Drug discovery from marine microalgae

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Doctor of Philosophy

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Abstract

Marine microalgae are protists that contribute up to 40% of global primary productivity. They have been shown to possess a broad range of biological activities, including antiproliferative (cancer cells), antimicrobial, antibiofilm and antiseizure activities that make them excellent candidates as new ocean medicines. They are also excellent sources of nutraceuticals and food supplements, can be easily cultivated in photo-bioreactors to obtain huge biomass and represent a renewable and poorly explored resource for human health benefits.

The general objective of this PhD project was to investigate eight marine microalgae strains (i.e. *Amphidinium carterae* FE102, *Leptocylindrus danicus* FE354, *Chaetoceros pseudocurvisetus* FE331, *Dunaliella tertiolecta* FE200, *Asterionellopsis glacialis* FE355, *Asterionellopsis glacialis* A4, *Skeletonema costatum* FE85, and *Odontella sinensis* B2) to identify secondary metabolites with anticancer, antimicrobial and antibiofilm properties which can become potential drug candidates. Different culturing conditions can lead to changes in biological activity so I also explored the potential of using this phenomenon during my thesis.

Of all the species tested, five displayed interesting biological activities and three of them were chemically studied in order to identify the source of the observed activity. From the bioassay-guided fractionation approach applied on *A. carterae* (Chapter 2), I isolated and identified a new marine polyketide with anticancer activity: amphidinol 22 (Chapter 3). From the extracts of *L. danicus*, I detected two different metabolites with potent antibiofilm activity (Chapter 4). Furthermore, the source of the antibacterial activity observed in *C. pseudocurvisetus* was unveiled (Chapter 4). The biological activity of the other species was assessed to identify those with potentially interesting bioactivity (Chapter 5).

There are still very few studies on bioactive metabolites from marine microalgae and this project allowed me to further exploit this niche and also to travel to other research institutions in order to complete such a multidisciplinary work.
The MarPipe project and related complementary activities

My PhD project was funded by the EU-H2020-MSCA-ITN MarPipe Project (2016 - 2020). MarPipe is a Research and Training Network of 11 academic and industrial partners based in 8 European countries working in collaboration to train young researchers in the field of marine drug-discovery. For further information, please consult: www.marpipe.eu

Within this project I participated in the following complementary training activities:

EU-MarPipe Project Short Scientific Course: Biodiscovery with zebrafish. Date: October 2017; Location: KU Leuven, Belgium; Organising committee: Prof. Peter de Witte, Dr. Annellii Ny, Dr. Aleksandra Siekierska

EU-MarPipe Project Technical Course: Scientific communication and grant writing skills. Date: May 2018; Location: CNR Naples, Italy; Organiser: Dr. Donatella de Pascale

EU-MarPipe Project Short Scientific Course: Organic Structure Analysis. Date: December 2018; Location: Fundación MEDINA, Spain; Organising committee: Dr. Fernando Reyes Benitez, Prof. Marcel Jaspars, Dr. Rainer Ebel.

EU-MarPipe Project Short Scientific Course: Bioinformatics tools for new natural products discovery. Date: August 2019; Location: University College Cork, Ireland; Organising committee: Prof. Alan Dobson and Dr. Marnix Medema

EU-MarPipe Project internal presentations (updates on my PhD project): KU Leuven (Leuven, October 2017), National Research Council IBP-CNR (Naples, May 2018), Brussels Liaison Office CNR (Brussels, October 2018), Marbio UiT (Tromsø, February 2019), Stazione Zoologica Anton Dohrn (Naples, December 2019)


Gordon Research Conference (Proctor Academy, Andover, NH, USA) and Gordon Research Seminar (Proctor Academy, Andover, NH, USA). Abstract and poster presentation: "A new bioactive amphidinol from the athecate dinoflagellate Amphidinium carterae", 27th July - 2nd August 2019.
EU-MarPipe Project Secondment: Fundación MEDINA (Granada, Spain). Date: May to July 2018. High-throughput screening of microalgal extracts.


EU-MarPipe Project Secondment: Brugge (Brugge, Belgium). Date: October 2019. Legal skills, patenting, regulatory affairs and entrepreneurship.
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Chapter 1: Introduction

1.1 Background

According to Dyshlovoy and Honecker (Dyshlovoy and Honecker, 2015) approximately 60% of the drugs used in hematology and oncology have been discovered from natural sources, and one third of the most sold drugs are either natural compounds or semisynthetic derivatives. To date, more than 35,000 compounds isolated from marine organisms have been described (MarinLit database, Royal Society of Chemistry) compared to the 28,000 compounds reported in the literature until 2013 (Blunt et al., 2015), indicating that the number of marine-derived compounds has been increasing significantly over the last few years, at a rate of about 800-1000 compounds per year. There has been a growing interest in marine biodiscovery because potent marine natural compounds (e.g. alkaloids, polyketides, nucleosides, peptides, etc.) have already given rise to 9 approved marine-derived pharmaceuticals, and an additional 31 marine compounds are either in Phase I, II, and III of clinical pharmaceutical development (Mayer et al., 2020). Most of these compounds originate from macroorganisms such as sponges, mollusks and tunicates that are difficult to rear under aquaculture conditions. However, some of these compounds or their chemical scaffolds have been shown to be biosynthethised by associated microbes as discussed over the last years (Bibi et al., 2020; McCauley et al., 2020). For example, this is the case for haliochondrin B (Van Wagoner, Satake and Wright, 2014), okadaic acid (Tachibana et al., 1981; Dickey et al., 1990) and the scaffold that is currently used for the semi-synthesis of Yondelis, cyanosafracin B (Cuevas and Francesch, 2009). In my opinion, natural products originally isolated from macroorganisms could be either partially biosynthethised by the associated microorganisms taking advantage of their biosynthetic machineries, or are completely biosynthethised by the associated microorganisms. However, studies should be addressed for each individual case in order to confirm the biological origin of each natural product.

Microorganisms such as marine microalgae have received increasing attention due to the possibility of cultivating them in large quantities, thus overcoming the problem of biomass supply for chemical analysis and bioactivity characterization at the very beginning of the clinical pipeline (De Morais et al., 2015). This is coupled to an increasing need for additional compounds for use in medicine due to emerging diseases such as viral and antibiotic.
resistant bacterial infections, an increased incidence of cancer, and other human pathologies.

Microalgae are protists that contribute up to 40% of global productivity. They are at the base of aquatic food webs, have short generation times (doubling time = 5-8h for some species) and have colonized almost all biotopes, from temperate to extreme environments (e.g. cold environments and hydrothermal vents). Over the years, microalgae have found different applications oriented to industrial exploitation. John Volkman and co-workers were pioneers in the study of compounds such as lipids, sterols and ketones from marine microalgae, in particular from *coccolithophorid* species (Volkman *et al.*, 1980, 1981). Years later, they evaluated the fatty acid composition and potential use of microalgae to produce food supplements for the marine aquaculture industry (Volkman *et al.*, 1989; Brown *et al.*, 1997), highlighting the successful use of mixed algal diets for mollusc growth and linking it to the fatty acid total content and composition. Renè H. Wijffels and coworkers showed the potential use of microalgae for the production of high value-added products such as carotenoid pigments and vitamins (Carballo-Cárdenas *et al.*, 2003; Barbosa *et al.*, 2005; Kleinegris *et al.*, 2011), and they also enormously contributed to the field of microalgal culturing in photobioreactors at the laboratory and plant scales (Borowitzka, Borowitzka and Kessly, 1990; Barbosa *et al.*, 2003; Barbosa, Albrecht and Wijffels, 2003; Zijffers *et al.*, 2008; Benvenuti *et al.*, 2015). Microalgae can therefore play an important role in the production of several compounds with possible applications in various biotechnological sectors (i.e. food, energy, health, environment and biomaterials) (Lauritano *et al.*, 2016; Romano *et al.*, 2017).

However, most of the studies reporting the use of microalgae in the drug discovery pipeline have only been published in the last decade (Table 1.1). As reported in these studies, microalgal bioactivities and hence the presence/concentration of bioactive compounds may differ for different clones and can vary depending on the culturing conditions (e.g. nutrient availability, temperature, light intensity, etc.) (Ingebrigtsen *et al.*, 2016; Lauritano *et al.*, 2016, 2018) and growth phase (Ribalet *et al.*, 2007). Unfortunately, the information available in the literature regarding bioactive metabolites from marine microalgae is still scarce. Most of the reported studies were performed by testing microalgal raw extracts or complex fractions, not isolated pure compounds. Marine microalgae have also been considered a blooming reservoir of natural products, with the potential to produce isoprenoids, complex polyketides, non-ribosomal peptides, polyunsaturated fatty acids,
oxylipins, alkaloids, and aromatic secondary metabolites (Sasso et al., 2012). They are therefore a potential niche for the discovery of new bioactive metabolites. Hereafter we report what is known on antiproliferative (on cancer cells), antibacterial and antifungal activities in marine microalgae, as well as the reported bioactive metabolites found. Such bioactivities are also the ones explored within the context of this doctoral thesis.

1.1.1 Marine microalgae with antiproliferative activity on cancer cells

Table 1.1 provides a synopsis of the microalgal extracts, fractions or compounds assessed for their antiproliferative activity on cancer cells until now. Unfortunately, most studies are based on the biological activity of raw extracts, rather than of pure compounds. Microalgae are great producers of compounds such as xanthophylls and polyunsaturated fatty acids, but these compounds are active only at high concentrations. The range of concentrations used for testing in the assays is in the µg/mL scale.

Table 1.1. Active microalgal species, active fraction/compounds tested, cell lines against which these have proven to be effective and active concentration of the fraction/compounds (CV stands for cell viability).

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Fraction/Compound</th>
<th>Target cells</th>
<th>Active Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thalassiosira rotula</em>, <em>Skeletonema costatum</em> and <em>Pseudonitzschia delicatissima</em> (diatoms)</td>
<td>Commercial source Polyunsaturated Aldehydes (PUAs)</td>
<td>Colon adenocarcinoma (Caco2) Lung adenocarcinoma (AS49) Colon adenocarcinoma (COLO 205)</td>
<td>11 to 17 µg/ml (arrest of cell growth) 0.22 to 1.5 µg/ml (CV of 80% to 0%)</td>
<td>(Miralto et al., 1999) (Sansone et al., 2014)</td>
</tr>
<tr>
<td><em>Chlorella ellipsoidea</em> (green alga)</td>
<td>Carotenoid extract</td>
<td>Colon carcinoma (HCT-116)</td>
<td>40 µg/ml (IC50)</td>
<td>(Kwang, Song and Lee, 2008)</td>
</tr>
<tr>
<td><em>Synedra acus</em> (diatom)</td>
<td>Chrysolaminaran (polysaccharide)</td>
<td>Colorectal adenocarcinoma (HT-29 and DLD-1)</td>
<td>54.5 and 47.7 µg/mL (IC50 for HT-29 and DLD-1)</td>
<td>(Kusaikin et al., 2010)</td>
</tr>
<tr>
<td><em>Dunaliella tertiolecta</em> (green alga)</td>
<td>Violaxanthin (carotenoid already identified in <em>C. ellipsoidea</em>)</td>
<td>Breast adenocarcinoma (MCF-7)</td>
<td>40 µg/ml (to observe cytostatic activity)</td>
<td>(Pasquet et al., 2011)</td>
</tr>
<tr>
<td>Organism</td>
<td>Extraction</td>
<td>Tumor Type</td>
<td>IC50/EC50</td>
<td>CV</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>------------</td>
<td>----------</td>
<td>----</td>
</tr>
<tr>
<td>Cocconeis scutellum (diatom)</td>
<td>Eicosapentaenoic Acid (EPA) *On the market as nutraceutical</td>
<td>Breast carcinoma (BT20)</td>
<td>Not clarified</td>
<td>(Nappo et al., 2012)</td>
</tr>
<tr>
<td>Chaetoseros sp., Cylindrotheca closterium, Odontella aurita and Phaeodactylum tricornutum (diatoms)</td>
<td>Fucoxanthin (carotenoid) *On the market as nutraceutical</td>
<td>Promyelocytic leukemia (HL-60), Caco-2, colon adenocarcinoma (HT-29), DLD-1 and prostate cancer (PC-3, DU145 and LNCaP)</td>
<td>29.78 µg/ml (CV of 17.3% for HL-60) 10.01 µg/ml (CV of 14.8%, 29.4% and 50.8% for Caco-2, DLD-1 and HT-29) 13.18 µg/ml (CV of 14.9%, 5.0% and 9.8% for PC-3, DU145 and LNCaP)</td>
<td>(Peng et al., 2011)</td>
</tr>
<tr>
<td>Chaetoceros calcitrans (diatom)</td>
<td>EtOH extract AcOEt extract</td>
<td>MCF-7 Breast adenocarcinoma (MDA-MB-231)</td>
<td>3.00 µg/mL (IC50) 60 µg/mL (IC50)</td>
<td>(Nigjeh et al., 2013) (Goh et al., 2014)</td>
</tr>
<tr>
<td>Amphidinium carterae (dinoflagellate)</td>
<td>CH3Cl extract Hexane extract AcOEt extract</td>
<td>HL-60 HL60, Skin melanoma (B16F10), A549</td>
<td>50 µg/mL (CV of 40%) 25-50 µg/mL (CV between 90-50%)</td>
<td>(Samarakoon et al., 2013)</td>
</tr>
<tr>
<td>Eleven strains of benthic diatoms Ostreopsis ovata Amphidinium operculatum</td>
<td>MeOH extract</td>
<td>HL-60</td>
<td>50 µg/mL (CV of 48% for O. ovata and 58% for A. operculatum)</td>
<td>(Rahman Shah et al., 2014)</td>
</tr>
<tr>
<td>Navicula incerta (diatom)</td>
<td>Stigmasterol (phytosterol)</td>
<td>Liver hepatocellular carcinoma (HepG2)</td>
<td>8.25 µg/mL (CV of 54%)</td>
<td>(Kim et al., 2014)</td>
</tr>
<tr>
<td><strong>Phaeodactylum tricornutum</strong> (diatom)</td>
<td>Nonyl-8-acetoxy-6-methyloctanoate (NAMO, fatty alcohol ester)</td>
<td>HL-60</td>
<td>2.3 µg/mL (IC₅₀)</td>
<td>Phaeodactylum tricornutum (diatom)</td>
</tr>
<tr>
<td>Skeletonema marinoi FE6 (1997 Adriatic Sea) FE60 (2005, Adriatic Sea) (diatoms)</td>
<td>Hydrophobic fraction and PUAs</td>
<td>Caco2 (A2058 not affected)</td>
<td>11 to 17 µg/ml (PUAs)</td>
<td>Skeletonema marinoi FE6 (1997 Adriatic Sea) FE60 (2005, Adriatic Sea) (diatoms)</td>
</tr>
<tr>
<td>Canadian marine microalgal pool</td>
<td>Aqueous extract</td>
<td>A549, lung carcinoma (H460), prostate carcinoma (PC3, DU145), stomach carcinoma (N87), MCF-7, pancreas adenocarcinoma (BxPC3) and osteosarcoma (MNN)</td>
<td>5000 µg/mL (CV between 30% and 80% depending on the cell line)</td>
<td>Canadian marine microalgal pool</td>
</tr>
<tr>
<td>Chlorella sorokiniana (green alga)</td>
<td>Aqueous extract</td>
<td>A549 and lung adenocarcinoma (CL1-5)</td>
<td>0.0156 to 1 µg/mL (CV reduced down to 20% progressively)</td>
<td>Chlorella sorokiniana (green alga)</td>
</tr>
<tr>
<td>Skeletonema marinoi (diatom)</td>
<td>Monoacylglycerides (1-MAG)</td>
<td>Histiocytic lymphoma U-937, HCT-116 and mesenchymal progenitor model cells MePR-2B</td>
<td>25µg/ml (CV of U-937 reduced to 20-60% depending on the MAG used)</td>
<td>Skeletonema marinoi (diatom)</td>
</tr>
</tbody>
</table>

1.1.1A Antiproliferative potential of microalgal extracts/fractions against cancer cells.

The use of marine microalgae as a source to discover potential anticancer agents is still a poorly explored field. Kwang et al. (2008) tested the antiproliferative effect of the carotenoids extracted from the green algae *C. ellipsoidea* and *C. vulgaris* on a human colon carcinoma cell line (HCT116). The cultures of the *Chlorella* strains used (i.e. *C. vulgaris* KMCC C-024 and *C. ellipsoidea* KMCC C-020) were acquired from the Korea Marine
Microalgae Culture Center in Busan, Korea. Those extracts were analysed using high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) in order to investigate the carotenoid composition. It was found that the carotenoid extract from *C. ellipsoidea* was mainly composed of violaxanthin, while lutein was the main component in the case of *C. vulgaris*. The half maximal inhibitory concentration (IC$_{50}$) value was 40.73 ± 3.71 µg/mL for *C. ellipsoidea* and 40.31 ± 4.43 µg/mL for *C. vulgaris*, after 24h of treatment. An apoptotic effect on the cells was confirmed by annexin V-fluorescein assay for both *C. ellipsoidea* and *C. vulgaris* carotenoid extracts and was higher in the case of *C. ellipsoidea* (2.5-fold). These studies suggest that xanthophylls found in *Chlorella* species possess antiproliferative activity against colon carcinoma cells mediated by an apoptosis mechanism.

Nigjeh et al. (2013) evaluated the biological activity of the indigenous Malaysian diatom *Chaetoceros calcitrans* UPMAAHU10 on breast adenocarcinoma (MCF7), breast epithelial (MCF10A) and peripheral blood mononuclear cells (PMBC). The antiproliferative activity of the ethanol extracts from the diatom was assessed on those cell lines. The half maximal inhibitory concentration (IC$_{50}$) values of the ethanolic extract screened on MCF-7 cells were 3.00 ± 0.65 µg/mL for 24h and 2.69 ± 0.24 µg/mL after 72h, while IC$_{50}$ values on MCF-10A cells were 12.00 ± 0.59 µg/mL after 24h and 3.30 ± 0.36 µg/mL after 72h of treatment. *C. calcitrans* extract had no effect on PMBC cells at the concentrations tested. Annexin V/propidium iodide analyses indicated apoptosis induction in MCF-7 cells after treatment with the extract. An increase in some proapoptotic protein transcripts (i.e. Bax, caspase 3 and caspase 7) supported the conclusion that apoptosis was the mechanism of cell death. The authors concluded that crude ethanol extracts of *C. calcitrans* have the potential to be used for breast cancer treatment, as such extracts present IC$_{50}$ values as low as 3 µg/mL.

On the other hand, Goh et al. (2013) screened the hexane, dichloromethane, ethyl acetate and methanol extracts from the same clone of *C. calcitrans* (UPMAAHU10) on a wide range of cancer cell lines: human breast adenocarcinoma (MDA-MB-231), MCF7, mouse breast carcinoma (4T1), Liver hepatocellular carcinoma (HepG2), cervix epithelial carcinoma (HeLa), human prostate carcinoma (PC3), human lung adenocarcinoma (A549), human colon adenocarcinoma (HT29), and human ovarian adenocarcinoma (CAOV3). Mouse embryo fibroblast (3T3) cell line was used to measure cytotoxicity against non-tumorigenic cells. From all the extracts, only the crude ethyl acetate extract displayed biological activity by reducing the cell viability of the MDA-MB-231 cancer cell line by 50% at 60 µg/mL (IC$_{50}$).
after 72h. However, no significant effects were observed on the other tested cell lines and no toxicity was observed on the normal 3T3 cell line. The results observed within this study were not as promising as the ones observed by Nigjeh et al. (2013). The IC$_{50}$ value for the ethyl acetate extract was 60 µg/mL, a value of concentration 20 times higher compared to the ethanolic extract of *C. calcitrans* (considering it is the same clone). Further studies should be performed in order to identify and describe the source of the biological activity in both studies, and answer why the ethanolic extract is much more active than the other extracts.

Samarakoon et al. (2013) tested the activity of various fractions from the diatom *Phaeodactylum tricornutum* Bohlin, the green alga *Chlorella ovalis* Butcher and the eustigmatophytes *Nannochloropsis oculata* Droop, which were provided by the Korea Marine Microalgae Culture Center (KMMCC). Fractions from the dinoflagellate *Amphidinium carterae* Hulburt, collected from the Jeju Island, were also screened. Crude methanol extracts were prepared from the biomass, while n-hexane, chloroform, ethyl acetate, and water were used in a solvent-solvent partition chromatography in order to obtain the fractions to be tested. The biological activity was assessed on different cancer cell lines: HL-60 (Human promyelocytic leukemia cells), B16F10 (Mouse melanoma tumor cells), A549 (Adenocarcinomic human alveolar basal epithelial cells) and mouse monocyte macrophage cell line (RAW 264.7). *A. carterae* chloroform fraction displayed the most potent activity, reducing HL60 cell viability by 50% at a concentration of 50 µg/mL after 24h, while the extracts and fractions of the other species did not display any significant activity. The authors concluded that further purification processes are necessary to determine the identity of the bioactive components.

Shah et al. (2014) cultivated eleven different strains of benthic dinoflagellates (*Amphidinium carterae*, *Prorocentrum rhathymum*, *Symbiodinium* sp., *Coolia malayensis* strain 1, *Ostreopsis ovata* strain 1, *Ostreopsis ovata* strain 2, *Coolia malayensis* strain 2, *Amphidinium operculatum* strain 1, *Heterocapsa psammophila*, *Coolia malayensis* strain 3 and *Amphidinium operculatum* strain 2) isolated from the coast of Jeju Island (South Korea) in 2011. The dinoflagellate methanol extracts were then screened against RAW 264.7 (murine macrophage cell line) and HL-60 (human promyelocytic leukemia cell line) cells. From all the extracts, only *Ostreopsis ovata* 1 and *Amphidinium operculatum* 1 extracts displayed significant growth inhibition effects against the cancer cell lines tested, reducing cell viability by 40% and 60% (compared to the control) at a concentration of 50 µg/mL
after 24h of treatment. These studies covered the antiproliferative activity of benthic Korean dinoflagellates on leukemia cells.

Lauritano and coworkers (2016) studied a broad panel of biological activities for 32 different microalgal species (i.e. 21 diatoms, 7 dinoflagellates, 3 chryptophites and 1 haptophyta). The authors found that hydrophobic extracts from *Skeletonema marinoi, Alexandrium minutum, Alexandrium tamutum* and *Alexandrium andersoni* cultured under different conditions (control, nitrogen starvation and phosphate starvation) were active against melanoma cancer cell line A2058 at a concentration of 100 μg/mL after 72h of treatment. However, all *Alexandrium* species were found to be toxic when tested against normal lung fibroblast MRC5 cell line. Two different strains of *Skeletonema marinoi* (i.e. FE6 and FE60 strains from the Adriatic Sea) were tested on A2058 and MRC5 cell lines. Results showed that only the extract from the FE60 strain cultured under nitrogen-starvation conditions was active against the A2058 cell line. The hydrophobic extract of the nitrogen starved FE60 strain reduced cell viability to 60% at a concentration of 50 μg/mL and to 10% at 100 μg/mL, after 72h of exposure to the extract. These high-throughput screening studies not only highlighted that different clones from the same species could present different activities, but also introduced the use of different culturing conditions as a tool to trigger the production of different metabolites, changing the observed bioactivity of the microalgae extracts.

Somasekharan et al. (2016) studied the antiproliferative effect of raw marine microalgal material from Canada (dried powder) on eight different cancer cell lines. Aqueous extract was prepared from the powder and then tested on A549, H460 (lung adenocarcinoma cell lines), PC3, DU145 (prostate cancer cell lines), N87 (stomach cancer cell line), MCF7 (breast cancer cell line), BxPC3 (pancreas cancer cell line) and MNNG (bone cancer cell line). The cells were treated with the extract for 72h at a concentration of 1, 2 and 5 mg/mL. The extract did not show any significant activity at 1-2 mg/mL except for the MNNG cell line (50% reduction in cell viability at 2 mg/mL). At 5 mg/mL proliferation of almost all the cell lines was significantly inhibited. In these studies, the authors used a different approach by extracting biomass from pooled microalgae instead of individual species. However, the active concentrations observed are in the range of 1 to 5 mg/mL, much higher than the concentrations observed for single species (μg/mL) covered within this chapter. The concentration of different active metabolites present in pools of microalgae may not be enough to yield significant bioactivity results.
Finally, Lin et al. (2017) studied the effects of hot water extracts from the green alga *Chlorella sorokiniana* (marine strain) on lung adenocarcinoma cell lines A549 and CL1-5. Results indicated dose-dependent reduction of cell viability on both A549 and CL1-5 cell lines when treated for 24h, and IC$_{50}$ values of around 100 and 250 ng/mL. The authors also studied the mechanism of action of *C. sorokiniana* extract using Annexin V/Propidium Iodide staining but cell cycle arrest did not occur. However, an increment in the number of cells in sub-G1 phase was observed, which is a phenomenon that typically indicates apoptosis. Protein expression of the cleaved and activated forms of caspase 9, caspase 3 and PARP was upregulated in both cell lines after treatment. Activation of caspase 9 and caspase 3 suggested that the main pathway involved in apoptosis was the mitochondrial pathway. In addition, Bax/Bcl-2 ratio (pro/antiapoptotic proteins) increased after 24 h of treatment which is another sign of apoptosis. These promising studies showed the unprecedented potential of *C. sorokiniana* aqueous extract, with IC$_{50}$ values within the nanoscale concentrations and experiments confirming apoptosis as the mechanisms of action for the antiproliferative activity observed.

The studies from this section evaluated the *in vitro* antiproliferative activity of several extracts and fractions from marine microalgae on different cancer cell lines. The most interesting results were observed on the treatments with ethanol extract from *C. calcitrans* (Nigjeh et al., 2013) and aqueous extract from *C. sorokiniana* (Lin et al., 2017), where the active concentrations were the closest to nanograms/mL and hence it is worth proceeding to identify the source of the bioactivity for each microalga. Unfortunately, even if in some cases the authors performed studies on the mechanism of action of the extracts and fractions to better characterize the biological activity, they did not perform studies on the identification of the molecules which can be the source of the bioactivity observed. From the experience acquired during my PhD project, I think that further development of those studies was hampered by the low concentration of secondary metabolites in marine microalgae, especially in diatoms, making it difficult to isolate those metabolites. In most of the cases motivation to isolate the compounds could be lost when the bioactivity was already low for the raw extracts because for these situations the most probable outcome is the loss of resources and time.

1.1.1B Antiproliferative potential of microalgal compounds against cancer cells

To my knowledge Miralto et al. (1999) were the first to test natural products isolated from diatoms on human cancer cells. Three polyunsaturated aldehydes (PUAs, Figure 1.1) were
isolated from the marine diatoms *Thalassiosira rotula*, *S. costatum* and *P. delicatissima* by extraction of the biomass with dichloromethane and further fractionation using thin layer silica gel chromatography (TLC). The Authors found that 2-trans-4-cis-7-cis-decatrienial (1), 2-trans-4-trans-7-cis-decatrienial (2) and 2-trans-4-trans-decadienial (3) had antiproliferative activity on human colon adenocarcinoma Caco2 cell line, reducing cell viability to almost 0% at concentrations of about 11-17 µg/mL. In addition, a TUNEL assay was performed to check DNA fragmentation and verify that the mechanism behind cell viability arrest was apoptosis. Sansone *et al.* (2014) also assessed the antiproliferative effect of the PUAs 2-trans-4-trans-decadienial (3), 2-trans-4-trans-octadienal (4) and 2-trans-4-trans-heptadienal (5) on the adenocarcinoma lung A549 and colon COLO 205 cell lines. The PUAs were commercially available and purchased from Sigma-Aldrich Inc. (Milan). The Authors treated the cells with these three polyunsaturated aldehydes at different exposure times (i.e. 48 and 72h) and concentrations (i.e. 2, 5 and 10 µM). 3 induced a stronger effect (after only 24 hours) when used as a treatment at a concentration 10 µM, inducing a decrease in cell viability of 18% and 26% for A549 and COLO205 cell lines, respectively. In the case of 4, 72h of treatment with 10 µM of the compound decreased cell viability by 35% and 41% on A549 and COLO 205 cells, respectively. 5 also required 72h of treatment, but it managed to reduce the cell viability down to 0% at a concentration of 10 µM on A549 cells. Sansone *et al.* (2014) also tested the 3 PUAs on normal lung/bronchus epithelial BEAS-2B cell line to check cytotoxicity on healthy cells. No toxicity against the BEAS-2B cell line was observed for the PUAs tested. These studies successfully complement the ones performed by Miralto *et al.* (1999) by going deeper on the profiling of the anticancer activity of PUAs.

![Figure 1.1](image_url) Polyunsaturated aldehydes. 2-trans-4-cis-7-cis-decatrienial (1), 2-trans-4-trans-7-cis-decatrienial (2), 2-trans-4-trans-decadienial (3), 2-trans-4-trans-octadienal (4) and 2-trans-4-trans-heptadienal (5)

Kusaikin *et al.* (2010) isolated one polysaccharide of the chrysolaminaran family (Figure 1.2) from a clone of the diatom *Synedra acus* isolated from Lake Baikal. These storage polysaccharides are well known to be the most common biopolymers in the world
synthesized by diatoms. Antitumor activity of the chrysolaminaran extracted from *S. acus* was studied in HTC116 and DLD1 human colon cancer cell lines. Cancer cells were treated with 25, 50 and 100 µg/mL of the chrysolaminaran for 72 hours. IC₅₀ values were determined for each cell line: 54.5 µg/mL for HCT116 and 47.7 µg/mL for DLD1. The polysaccharide did not show any toxicity on HTC116 and DLD1 cell lines even at concentrations above 200 mg/mL, indicating that it blocked proliferation without being lethal to the cells.

![Chrysolaminaran monomer](image1)

**Figure 1.2.** Chrysolaminaran monomer.

Pasquet *et al.* (2011) performed an anticancer screening of extracts from the green alga *Dunaliella tertiolecta* (strain CCMP364) on four different cancer cell lines: MCF-7, MDA-MB-231, A549 and LNCaP. The authors produced a fraction from the microalgal biomass identified mainly as the epoxycarotenoid violaxanthin (95%, Figure 1.3) and tested it against MCF7 cancer cells. The fraction possessed significant activity against MCF7 cancer cells. It showed a dose-dependent trend in the range of concentrations tested (0.1 µg/mL to 40 µg/mL, and 72h of exposure time), and inhibited the growth of MCF7 cancer cells by 50% at a concentration of 11.7 µg/mL (IC₅₀). In addition to these results, the DNA of nontreated and treated cells was extracted and analysed using standard electrophoresis. Despite indications of early apoptosis (phosphatidylserines translocation detected using annexin-V-Alexa 568 fluorochrome), the fraction containing 7 did not cause any DNA fragmentation. This study gives an insight on the possible use of epoxycarotenoids for treatment or prevention of cancer.

![Violaxanthin](image2)

**Figure 1.3.** Violaxanthin
Nappo et al. (2011) screened the butanol and diethylether extracts from the marine diatom *Cocconeis scutellum*, isolated from the island of Ischia (Italy), on the following cells lines: BT20 (human breast cancer), MB-MDA468 (human breast cancer), LNCaP (human prostate adenocarcinoma cells), COR (Epstein Barr Virus-transformed B cells isolated from human tonsils), JVM2 (lymphoblast immortalized with Epstein-Barr virus) and BRG-M (Burkitt’s lymphoma cells). Fractionation of the diethyl ether extract, which resulted the most active, yielded three fractions with different activities. Fractions 1-2 did not significantly reduce cell viability compared to the control, but fraction 3 (81.7% of fatty acids and 2.3% of 4-methylcholesterol) reduced cell viability to 56.2%. Within fraction 3, fatty acids were the only components that could be associated to the observed activity. In particular, eicosapentaenoic acid (EPA, Figure 1.4) was the only fatty acid present reported to possess such activity (Chajès et al., 1995). Activation of caspases 8 and 3 was confirmed by western blot analysis, indicating apoptosis as the mechanism of action activated by the fraction on the cancer cells. The authors concluded that it was not clear whether 8 was the only compound involved in inducing apoptosis in BT20 cells or if there was a synergic association among more compounds in the fraction.

![Figure 1.4. Eicosapentaenoic acid, EPA.](image)

One of the most studied compounds that can be found in both macro- and microalgae is fucoxanthin (Figure 1.5), a pigment from the family of the xanthophylls and the main carotenoid found in brown algae (Kong et al., 2016). Kadekaru and co-workers (Kadekaru, Toyama and Yasumoto, 2008) evaluated 9 toxicity by an oral treatment (10 mg/kg and 50 mg/kg) in rats, for a period of 28 days. 9 did not display any sign of toxicity and it was hence considered safe as a pharmaceutical ingredient. Ishikawa and co-workers (Ishikawa et al., 2008) performed a similar analysis in mice using a metabolite derived from 9, fucoxanthinol, using a higher dose (200 mg/kg) to maintain the previous conditions. However, the compound did not display any toxicity. Peng et al. (2011) summarized the
studies from the bibliography related to 9, indicating the microalgae species known to produce this carotenoid (i.e. *Chaetoseros* sp., *Cylinrotheca closterium*, *Odontella aurita* and *Phaeodactylum tricornutum*) and the observed biological activities (e.g. antioxidant, antiinflammatory, anticancer, antidiabetic, skin protective, bone protective, etc.). Some of the most interesting studies regarding the anticancer activity are summarised below.

In their studies, Hosokawa and co-workers (*Hosokawa et al.*, 1999) observed that 9 had strong antiproliferative activity and could also induce apoptosis on HL-60 cells. When treated with 11.3 and 45.2 µM of 9 after 24h, cell viability was reduced to 46.0% and 17.3%, respectively. Authors also tested 9 against three human colon cancer cell lines (Caco-2, DLD-1 and HT-29), observing a dose-time dependent trend. The viability of Caco-2 cell line was more affected compared to the other two cell lines. Kotake-Nara and co-workers (*Kotake-nara et al.*, 2001) studied the effects of 15 different carotenoids on three different prostate cancer cell lines (PC-3, DU145 and LNCaP). They stated that 9 presented one of the highest antiproliferative effects amongst all the carotenoids tested. The reported percentages of viable cells after 72h of treatment with fucoxanthin at a concentration of 20 µM were 14.9% for PC-3, 5.0% for DU145 and 9.8% for LNCaP, respectively. As a conclusion, 9 is a well-studied compound with low toxicity and potential positive effects for human health.

![Figure 1.5. Fucoxanthin](image)

The sterol molecule known as stigmasterol (Figure 1.6) was isolated from the methanol:dichloromethane (1:1) extract of the benthic diatom *Navicula incerta* (KMMCC B-001,) provided by the Korea Marine Microalgae Culture Center. Kim et al. (2014) studied the antiproliferative effect of 10 on the HepG2 cell line at concentrations of 5, 10 and 20 µM. The compound reduced cell viability by 40%, 43% and 54%, respectively, which indicated a dose-dependent trend within the range of concentrations tested. They also studied apoptosis by controlling morphological changes, fluorescence-activated cell sorting, apoptosis pathways analysis, gene expression levels and also flow cytometric...
measurement of cell cycle arrest. The studies evidenced the apoptosis induction capabilities of 10, the only reported sterol-like molecule from marine microalgae with antiproliferative activity on cancer cells.

![Stigmasterol](image1)

**Figure 1.6. Stigmasterol**

Samarakoon et al. (2014) assessed the antiproliferative activity of nonyl-8-acetoxy-6-methyloctanoate (NAMO, Figure 1.7), a compound isolated from the diatom *Phaeodactylum tricornutum*, against three different cell lines: human promyelocytic leukemia cell line (HL60), a human lung carcinoma cell line (A549) and a mouse melanoma cell line (B16F10). 11 was tested against the different cell lines at a concentration of 25 and 50 µg/mL for 48h. Antiproliferative activity was observed only on HL60 leukemia cells, at both concentrations tested. HL-60 cells growth was reduced by 70% when treated at 50 µg/mL. In addition, 11 induced DNA damage and increased apoptotic body formation. Cell cycle arrest and accumulation of cells in the sub-G1 phase were proportional to the concentration of 11 used in the treatment. The authors also observed activation of the pro-apoptotic protein Bax, suppression of the anti-apoptotic Bcl-x and an increment in the expression of both caspase-3 and p53, both of which as apoptotic-related proteins.

![Nonyl-8-acetoxy-6-methyloctanoate](image2)

**Figure 1.7. Nonyl-8-acetoxy-6-methyloctanoate, NAMO.**

Andrianasolo et al. (2008) isolated two different monogalactosyl glycerols (Figure 1.8, 12 and 13) from the diatom *Phaeodactylum tricornutum* (Bohlin clone Pt1 8.6, CCMP2561) and tested them against immortal mouse epithelial cells (wild type W2 and apoptosis disabled D3). The antiproliferative assay performed on W2 and D3 mouse epithelial cell lines is one of the approaches to evaluate if apoptosis is the mechanism of action. The minimum values
that were considered to induce apoptosis with this test were a death rate of 20% on W2 cell line and a growth rate of 10% or higher on D3 cell line. For compound 12 (52 µM) the W2 death rate was 18% ± 1% and the D3 growth rate was 10% ± 1%. For 13 (64µM) the W2 death rate was 18% ± 1% and the D3 growth rate was 14% ± 1%. The results confirmed specific apoptotic activity against W2 cell line by treatment with either 12 or 13.

![Figure 1.8. Monogalactosyl glycerols](image)

Miceli et al. (2019) screened the methanolic extracts of eight different microalgal species (i.e. the diatoms Skeletonema marinoi, Skeletonema japonicum, Chaetoceros affinis and Thalassiosira rotula, the green algae Tetraselmis suecica and Dunaliella salina, and the dinoflagellates Amphidinium carterae and Alexandrium tamarense) against the hematological cancer cell line U-937 and the colon cancer cell line HCT-116. Human mesenchymal progenitor model MePR-2B cell line was used in order to assess the cytotoxicity against a normal cell line. Bioassay-guided fractionation of the methanol extract from S. marinoi (most active) allowed the isolation and identification of several compounds with 1-monoacylglyceride structure (i.e. 1-MAG-C16:3, 1-MAG-C16:2, 1-MAG-C16:1) (Figure 1.9). The biological activity of the pure compounds was assessed and compared with the commercially available monoacylglycerides 1-palmitoleoyl-rac-glycerol (1-MAG-C16:1), 1-palmitoyl-rac-glycerol (1-MAG-C16:0), 1-stearoyl-rac-glycerol (1-MAG-C18:0) and the synthetic derivative 1-monoarachidonoyl-glycerol (1-MAG-ARA). All the compounds tested were significantly active against the U-937 cell line after 24 hours of treatment with a concentration of 25 µg/mL, reducing cell viability to values from 20% to 60%. The cell viability of the normal cell line MePR-2B was not affected when treated under the same conditions but it was slightly reduced after 48 hours of treatment.
Most of the compounds reviewed in this section are fatty acids, fatty acid related compounds or pigments, so the chemical diversity of the microalgal compounds with antiproliferative activity on cancer cells is very narrow. In addition, and considering that the most active compounds (PUAs and NAMO) from microalgae were active at concentrations of µg/mL, further pharmaceutical development is far from being considered. I can state it because the concentrations triggering the activity are much higher than the standard in vitro concentrations (IC₅₀ 0.365–272 ng/mL) of the active principles in marketed drugs (Martínez Andrade et al., 2018). In fact, from all the compounds only the carotenoids (violaxanthin and fucoxanthin) have found a niche in industry, mainly as additives (colourants) or nutraceuticals. For instance, fucoxanthin-based products such as “Solaray Fucoxanthin Special Formula Vegetarian Capsules” or “BRI NUTRITION® Fucoxanthin Capsules” are commercialized as dietary supplements. As a final conclusion, attempting the isolation of secondary metabolites that can lead to anticancer drugs from microalgae was rather risky from the very beginning, considering the results from the reviewed bibliography. As I mentioned in the previous section, the concentration of secondary metabolites in microalgae is low according to my experience, which added an extra difficulty to the task. This is why I also looked for antibacterial activity in the species tested within my project, an idea that was born as a backup plan.

1.1.2 Marine microalgae with antimicrobial properties

Table 1.2 provides a synopsis of the microalgal fractions, extracts and compounds that have presented antimicrobial activity until now. Some of the results are expressed in terms of inhibition diameter and others in terms of growth inhibition, depending on the type of assay performed.

Table 1.2. Summary of the antimicrobial microalgal species, active fraction/compounds tested, target microorganisms and references from the literature.

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Fraction/Compound</th>
<th>Target organisms</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amphidinium spp.</em></td>
<td>Amphidinols</td>
<td>Red blood cells, cancer cells and several fungi</td>
<td>-</td>
<td>Several (please</td>
</tr>
<tr>
<td>Extractant</td>
<td>Antimicrobial Activity</td>
<td>Activity against</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------------</td>
<td>---------------------------------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Gambierdiscus toxicus</strong></td>
<td>Gambieric acids A and B</td>
<td>Several fungal strains</td>
<td><strong>0.2-6.25 μg/mL (MIC)</strong></td>
<td>(Nagai et al., 1993)</td>
</tr>
<tr>
<td>(dinoflagellate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Skeletonema costatum</strong></td>
<td>Organic sub-fraction</td>
<td>Listonella anguillarum</td>
<td>Inhibition diameter: <strong>15 mm</strong></td>
<td>(Naviner et al., 1999)</td>
</tr>
<tr>
<td>(diatom)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phaeodactylum tricornutum</strong></td>
<td>6Z, 9Z, 12Z-hexadecatrienoic acid</td>
<td>Staphylococcus aureus</td>
<td><strong>20-40 μM (IC50)</strong></td>
<td>(Desbois et al., 2008)</td>
</tr>
<tr>
<td>(diatom)</td>
<td>Palmitoleic acid</td>
<td></td>
<td><strong>10–20 μM (IC50)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Thalassiosira rotula</strong></td>
<td>Paste of biomass</td>
<td>Staphylococcus aureus and Bacillus pumilus</td>
<td>Inhibition diameters:</td>
<td>(Qin et al., 2013)</td>
</tr>
<tr>
<td>(diatom)</td>
<td>Water extract</td>
<td></td>
<td><strong>14.2-14.5 mm</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexane:tBME extract</td>
<td></td>
<td><strong>7.8-9.7 mm</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform:methanol extract</td>
<td></td>
<td><strong>9.5-13.8 mm</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Skeletonema costatum</strong></td>
<td>Ethanol extract</td>
<td>Streptococcus pyogenes</td>
<td><strong>18.1 mm</strong></td>
<td>(Sushanth and Rajashekhar, 2015)</td>
</tr>
<tr>
<td><strong>Nannochloropsis oceanica</strong></td>
<td>Hexane extract</td>
<td></td>
<td><strong>17.4 mm</strong> (Inhibition diameters)</td>
<td></td>
</tr>
<tr>
<td>(Ochrophyta)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dunaliella tertiolecta</strong></td>
<td>Methanol extract</td>
<td>Pseudomonas aeruginosa</td>
<td><strong>Mtg0 values:</strong></td>
<td>(Pane et al., 2015)</td>
</tr>
<tr>
<td>(green alga)</td>
<td></td>
<td>Staphylococcus aureus</td>
<td><strong>5.6×10⁹ cells/mL</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>6.2×10⁹ cells/mL</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Skeletonema costatum</strong></td>
<td>Amberlite resin (XAD16N) extract</td>
<td>Mycobacterium bovis Mycobacterium tuberculosis</td>
<td><strong>800 μg/mL (91% to 99% of growth inhibition)</strong></td>
<td>(Lauritano et al., 2018)</td>
</tr>
<tr>
<td>and Chaetoceros pseudocurvisetus</td>
<td>Acetone:dichloromethane extract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diatoms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chrysothamnium taylorii</strong></td>
<td>Chrysophaentin analogs</td>
<td>Staphylococcus aureus</td>
<td><strong>10-40 μg/mL (MIC)</strong></td>
<td>(Davison and Bewley, 2019)</td>
</tr>
<tr>
<td>(Ochrophyta)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.2A Antimicrobial microalgal extracts/fractions.

Hereafter I provide a more detailed description of the marine microalgal extracts/fractions and their antimicrobial activities reported in the literature (Table 1.2)

Naviner et al. (1999) studied the effect of a fraction from the ethanolic extract of the diatom *S. costatum* on 9 different gram-negative bacteria (*Aeromonas salmonicida, Aeromonas hydrophila, Serratia liquefasciens, Yersinia ruckeri, Vibrio alginolyticus, Vibrio*...
mytili, Listonella anguillarum, and two species of Vibrio pathogens from bivalve larvae). The antibacterial activity of the fraction was evaluated using the agar-disc diffusion method and was compared to four reference antibiotics (oxolinic acid, flumequin, chloramphenicol and oxytetracycline). The inhibition diameter of the fraction against L. anguillarum, Vibrio mytili T, Vibrio spp. S322 and Vibrio spp. VRP was between 9 and 15mm after 48h of treatment, while all the reference antibiotics presented inhibition diameters superior to 15mm for the same bacteria. The authors suspect that the origin of the observed activities are the fatty acids detected in the fraction, but more studies are necessary to validate this hypothesis.

Qin et al. (2013) tested the antimicrobial activity of the marine diatom T. rotula using microalgal biomass and different extracts from the biomass. The biomass and extracts were tested for its antimicrobial activity against Bacillus pumilus, Micrococcus luteus, Escherichia coli, Enterococcus cloacae, Vibrio natrigens, Vibrio harveyi, Vibrio fischerii and Staphylococcus aureus, by the agar disk diffusion method. The extracts from T. rotula showed the highest activity against S. aureus and B. pumilus. After 18h of treatment with the paste of biomass, water extract, hexane:tBME extract and chloroform:methanol extract from T. rotula, the inhibition diameters were 14.2 mm, 7.8 mm, 9.5 mm and 7.3 mm for S. aureus and 14.5 mm, 9.7 mm, 13.8 mm, and 7.5 mm for B. pumilus, respectively. The authors also pointed out the importance of the extraction methods, reporting that they obtained better results when biomass was extracted by sonication with the addition of glass beads.

Sushanth and Rajashekhar (2015) tested the antimicrobial activity of various organic extracts (i.e. methanol, ethanol and hexane extracts) from the diatoms Chaetoceros calcitrans and Skeletonema costatum, the Cyanobacterium Chroococcus turgidus, and the green alga Nannochloropsis oceanica. For these studies the authors used seven bacterial pathogens (i.e. Escherichia coli, Streptococcus pyogenes, Bacillus subtilis, Proteus vulgaris, Klebsiella pneumonia, Salmonella typhi and Staphylococcus aureus) and four fungal pathogens (i.e. Candida albicans, Fusarium moniliforme, Aspergillus flavus and Aspergillus niger). The in vitro antimicrobial activity of the different extracts was assessed using the agar-disc diffusion method with treatments of 24h for bacteria and 48h for fungi. The most active extracts were the hexane extract from C. turgidus (21.4 mm inhibition diameter against E. coli), the ethanol extract from S. costatum (18.1mm inhibition diameter against S. pyogenes) and the hexane extract from N. oceanica (17.4 mm inhibition diameter against S. pyogenes). Antibacterial activity of C. calcitrans was quite similar when extracted with
ethanol or methanol, but for *S. marinoi* the activity profile was different depending on the solvent used for the chemical extraction. *S. marinoi* ethanol and methanol extracts also presented similar antifungal activity against *F. moniliforme* but it was low compared to the antibacterial activity. These studies presented antimicrobial activity results on the extracts of two diatoms, and also highlights that the use of different solvents for biomass extraction plays an important role on the biological activity observed.

External otitis is the inflammation or infection of the external auditory canal mainly caused by bacteria. Pane *et al.* (2015) tested methanol extracts of *D. tertiolecta* against the main bacteria involved in the development of external otitis, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The Minimum Inhibitory Concentration (MIC) of the extract from *D. tertiolecta* was determined using the broth microdilution method, according to the Clinical & Laboratory Standards Institute guidelines (Wayne, 2015). MIC₉₀ values for *D. tertiolecta* were 5.6×10⁹ cells/mL and 6.2×10⁹ cells/mL against *P. aeruginosa* and *S. aureus*, respectively.

Lauritano *et al.* (2018) tested 46 different species of marine microalgae (mainly the diatoms *Skeletonema* sp. and *Chaetoceros* sp. and the dinoflagellate *Alexandrium* sp.) looking for possible antibacterial and antifungal activities, and in particular for antituberculosis activity. The authors used three different culture conditions (i.e. control conditions, phosphate starvation conditions and nitrogen starvation conditions) and two different methods of extraction, one based on solid-phase extraction with an amberlite resin and the other based on a liquid-liquid extraction. Results showed that bioactivity was dependant on both culturing conditions and extraction methods used. In particular, *S. costatum* and *C. pseudocurvisetus* showed activity only in phosphate starvation conditions when using Amberlite resin method of extraction. At 800 μg/mL, extracts of *S. costatum* inhibited the growth of *M. bovis* up to 91% while extracts of *C. pseudocurvisetus* displayed a 99% inhibition activity against *M. tuberculosis*. When applying the liquid-liquid extraction method, both control and phosphate starvation condition extracts were active. In particular, control extracts of *S. costatum* showed a concentration dependent inhibition against both *Mycobacterium* strains while the phosphate starvation extract was active only at the highest concentration (800 μg/mL). For *C. pseudocurvisetus*, extracts from the control conditions inhibited the growth of both bacterial strains only at the highest concentration. This was the first report of microalgae (*S. costatum* and *C. pseudocurvisetus*) showing antitubercular activity.
The studies from this section evaluated the \textit{in vitro} antimicrobial activity of several extracts and fractions from marine microalgae. Unlike the previous section regarding anticancer activity, it is difficult to compare the results on antimicrobial activity because of the different methods used to measure such bioactivity. While agar disk diffusion methods provide results in terms of diameter, the microdilution broth and MIC assays give them in terms of concentration. Also in this case, the authors did not perform any study on the identification of the molecules responsible for the antimicrobial activity. However, the studies from Lauritano et al. (2008) served as the starting point of a part of Chapter 4 regarding the antibacterial activity of the diatom \textit{C. pseudocurvisetus}.

1.1.2B Antimicrobial microalgal compounds.

In this section I describe the biological activity of compounds from microalgae which have been demonstrated to possess antimicrobial activity in the literature (Table 1.2).

Amphidinols (Figure 1.10) are a family of polyketides produced by the family of marine dinoflagellates of the genus \textit{Amphidinium spp}. The first amphidinol was discovered in 1991 by Satake and co-workers (Satake \textit{et al.}, 1991a) and since then, almost all the isolated amphidinol-related compounds have displayed antifungal or/and haemolytic activity. The background and activity of these compounds will be deeply covered in chapter 2 of this doctoral thesis.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{amphidinol.png}
\caption{Amphidinol 22, an example of amphidinol. This compound was isolated within this PhD project.}
\end{figure}

Gambieric acids A and B (Figure 1.11; 15 and 16) are two polyethers isolated from the toxic marine dinoflagellate \textit{Gambierdiscus toxicus} (Nagai \textit{et al.}, 1993). They are closely related to brevetoxins, a class of potent marine neurotoxins closely related to ciguatoxins and associated to gastrointestinal, neurological and cardiovascular harmful effects on humans (Baden and Adams, 2000; Naar \textit{et al.}, 2009). 15 and 16 biological activity was assessed against several bacteria and fungi. The compounds did not show any activity against bacteria at the highest concentration tested, but they were really active against
filamentous fungi, having minimum inhibitory concentration (MIC) values superior to amphotericin B meaning in the range of 0.3 to 3 μg/mL. However, cytotoxicity on mouse lymphoma cells P388 was also high at ng/mL so the activity observed was not specific against fungi and indicates that the compound is probably as toxic as brevetoxins.

Desbois et al. (2008) isolated two antibacterial free fatty acids (Figure 1.12) from the diatom P. tricornutum. A bioassay-guided screening method allowed them to isolate (6Z, 9Z, 12Z)-hexadecatrienoic acid (14) and palmitoleic acid (15), two free fatty acids active against S. aureus from the alga P. tricornutum (SAG 1090-6). To determine the antibacterial properties of the compounds, the 50% inhibitory concentration (IC₅₀) was measured by an antibacterial broth microdilution assay. Minimum bactericidal concentration (MBC) values for 17 and 18 were 40-80 μg/mL and >640 μg/mL, respectively. This is the first study that demonstrated the bactericidal activity of the unusual fatty acid 17. The authors also compared their data with other fatty acids of equal length such as the palmitic acid (saturated analogue of 15) and concluded that bactericidal potential of these compounds was directly proportional to the number of double bonds in the molecule (unsaturation degree).
Davison and Bewley (2019) isolated four new brominated chrysophaentin-related compounds (Figure 1.13; 19, 20, 21 and 22) from the rare marine chrysophyte *Chrysophaeum taylorii* (clone NIES-1699). The chrysophaentins have been previously described as compounds with known antibiotic activity against gram-positive bacteria. *C. taylorii*, the microalga that produced these four new metabolites, was collected from the Island of Iriomote in Okinawa (Japan). Antibacterial activity of the isolated compounds was assessed against *S. aureus* (ATCC 29213) as described in the Clinical and Laboratory Standards Institute guidelines M07-A9 (Wayne, 2012). All four compounds were found to be active. Compounds 19 to 22 presented a minimum inhibitory concentration (MIC) value of 10 μg/mL, 40 μg/mL, 10 μg/mL and 40 μg/mL, respectively. The structural novelty of the compounds relies on the presence of brominated positions when compared to the known chrysophaentins. However, the authors observed that bromination of the B ring observed in compounds 20, 21, and 22 barely affected their bioactivity compared to compound 19, and bromination of the 2-position of the A ring also did not significantly change their bioactivity.

![Figure 1.13. Brominated chrysophaentin-related compounds.](image)

Four classes of compounds from marine microalgae assessed for antimicrobial activity were reviewed in this section. The antifungal activity of the amphidinols are discussed extensively the chapter 3 of this thesis. Redarging the other compounds, gambieric acids presented high cytotoxicity in addition to the antifungal activity, implying that they are probably toxic compounds and cannot undergo further development. MBC value of the polyunsaturated fatty acid HTA was much higher compared to the values of the penicillins, cephalosporins and aminoglycosides against *S. aureus* (Reimer, Stratton and Reller, 1981). The same happened with the brominated chrysophaentins, which presented MIC values on *S. aureus* that were much higher compared to known antibiotics. It seems like marine
microalgae have not provided much in terms of antimicrobial compounds, and my thesis was an attempt to further explore this possibility assuming the risk of not finding anything at the very end, but learning a lot during the whole process.

1.1.3 Criteria for microalgae selection and culturing conditions

Marine microalgae species were selected for my drug discovery project based on previous results from the EU-FP7-Pharmasea project (http://www.pharma-sea.eu/), in particular the results generated by my internal supervisor Chiara Lauritano and coworkers (Lauritano et al., 2016, 2018). I have also considered the information available in the literature about the microalgae that have already shown any biological activity (Sections 1.1.1 and 1.1.2). Using these criteria, the probability to find bioactivity between the selected species was higher. However, one of the species was selected since it was freshly isolated and/or no information on biological activity was found in the literature, and then it was randomly selected. I considered that random selection is also valid under the circumstances of my project, since you never know which kind of metabolites a species can produce until you investigate it. All the species selected were already available at the Stazione Zoologica Anton Dohrn, and were isolated from the Gulf of Naples in the Mediterranean Sea, from the Southern Bight in the Northern sea, bought from the National Center of Marine Microalgae and Microbiota (NCMA Bigelow) or bought from the Roscoff Culture Collection (RCC). The background information about the species selected will be discussed in each of the following chapters. A table summarising the criteria for the selection of each species is presented below (Table 1.3).

Table 1.3. Summary of the microalgal species selected, biological activity reported, criteria for the selection, compounds already isolated and references from the literature.

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Activity reported</th>
<th>Criteria</th>
<th>Isolated compound?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amphidinium carterae</em> (dinoflagellate)</td>
<td>Antifungal activity</td>
<td>Pharmasea</td>
<td>No</td>
<td>Non-published results</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative activity (cancer cells)</td>
<td>Literature</td>
<td>No</td>
<td>(Samarakoon et al., 2013)</td>
</tr>
<tr>
<td><em>Chateroceros pseudocurvisetus</em> (diatom)</td>
<td>Antitubercular activity</td>
<td>Pharmasea</td>
<td>No</td>
<td>(Lauritano et al., 2018)</td>
</tr>
</tbody>
</table>
In my studies, I based my selection of the control culturing conditions on the vast experience of the researchers from my institution (Stazione Zoologica Anton Dohrn) on maintaining and growing microalgae as part of a long tradition of studies in the field of microalgal biology and ecology. I selected the conditions in which most microalgae from the culture collection were able to grow reaching high cell densities at the stationary phase. I used Guillard’s F/2 medium for diatoms, but silica depleted Guillard’s F/2 was used for green algae and Keller medium was used for dinoflagellates. The deletion of the silica from the Guillard’s F/2 recipe in the case of green algae is because they do not have the unique silica cell wall of diatoms, and hence green algae do not use any silica enrichment. I used these media because the SZN has many years of experience in using these growth media even if some studies also use Conway’s medium for dinoflagellates and green algae (Pasquet et al., 2011; Nigjej et al., 2013; Samarakoon et al., 2014). Regarding light intensity and photoperiod, 110 µmol·m²·s⁻¹ and 12h:12h light:darkness were selected as control conditions. In the literature, light intensities and photoperiods used were not homogeneous, being quite different between the different authors. In addition, not everyone reported these variables. Finally, the temperature used in my institution agrees with the most used temperature in the literature, 20°C.

Microalgal metabolism is characterized for its plasticity, so physical and biochemical variations on the culturing conditions can trigger or block different genes related to the biosynthesis of metabolites. For example, studies performed on the green alga *Chlamydomonas reinhardtii* suggested an increase in the production of triacylglycerols,
steryl esters, and wax esters when the alga was cultured under nutrient limitation (phosphorus and nitrogen), and a substantial reduction in biomass production was also noted (Kamalanathan et al., 2016). Phosphorus limitation on the marine microalga *Nannochloropsis oceanica* triggered phospholipid-recycling mechanisms that induced a variation of the lipid composition of the microalga, which compensated the lack of phospholipids in exponential growth by producing sulfoquinovosyldiacylglycerol and diacylglyceroltrimethylhomoserine (Mühlroth et al., 2017). Other studies performed on *Nannochloropsis* sp. indicated variations on maximum cell density and total lipid content achieved by the alga when cultured under different photoperiods (24:0, 18:6 and 12:12 light/darkness) and light intensities (50, 100 and 200 µmol·m⁻²·s⁻¹). The higher amount of lipid total content was achieved when using a photoperiod of 18:6LD at 100 µmol·m⁻²·s⁻¹ (Wahidin, Idris and Shaleh, 2013).

It is then clear that microalgal metabolism is affected by the culture conditions, so microalgal bioactivities and hence the presence/concentration of bioactive compounds may also depend on the physical and nutrient regime modifications on such culture conditions. For instance, Ingebrigtsen et al. (2016) demonstrated that the bioactivity of various marine microalgal extracts (i.e. the diatoms *Attheya longicornis*, *Chaetoceros socialis*, *Chaetoceros furcellatus*, *Skeletonema marinoi* and *Porosira glacialis*) with anticancer activity against melanoma cells A2058, changed when they were cultured under different light and temperature conditions. Lauritano et al. (2016) showed that the diatom *Skeletonema marinoi* had anticancer activity exclusively when cultured under nitrogen starvation conditions indicating that nutrient limitation played an important role in the expression of its biological activity. Extracts of another two different diatoms (i.e. *Skeletonema costatum* and *Chaetoceros pseudocurvisetus*) were tested against *Mycobacterium tuberculosis* and *Mycobacterium bovis* (Lauritano et al., 2018), demonstrating that the bioactivity changed when the species were cultured under control and phosphate starvation conditions. It was also observed that when the authors used different extraction methods (i.e. Solid phase extraction with Amberlite resin XAD16N and liquid-liquid extraction with acetone and dichloromethane), the biological activity was also different, underlining the importance of chemical extraction methods. This is the reasoning behind using different culturing conditions to trigger changes in the bioactivity of the microalgal extracts. The use of stressful conditions such as nutrient depletion or alteration of light cycles/intensity can promote the production of different metabolites, and this
phenomenon is in line with principles of the “One Strain-MAny Compounds” (OSMAC) approach, that has been extended also to marine microorganisms and considers both nutrient regime changes and physical changes (Romano et al., 2018). I then used this potential tool for the studies performed within this doctoral thesis. In particular, I selected the phosphorus depletion and the 24h:0h (Light:darkness) photoperiod as nutrient regime and physical alterations of the culturing conditions, respectively.

Finally, marine microalgae generally show a sigmoid-like growth pattern which includes an exponential phase when algae are growing at a faster rate than they are dying, a stationary phase when the number of cells replicated is equal to the number of cells that die, and a senescent phase when most cells are dying. Charles Vidoudez and Georg Pohnert (Vidoudez and Pohnert, 2008) demonstrated that in the stationary phase the concentration of secondary metabolites was the highest (in their case for polyunsaturated aldehydes in Skeletonema marinoi). In addition, most of the authors in the literature looking for bioactivity in marine microalgae usually harvest the biomass at the stationary phase since this is the period when most secondary metabolites with different ecological functions and potential biotechnological interest are produced (Desbois et al., 2008; Pasquet et al., 2011; Nigjeh et al., 2013; Rahman Shah et al., 2014; Sushanth and Rajashekhkar, 2015; Lauritano et al., 2016). In my opinion, the motivation for harvesting at the stationary phase is the availability of resources and biosynthetic machinery that are not being used as much for cell proliferation as they are in the exponential phase. I then decided to harvest the biomass from the microalgal cultures at the stationary phase.

1.1.4 Isolation of bioactive secondary metabolites from marine microalgae

The first step in the isolation of metabolites from a natural source is their chemical extraction. Most of the studies from this chapter (sections 1.1.1 and 1.1.2) used an organic solvent in order to produce a raw extract from marine microalgae, usually methanol, ethanol or hexane. The studies from Sushanth and Rajashekhkar (2015) are an example of the role of the solvent used for the extraction on the biological activity detected later on. Within my studies, the idea was following the classical bioassay guided fractionation approach in order to isolate bioactive metabolites, so I did not have a particular target family of compounds to be extracted, which can affect the decision of the solvent to be used. Methanol was the solvent selected due to several characteristics: it provides the best yield of phytochemicals such as phenolics, flavonoids, alkaloids and terpenoids, including both polar and non-polar components (Truong et al., 2019); it is relatively unexpensive and
free of regulations when compared to other less toxic solvents such as ethanol; its boiling point is fairly low in order to evaporate it under reduced pressure at temperatures that do not degrade thermolabile metabolites. Sonication in order to break the cells was also considered for the extractions.

Raw extracts can be screened using biological assay platforms in order to select those with the strongest bioactivity. As I already mentioned, I think that the further development of studies on the isolation of bioactive microalgal compounds from this chapter was hampered by the low concentration of secondary metabolites in marine microalgae, especially in diatoms. This low concentration of metabolites is the reason why I also decided to establish higher thresholds of testing concentrations in the bioassay platforms, in order to avoid losing metabolites of interest.

Regarding successive steps, studies from this chapter regard those in which the authors isolated bioactive compounds and described processes of isolation applying mainly liquid chromatography methods (Andrianasolo et al., 2008; Kusaikin et al., 2010; Pasquet et al., 2011; Desbois and Lawlor, 2013; Kim et al., 2014). The fractions generated in each step were tested for their biological activity, and the fractions that showed bioactivity were the ones selected for further fractionation. This cycle was repeated until isolation of the component responsible for the observed activity. This is how I also proceeded in my project.

1.2 Aim of the thesis

Cancer includes a large group of pathologies related to the unrestrained proliferation of cells in the body (https://www.cancer.gov/). There are more than 200 different types of cancers and some cancers may eventually spread into other tissues causing metastases that are often lethal. Cancer is the leading cause of death globally, largely because of aging and growth of the world population. According to the European Cancer Observatory (https://ecis.jrc.ec.europa.eu/), estimations for the four most common types of cancer in the European Union are: 378445 cases of colorectum cancer, 364601 cases of lung cancer (including trachea and bronchus cancer), 404920 cases of breast cancer and 164450 cases of bladder cancer (in 2018).

On the other hand, since the first reported uses of penicillin in 1942, antibiotics have been used worldwide to save millions of lives from infectious diseases. Unfortunately, overuse, inappropriate prescriptions, extensive agricultural use and availability of a limited number
of antibiotics are factors causing the fast emergence of resistant bacteria (Ventola, 2015). For example, according to a factsheet of the World Health Organization (http://www.who.int/), in 2016 10.4 million people were infected with tuberculosis and 1.7 million died from the disease. In addition, there were 600000 new cases of multidrug-resistant tuberculosis with resistance to rifampicin, the most effective first-line drug. This is a public health crisis and a health security threat.

The demand for new drugs for the treatment of such diseases has triggered a growing interest in marine sources for the biodiscovery of new molecules with biological activity which can have the potential to become marketed drugs (Jaspars et al., 2016). Among all marine organisms, microalgae represent a renewable and still poorly explored resource for drug discovery. They can be easily cultivated in photo-bioreactors to obtain huge volumes of biomass, avoiding difficult harvesting practices as in the case for sponges, tunicates and other marine macro-organisms. The general objective of my PhD project was to investigate different marine microalgae strains (diatoms, green algae and dinoflagellates) in order to identify secondary metabolites with anticancer and antimicrobial properties which can become potential drug candidates. During my PhD project I aimed to accomplish the following tasks:

1. Select microalgal species from the Stazione Zoologica Anton Dohrn culture collection or freshly isolated species, based on an extensive literature review from recent bibliography and the results from the EU-FP7 project Pharmasea (A project ended in 2017 focused on drug discovery from marine microorganisms; http://www.pharma-sea.eu/).

2. Increase the possibility of finding bioactive compounds using variations in media composition (nutrient starvation or depletion) and/or other fermentation conditions (e.g. light) which can trigger different metabolic pathways leading to the production of new compounds.

3. Apply analytical methods for microalgal biomass extraction and isolation of bioactive compounds from marine microalgal extracts

4. Test microalgal extracts on several anticancer and antimicrobial cell platforms and performed bioassay-guided fractionation to identify and study fractions/compounds with anticancer or antimicrobial activity.
5. Scale-up culturing volumes from small (30 mL Flask), to medium-large volumes (10L carboys) to produce larger amounts of biomass for chemical and bioactivity analyses.

6. Chemical characterization of compounds of interest using NMR and HRMS techniques.

There are still very few studies on the biological activity of marine microalgae and even fewer that take into consideration the relationship between bioactivity and culturing conditions of marine microalgae. This project gave me the opportunity to exploit this poorly explored niche and also to travel to other research institutions in order to complete several parts of the project. At the Stazione Zoologica Anton Dohrn, I grew the marine microalgae using various culturing conditions, producing the biomass necessary for the biological and chemical studies. At Fundación MEDINA (Granada, Spain) as well as in Marbio (UiT, Tromsø, Norway), I performed chemical extractions, biological assays, high-throughput screening and chemical characterization of the compounds of interest. So far, I managed to isolate and fully describe one new compound from the dinoflagellate A. carterae and to study the biological activity of many other marine microalgal species. All the details about the research performed within my doctoral thesis will be fully explained in the following chapters.
Chapter 2: Biological and chemical analysis of the harmful dinoflagellate *Amphidinium carterae*

2.1 Introduction

*Amphidinium carterae* is an athecate dinoflagellate present in both temperate and tropical waters (Tomas *et al.*, 1996). This toxic species has been associated with harmful algal blooms in the Mediterranean Sea (Pagliara and Caroppo, 2012) and is also well-known for the production of several cytotoxic macrocyclic compounds known as amphidinolides (Kobayashi *et al.*, 1991; Kobayashi, 2008).

Pagliara and Caroppo (2012) studied the effect of a Mediterranean strain of *A. carterae* on the embryos of the sea urchin *Paracentrotus lividus*. They observed that exposure to the living alga had no effect on the development of the embryo, but there was a 60% and 100% embryo growth inhibition after treatment with 3.75 mg/mL and 7.5 mg/mL of *A. carterae* cell lysate, respectively. In addition, they calculated the LC$_{50}$ value (needed concentration to reach 50% lethality) of the lysate for the brine shrimp species *Artemia salina*. When *A. salina* was exposed to 3.67 mg/mL (LC$_{50}$) of lysate for 24 hours, the lethality towards the shrimp was 50%. The antioxidant activity of *A. carterae* was also evaluated in LPS-induced macrophages (cell line RAW 264.7), which are macrophages with active secretion of cytokines (related to inflammatory processes) triggered by the action of lipopolysaccharide (LPS), an outer component of the gram-negative bacterial membrane. Shah and co-workers (Rahman Shah *et al.*, 2014) observed an 80% reduction in nitric oxide production in RAW 264.7 macrophages after treatment with the methanolic extract of *A. carterae* at a concentration of 50 µg/mL, without cell viability being affected. They also tested the anticancer potential of the extract in a human promyelocytic leukemia cell line (HL-60) but the activity found was rather low, with 20% growth inhibition after treatment with 50 µg/mL of raw extract.

So far, the genus *Amphidinium* has displayed a variety of different biological activities, and various bioactive marine natural products responsible for such bioactivities have been
isolated from different species, including amphidinols, amphidinolides, amphirionins and karatungiols (Figure 2.1; 20, 21, 22 and 23, respectively) (Echigoya et al., 2005; Washida et al., 2006; Kobayashi, 2008; Nuzzo et al., 2014; Kumagai et al., 2015; Satake et al., 2017a). All the isolated bioactive compounds present a linear or cyclic polyketide skeleton but not all *Amphidinium* species have the ability to synthesize polyketides though their enzymatic machinery (Lauritano et al., 2017).

All the isolated bioactive compounds present a linear or cyclic polyketide skeleton but not all *Amphidinium* species have the ability to synthesize polyketides though their enzymatic machinery (Lauritano et al., 2017).

![Amphidinol 18 (20)](image1)

![Amphidinolide A (21)](image2)

![Amphirionin-S (22)](image3)

![Karatungiol A (23)](image4)

**Figure 2.1.** Examples of bioactive marine natural products isolated from *Amphidinium* sp.

The aim of this chapter of the thesis was to screen *A. carterae* clone FE102 for various bioactivities useful for the treatment of human pathologies, and further isolate the compounds responsible for such activities. This clone was promising since a sequence encoding a β-ketosynthase, an enzyme involved in polyketide synthesis, was found in its transcriptome (Lauritano et al., 2017). Extracts from FE102 were already found to be active against the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans*, within the framework of the EU-FP7 PharmaSea project, suggesting the production of potentially active secondary metabolites in *A. carterae*.

Throughout this chapter I describe the methodologies applied to *A. carterae* (FE102) in order to characterize the biological activity of raw extracts and fractions generated from the dinoflagellate. I also performed a preliminary chemical analysis (dereplication) in order to identify possible bioactive compounds in the fractions. The outputs from the experiments are shown and discussed.
2.2 Methods

2.2.1 Culturing and harvesting

A. carterae (FE102) was bought from NCMA Bigelow (code CCMP 1314) in September 2011 has been maintained since then in the culture collection of the Stazione Zoologica Anton Dohrn (SZN). The medium used to maintain and grow this dinoflagellate was Keller’s medium (Keller et al., 1987). A. carterae from the SZN culture collection was cultured in control conditions (Keller’s medium, 20°C, 110 µmol·m⁻²·s⁻¹ light intensity and 12h:12h light:darkness photoperiod), first in a volume of 2L to build the growth curve, and then in 10L for biomass harvesting until a total of 180L were harvested. Cultures were grown at 20°C, 110 µmol·m⁻²·s⁻¹ light intensity, 12h:12h (light:darkness) photoperiod and continuous aeration. The 10L cultures were monitored every 24 hours by counting the cells. Once in the stationary phase was reached, 10L cultures were centrifuged at 3000 rpm, at 4ºC and for 10 minutes in order to harvest the cells and discard the supernatant. The biomass harvested was stored at -80°C. Further details regarding medium preparation, inoculation, growth curves, scaling-up processes and harvesting are fully explained in the General Methodologies section (M.1, M.2 and M.3) of this thesis.

2.2.2 Chemical extraction, preliminary screening and dereplication

The chemical extraction of the microalgal biomass and the preliminary screening of the extracts were performed using the Fundación MEDINA methods as reported in the General Methodologies section (M.4, M.5.1 and M.5.2). A. carterae (FE102) was screened against a panel of five different cancer cell lines (i.e. human lung carcinoma A549 ATCC® CCL-185™, human skin melanoma A2058 ATCC® CRL-11147™, hepatocyte carcinoma HepG2 ATCC® HB-8065™, breast adenocarcinoma MCF7 ATCC® HTB-22™ and pancreas carcinoma MiaPaca-2 ATCC® CRL-1420™), two gram-positive bacteria (Staphylococcus aureus MRSA MB5393, Staphylococcus aureus MSSA ATCC29213), two gram-negative bacteria (Escherichia coli ATCC25922 and Klebsiella pneumoniae ATCC700603), Mycobacterium tuberculosis H37Ra and the fungus Aspergillus fumigatus ATCC46645, as reported in sections M.5.1 and M.5.2.

The crude extract was analysed by HPLC-UV-HRESIMS on an Agilent 1200 RR coupled to a Bruker maXis QToF spectrometer with electrospray ionization, as reported by Martin and co-workers (Martín et al., 2014). Analysis was performed using a Zorbax SB-C8 (2.1 x 30 mm, 5µm) column at 40°C and with a flow rate of 300 µL/min. For the mobile phase, two
solvents were used. Solvent A was 10% acetonitrile and 90% water with trifluoroacetic acid and ammonium formate at a concentration of 1.3 mM, while solvent B was 90% acetonitrile and 10% water with trifluoroacetic acid and ammonium formate at a concentration of 1.3 mM. Gradient for the method is detailed in Table 2.1. Full diode array UV scans from 200 to 900 nm were collected in 4 nm steps at 0.25 s/scan. The mass spectrometer was operated in positive ESI mode. The instrumental parameters were 4 kV capillary voltage, drying gas flow of 11 L min⁻¹ at 200 °C, and nebulizer pressure of 2.8 bar. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Pre-run calibration was made by infusion with the same TFA-Na calibrant. Mass spectra were collected as full scans from 50 m/z to 2000 m/z. Data were analysed using the platform available at Fundación MEDINA (Pérez-Victoria, Martín and Reyes, 2016) and compared with the data available in the Dictionary of Natural Products and PubChem databases.

Table 2.1. Gradient used for the HPLC-UV-HRESIMS method. Time, flow rate and percent of the solvents are reported.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (µL/min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

2.2.3 Preliminary analysis of fractions

A ten milligrams aliquot from the microalgal crude extract was fractionated by semipreparative HPLC-DAD using a Zorbax SB-C8 (4.6 mm × 150 mm, 5 µm particle size) column with a flow of 3.6 mL/min and a ddH₂O:CH₃CN gradient (95:5 to 0:100 in 80 min) as eluent. Seven fractions (F1 to F7) were collected, dried, dissolved in 100% DMSO and tested against *A. fumigatus* and *C. albicans* as reported in the General Methodologies section (M.5.2). Each fraction was individually analysed by HPLC-UV-MS following the methodology reported in section 2.2.2 (Martín *et al.*, 2014).
2.3 Results and discussion

2.3.1 A. carterae (FE102) cultures and growth data

The growth curve of A. carterae in control conditions is reported in the figure below (Figure 2.2).

![Growth Curve](image)

Figure 2.2. A. carterae growth curve in control conditions.

According to the data collected, the stationary phase was fixed from day 10 to day 13 in the case of control conditions. Ten litres carboys were used to grow A. carterae in control conditions and collect biomass for further experiments. The biomass was collected by centrifugation, as reported in methods. The following table (Table 2.2) summarises the maximum cell concentration reached, the harvest day and the weight of the wet biomass (in triplicate). All samples were freeze-dried and stored at -80°C.

Table 2.2. A. carterae samples in triplicate reporting wet weight, harvest day and maximum concentration reached.

<table>
<thead>
<tr>
<th>A. carterae simples</th>
<th>Maximum concentration (cells/mL)</th>
<th>Harvest day</th>
<th>Wet weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>$1.90 \times 10^5$</td>
<td>13</td>
<td>4.73</td>
</tr>
<tr>
<td>Control 2</td>
<td>$1.86 \times 10^5$</td>
<td>13</td>
<td>4.59</td>
</tr>
<tr>
<td>Control 3</td>
<td>$2.26 \times 10^5$</td>
<td>13</td>
<td>3.16</td>
</tr>
</tbody>
</table>
Preliminary studies made during the EU-FP7 project PharmaSea showed that concentration of the active metabolite was very low and hence a considerable quantity of biomass was needed to extract sufficient material for further work. An additional 150L of cultures were prepared to harvest enough biomass for the isolation and characterization steps. The culturing conditions were the same for all the extra cultures, and so was the harvest time.

2.3.2 Preliminary bioactivity screening and dereplication of *A. carterae* (FE102)

Total biomass (from 180L) was extracted as reported in the methods, and yielded 50 mg of extract for each litre of microalgal culture, approximately. The anticancer activity of the crude extract was assessed against the cancer cell lines A549, A2058, HepG2, MCF7 and MiaPaca-2, while its anti-infective potential was assessed against *E. coli, K. pneumonia, MRSA, MSSA, M. tuberculosis* and *A. fumigatus*. The bioactivity results are expressed in terms of percentage of growth inhibition and are summarised in Table 2.3.

Table 2.3. Anticancer activity was tested at a concentration of 175 µg/mL, while antibacterial and antifungal activity were tested at a concentration of 560 µg/mL. The given values are the mean of three biological replicates and two technical replicates.

<table>
<thead>
<tr>
<th>Anticancer screening</th>
<th>Cancer cell line</th>
<th>A549</th>
<th>A2058</th>
<th>HepG2</th>
<th>MCF7</th>
<th>MiaPaca-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Growth Inhibition</td>
<td>100±0</td>
<td>100±1</td>
<td>100±0</td>
<td>100±0</td>
<td>100±1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibacterial screening</th>
<th>Bacteria</th>
<th><em>E. coli</em></th>
<th><em>K. pneumoniae</em></th>
<th>MRSA</th>
<th>MSSA</th>
<th><em>M. tuberculosis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Growth Inhibition</td>
<td>37±8</td>
<td>19±10</td>
<td>95±10</td>
<td>88±18</td>
<td>83±16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antifungal screening</th>
<th>Fungus</th>
<th><em>A. fumigatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Growth Inhibition</td>
<td>100±1</td>
<td></td>
</tr>
</tbody>
</table>

Marine dinoflagellates belonging to the genus *Amphidinium* are well-known producers of different toxins (Karafas et al., 2017), such as amphidinolides (Kobayashi, 2008). The bioassay results show that the *A. carterae* (FE102) extract has unspecific bioactivity and general cytotoxicity, especially against eukaryotic organisms. It is therefore not surprising that the observed activity matches with the known toxicity of closely related species.
Dereplication of the raw extract using qToF-Mass spectrometry revealed the presence of two known and one unidentified natural product belonging to the family of the amphidinols. These compounds were identified by their retention time and characteristic absorbance patterns at 260 nm, 270 nm and 280 nm (Figure 2.3) due to the conjugated triene in their structures. One of the known compounds presented a retention time of 3.52 min, a mass peak [M+H]+ of 1359.8326 m/z and a suggested molecular formula C\textsubscript{71}H\textsubscript{122}O\textsubscript{24}. This compound was putatively identified as amphidinol 18 according to the information available in the dictionary of natural products database when it was fed with the previous data (accurate mass, UV maxima and taxonomic information). In addition, the MEDINA-HRMS library (Pérez-Victoria, Martín and Reyes, 2016) that was automatically populated with the accurate mass, UV maxima and HRMS spectra of the component identified the compound as amphidinol 18, firstly isolated by Nuzzo et al. (2014) from another clone of *Amphidinium carterae* (CCMP121). The chemical data from amphidinol 18 were already in the MEDINA-HRMS internal database from *A. carterae* samples of the EU-FP7-PharmaSea project. The other known compound presented a retention time of 3.22 min, a mass peak [M+NH\textsubscript{4}]\textsuperscript{+} of 1356.8289 m/z and a suggested molecular formulae C\textsubscript{71}H\textsubscript{122}O\textsubscript{27}S. In the same way as for amphidinol 18, it was then identified as amphidinol 19, also isolated by Nuzzo et al. (2014) from the same clone mentioned above. A potentially new amphidinol (AM) with an accurate mass of 1644.9437 m/z (RT 3.36 min) was detected and assigned a suggested molecular formula C\textsubscript{77}H\textsubscript{144}O\textsubscript{36}, later discarded because it did not match the NMR data. The HPLC-UV-HRMS (QToF) software was refined only for compounds with a mass below 1000 m/z and therefore the molecular formulae were only putatively assigned until they were confirmed by comparison of the data with the databases or NMR data.

![Figure 2.3. Absorbance pattern typically observed within the amphidinols (Amphidinol 18 shown here). Three maximum peaks of absorbance are observed at the wavelengths 260 nm, 270 nm and 280 nm.](image)
A preliminary analysis of the FE102 fractions, obtained by semipreparative HPLC-DAD fractionation of a 10 mg aliquot from the raw extract, was performed as reported in the methods section. A total of seven fractions (named F1 to F7) were collected, dried under nitrogen-stream, dissolved in pure DMSO and tested against the fungi *A. fumigatus* and *C. albicans* (Figure 2.4). These fungal species were selected as targets since Nuzzo *et al.* (2014) had already reported the activity of amphidinols 18 and 19 against these organisms.

The only fraction in the chromatogram found to be active was fraction F4. Analysis of the fractions through HPLC-UV-HRESIMS allowed to identify fractions F1 and F4 mainly as amphidinol 19 (AM19, \( C_{71}H_{122}O_{27}S \)) and amphidinol 18 (AM18, \( C_{71}H_{122}O_{24} \)), respectively. The compounds were identified in the same way as for the dereplication of the raw extract. Fractions F3, F5, F6 and F7 also contained other possible amphidinol-related compounds (they featured amphidinol’s UV pattern and similar retention time) that were not studied since their concentrations within the whole extract were rather low or very low, precluding further chemical analyses. F2 contained a new amphidinol with an accurate mass of 1644.9437 m/z and a putative molecular formula \( C_{77}H_{144}O_{36} \).
2.4 Conclusion

Within this chapter I showed the bioactivity results of *A. carterae* clone FE102 (Bigelow CCMP1314) and a preliminary chemical analysis of the extract from the dinoflagellate. The extract from FE102 displayed unspecific bioactivity and general cytotoxicity, especially against eukaryotic organisms. It was also possible to observe antibacterial effects on Gram+ bacteria and *M. tuberculosis*. I was also able to detect a new compound belonging to the family of the amphidinols, with one of the highest molecular weights compared to its related compounds, and which could find biomedical applications in the future.

Although my *A. carterae* clone FE102 showed a sharp toxic profile, it may still be interesting since it may be able to produce compounds (including the new amphidinol I detected) with potential biomedical applications within a specific range of concentrations (safe windows). These compounds can also be derivatised to avoid toxicity when it comes to prevent failure during clinical trials and successful drug development (Smith, 2011).

Dinoflagellates are known to be strong producers of secondary metabolites, most of which are biologically active compounds classified as toxins (Caruana and Amzil, 2018). In humans, exposure to these toxins can lead to gastrointestinal and neurological syndrome (i.e. paralytic shellfish poisoning—PSP, amnesic shellfish poisoning—ASP, diarrheic shellfish poisoning—DSP, neurologic shellfish poisoning—NSP, and ciguatera fish poisoning—CFP) (Caruana and Amzil, 2018). However, several studies have reported that toxins from dinoflagellates have biological activities which are or could be of interest for possible human health applications (Assunção, Catarina Guedes and Xavier Malcata, 2017). For instance, the potent neurotoxin tetrodotoxin, also found in the dinoflagellate *Alexandrium tamarense* (Kodama *et al.*, 1996), is now undergoing phase 3 clinical trials for cancer chemotherapy associated pain relief and is the active principle of a drug called Halneuron®, produced by WEX Pharmaceuticals ([https://wexpharma.com/](https://wexpharma.com/)). Halneuron® has already been tested on more than 500 patients and it has shown varying degrees and duration of pain relief. Nonetheless even if the extracts from the alga were toxic, I decided to explore the bioactivity of these extracts and in doing so I discovered a new amphidinol which is described in detail in the following Chapter.
Chapter 3: Amphidinol 22, a new bioactive metabolite from *Amphidinium carterae*

3.1 Introduction

Polyketides from marine dinoflagellates have been widely studied (Rein and Borrone, 1999; Van Wagoner, Satake and Wright, 2014), especially those coming from strains of the genus *Amphidinium* (Kobayashi and Kubota, 2007). Amphidinols (AM) are a family of 23 linear polyketides discovered thirty years ago (Satake et al., 1991b) from *Amphidinium* sp. All amphidinols discovered so far were tested for their antifungal activity and it was observed that not all of them were active at the tested concentrations. For instance, the short side-chain amphidinols AM6 and AM2 were found to be active against *Aspergillus niger* at 6 µg per disk (Morsy et al., 2008). Echigoya and co-workers observed strong antifungal activity on *A. niger* for AM2, AM4 and AM9 (44.3, 58.2 and 32.9 µg per disk, respectively) while the activity for its respective sulfate ester derivatives AM11, AM12 and AM13 was rather low (>100–256.6 µg per disk) (Echigoya et al., 2005). AM18 displayed strong activity against the fungus *Candida albicans* (MIC 9 µg/mL) while the antifungal activity of its sulfate ester derivative AM19 was absent (Nuzzo et al., 2014). AM20 and M21 were the largest amphidinol homologues ever characterized, but they did not display any antifungal activity in *A. niger* even at the highest concentration tested, 15 µg per disk (Satake et al., 2017a).

Considering all these examples, the trend on the structure-bioactivity relationship indicates that long chain amphidinols and the sulfated derivatives displayed weaker antifungal activities. The trend observed for the haemolytic activity on human erythrocytes is similar, as observed in the results from Echigoya and co-workers (2005). Satake et al. (2017) proposed membrane permeabilization (formation of channels) as the mode of action, and two different models to explain the difference in bioactivity of the amphidinols. While short-chain amphidinols form a “spike” with a sterol molecule (such as cholesterol in human cells or ergosterol in fungi) in order to penetrate cell membranes forming a barrel-stave channel, the long chain amphidinols are folded in a carpet-model bound to the lipidic portion of the membrane bilayer. These models also explain why the amphidinols with voluminous polar substituents in the chain (as the sulfate group OSO$_3$Na) do not
display strong bioactivity, since such moieties will be repelled by the lipidic fraction of the bilayer.

Throughout this chapter I describe the methodologies applied to A. carterae (FE102) extracts in order to isolate a new marine natural product from the family of the amphotinols, amphidinol 22. Hereafter we describe the chemical and biological characterization of amphidinol 22. The outputs from the experiments are shown and discussed.

3.2 Methods

3.2.1 Reversed-phase flash chromatography

Chromatographic conditions selected: RP18 resin (reversed phase C18) Column chromatography (low resolution), 40x100mm of resin, 5600 mg extract. The method consisted in a gradient of ddH$_2$O (A) and acetonitrile (b) reported in Table 3.1. Wavelengths measured were 210 and 260 nm. These conditions were selected empirically according to the test injections on a HPLC-DAD system.

Table 3.1. Gradient used for the flash fractionation method. Time, flow rate and percent of the solvents are reported.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>40</td>
<td>18</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>18</td>
<td>0</td>
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</tbody>
</table>

The A. carterae microalgal extracts were fractionated through low resolution reverse phase chromatography using a CombiFlash® Rf chromatography instrument. This instrument requires the column to be prepared manually in advance. The stationary phase of the column consisted in 40x100 mm of RP18 resin and the samples was packed in the head of the column as slurry prepared using more RP18 resin and methanol. The extract was dissolved in methanol and added to a 500 mL rounded flask together with the RP18 resin at a rate of 3 grams of resin for each gram of extract (3:1, Resin:Extract). The prepared slurry was then vacuum dried using a rotary evaporator and stored in a filter paper cone. To assemble the column, a RediSep® Rf Cartridge was used as a support. A RediSep® Frit (filter) was placed at the bottom of the cartridge and the RP18 resin was added and
compacted using methanol. Another frit was placed at the top of the stationary phase to seal it and a plunger was placed at the top of the column. Using the Combiflash instrument, the stationary phase was conditioned using the initial conditions of the method for the fractionation. Once the stationary phase was conditioned, the plunger was removed and the dried slurry was added to the top of the second frit filter and sealed with a third and final frit filter. At this point, the plunger was placed again at the top of the column and the Combiflash instrument was ready to start the fractionation. 80 fractions of 18 mL each were collected.

3.2.2 Semipreparative HPLC-DAD purification
Fractions 19 and 20 from the flash chromatography (17.7 mg, eluted at 48% B) were pooled and further fractionated by semi-preparative reversed-phase HPLC-DAD using a Gilson Automated Preparative HPLC System with a Zorbax SB-C8 (9.4 x 250 mm, 5 µm particle size) column, a flow of 3.6 mL/min and mqH₂O:CH₃CN (gradient 75:25 to 68:32 in 36 minutes) as eluents. Two sub-fractions (FH1 and FH2) were collected and analysed by HPLC-UV-HRMS as in Section 2.2.2 in order to assess the purity and annotate the mass of the components.

3.2.3 Nuclear magnetic resonance (NMR) and HPLC-UV-HRESIMS analysis
NMR spectra were acquired using a Bruker Avance 500 MHz spectrometer with pulsed field gradient and referenced to the solvent signals (CD₃OD, at δH 3.31 ppm and δC 49.0 ppm). The instrument was equipped with a 1.7 mm TCI MicroCryoProbeTM (Bruker Biospin, Fällanden, Switzerland). HPLC-UV-HRMS analysis allowed to obtain the accurate mass of the compound as well as the full mass/UV spectra, with the conditions reported in Section 2.2.2.

3.2.4 Amphidinol 22 identification
Amphidinol 22 was isolated as a pale yellow, amorphous solid; UV data in MeOH: λmax 282, 270 and 260 nm; NMR data in CD₃OD is presented in Table 3.2; HRESIMS m/z 1662.9705 [M + NH₄]⁺ (calcd for C₈₄H₁₄₄O₃₁N⁺, 1662.9717, Δ −0.7 ppm); 1645.9515 [M + H]⁺ (calcd for C₈₄H₁₄₁O₃₀⁺, 1645.9451, Δ 3.9 ppm); 1627.9371 [M−H₂O + H]⁺ (calcd for C₈₄H₁₃₉O₃₀⁺, 1627.9346, Δ 1.5 ppm). ¹H NMR (500 MHz), ¹³C NMR (125 MHz), HSQC, COSY, HMBC and NOESY are available in the section at the end of this chapter (Section 2.5 NMR spectra; Figures S1, S2, S3, S4, S5 and S6, respectively).
3.2.5 Statistical Analysis

Statistical differences between treated and control cells for the assays performed on amphidinol 22 were determined by Student’s t-test using GraphPad Prim statistic software, V4.00 (GraphPad Software, San Diego, California, USA). Data were considered significant when at least p was <0.05 (* for p < 0.05, ** for p < 0.01, and *** for p < 0.001).

3.3 Results and discussion

3.3.1 Isolation of the new amphidinol and structural elucidation

The crude extract from *A. carterae* FE102 (5.6 g) was fractionated by flash reversed-phase chromatography. The resulting fractions 19 and 20 were pooled together and further fractionated by semipreparative HPLC-DAD. Two subfractions (FH1 and FH2, Figure 3.1) were collected, dried under nitrogen stream, weighted and analysed by HPLC-UV-HRESIMS.

![Figure 3.1. Chromatogram of the purification of the new amphidinol (peak FH1). The wavelengths selected for the chromatogram were 210 nm (in blue) and 260 nm (in pink).](image)

The pure fraction FH1 weighed 1.7 mg and presented an accurate mass of 1644.9437 m/z and a putative molecular formula of C_{77}H_{144}O_{36} (Unsaturation number: 6). On the other hand, FH2 weighed 1.1 mg and was identified as amphidinol 18 by populating the MEDINA-HRMS library with the data of the component (accurate mass, UV data and HRMS spectra), as already done above for the raw extract and fractions.
From now onwards, fraction FH1 will be called amphidinol 22. After freeze-drying, the compound was observed as an amorphous solid with a pale-yellow tone. The UV absorption maxima at 260, 270 and 280 nm from the HPLC-UV-HRESIMS analysis confirmed the presence of the conjugated triene typical of amphidinols. The LC-UV trace and UV spectra are reported in the figure below (Figure 3.2):

![LC-UV trace and UV spectra of amphidinol 22](image)

**Figure 3.2.** LC-UV trace and UV spectra of amphidinol 22

The small peak observed in the UV trace was identified as a plasticizer present in the 96-deep well plates used during the semipreparative HPLC-DAD purification.

Regarding HRESIMS data, the accurate mass calculated by the software of the instrument was 1644.9437 Da, very close to the calculated exact mass 1644.9379 Da. Three adducts with \( m/z \) 1662.9705 \([M + NH_4]^+\) (calcd for \( C_{84}H_{140}O_{31}N^+ \), 1662.9717, \( \Delta -0.7 \) ppm), 1645.9515 \([M + H]^+\) (calcd for \( C_{84}H_{141}O_{31}^+ \), 1645.9451, \( \Delta 3.9 \) ppm) and 1627.9371 \([M-H_2O + H]^+\) (calcd for \( C_{84}H_{139}O_{30}^+ \), 1627.9346, \( \Delta 1.5 \) ppm) were observed in the mass spectrum (Figures 3.3 and 3.4) and confirmed that the molecular formula of amphidinol 22 was \( C_{84}H_{140}O_{31} \) (degrees of unsaturation = 15). The HRESIMS data and the molecular formula obtained
from the elucidation with the NMR spectra were well correlated (please check below in this section). The molecular formula $C_{77}H_{144}O_{36}$ calculated by the HRMS software was then discarded.

**Figure 3.3.** HRESIMS spectra of amphidinol 22
Figure 3.4. Expansions of the peaks corresponding to the reported adducts in the HRESIMS spectrum of amphidinol 22

A set of NMR spectra (\(^1\)H, \(^{13}\)C, HSQC, COSY, HMBC and NOESY) were acquired as reported in the methods in order to perform the structural elucidation of the new molecule.

To start with, \(^{13}\)C NMR spectrum revealed the presence of 79 different carbon signals of which 20 signals corresponded to \(sp^2\) carbons, corresponding to 10 unsaturations (no carbon signals in the \(^{13}\)C NMR spectrum presented the shift of carbonyl groups) present in the molecule out of 15 unsaturations calculated for the molecular formula \(C_{84}H_{140}O_{31}\). In the region between \(\delta 55.11\) and \(\delta 82.36\) ppm of the \(^{13}\)C NMR spectrum, 33 carbon signals were found. These signals are more likely to correspond to oxygenated carbons, two of them presenting a particularly shielded chemical shift (\(\delta^{13}\)C of 55.11 and 62.35 ppm), found in epoxide groups. On the other hand, the high degree of overlap in the \(^1\)H NMR spectrum interfered with the establishment of the multiplicity of the proton signals, especially in the region of the oxygenated protons (\(\delta H\) from 3 to 4 ppm). The multiplicity of only a few signals in the outermost regions of \(^1\)H spectrum could be determined.

The 2D-NMR hetero/homo-nuclear experiments performed (i.e. HSQC, COSY, HMBC and NOESY) were then used for the identification of the planar structure of amphidinol 22.

The HSQC spectrum revealed carbon overlap at the signals at \(\delta_C 30.41\) (2 \(CH_2\)) and 33.59 (CH and \(CH_2\)), and \(\delta 36.68\) (2\(CH_2\)), 74.63 (2 \(CH\)) and 76.77 (2 \(CH\)), each of them accounting for two carbons. It also allowed to indicate that the signals \(\delta 136.85, \delta 139.30, \delta 144.65\) and \(\delta 151.35\) were four \(sp^2\) quaternary carbons, since they did not present correlations with any proton in the spectrum. The total number of carbons identified in the molecule was then 84, matching with the proposed molecular formula \(C_{84}H_{140}O_{31}\). A table listing all the carbons and their respective proton assignments (Table 3.2) was generated with the mixed information from the \(^1\)H, \(^{13}\)C, and HSQC spectra.

Table 3.2. NMR data of amphidinol 22 (500 MHz) in CD\(_3\)OD. The multiplicity of the proton signals as well as their coupling constants \(J\) in Hz, for double bonds are included.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(\delta^{13})C</th>
<th>(\delta^1H,) mult, (J) (Hz)</th>
<th>Carbon</th>
<th>(\delta^{13})C</th>
<th>(\delta^1H,) mult, (J) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.09</td>
<td>0.91, t, 7.4</td>
<td>43</td>
<td>41.90</td>
<td>1.97, m; 1.51, m</td>
</tr>
<tr>
<td>2</td>
<td>23.58</td>
<td>1.42, m</td>
<td>44</td>
<td>71.79</td>
<td>3.85, m</td>
</tr>
<tr>
<td>3</td>
<td>35.83</td>
<td>2.06, m</td>
<td>45</td>
<td>36.68</td>
<td>1.67, m; 1.58, m</td>
</tr>
<tr>
<td>4</td>
<td>136.10</td>
<td>5.69, m</td>
<td>46</td>
<td>36.68</td>
<td>2.20, m; 2.11, m</td>
</tr>
<tr>
<td>5</td>
<td>131.23</td>
<td>6.05, dd, 15.2, 10.5</td>
<td>47</td>
<td>139.30</td>
<td>Null</td>
</tr>
</tbody>
</table>
In addition to the previous information, the COSY and the HMBC spectra were acquired to obtain more information regarding the connectivity of the molecule and helped me to solve the overlapping observed in the $^1$H spectrum.

I built four spin systems (Figure 3.5) for the proposed structure: A (from H1 to H33), B (from H37 to H46), C (from H48 to H58) and, finally, D (from H60 to H78) using the COSY spectrum and the previously discussed data.
Figure 3.5. Spin systems established based on the COSY spectrum signals and supported by a comparative approach with the data known from amphidinols 18 and 19. The spin systems appear in different colors as follows: A (black), B (blue), C (orange) and D (green).

Most of the COSY signals were visible and easy to correlate to the protons from the list (Table 3.2). However, there was a fragment of the molecule (C62 to C65) that was particularly difficult to resolve because of extensive overlap. A comparative approach using data available of the already known amphidinols 18 and 19 (Nuzzo et al., 2014) permitted me to complete spin system D due to the similarity of the chemical shifts with those of amphidinol 22. On the other hand, HMBC spectrum correlations were used to connect the spin systems, and they were also used when the overlapping of the signals was considerable and it was difficult to establish the connectivity only with the COSY correlations. In addition, cross-peaks on the HMBC spectrum at δ 3.14/81.54 (H20/C16), δ 4.22/74.63 (H23/C27), δ 3.48/79.21 (H55/C51) and δ 3.74/70.41 (H66/C62) corresponded to long range through-oxygen correlations and were used to spot four ether bridges along the structure. These four established ether bridges, the previously mentioned epoxide group at C10-C11 (δ\textsuperscript{13}C of 55.11 and 62.35 ppm) and the 10 double bonds corresponding to the sp\textsuperscript{2} carbons, fulfilled the total number of unsaturations (15). This allowed me to link the other oxygenated carbons (δ\textsuperscript{13}C 60-85) to hydroxyl moieties (Figure 3.6).
Figure 3.6. Spin systems confirmed with the HMBC correlations. The HMBC correlations confirming the bonds where the COSY did not provide enough information are highlighted as red arrows (Confirmatory HMBC correlations).

4-bond long distance COSY correlations were observed between H35-H80 (δ 5.72/1.84), H35-H81 (δ 5.72/5.05-4.85), H37-H81 (δ 2.27-2.21/5.05-4.85) and H80-H33 (δ 1.84/2.29), suggesting the proximity of these protons. A summary of all the visible correlations in the COSY and NOESY spectra is tabulated below (Table 3.3).

Table 3.3. 2D-NMR COSY and HMBC correlations. (ld) in the COSY signals stands for long distance.
7  
5.61, dd, 15.2, 8.6  
H6, H8  
C5, C8, C9  
49  
4.56, dd, 8.9, 1.7  
H48, H50  
C47, C48, C51

8  
4.26, ddd, 6.6, 6.6, 6.6  
H7, H9  
C6, C7, C9, C10  
50  
3.68, dd, 9.5, 1.9  
H49, H51  
C48, C51, C52

9  
1.75, m, 2H  
H8, H10  
C7, C10, C11, C12  
51  
3.95, m  
H50, H52  
C50, C52, C53, C55

10  
3.00, ddd, 6.2, 5.6, 2.1  
H9, H11  
C9, C11, C12  
52  
4.05, m  
H51, H53  
C54

11  
2.72, dd, 5.2, 2.1  
H10, H12  
C9, C10, C12, C13  
53  
3.97, m  
H52, H54  
- 

12  
3.41, m  
H11, H13  
C10, C11, C13, C14  
54  
1.76, m  
H53, H55  
C52, C53, C55, C56

13  
1.62, m; 1.48, m  
H12, H14  
C11, C14, C15  
55  
3.48, m  
H54, H56  
C51, C53, C56, C57

14  
1.77, m; 1.44, m  
H13, H15  
C13  
56  
3.60, m  
H55, H57  
C54, C55, C57, C58

15  
1.89, m; 1.44, m  
H14, H16  
C13, C14, C16, C17  
57  
1.96, m; 1.55, m  
H56, H58  
C55, C56, C58, C59

16  
3.07, m  
H15, H17  
C14, C15, C17, C18  
58  
2.41, m; 2.09, m  
H57  
C56, C57, C59, C60, C84

17  
3.38, m  
H16, H18  
C15, C16, C19  
59  
null  
-  
- 

18  
3.39, m  
H17, H19  
C17  
60  
4.18, d, 8.9  
H61  
C58, C59, C61, C62, C84

19  
4.08, m  
H18, H20  
C17, C18  
61  
3.35, m  
H60, H62  
C59, C60, C63

20  
3.14, br d, 8.9  
H19, H21  
C16, C18, C19, C21, C22  
62  
4.04, m  
H61, H63  
C63, C64

21  
3.87, m  
H20, H22  
C19, C20, C22, C23  
63  
2.08, m; 1.55, m  
-  
C62, C64, C65

22  
2.05, m; 1.74, m  
H21, H23  
C20, C21, C23  
64  
4.05, m  
H63  
C63

23  
4.22, m  
H22, H24  
C21, C22, C24, C25, C27  
65  
4.04, m  
H66  
C63, C64

24  
3.64, m  
H23, H25  
C25, C26  
66  
3.74, br d, 9.9  
H65, H67  
C62, C64, C65, C67, C68

25  
3.92, m  
H24, H26  
C24, C26  
67  
3.97, m  
H66, H68  
C66, C68, C69
| 26 | 1.76, m | H25, H27 | C25, C27 | 68 | 4.37, dd, 7.6, 2.9 | H67, H69 | C69, C70 |
| 27 | 3.54, m | H26, H28 | C25, C28, C29 | 69 | 5.63, dd, 16.5, 8.0 | H68, H70 | C68, C70, C71 |
| 28 | 3.71, m | H27, H29 | C26, C29, C30 | 70 | 5.80, m | H69, H71 | C68, C69, C71, C72 |
| 29 | 1.70, m; 1.38, m | H28, H30 | C27, C30, C31, C79 | 71 | 2.19, m | H70, H72 | C69, C70, C72 |
| 30 | 1.97, m | H29, H31, H79 | C28, C29, C31, C79 | 72 | 2.21, m | H71, H73 | C71, C73, C74 |
| 31 | 3.12, dd, 7.6, 2.8 | H30, H32 | C29, C30, C33, C79 | 73 | 5.78, m | H72, H74 | C71, C72, C75 |
| 32 | 3.85, m | H31, H33 | C33, C34 | 74 | 6.10, dd, 15.2, 10.4 | H73, H75 | C72, C76 |
| 33 | 2.29, m | H32 | C31, C32, C34, C35, C80 | 75 | 6.21, dd, 15.7, 10.2 | H74, H76 | C76, C77 |
| 34 | null | - | - | 76 | 6.13, dd, 15.7, 10.2 | H75, H77 | C75, C77, 78 |
| 35 | 5.72, br s | H80 (ld), H81 (ld) | C33, C34, C37, C80, C81 | 77 | 6.35, ddd, 16.9, 10.2, 10.2 | H76, H78 | C75, C76 |
| 36 | null | - | - | 78 | 5.15, dd, 17.0, 1.0; 5.01, dd, 10.2, 1.0 | H77 | C75, C76, C77 |
| 37 | 2.27, m; 2.21, m | H38, H81 (ld) | C35, C38, C39, C81 | 79 | 0.97, d, 6.8 | H30 | C29, C30, C31 |
| 38 | 3.80, m | H37, H39 | C36, C37, C40 | 80 | 1.84, br s | H33 (ld), H35 (ld) | C33, C34, C35, C36, C37, C81 |
| 39 | 1.63, m; 1.27, m | H38, H40 | C37, C38, C40, C41, C82 | 81 | 5.05, br s; 4.85, br s | H37 (ld) | C35, C36, C37, C80 |
| 40 | 2.12, m | H39, H41, H82 | C39, C41, C82 | 82 | 0.90, d, 7.4 | H40 | C39, C40, C41 |
| 41 | 3.33, m | H40, H42 | C39, C40, C42, C82 | 83 | 1.74, br s | H48 (ld) | C46, C47, C48 |
| 42 | 3.64, m | H41, H43 | C41, C43, C44 | 84 | 5.07, br s; 4.98, br s | - | C58, C59, C60 |

HMBC correlations allowed me to link the spin systems of amphidinol 22. Spin systems A and B were linked by the cross-peaks at δ 2.29/18.51 (H33/C80), δ 2.29/129.54 (H33/C35),
δ 2.29/136.85 (H33/C34), δ 1.84/46.06 (H80/C33), δ 1.84/136.85 (H80/C34) and δ 1.84/129.54 (H80/C35), together with the signals at δ 2.27–2.21/115.77 (H37/C81), δ 2.27–2.21/144.65 (H37/C36), δ 2.27–2.21/129.54 (H37/C35), δ 5.05–4.85/129.54 (H81/C35), δ 5.05–4.85/144.65 (H81/C36) and δ 5.05–4.85/47.72 (H81/C37). HMBC signals H80/C35 and H80/C36, and the previously reported long-distance COSY correlations confirmed the location of CH-35.

HMBC CH$_2$-46 cross-peaks at δ 2.20–2.11/126.24 (H46/C48), δ 2.20–2.11/139.30 (H46/C47) and δ 2.20–2.11/17.39 (H46/C83), in addition to those of the CH-48 signals at δ 5.48/17.39 (H48/C83) and δ 5.48/36.68 (H48/C46), allowed me to connect the spin systems B and C. HMBC correlations of the CH$_3$-83 signals at δ 1.74/36.68 (H83/C46), δ 1.74/139.30 (H83/C47) and δ 1.74/126.24 (H83/C48) confirmed the link.

Spin systems C and D were linked by the CH$_2$-58 cross-peaks at δ 2.41–2.09/151.35 (H58/C59), δ 2.41–2.09/76.77 (H58/C60) and δ 2.41–2.09/113.21 (H58/C84), together with the CH-60 cross-peaks at δ 4.18/151.35 (H60/C59), δ 4.18/27.89 (H60/C58) and δ 4.18/113.21 (H60/C84). The cross-peaks CH$_2$-84 signals at δ 5.07–4.98/27.89 (H84/C58), δ 5.07–4.98/151.35 (H84/C59) and δ 5.07–4.98/76.77 (H84/C60) confirmed the link.

With all the data interpreted and the information gathered I was able to link the spin systems (Figure 3.7) and establish the planar structure of amphidinol 22 (Figure 3.8).

![Figure 3.7](image-url)  
Figure 3.7. HMBC correlations that allowed to link the spin systems (highlighted as red arrows).
To partially determine the three-dimensional structure of amphidinol 22, the magnitude of the coupling constants of the signals belonging to the $\Delta^4$, $\Delta^6$, $\Delta^{69}$, $\Delta^{73}$ and $\Delta^{75}$000 double bonds were measured, and were found to be higher than 15 Hz in all cases (Table 3.2), hence they were proposed to be in the $E$ configuration. The cross-peaks H33/H35 and H46/H48 in the NOESY spectrum confirmed the $E$ configuration in the $\Delta^{34}$ and $\Delta^{47}$ double bonds. It was deemed too complex to determine the configuration of each stereocentre. However, amphidinol 22 is likely to share the relative configuration of the common substructure reported for AM18, AM19, AM20 and AM21 (Figure 3.9) because of the similarities in the chemical shifts and coupling constants around this part of the molecules (Nuzzo 2014, Satake 2017).
the 2D-NMR ROESY (rotating Overhauser enhancement spectroscopy) experiment should generate a larger number of cross-peaks in the case of medium-size molecules such as amphidinol 22. We could then have more information on H-H proximities all along the molecule that will add valuable information for the determination of the relative stereochemistry.

3.3.2 Biological activity of amphidinol 22

The biological activity of amphidinol 22 was evaluated in a platform consisting of five different cancer cell lines (i.e. A549, A2058, HepG2, MCF7 and MiaPaca2) and five different pathogenic microorganisms (i.e. A. fumigatus, C. albicans, MRSA, MSSA, M. tuberculosis and M. bovis). The assays were performed as reported in General Methodologies (Sections M.5.1 and M.5.2).

The anticancer activity of amphidinol 22 was assessed using different concentrations (from 50 µM to 0.098 µM) and the compound presented IC₅₀ values of 8 µM, 16.4 µM, 6.8 µM, 16.8 µM and 8.6 µM on the A549, A2058, HepG2, MCF7 and MiaPaca2 cell lines, respectively. The cell viability at the different concentrations (Figure 3.10) indicated a general cytotoxic profile.

Amphidinol 2, first isolated by Paul and co-workers (Paul et al., 1995), was the only amphidinol with reported antiproliferative properties on cancer cells (Espiritu, Tan and Oyong, 2017). The IC₅₀ values ranged from 1 to 7 µM when the anticancer activity was evaluated against HCT116 (colon carcinoma), HT29 (colon adenocarcinoma) and MCF7 cancer cell line, with values similar to the ones displayed by amphidinol 22. After treatment with AM2, a 100-fold up-regulation of the early apoptotic markers cfos/cjun was also observed.
Figure 3.10. Percentage growth inhibition of A549 (lung), A2058 (skin), HepG2 (liver), MCF7 (breast) and Miapaca-2 (pancreas) cancer cell lines after incubation for 72 h with 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.250, 12.5, 25, 50 µM of amphidinol 22 (** for $p < 0.01$ and *** for $p < 0.001$, Student’s t-test). Experiments were performed in triplicate.

Other compounds from *Amphidinium* spp. have also demonstrated potent cytotoxic effects, such as the amphidinolide macrolides. In particular, amphinolides N and H exhibited the most potent activities, being extremely cytotoxic against L1210 murine leukemia cells ($IC_{50}$ values of 0.05 and 0.48 ng/mL), and KB human epidermoid carcinoma cells ($IC_{50}$ values of 0.06 and 0.52 ng/mL) (Kobayashi and Tsuda, 2004). The activity of the amphidinolide H was explained by a covalent binding mechanism on the actin Tyr200 subdomain, stabilizing the actin filament (Usui *et al.*, 2004). On the other hand, amphidinolide N seems to have much more affinity for the mitochondria of the malignant cells rather than for the cytoskeletal structures (Kobayashi and Tsuda, 2004).

The antifungal activity of amphidinol 22 was assessed against the fungi *C. albicans* and *A. fumigatus* (Figure 3.11). The compound showed antifungal activity with a MIC value of 64 µg/mL for both fungi, which was the highest concentration tested in the assays performed.

So far, no antibacterial activity was reported before for any of the amphidinols. However, the antibacterial activity of amphidinol 22 was assessed as well. The compound was tested against Gram-positive bacteria (*Staphylococcus aureus* MRSA, *Staphylococcus aureus*...
MSSA), *Mycobacterium tuberculosis* and *Mycobacterium bovis*, but no growth inhibition was observed and hence the antibacterial activity was nonexistent (Figure 3.11).

![Figure 3.11. Percentage of growth inhibition of Staphylococcus aureus (MRSA), methicillin sensitive Staphylococcus aureus (MSSA), Mycobacterium tuberculosis, Candida albicans and Aspergillus fumigatus after incubation for 20 to 30 h (depending on the microorganism) with 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 µM of Amphidinol C (** for p <0.01 and *** for p < 0.001, Student’s t-test). Experiments were performed in triplicate.](image)

### 3.3 Conclusions

Amphidinol 22 displays four six-membered heterocycles and an epoxide moiety in its structure, features that have not been previously observed in the structure of the other amphidinols, which usually have two or three heterocycles and do not present epoxide moieties. However, this new member of the family is in line with the observed structures so far, being quite similar to its congeners.

Regarding its biological activity, the compound presented cytotoxicity against all the tested cancer cell lines (i.e. A549, A2058, HepG2, MCF7 and MiaPaca2), with IC₅₀ values similar to amphidinol 2 (6.8-16.8 µM), the only amphidinol that was assessed for antiproliferative activity on cancer cells. The observed biological activity was not spectacular considering that other metabolites from *Amphidinium* sp., such as the amphidinolides, have displayed antiproliferative activity on cancer cell lines at the nanomolar scale. On the other hand, the antimicrobial activity was low to absent. This was expected considering the structure of amphidinol 22, which features a long chain that only allows a much weaker membrane
permeabilization mode of action, the carpet model, as explained in the introduction of this chapter (Section 3.1).

In general, the biological activity of the compound was disappointing, but it was an excellent exercise for my PhD project. The isolation and characterization of the compound encompassed almost the whole preclinical drug discovery pipeline, providing me with the big picture of the issue and with excellent experience on biological assays, separative liquid chromatography and structural elucidation of natural products.

The results of this work were published in the journal Marine Drugs (Martínez et al., 2019).
3.4 NMR spectra

Figure S1. $^1\text{H}$ NMR spectrum of amphidinol 22 (500 MHz) in CD$_3$OD.
Figure S2. $^{13}$C NMR spectrum of amphidinol 22 (125 MHz) in CD$_3$OD.
Figure S3. HSQC spectrum of amphidinol 22.
Figure S4. COSY spectrum of amphidinol 22.
Figure S5. HMBC spectrum of amphidinol 22
Figure S6. NOESY spectrum of amphidinol 22.
Chapter 4: Bioactive metabolites from marine diatoms

4.1 Introduction

Diatoms, one of the largest and ecologically most significant microalgal groups on Earth, have proven to be producers of natural compounds with anticancer (Martínez Andrade et al., 2018), antibacterial (Lauritano et al., 2016, 2018), anti-epileptic (Brillatz et al., 2018) and antibiofilm (Lauritano et al., 2016; Cepas et al., 2019) properties. However, until now no compounds from diatoms are commercialized as approved drugs or are undergoing clinical trials (Mayer et al., 2020). So far, only a few compounds with demonstrated biological activity have been isolated from diatoms (Miralto et al., 1999; Andrianasolo et al., 2008; Desbois et al., 2008; Kusaikin et al., 2010; Kim et al., 2014; Samarakoon et al., 2014; Miceli et al., 2019) and their bioactivity is not particularly strong when compared to compounds present in the marine clinical pipeline. This chapter is focused on two different marine diatoms: *Leptocylindrus danicus* and *Chaetoceros pseudocurvisetus* that may produce compounds of interest for this pipeline.

*Leptocylindrus danicus* is a diatom first identified in South-Eastern Australia and considered to be an important member of the phytoplankton community (Ajani et al., 2016), since it forms major blooms in coastal waters and is present throughout the world’s oceans (Nanjappa, Kooistra and Zingone, 2013). It belongs to the genus *Leptocylindrus*, the only member in the family Leptocylindraceae. During the EU-FP7 PharmaSea project, extracts from two species belonging to the genus *Leptocylindrus* (i.e. *L. danicus* clone FE322 and *Leptocylindrus aporus* clone FE332) strongly inhibited the formation of *Staphylococcus epidermidis* biofilms at 50 µg/mL using the crystal violet assay (Lauritano et al., 2016). However, the compound/s responsible for this activity were not isolated. In addition, *L. danicus* FE322 was tested for antibacterial and anticancer activity by Lauritano et al. (2018) but no biological activity was observed.

Studies on the antibiofilm activity of extracts of marine microalgae are scarce, but in addition to Lauritano and co-workers, Cepas et al. (2019) carried out an outstanding high throughput screening project on a library of 675 extracts from microalgae and
cyanobacteria that were tested for their antibiofilm activity on gram-negative bacteria (i.e. *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*), gram-positive bacteria (i.e. *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus hominis*) and *Candida* sp. fungi (i.e. *Candida albicans* and *Candida parapsilosis*). The authors observed that extracts from the phylum Cercozoa were very active against targeted bacteria and fungi with minimum biofilm inhibitory concentrations (MBIC) ranging from 32 µg/mL to 128 µg/mL, while they observed rather weak antibiofilm activity from extracts of Chlorophyta and Charophyta phyla, which presented MBIC values of 2-4 mg/mL. However, the phylum Bacillariophyta, which includes *Leptocylindrus* sp., was not studied. López and Soto pointed out the importance of microalgal compounds for the prevention of biofilms originating from infections, highlighting the activity of *Chlorella vulgaris* in processes related to Quorum sensing (QS) inhibition (López and Soto, 2020), a communication system observed within biofilms (Roy et al., 2018).

*Chaetoceros pseudocurvisetus* is another marine microalga belonging to the class of the diatoms. The genus *Chaetoceros* was introduced in 1844 and is one of the largest genera within the marine diatoms with 376 species names registered within the AlgaeBase database (https://www.algaebase.org/). *Chaetoceros* species are easily identified by light microscopy because of their particular features such as the formation of cell chains, the separation of their cells by apertures and their long setae protruding from the corners of each cell. However, it is often difficult to differentiate among *Chaetoceros* species so taxonomists have been using a combination of morphological and molecular data in order to classify them (Li et al., 2017) *C. pseudocurvisetus* is a diatom originally reported as a tropical/subtropical species (Cupp, 1943). Their blooms have been reported to be common in warm waters of Japanese coastal regions, from autumn to spring (Kuwata, Hama and Takahashi, 1993). This species was also reported to be present in the Gulf of Naples at the long-term monitoring station MareChiara (LTER-MC) in 1999 (Sarno and Zingone, 2008).

During the EU-FP7 Pharmasea project, extracts from *C. pseudocurvisetus* (FE-331, isolated from the Gulf on Naples LTER-MC in September 2014) were tested for their antibacterial activity against two *Mycobacterium* pathogenic species (i.e. *M. tuberculosis* and *M. bovis*); extracts were shown to have growth inhibitions rates higher than 90% when tested at a concentration of 800 µg/mL (Lauritano et al., 2018). To date, there are very few marine
microalgae reported to display antibacterial activity. Further details can be found in the introduction chapter of this thesis (Chapter 1, Section 1.1.2B).

This chapter of my thesis comprises of the screening of the extracts from the marine diatoms *L. danicus* (clone FE354) and *C. pseudocurvisetus* (clone FE331), and the isolation attempts of the metabolites responsible for their biological activity. A freshly isolated clone of *L. danicus* (FE354) was screened using the antiproliferative (cancer cells), antibacterial and antibiofilm platforms available at the Marbio biodiscovery laboratory (UiT, Tromso, Norway), since the old clone (*L. danicus* FE322) tested by Lauritano et al. 2018 within the EU-FP7 project Pharmasea was no longer available. A bioassay-guided fractionation approach supported by high-resolution mass spectroscopy (HRMS) was used to attempt the isolation of the secondary metabolites that may be responsible for the observed biological activity. To our knowledge, this is the first study on the isolation of antibiofilm metabolites from a marine diatom.

On the other hand, *C. pseudocurvisetus* (clone FE331) was screened using the anticancer and antimicrobial assay platforms available at the Fundación MEDINA (Granada, España) research centre. A bioassay-guided fractionation approach supported by high-resolution mass spectroscopy (HRMS) was used for the isolation of a polyunsaturated fatty acid compound that may be responsible for the activity already reported by Lauritano *et al.* (2018), the first study on the antitubercular activity of diatoms. My studies are the first attempt for the isolation of the potential metabolites that may be responsible for the observed activity and also the second attempt to find other interesting biological activities or bioactive metabolites produced by *C. pseudocurvisetus*.

### 4.2 Methods

#### 4.2.1 Growth and biomass production

*L. danicus* (clone FE354) was isolated from the Gulf of Naples (Long term monitoring station MareChiara) in February 2017 and maintained in the Stazione Zoologica Anton Dohrn (SZN) culture collection. *C. pseudocurvisetus* (clone FE331) was isolated from the Gulf of Naples (Long term monitoring station MareChiara) in September 2014 and maintained in the SZN culture collection. *L. danicus* was identified by light microscopy and 28S DNA sequencing (100% sequence identity with KC814830.1), while *C. pseudocurvisetus* was identified by light microscopy and 18S DNA sequencing (100% sequence identity with MG972304.1). The alga was grown in Guillard’s F/2 medium (Guillard, 1975) in 2L and 10L polycarbonate
carboys. For phosphate starvation conditions, phosphate salt (NaH$_2$PO$_4$·H$_2$O) was removed from the Guillard recipe. Cultures were grown at 20°C, 110 µmol·m$^{-2}·$s$^{-1}$ light intensity, 12h:12h (light:darkness) photoperiod and continuous aeration through 0.2 µm filters. For light stress conditions, the photoperiod was set at 24h:0h (light:dark). The 2L and 10L cultures were monitored every 24 hours by counting the cells. The initial cell concentration was approximately 5000 cells/mL for all experiments. Growth curves were built from the data of the 2L cultures. Biomass was harvested from the 10L cultures during the stationary phase (on the same day and at the same time of day for each replicate to avoid possible interference due to intrinsic circadian rhythms) by centrifugation for 10 minutes at 4°C and 2300 rpm. All experiments were performed in triplicate. Microalgal biomasses were kept at -80°C until chemical extraction. Further details of the medium preparation, inoculation, growth curves, scaling-up processes and harvesting are detailed in the General Methodologies Section (M.1, M.2 and M.3)

4.2.2 Chemical extraction
Chemical extraction of the *L. danicus* biomass was performed using the Marbio method as reported in the General Methodologies (section M.4). On the other hand, chemical extraction of the *C. pseudocurvisetus* biomass was performed using the Fundación MEDINA method as indicated in the General Methodologies section (Section M.4). Aliquots from the raw extracts were lyophilized and dissolved in DMSO for testing.

4.2.3 Anticancer assays
The cell viability of three different human cancer cell lines (i.e. A2058 ATCC CRL-11147, HT29 ATCC HTB-38, MCF7 ATCC HTB-22) after treatment with the *L. danicus* samples was assessed using the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. In addition, the lung fibroblast cell line MRC5 ATCC CCL-171 was used to assess toxicity in healthy human cells. The anticancer activity was assessed after 72h of treatment with the raw extracts of *L. danicus*. The extracts were tested as biological and technical triplicates. Further details of the anticancer assay protocol can be found in the methods section of this thesis (General methodologies, Section M.5.3)

Biological triplicates of the raw extracts of *C. pseudocurvisetus* in three different culture conditions (i.e. 12h:12h photoperiod and medium repletion Control conditions, phosphate starvation conditions, and 24h:0h photoperiod light stress conditions) were screened
against a panel of 5 different cancer cell lines (i.e. human lung carcinoma A549 ATCC® CCL-185™, human skin melanoma A2058 ATCC® CRL-11147™, hepatocyte carcinoma HepG2 ATCC® HB-8065™, breast adenocarcinoma MCF7 ATCC® HTB-22™ and pancreas carcinoma MiaPaca-2 ATCC® CRL-1420™). The extracts were screened for their anticancer activity as reported in Audoin and co-workers (Audoin et al., 2013) by colorimetric MTT assay, using a treatment of 72h at an extract concentration of 175 µg/mL for the cancer cell lines. Further details about the biological assays can be found in the General Methodologies section of this thesis (Section M.5.1).

4.2.4 Antimicrobial assays
Leptocylindrus danicus was tested for its antibacterial activity. Five different bacteria were used for the antibacterial MIC assays: Enterococcus faecalis ATCC 29212, Escherichia coli ATCC25922, Pseudomonas aeruginosa ATCC27853, Staphylococcus aureus ATCC25923, Streptococcus agalactiae ATCC12386. Raw extracts of L. danicus were tested as technical and biological triplicates. Further details about the antibacterial assay protocol can be found in the methods section of this thesis (General Methodologies, Section M.5.4).

Biological triplicates of the raw extracts of C. pseudocurvisetus in three different culture conditions (i.e. 12h:12h photoperiod and medium repletion control conditions, phosphate starvation conditions, and 24h:0h photoperiod light stress conditions) were screened against a panel of five different pathogenic bacteria (i.e. Gram-negative bacteria Escherichia coli ATCC25922 and Klebsiella pneumoniae ATCC700603, Gram-positive bacteria Staphylococcus aureus MRSA MB5393 and MSSA ATCC29213, M. tuberculosis H37Ra) and the fungus A. fumigatus ATCC46645. Extracts were screened for their antimicrobial activity as reported in Audoin and co-workers (Audoin et al., 2013), using a treatment of 20-30h (depending on the microorganism) at an extract concentration of 560 µg/mL. Further details about the biological assays can be found in the General Methodologies section of this thesis (Section M.5.2).

4.2.5 Antibiofilm assays
L. danicus extracts were assessed for their antibiofilm activity. Two different bacteria were used for the crystal violet biofilm assay: the biofilm forming bacterium Staphylococcus epidermidis and the non-biofilm forming bacterium Staphylococcus haemolyticus. The non-biofilm forming bacterium was used as a control. The extracts were tested as biological and technical triplicates (9 replicates in total for each extract) at different concentrations (100,
50, 25 and 12.5 µg/mL). Further details about the antibiofilm crystal violet assay protocol can be found in the methods section of this thesis (General methodologies, Section M.5.5)

### 4.2.6 Flash fractionation

The crude microalgal extract of *L. danicus* in control conditions (770 mg) was fractionated using a Biotage SP4 flash chromatography system. The column used for the fractionation was prepared using 6.5 grams of a polystyrene-divinylbenzene based resin (Diaion® HP-20SS) that was pre-treated by soaking in methanol for a minimum of 20 minutes and washed with milli-Q water once inside the column. The extracts were dissolved in methanol and mixed with 2 grams Diaion® HP-20SS resin to produce a slurry that was dried under reduced pressure. The resulting resin imbued with the extract was introduced into the column. Eight different fractions were collected by applying a first gradient using H₂O (solvent A) and MeOH (solvent B) over a period of 36 minutes (Table 4.1), followed by a second gradient MeOH:acetone to acetone in two steps (50:50, 0:100) over 18 minutes (F7 was collected in 6 minutes while F8 was collected in the final 12 minutes). Flow rate was set at 12 mL/min and the UV detector was fixed at a wavelength 254 nm. Each fraction was then evaporated at 40°C under reduced pressure. They were then lyophilized in order to remove humidity and remaining traces of solvent.

**Table 4.1. Gradient applied for the flash fractionation of *L. danicus* raw extract**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
<th>Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6</td>
<td>12</td>
<td>95</td>
<td>5</td>
<td>F1</td>
</tr>
<tr>
<td>6-12</td>
<td>12</td>
<td>75</td>
<td>25</td>
<td>F2</td>
</tr>
<tr>
<td>12-18</td>
<td>12</td>
<td>50</td>
<td>50</td>
<td>F3</td>
</tr>
<tr>
<td>18-24</td>
<td>12</td>
<td>25</td>
<td>75</td>
<td>F4</td>
</tr>
<tr>
<td>24-30</td>
<td>12</td>
<td>0</td>
<td>100</td>
<td>F5</td>
</tr>
<tr>
<td>30-36</td>
<td>12</td>
<td>0</td>
<td>100</td>
<td>F6</td>
</tr>
</tbody>
</table>

*C. pseudocurvisetus* control extract was fractionated through low resolution reverse phase chromatography using a CombiFlash® Rf chromatography instrument. This instrument requires the column to be prepared manually in advance. The stationary phase of the column contained 40x100 mm of RP18 resin, with the head of the column consisting of a dried slurry prepared using the RP18 resin and the microalgal extract. The extract was
dissolved in methanol and added to a 500 mL rounded flask together with the RP18 resin at a rate of 3 grams of resin for each gram of extract (3:1, RP18:extract). The prepared slurry was then vacuum dried using a rotary evaporator and transferred into a cellulose filter paper reservoir. To assemble the column, a RediSep® Rf Cartridge was used as a support. A RediSep® Frit (filter) was placed at the bottom of the cartridge and the RP18 resin was added and compacted using methanol. Another frit was placed at the top of the stationary phase to seal it and a plunger (device to attach the column to the conduits of the instrument) was placed at the top of the column. Using the Combiflash interface, the stationary phase was conditioned using the initial conditions of the method (details are explained below) for the fractionation. Once the stationary phase was conditioned, the plunger was removed and the prepared slurry was added at the top of the second frit filter and sealed with a third frit filter. At this point, the plunger was placed again at the top of the column and the Combiflash instrument was ready to start.

On the other hand, the conditions used for the chromatography were the following: RP18 resin (reversed phase C18) column chromatography (low resolution), 10-11cm height of resin, 500 -1500 mg extract, 5-100B MeOH-mqH₂O gradient (B=MeOH) during 50 minutes. Flow rate: 18mL/min. The wavelengths selected were 210 and 260 nm. Seven fractions were collected and pooled according to the chromatographic profile. Biological activity of the fractions was assessed, and the active fractions were chemically analysed by HPLC-UV-HRESIMS (Section 4.2.7)

4.2.7 General chemical analysis procedures
UPLC-UV-HRMS analysis was used to perform the chemical analysis of the fractions from L. danicus. Spectra were acquired on an Acquity UPLC and LCT premier Time-of-Fight MS using ESI+ ionization (Waters) (Michael et al., 2020). An aliquot of 5 µL of the fractions dissolved in methanol was injected into a Waters Acquity BEH C18 column (1.7 µm, 2.1x100 mm) with the thermostat set at 40°C. Ultra-pure water (solvent A) and acetonitrile (Solvent B) were used as mobile phase supplemented with 0.1% formic acid, and with a flow rate of 0.45 mL/min. The gradient went from 10%B to 100%B in 12 minutes. The system was operated in positive mode (ESI+) performing 10 scans per second in the mass range of m/z 50-2000, and the conditions were set as follows: 0.8kV and 40V capillary and cone voltages, respectively; 350°C and 120°C desolvation and ion source temperatures, respectively; 800L/h N₂ desolvation gas and 50L/h N₂ cone gas; UV detector wavelength range of 190nm to 500 nm. To lock mass correction, a 50% H₂O: acetonitrile solution of
Leucine-enkephalin (100 pg/µL) with 0.1% of formic acid was used. The software MassLynx version 4.1 was used to process the MS data. ChemSpider, Dictionary of Marine Natural Products and Reaxys were used as databases to compare the acquired data.

*C. pseudocurvisetus* fractions were analyzed by HPLC-UV-HRESIMS on an Agilent 1200 RR coupled to a Bruker maXis QToF spectrometer with electrospray ionization, as reported by Martin *et al.* (2014). Analyses were performed using a column Zorbax SB-C8 (2.1 x 30 mm, 5µm) at 40°C and with a flow rate of 300 µL/min. For the mobile phase, two solvents were used. Solvent A was 10% acetonitrile and 90% water with trifluoroacetic acid and ammonium formate at a concentration of 1.3 mM, while solvent B was 90% acetonitrile and 10% water with trifluoroacetic acid and ammonium formate at a concentration of 1.3 mM. The method used consisted in a gradient that started at 10%B and went to 100%B in six minutes, was maintained two minutes at 100%B and finally was returned to 10%B in two minutes to reach the initial conditions. Full diode array UV scans from 200 to 900 nm were collected in 4 nm steps at 0.25 s/scan. The mass spectrometer was operated in positive ESI mode. The instrument parameters were 4 kV capillary voltage, drying gas flow of 11 L min⁻¹ at 200 °C, and nebulizer pressure of 2.8 bar. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Pre-run calibration was made by infusion with the same TFA-Na calibrant. Mass spectra were collected as full scans from 50 m/z to 1500 m/z. Data were analysed using the platform available at Fundación MEDINA (Pérez-Victoria, Martín and Reyes, 2016) and compared with the data available in the Dictionary of Natural Products database and PubChem.

4.3 Results and discussion

4.3.1 Cultures and growth data

The growth curves of *L. danicus* (FE354) in control conditions (replete nutrient supply) and 12h:12h photoperiod), phosphate starvation conditions (phosphate depletion) and light stress conditions (24h:0h photoperiod) are reported in the figures below (Figure 4.1 and Figure 4.2)
Figure 4.1. *L. danicus* (FE354) growth curve in control conditions (2 Liters).

Figure 4.2. *L. danicus* (FE354) growth curve in phosphate starvation conditions (2 Liters).

The growth curves of *C. pseudocurvisetus* (FE331) in control conditions (replete nutrient conditions and 12h:12h photoperiod), phosphate starvation conditions (phosphate depletion) and light stress conditions (24h:0h photoperiod) are reported in the figures below (Figure 4.3, Figure 4.4 and Figure 4.5).
**Figure 4.3.** *C. pseudocurvisetus* (FE331) growth curve in control conditions (2 Liters).

**Figure 4.4.** *C. pseudocurvisetus* (FE331) growth curve in phosphate starvation conditions (2 Liters).
Figure 4.5. *C. pseudocurvisetus (FE331)* growth curve in light stress conditions (2 Liters).

According to the data collected, the stationary phase of *L. danicus* was fixed from day 7 to day 11 in the case of control conditions and from day 4 to day 7 in the case of the phosphate starvation conditions. However, it is important to mention that when *L. danicus* control cultures were scaled up to 10L volume the diatom reached the senescent phase much faster, often after day 5. In the case of *C. pseudocurvisetus*, the stationary phase was fixed from day 4 to day 6 for control conditions, from day 3 to day 9 for phosphate starvation conditions and from day 5 to day 8 for light stress conditions. Setting the light time to 24h led to a 2-fold increase in cell concentrations compared to control conditions. Phosphate enrichment of the media was crucial for a correct development of the microalga since algal concentrations in control conditions was 3-fold higher compared to concentrations in phosphate starvation conditions.

Ten litre carboys were used to grow *L. danicus* in two different culture conditions (control and phosphate starvation) and *C. pseudocurviset us* in three different culture conditions (control, phosphate starvation and light stress) to produce biomass necessary for the biological and chemical studies. *L. danicus* studies were performed in the Marbio Laboratories at the University of Tromsø (Norway), while *C. pseudocurviset us* studies were performed at the research institution Fundación MEDINA (Granada, Spain). The following table (Table 4.2) summarises the maximum concentrations reached, harvest day and weight of wet biomass produced for each species and culture condition. All samples were further freeze-dried and stored at -80°C.
Table 4.2. *L. danicus* and *C. pseudocurvisetus* samples wet weight, harvest day and maximum concentrations reached. The data presented are biological triplicates in control and phosphate starvation conditions.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Maximum concentration (cells/mL)</th>
<th>Harvest day</th>
<th>Wet weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. danicus</em> Control 1</td>
<td>1.75x10^5</td>
<td>4</td>
<td>5.28</td>
</tr>
<tr>
<td><em>L. danicus</em> Control 2</td>
<td>1.62x10^5</td>
<td>4</td>
<td>5.11</td>
</tr>
<tr>
<td><em>L. danicus</em> Control 3</td>
<td>1.72x10^5</td>
<td>4</td>
<td>5.46</td>
</tr>
<tr>
<td><em>L. danicus</em> P-starvation 1</td>
<td>7.75x10^4</td>
<td>4</td>
<td>1.85</td>
</tr>
<tr>
<td><em>L. danicus</em> P-starvation 2</td>
<td>4.58x10^4</td>
<td>4</td>
<td>0.77</td>
</tr>
<tr>
<td><em>L. danicus</em> P-starvation 3</td>
<td>4.14x10^4</td>
<td>4</td>
<td>0.87</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Control 1</td>
<td>1.97x10^5</td>
<td>6</td>
<td>7.03</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Control 2</td>
<td>1.75x10^5</td>
<td>6</td>
<td>7.86</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Control 3</td>
<td>1.88x10^5</td>
<td>6</td>
<td>7.14</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> P-starvation 1</td>
<td>4.04x10^4</td>
<td>3</td>
<td>3.47</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> P-starvation 2</td>
<td>3.72x10^4</td>
<td>3</td>
<td>3.59</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> P-starvation 3</td>
<td>4.85x10^4</td>
<td>3</td>
<td>3.41</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Light stress 1</td>
<td>4.08x10^5</td>
<td>6</td>
<td>6.17</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Light stress 2</td>
<td>4.59x10^5</td>
<td>6</td>
<td>5.81</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Light stress 3</td>
<td>4.25x10^5</td>
<td>6</td>
<td>6.52</td>
</tr>
</tbody>
</table>
For both diatoms, biomass production was much lower in the phosphate starvation conditions for the 10L cultures. Phosphate enrichment of the media was important for the correct development of the cultures in laboratory conditions, especially to attain high concentrations of biomass. *L. danicus* biomass yield in the control conditions was approximately 3 to 5-fold higher compared to phosphate starvation conditions. On the other hand, *C. pseudocurvisetus* maximum cell concentration was reached in light stress conditions, which was 2-fold higher compared to control conditions and 10-fold higher when compared to phosphate starvation conditions.

**4.3.2 Preliminary bioactivity screening**

*L. danicus* biomass was extracted as explained in the methods section. Pellets were freeze-dried before chemical extraction. The approximate extract yield per gram of wet pellet was 76 mg extract/g pellet for the control conditions and 80 mg extract/g pellet for the phosphate starvation conditions. One extract for each biological replicate and for each growth condition was obtained, hence a total of 6 extracts were tested for anticancer, antibacterial and antibiofilm activity at the Marbio biological screening platform at the University of Tromsø (Norway).

The antiproliferative activity of raw extracts of *L. danicus* was assessed against three cancer cell lines (i.e. skin cancer cell line A2058 ATCC CRL-11147, colon cancer cell line HT29 ATCC HTB-38, breast cancer cell line MCF7 ATCC HTB-22), and the lung fibroblast cell line MRC5 ATCC CCL-171 was used to assess toxicity in healthy cells. The results are expressed in terms of percentage of cell viability and are shown in the table below (Table 4.3).

**Table 4.3.** Cell viability for the *L. danicus* extracts tested on all cell lines. Each value represents the mean of three technical replicates performed for each extract and cell line.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>Conc. (µg/mL)</th>
<th>MRC5</th>
<th>A2058</th>
<th>HT29</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL1</td>
<td>LD1</td>
<td>100</td>
<td>122±8</td>
<td>127±5</td>
<td>136±6</td>
<td>173±16</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL2</td>
<td>LD2</td>
<td>100</td>
<td>104±1</td>
<td>108±12</td>
<td>136±11</td>
<td>147±4</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL3</td>
<td>LD3</td>
<td>100</td>
<td>132±15</td>
<td>131±5</td>
<td>148±10</td>
<td>151±6</td>
</tr>
</tbody>
</table>
Leptocylindrus danicus - P1 | LDP1 | 100 | 122±11 | 119±7 | 121±25 | 152±18
Leptocylindrus danicus - P2 | LDP2 | 100 | 102±6  | 92±10 | 109±7  | 125±8
Leptocylindrus danicus - P3 | LDP3 | 100 | 107±4  | 115±5 | 123±5  | 125±13

Extracts did not show any antiproliferative activity. In some cases, cell viability was even higher than 150% when compared to control wells, so further studies were discontinued. However, it is interesting how some extracts are promoting the growth of cancer cells and these results may be worth investigating.

MIC antibacterial assays were performed on the gram-positive bacteria *E. faecalis* ATCC 29212 and *S. aureus* ATCC25923, and on the gram-negative bacteria *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *S. agalactiae* ATCC12386. The results (Table 4.4) are expressed in terms of bacterial cell viability.

**Table 4.4.** Bacterial cell viability for *L. danicus* extracts tested on all pathogenic microorganisms. Each value represents the mean of three technical replicates performed for each extract and bacterium.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>Conc. (µg/mL)</th>
<th><em>E. faecalis</em></th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>S. aureus</em></th>
<th><em>S. agalactiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL1</td>
<td>LD1</td>
<td>100</td>
<td>60±1</td>
<td>126±2</td>
<td>121±3</td>
<td>56±1</td>
<td>153±6</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL2</td>
<td>LD2</td>
<td>100</td>
<td>68±3</td>
<td>134±18</td>
<td>131±2</td>
<td>129±7</td>
<td>159±0</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> CTRL3</td>
<td>LD3</td>
<td>100</td>
<td>58±3</td>
<td>128±6</td>
<td>121±5</td>
<td>102±5</td>
<td>144±15</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> - P1</td>
<td>LDP1</td>
<td>100</td>
<td>64±1</td>
<td>122±6</td>
<td>132±0</td>
<td>97±7</td>
<td>142±6</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> - P2</td>
<td>LDP2</td>
<td>100</td>
<td>46±2</td>
<td>106±4</td>
<td>132±10</td>
<td>102±8</td>
<td>144±21</td>
</tr>
</tbody>
</table>
The antibacterial MIC assay results show that extracts from *L. danicus*, in both conditions tested, slightly arrested bacterial growth only for *E. faecalis*. In general, the results observed were not promising and hence studies on the antibacterial activity of these extracts were discontinued.

Antibiofilm activity was also assessed using two different bacteria and the crystal violet biofilm assay: the biofilm forming bacterium *S. epidermidis* and the non-biofilm forming bacterium *S. haemolyticus*. As mentioned in the methods section (3.2.5) the results are expressed in term of OD$_{600}$ and samples were considered active when values were lower than 0.09 units. After the first round of screening, the raw extracts were tested at different concentrations (100, 50, 25 and 12.5 µg/mL) because the activity was particularly strong at 100 µg/mL. The results are shown in the table below (Table 4.5). The wells showing *S. haemolyticus* proliferation did not have a biofilm.

**Table 4.5.** OD$_{600}$ for the *L. danicus* extracts tested at four different concentrations on *S. epidermidis*. Each value represents the mean of three technical replicates. Results highlighted in **green** were considered active (lower than 0.09).

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Code</th>
<th>Concentrations (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL1</td>
<td>LD1</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td>CTRL2</td>
<td>LD2</td>
<td>0.09±0.00</td>
</tr>
<tr>
<td>CTRL3</td>
<td>LD3</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> -P1</td>
<td>LDP1</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> -P2</td>
<td>LDP2</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td><em>Leptocylindrus danicus</em> -P3</td>
<td>LDP3</td>
<td>0.06±0.01</td>
</tr>
</tbody>
</table>

Extracts from both culture conditions had biological activity at a concentration of 100 µg/mL, but samples in phosphate starvation conditions were more active, inhibiting biofilm growth.
formation also at 50 µg/mL. The biological activity seems to be strictly antibiofilm, since no antibacterial effect was observed in the MIC assays even at the highest concentration tested, indicating that the compounds are inhibiting biofilm formation but not bacterial growth.

*C. pseudocurvisetus* biomass was extracted as explained in the methods section. The approximate extract yield per gram of wet pellet was 38 mg extract/g pellet for the control conditions, 34 mg extract/g pellet for the phosphate starvation conditions and 43 mg extract/g pellet for the light stress conditions. Each sample of microalgal biomass from the diatom *C. pseudocurvisetus* (Table 4.2) was extracted and their biological activities were assessed in the anticancer and antimicrobial high-throughput screening platforms at Fundación MEDINA (Granada, Spain)

The antiproliferative activity of raw extracts of *C. pseudocurvisetus* was assessed against five different cancer cell lines (human lung carcinoma A549 ATCC® CCL-185™, human skin melanoma A2058 ATCC® CRL-11147™, hepatocyte carcinoma HepG2 ATCC® HB-8065™, breast adenocarcinoma MCF7 ATCC® HTB-22™ and pancreas carcinoma MiaPaca-2 ATCC® CRL-1420™) as indicated in the methods section of this chapter (Section 4.2.3). Liver HepG2 cell line was used as an indicator of human hepatic cell toxicity, but healthy cells were not tested. Results of the biological assays were expressed in terms of percentage of cell viability and are shown in the table below (Table 4.6).

**Table 4.6.** Cell viability for the *C. pseudocurvisetus* extracts tested on all cell lines. Each value represents the mean of two technical replicates performed for each extract and cell line. Most interesting results are highlighted in green.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>Conc. (µg/mL)</th>
<th>A549</th>
<th>A2058</th>
<th>HepG2</th>
<th>MCF7</th>
<th>MiaPaca-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pseudocurvisetus</em> Control 1</td>
<td>CPC1</td>
<td>175</td>
<td>76±2</td>
<td>6±1</td>
<td>2±1</td>
<td>54±1</td>
<td>54±1</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Control 2</td>
<td>CPC2</td>
<td>175</td>
<td>92±4</td>
<td>7±1</td>
<td>1±0</td>
<td>46±3</td>
<td>46±3</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> Control 3</td>
<td>CPC3</td>
<td>175</td>
<td>86±3</td>
<td>3±1</td>
<td>1±0</td>
<td>51±2</td>
<td>51±2</td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> -P1</td>
<td>CPP1</td>
<td>175</td>
<td>98±3</td>
<td>50±1</td>
<td>23±4</td>
<td>110±1</td>
<td>110±1</td>
</tr>
</tbody>
</table>
The results show that CPC extracts caused cell growth inhibition in all cell lines tested except for the A549 cell line, and antiproliferative activity was particularly strong against A2058 and HepG2 cells. CPL extracts showed antiproliferative activity only against A2058 and HepG2 cells, with HepG2 more sensitive to the extracts. Finally, CPP extracts showed activity only against HepG2 cells. In general, it seems that stressing the microalga led to weaker bioactivities when extracts were tested in vitro. Even if CPC samples seem to present high toxicity against almost all cell lines tested, it would be interesting to identify the chemicals responsible for the observed bioactivity. However, such studies were not developed in this thesis.

Antimicrobial assays were performed on five different pathogenic bacteria (i.e. Gram-negative bacteria *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603, Gram-positive bacteria *S. aureus* MRSA MB5393 and MSSA ATCC29213, *M. tuberculosis* H37Ra) and the fungus *A. fumigatus* ATCC46645. Results are expressed in terms of cell viability of each microorganism (Table 4.7).

**Table 4.7.** Cell viability for all pathogenic microorganisms treated with the *C. pseudocurvisetus* extracts. Each value represents the mean of two technical replicates performed for each extract and bacterium. EC, KP, MT and AF stand for *E. coli*, *K. pneumoniae*, *M. tuberculosis* and *A. fumigatus*. Most interesting results are highlighted in green.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Code</th>
<th>Conc. (µg/mL)</th>
<th>EC</th>
<th>KP</th>
<th>MRSA</th>
<th>MSSA</th>
<th>MT</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pseudocurvisetus</em> -P2</td>
<td>CPP2</td>
<td>175</td>
<td>94±4</td>
<td>63±1</td>
<td>61±6</td>
<td>100±0</td>
<td>100±0</td>
<td></td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> -P3</td>
<td>CPP3</td>
<td>175</td>
<td>88±3</td>
<td>60±5</td>
<td>44±8</td>
<td>86±3</td>
<td>86±3</td>
<td></td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> L1</td>
<td>CPL1</td>
<td>175</td>
<td>92±4</td>
<td>29±10</td>
<td>9±2</td>
<td>104±1</td>
<td>104±1</td>
<td></td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> L2</td>
<td>CPL2</td>
<td>175</td>
<td>105±6</td>
<td>55±6</td>
<td>15±3</td>
<td>108±5</td>
<td>108±5</td>
<td></td>
</tr>
<tr>
<td><em>C. pseudocurvisetus</em> L3</td>
<td>CPL3</td>
<td>175</td>
<td>104±1</td>
<td>47±4</td>
<td>5±2</td>
<td>92±4</td>
<td>92±4</td>
<td></td>
</tr>
</tbody>
</table>
According to the results, *C. pseudocurvisetus* presented stronger activity against the gram-positive bacteria (MRSA and MSSA) and *M. tuberculosis*. The antimicrobial activity follows the same pattern that was observed for the anticancer activity, being control extracts the most active followed by the light stress extracts, and lastly the phosphate starvation extracts. It is not surprising to observe bioactivity on *M. tuberculosis*, since the antitubercular effects of this species was already observed by Lauritano et al. (2018).

Authors extracted the biomass from control cultures of *C. pseudocurvisetus* using an acetone/chloroforms extraction, tested them against *M. tuberculosis* at a concentration of 800 µg/mL and observed strong inhibition of bacterial growth (above 90% of inhibition). They also tested the extracts from phosphate starvation cultures that were obtained using a specific method involving amberlite resin, which displayed 99% inhibition of *M. tuberculosis* at 800 µg/mL. Finally, they performed a dereplication based on LC-UV-HRESIMS on the phosphate starvation extracts and identified the following components:

- Phaeophorbide A (C_{35}H_{36}N_{4}O_{5}), a decomposition product of chlorophyll, known to possess several bioactivities including anticancer, antiinflammatory, antioxidant, antiviral and antiparasite activity (Saide et al. 2020). Most of the time the activity was observed only in combination with photodynamic therapies.
- C_{36}H_{60}O_{8} and C_{36}H_{60}O_{7}, identified as triterpene glycosides.
- C_{16}H_{28}O_{3}, a component with a molecular formula not associated to any bioactive component in the Dictionary of Natural Products database.
- A component of the main UV peak that did not ionize in the analytical conditions tested, perhaps a fatty acid because of its retention time.

However, Lauritano et al. (2018) did not fractionate the extract to perform biological assays on fractions/pure compounds and hence more studies were necessary to identify the source of the antitubercular activity.

4.3.3 Effects of the variations on culturing conditions to bioactivity

Different culturing conditions can be used as a tool to trigger or alter the production of different metabolites that contribute to the biological activity of raw extracts, as previously observed for marine microalgae (Ingebrigtsen et al., 2016; Lauritano et al., 2016).

Two different Leptocylindrus species from the Gulf of Naples (L. aporus FE332 and L. danicus FE322) were already found to be active against biofilms using the same assay (Lauritano et al., 2016); the authors also found stronger activities at 50 µg/mL testing extracts from cultures grown under nutrient starvation (nitrogen and phosphate starvation), and for both clones tested. In my studies, the phosphate starvation extract presented higher bioactivity values compared to the control extracts.

On the contrary, the phosphate starvation and light stress conditions caused a reduction on the biological activity of the extracts from C. pseudocurvisetus, being control extracts the most active.

4.3.4 Flash fractionation and biological activity assessment of the fractions

For L. danicus, I decided to proceed with the experiments using only the control extract, for practical reasons. The quantity of extract obtained from 30L of culture in phosphate starvation was much lower compared to control conditions, and hence the material was not sufficient for the isolation of the metabolites responsible for this activity. Control extracts from L. danicus were fractionated (770 mg in total) according to the procedure described in the “Flash fractionation” section 3.2.6 of this chapter. Eight fractions were generated using the Biotage SP4 system and the chromatograms are presented below (Figure 4.6).
Figure 4.6. *L. danicus* CTRL extract flash fractionation chromatograms. The upper chromatogram corresponds to the first gradient (MeOH:H$_2$O) and the lower to the second gradient (MeOH:acetone). The fractions collected are pools of the subfractions shown in the chromatograms.

The fractions were then dried under reduced pressure, lyophilized and weighed (Table 4.8) in order to prepare aliquots used for the assessment of their antibiofilm activity, using the same procedure as the one used for the raw extracts (*Antibiofilm assays*, section 3.2.5) and thus following the classical scheme of bioassay guided fractionation.

**Table 4.8.** Name and weight (in milligrams) from the different fractions from *L. danicus* produced by flash fractionation

<table>
<thead>
<tr>
<th><em>L. danicus</em> control fractions</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. danicus</em> CTRL – F1</td>
<td>207</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F2</td>
<td>58</td>
</tr>
</tbody>
</table>
In this second phase of screening, fractions from *L. danicus* were tested at two different concentrations (25 and 12.5 µg/mL). The wells showing *S. haemolyticus* proliferation did not present biofilms. The results are shown in the table below (Table 4.9).

**Table 4.9.** OD<sub>600</sub> for the *L. danicus* CTRL fractions tested at 25 µg/mL and 12.5 µg/mL. Each value represents the mean of three technical replicates. Results highlighted in green were considered active (lower than 0.09).

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>25 µg/mL</th>
<th>12.5 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. danicus</em> CTRL – F1</td>
<td>LDC-F1</td>
<td>0.31±0.06</td>
<td>0.31±0.03</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F2</td>
<td>LDC-F2</td>
<td>0.30±0.04</td>
<td>0.32±0.04</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F3</td>
<td>LDC-F3</td>
<td>0.05±0.00</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F4</td>
<td>LDC-F4</td>
<td>0.04±0.00</td>
<td>0.05±0.00</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F5</td>
<td>LDC-F5</td>
<td>0.11±0.00</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F6</td>
<td>LFC-F6</td>
<td>0.22±0.01</td>
<td>0.22±0.00</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F7</td>
<td>LDC-F7</td>
<td>0.32±0.03</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td><em>L. danicus</em> CTRL – F8</td>
<td>LDC-F8</td>
<td>0.30±0.03</td>
<td>0.29±0.01</td>
</tr>
</tbody>
</table>

As shown in table 4.9, both fractions LDC-F3 and LDC-F4 (50% methanol and 75% methanol from the flash fractionation gradient) were active at 25 µg/mL. LDC-F4 antibiofilm activity was even higher considering it was also active at 12.5 µg/mL with an OD<sub>600</sub> value of 0.05. It
is interesting to note that fraction LDC-F4 is still a complex mixture of compounds, and that the bioactivity of a pure compound isolated from this fraction could therefore be much higher. It would not be surprising to obtain pure compounds active at concentrations in the nanogram scale.

Proceeding with the other diatom, *C. pseudocurvisetus* control extract (1.44 grams) was fractionated as described in “Flash fractionation” section 4.2.4 of this chapter. 50 subfractions were collected and pooled together according to the chromatographic profile to obtain 6 different fractions (Figure 4.7)

![Figure 4.7. C. pseudocurvisetus control extract flash fractionation chromatograms. The fractions collected are pools of the subfractions shown in the chromatograms. Blue line represents the B solvent gradient, red line is the absorbance at 210 nm and pink line is the absorbance at 260 nm](image)

The fractions were then dried under reduced pressure, lyophilized and aliquoted in order to be used for the assessment of their antimicrobial activity, using the same protocol as the one used for the raw extracts (*Bioactivity screening*, section 4.2.3)

**Table 4.10.** Name and weight (in milligrams) from the different fractions from *C. pseudocurvisetus* produced by flash fractionation

<table>
<thead>
<tr>
<th><em>C. pseudocurvisetus</em> control fractions</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPC-F1</td>
<td>852</td>
</tr>
<tr>
<td>CPC-F2</td>
<td>59</td>
</tr>
</tbody>
</table>
Fractions from *C. pseudocurvisetus* (Table 4.10) were tested against MRSA, MSSA and *M. tuberculosis*. Results are expressed in terms of cell viability of the microorganisms and are reported below (Table 4.11).

**Table 4.11** Cell viability of the different pathogens treated with *C. pseudocurvisetus* control (CPC) fractions at a concentration of 100 µg/mL. Each value represents the mean of two technical replicates. Most significant results are highlighted in green.

<table>
<thead>
<tr>
<th>Fraction name</th>
<th>Conc. (µg/mL)</th>
<th>MRSA</th>
<th>MSSA</th>
<th>MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPC-F1</td>
<td>100</td>
<td>99±6</td>
<td>110±1</td>
<td>99±1</td>
</tr>
<tr>
<td>CPC-F2</td>
<td>100</td>
<td>114±5</td>
<td>152±8</td>
<td>97±0</td>
</tr>
<tr>
<td>CPC-F3</td>
<td>100</td>
<td>103±7</td>
<td>96±4</td>
<td>95±4</td>
</tr>
<tr>
<td>CPC-F4</td>
<td>100</td>
<td>85±1</td>
<td>80±11</td>
<td>99±3</td>
</tr>
<tr>
<td>CPC-F5</td>
<td>100</td>
<td>0±0</td>
<td>-1±2</td>
<td>96±2</td>
</tr>
<tr>
<td>CPC-F6</td>
<td>100</td>
<td>120±11</td>
<td>20±7</td>
<td>94±1</td>
</tr>
</tbody>
</table>

Of all the fractions tested, only CPC-F5 and CPC-F6 displayed significant activity. In particular, CPC-F5 completely inhibited the growth of MRSA and MSSA at the concentration tested, while CPC-F6 only inhibited the growth of MSSA by 80%. However, it is surprising to see that no inhibition was observed for *M. tuberculosis* and further experiments are necessary to understand why the biological activity was no longer present in the fractions. Perhaps the biological activity observed for the raw extract could be due to a synergic effect between two or more compounds, or the concentration tested was not high enough to
trace the bioactivity. CPC-F5 was then chemically analysed to look for components that may be responsible for the activity against gram-positive bacteria.

4.3.5 Chemical analysis of the active fractions from *L. danicus* (FE354)

Fractions LDC-F3 and LDC-F4 were analysed by UPLC-UV-HRMS as specified in the general chemical analysis procedures of this chapter (Section 3.2.7). The chromatograms related to the HRMS and UV data are presented below (Figures 4.8 and 4.9).
Figure 4.8. BPI (upper) and UV (lower) chromatograms of fraction LDC-F3. The base peak intensity chromatogram (BPI) was generated from the mass spectroscopy data of the highest peak at a given retention time when the instrument was operated at low ionization energy (6eV). UV chromatogram was generated from the UV spectra absorbance at a given retention time in the full range of 190nm to 500 nm.
Figure 4.9. BPI (upper) and UV (lower) chromatograms of fraction LDC-F4. The base peak intensity chromatogram (BPI) was generated from the mass spectroscopy data of the highest peak at a given retention time when the instrument was operated at low ionization energy (6eV). UV chromatogram was generated from the UV spectra absorbance at a given retention time in the full range of 190nm to 500 nm. The larger peaks in common with LDC-F3 are highlighted, and the UV maxima values for both peaks are specified.
Since LDC-F3 and LDC-F4 fractions were both active, they were compared in order to find common peaks, most likely to be the source of the biological activity. A difference in the concentration of active metabolites in the fractions could explain the bioactivity differences of both fractions (Table 4.9). Two UV peaks with retention times of 7.26 (peak 1) and 7.44 (peak 2) were thus highlighted (Figure 4.8 and 4.9) as they were present in both spectra at different intensities, and were more abundant in fraction LDC-F4, matching the previous statement on the bioactivity of the fractions. The higher concentration of the putatively active peaks in fraction LDC-F4 is consistent with the stronger bioactivity when compared with fraction LDC-F3.

The masses for peak 1 and peak 2 on the HRMS spectra are m/z 277.21548 and m/z 301.21457. Both peaks presented dimers with an m/z 553.42580 and m/z 601.42635, respectively (Figures 4.10 and 4.11). The outputs of the analysis with the MassLynx version 4.1 software were two molecular formulae assigned to the peaks: C_{20}H_{28}O_{2} (unsaturation degree 7) and C_{18}H_{28}O_{2} (unsaturation degree 5), corresponding to peak 1 (adduct [M+H]^+ m/z 301.21457) and peak 2 (adduct [M+H]^+ m/z 277.21548), respectively.
Figure 4.10. High resolution mass spectrum extraction from fraction LDC-F4, where the mass peaks from the compound with molecular formula C_{20}H_{28}O_{2} can be observed. The instrument was operated in positive mode (ESI+) at low ionization energy (6eV).

Figure 4.11. High resolution mass spectrum extraction from fraction LDC-F4, where the mass peaks from the compound with molecular formula C_{18}H_{28}O_{2} can be observed. The instrument was operated in positive mode (ESI+) at low ionization energy (6eV).

When the elemental composition of the peaks was introduced into databases such as Dictionary of Marine Natural Products, ChemSpider or Reaxys, most of the outputs were either terpenoids or fatty acids/fatty acid derivatives, but the number of hit compounds was high. Even though the data was not sufficient to identify the compounds since NMR spectra were needed, there were several clues that can be useful for the identification of the peaks. Firstly, the fractionation method used to obtain the active fractions is similar to the approach used by Cutignano and co-workers (Cutignano et al., 2015) because they are both based on polystyrene-divinylbenzene resins and descending polarity solvents, and hence the separation of compounds should be rather similar. Here our compounds are more polar when compared to fatty acids, starting to elute at a 50% of MeOH (LDC-F3), while with the Cutignano method the fatty acids start to elute when they used 100% of
organic eluent. In addition, the UV (λ, wavelength) maxima values of the compounds (Figure 4.12, ≈230nm) restrict the presence of conjugation in the molecules to a maximum of two conjugated double bonds. Compounds of the family of the polyunsaturated fatty acids typically have UV (λ) maxima at wavelengths around 210 nm (Sahi et al., 2019) because they are not conjugated. In addition, fractions LDC-F3 and LDC-F4 presented a resin-like appearance, a feature that is not expected in regular fatty acids. As a conclusion from these arguments, regular fatty acids should not be contemplated as the possible identity of the substances isolated.

![Figure 4.12. UV pattern for peak 1 (left) and peak 2 (right). The wavelength of the spectra ranges from 190 nm to 500 nm.](image)

Since our compounds have a low number of carbons and display a high degree of unsaturation (7 degrees for C_{20}H_{28}O_{2} and 5 degrees C_{18}H_{28}O_{2}), the possibilities to find structures in which the double bonds will be the only type of unsaturation are rather low, because such compounds should present higher conjugation in their structures and hence higher UV (λ) maxima values. Compounds are then most likely to present cyclic structures together with double/triple bonds. Further purification and NMR spectra are necessary to perform the full structural elucidation of the pure compounds.
Molecular networking is a useful tool successfully applied for the detection and the isolation of bioactive natural products belonging to the same family. Evolution applied to biosynthesis often increases the diversity of molecules through attaching novel moieties to base chemical backbones (common structure), which enables to correlate a family of compounds between each other (Quinn et al., 2017). Those compounds will present similar fragmentation patterns, and molecular networking produces an MS/MS similarity map that allows the visualization of clusters of structurally similar molecules (Watrous et al., 2012). This would be then helpful to establish structural similarities between the potentially active metabolites found in my fractions, and also identify molecules that may belong to the same family.

Unfortunately, the quantity of the fractions available was not sufficient to complete the experiments while I was working at the Marbio facilities in Norway. These investigations are still ongoing in collaboration with the group of professor Jeanette H. Andersen. We had planned to deliver the final results for this doctoral thesis but unfortunately, my experimental work was forced to stop due to the COVID-19 crisis.

4.3.6 Chemical analysis of the active fraction from C. pseudocurvisetus (FE331)
Fraction CPC-F5 was analysed by UPLC-UV-HRMS as specified in the general chemical analysis procedures of this chapter (Section 4.2.5). UV-HRMS spectrum is shown in the figure below, together with the HRESIMS of the main component found in the fraction (Figure 4.13)
The major compound in the spectra presented an accurate mass of 302.2243, retention time 6.25 (in the range of fatty acids) and a particularly intense peak in HRESIMS corresponding to the adduct [M+NH₄]⁺ m/z 320.2583. The UV spectrum in MeOH displayed its λₘₐₓ at ≈ 210 nm. When the dictionary of natural products database was fed with the previous data together with the taxonomic information, the main hit was eicosapentaenoic fatty acid (EPA, Figure 4.14). In addition, the MEDINA-HRMS library (Pérez-Victoria, Martín and Reyes, 2016) was automatically populated with the accurate mass, UV maxima and HRESIMS spectra of the component and it was identified as 8. Microalgae are well-known producers of polyunsaturated fatty acids and 8 is predominant among diatoms (Ramesh Kumar et al., 2019). On the other hand, significant amounts of 8 were found on the closely-related species Chaetoceros muelleri (Liang, Beardall and Heraud, 2006). It was concluded that the main component present in fraction CPC-F5 is eicosapentaenoic acid 8.

Regarding the biological activity observed for the fraction, Thien Le and Desbois demonstrated that 8 displayed activity against two species of pathogenic gram-positive bacteria (i.e. Bacillus cereus and S. aureus). Authors showed minimum inhibitory concentration (MIC) values of 64 µg/mL for both bacteria, a result consistent with the 100% growth inhibition of the fraction CPC-F5 when it was used as treatment for MRSA and MSSA at a concentration of 100 µg/mL (Thien Le and Desbois, 2017).

4.4 Conclusion

L. danicus is shown to possess potent antibiofilm activity against the pathogenic bacterium S. epidermidis, whereas it seems to lack anticancer and antibacterial properties. The
Chapter also highlights the importance of using different culture conditions in order to trigger the production of secondary metabolites that can display interesting biological activities, since extracts obtained from cultures grown under phosphate starvation conditions were found to be more active compared to control nutrient repletion conditions. Once raw extracts from *L. danicus* were fractionated, the fractions were even more potent due to the concentration of the secondary metabolites responsible for the antibiofilm activity. Chemical analysis of the fractions presents a first insight on the nature of the compounds responsible for this antibiofilm effect, but further purification and studies on the pure compounds are necessary to present the final outputs (the identity of the pure compound/s and their biological activity). To my knowledge, this is the first study to attempt the identification of secondary metabolites with antibiofilm activity in diatoms and to present potent activity at the lowest concentrations tested in fractions generated from the raw extracts of microalgae.

*C. pseudocurvisetus* displayed potent antiproliferative activity against all cell lines tested, and antimicrobial activity against gram-positive bacteria (i.e. MRSA and MSSA) and *M. tuberculosis*. In this case the stressful conditions seemed to reduce the potency of the raw extract, a result that contrasted the observations for *L. danicus*. The control condition extract was fractionated and the fractions were consequently tested on the bioassay’s platform. As a result, one of the fractions resulted bioactive against gram-positive bacteria, but the track of the activity against *M. tuberculosis* was lost. Chemical analysis of the active fraction concluded that the main component was EPA, a polyunsaturated fatty acid known for its antibacterial activity against gram-positive bacteria, a result consistent with the bioactivity observed for the fraction. This study also attempted to identify the metabolites responsible for the antitubercular activity observed for the raw extracts of *C. pseudocurvisetus*, but further studies will be necessary to accomplish this task since the activity was lost during the bioassay-guided fractionation procedure, something rather frequent when using this approach for drug discovery. Finally, a significant antiproliferative activity on cancer cell lines was observed, and hence it is worth studying the source of such biological activity in the future.
Within this chapter I report the biological activity of other microalgal extracts that were tested for their anticancer, antimicrobial and antibiofilm activity using the different platforms at the research centre Fundación MEDINA (Granada, Spain) and the Marbio group laboratories (The arctic university of Norway, Tromsø, Norway). The studied microalgae were the green alga *Dunaliella tertiolecta* (FE200) and the diatoms *Asterionellopsis glacialis* (FE355), *Asterionellopsis glacialis* (A4), *Skeletonema costatum* (FE85) and *Odontella sinensis* (B2). Methods and results are presented and discussed. No chemical analyses were performed for these microalgae.

5.1 Methods

5.1.1 Microalgal growth and biomass production

*Dunaliella tertiolecta* (FE200) was bought from NCMA Bigelow (code CCMP 1320) in September 2010. *Skeletonema costatum* (FE85) was bought from the Roscoff Culture Collection (code RCC1716). *Asterionellopsis glacialis* (FE355) was isolated from the Gulf of Naples (Long term monitoring station MareChiara) in July 2017. *Asterionellopsis glacialis* (A4) and *Odontella sinensis* (B2) were isolated from the Northern Sea (Southern Bight) in September 2018. All the microalgae were maintained in the Stazione Zoologica culture collection. The microalgae isolated from the Gulf of Naples and the Northern Sea were identified by light microscopy and 28S DNA sequencing (Blastn 100% sequence identity FE355 *Asterionellopsis glacialis* KC969865.1; Blastn 100% sequence identity A4 *Asterionellopsis glacialis* KC969865.1; Blastn 100% sequence identity B4 *Odontella sinensis* MG835793.1). The diatoms were grown in Guillard’s F/2 medium (Guillard, 1975) while the green alga *D. tertiolecta* was grown in modified Guillard’s F/2 medium (Silicates depleted). All algae were grown in ten-litre polycarbonate carboys for biomass production. For phosphate starvation conditions, phosphate salt (NaH₂PO₄ · H₂O) was removed from both media recipes. The growth conditions were set in a climate chamber at 20°C, on a 12:12 h (light:dark) photoperiod at 110 μmol photons·m⁻²·s⁻¹ and with filtered air (through 0.2 μm membranes) supply. For light stress conditions, photoperiod was set at 24h:0h (light:dark). Initial cell concentration was approximately 5000 cells/mL for all experiments except for...
cultures of *O. sinensis*, which started at 500 cells/mL. 2L cultures were used to build the growth curves while 10L cultures were used to harvest the biomass necessary for the bioassays. Biomass was harvested during the stationary phase (on the same day and at the same time of day for each replicate to avoid possible interference due to intrinsic circadian rhythms) by centrifugation for 10 minutes at 4°C and 2300 rpm. All experiments were performed in triplicates. Microalgal biomasses were kept at -80°C until chemical extraction. Further details regarding medium preparation, inoculation, growth curves, scaling-up processes and harvesting are detailed in the General Methodologies section (Sections M.1, M.2 and M.3) of this thesis.

5.1.2 Chemical extraction
The chemical extraction of the microalgal biomasses was performed as indicated in the General Methodologies section.

*D. tertiolecta* (FE200) and *A. glacialis* (FE355) were extracted following the Fundación MEDINA extraction method (Section M.4). *Asterionellopsis glacialis* (A4) and *Odontella sinensis* (B2) were extracted following the Marbio extraction method (Section M.4). Aliquots from the dried extracts were lyophilized and dissolved in DMSO for testing, after both extraction methods.

5.1.3 Bioactivity screening
At Fundación MEDINA, the raw extracts were screened against a panel of 5 different cancer cell lines (*i.e.* human lung carcinoma A549 ATCC® CCL-185™, human skin melanoma A2058 ATCC® CRL-11147™, hepatocyte carcinoma HepG2 ATCC® HB-8065™, breast adenocarcinoma MCF7 ATCC® HTB-22™ and pancreas carcinoma MiaPaca-2 ATCC® CRL-1420™), five different pathogenic bacteria (*i.e.* Gram-negative bacteria *Escherichia coli* ATCC25922 and *Klebsiella pneumoniae* ATCC700603, Gram-positive bacteria *Staphylococcus aureus* MRSA MB5393 and MSSA ATCC29213, and *M. tuberculosis* H37Ra) and the fungus *A. fumigatus* ATCC46645. The extracts were screened for their anticancer and antimicrobial activity as reported in Audoin *et al.* (2013), using a treatment of 72h at an extract concentration of 175 µg/mL for the cancer cell lines, and a treatment of 20-30h (depending on the microorganism) at an extract concentration of 560 µg/mL for the pathogenic bacteria. Anticancer assays were developed using the MTT method, while the antibacterial activity was measured either by the optical density at the initial time (T₀) and
the final time (Tf) or by using the REMA method (Palomino et al., 2002) depending on the pathogenic microorganism.

Further details about the Fundación MEDINA biological assays can be found in the General Methodologies section of this thesis (Sections M.5.1 and M.5.2). All extracts were tested as biological triplicates and technical duplicates.

At Marbio, cell viability of three different human cancer cell lines (i.e. A2058 ATCC® CRL-11147™, HT29 ATCC® HTB-38™, MCF7 ATCC® HTB-22™) after treatment with the raw extracts was assessed using the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. In addition, the lung fibroblast cell line MRCS ATCC CCL-171 was used to assess toxicity in healthy human cells. The anticancer activity was assessed after 72h of treatment with the raw extracts. For the antibacterial MIC assays, five different bacteria were used (i.e. Enterococcus faecalis ATCC® 29212, Escherichia coli ATCC® 25922, Pseudomonas aeruginosa ATCC® 27853, Staphylococcus aureus ATCC® 25923, Streptococcus agalactiae ATCC® 12386). Optical density of the bacteria was measured at 600nm (OD$_{600}$) and the OD$_{600}$ values were compared to the mean of the negative controls (wells with only bacteria and media) to measure the antibacterial activity. Lastly, for the crystal violet antibiofilm assay, two different bacteria were used: the biofilm forming bacterium Staphylococcus epidermidis and the non-biofilm forming bacterium Staphylococcus haemolyticus. The non-biofilm forming bacterium was used as a control. Bacteria were grown for 24h using the raw extracts as treatment. Biofilm formation should not be observed in the control wells where Staphylococcus haemolyticus proliferated (cloudy wells).

The concentration of extracts used for all the Marbio assays was 100 µg/mL. Further details about the Marbio anticancer, antibacterial and antibiofilm assays can be found in the General Methodologies section of this thesis (Sections M.5.3, M.5.4 and M.5.5). All extracts were tested as biological and technical triplicates.

5.2 Dunaliella tertiolecta

5.2.1 Introduction

The marine microalga Dunaliella tertiolecta is a flagellated green alga that belongs to the class Chlorophyceae. Dunaliella species are considerably interesting because of their high salinity tolerance and their ability to produce commercially valuable products such as carotenoids. Previous studies from the 1990’s reported a direct correlation between
salinity levels and carotenoid production in *Dunaliella salina*, indicating an enhanced carotenogenesis when salinity levels were higher. However, the proportion of pigments changed dramatically as salinity rises, with beta-carotene as the main product at 25% (w/v) NaCl concentration (Borowitzka, Borowitzka and Kessly, 1990). In more recent studies on *Dunaliella tertiolecta*, a mutagenesis approach using ethyl methanesulfonate (EMS) treatment was used to generate a library of *D. tertiolecta* mutants which were screened to find clones with enhanced production of zeaxanthin (E161h), a food additive/colouring agent approved by the European Union (Kim et al., 2017). Zeaxanthin is a xanthophyll carotenoid present in higher plants with a high antioxidant potential; it has a role in protecting cell membranes and lipoproteins against oxidative stress and has also been found to prevent age-related macular degeneration and coronary heart diseases (Murillo, Hu and Fernandez, 2019). Zeaxanthin is related to Violaxanthin, another xanthophyll carotenoid which is rapidly and reversibly de-epoxidized into zeaxanthin in plants under high light stress (Havaux, Dall’Osto and Bassi, 2007). Violaxanthin was isolated from the *D. tertiolecta* clone CCMP364 by Pasquet et al. (2011). *D. tertiolecta* clone CCMP364 had previously shown anticancer activity and was submitted to a bioassay guided fractionation approach that spotted violaxanthin as the source of the anticancer activity observed at the level of raw extract and fractions (Pasquet et al., 2011). Within my studies I aimed to use the clone *D. tertiolecta* FE200, bought from NCMA Bigelow (code CCMP1320) in September 2010, to isolate potential bioactive secondary metabolites using a bioassay guided fractionation approach.

5.2.2 Growth curves and biomass production

The growth curves of *D. tertiolecta* cultured under control, phosphate starvation and light stress conditions are reported in the figures below (Figures 5.1, 5.2 and 5.3).
Figure 5.1. *D. tertiolecta* growth curve in control conditions (2 Liters).

Figure 5.2. *D. tertiolecta* growth curve in phosphate starvation conditions (2 Liters).
According to the data collected, the stationary phase was fixed from day 21 to day 57 for control conditions, from day 3 to day 13 for phosphate starvation conditions, and from day 18 to day 46 for light stress conditions. Regarding phosphate starvation conditions, the green alga barely reached 1% of the maximum concentration in the stationary phase of the control conditions, indicating that *D. tertiolecta* strongly depends on the concentration of phosphates for its growth. When the green alga was cultured under light stress conditions, the growth curve becomes slightly shorter. It is important to highlight the impressively long growth curve observed for the control and light stress conditions. It may be interesting to test and analyse different samples collected all along the curves in order to monitor the variability in its metabolism linked to the growth phase, as in the studies of Vidoudez and Pohnert (2008) on *Skeletonema marinoi*.

Ten litter carboys were used to grow *D. tertiolecta* in the three different culture conditions and collect biomass for further experiments. The following table (Table 5.1) summarises the maximum concentration reached, the harvest day and the weight of the wet biomass. All the samples were freeze-dried and stored under -80°C.

**Table 5.1.** *D. tertiolecta* samples in triplicate with wet weight, harvest day and maximum concentration reached.
### Table 5.1

<table>
<thead>
<tr>
<th><strong>D. tertiolecta samples</strong></th>
<th><strong>Maximum concentration (cells/mL)</strong></th>
<th><strong>Harvest day</strong></th>
<th><strong>Wet weight (grams)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>8,21x10^5</td>
<td>7</td>
<td>2.53</td>
</tr>
<tr>
<td>Control 2</td>
<td>5,39x10^5</td>
<td>7</td>
<td>2.28</td>
</tr>
<tr>
<td>Control 3</td>
<td>4,69x10^5</td>
<td>7</td>
<td>2.42</td>
</tr>
<tr>
<td>Phosphate starvation 1</td>
<td>2,61x10^4</td>
<td>6</td>
<td>0.23</td>
</tr>
<tr>
<td>Phosphate starvation 2</td>
<td>2,21x10^4</td>
<td>6</td>
<td>0.52</td>
</tr>
<tr>
<td>Phosphate starvation 3</td>
<td>5,16x10^4</td>
<td>6</td>
<td>0.36</td>
</tr>
<tr>
<td>Light stress 1</td>
<td>2,19x10^6</td>
<td>7</td>
<td>6.55</td>
</tr>
<tr>
<td>Light stress 2</td>
<td>2,11x10^6</td>
<td>7</td>
<td>5.30</td>
</tr>
<tr>
<td>Light stress 3</td>
<td>2,13x10^6</td>
<td>7</td>
<td>5.07</td>
</tr>
</tbody>
</table>

### 5.2.3 Extraction and raw extracts bioassays

The chemical extractions of the microalgal biomass were performed as reported in the methods section (Section 5.1.2). Pellets were freeze-dried before chemical extraction. The approximate extract yield per gram of wet pellet was 62 mg extract/g pellet for the control conditions, 42 mg extract/g pellet for the phosphate starvation conditions and 56 mg extract/g pellet for the light stress conditions. Each sample of microalgal biomass from the table above (Table 5.1) was extracted and their biological activities were assessed in the anticancer and antimicrobial high-throughput screening platforms at Fundación MEDINA (Granada, Spain).

The tables below (Table 5.2 and table 5.3) summarise the results regarding the anticancer and antimicrobial activity of the *D. tertiolecta* raw extracts produced from control (CTRL), phosphate starvation (-P) and light stress (L) cultures.

**Table 5.2.** Cell viability for the *D tertiolecta* extracts tested on all cancer cell lines. Each value represents the mean of two technical replicates performed for each extract and cell line. Most interesting results are highlighted in green.
Table 5.3. Cell viability for all pathogenic microorganisms treated with the extracts. Each value represents the mean of two technical replicates performed for each extract and bacterium. EC, KP, MT and AF stand for *E. coli*, *K. pneumoniae*, *M. tuberculosis* and *A. fumigatus*. Most interesting results are highlighted in green.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>Conc. (µg/mL)</th>
<th>A549</th>
<th>A2058</th>
<th>HepG2</th>
<th>MCF7</th>
<th>MiaPaca-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. tertiolecta</em> CTRL1</td>
<td>DTC1</td>
<td>175</td>
<td>87±4</td>
<td>52±0</td>
<td>12±4</td>
<td>92±5</td>
<td>92±5</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> CTRL2</td>
<td>DTC2</td>
<td>175</td>
<td>74±1</td>
<td>49±2</td>
<td>17±5</td>
<td>96±6</td>
<td>96±6</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> CTRL3</td>
<td>DTC3</td>
<td>175</td>
<td>81±4</td>
<td>50±2</td>
<td>8±4</td>
<td>74±7</td>
<td>74±7</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> -P1</td>
<td>DTP1</td>
<td>175</td>
<td>81±3</td>
<td>81±1</td>
<td>68±9</td>
<td>89±0</td>
<td>89±0</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> -P2</td>
<td>DTP2</td>
<td>175</td>
<td>81±0</td>
<td>76±1</td>
<td>56±3</td>
<td>96±0</td>
<td>96±0</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> -P3</td>
<td>DTP3</td>
<td>175</td>
<td>71±2</td>
<td>82±2</td>
<td>54±5</td>
<td>86±4</td>
<td>86±4</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> L1</td>
<td>DTL1</td>
<td>175</td>
<td>74±2</td>
<td>75±2</td>
<td>52±4</td>
<td>93±2</td>
<td>93±2</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> L2</td>
<td>DTL2</td>
<td>175</td>
<td>91±4</td>
<td>85±3</td>
<td>76±6</td>
<td>101±0</td>
<td>101±0</td>
</tr>
<tr>
<td><em>D. tertiolecta</em> L3</td>
<td>DTL3</td>
<td>175</td>
<td>90±4</td>
<td>79±1</td>
<td>71±6</td>
<td>97±2</td>
<td>97±2</td>
</tr>
</tbody>
</table>
The data reveals that, in general, the biological activity of the extracts is stronger when the green alga is cultured under control conditions. At 175 µg/mL, *D. tertiolecta* control extracts inhibit the growth of the liver cancer cells HepG2 (less than 20% of cell viability) while at 560 µg/mL, it inhibits the growth of the gram-positive bacterium MSSA (less than 30% of cell viability). Lower but noticeable activity was also observed for the cancer cell line A2058 (around 50% cell viability), and for the fungus *A. fumigatus* (around 40% cell viability).

*D. tertiolecta* (strain CCMP364) was already tested for anticancer activity by Pasquet et al. (2011), resulting in 50% growth inhibition of the breast cancer MCF7 cell line when the ethanol and dichloromethane extracts were used as treatment at 50-60 µg/mL. However, activity against MCF7 cell line was not observed within my studies on *D. tertiolecta*. This could be due to the different metabolism of the clone used (CCMP 1320), the harvesting time or the extraction method used. Further studies should be performed in order to identify the source of bioactivity against the cancer cell line HepG2 and the gram-positive bacterium MSSA. In addition, samples at different points of the growth curve could be collected and tested as a stronger biological activity could be observed.

### 5.3 Asterionellopsis glacialis

#### 5.3.1 Introduction

The marine microalga *A. glacialis* is a diatom that belongs to the class Bacillariophyceae. Cells are three-cornered in shape with an enlarged narrow region and very delicate transapical striae. Cells are usually united as star-like colonies. *A. glacialis* can be found either in neritic and benthic zones (Cupp, 1943; McLachlan and Brown, 2006). Rörig and co-workers studied the antimicrobial activity of two surf-zone Brazilian species of *A. glacialis* against several bacteria and fungi using a disk diffusion test, showing that neither of the two species presented any significant antimicrobial activity on all the pathogenic organisms.
tested (Rörig et al., 2017). Apart from these studies on the activity against different bacteria, the biological activity of this species has been poorly explored. Within my studies I tested two different clones of *A. glacialis* (i.e. *A. glacialis* FE355 from the gulf of Naples and *A. glacialis* A4 from the Northern Sea).

5.3.2 Growth curves and biomass production

The growth curves of *A. glacialis* FE355 cultured under control, phosphate starvation and light stress conditions, and the growth curve of *A. glacialis* A4 cultured under control conditions are reported in the figures below (Figures 5.4, 5.5, 5.6 and 5.7).

![A. glacialis (FE355) CTRL](image1)

**Figure 5.4.** *A. glacialis* FE355 growth curve in control conditions (2 Liters).

![A. glacialis (FE355) -P](image2)
According to the data collected, the *A. glacialis* FE355 stationary phase was fixed from day 4 to day 9 for control conditions, from day 4 to day 12 for phosphate starvation conditions, and from day 3 to day 11 for light stress conditions. On the other hand, *A. glacialis* A4 stationary phase in control conditions was fixed from day 6 to day 10. *A. glacialis* FE355 performed worst when it was cultured under phosphate starvation and light stress conditions, with maximum cell concentrations at least 3-fold and 2-fold lower compared to
the control conditions, respectively. Both clones had rather similar growth rates in control conditions, but maximum cell concentration was reached by *A. glacialis* FE355.

Ten litter carboys were used to grow clone FE355 in the three different culture conditions and clone A4 in control conditions in order to collect biomass for bioactivity assessment. The following table (Table 5.4) summarises the maximum concentrations reached, harvest day and weight of the wet biomass. All samples were freeze-dried and stored under -80°C.

Table 5.4: *A. glacialis* samples in triplicate with wet weight, harvest day and maximum concentration reached.

<table>
<thead>
<tr>
<th><em>A. glacialis</em> samples</th>
<th>Maximum concentration (cells/mL)</th>
<th>Harvest day</th>
<th>Wet weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE355 Control 1</td>
<td>3.34x10⁵</td>
<td>5</td>
<td>3.02</td>
</tr>
<tr>
<td>FE355 Control 2</td>
<td>3.83x10⁵</td>
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</tr>
<tr>
<td>FE355 Control 3</td>
<td>3.56x10⁵</td>
<td>5</td>
<td>3.89</td>
</tr>
<tr>
<td>FE355 Phosphate starvation 1</td>
<td>7.00x10⁴</td>
<td>4</td>
<td>0.66</td>
</tr>
<tr>
<td>FE355 Phosphate starvation 2</td>
<td>1.05x10⁵</td>
<td>4</td>
<td>1.27</td>
</tr>
<tr>
<td>FE355 Phosphate starvation 3</td>
<td>6.08x10⁴</td>
<td>4</td>
<td>0.62</td>
</tr>
<tr>
<td>FE355 Light stress 1</td>
<td>2.40x10⁵</td>
<td>5</td>
<td>6.55</td>
</tr>
<tr>
<td>FE355 Light stress 2</td>
<td>2.70x10⁵</td>
<td>5</td>
<td>5.30</td>
</tr>
<tr>
<td>FE355 Light stress 3</td>
<td>2.48x10⁵</td>
<td>5</td>
<td>5.07</td>
</tr>
<tr>
<td>A4 Control 1</td>
<td>3.05x10⁵</td>
<td>5</td>
<td>4.52</td>
</tr>
<tr>
<td>A4 Control 2</td>
<td>2.85x10⁵</td>
<td>5</td>
<td>5.09</td>
</tr>
<tr>
<td>A4 Control 3</td>
<td>2.91x10⁵</td>
<td>5</td>
<td>4.89</td>
</tr>
</tbody>
</table>

5.2.3 Extraction and raw extracts bioassays

The chemical extraction of the *A. glacialis* A4 biomass was performed at Marbio as reported in the methods section (Section 5.1.2). Pellets were freeze-dried before chemical extraction. The approximate extract yield per gram of wet pellet was 70 mg extract/g pellet.
for A4 cultured under control conditions. The biological activity of each A4 extract was assessed using the anticancer, antibacterial and antibiofilm screening platforms at Marbio (Tromsø, Norway). Unfortunately, no biological activity was observed for *A. glacialis* A4 in any of the platforms at the concentrations tested.

The chemical extraction of the *A. glacialis* FE355 biomass was performed at Fundación MEDINA, as reported in the methods section (Section 5.1.2). Pellets were freeze-dried before chemical extraction. The approximate extract yield per gram of wet pellet was 42 mg extract/g pellet for FE355 in control conditions, 71 mg extract/g pellet in phosphate starvation conditions and 58 mg extract/g pellet in light stress conditions. The biological activity of each FE355 extract was assessed using the anticancer and antimicrobial high-throughput screening platforms at Fundación MEDINA (Granada, Spain).

The tables below (Table 5.5 and Table 5.6) summarise the results regarding the anticancer and antimicrobial activity of the *A. glacialis* FE355 raw extracts produced from control (CTRL), phosphate starvation (-P) and light stress (L) cultures.

**Table 5.5.** Cell viability for the FE355 extracts tested on all cancer cell lines. Each value represents the mean of two technical replicates performed for each extract and cell line. Most interesting results are highlighted in green.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract code</th>
<th>Conc. (µg/mL)</th>
<th>A549</th>
<th>A2058</th>
<th>HepG2</th>
<th>MCF7</th>
<th>MiaPaca-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. glacialis</em> FE355 CTRL1</td>
<td>AGC1</td>
<td>175</td>
<td>71±7</td>
<td>35±3</td>
<td>12±1</td>
<td>71±3</td>
<td>77±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 CTRL2</td>
<td>AGC2</td>
<td>175</td>
<td>78±4</td>
<td>50±0</td>
<td>10±2</td>
<td>70±4</td>
<td>75±6</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 CTRL3</td>
<td>AGC3</td>
<td>175</td>
<td>87±6</td>
<td>54±1</td>
<td>21±3</td>
<td>97±2</td>
<td>82±2</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P1</td>
<td>AGP1</td>
<td>175</td>
<td>99±5</td>
<td>83±2</td>
<td>70±6</td>
<td>99±3</td>
<td>99±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P2</td>
<td>AGP2</td>
<td>175</td>
<td>96±4</td>
<td>84±5</td>
<td>78±10</td>
<td>80±2</td>
<td>76±3</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P3</td>
<td>AGP3</td>
<td>175</td>
<td>93±4</td>
<td>83±1</td>
<td>76±8</td>
<td>86±4</td>
<td>99±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 L1</td>
<td>AGL1</td>
<td>175</td>
<td>108±3</td>
<td>78±4</td>
<td>56±9</td>
<td>93±1</td>
<td>99±3</td>
</tr>
</tbody>
</table>
A. glacialis FE355 L2 | AGL2 | 175 | 93±19 | 93±2 | 53±1 | 75±3 | 85±3
A. glacialis FE355 L3 | AGL3 | 175 | 101±8 | 80±0 | 51±3 | 96±0 | 111±3

**Table 5.6.** Cell viability for all pathogenic microorganisms treated with the FE355 extracts. Each value represents the mean of two technical replicates performed for each extract and bacterium. EC, KP, MT and AF stand for *E. coli*, *K. pneumoniae*, *M. tuberculosis* and *A. fumigatus*. Most interesting results are highlighted in green.

<table>
<thead>
<tr>
<th>Extract name</th>
<th>Extract Code</th>
<th>Conc. (µg/mL)</th>
<th>EC</th>
<th>KP</th>
<th>MRSA</th>
<th>MSSA</th>
<th>MT</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. glacialis</em> FE355 CTRL1</td>
<td>AGC1</td>
<td>560</td>
<td>66±1</td>
<td>55±0</td>
<td>-4±1</td>
<td>-4±3</td>
<td>3±3</td>
<td>22±2</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 CTRL2</td>
<td>AGC2</td>
<td>560</td>
<td>71±2</td>
<td>67±4</td>
<td>-5±0</td>
<td>-2±0</td>
<td>1±0</td>
<td>27±2</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 CTRL3</td>
<td>AGC3</td>
<td>560</td>
<td>68±1</td>
<td>64±2</td>
<td>-4±0</td>
<td>-2±1</td>
<td>4±8</td>
<td>29±2</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P1</td>
<td>AGP1</td>
<td>560</td>
<td>84±1</td>
<td>89±0</td>
<td>94±5</td>
<td>106±4</td>
<td>77±6</td>
<td>92±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P2</td>
<td>AGP2</td>
<td>560</td>
<td>84±1</td>
<td>79±6</td>
<td>70±4</td>
<td>83±4</td>
<td>94±4</td>
<td>74±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 -P3</td>
<td>AGP3</td>
<td>560</td>
<td>84±1</td>
<td>82±1</td>
<td>92±1</td>
<td>110±4</td>
<td>118±7</td>
<td>83±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 L1</td>
<td>AGL1</td>
<td>560</td>
<td>67±0</td>
<td>54±1</td>
<td>35±2</td>
<td>22±2</td>
<td>36±9</td>
<td>34±1</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 L2</td>
<td>AGL2</td>
<td>560</td>
<td>71±0</td>
<td>88±1</td>
<td>-4±0</td>
<td>-4±1</td>
<td>-2±0</td>
<td>23±0</td>
</tr>
<tr>
<td><em>A. glacialis</em> FE355 L3</td>
<td>AGL3</td>
<td>560</td>
<td>68±1</td>
<td>65±6</td>
<td>-2±0</td>
<td>-2±1</td>
<td>2±6</td>
<td>29±0</td>
</tr>
</tbody>
</table>

The data reveals that the anticancer activity of the *A. glacialis* FE355 extracts is stronger when the green alga is cultured under control conditions. At 175 µg/mL, FE355 control extracts inhibit the growth of liver cancer cells HepG2 by 10-20% and slightly inhibits the growth of melanoma cells A2058 by 40-50%, while in light stress conditions it slightly affects the growth of HepG2 (cell viability ≈ 50%). No antiproliferative activity was observed for the phosphate starvation conditions.

Regarding the antimicrobial activity, it is interesting to see that both control and light stress extracts of FE355 completely inhibit the growth of MRSA, MSSA and *M. tuberculosis*, and
inhibit the growth of the fungus *A. fumigatus* by approximately 70% at the concentrations tested. Phosphate starvation extracts were inactive for all the organisms tested. However, the results observed using the antimicrobial platform are really positive, considering that Rorig et al. (2015) reported that the clones of *A. glacialis* they isolated from Brazilian waters were not active against any of the pathogenic microorganisms they tested. The study of the transcriptomes of and active and inactive microalgal samples were planned to be included within this doctoral thesis, but this was not possible due to the COVID-19 crisis. Further studies should be performed in order to identify the source of the different bioactivities observed for clone *A. glacialis* FE355.

### 5.4 Other tested microalgae without biological activity

Extracts from cultures of the diatom *Skeletonema costatum* (FE85) in control, phosphate starvation and light stress conditions were also tested. The biological activity of these extracts was studied at Fundación MEDINA (Granada, Spain) using the methods reported in Section 5.1.3. However, no antimicrobial nor anticancer activity were observed at the concentrations tested for any of the culture conditions.

On the other hand, extracts from the diatom *Odontella sinensis* (B2) in control conditions were tested at the platforms available at Marbio (Tromsø, Norway) using the methods reported in Section 5.1.3. However, no anticancer, antibacterial nor antibiofilm activity was observed at the concentrations tested.

### 5.5 Conclusions

The biological activity of the microalgae *Dunaliella tertiolecta* (FE200), *Asterionellopsis glacialis* (clone FE355 and clone A4), *Skeletonema costatum* (FE85) and *Odontella sinensis* (B2) was evaluated for this thesis. Promising results were observed for the green alga *D. tertiolecta* and the diatom *A. glacialis* (clone FE355) and they are hence worth further investigation as potential sources of bioactive secondary metabolites. The different bioactivities observed when culture conditions were changed denotes the importance of using the One Strain MAny Compounds (OSMAC) approach to trigger different pathways that can lead to a different metabolism, and ultimately to new active secondary metabolites. In addition, since the concentration of metabolites have been demonstrated to be dependent on the growth phase in previous studies (Vidoudez and Pohnert, 2008), the evaluation of the biological activity at different points of particularly long growth curves (such as the one of *D. tertiolecta*) should be considered in the future.
Chapter 6: Conclusions

As already stated in this doctoral thesis, marine microalgae represent a renewable and still poorly explored resource for biodiscovery of secondary metabolites which can potentially become drug candidates. The general objective of my PhD project was to investigate eight different marine microalgal strains (diatoms, green algae and dinoflagellates) in order to isolate and characterize secondary metabolites (chemically and biologically) with anticancer, antimicrobial and antibiofilm properties. During the project, five out of the eight microalgal clones tested were found to be biologically active in one or more of the bioassay platforms utilized and hence are worth being further explored for their potential as producers of bioactive compounds. In addition, I aimed to exploit culturing conditions which can lead to changes in the metabolism of the microalgae and hence trigger the production of different metabolites that perhaps present interesting biological activities. Phosphate depletion and an altered light cycle (photoperiod 24h:0h) were the stressful conditions I selected to trigger such effects, based on the background information from previous investigations (Samarakoon et al., 2013; Ingebrigtsen et al., 2016; Lauritano et al., 2016). So far, I have observed contrasting differences in the biological activity of the microalgae that I cultured in different conditions. While this approach led to an increment in the biological activity for the studies performed on L. danicus when cultured in phosphate depletion, the biological activity of the other microalgae became weaker with the use of stressful conditions. The main problem I faced when I cultured microalgae under phosphate depletion was the yield of biomass harvested, that in some cases was down to 5-fold lower compared to control conditions. Having enough material to perform the bioassay guided fractionation is critical in order to isolate the compounds responsible for the bioactivity. In addition, I observed that secondary metabolites are really found in low concentrations in the extracts from marine microalgae I investigated, which made it even more difficult to extract sufficient biomass for further chemical investigations. However, the use of different culturing conditions triggering differences in the biological activity that may facilitate the identification of the biosynthetic pathways involved in the production of bioactive compounds by using comparative transcriptomics on the microalgae in control conditions and in stressful conditions.
Within this doctoral thesis I managed to identify and characterize a new polyketide from the family of the amphidinols, named amphidinol 22. It was isolated from *A. carterae*, a well-known producer of such compounds. Amphidinol 22 presented only a few structural features compared to the other members of the family, and noticeable antiproliferative activity on all the cancer cell lines that I used for the assessment of the biological activity (i.e. A549, A2058, HepG2, MCF7 and MiaPaca2). Antifungal activity was very weak, a result that is in line with those of the long-chain amphidinols AM20 and AM21 (Satake *et al.*, 2017b). This was expected considering the structure of amphidinol 22, which features a long chain that only allows the much weaker membrane permeabilization mode of action explained by Satake and coworkers (2017), the carpet model. Even if bioactivity was not worth highlighting, this work demonstrated how potent dereplication and bioassay-guided fractionation approaches are for the isolation of new/novel bioactive secondary metabolites from marine sources.

*L. danicus* extracts showed potent antibiofilm activity against the pathogenic bacterium *S. epidermidis*, whereas it seems to lack anticancer and antibacterial activities. I detected two metabolites most likely to be the compounds responsible for the strong antibiofilm activity observed for the raw extracts of *L. danicus*. This work also highlights the importance of using different culture conditions in order to trigger the production of secondary metabolites that can display interesting biological activities, since extracts obtained from cultures grown under phosphate starvation conditions were found to be more active compared to control phosphate replete conditions. In addition, the clone tested within these studies displayed the same biological effects on the antibiofilm formation as the clones tested by Lauritano *et al.* (2016), both of which were isolated from the same location in the Gulf of Naples (long term station MareChiara LTER-MC). To my knowledge, this is the first study to attempt the isolation and identification of antibiofilm metabolites from diatoms.

*C. pseudocurvisetus* was found to possess potent antiproliferative activity against all cell lines tested, and antimicrobial activity against gram-positive bacteria (i.e. MRSA and MSSA) and *M. tuberculosis*. The biological activities observed for this diatom merits further investigation, in particular because of its anticancer activity. Using different culturing conditions led to differences in the biological activity of the samples from *C. pseudocurvisetus*, even if this time I observed a reduction in the potency of the extracts when the microalga was stressed in culture. Last generation dereplication tools such as
high-resolution mass spectroscopy (HRMS) combined with the information from internal (Pérez-Victoria, Martín and Reyes, 2016) and external databases (PubChem, Dictionary of marine natural products) allowed to identify the source of bioactivity as eicosapentaenoic polyunsaturated fatty acid without undergoing expensive and time-consuming work such as the isolation and characterization of a compound.

Regarding the other microalgal species tested, the green alga D. tertiolecta and the diatom A. glacialis (clone FE355) seemed to be promising sources of secondary metabolites worthy of further investigations. D. tertiolecta displayed a particularly long growth curve (considering that nutrient availability was not altered along the curve), and hence it could be interesting to study the variability of the biological activity along the curve, to establish the harvesting time when bioactive secondary metabolite production is strongest. As for C. pseudocurvisetus the biological activity was altered when using different culture conditions for A. glacialis, and also here the bioactivity was reduced when the microalga was stressed in culture.

Overall, of the eight microalgal strains tested, I isolated one new compound with antiproliferative activity on cancer cells, isolated a known fatty acid which presented an already studied antibacterial activity, detected two potentially active compounds from a microalgal fraction with strong antibiofilm activity, and identified another three strains with mild biological activity (Table 6.1). I also observed variations in the bioactivity by using stressful culture conditions such as nutrient depletion or light cycle alterations. The use of this OSMAC-like (One Strain Many Compounds) approach combined with tools such as bioassay-guided fractionation and HRMS-based dereplication can be applied to create high-throughput screening platforms to improve the biodiscovery pipeline from marine microalgae.

**Table 6.1. Final outcomes of my PhD project**

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Activity on raw extracts</th>
<th>Activity on fractions or compound</th>
<th>Isolated compound?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amphidinium carterae</em> FE102</td>
<td>Antiproliferative activity (cancer) and antimicrobial activity</td>
<td>Antiproliferative activity and antifungal activity</td>
<td>Amphidinol 22</td>
</tr>
<tr>
<td><strong>Chateroceros pseudocurvisetus FE331</strong></td>
<td>Antiproliferative activity (cancer)</td>
<td>Antibacterial activity</td>
<td></td>
</tr>
<tr>
<td><strong>Leptocylindrus danicus FE354</strong></td>
<td>Antibiofilm activity</td>
<td>Antibiofilm activity</td>
<td>Two compounds detected with potential antibiofilm activity</td>
</tr>
<tr>
<td><strong>Dunaliella tertiolecta FE200</strong></td>
<td>Antiproliferative activity (cancer)</td>
<td>Antimicrobial activity</td>
<td>-</td>
</tr>
<tr>
<td><strong>Asterionellopsis glacialis (FE355 and A4)</strong></td>
<td>Only FE355: Antiproliferative activity (cancer)</td>
<td>Antimicrobial activity</td>
<td>-</td>
</tr>
<tr>
<td><strong>Skeletonema costatum (FE85)</strong></td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td><strong>Odontella sinensis (B4)</strong></td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
</tbody>
</table>

Regarding the future outcomes, a project on the isolation of the antibiofilm metabolites from the fractions of *L. danicus* is still ongoing with the University of Tromsø (Norway), since the biological activity observed in those fractions was really promising. Structural elucidation and more specific biological assays will be performed on the pure compounds once they are isolated and the antibiofilm activity is confirmed. Further development of amphidinol 22 is not contemplated since the bioactivities were not potent enough to be considered for human health applications. On the other hand, the antiproliferative activity on cancer cell lines observed with the extracts of *C. pseudocurvisetus* will be further studied, since the extracts from this microalga exhibited a strong cytotoxic profile in all the cell lines tested and the antibacterial activity only led to the isolation of a well-known polyunsaturated fatty acid. Finally, the other bioactive microalgal extracts will be considered for future projects on drug discovery from microalgae.
General Methodologies

M.1. Microalgal medium preparation

In this section I provide all the information related to medium preparation for each of the 8 studied species studied: *Amphidinium carterae* (FE102), *Leptocylindrus danicus* (FE354), *Chaetoceros pseudocurvisetus* (FE331), *Dunaliella tertiolecta* (FE200), *Asterionellopsis glacialis* (FE355), *Asterionellopsis glacialis* (A4), *Skeletonema costatum* (FE85), and *Odontella sinensis* (B2). All the media used are based on enriched seawater recipes.

The diatoms (i.e. *L. danicus*, *C. pseudocurvisetus*, *A. glacialis*, *S. costatum* and *O. sinensis*) were cultured using Guillard’s F/2 medium. Modified Guillard’s F/2 medium without silicates was used for *D. tertiolecta*. Keller’s K medium was used for *A. carterae*. Components and concentrations for Guillard’s F/2 and Keller’s K media are reported in the table below (Table M1).

Table M1. Description of all the nutrients and their final concentrations in molarity for each of the main media used. *Omitted for the green alga (*D. tertiolecta*).

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Guillard’s F/2 medium (M)</th>
<th>Keller’s K medium (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>NaNO₃ (8.82 x 10⁻⁴)</td>
<td>NaNO₃ (8.82 x 10⁻⁴)</td>
</tr>
<tr>
<td>Phosphate</td>
<td>NaH₂PO₄ · H₂O (3.62 x 10⁻⁵)</td>
<td>NaH₂PO₄ · H₂O (3.62 x 10⁻⁵)</td>
</tr>
<tr>
<td>Silicate</td>
<td>Na₂SiO₃ · 9H₂O (0.53 - 1.06 x 10⁻⁴) *</td>
<td>Na₂SiO₃ · 9H₂O (5.04 x 10⁻⁵)</td>
</tr>
<tr>
<td>Trace metals</td>
<td>FeCl₃ · 6H₂O (1.17 x 10⁻⁵)</td>
<td>FeCl₃ · 6H₂O (1.17 x 10⁻⁵)</td>
</tr>
<tr>
<td></td>
<td>Na₂EDTA (1.17 x 10⁻⁵)</td>
<td>Na₂EDTA (1 x 10⁻⁴)</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ · 5H₂O (3.93 x 10⁻⁸)</td>
<td>CuSO₄ · 5H₂O (1 x 10⁻⁸)</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄ · 7H₂O (7.65 x 10⁻⁸)</td>
<td>ZnSO₄ · 7H₂O (8 x 10⁻⁸)</td>
</tr>
<tr>
<td></td>
<td>CoCl₂ · 6H₂O (4.20 x 10⁻⁸)</td>
<td>CoCl₂ · 6H₂O (5 x 10⁻⁸)</td>
</tr>
<tr>
<td></td>
<td>MnCl₂ · 4H₂O (9.10 x 10⁻⁷)</td>
<td>MnCl₂ · 4H₂O (9 x 10⁻⁷)</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄ · 2H₂O (2.60 x 10⁻⁸)</td>
<td>Na₂MoO₄ · 2H₂O (3 x 10⁻⁸)</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Thiamine · HCl (2.96 x 10⁻⁷)</td>
<td>Thiamine · HCl (2.96 x 10⁻⁷)</td>
</tr>
<tr>
<td></td>
<td>Biotin ((2.05 \times 10^{-9}))</td>
<td>Biotin ((2.05 \times 10^{-9}))</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Cyanocobalamin ((3.69 \times 10^{-10}))</td>
<td>Cyanocobalamin ((3.69 \times 10^{-10}))</td>
</tr>
<tr>
<td>Other additions</td>
<td>TRIZMA ((1 \times 10^{-3}))</td>
<td>TRIZMA ((1 \times 10^{-3}))</td>
</tr>
<tr>
<td></td>
<td>(\text{NH}_4\text{Cl} (5 \times 10^{-5}))</td>
<td>(\text{NH}_4\text{Cl} (5 \times 10^{-5}))</td>
</tr>
<tr>
<td></td>
<td>(\text{H}_2\text{SeO}_3 (1 \times 10^{-8}))</td>
<td>(\text{H}_2\text{SeO}_3 (1 \times 10^{-8}))</td>
</tr>
</tbody>
</table>

More specific details on the composition can be found in the original publications (Guillard, 1975; Keller et al., 1987). The final amounts of some substances can be changed in order to prepare mediums with altered nutrient availability. In the experiments I conducted, some of the microalgae selected were grown both under replete nutrient conditions (control) and phosphate-limited conditions (medium without addition of \(\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}\)).

Filtered seawater and Milli-Q water were the base of the preparation. Fresh sea water was taken weekly from the gulf of Naples and filtered according to the protocol shown below. Milli-Q water is obtained from the system Millipore Synergy 185 (Figure M1). 5% of Milli-Q water was added in other to adjust the salinity of the sea-water. The following material was used for the preparation of filtered sea-water:

- Millipore filtration apparatus (90mm filter holder, 275 psi max inlet)
- Millipore 0.22 µm filter (White GSWP, 90mm)
- Millipore glass fiber pre-filter
- KNF Neuberger N811 KN.18 vacuum pump
- Trap (glass Erlenmeyer with vacuum adapter)
- Schott DURAN 10L glass carboy with vacuum adapter
- Silicone hoses
The filtration apparatus (Figure M2) was assembled placing the filters inside the core. The 0.22 µm filter was placed at the bottom and the pre-filter on the top. The apparatus was then sealed by tightening the screws. A little valve was placed in the apparatus to eliminate air from the system. The hoses were attached to the apparatus, one to the sea-water recipient and to the upper part of the apparatus. Another hose was attached to the bottom side of the apparatus and to the upper part of the glass carboy. The third hose was attached to the vacuum adapter of the glass carboy and to the trap. There was a tube which connected the vacuum pump and the trap, closing the system. When the system was closed the faucet of the sea-water plastic recipient was opened and water started to drop into the glass carboy. At this point the pump can be switched on to filter faster.
Water was collected in the Schott DURAN 10L glass carboy with a vacuum adapter. Filtered seawater was stored in 10L carboys (Figure M1) or 2L bottles. An aeration cap was necessary since bubbling was required to avoid sedimentation of the biomass on the bottom surface of the carboys/bottles. At this point, salinity can be adjusted by removing 5% of the volume and adding an equal volume of milliQ-water. Then all nutrients except for the vitamins can be added to seawater (vitamins should be prepared and filter-sterilized separately, since they are thermolabile).

For the sterilization process, the autoclave FEDEGARI Model FVG3 (Figure M1) was used. The parameters of the program were 121º C temperature, 3-3.5 bar (g) pressure and 20-40 minutes time depending on the volume. The bottles containing nutrients were dropped inside the autoclave with the cap a little opened. When the program ended it was necessary to open the autoclave and close the sterilized medium bottles/carboys. After reaching room temperature, the vitamins were added to the medium that was then ready for use.

M.2 Microalgae inoculation, growth, scale-up and harvesting processes

The inoculation, growth and scale-up processes were done under aseptic conditions using a laminar flow hood (Folabo instruments CLEAN FLUX O/130). To start a microalgal culture and perform the further scale-up, it was necessary to start with a small volume of the microalgal strain. All the microalgal species were taken from the Stazione Zoologica Anton Dohrn culture collection. The first step was the preparation of a stock culture in a volume of 25 mL. Up to 5 drops (depending on the concentration) of microalgal inoculum were added to a 30 mL aseptic cultivation flask together with 25 mL of medium, under laminar flow hood. The microalgae were grown in a chamber at 20°C, 110 µmol·m⁻²·s⁻¹ light intensity and 12h:12h (light:darkness) photoperiod. This stock culture can be transferred to superior volumes (125 mL, 200mL and 500mL), scaling up the culture as many times as necessary depending on the concentration of microalgae required. Inoculation from this stock cultures was performed in 2L bottles and 10L carboys for different purposes: 2L experiments were performed in order to determine the growth curves for each microalgal species and each culture condition, while the 10L experiments were used to produce enough biomass to test bioactivity in the anticancer and antimicrobial bioassays, and also perform the chemical analyses.

To start an experiment, the mean value of cells concentration in the stock cultures (inoculum) was assessed by using the ZEISS Axioskop 2 MOT microscope and a 1 mL
Sedgewick Rafter counting chamber. A 3mL sample fixed with 3 drops of Lugol solution was used for counting. Once the concentration was calculated, it was possible to also calculate the volume of inoculum to be transferred to the final volume of the experiments. The 10L triplicates performed during my experiments started at a concentration of 5000 cells/mL, as well as the 2L cultures used to build the growth curves.

Before starting cultures of microalgae in 2L or 10L volumes for the experiments, the inoculum was allowed to reach the late exponential or early stationary phase, when most cells are viable and actively growing. The inoculation was achieved by transferring the calculated volume of stock culture (inoculum) to the final volume of prepared and sterilized media, under a laminar flow hood. After inoculation, bottles and carboys were submitted to continuous aeration to avoid sedimentation of the cells on the bottom surface inside a climatic chamber with the conditions mentioned above: 20°C, 110 µmol·m⁻²·s⁻¹ light intensity and 12h:12h (light:darkness) photoperiod. Using the complete formulation of the media and the above conditions was considered as control conditions. Microalgae from this thesis were grown in control conditions, phosphate starvation and light stress (24h:0h photoperiod). In phosphate starvation conditions, the media was prepared without NaH₂PO₄·H₂O enrichment, while in light stress conditions the climate chamber was set up to provide light for 24 hours/day. No further nutrients were added during growth.

Biomass was harvested at the stationary phase since Charles Vidoudez and Georg Pohnert (Vidoudez and Pohnert, 2008) demonstrated that highest concentrations of secondary metabolites occurred in this phase (Figure M3, for polyunsaturated aldehydes in Skeletonema marinoi). In fact most most studies searching for bioactivity in marine microalgae usually harvest algae at the stationary phase, when most secondary metabolites of potential biotechnological interest are produced (Desbois et al., 2008; Pasquet et al., 2011; Nigjeh et al., 2013; Kim et al., 2014; Rahman Shah et al., 2014).
Even if growth curves were made to detect the stationary phase, the 10L cultures were monitored every day by counting the cells in order to ensure the harvesting at the stationary phase. To harvest the microalgal biomass I used the Beckman Coulter Allegra® 6R centrifuge. I centrifuged in 500mL bottles at 2300 rpm, 4°C and 10 minutes for all the microalgae cultures in 10L. All the biomass was transferred to 50mL falcons and stored at -80°C.

The following material (Figure M4) was used for the inoculation, growth and scale-up processes:

- Pipetor STAR LAB
- Aseptic pipetes (5 mL, 10 mL, 25 mL and 50 mL)
- Aseptic cultivation flasks w/ air filter (30 mL, 250 mL, 500 mL and 1L)
- Autoclaved carboys w/ aeration system (2L and 10L)
- Microscope ZEISS Axioskop 2 MOT for counting.
- Microscope ZEISS Axiovert 200 for observing.
- Sedgewick Rafter counting chamber from Hausser Scientific Co.
- 5% iodine Lugol solution
M.3 Construction of microalgae growth curves

Microalgal growth generally features a sigmoid-like pattern which includes an exponential phase when algae are growing at a faster rate than they are dying, a stationary phase when the number of cells replicated is equal to the number of cells that die, and a senescent phase when most cells are dying (Figure M5).

To build a growth curve it was necessary to measure microalgal cell concentrations over a period encompassing the life cycle of the cultures. The volume selected to obtain the growth curves was 1L. For each species and culture condition (control, phosphate starvation and light stress), one culture was used. A sample was collected every 24 hours starting from the day of inoculation. The mean value of cell concentrations in the cultures was determined by using the ZEISS Axioskop 2 MOT microscope and a 1 mL Sedgewick...
Rafter counting chamber. A 3mL sample fixed with 3 drops of Lugol solution was used for counting.

Cell counts were performed every day to follow the time-lapse of the different phases that microalgae undergo (exponential, stationary and senescent phases). In my case I was aiming to establish when the different species reached the stationary phase. ZEISS Axiovert 200 Microscope was also used to observe the cells in vivo, controlling if cells were healthy (regular shape and size)

M.4 Chemical extraction from microalgal biomass

Chemical extraction of microalgal biomass was required to obtain a substratum enriched in secondary metabolites and also to remove salt and water from my samples. Protocols such as the one described by Cutignano et al. (2015) resulted in a good separation of terpenes, alkaloids and polyketides which are known to represent the most common classes of metabolites found in extracts of marine organisms.

For the extraction of microalgal biomass, I used two different protocols: FUNDACION MEDINA (Granada, Spain) extraction protocol and Marbio (The arctic university of Norway, Tromsø, Norway) extraction protocol.

According to MEDINA’s protocol, 3mL of methanol (HPLC grade, Sigma Aldrich) per gram of wet biomass were added to the 50mL falcon tubes containing the microalgal samples. Samples were vortexed for 1 min and sonicated in 3 bursts of 30 secs. They were then transferred to 40 mL EPA glass vials and agitated for 2h at room temperature with the Kuhner ISF4-X Climo-Shaker. Samples were then transferred to 15 mL Corning tubes and they were centrifuged at room temperature and 3000 rcf for 20’. The supernatant was then transferred to weighted 20 mL EPA glass vials and the solvent was evaporated under nitrogen stream until all the solvent was removed (overnight). Raw extracts were then lyophilized and weighted. Aliquots were prepared in 1.6 flat HPLC glass vials for the biological assays.

According to Marbio’s protocol Methanol (HPLC grade, Sigma Aldrich) was added into the 50 mL falcon tubes containing the biomass to reach two times the volume of the dry material (liophilized biomass). The tubes were vortexed to ensure that methanol completely soaked the material, that was then agitated for 2 hours at 20°C using a Universal orbital Shaker SM. The organic mixture was then filtered using regular cellulose paper filter,
the extract was transferred to a 250 mL rounded flask and evaporated under reduced pressure at 35°C using a Laborota Rotary Evaporator. Samples were aliquoted in 1.6 mL flat HPLC glass vials for the biological assays.

**M.5 Biological assays**

For the biological assays, I used protocols from the two different institutions where I worked: FUNDACION MEDINA (Granada, Spain) and Marbio (The arctic university of Norway, Tromsø, Norway). Here I detail the procedures I used in both platforms.

**M.5.1 Fundación MEDINA anticancer assays**

Microalgal extracts (biological triplicate) were screened against a panel of 5 different cancer cell lines (i.e. Lung cell line A549, skin cell line A2058, liver cell line HepG2, breast cell line MCF7 and pancreas cell line MiaPaca-2). The extracts were dissolved in 100% DMSO to use them as stock solutions. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were carried out to assess the cell viability at different concentrations after 72 hours of treatment with the extracts.

The medium composition was different for each type of cell line. A549 cells were grown in Ham’s F12K medium with 2mM Glutamine, 10% Foetal Bovine Serum (FBS), 100U/mL penicillin and 100 µg/mL streptomycin. A2058 and HepG2 were grown in ATCC formulated Eagle’s M essential medium (MEM) with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 µM MEM-non-essential aminoacids. MCF-7 cells were grown in the previous medium supplemented with 0.01 mg/mL of bovine insulin. MiaPaca-2 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS, 100U/mL penicillin and 100 µg/mL streptomycin. The cells were seeded in 96 well plates at a cell density of 1.000.000 cells/plate, and incubated for 24h at 37°C, 90% humidity and 5% CO2.

After 24h, the medium was removed and cells were treated by adding stock and fresh medium to the extracts (1 µL of stock in 200 µL of medium). The plates were then incubated for 72h at 37°C, 90% humidity and 5% CO2. The first and last column of the 96-well plates were used as controls and to calculate the standard curve. The first column alternated positive and negative controls, using methyl methanesulfonate (MMS) 8mM as the positive control and DMSO 100% (diluted 1:200 with MEM) as the negative control. The standard consisted of a dose-response curve using doxorubicin at 5 mM with 8-point serial dilutions (1:2 dilutions).
After the incubation period the medium was removed and the wells were washed with 100 µL of PBS 1X using a Multidrop™ Combi Reagent Dispenser. The MTT tetrazolium salt solution was prepared by diluting MTT in PBS 1X (5 mg/mL) and then in culture medium (without phenol red) to reach a concentration of 0.5 mg/mL. 100 µL of MTT solution were added to each well using the Multidrop™, and the plates were incubated for 3 hours at 37ºC. Viable cells with active metabolism reduce the MTT tetrazolium salt to an insoluble purple formazan product with a maximum of absorbance at 570 nm. The supernatant was removed and 100 µL of DMSO 100% were added to each well to dissolve the formazan precipitates. Finally, absorbance intensity was measured at 570 nm using a Perkin Elmer Wallac 1420 VICTOR2™ multilabel plate reader and the resulting data were analysed using Genedata Screener® software. The screening was performed using 2 technical replicates for each sample.

**M.5.2 Fundación MEDINA antimicrobial assays**

Microalgal extracts (biological triplicate) were also screened against a panel of 6 different pathogenic microorganisms (i.e. *Escherichia coli*, *Klebsiella pneumoniae*, methicillin resistant *Staphylococcus aureus*, methicillin sensitive *Staphylococcus aureus Mycobacterium tuberculosis* and *A. fumigatus*). The extracts were dissolved in 100% DMSO to use them as stock solutions.

Gram-negative bacteria *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603, and Gram-positive bacteria *S. aureus* MRSA MB5393 and MSSA ATCC29213 were used for the antibacterial MIC assays, performed as reported in Audoin et al. (2013). Thawed stock inocula suspensions from cryovials of each microorganism (MRSA, MSSA, *E. coli* and *K. pneumoniae*) were streaked onto Luria-Bertani agar plates (LBA, 40 g/L) and incubated at 37 °C overnight to obtain isolated colonies. Single colonies of each microorganism were inoculated into 10 mL of Luria-Bertani broth medium (LB, 25 g/L in 250 mL Erlenmeyer flasks) and incubated overnight at 37 °C with shaking at 220 rpm. The inocula were then diluted in order to obtain a concentration of approximately $1.1 \times 10^6$ CFU/mL (MRSA and MSSA) or $5–6 \times 10^5$ CFU/mL (*E. coli* and *K. pneumoniae*). For the assay, 98.6 µL/well of the diluted inoculum were mixed with 1.6 µL/well of each extract stock (100% DMSO). Aztreonam for *E. coli*, gentamycin for *K. pneumoniae*, and vancomycin for *S. aureus* were used as positive controls. Absorbance was measured at 612 nm with a Tecan Ultra Evolution spectrophotometer (Tecan, Durham, USA) at T0 (zero time) and immediately after that, plates were statically incubated at 37 °C for 20 h. After this period, the assay
plates were shaken using the DPC Micromix-5 and once more the absorbance at OD612 nm was measured at Tf (final time). Growth controls (bacteria + medium) and blank controls (medium) were also measured at T0 and Tf. The screening was performed using 2 technical replicates for each sample. The bacterial cells viability was calculated following the equation below.

\[
\% \text{Cell viability} = \frac{[(T_f \text{sample} - T_0 \text{sample}) - (T_f \text{blank} - T_0 \text{blank})]}{[(T_f \text{growth} - T_0 \text{growth}) - (T_f \text{blank} - T_0 \text{blank})]} \times 100
\]

Antifungal activity was assessed against *C. albicans* ATCC64124, grown in modified RPMI-1640 medium which was prepared as follows: 20.8 g of RPMI powder (Sigma) were poured into a 2L flask, together with 13.4 g of yeast nitrogen base, 1.8 L of milli-Q water, 80 mL of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer 1 M and 72 mL of glucose 50%. The volume was adjusted to 2L using milli-Q water and filtered. *C. albicans* was inoculated and the optical density at 660 nm of this first culture was adjusted to 0.25 using the growth medium. This culture was then diluted 1:10 and kept on ice until inoculation of the 96-well microtiter plates. For the assay, 90 μL of the 1:10 inoculum were mixed with 1.6 μL of microalgal extract solution in 100% DMSO and 8.4 μL of RPMI-1640 modified medium in each well. Amphotericin B was used as positive control. Absorbance was measured at 612 nm with a Tecan Ultra Evolution spectrophotometer (Tecan, Durham, USA) at T0 (zero time) and immediately after that, plates were statically incubated at 37 °C for 20 h. After this period, the assay plates were shaken using the DPC Micromix-5 and once more the absorbance at OD612 nm was measured at Tf (final time). Growth controls (bacteria + medium) and blank controls (medium) were also measured at T0 and Tf. The screening was performed using 2 technical replicates for each sample. The bacterial cells viability was calculated following the equation above.

The antitubercular activity of the extracts was determined using the REMA method (Palomino et al., 2002) as reported in Audoin *et al.* (2013). *M. tuberculosis* H37Ra ATCC 25177 was grown for 15-21 days in Middlebrook 7H9 broth (Becton Dickinson ref 271310) supplemented with 10 % ADC enrichment (Becton Dickinson ref. 211887) containing albumin, dextrose, and catalase; 0.5% glycerol as a carbon source; and 0.25% Tween 20 to prevent clumping. *M. tuberculosis* was inoculated into the media and dilutions were made to reach a final bacterial suspension concentration of 5×10^5 CFU/mL for the assay. A volume of 98.4 μL of the inoculum was added to each well of a 96-well microtiter plate containing
1.6 μL of extracts (100% DMSO, 10-point serial dilutions 1:1). Streptomycin was used as a positive control. Growth controls were also included (medium + bacteria). Plates were statically incubated for 7 days at 5% CO₂, 95% humidity and 37°C.

After incubation, 30 μL of 0.02% resazurin and 15 μL of Tween 20 were added to each well, incubated for 24 hours and assessed for colour development. A change from blue to pink indicates reduction of resazurin to resorufin and therefore microbial growth. The wells were read for colour change and the data were quantified by measuring fluorescence (excitation 570 nm, emission 615 nm) using a Perkin Elmer Wallac 1420 VICTOR2™ multilabel plate reader. For the Resazurin Solution, Resazurin sodium salt (C₁₂H₆NO₄Na; R7017, Sigma-Aldrich) stock solution of 0.02 g was dissolved in 100 mL of sterile distilled water and sterilized by filtration. The screening was performed using 2 technical replicates for each sample.

Antifungal activity against *A. fumigatus* ATCC46645 was also tested using the REMA methods. *A. fumigatus* ATCC46645 was grown in modified RPMI-1640 medium, prepared as for *C. albicans*. The inoculum concentration size for the assay was 2.5 × 10⁴ CFU/mL (determined by counting in a Neubauer chamber). A volume of 98.4 μL of the inoculum was added to each well of a 96-well microtiter plate containing 1.6 μL of extracts (100% DMSO, 10-point serial dilutions 1:1). Vancomycin was used as positive control. Growth controls were also included (fungi + medium). Plates were statically incubated for 30 h at 5% CO₂, 95% humidity and 37°C. The assays were set up in duplicate and developed as explained above for *M. tuberculosis*.

**M.5.3 Marbio anticancer assays**

Microalgal extracts (biological triplicates) were screened against a panel of 5 different cancer cell lines (i.e. Skin cell line A2058 ATCC CRL-11147, colon cell line HT29 ATCC HTB-38, breast cell line MCF7 ATCC HTB-22 and healthy lung cell line MRC5 ATCC CCL-171). Extracts were dissolved in 2.5% DMSO to use them as stock solutions. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays were carried out to assess the cell viability at different concentrations after 72 hours of treatment with the extracts (3 technical replicates).

The medium composition was different for each type of cell line. A2058 and HT29 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, 10 μg/mL gentamycin and 2 mM L-alanyl-L-glutamine. MCF7 and MRC5 cells were grown in
Minimum Essential Medium (MEM) with Earle's Balanced Salts supplemented with 10% FBS, 2mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, 100 µM MEM-non essential aminoacids and 0.15% NaHCO₃ salt. The cells were seeded in 96 well plates at a cell density of 2000 cells/well (cancer cells) or 4000 cells/well (healthy cells), and incubated for 24h at 37°C, and 5% CO₂. To calculate the concentration of cells, they were counted using a haemocytometer (Burker chamber).

After 24h, the medium was removed and cells were treated by adding stock and fresh medium to the extracts (10 µL of stock in 90 µL of medium). The plates were then incubated for 72h at 37°C, and 5% CO₂. Sterile medium was used as a negative control and 10 % DMSO (10 µL of DMSO in 90 µL of medium) was used as positive control.

After 72 hours of incubation, 10 µL of CellTiter 96® AQueous One Solution (Promega) per well was added, and the plates were incubated again for 1 hour. Absorbance was measured at 485 nm, using the DTX 880 Multimode Detector and the Multimode Analysis Software (Beckman Coulter, USA). Cell viability was calculated according to the equation below.

\[
Cell\ viability\ (\%) = \frac{[Abs(Ext) - Abs(\text{Pos})] \times 100}{[Abs(Neg) - Abs(\text{Pos})]}
\]

Abs(Ext) stands for the absorbance measured for the wells of treatment, Abs(Pos) is the absorbance measured for the positive control (10% DMSO) wells and Abs(Neg) is the absorbance measured for the negative control (sterile media) wells.

**M.5.4 Marbio antibacterial assays**

Microalgal extracts (biological triplicate) were also screened against a panel of 6 different pathogenic bacteria (i.e. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus agalactiae* ATCC 12386). The extracts were dissolved in 2.5% DMSO to use them as stock solutions.

*S. aureus, E. coli* and *P. aeruginosa* were grown in Müller-Hinton broth (MH, Becton, Dickinson and Company), while *E. faecalis* and *S. agalactiae* were grown in Brain heart infusion broth (BHI, Sigma-Aldrich). To prepare the stock cultures, a scoop of each bacterium was transferred into 8 mL of growth medium and incubated overnight at 37°C. From those initial cultures, 2 mL were transferred into new flasks containing 25 mL of fresh medium. Cultures were then incubated until they reached 0.5 McFarland standard of turbidity at 600 nm (1.0 x 10⁸ CFU/mL) with agitation at 37°C. Approximate time of
incubation was 2.5 hours for *S. aureus* and *P. aeruginosa*, and 1.5 hours for *E. coli*, *E. faecalis* and *P. agalactiae*. After incubation, the bacterial solution was further diluted 1:1000 using MH or BHI.

For the assay, 50 μL of the diluted inoculum (1:1000) were mixed with 50 μL of each extract stock (2.5% DMSO) in each well of the plate. Positive control was 50 μL of medium and 50 μL of autoclaved Milli-Q water, and negative control was 50 μL of autoclaved Milli-Q water and 50 μL of bacterial inoculum (1:1000). The plates were incubated at 37°C for 20-24 hours. Then, optical density was measured at 600 nm with a Tecan Victor Multilabel Counter (Perkin Elmer). The screening was performed using 3 technical replicates for each sample. Controls were performed routinely to monitor regular growth of the bacteria by testing gentamycin at concentrations ranging from 16 to 0.01 μg/mL. The minimum inhibitory concentration (MIC) was calculated and compared to previous values. Bacterial viability was calculated according to the following formula:

\[
\text{Bacterial viability (\%)} = \frac{[\text{OD}_{600}(\text{Ext}) - \text{OD}_{600}(\text{Pos})] \times 100}{\text{OD}_{600}(\text{Neg}) - \text{OD}_{600}(\text{Pos})}
\]

OD600(Ext) stands for the absorbance measured for the wells of treatment, OD600(Pos) is the mean absorbance measured for the positive control wells and OD600(Neg) is the mean absorbance measured for the negative control wells.

**M.5.5 Marbio antibiofilm assays**

Antibiofilm activity was assessed using the crystal violet biofilm assay. Two bacteria were used for testing: the biofilm forming bacterium *Staphylococcus epidermidis* ATCC 35984 and the non-biofilm forming bacterium *Staphylococcus haemolyticus* (clinical isolate). The non-biofilm forming bacterium was used as a control. A scoop of both bacteria was inoculated into 5 mL of tryptic soy broth (TSB) overnight at 37 °C. The resulting bacterial cultures were diluted 1:100 in TSB with 1% glucose (induces the biofilm formation) and 50μL were added to each well of the assay plate. Then, 50 μL of the extracts diluted in milli-Q water (2.5% DMSO) were added to the wells. Positive control was 50 μL of *S. epidermidis* culture and 50 μL of sterile Milli-Q water. Negative control for the screening was 50 μL of *S. haemolyticus* culture and 50 μL of sterile Milli-Q water. After 24 hours, all wells were rinsed with distilled water, and a 0.1% crystal violet solution was added to each well of the plate for 10 minutes. Wells were rinsed again with distilled water, and the remaining crystal violet (staining the biofilm) was diluted with 100 μL of 70% ethanol. The results are
expressed in terms of optical density measured at 600 nm and a sample was considered active when the OD$_{600}$ was under 0.9 units. The extracts and fractions were tested as biological and technical triplicates (9 replicates in total for each extract) at different concentrations (100, 50, 25 and 12.5 µg/mL). Biofilm formation should not be observed in the control wells where *Staphylococcus haemolyticus* proliferated (cloudy wells)
Publications from my thesis work

**Amphidinol 22, a New Cytotoxic and Antifungal Amphidinol from the Dinoflagellate *Amphidinium carterae***

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**Abstract:** Due to the unique biodiversity and the physical-chemical properties of their environment, marine microorganisms have evolved defense and signaling compounds that often have no equivalent in terrestrial habitats. The aim of this study was to screen extracts of the dinoflagellate *Amphidinium carterae* for possible bioactivities (i.e., anticancer, anti-inflammatory, anti-diabetes, antibacterial and antifungal properties) and identify bioactive compounds. Anticancer activity was evaluated on human lung adenocarcinoma (A549), human skin melanoma (A2058), human hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF7) and human pancreas carcinoma (MiaPca-2) cell lines. Antimicrobial activities were evaluated against Gram-positive bacteria (*Staphylococcus aureus* MRSA and *MSSA*), Gram-negative bacteria (i.e., *Escherichia coli* and *Klebsiella pneumoniae*), *Mycobacterium tuberculosis* and the fungus *Aspergillus fumigatus*. The results indicated moderate biological activities against all the cancer cell lines and microorganisms tested. Bioassay-guided fractionation assisted by HRMS analysis allowed the detection of one new and two known amphidinols that are potentially responsible for the antifungal and cytotoxic activities observed. Further isolation, purification and structural elucidation led to a new amphidinol, named amphidinol 22. The planar structure of the new compound was determined by analysis of its HRMS and 1D and 2D NMR spectra. Its biological activity was evaluated, and it displayed both anticancer and antifungal activities.

**Keywords:** marine microalgae; dinoflagellates; marine natural products (MNP); bioactive compounds; blue biotechnology; amphidinol; antifungal; anticancer

1. Introduction

Many microalgae, including dinoflagellates, are known to produce compounds with a wide range of biological and biochemical properties [1]. The biodiversity of marine phytoplankton species leads to a great metabolic variety that renders them a huge reservoir of new bioactive compounds with multiple possible pharmaceutical applications [2] (e.g., cytotoxic, anticancer, antibiotic, antifungal, immunosuppressor and neurotoxic activities [3–10]). Bioactive compounds of microbial origin can be sourced directly from primary metabolism (e.g., proteins, fatty acids, vitamins and pigments) or
Bibliography


