Bioprospection of cyanobacterial strains for antifouling compounds

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2021

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O presidente do Júri,

Porto, ____/____/_____

To all my friends and family.

Acknowledgments

This work was very challenging, especially given the world situation throughout 2020-2021, and without the support of some wonderful people I'm positive I wouldn't be able to finish it.

First, I would like to thank my supervisor Dr. Marco Preto, who helped me immensely through the development of this work, his thoroughness and availability with each and every single aspect of the work we did is something that inspired me to always go a bit further. He always proved very helpful and supportive, even in subjects beyond the dissertation. I'm very glad that our paths got to cross.

I would also like to thank my co-supervisor Prof. Dr. Vitor Vasconcelos, who helped me sort out the dissertation paperwork and agreed to accept me as a student, giving me the wonderful opportunity to work at CIIMAR.

I'd also like to thank the colleagues of CIIMAR staff for being so welcoming, in special Dr. Joana Almeida, Catarina and Sandra, who helped me immensely in all sorts of doubts about the assays performed and taught me a lot.

A huge thanks to the NASCEM project, for the funding provided for this project. This research was supported by national funds through FCT–Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020, under the project NASCEM PTDC/BTA-BTA/31422/2017 (POCI-01-0145-FEDER-031422) financed by FCT, COMPETE 2020 and PORTUGAL 2020.

To all my friends, especially Thiago, Feliphe, Mariana and Marcos Aragão, they were crucial to keep me focused and well enough to reach the finish line, I'm forever grateful to have you in my life.

To my sister, Beatriz, who accompanied me in this crazy journey of moving to another continent and consistently helping me with everything.

Finally, to my parents, Aurimar and Fabiane, for believing in me in every single step of the way, without their support I don't really know if I would have gone this far, I love you all.

Resumo

Bioincrustação é um processo natural de colonização de organismos quando uma superfície (como o casco de uma embarcação) é submersa em água. Este fenómeno está associado com uma panóplia de desafios para o setor marítimo, como um maior consumo de combustível devido ao aumento de arrasto promovido pelos seres incrustados, além do transporte de espécies invasoras para outras regiões do oceano que podem causar danos irreversíveis ao ecossistema da região afetada. Soluções eficazes para esta problemática como tintas à base de tri-butilestanho (TBT) foram provadas altamente tóxicas e seu uso foi banido. Apesar de novas alternativas de tintas com atividade anti-incrustante estarem disponíveis no mercado (como revestimentos à base em Cu^{2+}), elas ainda apresentam toxicidade ao ambiente e sua eficiência deixa a desejar quando comparada aos revestimentos à base de TBT. Uma estratégia empregada para a descoberta de novos compostos anti-incrustantes com baixa toxicidade é a prospecção de produtos naturais sintetizados por organismos incrustantes, uma vez que bilhões de anos de evolução podem revelar em compostos potentes e interessantes usados como estratégia para prosperar em um ambiente altamente competitivo. Cianobactérias são capazes de produzir compostos naturais com atividades interessantes e são um dos grupos responsáveis pela colonização inicial da bioincrustação, realizando esta tarefa através da formação de biofilmes. Este facto levanta a hipótese da possibilidade destas bactérias serem capazes de produzir compostos anti-incrustantes efetivos. Desta forma, o objetivo do presente trabalho foi descobrir novos compostos anti-incrustantes de cianobactérias produtoras de biofilmes e/ou géneros poucos representados na investigação de compostos naturais através de resultados guiados por bioensaios anti-micro e macro-incrustação.

Palavras-chave: Bioincrustação, produtos naturais, metabólitos secundários, cianobactéria.

Abstract

Biofouling is a natural occurring process of adhesion of organisms when a surface (such as a ship's hull) is submerged in water. This phenomenon is associated with several challenges to the maritime industry, such as a higher consumption of fuel due to the increased drag provided by the fouled organisms, and the transportation of several alien species to another region of the ocean that may disturb and cause irreversible damage to this region's ecosystem. Effective solutions such as tributiltyn-based (TBT) paints were proven to be highly toxic to the environment and because of that their use was banned. Although new alternative paints with anti-fouling properties are being used (such as Cu^{2+}-based paints), they still present some toxicity to the environment and their efficacy are lacking when compared to TBT paints. A strategy employed to discover new anti-fouling compounds with associated low toxicity is to prospect natural products produced by fouling organisms, since millions of years of evolution may unravel interesting and potent compounds used as a strategy to thrive in a highly competitive environment. Cyanobacteria produce interesting natural products and are early colonizers of immersed surfaces, doing so by forming biofilms, meaning that it is possible that they can produce effective anti-fouling compounds. Thus, the aim of this work was to unravel new anti-fouling compounds from biofilm-forming cyanobacteria and/or poorly represented genera in natural products prospection through results obtained in anti-micro and macrofouling bioassays.

Keywords: Biofouling, natural products, secondary metabolites, cyanobacterial.

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Abbreviation list

AF – Antifouling

BBE – Blue Biotechnology and Ecotoxicology Laboratory

BMAA – β-Methylamino-L-alanine

BOGA – Aquatic Organisms Bioterium

CECT – Spanish Type Culture Collection

CEMUP – Centro de Materiais da Universidade do Porto

CIIMAR – Interdisciplinary Centre of Marine and Environmental Research

DCOIT – Dichloro-octyl-isothiazolin

DMSO – Dimethyl Sulfoxide

EPS – Extracellular polymeric substances

GNPS – Global Natural Products Social Molecular Network

LC-MS – Liquid Chromatography – Mass Spectrometry

HPLC – High Performance Liquid Chromatography

ISSG – Invasive Species Specialist Group

LEGE – Laboratório de Ecotoxicologia, Genómica e Evolução

LEGE-CC – Blue Biotechnology and Ecotoxicology Culture Collection

MeOH – Methanol

MTA – Material Transfer Agreement

MS – Mass Spectrometry

NRP – Non-ribosomal peptide

NRPS – Non-ribosomal peptide synthetase

OD – Optical density

PK – Polyketide

PKS – Polyketide synthase

QS – Quorum-sensing

TBT – Tributyl-tin

TCMTB – Thiocyanatomethylthio-benzothiaole

UV – Ultraviolet

WP 14 – Work plate 14

WP 16 – Work plate 16

“La utopía está en el horizonte. Camino dos pasos, ella se aleja dos pasos y el horizonte se corre diez pasos más allá. Entonces, ¿para qué sirve la utopía? Para eso,sirve para caminar.”

Eduardo Galeano

1 Introduction

The use of nature and natural products is tightly linked with humankind. Through observation and empiric experiences, soon it became clear to humans that from these interactions some plants were believed to have special beneficial properties, sometimes therapeutic and healing and other times harmful such as poisonous plants (Farnsworth, 2007). These properties are often related to bioactive compounds present in these organisms, and with the progress of science, it became quite clear that the isolation of these molecules could be extremely useful for society as a whole.

Even though science has come a long way in finding and isolating very important bioactive compounds, the main focus point in regards to prospection for new secondary metabolites remained for many years in terrestrial organisms (Altmann, 2017). Although this approach was successful in many ways, it failed to acknowledge the huge potential and diversity of bioactive compounds produced by marine organisms until 40 years ago (Altmann, 2017), when researchers started to prospect and isolate these molecules from a number of organisms, such as corals, microalgae, cyanobacteria, sponges and many others (Blunt et al., 2018).

Indeed, prospecting compounds produced by marine organisms proved very promising, since the environmental pressure that marine organisms endure is very distinct when compared to that of terrestrial organisms. In addition to this, the large size of the ocean and its varying conditions such as temperature, pressure and salinity could mean that an extensive library of molecules with a vast array of activity can be identified and possibly isolated (Blunt et al., 2018; Xie et al., 2018). This is especially true when billions of years of evolution is taken in consideration, meaning that organisms may produce a plethora of new effective chemicals that were used as a response to the environmental pressure the organism is inserted (Qi & Ma, 2017; Skropeta & Wei, 2014). Another interesting aspect of marine synthesized compounds is that they need to be highly potent to have any sort of effect, since they are easily diluted and dispersed when released in to the water (Haefner, 2003).

This prospection strategy can be especially effective if the organisms suffer direct environmental pressure from the problems that are trying to be solved by researchers. For instance, biofilm-forming cyanobacteria compete directly with micro- and macro-organisms when it comes to adhering and thriving in a marine surface, be it man-made or not, meaning it is possible that these organisms are capable of producing molecules that hinder other organism’s ability to adhere on the surface. Indeed, this

was proven true with the isolation of several compounds that have anti-adhering properties, not only from cyanobacteria but from other adhering organisms such as sponges and tunicates (Pech-Puch et al., 2020; Sang et al., 2019; Tan et al., 2010).

The process consisting in the adhesion of micro- and macro-organisms in artificial or natural underwater surfaces is called biofouling, and creates a complex community composed by bacteria, microalgae, algae, barnacles, mussels and several other organisms (Abarzua & Jakubowski, 1995; Nurioglu et al., 2015) .

The formation of these communities occurs in phases, where the first step is the adsorption of proteins and other macromolecules present in the ocean water into a surface such as the ship's hull, providing a favorable surface where microorganisms such as biofilm-forming bacteria can easily have access to the adsorbed nutrients and thus forming what it is called microfouling. In addition, the biofilm formed promotes a suitable surface for the larvae and spores of some organisms like mussels and barnacles to settle on, creating a visible coat of organisms on the ship's hull known as macrofouling (Nurioglu et al., 2015).

The attachment of such organisms in naval ships produces an increase in fuel consumption due to the consequent increase of drag induced by the formation of these types of communities (Dahlbäck et al., 2010), averaging a cost of 60€ billion per year according to a 2011 study (Schultz et al., 2011), and also increasing the emission of greenhouse gases such as CO_2 and SO_2 (Davidson et al., 2016).

Another problem associated with marine biofouling is the likely possibility of these attached organisms to be carried into other regions and invading this newly presented ecosystem and potentially causing serious damage to the homeostasis of the area (Charles et al., 2018; Seebens et al., 2013).

Strategies to prevent these organisms to attach to the ship's hull are more than 2000 years old, with coatings based on whale oils, lead, sulfur and arsenic. The constant evolution of the maritime transportation demanded that the needs for a powerful anti-foulant paint to be met (Castro et al., 2011)

The first modern and most effective solution for the anti-fouling problem was paint-based tributyl-tin (TBT) (Schultz et al., 2011; Thomas & Brooks, 2010). However, the toxicity towards non-target organisms in these paints was not accounted for, greatly polluting ocean waters and severely damaging sea life (Thomas & Brooks, 2010). Indeed, one of the very first reports of the toxicity towards non-targets was described in Arcachon Bay, where high levels of tin and organotin were identified in oysters and manifested in anomalies such as shell malformation and poor larval development (Alzieu et al., 1986).

These results caught attention of the International Marine Organization, which led to the prohibition of the application of TBT paints in all vessels by 2003, and the total absence of these paints by 2008 (Pangam et al., 2009; Schultz et al., 2011; Thomas & Brooks, 2010).

The ban of TBT led to a new search for a biocide that could replace it. Copper (Cu)-paints were the natural replacement, since it was already used as a cheaper alternative to TBT-paints or as a co-biocide to boost its effectiveness (Nichols, 1988). Although it presented less toxicity than TBT paints, it still greatly affected the environment through undesired effects on non-target species through the accumulation of Cu in coastal regions (Bao et al., 2011; Carson et al., 2009).

Despite the already proven toxicity, the commercially available paints are often Cu-based paints supplemented with substances called “boosters”, in order to boost its effectiveness and target organisms resistant to the action of Cu-paints (Dafforn et al., 2011; Reed & Moffat, 1983; Thomas & Brooks, 2010). Common examples of boosters utilized by the industry include Irgarol 1051®, thiocyanatomethylthio-benzothiaole (TCMTB), dichloro-octyl-isothiazolin (DCOIT, Sea Nine 211®) and Diuron (Dafforn et al., 2011). Most of these boosters are often herbicides, since the biggest offenders in terms of resistance to Cu are algae and diatoms, and boosters like Diuron and Irgarol target the photosynthetic ability of these organisms (Dafforn et al., 2011; Thomas & Brooks, 2010). Studies already link these boosters to a high toxicity, and its use is already heavily regulated (Thomas & Brooks, 2010; Viana et al., 2020).

Given the available options in the market, new anti-fouling (AF) paints that are environmental-friendly while being cost effective and an efficient anti-foulant, must be a priority for the maritime transport industry.

A great strategy to find suitable answers for the biofouling problem is through mimicking the survival approach of the organisms that directly suffer from the pressure of fouling, a strategy also known as biomimetics (Chen et al., 2021; Sánchez-Lozano et al., 2019). There are two prominent strategies utilized by marine organisms to prevent fouling, which can be called passive and active inhibition. Some mollusks shells and shark skin present microstructures that hamper the attachment of fouling organisms, this type of anti-fouling is called passive inhibition (Chen et al., 2021; Sánchez-Lozano et al., 2019).

The active inhibition is typically presented by sessile organisms, such as corals, sponges, algae and cyanobacteria. This type of inhibition is manifested through the production of secondary metabolites that hinder the ability of the foulers to attach to the surface (Chen et al., 2021; Gu et al., 2020).

Active inhibition is manifested through a plethora of mechanisms, one of the most well documented strategies to prevent the attachment by foulers is through the disruption of quorum-sensing (QS) (Mieszkin et al., 2013). A vital step for the formation of the biofilm is the cell-to-cell communication between bacteria that is commonly known as QS, and disrupting this phenomenon may prevent microfouling (Dahms & Dobretsov, 2017; Mieszkin et al., 2013).

Other more direct active inhibition strategies encompass direct toxicity against fouling organisms, such as anti-diatom, anti-larval and anti-bacterial activities (Dahms & Dobretsov, 2017; Dahms et al., 2006).

In regards to the active inhibition, one of most the promising candidates for the prospection of molecules for antifouling purposes are cyanobacteria, since the methodology for the growth and upscaling of these microorganisms is well established and usually have a relatively low cost and low maintenance (Dahms et al., 2006). Another reason to prospect cyanobacteria for AF compounds is the capability of some of the cyanobacteria to form biofilms, meaning that it is likely that the production of AF compounds could be used as a part of a mechanism to survive in a competitive environment (Antunes, et al., 2019).

Indeed, cyanobacteria have shown that they are capable of synthesizing AF compounds, as it was proven with the discovery of portoamides and many other examples (Antunes, et al., 2019; Dahms et al., 2006; Fathoni et al., 2020; Tan & Goh, 2009).

Thus, the main goal with this work was to prospect new cyanobacterial strains with promising antifouling activity. This was done by fractioning the methanolic extracts of said strains and testing through bio-guided assays. The ultimate goal of this work was isolating and characterizing any molecules responsible for the activity observed in the bioassays.

1.1. Cyanobacteria

1.1.1 General characteristics and morphology

Cyanobacteria or Cyanophyta are a diversified group of gram-negative prokaryotes, although being also known as blue-green algae and share their photoautotrophic photosynthetic oxygenizing capabilities with most algae, they differ from algae for the lack of membrane-enclosed chloroplasts and the fact that they are all prokaryotes (Broady, 2019). They are thought to be one of the oldest photosynthetic

organisms, and are tightly associated with the evolution of modern eukaryotic photosynthetic organisms (Kultschar & Llewellyn, 2018).

Morphologically they can be filamentous or unicellular organisms, and have rod, spherical and spiral shapes. Taxonomically cyanobacteria are divided in five major sections based on the aforementioned morphological characteristics as well as other physiological traits. The orders Chroococcales and Pleurocapsales comprise of unicellular single or aggregate cells that reproduce by budding or binary fission (former order) and multiple fission or both binary and multiple fission (latter order). The last three orders (Oscillatoriales, Nostocales and Stigomatales) are composed by filamentous bacteria that present trichomes, and share the reproductive method which is the formation of motile fragment called hormogonia. They differ in the cell differentiation capabilities, with Nostocales and Stigomatales being capable of producing different cell-types such as heterocysts, whereas Oscillatoriales are composed solely by vegetative cells (Broady, 2019; Kultschar & Llewellyn, 2018).

Picocyanobacteria, which encompasses five genera (*Synechococccus*, *Cyanobium*, *Synechocystis*, *Cyanothece* and *Cyanobacterium*), are the most abundant photosynthetic organisms on earth, and some of the strains in this work pertain in this group (Jasser et al., 2017; Huang et al., 2012).

Cyanobacteria are nearly ubiquitous, being able to adapt and evolve to inhabit aquatic and terrestrial habitats even under extreme conditions such as extreme values of temperature and pH, as well as other abiotic stresses like high levels of salt or intense UV radiation (Seckbach, 2007).

The aforementioned diversity and adaptability presented by cyanobacteria makes them a strong candidate for bioprospection of new secondary metabolites, since it is quite plausible that in order to thrive in competitive and extreme conditions, these prokaryotes developed a substantial library of surviving strategies, many of which are proven or thought to be promoted by the synthesis of such metabolites (Carmichael, 1992; Harada, 2004).

1.1.2 Cyanobacteria and secondary metabolites

Cyanobacteria are well known to be able to synthesize natural products due to their relation with harmful algal blooms and another toxin-related incidents (George, 1878; Osborne et al., 2001), whereas it was identified the presence of chemicals now known as cyanotoxins (such as microcystins and cylindrospermopsins) (Carmichael,

1992; Sanseverino et al., 2017), meaning that these metabolites could be indicators of other products or even have beneficial properties at lower concentrations.

The first successful isolation of interesting secondary metabolites from cyanobacteria was accomplished in the late 1970s by Richard E. Moore and colleagues, majusculamides from *L. majuscula* (Marner et al., 1977). Since then, the prospection of natural products from cyanobacteria has grown exponentially, and the search for these molecules resulted in a huge library of new secondary metabolites with a plethora of biological activity, ranging from health-related activities such as antibiotic, immunostimulant, cytotoxic and anti-inflammatory (Blunt et al., 2018; Lopes et al., 2020; Singh et al., 2011), to industrial use such as antifouling, production of biocides (such as herbicides and pesticides) and bioremediation purposes (Årstøl & Hohmann-Marriott, 2019; Berry, 2008; Rastogi & Sinha, 2009).

The order Oscillatoriales are responsible for nearly 50% of isolated cyanobacteria compounds, and the genus *Lyngbya* accounts for 35% of all natural products isolated from these bacteria. From the *Lyngbya genus*, *L. majuscula* is the most important species in terms of isolation of secondary metabolites, accounting for more than half of compounds isolated inside the genus (Demay et al., 2019, Tidgewell et al., 2010). This is likely due to the fact that this genus is easily harvested in large quantities directly from the environment it inhabits, meaning that *Lyngbia* is suffering from direct environmental pressure and responding to it by producing its secondary metabolites (Osborne et al., 2001; Tripathi et al., 2010). Additionally, due to the easy harvest, there is no need to grow and cultivate the genus, making it easier for researchers to study, while other species generally need to be isolated and grown in a monoculture environment in order to extract its natural products, often leading to low production (or no production at all) of such compounds given they are not subjected to any environmental stress, these factors lead to an overrepresentation of the *Lyngbia* genus.

Most of secondary metabolites isolated from cyanobacteria are non-ribossomal peptides (NRPs), polyketides (PKs) or a hybrid of the two, such as peptoketides or ketopeptides (Tidgewell et al., 2010).

The presence of these biosynthetic gene clusters is a powerful tool to find and identify the strains that are able to produce secondary metabolites, and even identify the natural product synthesized by this strain (Ehrenreich et al., 2005).

Synthesis of these molecules occur in a modular multienzymatic domains such as polyketides synthases (PKSs) and non-ribosomal peptides synthases (NRPSs) (Kehr et al., 2011, Tidgewell et al., 2010), the complexity of these domains are responsible for the high diversity of secondary metabolites produced by cyanobacteria.

1.2 Biofouling: an in-depth look

As previously mentioned, the biofouling process is composed of four phases, the first step towards the establishment of a fouling community is called biochemical conditioning, which represents the adsorption of mostly macromolecules seconds after surface immerses in seawater (Wahl, 1989).

The adsorption film (or conditioning film) composed of proteoglucans, polysaccharides and glycoproteins create a perfect environment for microorganism settlement to thrive, initiating the second step of the biofouling process only hours after the immersion of the surface: the bacterial colonization. This phase is divided in two, the first is comprised of a reversible bacterial attachment through electrostatic and hydrodynamic interactions between the surface and the microorganism, the second is a permanent adherence to the surface through covalent bonds between the bacteria and the substrate (Nurioglu et al., 2015; Wahl, 1989). Although this phase is mainly represented by bacteria, other organisms such as diatoms, cyanobacteria, dinoflagellates, protozoa and fungi also can attach through mechanisms such as mucilage secretions (Molino & Wetherbee, 2008).

The success of the new attached heterogeneous colony is highly dependent on the production of extracellular polymeric substances (EPS), a very important step for the formation of the biofilm (Antunes, et al., 2019; Flemming & Wingender, 2010).

The third step comprises of the colonization of the surface by eukaryotes, such as yeasts, diatoms, macroalgae and protozoa spores. These organisms adhere to the first heterogenous film and within weeks generate a secondary film (Nurioglu et al., 2015; Wahl, 1989).

The fourth and final step is the macrofouling, or the colonization of the hard foulers, this step occurs several weeks after immersion and creates a visible layer of organisms such as corals, mussels and tunicates (Nurioglu et al., 2015; Wahl, 1989).

This adhesion is a protein-mediated process, and interestingly marine organisms have developed distinct strategies when it comes to attaching to the surface. For instance, adhesion of mussels is carried out through the secretion of a proteinaceous byssus by byssal glands located in the mussel's feet (Waite, 2017). On the other hand, barnacles secrete sticky adhesive proteins from cement glands in order to fixate on a surface (So et al., 2019). The strategy employed by to adhere into a surface ascidians is the mucus secreted by its papillae (Li et al., 2021).

A schematic of the biofouling timeframe is represented below in **Figure 1**.

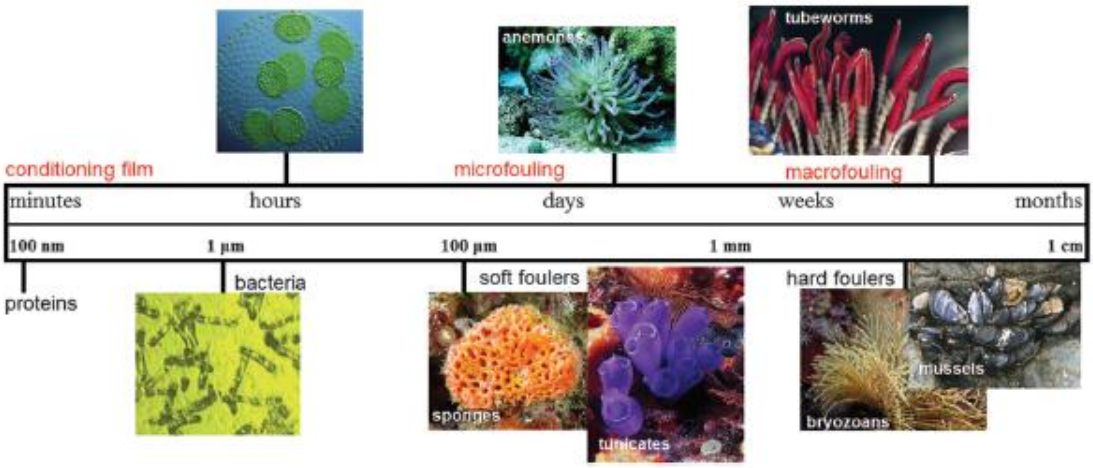


Figure 1. Timeframe of phases of an evolving marine biofouling surface. (Nurioglu et al., 2015)

1.3 Cyanobacteria chosen for the present study

Twelve strains from the LEGE Culture Collection (LEGE-CC) were chosen for the present work (**Table 1**). While some strains were simply chosen for didactic purposes, such as learning the extraction techniques in a strain with a readily available amount of previously grown mass, some particular characteristics were favoured when selecting most of the strains: The novelty factor and the ability to form a biofilm.

The novelty factor brings never-before tested strains extracts, meaning that any results coming from these strains are unprecedented. Also, some genera of cyanobacteria were almost completely ignored in regards to prospecting new molecules due to the bothersome work needed to grow these genera, which indicates that any molecules isolated from these genera have a fair probability of being a new, undiscovered natural product.

The formation of biofilm by cyanobacteria indicates the ability of the organism to foul surfaces when in a natural habitat, meaning it can be possible that they are capable of producing AF compounds based on the environmental pressure it suffered when fouling a surface.

Table 1 - LEGE codes and strains used in the present study.

LEGE Code	Order	Species	Location of sampling
LEGE 00049	Oscillatoriales	Unindentified *Oscillatoriales*	Al Massira, Morocco (Freshwater)
LEGE 03282	Nostocales	*Cuspidothrix issatschenkoi*	Montargil, Portugal (Freshwater)
LEGE 06005	Synechococcales	*Synechocystis sp.*	São Pedro de Moel, Portugal (Marine)
LEGE 06009	Synechococcales	*Nodosilinea sp.*	Caldas da Rainha, Portugal (Marine)
LEGE 06010	Synechococcales	*Nodosilinea sp.*	Caldas da Rainha, Portugal (Marine)
LEGE 06077	Nostocales	*Nostoc sp.*	Seixas, Portugal (Estuarine)
LEGE 06108	Synechococcales	*Toxifilum mysidocida*	Lagos, Portugal (Marine)
LEGE 06139	Synechococcales	*Cyanobium sp.*	Arcozelo, Portugal (Marine)
LEGE 10388	Synechococcales	unidentified colonial *Synechococcales*	Vila Nova de Mil Fontes, Portugal (Marine)
LEGE 11430	Oscillatoriales	*Phormidesmis sp.*	Matosinhos, Portugal (Marine)
LEGE 99043	Nostocales	*Cylindrospermopsis raciborskii*	Ferreira do Alentejo, Portugal (Freshwater)
LEGE XX601	Unindentified	Unindentified	Portugal

One of the aims in the cyanobacteria chosen was diversity, as seen in Table 1 several cyanobacterial orders are represented, and since one of the goals of this study is to found new metabolites, a decision was made to prospect newly isolated cyanobacteria (such as LEGE XX601) and never published strains (such as LEGE 10388 and LEGE 06108).

LEGE 06139, LEGE 06077, LEGE 00049 and LEGE 03282 were chosen for being previously studied in numerous projects by fellow study groups, in particular the NoMorFilm, which envisioned the prospection of secondary metabolites from these strains that inhibited biofilm formation, but with the focused scope of medical uses only, raising the possibility that maybe these strains have an effective antifouling capability.

Indeed, in previous studies our research group was able to identify antifouling properties in LEGE 06077 in an unpublished result. Additionally, its crude methanolic extract displayed cytotoxic behaviour in L929 mouse fibroblasts *in vitro* (Lopes et al., 2011). Also, the known neurotoxin β-N-methylamino-L-alanine (BMAA) produced by cyanobacteria was also detected in LEGE 06077 (Cervantes Cianca et al., 2012), although it is not known if the antifouling capabilities of LEGE 06077 is due to BMAA.

Despite the positive results, the molecule(s) responsible for the activities of LEGE 06077 are still unknown, and since this strain has been domesticated for a long time, the bioassays were performed in order to guarantee the activity previously reported by our group's study, as well as provide new information since the microfouling bioassay has never been performed in this strain. Given that possibility, the bioassays were performed once more in order to confirm that the compound is still produced in order to carry out the upscaling process and attempt to isolate the metabolite.

The cyanobacterium LEGE 99043 was also chosen for the present study. It is known that *Cylindrospermopsis raciborskii* is capable of producing toxic compounds such as cylindrospermopsin, saxitoxins and gonyautoxins, and some undiscovered compounds are thought to be produced by this species (Burford et al., 2016; Lagos et al., 1999).

Although LEGE 99043 has already some interesting molecules that may be responsible for the antifouling property, it is worth noting that the assay in *Mytilus galloprovincialis* has never been done to the best of our knowledge. In addition, there seems to be a direct correlation between its geographical distribution and the production of toxins. For instance, European strains do not seem to be able to produce cylindrospermopsin (Burford et al., 2016), meaning it is possible that the antifouling properties is tied to a new, undiscovered molecule.

LEGE 06005, LEGE 06009 and LEGE 06010 dichloromethane extracts showed some toxicity against *Artemia salina* (Frazão et al., 2010). Additionally, some KS domains were identified in LEGE 06009 through PCR amplification (Brito et al., 2015).

1.4 Targets chosen for the bioassay

For the microfouling assays five bacteria from the Spanish Type Culture Collection (CECT) were chosen, *Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570 and *Roseobacter litoralis* CECT 5395. All of these bacteria are marine bacteria and are known to have a role in the early settlement steps of the fouling process, and have been the standard protocol of our study group for assessing the anti-fouling properties of the compound (Antunes, et al., 2019; Martín-Rodríguez et al., 2015).

Regarding the macrofouling assay, the model animal chosen was *Mytilus galloprovincialis*, a mussel known to be an invasive species that is often transported to other regions through ballast water and fouling the hull of the ship (Antunes et al., 2019). Indeed, the Invasive Species Specialist Group (ISSG) issued a paper that considers *M. galloprovincialis* as one of the 100 worst invasive species (Lowe et al., 2000), making them a very promising model for anti-fouling assays.

The mussel adherence strategy to fixate in a surface is by secreting a proteinaceous glue denominated byssus threads from the byssal glands presented in the mussel's foot (Waite, 2017).

1.5 Aims of the Work

Considering the ever-increasing need for new, effective and eco-friendly strategies to tackle the biofouling problem, the main aim of this work was to screen through a plethora of cyanobacterial strains in order to find new molecules with anti-fouling properties through a bioassay-guided approach. The AF activity was assessed as the inhibition of growth of bacteria strains present in the early stages of biofouling. In addition to that, the AF activity was also measured by the eventual inhibition of the capacity of *Mytilus galloprovincialis* in forming byssal threads, structures responsible for the attachment of this organism.

In case of a positive hit, the attempt to identify, isolate and characterize the molecule(s) responsible for the activity was also an aim.

To achieve the main objective, specific objectives were established:

- Production of cyanobacterial strains biomass from to the LEGE Culture Collection.
- Production of methanolic extracts from the grown cyanobacteria, as well from cyanobacterial strains previously grown by our research group.
- Performing of microfouling anti-settlement bioassay with five strains of marine bacteria known to be early settlers (*Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570 and *Roseobacter litoralis* CECT 5395), using inhibition of growth as confirmation of activity.
- Performing of macrofouling anti-settlement bioassay with *M. galloprovincialis* larvae, using the inhibition of attachment as a confirmation of activity.
- Profiling of promising fractions LC-MS spectra in order to tentatively identify the molecule responsible for the activity.

2 Materials and Methods

2.1 Growth and upscaling of cyanobacteria strains

Three cyanobacterial strains *Nostoc sp.* LEGE 06077, *Synechococcales* sp. LEGE 10388 and *Phormidesmis* sp. LEGE 11430 (LEGE-CC) were cultured and upscaled in Z8 (LEGE 06077) and Z8 salt water medium (LEGE 10388; LEGE 11430) (Kotai, 1972).

In order to grow the selected cyanobacteria an internal MTA (Material Transfer Agreement) form was filled to guarantee correct and ethical usage of the strains.

The inoculum of the grown cyanobacteria was transferred from the LEGE-CC to a starter culture prepared in a 50 mL culture flask, which was taken to the cyanobacteria cultivation room at BOGA (Bioterium of Aquatic Organisms) where they were incubated at 25 ºC with a 14/10h light-dark cycle. After a month of growth, a large aliquot of the starter culture was transferred to a 400 mL culture flask, while preserving the starter culture in case of any contaminations. They were then taken to BOGA to be incubated at the same conditions for another month. The contents of the 400 mL culture flask were then transferred to a 6 L culture flask containing 4 L of the respective medium, they were once again taken to BOGA to be incubated for another month.

Since LEGE 06077 had already previously documented activity by our research group, it was decided to grow it further in order to increase the harvested biomass and consequently have more material to work in order to isolate the compound responsible for the AF activity. A large aliquot of the 6 L culture flask was transferred to another 6 L culture flask and grown for another month. The contents of this 6 L culture flask were then transferred to a 20 L culture flask.

LEGE 11430 and 10388 were harvested through filtration using a 20 µm sieve, since these strains presented a very apparent biofilm that was the vast majority of the biomass. LEGE 06077 was harvested using Thermo Scientific Sorvall BIOS 16 Centrifuge at 4.700 rpm for 10 minutes twice. The biomass from the sieve and the pellets formed by the centrifugation were transferred to an 80 mL cup, frozen at -80 ºC and then freeze-dried. The freeze-dried biomass was used to proceed with the methanolic extractions of the potential molecules with AF activity.

Additionally, the biomass of nine previously grown and freeze-dried cyanobacterial strains *Synechocystis* sp. LEGE 06005, *Leptolyngbya mycoidea* LEGE 06009, *Nodosilinea* sp. LEGE 06010, *Toxifilum mysidocida* LEGE 06108, *Cyanobium* sp. LEGE 06139 and *Cylindrospermopsis raciborskii* LEGE 99043, *Oscillatoriales* sp.

LEGE 00049, *Cuspidothrix issatschenkoi* LEGE 03282 and LEGE XX601 as a yet to be classified species were also used for extraction.

2.2 Methanolic extractions of the cyanobacteria

Methanolic extractions were performed by using 2.5 (± 0.2) g of the freeze-dried biomass of each strain aforementioned (**Table 1**). This biomass was grounded using a mortar and pestle and transferred to an Erlenmeyer.

Extraction was carried out by adding 50 mL of methanol to the grounded biomass and then submitting the Erlenmeyer to an ultrasonic bath for five minutes (with care to keep temperature below 30 ºC). The supernatant was then decanted into a round bottom flask connected to a filtering system composed of cotton, paper filter and a Buchner funnel with a vacuum adapter (**Figure 2**). Afterwards the previous step was repeated twice, only changing the amount of methanol added (from 50 mL to 25 mL).

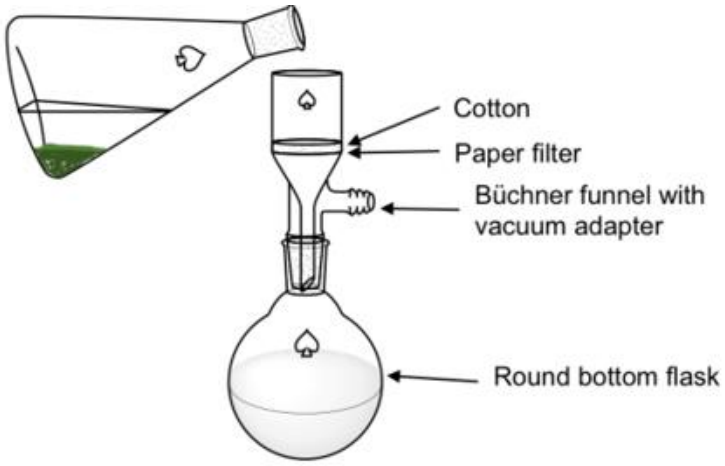
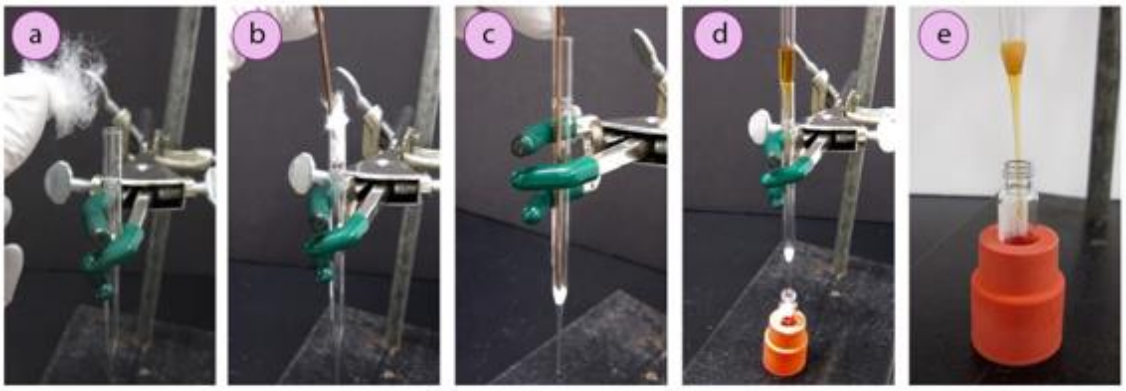


Figure 2. Representation of the methanolic extraction of cyanobacterial strains. Source: MeOH extraction SOP V1.2019 protocol from BBE, CIIMAR).

The decanted supernatant in the round bottom flask was taken to a rotary evaporator in order to concentrate the solution and remove excess methanol. The concentrated content was then filtered through a Pasteur pipette filled with cotton to capture any of the reminiscent non-soluble particles (**Figure 3**). The filtered liquid was collected in a 20 mL vial and once again taken to the rotary evaporator in order to remove the solvent.



(https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Book%3A_Organic_Chemistry_Lab_Techniques_(Nichols)/01%3A_General_Techniques/1.05%3A_Filtering_Methods/1.5F%3A_Pipette_Filtration_(Microscale))

Figure 3. Filtration of the cyanobacterial methanolic extracts through the Pasteur pipette.

2.3 High Performance Liquid Chromatography (HPLC) fractioning of the extracts

In order to prepare the extracts for the fractioning an aliquot of 40 mg were taken from each methanolic extract and stored in a 2 mL HPLC glass vial, 1 mL of LC-MS (Liquid Chromatography-Mass Spectometry) grade methanol was added to solubilize the extracts and to achieve a 40 mg/mL solution. The glass vials were loaded on the Water Alliance e2695 HPLC system and each extract was fractioned in eight fractions using a 10% Acetonitrile-90% Ultra-pure Water gradient as starting condition, with the concentration of acetonitrile steadily increasing by 10% in each fraction according to our research group optimized protocol, for a final gradient of 100% Acetonitrile.

The fractions were collected by an automatic collector in deep-well plates, had their solvents removed in a CentriVap or rotaevaporator, depending on the availability, and then were resuspended in 500 µL of DMSO to transfer the fractions to a mother plate, which was stored in a -80 ºC freezer and added to the BBE's fractions library.

In this work, two work plates (work plate 14 and work plate 16) were prepared using an aliquot of 100 µL of each fraction in order to test the potential bioactivity of each fraction, the decision to divide in to two work plates was taken due to all of the biomass not being readily available. So, to carry on with the work, it was decided to manufacture a work plate containing fractions of most strains that had the biomass readily available.

2.4 Microfouling anti-settlement bioassays

The microfouling anti-settlement bioassay was carried out as described by Antunes *et al.* (2019), using five marine bacteria as targets (*Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570 and *Roseobacter litoralis* CECT 5395).

In a 200 µL 96-well plate, 196 µL of the marine bacteria inoculum in Marine Broth (Difco) medium was administered in each well (200 µL was administered in the blank wells) and had their initial optical density measured at 600 nm (OD_{600}) after a shake cycle of 30 seconds using the Biotek Plate Reader, where OD_{600} values between 0.1-0.14 were the desired ones to start the assay.

After measuring the bacteria's initial OD_{600}, 4 µL of each extract's fraction at a 10 µg/mL concentration was administered in each well, each fraction was added four times in order to achieve four replicates per fraction. A negative control of 0.1% DMSO and a positive control with 1:100 penicillin-streptomycin-neomycin stabilized solution (P4083, Sigma-Aldrich, St. Louis, MO, USA) were also administered in the plates in a similar fashion as the fractions. The plates were then once again taken to the Biotek Plate Reader to have their initial OD_{600} measured, in order to establish the 0h conditions for the bioassay.

Afterwards, the 96-well plates were incubated for 24h (72h for *Roseobacter litoralis*) at 26 ºC and then had their OD_{600} measured once again, the potential inhibitory activity of each fraction was calculated as follows:

$$\%\ Growth\ inhibition = \left(\frac{Sample\ OD24h - Sample\ OD0h}{Ctrl(DMSO)24h - Ctrl(DMSO)0h}\right) x\ 100$$

2.5 Mussel Larvae Antisettlement Bioassays

Algae with mussel larvae (*Mytilus galloprovincialis*) were sampled at low tides at Memória beach, Matosinhos, Portugal (41°13'59" N; 8°43'28" W).

The mussel larvae with sizes ranging from 0.5 to 2 mm were screened with the help of a Pasteur pipette and a binocular magnifier (Olympus SZX2-ILLT) and transferred to a 2.5 mL 24-well plate filled with filtered sea water, where five mussel larvae were put in each well. Before collecting the mussel larvae, it was made sure that

they were active, this was done by confirming a foot exploring behaviour, as seen in **Figure 4**.

Figure 4. Mussel (*M. edulis*) presenting a foot exploring behaviour. A competent mussel larvae should have an active moving foot, used to establish an attachment to the surface the mussel is presented. Source: Bigelow.org

Solutions of the fractioned extracts of LEGE 06077, LEGE 99043 and LEGE 06139 were prepared in 15 mL falcon tubes by mixing the aforementioned extracts and 10 mL of filtered seawater to a final concentration of 10 µg/mL, these falcon tubes were mixed in a vortex and then 2.5 mL of its content was administered in each well, for a total of four replicates per fraction. The controls used for this bioassay were DMSO 0.1% as a negative control and 5µM $CuSO_4$ as a positive control.

The plates were incubated overnight in darkness at 18 ºC and the number of formed byssal threads (**Figure 5**) or absence of said threads was assessed with the help of a microscope. This assay must be performed with extreme caution, since the excessive handling of the larvae could compromise the byssal threads formed, thus harming the integrity of the registered results. In order to avoid any problems regarding mishandling the results, the analysis of the results was performed by Dr. Joana Almeida, the researcher responsible for the idealization and implementation of the macrofouling assay.

The antifouling activity of the fractioned extracts was evaluated by comparing the formation of the byssal threads formed by the mussels from the negative control in relation to the mussels exposed to the fractions.

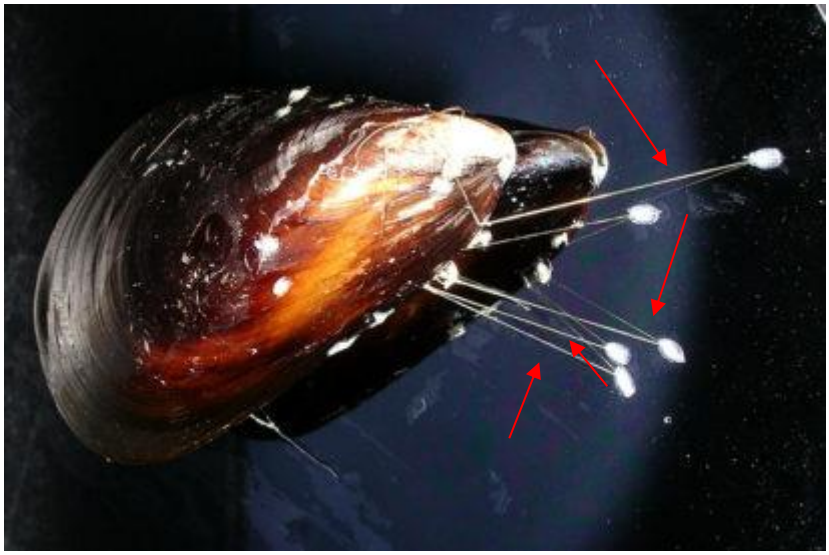


Figure 5. Mussel displaying the formed byssal threads (red arrows) for fixation on a surface. Antifouling activity is closely related with the mussel's capability of producing byssal threads. Source: Berkeley.edu.

2.6 HPLC-MS Characterization and GNPS analysis

The fractions with positive results in the bioassays and their adjacent fractions were submitted to a LC-MS analysis, where 15 µL of each fraction was dissolved in DMSO and transferred to a vial, the solvent was then dried and the contents of the vial were resuspended in MeOH MS-grade. The vials were then taken to CEMUP (Centro de Materiais da Universidade do Porto), where the MS spectra was generated.

The analysis of the LC-MS spectra was carried out using the Xcalibur™ software, which allowed to propose mass(es) of the molecule(s) responsible for the activity by comparing the peaks present in the fraction with positive hits and their respective adjacent fractions. Given that some of the strains studied in this work have already some cyanotoxins and other molecules documented from previous studies, it is possible to at least identify if there are peaks compatible with these compounds in the bioactive fractions, which may indicate that they are responsible for the activity.

Another strategy used to propose the possible masses for the active compounds was to upload the MS data obtained to GNPS (Global Natural Products Social Molecular Networking), where the spectra was compared to the site's database in order to evaluate if any known molecule can be identified based on the uploaded data.

3 Results

3.1 Microfouling antisettlement bioassay

In total, twelve strains with eight fractions each were tested in the microfouling assay. The bioassays were performed in two batches, the first using the work plate 14 (WP 14) and the second the work plate 16 (WP 16). Results are presented below:

3.1.1 *Cobetia marina*

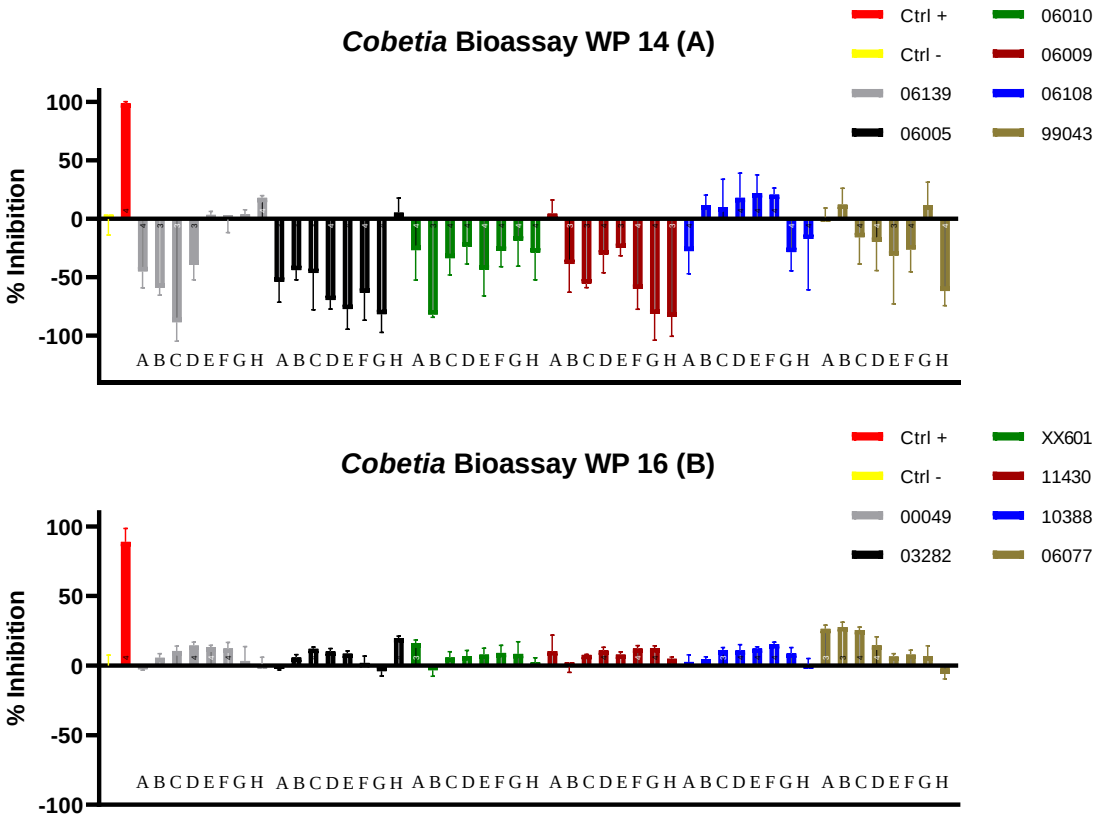


Figure 6. Graphics representing the microfouling inhibition bioassay in *Cobetia marina* CECT 4278 strain. **(A)** represents the WP 14, **(B)** represents WP 16. Each fraction of strain is represented by the letters underneath the columns. **Ctrl +**: 1:100 penicillin-streptomycin-neomycin stabilized solution, **Ctrl -**: 0.1% DMSO.

As seen in **Figure 6**, none of the tested fractions had a satisfactory inhibition in growth of *Cobetia*. Interestingly, many fractions in WP 14 (**Figure 6.A**) presented a stimulatory effect in growth of this strain, although that was beyond the scope of the work.

3.1.2 *Vibrio harveyi*

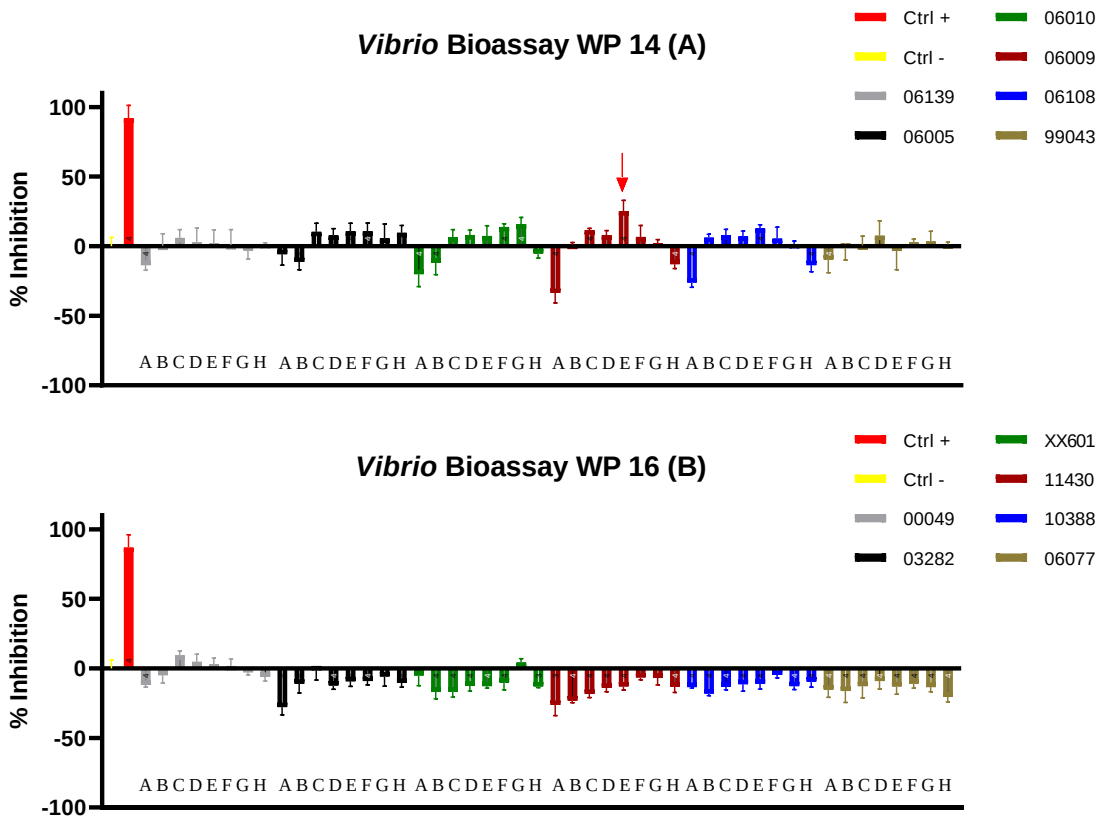
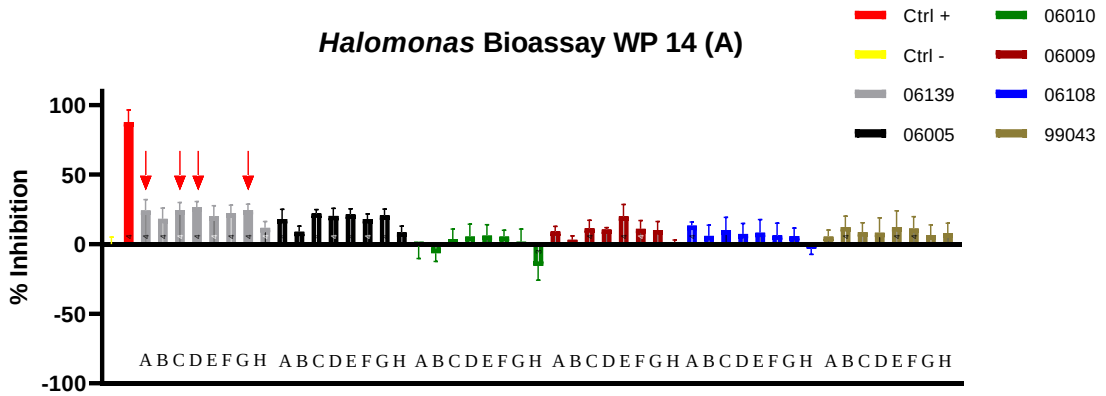


Figure 7. Graphics representing the microfouling inhibition bioassay in *Vibrio harveyi* CECT 525 strain. **(A)** represents the WP 14 **(B)** represents WP 16. Each fraction of strain is represented by the letters underneath the columns. **Ctrl +**: 1:100 penicillin-streptomycin-neomycin stabilized solution. **Ctrl -**: is 0.1% DMSO. Satisfactory results are indicated with a red arrow.

In the *Vibrio harveyi* bioassay, fraction E of the LEGE 06009 strain (**Figure 7.A**) presented the highest potential with 25.2% inhibition in *Vibrio*'s growth.

3.1.3 *Halomonas aquamarina*



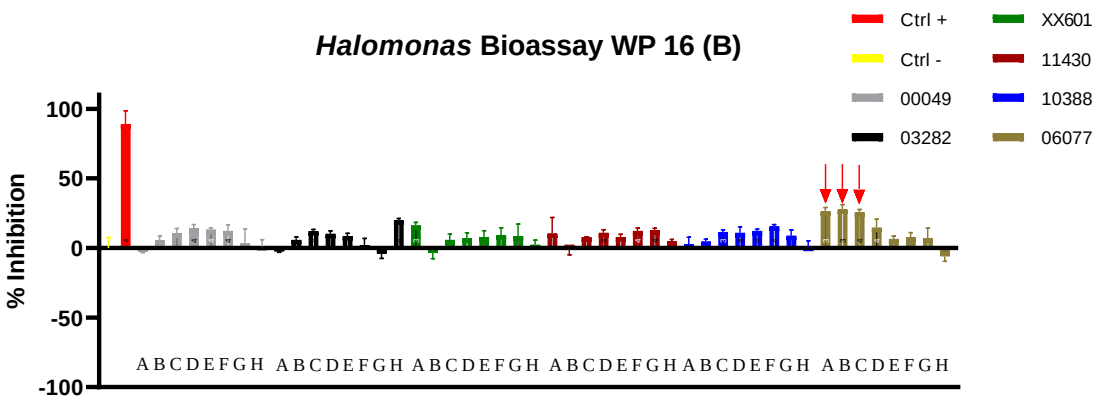
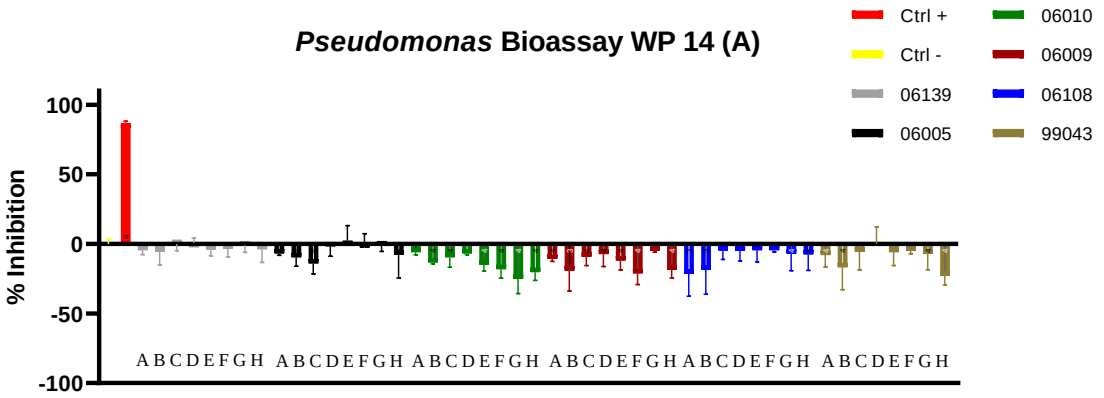


Figure 8. Graphics representing the microfouling inhibition bioassay in *Halomonas aquamarina* CECT 5000 strain. **(A)** represents the WP 14, **(B)** represents WP 16. Each fraction of strain is represented by the letters underneath the columns. **Ctrl +**: 1:100 penicillin-streptomycin-neomycin stabilized solution. **Ctrl -**: 0.1% DMSO. Satisfactory results are indicated with a red arrow.

LEGE 06139 presented a very interesting inhibitory result, with fractions A, C, D and G presenting a 24.21%, 24.54%, 26.36% and 24.42% inhibition (**Figure 8.A**), respectively. Additionally, LEGE 06077 A, B and C fractions also presented a similar result, with 26.42%, 27.73% and 25.63% inhibition, respectively (**Figure 8.B**).

3.1.4 *Pseudoalteromonas atlantica*



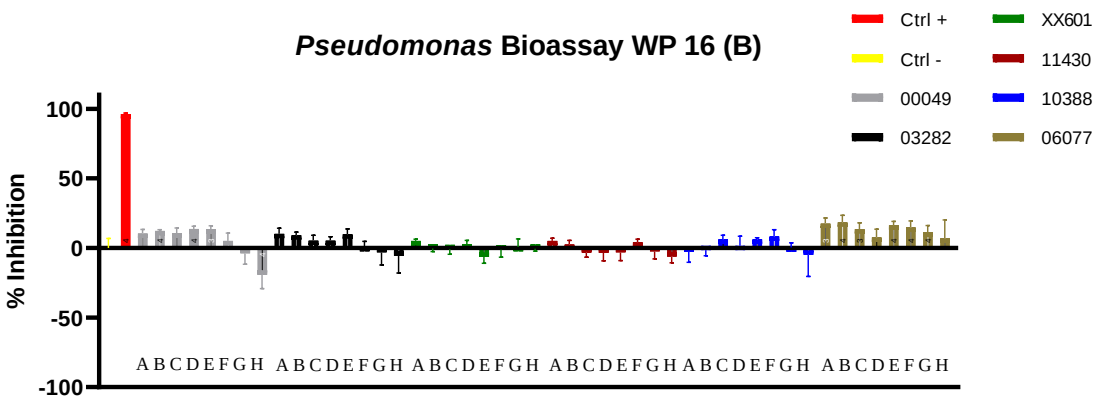


Figure 9. Graphics representing the microfouling inhibition bioassay in *Pseudoalteromonas atlantica* CECT 570 strain. (**A**) represents the WP 14, (**B**) represents WP 16. Each fraction of strain is represented by the letters underneath the columns. **Ctrl +**: 1:100 penicillin-streptomycin-neomycin stabilized solution, **Ctrl -**: 0.1% DMSO.

In the *Pseudomonas* bioassay, no interesting inhibition was observed in any of the fractions tested (**Figure 9**).

3.1.5 *Roseobacter litoralis*

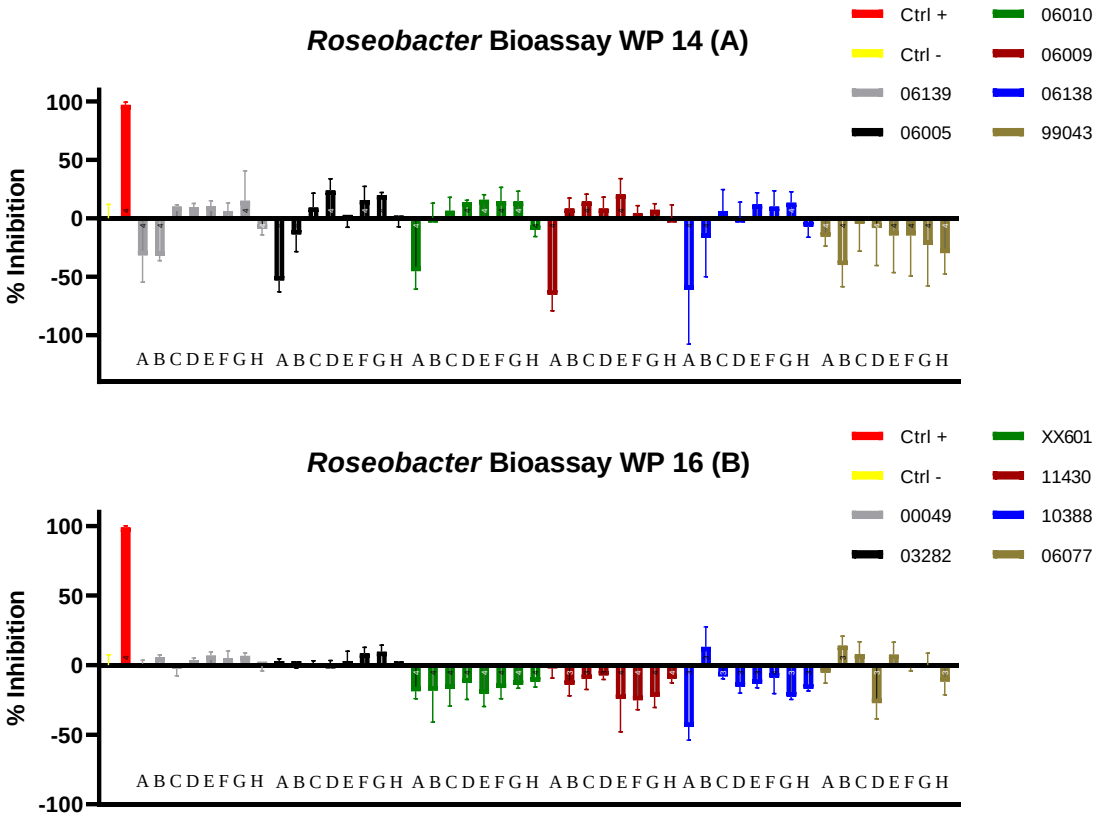
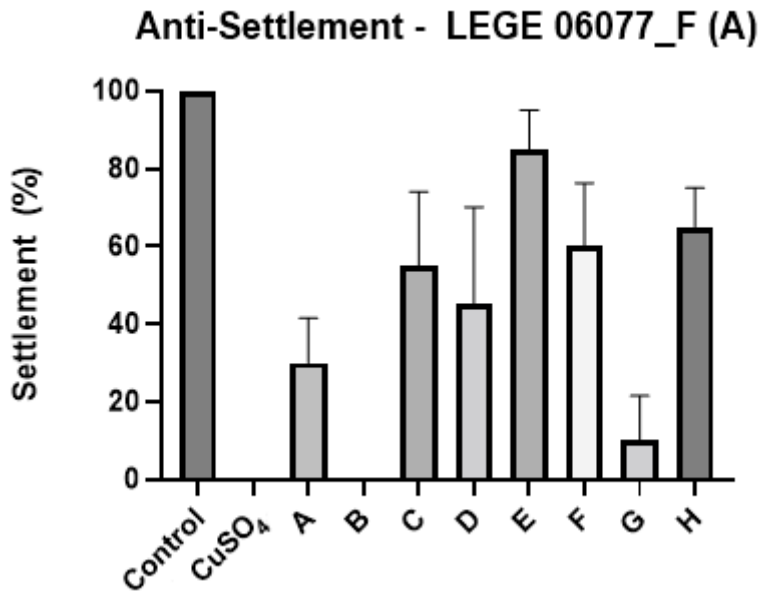


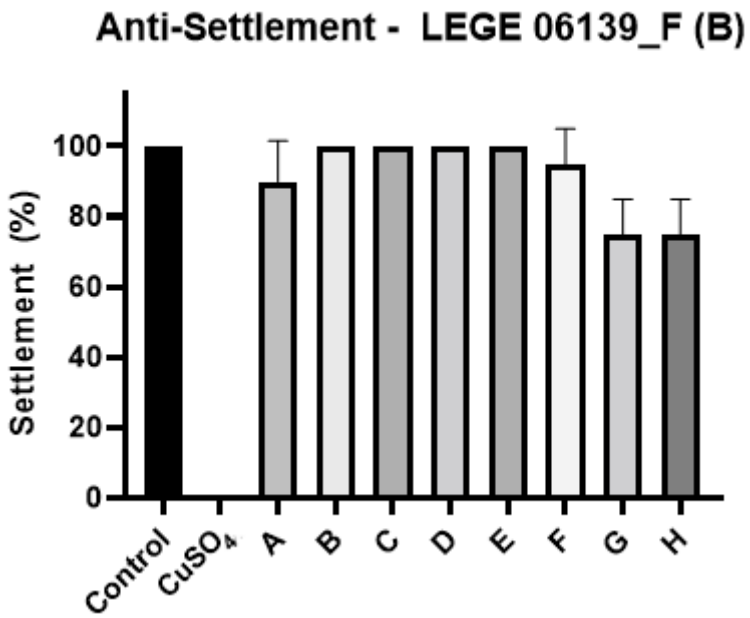
Figure 10. Graphics representing the microfouling inhibition bioassay in *Roseobacter litoralis* CECT 5395 strain. (**A**) represents the WP 14, (**B**) represents WP 16. Each fraction of strain is represented by the letters underneath the columns. **Ctrl +**: 1:100 penicillin-streptomycin-neomycin stabilized solution, **Ctrl -**: 0.1% DMSO.

In the *Roseobacter* bioassay, no interesting inhibition was observed in any of the fractions tested (**Figure 10**).

3.3 Macrofouling antisettlement bioassay

The results for each strain are presented in the graphics below (**Figure 11**):





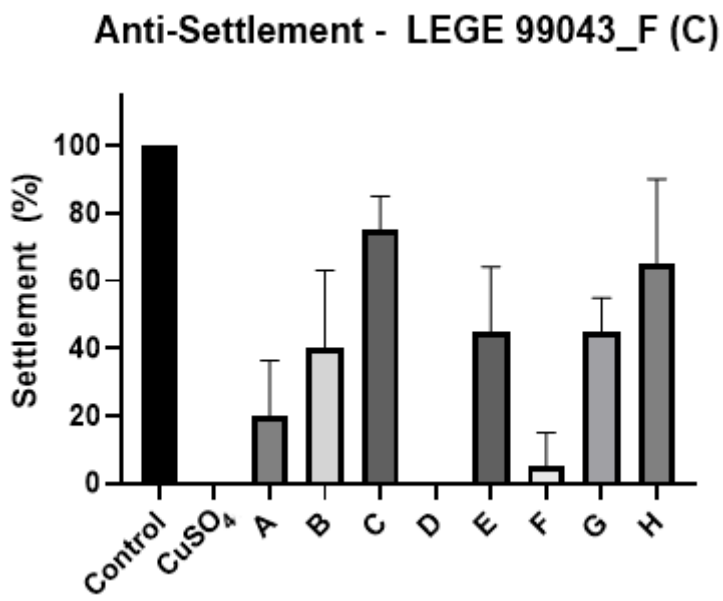


Figure 11. Graphics representing the macrofouling antisettlement bioassay in *M. galloprovincialis*. (**A**) represents LEGE 06077, (**B**) LEGE 06139 and (**C**) LEGE 99043. Each fraction of strain is represented by the letters underneath the columns. 5µM **$CuSO_4$** is a positive control, **Control** is 0.1% DMSO.

As seen in **Figure 11.A**, LEGE 06077 still maintained its antifouling properties that was previously observed by our research group. Fractions B and G displayed a powerful antifouling capability, completely hindering the *M. galloprovincialis* ability to settle when treated with B and reducing to 10% when treated with G.

Mussels treated with LEGE 06139 fractions did not present any interesting change in settlement (**Figure 11.B**). However, LEGE 99043 presented two fractions with satisfying antifouling properties, completely inhibiting settlement in fraction D and nearly inhibiting in fraction F.

3.4 LC-MS analysis and GNPS

Given the positive results obtained in the macrofouling bioassay, the fractions with anti-fouling activity in LEGE 06077 (B and G) and in LEGE 99043 (D and F) and their respective adjacent were taken to LC-MS analysis to propose the possible masses of the active compounds (See Appendix). This was done by comparing the MS spectra of the adjacent fractions with the MS spectra of the active fractions and highlighting peaks that are only present in the positive results.

In the case of LEGE 99043, the mass of the cyanotoxins associated with the species was assessed. Interestingly, none of the cyanotoxins documented to be

produced by *Cylindrospermopsis raciborskii* was observed in the LC-MS spectra, meaning it is possible that the molecule responsible for the antifouling activity is an entirely new compound, although this cannot simply be confirmed by analysing the spectra.

In regards of LEGE 06077, masses compatible with two compounds were documented in a previous work by a CIIMAR research group were identified, anaebaenopeptidin A and D (**Figure 12**). (Lopes et al., 2012).

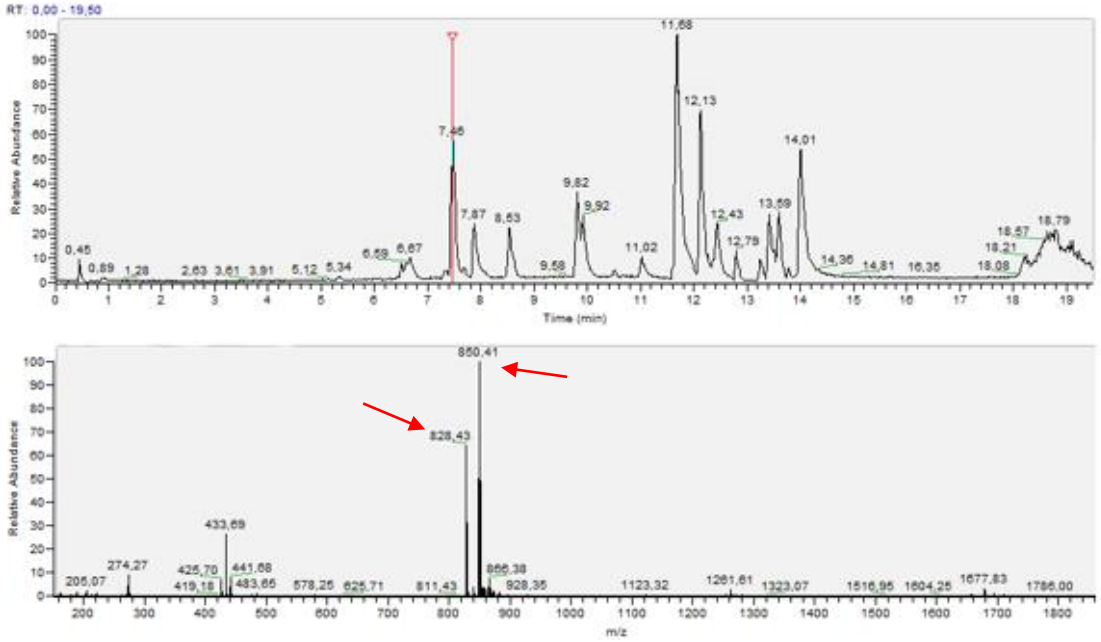


Figure 12. Chromatogram and MS spectra (minute 7.46) of LEGE 06077 B fraction. Red arrows indicate mass corresponding to anabaenopeptidins, [M+Na], [M+H].

Although uploading the LC-MS spectra of the bioactive fractions presented some library hits in the GNPS database, these appear to be misidentifications. For example, Baccatin III, a precursor of the anti-cancer drug paclitaxel, was a positive hit in both LEGE 06077's B fraction and LEGE 99043's D fraction, even though the biosynthesis of this molecule is only naturally observed in the bark of the *Taxus* species (Han et al., 2014).

Another example of a possible misidentification is the presence of acarbose, an anti-diabetic drug detected in both LEGE 06077's G fraction and LEGE 99043's F fraction.

The compounds identified in the GNPS run only seem to have a similar mass with the unidentified molecules in the bioactive fractions, and although it is possible that the molecules identified in the GNPS run may be produced by the strains, it is very unlikely. Still, other methods are required to guarantee any definitive response.

4 Discussion

Biofouling is characterized by the settlement of aquatic organisms in submerged surfaces (Wahl, 1989). Although some less toxic alternatives to TBT-based paints are already on market, new compounds that are less harmful to the environment are still in great demand (Nurioglu et al., 2015). Luckily, it's possible to look for natural products with antifouling effects, such as the ones produced by cyanobacteria, which have already proven to be a great source of antifouling compounds and other secondary metabolites (Dahms et al., 2006; Dobretsov et al., 2013).

In the present work, an assortment of bioassays was performed in order to test the antifouling ability of cyanobacteria's fractioned extracts, be it in micro or macrofouling.

The early steps of settlement by microorganisms are crucial for the formation of a more developed fouled surface (Nurioglu et al., 2015), therefore it would be interesting to find molecules capable of stopping biofouling in its early stages.

Five marine bacteria that are present in the early settlement were tested in the microfouling anti-settlement bioassay. In the *Cobetia marina*, *Roseobacter litoralis* and *Pseudoalteromonas atlantica* bioassays, no significant inhibition was observed in all of the fractions tested. Interestingly, some fractions presented a stimulatory effect in growth, as observed in **Figure 6.A**.

In the *Vibrio harveyi* bioassay, only the E fraction of LEGE 06009 presented activity, with a 25.2% inhibition growth (**Figure 7.A**).

In the *Halomonas aquamarina* bioassay, LEGE 06139 presented fractions with significant results. Indeed, fractions A, C, D and G of this strain had an inhibitory activity on this bacterial strain, with a growth inhibition of 24.21%, 24.54%, 26.36% and 24.42%, respectively (**Figure 8.A**). Additionally, fractions A, B and C of LEGE 06077 also presented an inhibition of growth of 26.42%, 27.73% and 25.63%, respectively (**Figure 8.B**).

Although all of the fractions with positive results were below the threshold considered by our research group as a satisfactory inhibition (30%+), it is probable that with further purification of the fractions the inhibition of said fractions is increased. Additionally, combining the molecules present in the fractions for a multi-compound approach may be a valid strategy in increasing the antifouling effects of these fractions. (Qian et al., 2009)

The macrofoulers are the biggest offender in regards to the problems associated with biofouling, being responsible for the increased drag and the more

problematic aspects of alien species invading a new environment (Abarzua & Jakubowski, 1995).

Due to time restrictions and problems caused by COVID-19 pandemic, only three strains of cyanobacteria were able to be tested. The choice was made to test LEGE 06077 for its previously documented activity by our research group, LEGE 06139 for its results in the *Halomonas* anti-settlement bioassay, and LEGE 99043 for its high potential in producing secondary metabolites, such as cyanotoxins (Antunes et al., 2012; Burford et al., 2016).

Fortunately, two out of the three strains tested had positive results. While LEGE 06139 (**Figure 11.B**) did not present any activity in the macrofouling assay, LEGE 06077 presented two fractions with high potential, inhibiting 100% of settlement in the B fraction, and reducing settlement to 10% in the G fraction (**Figure 11.A**). Additionally, LEGE 99043's D fraction also completely inhibited settlement, while F fraction almost completely inhibited settlement, with only 5% observed (**Figure 11.C**).

The strains with positive results are from two different orders, Synechococcales (LEGE 06139 and LEGE 06009) and Nostocales (LEGE 06077 and LEGE 99043). Nostocales are represented as the second order with most isolated marine cyanobacterial natural products, comprising of 24% of the total isolated molecules, most isolated from unknown species (Tidgewell et al., 2010). Picocyanobacteria in general were largely ignored in the bioprospection due to the difficulty of reaching desired densities and acquiring a satisfactory quantity of biomass in order to isolate compounds from these genera (Costa et al., 2015). This turns the picocyanobacteria genera in to a source of untapped secondary metabolites, making it possible that the activity detected in this work are from new molecules.

LEGE 06139 (*Cyanobium* sp.) presented a very interesting result in the *Halomonas* bioassay, but no significant activity against *M. galloprovincialis*. Although very little information is available about this strain, it was already confirmed in a previous study some activity against the growth of *Nannochloropsis* sp. when treated with its crude extract (Costa et al., 2015). Strains of *Cyanobium sp.* were also found to be able to produce cylindrospermopsin (Tavakoli et al., 2021), although due to the lack of activity in mussel larvae LEGE 06139 doesn't seem to able to produce it, at least not in significant quantities, since *M. galloprovincialis* are disrupted when exposed to cylindrospermopsin (Puerto et al., 2011).

LEGE 06009 (*Nodosilinea* sp.) only presented a single fraction with activity in the *Vibrio* bioassay. It was previously documented PKS genes in this strain, meaning they have biosynthetic gene clusters that could yield in interesting molecules (Brito et al., 2015). Additionally, LEGE 06009's crude extract showed an expressive result

against *Artemia salina*, although it was the dichloromethane extract and not the methanolic extract that was responsible for the activity (Frazão et al., 2010).

Based on the macrofouling anti-settlement bioassay results, it was decided to generate MS spectra of the bioactive fractions and their adjacent spectra (LEGE 06077 A, **B**, C, F, **G**, H; LEGE 99043: C, **D**, E, **F**, G), this way it would be possible to analyse which mass was present in the bioactive and absent in the non-active adjacent fractions, giving some information about the possible molecular mass of the substance responsible for the anti-settlement response.

LEGE 06077 (*Nostoc* sp.) is an heterocystous filamentous cyanobacteria from Nostocales order, and although Nostocales is well represented in regards to isolated secondary metabolites from marine cyanobacteria (Kultschar & Llewellyn, 2018), strains from estuarine regions like LEGE 06077 are still a source of untapped potential when it comes to prospection of new natural products.

Previous works have found in LEGE 06077's MS spectra specific peaks that could be related with known peptides, such as anabaenopeptin A and D, with 844.4 and 828.4 peaks, respectively (Lopes et al., 2012). In this work, analysis of the MS spectra of LEGE 06077's fractions revealed these exact two peaks in fractions A and B, with a peak compatible anabaenopeptin D being way stronger in the bioactive fraction. Although highly speculative, it may be possible that anabaenopeptin D is the molecule responsible for the antifouling activity in the B fraction.

Indeed, anabaenopeptins have a toxic effect towards crustaceans and fish, and are known protease and phosphatase inhibitors (I. S. Huang & Zimba, 2019; Spoof et al., 2016). These effects could probably disrupt *M. galloprovincialis* homeostasis. Additionally, it is known that LEGE 06077 is able to produce BMAA, a neurotoxin that could affect the mussel larvae (Cervantes Cianca et al., 2012).

Although it was possible to draw some conclusions about LEGE 06077's B fraction activity, the compounds that may be responsible for LEGE 06077's G fraction still remains completely unknown, indicating that it may be an undiscovered molecule.

LEGE 99043 is a strain of *Cylindrospermopsis raciborskii*, a cyanobacteria known to produce an impressive library of cyanotoxins and secondary metabolites, such as cylindrospermopsin, saxitoxins, gonyautoxin, and several variations of the aforementioned molecules (Burford et al., 2016; Lagos et al., 1999).

In alignment with the literature, no toxins previously documented to be produced by *Cylindrospermopsis raciborskii* were found to be produced by the European strain LEGE 99043 (Burford et al., 2016) when analysing the MS spectra. Despite this, it is confirmed that Portuguese strains produce a molecule capable of being toxigenic to

mice via intra-peritoneal injection, though it is a yet unknown compound (Saker et al., 2003).

Recent studies with LEGE 99043 also suggested an allelopathic behaviour from this strain, capable of inhibiting growth of the microalgae *Ankistrodesmus falcatus* (Leão et al., 2009), although no molecule is linked to this activity. Interestingly, when grown in a monoculture environment, no activity was found in the microfouling bioassay, indicating that the activity presented by the compound may exclusively affect eukaryotic organisms, and that the production of the molecule responsible for the allelopathic response is only expressed when LEGE 99043 is grown with other species, such as *A. falcatus*.

Apart from LEGE 06077's B fraction, which had activity against *M. galloprovincialis* and *Halomonas marina*, all other positive hits either exclusively inhibits a bacteria strain or the mussel larvae. This is one of the biggest shortcomings about prospecting anti-fouling compounds from marine organisms, more often than not the activity presented by these secondary metabolites are specific to a target (Chen et al., 2021; Qian et al., 2009). As mentioned above, a good strategy in order to overcome this problem would be to use a multi-compound approach, combining effective natural products that can complement each other's activity and, in that way, prevent an assortment of fouling organisms (Chen et al., 2021; Xie et al., 2019).

Additionally, most of the strains studied in prospection of natural products are cultivated as a monoculture under conditions that provide little to no stress to the strain, meaning that without the proper environmental pressure or chemical cues these bacteria have no reason to produce secondary metabolites (Leão et al., 2009; Morone et al., 2019). Indeed, an enormous amount of cryptic gene clusters that are responsible for the synthesis of secondary metabolites have already been identified in cyanobacteria species, but they are silenced for not having the right stimulus to produce the natural product (Dittmann et al., 2015). New strategies must be developed to induce the strains to produce these secondary metabolites

Another known shortcoming of prospection of AF compounds is the bioassays. Biofouling is an extremely complex community, with an enormous diversity of bacteria, diatoms, cyanobacteria, fungi, algae, and other organisms. In the present work and in most works that attempt and succeed to identify new AF molecules, only a handful of micro and macrofoulers are tested, which leads to the problem of finding molecules that only works in specific targets (Chen et al., 2021; Qian et al., 2009). Although quite challenging, new and effective bioassays need to be developed in order to find molecules with broader activities or new strategies to fight biofouling. For instance, approaches that impede the first step of the fouling process would be ideal, since the

first steps of the biofouling are the stepping stones for developing a complex community of foulers (Nurioglu et al., 2015). A great activity that could be further explored to prevent the first steps of fouling is the disruption of the QS, which hamper the ability of the formation of a biofilm and ultimately dismantle the first steps of the fouling process (Chen et al., 2021; Dobretsov et al., 2013).

Although the GNPS molecular network tool present a powerful and interesting tool for researchers, in the present work all the library hits obtained appeared to be false identifications, since most of the hits were either synthetic molecules or molecules isolated from very different organisms. It is possible that the MS spectra of the fractions fed to compare with the GNPS database needed to be fractioned further in order to present less peaks, thus facilitating the identification of the molecules present in the spectra and providing a more reliable library hit with more conclusive results.

Additionally, GNPS is still a very new database (Aron et al., 2012), meaning not a lot of spectra of molecules are available in its database, it is likely that in the future GNPS as a tool will be more reliable in terms of analysing complex MS spectra.

5 Future perspectives

In this work, it was possible to identify several fractions with bioactivity against a plethora of fouling agents. Also, it was possible to propose a probable agent responsible for one of the bioactive hits observed in LEGE 06077, anabaenopeptin D.

Due to time restraints, it was not possible to try to isolate and characterize any compound associated with the positive hits found in this work. However, this work opens ways to find new molecules with already proven activity through the performed bioassays.

Specifically, LEGE 06077 is already being grown in large scale in order to try to isolate and characterize the molecules that may be responsible for the bioactivity. LEGE 06139 has also proven an interesting inhibitor of *Halomonas aquamarina* throughout several fractions, and the identification of the factors responsible for this inhibition is another promising research avenue to be explored.

Also, LEGE 06139 and LEGE 06009 are both Synechococcales strains. This genus is poorly researched due to the bothersome work of the need to grow the strains in order to isolate secondary metabolites. For this reason, it is very likely that these molecules are yet to be discovered molecules, since the prospection of these genera is lacking. The results in this work present a promising new research path in prospecting this genus of cyanobacteria.

Overall, this work presents great opportunities for further prospection of antifouling compounds.

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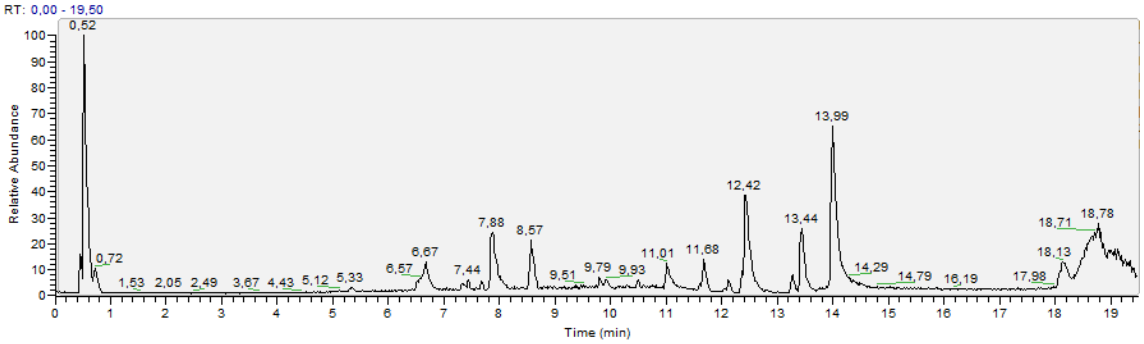
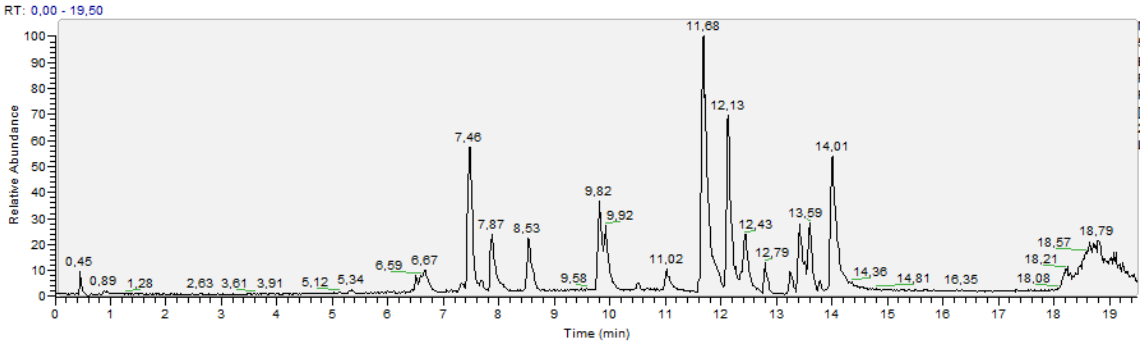
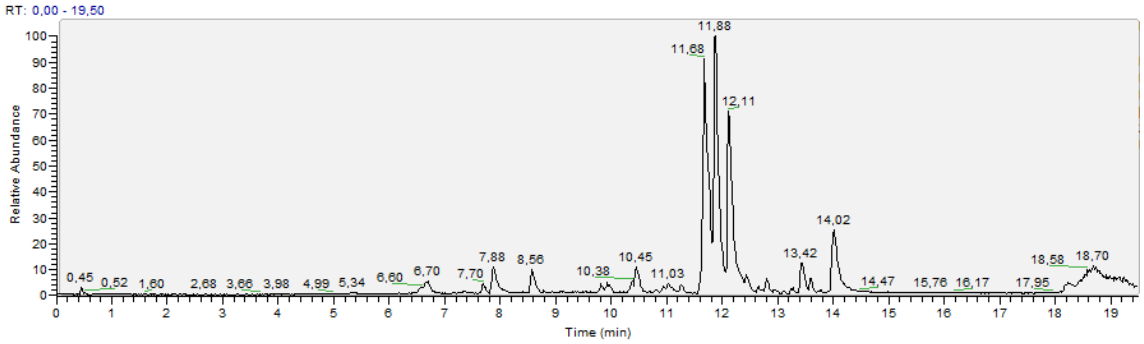
7 Appendix



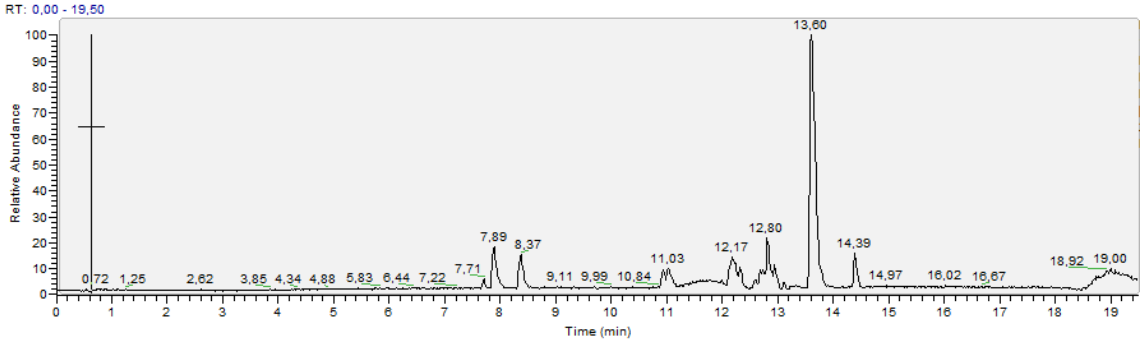
Supplementary figure 1. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction A.



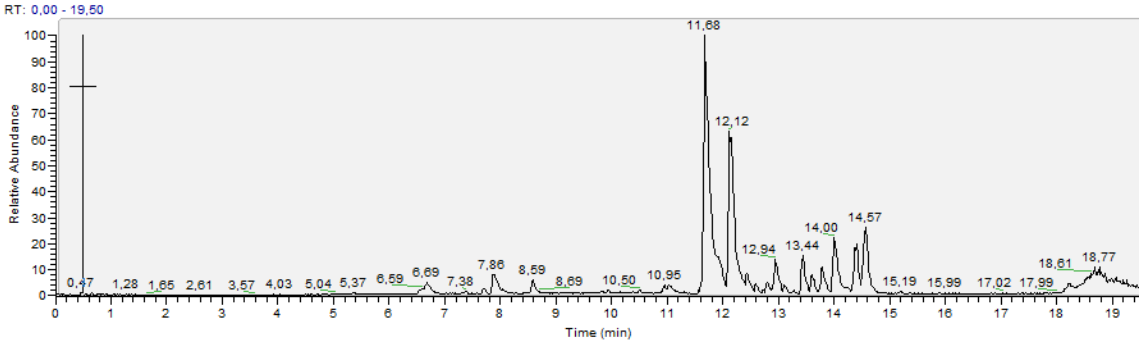
Supplementary figure 2. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction B.



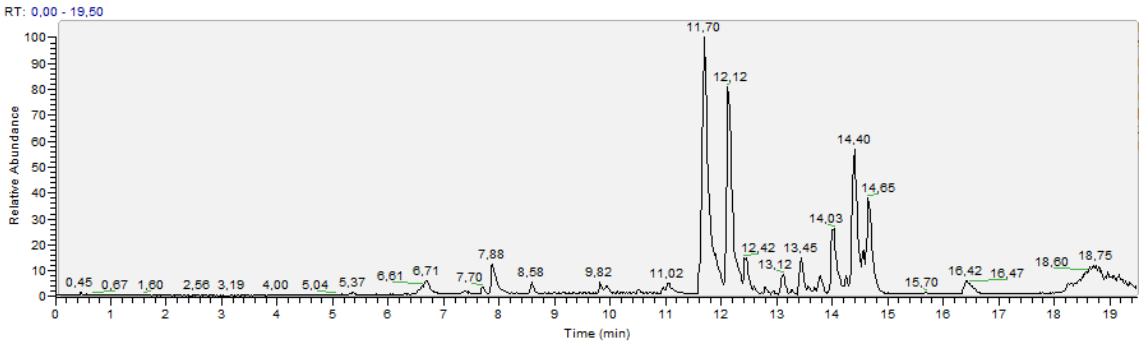
Supplementary figure 3. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction C.



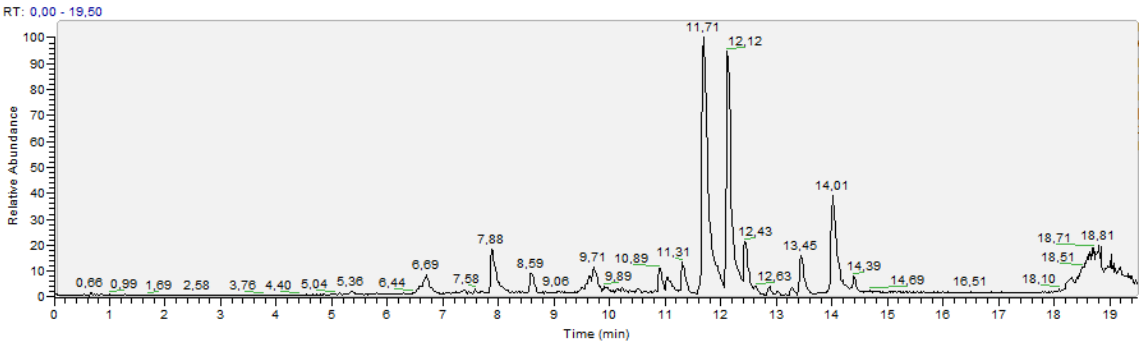
Supplementary figure 4. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction F.



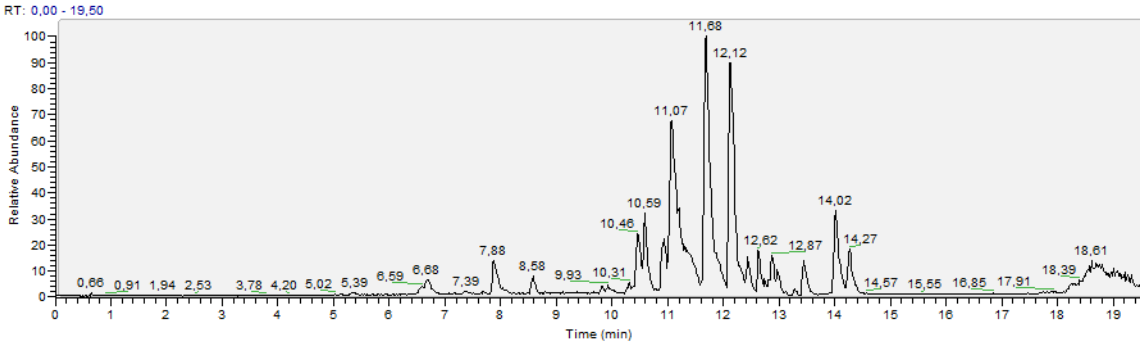
Supplementary figure 5. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction G.



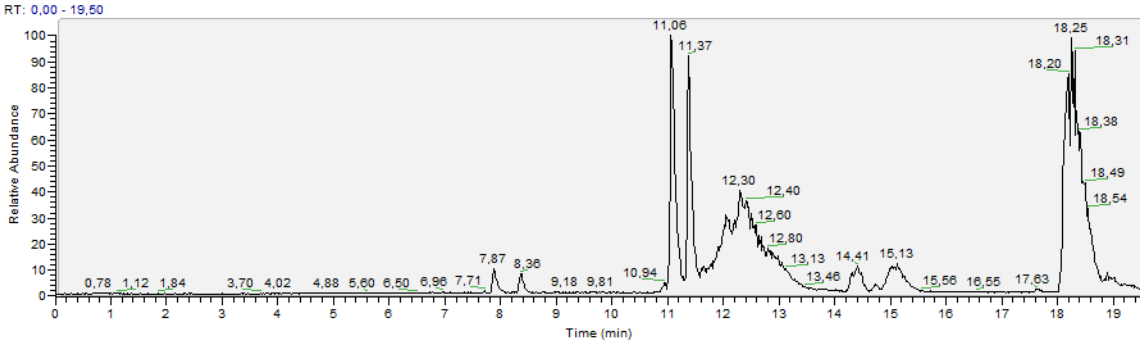
Supplementary figure 6. Base peak [M+] LC-MS chromatogram of LEGE 06077's fraction H.



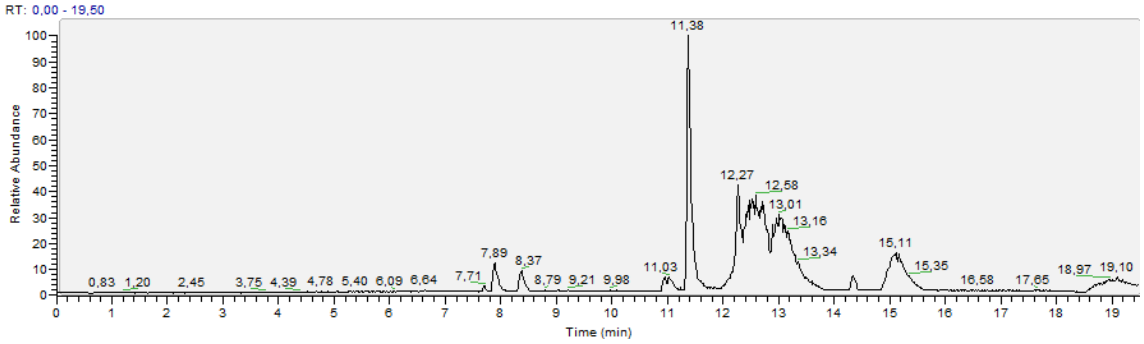
Supplementary figure 7. Base peak [M+] LC-MS chromatogram of LEGE 99043's fraction C.



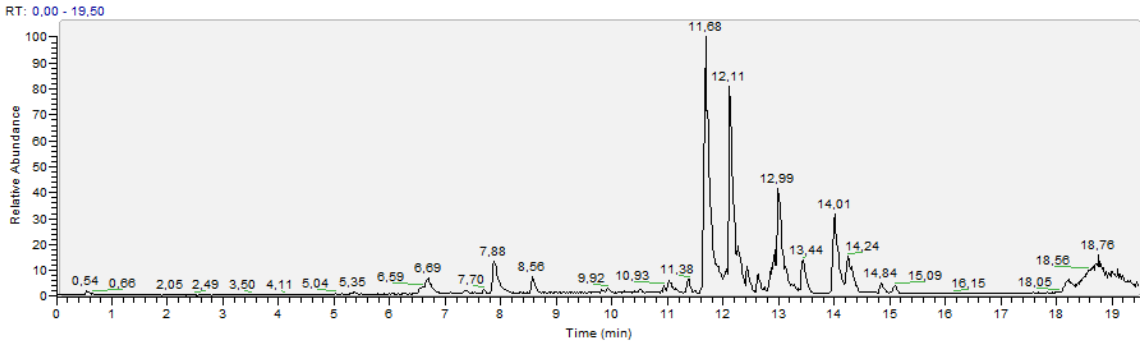
Supplementary figure 8. Base peak [M+] LC-MS chromatogram of LEGE 99043's fraction D.



Supplementary figure 9. Base peak [M+] LC-MS chromatogram of LEGE 99043's fraction E.



Supplementary figure 10. Base peak [M+] LC-MS chromatogram of LEGE 99043's fraction F.



Supplementary figure 11. Base peak [M+] LC-MS chromatogram of LEGE 99043's fraction G.