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Bioprospecting anti-inflammatory compounds in three diatoms, *Cylindrotheca closterium*,*Cylindrotheca fusiformis* and *Nanofrustulum shiloi*

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

Britta Kautzmann

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"Wisdom begins in wonder."

Socrates

"God, grant me the serenity to accept the things I cannot change,

Courage to change the things I can,

And wisdom to know the difference."

Reinhold Niebuhr

Resumo

As microalgas como produtores primários e na sua maioria fotossintéticos são consideradas organismos importantes para o equilíbrio ambiental, pela sua elevada capacidade de fixar CO_2, o que contribui para o sequestro global deste composto. As microalgas pela sua diversidade e composição bioquímica são ainda descritas como uma fonte de compostos bioativos, que se encontra pouco explorada, mas que tem diversas aplicações biotecnológicas.

Este grupo de organismos podem ser classificados de diversas formas, sendo a mais comum baseada no perfil de pigmentos, que lhes confere diferentes cores. Os grupos mais frequentemente descritos dividem-se em algas procarióticas, que engloba as "algas azuis" (*Cyanophyta*), algas eucarióticas, que inclui as algas verdes (*Chlorophyta*), castanhas (*Phaeophyceae*), castanho-douradas (*Chrysophyceae*), vermelhas (*Rhodophyta*), e mais dois grupos principais como dinoflagelados (*Dinoflagellata*) e as diatomáceas (*Bacillariophyceae*).

Os compostos provenientes da biomassa de microalgas são utilizados em várias indústrias, como a alimentar humana e animal (produção de rações), agrícola (fertilizantes), farmacológica e energética. Compostos como ácidos gordos essenciais, proteínas e carotenoides, entre outros, podem ser encontrados em microalgas, tendo a maioria sido reportada como contendo atividades antioxidantes e anti-inflamatórias. A investigação ligada à procura de novos compostos tem como principal objetivo encontrar novos ingredientes ativos para desenvolvimento de fármacos que possam ser utilizados em doenças comuns na população, como as doenças inflamatórias crónicas.

Um dos grupos de microalgas menos explorado na área biomédica e farmacêutica são as diatomáceas. Estes organismos, estão envolvidos por uma frústula bipartida, uma estrutura vítrea e translúcida formada por sílica e que forma a sua parede celular. As diatomáceas têm dois tipos de divisão celular, mitótica por divisão celular vegetativa e reprodução sexual, por meiose. A divisão celular vegetativa ocorre através da divisão da membrana plasmática na célula parental com a construção da respetiva segunda válvula nas células filhas.

O conteúdo celular das diatomáceas inclui pigmentos fotossintéticos como a clorofila, em particular as clorofilas *a* e *c1, c2* e *c3*, bem como a fucoxantina. Ambos os pigmentos são conhecidos pela sua forte capacidade antioxidante e anti-inflamatória. Foram ainda reportados na sua composição compostos bioativos com capacidade antioxidante, como fenólicos, ácidos gordos polinsaturados (PUFA), flavonoides, tocoferóis e alcaloides.

A produção de diatomáceas marinhas requer nutrientes, fornecimento de luz e um sistema de cultivo adequado. Os sistemas de produção podem ser divididos em 2 grupos: sistemas fechados e abertos. Sistemas abertos, que podem ser tanques abertos ou raceways (sistemas que possuem uma pá mecânica que movimenta a cultura), são mais utilizados, em instalações de larga escala, para produção de biocombustíveis e fertilizantes. Os sistemas fechados, que incluem os fotobiorreatores (PBRs), podem apresentar vários formatos, desde colunas, painéis planos e PBRs tubulares. São sistemas translúcidos, equipados com sistemas de aeração e menos sujeitos aos fatores externos. São usados na produção de compostos de alto valor comercial uma vez que sendo sistemas fechados permitem exercer um maior controlo sobre as condições de cultivo.

Neste sentido, o objetivo principal deste trabalho foi avaliar a capacidade antioxidante e anti-inflamatória para valorização da biomassa de três espécies de diatomáceas marinhas, nomeadamente *Cylindrotheca closterium*, *Cylindrotheca fusiforme* e *Nanofrustulum shiloi*. As microalgas *Cylindrotheca* eram provenientes da coleção do laboratório MarBiotech (CCMAR, Universidade do Algarve) *e Nanofrustulum shiloi*, foi fornecida como pasta ultracongelada pela empresa Necton (Olhão).

No que respeita às duas espécies de *Cylindrotheca* em estudo, numa primeira etapa, foi necessário proceder em condições laboratoriais ao aumento de escala para produção de 10 g de biomassa seca de cada espécie. Numa segunda fase, a biomassa das três microalgas foi extraída com diferentes solventes, nomeadamente hexano, acetato de etilo e etanol. Por usa vez, os extratos gerados foram usados para a determinação da sua capacidade antioxidante e anti-inflamatória. Para a determinação da atividade antioxidante, foram realizados ensaios de DDPH (2,2-Diphenyl-1-picrylhydrazyl) e ensaios de atividade redutora de ferro, como uma etapa de triagem para os consequentes testes anti-inflamatórios. Estes consistiram em ensaios de inibição de enzima conversora da angiotensina (ECA) e de inibição da ciclooxigenase-2 (COX-2). A última fase correspondeu à identificação tentativa dos compostos bioativos responsáveis pelas atividades.

Os extratos etanólicos de *C. fusiformis* e de *N. shiloi* demonstraram ser os mais ativo nos ensaios anti-inflamatórios, exibindo uma capacidade de inibição da COX-2 de 63.78% e de 67.73% para *N. shiloi* e *C. fusiformis*, respetivamente. No ensaio de inibição da ECA obtiveram-se valores de 58.93% e de 48.72% de inibição para *N. shiloi* e *C. fusiformis*, respetivamente, quando testados a uma concentração de 1 mg ml-1. Posteriormente o extrato etanólico de *Nanofrustulum shiloi* foi selecionado para ser fracionado por cromatografia líquida. As frações

contendo massa suficiente foram novamente analisadas e os resultados obtidos levaram à seleção final de 2 frações (F10, F13). Ambas demonstraram inibição da ECA de 82.76% (F 10) e de 92.47 % (F13) à concentração de 0.5 mg ml^{-1}. Ambas foram ainda testadas quanto à sua capacidade para inibir a ciclooxigenase-1 (COX-1), sendo a fração F10 inibidora seletiva da COX-2 (83.44% à concentração de 0.5 mg ml^{-1}) e a fração F13 apresentando uma inibição significativamente mais forte da COX-2 (81.93% à concentração de 0.5 mg ml^{-1}) do que da COX-1 (20.10% à concentração de 0.5 mg ml^{-1}).

As duas frações foram submetidas a cromatografia gasosa combinada com espectrometria de massa (GC-MS) para determinar os possíveis compostos ativos responsáveis pela atividade anti-inflamatória. Em ambas as frações foram detetados ácidos gordos polinsaturados (PUFAs) omega-3, com efeitos anti-inflamatórios conhecidos, que podem ter origem em compostos de natureza lipídica abundantes em microalgas como os acilgliceróis, fosfolípidos, glicolípidos, entre outros. Foram ainda observados carotenoides nas duas frações ativas, revelados pela intensa coloração laranja ou verde. Contudo, a sua análise por cromatografia líquida (HPLC), não possibilitou a identificação de carotenoides conhecidos. A presença de compostos com absorvância no comprimento de onda entre 230-260 nm indicam ainda a presença de compostos fenólicos.

À escala laboratorial as culturas de *C. fusiformis* apresentaram melhores resultados de crescimento do que *C. closterium*, sugerindo a sua aplicação para ensaios de produção a escala industrial. Os resultados referentes às propriedades antioxidantes e anti-inflamatórias, demonstram que os extratos etanólicos para as microalgas *C. fusiformis* e *N. shiloi* possuem atividade antioxidante e anti-inflamatória e, assim, potencial biotecnológico.

Palavras chave

Diatomácias, inibição da Enzima Conversora da Angiotensina, inibição da Ciclooxigenase-2, actividade antioxidante

Abstract

Microalgae are a source of bioactive compounds that is far from being exhausted. In addition to a wide range of possible applications in the food industry and agriculture, as well as in other industries as sources of novel pharmaceuticals, nutriceuticals, plastics and fuels, there is also a focus on finding new active ingredients against common ailments in which chronic inflammatory diseases are included. Besides the growth behaviour of 2 benthic diatoms, *Cylindrotheca closterium,* and *Cylindrotheca fusiformis,* under laboratory conditions, this work also investigated possible antioxidant and anti-inflammatory activities of extracts produced from these diatoms. Extracts of a third diatom, *Nanofrustulum shiloi*, were also carried out. After extraction of the biomass with 3 solvents of different polarities (hexane, ethyl acetate and ethanol), the antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DDPH) and iron reducing antioxidant activity assays in order to make a selection of the most suitable extracts for several anti-inflammatory tests. ACE inhibition assay and COX-2 inhibition assay were performed with the most potent antioxidant extracts, mainly the ethyl acetate and ethanol extracts of all three microalgae. The ethanolic extracts from *Cylindrotheca fusiformis* and *Nanofrustulum shiloi* emerged as the most active from this series of tests. Furthermore, the *N. shiloi* ethanolic extract was seletected to be fractionated in order to narrow down the search for the compound(s) responsible for the detected activity. Ultimately, this resulted in 2 fractions with strong COX-2 inhibition activity, one fraction with selective COX-2 inhibition compared to COX-1, and both with good ACE inhibitory capacity. For the tentative identification of the compounds responsible for the observed activities, the fractions were subjected to chemical characterization by GC-MS after derivatization. Various fatty acids, some *n*-3 PUFAs with known anti-inflammatory properties were identified, leading us to believe that the responsible compound might be lipidic in nature, either an acylglycerolipid, phospholipid, or glicolipid, among others, all abundant in microalgae. Analysis by LC-MS is recommended to better identifiy the anti-inflammatory active compounds as GC-MS is limited to volatile or semi-volatile compounds. In conclusion both *N. shiloi* and *C. fusiformis* show great potential as sources of anti-inflammatory compounds although further studies for a more precise identification and possible use of the active compounds are needed.

Keywords

Diatoms, ACE-inhibition, COX-2 inhibition, bioactivity, antioxidant

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Abbreviations

AA- arachidonic acid

ACE - angiotensin converting enzyme

ALA - alpha-linolenic acid

ANOVA - analysis of variance

ASE - accelerated solvent extraction

CCMAR - Centro de Ciências do Mar, centre of marine science

CIDs - chronic inflammatory diseases

COX-1 - cyclooxygenases 1

COX-2- cyclooxygenases 2

COXi - cyclooxygenases -inhibitors

DGDG - digalactosyldiacylglycerol

DGLA - dihomogamma linolenic acid

DHA - docosahexaenoic acid

DMSO - dimethyl sulfoxide

DPPH - (2,2-diphenyl-1-picrylhydrazyl)

ELISA - enzyme immunosorbent assay

ELSD - evaporative light scattering detector

EPA - eicosapentaenoic acid

EPS - exopolysaccharides

EU - European Union

FA - fatty acids

GC - gas chromatography

GC-MS - gas chromatography - mass spectrometer

GLA - gamma-linolenic acid

GRAS – "Generally Recognized As Safe "

HAT - hydrogen atom transfer reaction

HPLC - high performance liquid chromatography

IL-1β - interleukin 1

IL-6 - interleukin 6

IL-8 - interleukin 8

LA - linoleic acid

LC - liquid chromatography

LC PUFAs - long chain polyunsaturated fatty acids

LC-MS - liquid chromatography - mass spectrometer

LPS - lipopolysaccharide

LTB4 - leukotriene B4

MA- mystiric acid

MAE - microwave assisted extraction

MGDG - monogalactosyldiacylglycerol

MS - mass spectrometer

MSTFA - N-methyl-N-(trimethylsilyl) trifluoroacetamide

MUFAs - mono-unsaturated fatty acids

NIST - National Institute of Standards and Technology

PA - palmitic acid

PBRs - photobioreactors

PC - phenolic compounds

PDA - photo diode array detector

PG - prostaglandin

PgE_1 - prostaglandin E_1

PGE_2 - prostaglandin E_2

PLE - pressurized liquid extractions

PUFAs - polyunsaturated fatty acids

RAS - renin-angiotensin system

rDNA - ribosomal deoxyribonucleic acid

ROS – reactive oxygen species

SET - single electron transfer reaction

SFA - saturated fatty acids

SFE - supercritical fluid extraction

SPS - sulphated polysaccharides

TAG - triacylglycerides

TCA - trichloroacetic acid

TMS - trimethylsilyl

TNA-α - tumor necrosis factor alpha

TXA - thromboxanes

UHPLC-MS-MS - ultra-high-performance liquid chromatography tandem mass spectrometer

UV - ultraviolet

VIS - visible light

1 Introduction

1.1 Microalgae

Microalgae are a highly diverse group of unicellular, mostly photosynthetic, prokaryotic or eukaryotic microorganisms, which are found in nearly every environment, but especially in freshwater and marine systems and wetlands (Tabarzad et al., 2020). Microalgae make important contributions to environmental protection. Their high capacity to fixate CO_2 contributes to global CO_2 sequestration (Prasad et al., 2021). In fact, it is estimated that microalgae are responsible for producing over 50% of the planet's annual oxygen needs (Chapman, 2013). Microalgae can be classificated based on various factors, but the most common is based on their pigment composition, more or less classified by their colours. The most known and largest groups are the prokaryotic "blue-green algae" (*Cyanobacteria*), the eukaryotic algae known as green algae (*Chlorophyta*), brown algae (*Phaeophyceae*), golden brown algae (*Chrysophyceae*), red algae (*Rhodophyta*), and two more main algae groups the dinoflagellates (*Dinoflagellata*) and the diatoms (*Bacillariophyceae*) (Guiry & Guiry, 2021). Their compounds are used for a wide range of applications as food, feed, agriculture fertilizer, cosmetics, biodiesel and nutraceuticals. Among other capabilities, microalgae can also be used for wastewater treatment. Research is also being carried out into the use of microalgae species that can absorb and accumulate heavy metals and even strontium to also treat nuclear wastewater (Liu et al., 2014; Wollmann et al., 2019). An enzyme has been found in green microalgae to degrade polyethylene terephthalate (PET), a plastic widely used in beverage bottles. (Kim et al., 2020).

Microalgae have been consumed by humans for centuries. Certain microalgae are harmless for human consumption as confirmed through their traditional use. In the United States, the Food and Drug Administration (FDA) has classified *Spirulina*, *Chlorella*, *Dunaliella, Haematococcus*, *Schizochytrium*, *P. cruentum* and *C. cohnii*, among others, as “Generally Recognized As Safe” (GRAS) food sources. For non-GRAS products, an approval program, some of which is extensive, must be completed in order to guarantee that consumption is safe (García et al., 2017). In the European Union (EU), the Novel Food Regulation (EU) 2015/2283 applies, so microalgae that have been on the market as a food or food ingredient and consumed to a significant degree before 15 May 1997 in the EU market, as *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Spirulina* sp., *Arthrospira platensis*, *Aphanizomenon flosaquae var. flosaquae*, and *Auxenochlorella protothecoid*es and microalgae

and/or their products that has been approved as novel food, as dried *Tetraselmis chui*, *Odontella aurita*, and astaxanthin-rich oleoresin from *Haematococcus pluvialis*, can be commercialized in the EU. For all microalgae species or products that have not yet been approved, a corresponding approval process must be carried out, which primarily determines the product safety for the consumer. A shortened approval procedure can be used for traditional foods from non-EU countries. Information on the approval status of a food can be found in the European Union's Novel Food Catalogue (European Comission of Food Safety, 2022; Fernandes & Cordeiro, 2021).

Various essential fatty acids, proteins and carotenoids, among other compounds, have been found in microalgae, mostly with antioxidant and anti-inflammatory activities. Just a few examples of the diversity of bioactive compounds are the "*omega*-3" or *n*-3 fatty acids produced from the marine species *Isochrysis galbana*, and astaxanthin with antioxidant activity from *Haematococcus pluvialis* or even polyhydroxyalkanoates for biodegradable plastics from *Spirulina* sp., both freshwater species (Fu et al., 2017; Lauritano et al., 2016; Ventura et al., 2017).

1.1.1 Diatoms

Diatoms, the most biodiverse microalgal group, are enclosed by a bipartite frustule, a glassy and translucent structure formed by silica in the cell walls; therefore, they are also called jewels of the sea (Saxena et al., 2021). Their biodiversity is estimated to be as high as 200,000 species, but even higher numbers have been suggested (Diatoms.org, 2022; Spaulding et al., 2021). They are responsible for 20% of the world's CO_2 fixation, which is the amount achieved by all rainforests in the world combined. Diatoms can be grouped relatively easily according to their shape and symmetry, as centrics (radial and polar) and pennates (raphid and araphid). The comparison with a Petri dish is often used to describe the radial centrics in particular. The two valves (thecas) of the diatoms are almost identical, with one valve being slightly smaller (hypotheca) and being encompassed by the larger half (epitheca). Among other positions, the thecas are connected in the overlapping area with the so-called pleural band. The different forms and symmetries are illustrated in Figure 1.1. Of these diatoms, only the raphid pennates can move on surfaces. This happens via a sliding mechanism with the release of a mucilage substance by the raphe, creating an adhesive film in which the pennate diatom moves (Kröger & Poulsen, 2008).

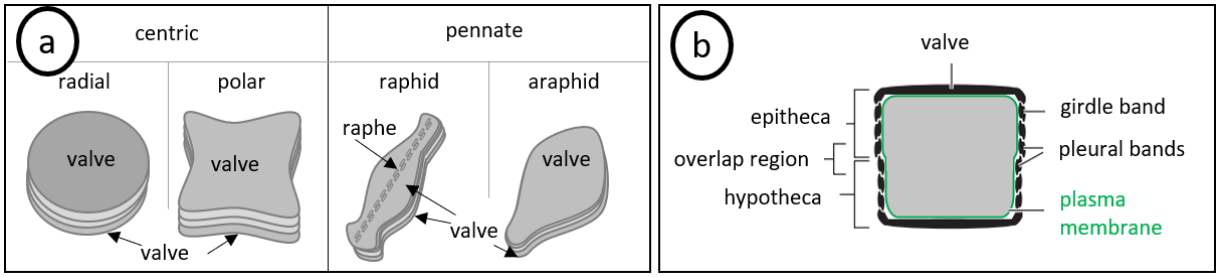

Figure 1.1: (a): Symmetry of diatoms; (b): general structure of diatoms (adapted from Kröger & Poulsen, 2008).

1.1.1.1 Reproduction of diatoms

Diatoms have two types of cell division: mitosis, a type of vegetative cell division, and meiosis, when sexual reproduction occurs. Vegetative cell division takes place through plasma membrane division of the parental cell with accompanying construction of the respective second valve (hypotheca) of the daughter cell. Both thecas of the parent cell become the respective epithecas of the daughter cells. When the parental thecas separate, two daughter cells are formed. The increase in cell volume up to the mature cell is accompanied by the gradual enlargement of the lateral sides of the respective hypotheca and the formation of girdle bands. Thus, as vegetative division progresses, the cell size of the population decreases, since the daughter cells that inherit the hypothecas become successively smaller than their siblings that inherit the epithecas. When a threshold cell size is reached and further reduction in size would lead to cell death, sexual reproduction occurs through a mechanism that has not yet been fully elucidated. Through meiosis, the original size is finally restored in the following generation (Figure 1.2). The vegetative division phase lasts longer (several months up to years) than the sexual reproduction phase (from few hours up to some weeks) (Kröger & Poulsen, 2008).

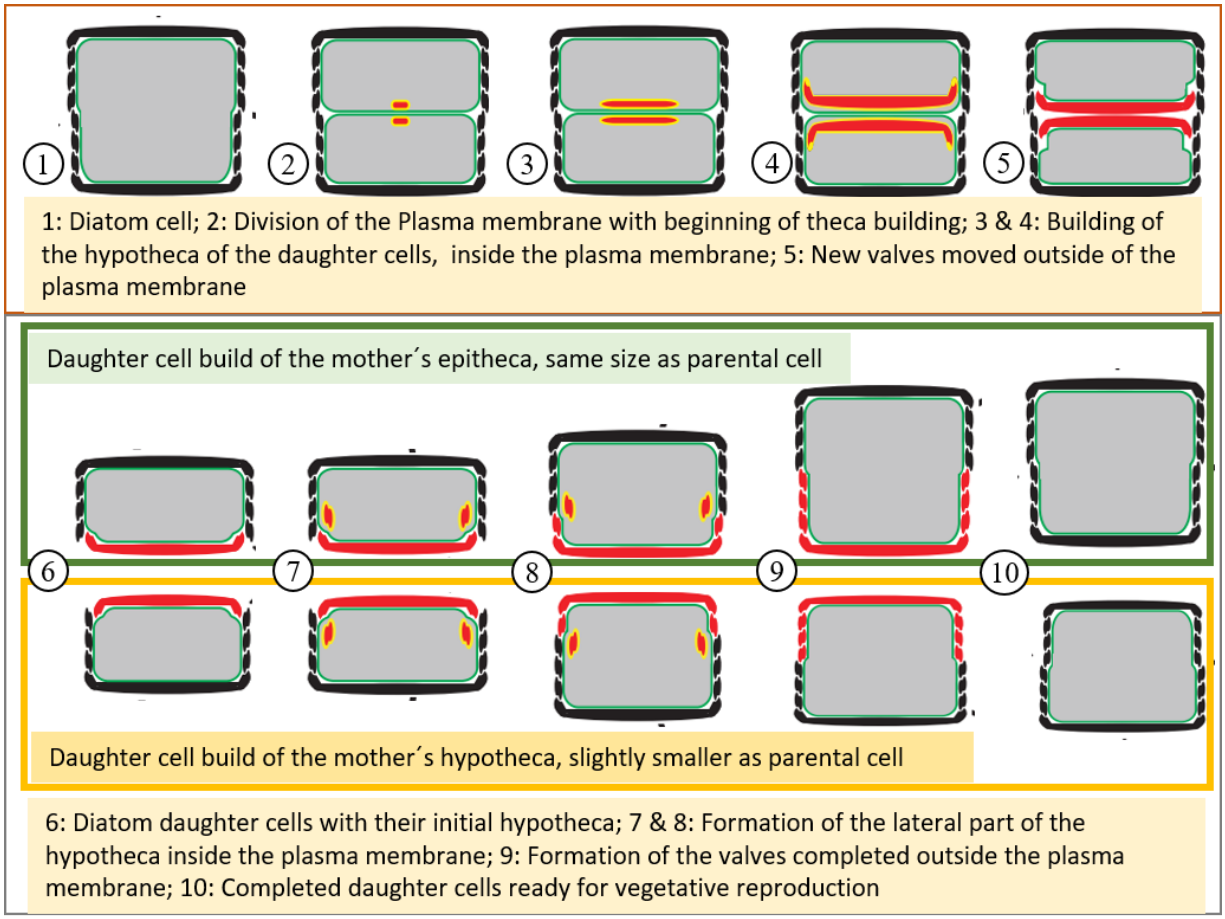

Figure 1.2: Diagram of vegetative cell division in diatoms by mitosis. Daughter cells are identical to the parental cell, except for the daughter cells inheriting the hypotheca of the parental cell. In this case, cells decrease slightly in size (adapted from Kröger & Poulsen, 2008).

1.1.1.2 Chemical composition of diatoms

Photosynthetic pigments of diatoms are green chlorophyll, in particular chlorophyll *a* and *c1*,*c2*, and *c3*. They do not contain chlorophyll *b*, although they are able to produce a golden brown carotenoid, fucoxanthin, one of the pigments that are known for their strong antioxidant and anti-inflammatory capacities (Seckbach & Gordon, 2019; Seth et al., 2021). Related to pigments, pheophorbide *a*, a chlorophyll *a* derivate, has also been reported to display anti-inflammatory activity (Subramoniam et al., 2012), which was later confirmed in a study with *Cylindrotheca closterium* (Lauritano et al., 2020). Another important compound class that is found abundantly in diatoms are lipids or components thereof. One of the most abundant lipids are triacylglycerols (TAG), which can be biosynthesized from fatty acids (FA). These FA can be saturated (SFA) or unsaturated fatty acids. The latter can be mono-unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), if they contain, respectively, one or more

double bonds. In particular long chain polyunsaturated fatty acids (LC-PUFAs) as, for example, the *n*-3 docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, are essential for cardiovascular health, brain development and several other metabolic processes, showing antioxidant and anti-inflammatory activities (Li et al., 2014). Glycolipids (GL), i.e., glycosylated lipids include the glyceroglycolipids (GL), which contain a glycerol backbone. GL constitute ~50 and ~30% of photosynthetic membranes lipids in microalgae and are predominant as neutral and uncharged galactosylglycerides. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) have been demonstrated to display anti inflammatory activities, with their activity being dependent on their FA (Costa et al., 2016). Polysaccharides, which can make up between 10 and 80% of the the microalgal biomass, seem to have anti-inflammatory activity, as demonstrated by enzymatic inhibition assays and their ability to decrease the levels of pro-inflammatory cytokines (Tabarzad et al., 2020). Other bioactive compounds from diatoms include phenolic compounds, flavonoids, tocopherols, alkaloids and carotenoids (including fucoxanthin and astaxanthin), mostly with antioxidant capacity (Bule et al., 2018). Their unique silicified frustules have promoted research on their use in nanotechnology and biomedicine as carriers for drug delivery (Mishra et al., 2017).

The marine diatoms included in this project are all eukaryotes belonging to the Phylum *Ochrophyta*, class *Bacillariophyceae*, order *Bacillariales* and the family *Bacillariaceae*. Among the selected species, two belong to the genus *Cylindrotheca*. All three species were isolated from the Algarve Coast by the Marbiotech group of Center of Marine Science (CCMAR), University of Algarve during the AlgaRed+ project. This project was funded by the European Union as a "Cross-border NETWORK for the development of innovative products with microalgae", which had the objective to characterise microalgal species inhabiting the coast of Cádiz, Huelva and the Algarve (Universidad de Cordoba, 2021).

1.1.1.3 *Cylindrotheca closterium*

Cylindrotheca closterium (Ehrenberg) Reimann & J.C.Lewin, 1964 (Diatombase, 2008a) is a needle-shaped diatom with slightly ball-shaped ends. The central part of the cell body is enlarged like a belly, having a length size of 25-260 μm (Ryabushko et al., 2019) (Figure 1.3). *C. closterium* shows high motility (Kingston, 2009). This alga is already known for its high

fucoxanthin content (Wang et al., 2018), being able to biodegrade phthalate acid esters (Li et al., 2015), thus demonstrating its potential ecological impact.

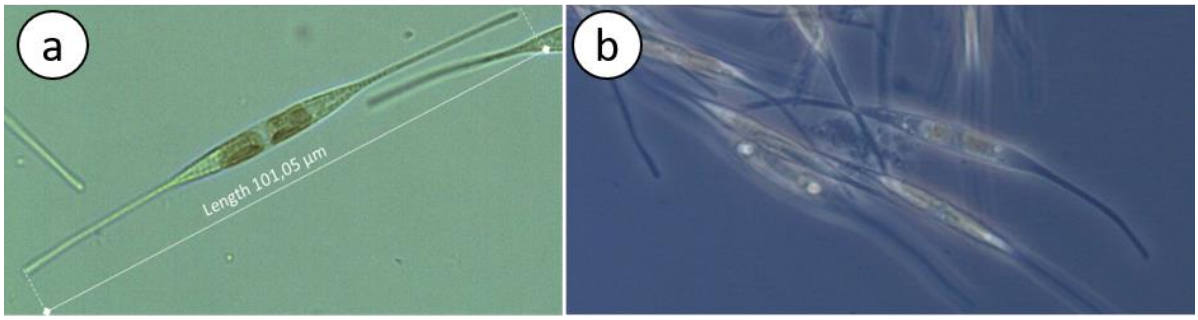

Figure 1.3: Light microscopy observation (400 x magnification) of *Cylindrotheca closterium* (a). Fluorescence microscopy observation (400 x magnification) of *Cylindrotheca closterium* (b) (Photos by Britta Kautzmann).

1.1.1.4 *Cylindrotheca fusiformis*

Cylindrotheca fusiformis Reimann & J.C.Lewin, 1964 has a filigree elongated S-spindle shape, which can be compressed or stretched depending on the stage of development (Figure 1.4). Its length ranges from 25-50 µm (Diatombase, 2008b). The silica-precipitating peptides of *Cylindrotheca fusiformis* were investigated in order to create artificially silica nanospheres (Kröger et al., 2001).

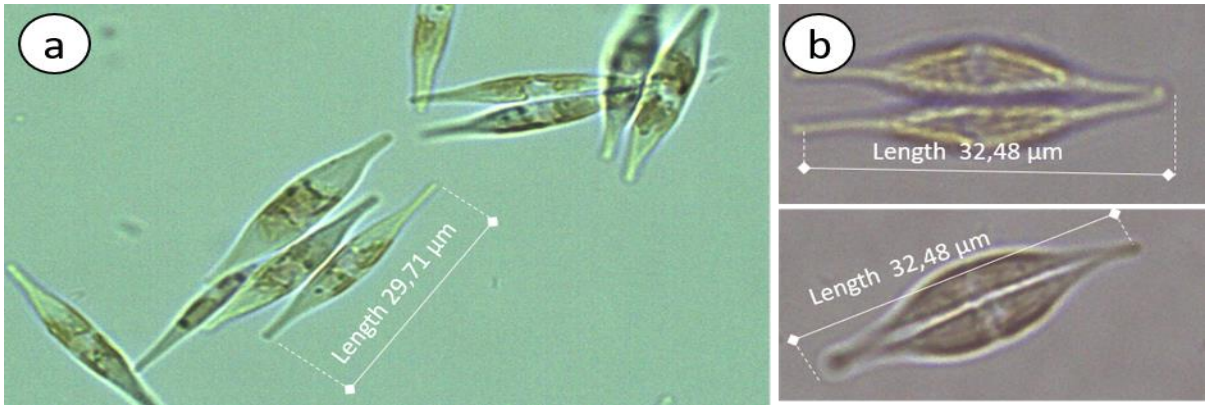

Figure 1.4: Light microscopy observation (400 x magnification) of *Cylindrotheca fusiformis* (a). *Cylindrotheca fusiformis* undergoing vegetative division (b) (Photos by Britta Kautzmann).

1.1.1.5 *Nanofrustulum shiloi*

Nanofrustulum shiloi (J.J.Lee, Reimer & McEnery; Round, Hallsteinsen & Paasche, 1999; Diatombase, 2008b) has a cylindric frustule with a diameter of 2.0-2.5 µm with rectangular-like cells that form chains interconnected by small spines (Figure 1.5) (Ruocco et al., 2018). In termos of their composition, most studies have reported on their fucoxanthin and lipid contents of this microalgae (Rampen & Sinninghe-Damsté, 2009; Roychoudhury et al., 2021).

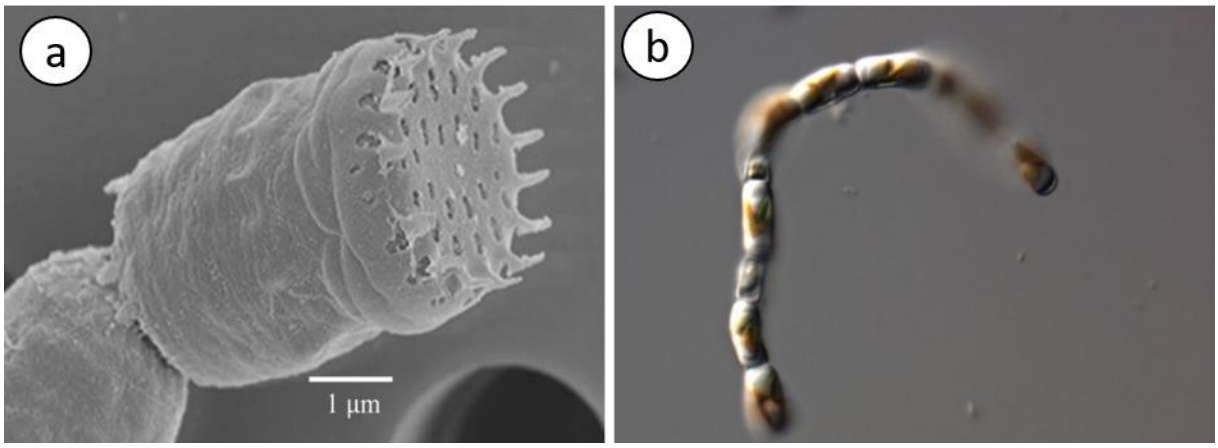

Figure 1.5: Scanning electron micrograph of *Nanofrustulum shiloi*, scale bar 1µm (photo addapted from Ruocco et al., 2018) (a); *Nanofrustulum shiloi* in chain (Photo by G. Bombo) (b)

1.1.2 Microalgae culture

It is relatively easy to cultivate marine diatoms due to their rapid growth rate and simple requirements such as adequate nutrient and light supply. Cultivation systems can be divided into 2 groups, closed and open systems. Open systems, such as open ponds and raceways, are more likely to be found in large scale production facilities. They are usually aerated or have a mechanic system (e.g., a paddlewheel) that physically moves the growth medium (Figure 1.6). These types of systems are mainly used for mass production of microalgae for biofuel and biofertilizer, as the rules for producing these products are not as strict and less cost intense volume of production (Chew et al., 2018).

Figure 1.6: Microalgal growth facilities at Necton S.A., Olhão, where small (a) and large (b) raceways are shown, both of them with the remnants of previous cultures (Photo Britta Kautzmann), (c): Centre-Pivot ponds for the culture of *Chlorella*, Japan (Photo adapted from FAO, Algae based Biofuels, 2010).

For high added-value products, as compounds for the pharmaceutical industry, closed systems are used to avoid contamination and to be able to better control the cultivation conditions. For this purpose, closed, translucent, transparent photobioreactors equipped with aeration systems and exposed to a light source are used, in which the microalgae are cultivated in the nutrient medium. These photobioreactors (PBRs) can have various designs, from columns (e.g., bubble columns or airlifts) to flat panel and tubular PBRs of different sizes (Figure 1.7) (Ahmad et al., 2021; FAO, 2010).

Figure 1.7: Closed systems for the production of microalgae at the facilities of Necton S.A., Olhão. Tubular PBRs (a,b) and vertical panels (c) known as "flat pannels" or "greenwalls" (Photos by Britta Kautzmann).

To cultivate diatoms under laboratory conditions, the f/2 growth medium, an enriched seawater medium, what in addition to nitrates and phosphates, also contains a mix of trace elements and vitamins, plus the silica required for the formation of the frustule in the form of sodium metasilicate, is often used (Guillard, 1975; Guillard & Ryther, 1962). Environmental conditions such as temperature (16-27°C with optimal values ranging from 18 to 24°C), salinity (12-40 g L^{-1} with optimal values ranging from 20-24 g L^{-1}) and pH (7-9 with optimal values ranging from 8.2 to 8.7) should be adapted to the selected species (Kim & Chojnacka, 2015). Species-related fine-tuning is often needed to maximize different target compounds production. As photosynthetic organisms, an appropriate supply of light is essential, and thus they can be cultivated using photoperiods composed of light and/or darkness periods with durations of

16h/8h or 24h/0h. Light intensity, depending on the type of diatom, can be between 40 and 200 µmol photons m^{-2} s^{-1} (Mortain-Bertrand et al., 1989; Prins et al., 2020). Diatoms are able to absorb light in the red, green, and blue zones of the electromagnetic spectrum due to their photosynthetically active pigments, such as chlorophylls and carotenoids, especially fucoxanthin (Diatoms.de, 2022). In order to be able to guarantee an even supply of nutrients and light as well as a good gas exchange, cultivation systems with aeration, in general compressed air, but also with the addition of CO_2, have proven to be effective. However, gas exchange can also be attained by shaking manually or using orbital shakers, in particular if small flasks are used at the beginning of the scale-up process (Figure 1.8) (Ahmed et al., 2021a).

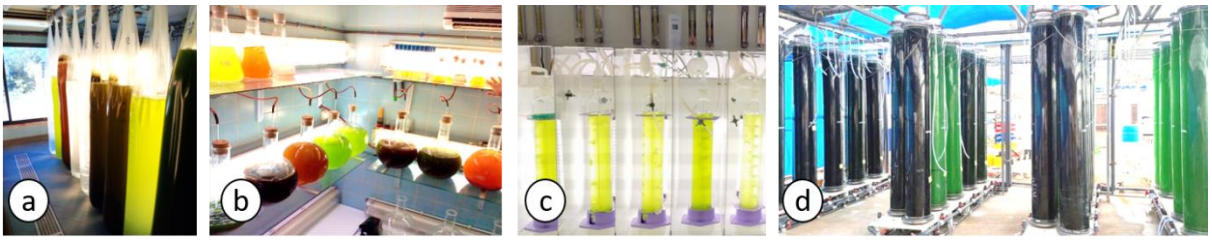

Figure 1.8: (a): Microalgae cultures in Bag-Batch cultures with aeration; (b): inoculum cultures, aereated, IPMA facilities, Olhão; (c): small scale 1-L bubble columns, MarBiotech Laboratory, University of Algarve, Faro (Photos by Britta Kautzmann), (d): Bubble columns at the Arizona Algae Products installations (Photo adapted from Arizona Algae Products LLC, 2022)

Normally the first step for a new culture is the selection and identification of the target species by their morphology using light microscopy and, if necessary, via 18S (small subunit; SSU) rDNA sequencing (Khaw et al., 2020); although other sequences might be needed to identify diatoms down to the species level (e.g., ITS1 and ITS2 sequences) (Beatrice-Lindner et al., 2018; Hadi & Al., 2016). The diatom cultures start on Petri dishes with solid (based on f/2 or similar) medium on agar base and with extra silica added. After that, the cultures are switched to liquid media, in general the same nutrient combination as before, such as f/2 with added silica, using sterilized seawater. The volume is increased regularly to scale up the cultures (Ahmed et al., 2021b). Monitoring of culture growth can be performed by cell counting in special counting chambers under the microscope, by measuring the optical density at 750 nm by spectrophotometry and/or gravimetric determination of dry weight (Lu et al., 2017). In microalgal cultures whose cells tend to form aggregates, there can be difficulties with the homogeneous distribution of the individual cells and thus non-representative results during monitoring can occur. The Sedgewick Rafter counting chamber with a sample volume of 1 ml

is particularly indicated for phytoplankton cell counting, diatoms in particular (Figure 1.9) (CSIRO, 2010).

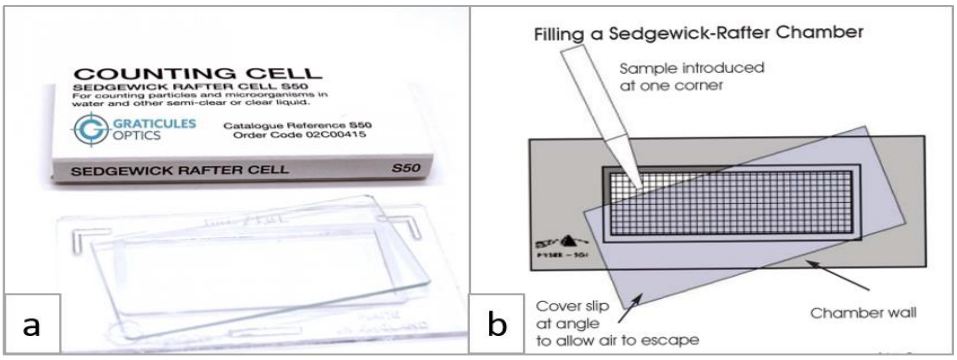

Figure 1.9: Sedgewick Rafter counting cell for 1 ml of sample, cell chamber size: 50 mm x 20 mm x 1 mm (a); filling of the chamber (b) (Images adapted from Graticules Optics, 2022).

The monitoring of growth of monoalgal cultures in well controlled environment is essential, as the content of bioactive compounds in microalgae depends, among other things, on their growth phase (lag, exponential, stationary and death phases), which is important when determining the time of harvest (Ahmed et al., 2021b; Kingston, 2009).

1.1.3 Harvesting

Microalgal biomass harvesting is the separation of the cells from the media and should be done best before the onset of the death phase. The method chosen depends on the volume, technical equipment and target compounds. Filtration, flotation, sedimentation, flocculation with chemical or natural agents and centrifugation are methods that can be used.

On a laboratorial scale and for high value products as pharmaceuticals, centrifugation is the method most used (Barros et al., 2015; Zhu, Li and Hiltunen, 2018; Rakesh, Tharunkumar and Sri, 2020) . Lyophilisation, or freeze drying, as a pre-treatment for subsequent extraction of bioactive compounds, increases the active ingredient yield of the extraction (Unterlander et al., 2017).

1.1.4 Extraction methods

There are different extraction methods to obtain the bioactive substances from microalgae such as solvent-based conventional and accelerated extraction methods, using the chemical concept that "similar dissolves similar". With conventional methods, such as solid-liquid extraction, Soxhlet, maceration, or liquid-liquid processes, larger amounts of organic solvents are used, which are neither environmentally friendly nor healthy, and the processes are also time-consuming and labour-intensive. Therefore, alternative methods were sought to save solvent, time and labour.

Accelerated methods use energy, pressure or electrical voltage, and combinations thereof. Pressurized liquid extractions (PLE) include also accelerated solvent extraction (ASE). In Microwave Assisted Extraction (MAE), the sample solvent mixture is quickly heated through the action of microwaves, thus accelerating the extraction. With Ultrasound Assisted Extraction (UAE), the cell structure is broken due to a cavitation effect and the individual compounds can be quickly released into the solvent. However, both methods can only be used to a limited extent with heat-sensitive target substances. Supercritical Fluid Extraction (SFE) is a newer green alternative, replacing organic solvents with supercritical fluids, mostly supercritical CO_2, sometimes with the addition of a co-solvent such as ethanol to extract a wider range of target substances. An example of a method using electro-technologies is Pulsed Electric Field extraction (PEF), which avoids the heating of the sample but can be combined with solvent extraction. Moderate Electric Field extraction (MEF) is another example of the previous technology (Kim and Chojnacka, 2015; Ahmed et al., 2021b; Kannaujiya et al., 2020). Automatization and combination of methods have led to a new extraction system, such as automated solvent extraction implemented as Energized Dispersive Guided Extractor (EDGE), which is based on pressurized liquid extraction. Up to 12 samples can be extracted in this apparatus in a short time with small amounts of solvents (Dharmarajan, 2019).

Solvents for the extractions should be safe and their negative impact on environment and health should be reduced to a minimum. Additionaly, they should be from fully renewable resources. In the food and pharmaceutical industry, where the safety of the ingredients is extremely important, they should be free of solvent used during the extraction. In any case, only food-grade solvents should be used.

Water is the most eco-friendly solvent; however, it is only suitable for polar compounds. Agro-solvents or bio-solvents as ethanol, obtained from fermentation of cereals, for example, or terpenes, such as D-limonene (with high solvent power particularly for fats and oils) extracted from citrus fruits or α-pinene extracted from pine, ethyl acetate and 2-methyloxolane are biodegradable, renewable and safe to use as solvents (Claux et al. 2021; Chemat, Vian & Cravotto, 2012). Natural deep eutectic solvents (NADES), liquid solvents as a result of mixtures of natural solids, are a new non-toxic alternative to petrochemical solvents. Furthermore, in food production in the EU, acetone, butane and propane are allowed (Choi & Verpoorte, 2019). Upon decreasing solvent polarity, the range of alternatives decreases significantly. The often-used hexane is very difficult to be replaced. If a replacement of the solvent is not possible, the extraction method might be changed, or at least the used volume should be kept to a minimum and in the finale product even small traces of the solvent must not be found. Good examples of green extraction methods are supercritical fluid extraction (SFE), pressurized hot water extraction (PHWE) and pulse electric field extraction (PEF). Of course, during the work with solvents, all safety regulations and recommendations should be followed (Dharmarajan, 2019; Inglis et al., 2011; Parniakov et al., 2015).

1.1.5 Bioactive compounds from microalgae

Microalgal products with high added value can be found especially in the pharmaceutical field, even in biopharmaceutical production, where in a biotechnological process, microalgae modified by genetic engineering are used as a host for the production of biopharmaceuticals such as hormones, enzymes, vaccines and others. In particular, the green microalga *Chlamydomonas reinhardtii* has been the focus of research for a successful production pipeline of biopharmaceuticals (Rosales-Mendoza et al., 2020).

Economic aspects can also play a role in the research of microalgae. Fucoxanthin serves as a good example. This carotenoid pigment is currently priced with € 1200.00 g^{-1}, around 200 times the price of gold (*Gold Price*, 2022; Merk, 2022). Substances for the treatment of common diseases could be particularly interesting. There is a great need for this worldwide, since these diseases can, in the worst case, lead to death if left untreated, and some of the conventional drugs have undesirable side effects.

1.2 Chronic inflammatory diseases

Chronic inflammatory diseases (CIDs) are a leading cause of death worldwide (WHO, 2021; Porcelli, 2018). Thus, it has become quite important to find new treatment options with less side effects. Acute inflammation is a natural and important reaction of the body´s immune defence system elicited by pathogens, trauma, aging and other agents. The damaged cells release various biochemical signals in order to set the inflammatory cascade in motion and achieve repairing of the damaged tissue (Bosma-Den Boer et al., 2012; Schmid-Schönbein, 2006). If the regular inflammatory process is disturbed, by missing the feedback signal to end the inflammatory cascade, for example, it can cause abnormal inflammatory activity. This can lead to chronic inflammatory diseases, including various autoimmune diseases, whereby the body's own defence is directed against its own tissues, such as rheumatoid arthritis, cardiovascular and intestinal diseases, systemic lupus erythematosus, diabetes and also cancer. Inflammation is a complex mechanism of up- and down-regulation of a variety of different pro- and anti-inflammatory factors. There is no simple off switch for the inflammatory process, or if there is one, it has not yet been found. Pro-inflammatory factors include proinflammatory cytokines such as interleukin 1 (IL-1β), 6 (IL-6) and 8 (IL-8), tumor necrosis factor alpha (TNA-α) and prostaglandin E_2 (PGE_2). Other factors involved in the inflammatory process include enzymes such as cyclooxygenases 1 and 2 (COX1 and COX2) (Ahmadi et al., 2022) and also the angiotensin converting enzyme (ACE) (Cantero-Navarro et al., 2021). Their role in this process and the importance of developing inhibitors able to block their activity is discussed below.

1.2.1 Anti-flammatory activity

1.2.1.1 COX inhibitors

Cyclooxygenases catalyse the conversion of arachidonic acid (AA), e.g., released from damaged cell membranes, into prostaglandins and thromboxanes (TXA) via oxidation (Figure 1.10) (Jara-Gutiérrez & Baladrón, 2021). Prostaglandins perform a large number of functions in the body. AA metabolism might vary in different cells, tissues or organs due to, for example, the occurrence of diverse cyclooxygenases. In platelets, AA is transformed by COX-1 into thromboxane A with a proaggregatory and vasoconstrictive effect. In the gastric mucosa,

COX-1 forms prostaglandins PGE2 and PGI2 which have a gastric protection effect and, in endothelial cells, AA becomes prostacyclin through the action of COX-1 and COX-2 with a vasodilating effect and reduction of platelet aggregation. However, if COX-2 is stimulated by inflammatory messengers, the production of PGE2 increases, which then has a pro-inflammatory effect (Jara-Gutiérrez & Baladrón, 2021; Kam & See, 2000). With the aim of reducing chronic inflammation, non-steroidal anti-inflammatory drugs (NSAIDs) are used as COX-inhibitors, which are mostly non-selective COX-inhibitors (COXi), such as acetylsalicylic acid (Aspirin), Ibuprofen and Naxproxen (Pasero & McCaffery, 2001). For a long time, COX-1 was seen as a type of housekeeping enzyme that helped maintain homeostatic functions, but recent research has shown its involvement in neuroinflammatory diseases in which selective COX-1 inhibitors may have an important therapeutic effect (Calvello et al., 2017). Non-selective COXi can lead to ulcers and gastric bleedings, and selective COX-2 inhibitors might increase the probability of stroke and heart attack (Pasero & McCaffery, 2001).

For the screening of COX inhibitors, assays are usually used which measure the ability of a substrate/extract to inhibit the COX enzymatic activity. The measured change is displayed via colourimetry and measured via absorption in a spectrophotometer (Sharma et al., 2008). An enzyme immunosorbent assay (ELISA) is another possible screening assay to find COX inhibitors. A competitive sandwich ELISA test determines the amount of the product, prostaglandins, converted by the relevant COX enzyme (COX-1 or COX-2). The colour intensity is inversely proportional to the quantity of prostaglandins formed, or proportional to the inhibitory capacity of the substance tested. This type of test is done in 2 phases: first, the reaction of the COX on the substrate, arachidonic acid, and the test samples; in the second phase, an ELISA is used to determine the inhibitory capacity. In order to check whether the COX-2 inhibitor is selective, a COX-1 inhibition test is also carried out. If there is no COX-1 activity, it is a selective COX-2 inhibitor (Cuendet et al., 2006).

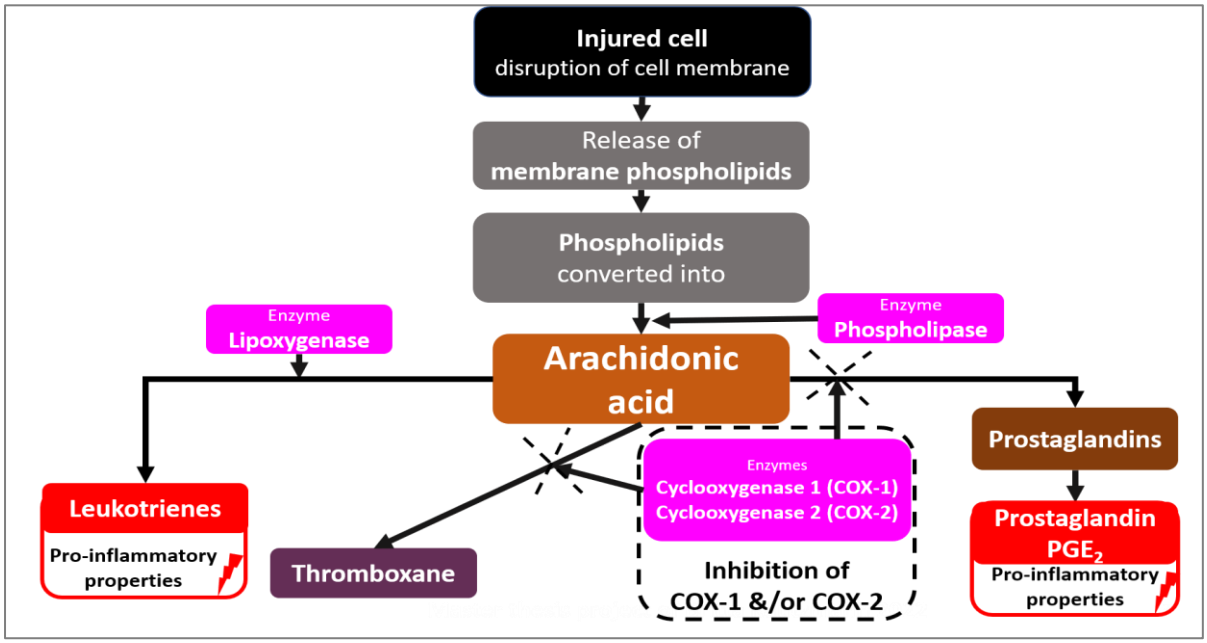

Figure 1.10: Simplified diagram of anti-inflammatory action mode of COX inhibitors (adapted from Jara-Gutiérrez & Baladrón, 2021).

1.2.1.2 ACE inhibitors

The renin-angiotensin system (RAS) is a regulatory circuit with various hormones and enzymes that essentially control the body's volume balance and is also one of the most important blood pressure-regulating systems in the body. RAS angiotensin II is not just the main responsible to keep homeostasis in the cardiovascular system, it also stimulates the release of NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidase, which is strongly involved in the formation of reactive oxygen species (ROS) in several tissues (Wen, 2012). The potent vasoconstrictor hormone angiotensin II originates from angiotensinogen from the liver, which then becomes angiotensin I with the help of renin present in the kidney. The angiotensin converting enzyme (ACE) acts as a peptidase and converts angiotensin I into angiotensin II (Figure 1.11). ACE inhibitors (ACEi) have, in addition to their well-known and well-used effect of vasodilation in antihypertension drugs, strong anti-inflammatory impact on arthritis, pancreatitis, and vascular inflammation, being expressed as reduced release of pro-inflammatory cytokines and reduced cell activation of inflammation-relevant cells, particularly macrophages (Kortekaas et al., 2014; Ranjbar et al., 2019). Vascular inflammation can lead to heart attack and stroke, among other diseases (Emsley & Tyrrell, 2002).

Among the many bioactive substances in microalgae, peptides have been shown in various studies to be ACE inhibitors (Jiang et al., 2021b; Samarakoon & Jeon, 2012). ACE inhibitors can be screened using specific assays that measure the ability of a substance/extract to inhibit the activity of an enzyme in relation to a substrate (Kreutter, Schwaller & Reymond, 2021).

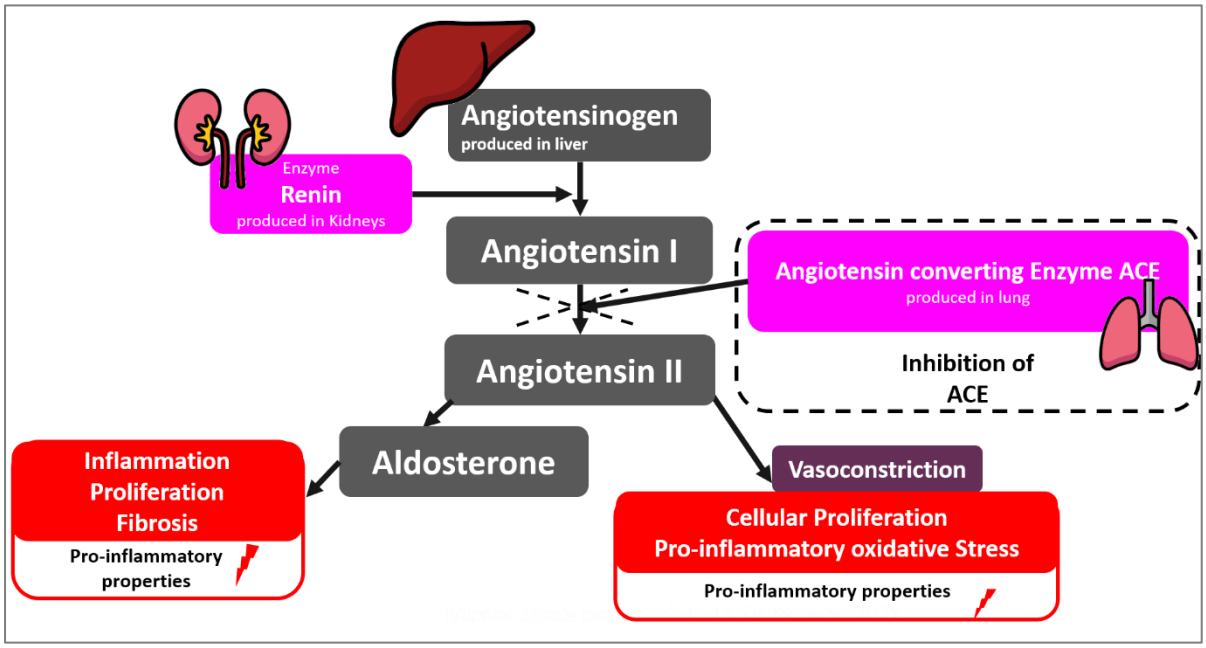

Figure 1.11: Simplified diagram of anti-inflammatory action mode of ACE inhibitors (adapted from Kortekaas et al., 2014 and Ranjbar et al., 2019).

1.2.2 Antioxidant activity

For a pre-screening for anti-inflammatory activities, bioprospecting for antioxidant active molecules is indicated, as several studies have shown that antioxidant compounds display also anti-inflammatory properties (Arulselvan et al., 2016). A balance between endogenous antioxidants and reactive oxygen species (ROS) is important to prevent damage from the latter to cells and tissues. Antioxidants can be divided in two different classes: enzymatic and non-enzymatic (Figure 1.12). Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx), break down the free radicals by multi-step processes into less toxic or harmless products. SOD needs the presence of metal ion cofactors such as copper (Cu), zinc (Zn), or manganese (Mn) for this process (Nimse & Pal, 2015). Non-enzymatic antioxidants include flavonoids, phenolic acids, vitamins and derivates, carotenoids, enzyme cofactors and others (Carocho & Ferreira, 2013). Polysaccharides from microalgae have been shown to possess antioxidant activity. This effect does not seem to be due to a single

mechanism of action, but to the interaction of various factors, such as the type of monosaccharides, molecular weight, the configuration of the structure and other structural properties (Sun et al., 2014).

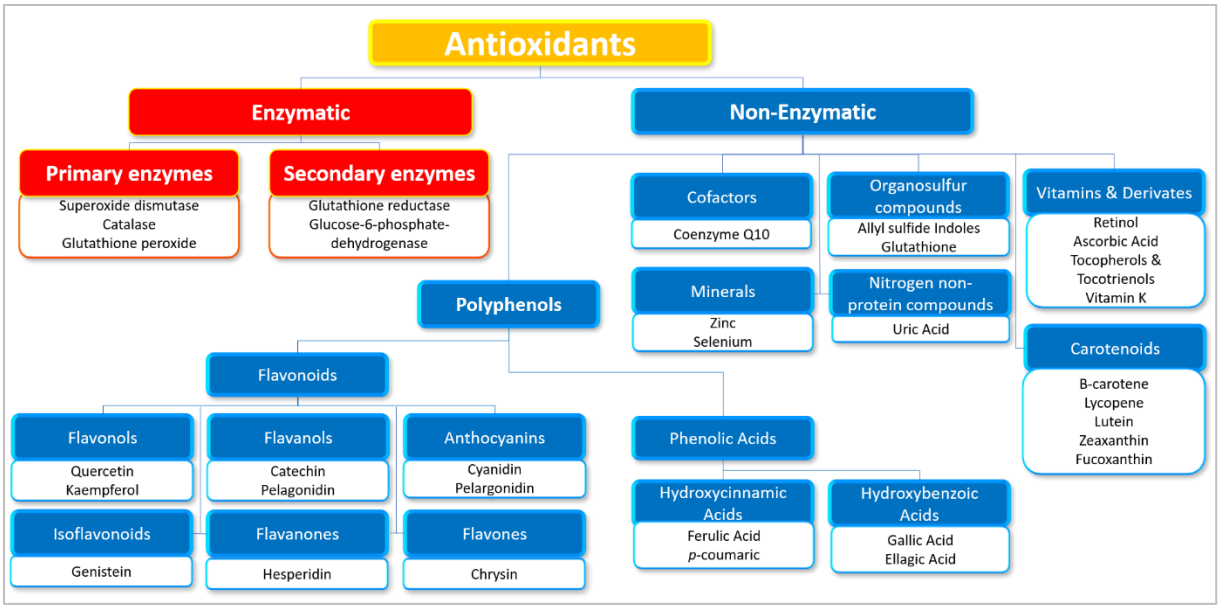

Figure 1.12: Simplified classification of antioxidants. Examples belonging to each class and subclass of compounds displaying this bioactivity are also given (adapted from Carocho and Ferreira, 2013).

1.3 Untargeted metabolomics of bioactive extracts

Microalgal extracts contain an immense number of different bioactive compounds, many of which have already been discovered and analysed accordingly, but microalgae still offer a large source of unknown substances, and analysing them is the goal of many studies. When it comes to identifying substances with certain properties, various screening assays are carried out in order to analyse the most promising extracts in more detail at a later stage, thus obtaining a pre-selection. Compounds are best separated using High Performance Liquid Chromatography (HPLC), a process in which the sample in a solvent, known as the mobile phase, is passed through a column containing a solid filler, the stationary phase. The separation of the individual compounds takes place due to the different interactions between the mobile and stationary phases. With preparative liquid chromatography, the sample can be divided into different fractions and thus be supplied to undergo further treatment and analysis.

In Gas Chromatography (GC), the mobile phase is a gas, and the column is heated to relatively high temperatures to elute compounds. Advantages of GC are the ability to detect volatile and semivolatile metabolites, consistency of retention times and a free available universal database

of mass spectra that can be used to identify compounds in the sample, if a Mass Spectrometer (MS) is available as detector. But GC has also some limitations with regard to the substances that can be analyzed, which must be volatile and thermostable and have a molecular weight of less than 1250 Da. Derivatization can help make samples more volatile, but the formation of byproducts and structural alterations due to the derivatization process should be considered. A typical method employs MSTFA (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide) as a derivatization reagent where trimethylsilyl (TMS) esters are formed from the carboxylic group, but also from amino and hydroxyl groups, among others (Bowden et al., 2009). After injection into GC, the sample is vaporized and carried through the stationary phase with the mobile phase. The separation also takes place here due to interaction with the stationary phase, which should be selected in relation to the volatility and the functional groups of the sample material.

A mass spectrometer (MS) consists of an ion source, a mass analyzer and a detector. The analyte is ionized in the ion source. Electron impact (EI) is mostly used in combination with gas chromatography, as well as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in combination with HPLC. In the analyzer or mass selector, the ions are separated according to their mass (more precisely: mass/charge ratio, i.e. m/q). There are several methods for this, which differ in the resolution. As a detector a photomultiplier, a secondary electron multiplier (SEV) or a Faraday collector is usually used (Awad et al., 2015). A MS with combined preceding GC, is well indicated to analyse volatile substances. This combination is especially useful for the detection of fatty acids methyl esters (derivatized fatty acids), TMS-sugar (mono-/disaccharides), lactate, glycerol, pentacetyl glucose, vitamins and micronutrients. A spectral library as database is usually consulted in order to be able to identify the detected substances with the greatest possible accuracy, in particular the free software of the Automated Mass Spectral Deconvolution and Identification System (AMDIS) from the National Institute of Standards and Technology (NIST).

MS in combination with the upstream HPLC, which ensures the separation of the compounds to be analysed, brings the possibility of drawing conclusions about the structure of individual substances that are not volatile or that are thermolabile and therefore cannot be analysed by GC-MS (Ren et al., 2018). Large online libraries of compounds' structures are also available for this purpose. Especially with more complex formulas there are often different combinations possible and thus also many possible substances that can result from the combination of the corresponding molecules/atoms (Pérez et al., 2021). Comparing to GC-MS, the sample

preparation is more simple, derivation is not needed and more types of metabolites are detectable. But also ion suppression occurs and without the forming of ion adducts, metabolites could not be detected (Ren et al., 2018).

2 Objectives of the work

The main objective of this work is the valorization of the biomass of three marine diatoms, *Cylindrotheca closterium, Cylindrotheca fusiformis* and *Nanofrustulum shiloi*, through the identification of compounds with anti-inflammatory potential for use in the pharmaceutical industry.

To achieve this main objective, specific goals were set, namely:

- Scale-up and cultivation of *Cylindrotheca closterium* and *Cylindrotheca fusiformis*, under laboratorial conditions
- Screening for bioactive compounds with anti-inflammatory properties based on ACE-, and COX - inhibition assays of the 3 diatoms
- Tentative identification of the bioactive compounds

3 Material and Methods

3.1 Upstream

3.1.1 Microalgal strains

The microalgae strains of *Cylindrotheca closterium* and *Cylindrotheca fusiformis* were provided as monocultures from the culture collection of the MarBiotech laboratory, CCMAR, University of the Algarve. Biomass of *Nanofrustulum shiloi* was provided in the form of deep-frozen paste by Necton S.A., Olhão. This biomass was produced in outdoor tubular photobioreactors at Necton during the summer. Nutribloom® Plus (NB+) and silica were used as nutritive solution, with a final concentration of 2 mM and 0.11 mM, respectively.

3.1.2 Culture of Microalgae

Culture scale-up was attained by transferring an inoculum (10%–30%) to a larger flask by adding additional culture media (Figure 3.1). Cultivation under laboratorial conditions was done until a total biomass of 10 g per species was obtained.

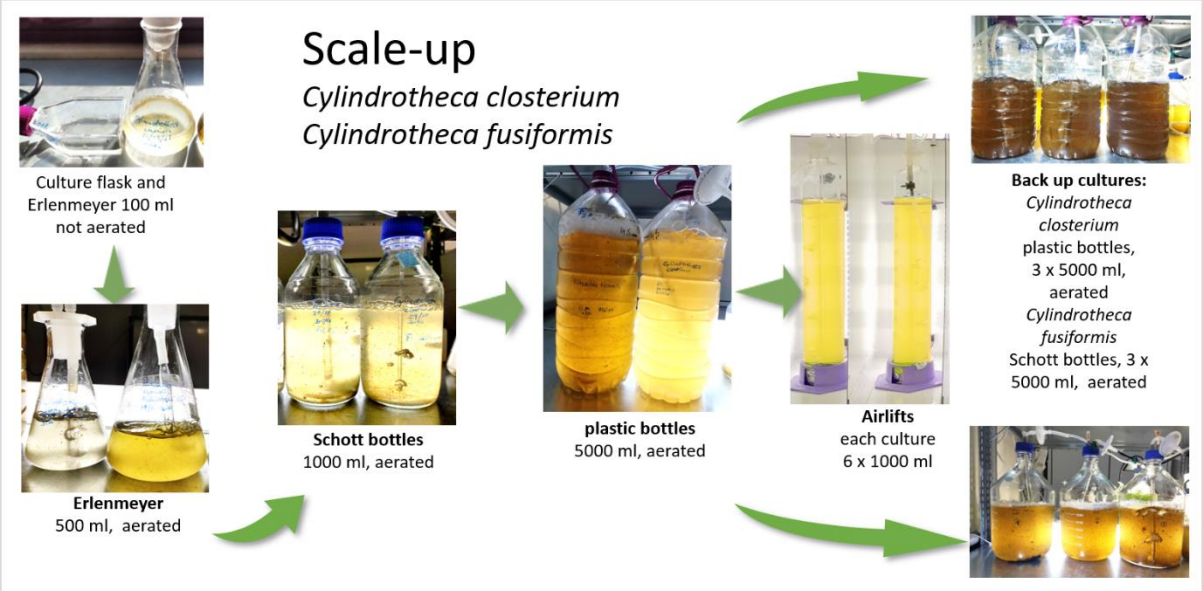

Figure 3.1: Scale-up of microalgae cultivation in the laboratory

The culture of *C. closterium* was performed in liquid f/2 medium containing silica (0.003 mg l^{-1}) (Guillard, 1975; Guillard & Ryther, 1962), in a 100-mL Erlenmeyer. Culture of *C. fusiformis* was performed on a solid agar medium supplemented with modified Algal nutritive solution with silica. The general culture conditions were (if not described differently elsewhere): 24h illumination by LED lamps (HITAKA, 30 W, 5000K; LUMECO, 22W, 4000K) at ≈80 µmol photons m^{-2} s^{-1}. Temperature was set at 20-24°C. The culture medium f/2 with added silica was prepared with autoclaved seawater with a salinity of 30-31. All material was sterilized chemically or by autoclaving. The first scale-up of *C. fusiformis* was carried out successively in four plastic tissue culture flasks (area: 25 cm^2), and two 100-ml Erlenmeyer flasks (culture volume in flask: 50 ml), resulting in a 5-fold increase in volume. Scale-up of *C. closterium* was carried out in 2 x 100-ml Erlenmeyer flasks, resulting in a 4-fold increase in volume under general culture conditions; cells were maintained in suspension through orbital shaking. Upon 5 days of culture, the next scale-up step consisted in the transfer of both cultures to 500-ml Erlenmeyer flasks, resulting in a 5-fold volume increase under general culture conditions. Cells' suspension was achieved by aeration with pressurized air via air stone diffusors at the bottom of the flasks. Monitoring of culture growth was implemented by cell counting in a Sedgewick Rafter counting chamber (CSIRO, 2010; Mcalice & Darling, 1971)) under a 400x magnification every two days. After 10 days, culture scale-up was done by transferring the cultures to 1000-ml Schott bottles, resulting in a 5-fold volume increase. In an attempt to increase biomass productivity of *C. closterium* the amount of trace metals of the standard f/2 solution was increased 5 fold, adapted to a medium suitable for intensive growth of concentrated

C.closterium cultures (Ryabushko et al., 2016). Agitation by aeration with pressurized air via pipettes inserted close to the flask bottom was used. Monitoring of culture growth was carried out as described before.

Scale-up to 5000 ml was carried out by a 5-fold increase in culture volume. Growth media and conditions were kept as described before (adapted *C. closterium* medium). The following step of the scale-up process of both cultures was done after 10 days. For *C. closterium*, 3 x 5000-ml plastic bottles were used, whereas for *C. fusiformis*, 3 x 5000-ml Schott bottles were preferred, resulting in a 5-fold culture volume increase. Selection was made due to the limited availability of the glass bottles, the decision which diatom was cultivated in the glass bottles was arbitrary. Cell counting and optical density were used to monitor culture growth.

Final scale-up of both cultures after 8 days was attained by inoculating 6 x 1000-ml airlifts for each diatom, using a 3-fold culture volume increase in each airlift. General growth conditions were used as described before (adapted *C. closterium* medium), with the exception of a change in the light intensity, which was increased to ≈120 µmol photons m^{-2} s^{-1}, whereas aeration was achieved by bubbling pressurized air. Backup cultures were kept in a semi-continuous culture mode in 3 x 5000-ml plastic bottles for *C. closterium* and 3 x 5000-ml Schott bottles for *C. fusiformis*.

3.1.3 Harvesting

The content of each airlift was transferred to 1000-ml centrifugation flasks and biomass was harvested at 5000 × *g* during 5 minutes at 20°C in an Avanti JXN-26 centrifuge from Beckman Coulter, USA. The supernatant was decanted and the pellets were transferred to 50-ml Falcon tubes. Similarly to the first centrifugation, samples were again centrifuged at 5000 × *g* for 5 minutes (Megafuge 16R centrifuge ThermoFischer, USA). The supernatant was decanted again and the culture pellets were frozen at -20°C after which they were lyophilized. The deep-frozen biomass of *Nanofrustulum shiloi* was lyophilized at -80°C, during 24 h under vacuum in a Labanco 6l benchtop freeze dry system (USA), under the same conditions as the harvested biomass.

3.2 Biomass extraction and bioactivity analysis

3.2.1 Chemicals

For the extraction, commercial grade hexane (VWR, Portugal), analytical reagent grade (>= 99.8%) ethyl acetate and laboratory reagent grade (>= 99%) ethanol (Fisher Chemicals, Portugal) were used. Other reagents included analytical reagent grade (>= 99.8%) dimethyl sulfoxide (DMSO), HPLC gradient grade (>=99,9%) Acetonitrile (Fisher Chemicals, Portugal), and pure methanol and iron (III) chloride hehahydrate (Valente & Ribeiro, Portugal). Milli-Q-water was obtained through Milli-Q® Advantage A10 Ultrapure Water Purification System and destilled water by Elix/Gradient Water Purification System both equipments from Merck (Germany). Potassium ferricyanide 98% pure was supplied by Arcos organics (Spain). From Sigma-Alderich, Merk (Germany) were obtained the angiotensin converting enzyme (ACE)-Activity Assay kit, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Captoril, Ibuprofen (≥98% (GC)), gaalic acid and trichloroacetic acid. The Cyclooxygenase (COX) Inhibitor Screening Assay Kit was obtained from Cayman Chemical (USA). The positive control used was Ibuprofen (≥98% (GC)) from Sigma–Aldrich (Munich, Germany).

3.2.2 Extraction of Microalgae

In order to break the frustules, the vitreous cell walls, the lyophilized biomass of the respective microalgae were ground into finest powder in portions of 10 g in a planetary ball mill PM 100 (Retsch GmbH, Germany) with 5 stainless steel mill balls of 20 mm diameter at room temperature, 450 rpm for 5 min with invervals of 30 sec each minute to avoid temperature upshifts.

Extrations were made in an automated pressurized liquid extractor EDGE by CEM (C.E.M Corporation, 2017). In order to prevent possible degradation of heat-sensitive compounds, the chosen extraction temperature was set at 30°C, the mimum temperature allowed by the equipment. Solvents of 3 different polarities in order to widen the range of compounds which could be extracted related to their polarity (Ramluckan et al., 2014), with the lowest possible impact on health and the environment were selected, namely ethanol (polarity index (PI) 5.2), ethyl acetate (PI 4.4) and *n*-hexane (PI 0.1) (Ramluckan et al., 2014; SPEX, 2022). Good ventilation was ensured in the work area. For the extraction, 1 g dry biomass was extracted with 20 ml of the selected solvent at 30°C, for 10 min. Extractions were repeated twice in a total of

3 extration cycles, which were pooled, leading to a total of 60 ml extract. The extract was concentrated under vacuum in a rotary evaporator and transferred into 4-ml amber vials and then dried under a gentle nitrogen flow. The dry weight of the extract was determined and the amount of dimethyl sulfoxide (DMSO) required to produce a 50 mg ml^{-1} concentration was added accordingly. Extracts were stored at -80°C under an inert nitrogen atmosphere. DMSO, was chosen to dissolve the extracts as it is a solvent appropriate for both polar and non-polar compounds.

3.2.3 Antioxidant activity assays

There are several antioxidant activity assays for use *in vitro* or *in vivo*. In the hydrogen atom transfer reaction (HAT), the ability of the antioxidants to donate a H-atom is measured. In this relatively fast reaction, of seconds to minutes, the weaker the bond between the antioxidant and the H atom, the higher the antioxidant effect. In the single electron transfer reaction (SET), it is mostly measured the capacity of ionization of the antioxidant. This type of reaction depends on the pH and is in general slower than the HAT-reaction (Coulombier, Jauffrais & Lebouvier, 2021). In a spectrophotometric-SET based assay, the oxidant changes colour as it is reduced. The DPPH assay (2,2-Diphenyl-1-picrylhydrazyl) is a decolourizing assay to indicate free radical scavengers, where the antioxidant as radical scavenger reacts with the purple DPPH radical and reduced it. The stronger the antioxidant effect, the lighter the colouration will be, down to a weak yellow. The absorbance is measured at 515 nm. Another colourimetric method is the ferric ion reducing antioxidant power assay (FRAP), where the increase in colour indicates an electron-transfer reaction. This assay measures the capacity of the antioxidant to reduce ferric-TPZT to ferrous-TPTZ in an acid medium (pH 3.6). As the ferrous complex has an intensive blue colour, the increase in absorbance is measured at 593 nm (Shahidi, 2015; Coulombier, Jauffrais & Lebouvier, 2021). Other assays include measuring lipid peroxidation inhibition, free radical quantification or, in general, antioxidant activity. Antioxidant capacity can also be detected by electrochemical techniques as cyclic voltammetry, amperometry or biamperometry (Moharram & Youssef, 2014).

3.2.3.1 DPPH radicale scavenging activity assay

Pre-screening of extracts with antioxidant properties was done by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay to determine the presence of compounds with radical scavenging activity. The following solutions were prepared: as positive control a solution of gallic acid with 1 mg ml^{-1} in DMSO; different concentration of the extracts in DMSO (5 & 1 mg ml^{-1}) and the DPPH solution with 0.48 mg ml^{-1} in methanol. As negative control 100% DMSO was used. The set up of the 96 wells plate were as follows: 22 µl of extract / negative control / positive control and 200 µl of DPPH solution, except for the colour control. For this control, 200 µl methanol instead of DDPH solution. Plates were gently shaken to mix well the reagents and incubated in the dark for 30 minutes at room temperature, followed by reading in a spectrophotometer plate reader Synergy 4 from Biotek (Agilent, USA) at 515 nm. The results were expressed as percentage of inhibition or as DPPH scavenger activity in percentage, compared to the negative control.

3.2.3.2 Iron reducing power assay (Ferricyanide/prussian blue assay)

The performance of this assay was based on the protocol described by (Reis et al., 2012). Firstly, the following solutions were prepared in destilled water: iron (III) chloride ($FeCl_3$-$6H_2O$) 0.1%, potassium ferricyanide 1% and trichloroacetic acid (TCA) 10%. Gallic acid in 1 mg ml^{-1} in DMSO was used as positive control and different concentration of the extracts in DMSO (5, 2.5, 1, 0.5, 0.25 and 0.125 mg ml^{-1}) were applied.

The set up of the 96 wells plate were as follows:

Negative control (DMSO): 50 µl DMSO, 50 µL destilled water, 50 µl potassium ferricyanide (1% solution); positive control: 50 µl gallic acid solution, 50 µl destilled water, 50 µl potassium ferricyanide (1% solution); assay/extract: 50 µl extract, 50 µl destilled water, 50 µl potassium ferricyanide (1% solution); colour control: 50 µl extract, 160 µl distilled water. All assays and controls were done in triplicate. Plates were carefully shaken to mix the reagents and then incubated for 20 min at 50°C. Afterwards, except for the colour control wells, 50 µl of TCA-solution and 10 µl of iron (III) chloride solution were added and the plates incubated again at 50°C for 10 min. The absorbance was read in plate spectrophotometer reader at 700 nm. The reducing power was calculated relatively to the negative control and the IC_{50} (half maximal inhibitory concentration) values for the tested extracts were determined.

The most promising extracts, with the strongest antioxidant activity, were subjected to 2 anti-inflammatory assays.

3.2.4 Anti-inflammatory assays

3.2.4.1 ACE inhibition assay

A fluorometric method was used, often based on various commercially available kits for screening ACE inhibitors, using ACE-specific fluorogenic substrates. More specifically, ACE reacts with its substrate and a new product is formed which can be quantified with a fluorescence plate reader at a specific excitation and emission wavelength. A positive control, i.e., a known ACE inhibitor, was used, at various concentrations, to calibrate the results of this procedure. The data was then analysed and the inhibition capacity or, accordingly, the activity in percent was calculated. The kits usually contained the substrate, enzyme, buffer and positive control in the appropriate amounts for the specified number of tests that needed to be carried out (Kreutter, Schwaller and Reymond, 2021). The screening of ACE-inhibitors was done using a fluorometric method on 96 well plates, following the protocol of Sentandreu and Toldrá (2006). First the 3 buffer solutions based on Tris-base buffer with a pH of 8.3 were prepared. The enzyme stock solution of ACE in buffer with a concentration of 150 µg ml^{-1} and also de substrate stock solution in a concentration of 10 mM were prepared. On the experiment day, the working solutions were prepared. As positive control captopril with a concentration of 200 µM dissolved in DMSO was used, and as negative control, DMSO solution in same concentration as used as solvent for the positive control and for the extract samples. An aliquot of 50 µl of positive or negative control or samples were added in triplicate to the well plate. Afterwards, 50 µl of ACE working solution were added and the plate was incubated at 37°C for 10 min, as well as the substrate working solution. Aliquots of 200 µl of substrate were added to the working solution in each well by means of a multi-channel pipette to process all wells in less than 2 minutes. The plate was read in a fluorometer Synergy 4 from Biotek (Agilent, USA) at the excitation wavelength of 355-375 nm. Fluorescence emission was read at a wavelength range of 400-430 nm at 37°C every 5 minutes during a period of 40 minutes. The results obtained were analysed and, based on the kinetic curves, the ACE-inhibitory activity for the extracts tested were determined (Sentandreu & Toldrá, 2006).

3.2.4.2 COX inhibition assay

The screening of COX-2 inhibitors was done by an ELISA test from Cayman Chemicals (Cayman chemicals, 2018). For the first part of the test, the selected extract, arachidonic acid and the COX enzyme were mixed to elicit a reaction in which prostaglandin will be produced according to the COX inhibitory capacity of the extract to be tested. In the second step, the prostaglandin content of the mixture is determined using an ELISA procedure. All procedures were done according to the manufacturer's protocol. The most promising extracts, with the strongest anti-inflammatory activity, was subjected to further analyses to tentatively identify the compounds responsible for the observed activities.

3.3 Untargeted metabolomics

3.3.1 Fracionating by preparative HPLC

For fractionation, a larger amount of *N. shiloi* ethanolic extract was prepared. The process was similar to the previous extraction process, with the exception that after drying in the vial, the extract was dissolved in acetonitrile (HPLC grade) at a concentration of 100 mg ml^{-1} and filtered with a 0.45-µm nylon filter. Liquid chromatography (LC) was a LC-20AP from Shimadzu with a SPD-M40 photo diode array detector (PDA) and ELSD LTII low temperature evaporative light scattering detector (ELSD). Compounds that are not detectable in ultraviolet (UV) or visible (VIS) range can be observed with ELSD, as this one is a more universable detector. All LC data was treated by the Shimadzu system software LabSolution version LCGC 2020.

Since the goal was primarily to be able to fractionate peaks corresponding to pure compounds, a time-based mode with collection of 26 fractions, each with 10 ml volume was chosen. A preparative column (Luna 5u C18(2) 100A, 250 x 10.0 mm from Phenomenex (Germany)) was used. Fractionation was carried out with a flowrate of 4.73 ml min^{-1}, injection volume of 473 µL, starting with 65% acetonitrile and 35% Milli-Q water, reaching 100% acetonitrile after 30 minutes and keeping this condition for 25 minutes and back to % acetonitrile and 35% Milli-Q water for the last 5 minutes. The fractions were automatically collected in 10-ml vials, starting at minute 2.5 and ending at minute 57.5. In order to obtain an amount sufficient for the subsequent assays, this procedure was carried out 4 times. All fractions were concentrated under a gentle nitrogen flow in a sample concentrator SBHCONC/1 from Stuart (Cole-Parmer,

Staffordshire, UK). The yield of each fraction was determined before re-dissolving in acetonitrile at a concentration of 0.5 mg ml^{-1}.

Fractions were re-tested on the previous described antioxidant assays, followed by anti-inflammatory assays. In addition to the fractions, the DPPH assay and the iron reducing power assay were also carried out with the previous extract at the same concentration of 0.5 mg ml^{-1}. For this purpose, the extract was dissolved in acetonitrile and filtered into an amber glass vial using a 0.45-µm nylon syringe filter.

The fractions with the most promising results from the antioxidant assays were subjected to ACE and COX-2 assays. All assays were performed according to the protocols previously used for the extracts. A COX-1 assay was performed on the two fractions with the best results, using the same protocol and assay kit that was used for the COX-2 assay, in order to check for possible selective COX inhibition.

LC-MS should have been used as the next step for the tentative identification of the active compounds. However, due to a technical problem in the equipment, this plan could not be followed, so GC-MS was used, as well as additional analyzes of carotenoids by HPLC.

3.3.2 Comparative carotenoid profile analysis by HPLC

Carotenoids were analysed by HPLC following the protocol described in Schüler et al. (2020) and Couso et al. (2012). For this purpose, the carotenoids lutein, fucoxanthin, astaxanthin, cantaxanthin and β-carotene were prepared with HPLC grade methanol in the concentration of 0.5 mg/mL, and also a mix of the freshly prepared solutions of lutein, fucoxanthin and β-carotene with the ratio 1:1:1. For identification purposes only, existing methanol solutions of neoxanthin and violaxanthin with unknown concentrations were also injected. In the previously used LC-20AP from Shimadzu with SPD-M40 photo diode array detector (PDA), a Purospher STAR RP-18 endcapped (250 x 2.1 mm, 5 µm) column from Merck (Germany) was installed Chromatography was performed at room temperature using a flow rate of 1.0 ml min^{-1} with a spectral range of 190-750 nm. As eluents, the following mobile phases were used: A, acetonitrile : Milli Q water in a ratio 9:1 (v/v) (both filtered with a 0.45-µm filter and in HPLC grade); and B, ethyl acetate in HPLC grade (also filtered with a 0.45-µm filter). The gradient programm was set as described in Table 3.1 (Couso et al., 2012; Schüler et al., 2020).

Table 3.1: Gradient program for carotenoid HPLC analysis following the protocol adapted from Schüler et al. (2020) and Couso et al. (2012).

Time (min)	**Flow type**	**%Mobile phase A** Acetonitrile:H_2O (9:1 v/v))	**%Mobile phase B** Ethyl acetate
0	initial	100	0
0-16	gradient	40	60
16-30	constant	40	60
30-32	gradient	0	100
32-35	gradient	100	0
35-40	constant	100	0

3.3.3 Tentative identification of compounds by GC-MS

The two fractions with the best antioxidant and anti-inflammatory activities were injected in a GC-MS, a 456-GC TQ from Bruker (USA) with a ZB-5ms plus column from Phenomenex (Germany), to obtain the tentative identification of compounds present. Fractions were derivatized with N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) to increase the volatility of the compounds present in the fractions/extracts. MSTFA introduces non-polar trimethylsilyl protecting groups (TMS) into polar compounds, thus reducing polar interactions between analyte molecules. This allows these molecules to vaporize at lower temperatures and prevents thermal decomposition reactions in the injector or on the GC column. With this technique, a wider range of compounds can be determined (Urbach, 2012). For this, 50 µl of each fraction were dried in 1.5 ml glass vials with 200 µl glass inserts under gentle nitrogen flow and redissolved with 50 µl of derivatization solution which consisted on a mix of 10 µl of 0.25 ml of MSTFA with 10 mg of NH_4I, and 15 µl of β-mercaptoethanol, and 90 µl of pure MSTFA. The capped vials were briefly vortexed and then placed in the heating block set at 70°C for 30 min. Subsequently, they were injected into the GC-MS. The GC conditions were as follows, injection temperature was 250°C, the used gas for the mobile phase was helium with a flow rate of 1 ml min^{-1} and the sample injection volume was 1 µl. The oven temperature increased from 60°C (hold 1 min) to 310°C with 4°C min^{-1} steps, and kept for 6 min at 310°C. The MS the conditions were: transfer line temperature of 220°C and source temperature of 260°C, for the spectrum acquisition a full scan mode was choosen with a m/z rate range of 50-1200. The library of the National Institute of Standards and Technology (NIST, 2022) was used as a reference in order to identify the compounds as accurately as possible.

3.4 Statistical analyses

Statistical analyses were performed using statistical software RStudio, version 2022.02.0. For sample comparison, one/two-way ANOVA and pairwise t-tests were used. For multiple comparisons, the Tukey test was used. Differences were considered significant at $p<0.05$ unless if other p values are specified.

4 Results & Discussion

4.1 Culture scale-up and biomass production

The *Cylindrotheca closterium* and *Cylindrotheca fusiformis* cultures were scaled up successfully. The usual f/2 nutrient solution with the addition of silicic acid was used and, in the case of *C. closterium*, the metal trace content was increased 5-fold compared to the f/2 standard metal trace amount. The first growth curves were obtained by cell counting (Figure 4.1).The growth behaviour of both cultures was compared to studies with similar growth conditions (Kingston, 2009; Pinela Raposo, 2020). The growth curves obtained in 1-l bottle cultures show an expected growth behaviour of the *C. closterium*, similar to the reported results, whereas the *C. fusiformis* growth rate was much better than expectated reaching a cell concentration 5-fold higher than that of *C closterium*.

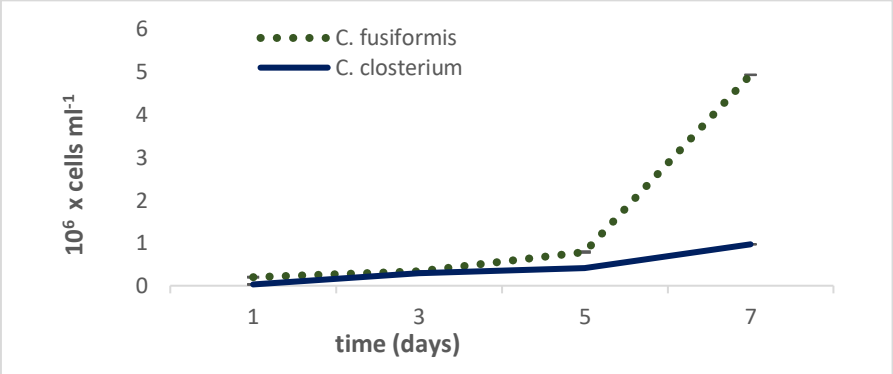

Figure 4.1: Growth of *Cylindrotheca closterium* cultivated in aerated 1-l Schott flasks using f/2 media + silica + 5 × metal trace solution and *Cylindrotheca fusiformis* in aerated 1-l Schott flasks using f/2 media + silica expressed as cell counts, n=3.

Upon scale-up to the airlifts, a even better growth performance could be confirmed for *C. fusiformis*. A desired improved growth behavior of *C. closterium* through the adapted medium for intensive *C. closterium* cultures could not be obtained even under standard f/2 medium with added silica as suggested by the literature (Suman et al., 2012). The monitoring was carried out by determining dry biomass, optical density and cell counting (Figure 4.2). As the airlifts have a smaller diameter and greater height than the previously used 5-l bottles or glass bottles, there was higher sediment formation and also deposits on the inner walls of the PBR. However, the airlifts exert more turbulence and therefore greater gas exchange, and the individual algae are more exposed to the light. Excessive light exposing can lead to photoinhibition with an increase in reactive oxygen species (ROS) and a less adequate response to the oxidative stress, which could affect biomass production (Gupta et al., 2019; Maltsev et al., 2021). At the end of the airlift culture, the *C. closterium* culture was contaminated with *C. fusiformis* and had to be eliminated. The backup culture in the 5-l plastic bottles were able to compensate for this.

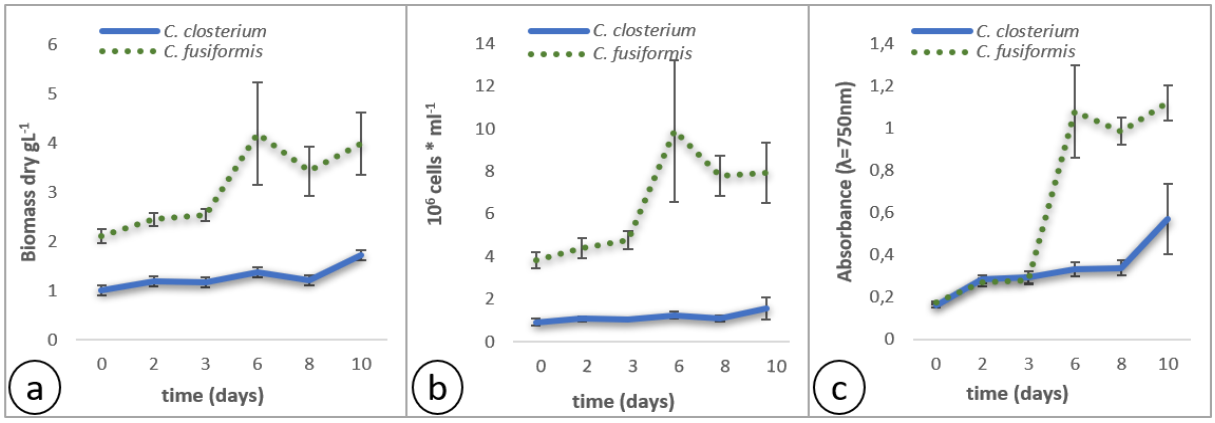

Figure 4.2: Growth curves of *C. closterium* and *C. fusiformis* monitored by (a) dry weight (g l^{-1}); (b) by cell counting (cells ml^{-1}), and (c) by optical density with absorbance measured at 750nm over time: days of culture in the airlifts, *n*=6.

When monitoring the culture growth via dry weight, it was noticed that the colour of the filtered algae began to change even at room temperature during the processing of the biomass. From a colour similar to ground coffee brown to a green tinge, the colour changed completely to green upon drying at 100°C. This observation is consistent with the presence of carotenoids in this biomass, especially fucoxanthin, because when exposed to light and/or heat, it undergoes degradation and the orange-brownish colour fades out, leaving the green of chlorophyll as the dominant colour (Kartikaningsih et al., 2017) (Figure 4.3).

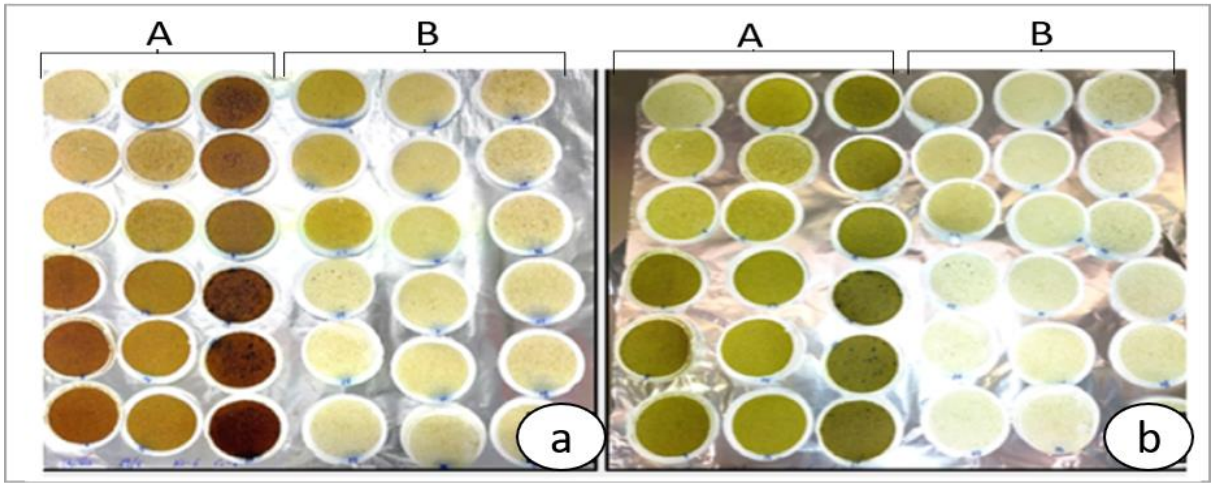

Figure 4.3: Filters with samples of *Cylindrotheca fusiformis* (A) and *Cylindrotheca closterium* (B) before (a) or after (b) drying in an oven at 100°C for 48h.

4.2 Biomass extraction and bioactivity analysis

4.2.1 Extraction yield

By far, the best relative extraction yields (9.92-18.53%) were achieved with *Cylindrotheca fusiformis* with all 3 solvents. The lowest yields were obtained for *Cylindrotheca closterium* (3.35%) and *Nanofrustulum shiloi* (5.20%), especially when extracted with hexane (Table 4.1).

Table 4.1: Relative and absolut extraction yield of the microalgae related to their extraction solvent, extracted with EDGE extractor with 30°C extraction temperature.

Solvent	Microalgae	dry biomass (g)	yield (mg)	yield (%)
Hexane	*Nanofrustulum shiloi*	2.020	105	5.20
	Cylindrotheca fusiformis	2.007	199	9.92
	Cylindrotheca closterium	1.016	34	3.35
Ethyl acetate	*Nanofrustulum shiloi*	2.026	132	6.52
	Cylindrotheca fusiformis	1.000	117	11.7
	Cylindrotheca closterium	2.024	142	7.02
Ethanol	*Nanofrustulum shiloi*	6.054	443	7.31
	Cylindrotheca fusiformis	1.009	187	18.53
	Cylindrotheca closterium	1.009	139	13.78

4.2.2 Antioxidant and anti-inflammatory activity

In diatoms, extracts are often rich in phenolic compounds, fatty acids (polyunsaturated fatty acid, PUFA), flavonoids, tocopherols, alkaloids and carotenoids (including fucoxanthin and astaxanthin), as reported in several studies that investigated their antioxidant and anti-inflammatory capacity. Flavonoids, phycobiliproteins (phycoerythrin, phycocyanin, allophytocyanin), alkaloids, polar lipids (glycolipids, phospholipids and PUFAs) and polysaccharides (sulphated polysaccharides (SPS) and exopolysaccharides (EPS)) are likely to be present in ethanol extracts (Ahmed et al., 2021b; Kannaujiya et al., 2020; Kim and Chojnacka, 2015; Tabarzad, Atabaki & Hosseinabadi, 2020). In the ethyl acetate extracts from all strains, *β*-carotene and other carotenoids (e.g., diadionochrome, diadinoxanthin, diotoxanthin, violaxanthin, zeaxanthin and fucoxanthin), chlorophyll *a* and chlorophyll *c1*, *c2* and *c3* are expected to be found (Wang et al., 2018). In the less polar solvent, hexane, unpolar lipids (e.g., triacylglycerols) and tocopherols are expected as these are more lipophilic (Péres et al., 2006).

The various antioxidant and anti-inflammatory assays were used as indication to select the extract or fractions with the most promising results for further analysis in order to bioprospect for and identify compounds with anti-inflammatory activity.

4.2.2.1 Antioxidant assays of the extracts

4.2.2.1.1 DPPH assay

In this study the DPPH assay and iron reducing activity assay, showed very different results in some cases, with the low activities of the hexane extracts being noticeable as a common feature. Overall, an effect of the extracting solvent polarity on the antioxidant properties ($p < 0.05$) were observed. The mechanisms of action of the two used antioxidant assays differ from each other. The DPPH assay measures the ability of a substance to determine how well it can stabilize the free DPPH radical by donating an electron to the radical (single electron transfer reaction (SET)) or a hydrogen radical (hydrogen atom transfer reaction (HAT)). Whether it is a HAT, a SET reaction or even both depends on the tested antioxidant substance, the solvent and the pH of the solution. The iron reducing antioxidant power assay is always a SET reaction and the capacity of ionization of the antioxidant is measured (Xie & Schaich, 2014). The strongest

activities in the DPPH assay were shown by the ethyl acetate extracts of *C.closterium* and *N. shiloi* (Figure 4.4). This fits with expectations of finding powerful antioxidants such as the carotenoids and chlorophylls in ethyl acetate extracts.

4.2.2.1.2 Iron reducing antioxidant power assay

Extracts of *C. closterium* showed a particularly strong iron reducing capacity, with very low IC_{50} values: 0.203 mg ml^{-1} ± 0.100 for the ethyl acetate extract and 0.286 mg ml^{-1} ± 0.188 for the ethanolic extract.

Based on statistical analyses and the shown significant differences all non-hexane extracts were selected for further analysis of anti-inflammatory activities (Table 4.3).

Table 4.3: IC_{50} of the Iron reducing antioxidant power of 3 different extracts prepared from the three microalgae under study. Values are the mean and standard deviation of three independent assays performed at different days, *n*=3.

Microalgae (extract dissolved in DMSO)	**Extraction Solvent**	**IC_{50}** (mg ml^{-1}) **(means** ± SD)
Nanofrustulum shiloi	Hexane	**1.613** ± 0.181
	Ethyl Acetate	**0.917** ± 0.000
	Ethanol	**1.727** ± 0.022
Cylindrotheca fusiformis	Hexane	**2.681** ± 0.654
	Ethyl Acetate	**0.516** ± 0.256
	Ethanol	**0.529** ±0.286
Cylindrotheca closterium	Hexane	**2.293** ± 0.413
	Ethyl Acetate	**0.203** ± 0.100
	Ethanol	**0.286** ± 0.188

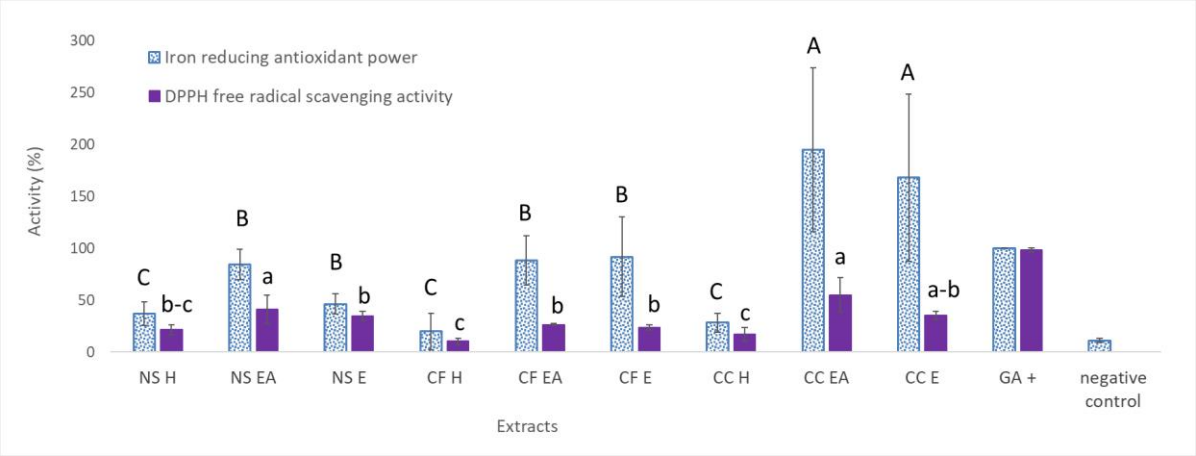

Figure 4.4: Comparison of antioxidant capacities of three different microalgae, *Nanofrustulum shiloi* (NS), *Cylindrotheca fusiformis* (CF), and *Cylindrotheca closterium* (CC) in three different extraction solvents, namely hexane (H), ethyl acetate (EA) and ethanol (E). Extract concentrations for DPPH and for iron reducing power assays were 5 and 1 mg ml^{-1}, respectively. The positive and negative controls corresponded to gallic acid and DMSO, respectively. Values are the mean and standard deviation of three independent assays performed at different days.

4.2.2.2 Anti-inflammatory assays of the extracts

4.2.2.2.1 ACE inhibition

In the ACE inhibitor assay, only 3 extracts were able to inhibit this enzyme, with the strongest inhibitory effect being found in the *N. shiloi* ethanol extract with 58.93% inhibition (Table 4.4).

Table 4.4: Angiotensin converting enzyme (ACE) assay of microalgae with ACE inhibition capacity, 6 replics, *n*=1.

Microalgae (extract concentration in DMSO: 1 mg ml^{-1})	**Extraction Solvent**	**ACE inhibition** (%)
Nanofrustulum shiloi	Ethyl Acetate	56.97
	Ethanol	**58.93**
Cylindrotheca fusiformi	Ethyl Acetate	n.a.
	Ethanol	48.72
Cylindrotheca closterium	Ethyl Acetate	n.a.
	Ethanol	n.a.

n.a.: not active

4.2.2.2.2 COX-2 inhibition

The best COX-2 inhibitory effects were shown by the ethanol extracts from *N. shiloi* and *C. fusiformis* with 63.78 ± 2.10% and 67.73 ± 3.72%, respectively (Table 4.5). The decision which extract should be analyzed further fell on the ethanol extract from *N. shiloi*. Even if the ethanol

extract from *C. fusiformis* delivered the best result in the COX-2 assay, and there was virtually a tie between these two extracts, a factor that came into the decision-making process was that a larger amount of extract from *N. shiloi* and also lyophilized biomass were available, which also facilitated further analysis in terms of quantity.

Table 4.5: COX-2 inhibition assay of 3 different microalgae each with 2 different extract solvents, 4 replics, *n*=1.

Microalgae (extract concentration in DMSO: 1 mg $ml^{-1)}$	**Extraction Solvent**	**COX-2 inhibition (%)** (means ± SD)
Nanofrustulum shiloi	Ethyl Acetate	**49.3** ± 1.2
	Ethanol	**63.8** ± 2.1
Cylindrotheca fusiformis	Ethyl Acetate	**62,3** ± 6.1
	Ethanol	**67.7** ± 3.7
Cylindrotheca closterium	Ethyl Acetate	**27.7** ± 3.9
	Ethanol	**32.5** ± 4.5

Based on a study by Delbrut et al. (2018), for the extraction of polyunsaturated fatty acids (PUFAs) and fucoxanthin, ethanol is indicated as a solvent able to extract relatively high amounts of the xantophyll fucoxanthin and PUFAs (Delbrut et al., 2018). Besides the PUFAs, further polar lipids like galactolipids and phosphatidylcholines can be expected to be present in the ethanol extract and it has been reported, that this type of compounds have anti-inflammatory activity (Harraz et al., 2019; Plouguerné et al., 2014; Yi et al., 2017). A previous work with *N. shiloi* biomass, from the same production as that provided for this study, through the AlgaRed+ project, reported a fucoxanthin concentration of 2.25 ± 0.02 µg mg^{-1} (Table 4.6) (Pinela Raposo, 2020). From this it can be concluded that fucoxanthin is likely to be found in the examined ethanol extract of *N. shiloi*. It has also been reported that chlorophyll *a* and its degradation product pheophytin *a* have anti-inflammatory capacities, assumedly related with the inhibition of gene expression of TNF-α, COX-2 and iNOS (Subramoniam et al. 2012).

Table 4.6: Biomass composition, carotenoid and chloropyll contents of *Nanofrustulum shiloi* (adapted from Pinela Raposo 2020). Values are given as mean ± standard deviation, *n* = 3.

Biomass composition			
Proteins (%)	Lipids (%)	Carbohydrates (%)	Ash (%)
21.93 ± 1.28	8.52 ± 1.05	37.29 ± 0.65	32.26 ± 0.75
Carotenoids content			
Fucoxanthin (µg mg^{-1})	Diatoxanthin (µg mg^{-1})	Diadinoxanthin (µg mg^{-1})	β-carotene (µg mg^{-1})
2.25 ± 0.02	0.10 ± 0.00	0.16 ± 0.02	0.19 ± 0.02
Chlorophylls content			
Chlorophyll *a* (µg mg^{-1})		Chlorophyll *c* (µg mg^{-1})	
3.80 ± 0.28		0.53 ± 0.06	

4.2.3 Fractionation

The chromatograms of the analytical LC showed mainly 2 large peaks, detected by the ELSD as well as by the PDA with nearly the same retention time, although values detected by the ELSD had a slightly delay caused by the system configuration (Figure 4.5). Since the goal was primarily to be able to fractionate the extracts and isolate pure compounds, a time-based mode with collection of 26 fractions was chosen, based on the obtained chromatograms (Figure 4.6). The collection of the fractions started at 2.5 minutes and ended at 57.5 minutes. To ensure that no compounds that might not be detectable by PDA or ELDS were lost, fractions were collected even from parts of the chromatogram where no peaks were observed.

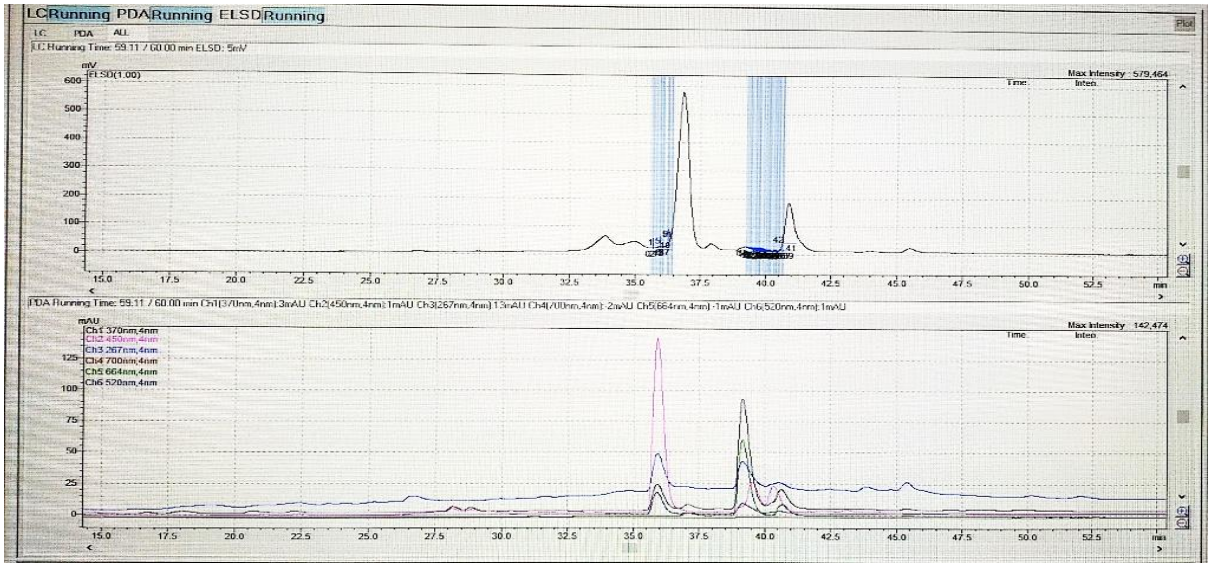

Figure 4.5: Chromatogram of analytical LC with low temperature evaporative light scattering detector (ELSD) (top) and photo diode array detector (PDA) (bottom) of *Nanofrustulum shiloi* ethanolic extract. (HPLC conditions: Column: Luna 5u C18(2) 100A, 250 x 10.0 mm from Phenomenex (Germany), flowrate of 4.73 ml min^{-1}, injection volume: 473 µL, initial: 65% acetonitrile and 35% Milli-Q water ; minutes 0-30, gradient: 100% acetonitrile; minutes 30-55, constant: 100% acetonitrile; minutes 55-60, gradient: 65 % acetonitrile and 35% Milli-Q water).

Figure 4.6: *Nanofrustulum shiloi* ethanol extract chromatograms at 3 different wavelengths, 250nm, 370 nm and 450 nm, with the fractionation scheme, marked with circles 1-26 on the light/dark blue fraction sections. Main peaks collected are marked with triangles 1-6. (HPLC conditions: Column: Luna 5u C18(2) 100A, 250 x 10.0 mm from Phenomenex (Germany), flowrate of 4.73 ml min^{-1}, injection volume: 473 µL, initial: 65% acetonitrile and 35% Milli-Q water; minutes 0-30, gradient: 100% acetonitrile; minutes 30-55, constant: 100% acetonitrile; minutes 55-60, gradient: 65 % acetonitrile and 35% Milli-Q water; automatic fraction collection starting at minute 2.5 and ending at minute 57.5.)

4.2.3.1 Antioxidant assays of the fractions

Various criteria flowed into the selection of the fractions that were subjected to the two antioxidant assays: major peaks present in the chromatograms, fraction yield, and colour and its intensity. With regard to the peaks in the analytical LC chromatogram, fractions 1, 5, 10, 13 and 15 were selected, and because of their high yield: 1, 9, 10, 11, 12 and 14 (Table 4.7). Some of the fractions obtained showed very different colours and colour intensities, in particular fraction number 10 (F10) with a bright golden-orange colour and fraction number 13 (F13) with an intensive olive-green colour. Fractions 16-19 were also selected becaused of their light green colour (Figure 4.7).

Table 4.7: Preparative LC fractionation yields of *Nanofrustulum shiloi* ethanolic extract, after 4 cycles with each 10 ml per fraction, totally 40 ml for each fraction.

Fraction number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Yield (µg)	2.252	1.022	0.877	0.740	0.802	0.787	0.986	1.003	2.928
Fraction number	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Yield (µg)	4.25	17.1	1.76	1.429	4.642	0.652	0.806	0.645	0.853
Fraction number	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	
Yield (µg)	0.933	0.658	0.583	0.658	0.759	0.482	0.783	0.604	

Figure 4.7: Colour of the fractions of *N. shiloi* ethanol extract, at a concentration of 0.5 mg ml^{-1}.

Iron reducing antioxidant and the DPPH free radical scavenging activity assays (Figure 4.8) were performed on a total of 12 different fractions (1, 5, 9, 10 - 19), with fractions 17 & 18 being pooled together due to their low yield. In addition, the raw *N. shiloi* ethanolic extract was also analyzed at the same concentration of the fractions for comparison. Fractions 10, 13, and 15 have shown significantly higher ($p< 0.00001$) iron-reducing antioxidant power compared with the raw *N. shiloi* ethanol extract. Similar results were obtained for the DPPH free radical scavenging activity, where fractions 9, 10, 11, 13, 14,15, and 16 showed significantly higher ($p< 0.001$) activity. Based on the higher activity of the mentioned fractions compared to the raw extract, a positive synergistic effect of compounds related to antioxidant activity in the raw extract could not be detected. The more purified compound configuration in the fractions, caused by the separation through the preparative LC, showed stronger results which could be caused by the reduced impact of negative synergistic effects, or by the increased concentration of the active compounds in the respective fractions.

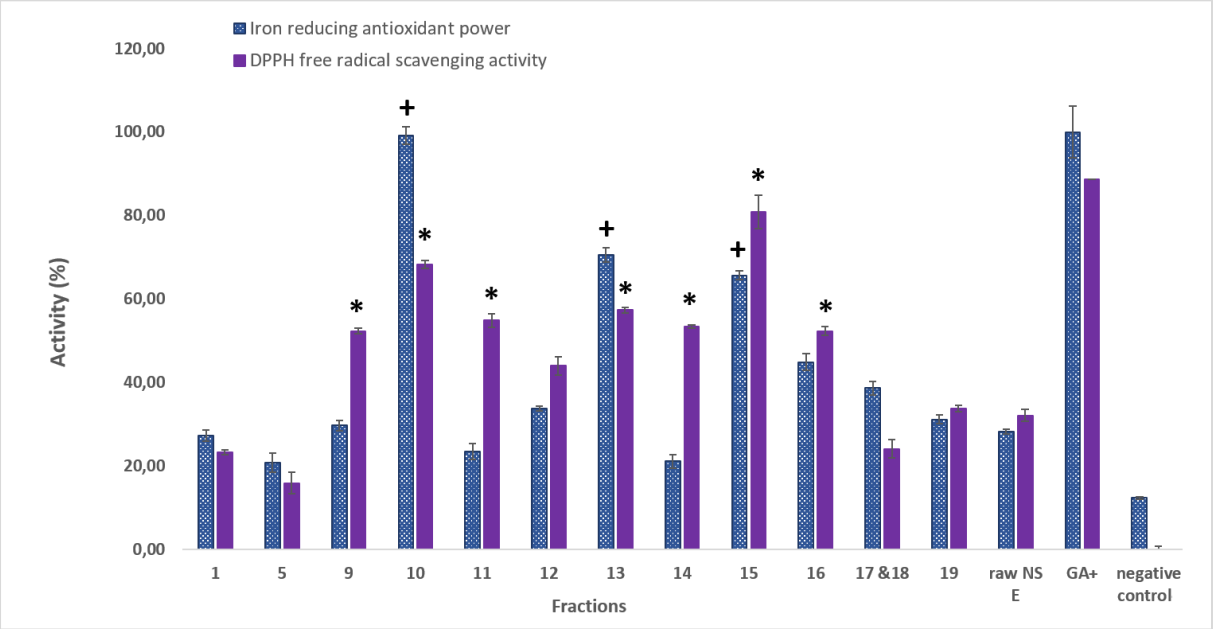

Figure 4.8: Antioxidant activity of *Nanofrustulum shiloi* ethanol extract fractions 1, 5, 9 -16, 17&18, 19 and raw extract (raw NS E) at 0.5 mg ml^{-1}. Gallic acid was used as positive control (GA+) and DMSO, at the same concentration of that used in the fractions, was used as a negative control. Based on Iron reducing antioxidant power assay,$^{++}$ indicates significant differences compared to the raw extract, $p<0.00001$ (pairwise *t*-test) and for the DPPH assay bars labelled with * indicate a level of significance of $p<0.001$ (pairwise *t*-test), $n=3$.

4.2.3.2 Anti-inflammatory assays of the fractions

4.2.3.2.1 ACE-inhibition

Fractions 10, 11, 13, 14 and 15 were selected to be tested for ACE inhibition activity at concentrations of 0.5 mg ml^{-1} based on their previously shown antioxidant activity (Fig. 4.8) or their high yield. A measurable inhibitory effect on ACE could only be determined in fractions 10 and 13 with 82.75% and 92.47%, respectively, compared to an activity of 48.63% of the crude extract. Since the inhibitory effect of the crude extract is significantly lower than that of the individual fractions, a synergistic effect due to the mixture of substances in the crude extract can be ruled out and a higher concentration of the inhibiting compounds, or a decreased impact of negative synergistic effects of the compound mixture in fractions F10 and F13 can be assumed.

4.2.3.2.2 COX- inhibition

The same 5 fractions were also analysed for COX-2 inhibition ability. Fraction F14 did not present inhibition capacity while the strongest inhibitory effect was again found in fractions F10 and F13, with 83.44 % ± 9.51 % and 81.89 % ± 9.18 %, respectively (concentration of 0.5 mg ml^{-1}) (Figure 4.9). These results are significative higher than the value for the raw *N. shiloi* ethanol extract with 63.78 % ± 2.10 % at the concentration of 1.0 mg ml^{-1}. As in the ACE assay, an enrichement in inhibitory compounds in fractions 10 and 13 can be assumed, because they displayed higher activity compared to the raw extract at a lower concentrations. To check for selectivity between COX-2 and COX-1 inhibition, a COX-1 inhibition assay was also performed on these two fractions. Fraction 10 showed no COX-1 inhibition, and fraction 13 showed only a slight inhibition of 20% ± 2% at a concentration of 0.5 mg ml^{-1} (results not shown). Therefore, it can be concluded that both these fractions contain selective COX-2 inhibitors. Fractions 10 and 13, were thus selected for chemical characterization by GC-MS.

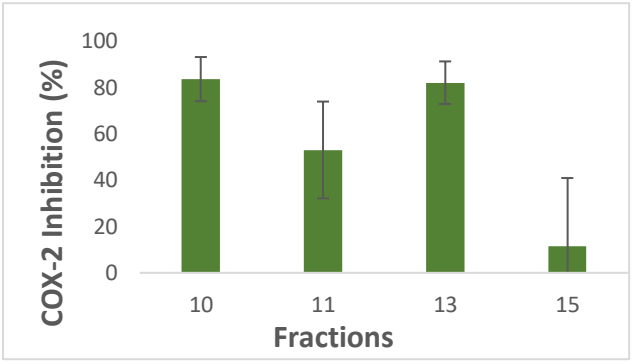

Figure 4.9: COX-2 Inhibition activity of *Nanofrustulum shiloi* ethanol extract fractions, tested at 0.5 mg ml^{-1}, *n*=3.

4.2.4 GC-MS Analyses

In the non-derivizated fractions GC-MS analyses show mostly the presence of fatty acids. Fraction F10 was mostly composed of mystiric acid (MA) which represented 93.26% of the whole area of the chromatogram, with minor amounts of gamma-linolenic acid (GLA, 4.01%), palmitoleic acid and alpha-methyl linolenate (1.12% and 0.89%, respectively). In the non derivatized fraction F13 only 3 different compounds were detected by the GC-MS analysis. First and foremost, with a proportion of 93.94%, palmitic acid (PA) and a relatively small proportion of the *n*-3 arachidonic acid methyl ester, representing 2.8% and phthalic acid 3.25%

of the whole chromatogram. In order to increase the volatility of the compounds present in the fractions and thus improve their detection limit by GC-MS, derivatization with MFSTA was performed on both fractions, which resulted in a higher number of compounds being detected in both fractions (Table 4.8). After derivatization, the same FAs were found as the main components in both fractions, as previously in the non-derivatized analyses, namely myristic acid with 59.75% in F10 and PA with 44.47% in F13. Other FA were now detectable at higher proportions such as arachidonic acid (AA), 17.2% and 16.0%, in F10 and F13, respectively, , representing a significant change from the non-derived variant. In addition, alpha-linolenic acid (ALA) was found in F13 with 10.7%, and in F10 only a percentage of 2.21% (Table 4.8).

Table 4.8: Results of the GC-MS analysis of fraction F10 and F13, non derivatized and derivizated.

Fraction	compound name	Formula	%
Fraction 10- non-derivizated	Myristic acid	$C_{14}H_{28}O_2$	93.3
	gamma-Linolenic acid	$C_{16}H_{20}$	4.01
	Palmitoleic acid	$C_{16}H_{30}O_2$	1.12
	alfa-Methyl linolenate	$C_{18}H_{30}O_2$	0.89
	Naphthalene derivat	$C_{19}H_{32}O_2$	0.47
Fraction 13 -non-derivizated	Palmitic acid	$C_{14}H_{28}O_2$	93.9
	Phthalic acid, di(oct-3-yl) ester	$C_{16}H_20$	3.25
	omega-3 Arachidonic acid methyl ester	$C_{21}H_{34}O_2$	2.81

derivizated fractions:

compound name	Type of compound	Formula	Fraction 10 (%)	Fraction 13 (%)
Myristic acid	SFA	$C_{14}H_{28}O_2$	59.75	1.54
Phthalate derivate		$C_{24}H_{38}O_2$	-	0.14
beta-D-Galactopyranose	Hexose, Monosaccharide	$C_6H_{12}O_6$	-	0.11
10-Heptadecenoic acid, (Z)-		$C_{17}H_{32}O_2$	-	3.41
1-Hexadecanol	16-C fatty alcohol, reduction from palmitic acid	$C_{186}H_{34}O_2$	-	0.16
1-Monolinolein	1-monoglyceride	$C_{21}H_{38}O_2$	0.153	-
1-Monopalmitin	1-monoglyceride	$C_{19}H_{38}O_2$	-	0.22
1-Octadecanol	long-chain primary fatty alcohol, Stearyl alcohol	$C_{18}H_{38}O$	-	0.53
2,2'-Trithiobisethanol, o,o'-		$C_4H_{10}O_2S_3$	0.26	0.39
2,3-Butanediol	Glycol	$C_4H_{10}O_2$	0.30	-
2-Butenedioic acid (E)-	Polyethylene, grafted with fumaric acid	$C_6H_8O_4$	0.23	0.39
5-Dodecenoic acid, (Z)-	MUFA (12:1n-7)	$C_{12}H_{22}O_2$		0.40
9,12-Octadecadienoic acid (Z,Z)-	alpha-Linoleic acid	$C_{18}H_{32}O_2$	0.61	0.18
9-Hexadecenoic acid, (Z)-	Palmitelaidic acid	$C_{16}H_{30}O_2$	3.47	1.65
alpha-Linolenic acid	omega-3 LC PUFA (18:3n-3)	$C_{18}H_{30}O_2$	2.21	10.7
Arachidonic acid	omega-6 LC PUFA (20:4n-6)	$C_{20}H_{32}O_2$	17.2	16.0
Docosahexaenoic acid	DHA, omega-3 LC PUFA, (22:6n-3)	$C_{22}H_{32}O_2$	1.24	-
Dodecanoic acid	Lauric acid, SFA	$C_{12}H_{24}O_2$	0.13	-
Eicosapentaenoic Acid	EPA, omega-3 LC PUFA (20:5n-3)	$C_{20}H_{30}O_2$	0.45	1.89
Ethylene glycol		$C_2H_6O_2$	0.14	4.74
Glycerol	Glycerin, trihydroxyalcohol	$C_3H_8O_3$	1.46	-
Napthalene derivate		$C_2H_5NO_2$	1.14	1.35
Nonanoic acid	straight-chain saturated fatty acid	$C_9H_{18}O_2$	0.42	0.58
Octadecyl methyl ether		$C_{21}H_{42}O_2$	0.07	-
Oleic Acid, (Z)-	MUFA, (18:1n-9)	$C_{18}H_{34}O_2$	-	0.17
Palmitic Acid	LC SFA	$C_{16}H_{32}O_2$	0.67	44.47
phosphate		O_4P^{-3}	0.49	0.73
Propanoic acid		$C_3H_6O_2$	1.59	3.26
Propylene glycol		$C_3H_8O_2$	3.23	3.62
Stearic acid	LC SFA	$C_{18}H_{36}O_2$	0.15	0.30
Benzoates			-	0.07
not identifyed			4.62	3.10

LC SFA: long chain saturated fatty acid; MUFA: mono-unsaturated fatty acid; LC PUFA: long chain polyunsaturated fatty acid; Formulas and information to type of compounds were obtained from NIH-NLM, 2022.

To compare the structures of the SFA, MUFA and PUFAs most frequently detected in the GC-MS analyzes of the fractions, the formulas were shown in 2D skeletal form (Figure 4.11).

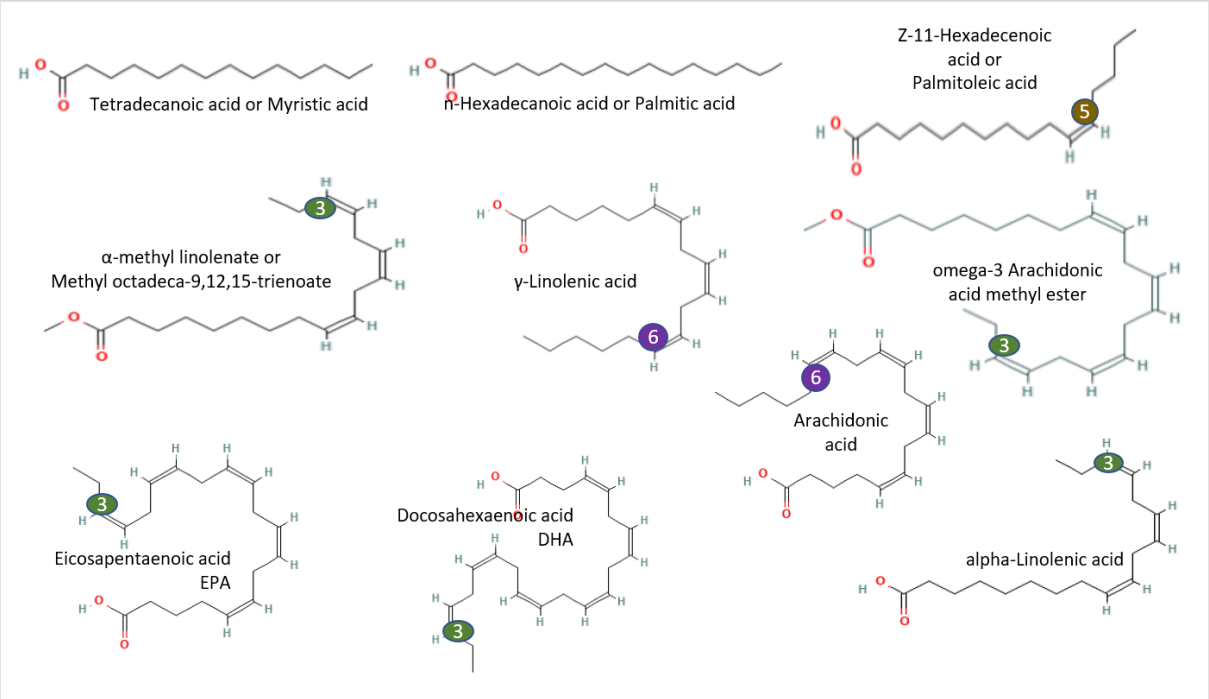

Figure 4.11: Images of the skeletal formulas of the main fatty acids detected with GC-MS in the 2 active fractions (F10 & F13) of *Nanofrustulum shiloi* ethanol extract, formulas marked with (3) are PUFAs-n-3; (6) PUFAs-n-6; (5) MUFA n-5 (adapted from NIH-NLM, 2022).

COX inhibition

Almost all of the various compounds detected have demonstrated anti-inflammatory effects in multiple studies, but not via the mechanism of action tested in this study (e.g., COX-2 inhibition). MA, for example, is a straight chain saturated fatty acid found in many plants and animals. Both *in vivo* and *in vitro* anti-inflammatory assays with MA have demonstrated its anti-inflammatory activity. It is assumed that the anti-inflammatory mode of action is related to the production of the anti-inflammatory interleukin-10 (IL-10), which is promoted by MA (Alonso-Castro et al., 2022). Since an explicit test regarding COX-2 inhibition by MA was not carried out, it cannot be directly concluded from this whether MA is responsible for the anti-inflammatory properties found in F10. Another anti-inflammatory mechanism is also found for PA, the main compound found by GC-MS in fraction 13. This is a saturated fatty acid also common in plants, animals and microorganism. Its anti-inflammatory effect was studied in lipopolysaccharide (LPS) stimulated mouse peritoneal macrophages, where PA was shown to have an effect on the regulation of the inflammatory cytokines TNF-α and IL-6 and a reduction in COX-2 enzyme expression and thus having an additional anti-inflammatory effect (Lee et

al., 2010). Ringbom et al. (2001) tested several types of fatty acids using similar testing methods as those done with the diatom extracts of this study and the resulting fractions. The two major fatty acids found in F10 and F13, MA and PA, did not show any COX-2 inhibition when applied at concentrations up to 500 μM (Ringbom et al., 2001). In order to clarify whether higher concentrations of MA or PA could ultimately bring about COX-2 inhibition, a corresponding analysis with different concentrations of this FA with the same assays and under the same conditions as these in the analysis of the extracts or fractions would have to be carried out.

Gamma-linolenic acid (GLA) ($C_{18}H_{30}O_2$), an *n*-6 PUFA, was detected as the second most abundant compound in non-derivizated F10, representing around 4% of the whole chromatogram of this fraction. Studies have shown that GLA is a precursor of dihomogamma linolenic acid (DGLA) ($C_{20}H_{34}O_2$), which competes with AA as a COX substrate and is metabolized to prostaglandin E_1 (PgE_1) which has an anti-inflammatory effect. This way of action would be dectectable in the COX-2 inhibition assay. Therefore GLA could contribuite to the COX-2 inhibition effect detectect in F10. GLA has a double form of effectiveness: the formation of pro-inflammatory substances is reduced and at the same time the production of anti-inflammatory substances is promoted by GLA (Alonso-Castro et al., 2022).

GC-MS analyses of derivatized or non-derivatized extracts or fractions always leads to the breakage of some structures that are thermolabile or have high boiling points. Thus, it is possible that the identified compounds were in fact part of more complex structures, common in microalgae that decomposed during the analyses. The finding of galactopyranose, different glycols and different FAs in F13 suggests that this fraction could contain galactolipids, a form of glycerolipids, which contain significant amounts of EPA accompanied with C16 fatty acids. Monogalactosyldiacylglycerol (MGDG) and digalactosylglycerols (DGDG), also named as the main photosynthetic glycerolipids, have shown by different studies to possess anti-inflammatory activities (Artamonova et al., 2017). Other polar lipids, as glycolipids and phospholipids, could also be present in these fractions, as phosphate, glycerol, and various glycols, in addition to various PUFAs can also be observed in the analysis of derivatised F10 (Plouguerné et al., 2014).

Not all of the fatty acids found in the GC-MS analyzes have shown anti-inflammatory effects. Above all, arachidonic acid, as a substrate of the COX-1 and COX-2 enzymes, and thus the starting product of the pro-inflammatory series 2 prostaglandins, especially PGE2, promotes inflammatory processes. Thus, a high proportion of 17.2% in F10 and 16.0% in F13 should be viewed critically (Figure 4.12) (Das, 2013; Waehler, 2021).

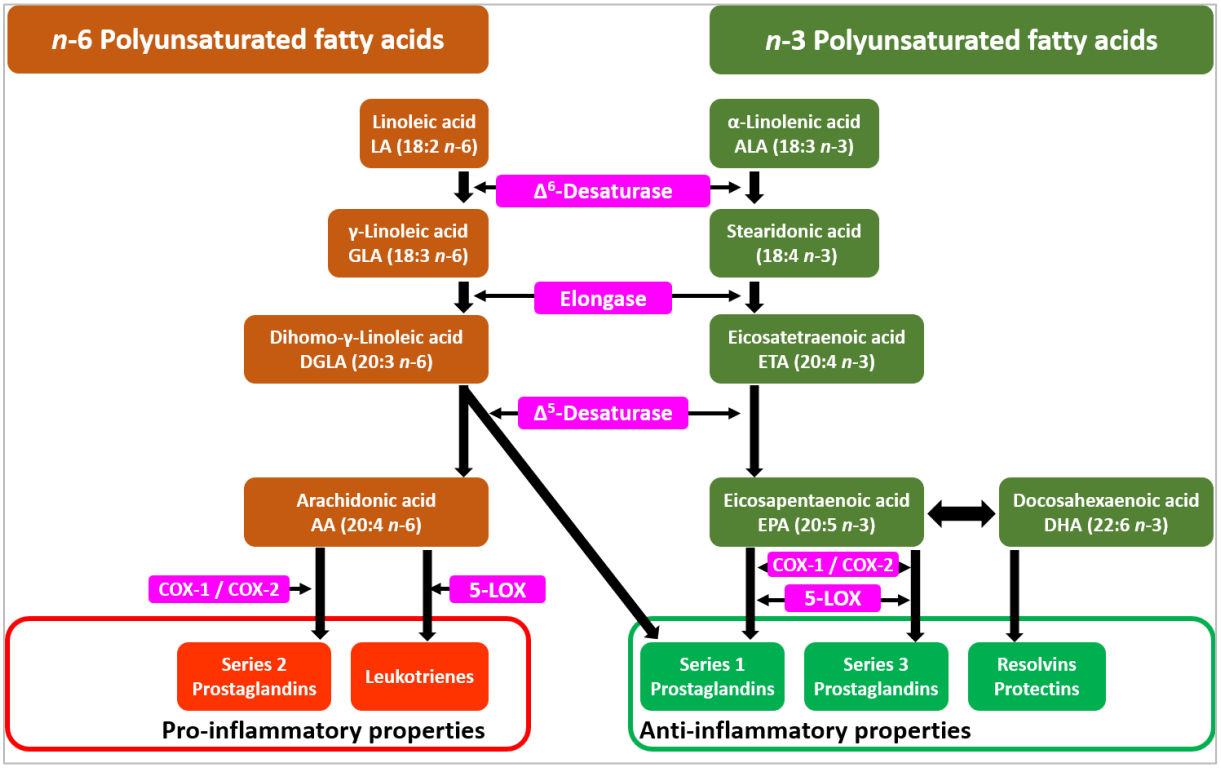

Figure 4.12: Simplified diagram of interligations of *n*-3/ *n*-6 polyunsaturated fatty acids in relation of inflammatory effects, COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2, 5-LOX: 5-lipoxygenase (adapted from Waehler, 2021; Das, 2013).

The most unusual assumption could also be the possible action of aryl-propanoic acid derivatives, since by GC-MS analyses of the derivizated fractions naphthalene derivatives and benzoates in addition to propanoic acid were found. Aryl-propanoic acid derivatives, also called profanes, include the nonsteroidal anti-inflammatory drugs (NSAIDs) Naproxen and Ibuprofen (chemically called 2-(4-isobutyl phenyl) propionic acid), with the latter used as a positive control in the COX assays in this study (Figure: 4.13) (Dhall et al., 2016). Profenes are usually produced synthetically in laboratories. If profanes could be detected in the studied diatoms, they could have their origin in contamination of the sea water used in the preparation of the culture medium by residues of the mentioned NSAIDs. Alternatively, *de novo* biosynthesis in diatoms could be suggested, which has not yet been discovered or proven.

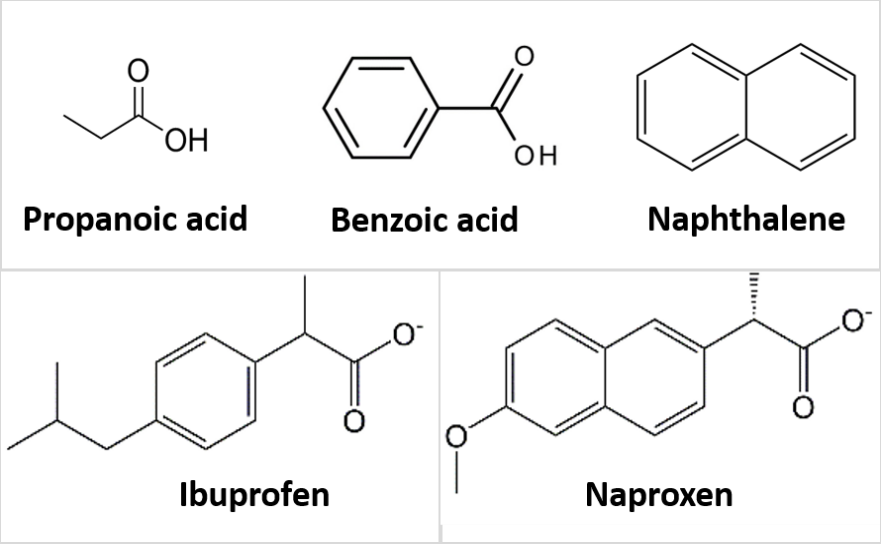

Figure 4.13: Skeletal formulas of compounds detected with GC-MS in 2 fractions (10 & 13) of *N. shiloi* ethanol extract after derivization (propanoic acid, benzoic acid and naphthalene) and of two anti-inflammatory drugs with COX inhibition, Ibuprofen and Naproxen (adapted from NIH-NLM, 2022).

The anti-inflammatory effect of *n*-3 PUFAs, also called "omega-3 PUFAs", has been confirmed several times in different studies. Various mechanisms of action have been described, such as changes in cell membrane composition, impact on expression of pro- and/or anti-inflammatory gens (with a corresponding diminishing or enhancing effect), bioactive metabolites with anti-inflammatory effects and competitive substitution of *n*-6 PUFAs. The latter has been observed in the COX-2 substrate competition, when *n*-3 PUFAs replace arachidonic acid, thereby reducing the production of the pro-inflammatory prostaglandin E_2 (Margină et al., 2020). This effect is expected in relation with EPA and the *n*-3 arachidonic acid methyl ester in the analysed fractions of this study.

Alpha-linoleic acid (ALA), an *n*-3 PUFA present in F10 in small amounts, has been shown to act as anti-inflammatoy compound. ALA metabolites appear to inhibit the production of pro-inflammatory substances such as prostaglandin E_2 (PGE_2), leukotriene B4 (LTB4), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). In addition, it is assumed that they influence the expression of a gene that has an inflammatory effect (DrugBank Online, 2022). Strong COX-2 inhibiton by EPA with low IC_{50} values (7.1 μM) has been shown through COX-2 inhibition assays. DHA, ALA, and linoleic acid (LA) showed also low IC_{50} values, respectively, 9.8, 12 and 98 μM (Ringbom et al., 2001). In a study by Robertson et al. (2015), it was shown that long chain *n*-3 PUFAs such as EPA can inhibit the production of the cytokines

IL-6 and IL-1β by human macrophages and demonstrated a restrictive effect of inflammatory gene expression.

ACE inhibition

It is reported that microalgal peptides molecular docking to ACE can cause ACE inhibitory effects. Two different mechanisms of action have been described, competitive and non-competitive inhibition. In the case of the competitive mechanism, there is a competition between the substrate, angiotensin I, and the peptide; if the peptide docks onto the active side of the ACE, the enzyme is blocked. In the non-competitive mechanism, the peptide binds to an inactive side of the ACE and thus changes the configuration of the enzyme. This prevents angiotensin I from binding to the active side of the enzyme and the formation of angiotensin II is thus prevented. The corresponding active peptides have not only shown strong ACE inhibition in these tests, but also a strong antioxidant effect by the DPPH assay (Jiang et al., 2021a). The investigated ACE-inhibiting peptides were short-chain peptides with 2-12 amino acids. The N-domain and C-domain of ACE each contain a zinc cofactor-binding active site, therefore inhibition of ACE by metal chelators would be possible (Jiang et al., 2021a). Pekooh et al. (2022) showed that PUFAs, especially EPA, DHA, ALA and AA, have strong binding activities to the binding sites of ACE and thus can contribute to ACE inhibition. The inhibitory effect found for EPA, DHA and AA was almost as high as that of the enalaprilat used as a positive control (Das, 2008; Pekkoh et al., 2022).

Anti-inflammatory PUFAs, proven in studies, were found in the derivatized fractions, mainly ALA with 2.21% (F10) or 10.7% (F13), and, in small amounts, DHA and EPA, resulting in a total of 3.90 % (F10) and 12.59% (F13), which seems relatively low compared to the values of the pro-inflammatory acting AA with 17.2% (F10) and 16.0% (F13). Even if the PUFAs mentioned support the anti-inflammatory effect found, other bioactive compounds that have not yet been analysed, can be responsible for the strong inhibition of COX-2 and ACE shown.

The FAs found in the GC-MS analysis of the derivatised fractions, in particular the PUFAs, are probably also responsible for the high antioxidant activity of the fractions in the assays. It is important to note that although PUFAs can easily de-radicalize ROS, they oxidize themselves in the process. PUFAs such as LA are particularly susceptible to oxidation as the multiple double bonds facilitate the stripping of an H atom by a hydroxyl radical (•OH), which has an unpaired electron. This reaction then forms a lipid radical and water. If the lipid radical now reacts with oxygen, a lipid peroxyl radical is formed. This can now in turn react with another intact PUFA and thus form another lipid radical, a process called lipid peroxidation that takes

place in a chain reaction leading to cell damage. It can be said that in terms of reaction, PUFAs might function as antioxidants, but they may pose other problems as indicated above (Skrip & Mcwilliams, 2016). Therefore, it is important to examine the antioxidant agents in the fractions more closely.

The strong coloring of fractions 10 and 13 can lead to the assumption that pigments, such as carotenoids and chlorophyll, are present in these fractions. F10 was mainly yellow to dark orange/reddish as most carotenoids and the dark olive color of F13 could be caused by a large amount of chlorophyll. Since carotenoids cannot be analysed easily by GC-MS, HPLC analyses were performed. The chromatogram of F10 contained three major peaks in the first 6.5 minutes of the analysis (Figure 4.14), but none of the retention times coincided with that of the pure carotenoids used as standards (Table 4.9).

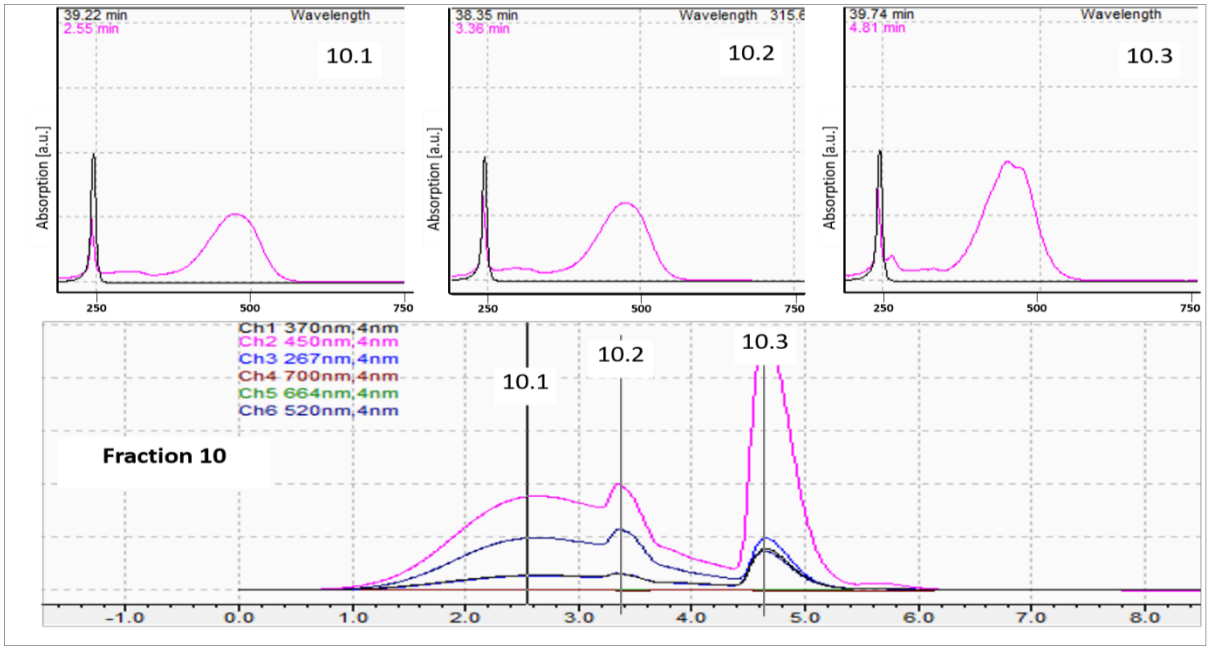

Figure 4.14: LC-Chromatogram of fraction 10 with absorption spectrum profiles

Table 4.9: HPLC retention times of peaks of 7 carotenoids and 2 fractions of *Nanofrustulum shiloi* ethanol extract

Carotenoid	retention time (min)	**Fraction 10 peaks**	retention time (min)	**Fraction 13 peaks**	retention time (min)
Cantaxanthin	3.59 / 3.94	**10.1**	2.55	**13.1**	3.58
Neoxanthin	3.85	**10.2**	3.36	**13.2**	10.67
Fucoxanthin	10.90	**10.3**	4.81	**13.3**	11.97
Violaxanthin	14.12			**13.4**	13.01
Astaxanthin	16.36			**13.5**	13.67
Lutein	18.85				
β-carotene	30.88				

F13 had 5 peaks (Figure 4.15), three of which had retention times similar to those of cantaxanthin and fucoxanthin but the absorbance profiles of the samples peaks and those of the pure standards do not appear to match (Figure 4.16).

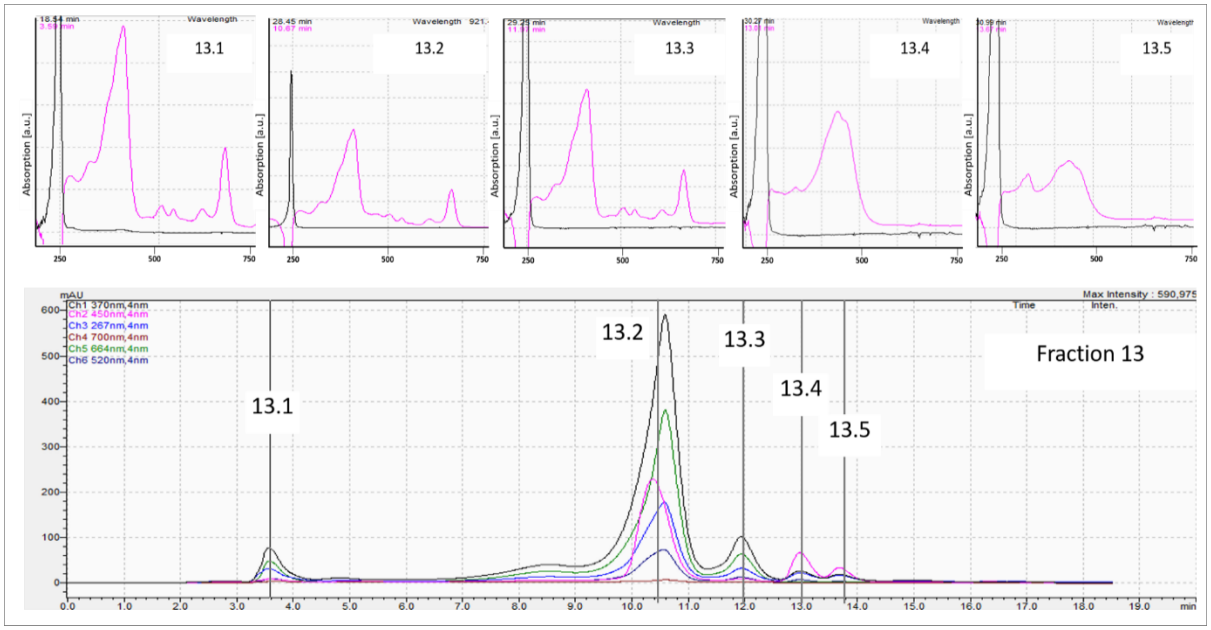

Figure 4.15: LC-Chromatogram of fraction 13 with absorption spectrum profiles

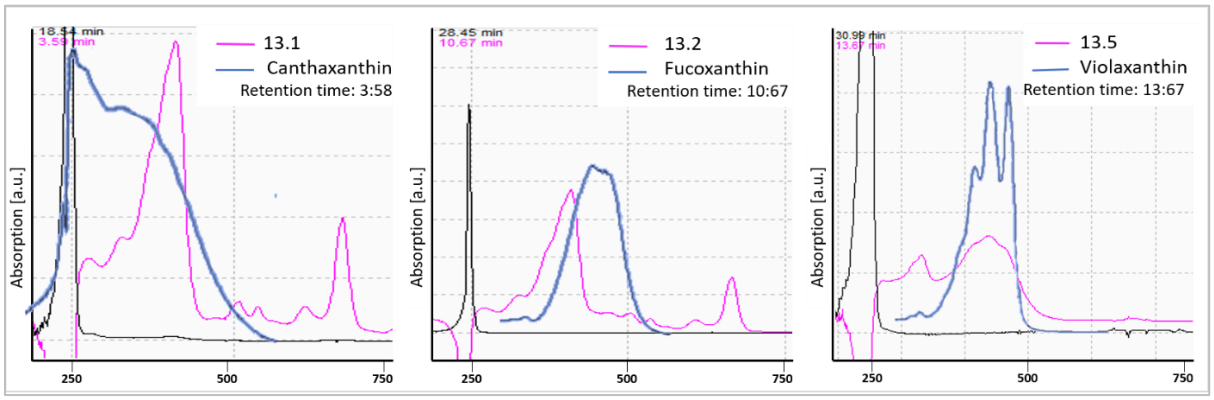

Figure 4.16: Peaks absorption spectrum profiles of three peaks of the chromatogram of F13, in comparision with carotenoid spectrum profiles with retention times as similar as possible with that of a carotenoid used as standard.

Since the retention times and the respective spectrum of the peaks in the fractions and those of pure carotenoids did not match, comparisons were made with spectra found in the literature, such as those of chlorophyll *c1* and *c2*, and the carotenoids diadinoxanthin, diatoxanthin, violaxanthin, antheraxanthin and zeoxanthin (Figure 4.17). Again, no matches were found.

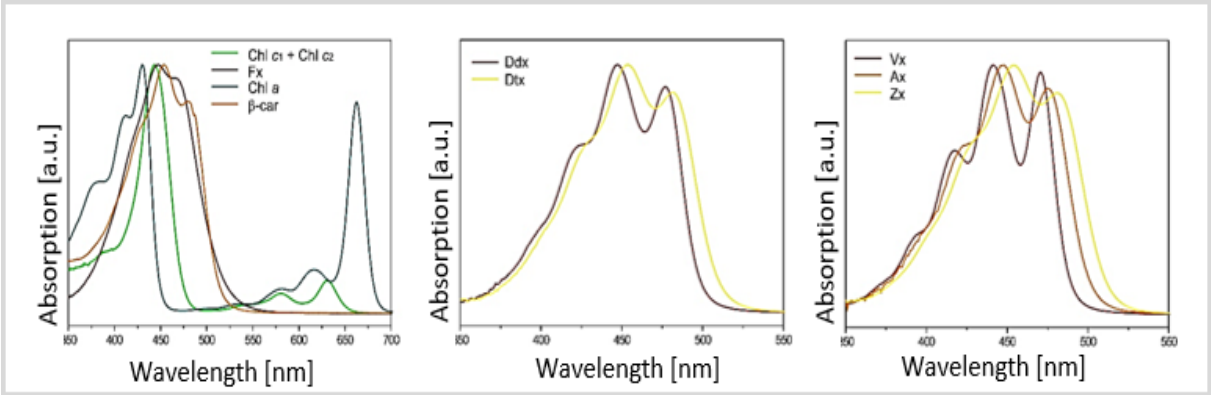

Figure 4.17: Carotenoids'and Chlorophylls' absorption spectrum profils; Chl *c1*, *c2:* Chlorophyll *c1* & *c2* Fx: Fucoxanthin; Chl *a*: Chlorophyll *a*; β-car: β-carotene; Ddx: Diadinoxanthin; Dtx: Diatoxanthin; VX: Violaxanthin, AX: Antheraxanthin; Zx: Zeoxanthin (adapted from Kuczynska, Jemiola-Rzeminska, and Strzalka 2015).

The color of the fractions does not necessarily have to be caused by carotenoids and chlorophyll. Nature offers other colour-intensive compounds, such as flavonoids. They have been used to dye fabrics for centuries (Deveoglu & Karadag, 2019) and are an integral part of traditional medicine, as they mainly have antioxidant and also strong anti-inflammatory effects (Ginwala et al., 2019). Phenolic compounds (PC) are secondary metabolites. PC include flavonoids and non-flavonoids as also phenolic acids (la Rosa et al., 2019). PC often have two wavelength ranges of UV-vis absorbtion (Table 4.10 and Figure 4.18) (Versari et al., 2012), which would match with absorption peaks of fraction 10 and 13. Goiris et al. (2014) screened microalgae for flavonoids in their work in 2014 and studied the corresponding biosynthetic pathways. Probably one of the most suitable analyzes for the accurate determination of phenolic compounds is UHPLC-MS-MS (Goiris et al., 2014). Hence, further analysis are needed to confirm the identity of the compounds present in the active fractions.

Table 4.10: Orientation values of UV-vis absorption spectra of phenolic compounds, especially in the case of anthocyanins, depending on the pH (adapted from Versari, Parpinello, and Laghi, 2012).

UV–vis portion of the electromagnetic spectrum of phenolic compounds	
phenolic compound	**absorbance**
anthocyanins	267–275 nm and 475–545 nm
benzoic acids	235–305 nm
hydroxycinnamic acids	227–245 nm and 310–332 nm
flavonols	250–270 nm and 350–390 nm
flavan-3-ols (catechins)	280 nm

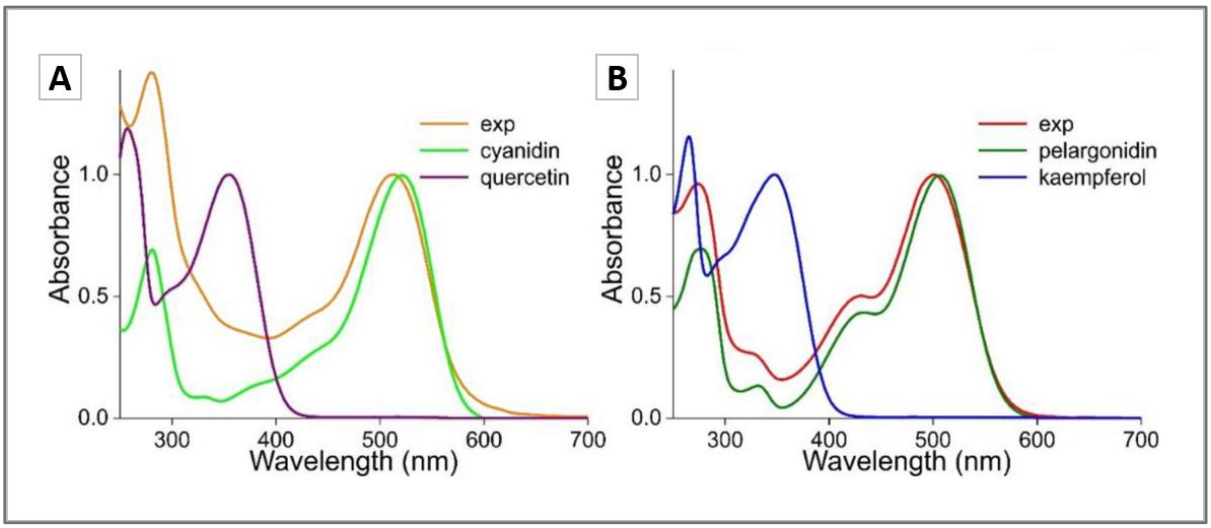

Figure 4.18: Absorbance spectra of example flavonoids, A: Cyanidin, quercetin and an example from a flower extract, B: Pelargonidin, Kaempferol and an example from a flower extract (adapted from Cheung et al. 2019).

5 Conclusion and further Perspectives

The aim of this dissertation was to valorize the biomass of three marine diatoms, *Cylindrotheca closterium*, *Cylindrotheca fusiformis* and *Nanofrustulum shiloi*, which was done through the identification of their potential for producing compounds with anti-inflammatory activity for the pharmaceutical industry.

During the scale-up and cultivation of *C. closterium* and *C. fusiformis*, under laboratorial conditions, *C. fusiformis* cultures showed better growth performance than *C. closterium* cultures and, from this point of view, *C. fusiformis* would be recommended for cultivation rather than *C. closterium*.

The results obtained in relation to anti-inflammatory potential, suggest that all 3 investigated diatoms have compounds with anti-inflammatory properties. In particular, the ethanolic extracts of *C. fusiformis* and *N. shiloi* showed good to very good anti-inflammatory activities in relation to inhibition of COX and ACE. Based on these results, it seems appropriate to further investigate both microalgal extracts. The results of the GC-MS analysis suggest that various *n*-3 and *n*-6 PUFA containing compounds are among the substances responsible for the ACE and COX-2 inhibitory effects. In order to achieve the most comprehensive possible detection of compounds and thus identify those responsible for the anti-inflammatory activities more precisely, the use of HPLC-MS or UHPLC-MS-MS is indicated for further analyses. Not only *in vitro* assays, but also extensive *in vivo* assays should be used to study the anti-inflammatory and antioxygen mechanism of action in more detail.

6 References

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