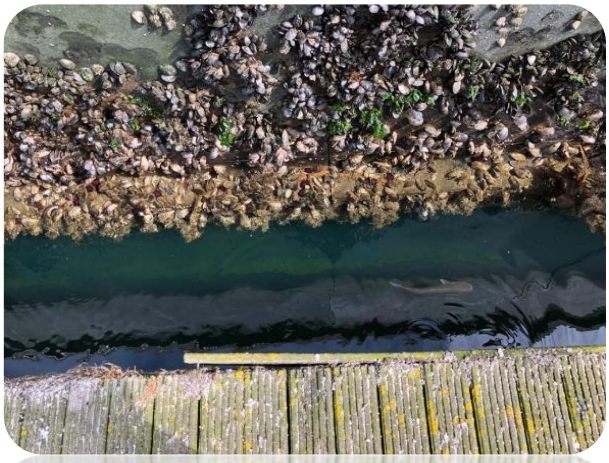

Exploring microalgal derivatives for antifouling application: bioactivity and ecotoxicity

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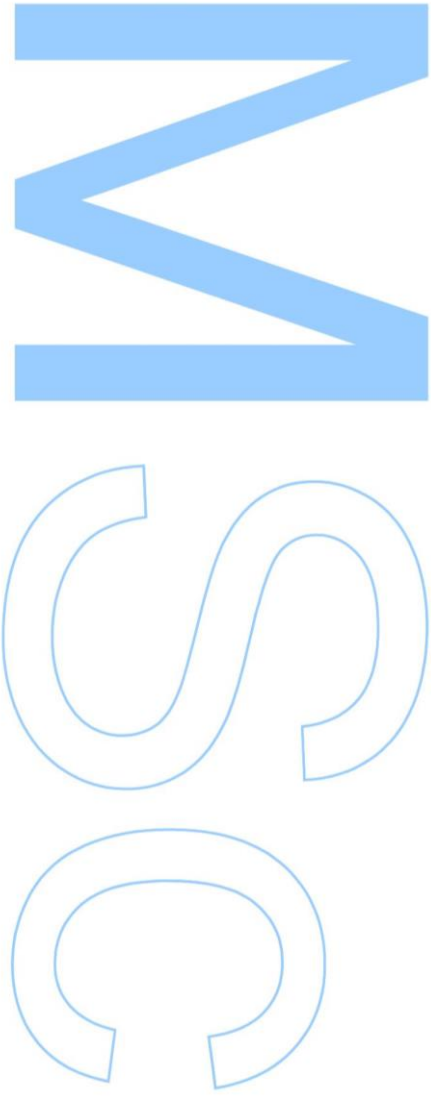

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Resumo

A bio-incrustação marinha representa um enorme desafio para as indústrias marítimas e é também uma séria ameaça ecológica, sendo assim um problema que merece atenção urgente da comunidade científica. Os produtos naturais marinhos possuem estruturas químicas únicas que lhes fornecem uma ampla variedade de atividades biológicas que podem ter diversas aplicações biotecnológicas. Adicionalmente, os produtos naturais extraídos de organismos marinhos já mostraram ser alternativas anti-incrustantes promissoras. Não obstante, o potencial anti-incrustante de produtos naturais extraídos de microalgas tem vindo a ser negligenciado. O facto de os novos produtos naturais serem encontrados principalmente em microrganismos, de que algumas substâncias encontradas em microrganismos já mostraram ter propriedades anti-incrustantes, e o facto de os microrganismos fotossintéticos serem altamente diversos, tornam as microalgas boas candidatas para a bioprospecção anti-incrustante sustentável e ecológica.

Este trabalho pretendeu explorar novas substâncias com potencial anti-incrustante, a partir de 14 estirpes de microalgas disponíveis na coleção LEGE-CC, no CIIMAR. Foram produzidos extratos metanólicos, que em seguida foram fracionados e testados *in vivo* por meio de bioensaios anti-fixação de larvas, anti-bacterianos e anti-diatomáceas. As estirpes LEGE 16726M, LEGE 15824M, LEGE 14699M e LEGE 19954M mostraram ter componentes bioactivos que inibiram exclusivamente a fixação das larvas de *Mytilus galloprovincialis*. Todas as estirpes, exceto a estirpe LEGE 191004M, mostraram ter alguma atividade exclusivamente contra o crescimento de bactérias marinhas. Outras estirpes apresentaram frações que inibiram significativamente o crescimento de bactérias e diatomáceas: estirpe LEGE 191004M (fração F), estirpe LEGE 19954M (fração F) e estirpe LEGE 16866M (frações E e F).

A fração E da estirpe LEGE 15824M e a fração C da estirpe LEGE 14699M foram ambas capazes de inibir a fixação de larvas de *M. galloprovincialis*, inibir o crescimento bacteriano, e inibir o crescimento de diatomáceas, mostrando ainda baixa ecotoxicidade para a espécie não-alvo *Artemia salina*. Estas frações, consideradas as mais promissoras tendo em conta a sua ação abrangente contra vários níveis da comunidade incrustante, demonstram grande potencial biotecnológico como agentes ativos de novos revestimentos marinhos anti-incrustantes sustentáveis.

Palavras-Chave: Bioincrustação, Agentes Anti-incrustantes, Produtos Naturais, Microalgas, Bioensaios

Abstract

Marine biofouling is an economic hardship for maritime-based industries and also an ecological threat, making it a challenge for science and research. Marine natural products have unique chemical structures that provides them with a wide range of biological activities for various biotechnological applications, and these natural products extracted from marine organisms have already proven to be promising antifouling alternatives. However, the antifouling potential of marine microalgae metabolites is still underexplored. Considering that microorganisms are a promising source of new natural products, and that some substances found on microorganisms were already shown to have antifouling properties, plus the fact that photosynthetic microorganisms are highly diverse, we conclude that microalgae are good candidates for sustainable and eco-friendly antifouling bioprospection.

Here, we explored new natural derivatives for antifouling purposes from 14 microalgae strains available in the BBE Group Collection (LEGE-CC), at CIIMAR. Methanolic microalgae extracts were produced, fractioned, and tested *in vivo* through anti-settlement, anti-bacterial and anti-diatom bioassays. Strains LEGE 16726M, LEGE 15824M, LEGE 14699M and LEGE 19954M were proven to have fractions that exclusively inhibited *Mytilus galloprovincialis* larvae settlement. All strains except strain LEGE 1191004M were proven to have fractions that exclusively inhibited bacterial growth. Other strains had fractions that exclusively inhibited bacteria and diatom growth, namely strain LEGE 191004M (fraction F), strain LEGE 19954M (fraction F) and strain LEGE 16866M (fractions E and F).

Fraction E of strain LEGE 15824M and fraction C of strain LEGE 14699M were both capable of inhibiting simultaneously the settlement of *M. galloprovincialis* larvae, bacterial growth, and diatom growth; and also showed no ecotoxicity to the non-target species *Artemia salina*. These fractions were considered the most promising considering their broad range bioactivity against different levels of the biofouling community, thus showing biotechnological potential as new active ingredients for new sustainable antifouling marine coatings.

Keywords: Biofouling, Antifouling, Natural Products, Microalgae, Bioassays

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Abbreviations

AF – Antifouling

BBE – Blue Biotechnology and Ecotoxicology

CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental

IMO - International Maritime Organization

EPS – Extracellular Polymeric Substances

NIS – Non-Indigenous Species

TBT – Tributyltin

nAChR - Nicotinic acetylcholine receptor

HPLC – High performance liquid chromatography

DMSO – Dimethyl sulfoxide

LC – Liquid chromatography

MS – Mass spectrometry

1. Introduction

1.1. Marine biofouling: definition and challenges

Marine biofouling can be succinctly defined as the natural colonization of submerged structures and surfaces in the marine environment (Wahl 1989) (Fig.1), by a wide range of thousands of micro and macro organisms which includes bacteria, microalgae, fungi, protozoa, bryozoans, macroalgae, ascidians, molluscs, and crustaceans (Fig.2) (Satheesh, Ba-akdah et al. 2016, Wang, Wu et al. 2017, Réveillon, Tunin-Ley et al. 2019).

The marine environment is extremely diverse, bursting with life. According to Harder, T. (2009), a single millilitre of seawater, depending on the season of the year and the location from where it's collected, can contain millions of bacteria, viruses, thousands of fungi cells and up to one hundred larvae. Consequently, this makes submerged surfaces greatly exposed to colonization.

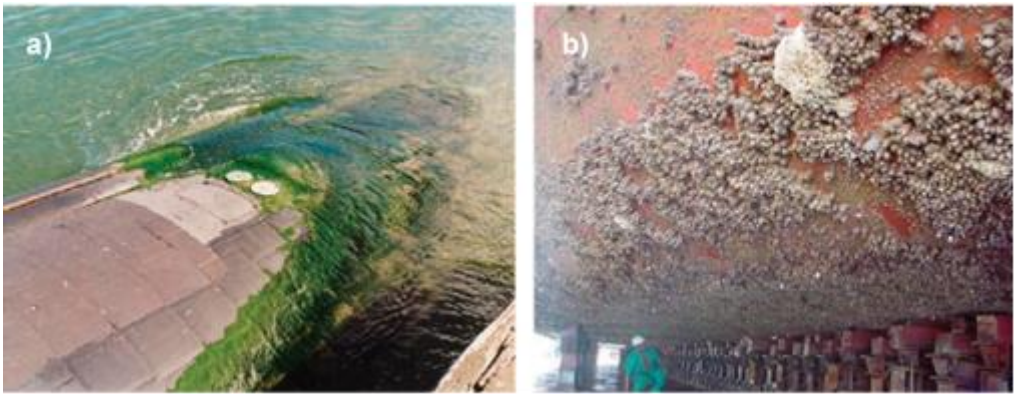


Figure 1- Biofouling examples. a) Biofouling by *Ulva sp*.; Biofouling by barnacles (Callow and Callow 2011)

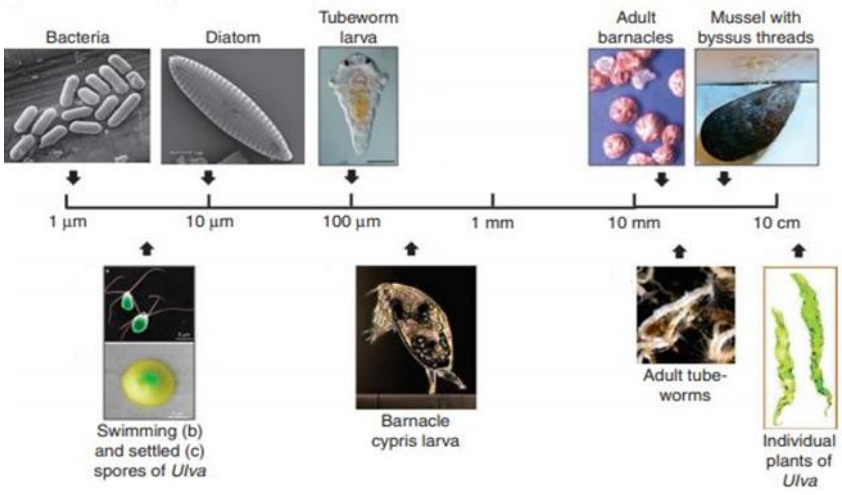


Figure 2- Diversity and comparative sizes of the principal marine biofouling organisms (Callow and Callow 2011)

The establishment of these underwater communities (Fig.3) is a complex process that can be divided into distinct stages, as described by Gu et al. (2020).

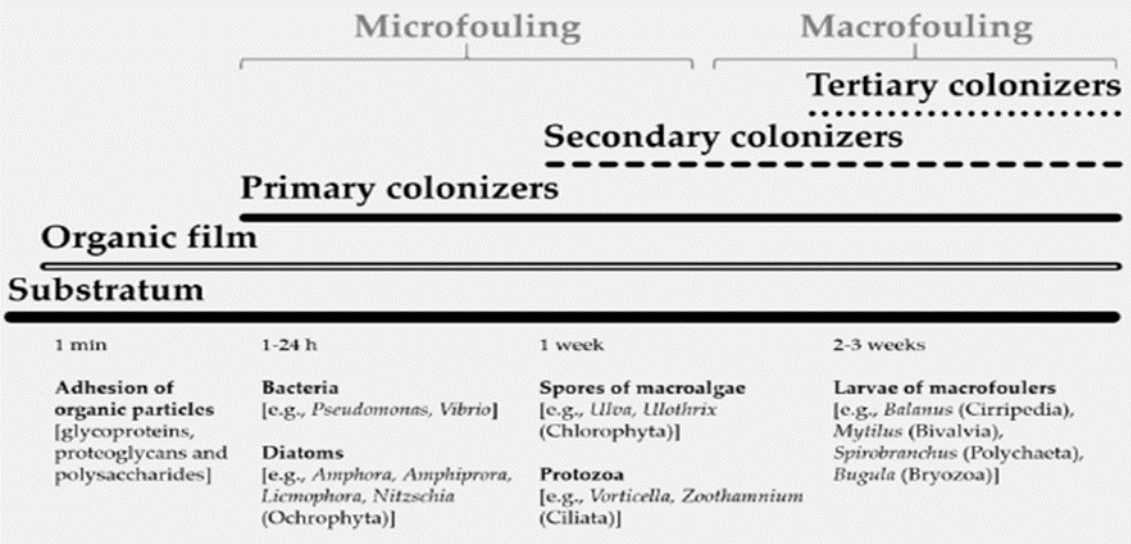


Figure 3- Biofouling establishment (Vinagre, Simas et al. 2020).

As stated, the first colonization stage begins with the formation of the conditioning film. In this stage, after a pristine surface is submerged in water, this surface begins being covered by a thin layer of organic molecules and nutrients, such as proteins, polysaccharides, and lipids (Gu, Yu et al. 2020, Hakim, Lekchiri et al. 2020). The formation of the conditioning film is of the outmost importance, since it's responsible for several changes on the surface's chemical and physical properties, including, for example, the roughness of the surface, its chemical composition, and chemical properties such as hydrophobicity and hydrophilicity (Talluri, Winter et al. 2020), thus conditioning and affecting subsequent microorganism attraction and adhesion.

The conditioning film stage is then followed by the microfouling stage. This stage consists in the adhesion and proliferation of bacteria, microalgae, fungi, and protozoa, the five most important microfouling organisms, on the underwater surfaces (Pradhan, Kumar et al. 2019). Microorganisms adhere to surfaces by different means. For example, bacterial adhesion is influenced by diverse factors. Floating bacterial cells are subjected to factors such as electrostatic interactions, the force of gravity and the flow of water (Pradhan, Kumar et al. 2019), which promotes their reversible adhesion to underwater structures. Additionally, bacteria make use of a variety of extracellular structures (*e.g.* flagella, pili, fimbriae) and extracellular sensorial proteins) to attach to surfaces where a conditioning film was previously formed, a process that is also reversible (Renner and Weibel 2011). However, some fouling microorganisms do not possess the extracellular

structures that bacteria do. In this case, they make use of only gravity and water flow. Finally, the last stage of the microfouling process consists in the irreversible adhesion of microorganisms to the underwater surface. This is achieved by the microorganism secretion of extracellular polymeric substances, also known as EPS. EPS are composed of lipopolysaccharides, lipids, nucleic acids, and proteins (Flemming and Wingender 2010), although its composition varies from specie to specie. The secretion of these substances leads to the creation of a biofilm, which can be defined as a microbial community strongly adhered to a certain surface by a complex network of secreted EPS (Karygianni, Ren et al. 2020). Biofilm characteristics, including its physical properties, biotic constitution, and the chemical signals it produces, have been described as being able to stimulate or inhibit the settlement of a specific macrofouling community (Almeida and Vasconcelos 2015), which makes this stage also crucial for the whole process of biofouling to happen.

Finally, in the macrofouling stage, planktonic invertebrate larvae of benthic organisms (such as molluscs, barnacles, polychaetes, bryozoans, and tunicates) colonize surfaces where a biofilm was previously formed (Agostini, Macedo et al. 2019). Biofilm is also responsible for providing these larvae a safe source of nutrition, promoting their growth and a successful colonization of the submerged surfaces, which can lead to organisms living several years attached to them (Gu, Yu et al. 2020). Additionally, the topology of the surface, its wettability, exposure to light, chemistry, colour of the substrate and streaming conditions are also important factors in determining macroorganism adhesion to surfaces (Almeida and Vasconcelos 2015).

Although its natural origin, marine biofouling represents at the same time a serious ecological and economic threat, being capable of jeopardizing several industries and ecosystems. In fact, biofouling of container-ship hulls, cruises and recreational boats is one of the most prominent factors of non-indigenous species (NIS) introduction to new environments. As pertinently stated by Parretti, Canning-Clode et al. (2020), the establishment of transcontinental maritime routes makes it possible for several organisms to disperse all around the world (Fig.4), thereby enabling these invasive species to cause significative changes on native species habitats, number of individuals, changes on the ecological communities and interfering with the cycle of nutrients.

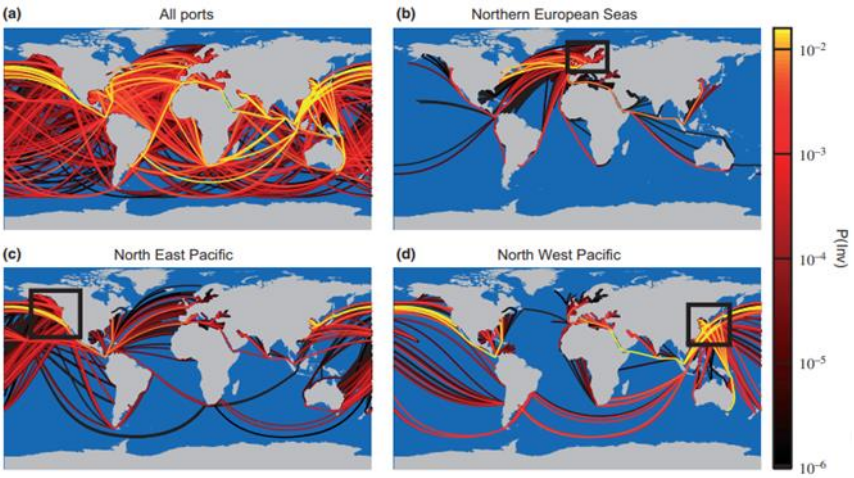


Figure 4- Principal pathways of invasive species introduction to new habitats. Colour coding indicates invasion probability (Seebens, Gastner et al. 2013)

Biofouling of ship hulls also contributes to an increase of the hull's roughness, which promotes a phenomenon called hull drag. Hull drag increases fuel consumption and, consequently, the emission of several harmful greenhouse gases, like carbon dioxide (CO_2), carbon monoxide (CO) and sulphur dioxide (SO_2) (Saha, Goecke et al. 2018, Agostini, Macedo et al. 2019), which can have a real contribution to climate change (Fig.5).

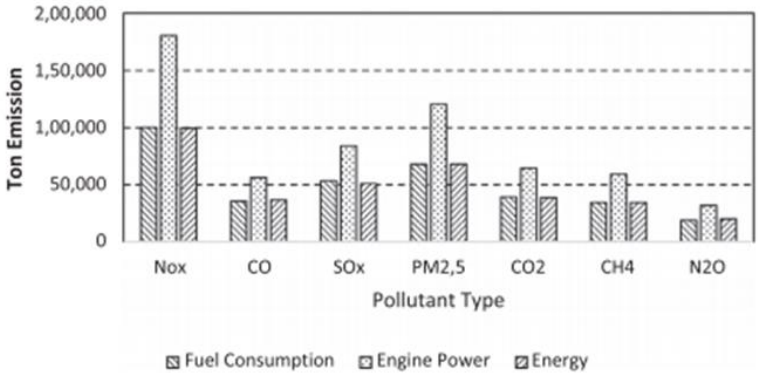


Figure 5- Total amounts of NO_x, SO_x, PM2.5, CH_4 and N_2O emitted by a nine-ship fleet during a three year period using three different estimation methods (Bilgili and Celebi 2018).

Additionally, marine biofouling can increase the corrosion and erosion of submerged surfaces, being also responsible for pipeline clogging (Fig.6) (Ozkan and Berberoglu 2013). Another interesting implication of biofouling is its contribution on increasing the weight of structures like oil platforms, making them less resistant to earth quicks and tsunamis (Gu, Yu et al. 2020).

All of these problems and damages caused by marine biofouling can cost more than one hundred billion euros per year to repair and solve, a very significant value (Chapman, Hellio et al. 2014), hence causing major economic impacts on several maritime-based industries. For this reason, the development of antifouling (AF) strategies to combat the previously mentioned adversities is of the outmost importance.

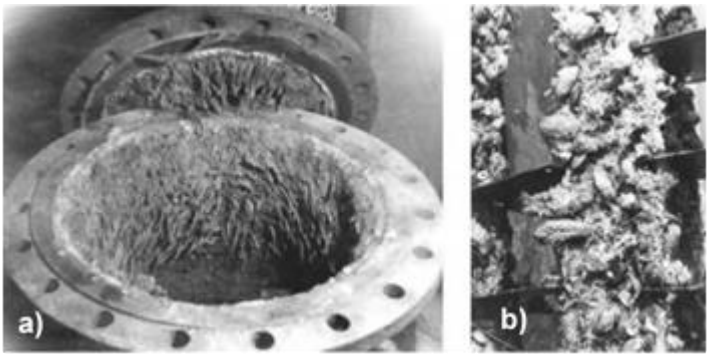


Figure 6- Biofouling on pipes (a) and in another submerged structure (b)(Railkin 2003).

1.2. Antifouling Strategies: A brief history of antifouling coatings

Since the ancient Greeks and Romans, mankind has tried to prevent marine biofouling by using diverse antifouling strategies, such as coating ships with lead sheets and using copper nails to put them into place (Readman 2006). At the end of the nineteenth century, paints with antifouling properties started to appear. Copper-based paints and mercury-based paints were among the first being used for antifouling purposes with relative success, having, however, showed dubious efficacy, durability, as well as presenting a real ecological and health threat, by promoting metal accumulation on living organisms and the pollution of the marine environment with toxic substances (Abioye, Loto et al. 2019, Gu, Yu et al. 2020).

The next chemical compound to be widely used as an antifouling substance added to paints was tributyltin (TBT) (Fig. 7), in the end of the twentieth century. Although this substance was reportedly a powerful biocide, it also represented, in several ways, a real threat. TBT was shown to be toxic to non-target organisms, causing deleterious effects on marine ecosystems. Tributyltin acted as an endocrine disruptor, causing a phenomenon known as imposex (Abioye, Loto et al. 2019, Martínez, Codina et al. 2020), which can be described as the development of male genitalia by female gastropods due

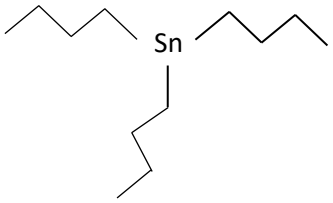


Figure 7- Tributyltin chemical structure.

to their exposition to pollutants. Because of this, TBT was banned for antifouling purposes in 2008 by the International Maritime Organization (IMO).

Substances with biocidal capability like irgarol, chlorothalonil, dichlofluanid, and diuron were also used in paints, until being proved that they were damaging as well (Chapman, Hellio et al. 2014, Saha, Goecke et al. 2018). Additionally, acrylic, fluoride, silicate, or silicone-based paints were also used for antifouling purposes, but all of these materials have adverse effects on the marine environment because of the pollutants they release and/or are not economic efficient (Coneski, Weise et al. 2013, Azemar, Faÿ et al. 2015, Abioye, Loto et al. 2019).

Consequentially, the discovery of novel environmental-friendly antifouling substances is an emerging and promising field of studies since there is an urgent need to find more sustainable alternatives to biofouling control.

1.3. Aquatic Natural Products – Applications and Prospection for Antifouling Purposes

Aquatic natural products are known to usually show a broad range of biological activities due to their unique chemical structures (Hu, Chen et al. 2015, Réveillon, Tunin-Ley et al. 2019). In fact, multiple studies have proven that substances extracted from organisms such as macroalgae, bacteria, cyanobacteria, microalgae, sponges, cnidarians, bryozoans, molluscs, tunicates, and echinoderms show a wide range of possible biotechnological applications (Carroll, Copp et al. 2020).

As a matter of fact, aquatic natural products were demonstrated to have, for instance, anti-obesity and antihypertensive activity (Seca and Pinto 2018), allelopathic activity (Sudatti, Duarte et al. 2020), anticancer activity (Khalifa, Elias et al. 2019), and were even studied for a possible use in the cosmetic industry (Alves, Sousa et al. 2020). Microorganisms are a great source of aquatic natural compounds (Fig.8), and in 2017, newly discovered natural products extracted from marine microorganisms like cyanobacteria, marine bacteria and microalgae represented 57% of the total discovered compounds (Carroll, Copp et al. 2020).

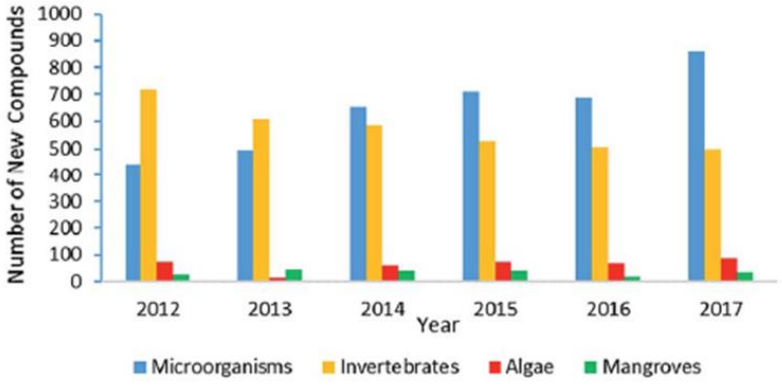


Figure 8- Number of new marine natural products found from 2012-2017 (Carroll, Copp et al. 2020)

In particular, certain molecules, such as terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides, and lactones, extracted from a large range of marine organisms were confirmed to show antifouling activity (Wang, Huang et al. 2017, Salama, Satheesh et al. 2018, Antunes, Pereira et al. 2019, Réveillon, Tunin-Ley et al. 2019, Darya, Sajjadi et al. 2020) and, to date, numerous molecules have shown varying degree of activity against a wide range of biofouling organisms, being capable of interfering with the various stages of marine biofouling (Réveillon, Tunin-Ley et al. 2019).

1.4. Microalgae-derived antifouling substances

Among microorganisms, microalgae have been shown to be a promising source of compounds. In one hand, photosynthetic microorganisms are highly diverse, and occupy a wide range of habitats, both marine and freshwater (Barra, Chandrasekaran et al. 2014). This high diversity can, in fact, be indicative of a wide range of different natural products waiting to be discovered and studied regarding their antifouling capacity. Also, promising compounds can be easily and sustainably obtained in a short period of time via large-scale controlled cultures (Réveillon, Tunin-Ley et al. 2019). Nevertheless, the antifouling potential of substances extracted from microalgae has long been neglected.

In the last years, several new compounds were identified with different attributed activities that are associated with antifouling properties. A few examples of these described antifouling compounds are given in Table 1.

Table 1- Examples of antifouling compounds extracted from microalgae.

Compound	Source Organism	Target Organisms	Antifouling activity	References
13-desmethyl spirolide-C	*Alexandium ostenfeldii*	*C. savignyi (1);* *M. galloprovincialis (2);* *S. caraniferus (3);*	(1,3) inhibition of larvae settlement and metamorphosis; (2) inhibition of larvae and embryo development;	(Hauser, Hepler et al. 2012, Brooke, Cervin et al. 2018)
portimine	*Vulcanodinium rugusom*	*C. savigny (1)i;* *M. galloprovincialis (2);* *S. caraniferus (3);* *A. improvisus (4);*	(1,3,4) inhibition of larvae settlement and metamorphosis; (2) inhibition of larvae and embryo development;	(Selwood, Wilkins et al. 2013, Brooke, Cervin et al. 2018)
pinnatoxin-F	*Vulcanodinium rugosum*	*C. savignyi (1);* *M. galloprovincialis (2);* *S. caraniferus (3);*	(1,3) inhibition of larvae settlement and metamorphosis; (2) inhibition of larvae and embryo development;	(Selwood, Miles et al. 2010, Brooke, Cervin et al. 2018)
gymnodimine-A	*Karenia selliformis*	*C. savignyi (1);* *M. galloprovincialis (2);* *S. caraniferus (3);* *A. improvisus (4);*	(1,3,4) inhibition of larvae settlement and metamorphosis; (2) inhibition of larvae and embryo development;	(Hauser, Hepler et al. 2012, Brooke, Cervin et al. 2018)

Gymnodimine-A is a powerful phycotoxin produced by the microalgae *Karenia selliformis*, that is responsible for the contamination of various types of shellfish and causing food poisoning (Kharrat, Servent et al. 2008). When tested against the tunicate *C. savignyi,* the tubeworm *S. caraniferus* and the bay barnacle *A. improvisus*, this toxin was capable of interfering with normal larvae development, metamorphosis, and settlement. Regarding *M. galloprovincialis*, gymnodimine-A was capable of inhibiting larvae and embryo development. Portimine, also a toxin, produced by *Vulcanodinium rugosum,* was shown to have the same antifouling effects. *V. rugosum* also produces the toxin pinnatoxin-F, known for being accumulated in shellfish (Selwood, Wilkins et al. 2013), that was also capable of interfering with normal larvae development, metamorphosis, and settlement of *C. savignyi, S. caraniferus* and *A. improvisus. Alexandium ostenfeldii*, a species of microalgae found in phytoplankton blooms (Hauser, Hepler et al. 2012) produces 13-desmethyl spirolide-C, a toxin that showed the same antifouling capacity as pinnatoxin-F. All these compounds, spirolides, pinnatoxins, gymnodimines and portimines, belong to the cyclic imines group, and are capable of blocking nicotinic acetylcholine receptors (nAChRs) (Selwood, Wilkins et al. 2013).

Compounds in Table 1 only target organisms responsible for the macrofouling stage. As previously stated, information about antifouling compounds extracted from microalgae is scarce and there is a real need of research of new antifouling substances extracted from these group of microorganisms and that are capable of inhibiting marine biofouling in all its stages.

1.5. Antifouling bioassays

To access the antifouling potential of a certain substance, several bioassays might be conducted to cover the ability of the substance in study to inhibit marine biofouling at different stages, including microfouling and macrofouling (Almeida and Vasconcelos 2015). Additionally, a broad number of organisms is involved on the marine biofouling process. Consequently, the diversity of organisms tested in these bioassays should reflect, at a certain level, the diversity of the marine biofouling community (Briand 2009).

To evaluate the capability of a certain compound to inhibit the microfouling process, bioassays are performed using organisms involved in the biofilm production, marine bacteria, and diatoms (Balqadi, Salama et al. 2018, Pradhan, Kumar et al. 2019). These bioassays might evaluate microorganism growth (Antunes, Pereira et al. 2019), capacity of attachment and adhesion strength (Briand 2009).

On the other hand, to evaluate the capability of a certain compound to inhibit the macrofouling process, bioassays are performed using prominent biofouling macroorganism, such as macroalgae (De Nys, Steinberg et al. 1995), barnacles (Feng, He et al. 2018), mussels (Almeida, Correia-da-Silva et al. 2017), tunicates, tubeworms (Brooke, Cervin et al. 2018), and other invertebrates. For anti-macrofouling bioactivity, bioassays are used to determine how antifouling substances are capable of interfering with larvae settlement, adhesion strength, behaviour, spore settlement (in the case of macroalgae), and at the same time determine the toxicity of the studied substance (Briand 2009).

Together, these bioassays help to enlighten the way that these potential antifouling substances act on a wide range of biofouling organisms. Nonetheless, marine biofouling is a very complex process, that it’s not possible to completely recreate in a laboratory-controlled environment, and it is important to note that the knowledge obtained from these bioassays is not completely representative of a real *in situ* situation. So, for a full assessment of the efficacy and suitability of a new antifouling substance, a field proof-of-concept test in the marine environment is needed.

2. Aims and Work Approach

Considering the need for more sustainable and effective antifouling products, in this work, a set of underexplored freshwater microalgae strains available in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC), at CIIMAR (Interdisciplinary Centre of Marine and Environmental Research) was explored for antifouling potential, considering the potential of natural products extracted from microorganisms against marine biofouling at different levels. For that purpose, microalgal biomass was produced by cultivating the several microalgae strains using scale-up techniques, and the dried biomass was used to prepare extracts by methanolic extraction and fractions using HPLC techniques. The antifouling activity of the produced microalgal extracts and fractions was tested against prominent microfouling and macrofouling species, namely marine bacteria and diatom strains and the adhesive larvae of the mussel *Mytilus galloprovincialis*. The most promising antifouling fractions were subjected to chemical elucidation and ecotoxicity assays.

To reach this main objective, following the described work approach, specific objectives were established:

- Microalgal strains culture, according to the procedures of BBE Guidelines for microorganismal strains cultivation Standard Operational Procedure (SOP), and biomass processing;
- Production of microalgal fractions by methanolic extraction;
- Screening for antifouling bioactivity of the obtained microalgal fractions using antibacterial, anti-microalgal and anti-settlement bioassays;
- Evaluate the potential ecotoxicity of the most promising antifouling strains against non-target species;
- Analyse the most promising fractions by LC-MS to enlighten chemical evidences on fractions constituents.

3. Materials and Methods

3.1. Microalgal culture and biomass production

A set of 14 microalgal strains from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) was selected using diversity and growth conditions criteria. The 14 used microalgal strains belong to the *Chlorophyta* and *Ochrophyta* phyla and were sampled from 2014 to 2019 from freshwater environments in Portugal, except one, that was sampled from a terrestrial environment (Appendix I). Microalgal biomass was obtained by cultivating each of the 14 studied microalgae species on 50mL, 500 mL and 5 L volumes, consecutively, on Z8 medium (Kotai 1972). Each microalgae strain grew during a one-month period in each of the growing stages, at a temperature of 24ºC, under a fluorescent lamp light (photoperiod of 16 hours of light followed by 8 hours of darkness), with aeration on the last stage of growth (5L).

3.2. Methanolic extraction and Fractioning

Microalgal biomass of each strain was collected using a sieve or by centrifugation (*Megafuge*™ 16, *Thermo Fisher*) at 6000 g, for 10 minutes, at 4ºC. The collected biomass was frozen and lyophilized.

The freeze-dried biomass was grinded using a mortar and pestle, transferred to an Erlenmeyer flask, with 50 mL of methanol and underwent through an ultrasonic bath for 5 minutes, not allowing the temperature of the flask to surpass the 30ºC mark. After that, the Erlenmeyer flask was left resting for a few minutes, allowing for the cellular debris produced by the ultrasonication step to settle down on the bottom of the flask. Finally, the supernatant was decanted to a vacuum filtering system (Fig. 9). This process was repeated two additional times, using 25 mL of methanol. The methanolic extract collected in the round-bottom flask was then concentrated in a rotary evaporator, at 30ºC, and transferred to a pre-weighted 20 mL vial flask.

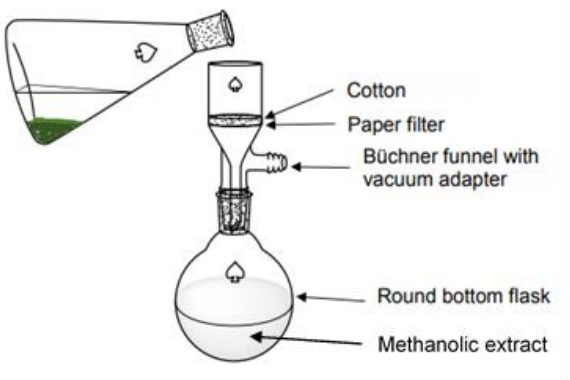


Figure 9- Vacuum filtering system

The remaining methanol was evaporated using a rotary evaporator and, finally, the vial was left in high vacuum conditions to dry completely. After that, each methanolic extract was separated into 8 fractions by high performance liquid chromatography (HPLC). For HPLC, 40 mg of methanolic extract were transferred to an empty 2 mL glass vial, with 1 mL of LC-MS grade methanol, homogenized using a vortex and a sonicator. The HPLC separation of the methanolic extract was done on the Water Alliance e2695 system, and fractions were collected in 48-deep well plates. The obtained fractions were stored in the freezer, lyophilized, and the remaining content of each well was dissolved in DMSO (dimethyl sulfoxide) at a 5 mg/mL concentration (extracts stock solutions).

3.3. Antifouling Bioassays

3.3.1. Mussel larvae anti-settlement bioassay

In order to access each fraction ability to interfere with the macrofouling stage, anti-settlement bioassays were performed. Mussel juveniles (*M. galloprovincialis*) aggregates were collected during low tide periods at Memória beach, Matosinhos, Portugal (41°13'50.5"N 8°43'17.7"W) and transported to the laboratory in controlled conditions. Healthy mussel larvae showing a functional foot and exploratory behaviour were selected and isolated from the collected aggregates using a binocular magnifier.

After selection, larvae were exposed to each of the obtained fractions, at a concentration of 10 µg/mL. Bioassays were conducted using 24-well microplates with 4 replicates (4 wells) per condition and 5 larvae per well. Test solutions were prepared using filtered seawater. A negative control, containing filtered seawater and DMSO (10 µg/mL) and a positive control (containing $CuSO_4$ (10 µg/mL), a powerful antifouling substance) were included. The bioassay was conducted for 15h, at 18ºC, in the darkness. After the 15h incubation period, the anti-settlement activity of the fractions was evaluated by accessing the presence or absence of byssal threads produced by each individual mussel larvae.

3.3.2. Anti-bacterial bioassay

For this bioassay, five strains of marine fouling bacteria from the Spanish Type Culture Collection (CECT): *Cobetia marina* (CECT 4278), *Vibrio harveyi* (CECT 525), *Roseobacter litoralis* (CECT 5395), *Halomonas aquamarina* (CECT 5000), and *Pseudoalteromonas atlantica* (CECT 570) (Almeida, Correia-da-Silva et al. 2017) were used as target microfouling species. The bacteria were inoculated in Marine Broth medium at an initial density of 0.10-0.14 (OD_{600}), and were then incubated for a 24h period, at a temperature of 26ºC, in 96-well plates (4 replicates per condition) with the microalgal fractions at a concentration of 100 µg/mL. Positive and negative controls were

used in this bioassay, containing, respectively, a penicillin-streptomycin-neomycin stabilized solution (100 µg/mL), an antibiotic solution, and DMSO (100 µg/mL). Bacterial growth in the presence of the compounds was measured by reading the optical density at OD_{600}.

3.3.3. Anti-diatom bioassay

Fractions that significantly inhibited bacterial growth in more than 40%, that inhibited bacterial growth and larvae settlement at the same time, and a fraction that inhibited completely larvae settlement were tested in this bioassay. For this bioassay, it was used a well-known marine biofouling diatom species, *Navicula sp*, purchased from the Spanish Collection of Algae. The diatoms were inoculated in f/2 medium at an initial diatom concentration of around $2–4\times10^6$ cells ml^{-1}, being counted using a Neubauer Improved counting chamber. They were grown in 96 well flat-bottom plates for 10 days at a temperature of 20ºC, with the microalgal test fractions at a 100 µg/mL concentration (4 replicates per condition). Differences between cell densities among treatments were counted with Neubauer Improved counting chambers. The negative and positive controls were, respectively, f/2 medium with DMSO (100 µg/mL) and cycloheximide (100 µg/mL).

3.3.4. Ecotoxicity bioassays – *Artemia salina*

The standard ecotoxicity test evaluating *Artemia salina* nauplii mortality was used to determine the toxicity of the most promising tested fractions (Fraction E of strain LEGE 15428M and fraction C of strain LEGE 14699M) to non-target marine organisms. First, *A. salina* eggs were allowed to hatch in nutrient-enriched seawater for a 48h period, at 25ºC. The bioassay was then carried out in 96-well plates, using 10-15 *A. salina* nauplii per well. Each fraction was tested at a 10 µg/mL concentration, using test solutions prepared with filtered seawater. The bioassay included potassium dichromate ($K_2Cr_2O_7$) (13,6 µM) as positive control and DMSO (10 µg/mL) as negative control. The bioassays ran in the dark, at 26° C, and mortality was accessed after 24h and 48h of exposure.

3.4. LC-MS analysis and Molecular Networking

Fraction E of strain LEGE 15428M and fraction C of strain LEGE 14699M were analysed using liquid chromatography and mass spectrometry (LC-MS) techniques and the obtained results were used in the construction of a molecular network. Molecular networking is a bioinformatic tool that gives aid in analysing and interpreting data from mass spectrometry analysis. Using molecular networking, we are able to identify possible similarities among different mass spectra (Vincenti, Montesano et al. 2020). In this approach, processed data is presented in the form of nodes, which can be linked. As stated by Vincenti, Montesano et al. (2020), each node represents an ion, to which is

associated an unique spectrum, and the link between nodes indicates similarity between ions. Fraction E of strain LEGE 15428M and fraction C of strain LEGE 14699M were analysed to enlighten which molecules are found in each fraction.

Raw mass spectrometry files were converted to the (.mzML) format using the MSConvert software. Then, the converted files were uploaded to the GNPS (Global Natural Product Social Molecular Networking) website, a software that contains an online mass spectrometry database and that is capable of analysing mass spectrometry data and organize them according to the molecular networking principles described above. The obtained molecular networks were visualised with Cytoscape.

3.5. Statistical analysis

Datasets from anti-settlement, antibacterial and anti-microalgal bioassays were analysed by one-way analysis of variance (ANOVA) followed by a multi-comparisons Dunnett's test against negative control ($p < 0.01$).

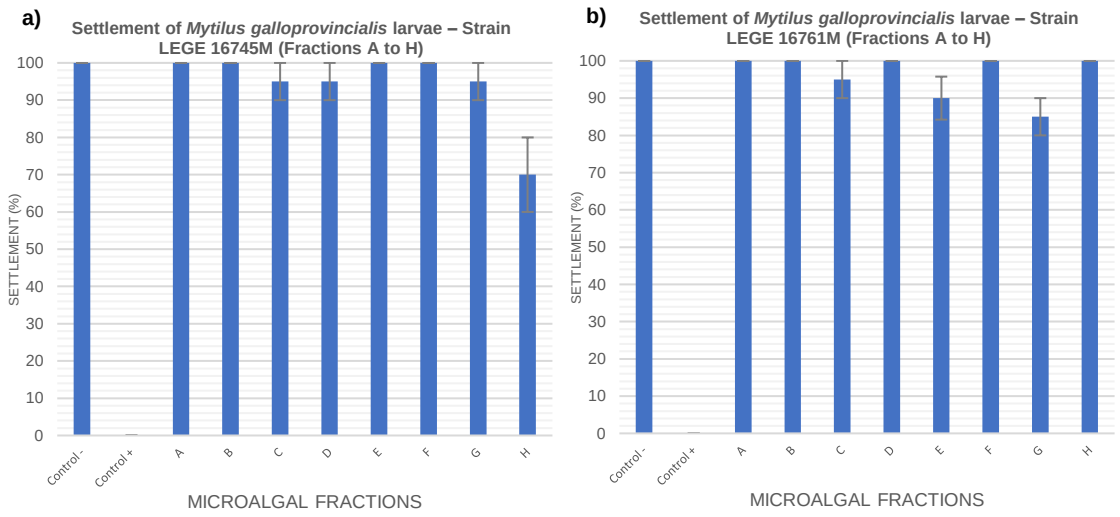
4. Results

4.1. Microalgae production, methanolic extraction and fractioning

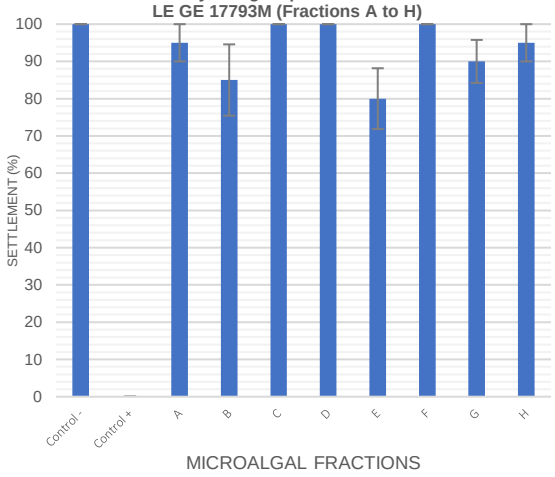
Microalgae culture presented different degrees of success, depending on the cultivated strain. At the end of three months, strains LEGE 16854M, LEGE 16761M, LEGE 16726M and LEGE 14699M produced great quantities of biomass that were easily collected using a sieve. Strain LEGE 16854M was particularly productive in terms of biomass (250 mL of wet biomass). The remaining strains were collected by centrifugation and the quantities of biomass they were capable of producing were, comparatively, considerably smaller (less than 25mL of wet biomass). In this case, biomass sedimented at the end of the falcon tubes in the form of pellets, after centrifugation. Losses of biomass occurred in these cases due to the difficulty of sedimentation of these strains since a portion of the microalgal cells remained in suspension. Methanolic extraction also presented some difficulties. Viscosity of the extracts of several strains interfered with the efficiency of the vacuum filtration, leading to some losses of raw methanolic extract. The 14 produced strains originated 112 different fractions.

4.2. Anti-settlement bioactivity

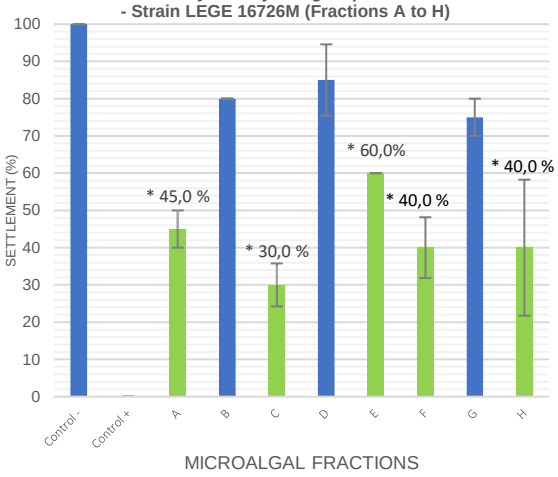
From the 112 fractions belonging to the 14 microalgal strains tested (Fig. 10), 19 demonstrated anti-settlement activity when compared to negative control (filtered seawater) by significantly inhibiting *M. galloprovincialis* adhesive larvae settlement, namely fractions A, C, E, F and H of strain LEGE 16726M (Fig.10d) ; fractions B, E and G of strain LEGE 15824M (Fig.10e); fractions C, D, F and H of strain LEGE 14699M (Fig.10f); fractions B and E of strain LEGE 16854M (Fig.10g); fraction B of strain LEGE 19984M (Fig.10j); fractions A, B and C of strain LEGE 19954M (Fig.10l); fraction G of strain LEGE 17946M (Fig.10m). No mortality was observed in any of the conditions tested including in the positive control.



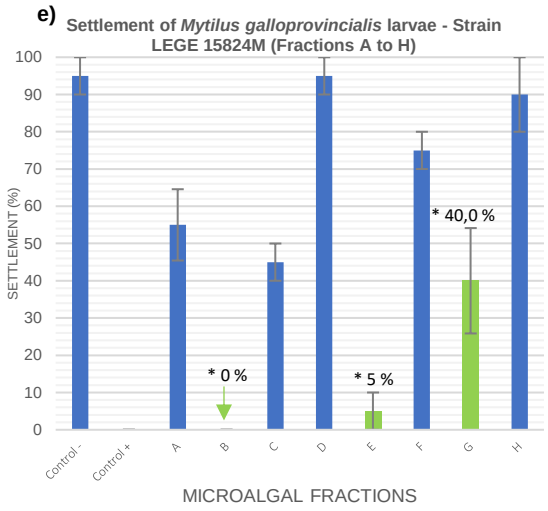
c)


Settlement of Mytilus galloprovincialis larvae - Strain LE GE 17793M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS
Control -
Control +
A
B
C
D
E
F
G
H

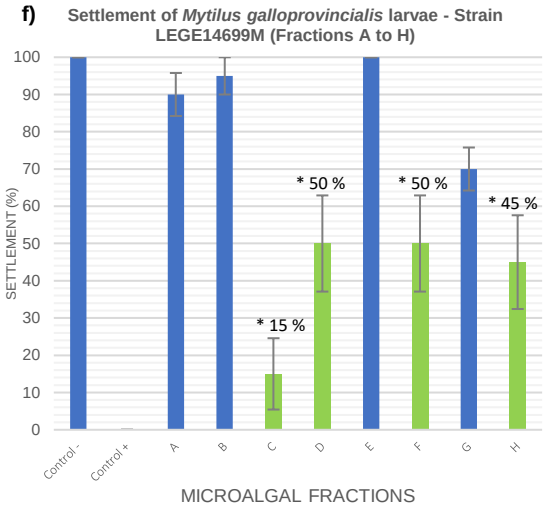
d)


Settlement of Mytilus Mytilus galloprovincialis larvae - Strain LEGE 16726M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS
* 45,0 %
* 30,0 %
* 60,0%
* 40,0 %
* 40,0 %

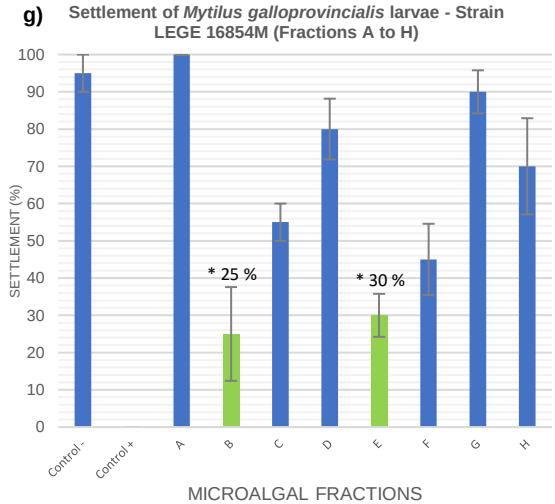
e)


Settlement of Mytilus galloprovincialis larvae - Strain LEGE 15824M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS
* 0 %
* 5 %
* 40,0 %

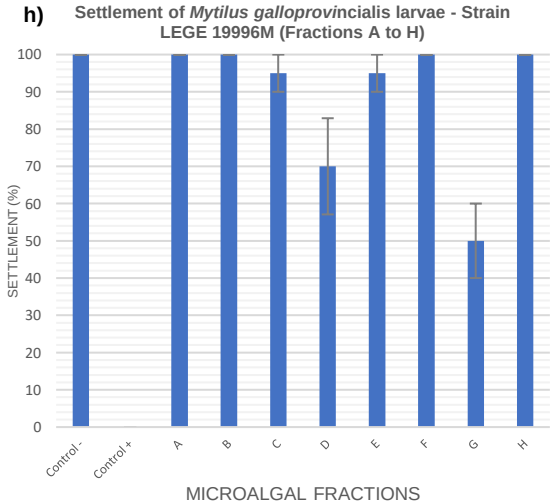
f)


Settlement of Mytilus galloprovincialis larvae - Strain LEGE14699M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS
* 15 %
* 50 %
* 50 %
* 45 %

g)


Settlement of Mytilus galloprovincialis larvae - Strain LEGE 16854M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS
* 25 %
* 30 %

h)


Settlement of Mytilus galloprovincialis larvae - Strain LEGE 19996M (Fractions A to H)
SETTLEMENT (%)
MICROALGAL FRACTIONS

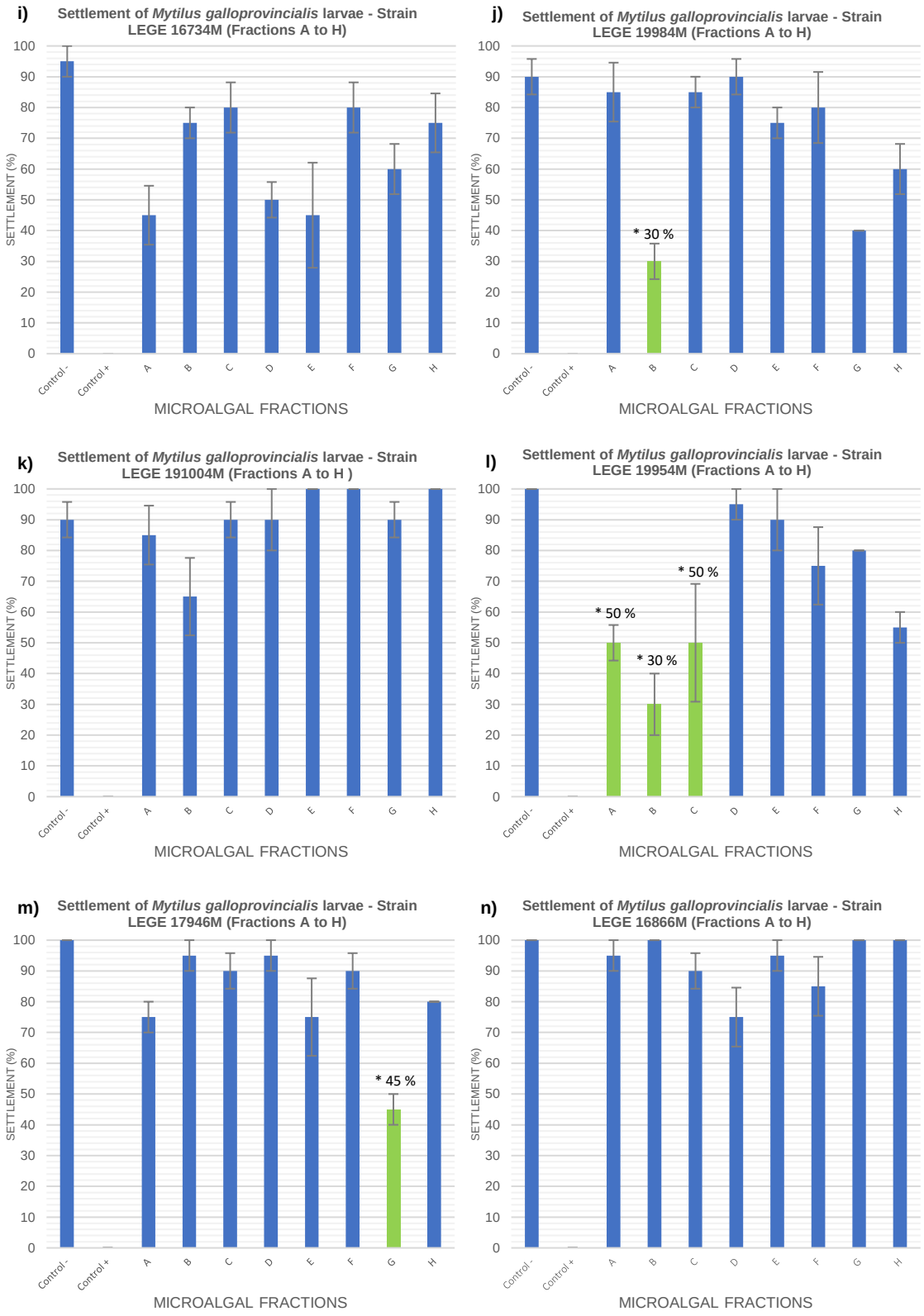


Figure 10 - Anti-settlement activity of the different microalgae strains and fractions (A-H) against *Mytilus galloprovincialis* larvae. a) LEGE 16745M; b) LEGE 16761M; c) LEGE 17793M; d) LEGE 16727M; e) LEGE 15824M; f) LEGE 14699M; g) LEGE 16854M; h) LEGE 19996M; i) LEGE 16734M; j) LEGE 19984M; k) LEGE 191004M; l) LEGE 19954M; m) LEGE 17946M; n) LEGE 16866M. Significantly active fractions are highlighted in green. Control -: filtered seawater; Control +: CuSO4 at 5µM. * indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

4.3. Anti-bacterial bioactivity

Regarding antibacterial bioactivity of the 112 microalgal fractions against the studied marine fouling bacteria *C. marina*, *V. harveyi*, *R. litoralis*, *H. aquamarina* and *P. atlantica*, several significant hits were found. Fractions C (Fig.11b) and F (Fig.11a) of strain LEGE 16745M inhibited in 38% and 51,5% the growth of *V. harveyi* and *C. marina*, respectively; Fraction E (Fig.11a) of strain LEGE 16761M inhibited in 20% the growth of *H. aquamarina*; Fractions E, G (Fig.11a) and F (Fig.11b) of strain LEGE 17793M inhibited in 25%, 48,8% and 25,7% the growth of *H. aquamarina, C. marina* and *V. harveyi*, respectively; Fraction G (Fig. 11a) of strain LEGE 16726M inhibited in 46,4% the growth of *C. marina*.

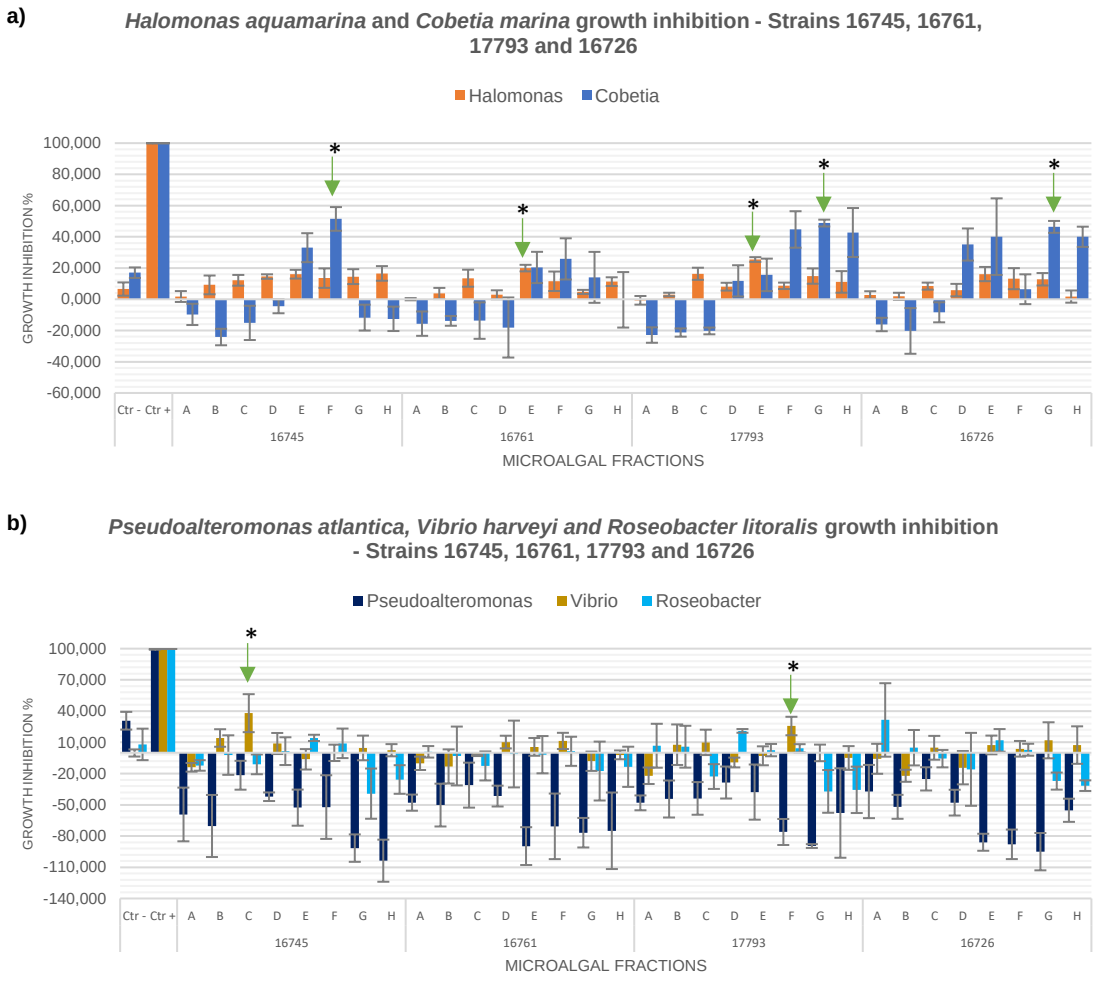


Figure 11- Bacterial growth inhibition – LEGE 16745M, LEGE 16761M, LEGE 17793M and LEGE 16726M. a) *Halomonas aquamarina* and *Cobetia marin*a growth inhibition. b) *Vibrio harveyi*, *Roseobacter litoralis* and *Pseudoalteromonas atlantica* growth inhibition. Significantly active fractions are signalized with a green arrow. Control -: DMSO (2%); Control +: penicillin-streptomycin-neomycin solution (2%). * indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

Fractions D, E and G (Fig. 12a) of strain LEGE 15824M inhibited in 22,7%, 20,9% and 18,8%, respectively, the growth of *H. aquamarina*; Fraction C (Fig. 12b) of strain LEGE 14699M inhibited in 31,3% the growth of *V. harveyi* and fractions E and F (Fig. 12a) of the same strain inhibited the growth of *H. aquamarina* in 27,6% and 19,1%, respectively; Fraction C of LEGE 16854M inhibited the growth of *V. harveyi* (Fig. 12b) in 19,6% and in 24,8% the growth of *H. aquamarina* (Fig.12a), fraction D (Fig. 12b) inhibited the growth of *V. harveyi* in 20% and fraction E (Fig. 12a) inhibited the growth of *H. aquamarina* in 30,2%; Fraction F of strain LEGE 19996M inhibited in 23,8% the growth of *H. aquamarina* and in 49,9% the growth of *C. marina* (Fig.12a).

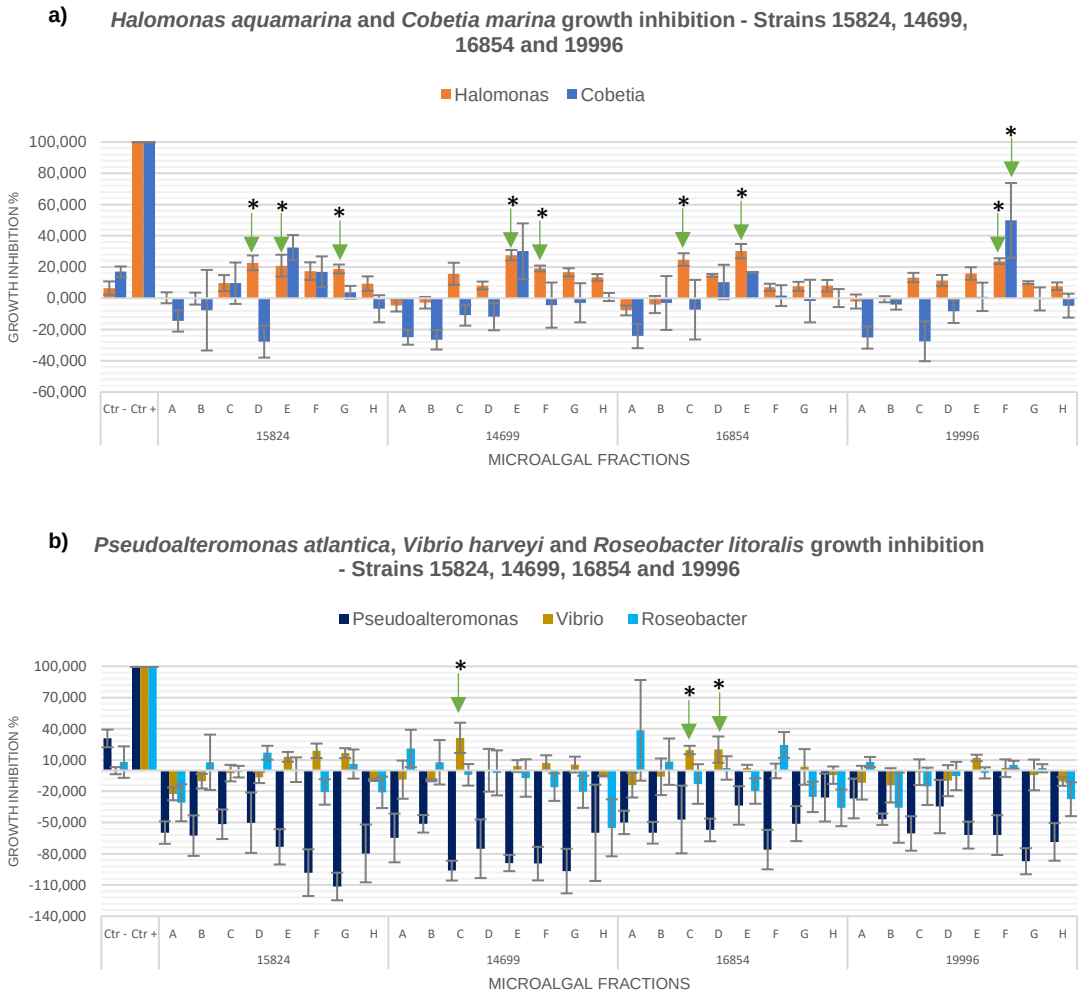


Figure 12- Bacterial growth inhibition – LEGE 15824M, LEGE 14699M, LEGE 16854M and LEGE 19996M. a) *Halomonas aquamarina* and *Cobetia marina* growth inhibition. b) *Vibrio harveyi*, *Roseobacter litoralis* and *Pseudoalteromonas atlantica* growth inhibition. Significantly active fractions are signalized with a green arrow. Control -: DMSO (2%); Control +: penicillin-streptomycin-neomycin solution (2%). * indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

Fraction D (Fig.13a) of strain LEGE 16734M inhibited the growth of *H. aquamarina* in 26,6%. Fraction E of the same strain inhibited the growth of *H. aquamarina* in 25,7%(Fig.13a) and in 79,1% (Fig.13b) the growth of *R. litoralis*. Fractions A, B, C, D, E, F, G and H of strain LEGE 19984M inhibited the growth of *H. aquamarina* in 21,6%, 36,3%, 36,1%, 39,1%, 48,3%, 40,5%, 36,8% and 36,1%, respectively. Fraction F of the same strain was also capable of inhibiting the growth of C. marina in 54.1% (Fig.13a). Fraction F (Fig.13a) of strain LEGE 191004M inhibited the growth of *C. marina* in 53,5%; Fractions E and F (Fig.13a) of strain LEGE 19954M inhibited the growth of *H. aquamarina* in 19,6% and the growth of *C. marina* in 50,7%, respectively.

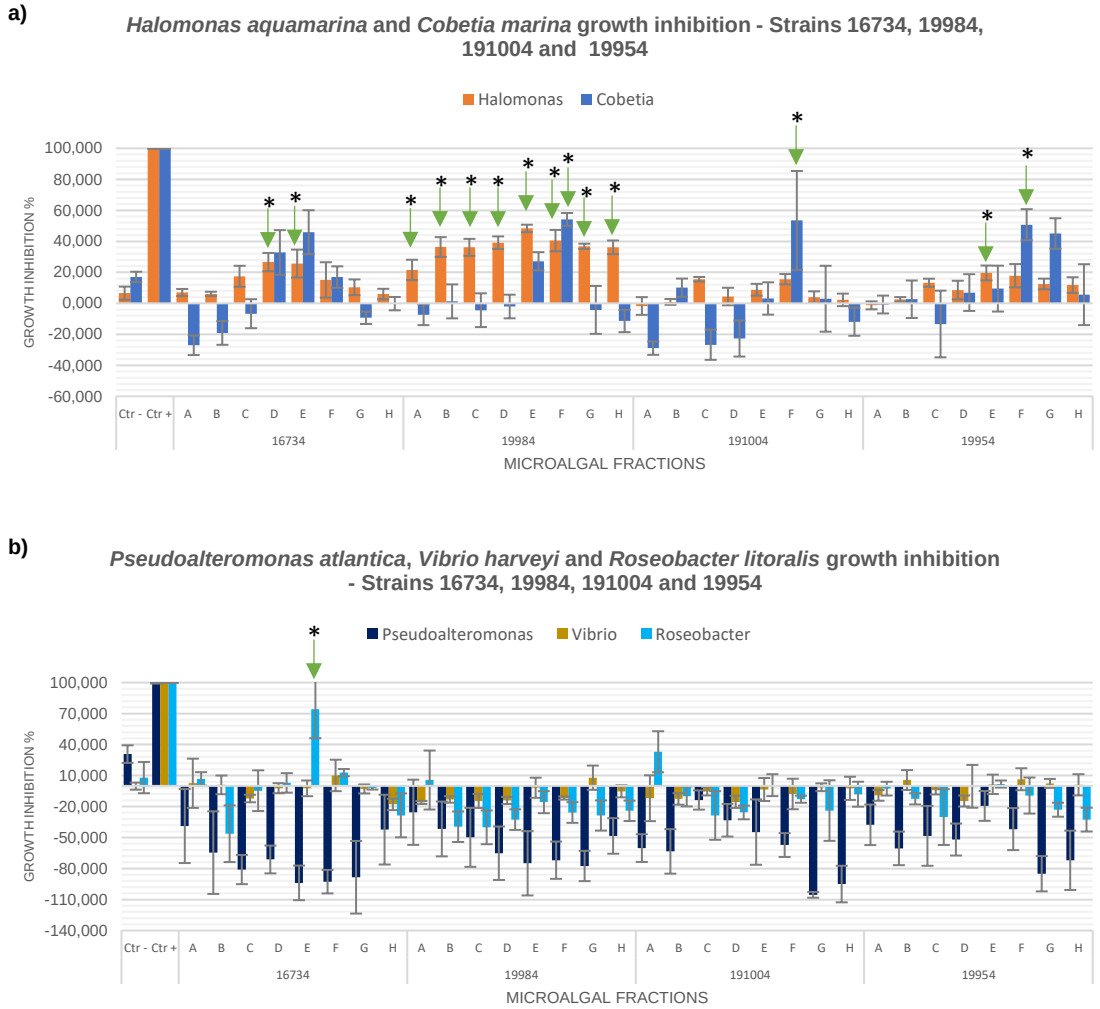


Figure 13- Bacterial growth inhibition – LEGE 16734M, LEGE 19984M, LEGE 191004M and LEGE 19954M. a) *Halomonas aquamarina* and *Cobetia marina* growth inhibition. b) *Vibrio harveyi, Roseobacter litoralis* and *Pseudoalteromonas atlantica* growth inhibition. Significantly active fractions are signalized with a green arrowControl -: DMSO (2%); Control +: penicillin-streptomycin-neomycin solution (2%).* indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

Fractions A, E, F and H of strain LEGE 17946M inhibited the growth of *C. marina* in 40,2%, 34,5%, 34,3% and 35,9%, respectively (Fig.14a). Fractions C and D of the same strain inhibited the growth of *V. harveyi* in 24,4% and 20,5%, respectively (Fig.14b). Fraction E was also capable of inhibiting in 22,1% the growth of *V. harveyi* (Fig.14b).

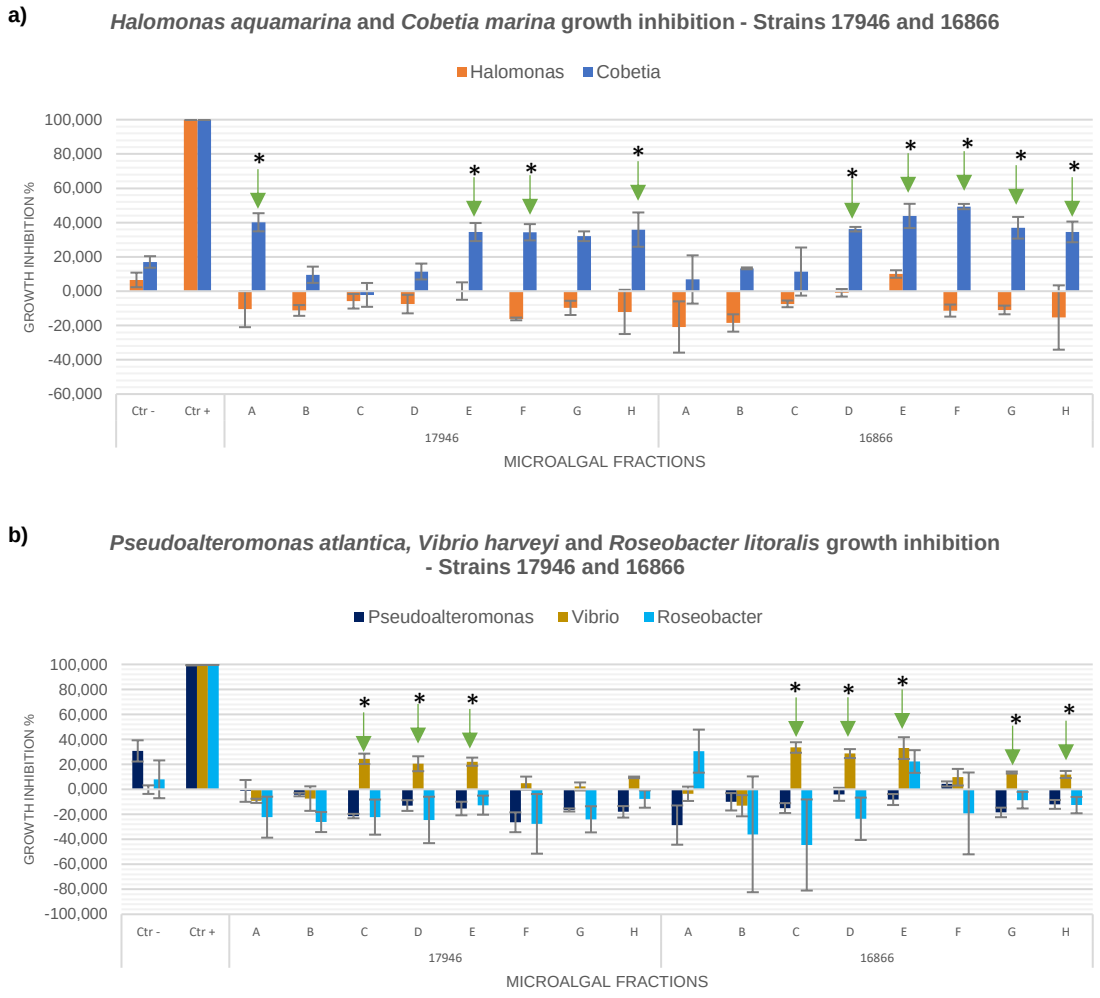


Figure 14- Bacterial growth inhibition – LEGE 17946M, LEGE 16866M. a) *Halomonas aquamarina* and *Cobetia marina* growth inhibition. b) *Vibrio harveyi, Roseobacter litoralis* and *Pseudoalteromonas atlantica* growth inhibition. Significantly active fractions are signalized with a green arrowControl -: DMSO (2%); Control +: penicillin-streptomycin-neomycin solution (2%). * indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

Regarding fraction LEGE 16866M, fraction C was capable of inhibiting the growth of *V. harveyi* in 33,5%. Fraction D inhibited the growth of *C. marina* (Fig.14a) in 36,1% and 28,6% in *V. harveyi* (Fig.14b). Fraction E inhibited the growth of *C. marina* (Fig.14a) in 43,9 % and 33% in *V. harveyi* (Fig.14b). Fraction F was capable of inhibiting the growth of *C. marina* in 49,3% (Fig.14a). Fraction G inhibited the growth of *C. marina* (Fig.14a)

in 37 % and 13,5% in *V. harveyi* (Fig.14b). Fraction H inhibited the growth of *C. marina* (Fig.14a) in 34,6 % and 11,9% in *V. harveyi* (Fig.14b).

None of the fractions were capable of inhibiting *P. atlantica* growth. Thirteen fractions out of the 112 were capable of significantly inhibiting *V. harveyi* growth and only 1 significantly inhibited the growth of *R. litoralis*. Sixteen fractions significantly inhibited the growth of *C. marina* and twenty-one the growth of *H. aquamarina*. All fractions of strain LEGE 19984M inhibited *H. aquamarina* growth.

4.4. Anti-diatom bioactivity

Regarding the 14 tested fractions (Fig. 15), caused significant diatom growth inhibition: Fraction E of LEGE 15824M strain (44%); fraction C of LEGE 14699M strain (24,9%); fraction F of LEGE 191004M strain (25,4%); fraction F of LEGE 19954M strain (58,8%), and fractions E (97,7%) and F (34,7%) of strain LEGE 16866M.

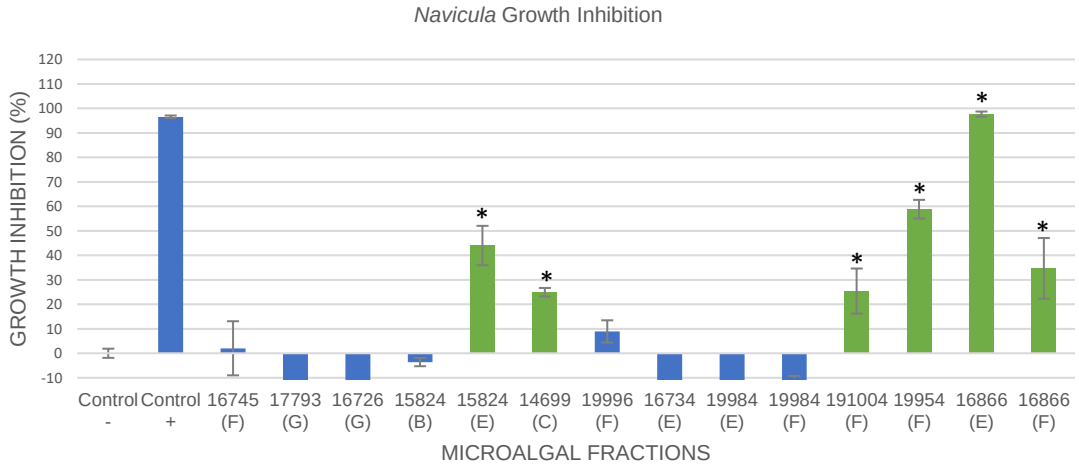


Figure 15-Anti-diatom activity against *Navicula sp*. Significantly active fractions are shown in greenControl -: DMSO (2%); Control +: cycloheximide (2%). * indicates significant differences at $p < 0.01$ (Dunnett test), against the negative control.

4.5. Exploring the most promising fractions

4.5.1. Ecotoxicity

In this bioassay we determined *A. salina* nauplii mortality when incubated with the most promising fractions. The two selected fractions for testing both showed to be non-toxic, as no significant differences were found, when compared to the negative control (Fig.16). Fraction E of strain LEGE 15824M showed an 8,37% mortality rate and fraction C of LEGE 14699M strain a 6,59% mortality rate.

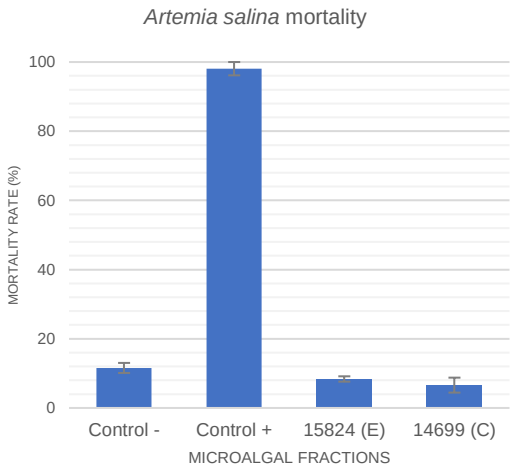


Figure 16- *Artemia salina* mortality.Fraction E of strain LEGE 15824M (8,37%) and fraction C of LEGE 14699M (6,59%) strain. Control -: DMSO in filtered seawater (1%); Control +: $K_2Cr_2O_7$ (13,6 µM).

4.5.2. Spectrometric analysis of the most promising fractions

The chromatograms obtained from the LC-MS analysis of the two most promising fractions (MS filter and UV filter - Fig.17 and Fig. 18) showed some aspects about their chemical composition. The MS filter graphic showed a region of similar retention times between the three different tested samples, the blank sample, fraction E of strain LEGE 15824M and fraction C of LEGE 14699M, the region between 12 and 12,71 minutes. The blank sample and the fraction E of strain LEGE 15824M sample share an exact peak at 7,66 minutes. On the other hand, the UV filter graphic (Fig.18) showed some unique well-defined peaks in fraction E of strain LEGE 15824M: 7,54 min; 11,46 min; 17,17 min; 12,50 min; 14,15 min; 14,54 min. Fraction C of LEGE 14699M strain showed some small peaks at 7,86 min; 8,24 min; 8,68 min; 9,02 min. Molecular networking analysis produced a molecular network of 855 nodes and 75 clusters that deserves further analyses (data not known).

A complete elucidation of this set of results is required.

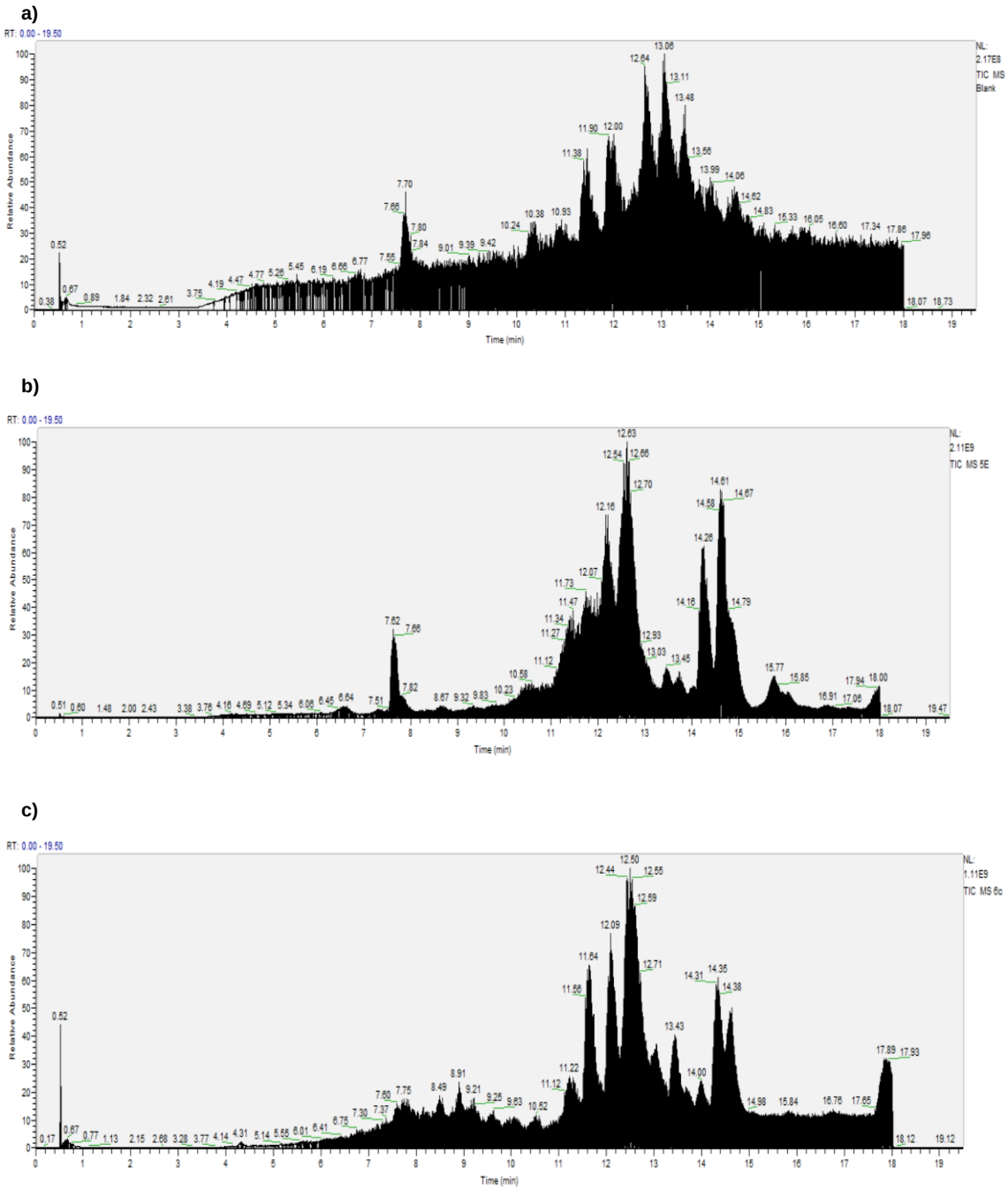
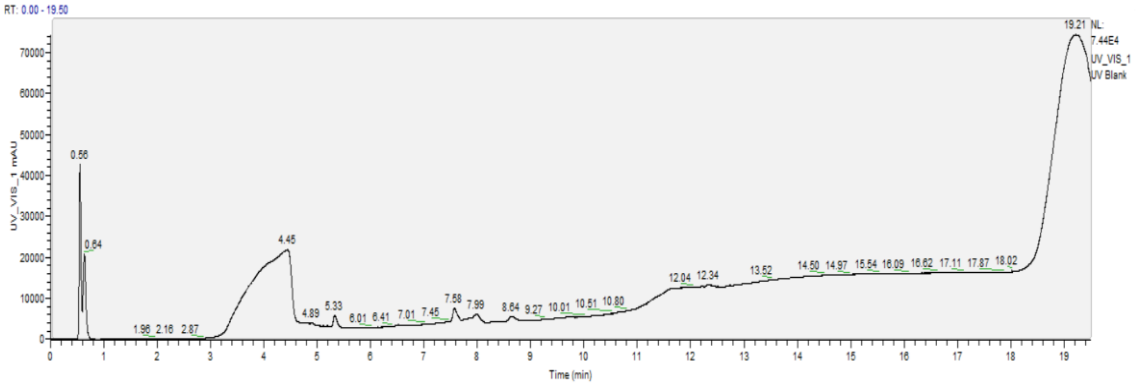
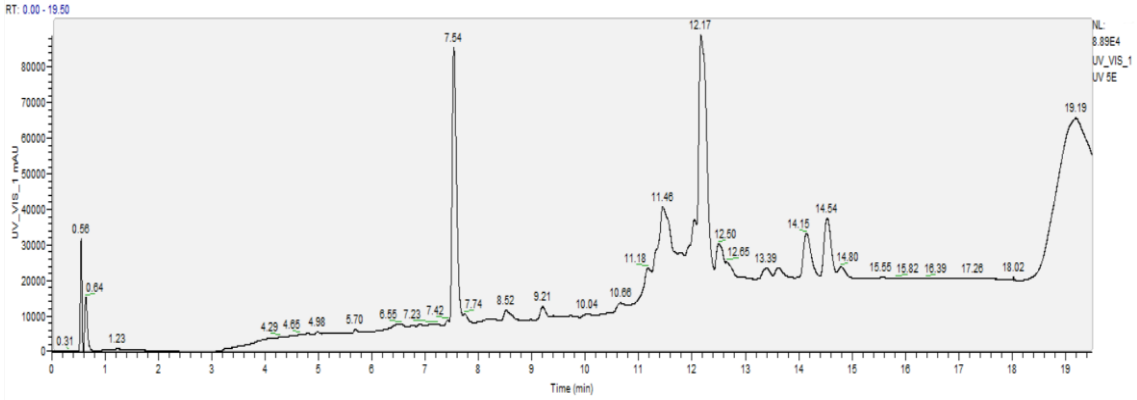


Figure 17- MS filter chromatogram. a) Blank; b) Fraction E of LEGE 15824M strain; c) Fraction C of LEGE 14699M strain.

a)



b)



c)

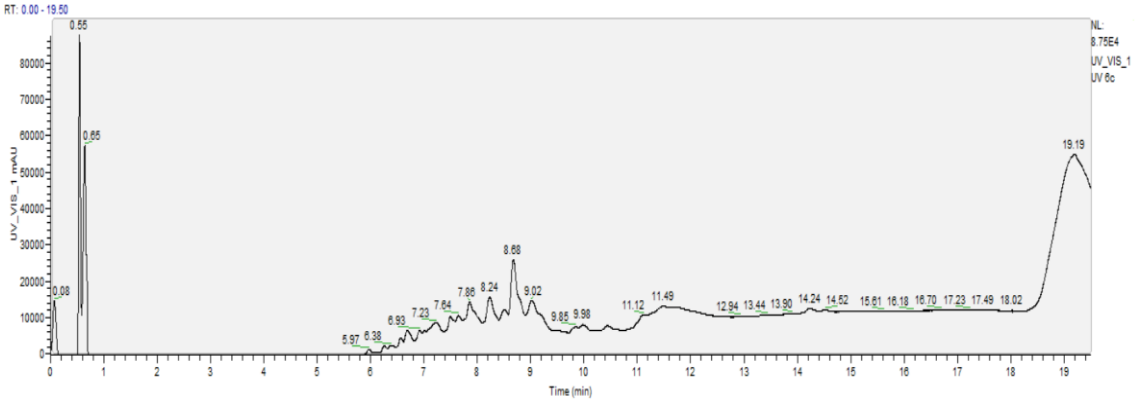


Figure 18- UV filter chromatogram. a) Blank; b) Fraction E of LEGE 15824M strain; c) Fraction C of LEGE 14699M strain.

5. Discussion

In this work, 14 strains of microalgae were studied regarding their antifouling capacity. Methanolic extracts of each strain were fractioned into 8 fractions by HPLC techniques and each obtained fraction was subject of antifouling bioassays. In table 2 is displayed the conglomerate of results obtained from all the performed bioassays.

Table 2- Antifouling activity and ecotoxicity data from the 14 microalgal strains tested. 1 – *Vibrio harveyi*; 2 – *Halomonas aquamarina*; 3 – *Cobetia marina*; 4 – *Pseudoalteromonas altlantica*; 5 – *Roseobacter litoralis*; (-) – no information. Decoding examples: Fraction LEGE 16745M presented a C1 positive result – This means that, regarding strain LEGE 16745M, fraction C was capable on inhibiting *Vibrio harveyi* growth. In the case of strain LEGE 191004M there was F3 positive result. This means fraction F of strain LEGE 191004M was capable of inhibiting *Cobetia marina* growth.

Microalgal Strain	Anti-settlement bioassay (*Mytilus galloprovincialis*)	Anti-bacterial bioassays	Anti-diatom bioassay (*Navicula sp.*)	Toxicity
LEGE 16745M	Negative	Positive (C_1 - 38%; F_3 - 51,5 %)	Negative	-
LEGE 16761M	Negative	Positive (E_2 – 20%)	-	-
LEGE 17793M	Negative	Positive (F_1 – 25,7%; E_2 – 25%; G_3 – 48,8%)	Negative	-
LEGE 16726M	Positive (A – 45%; C – 30%; E – 60%; F – 40%; H – 40%)	Positive (G_3 – 46,4%)	Negative	-
LEGE 15824M	Positive (B – 0%; E – 5%; G – 40%)	Positive (D_2 – 22,7%; E_2 – 20,9%; G_2 – 20,9%)	E (44%)	Non-toxic (E)
LEGE 14699M	Positive (C – 15%; D – 50%; F – 50%; H – 50%)	Positive (C_1 – 31,3%; E_2 – 27,6%; F_2 – 19,1%)	C (24,9%)	Non-toxic (C)
LEGE 16854M	Positive (B – 25%; E – 30%)	Positive (C_1 – 19,6%; D_1 – 20%; C_2 – 24,8%; E_2 – 30,2%)	-	-
LEGE 19996M	Negative	Positive (F_2 – 23,8%; F_3 – 49,9%)	Negative	-
LEGE 16734M	Negative	Positive (D_2 – 26,6%; E_2 – 25,7%; E_5 – 74%)	Negative	-
LEGE 19984M	Positive (B – 30%)	Positive (A_2 – 21,6%; B_2 – 36,3%, C_2 – 36,1%, D_2 – 39,1%, E_2 – 48,3%, F_2 – 40,5%, G_2 – 36,8%, H_2 – 36,1%, F_3 – 54,1%)	Negative	-
LEGE 191004M	Negative	Positive (F_3 – 53,5%)	F (25,4%)	-
LEGE 19954M	Positive (A – 50%; B – 30%; C – 50%)	Positive (E_2 – 19,6%; F_3 – 50,7%)	F (58,8%)	-
LEGE 17946M	Positive (G – 45%)	Positive (A_3 – 40,2%; E_3 – 34,5%; F_3 – 34,3%, H_3 – 35,9%; C_1 – 24,4%; D_1 – 20,5%; E_1 – 22,1%)	-	-
LEGE 16866M	Negative	Positive (D_3 – 36,1%; E_3 – 43,9%; F_3 – 49,3%; G_3 – 37%, H_3 – 34,6 %; C_1 – 33,5%; D_1 – 28,6%; E_1 – 33%; G_1 – 13,5%; H_1 – 11,9%)	E (97,7%), F (34,7%)	-

Considering the overall results, there was a significant number of positive hits across the different carried out bioassays. Regarding the anti-settlement bioassays, strains LEGE 16726M, LEGE 15824M, LEGE 14699M and LEGE 19954M showed to have fractions that exclusively inhibited the settlement of *M. galloprovincialis* larvae. With

the exception of strain LEGE 191004M, all strains have fractions that exclusively inhibited bacterial growth. Other strains had fractions that exclusively inhibited bacteria and diatom growth: strain LEGE 191004M (fraction F), strain LEGE 19954M (fraction F) and strain LEGE 16866M (fractions E and F).

Additionally, Fraction E of strain LEGE 15824M and fraction C of strain LEGE 14699M were both capable of significantly inhibiting the settlement of *M. galloprovincialis* larvae, inhibiting bacterial growth, inhibiting diatom growth, and were proven to be non-toxic.

Regarding the anti-settlement bioassays, it´s important to note that *M. galloprovincialis* proved to be an appropriate test organism for antifouling studies and that $CuSO_4$ at a 5µM concentration is indeed a powerful antifouling substance, and a good positive control, since it totally inhibited larvae settlement (Almeida, Correia-da-Silva et al. 2017, Antunes, Pereira et al. 2019, Pereira, Almeida et al. 2020, Pereira, Gonçalves et al. 2021). Fractions B and E obtained from the green microalgae *Scenedesmus obliquus* (LEGE 15824M), fraction C obtained from the green filamentous microalgae *Tribonema vulgare* (LEGE 14699M), and fraction B obtained from the green filamentous microalgae *Oedogonium sp.* (LEGE 16854M) presented the most promising results in inhibiting larvae settlement (settlement < 30%). At the best of our knowledge, none of the three species are known to have been studied for antifouling applications in the literature.

Then, all of the obtained 112 fractions from the 14 studied strains were subjected to antibacterial assays to determine their potential in inhibiting the growth of several bacteria involved in biofilm formation. Results showed that several fractions were capable of inhibiting bacterial growth, but no fraction was capable of inhibiting *P. atlantica* growth. Similar results were obtained in other antifouling studies (Almeida, Correia-da-Silva et al. 2017, Pereira, Gonçalves et al. 2021). This gram-negative bacterium showed to not be susceptible to the tested substances. On the other hand, *C. marina* growth presented higher percentages of growth inhibition than any other of the tested marine bacteria. In other antifouling studies, on the contrary, *C. marina* showed to be not very sensitive to studied antifouling compounds (Almeida, Correia-da-Silva et al. 2017, Pereira, Almeida et al. 2020). *C. marina* is a gram-negative bacterium, having two phospholipid membranes, thus making this bacterium more resistant to antibiotics and other drugs. Consequently, it´s interesting that the tested substances were capable of inhibiting it´s growth at this level.

The most promising fractions were then subject to anti-diatom bioassays. The diatom specie used in this work, *Navicula sp.*, was also used in several other antifouling studies (Antunes, Pereira et al. 2019, Pereira, Gonçalves et al. 2021), and cycloheximide, a protein synthesis inhibitor, showed to be effective as a positive control. Fraction F extracted from the green microalgae *Monoraphidium contortum* (LEGE 191004M), fraction F of the green microalgae *Stichococcus* sp. (LEGE 19954M) and fractions E and F of the green microalgae *Pediastrum simplex (*LEGE 16866M) were capable on inhibiting bacterial and diatom growth. Also, none of these species are known to have been studied for antifouling applications in the searched literature.

Two fractions stood out from the 112 initial ones, fraction E obtained from the green microalgae *S. obliquus* (LEGE 15824M) and fraction C obtained from the green filamentous microalgae *T. vulgare* (LEGE 14699M). Both fractions were capable of inhibiting the settlement of *M. galloprovincialis* larvae, inhibiting bacterial and diatom growth and, at the same time, were found to be non-toxic to *A. salina* individuals, an organism widely used in ecotoxicity bioassays in an antifouling search context (Almeida, Correia-da-Silva et al. 2017, Pereira, Gonçalves et al. 2021). As previously stated, none of the two species are known to have been studied for antifouling applications in the searched literature.

These two fractions were then analysed to better understand their chemical composition. Liquid chromatography and mass spectrometry (LC-MS) techniques were performed, and the obtained results were used in the construction of a molecular network (data not shown). The obtained chromatograms showed unique peaks in each fraction, and the molecular network showed a broad range of substances waiting to be identified and others to be discovered, possibly, for the first time. Further analysis is required and will be considered in future work.

In this work, we started testing first the mussel anti-settlement bioassays, then the anti-bacterial bioassays, then the anti-diatom bioassays and finally the ecotoxicity bioassays. By beginning testing the fractions on more complex living beings, we can have an idea if these fractions would be of interest on the other antifouling tests, which was proven to be true in certain fractions. Additionally, is worth mentioning that bacteria were the most susceptible organisms. Further studies should follow this approach.

In short, two fractions out of the initial 112 proved to be able to have antifouling capacity both on the macrofouling and microfouling stages without causing significant mortality on non-target organisms, thus making them the most promising analysed fractions: fraction E of strain LEGE 15824M and fraction of strain LEGE 14699M.

6. Conclusions and Future Perspectives

In conclusion, the path of discovering novel natural antifouling substances which are environmentally friendly is a long one. The two mentioned fractions appear to have a real potential, being able to stunt the growth of important microorganisms involved in the biofilm production and preventing the adhesion of a significant macrofouling organism (*M. galloprovincialis*), but additional and more thorough investigation is needed. Nonetheless, these two fractions presented promising results, and it is important to note that none of the organisms from which they were extracted are described as having been studied for antifouling applications. Additionally, future studies in this field should continue to focus on diverse microalgae, since high diversity improves the chances of finding novel chemical compounds of interest.

However, complementary studies have to be conducted in order to enlighten the chemical composition of the most promising fractions, and to fully clarify the molecular structure of the compounds involved in the antifouling activity. Then, EC_{50} and LC_{50} need to be assessed to elucidate the concentrations at which the compounds are effective and non-toxic. Finally, in the future, if the fractions prove to be worth it, they have to be incorporated in paints to access its antifouling capacity in coatings and to discover its durability and efficacy when it is coating an underwater surface.

It is important to note that it is possible that single type of molecule alone cannot act against a wide range of biofouling agents and that multiple molecules act together in the antifouling process. This knowledge, consequently, is essential to comprehend the specific way in which a compound acts and how it prevents adhesion and microorganism growth specifically.

Overall, this work disclosed the promising broad antifouling activity of microalgal derivatives that have not been previouly reported, and showed the potential of these underexplored microorganisms as source of efficient and sustainable antifouling agents.

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Appendix I

Microalgal characterization

LEGE Code	Strain ID (Only by Morphology)	Phylum	Class	Order	Genus	Sampling Date	Habitat|Sample Description	Location	Country	Environment
LEGE 19996M	*Ankistrodesmus gracillis*	Chlorophyta	Chlorophyceae	Sphaeropleales	Ankistrodesmus	2019	plancton	Parque de Stº António, Aveiro	Portugal	aquatic, freshwater
LEGE 16726M	*Haematococcus pluvialis*	Chlorophyta	Chlorophyceae	Chlamydomonadales	Haematococcus	2016	pavement, scraping	Coimbra	Portugal	subaerial, terrestrial
LEGE 15824M	*Scenedesmus obliquus*	Chlorophyta	Chlorophyceae	Sphaeropleales	Scenedesmus	2015	aquarium water	Unknown	Portugal	aquatic, freshwater
LEGE 19984M	*Chlamydomonas* sp.	Chlorophyta	Chlorophyceae	Chlamydomonadales	Chlamydomonas	2019	river	Rio Tua, Mirandela	Portugal	aquatic, freshwater
LEGE 16734M	*Kirchneriella irregularis*	Chlorophyta	Chlorophyceae	Sphaeropleales	Kirchneriella	2016	water tank	Malhada Quente, Serra de Monchique	Portugal	aquatic, freshwater
LEGE 16745M	*Monoraphidium contortum*	Chlorophyta	Chlorophyceae	Sphaeropleales	Monoraphidium	2016	ditch	Exploratório - Centro Ciência Viva de Coimbra, Coimbra	Portugal	aquatic, freshwater
LEGE 16761M	*Coelastrum polychordum*	Chlorophyta	Chlorophyceae	Sphaeropleales	Coelastrum	2016	floodgate wall, scraping	Rio Douro	Portugal	aquatic, freshwater
LEGE 16854M	*Oedogonium* sp.	Chlorophyta	Chlorophyceae	Oedogoniales	Oedogonium	2016	water tank	Relva de Trás, Serra de Monchique	Portugal	aquatic, freshwater
LEGE 19954M	*Stichococcus* sp.	Chlorophyta	Trebouxiophyceae	Prasiolales	Stichococcus	2019	river	Rio Tua, Mirandela	Portugal	aquatic, freshwater
LEGE 191004M	*Monoraphidium contortum*	Chlorophyta	Chlorophyceae	Sphaeropleales	Monoraphidium	2019	river	Rio Mondego, Coimbra	Portugal	aquatic, freshwater
LEGE 16866M	*Pediastrum simplex*	Chlorophyta	Chlorophyceae	Sphaeropleales	Pediastrum	2016	floodgate wall, scraping	Rio Douro	Portugal	aquatic, freshwater
LEGE 14699M	*Tribonema vulgare*	Ochrophyta	Xanthophyceae	Tribonematales	Tribonema	2014	plankton	Coimbra	Portugal	aquatic, freshwater
LEGE 17946M	*Dictyosphaerium* sp.	Chlorophyta	Trebouxiophyceae	Chlorellales	Dictyosphaerium	2016	ditch	Exploratório - Centro Ciência Viva de Coimbra, Coimbra	Portugal	aquatic, freshwater
LEGE 17793M	*Chlorolobion braunii*	Chlorophyta	Chlorophyceae	Sphaeropleales	Chlorolobion	2017	lake	Mira	Portugal	aquatic, freshwater