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# **Identification of marine antifouling compounds from marine resources**

**Institute of Marine Biotechnology  
(University Malaysia Terengganu)**

Travail de fin d'études réalisé par  
Collard Olivier en vue de l'obtention du grade académique  
de Bachelier en chimie

Orientation : biotechnologie

Promoteur : Déborah Lanterbecq

Maître de stage : Noraznawati Binti Ismail

Année académique 2018 – 2019

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## **List of acronyms**

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AMS: Air Membrane Surface

DDT: Diffusion Disc Test

EPS: Extracellular Polymeric Substance

FT-IR: Fourier-Transform Infrared Spectroscopy

GC-MS: Gaz Chromatography: Mass Spectrometry

HPLC: High Performance Liquid Chromatography

IMAP: Insoluble matrix antifouling Paint

NMR: Spectroscopy: Nuclear Magnetic Resonance spectroscopy

Rf: Retention factor

SMAP: Soluble matrix antifouling Paint

TLC: Thin Layer Chromatography

## **Glossary**

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**Biocide:** a chemical substance or microorganism intended to destroy, deter, render harmless, or exert a controlling effect on any harmful organism by chemical or biological means.

**Biofouling:** the accumulation of microorganisms, plants, algae or animals on wetted surface (including organisms)

**Brownian motion:** the random motion of particles suspended in a fluid liquid or gas resulting from their collision with the fast-moving molecules in the fluid

**Epibiosis:** any relationship between two organisms in which one grows on the other but is not parasitic on it.

**Topography:** the study of the shape and features of a surface

# **1 Presentation of the company**

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## **1.1 University Malaisia Terengganu (UMT)**

### **1.1.1 History**

The university of Terengganu Malaysia (UMT) is a university located in Malaysia (Figure 1).

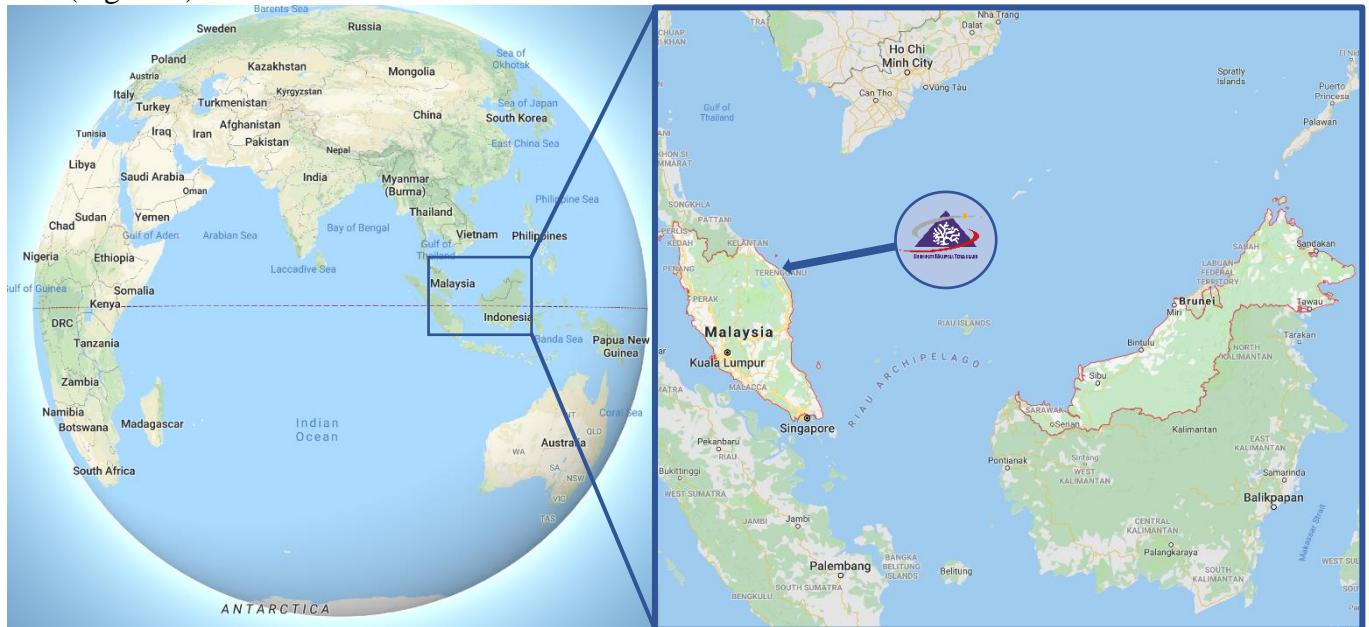


Figure 1 Localization of UMT in the world ([www.google.com](http://www.google.com))

UMT was first a marine science station from the Faculty of the Fisheries Science and Marine Science. In June 1996, the Faculty of Fisheries Science and Marine Science has been transferred to Terengganu and upgraded to a branch campus to be renamed as University Pertanian Malaysia Terengganu (UPMT). Later, on 1 May 2001, the UPMT was given autonomy and was renamed the Malaysian Science and Technology University College (KUSTEM) on 20 June 2001. On 1 February 2007, the name KUSTEM was changed again to become University Malaysia Terengganu (UMT), which is the present name.

### **1.1.2 The UMT**



The University of UMT (Figure 3)(Figure 2) is focused on Fisheries and Marine Science. It proposes a wide range of Fisheries and Marine Science programmes such as fisheries and aquaculture sciences or marine and environmental sciences as well as maritime business and management.

Figure 2 Waist band of the UMT

The campus is composed of many different facilities (laboratories, hatcheries Figure 4, auditoriums, stores, sport complex) thus providing a suitable working place for students and researchers.

The University also possesses many other external sites out of the campus, as Bidong Island (Figure 5) which is a nature reserve providing an enormous amount of sampling resources for the research, or Kenyir lake (Figure 6) research centre placed next to the Kenyir lake which is the biggest manmade lake in Southern Asia.



Figure 3 UMT campus ([www.umt.edu.my](http://www.umt.edu.my)) located in Kuala Terengganu



Figure 4 Hatchery of the university (<http://www.umt.edu.my>) located in Kuala terengganu



Figure 5 Bidon Island (<http://coraldivestore.com>) located in Kuala Terengganu



Figure 6 Kenyir lake located in the state of Terengganu (<http://www.dronestagr.am>)

### **1.1.3 Research themes in UMT**

The university research is separated into different institutes and programs (Figure 7) (<https://www.umt.edu.my>):

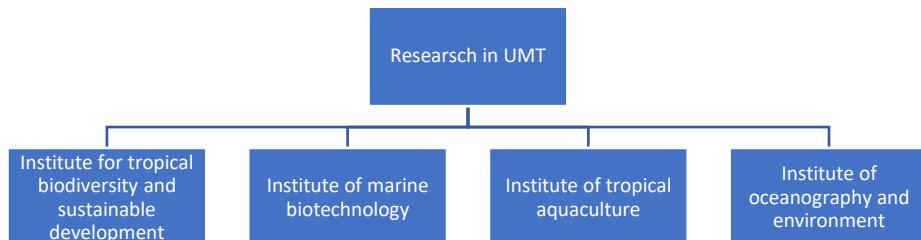


Figure 7 Organigram of the research in the UMT

- **The Institute of tropical biodiversity and sustainable development** is an institute established in 2013 and situated near the Kenyir lake. The main objective of this research institute is to preserve and study the ecosystem of this lake which is put in danger by different threats and problems like overfishing, development of tourism industry, etc... Another goal is to let Kenyir become a famous tourist destination with a model of rural development focusing on environmental sustainability.

- **The Institute of tropical aquaculture** is one of the 21 excellent centres of aquaculture in the world and has been established in 2004. It aims to increase the production of important fishes, shrimps and other aquatic species through research and development (R&D).
- **The Institute of oceanography and environment** focuses its activities on oceanic and marine related research and post-graduate training.
- **The institute of marine biotechnology** was established in 2006. Its main mission is to spearhead the exploration of new resources through sustainable management by merging expertise and advanced biotechnology applications.

#### **1.1.4 The Institute of Marine Biotechnology (IMB)**

The Institute of Marine Biotechnology is divided in different research programs (Figure 8) (<https://www.umt.edu.my>):

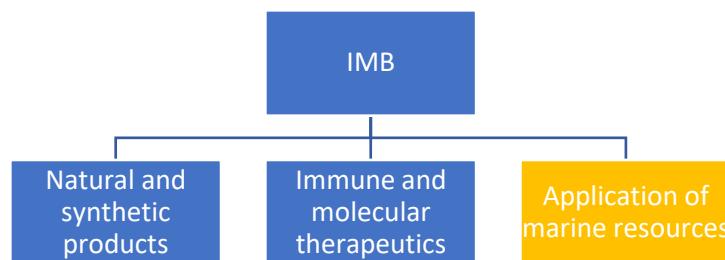


Figure 8 Organigram of the research program in the IMB

- **Natural and synthetic products.** This program's goal is to spearhead the production of enhanced fractions and small molecules from the prepared powdered biota. The molecules are then isolated, and their structure is determined.
- **Immune and molecular therapeutics.** This program develops high-throughput screening assays using cell-free, cell-based and whole organism model systems in order to serve as platforms to test the small molecules prepared from Natural and Synthetic products research program.
- **Application of Marine Resources.** The goal of the program is to carry out sustainable collection and identification of marine microorganisms with wide potentials to produce secondary metabolites as drug candidates. In addition, the researchers are also actively identifying new algae as important sources of high value bioproducts for edible (anti-oxidative agents) and non-edible (biofuels) applications. My work was conducted in the context of this research program

## **1.2 Petronas**



**PETRONAS**

*Figure 9 company designator  
([www.petronas.com](http://www.petronas.com))*

Petronas is the founder of the project that will be described in this work. It is a huge and very important Malaysian oil and gas company founded on August 1974 (Figure 9)(Figure 11) It is ranked among Fortune Global 500's largest corporations in the world and also as the 8<sup>th</sup> most profitable company in the world and the most profitable in Asia ([https://www.petronas.com/](http://www.petronas.com/)).

Petronas is also a company that put effort into reducing their carbon impact as well as their environmental impact. The company deals with a lot of problems due to the attachment of organisms on their marine structures (also known as biofouling) (Figure 10). UMT

collaborates with the company in order to find new environmental-friendly compounds to prevent the biofouling formation on their structures while reducing their environmental impact on the marine ecosystem .



*Figure 10 Pictures of the attachment of organism under an oil platform (<http://awesomedesign.com>)*



*Figure 11 Pictures of the different installations of Petronas in Malaysia ([www.petronas.com](http://www.petronas.com)), oil platform (1), main office situated in Kuala Lumpur (2), oil installation situated in Terengganu (3).*

### **1.3 Motivation for the University choice**

Marine biology is a subject which has fascinated me from an early age. I have always liked to observe marine organisms in aquariums, as well as studying them in a laboratory. UMT was the perfect choice for me as it has a very large amount of different marine research programs, especially the field of marine biotechnology, for which I have a lot of interest. The university is also located next to the sea where I could observe a large range of different organisms.

Furthermore, I have always been sensible to the climate changing and the increase of pollution in the world. I have always wanted to act in order to find solutions that would at least improve the situation of those world threats. By working on the project of this lab, I have the feeling to contribute to a better world.

## **2 Introduction**

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Biofouling is the accumulation of microorganisms, plants, algae or animals on wetted surfaces. It plays an important role in marine ecosystems such as an efficient filter of plankton and detritus, it is also an important source of food for many marine animals as well as for man [1]. However, when interacting with manmade structures, they incur in severe costs to navigation and other maritime activities. For example, biofouling makes ship hulls irregular and rough, increasing drag and decreasing manoeuvrability and cruising speed. Fouling is also known to cause destruction of metallic surface by accelerating the corrosion rate (Example of the formation of biofouling on a ship's hull (Figure 12).



*Figure 12 Example of the formation of biofouling on a ship's hull*

Those effects are even worst in tropical environments where fouling development is much faster. This phenomenon costs marine, shipping and other global industries, billions of dollars every year. One of the most efficient methods to prevent marine fouling is the use of tributyl tin (TBT) based antifouling paint, but this chemical compound has harmful effects on many non-target organisms (e.g., shell malformation in bivalves, depletion of oyster population, imposex in gastropods and others). TBT has been labelled as the most lethal substance to have been deliberately introduced into the marine environment [2]. Marine Environment Protection Committee banned the application of TBT for marine applications since 1 January 2008 [3]. After the ban of TBT, copper paints and many other compounds as chlorothalonil, zinc parathion, pyridine and more, are used as an alternative, but most of them are under strict regulations in various regions of the world due to the possible negative effects on marine ecosystems [3]. Because of this environmental issue, an environmentally friendly alternatives to toxic biocides in antifouling paints are urgently needed.

### **2.1 Objectives**

The main objectives of this work are to investigate new environmentally friendly antifouling compounds produced by free-living marine bacteria or bacteria hosted by organisms such as sponges and seaweeds.

The objectives of this project are:

- Isolation of free-living marine bacteria or bacteria hosted by marine organism
- Measurement of the antibacterial activity of their secondary metabolite using Disc Diffusion Test (DDT).
- Separation and identification of the chemical nature of the compounds using High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC)

### **3 Theoretical part**

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#### **3.1 Bio-fouling**

##### **3.1.1 The process**

Biofouling or biological fouling is a gradual accumulation (attachment and growth) of microorganisms, plants or animals, on artificial and natural wetted solid surfaces such as pipes, underwater equipments and so on. It is a really important process in the marine ecosystem as it provides food for many organisms and filter detritus and plankton from the seawater. but when it interferes with manmade structures it brings a lot of problems and costs that will be discussed later.

Its natural formation process in the sea can be divided in 3 major steps [4] (Figure 14):

##### **Conditioning film (step 1 Figure 14)**

First, a layer of conditioning film is formed on the hard surface (Figure 14: step 1). It is composed of organic materials such as proteins, polysaccharides and proteoglycans naturally present in the seawater. This step lasts a really short time (1 minute) and provides a stickier surface so that the microorganisms can adhere to it.

##### **Microfouling (step 2 Figure 14)**

This step is the formation of a primary film by biofilm-forming bacteria and diatoms.

The biofilm is formed as bacteria, microalgae and protozoa adhere to the surface (Figure 14 step 2). The adhesion of the microorganisms on the surface involves 2 steps:

- 1) Reversible absorption: this adhesion is caused by physical effect such as van der Waals forces, electrostatic interaction, gravity, water flow and Brownian motion\*.
- 2) Irreversible adhesion: this adhesion mainly occurs through a biochemical effect such as secretion of Extracellular Polymeric Substance (EPS) which includes a variety of extracellular carbohydrates, proteins, nucleic acids, glycoproteins, phospholipids and other surfactants forming the biofilm.

The biofilm then formed by the microorganism is composed of 5-25% of living organisms, 75-95% of matrix (itself composed of 95-99% of water). The dry weight of the matrix is mainly composed of acidic exopolysaccharides as well as lipopolysaccharides around the bacterial cell [5].

This biofilm provides the microorganism with a good protection from predators, toxins and environmental changes, as well as an easier capture of the necessary nutrients. The existence of adhesive substances EPS in the biofilm and the roughness of irregular microbial colonies help to trap more particles and organisms as well.

\*(Brownian motion is the random motion of particles suspended in a fluid liquid or gas resulting from their collision with the fast-moving molecules in the fluid).

### Macrofouling (step 3 Figure 14)

After the formation of the biofilm, larvae or spores such as barnacles, mussels, polychaete worms, bryozoans and seaweed (Figure 13) (also called macrofoulers) will attach to the surface after 3-4 days (Figure 14 step 3). It is also important to mention that few macrofoulers (as some species of bryozoans for example) adhere to the surface before the formation of biofilm.



Figure 13 The most important macrofouling species including barnacles (A), mussels (B), polychaete worms (C), bryozoans (D) and seaweed (E). Source of pictures a respectively (<https://en.wikipedia.org>) (<https://mnogolok.info>) (<http://www.snipview.com>) (<https://fishhawklakerealtor.com>) (<https://phys.org>)

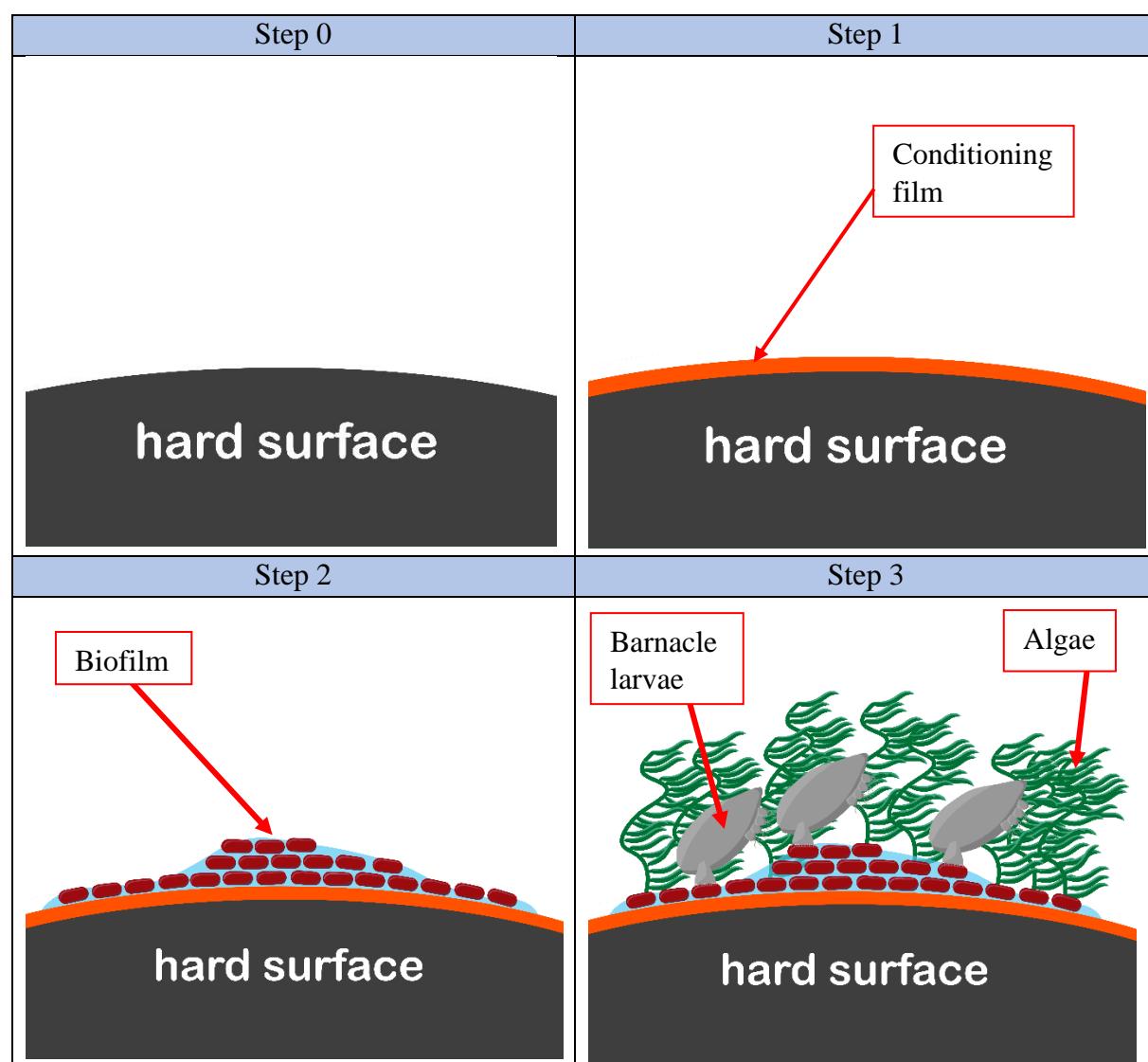


Figure 14 Steps of the biofouling process (Collard Olivier)

After several months, the biofouling is made of thousands of different organisms such as bacteria, phytoplankton, molluscs, fungi and more.

The biofouling formation speed is affected by different factors. Some of them depend of the environment and thus can hardly be modified [6]:

- **Temperature.** The biofouling process is known to be heavier in regions with high water temperature. In tropical climate, where the climate doesn't change significantly and stay warm, biofouling may continue without interruption. In regions where marked seasonal variations in temperature occur, the reproduction and the growth of many species are completely suppressed during the low-temperature period.
- **Salinities.** Low salinities affect negatively the growth rate and the maximum size attained by biofouling organisms. However, some species live better in such conditions.
- **Solar radiation.** The amount of solar radiations affects directly the rate of photosynthesis of the plants and thus controls the nutrition of the animals and the growth of algae.
- **Pollution.** Polluted water can decrease the biofouling process speed either directly by being toxic to the organism or indirectly by reducing the amount of oxygen or solar radiation available.
- **Water depth.** The depth affects the speed of the biofouling process as well. However, the colonised ships are most of the time sailing in superficial waters so that the depth factor rarely changes and represents little influence on biofouling process.
- **Interaction between different organisms.** Some organisms produce secondary metabolites in order to inhibit the attachment of other organisms on themselves. This phenomenon is very interesting and is the first key feature of the current project. It will be longer discussed in a following section.

Other factors concern the ships characteristics and can be modified [6]:

- **Ship's speed.** Biofouling can not occur significantly on the ship surface if its speed is higher than 11,5 km/h. this parameter can be easily modified but it depends mostly on the kind of ship and its activity
- **Ship surface.** the nature of the support clearly affects the biofouling process. It depends on the coating surface properties and thus can be modified and optimized for antifouling purposes. This factor is the second key feature of the current project and will be longer discussed in a following section.

### **3.1.2 The biofouling and manmade marine structure**

When biofouling interacts with manmade marine structures it can induce huge harmful effects leading to severe costs (Figure 15).



Figure 15 Effect of the biofouling on boat's hull (A)(<http://lovesail.com>) and boat's propeller (B) ([www.kyl.com](http://www.kyl.com))

On ships's hulls, biofouling results in increase in roughness, turbulence, and extra weight. Moreover, it diminishes the ship's manoeuvrability, laminar flow and carrying capacity. If the ship's is not or poorly treated, marine fouling can add up to 150 Kg per square meter in only six months at sea (Figure 15).

This increase of the boat weight and the hydrodynamic drag can increase the fuel consumption up to 40-50% and increasing the voyage overall cost as much as 77%. Adding to other costs such as paint removal and repainting, hull cleaning and a variety of associated and interrelated environmental compliance regulations, biofouling is a serious and very expensive worldwide problem, costing the US Navy alone an estimated 2,1 billion dollars annually (<https://smallbusinessonlinecommunity.bankofamerica.com> 2008).

Adding to the economic problem, biofouling on ship also introduce species (invasive or non-invasive) into environments where they were not naturally present, leading to even more environmental threat.

In the oil sector (for example in Petronas's installation), biofouling is also a serious threat for structures such as oil platforms [1]. Biofouling promotes corrosion and increases the mass of their underwater structures, distorting their original configuration that lead to a weakening of their structure.

Biofouling is also a big problem when it comes to aquaculture. In shellfish aquaculture, biofouling induces biological competition, environmental modification, physical damages to the organism, etc... In finfish aquaculture, most of the problems come from the biofouling of the infrastructure, which restrict water exchange, increase disease risk and cause deformation of cages and structures. Therefore, biofouling brings cost estimated between 5-10% of the production cost [7].

Biofouling is thus a big economic and environmental problem that constantly needs treatment (antifouling treatment) in order to reduce its formation.

## **3.2 Antifouling**

Biofouling has been recognized as problematic for more than 2000 years and many kinds of antifouling methods have been investigated over time [6].

To date, different methods exist to prevent the formation of biofouling. these methods are divided in three different categories:

- Chemical method
- Physical method
- Biological method

### **3.2.1 Chemical methods**

Chemical methods use biocides contained in a matrix that is applied on the surface. When the surface is in contact with seawater, the matrix will gradually release the biocides into the seawater killing the biofouling organisms.

Chemical methods are separated in two categories:

- Traditional chemical antifouling methods
- Modern chemical antifouling methods

#### **3.2.1.1 Traditional antifouling chemical methods**

Traditional antifouling chemical methods comprise:

- Insolubles matrix antifouling paints (IMAP)
- Soluble matrix antifouling paints (IMAP)

##### **1) Insoluble matrix antifouling paints (IMAP)**

Insoluble antifouling paints (Figure 16) have a polymer matrix (For example vinyl polymer) that will not erode in water, only toxic materials will dissolve in seawater resulting in an empty matrix called “each layer”. The advantage of the use of these paints is that the structures is mechanically strong and stable to oxidation and photodegradation. However, the paint has its limit. Since the matrix is insoluble, the thickness of the paint layer must not exceed a certain amount depending on the type of matrix used. At a certain point the leach layer will be so thick that the seawater will not be able to penetrate any deeper into the matrix and the rate of release of the biocide will fall under the minimum value required for antifouling [6] that depend on the biocide contained in the matrix.

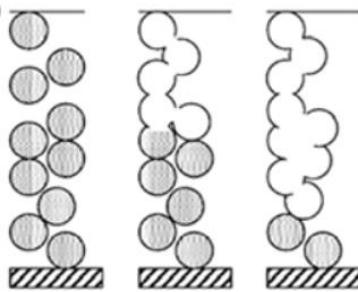


Figure 16 Diagram of insoluble matrix antifouling paint [6]

## 2) Soluble matrix antifouling paints (SMAP)

Soluble matrix antifouling paints (Figure 17) have a soluble matrix containing biocides. The biocides will be released gradually in the seawater, leaving a leach layer that will be washed away when the boat moves into the water. The advantage of this method is that the biocide contained deeply inside the paint layer can be easily exposed to the sea water and thus the lifespan of the coating is much better than the one of insoluble matrix antifouling paints. However, mechanical properties are inferior than the ones of insoluble matrix antifouling paints. Moreover, if the boat is not moving for a long time, the leach layer will stay and the biocides will be trapped in the film formed by the leach layer causing a decrease of the biocide effect [6].

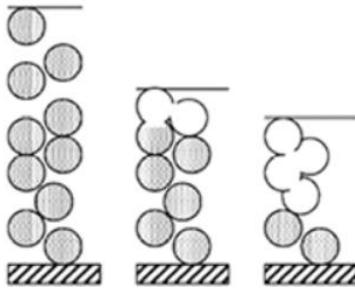


Figure 17 Diagram of Soluble matrix antifouling paint [6]

### **3.2.1.2 Modern chemical antifouling methods (MCAM)**

Modern chemical antifouling method (Figure 18) are more complicated than the traditional ones but they present a lot of advantages. One of the most popular MCAM is the tributyltin self-polishing copolymer coating (TBT-SPC paints) (Figure 19). To summarize this technology, the biocide (for example TBT) is bound to the polymer backbone by an ester link. This carboxyl-TBT linkage is easily hydrolysed in slightly alkaline environment, such as in seawater, thus releasing the biocide. This method allows to have no leach layer and therefore the biocide is released whatever the boat is moving or not [6].

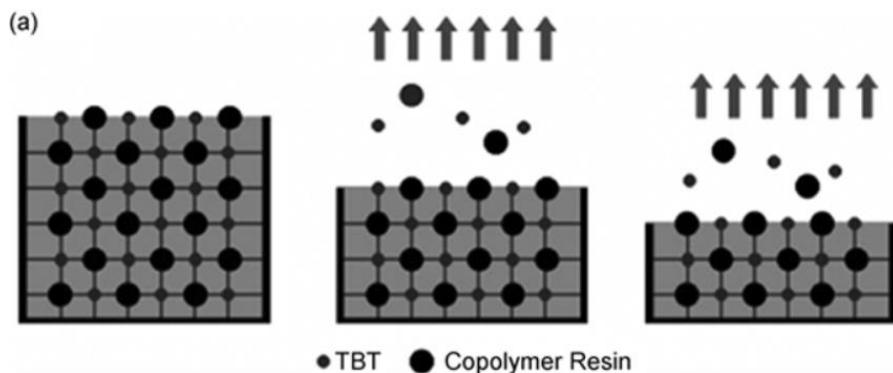


Figure 18 diagram of the TBT-SPC system [6]

The polymer chemistry can be changed to control the polishing rate and thus the biocide release. This method has high mechanical properties, short drying time and high stability to oxidation as well. This type of paint have as well a very long lifespan (4-5 years) compare to the IMAP and SMAP (6-24 months)

TBT-SPC have been the most successful coatings in combating biofouling on ships. It was used by up to 70% of the world fleet in 2004 [6]. However, TBT and its related agents were found to be highly toxic on non-target organisms because of their lipophilicity, which allows them to penetrate the biological membranes. For that reason, TBT-SPC paints have been totally forbidden in 2008 by the International Maritime Organization [3].



Figure 20 Bottle of an old antifouling paint that used TBT before its abolition ([www.sailfeed.com](http://www.sailfeed.com))

Figure 19 Application of the antifouling paint on a hull's boat ([www.coating.co.uk](http://www.coating.co.uk))

After the banishment of TBT and all its derived products, copper and inorganic zinc have become the principal biocide components of most antifouling paints. However, following the European Union laws, they are toxic to aquatic organisms, with long-term harmful effects on the environment. Their use are thus under strict regulation. Moreover, in the food industry (for example in aquaculture), the use of copper and zinc as antifouling compounds is strongly unwelcomed for health and marketing perspectives [8].

Chemicals methods are the most effective antifouling methods but, because of all the environmental consequences and their toxicity leading to banishment and restriction, it is not a durable method for the future. Thus, researchers have focussed their studies on other "more" environmental friendly methods.

### **3.2.2 Physical method**

- 1) **Antifouling by electrolysis.** Electrolysis of seawater produces different compounds such as hypochlorous acid (HCLO), ozone bubbles, hydrogen peroxide or bromine. Those different compounds possess a strong oxidizing ability and will spread all over the ship's hull and eliminate areas of fouling organisms. However, some of these systems are not highly efficient because of large voltage drop across the surface. Moreover, it intensify the corrosion problems of steel [4].
- 2) **Antifouling with vibration methods.** Vibration methods such as acoustic technology have also been confirmed to have an antifouling effect [9]. However, the huge power consumption of these methods is difficult to overcome.
- 3) **Antifouling with radiation methods.** Other studies have evaluated magnetic field, ultraviolet radiation and radioactive coatings as having antifouling activities. but these methods have no practical applications [6] .
- 4) **Antifouling by modification of surface topography and hydrophobic properties**  
**Surface characteristics** like roughness, topography, hydrophobic behaviour and lubricity can also have some antifouling applications [4].

### **3.2.3 Biological methods**

The biological methods involve the use of variety of compounds secreted by cells and inhibiting the growth of biofouling organisms. These secretions are environmentally friendly and biodegradable, which make them a good substitute for traditional biocides. These methods stir the interest of researchers and could replace chemical methods in a near future.

## **3.3 Biological environmental friendly antifouling compounds**

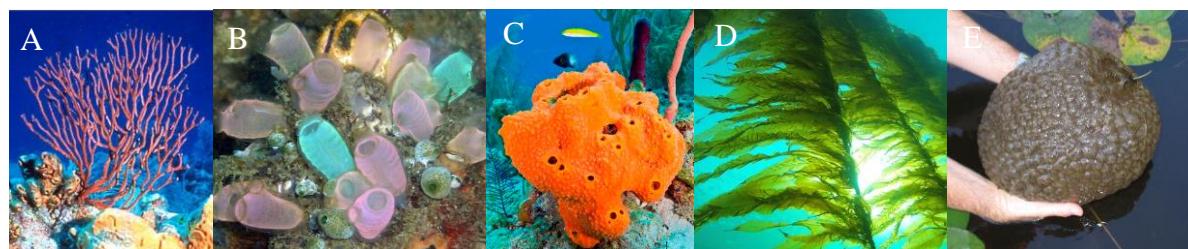
### **3.3.1 Organisms producing antifouling compounds**

The search for natural antifouling products has grown a lot since the need of an alternative for toxic biocides has become urgent. Researchers especially focus on marine microorganisms and macroorganisms as they are known to produce an enormous amount of bioactive compounds that could possibly serve as antifouling products [2].

The idea of this project comes from the fact that marine organisms such as sponges, seaweed, and so on..., present also a surface submerged in the water , which is ideal for the Biofouling formation. Surprisingly, most of them present no or very few biofouling formations on their surface.

In densely populated marine environments, space is often a limiting factor. When free space is not available, one organism grows on top of another one. This process is called epibiosis [2]. This process can interfere with vital processes such as respiration, nutrient absorption and sensing. Therefore, organisms use different defensive methods which include surface sloughing, possession of spine, production of mucus, low surface energy, but mainly secondary metabolites production. Those secondary metabolites are great candidates as they are natural antifouling compounds with low-toxicity and biodegradable [10]. However, those secondary metabolites are produced in small quantity and as a complex mixture. To be able to produce an efficient paint, a large number of organisms (such as sponges and corals) would have to be collected from the sea in order to get enough compounds. This scenario would be dramatic for the biodiversity conservation. Some studies have revealed that antifouling compounds retrieved in some organisms are in fact produced by microbes associated with the marine organism itself [2].

Macroorganisms, such as gorgonians, ascidians, sponges, algae (seaweed) and bryozoans (Figure 21) represent a promising source of antifouling metabolites as they host a large number of different bacteria and fungi.



*Figure 21 Gorgonians (A), Ascidiants (B), Sponges (C), Seaweed (D) Bryozoans (E), which are a great potential source of antifouling metabolites*

Sponges have been the ones to capture researchers attention due to their close relationships with a wide variety of microbes and the presence of a large amount of biologically active secondary metabolites [11].

The candidate microorganism, producing the compounds of interest, could thus be grown on large-scale culture in order to produce a sufficient amount of antifouling compounds (useful for the production of antifouling paints).

However, the isolation of microbes from macroorganisms is not an easy task as some of them need really specific conditions for their growth or need a stress in order to produce their secondary metabolites.

Fortunately, microorganisms associated with marine organisms are not the only source of antifouling compounds production. Some free living bacteria in the sea shows antifouling properties as well, but seem to be less efficient in term of efficacy than symbiotic ones [3]. Other non-marine resources like the leaf of some trees constituting of mangrove show some antifouling activities too.

### 3.3.2 Nature and properties

secondary metabolites produced by microorganisms can have a wide range of chemical structures [12][13] (Table 1).

Fatty acids	Lactones
Fatty acids are composed of a carboxylic acid followed by a long chain of carbon	Lactones are cyclic carboxylic esters, containing a 1-oxacycloalkan-2-one structure (C=O)-O-
Terpenes	Steroids
Terpenes are hydrocarbons composed of the combination of isoprene units.	Steroids are organic compounds composed principally of a core containing 17 carbons bonded in four fused rings
Benzenoids	Phenyl Ethers
Benzenoids are compounds containing at least one benzene ring.	Phenyl ethers are composed of phenyl groups bounded to other chemical groups by an ester
Polyketides	Alkaloids
Polyketides contain alternating carbonyl and methylene groups	Alkaloids are organic compounds that mostly contain basic nitrogen atoms
Nucleosides	Peptides
Enzymes	Enzymes are a chemical produced by cell that generally speed up specific chemical reaction

Table 1 Different types of chemical structures that are known to show antifouling activities (Kai-Ling Wang et al. 2017)

Those compounds can act differently on fouling organisms. Those antifouling effects can be divided in 7 major categories [14] (Figure 22).

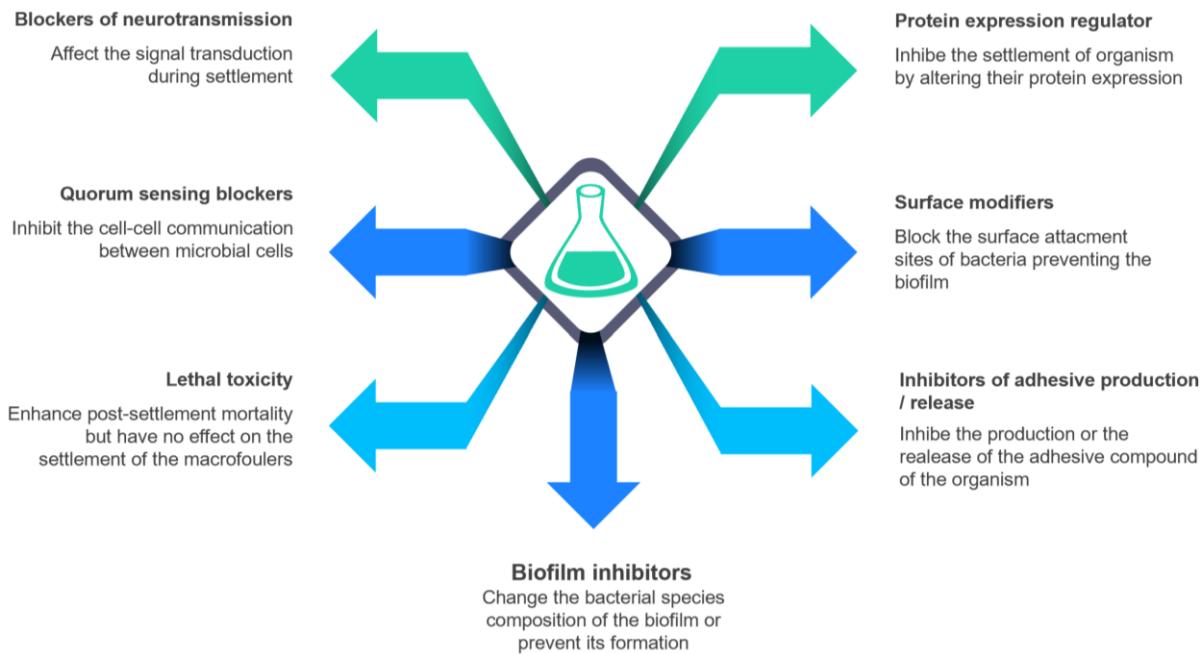


Figure 22 General mode of action of antifouling compound on fouling organisms

The current work (subject of my bachelor thesis) focuses only on the biofilm inhibitors antifouling compounds because these antifouling effects present some advantages compares to the others. First, as shown in the section 3.1.1 explaining the process of biofouling formation, biofilms are the basis for further settlement of invertebrate larvae in marine environment. Compounds disrupting or inhibiting the biofilm formation would prevent the settlement of many fouling organisms compared to those compounds that disrupting directly the fixation of one specific macrofouler. Secondly, biofilm inhibitors compounds are easier to screen: it is easier and faster to see the effects of compounds on fast growing bacteria in a Petri dish than on the settlement of larvae and algae, which need more time and attention to occur and requires specific lab equipment.

Therefore, the antifouling activity of a compound will be determined by measuring its antibacterial activity on easy to culture gram-positive and gram-negative bacteria. Secondary metabolites that show antibacterial activity are considered to be antifouling compounds since they would disrupt the bacteria forming the biofilm.

### 3.4 Isolation and Identification of marine antifouling compounds from marine resources

To isolate and identify antifouling compounds of the sea, different steps are needed (Figure 23).

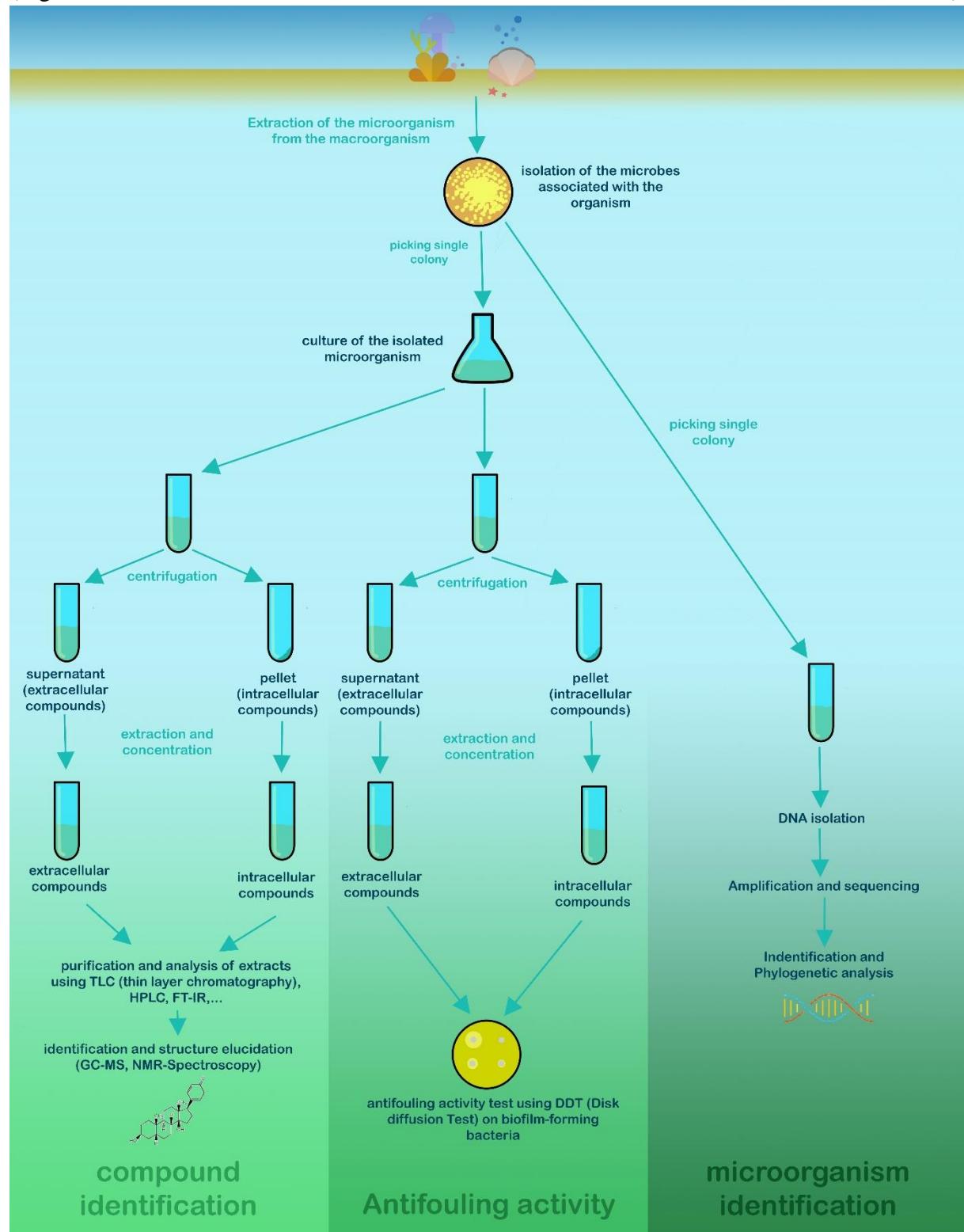


Figure 23 Diagram of the process to isolate, identify and measure the antifouling activity from a compound produced by a microorganism (Olivier Collard)

- 1) Firstly, the crude extract of the organism is extracted by mixing the powdered organism with an organic solvent which is then filtered and concentrated. The antibacterial activity of the crude extract is then determined using a Disc diffusion test (DDT). The disc diffusion test consist of seeing the effect of a substance (loaded on a sterile disk) on the growth of different bacteria in a petri dish.
- 2) If the crude extract of the organism show antibacterial activities, The microbes associated to this organism are isolated and culture on solid media to obtain single colonies.
- 3) Single colonies are cultivated in order to produce different types of secondary metabolites. Different culture methods can be used given that some bacteria produce their secondary metabolites only when a stress is induced or if they need an environment that mimic the chemical and physical characteristics of the natural environment.
- 4) The medium is then centrifuged to separate the supernatant (extracellular metabolite) from the pellet (intracellular metabolite).
- 5) The potential antibacterial activity is also tested by antibacterial assay using Diffusion Disc Test (DDT).

If the results are positive, meaning that the bacteria have antibacterial activity, the antifouling activity of the compound will be measured using a DDT on biofilm forming bacteria

- 6) If the secondary metabolites show antibacterial activities, the secondary metabolites will be concentrated using a rotavapor to be purified and analysed using thin layer chromatography (TLC), HPLC FT-IR, GC-MS, NMR (nuclear magnetic resonance) spectroscopy.
- 7) DNA from single colonies that have shown antifouling activity is also isolated and sequenced to identify the bacteria and make a phylogenetic analysis

The bacteria identifies as producing antifouling compounds can then be cultured in a bioreactor in order to produce a large amount of antifouling compounds that will then be integrated into a paint to be tested on the field [3].

For this work, only the steps 1 to 5 will be realised. In the step 5, only the Thin Layer Chromatography (TLC) will be realised.

### 3.4.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (Figure 24) is a chromatographic technique used to separate components of a mixture

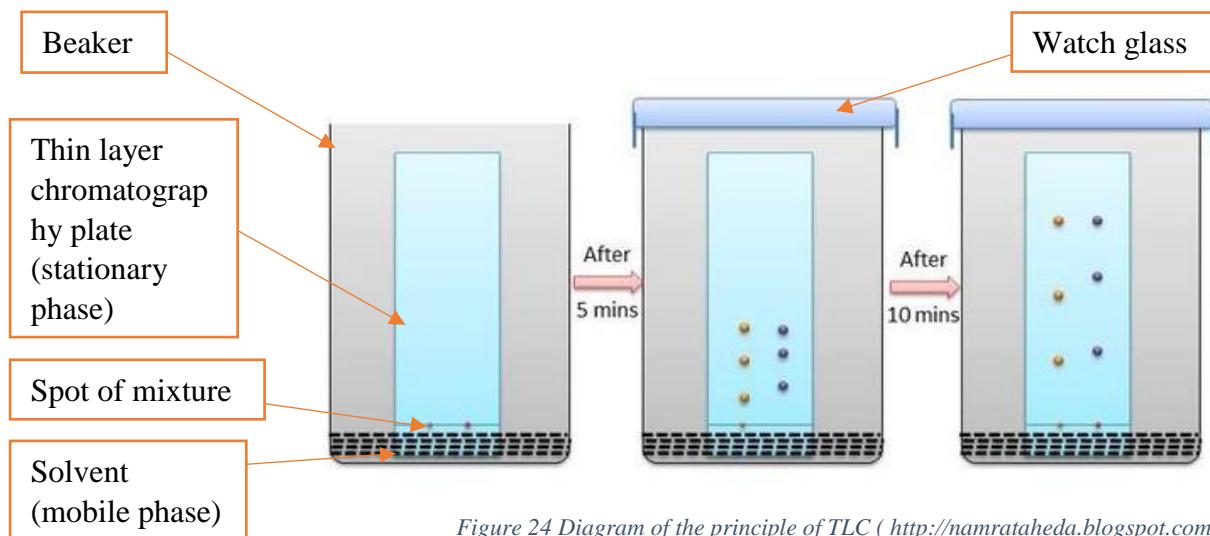


Figure 24 Diagram of the principle of TLC (<http://namrataheda.blogspot.com>)

The mixture is firstly deposited on a thin layer chromatography plate. The TLC plate is then placed in a beaker which contains a certain level of solvent without reaching the spot mixture. A watch glass is then placed on the beaker to allow the solvent to saturate the atmosphere inside the beaker.

The solvent will then ascend in the TLC plate taking with him the different compounds of the mixture.

The distance that the compounds will reach in the TLC plate depends on the affinity the compounds have with the stationary phase and the mobile phase.

If the compounds have more affinity with the mobile phase, they will travel a long distance in the TLC plate

If the compounds have more affinity with the stationary phase, they will travel only a short distance in the TLC plate.

After the separation is completed, compounds appear as vertically separated spots. Each spot has a retention factor ( $R_f$ ) which is equal to the distance the spot has migrated over the total distance covered by the solvent.

$$R_f = \frac{\text{distance travelled by sample}}{\text{distance travelled by solvent}}$$

The  $R_f$  can be used to identify the compound considering the type of stationary phase and mobile phase that have been used.

## 4 Equipment and method

5 species of marine macroorganisms have been studied in this work.

- *Styliessa carteri* (sponges)
- *Sargassum*. Sp (seaweed)
- *Aconthaster planci* (Corn of Thorn)
- *Diadema setosum* (Sea urchin)
- *Gracilaria fisheri* (seaweed)

32 marine bacteria provided by the Institute of Marine Biotechnologie (IMB)'s samples bank ,which is composed of freezer where the sample are stocked, were included in the study as well (31 free-living bacteria and 1 associated to marine organism host).

### 4.1 Samples collection

5 kg of sponges of the species of *Styliessa carteri* were collected in the sea around Bidong Island (Figure 25) on 6<sup>th</sup> March 2019. during the day at 11 m depth.



Figure 25 Localisation of the sampling area where the sponges *Styliessa carteri* were collected (<https://www.google.be/maps>)

Sponges were then cut and washed from all parasite organisms present in the sponges (Figure 26, Figure 27).



Figure 26 Extraction of the sponges from the sea at bidong Island (Olivier Collard)



Figure 27 Cleaning and cutting process of the sponges (Olivier Collard)

A small part of the sample was taken in order to make the microorganism extraction detailed in section 4.4. the rest of the sample was placed in a freezer at -25°C. When the samples were completely frozen, they were blended in order to get a paste. This paste was placed back in a freezer at -80°C for one day. The frozen paste was then warped in aluminium and placed into a freeze-dryer (Figure 28) for 4-5 days to obtain it into a dry powder.

A freeze-dryer is an equipment that uses low temperature and low pressure dehydration process in order to remove the ice by sublimation (Figure 29 blue arrow). The freeze-drying process is also known as lyophilisation or cryodesiccation.

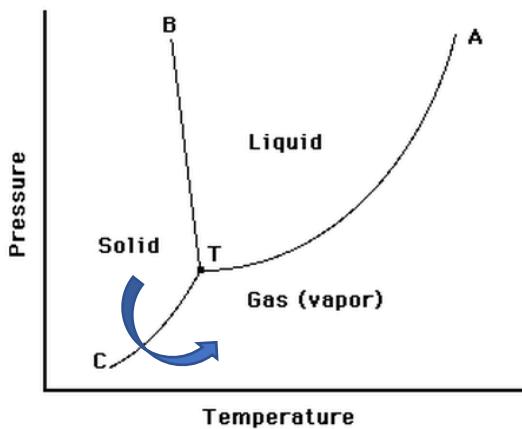


Figure 29 Graph of the change of state of water showing the way of sublimation (<http://dwb5.unl.edu>)



Figure 28 Freeze-dryer used in the lab (LABCONCO) (Olivier Collard)

This technique allows to conserve the sample for a longer time and to get a better quality of the sample when it is rehydrated.

The samples from *Sargassum*. sp, *Aconthaster planci* and *Diadema setosum* were taken from the IMB's bank sample under the form of a powder.

The samples from *Gracilaria fisheri* were taken from the IMB's sample bank under the form of dried seaweed.

## **4.2 Extraction of the crude extract from *Sargassum*. sp, *Aconthaster planci*, *Diadema setosum* and *Styliasa carteri***

200 g of dried powder is mixt with an organic solvent with a ratio of 10:1 (organic solvent in ml:dried powder in g) in a 2L flask and placed on a shaking platform for 2 days at room temperature. The nature of the solvent depends on the compound to be extracted (Table 2)

Solvent	Compound extracted	Bibliographic reference
Methanol	Semi polar/ polar	[15][16][17]
Ethanol	Polar	[16]
Chloroform	Semi-polar	[16][17]
Ethyl acetate	Semi-polar	[16]
Acetone	Non-polar	[16]

Table 2 Organic solvent used for the extraction

The solution is then filtered using a Whatman 2 filter paper and concentrated using the rotavapor at 38°C. 200 ml of methanol are then added back in the concentrated solution and left overnight in order to allow the precipitation of the salt present in the solution. The methanol added is then evaporated using a rotavapor at 38°C. The desalting process is repeated until no salt remains in the solution. The solution can then be conserved in a cold room at -4°C until needed for Disc diffusion Test (see section 4.10).

## **4.3 Extraction of the crude extract from *Gracilaria.fisheri***

100g of dried sea weed is cut into small pieces with a scissor. The compound is then extracted with a ratio 4:1 (organic solvent in ml:dried seaweed in g) in a 500ml flask. 4 different organic solvents were tested (Acetone, Ethanol, Ethyl acetate and Chloroform). The bottle is then left on a shaking plate for 2 days at room temperature. The following steps are then the same then the ones used for *Sargassum*. sp, *Aconthaster planci*, *Diadema setosum* and *Styliasa carteri*.

## **4.4 Extraction and isolation of the microorganisms from sponges**

A 20g piece of the sponges collected in the sea is taken and placed in a 50 ml centrifuge tube containing 30 ml of sterile seawater. The bacteria are taken from the sample using a sterile cotton swab and inoculated on a marine agar medium (Annexes A4-A6) for 1 day at 37°C. The marine agar medium is a nutrient medium with a high concentration of salt in order to let grow the bacteria from the sea and also to prevent contamination of bacteria coming from the air at the same time. Bacteria are isolated from 3 different anatomic parts of the sponges, root surface, upper part surface and inner surface.

After incubation, single colonies evenly distributed on the plate are randomly selected and taken and culture on separate new plates. The plates are then incubated 1 day at 37°C.

## **4.5 Culture methods of the selected microorganisms**

### **4.5.1 Classical methods**

Each isolated bacterium are cultured in a flask containing marine broth for 2 days at 37°C.

#### **4.5.2 Air membrane surface (AMS)**

In order to induce a stress and to induce bacteria to produce more secondary metabolites an AMS culture is used (Figure 30)

Bacteria from the pure cultures are first cultured in a small bottle containing 3 ml of marine broth and incubated at 37°C for 1 day.

Under a laminar flow, A sterile centrifuge cap is placed in a Petri dish using a sterile clamp. 8 ml of marine broth is placed in the centrifuge cap. Then, a membrane is placed gently on top of the centrifuge cap (the membrane should be in contact with the medium). The bacteria that have been incubated overnight is culture on the membrane using a sterile cotton swab and incubated at 37°C for 5 days. 2 AMS cultures are realized per bacteria in order to get enough amount for the following step.

The membrane will create a gradient of oxygen and nutrient trough the membrane inducting stress to the bacteria.

The bacteria present on the top of the membrane will have plenty of oxygen but a small amount of nutrients. The bacteria on the bottom of the membrane will have plenty of nutrients but a small amount of oxygen. The compound produced by the bacteria will diffused in the broth, which will be collected at the end of the incubation period.

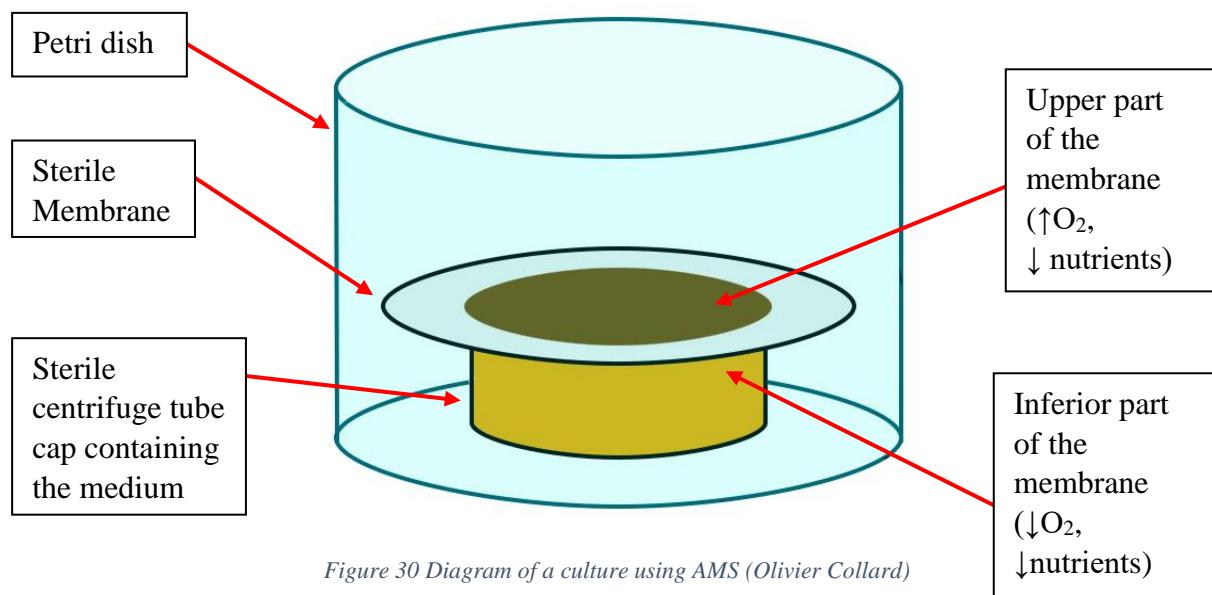


Figure 30 Diagram of a culture using AMS (Olivier Collard)

The collected broth (16 ml) is put conserved in a -80°C freezer for 1 day then in a freeze dryer for 2 days, in order to get the dry sample. Then, the dried sample is diluted in 1,5 ml of 100% methanol to be used for a DDT test.

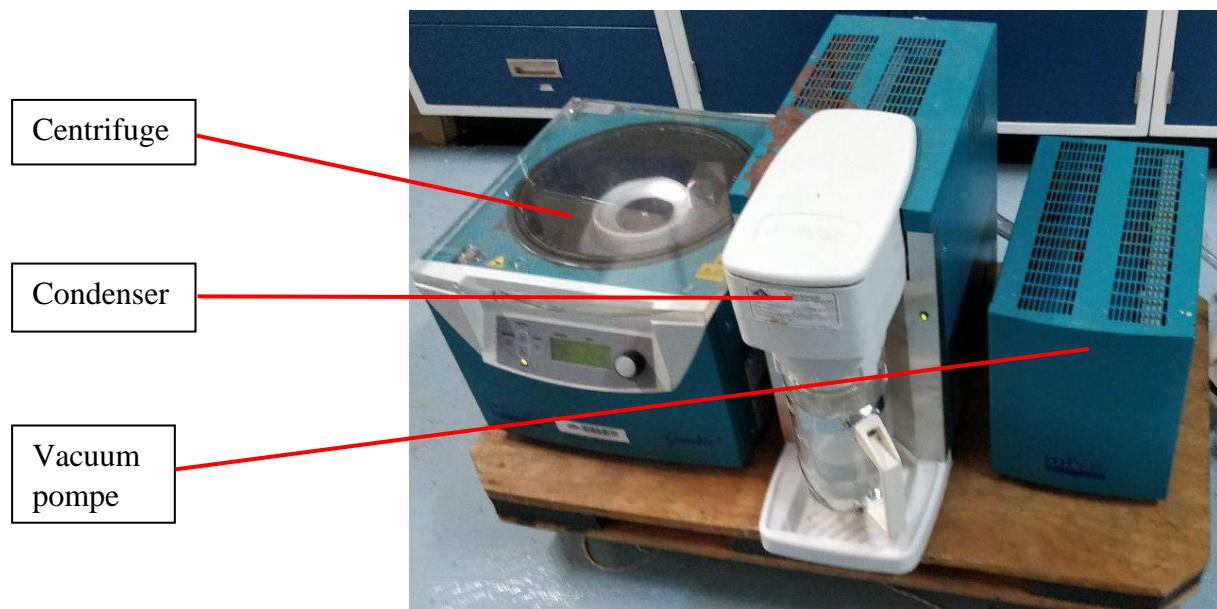
#### **4.6 Separation of the intracellular and the extracellular secondary metabolites .**

50 ml of the bacteria incubated into marine broth are transferred in a 50 ml centrifuge tube and centrifuged at 11000 rpm at 4° for 20 minutes. The supernatant is separated from the pellet and filtered using a 0,2 µm syringe filter, a needle 18G<sup>1/2</sup> and a 20 ml syringe in order to eliminate the remaining cell that would have stayed in the supernatant.

#### **4.7 Extraction of secondary metabolites from the pellet using a methanol extraction method.**

The pellet is placed in a 50 ml centrifuge tube containing 10 ml of methanol 100% and sonicated with a ultrasonic bath (Hawashin model: Powersonic 510; series: 510H230414) for 60 minutes at 40°C. The solution is then centrifuged at 11000 rpm at 4°C for 20 minutes to separate bacteria debris from methanol. Methanol is then removed and put in a 50 ml centrifuge tube covered by an aluminium paper pierced with holes. Then, the centrifuge tube is placed in the centrifugal evaporator (Mivac model: DUP-23050-A00; serial: DUP08100251) (Figure 31) for 7 hours in order to concentrate the compound.

The centrifugal evaporator is composed of a centrifuge, a vacuum pump and a condenser.



*Figure 31 Centrifugal evaporator miVac used in the lab*

The vacuum pomp reduces the pressure inside the centrifuge below the boiling point of the solvent allowing the solvent to evaporate at low temperature. The centrifuge then spins in order to prevent the solvent of “bumping” out of the centrifuge tube. Finally, the condenser recovers the solvent evaporated in a container.

The concentrated compound is then placed in a cold room at -4°C until needed.

#### **4.8 Extraction of the secondary metabolite from the pellet using chloroform extraction**

5 ml of water and 5 ml of chloroform are added to the pellet and put into the ultrasonic bath for 60 minutes at 40°C. After 60 minutes, the chloroform is removed and put in another 50 millilitres centrifuge tube. This step is then repeated 2 times by adding 5ml of chloroform to the aqueous phase; the tube is shaked vigorously, then left for 10 minutes.

The fifteen millilitres of chloroform obtained after the extraction is then placed in the centrifugal evaporator in order to concentrate the sample. The sample is then placed in a cold room at -4°C until needed.

#### **4.9 Extraction of the secondary metabolites from the supernatant**

The supernatant is concentrated using a centrifugal concentrator overnight. 10 ml of methanol is then added to the concentrated compound and left overnight in a cold room in order to precipitate the salt originally present in the broth. Then, the methanol is separated from the salt using a Whatman 1 filter paper. The solution is then put back in the centrifugal concentrator for 5-6 hours in order to evaporate the methanol. The concentrated solution is put in a cold room to be conserved until needed.

#### **4.10 Disc Diffusion Test (DDT)**

To test the antibacterial activity of a compound a disk diffusion test is realized on 4 bacteria *E.coli*, *Salmonella. sp*, *B.cereus*, *S. uberis* (Table 3).

Name	Gram	Genus
<i>E.coli</i>	-	Bacillus
<i>Salmonella. sp</i>	-	Bacillus
<i>B.cereus</i>	+	Bacillus
<i>S.uberis</i>	+	Streptococcus

Table 3 Bacteria used to measure the antibacterial activity of the compound

Under a laminar flow, 30 µl of the compound contained in methanol is loaded on four discs (one disc for each bacteria culture) on a sterile aluminium plate. Four other discs are loaded with 30 µl from the organic solvent used for the extraction to serve as a negative control. The discs are then left 30 minutes for them to dry. If the time to dry is too long the disc is left overnight under an UV flux. When the disc is dried, it is turned over and the same procedure is repeated three times (only one time for the crude extract).

For the positive control, a disc of streptomycin (10µg) is used for *E.coli* and *B.cereus* and a disc of Kanamycine (30µg) is used for *S.uberis* and *Salmonella. sp*.

Each bacterium is cultured on a plate containing marine agar medium. On each plate, the disc containing the compound, the positive control and the negative control are placed. The medium is then incubated at 37°C overnight. After the incubation, the diameter of the inhibition zone of the extracted compound is measured with a measuring stick.

#### **4.11 Thin Layer Chromatography (TLC)**

For TLC, a silica gel 60 F<sub>254</sub> (20 X 20 cm) was used as stationary phase. The plate is first cut into smaller pieces (6,66 X 3 cm) (Figure 32).

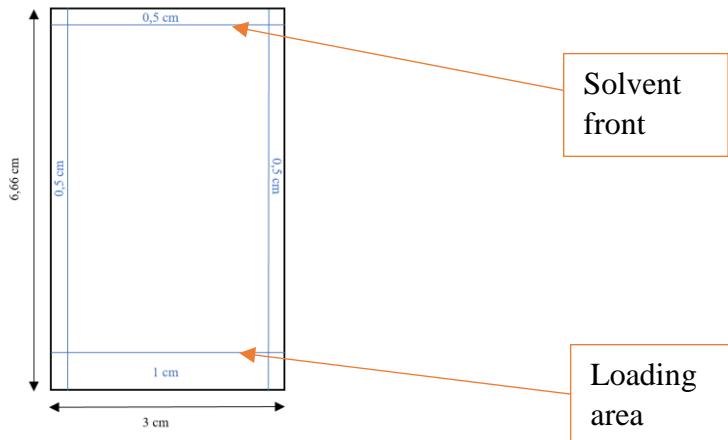


Figure 32 Measure of a TLC plate used for this method (Olivier Collard)

The samples are then loaded 1 cm above the bottom edge on the plate and left a few seconds to dry. The plate is then placed into a beaker, containing the appropriate mobile phase and 1 drop of formic acid. The formic acid is used to get a better resolution of the spot obtained by the TLC. The beaker is then covered by a watch glass.

2 solvent system will be used in this work:

- Hexane/ethyl acetate (7:3), (8:2), (9:1)
- Chloroform/methanol (7:3), (8:2), (9:1)

The plate is left around 10 minutes into the beaker to let the mobile phase migrating through the plate.

When the migration of the mobile phase in the silicate plate is completed, the plate is dried and placed under UV (254 nm) to observe aromatic and highly conjugated compounds. The silica plate possesses fluorescent molecules that will fluoresce when exposed to UV, aromatic and highly conjugated compounds absorb UV light and thus will appear as black spots on the plate.

An anisaldehyde – sulfuric acid reagent is then sprayed on the plate. To prepare this reagent, 0,5 ml anis aldehyde, 10 ml of acetic acid, 85 ml of methanol and 5 ml of sulfuric acid are mixed in a 100 ml measuring flask. The solution is then conserved in a 500 ml bottle, covered by aluminium and placed away from the light. After the application of the reagent, the plate is heated at 110°C using a hairdryer. The reagent reveals the presence of different compounds:

- Purple colour indicates the presence of various terpenoid derivatives
- Blue colour indicates the presence of terpenes
- pink/red colour indicates phenol derivatives.

The distance travelled by the solvent and the distance travelled by the compound is then measured with a measuring stick in order to calculate the retention factor ( $R_f$ ) using the formula described in point 3.4.1.

## 5 Results and discussion

### 5.1 Macroorganism's crude extracts

#### 5.1.1 DDT

A DDT test has been realised on crude extract of different marine organisms, *Sargassum. sp* (seaweed), *Aconthaster planci* (Crown of Thorn), *Diadema setosum* (Seas Urchin) and *Styliissa carteri* (sponges that have been extracted near Bidong island, see section 4.1). Antibacterial activity of these extracts were tested against different bacteria (*E.coli*, *Salmonella*, *S.uberis*, *B.cereus*)

The species *Sargassum. sp*, *Aconthaster planci* and *Diadema setosum* were taken from the IMB's sample bank in the form of a dried powder.

##### 5.1.1.1 Result

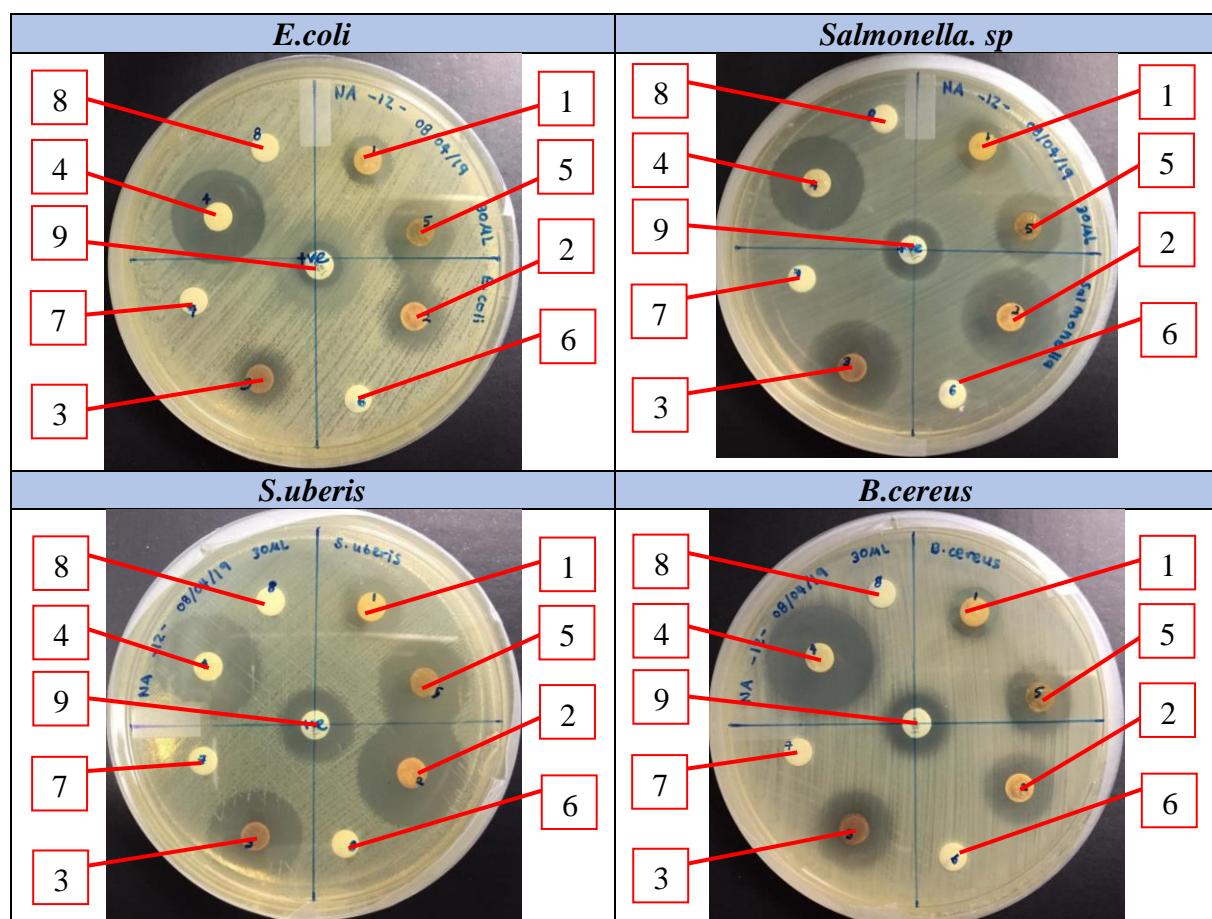


Figure 33 Result of the antibacterial activities of the 4 species tested extract (*Sargassum. Sp*, *Aconthaster planci*, *Diadema setosum* and *Styliissa carteri*).

The following table show the result of the measurement of the inhibition zone in Figure 33 realized with a measuring stick.

N°	Disk containing tested compounds	<i>E.coli</i> (cm)	<i>Salmonella.</i> <i>sp</i> (cm)	<i>B.cereus</i> (cm)	<i>S.uberis</i> (cm)
1	Sea weed ( <i>Sargassum sp</i> )(extracted with chloroform method)	1,1	1,2	1,2	1,4
2	Crown of Thorn ( <i>Aconthaster planci</i> ) (extracted with chloroform)	1,3	2,2	1,5	2,5
3	Sea Urchin ( <i>Diadema setosum</i> ) (extracted with chloroform)	1,2	2,1	1,7	2,1
4	Sea Urchin ( <i>Diadema setosum</i> ) (extracted with 95% ethanol)	2	2	2,2	2,4
5	Sponges ( <i>Styliissa carteri</i> ) (extracted with methanol)	1,6	1,5	1,5	2
6	Negative control (chloroform)	/	/	/	/
7	Negative control (95% ethanol)	/	/	/	/
8	Negative control (methanol)	/	/	/	/
9	Positive control (streptomycin)	1	1,4	1,3	1,5

Table 4 Inhibition's diameter of each disk in cm of the DDT realized on *Sargassum*. *Sp*, *Aconthaster planci*, *Diadema setosum* and *Styliissa carteri*. / = no inhibition zone

An antibacterial study has been realized on *Gracilaria fisheri* too.

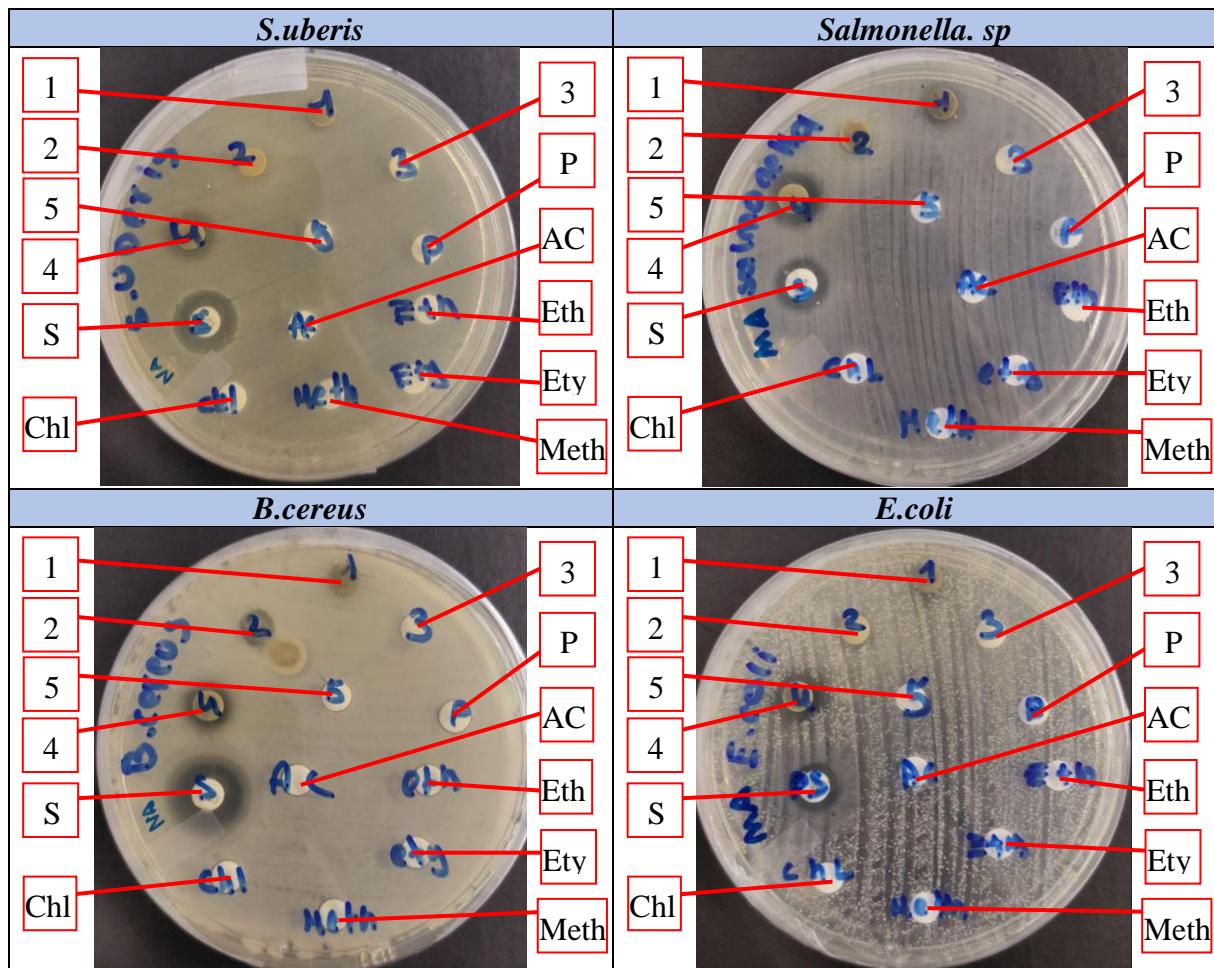


Figure 34 Result of the DDT realized on the crude extract of *G. fisheri* extracted with different solvents (acetone, ethyl acetate, chloroform and ethanol).

The following table show the result of the measurement of the inhibition zone in Figure 34 realized with a measuring stick.

N°	Disk containing test compound	<i>E.coli</i> (cm)	<i>Salmonella. sp</i> (cm)	<i>B.cereus</i> (cm)	<i>S.uberis</i> (cm)
<b>1</b>	<i>Gracilaria fisheri</i> (chloroform)	/	1	/	/
<b>2</b>	<i>Gracilaria fisheri</i> (acetone)	/	/	/	/
<b>3</b>	<i>Gracilaria fisheri</i> (ethanol)	/	/	/	/
<b>4</b>	<i>Gracilaria fisheri</i> (ethyl acetate)	1	1,1	0,8	1
<b>5</b>	5 DDT realized on a bacteria associated to sponges that will be discussed later.				
<b>P</b>	Penicillin (positive control)	/	/	/	/
<b>S</b>	Streptomycin (positive control)	0,9	1,1	1,4	1,3
<b>AC</b>	Acetone (negative control)	/	/	/	/
<b>Eth</b>	Ethanol (negative control)	/	/	/	/
<b>Chl</b>	Chloroform (negative control)	/	/	/	/
<b>Ety</b>	Ethyl acetate (negative control)	/	/	/	/
<b>Meth</b>	Methanol (negative control)	/	/	/	/

Table 5 Inhibition's diameter of each disk in cm for the DDT realized on *Sargassum. Sp*, *Aconthaster planci*, *Diadema setosum* and *Styliissa carteri*. / = no inhibition zone

### 5.1.1.2 Discussion

Every organism show antibacterial activity (Figure 33, Figure 34, Table 4, Table 5). The extracts from *Diadema setosum* (extracted with 95% ethanol) show particularly strong antibacterial activity. This extract show a very large inhibition zone on all the tested bacteria. On the other hand, the extract from *Sargassum. sp* have a very small inhibition zone compared to the others.

Extracts from *Aconthaster planci* induce a strong inhibition zone for the growth of *Salmonella* and *S. uberis*, but a moderate zone for *E. coli* and *B.cereus*.

Sample of *Diadema setosum* have been extracted with 2 different solvents (chloroform, disc 3 and 95% ethanol disc 4). The crude extracts realized with 95% ethanol show a bigger inhibition zone than the crude extracts realized with chloroform. Ethanol is more polar than chloroform, it shows the presence of strongly polar antibacterial compounds that are not able to be extracted with chloroform.

Extracts from *Styliissa carteri* possess moderate inhibition zone for *E. coli*, *salmonella* and *B. cereus* and a strong inhibition zone for *S. uberis* as well.

Extracts from *G.fisheri* show an inhibition zone on gram-positive and gram-negative bacteria with the ethyl acetate crude extracts and on *salmonella* with the chloroform crude extracts (Figure 34. Table 5). However. Compared to the other species, extracts from *G.fisheri* show a very small inhibition zone. The fact that this particular sample was not in powder form for the extraction could have led to a decrease in compounds extraction efficiency and thus a low antibacterial activity.

## 5.1.2 TLC

The quality of the separation method using TLC on silicate gel (60 F<sub>254</sub>) depends on the solvent system and the sample origin as well. Thus, different solvent systems have been tested on these samples in order to find the best one on each sample type.

The TLC studies have not been realized on *G.fisheri* due to the late acquisition of the dried samples.

### 5.1.2.1 Results

#### 5.1.2.1.1 Hexane/ethyl acetate solvent

Hexane/ethanol acetate is a non-polar solvent system and thus separates non-polar compounds [18].

After testing different ratios for the solvent system hexane/ ethanol acetate, the ratio 8:2 gave the best separation overall.

S = compounds from *Styliissa carteri* (Sponges)(100% methanol)

SW = compounds from *Sargassum. Sp* (Seaweed)(Chloroform)

CT = compounds from *Aconthaster planci* (Corn of Thorn) (Chloroform)

SU1 = compounds from *Diadema setosum* (Sea Urchin)(Chloroform)

SU2 = compounds from *Diadema setosum* (Sea Urchin) (95% ethanol)

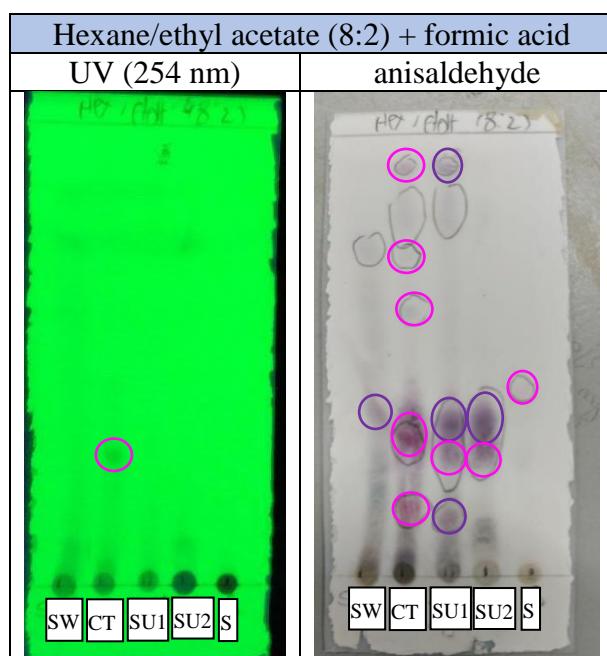


Table 6 Result of the TLC realized with the mobile phase Hexane/ethyl acetate (8:2). the different spots are marked as follow: pink spot (pink circle), purple spot (purple circle), spot visible under UV but not with anis aldehyde (yellow circle)

The following table show the result of the measurement and revelation realized on the TLC plate Hexane/ethyl acetate (8:2) (Table 6)

Extract from:	Spot N°	UV revelation	Compounds nature	Distance of the sample (cm)	Distance of the solvent (cm)	Rf
<i>Sargassum. sp</i> (Seaweed)(SW) (chloroform)	1	No	Terpenoid	3,9	5,35	0,729
<i>Aconthaster.planci</i> (Corn of Thorn)(CT) (chloroform)	1	No	Phenol derivative	0,8	5,35	0,150
	2	Yes	Phenol derivative highly conjugated or with an aromatic group	1,6		0,299
	3	No	Phenol derivative	3,2		0,598
	4	No	Phenol derivative	3,8		0,710
	5	No	Phenol derivative	4,9		0,916
<i>Diadema setosum</i> (SeaUrchin)(SU1) (chloroform)	1	No	Terpenoid	0,7	5,35	0,131
	2	No	Phenol derivative	1,4		0,262
	3	No	Terpenoid	1,8		0,336
	4	No	Phenol derivative	4,9		0,916
<i>Diadema setosum</i> (SeaUrchin)(SU2) (95% ethanol)	1	No	Phenol derivative	1,5	5,35	0,280
	2	No	Terpenoid	1,8		0,336
<i>Styliissa carteri</i> (Sponges)(S) (methanol)	1	No	Phenol derivative	2,, 2	5,35	0,411

Tableau 7 Results of the TLC realized with the mobile phase hexane/ethyl acetate (8:2). Pink number are for pink spot, purple numbers are for purple spot, blue number are for blue spot and yellow number are for spot that are visible under UV but not with anisal

### 5.1.2.1.2 Chloroform/methanol solvent

The chloroform/methanol is a semi-polar solvent system already used for TLC studies on sponges [19]. the solvent system has been tested in different proportion in order to find the best ratio which is (9:1)

S = compounds from *Styliissa carteri* (Sponges)

SW = compounds from *Sargassum. sp* (Seaweed)

CT = compounds from *Aconthaster planci* (Corn of Thorn)

SU1 = compounds from *Diadema setosum* (Sea Urchin) (Chloroform)

SU2 = compounds from *Diadema setosum* (Sea Urchin) (95% ethanol)

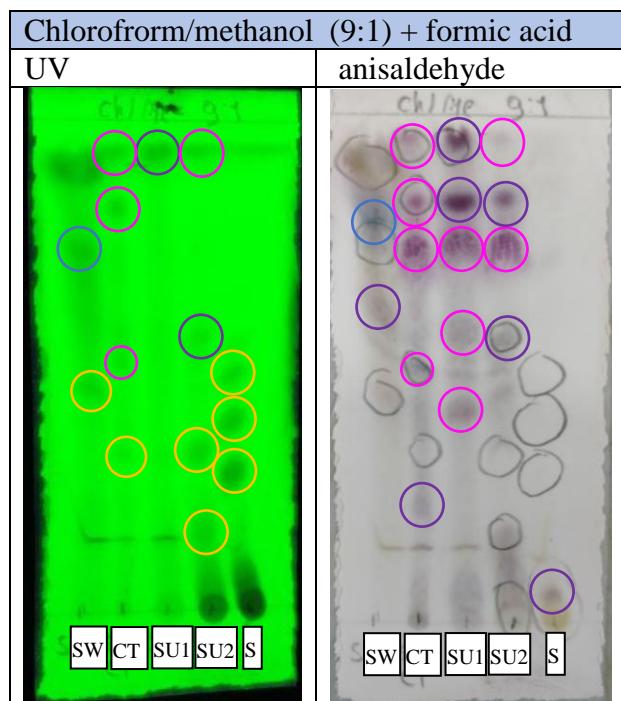


Table 8 Result of the TLC realized with the mobile phase chloroform/methanol (9:1). the different spots are marked as follow: pink spot (pink circle), purple spot (purple circle), spot visible under UV but not with anis aldehyde (yellow circle) and blue spot (blue circle)

The following table show the result of the measurement and revelation realized on the TLC plate Chloroform/methanol (9:1) (Table 8).

Spot N°	UV revelation	Compounds nature	Distance Of the sample (cm)	Distance of the solvent (cm)	Rf	
Seaweed ( <i>Sargassum sp</i> )(SS) (chloroform)	1	Yes	Aromatic/ highly conjugated compound	2,4	5,1	0,471
	2	No	Terpenoid	3,4	5,1	0,667
	3	yes	Terpene highly conjugated or with an aromatic group	4,3	5,1	0,843
Corn of Thorn ( <i>Aconthaster planci</i> )(AP) (chloroform)	1	No	Terpenoid	1,2	5,1	0,235
	2	Yes	Aromatic/ highly conjugated compound	1,8	5,1	0,353
	3	No	Phenol derivative	2,7	5,1	0,529
	4	No	Phenol derivative	4	5,1	0,784
	5	Yes	Phenol derivative highly conjugated or with an aromatic group	4,5	5,1	0,882
	6	Yes	Phenol derivative highly conjugated or with an aromatic group	5,1	5,1	1,000
SeaUrchin 1 ( <i>Diadema setosum</i> )(DS) (chloroform)	1	No	Phenol derivative	2,3	5,1	0,451
	2	No	Phenol derivative	3,2	5,1	0,627
	3	No	Phenol derivative	4	5,1	0,784
	4	No	Terpenoid	4,4	5,1	0,863
	5	Yes	Terpenoid derivative highly conjugated or with an aromatic group	5,1	5,1	1,000
SeaUrchin 2 ( <i>Diadema setosum</i> )(DS2) (95% ethanol)	1	Yes	Aromatic/highly conjugated compound	1	5,1	0,196
	2	Yes	Aromatic/highly conjugated compound	1,7	5,1	0,333
	3	Yes	Terpenoid highly conjugated or with aromatic group	3	5,1	0,588
	4	No	Phenol derivative	4	5,1	0,784
	5	No	Terpenoid	4,5	5,1	0,882
	6	Yes	Phenol derivative	5,1	5,1	1,000
Sponges ( <i>Styliasa carteri</i> ) (SC) (methanol)	1	?	Phenol derivative	0,3	5,1	0,059
	2	Yes	Aromatic/highly conjugated compound	1,6	5,1	0,314
	3	Yes	Aromatic/highly conjugated compound	2,1	5,1	0,412
	4	Yes	Aromatic/highly conjugated compound	2,6	5,1	0,510

Table 9 Result of the TLC realized with the mobile phase chloroform/methanol (9:1). Pink number are for pink spot, purple numbers are for purple spot, blue number are for blue spot and yellow number are for spot that are visible under UV but not with anis aldehyde.

### **5.1.2.2 Discussion**

A very few compounds were extracted from *Sargassum sp.* (SS) using a non-polar solvent system or a semi-polar solvent system. The small amount of compounds in the crude extract could explain the poor antibacterial properties observed after the DDT test (see point 5.1.1.1).

Extracts from *Aconthaster planci* (AP) shows a large amount of different phenol derivatives with different polarities if extracted with hexane/ethyl acetate (AP. spots 1,2,3,4,5) and chloroform/methanol (AP. spot 3,4,5,6,). Some of them are highly conjugated or present an aromatic group (Hexane/ethyl acetate AP spot 2) (Chloroform/methanol AP spot 5 and 6).

Extracts from *Diadema setosum* (DS) obtained with the solvent system hexane/ethyl acetate shows more different compounds when the extraction is realized with chloroform than with 95% ethanol. Chloroform is a mid-polar solvent used mainly to extract fatty acids [20] and ethanol is a polar solvent. Thus, considering that hexane/ethyl acetate is a non-polar solvent system, it is normal that it shows a better separation efficiency with a crude extract that contains high amount of non-polar to mid-polar compounds.

With the Chloroform/methanol solvent system, good results are visible on both crude extracts. Both show terpenoid and phenol derivatives. However, the results are different (the compounds have different Rf). Furthermore, a black spot is still visible under UV at the loading area of the crude extracts realized with 95% ethanol indicating the presence of more compounds that have not migrated into the plate. For this crude extract, a more polar solvent system is needed. One phenol derivative possesses the same Rf on both crude extracts from *Diadema setosum* (DS1 spot 3, DS2 spot 4) as well as on *Aconthaster planci* (AP. spot 4), indicating the presence of a similar phenol derivative in those crude extracts.

Extracts from *Styliissa carteri* (SC) show only the presence of one phenol derivative (SC spot 1) with the solvent system hexane/ethyl acetate. However, a dark spot situated at the loading area is visible under UV. indicating that most of the sample did not migrate with the non-polar solvent system. Methanol is a polar solvent, but some non-polar solvents are fairly soluble in methanol, explaining the presence of only one spot on the TLC plate.

With the solvent system Chloroform/methanol, the crude extract reveals 3 compounds highly conjugated/with aromatic group that do not belong to terpenoids or phenol derivatives chemical (it is not coloured by anisaldehyde). However, the loading zone still reveals a dark spot under UV, indicating the presence of compounds that have not been separated by the solvent system. As it was suggested for the extract from *Diadema setosum*, another more polar solvent system would be necessary to fully separate this sample. The crude extracts also presents a phenol derivative (Chloroform/methanol SC spot 1) which is visible with anisaldehyde near the loading zone. However, as the spot is too close to the loading zone, it is not possible to determine if the compound is visible under UV or not.

Anisaldehyde and UV revealed a limited range of chemical structures and there is a high probability that the samples possess much more compounds (but they were not revealed by the TLC). As it was said in the objective, HPLC analyses should have been done on the samples but it was not possible during the time of my stay.

## **5.2 Bacteria Analysis**

### **5.2.1 Result**

#### **5.2.1.1 Free living bacteria extracts**

All free-living bacteria that were tested in the current study come from the samples bank of IMB.

31 bacteria have been taken from the bank and culture on marine agar. 15 bacteria have grown on the marine agar and have then been cultured in marine broth for 2 days in order to be used for the secondary metabolites extraction.

- TB are bacteria that have been collected in the city of Tok Bali in the North of Malaysia
- C.V are bacteria that have been collected with a medium containing crystal violet. However, the location of the sampling is unknown

The compounds of TB18, TB9, TB13 and TB17 have been extracted with chloroform following the method described in 4.8 for the pellet and 4.9 for the supernatant.

For the other bacteria. the extraction has been realised with the method described in 4.7 for the pellet and 4.9 for the supernatant.

The following table show the result of the DDT of the 15 bacteria realized on the growth of *E. coli*, *B. cereus*, *S. uberis* and *Salmonella*

	Intracellular compounds				Extracellular compounds			
	<i>E. coli</i>	<i>Sal</i>	<i>B. cereus</i>	<i>S. uberis</i>	<i>E.coli</i>	<i>Sal</i>	<i>B. cereus</i>	<i>S. uberis</i>
<b>TB18</b>	-	-	-	-	-	-	-	-
<b>TB9</b>	-	-	-	-	-	-	-	-
<b>TB13</b>	-	-	-	-	-	-	-	-
<b>TB17</b>	-	-	-	-	-	-	-	-
<b>TB24</b>	-	-	-	-	-	-	-	-
<b>TB36</b>	-	-	-	-	-	-	-	-
<b>TB15</b>	-	-	-	-	-	-	-	-
<b>TB25</b>	-	-	-	-	-	-	-	-
<b>TB14</b>	-	-	-	-	-	-	-	-
<b>TB5</b>	-	-	-	-	-	-	-	-
<b>TB29</b>	-	-	-	-	-	-	-	-
<b>TB31</b>	-	-	-	-	-	-	-	-
<b>TB37</b>	-	-	-	-	-	-	-	-
<b>C.V 2.9</b>	-	-	-	-	-	-	-	-
<b>C.V 2.5</b>	-	-	-	-	-	-	-	-

Table 10 Result of the DDT realised with the extracellular compound and the intracellular compound obtained with a classical culture of different free-living bacteria. (-) = negative result, (+) = positive result, (/) = the bacteria didn't growth in the broth.

### **5.2.1.2 Bacteria associated with macro organism.**

The bacteria studied in this part have been extracted from the root surface of the sponge *Styliissa. carteri* that have been collected near Bidong Island. These bacteria of the sponges have been extracted using the method described in the section “method and equipment” (point 4.1)

Eighteen bacteria out of a total of 20 have grown on marine agar after their isolation and cultured in a marine broth for 2 days. Then, the compounds have been extracted from the pellet and the supernatant following the method described in 4.7 and 4.9, respectively.

The bacteria hosted by jelly fishes have been provided by the sample bank of IMB. They first have been culture on a marine agar and incubated for 1 day at 37°C to be then cultured in a marine broth for 2 days at 37°C. The used extraction method was the same than the sponge’s bacteria.

#### **5.2.1.2.1 Classical culture**

The following table show the antibacterial activity of the different bacteria on the growth of *E. coli*, *Salmonella. sp* , *B. cereus*, *S. uberis*.

Jelly fish	JF 1.6.2	Intracellular compound				Extracellular compound			
		<i>E. coli</i>	<i>Sal.sp</i>	<i>B. cereus</i>	<i>S. uberis</i>	<i>E. coli</i>	<i>Sal.sp</i>	<i>B. cereus</i>	<i>S. uberis</i>
Sponge ( <i>Styliissa carteri</i> )	JF 1.6.2	-	-	-	-	-	-	-	-
	<b>B2</b>	-	-	-	-	-	-	-	-
	<b>B3</b>	-	-	-	-	-	-	-	-
	<b>B4</b>	-	-	-	-	-	-	-	-
	<b>B5</b>	-	-	-	-	-	-	-	-
	<b>B7</b>	-	-	-	-	-	-	-	-
	<b>B8</b>	-	-	-	-	-	-	-	-
	<b>B9</b>	-	-	-	-	-	-	-	-
	<b>B10</b>	-	-	-	-	-	-	-	-
	<b>B11</b>	-	-	-	-	-	-	-	-
	<b>B12</b>	-	-	-	-	-	-	-	-
	<b>B13</b>	-	-	-	-	-	-	-	-
	<b>B14</b>	-	-	-	-	-	-	-	-
	<b>B15</b>	-	-	-	-	-	-	-	-
	<b>B16</b>	-	-	-	-	-	-	-	-
	<b>B17</b>	-	-	-	-	-	-	-	-
	<b>B18</b>	-	-	-	-	-	-	-	-
	<b>B19</b>	-	-	-	-	-	-	-	-
	<b>B20</b>	-	-	-	-	-	-	-	-

Table 11 Result of the DDT realised with the intracellular and extracellular compound obtained with the supernatant and the pellet. (-) =negative result, (+) = positive result, (/) = the bacteria didn’t growth in the broth.

### **5.2.1.2.2 AMS culture**

The following table show the antibacterial activity on *E. coli*, *Salmonella*, *B. cereus*, *S. uberis* of the supernatant obtained by AMS culture of the sponge's bacteria

	Extracellular compound			
	<i>E.coli</i>	<i>Salmonella. sp</i>	<i>B. cereus</i>	<i>S. uberis</i>
<b>B2</b>	-	-	-	-
<b>B3</b>	-	-	-	-
<b>B4</b>	-	-	-	-
<b>B5</b>	-	-	-	-
<b>B7</b>	-	-	-	-
<b>B8</b>	-	-	-	-
<b>B9</b>	/	/	/	/
<b>B10</b>	/	/	/	/
<b>B11</b>	/	/	/	/
<b>B12</b>	-	-	-	-
<b>B13</b>	/	/	/	/
<b>B14</b>	-	-	-	-
<b>B15</b>	-	-	-	-
<b>B16</b>	/	/	/	/
<b>B17</b>	-	-	-	-
<b>B18</b>	-	-	-	-
<b>B19</b>	/	/	/	/
<b>B20</b>	/	/	/	/

Table 12 Result of the DDT realized with the supernatant of the sponge's bacteria cultured with AMS. (-) = negative result, (+) positive result, (/) = the bacteria didn't grow on AMS

### **5.2.2 Discussion**

The extracts from free-living bacteria (Table 10) show negative results with DDT tests for both gram-positive and gram-negative bacteria. Some studies reveal that free-living sea bacteria in the sea are less likely to produce antibacterial compounds than the symbiotic ones [21]. Thus, the chance to get antibacterial activity from free living bacteria is much more weaker than with bacteria associated with macro organisms.

The extracts from the sponges *Styliissa carteri* have shown antibacterial activity with the crude extracts (see section 5.1.1.1). However, compounds of the bacteria extracted from the sponge's root surface have shown negative result (no influence) on the growth of the tested bacteria for the classical culture (Table 11). Those results can come from multiple reasons:

- 1) Most of the bacteria hosted by a macro organism does not grow on standard ZoBell medium [20] (A4) because those bacteria need very specific conditions to growth. Zobell marine agar has been used to culture the bacteria and isolate the single colonies. Thus, it is possible that the sponges possess antifouling bacteria but that did not grow on the marine agar medium.

- 2) Studies show that bacteria produce only a part of their microbial compounds during classic in vitro fermentation. Many biosynthetic genes remain silent and are not expressed [22] under routine laboratory conditions. These observations have been confirmed by several sequencing project of different microorganisms [23]. In the classical culture, bacteria were cultured in a simple marine broth which could have led to a poor activation of secondary metabolite production. Several strategies exist to try to overcome this limitation problem.
- The OSMAC (one strain-many compounds) approach is one of them. The concept of this approach is to culture bacteria by changing many cultivation parameters (for example the medium, aeration, addition of enzymes inhibitors) in order to maximize the diversity of produced compounds [24].
  - By Epigenetics modification, microorganisms are treated with epigenetic modification such as histone deacetylase or DNA methyl transferase in order to force the transcription of silent genes that could lead to the production of new compounds [25].
  - Co-cultivation (or mixed fermentation) of two or more different microorganisms is also a good method in order to try to mimic the coexistence within a microbial communities that could lead to the production of more diversified microbial compounds [22].
  - Culture on support that mimic their natural support (for example, pieces of commercial sponges in the broth for sponge's bacteria) is also a possibility that could also activates the production of different secondary metabolites.
- 3) The antifouling compounds can also be produced by fungi associated with the sponges [26]. Different media, method and culture conditions are necessary to isolate and culture them, and thus the study of the fungi could not have been done in this work.
- 4) The antifouling compounds are not produced by the microorganisms but by the sponge itself, and the microorganism simply does not produce any antifouling compounds.

For the AMS, a stress has been induced on the bacteria which may improve their secondary metabolites production [27]. 11 out of 18 bacteria isolated from sponges were able to grow on the membrane, but the results shown by the AMS of those 11 bacteria remain negative (as for the classical culture) (Table 12).

Because of the negative results given by the DDT test, no TLC or HPLC studies have been done on the extracellular or intracellular compounds isolated from the bacteria.

## 6 Conclusion

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The crude extract of 5 macroorganisms species (*Diadema setosum*, *Styliissa carteri*, *Sargassum sp*, *G.fishereri*, *Aconthaster planci*) have shown antibacterial activites. *Diadema setosum* shows a strong antibacterial activities while *G.fishereri* and *Sargassum.sp* have a weak antibacterial activities compared to the others.

The TLC studies of the crude extracts shows that *Aconthaster planci* shows mostly the presence of phenol derivative. *Diadema setosum* show phenol derivative as well as terpenoid. *Sargassum.sp* show very few compounds compared to the others which could explain its weak antibacterial activities. *Styliissa carteri* show polar compound that doesn't belong to the chemical structure of terpenoid or phenol derivative. No TLC have been done on *G.fishereri* due to the late acquisition of the sample.

An HPLC should have been done on each crude extract in order to analyse more deeply their composition but due to a lack of time, these studies could not have been realised.

All the free-living bacteria tested in this work show negative result to the production of antibacterial compound. Bacteria have been isolated from the root surface of *Styliissa carteri* and show no production of antibacterial compounds with the classical culture and the AMS culture.

the discovery of antifouling compound is just one step in order to produce an antifouling paint. Following this work, the antifouling compound would have to be integrated in a paint (IMAP or SMAP for example) and applied on a surface that will be placed in the sea in order to see its effect on the biofouling formation. If the antifouling paint show effect on the formation of biofouling other characteristics would have to be verified before its use. Firstly, the natural compounds in the paint may not be degraded to rapidly otherwise it will decrease their efficiency. Secondly, the paint must fulfil the same requirement as the already existing paint such as mechanical properties, stability and release characteristic. Thirdly, the cost must be equal or lower than the existing environmentally friendly paint developed by other lab in order to be commercially competitive.

## 7 References

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**NUTRIENT  
BROTH**

**Code:**

# Dehydrated Culture Media

Sector: Industrial

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CM0001

*A general purpose fluid medium for the cultivation of micro-organisms not exacting in their nutritional requirements. Blood, serum, sugars, etc., may be added as required for special purposes.*

**Typical Formula\***

gm/litre

'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2 @ 25°C	

\* Adjusted as required to meet performance standards

**Directions**

Add 13g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

**Description**

Lab-Lemco beef extract is combined with peptone and sodium chloride to form the basic bouillon described by Loeffler and other early bacteriologists. Yeast extract is added to provide vitamins and minerals to help speed the growth of most organisms.

Nutrient Broth can be enriched with other ingredients such as carbohydrates, blood etc., for special purposes. See also Nutrient Broth No.2 CM0067.

**Storage conditions and Shelf life**

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.  
Store the prepared medium below 25°C.

**Appearance**

Dehydrated medium: Straw coloured, free-flowing powder  
Prepared medium: Straw coloured solution

**Quality control**

**Positive controls:**

**Expected results**

*Staphylococcus aureus* ATCC® 25923\*

Turbid growth

*Escherichia coli* ATCC® 25922 \* Turbid growth

**Negative control:**

Uninoculated medium

No change

\* This organism is available as a Culti-Loop®

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## MARINE BROTH

**CAT N°: 1217**

For the isolation and enumeration of heterotrophic marine bacteria

### FORMULA IN g/l

Sodium Chloride	19.40	Ferric Citrate	0.10
Magnesium Chloride	8.80	Potassium Bromide	0.08
Bacteriological Peptone	5.00	Strontium Chloride	0.034
Sodium Sulfate	3.24	Boric Acid	0.022
Calcium Chloride	1.80	Disodium Phosphate	0.008
Yeast Extract	1.00	Sodium Silicate	0.004
Potassium Chloride	0.55	Sodium Fluoride	0.0024
Sodium Bicarbonate	0.16	Ammonium Nitrate	0.0016

**Final pH 7.6 ± 0.2 at 25°C**

### PREPARATION

Suspend 40.20 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense into appropriate containers and sterilize in autoclave at 121°C for 15 minutes. The prepared medium should be stored at 2-8°C. The color is amber, slightly opalescent. It may present a light precipitation.

The dehydrated medium should be homogeneous, free-flowing and beige in color. If there are any physical changes, discard the medium.

### USES

MARINE BROTH is similar to Marine Agar (Cat. 1059), lacking the agar, but containing all the nutrients necessary to cultivate the majority of marine bacteria.

Since the marine environment has environmental conditions completely different to those of other environments, its microflora is also very different. Marine Microorganisms are capable of surviving at very low temperatures and in high salinity levels.

Both Marine Agar and Marine Broth (Cat. 1217) are prepared according to ZoBell, containing almost double the mineral content of sea water. The high salt content helps to simulate sea water. Bacteriological peptone provides nitrogen, vitamins, minerals and amino acids essential for growth. Yeast extract is a source of vitamins, particularly of the B-group. Bacteriological agar is the solidifying agent.

Dispense 50 ml of the broth in 250 ml Erlenmeyer flasks. Inoculate and incubate at 20-25°C for 24-72 hours.

### MICROBIOLOGICAL TEST

The following results were obtained in the performance of the medium from type cultures after incubation at a temperature of 20-25°C and observed after 24 - 72 hours.

Microorganisms	Growth
<i>Vibrio fischeri</i> ATCC 7744	Good

## Zobell Marine Agar 2216

M384

### Intended use

Zobell Marine Agar 2216 is recommended for cultivation, isolation and enumeration of heterotrophic marine bacteria.

### Composition\*\*

Ingredients	Gms / Litre
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000
Final pH ( at 25°C)	7.6±0.2

\*\*Formula adjusted, standardized to suit performance parameters

### Directions

Suspend 55.25 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and Pour in sterile Petri plates.

### Principle And Interpretation

Microorganisms in an aquatic environment may occur at all depths ranging from the surface region to the very bottom of the ocean trenches. The top layers and the bottom sediments harbour higher concentration of microorganisms (1). Marine microorganisms are vital to ecological cycles because they form the foundations of many food chains (2). Zobell Marine Agar formulated by Zobell (3), has a composition that mimics seawater (4) and thus helps the marine bacteria to grow abundantly. This medium has been used for the growth of marine bacteria (5, 6).

Zobell Marine Agar 2216 contains the nutrients, which are required for the growth of marine bacteria. These media have minerals as in seawater (7) and peptone and yeast extract as the sources of nutrients for the marine bacteria as reported by Jones (8). High amount of salt content is used to simulate seawater. Other minerals are used to mimic the mineral composition of seawater.

Pour plate and spread plate techniques can be used for enumeration. In the pour plate technique, the agar must be cooled to 42°C before inoculation to support thermo-sensitive nature of most marine bacteria. In spread plate technique, the medium is poured while still hot and allowed to cool and solidify before inoculation.

### Type of specimen

Marine water samples

### Specimen Collection and Handling:

For marine water samples follow appropriate techniques for handling specimens as per established guidelines (5,6). After use, contaminated materials must be sterilized by autoclaving before discarding.

## **Warning and Precautions :**

Read the label before opening the container. Wear protective gloves/protective clothing/eye protection/ face protection. Follow good microbiological lab practices while handling specimens and culture. Standard precautions as per established guidelines should be followed while handling specimens. Safety guidelines may be referred in individual safety data sheets.

## **Limitations**

1. The medium is recommended for the isolation of marine bacteria. Further biochemical and seological testing must be carried out for further identification.

## **Performance and Evaluation**

Performance of the medium is expected when used as per the direction on the label within the expiry period when stored at recommended temmperature.

## **Quality Control**

### **Appearance**

Cream to yellow homogeneous free flowing powder

### **Gelling**

Firm, comparable with 1.5% Agar gel.

### **Colour and Clarity of prepared medium**

Yellow coloured opalescent gel forms in Petri plates.

### **Reaction**

Reaction of 5.53% w/v aqueous solution at 25°C. pH : 7.6±0.2

### **pH**

7.40-7.80

### **Cultural Response**

M384: Cultural characteristics observed after an incubation at 20-25°C for 40-72 hours .

<b>Organism</b>	<b>Inoculum</b>	<b>Growth</b>	<b>Recovery</b>
	(CFU)		

### **Cultural Response**

*Vibrio fischeri* ATCC 7744 50-100 good-luxuriant >=50%

*Vibrio harveyi* ATCC 14126 50-100 good-luxuriant >=50%

## **Storage and Shelf Life**

Store below 10-30°C in a tightly closed container and the prepared medium at 20-30°C. Use before expiry date on the label. On opening product should be properly stored dry, after tightly capping the bottle inorder to prevent lump formation due to the hygroscopic nature of the product. Improper storage of the product may lead to lump formation. Store in dry ventilated area protected from extremes of temperature and sources of ignition. Seal the container tightly after use. Use before expiry date on the label.

Product performance is best if used within stated expiry period. .

## **Disposal**

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques (9,10).

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Revision : 02 / 2017

#### **Disclaimer :**

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# Diary

week	Sunday	Monaday	Tuesday	Wednesday	Thursday	Friday	Saterday
17/02/19			1) Cultur of new bacteria coming from the IMB's sample bank on Marine Agar (1)	1) Culture in a nutrient broth of the bacteria that didn't growth on Marine Agar (2)	1) Cultur of the bacteria (2) on Marine Agar 2) Cultur of bacteria (1) in Marine Broth		
24/02/19	1) Chlorofor m extraction of the pellet of the bacteria (1) 2) Filtration of the supernatant of bacteria (1)	1) Cultur of new bacteria coming from the IMB's sample bank on Marine Agar	1) continuatio n the extraction of bacteria (1) 2) Cultur of the bacteria used for the disc diffusion test (E.coli, Salmonella, S.uberis, B.cereus)	1) Finishing the extraction of bacteria (1)	1) Loading of the compound of bacteria (1) on steril disc	1) Realisation of the DDT test with bacteria (1)	1) Observatio n of the result of The DDT of the bacteria (1)
3/03/19	Horseshoe crab research lab Promotion in primary school	National holiday	1) Cultur of new bacteria in Marine Broth (4)	1) Sampling of sponges <i>Styliissa carteri</i> around Bidong Island 2) Cuting sponges into pieces and cleaning from parasite	1) Extraction of the compound of the bacteria (4)		

10/03/19	1) Continuation on the extraction of the bacteria (4)	1) Continuation on the extraction of the bacteria (4)	1) Continuation on the extraction of the bacteria (4) 2) Culture of new bacteria in Marine Broth (5)	1) Continuation on the extraction of the bacteria (4) 2) Culture of bacteria coming from the sponges <i>Styliissa carteri</i>	1) Continuation on the extraction of the bacteria (4) 2) Extraction of the compound from the bacteria (5)		
17/03/19	Breast cancer event Holiday	1) Continuation of the extraction of the compound from the bacteria (5) 2) Culture of bacteria coming from the sponges <i>Styliissa carteri</i> in marine broth (6) 3) Culture of the bacteria used for the disc diffusion test (E.coli, Salmonella, S.uberis, B.cereus)	1) Continuation of the extraction of the compound of the bacteria (5) 2) Extraction of the crude extract of the sponges	1) Continuation of the extraction of the compound of the bacteria (5) 2) Loading of the compound of bacteria (4) on steril disc	1) Continuation of the extraction of the compound of the bacteria (5) 2) Loading of the compound of bacteria (4) on steril disc	1) Realisation of the DDT test with bacteria (4)	1) Observation of the result of the DDT of the bacteria (4)

	1) Extraction of the compound of the bacteria (6) 2) Cultur of new bacteria from the sponges <i>Styliissa carteri</i> in marine broth (7)	1) Continuation of the extraction of the compound coming from the bacteria (6)	1) Continuation of the extraction of the compound coming from the bacteria (6) 2) Extraction of the compound coming from the bacteria (7)	1) Continuation of the extraction of the compound coming from the bacteria (6) 2) Extraction of the compound coming from the bacteria (7)	1) Continuation of the extraction of the compound coming from the bacteria (7) 2) Loading the compound of the bacteria (6) on the disc		
24/03/19	1) Continuation of the extraction of the compound coming from the bacteria (7) 2) Realisation of the DDT test with bacteria (6) 3) Cultur of bacteria (8) from sponges in a small amount of Marine Broth for AMS	1) Loading the compound of the bacteria (7) on the disc 2) Realisation of AMS with the bacteria (8)	1) Realisation of DDT with bacteria (7)				1) Extraction of the broth from the AMS of the bacteria (8) and put in -80°C
31/03/19	1) Put the broth of the AMS of the bacteria (8) in the freeze-dryer	1) Research for TLC solvent system for <i>Styliissa carteri, Sargassum. sp, Aconthaster planci, Diadema setosum</i>	1) Research for TLC solvent system for <i>Styliissa carteri, Sargassum. sp, Aconthaster planci, Diadema setosum</i>	1) Realisation of the DDT with the bacteria (8) 2) Preparation of the reagent anisaldehyde for TLC	1) Realisation of TLC with <i>Styliissa carteri, Sargassum. sp, Aconthaster planci, Diadema setosum</i>		
7/04/19							

14/04/19	1) Realisation of TLC with <i>Styliissa carteri</i> , <i>Sargassum. sp</i> , <i>Aconthaster planci</i> , <i>Diadema setosum</i>	1) Realisation of TLC with <i>Styliissa carteri</i> , <i>Sargassum. sp</i> , <i>Aconthaster planci</i> , <i>Diadema setosum</i>	1) Realisation of TLC with <i>Styliissa carteri</i> , <i>Sargassum. sp</i> , <i>Aconthaster planci</i> , <i>Diadema setosum</i>		1) Realisation of TLC with <i>Styliissa carteri</i> , <i>Sargassum. sp</i> , <i>Aconthaster planci</i> , <i>Diadema setosum</i> 2) Cultur of other bacteria coming from <i>Styliissa carteri</i> in a small amount of Marine Broth for AMS (9)	3) Cultur of the bacteria (9) on AMS	
21/04/19	1) Realisation of TLC with <i>Sonneratia caseolaris</i>	Horseshoe crab research lab Promotion in primary school	Horseshoe crab research lab Promotion in primary school	1) Extraction of the broth from the AMS of the bacteria (9) and put in -80°C	1) Put the broth of the AMS of the bacteria (9) in the freeze-dryer		
28/04/19	1) Realisation of the DDT with the bacteria (9)+(5) 2) Cultur of other bacteria coming from <i>Styliissa carteri</i> in a small amount of Marine Broth for AMS (10)	1) Cultur of the bacteria (1°) on AMS	1) Research on the method to obtain the crude extract of <i>Gracillaria changii</i>	1) Research on the method to obtain the crude extract of <i>Gracillaria changii</i>	1) Extraction of the crude extract from <i>Gracillaria changii</i>		

5/05/19	1) Extraction of the crude extract from <i>Gracillaria changii</i>	1) Extraction of the crude extract from <i>Gracillaria changii</i>	1) Extraction of the crude extract from <i>Gracillaria changii</i>	1) Extraction of the crude extract from <i>Gracillaria changii</i>	2) Realisation of DDT with the crude extract of <i>Gracillaria changii</i>	1) Observation of the result of the DDT realised with the crude extract of <i>Gracillaria changii</i>	
12/05/19	/	/	/	/	/		

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## Summary

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This work has been realized in the University Malaysia Terengganu situated in Malaysia. Biofouling is the fixation of organisms on wetted surfaces. It starts by the formation of a bacterial layer (biofilm) on the surface, on which macroorganisms fix themselves later. Biofouling is a huge problem for marine construction since hundreds of years and lead to important cost, as well as many environmental problems. Marine bacteria (free-living in the seawater and hosted by marine macroorganisms) are shown by different studies to produce a large diversity of secondary metabolites. Those secondary metabolites are potential antifouling environmental friendly compound. The goal of this work is to isolate those bacteria, culture them and test the antibacterial ability of their secondary metabolite using Disc Diffusion Test (DDT). The secondary metabolites that show antibacterial activity are then analysed using different chemical structures analysing methods (in this work, only Tin Chromatography Layer has been done). The bacteria that produce antibacterial compounds could then be cultured in a bioreactor in order to produce a large amount of antifouling compounds that would be applied on surfaces.

Ce travail a été réalisé à l'Université Malaisie Terengganu situé en Malaisie. Le Biofouling est le phénomène de fixation d'organismes sur des surfaces humides. Cela débute par la formation d'un couche bactérienne (biofilm) sur laquelle des macroorganismes se fixent plus tard. Le Biofouling est un énorme problème lorsqu'il touche aux constructions marines ayant pour conséquence d'importants coûts mais également d'importants problèmes environnementaux. Il a été démontré que les bactéries marines (vivant librement dans l'eau ou en symbiose avec des macroorganismes marins) produisent une grande diversité de métabolites secondaires. Ces métabolites secondaires présentent un potentiel élevé de composé antifouling sans impact environnemental. Le but de ce projet est donc d'isoler, cultiver et tester les propriétés antibactériennes de ces métabolites secondaires par l'application de tests de discs de diffusion. Les métabolites secondaires montrant des propriétés antibactériennes sont ensuite analysés par différentes méthodes d'analyses de la structure chimique des composés (dans ce travail, seul la chromatographie sur couche mince a été réalisée). Les bactéries produisant des composés antifouling peuvent ainsi être cultivées en bioréacteurs pour produire une grande quantité de ces composés qui seront ensuite appliqués sur la surface sous forme de peinture.