# Searching for cyanobacterial natural antifouling compounds against *Mytilus galloprovincialis* settlement

Susana Luísa Amaral da Cruz
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**Supervisor**
Prof. Dr. Vitor Vasconcelos, Professor at Faculdade de Ciências da Universidade do Porto and Researcher at CIIMAR – Porto, Portugal

**Co-supervisor**
Dr. Joana Reis Almeida, Post-Doc researcher at CIIMAR – Porto, Portugal

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

AF – Antifouling
AU – Absorbance Units
C (-) – Filtered Seawater Control
C (DMSO) – Filtered Seawater with 0.1% DMSO Control
CEMUP – Centro de Materiais da Universidade do Porto
$CO_2$ – Carbon Dioxide
Cu – Copper
$CuSO_4$ – Copper Sulfate
DCM – Dichloromethane
DMSO – Dimethyl Sulfoxide
$EC_{50}$ – Effective Concentration
EtOAc – Ethyl Acetate
HPLC – High Performance Liquid Chromatography
IMO – International Maritime Organization
LC – Liquid Chromatography
$LC_{50}$ – Lethal Concentration
LC-MS – Liquid Chromatography–Mass Spectrometry
LEGE – Laboratório de Ecotoxicologia, Genómica e Evolução
MeCN – Acetonitrile
MeOH – Methanol
$N_2$ – Nitrogen
NMR – Nuclear Magnetic Resonance
PSII – Photosystem-II
$Sn^{4+}$ – Tin
TBT – Tributyltin
TPT – Triphenyltin
U – Mann-Whitney value
VLC – Vacuum Liquid Chromatography
TLC – Thin Layer Chromatography

# Abstract

In aquatic environments, every natural or artificial underwater surface is subjected to a successional colonization by micro and macroorganisms in a process called biofouling. Biofouling represents one of the current issues in the marine environment due to the biocidal properties of the effective antifouling (AF) agents inducing toxic responses in organisms (*e.g.* tributyltin (TBT), booster biocides) and also due to the strong negative impact on the economical level leading to large investments worldwide estimated at 150 billion USD per year. Thus, there is a growing need of alternative non-toxic and environmentally friendly AF coatings based on natural bioactive compounds.

Cyanobacteria is a group of prokaryotic microorganisms that produce a wide range of secondary metabolites with recognized bioactivity towards a wide range of biological responses and although not so explored in terms of potential AF properties. Regarding this, the aim of this work was to test several strains of cyanobacteria for potential AF properties using anti-settlement bioassays with adult, juvenile and plantigrade larvae of the biofouling mussel *Mytilus galloprovincialis*.

This study was conducted by animal exposure for 15 hours to extracts of different cyanobacterial strains using experimental sets adapted to each developmental stage. Post-exposure settlement was determined by the production/non-production of byssal threads by each test individual, determining the potential of each strain to inhibit mussel adhesion. Promising extracts were then fractioned by a gradient of polarity and new assays were performed testing each obtained fraction. Active fractions continued to be fractioned and tested in order to obtain an AF substance was achieved and characterized.

Results showed some promise cyanobacteria crude extracts and fractions with anti-settlement activity of both juvenile and plantigrade larvae of *Mytilus galloprovincialis.* Despite this fact, results revealed different levels of sensibility from the two development stages as plantigrade displayed lower settlement percentages in almost every bioassay performed. LEGE06077 strain (Nostoc sp.) revealed high levels of thread inhibition throughout the project, from preliminary assays with adult individuals to fraction and sub-fraction bioassays using the two earlier stages. Cultured biomass from LEGE06077 was submitted to three fractioning processes through VLC and HPLC. Final assays showed positive results in plantigrade stage, induced by two sub-fractions that were then characterized by $^{1}$NMR.

On the basis of these results, it can be concluded that this bioassay-guided screening showed effectiveness in the detection of new potential natural AF agents.

Keywords: Biofouling; Antifouling; Natural Compounds; Cyanobacteria; *Mytilus galloprovincialis*

# Resumo

Em ambientes aquáticos, qualquer superfície submersa é sujeita a uma colonização sucessiva por micro e macroorganismos, num processo chamado biofouling. Biofouling representa, atualmente, um sério problema no ambiente marinho devido aos agentes antifouling usados (como tributilestanho (TBT)) que contêm propriedades biocidas e induzem respostas tóxicas em organismos marinhos. Este fenómeno causa também impactos a nível económico muito negativos com perdas de 150 biliões de dólares por ano. Deste modo, há cada vez mais a necessidade de agentes antifouling alternativos não tóxicos, ambientalmente aceitáveis e com base em compostos naturais bioativos.

As cianobactérias são um grupo de microorganismos procarióticos que produzem uma vasta gama de metabolitos secundários já identificados como bioativos e que induzem variadas respostas biológicas. No entanto este grupo continua sem ser explorado ao nível de propriedades anti-incrustantes. Com base nisto, o objetivo deste projeto foi testar várias estirpes de cianobactérias em bioensaios de anti-fixação com o intuito de encontrar substâncias com potenciais características antifouling. Para tal foram utilizados indivíduos adultos, juvenis e larvas plantigrade da espécie *Mytilus galloprovincialis.*

Este estudo foi conduzido por exposição do animal durante 15 horas a um meio enriquecido com diferentes extratos de cianobactéria, utilizando desenhos experimentais adaptados a cada uma das fases de desenvolvimento. Após exposição, a produção/não produção de fiadas de bisso por cada um dos indivíduos determinou a sua fixação e o potencial de inibição de cada estirpe. Extratos promissores foram fracionados por um gradiente de polaridade e novos bioensaios foram realizados com as frações obtidas. Frações ativas foram continuadamente fracionadas até compostos com propriedades antifouling terem sido alcançados e caracterizados.

Os resultados obtidos demonstraram alguns extratos brutos e frações promissores com atividade anti-incrustante em indivíduos juvenis e larvas plantigrade. Apesar deste facto, os resultados revelaram também diferentes níveis de sensibilidade da parte destes dois estados de vida, com as larvas plantigrade a obterem percentagens de fixação mais baixas do que os juvenis em quase todos os bioensaios realizados. LEGE06077 (*Nostoc* sp.) demonstrou níveis elevados de inibição de produção de fiadas ao longo de todo o

projeto, desde os bioensaios preliminares com indivíduos adultos, até aos testes com frações e sub-frações usando os dois estados de vida anteriores. Biomassa cultivada de LEGE06077 foi submetida a três processos de fracionamento, através de cromatografias líquidas por vácuo e cromatografias líquidas de alta performance. Bioensaios finais mostraram resultados positivos no estado larvar, induzidos por duas sub-frações que foram posteriormente caracterizadas por espectroscopia de ressonância magnética nuclear ($^{1}$H).

Com base nestes resultados, pode ser concluído que estes bioensaios se mostraram eficazes na deteção de novos compostos naturais com propriedades antifouling.

Palavras-chave: Biofouling, Antifouling, Compostos Naturais, Cianobactérias; *Mytilus galloprovincialis*

# **1.** Introduction

## 1.1 Biofouling

Biofouling can be described as an undesirable accumulation and fixation of microorganisms, plants or animals on artificial underwater surfaces (Yebra, Kiil et al. 2004). Particularly in aquatic environments, every natural or artificial surface, at least partially underwater is easily and rapidly colonized by micro and macro organisms (Dobretsov, Dahms et al. 2006). This process is generally described as a four-step process that can be initiated within minutes from the material immersion (Rosenhahn, Schilp et al. 2010), (Fig. 1).

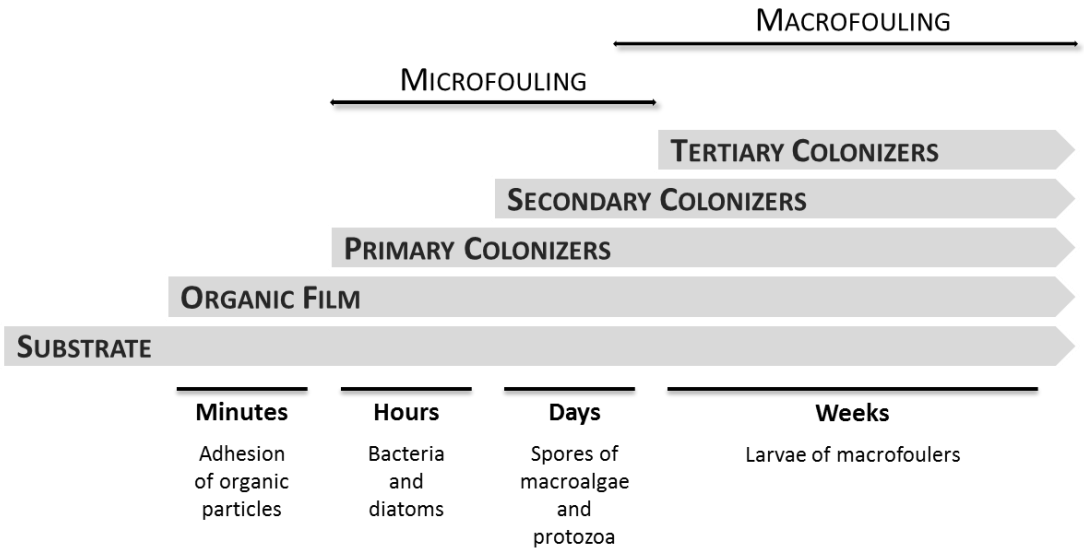


Fig. 1 - Biofouling process. Adapted from (Abarzua and Jakubowski 1995).

The first stage is the prompt adsorption of organic molecules and inorganic compounds to the newly submerged surfaces, creating a conditioning film (Martín-Rodríguez, González-Orive et al. 2014). This modified surface will then be colonized by bacteria and diatoms, forming a complex biofilm that will be essential for further species settlement (Gittens, Smith et al. 2013). The third biofouling step is the arrival of secondary colonizers like protozoa and algae spores that will lead to the fourth stage, the settlement and growth of macrofouling organisms like marine invertebrates and multicellular algae (Harris, Tozzi et al. 2013). This represents a critical survival process for many organisms

given that it is an essential step in their life cycles, normally after an initial planktonic existence (Chambers, Stokes et al. 2006).

The initial biofilm establishment appears to be a fundamental stage functioning as a subtract indicator to the following micro and macrofoulers (Chambers, Stokes et al. 2006, Dobretsov, Dahms et al. 2006). These biofilms are aggregations of microorganisms set in a gelatinous matrix of extracellular polymers which contribute to surface chemical composition modification and also provides nutrient access, predator protection and higher biocide resistance (Yebra, Kiil et al. 2004). The formation of the conditioning layer and subsequent biofilm are key stages that depend on the surrounding environmental conditions (Semiao, Gazzola et al. 2012). This means that the biofilm structure, like its chemical or biotic composition can be very diverse, fact that will also influence the fouling communities that will settle in the following stages (Dobretsov, Dahms et al. 2006).

Although divided into these four steps, biofouling is considered to be a very dynamic and complex process that may be not entirely sequential (Martín-Rodríguez, González-Orive et al. 2014). Environmental characteristics and species interactions are its two major components (Briand 2009).

Despite the biological importance of this process, biofouling brings several inconveniences to maritime industries, where men-made constructions are heavily colonized (Gittens, Smith et al. 2013). The naval industry is particularly susceptible to biofouling that occurs on ship's hulls representing economic losses of millions of dollars every year (Schultz, Bendick et al. 2011). The roughness created by the fouling organisms, including invertebrates, leads to a higher frictional resistance which decreases the ships top speed and range, and that can only be compensated by an increase in fuel consumption (Callow and Callow 2002). In addition to the performance losses and the higher pollution outputs, biofouling accelerates material degradation leaving ships more vulnerable to chemical phenomenon like oxidation and corrosion (Rosenhahn, Schilp et al. 2010). This means that more frequently dry-docking operations are required because of the higher deterioration rate of the hulls (Chambers, Wharton et al. 2014), producing a significant amount of toxic waste (Yebra, Kiil et al. 2004)

Other industries, like aquacultures may also be jeopardized by biofouling as well as activities involving cooling water systems, where the fouling organisms can impair the heating exchanges (Rosenhahn, Schilp et al. 2010). In countries with drinking water shortage, water purification or reverse osmosis membranes can be rapidly degraded by biofouling, causing its frequent replacement and therefor an increase in the final water cost

(Rosenhahn, Schilp et al. 2010).

Biofouling brings not only economic concerns but an important ecological threat as well. The fixation of organisms in ships hulls might contribute to the introduction of new species in different habitats, endangering global biodiversity (Briand 2009).

## 1.2 Antifouling

In order to mitigate these ecological and economic issues, several substances were applied for the prevention of biofouling through history.

Going back 2000 years, ship hulls were covered with lead, whale oils, sulfur and arsenic to prevent the fouling of these organisms. Several recipes were then patented and commercialized and by the 18th century, with the evolution of wooden hulls to metal, the demand for antifouling (AF) substances increased even more (Castro, Westphal et al. 2011).

For this reason, copper and zinc derivatives were implemented in the navy industry. However its short durability led to a continuum research for other substances that could persist longer. So, in the 60's, the first organotin –based AF substance was introduced in the market (Chapman and Guillette 2013).

The AF process is conducted by a progressive release of the biocide paint from ship hull's to the environment which contributes to a large input of these substances in enclosed spaces like docs and harbors (Yebra, Kiil et al. 2004). Suspension particles are especially damaging when concerning non-selective filter-feeders (Parks, Donnier-Marechal et al. 2010).

The tributyltin (TBT) or triphenyltin (TPT) were the main active ingredients of these new paints that rapidly became of common use (Qian, Xu et al. 2010). Its high efficiency and long durability provided a huge economical advantage above the until then used biocides (Castro, Westphal et al. 2011).

It was only in the 80's that the consequences caused by organotin derivatives, like TBT were discovered. The decline and extinction of mollusk populations, in coastal areas was for the first time associated with TBT exposure attributed to a phenomenon called imposex (Abidli, Lahbib et al. 2013). Imposex or pseudohermaphroditism is the superimposition of male characteristics in female individuals (Chapman and Guillette

2013). TBT acts as endocrine disruptor that can increase the testosterone hormone and therefor led to the development on penis and vas deferent in female aquatic gastropods (Santos, Vieira et al. 2004). In extreme cases these new male organs can overgrow the female ones causing sterility (Bettin, Oehlmann et al. 1996).

Tin ($Sn^{4+}$) organic derivatives as TBT are characterized by the presence of covalent bonds between three carbon atoms and a tin atom. Organotin toxicity is correlated with the presence and number of organic groups in the molecule (Sousa, Ikemoto et al. 2009, Sousa, Pastorinho et al. 2014). In aquatic environment, TBT is easily removed from the water column and adsorbed to dissolved particles or sediments because of its high specific gravity, low solubility and high octanol-water partition coefficient ($k_{ow}$) of 4.4 (Antizar-Ladislao 2008). Thus, it is rapidly absorbed by organic material including bacteria, algae and zooplankton from where it accumulates through the food chain. Also, TBT adsorption onto sediments is a reversible process, as these sediments act like a long term source of the compound, that is constantly resuspended it into the water column moved by hydrodynamics (Filipkowska, Kowalewska et al. 2014). TBT's half-life can be up to 5 months in aerobic conditions and reach tens of decades under anaerobic conditions (Santos, Vieira et al. 2004, Sousa, Ikemoto et al. 2009). It is the most known toxic organotin and it affects both target and non-target organisms (Santos, Enes et al. 2009), being responsible for several toxic effects like growth, development and reproduction deficiencies in aquatic species like the bivalve *Mytilus edulis* and *Scrobicularia plana* (Smith, Bolam et al. 2008).

Given the recognized TBT toxic effects, its wide distribution, high hydrophobicity and long persistence (Santos, Enes et al. 2009), TBT-based paints restriction began in the 90's, not showing significant results until September 2008 when the International Maritime Organization (IMO) totally banned the use of organotin compounds (Cho, Lam et al. 2014). The coatings containing TBT had to be removed and traffic was restricted to ships TBT free (Smith, Bolam et al. 2008, Sousa, Laranjeiro et al. 2009, Castro, Westphal et al. 2011). Despite these restrictions, several developing countries continue to use these very effective AF paints, as well as other countries that didn't join the IMO (Cruz, Moreira et al. 2014).

However, prior restriction organotin levels reached up to 500ng/L in European and North American harbors, values that dropped down to a maximum of 100ng/L in water samples, after the TBT's prohibition by the IMO (Antizar-Ladislao 2008).

### 1.2.1 Biocides and Boosters

After the ban of TBT, the demand for new biocides began to grow. Copper-based alternatives have become the most used compounds in AF paints after the TBT prohibitions (Dafforn, Lewis et al. 2011), gaining importance in this market.

Copper (Cu) is an essential metal needed at small levels to allow the proper functioning of all organisms. However it shows high toxicity when presented in high concentrations and therefor it has been used as an AF compound (Guardiola, Cuesta et al. 2010), normally in the form of cuprous oxide in a silicone polymer matrix (Gittens, Smith et al. 2013). Although it has proven to be a more eco-friendly alternative to TBT, copper-based biocides have also shown some environmental consequences, like effects on non-target species and significant concentrations accumulated in coastal areas (Vivien W.W. Bao, Kenneth M.Y. Leung et al. 2011). Copper toxicity to aquatic organisms is also correlated to factors like pH, salinity, among others that influence its speciation, which concerns the metal chemical state. This means that different Cu molecules can present distinct bioavailable forms and so different levels of toxicity (Reeder, Schoonen et al. 2006, Castro, Westphal et al. 2011). The use of copper-based biocides has also a negative impact because of the colonization advantage that is given to the metal tolerant species. This means that invasive species became able to settle and survive in copper polluted areas and native species began to disappear. In conclusion, the toxic tolerance levels are inversely proportional to the biodiversity presented by the polluted region and loss of native species might occur (Dafforn, Lewis et al. 2011). Thus, the entrance of these compounds in trophic chains through aquatic organism can be a seriously problem especially concerning species with high commercial importance and edible for humans (Dafforn, Lewis et al. 2011).

To control the Cu resistance displayed by some biofouling organisms and enlarge its action, most of the biocides paints were supplemented with new substances called “boosters” (Yebra, Kiil et al. 2004, Parks, Donnier-Marechal et al. 2010). These are generally agrochemicals (Omae 2003), and are used to control several species like algae and diatoms that present a higher resistance to copper (Guardiola, Cuesta et al. 2010). Organic boosters (i.e. Irgarol 1051, Diuron and Sea Nine) started to be incorporated into AF paints (Vivien W.W. Bao, Kenneth M.Y. Leung et al. 2011). According to the Biocides Directive (98/8/EC), biocides are active substances or preparations that are intended to destroy, deter, render harmless and exercise control or prevent the action of any other

harmful organism through chemical or biological means. Although the use of booster biocides was meant to be a less toxic alternative to TBT-based paints, some studies show that substances like Irgarol 1051 can be more harmful to several species than the organotins compounds (Balakrishnan, Takeda et al. 2010). Irgarol 1051 and Diuron are the two major booster biocides used worldwide (Gatidou, Thomaidis et al. 2007) and its toxicity levels to the environment have been of great public concerning (Gatidou, Thomaidis et al. 2007).

Irgarol 1051 is a ubiquitous herbicide (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine) that interferes with photosynthetic activity of autotrophs like algae and diatoms. It can act both on fresh and sea water and can rapidly dilute and disperse into the aquatic surroundings becoming bioavailable in larger areas. Significant concentrations can be found either on the water column as well as sediments which make each particle a source of continuum release of the toxic (V.Thomas, McHugh et al. 2002, Yebra, Kiil et al. 2004).

This booster biocide interferes with the transport of captured electrons within chloroplasts of the photosystem-II (PSII) thus inhibiting photosynthesis (Konstantinou and Albanis 2004). This brings severe consequences to marine biodiversity given that primary producers like phytoplankton communities can be seriously affected by even small concentrations like 50 ng/l (Liu, Pacepavicius et al. 1999, Yebra, Kiil et al. 2004, Zamora-Ley, Gardinali et al. 2006). Adding to the factors mentioned above, Irgarol 1051 has a persistence due to its long half-life time (about 200 days) which enhances its toxicity (Zhang, Leung et al. 2008), however might be degraded by slow biodegradation, hydrolysis and sunlight photodegradation. The major and more stable product of this degradation is M1 (2-methylthio-4-tert-butylamino-6-amino-s-triazine) that has been found as well in high concentrations in coastal areas (Gatidou, Thomaidis et al. 2007, Zhang, Leung et al. 2008). Although M1 is also considered a highly toxic compound, some studies show that this by-product is not as dangerous to the environment as Irgarol 1051 (Yebra, Kiil et al. 2004). However, there is not much information yet about M1 and its risks to the environment and further studies should be conducted. The Irgarol 1051 use as already been restricted in several countries but due to its high persistence, dangerous concentrations of this chemical continue to be found in coastal areas (Chambers 2008).

Diuron (1-(3,4-dichlorophenyl)-1,1-dimethylurea) is a substituted urea based herbicide that has been also used as an AF substance (Yebra, Kiil et al. 2004). It is a non-ionic compound with moderate solubility and high resistance to degradation (V.Thomas,

McHugh et al. 2002). Like Irgarol 1051, Diuron interferes with the photosynthesis process and studies show that it has a similar toxicity to the previous booster biocide, also affecting non-target species (Konstantinou and Albanis 2004). Considerable concentrations can be found in the marine environment, however not only from AF usage, given that agricultural activities can also be a great source of Diuron inputs (Dafforn, Lewis et al. 2011). Diuron is considered a hazard substance by the European commission and its use has been restricted in several countries like UK and France (Balakrishnan, Takeda et al. 2010).

4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) is the active compound of Sea Nine ® AF paint (Guardiola, Cuesta et al. 2010). It is a compound from natural origin and has high anti-microbial activity and a similar toxicity to both phytoplankton and zooplankton with effects in a wide range of bacteria, fungi and algae (Castro, Westphal et al. 2011, Dafforn, Lewis et al. 2011). Sea Nine® won the U.S "Green Chemistry Challenge Award'' and it is considered to be the less armful booster biocide (Konstantinou and Albanis 2004).

It has low environmental risk due to its rapid degradation with a half-life time of less than 24h (Dafforn, Lewis et al. 2011). Its degradation products can be up to five times less toxic than DCOIT and its toxicity can rapidly be reduced to non-significant levels (Yebra, Kiil et al. 2004). Furthermore it binds strongly to sediments becoming immobile and less bioavailable (Gatidou, Thomaidis et al. 2007). Despite the environmental advantages shown by DCOIT, some studies demonstrate that it can also affect non-target species like sea urchin (Kobayashi and Okamura 2002) and even be more toxic then Irgarol 1051 and Diuron to some organisms. Because of this, Sea Nine® has been given, in some countries, the same regulatory status as Diuron (Konstantinou and Albanis 2004).

Chorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum fungicide commonly used in agriculture (Guardiola, Cuesta et al. 2010). It can be acutely toxic to some organism like fish and due to its high toxicity at low concentrations and persistency (with a half-life time of four weeks) its use has been banned in some countries (Castro, Westphal et al. 2011, Dafforn, Lewis et al. 2011).

Dichlofluanid (N-dichlorofluoromethylthio-N0-dimethyl-N-phenylsulphamide) is a fungicide considered to be one of the less toxic AF booster biocides (Parks, Donnier-Marechal et al. 2010). It has a high degradation rate, especially by photodegradation, even when bond to sediments (Hamwijk, Schouten et al. 2005, Castro, Westphal et al. 2011). The use of dichlofluanid has become more popular as an AF substance since the implemented restrictions to the use of Diuron, Irgarol 1051 and Chorothalonil (Parks, Donnier-Marechal et al. 2010). However not many Dichlofluanid and its by-products

information is available so further studies should also be conducted (Parks, Donnier-Marechal et al. 2010).

The most important metal-based booster biocides are the salts of pyrithione, mainly zinc and copper (Yebra, Kiil et al. 2004). They have algaecide, fungicide and bactericide effects and are quite toxic to aquatic plants and animals (Guardiola, Cuesta et al. 2010). Despite its toxicity, pyrithiones have rapid degradation rates, especially to sunlight which results in less toxic compounds. It has low water solubility and low accumulation on sediments due to its easy reduction under anaerobic conditions. However, polluted or deep water areas with no UV light penetration may present higher pyrithiones concentrations (Yebra, Kiil et al. 2004).

### 1.2.2 Natural Antifouling

With all these ecological consequences and biocide restrictions, new and more environmentally friendly AF compounds are needed (Qian, Xu et al. 2010). One of the approaches currently in study is by using biomimetics, replicating mechanisms already used by nature (Gittens, Smith et al. 2013). In fact, several marine animals have developed the capacity to maintain its body surface free from biofouling. This colonization by other organisms (epibiosis) lead to respiration problems, drag increasing, nutrient absorption among others (Yebra, Kiil et al. 2004). Two different inhibition mechanisms are described: passive and active inhibition (Gademann 2007). Sharks, rays and other species of Elasmobranchii, have their skin surrounded by placoid scales that inhibit the fixation of fouling organism (Magin, Cooper et al. 2010). Its body surface it's naturally modified to prevent colonization and therefore to passively act against biofouling. Sessile organisms like sponges, cnidarian and algae can control biofouling through secondary metabolites that act enzymatically as chemical defenses (Limna Mol, Raveendran et al. 2009, Chapman, Hellio et al. 2014). This ability to prevent settlement and fixation by other organisms is now being explored as AF technology (Fusetani 2003, Soliman, Mohamed et al. 2014) for the substitution of the TBT-based AF or booster biocides that were used in the last decades (Soliman, Mohamed et al. 2014). Many marine compounds with bioactivity have been identified and isolated in the last decades from marine bacteria, algae, sponges and others. (Tan, Goh et al. 2010, Xua, Hea et al. 2010).

This bioactivity might be exerted by different mechanisms of action in the target biofouling species that are being explored. The capacity of inactivate surface key proteins and peptides in the adhesion process (Gittens, Smith et al. 2013); the multicellular control exhibited by some marine bacteria interfering with the thickness of biofilms therefore preventing biofouling in its first stages; spore germination of some macroalgae and the important bacteria regulatory system, quorum sensing, can also be prevented by biogenics produced by other organism (Bhadury and Wright 2004, Dobretsov, Teplitski et al. 2011); protection from fouling of host's soft body by epibiotic bacteria living in association (Dobretsov, Dahms et al. 2006); among others.

Despite the proved efficacy of natural products as AF substituents, the large amount of organisms involved in the biofouling process acts as an obstacle to natural AF, given that secondary metabolites usually inhibits only a restricted group of fouler organisms (Camps, Briand et al. 2011). As so, synergies between different compounds should be studied to better understand and replicate the natural defenses present in marine ecosystems (Chambers, Stokes et al. 2006).

Also, despite its natural presence in the environment, some marine compounds proved to be highly toxic, as happens with some natural-based booster biocides (e.g. Sea nine@) (Callow and Callow 2002). Therefore before approval, any natural AF has to be intensely studied and its properties established. Its stability, solubility, $LC_{50}/EC_{50}$ ratio and degradation speed should be all well determined before its entrance in the market and also at least its main mechanism of action in one or more target species (Qian, Xu et al. 2010).

These natural AF compounds would have to be incorporated in a paint matrix and a progressive releasing process should also be created (Chambers, Wharton et al. 2014). The biological material supply appears to be another issue in the progress of natural AF paints, given that some species are hard to cultivate in large amounts in short periods of time (Dobretsov, Dahms et al. 2006, Fusetani 2011).

All these requirements bring economic issues to this type of research (Dafforn, Lewis et al. 2011), and the costs of this technology should also be sustainable (Yebra, Kiil et al. 2004).

Microorganisms are known to produce several active compounds and new applications are constantly being discovered (Gademann 2007), pointing them as a sustainable and easily cultivable source of compounds with biotechnological and pharmaceutical interest (Costa, Garcia et al. 2014).

In this context, organisms like cyanobacteria that can be cultivated in high scales (Xua, Hea et al. 2010) and that produce a wide range of secondary metabolites with recognized bioactivity towards a wide range of biological responses, can represent one practical solution for the demand of new green AF products (Gademann 2007).

## 1.3 Cyanobacteria

Cyanobacteria are a vast group of gram-negative and photoautotroph bacteria that can be found in almost every habitat. From terrestrial habitats, to hot spring waters and artic environments, these prokaryotic organisms can survive and dominate (Lopes, Fernández et al. 2010, Lopes, Ramos et al. 2012). Once called blue-green algae due to a blue photosynthetic pigment, phycocyanin, that combined with the existing chlorophyll *a* returns a blue and green color (Madigan, Stahl et al. 2010), it is assumed that they were the first oxygenic photosynthetic organism in our planet, being responsible by the formation of the present atmosphere (Madigan, Stahl et al. 2010). In our days, cyanobacteria are responsible, on a global scale for a significant percentage of fixed $CO_2$ and $N_2$ (Shih, Wu et al. 2013). These primary producers have a photosynthetic system similar to eukaryote organisms with the presence of chlorophyll *a* and photosystems I and II (Willey, Sherwood et al. 2008). Although the majority of this group presents as photoautotrophs, some cyanobacteria can grow in dark conditions by using sugar like glucose as energy source (Madigan, Stahl et al. 2010).

As an estimated 3 billion years old group, cyanobacteria must have endured a high evolution and adaptation process throughout time and this is the cause for their great biodiversity (Leão, Ramos et al. 2013).

This group's classification has been very controversial (Oren 2011). Cyanobacteria were first considered blue-green algae under the Botanical Code, based on their morphologically characteristics (Palinska, Thomasius et al. 2006). However, these phenotypical characteristics are susceptible to be changed by environmental factors and data collected from cyanobacterial cultures can be distinct from organisms growing in nature because culture conditions can have strong influence over their morphological features (Zapomělová 2006, Komárek 2011).

Recently, biochemical, genetic and physiological analyses have proven cyanobacteria to be prokaryotic organisms, revealing the presence of a peptidoglycan cell wall, a typical feature from gram-negative Eubacteria (Vincent 2009). This forced the classification system to be reconsidered and so cyanobacteria began to be characterized under the Bacterial Code (Komárek 2006). The two classification methods are not easily combined and both molecular and morphological features should be taken in consideration when classifying cyanobacteria (Komárek and Mares 2012). Table I shows a botanical taxonomic classification in which cyanobacteria are distributed amongst five different groups that correspond to sub-sections of Phylum BX, which corresponds to cyanobacteria in the bacteriological scheme (Castenholz, Wilmotte et al. 2001, Vincent 2009).

Table I - Cyanobacteria classification (Vincent 2009)

| Order | Characteristics | Genera |
|---|---|---|
| Chroococcales | Coccoid cells that reproduce by binary fission or budding | *Aphanocapsa, Aphanothece, Gloeocapsa, Merismopedia, Microcystis,Synechococcus, Synechocystis* |
| Pleurocapsales | Coccoid cells, aggregates or pseudo-filaments that reproduce by baeocytes | *Chroococcidiopsis, Pleurocapsa* |
| Oscillatoriales | Uniseriate filaments, without heterocysts or akinetes | *Lyngbya, Leptolyngbya, Microcoleus, Oscillatoria, Phormidium, Planktothrix* |
| Nostocales | Filamentous cyanobacteria that divide in only one plane, with heterocysts; false branching in genera such as *Scytonema* | *Anabaena, Aphanizomenon, Calothrix, Cylindrospermopsis, Nostoc* |
| Stigonematales | Division in more than one plane; true branching and multiseriate forms; heterocysts | *Mastigocladus (Fischerella), Stigonema* |

Cyanobacteria from different genera may vary in size from 1 to 10µm and can be unicellular, form filaments or colonies (Gupta, Ratha et al. 2013). Several symbiotic relations with organisms like fungus and lichen have also been described (Willey, Sherwood et al. 2008).

Ecologically, cyanobacteria can be distribute over three groups: mat-forming, bloom-forming and picocyanobacteria (Vincent 2009).

Mat-forming cyanobacteria, like *Chroococcus* and *Leptolyngbya* create biofilms over rocks, sediments or plants that can reach up to several centimeters in thickness (Madigan, Stahl et al. 2010).

Bloom-forming, i.e. *Anabaena* and *Microcystis* occur mostly in eutrophic waters and represent a serious human health concern (Bláha, Babica et al. 2009). Under certain conditions, like proper temperatures, hydrodynamics and light conditions, these cyanobacteria begin to multiply and accumulate rapidly which can lead to the formation of blooms (Wang, Huang et al. 2010). These cyanobacteria blooms represent a serious threat to aquatic ecosystems and to any animal capable of drinking such waters. Recreation activities or fishing in cyanobacteria contaminated areas are two major concerns to human health (Vasconcelos, Morais et al. 2011). Although blooms occur mostly in freshwaters, estuary and marine environments can also be affected (Ismael 2012).

Picocyanobacteria, such as *Synechococcus* and *Prochlorococcus*, are believed to be the most abundant oxygenic photosynthetic organism on earth and have less than 2 µm of diameter (Willey, Sherwood et al. 2008). These cyanobacteria simplicity and small size provides them with a significant advantage in light capture and in low nutrient conditions (Vincent 2009).

Cyanobacteria present adaptable mechanisms through specialized cells like heterocytes. These structures are typical of filamentous species and are responsible for nitrogen fixation (Gupta, Ratha et al. 2013). Some species also produce resting spores called akinetes that are used for survival under stress conditions. Small motile fragments of filamentous cyanobacteria, called hormogonia are used for asexual reproduction and dispersal (Willey, Sherwood et al. 2008). These photosynthetic bacteria are also capable of synthesize UV protection compounds and can resist extreme light and nutrient conditions, being considered water quality indicators (Leão, Ramos et al. 2013).

Cyanobacteria can colonize and compete in almost every habitat known (Dvornyk and Nevo 2003) and for that reason they also had to develop several defense mechanisms

towards a wide range of organisms (Burja, Banaigs et al. 2001). These defenses include opposing herbivory, prevent plant growth, among others (Yadav, Sinha et al. 2011). For this purpose a high amount of secondary metabolites showing numerous modes of action such as photosynthesis inhibition, cellular paralysis or oxidative stress among others can be synthetized by these organisms (Gupta, Ratha et al. 2013). Studies show that species with a higher growth rate produce less chemical defenses than the ones with slower rates which can be explained by the greater and more extended need of protection from the last (Burja, Banaigs et al. 2001). These metabolites are also toxic to humans making cyanobacteria a human health problem (Martins, Fernandez et al. 2007). These cyanotoxins can include hepatotoxins, neurotoxins, cytotoxins and dermatotoxins among others (Yadav, Sinha et al. 2011). However, despite the potential danger that these compounds represent, several new properties have been discovered in the last years that made cyanobacteria become a valuable source of these unique bioactive natural products (Jones, Gu et al. 2009, Brito, Ramos et al. 2012). These metabolites can be aminoacids, lipopeptides, fatty acids, among others (Lopes, Fernández et al. 2010). Anti-viral activity, as well as anti-algae, anti-bacterial, fungicide and anti-tumor properties have been demonstrated by these cyanobacterial-derived compounds (Dahms, Ying et al. 2006).

Given that cyanobacteria appears to be a polyphyletic group with high diversity (Leão, Ramos et al. 2013), and that even different strains of the same species can produce different compounds, the biochemical potential of these photosynthetic bacteria is yet to be totally exploited (Zapomělová 2006). Adding to its great biodiversity cyanobacteria shows relative easy culture conditions (Dahms, Ying et al. 2006), appealing to the continuum research of these bioactive compound sources.

Recent studies have demonstrated that some cyanobacteria strains can prevent micro and macrofouling formation (Gademann 2007, Xua, Hea et al. 2010). The cyanobacteria *Lyngbya majuscula* has already been tested as an AF agent, showing positive outcomes (Tan, Goh et al. 2010). Further studies on this topic might show more promising results from other species given that cyanobacteria has already proven to have antibacterial activity, therefore preventing the early stages of biofouling process. This could lead to a possible replacement of the currently used booster biocides and other toxic substances like TBT, by a new and ecofriendly compound (Volk and Furkert 2006). Nevertheless, studies concerning AF properties against aggressive macrofoulers like mussels of the genus *Mytilus* are underexploited and ought to be conducted.

## 1.4 *Mytilus galloprovincialis* (Lamarck 1819)

The genus *Mytilus* represents marine bivalve mollusks, generically called mussels, that are widely distributed in intertidal and subtidal areas that play an important role in marine food webs and are also used for human consumption (Ciocan 2002, Osswald, S. Rellán et al. 2008). There are three species in the genus *Mytilus*: *M. edulis* L. 1758 (blue mussel), *M. galloprovincialis* Lamarck 1819 (Mediterranean mussel) and *M. trossulus* Gould 1850 (Baltic mussel) (Beaumont, Gjedrem et al. 2006). *M. edulis* and *M. galloprovincialis* are the most similar and their distribution areas frequently overlap which leads to hybridization resulting in fertile individuals (Bierne, David et al. 2002). Due to their easy culture conditions and appealing taste, mussels became very explored by aquaculture industries being considered today one of the most farmed aquaculture species (Wonham 2004), with approximately 1 800 000 tones produced in 2012 and valued at more than $2 billion USD (FAO 2013). Mussels beds can have up to 2400 individuals per $m^2$ distributed in multiples layers and provide habitat for several other species (O'Connor, Crowe et al. 2006). Studies show that they have a high ecological plasticity (Sukhotin and Portner 1999) and adaptability degree (Bhaby, Belhsen et al. 2014). *Mytilus* communities are constantly submitted to a wide range of variables like temperature, air and wave exposure (Steffani and Branch 2003). This variability tolerance combined with the species fast growth and reproduction rate provided it with great invasive capacity which led to its inclusion in the World's Worst 100 Invasive Alien Species (GISD 2012). *Mytilus* poses a severe threat worldwide not only because of its invasive characteristics but also due to its fouling potential (Briand 2009). With the constant increasing of world trades, many coastal areas have been subjected to the introduction of alien species by ship's hulls (Yebra, Kiil et al. 2004). *M. galloprovincialis,* in particular, has been very successful at establishing itself in new habitats and becoming a competitor with many indigenous species (Bownes and McQuaid 2006).

Mussels are sedentary filter feeders with 4 to 5 cm long, usually dark blue or brown (Aral 1999, GISD 2012). A 5cm mussel can filter about 5 litre per hour of water (FAO 2010). They accumulate and concentrate filtered food and for that reason are used as bioindicators (Osswald, S. Rellán et al. 2008). Both valves are identical in shape and size and become firmly closed when the animal is exposed to air by a posterior adductor

muscle (Beaumont, Gjedrem et al. 2006). They attached themselves to the hard substratum by producing about 50 to 100 threads in adults each with 3 to 4 cm, that combined form the byssus (Waite, Andersen et al. 2005). The threads emerge from the ventral gap, being secreted by glands in the mussel's foot and take less than five minutes to be formed (Dalsin and Messersmith 2005). The fixation of each thread to the substratum is made by an adhesion plaque that usually has 1 to 2 mm of diameter (Waite, Andersen et al. 2005). Multiple threads are constantly being produced when the individual is already settled, to enhance the byssus stability and to replace any broken or damaged one (Steffani and Branch 2003). The organism's settlement represents a fundamental key in the organism survival (O'Connor, Crowe et al. 2006), because it allows them to remain attached to the surface while filtering the surrounding water for phytoplankton and other organic matter (FAO 2010). They can detach and reattach in different locations by using the foot, a mobile organ, to scan the substratum and by producing new threads to form a new byssus (FAO 2010). If a direct connection was made amongst the mollusk and the hard substratum, the stiffness disparity between the two would cause the softer one to deform. This way the byssus acts like a common interface between the solid surface and the mussel's soft body preventing the deformation of the last (Waite, Andersen et al. 2005).

*Mytilus species* reproduce by external fertilization (Oyarzún, Toro et al. 2014) with both male and female spawning at the same time and millions of gametes being released into the surrounding waters (GISD 2012). This usually occurs when the water temperature rises which can be explained by the presence of higher food concentrations, given that gametogenesis has been proven to require a great amount of effort and food uptake by mussel individuals (Ros, Bressan et al. 1985). Sexual distinction of the animals is possible, however not totally assured, by gonad color analyses. Usually male mussels present white-yellowish gonads while female ones are red-yellowish (Ciocan 2002).

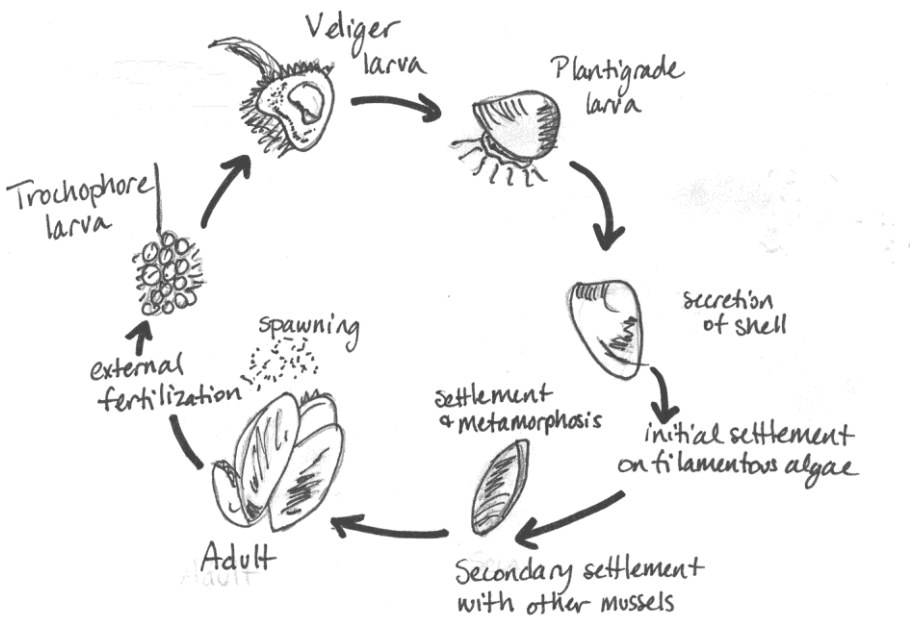


Fig. 2 - Mytilus life cycle, adapted from (Bertness 1999)

After fertilization, mussels life cycle (Fig. 2) start by different pelagic naupliar larval stages and the first settled mussel larvae are called pediveligers or veligers (Bayne 1976) which are selective about its settlement site (Carl, Poole et al. 2011). Settlement is described as the process in which larvae attach permanently to the substratum and after which metamorphosis occur (T. A. Lasiak and Barnard 1995) being a fundamental step for the organism survival (Robinson, Branch et al. 2007). After settlement, pediveligers metamorphose into plantigrades (Bayne 1976) that can reattach in several sites before becoming stablished and evolve as adult mussels (Carl, Poole et al. 2011).

Settlement and metamorphosis are two crucial steps in mussel life cycle and factors like temperature, salinity or predation can have a significant impact on the larvae growth and mortality rates (Chicharo and Chicharo 2000). This is especially relevant given the specie's importance in aquaculture industries (FAO 2010). Another pertinent aspect is the factors responsible for the larval settlement and metamorphosis. Besides substratum texture, local hydrodynamics among other physical factors (Robinson, Branch et al. 2007), studies show that these essential steps can be mediated by natural cues like biofilms (Bao, Satuito et al. 2007) or macroalgae (Yang, Satuito et al. 2007) and also induced by substances like GABA (gamma-aminobutyric acid) and epinephrine (García-Lavandeira, Silva et al. 2005).

## 1.5 Objectives

Considering the global need for effective eco-friendly AF compounds and also the great potential demonstrated by cyanobacteria in producing a wide range of promise secondary metabolites, the main aim of this work was to screen several cyanobacterial strains for their AF potential using a bioassay-guided approach in order to reach a promise AF substance. The AF activity was tested as the success of attachment (byssal threads production/non-production) of two life stages of the biofouling species *Mytilus galloprovincialis*.

In order to pursue this main objective, specific objectives were established:

- ✓ Cyanobacterial strains (LEGE culture collection) culture in large-scale;
- ✓ Production of organic and aqueous cyanobacterial extracts;
- ✓ Performing preliminary bioassays using adult mussels (*Mytilus galloprovincialis*), to validate a suitable AF bioassay.
- ✓ Performing AF bioassays with two earlier life stages of *M. galloprovincialis*, plantigrade larvae and juveniles, using the inhibition of attachment induced by cyanobacterial strains and fractions as endpoint;
- ✓ Fractioning of promising extracts using vacuum liquid chromatography (VLC) and high performance liquid chromatography (HPLC) techniques;
- ✓ Characterizing promising fractions by Nuclear Magnetic Resonance (NMR).

# 2. Material and methods

## 2.1 Cyanobacterial culture and biomass achievement

Cyanobacterial strains from Portuguese Atlantic estuaries, intertidal rocky beaches and freshwater waterways (LEGE culture collection) were cultured in aerated Z8 or $BG11_0$ liquid medium (Appendix 1) under laboratory conditions at 25ºC, light/dark cycle of 14/10 h and light intensity of approximately $25x10^{-6}$ E $m^{-2}$ $s^{-1}$ (Lopes, Ramos et al. 2012) .

The initial material was grown in 100ml Erlenmeyer flasks with 40ml of growth medium, being re-inoculated into larger recipients for up-scaling. Containers of 1L with 500ml, 6L with 4L and 20L with 15L of medium were used (Fig. 3). The material was collected and again inoculated repetitively until the required biomass was achieved (table II).

Fig. 3 - Cyanobacterial Culture Room

All the material used in the inoculation process was previously sterilized by autoclaving, dried in a 60ºC drying chamber and subjected to UV radiation for 20 minutes. The flasks with the Z8 components were sterilized as well before every inoculation. Also, filters with 0,22µm were used in the aeration system to minimize contamination.

When achieved maximum capacity, the biomass was collected by centrifuging (Thermo Fisher Scientific, Sorval Legend RT Centrifuge) at 4600 rpm in 7 minutes cycles or filtered through a planktonic net with 15µm depending on the cyanobacterial strain. Some strains form biofilms or large vesicles that could easily be filtered while others grew dispersed in the medium needing centrifugation.

After collection, the gathered material was stored at -80ºC and freeze-dried.

Table II - Cyanobacterial strains and achieved weights.

| Strain | Code | Growth Medium | Dry Weight (g) |
|---|---|---|---|
| *Phormidium autumnale* | LEGE07200 | Z8 | 4,8548 |
| *Leptolyngbya sp.* | LEGE07080 | Z8 | 4.0951 |
| *Nostoc sp.* | LEGE06077 | Z8 | 11,7174 |
| *Nostoc sp.* | LEGE06077 | $BG11_0$ | 3,0218 |
| *Leptolyngbya sp.* | LEGE06075 | Z8 | 9,3175 |
| *Oscillatoria sp.* | LEGE03272 | Z8 | 2.3419 |

Table III - Previously lyophilized strains from LEGE culture collection

| Strain | Code | Origin | Lyofilized Biomass (g) | Organic Extract (g) | Aqueous Extract (g) |
|---|---|---|---|---|---|
| *Cylindrospermopsis raciborskii* | LEGE99043 | Freshwater | 3 | 0,776 | 0,2414 |
| *Microcystis aeruginosa* | LEGE05195 | Freshwater | 1,7 | 0,2261 | 0,1535 |
| *Phormidium cf.animale* | LEGE6072 | Estuarine | 2,3 | 0,8088 | 0,1385 |
| *Leptolyngbya sp.* | LEGE06070 | Estuarine | 1,66 | 0,7574 | 0,2087 |
| *Microcoleus vaginatus* | LEGE07076 | Estuarine | 1,64 | 0,3831 | 0,0791 |
| *Nostoc sp.* | LEGE06077 | Estuarine | 0,65 | 0,0518 | 0,0543 |
| *Nodularia sp.* | LEGE06071 | Estuarine | 4,2 | 1,06 | 0,3084 |
| *Cyanobium sp.* | LEGE06068 | Estuarine | 4 | 0,681 | 0,8693 |
| *Synechocystis salina* | LEGE06079 | Estuarine | 2 | 0,3481 | 0,2301 |
| *Leptolyngbya sp.* | LEGE07084 | Estuarine | 2 | 0,4607 | 0,1432 |
| *Synechocystis cf. Salina* | LEGE07073 | Estuarine | 2,3 | 0,3171 | 0,718 |
| *Leptolyngbya aff. bijugata* | LEGE07085 | Estuarine | 0,8 | 0,0888 | 0,0977 |
| *Phormidium cf. chalybeum* | LEGE06078 | Estuarine | 2,7 | 0,5762 | 0,1791 |
| *Microcoleus chtonoplastes* | LEGE07092 | Estuarine | 1,88 | 0,4353 | 0,1765 |
| *Leptolyngbya sp.* | LEGE06069 | Estuarine | 2 | 0,1954 | 0,098 |

| | | | | | |
|---|---|---|---|---|---|
| *Leptolyngbya sp.* | LEGE07080 | Estuarine | 1,5 | 0,3858 | 0,1495 |
| *Leptolyngbya sp.* | LEGE07075 | Estuarine | 1,87 | 0,3786 | 0,1116 |
| *Synechocystis cf. Salina* | LEGE06083 | Estuarine | 2,4 | 0,3411 | 0,1031 |
| *Microcystis aeruginosa* | LEGE91094 | Freshwater | 21,67 | 3,486 | - |

As the time-frame for the achievement of sufficient biomass of the selected strains for testing was in some cases incompatible with the period of this thesis, some other strains were used from previously cultured and lyophilized material (table III).

## 2.2. Extracts preparation

### 2.2.1 Organic extraction

The extraction process was conducted according to Leão, Ramos et al. (2013) using the extraction apparatus shown in Fig. 4A. A Büchner funnel, with a Whatman No 1 filter paper and a cheese cloth inside was assembled into a vacuum adapter.

The pre-weighted lyophilized cyanobacteria biomass was placed in a stainless steel beaker and 40ml of a 2:1 mixture of dichloromethane and methanol (DCM:MeOH) were added per gram of biomass (Fig. 4B). The mixture was stirred for 15 minutes to achieve better extraction.

Solvent contents were decanted and filtered under vacuum through the cheese cloth and Whatman No 1 filter paper. The solvent was collected in a 500ml round bottom flask and evaporated under reduced pressure by a rotary evaporator (Rotavapor BÜCHI RE 111; Rotavapor BÜCHI R. 210). The biomass that remained in the cheese cloth was recovered, returned to the beaker and two more extractions were conducted at room temperature. The remaining biomass was then repetitively heat-extracted (<40 °C) with the help of a hotplate, until the extracted liquid became translucent.

After all the solvents evaporated, the dried extract was redissolved in a 1:1 mixture of isooctane:ethanol and transferred to a previously weighted vial. Using a $N_2$- stream the solvent was again evaporated, the crude extract weighed and stored at -20ºC (Fig. 4C).

### 2.2.2 Aqueous extraction

After the organic extraction, 20ml of ultrapure water were added to the remaining biomass and let to extract for 30 minutes. The mixture was then decanted and centrifuged. The collected supernatant precipitate was stored at -20ºC then freeze-dried, weighed and stored again.

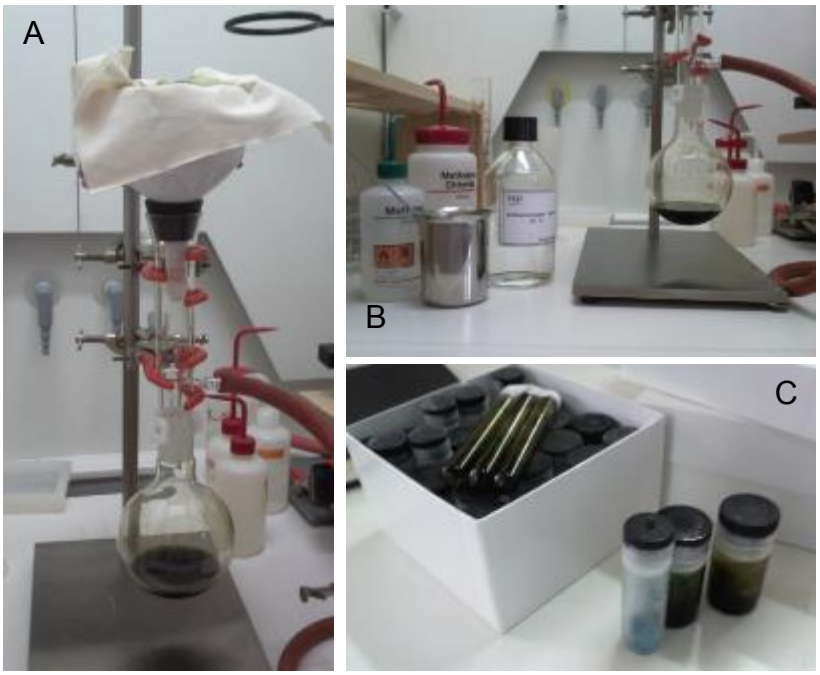


Fig. 4 - Extraction apparatus (A, B) and several organic and aqueous extracts (C).

AF bioassays were performed as describe in 2.4 and strains showing significant differences against the negative controls were fractioned for further testing.

## 2.3 Fractions production

Organic crude extracts were fractioned using a gradient of solvents from the non-polar hexanes (Hex) to 100% ethyl acetate (EtOAc) to 100% methanol (MeOH). A vacuum liquid chromatography (VLC) apparatus (Fig. 5) was assembled.

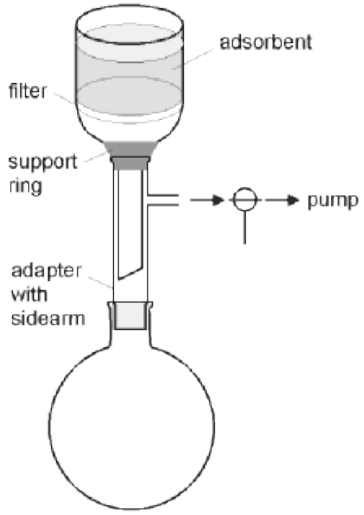


Fig. 5 Vacuum Liquid Chromatography apparatus (L. M. Harwood, C. J. Moody et al. 1999)

A silica gel (SiOH Gel) was added to the filtration funel and packed as better as possible by turning on the vacuum pump, tapping the funnel sides and adding the first mixture of solvents (90% Hex and 10% EtOAc).

Once the silica was properly packed a Whatman No 1 filter paper was placed on top of it.

This fractionation procedure was applied to 5 strains crude extracts, including 3 from previously lyophilized material (LEGE06079, LEGE07084, LEGE91094) and 2 from cultured cyanobacteria (LEGE06071 and LEGE06077). This last two strains (LEGE06077 and LEGE06071) were selected to sub-fractioning after the AF bioassay-based screening showed promise results for AF properties.

## 2.3.1 LEGE06077 sub-fractioning

### 2.3.1.1 Vacuum liquid chromatography (VLC)

Crude extract from LEGE06077 (1,25 g) was resuspended in chloroform, with sonication and transferred to the top of the column with a pausteur pipette. The vacuum was turn on and when the yellow part of the extract became near the bottom of the column, the first fraction started to be collected in a 500ml round bottom flask (Fig. 6). Solvents were sequentially added as shown in table IV. Ten fractions (A-J) were achieved,

evaporated in the rotary evaporator, redissolved in chloroform, dried with a $N_2$-stream and stored -80ºC until further use.

Table IV - Vacuum Liquid Chromatography (VLC) fractionation scheme used in LEGE06077

| Fraction | Solvent mixture | Volume (mL) | Biomass/fraction (mg) |
|---|---|---|---|
| A | 10% EtOAc (hex) | 250 | 68,8 |
| B | 20% EtOAc (hex) | 200 | 6,3 |
| C | 40% EtOAc (hex) | 200 | 11,5 |
| D | 50% EtOAc (hex) | 200 | 104,7 |
| E | 60% EtOAc (hex) | 200 | 75,4 |
| F | 80% EtOAc (hex) | 200 | 17,9 |
| G | EtOAc | 200 | 12,9 |
| H | 25% MeOH (EtOAc) | 250 | 336,1 |
| I | MeOH | 450 | 594,2 |
| J | MeOH | 200 | 190,4 |

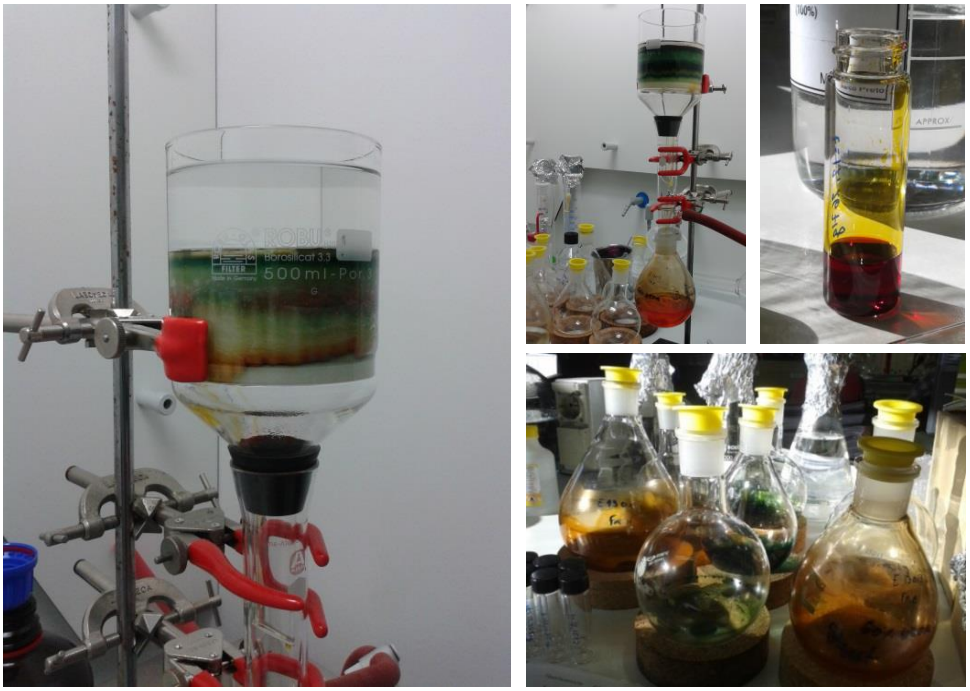

Fig. 6 - Fractioning LEGE06077 through Vacuum Liquid Chromatography (VLC).

All the fractions (A-J) were screened for AF activity and fraction H was selected for further VLC fractioning. LEGE06077/H (330mg) was dissolved in Hex:EtOAc (5:5) and the previous process was repeated with the solvent scheme showed in table V. Seven sub-fractions were obtained (LEGE06077/H/A – LEGE06077/H/G) and new bioassays were conducted pointing for sub-fraction H/F.

Table V - Vacuum Liquid Chromatography (VLC) fractionation scheme used in fraction H of LEGE06077

| Sub-fraction | Solvent mixture | Volume (ml) | Biomass/fraction (mg) |
|---|---|---|---|
| H/A | 50% EtOAc (hex) | 150 | 2,2 |
| H/B | 60% EtOAc (hex) | 100 | 1,3 |
| H/C | 80% EtOAc (hex) | 100 | 1,3 |
| H/D | 90% EtOAc (hex) | 100 | 1,5 |
| H/E | EtOAc | 100 | 2,1 |
| H/F | 20% MeOH (EtOAc) | 100 | 50,4 |
| H/G | MeOH | 100 | 161,4 |

2.3.1.2 High Performance Liquid Chromatography (HPLC)

Thus, the sub-fraction LEGE06077/H/F (50mg) was dissolved in methanol and injected in a high-performance liquid chromatography (HPLC) (Waters 1525 Binary HPLC Pump; Waters 2487 Dual λ Absorbance Detector) with a semi-preparative column (Phenomnex Synergy 10µ Hydro-RP 80A; 250x10.00 mm, 10 micron) to achieve new fractioning. Wavelength was measured at 254nm and 280nm with a flow rate of 3ml/min and 200µl of HF fraction solution were used in each injection. Table VI shows the eluent gradients used, and table VII the recovered fractions. Fractions were collected according to the peaks displayed in Fig. 7 (Empower $^{TM}$).

Table VI - High Performance Liquid Chromatography's (HPLC) eluent gradients for LEGE06077/H/F

| Time (m) | Solvent mixture | Flow Rate (ml/min) |
|---|---|---|
| 0 - 10 | 2% MeCN (water) | 3 |
| 10 -11 | Gradient to10% MeCN (water) | 3 |
| 11 - 15 | 10% MeCN (water) | 3 |
| 15 - 40 | Gradient to 100 %MeCN | 3 |
| 40 - 60 | MeCN | 3 |
| 60 - 65 | Gradient to 2% MeCN (water) | 3 |
| 65 - 90 | 2% MeCN (water) | 3 |

Table VII - Recovered fractions from LEGE06077/H/F

| Sub - Fraction | Time (m) | Biomass/fraction (mg) |
|---|---|---|
| H/F/ A | 2 - 10 | 0,9 |
| H/F/ B | 10 - 18 | 0,7 |
| H/F/ C | 18 - 26 | 1,1 |
| H/F/ D | 26 – 40 | 2,6 |
| H/F/ E | 40 - 60 | 24,7 |
| H/F/ F | 60 – 80 | 6,8 |
| H/F/ G | 80 - 90 | |

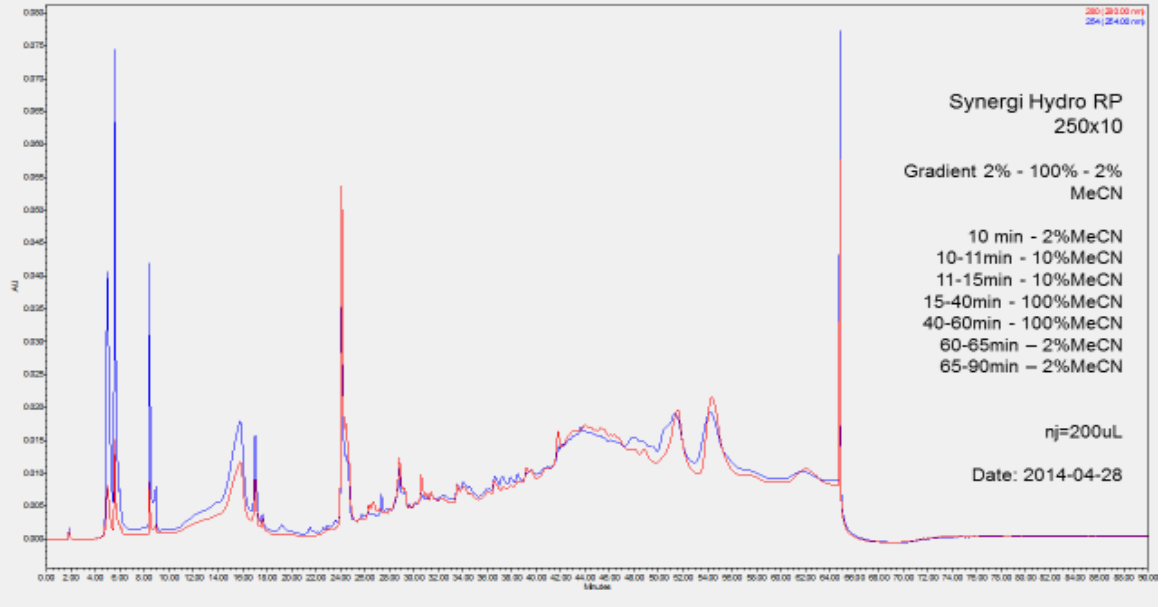


Fig. 7 - LEGE06077/H/F fractioning by High Performance Liquid Chromatography (HPLC). Absorbance units (AU) vs time (Minutes).

Seven sub-fractions were obtained (HFA – HFG) and new bioassays led, selecting two sub-fractions of HFB and HFC). However, as very few biomass was left from the previous chromatography, a proper characterization through $^{13}C$ NMR was not possible, although $^{1}H$ NMR analyses were performed.

To overcome the biomass shortage, another extraction was made with the remaining freeze-dried biomass of this strain and the new crude extract was directly purified by HPLC (Fig. 8). The aim was to recover directly the active AF compounds present in sub-fractions HFB and HFC without the initial VLC fractioning steps. The same semi-preparative column was used, as well as the same HPLC apparatus and eluents as previous. A small amount of crude extract was dissolved in 0,5ml of acetonitrile, centrifuged (Micro Star 17R), and 10µl were injected in the HPLC and run with a slightly different program than before. This preliminary injection was used to confirm the information about the required peek's retention time provided by the previous fractioning of LEGE06077/H/F. This was necessary because the crude extract represented a much more concentrated mixture then the already eluted fractions and the peek's position had to be confirmed. The following injections had higher volumes (50, 100 and 200) and were used to progressively optimize the eluent gradient and also start the purification process. The final program and recovering times are shown in table VIII.

Fig. 8 - High Performance Liquid Chromatography (HPLC) performed in new LEGE06077 crude extract and collected fractions.

Table VIII - High Performance Liquid Chromatography's (HPLC) eluent gradients for new LEGE06077 crude

| Time (m) | Solvent mixture | Flow Rate (ml/min) |
|---|---|---|
| 0 - 10 | 2% MeCN (water) | 3 |
| 10 - 11 | Gradient to 10% MeCN (water) | 3 |
| 11 - 15 | 10% MeCN (water) | 3 |
| 15 - 35 | Gradient to 100% MeCN | 3 |
| 35 - 75 | MeCN | 3 |
| 75 - 82 | Gradient to 2% MeCN (water) | 3 |
| 82 - 90 | 2% MeCN (water) | 3 |

The new recovered fractions (LEGE06077/a-f) were collected in round bottom 500ml flasks and evaporated (table IX).

Fig. 9 shows the obtained peaks used to guide the new fraction's collection.

Table IX - Recovered fractions from new LEGE06077 crude

| Fraction | Time (m) | Biomass/fraction (mg) |
|---|---|---|
| a | 0 - 11 | 2,9 |
| b | 11 - 15 | - |
| c | 15 – 20 | - |
| d | 20 - 23 | - |
| e | 23 - 28 | 0,5 |
| f | 28 - 90 | - |

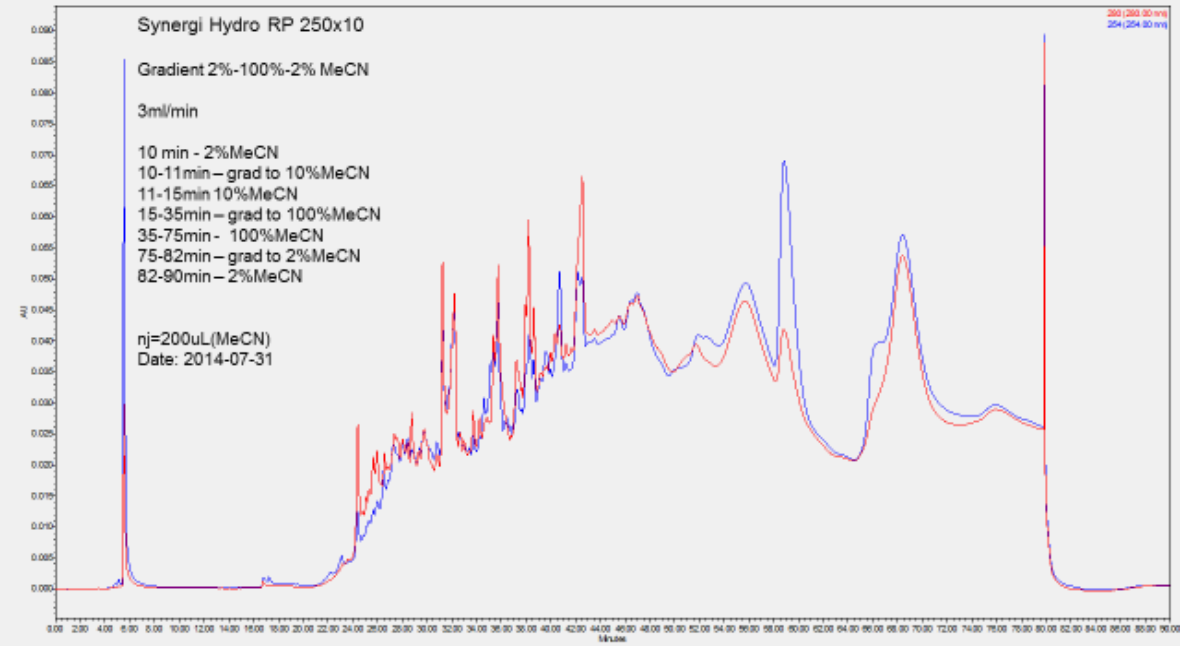


Fig. 9 - LEGE06077 crude extract fractioning by High Performance Liquid Chromatography (HPLC). Absorbance units (AU) vs time (Minutes).

## 2.3.2 LEGE06071 sub-fractioning

### 2.3.2.1 Vacuum liquid chromatography (VLC)

LEGE06071 (2,6g) was fractioned using the scheme presented in table XI. Eight (A – H) fractions were achieved and LEGE06071/A and LEGE06071/F were selected to further fractioning based on AF bioassays. Priority was given to sub-fractioning of LEGE06071/F as complementary tests simultaneously performed based on other biological activities also indicated some potential regarding this sub-fraction.

Table X - Vacuum liquid chromatography (VLC) fractioning scheme used in LEGE06071

| Fraction | Solvent mixture | Volume (ml) | Biomass/fraction (mg) |
|---|---|---|---|
| A | 10% EtOAc (hex) | 350 | 41 |
| B | 20% EtOAc (hex) | 250 | 7,1 |
| C | 40% EtOAc (hex) | 250 | 11,9 |
| D | 50% EtOAc (hex) | 250 | 190,9 |
| E | 60% EtOAc (hex) | 250 | 72,2 |
| F | 80% EtOAc (hex) | 250 | 52,3 |
| G | EtOAc | 250 | 28,2 |
| H | 25% MeOH (EtOAc) | 250 | 8,1 |

Only 36mg were available from LEGE06071/F that didn't represent enough material for another normal VLC, so a liquid chromatography by gravity was made. This type of column, having a much higher number of theoretical plates, allows for a very efficient separation (Fig. 10A).

The fractions were collected in 138 tubes, each with 3 ml (Fig. 10B) and the eluent mixtures are shown in table XII.

Table XI – Liquid chromatography (LC) scheme used in fraction LEGE06071/F

| Fraction | Eluent mixture | Volume (ml) |
|---|---|---|
| 1-42 | 50% EtOAc (hex) | 200 |
| 43-61 | 80% EtOAc (hex) | 100 |
| 62-82 | EtOAc | 100 |
| 83-102 | 90% EtOAc (MeOH) | 100 |
| 103-120 | 80% EtOAc (MeOH) | 100 |
| 121-138 | MeOH | 130 |

2.3.2.2 Thin Layer Chromatography (TLC)

As a large number of fractions were achieved, thin-layer chromatography (TLC) was performed for each tube, in order to evaluate their composition and merge the contiguous tubes with similar TLC patterns (Fig. 10C).

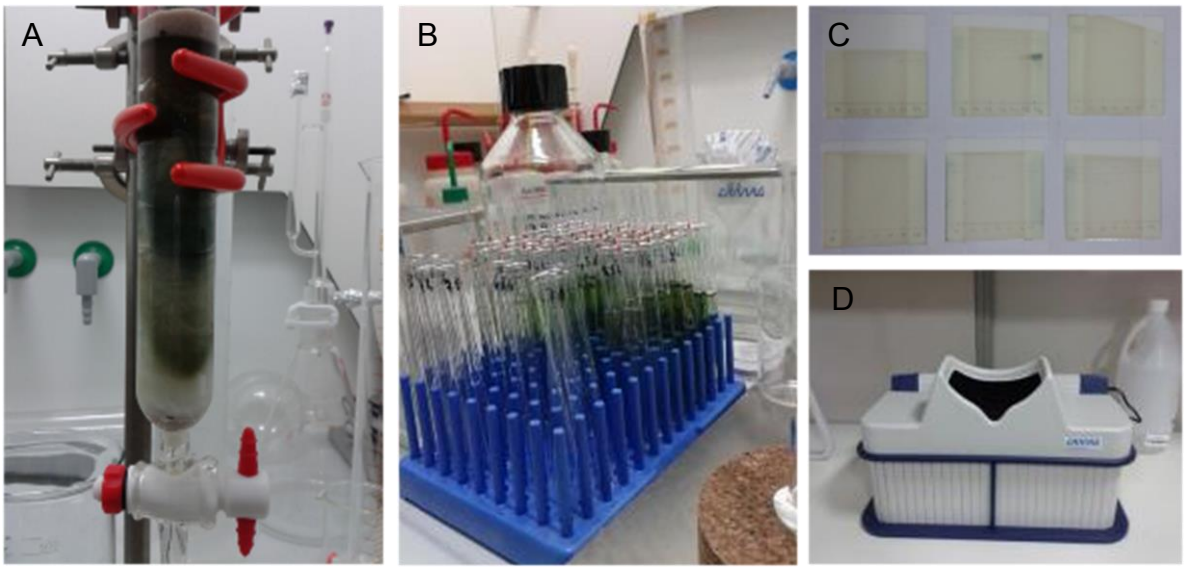


Fig. 10: A – LEGE06071/F liquid chromatography (LC) with a 13,5cm column. B – 138 fractions collected. C – TLC to merge fractions with the same compounds. D – UV chamber.

About 10µl were dotted in the bottom of a TLC plate that was then placed in a shallow pool of eluent. The initial eluent mixture was 75% Hex and 25% EtOAc and it was used from fraction 1 to 23. After that, a more polar mixture was made with 50% Hex and 50% EtOAc and used from fraction 22 to 138.

Although the distance that each colored compound completed could be seen by bare eye, the silica plate incorporated a highly reactive chromophore that in case of reaction with any colorless compounds would be seen in the UV chamber (254nm and 366nm) (Fig. 9D).

The TLC process allowed the merge of the 138 tubes into 11 fractions (LEGE06071/F/A – LEGE06071/F/K).

## 2.3.3 Nuclear magnetic resonance (NMR)

After every fractioning process, samples were sent to Centro de Materiais da Universidade do Porto (CEMUP) where $^{1}$H NMR analyses at 400 MHz were performed to determine the chemical structure of the compounds and to evaluate the effectiveness of the separation procedure, through the comparison of the NMR spectra of each obtained fraction. Checking the presence of each signal in each individual spectra enables to establish the distribution of compounds throughout the collected samples; in other words, if

a given signal (or set of signals) appears in the spectra of one of the fractions and in no other, means that the compound responsible for those peaks is contained solely in that sample.

## 2.4. Antifouling bioassays

### 2.4.1 Preliminary bioassays with *M. galloprovincialis* adults

Preliminary bioassays were conducted using adults of *Mytilus galloprovincialis* (1 cm shell length) and organic and aqueous extracts of previously lyophilized cyanobacterial biomass provided by the LEGE culture collection as previously mentioned.

Adult specimens of *M*. *galloprovincialis* were collected in Memória beach (Porto, North Portugal) and immediately transported to the laboratory. The animals were placed in aerated 30L aquaria with filtered sea water, at 17ºC and fed three times a week with commercial food. Before being used in bioassays, mussels were all cleaned from existing threads and trials were performed to select only competent animals (mussels that show foot crawling behavior).

Organisms were exposed for 15 hours to 60 ml medium with extracts of different cyanobacteria, in a final test solution concentration of 100 µg/ml.

Each cyanobacterial extract was tested with 15 individuals divided by 5 petri dish. A positive control was carried out with 5 µM $CuSO_4$ and a negative control with filtered seawater.

The AF potential of each strain was determined at the end of the trials by the success in attachment, given by the production/non-production of byssal threads by the mussels.

### 2.4.2 *M. galloprovincialis* plantigrade larvae and juvenile bioassays

Considering that preliminary bioassays with adults showed promising results but presented the constraint of requiring high amounts of extracts biomass to perform the trials, a new approach was designed using mussels plantigrade larvae and juveniles (between 0.2 and 0.3 cm). Improvements in cyanobacterial test solutions were also included to minimize the extracts usage.

#### 2.4.2.1 *M. galloprovincialis* sampling and preparation

Samples of intertidal sand and adult pedunculate barnacles were collected from rocky beaches of the north coast of Portugal, and immediately transported to the laboratory to be analyzed for plantigrade and juvenile mussels in a magnifier (LEICA EZ4).

#### 2.4.2.2 Cyanobacterial test solutions

Cyanobacterial organic extracts and fractions were redissolved in Chloroform (100%) or Isooctane-Ethanol (1:1) with sonication (50-100 mg/ml), except fractions H that were redissolved in 100% methanol in order to transfer the required biomass for the test solutions.

Test solutions were prepared according to Fig. 11, with final concentrations of 30 µg/ml for aqueous and organic extracts and 10 µg/ml for organic fractions in filtered seawater. Dimethyl sulfoxide (DMSO) was used as solvent as it provides high solubility potential, from non-polar to polar compounds, as is considered, almost, as a universal solvent.

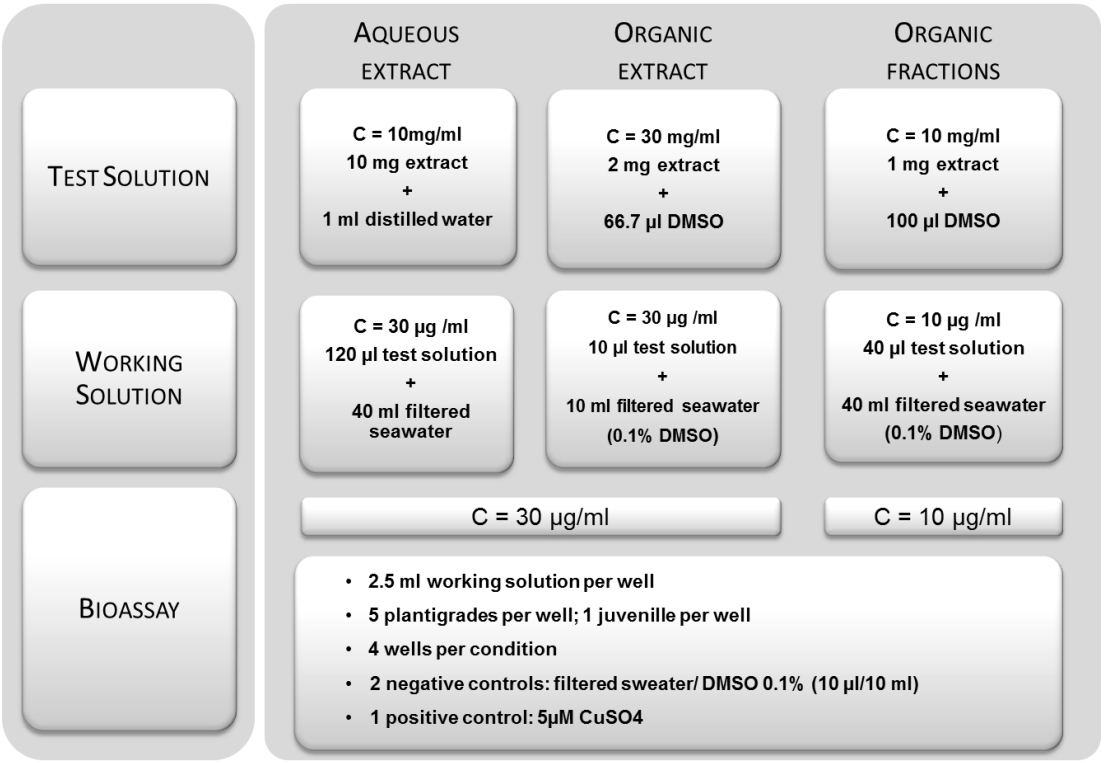


Fig. 11 - Scheme of test solution, working solution and bioassays preparation.

#### 2.4.2.3 AF bioassay

Plantigrade and juvenile *M. galloprovincialis* individuals were exposed in 24-well microplates to 2.5 ml of the cyanobacterial extracts- and fractions-enriched solutions for 15 hours in the darkness, to maximize byssal threads production (Carl, Poole et al. 2011). Four well replicates were used for plantigrades with a single well hosted 5 plantigrades, and twelve well replicates hosting only one juvenile. Two negative control conditions were used with filtered seawater only and filtered seawater with 0.1% DMSO. A positive control was used with a solution of 5 µM $CuSO_4$.

At the end of the exposure period the number of threads produced by each individual was counted using a magnifier with contrast light for juveniles and a microscope for plantigrades, to determine if the animal was fixated to the subtract (Fig. 12).

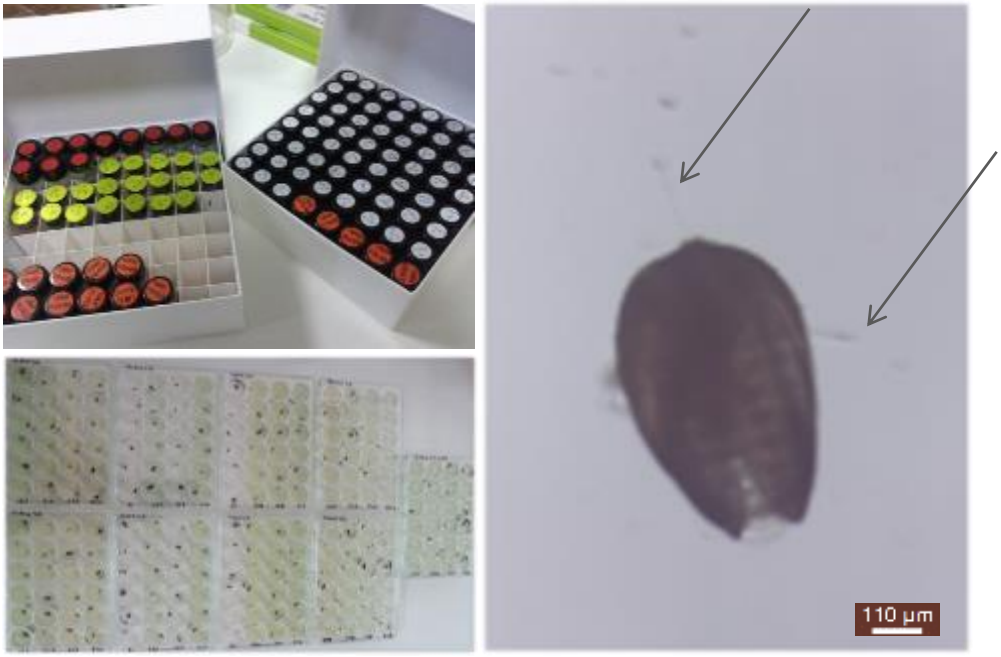


Fig. 12 - Bioassays: different working solutions being tested in plantigrade and juvenile *M. galloprovincialis* individuals.

### 2.4.3 Statistics

AF bioassays output data was expressed as the percentage of settlement considering the production of byssal threads by all the individuals in the same condition as 100% of settlement. All data were first checked for normality and homogeneity of variance by Kolmogorov-Smirnov and Barlett's tests, respectively. As these assumptions were not fulfilled, even after appropriate data transformations, non-parametric Mann-Whitney (U) test was applied to analyse significant differences between all the tested conditions (extracts, fractions and controls) and the solvent control. Statistical differences were considered significant at $p < 0.05$ and SPSS 21 software was used for the statistical analysis.

# 3. Results

## 3.1 Preliminary bioassays with *M. galloprovincialis* adults

Aqueous and organic extracts from eighteen cyanobacterial strains were tested in adult individuals as preliminary bioassays (Fig. 13). *M. galloprovincialis* adults showed altered responses regarding some strains and extracts when compared to mussels in control condition. LEGE06077, LEGE07084 and LEGE07085 organic extracts significantly inhibited settlement of *M. galloprovincialis* adults (U= 1.0, p= 0.013; U=3.0, p=0.031 and U= 2.0, p= 0.021). No effect was observed for aqueous extracts of the same strains (U= 12.5, p= 1.000; U=4.5, P=0.080 and U= 11.5, p= 0.811). LEGE99043 and LEGE06079 aqueous extracts induced a significant inhibition of settlement (U= 2.0, p= 0.020 and U= 3.0, p= 0.032) but no influence was observed concerning the organic extract (U= 10.0, p= 0.549 and U= 4.5, p= 0.080). Actually, none of the tested strains showed significant results in both organic and aqueous extracts. Some testing conditions like organic LEGE06072 appeared to induce settlement, showing higher percentages than negative control, however no significant differences were found (U= 7.5, p= 0.134). The positive control (5 µM $CuSO_4$) showed total settlement inhibition (no threads produced) by all the testing individuals (U= 0.0, p= 0.005).

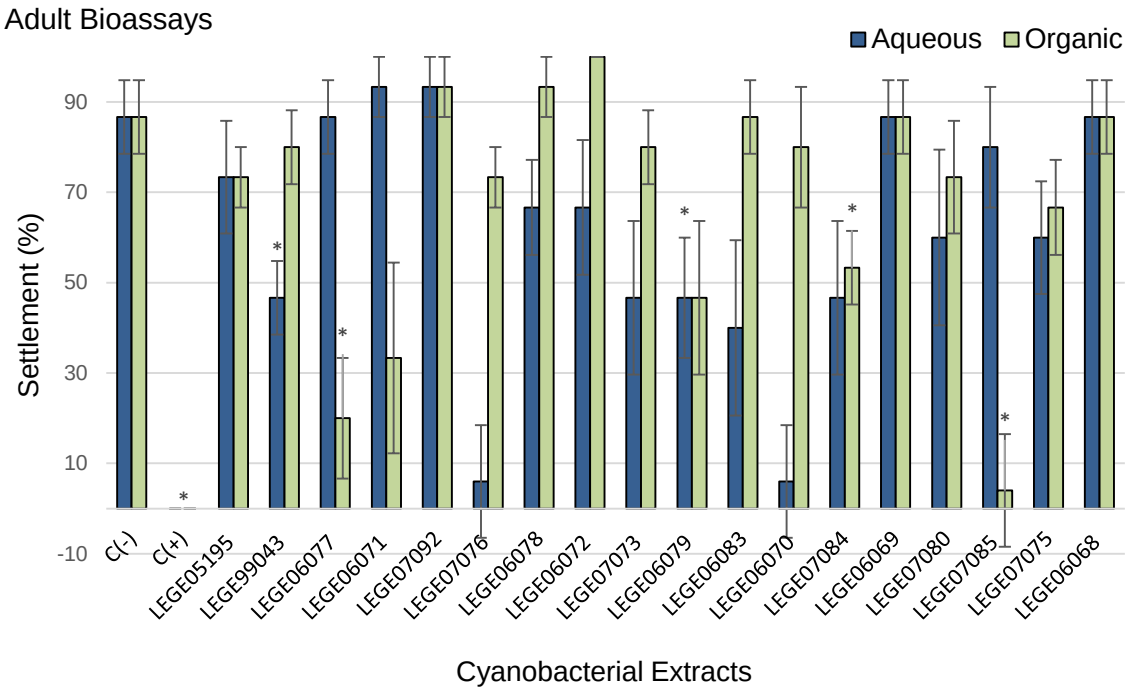


Fig. 13 - Percentage of settlement of *Mytilus galloprovincialis* adults after exposure to crude organic and aqueous extracts (100 µg/ml) of selected cyanobacterial strain from LEGE culture collection. C(-)= filtered seawater control; C(+)= positive control with CuSO4 5µM. *indicates significant differences against C(-) (Mann-Whitney U test, $p < 0.05$).

## 3.2 *M. galloprovincialis* plantigrade larvae and juvenile bioassays

### 3.2.1 Screening crude organic and aqueous extracts

Regarding the bioassays with the two early life stages, plantigrade larvae and juvenile mussels, using extracts of the same eighteen strains previously used (Fig. 14), organic extracts from strains LEGE05195, LEGE06077 and LEGE07075 significantly inhibited the settlement, of both plantigrade (U= 0.0, p= 0.019; U= 0.0, p= 0.017 and U= 0.0, p= 0. 019) and juvenile stages (U= 0.5, p= 0.025; U= 0.0, p= 0.017 and U= 0.0, p= 0. 015), when compared to DMSO control.

No significant differences were found between DMSO control and the negative control with filtered seawater only in both plantigrade larvae (U= 2.0, p= 0.05) and juvenile mussels (U= 8.0, p= 1.000), and so DMSO control was used as reference.

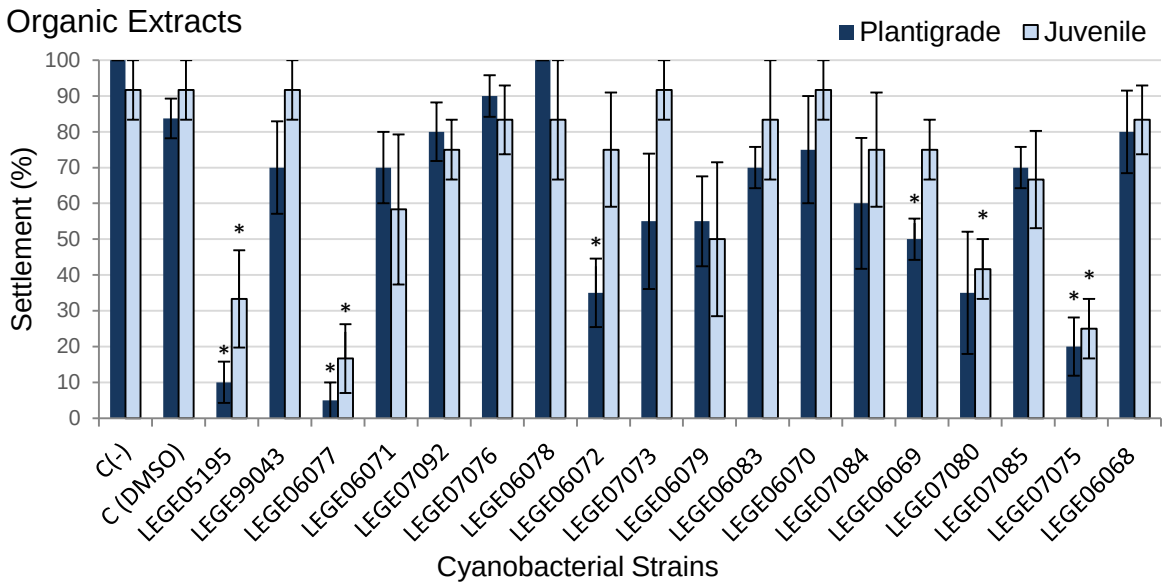


Fig. 14 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to crude organic extracts (30 µg/ml) of selected cyanobacterial strain from LEGE culture collection. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Bioassays with aqueous extracts showed higher effectiveness levels in plantigrade stages (Fig. 15). None of the tested strains induced significant differences against the control in juvenile mussels. LEGE06071 showed the highest potential in both life stages with only 5% settlement in plantigrade ($U= 0$, $p= 0.011$) and approximately 33% in juvenile, despite the last not being statistically significant ($U= 3.0$, $p= 0.129$).

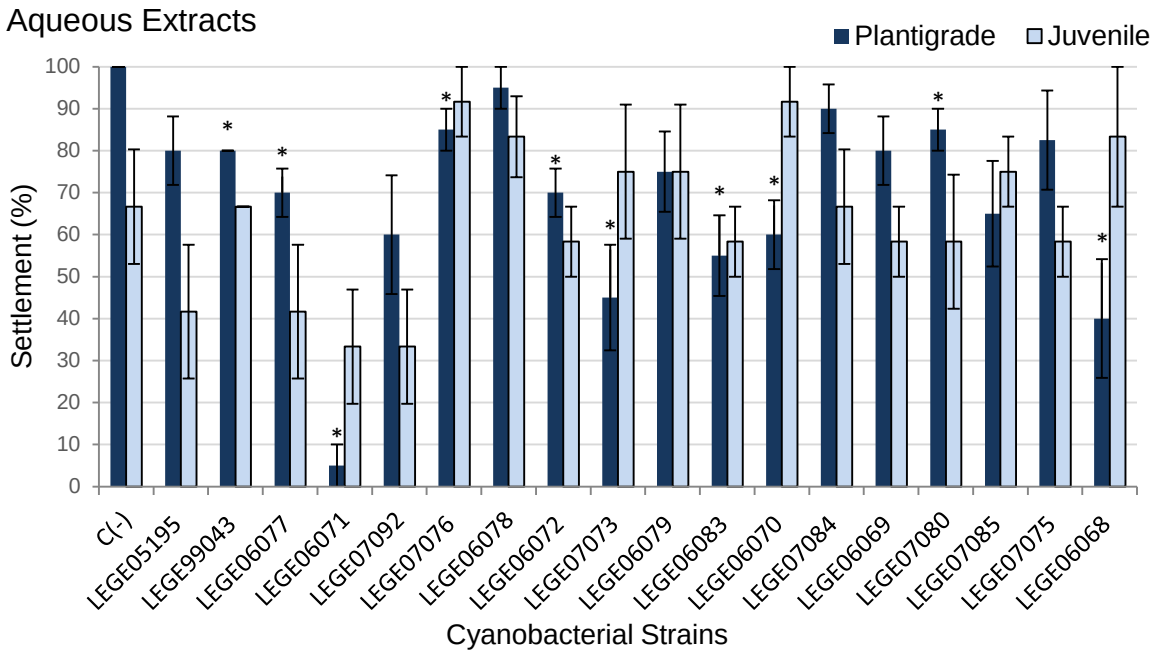


Fig. 15 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to crude aqueous extracts (30 µg/ml) of selected cyanobacterial strain from LEGE culture collection. C(-)= filtered seawater control. *indicates significant differences against C(-) (Mann-Whitney U test, $p < 0.05$).

### 3.2.2 Bioassays with fractions of selected strains

Several selected strains (LEGE07084, LEGE06079, LEGE91094, LEGE06071 and LEGE06077) were subjected to fractionation and further testing permitted to reach also some promise fractions.

LEGE07084 was successfully fractioned into nine fractions (Fig. 16). Statistical analyses were performed against DMSO control as no significant differences were found with filtered seawater control in both plantigrade and juvenile stages ($U= 6.0$, $p= 0.495$ and $U= 4.0$; $p= 0.752$). Low inhibition potential was showed by all fractions when concerning juvenile individuals except fraction D ($U= 1.5$, $p= 0.040$). Fractions C, D and F displayed the most significant settlement inhibition in plantigrade larvae ($U= 0.0$, $p= 0.015$; $U= 0.0$, $p= 0.017$ and $U= 0.0$, $p= 0.017$), with 5%, 15% and 20% of settlement, respectively.

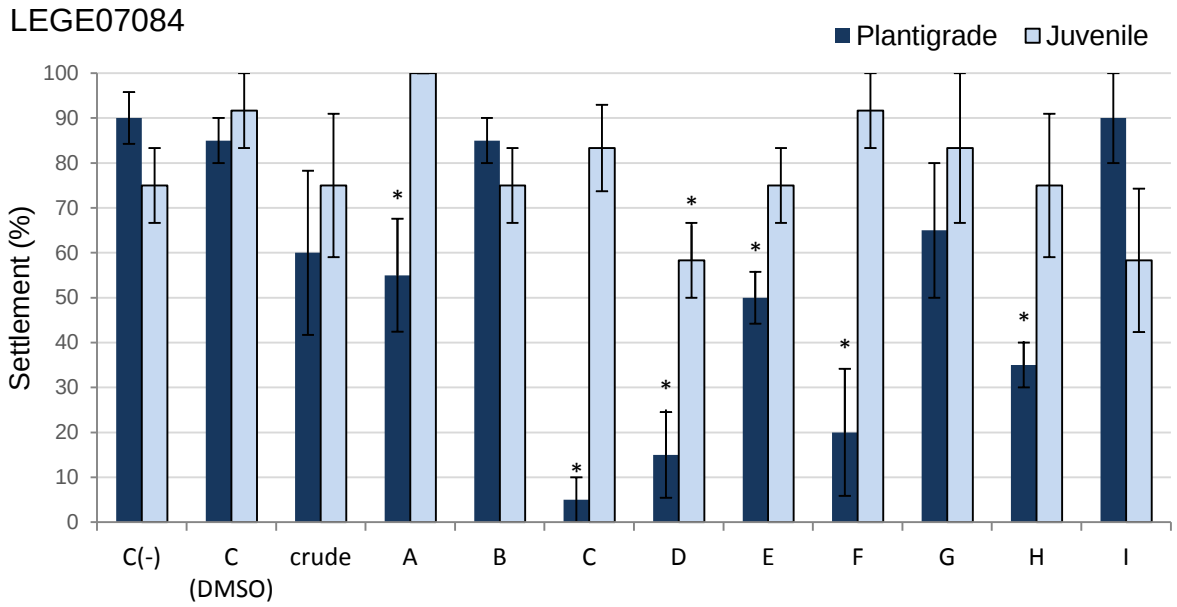


Fig. 16 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic fractions (10 µg/ml) of LEGE06084. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Regarding LEGE06079, no significant differences were found between filtered seawater control and DMSO control in both plantigrade (U= 2.0, p= 0.05) and juvenile mussels (U= 3.0, p= 0.096), and as so DMSO control was used as reference. Seven fractions were isolated and only fraction A induced significant inhibition of thread production in plantigrade larvae (U= 0.0, p= 0.017). None of the fractions showed significant differences on juvenile mussel's settlement compared to the DMSO control (Fig. 17).

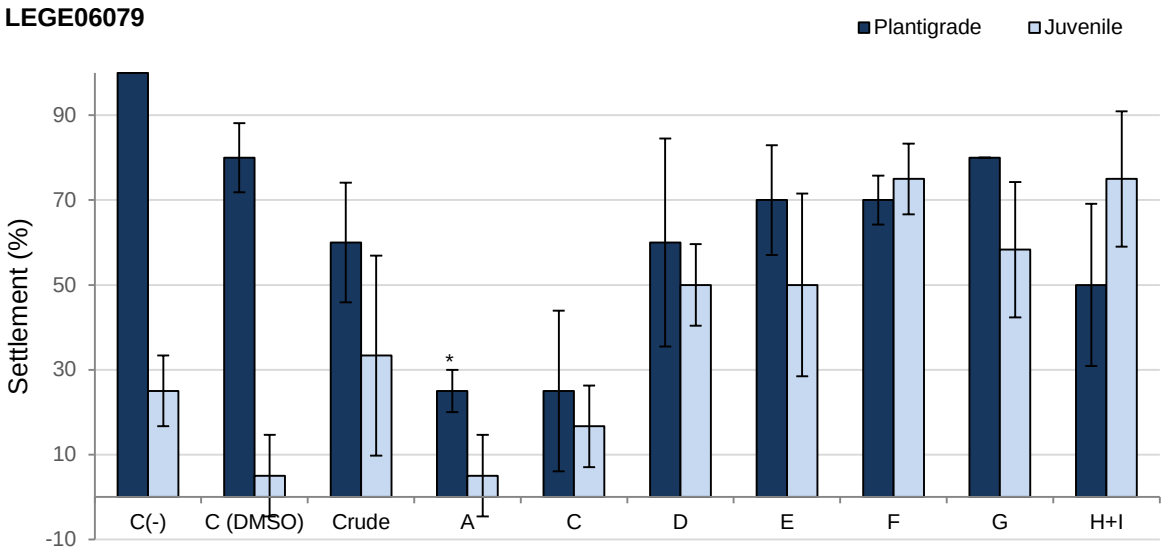


Fig. 17 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic fractions (10 µg/ml) of LEGE06079. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Nine fractions of LEGE91094 were obtained and tested (Fig. 18). Statistical analyses revealed no significant differences between the controls and DMSO control was used as reference. None of the tested fractions showed significant inhibition of settlement in juvenile mussels. Fractions, E, G and H presented significantly low percentages of settlement when compared to DMSO control ($U= 1.0$, $p= 0.036$; $U= 0.0$, $p= 0.019$ and $U= 1.0$, $p= 0.036$).

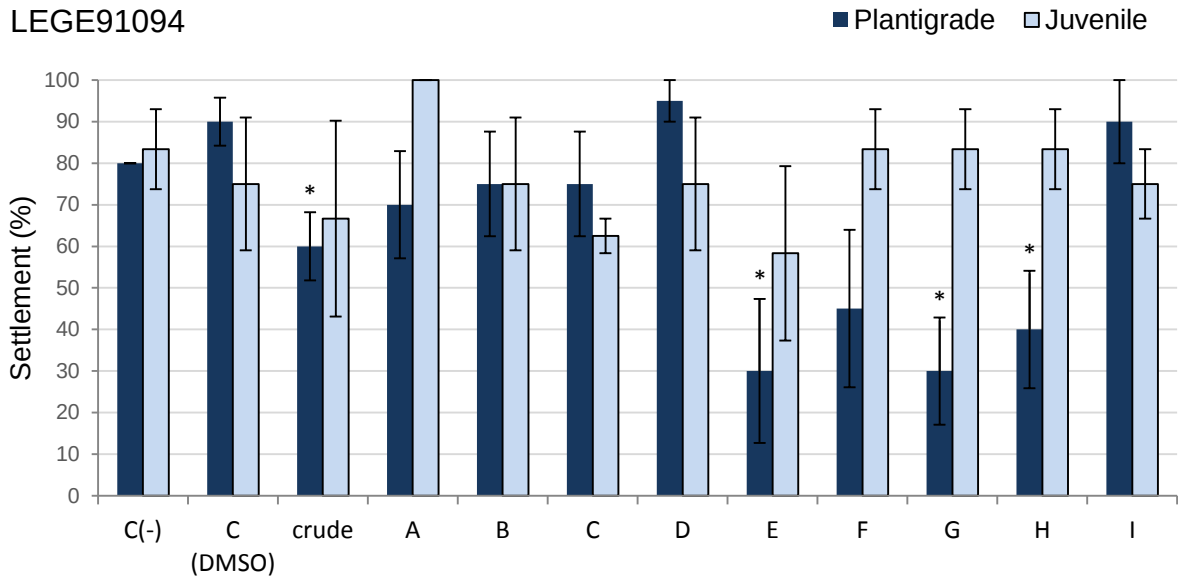


Fig. 18 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic fractions (10 µg/ml) of LEGE91094. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Nine fractions were obtained from LEGE06071 strain and only fraction A and F showed significant inhibition of settlement in plantigrade larvae and juvenile mussels, respectively (U= 0.0, p= 0.017 and U= 1.0, p= 0.032) (Fig. 19). Fraction A also induced a certain degree of inhibition of settlement in juveniles, despite not statically significant. (U= 5.0, p= 0.343). Controls also do not differ significantly in this bioassay in both plantigrade larvae and juvenile mussels (U= 2.0, p= 0.05 and U= 3.0, p= 0.096).

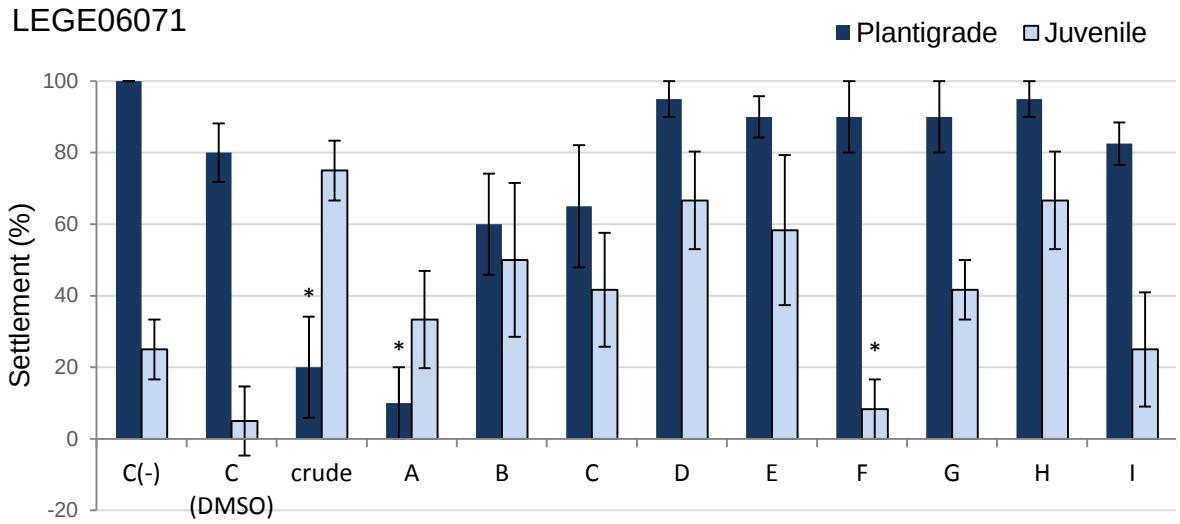


Fig. 19 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic fractions (10 µg/ml) of LEGE06071. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Ten fractions of LEGE06077 were successfully obtained as represented by NMR analyses performed where different compounds can be distinguished thus proving an effective separation (Fig. 20).

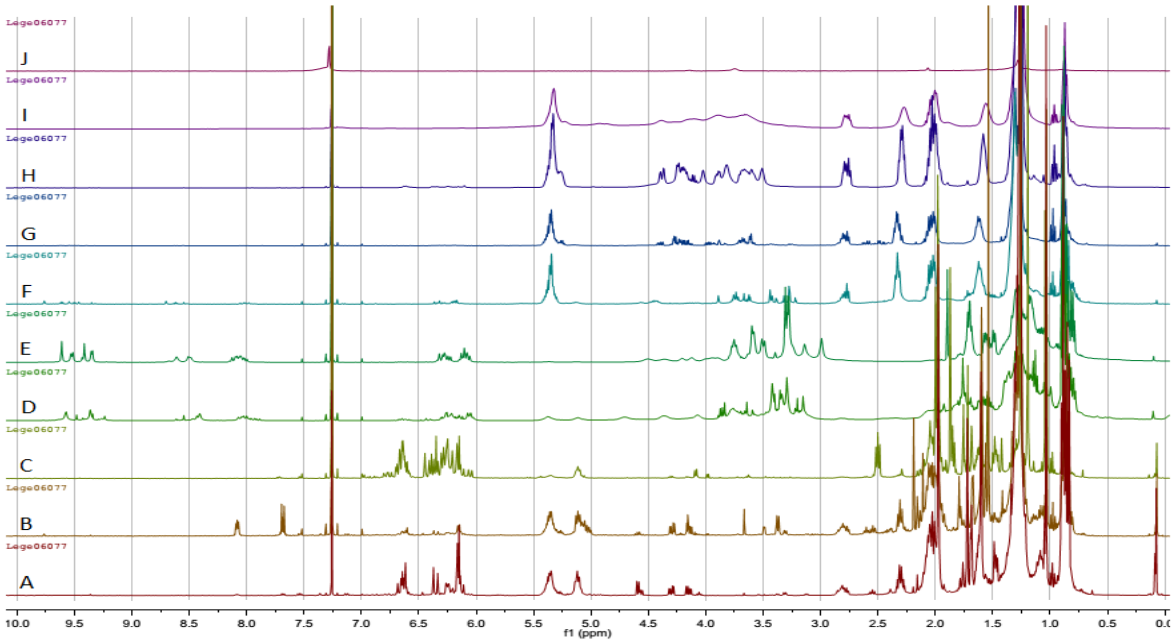


Fig. 20 - NMR spectrum of LEGE06077 fractions.

No statistical differences were found between the two control conditions in both plantigrade larvae and juveniles, and so results were analyzed against DMSO control. All LEGE06077 fractions (Fig. 21), except fraction B, showed high inhibition potential with fraction H reaching 15% and 25% of settlement inhibition in plantigrade and juvenile respectively (U= 0.0, p= 0.019 and U= 0.0, p= 0.011).

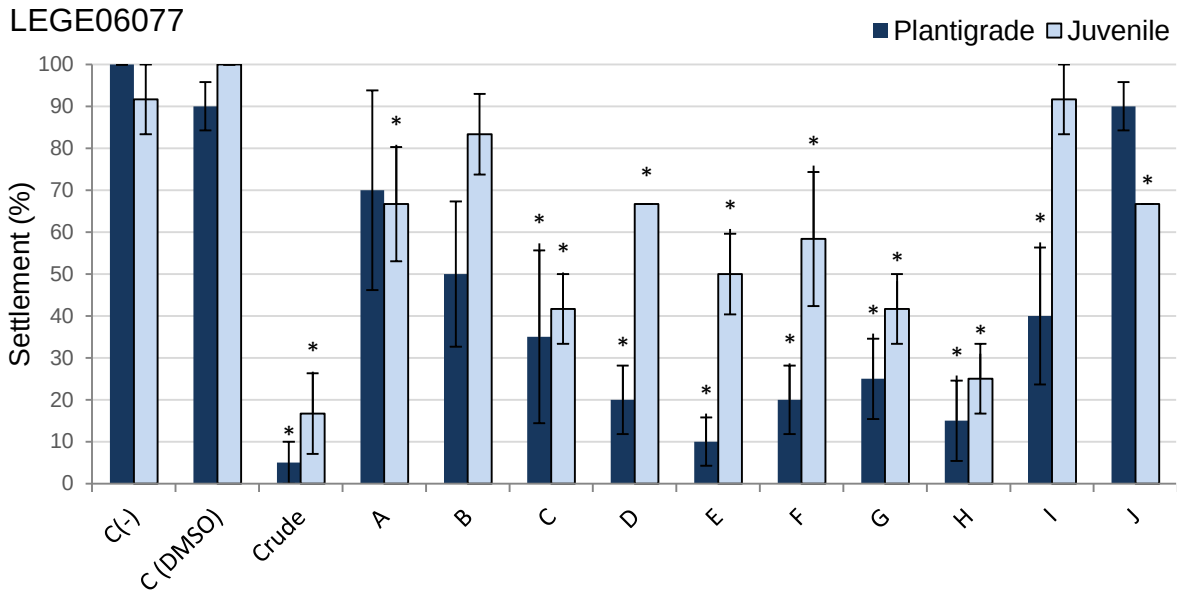


Fig. 21 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic fractions (10 µg/ml) of LEGE06077. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

### 3.2.3 Bioassays with selected sub-fractions

Due to the promising results provided by fraction H of LEGE06077 strain, this fraction was further fractioned and seven sub-fractions were successfully obtained as given by NMR analysis (Fig. 22). Bioassays using the obtained sub-fractions are showed in figure 23.

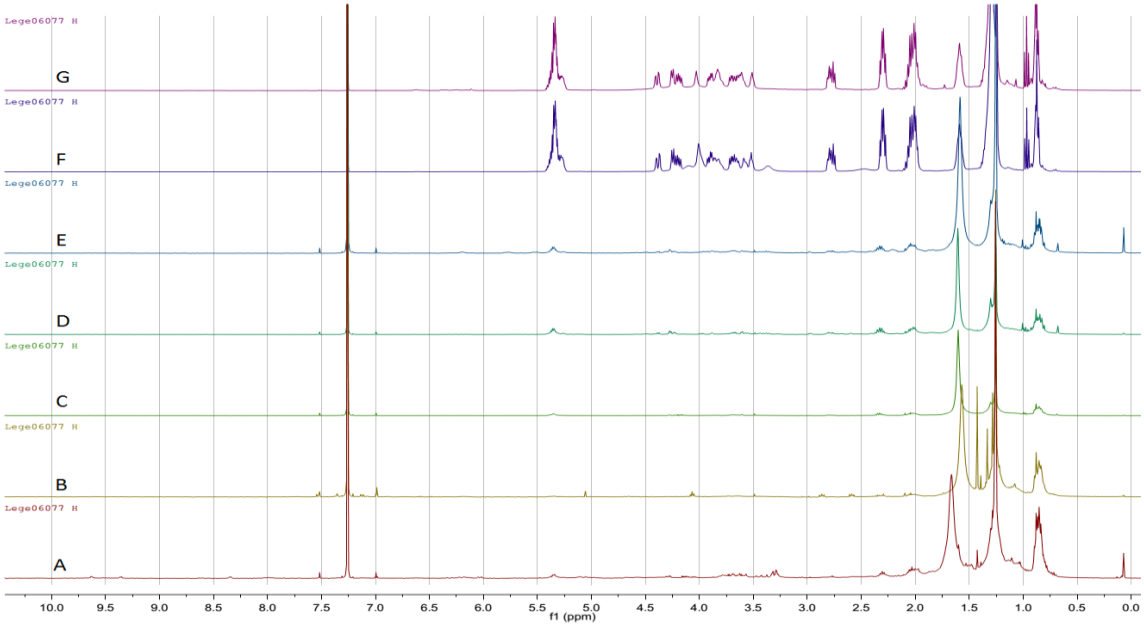


Fig. 22 – Nuclear Magnetic Resonance (NMR) spectrum of sub-fractions obtained from LEGE06077/H.

Control conditions were not significantly different in both stages, as DMSO control was adopted as reference. In general, juvenile mussels showed lower settlement levels then plantigrade larvae, however, none of the tested sub-fractions had significant inhibition effect on juveniles. Concerning plantigrade, three fractions HC, HF and HG, showed significant settlement inhibition compared to DMSO control ($U= 0.0$, $p= 0.019$; $U= 0.0$, $p= 0.036$ and $U= 0.0$, $p= 0.019$). From these three sub-fractions, HF showed the most concordant levels between life stages, with 40% of settlement inhibition in plantigrade and 50% in juvenile mussels.

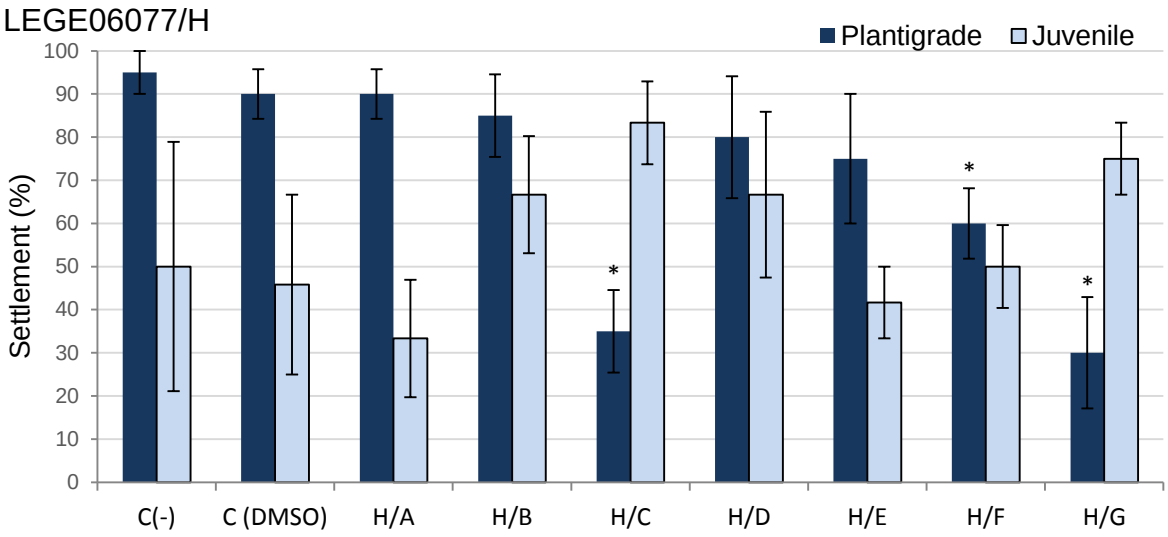


Fig. 23 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic sub-fractions (10 µg/ml) of LEGE06077/H. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

Sub-fraction LEGE06077/H/F was successful fractionated into seven new sub-fractions (Fig. 24). No statistical differences were found between DMSO control and filtered seawater control. None of the tested sub-fractions provided statistical relevant data in juvenile stage. Sub-fraction HFB and HFC presented significant inhibition levels in plantigrade larvae ($U= 0.0$, $p= 0.017$ and $U= 0.0$, $p= 0.017$) showing the lowest percentage of settlement, 35% in both sub-fractions (Fig. 25).

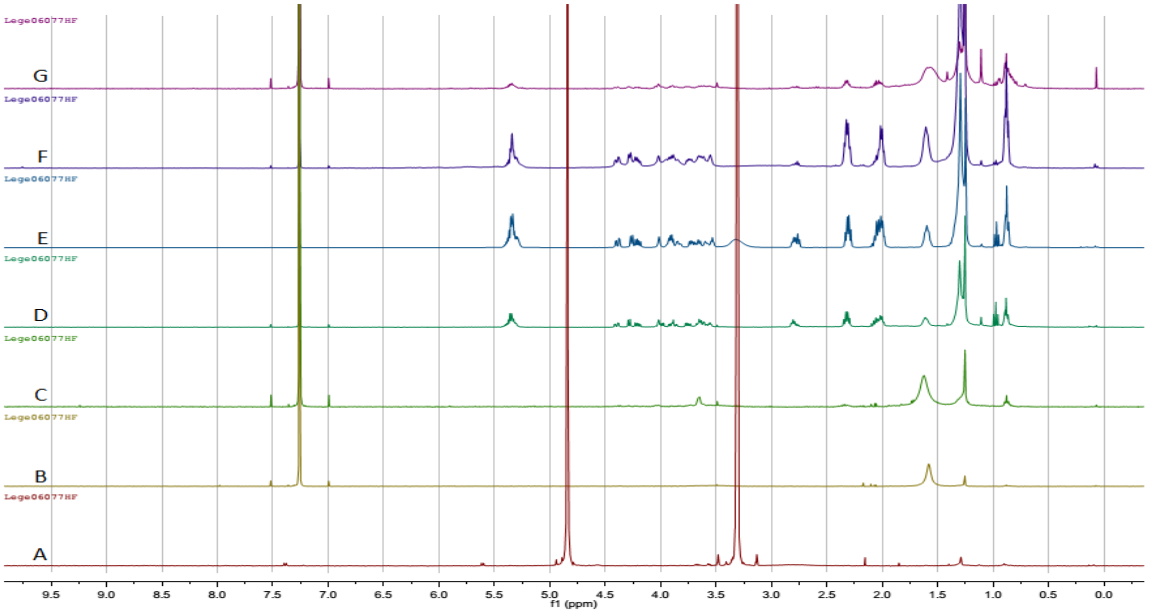


Fig. 24 - Nuclear Magnetic Resonance (NMR) spectrum of sub-fractions obtained from LEGE06077/H/F. Sub-fraction LEGE06077/H/F/A displays different satellite peaks from the other sub-fractions because, in this sub-fraction, methanol was used as solvent instead of chloroform.

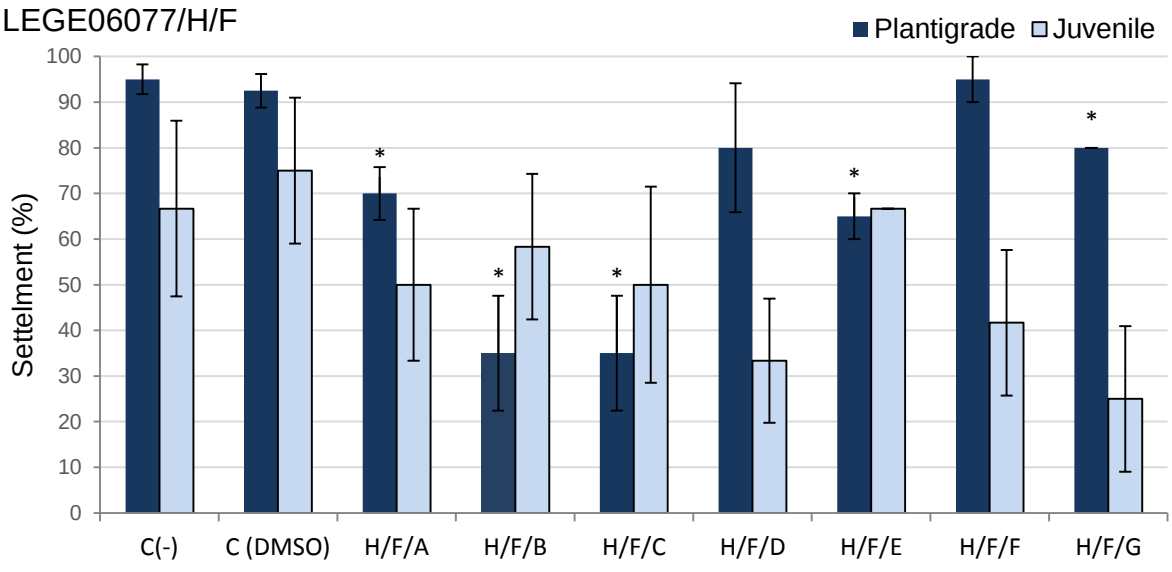


Fig. 25 - Percentage of settlement of *Mytilus galloprovincialis* plantigrade and juvenile after exposure to organic sub-fractions (10 µg/ml) of LEGE06077/H/F. C(-)= filtered seawater control; C(DMSO)= filtered seawater with 0.1% DMSO. *indicates significant differences against C(DMSO) (Mann-Whitney U test, $p < 0.05$).

## 3.3 Characterization through NMR

Given the results provided by sub-fraction LEGE06077/H/F/B and LEGE06077/H/F/C and the fact that these, by being achieved through three different fractionation processes would probably be constituted only by pure compounds, the following step was to characterized by NMR these two sub-fractions. Figures 26 and 27 show the NMR spectrum for the two sub-fractions with peaks highlighted in red representing solvent satellites and the ones in blue or grey characterize the compounds present in the tested samples. Grey peaks indicate compounds with lower intensities then blue. Characterization through $^{13}C$ NMR would be ideal to achieve better knowledge of the compounds structure. However, due to the biomass shortage only $^{1}H$ NMR analyzes were performed providing important but not complete information about the chemical structure of the compounds.

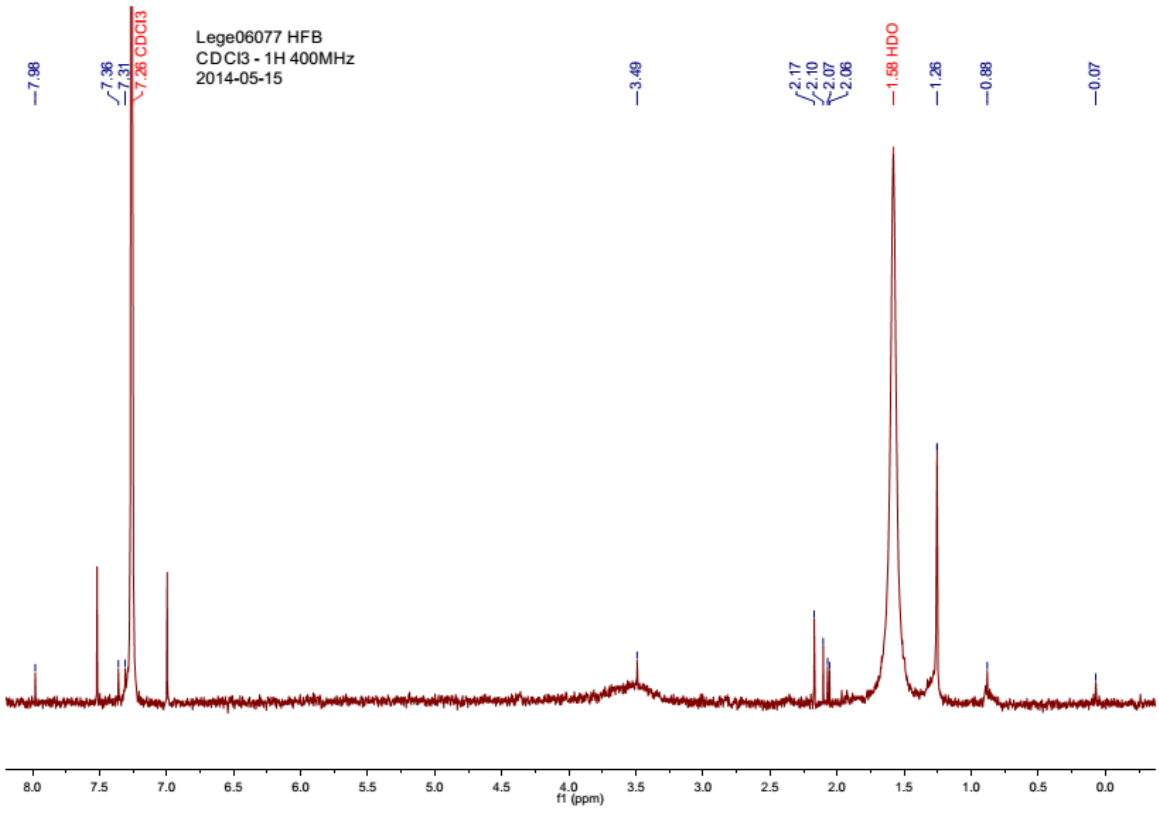


Fig. 26 - Nuclear Magnetic Resonance (NMR) spectrum of LEGE06077/H/F/B. Peaks highlighted in red represent solvent satellites and the ones highlighted in blue correspond to the compounds present in the sub-fraction.

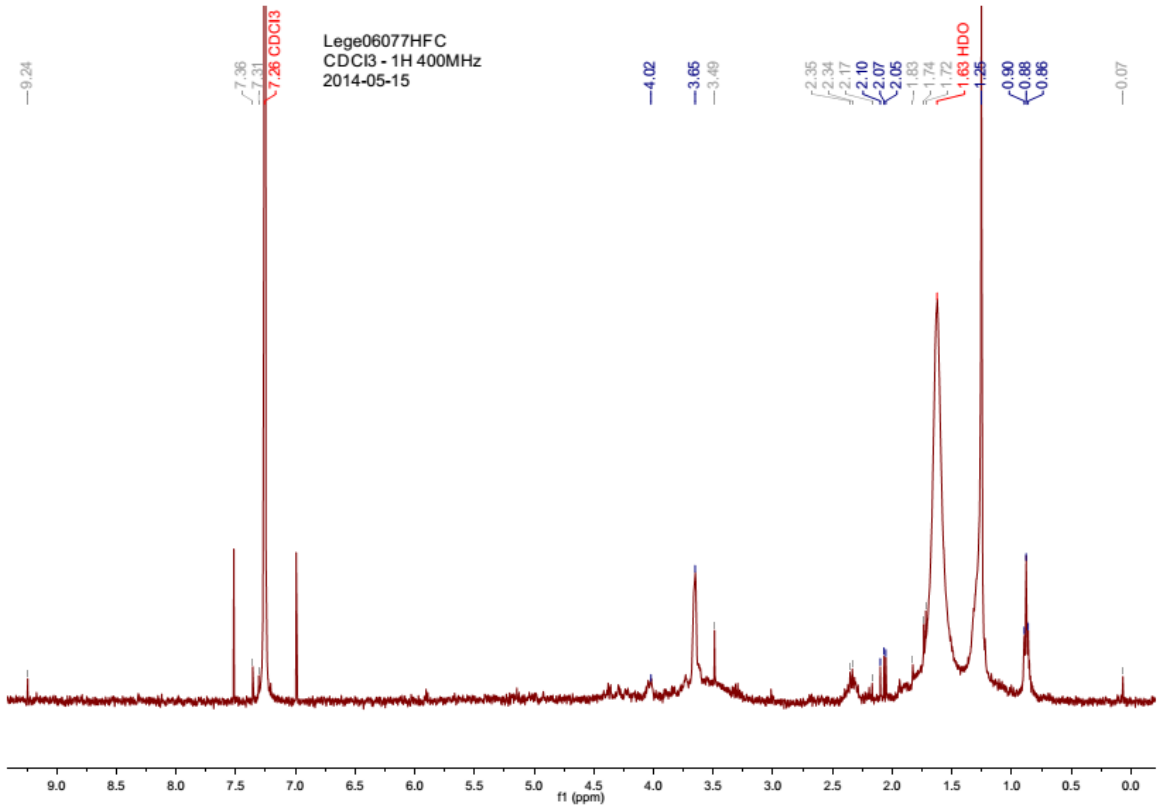


Fig. 27 - Nuclear Magnetic Resonance (NMR) spectrum of LEGE06077/H/F/C. Peaks highlighted in red represent solvent satellites and the ones highlighted in blue and grey correspond to the compounds present in the sub-fraction. Grey peaks signalize compounds with lower intensities then blue.

# 4. Discussion

Preliminary bioassays with mussel adults permitted to consider that *M. galloprovincialis* is a suitable species for an AF bioassay-guided approach showing differential responses in the production of threads when comparing mussel from control conditions and mussels exposed to cyanobacterial strains enriched-media. These bioassays also indicated that 5 µM $CuSO_4$ media is suitable to use as positive control in further AF bioassays as mussels showed total settlement inhibition in this condition.

*M. galloprovincialis* has been previously used in AF bioassays using other type of biological extracts like sponges and macroalgae and even pure compounds like capsaicin (Yang, Satuito et al. 2007, Qian, Xu et al. 2010). Copper has been used as an AF agent with high levels of effectiveness on organisms like mussels (Sera, Iida et al. 2000, Rosen, Rivera-Duarte et al. 2008, Brooks and Waldock 2009).

Still regarding the preliminary bioassay with mussel adults, only three strains showed effectiveness with organic extracts, LEGE06077, LEGE07084 and LEGE07085 from which only LEGE06077 had positive effect when comparing to the results from plantigrade larvae and juvenile mussels bioassays. This concordance between the three life stages was a good indicator of a promising strain and for that reason LEGE06077 was chosen to be fractionated and again tested.

Concerning aqueous extracts in adults, two different strains, LEGE99043 and LEGE06079, presented significant differences and AF potential indicating a differential separation of cyanobacteria constituents during the two extraction procedures, including potential active compounds. Only LEGE99043 also showed positive results in plantigrade larvae but not in juveniles. Conversely to what was observed with the organic extracts, aqueous extracts did not provide compliance results. None of the strains was statistically significant in all life stages which compromised the selection of promising strains for the next step of the project

After testing all the selected strains, the ones presenting better results and/or with enough biomass available were fractioned and further tests conducted. Due to the better results provided by organic extracts, only those were used in the succeeding assays.

LEGE07084 didn't show significant results in plantigrade or juvenile stages in organic extracts bioassays mostly because of the high variability among replicates. However it had shown statistically positive results when testing organic extracts in adult individuals. For these reasons and also due to biomass availability, it was fractionated and tested. Almost every fraction with significant inhibition was only effective on plantigrade stage, with fraction C displaying 5% settlement. Despite this value, none of the fractions showed results positive enough in both life stages, as to justify further fractionations and tests. This strain was already found to inhibited larval growth of the sea urchin *Paracentrotus lividus (*Lopes, Fernández et al. 2010), indicating biological activities other than AF caused by the active compounds.

Plantigrade individuals presented low settlement percentages when exposed to several strains, fractions and even sub-fractions revealing high levels of sensibility. In some cases, like for example LEGE91094 strain, the opposite was registered in juvenile stage, where thread production didn't show almost any influence by the cyanobacteria enriched medium. In fact, for some reason inherent to the bioassay and/or to the intrinsic characteristics of the species, even filtered seawater control levels showed abnormally low levels comparing to what was expected. This had a negative influence in some of the obtained test results, since statistical analyses were based on these control levels. In certain cases, like LEGE06071 bioassay, where only fraction F was significant in juvenile stage, fraction I inhibited 75% of settlement and fraction A 67% of settlement but were not considered significantly different from DMSO control. When concerning LEGE06079, none of the tested fractions induced significant results in juvenile, despite the settlement levels of only 5% and 16% showed by fraction A and C respectively. This means that, due to low settlement levels presented by negative controls in some bioassays, some fractions/sub-fraction's potential might have been undervalued.

Despite low settlement levels in some controls, it is a general point that juvenile individuals are not as sensitive as plantigrade larvae. This might happen because juveniles are a more advanced and evolved step in *M. galloprovincialis* development than plantigrade, and its settlement pressure is lower. Although plantigrade larvae are supposed to detach from an initial settlement site and search for more appropriate settlement areas (Carl, Poole et al. 2011), the establishing pressure it endures continues to be very high and can justify the greater settlement percentages showed by plantigrade larvae in negative control when comparing to juvenile. Moreover, the different responses

presented by the two tested life stages might be related with distinct responses to the external environmental stimulus also inherent to each developmental status and respective metabolic complexity.

LEGE06071 (*Nodularia* sp.) is an heterocystous cyanobacteria with some known activities like inducing acute effects on *Artemia salina* nauplii and inhibit larvae growth on *Paracentrotus lividus (*Lopes, Fernández et al. 2010*).* When testing this strain, fraction F presented high levels of settlement inhibition with one of the most positive results in juvenile stage in all bioassays. Despite not displaying the same outcome in plantigrade larvae, this fraction was selected due to the potential revealed in juvenile stage.

LEGE06077 was one of the most promising strains since preliminary assays. With the exception of fraction B, all LEGE06077 fractions tested provided significant results in one life stage or in several cases in all stages. Despite the higher inhibition levels displayed by fraction E on plantigrade larvae, fraction H had better results when evaluating the two life stages combined. In LEGE06077 bioassay, crude extract was the working solution that displayed better results in both stages with none of the its fractions achieving the same inhibition levels. This can be explained by synergies between different fractions that might enhanced the inhibition potential of the strain. By repeatedly fractionate these organic extracts and fractions, several synergies might have been lost. LEGE06077 (*Nostoc* sp.) is an heterocystous cyanobacteria and the good results obtained may be related to higher quantities of secondary metabolites produced as a more evolved specie (Lopes, Ramos et al. 2012). Studies show that this strain can also affect the growth of other marine cyanobacterial species like *Synechocystis salina*, *Nannochloropsis* sp and freshwater species such as *Microcystis aeruginosa* (Lopes and Vasconcelos 2011).

Regarding LEGE06077/H bioassay, again low settlement levels were presented by both controls in juvenile stage. This combined with high variability in thread production that represented high standard deviation values lead to no statistically significant differences between DMSO control and all of the tested sub-fractions. From the only three sub-fractions with significant results in plantigrade larvae, HF showed the highest homogeneity level between the two life stages and for that reason was chosen for fractionation and further tests.

None of the sub-fractions of LEGE06077/H/F had significant inhibition effect in juvenile individuals but sub-fractions HFB and HFC demonstrated good inhibition levels in plantigrade stage. Fraction LEGE06077/H was collected with a mixture of methanol and ethyl acetate which means that the compounds eluted had a medium polarity level. In the fractioning performed by HPLC, these two sub-fractions were obtained between minute 10 and 26 and a reversed-phase chromatography, where polar compounds are eluted first was used. This means that the active AF compounds present in sub-fraction HFB and HFC have polar characteristics.

Crude extracts used in organic bioassays, with plantigrade and juvenile, display different values from the ones present when testing each strain with its fractions. This fact can be justified by the different crude extracts used. Biomass was continuously being produced and after the bioassays testing all organic extract in plantigrade and juvenile, new crude extracts were needed. New biomass of the same strains was used and although conducting the same techniques and processes, the results provided by the new crudes were not completely concordant with the ones previously achieved. The differences obtained can be explained by slightly changes in culture conditions that lead the organism to produce different secondary metabolites. This can be used as an advantage point as a much greater variability of compounds can be obtained just by altering conditions like light or temperature to which cyanobacteria are exposed.

NMR outputs proved the fractioning processes to be successful but some difficulty was created in their analyses by the sample dilution level, particularly in sub-fractions LEGE06077/H/F/B and LEGE06077/H/F/C. Several peaks provided information about promising compounds but their intensity was very low due to the limited amount of organic biomass present in the tested samples

So far, screening cyanobacteria for AF compounds has provided positive results with several strains displaying promising inhibition levels when concerning the fouling mussel *M. galloprovincialis.* As more biomass continues to be cultured, new AF substances can be discovered.

# 5. Future perspectives

Despite the achievement of these two promising sub-fractions, several steps ought to be taken before the truly AF potential of the compounds can be reached, and that were not possible to complete in the time frame of this thesis. Because of the biomass shortage of sub-fraction LEGE06077/H/F/B and LEGE06077/H/F/C, new fractionations needed to be performed. Further steps forward include LC-MS analyses in order to identify and purify the active AF compounds. After the achievement of the pure active compounds, new bioassays should be lead to determine their effectiveness vs toxicity ($EC_{50}$ and $LC_{50}$). Characterization through carbon nuclear magnetic resonance spectroscopy will also be needed to accurately determine the structure of the present molecules.

In addition, synergies between different active compounds might be explored to achieve maximum inhibition and combinatorial chemistry may be applied by structural modification of the obtained compounds in order to improve their effectiveness.

In the meanwhile several other cyanobacterial strains continue to be cultured to large scale and new fractionations of promise extracts are being performed to be subjected to new series of tests and other potential AF compounds are expected to arise from these bioassay-guided approach.

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# 7. Appendix

## Appendix 1 - Z8 Medium (Kótai 1972)

### Composition of stock solutions:

**Solution A - 10 ml/l**

| Reagent | Name | Code | g/l | g/500mL |
|---|---|---|---|---|
| $NaNO_3$ | Sodium nitrate | N4 | 46.7 | 23.35 |
| $Ca(NO_3)_2.4H_2O$ | Calcium Nitrate Tetrahydrate | C1 | 5.9 | 2.95 |
| $MgSO_4.7H_2O$ | Magnesium Sulphate Heptahydrate | M2 | 2.5 | 1.25 |

**Solution B - 10 ml/l**

| Reagent | Name | Code | g/l | g/500 ml |
|---|---|---|---|---|
| $K_2HPO_4$ | Potassium phosphate dibasic | K1 | 3.1 | 1.55 |
| $Na_2CO_3$ | Sodium carbonate | N3 | 2.1 | 1.05 |

**Fe-EDTA Solution - 10 ml/l**

| Reagent | Name | ml/l | 800ml |
|---|---|---|---|
| $FeCl_3$* | Iron(III)chloride | 10 | 8ml |
| EDTA-Na** | Sodium EDTA | 9.5 | 7.6ml |

| Reagent | ml/l | ml/500ml |
|---|---|---|
| 1 a 12 | 10 | 5 |
| 13 e 14 | 100 | 50 |

**Micronutrients Solution – 1ml/l**

## Composition of basic solutions

* FeCl3 Solution

| Reagent | Code | 100ml | 200ml |
|---|---|---|---|
| $FeCl_3.6H_2O$ | F1 | 2.8g | 4.16g |
| HCl (0.1 N) | H2 | 100ml | 200ml |

** EDTA-Na Solution

| Reagent | Code | 100 ml | 200 ml |
|---|---|---|---|
| EDTA | E1 | 3.9 g | 7.8 g |
| NaOH (0.1 N) | N5 | 100 ml | 200 ml |

## Solutions 1-14

| Reagent | Code | g/l | g/100 ml | | Reagent | Code | g/l | g/100 ml |
|---|---|---|---|---|---|---|---|---|
| 1- $Na_2WO_4.2H_2O$ [a)] | N11 | 0.33 | 0.033 | | 8- $CuSO_4.5H_2O$ | C6 | 1.25 | 0.125 |
| 2-$(NH_4)_6Mo_7O_{24}.2H_2O$ | M4 | 0.88 | 0.088 | | 9- $NiSO_4(NH_4)_2SO_4.6H_2O$ | N6 | 1.98 | 0.198 |
| 3- KBr | K2 | 1.2 | 0.12 | | 10- $Cr(NO_3)_3.9H_2O$ | C5 | 0.41 | 0.041 |
| 4- KI | K3 | 0.83 | 0.083 | | 11- $V_2O_5$ | V1 | 0.089 | 0.0089 |
| 5- $ZnSO_4.7H_2O$ | Z1 | 2.87 | 0.287 | | 12- $Al_2(SO_4)_3K_2SO_4.24H_2O$ [b)] | A3 | 4.74 | 0.474 |
| 6- $Cd(NO_3).4H_2O$ | C2 | 1.55 | 0.155 | | 13- $H_3BO_3$ | H1 | 3.1 | 0.31 |
| 7- $Co(NO_3)_2.6H_2O$ | C4 | 1.46 | 0.146 | | 14- $MnSO_4.4H_2O$ [c)] | M3 | 2.23 | 0.223 |

a. Reagent numbered as N11 is $NaWO_3.2H_2O$
b. Reagent numbered as A3 is $Ka(SO_4)2.12H_2O$, the amount to weight should be the double indicated.
c. Reagent numbered as M3 is $MnSO_4$ mono and not tetra hydrated