOI, 1952
Several Columbus	13th – 15th centuries	Pitch and mixture with oils, resin or tallow Pitch and tallow	WHOI, 1952
Various	1618-1625	Copper, possibly with a mixture of cement, iron dust and a copper compound (sulphide) or arsenic ore	WHOI, 1952; Lunn, 1974
	18th century	Sacrificial wood sheathing on a layer of pitch and animal hair	Lunn, 1974
		Wood sheathing covered with mixture of tar, fat, sulphur and pitch, with numerous metallic nails arranged with their heads forming a type of metallic sheathing.	WHOI, 1952;
English	1758	Copper sheathing, which was abandoned for causing galvanic corrosion of iron, nails.	WHOI, 1952;
English	1786	Copper sheathing, using nails of copper and zinc alloy	Lunn, 1974
English	Early 19th century	Sir Humphrey Davy, after studying the copper corrosion process, demonstrated that copper dissolution in seawater prevented fouling	WHOI, 1952; Callow, 1990
Various	1758-1816	Suggested sheathing of zinc lead, nickel, arsenic, galvanized steel and alloys of antimony, zinc and tin, followed by copper-plated wood sheathing.	Callow, 1990
	1862	Wood sheathing covered with copper sheathing (abandoned due to cost)	WHOI, 1952;
Various	Mid 19th century	Paints containing toxicant (Cu, As or mercury oxide) dispersed in a polymeric binder (linseed oil, shellac, colophony)	WHOI, 1952;

Table 2.4: Types of antifouling paint used on steel hulls prior to 1960

Type of product and first used	Main component		Remarks	Reference
	Binder	Pigment		
First paint (Mid-19th century)	Linseed oil, Shellac varnish, tar, resins	Copper, arsenic or mercury oxides	Dispersion of toxicant in polymeric binder	WHOI, 1952 Callow, 1990
Insulating primer under antifouling paint (1847)	Idem, with preliminary insulating varnish coating	Copper, arsenic or mercury oxides	Insulation of hull from antifouling paint by application of varnish	Almeida *et al*, 2007
"Hot plastic paints" (1860)	Metallic soap composition	Copper sulphate	Similar to "Moravian"	Almeida *et al*, 2007
	Colophony	Copper compound	Italian "Moravian"	WHOI, 1952
Antifouling paint (1863)	Tar	Copper oxide	With naphtha or benzene	WHOI, 1952
Rust preventer (Late 19th century)	Shellac primer and Shellac antifouling paint	Different toxicants	Shellac type paints	WHOI, 1952
Spirit varnish paints (1908-1926)	Grade A "Gum Shellac"	Red mercury oxide or zinc oxide, zinc dust and India red	With alcohol, turpentine essence or pine tar oil	WHOI, 1952
"Cold plastic paints" (1926)	Coal tar or coal tar + colophony	Copper or mercury oxides	Easier to apply than hot plastic paints	WHOI, 1952 Almeida *et al*, 2007
First organo-metallic paints (1950-1960)	Acrylic esters or others	Copper compounds Copper compounds + co-biocides	Some seemed capable of resolving the problem of marine fouling seasonably well	Almeida *et al*, 2007

2.5 Antifouling methods

It is important to research ways to prevent the biofilm formation due to the banned of many toxicant based antifouling paints recently. Several physical/mechanical, physical/chemical and biological/biochemical principles for the prevention of biofouling were used in the last 30 years (Gerencser *et al.,* 1962, Loeb & Neihof 1975, Characklis 1981, Dhar *et al.,* 1981, Branscomb & Rittschof 1984, Fletcher & Baier 1984, Humphries *et al.,* 1986). However, in general antifouling methods can actually be classified into three large categories, which are chemical, physical and biological methods (Cao *et al.,* 2011).

2.5.1 Chemical antifouling methods

2.5.1. (a) Tributyltin self-polishing copolymer coatings.

In 1974, Milne and Hails have patented the first tributyltin self-polishing copolymer (TBT-SPC) technology, which provide an excellent antifouling activity that revolutionized the whole shipping industry (Yebra *et al.,* 2004). TBT-SPC paints are based on acrylic polymer with TBT groups bound on the polymer backbone by an ester. When in contact with seawater, the soluble pigment particles (such as ZnO) begin to dissolve. The polishing rate of the TBT-SPC paints can be control by manipulating the polymer chemistry. Therefore, it is possible to balance between high effectiveness and a long lifespan plus the coatings can be customized for ships with different condition of operation. TBT-SPC paints have high mechanical strength, high stability to oxidation and short drying times. In general, the TBT-SPC was widely applied as an antifouling coating in the shipping industry before it was banned due to the deleterious toxicant effects.

2.5.1. (b) Tin-free SPC-technology

There are two types of tin-free antifouling coatings, which are (i) controlled depletion systems (CDPs) and (ii) tin-free self-polishing copolymers (tin-free SPCs). CDPs incorporate modern reinforcing resins with the same antifouling mechanism as the conventional resin matrix paints. Tin-free SPCs has similar function to TBT-SPC but do not contain tin. The performance of tin-free SPCs is better in comparison to the CDPs. Tin-free SPCs react in a similar to organic tin SPCs, but their matrix material is mostly acrylic copolymer and non-tin metals such as copper, zinc, and silicon. As an example, the Exion series from Kansai Paint uses insoluble Zinc acrylate, which is hydrolyzed to soluble acidic polymer (Yebra *et al.,* 2004).

2.5.1. (c) Non-toxic antifouling technology

Although it seems like no alternative antifouling technology is capable to replace the biocide-based coatings in the meantime, there are some non-toxic approaches that have been reported, in example, the silicone and fluoropolymer coatings (Holland *et al.,* 2004). It appears that fluoropolymers and silicones possess the antifouling property by release. Some low surface energy coatings with modified acrylic resin and nano-SiO2 were also developed (Chen *et al.,* 2008). However, attached fouling organisms are not as easily released as claimed (Brady 2001; Holland *et al.,* 2004; Umemura *et al.,* 2007). A part from that, this method has many disadvantages, such as expensive, poor mechanical properties and difficult to recoat. Thus, the performance is limited which leads the focus of antifouling study to other methods.

2.5.2 Biological antifouling methods

Some organisms are able to secrete enzymes or metabolites that have low-toxicity and biodegradable to inhibit the growth of their competitors. Many attempts have been made by researchers to extract high concentrations of secondary metabolites for biological antifouling. The functional antifouling components have been reported in organisms such as fungi (Xiong *et al.,* 2009), sponges (Limna *et al.,* 2009) and some bacteria (Burgess *et al.,* 2003; Fernando & Carlos 2008). Various enzymes have been reported with antifouling properties such as oxidoreductase, transferases, hydrolase, lyase, isomerase and ligase (Dobretsov *et al.*, 2007; Jakob *et al.*, 2008; Chao *et al.,* 2010). In general, the function of enzymes for antifouling applications can be divided into the following four categories:

2.5.2. (a) Enzymes that degrade adhesive used for settlement

In macrofouling, protein and proteoglycans have an important role in the adhesion step. Proteases can hydrolyze peptide bonds at different sites. Thus, these enzymes can be used to degrade mucilage based on peptide to prevent biofouling. One example is the attachment of Ulva spores, barnacle cyprids and bryozoans were effectively inhibited by serine protease (Pettitt *et al.,* 2004; Dobretsov *et al.,* 2007) by reducing the adhesive effectiveness rather than any toxic effect (Nick *et al.,* 2008). However, the process is more complicated in microfouling (Pettitt *et al.,* 2004; Leroy *et al.,* 2008), because polysaccharide-based adhesive are as important as proteins during secondary adhesion. In general, glycosylase mediated the polysaccharide degradation and the process is difficult and quite complex (Chiovitti *et al.,* 2003). Glycosylase can

target only limited range or linkages. Therefore, it would be difficult to choose an appropriate glycosylase for broad-spectrum antifouling (Leroy *et al.,* 2008).

2.5.2. (b) Enzyme that disrupt the biofilm matrix

The varieties of EPS make biofilm very complex substances. Thus, a very broad combination of both hydrolyses and lyases are required to disintegrate their polymeric network (Jakob *et al.,* 2008). Biofilm are very adaptable to external conditions, the degradation of the crucial component will induce the generation of alternative components that will replace the original and establish a new network to proliferate the organism (Joao *et al.,* 2005). Tests have shown that even though alginase could detach a thin biofilm, it gave no effect on an identical biofilm that was already fully established (Joao *et al.,* 2005). In conclusion, the antifouling method of disrupting the biofilm matrix may not be suitable and effective due to the complexity and adaptability of the biofilm.

2.5.2. (c) Enzyme that generate deterrents and biocides

Recent antifouling compounds extracted from metabolites secreted by different marine animals or plants should be classified as deterrents rather than toxins (Krug, 2006; Krinstensen *et al.,* 2008). Some of the enzymes that possess such effect include glucose oxidase, hexose oxidase and haloperoxidase (Charlotte *et al.,* 1997; Krinstensen *et al.,* 2008). Glucose and hexose oxidase is used to generate hydrogen peroxide to induce oxidative damage in living cells (Imlay, 2003). Haloperoxidase catalyses the formation of hypohalogenic acids usually used in water treatment systems as disinfecting agents (Krinstensen *et al.,* 2008). Hypohalogenic acids have similar

characteristic as hydrogen peroxide that have high rate of decomposing into water and oxygen in seawater, this could be further study as potential nontoxic and biodegradable antifouling substances (Charlotte *et al.,* 1997).

2.5.2. (d) Enzyme that interfere with intercellular communication

As mentioned in section 2.2.1, quorum sensing plays an important role in biofilm formation. Some Gram-negative bacteria required N-acyl homoserine lactone (AHL) for quorum sensing mechanism. By first eliminating the AHL auto inducers may thus prevent the development of biofouling (Krinstensen *et al.,* 2008). AHL acylase able to degrade AHL and biofilm formation is inhibited by the increasing concentration of this enzyme. The settlement of Ulva spores and polychaete larvae was also affected by acylase to some extent (Callow & Callow, 2006; Huang *et al.,* 2008).

2.5.2. (e) Challenges for enzymatic antifouling methods

The temperature ranges of seawater from -2^0C to 30^0C can affect the enzyme activity and stability. It is very challenging to balance the effectiveness of the enzymatic antifouling coating and it lifespan because if the temperature is too high, the enzyme will decompose thus decrease the lifespan of the enzymatic antifouling coating. Apart from that, another crucial step in this antifouling method is to design an appropriate coating matrix that contains the enzyme for successful application. More study should be made to analyze the distribution of the enzyme and its amount because soluble enzymes will soon form a thick leaching layer.

2.5.3. Physical antifouling methods

2.5.3. (a) Antifouling by electrolysis and radiation

The most common physical method in preventing biofouling is to produce hypochlorous acid (HCIO), ozone bubbles, hydrogen peroxide or bromine through electrolysis of seawater (Chiang *et al.*, 2000; Tadashi & Tae, 2000; Yebra *et al.*, 2004;). Their strong oxidizing ability will spread all over the ship's hull and eliminate possible surface for fouling organism's attachment. Some of the systems are not highly efficient due to the large voltage drop across the surface that causes the corrosion problems of steel. Currently, titanium-supported anodic coating has been suggested with advantages such as having low decomposition tension, higher current efficiency, and lower energy consumption (Liang & Huang, 2000). Another method is by microcosmic electrochemical methods that use direct electron transfer between electrode and the microbial cells. This causes electrochemical oxidation of the intercellular substances, however it is expensive and the efficiency has not been established (Krinstensen *et al.*, 2008). Vibration method such as acoustic technology has also been reported (Sanford & Rittscho, 1984). Hydroids, barnacles and mussels can be inhibited to some extent by either external vibration sources or piezoelectric coating (Miloud & Mireille, 1995). However, this method requires huge power consumption. Other studies have evaluated magnetic fields, ultraviolet radiation and radioactive coatings (Yebra *et al.*, 2004), but these method are not practical in application. Another potential method is to use substrates with different color, which affect the attachment and growth of spores and worms (Swain *et al.*, 2006).

2.5.3. (b) Antifouling by modification of surface topography and hydrophobic properties

In recent years, varying surface characteristics, including surface roughness, topography, hydrophobic behavior, and lubricity, have been investigated for antifouling application. Studies had shown that fouling diatoms adhere more strongly to a hydrophobic polydimethylsiloxane (PDMSE) surface than to glass. Bacteria and Ulva spores adhere strongly on the surface with greater angle and hydrophilic surface. Moreover, hydrophilic surfaces are thought to be capable of antifouling. For example, surface with metal nanoparticles such as TiO2 have antifouling behavior, because the photocatalytic activities introduced by solar ultraviolet make the surface more hydrophilic so the biofilm is washed more easily (Dineshrama *et al.,* 2009). However, some species exhibit different adhesion behavior on the same set of surface highlighting the importance of differences in cell-surface interactions (Finlay *et al.,* 2002; Sitaraman *et al.,* 2006). Thus, it inspired the development of a surface that presents both hydrophilic and hydrophobic domains to settling cells and organisms. It has been shown that rougher surface increase adhesion of *Pseudomonas* (Scardino *et al.,* 2006). In conclusion, the identification of effective antifouling topographies typically occurs through trial-and-error rather than predictive models, thus these theories are not sufficient to explain the real situation. Therefore, these formulas are not expected to guide the development of antifouling methods.