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Exploring *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* as natural sources of high-value lipids

DOCTORAL THESIS

Tomásia Micaela Gomez Fernandes
DOCTORATE IN CHEMISTRY - SPECIALITY IN BIOCHEMISTRY

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ORIENTATION
Nereida Maria Abano Cordeiro

Exploring *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* as natural sources of high-value lipids

A thesis presented to University of Madeira with the aim of obtaining a Doctoral degree in Chemistry - Speciality in biochemistry

Tomásia Micaela Gomez Fernandes

Under the supervision of Doctor (PhD) Nereida Maria Abano Cordeiro

Faculty of Exact Sciences and Engineering

University of Madeira

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Resumo

A crescente demanda dos consumidores por fontes naturais e sustentáveis de compostos de valor acrescentado tem levado as comunidades científica e industrial a explorar a biodiversidade das microalgas e a sua diversidade química. Na bioprospecção de microalgas para a produção integrada de biomassa e compostos de valor acrescentado, diversos desafios são encontrados, como o cultivo de novas espécies, a identificação de novas moléculas e a otimização das condições de cultivo para a produção dos fitoquímicos desejados. Este estudo investiga o potencial de duas microalgas inexploradas, *Hemiselmis* cf. *andersenii* e *Pavlova pinguis*, como fontes de lípidos com valor acrescentado para aplicação na indústria nutracêutica e áreas relacionadas. A análise dos extratos lipídicos revelou uma grande diversidade de metabolitos sintetizados pela *H.* cf. *andersenii* e pela *P. pinguis*, nomeadamente ácidos gordos, esteróis, álcoois e monoglicerídeos. *H.* cf. *andersenii* demonstrou ser rica em ómega-3 polinsaturados. Enquanto, a *P. pinguis* revelou ser uma grande produtora de fitoesteróis. Para verificar a robustez das microalgas e induzir a acumulação dos compostos desejados, foram realizados estudos com diferente suplementação de azoto e de fósforo no meio de crescimento das microalgas. As condições repletas de azoto revelaram-se eficazes para modelar a composição lipídica das microalgas para as indústrias nutracêutica e farmacêutica. Aumentos dos teores de fósforo induziram um aumento da produtividade da biomassa e da produção de lípidos nutricionalmente relevantes. Na *P. pinguis*, baixos teores de fósforo mostraram-se eficazes para a produção de carotenoides e açúcares. A *H.* cf. *andersenii* mostrou-se promissora para a coprodução de carotenoides e lípidos. A análise multivariada permitiu visualizar os efeitos intra e interespecíficos dos nutrientes na composição lipídica das microalgas. A grande versatilidade de compostos de valor acrescentado produzidos pelas microalgas estudadas aliadas às suas características biológicas (ausência de parede celular rígida), fazem destas candidatas promissoras para futura produção à escala industrial.

Palavras-chave: Microalgas, Lípidos, Fitoquímicos, Nutracêutica, Compostos de valor acrescentado, Bioprospecção.

Abstract

The active seek of consumers for natural sustainable sources of high-value compounds has driven the scientific and industry communities to exploit microalgal biodiversity and its intrinsic chemical diversity. In the bioprospection of microalgae for integrated biomass and high-value compounds production, several challenges are found, like the cultivation of new species, the identification of new molecules, and the optimization of culture conditions to produce the desired phytochemicals. The present work aimed to investigate the potential of two unexplored microalgae, *Hemiselmis* cf. *andersenii* e *Pavlova pinguis*, as sources of high-value lipids for further application in nutraceutical and other related fields. The detailed analysis of lipid extracts revealed a great diversity of metabolites synthesized by *H.* cf. *andersenii* and *P. pinguis*, namely fatty acids, sterols, alcohols, and monoglycerides. *H.* cf. *andersenii* was shown to be a rich source of omega-3 polyunsaturated fatty acids. Meanwhile, *P. pinguis* was found to be a high-level producer of phytosterols. To evaluate the robustness of the microalgae and induce its production on high-value lipids, experiments were performed by inducing changes in nitrogen and phosphorus levels in microalgae growth medium. In both microalgae nitrogen-replete conditions were the most effective for modeling the lipid composition for nutraceutical and pharmaceutical industries. Increases in phosphorus concentrations prompted an increase in microalgal biomass productivity, as well as the production of nutritionally relevant lipids. In *P. pinguis*, low phosphorus concentrations were effective to produce carotenoids and sugars. *H.* cf. *andersenii* have shown to be suitable for the co-production of carotenoids and lipids. Multivariate analysis allowed to visualize the differential intra- and inter- specific effects of nitrogen and phosphorus on microalgal lipid composition. The great versatility of value-added compounds produced by the studied microalgae combined with its biological properties (absence of a rigid cell wall) make these microalgae promising candidates for future large-scale production.

Keywords: Microalgae, Lipids, Phytochemicals, Nutraceutical, High-value compounds, Bioprospection.

List of publications

Journal papers

Associated with this thesis

- Fernandes, T.; Martel, A.; Cordeiro, N. Exploring *Pavlova pinguis* chemical diversity: a potentially novel source of high value compounds. Sci. Rep. **2020**, 10, 339. https://doi.org/10.1038/s41598-019-57188-y.

- Fernandes, T.; Cordeiro, N. *Hemiselmis andersenii* and *Chlorella stigmatophora* as new sources of high-value compounds: a lipidomic approach. J. Phycol. **2020**, 56 (6), 1493–1504. https://doi.org/10.1111/jpy.13042.

- Fernandes, T.; Ferreira, A.; Cordeiro, N. Comparative lipidomic analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* in response to nitrogen-induced changes. Algal Res. **2021**, 58, 102417. https://doi.org/10.1016/j.algal.2021.102417.

- Fernandes, T.; Cordeiro, N. High-value lipids accumulation by *Pavlova pinguis* as a response to nitrogen-induced changes. Under review on Biomass Bioenergy. Manuscript ID: JBAB-D-21-00796.

- Fernandes, T.; Cordeiro N. Effects of phosphorus-induced changes on the growth, nitrogen uptake, and biochemical composition of *Pavlova pinguis* and *Hemiselmis* cf. *andersenii*. Under review on J. Appl. Phycol. Manuscript ID: JAPH-D-21-00565.

- Fernandes, T.; Cordeiro N. Microalgae as sustainable biofactories to produce high-value lipids: biodiversity, exploitation, and biotechnological applications. (Submitted manuscript). Under review on Mar. Drugs. Manuscript ID: marinedrugs-1413351.

Complementary work

- Fernandes, T.; Fernandes, I.; Andrade, C. A. P.; Cordeiro, N. Assessing the impact of sulfur concentrations on growth and biochemical composition of three marine microalgae. J. Appl. Phycol. **2020**, 32 (2), 967–975. https://doi.org/10.1007/s10811-019-01946-y.

- Fernandes, I.; Fernandes, T.; Cordeiro, N. Nutritional value and fatty acid profile of two wild edible limpets from the Madeira Archipelago. Eur. Food Res. Technol. **2019**, 245, 895–905. https://doi.org/10.1007/s00217-019-03234-y.

- Fernandes, I.; Leça, J. M. J. M.; Aguiar, R.; Fernandes, T.; Marques, J. C. J. C.; Cordeiro, N. Influence of crop system fruit quality, carotenoids, fatty acids and phenolic compounds in cherry tomatoes. Agric. Res. **2021**, 10 (1), 56–65. https://doi.org/10.1007/s40003-020-00478-z.

Oral presentations

Associated with this thesis

- Fernandes, T.; Cordeiro, N. Bioprospecting for new microalgal strains to health-promoting phytochemicals production: a lipidomic approach. Algaeurope 2020. Online event: December 1-4, **2020**.

Poster presentations

Associated with this thesis

- Fernandes, T.; Cordeiro, N. Modulation of lipid profiles in two marine microalgae under different phosphorus regimes for high-value lipids accumulation. XV Encontro de Química dos Alimentos. Funchal, Madeira Island: September 5-8, **2021**.

- Fernandes, T.; Cordeiro, N. Unravelling extracellular and intracellular monossacharide composition of three marine microalgae. AlgaEurope 2020. Online event: December 1-4, **2020**.

- Fernandes, T.; Martel, A.; Cordeiro, N. Gas Chromatography-Mass Spectrometry-based metabolite profiling of *Hemiselmis andersenii* and *Chlorella stigmatophora*: potential sources of health promoting phytochemicals. 2nd Food Chemistry Conference: Shaping the Future of Food Quality, Safety, Nutrition and Health. Seville, Spain: September 17 – 19, **2019**.

- Fernandes, T.; Cordeiro, N. Screening for biologically active metabolites in a marine microalga (*Pavlova pinguis*). Natural products in drug discovery and human health – PSE Meeting. Lisbon, Portugal: July 28 – 31, **2019**.

Complementary work

- Góis, A.; Fernandes, T.; Faria, D.; Andrade, CAP; Quintana, A.; Cordeiro, N. Adaptation of *Rhodomonas marina* to environmental induced stress conditions. AlgaEurope. Paris, France: December 3-5, **2019**.

Technical courses

- "Curso de formación de técnicos en colecciones de cultivo de microalgas" provided by Banco Español de Algas. Gran Canaria, Spain: September 12-14, **2017**.

List of abbreviations

μ	Growth rate
AA	Aliphatic alcohols
AH	After alkaline hydrolysis
ALA	9,12,15-Octadecatrienoic acid (C18:3ω3)
ANOVA	Analysis of variance
ARA	5,8,11,14-Eicosatetraenoic acid
ASG	Acylated steryl glycosides
AT	Austria
BE	Belgium
BEA	Spanish Bank of Algae
BG	Bulgaria
BH	Before alkaline hydrolysis
BHT	Butylated hydroxytoluene
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
C	Carbohydrates
CAGR	Compound anual growth rate
CH	Switzerland
Chl	Chlorophyll
CHO-K1	Chinese hamster ovary cells K1
CZ	Czech Republic
Car, C_t	Total carotenoids
CosIng	Cosmetic ingredient database
Con A	Concanavalin A
COX-2	Cyclooxygenase
d	Day
DA	Diacids
DCW	Dry cell weight
DE	Germany
DHA	4,7,10,13,16,19-Docosahexaenoic acid (C22:6ω3)
DK	Denmark
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
DOXP/MEP	1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	Dry weight
EE	Estonia
EETs	Epoxyeicosatrienoic acids
EL	Greece

EPA	5,8,11,14,17-Eicosapentaenoic acid (C20:5ω3)
ES	Spain
EU	European Union
FA	Fatty acids
FDA	Food and drug administration
FO	Faroe Islands
FR	France
FTIR	Fourier transform infrared spectroscopy
FTIR-ATR	Fourier transform infrared spectroscopy with attenuated total reflectance
FS	Free sterol
GC-MS	Gas chromatography-mass spectrometry
GPP	Geranyl pyrophosphate
GR	Greenland
H/H	Hypocholesterolaemic/hypercholesterolaemic fatty acids ratio
HCA	Hierarchical cluster analysis
HETEs	Hydroxyeicosatetraenoic acids
HN	High nitrogen
HP	High phosphorus
HPI	Health promoting index
HU	Hungary
IC	Iceland
IE	Ireland
iNOS	Nitric oxide synthase
IPP	Isopentenyl pyrophosphate
IS	Internal standard
IT	Italy
L	Lipids
LA	9,12-Octadecadienoic acid (C18:2ω6)
LCAA	Long-chain aliphatic alcohols
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LN	Low nitrogen
LP	Low phosphorus
LPS	Lipopolysaccharide
LV	Latvia
MG	Monoglycerides
MN	Medium nitrogen
MP	Medium phosphorus
MVA	Mevalonic acid
MUFA	Monounsaturated fatty acids

MW	Molecular weight
n.d.	Non detected
NL	The Netherlands
NO	Norway
OT	Other compounds
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
P/E	Protein / Enzymes
PG	Prostaglandins
Pig.	Pigments
PT	Portugal
PUFA	Polyunsaturated fatty acids
PUFA/SFA	Polyunsaturated to saturated fatty acids ratio
RCC	Roscoff culture collection
SA	6,9,12,15-Octadecatetraenoic acid
SE	Sterol fatty acid ester
SFA	Saturated fatty acids
SG	Steryl glycosides
SQDGs	Sulfoquinovosyl diacylglycerols
ST	Sterols
TFA	Total fatty acids
TMS	Trimethylsilyl derivatives
TNF-*α*	Tumor necrosis factor alfa
UK	United Kingdom
UV	Ultraviolet
V	Vitamins
Σω6/Σω3	Ratio between the total of omega-6 and omega-3 fatty acids
Σω3/Σω6	Ratio between the total of omega-3 and omega-6 fatty acids

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CHAPTER 1.

Introduction

This Chapter is based on the following publication:

Fernandes, T.; Cordeiro N. Microalgae as sustainable biofactories to produce high-value lipids: biodiversity, exploitation, and biotechnological applications. Under review on Mar. Drugs. Manuscript ID: marinedrugs-1413351.

Chapter 1. Introduction

Abstract

Microalgae are often called “sustainable biofactories” due to its dual potential to mitigate atmospheric carbon dioxide and produce a great diversity of high-value compounds. Nevertheless, the successful exploitation of microalgae as biofactories for industrial scale is dependent on choosing the right microalga and optimum growth conditions. Due to microalgae rich biodiversity, a screening pipeline should be developed to perform microalgal strain selection exploring their growth, robustness, and metabolite production. Current prospects in microalgal biotechnology are turning their focus to high-value lipids for pharmaceutic, nutraceutic, and cosmetic products. Within microalgal lipid fraction, polyunsaturated fatty acids and carotenoids are broadly recognized for their vital functions in human organisms. Microalgal derived phytosterols are still an underexploited lipid resource despite presenting promising biological activities. including neuroprotective, anti-inflammatory, anti-cancer, neuromodulatory, immunomodulatory, and apoptosis inductive effects. To modulate microalgal biochemical composition, according to the intended field of application, it is important to know the contribution of each cultivation factor, or their combined effects, for the wanted product accumulation. Microalgae have a vital role to play in future low-carbon economy. Since microalgal biodiesel is still costly, it is desirable to explore the potential of oleaginous species for its high-value lipids which present great global market prospects.

Keywords: Microalgal biodiversity, Industrial valorization, Microalgal biotechnology, High-value lipid, Polyunsaturated fatty acids, Phytosterol, Carotenoids

1. Introduction

Microalgae uses light energy and inorganic nutrients to produce oxygen and biomass rich in a diversity of value-added compounds (Figure 1.1) [1]. They can thrive in almost all environments and can be found in oceans, brackish water, freshwater, rocks, and soils [2]. Microalgal biotechnological historical data goes back to the Aztec population which harvested *Arthrospira* from lake Texcoco for food purposes [2,3]. *Arthrospira* has also been collected by local people surrounding Lake Chad and consumed as a nutritional supplement called “dihe” [2]. *Nostoc* species have been used by the Chinese as a food delicacy and for their properties for hundreds of years [2].

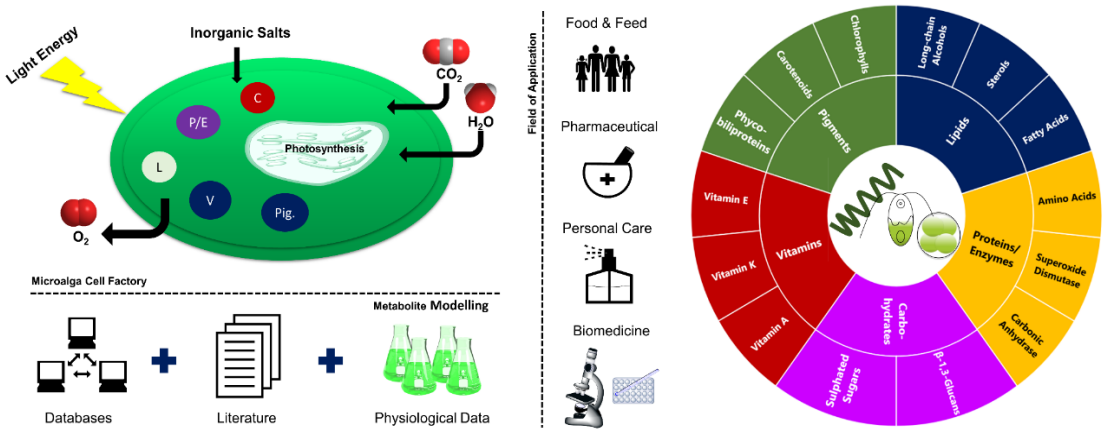

Figure 1.1. Uncovering the potential of a microalga as a bio-factory. P/E – Protein / Enzymes; C – Carbohydrates; L – Lipids; V – Vitamins; Pig. – Pigments.

Arthrospira platensis, *Aphanizomenon flosaquae* var. flosaquae, *Chlorella luteoviridis, Chlorella pyrenoidosa*, *Chlorella vulgaris*, and *Auxenochlorella protothecoides* have been on the market as a food or food ingredient and consumed to a significant degree before 15 May 1997 in European Union market, thus its access to the market is not subject to the Novel Food Regulation (EU) 2015/2283 [4]. Dried *Tetraselmis chuii*, *Odontella aurita*, and astaxanthin-rich oleoresin from *Haematococcus pluvialis* are microalgal products approved as novel food and, as the microalgae listed previously, are within the list of microalgae which can be commercialized in European Union (EU) [5]. Through Figure 1.2a it is possible to visualize that in most European countries, algae farmers mainly produce microalgal species belonging to Cyanobacteria and Chlorophyta phyla, except for Belgium (BE), Norway (NO), and Sweden (SE). This is consistent with the microalgae approved for human consumption which belong to Chlorophyta and Cyanobacteria phyla, except for *Odontella aurita* which belongs to Bacillariophyta phylum.

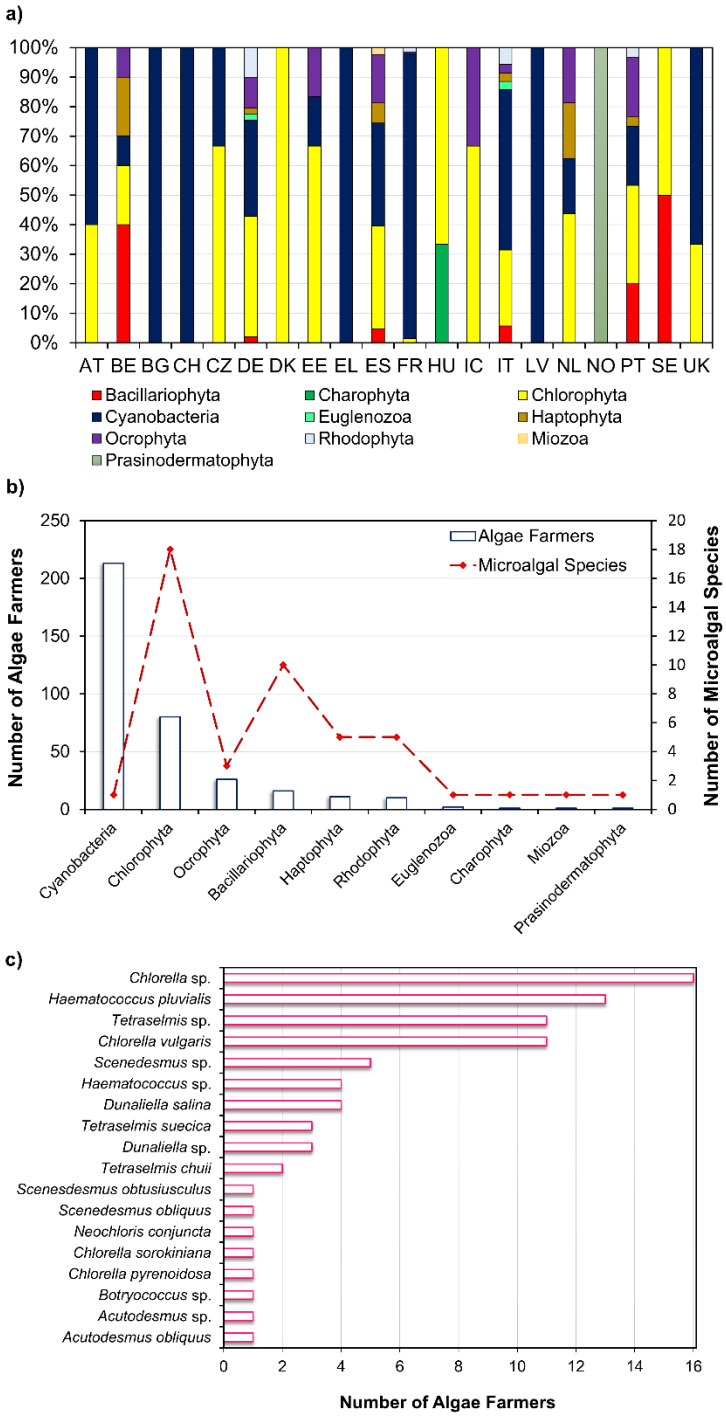

Figure 1.2. Microalgae production in Europe: a) Relative abundance of microalgae at phylum level produced by algae farmers (AT – Austria; BE – Belgium; BG – Bulgaria; CH – Switzerland; CZ – Czech Republic; DE – Germany; DK – Denmark; EE – Estonia; EL – Greece; ES – Spain; FO – Faroe Islands; FR – France; GR – Greenland; HU – Hungary; IC – Iceland; IE – Ireland; IT – Italy; LV – Latvia; NL – The Netherlands; NO – Norway; PT – Portugal; SE – Sweeden; UK - United Kingdom); **b)** Number of algae farmers against main microalgae phyla and diversity of species exploited; **c)** Diversity of Chlorophyta species produced by algae farmers. Based on EMODnet database [6].

Health, energy, and human nutrition are the three main applications for microalgal products [7]. However, the energy production from microalgae is experiencing a slow growth

compared to other segments [7]. Through Figure 1.3a it is possible to observe that both patents and research activities presented an exponential increase between 2004 and 2014. This trend continued for research activities, in contrast to patent publications. These waves of microalgae related research and development activities are mainly associated with energy-driven trends, and having as the driving force the high crude oil price [8]. After 2015 this driving force was lost due to the development and popularization of electric cars [8]. Fig. 1.3b shows that research activities were mainly focused on the biological sciences before 90's. From this decade forward biotechnology applied microbiology appeared in the main research categories meeting the slight increase in patent and research publications. Therefore, the co-production of several metabolites by microalgae and the growing interest in microalgae by researchers in the field of biotechnology brings the hope to meet the next wave of microalgae industry-related activities.

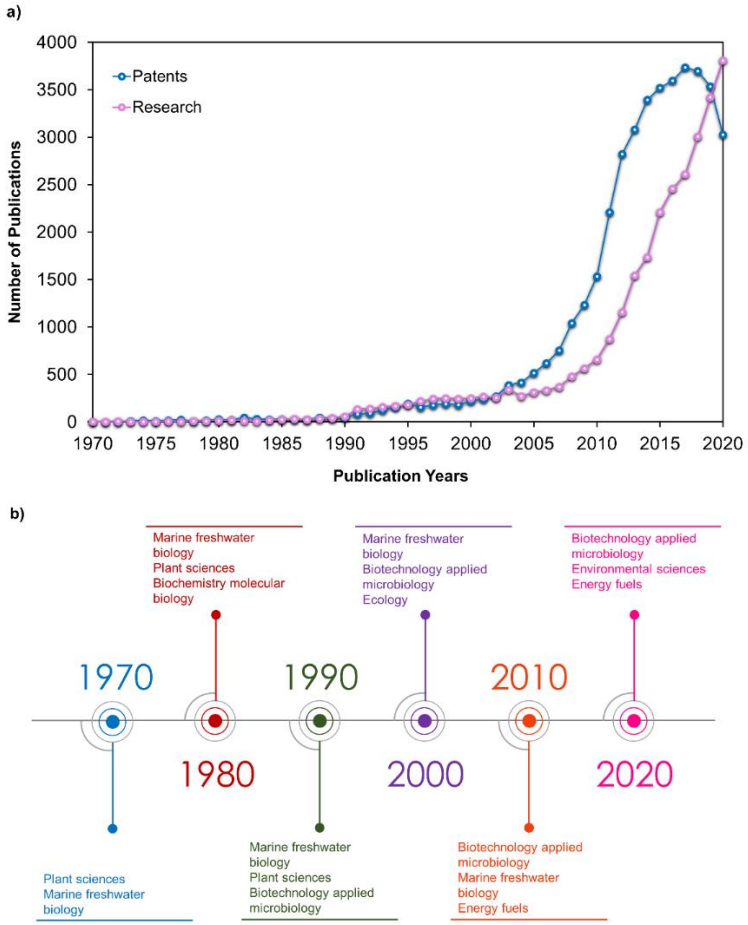

Figure 1.3. Patenting and research activity in the microalgal field: a) Numbers of microalgae-related patents and research publications against publication years; b) Timeline with the main research activities categories according to Web of Science. The information used to construct these plots using "microalgae" as topic can be found in Espacenet [9] and Web of Science databases [10].

According to the International Energy Agency [11], global energy-related carbon dioxide (CO_2) emissions remained at 31.5 Gt, despite the decline in 2020. CO_2 contributes up to 68% of total greenhouse gases which accumulation in the atmosphere has been considered as the main driver of climate changes [12]. Presenting higher carbon dioxide fixation efficiencies than

terrestrial plants, microalgae have a pivotal role to play in future low-carbon economy [12,13]. In literature, it is estimated that producing 280 t of microalgal dry biomass per ha per year using 9% of the incoming solar energy fixes roughly 513 t of CO_2 [14]. In this sense, microalgae are often called "sustainable biofactories" due to its dual potential to mitigate/ bioremediate atmospheric CO_2, and produce a wide array of high-value compounds, which can be further enhanced through induced changes in its growth conditions.

Through Fig. 1.2b it is possible to visualize that the divisions comprising the greatest diversity of microalgal species industrially exploited are Chlorophyta (18) and Bacillariophyta (10) phyla. According to Griffiths et. al [15] most microalgal species considered for biofuel production are either Chlorophyta or Bacillariophyta which may explain the previous observation. In the North-West European algae strategic initiatives, the bioenergy (*e.g.* biodiesel) market is the most mentioned. However, the lowest added value and the high production costs from high lipid microalgal species make this marked not viable economically [16]. Therefore, current prospects of algal biotechnology are turning their focus to high-value lipids production, namely polyunsaturated fatty acids (PUFA) which can be used in dietary supplements, functional food, pharmaceutical, and infant formula segments [17]. Some companies already produce ω3-PUFA with microalgal origin as dietary supplements or food ingredients (Oceans Alive (USA), Blue Biotech (Germany) Flora Health (USA) – *Nannochloropsis*; InnovalG (France) – *Odontella*) [18]. The global market size for ω3-PUFA in 2020 was estimated at US $ 16.2 billion, and its 2027 value projections are expected to reach US $ 36.9 billion at a compound annual growth rate (CAGR) of 12.5 over the forecast period of 2020-2027 [19].

Within Chlorophyta phylum it is possible to observe that most algae farmers have been focusing on the production of oleaginous microalgae species, namely from *Chlorella*, *Tetraselmis*, *Botryococcus*, *Scenedesmus* genera, which are more specialized for biofuel production [7]. *Chlorella* sp., *Haematococcus pluvialis*, *Tetraselmis* sp., and *Chlorella vulgaris* are on the first line of microalgal species produced by algae farmers (Fig. 1.2c). These microalgae have in common its versatility which allows to apply these in food, feed, energy production, and as a source of high-value molecules, which may reflect the efforts of algal farmers to target more than one market [16,20,21]. Furthermore, species belonging to *Haematococcus* and *Dunaliella* genera are mainly used for pigments exploitation namely for astaxanthin and ***β***-carotene production, respectively [7]. Protein is on the first line of development in human nutrition and health sectors, followed by pigments and lipids [7]. Having in account the large number of oleaginous species already produced by algae farmers, and the potentialities of microalgal lipids for high-value market, this review outlines the potential of microalgal high-value lipids for dietary supplements, cosmetics, and pharmaceutics, along with its health-promoting activities and optimization strategies.

2. Species selection and exploitation

Microalgae present a rich biodiversity comprising 40,000 – 50,000 described species with an estimation of nearly 800,000 existing species [22]. Chlorophyta comprises 6,952 algal species [23], and Bacillariophyta is the most diversified group within microalgae, with more than 10,000 diatom species being described [24]. Despite this great diversity only few microalgal species have been exploited for biotechnological applications, being produced by European algae farmers only 18 species for Chlorophyta and 10 species for Baccilariophyta phyla (Fig. 2b). A smaller number of species is recorded for the other phyla: Cyanobacteria, Ocrophyta, Haptophyta, Rhodophyta, Euglenozoa, Charophyta, Miozoa, and Prasinodermatophyta.

The successful exploitation of microalgae as biofactories for industrial scale is dependent on the selection of the right microalga considering specific culture conditions and desired product content [3]. This could be done through an exhaustive screen of scientific data through data bases and literature, followed by physiological data collection (Fig. 1.1). With respect to the product synthesized by microalgae, they can be applied in several fields from bioenergy (low-value market) to cosmetics and pharmaceutics (high-value market).

When exploiting the great biodiversity of microalgae as new natural sources of high-value phytochemicals, a screening-pipeline should be developed including the following key aspects: *i)* **growth** – for the successful improvement and progression of microalgae-based industries the discovery and improvement of new fast-growing strains is essential [25]. Thus, the study of maximum growth rate, maximum cell density, and dry cell biomass at different growth conditions, along with their amenability for heterotrophic/mixotrophic grow are critical features to predict the feasibility of microalgae for large scale production [26]; *ii)* **robustness** – the selected microalgae should withstand variable local climatic conditions, and be resistant to possible infections (*e.g.* other algae strains, grazers, bacteria or viruses) in order to prevent large scale crashes [27]. Nevertheless, strategies have been developed to combat and prevent contamination in microalgae cultivation like the use of extreme conditions to create an unfavorable environment for the competitive organisms of the microalgae [28,29], and the use of allelopathic approaches to control microalgae cultivation [30]; *iii)* **metabolite production –** in the production of microalgae for food and health purposes the potential toxicity of some species, as well as desired product accumulation should be considered, these are discussed below.

2.1. Phycotoxins

Phycotoxins are causative agents of seafood-borne poisoning syndromes (*e.g.* ciguatera fish poisoning) in humans [31]. From the wide microalgal diversity only around 200 species are health-threatening, with the main toxic microalgae belonging to dinoflagellates and diatoms groups [32]. Moreover, contamination of microalgae-based products resultant from unsuitable location of cultivation ponds (*e.g.* inflows of effluents containing pollutants) is a concern for human health [33]. Therefore, standard guidelines provided by international regulatory organizations, such as Food and Drug administration (United States of America) and European Food Safety Authority (Europe), guarantee that microalgae-based industries operate in

conformity with safety requirements [32]. Through Table 1.1 is possible to have a brief insight on the regulations and directives applied in European Union.

Table 1.1. Insight on the legislation available in European Union to regulate food market.

	Regulations and Directives	Brief description	Ref.
Food supplements	Directive 2002/46/EC	• Sets-out rules on food supplements to protect consumers • Ensure that products are not provided with misleading information	[34]
New (novel) foods	Regulation (EU) 2015/2283	• Rules for the placing of novel foods on the market in the EU	[35]
Feed additives	Regulation (EC) No 1831/2003	• Standardized procedure for authorizing feed additives Rules for labelling, placing on the market and use	[36]
Foods for specific groups	Regulation (EU) No 609/2013	• Sets-out compositional and labelling requirements for infant formulae Follow-on formulae intended for use by Community infants in good health	[37]
Nutrition and health claims on food	Regulation (EC) No 1924/2006	• Ensure a high level of consumer protection Give the consumer the necessary information to make choices Create equal conditions of competition for the food industry	[38]
Medicinal products	Regulation (EC) No 726/2004	• Guarantee high standards of quality and safety of medicines in the EU	[39]

2.2. High-value products

Microalgae can be produced targeting different fields of application such as food and feed, pharmaceutical, personal care, and biomedicine (Fig. 1.1). This versatility is derived from its ability to synthesize a multiplicity of metabolites distributed among pigments, vitamins, carbohydrates, lipids, and proteins/enzymes (Fig. 1.1). Besides, several biological properties attributed to microalgae, namely anti-inflammatory [40,41], anti-pyretic [41], and anti-cancer [42] activities, have been showing its potential to high-value compounds production.

Through Cosmetic Ingredient Database (CosIng) [43] it is possible to see that several substances with microalgal origin have already been authorized, namely the oils from *Odontella aurita*, *Nannochloropsis oceanica*, *Chlorella minutisssima*, *Chlorella protothecoides*, and *Haematococcus pluvialis*. These can be incorporated into cosmeceuticals functioning as skin conditioning-emollient, skin protecting, and anti-oxidant in cosmetic products [43]. Moreover, when analyzing research activities performed on microalgal production for food and health purposes, two main groups of metabolites stand out, fatty acids and carotenoids (Figure 1.4). These are mainly related with the lipid-soluble fraction of microalgae.

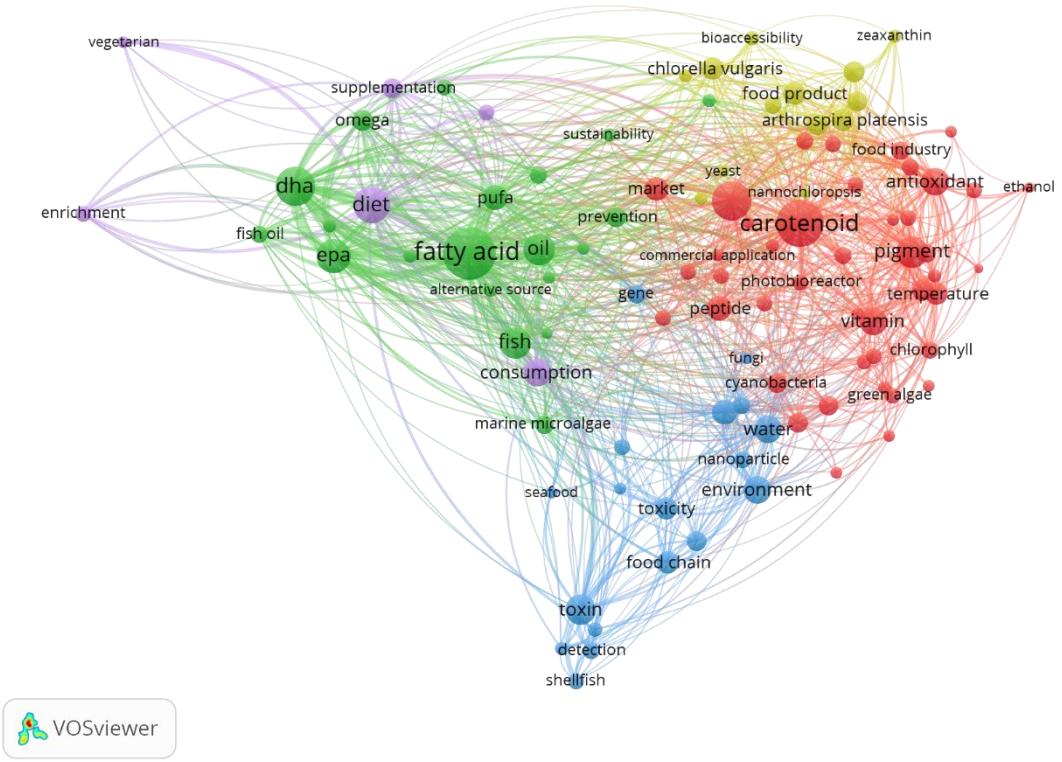

Figure 1.4. Concept's network obtained with VOSviewer software [44] for the research on "microalgae AND food AND health" in web of science database [10].

3. Polyunsaturated fatty acids exploitation from autotrophic microalgae

Polyunsaturated fatty acids (PUFA) are broadly known for their vital functions in the human organisms [45]. For instance, 4,7,10,13,16,19-docosahexaenoic acid (DHA, C22ω3) is enriched in human milk, plasma, and sperm, and along with 5,8,11,14-eicosatetraenoic acid (ARA, C20:4ω6) is concentrated in the membrane lipids of gray matter and in the visual elements of retina [46]. There is a lot of evidence that an adequate supply of these fatty acids improves visual acuity and infant cognitive development [46]. Moreover, several therapeutic properties have been attributed to the consumption of these fatty acids such as reduced risk of arthritis and cardiovascular diseases [47]. In contrast to microalgae, mammals do not have the ability to convert C18:1ω9 to the precursors of LC-PUFA biosynthesis pathway, and they poorly synthesize C20-C22 PUFA from dietary 9,12-octadecadienoic acid (LA, C18:2ω6) and 9,12,15-octadecatrienoic acid (ALA, C18:3ω3).

3.1. PUFA synthesis by microalgae

Long chain – polyunsaturated fatty acids (LC-PUFA) biosynthesis pathways by microalgae are initiated by Δ12 desaturation of 9-octadecenoic acid (C18:1ω9), producing LA, which might be further desaturated by a ω3-desaturase generating ALA, Figure 1.5 [48]. The ω3-pathway is initiated with the Δ6 desaturation of LA and leads to the synthesis of ω3-LC-PUFA 5,8,11,14,17-eicosapentaenoic acid (EPA, C20:5ω3) and DHA, whereas the ω6-pathway

initiates with the Δ6 desaturation of ALA and produces the ω6-LC-PUFA, ARA [48]. However, some EPA-producing Ocrophyta, such as *Nannochloropsis* sp., and *Monodus subterraneus*, are thought to preferentially synthesize EPA via the ω6-pathway by the action of a ω3-desaturase which catalyzes the conversion of ARA to EPA [48,49].

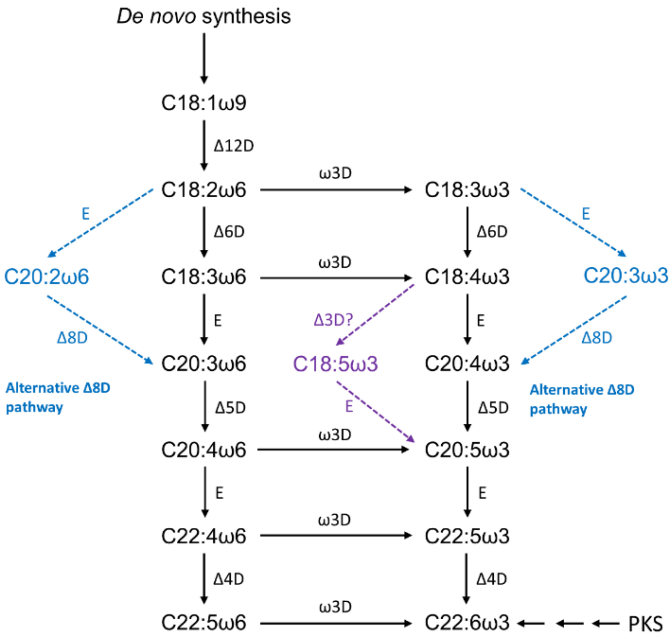

Figure 1.5. Biosynthesis of long-chain polyunsaturated fatty acids in microalgae [45,48,50]. ΔxD – "front-end" desaturase, adds a double bond at position x from carboxyl end; ωyD – "methyl-end" desaturase, adds a double bond at position y from methyl end; E – elongase which catalyzes carbon chain extension; PKS – Polyketide synthase.

As with other organisms, microalgal fatty acid composition is known to vary among the different phylogenetic groups [48]. In Fig. 1.5 is possible to visualize an alternative route for LC-PUFA biosynthesis, Δ8 desaturase pathway. According to Khozin-Goldberg [45] this pathway is known to exist in some microalgae, namely in *Isochrysis galbana* (Haptophyta), *Pavlova salina* (Haptophyta), and *Emiliana huxleyi* (Haptophyta), and the *Euglena gracilis* (Euglenozoa). For the DHA-producing *Isochrysis galbana* a gene encoding a C22-Δ4 polyunsaturated fatty acid specific desaturase has been isolated and characterized [51]. The *Lobosphaera incisa* (Chlorophyta) is a rare case in which AA is the major product of LC-PUFA biosynthesis in microalgae [45].

3.2. PUFA role in human health

From microalgal lipids, PUFA are the most studied for their pharmacological potential. In human health, C20-C22 PUFA play important roles in many physiological and pathological processes [47]. Moreover, most of PUFA health-benefits are due to their key roles as lipid mediators in inflammatory processes and as important compounds for growth and development.

EPA and DHA are parent compounds of specialized pro-resolving lipid mediators (protectins, resolvins, and maresins) that act as inflammatory brakes and promote the return of the affected site to homeostasis (Fig. 1.6) [52,53]. As with ω3-PUFA, ARA-derived lipoxins and their carbon 15 position epimers have beneficial effects on inflammation and resolution [52].

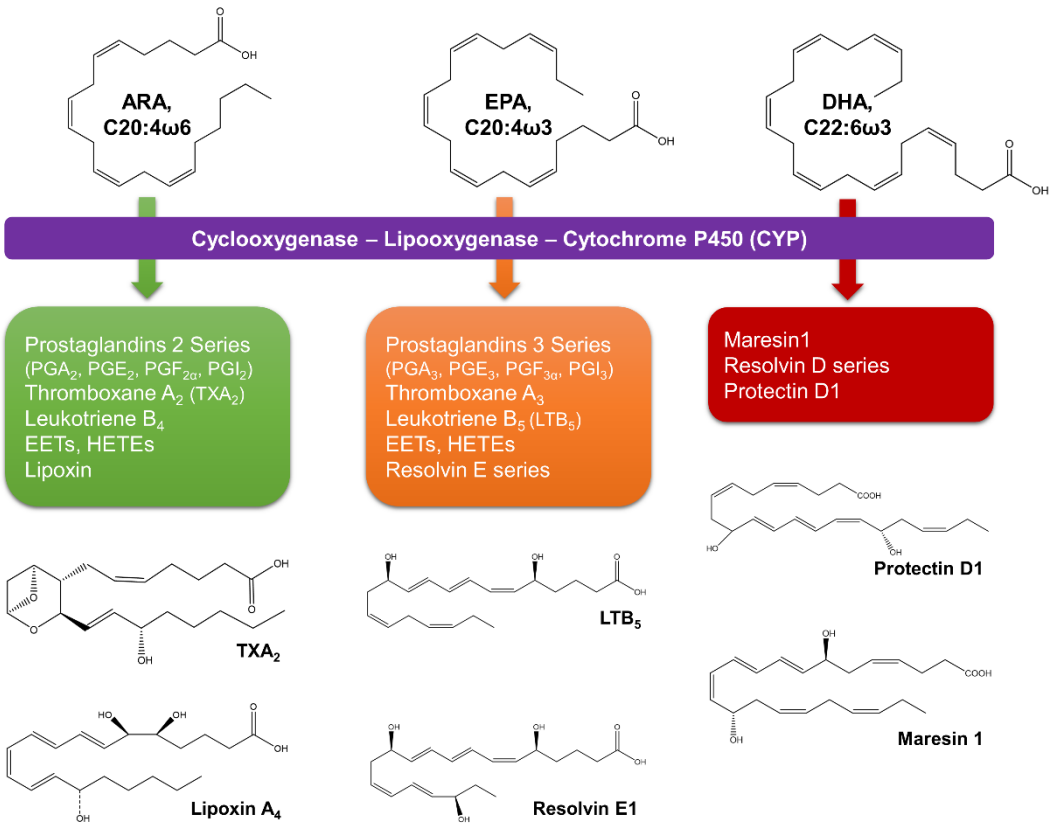

Figure 1.6. Lipid mediators of inflammatory process derived from arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [53,54]. EETs - epoxyeicosatrienoic acids; HETEs - hydroxyeicosatetraenoic acids; PG – prostaglandin.

EPA competitively inhibit the utilization of ARA by cyclooxygenasse/lipoxygenases to less pro-inflammatory mediators [55]. In contrast with eicosanoid products from ARA (prostaglandin E2, thromboxane A2, and leukotriene B4), EPA-derived eicosanoids (thromboxane A3, and leukotriene B5) are weak inducers of inflammation and have attenuated platelet – aggregating and vasoconstriction abilities [55]. In mammalian cells, ω6- and ω3-fatty acids are not interconvertible because they lack ω3-desaturase, therefore their balance in the diet is important [55]. In the western diet, the greatest amounts of ω6-fatty acids (ω6:ω3 of 20:1) drives to larger levels of ARA eicosanoids products (*e.g.* prostaglandins, thromboxanes, leukotrienes, and hydroxy fatty acids), causing an imbalance between pro- and anti-inflammatory molecules and shifting the physiological state to one that is proinflammatory, prothrombotic, and proaggregatory [55]. Thus, a balanced intake of ω6- and ω3-fatty acids is crucial.

3.3. Microalgae - PUFA enhancement strategies

Although the largest share of EPA/DHA oil market comes from wild fish, the declining fish stocks, and susceptibility to contamination by pollutants (like mercury) have turned the attention of PUFA exploitation to microalgae [56]. In this sense, the oxidative stability, sustainability, suitability for vegetarians, and the absence of fishy taste/smell are some of the advantages that make microalgae a feasible source for PUFA commercialization [56]. However, the immature production process is one of the weaknesses that must be surpassed for making PUFA exploitation from microalgae a feasible process [56].

New competitors of PUFA exploitation from microalgae are DHA producers belonging to thraustochytrids (*Traustochytrium* sp. and *Aurantiochytrium*) [56]. These unicellular heterothrophic organisms have already been authorized for food/feed/nutraceuticals and are marketed by DSM/Evonik and Source-Omega companies [56]. However, some disadvantages from the exploitation of these organisms for ω3-production (mostly produce DHA; the production chain increases pressure on arable land – since heterotrophic organisms need a sugar input to grow, and produces CO_2) are opportunities for the production of ω3-fatty acids from microalgae[56].

Since microalgae composition is known to vary with growth conditions, the study of strategies for microalgae PUFA enhancement is crucial to overcome the challenge of the undeveloped production process of phototrophic PUFA exploitation from microalgae. Although several strategies have been proposed to enhance microalgae lipids production, most of these have been projected for biofuel production. However, nowadays more attention have been driven towards PUFA production. Conventional approaches for enhancing microalgae lipid accumulation, include: nutrient stress (*e.g.* alterations in nitrogen, phosphorus, and carbon supply) or changes in cultivation conditions (*e.g.* light, and temperature) [57]. The main advantage of nutrient stress is its easy applicability at both lab and large scale cultivation, while cultivation conditions like light have high operational costs and are not easy to control at open cultivation systems [57]. Within nutrient regime alterations, nitrate limitation is a commonly employed strategy to enhance microalgal lipids quantity [57].

As essential constituent of proteins, nucleotides, vitamins, and coenzymes, any changes in nitrogen source and concentrations can trigger growth changes and biochemical remodeling in microalgae species [58–60]. Although the study of Huang et al [61] had the purpose of improving lipids properties of the microalgal strains *Tetraselmis subcordiformis* SHOU-S05, *Nannochloropsis oculata* SHOU-S14, and *Pavlova viridis* SHOU-S16, the insights on the fatty acid composition at different nitrogen concentrations enables to see some trends with respect to PUFA accumulation. Therefore, towards high nitrogen supplementations lipid content decreased whereas PUFA proportion increased for *N. oculata* and *P. viridis*. While for *T. subcordiformis* the highest PUFA percentage was registered at lower nitrogen supplementations as with the highest lipid content. As with Sukenik [62], this data seems to suggest that the growth conditions for maximization of PUFA production are similar to the conditions required to maximize biomass production for *N. oculata* and *P. viridis*.

Other cultivation conditions affecting microalgal growth and chemical diversity are salinity, light intensity, and photoperiod. Mitra et al. [63] studied the effects of these factors in *Nannochloropsis gaditana* CCNM1032 strain, and concluded that the most positive factor for fatty acid enhancement was the photoperiod. In this study maximal EPA productivities were achieved at 60 µmol photons m^{-2} s^{-1} and at photoperiod regime of 18h: 6h (light: dark), this observation was made for 1L cultures.

Two-stage cultivation, and combined nutrient and abiotic stresses are novel approaches used to enhance microalgal biochemical composition. In the two-stage cultivation, microalgae are first grown to gain higher biomass and then exposed to different cultivation conditions to trigger the accumulation of desired product content. For *Nannochloropsis gaditana* IMTE1 a two stage cultivation was studied by Xiao et al. [64]. This microalgal strain was firstly grown in batch culture for 6 days, washed and then transferred for a chemostat culture with a fixed dilution rate and adjusted nitrate concentrations. In these cultivation conditions the highest biomass (897.10 mg dw L^{-1}) and EPA content (2.62% dw) were obtained at high nitrogen supplementations.

When combining nutrient and abiotic stresses, it is important to know the importance of each factor for the desired product accumulation, as well as its synergistic effects [57]. With the purpose of optimizing ω3-fatty acid production by *Pavlova lutheri*, Carvalho and Malcata [65] studied the combined effects of dilution rate, light intensity and CO_2 concentration under continuous mode. The optimum conditions for EPA and DHA production were found in cultures supplied with 0.5% CO_2, at a dilution rate of 0.297d^{-1} and a light intensity of 120 µE m^{-2} s^{-1} [65]. Other combined nutrient and abiotic stresses are summarized in Table 1.2 for the following strains: *Chlamydomonas reinharditii* CC124, *Nannochloropsis gaditana* CCNM1032, *Phaeodactylum tricornutum* CS-29C, and *Chaetoceros muelleri* CS-176. The major disadvantage of the enounced novel approaches is that large scale trials are required.

4. Sterols as an underexploited lipid resource from microalgae

When linking microalgae with food and human health the predominant terms are fatty acids, and carotenoids (Fig. 1.4). Nevertheless, some microalgae are high-level producers of phytosterols which have been playing a key role in functional food market [66,67]. In addition, mixtures of phytosterols can function as skin conditioning in cosmetic products (creams and lipstick), and in pharmaceutics they are gaining interest for the production of therapeutic steroids [43,68].

Table 1.2. Summary of some strategies used for microalgae lipid enhancement and its impact on polyunsaturated fatty acids accumulation.

Microalga specie	Strain	Factors Used	Biomass	Lipid	PUFA content	Notes	Ref.
Chlamydomonas reinhardtii	CC124	Phosphorus supplementation under nitrogen deficiency			PUFA: 17.15 - 45.23µg mg^{-1}; DHA: 0.09-0.17 µg mg-1		[69]
Chlamydomonas reinhardtii	CC124	Acetate input (1, 2, and 4 g L^{-1} sodium acetate)	1.08 - 2.49 g L^{-1}		PUFA: 28.84 - 51.58 µg mg^{-1}; DHA: 0.03 - 0.09 µg mg^{-1}		[69]
Heterochlorella luteoviridis	BE002	Temperature (22, 27 and 32 °C) and $NaNO_3$ content (12, 24, 36, 48 or 60 mg L^{-1} of N-NO_3)	0.48 g L^{-1} d^{-1}*	82.5 - 99.1 mg g^{-1}	PUFA: 34.4 - 40.7% TFA	*Biomass productivity obtained at higher nitrogen conditions	[70]
Nannochloropsis oceanica	IMET1	Nitrogen-deficiency stress (60, 120, and 2200 µmol L^{-1} NO_3^{2-})	319.10 - 897.10 mg L^{-1}	34.04 - 56.17% dw	EPA: 1.77 - 2.62% dw*	The highest EPA amount was observed at 2200 µmol L^{-1} NO_3^{2-}, in contrast to, lipid content	[64]
Tetraselmis subcordiformis	SHOU-S05	Nitrogen supplementation (0, 0.22, 0.44, 0.88 and 1.76 mmol N·L^{-1})		13.40 - 29.77%	PUFA: 57.97 - 62.59% TFA EPA: 2.92 - 3.85% TFA	The highest values of PUFA, and EPA were obtained at 0.22 mmol N L^{-1}	[61]
Nannochloropsis oculata	SHOU-S14			22.5 - 35.85%	PUFA: 46.10 - 53.69% TFA EPA: 29.34 - 35.51% TFA	The highest values of PUFA and EPA were obtained at 1.76 mmol N L^{-1}	[61]
Pavlova viridis	SHOU-S16			26.45 - 32.10%	PUFA: 26.94 - 41.28% TFA EPA: 9.52 - 15.71% TFA DHA: 2.39 - 7.17% TFA	The highest values of PUFA and EPA were obtained at 1.76 mmol N L^{-1}	[61]

PUFA – Polyunsaturated fatty acids; DHA – 4,7,10,13,16,19-docosahexaenoic acid; EPA – 5,8,11,14,17-eicosapentaenoic acid; TFA – Total fatty acids; dw – dry weight.

Table 1.2. Summary of some strategies used for microalgae lipid enhancement and its impact on polyunsaturated fatty acids accumulation (Continuation).

Microalga specie	Strain	Factors Used	Biomass	Lipid	PUFA content	Notes	Ref.
Nannochloropsis gaditana	CCNM1032	Salinity (20, 30, 35, and 40 g L^{-1}), light intensity (60 and 150 µmol photons m^{-2} s^{-1}), and photoperiod (24/0, 18/6, 12/12, 6/18 and 0/24 light/dark hour)	45.01 mg L^{-1} d^{-1}*	14.63 mg L^{-1} d^{-1}*	EPA: 19.13 - 37.83% TFA	*Biomass and lipid productivities were obtained at a salinity gradient of 20 g L^{-1}	63
Phaeodactylum tricornutum	CS-29C	Nitrogen source (nitrate, ammonium, and urea) and ultraviolet (UV) radiation (UV-A: 315-400 nm; UV-B: 280-315 nm)			PUFA: 34.89 - 48.85% TFA EPA: 18.86 - 23.42% TFA DHA: 1.49 - 2.52% TFA		71
Chaetoceros muelleri	CS-176				PUFA: 29.26 - 36.76% TFA EPA: 9.61-14.23% TFA DHA: 0.75 - 1.42% TFA		71
Pavlova lutheri	SMBA60	CO_2 concentrations (0-2% v/v), light intensity (75 and 120 µE m^{-2} s^{-1}) and cultivation mode (batch and continuous)	0.900 g L^{-1}	132.5 mg L^{-1}	EPA: 3.61 mg L^{-1} d^{-1} DHA: 1.29 mg L^{-1} d^{-1}	Values obtained at 0.5% (v/v) CO_2, a dilution rate of 0.297 d^{-1}, and a light intensity of 120 µE m^{-2} s^{-1}	65

PUFA – Polyunsaturated fatty acids; DHA – 4,7,10,13,16,19-docosahexaenoic acid; EPA – 5,8,11,14,17-eicosapentaenoic acid; TFA – Total fatty acids; dw – dry weight.

Plant-derived phytosterols have been added to food products for its ability to reduce serum cholesterol levels and prevent coronary heart diseases [66,72]. *Nostoc commune* var. *sphaeroides* (Cyanobacteria) lipid extracts have been found to inhibit the expression of key regulatory genes involved in cholesterol and fatty acid biosynthetic pathways, this property could contribute to lower serum cholesterol as well as triglyceride concentrations [73]. Phytosterols occur in four common forms: as free sterols (FS), as fatty acid esters (sterol is esterified to fatty acid; SE), as steryl glycosides (bound to sugar with a glycosidic bond; SG), and as acylated steryl glycosides (sugar moiety is acylated with a fatty acid; ASG) [74]. Due to poor solubility of free phytosterols, major phytosterol/phytostanol products being marketed are in their conjugated forms (SE, SG, and ASG) as it is easier to add them into food products, Table1.3 [74]. Table 1.3 summarizes information with respect to some brands that commercialize phytosterol fortified food products (yoghurts, spreads, soft cheese, and drinks), and other phytosterol-based products (supplements, and paste). Although the raw materials used for phytosterols isolation for food industry being tall oil (fat-soluble by-product obtained from trees) and vegetable oil, the scarcity of land resources has been driven the attention of scientific and industrial communities towards the search of new sustainable natural sources of phytosterols [75,76]. The concentration of phytosterols in different vegetable oils was estimated by Yang et al. [77] and ranged between 142.64 (camellia oil) to 1891.82 (rice bran oil) mg $100g^{-1}$.

Table 1.3. Phytosterols marketed as low cholesterol agents.

Manufacturer	Brand	Products	Source	Ref.
Raisio group	Benecol	Soft cheese; Yoghurt drinks; Yoghurts; Spreads	Plant phytostanol esters	[78]
Upfield	Flora ProActiv	Spreads; Drinks	Plant sterols	[79]
Goodman Fielder	Logicol	Spread	Plant sterols	[80]
Archer Daniels Midland (ADM)	CardioAid	Powder; Paste soluble in oils and fats	Plant sterols	[81]
Cargill	CoroWise	Dietary foods*; Beverages; Supplements	Plant sterols (phytosterols and steryl esters)	[82]
Lipofoods	Lipophytol	Water-dispersible powder	Plant sterols (from soy or pine tree origin)	[83]

*Foods with plant sterols and commercialized include emulsified sterols (ES200); fine particle sterols (FP100); granular phytosterols (FP300); steryl esters (SE-C100); water dispersible steryl esters (WDSE-33).

The potential of Chlorophyta *Dunaliella tertiolecta* and *Dunaliella salina* as sources of phytosterols was studied by Francavilla et al. [84], in its study the highest yields of total sterols were 1.3% and 0.89% dw for *D. tertiolecta* and *D. salina*, respectively. Another promising microalgal strain for phytosterol production, investigated by Ahmed et al. [66], was *Pavlova lutheri* with phytosterol content reaching up to 5.1% dw. Comparing these values with the ones

previously mentioned for vegetable oils, it is possible to recognize that microalgae have the potential to become a useful alternative source of phytosterols to use as functional ingredients. Microalgal-derived phytosterols are mainly distributed among four groups: 4-desmethyl-Δ5-sterols; 4-desmethyl-Δ7-sterols; 4-methylsterols; and di-hydroxylated sterols [67]. With 4-desmethyl-Δ5-sterols being the predominant phytosterol in microalgae [67,85]. According to Moreau et al. [74], 4-desmethyl sterols and stanols have been shown to inhibit the uptake of cholesterol from the intestine, resulting in a decrease of serum cholesterol levels. This strengthens once more the potential of microalgae-phytosterols as a novel industrial application.

4.1. Sterol synthesis by microalgae

For microalgae there are described two distinct and compartmentalized pathways for isoprenoid synthesis, these are *i)* the mevalonic acid (MVA) pathway, in the cytosol; and *ii)* the 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate (DOXP/MEP) pathway, in the plastid [67,86]. In general, microalgae possess both DOX/MEP and MVA pathways [86]. Exceptions include Cyanobacteria, Chlorophyta, as well as the *Haslea ostrearia* (Bacillariophyta), and the *Cyanidioschyzon merolae* (Rhodophyta), which produce sterols only from the MEP/DOXP pathway [87,88]. Figure 1.7 shows a generalized overview of sterol biosynthesis pathway for microalgae. Therefore, sterol biosynthetic pathway can be split into three main stages *i)* biosynthesis of isoprenoid precursors – isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP); *ii)* biosynthesis of polyprenyl pyrophosphates like farnesyl pyrophosphate (FPP); and *iii)* squalene (precursor of all phytosterols) formation – dimerization of FPP [87]. According to Sasso et al. [87] there is evidence for transport of IPP and polyprenyl pyrophosphates, like FPP and geranyl pyrophosphate (GPP), across plastid membranes. Therefore, in algae such as Chlorophyta, in which sterols are only produced through MEP/DOXP pathway, IPP synthesized in the plastids could be exported to cytosol for the formation of sterols [89].

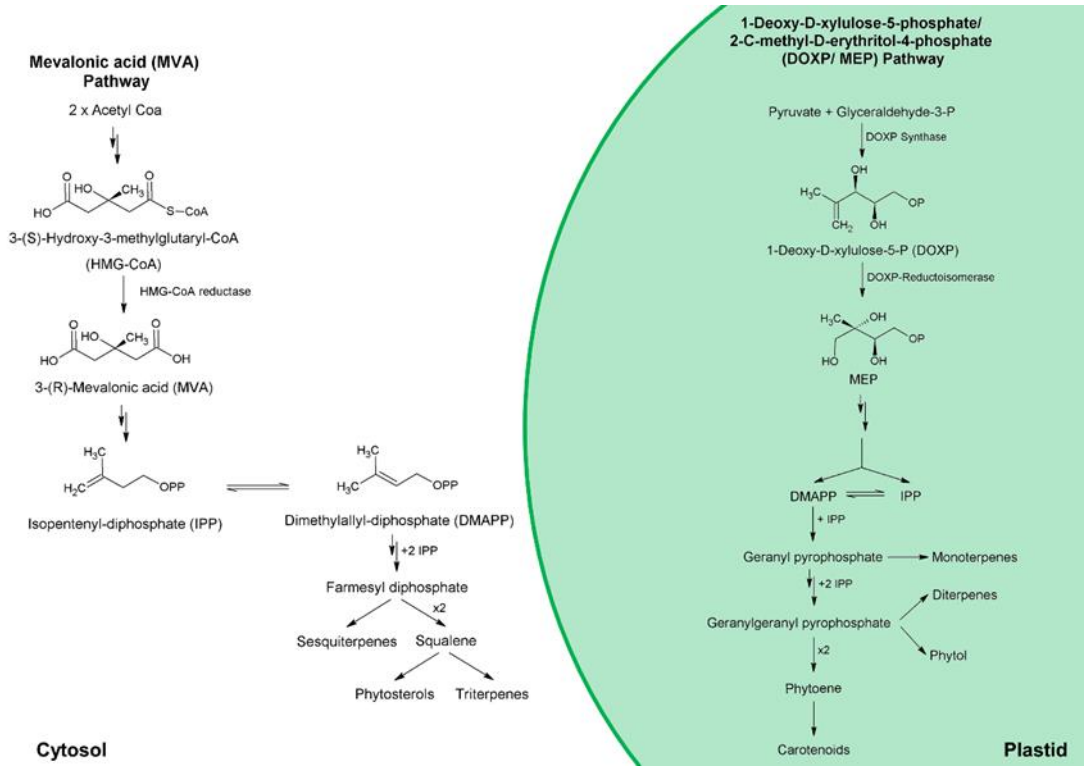

Figure 1.7. Generalized sterol biosynthesis pathway for microalgae [86,87].

4.2. Microalgae-derived phytosterols biological activities

Phytosterols synthesized by microalgae have shown interesting biological activities including neuroprotective, anti-inflammatory, anti-cancer, neuromodulatory, immunomodulatory, and apoptosis inductive effects. From Table 1.4 it is possible to visualize that the predominant biological activity studied for microalgal-derived phytosterols was anti-inflammatory. The anti-inflammatory potential of phytosterols can be assessed *in vitro* through determination of nitric oxide (NO), prostaglandins (PG) and cytokines production, and/or expression of nitric oxide synthase (iNOS), and cyclooxygenase (COX-2), after cell treatment with inflammation stimulation agents such as concanavalin A (Con A) and lipopolysaccharide (LPS) [90–94]. Promising results were obtained for a sterol rich fraction of *N. oculata* which was found to inhibit NO production, and down-regulate LPS-stimulated protein levels of inducible iNOS and COX-2 in a dose-dependent manner [92]. Moreover, a study performed for *D. tertiolecta*, testing several mixtures of phytosterols, showed that ergosterol and a mix of ergosterol and 7-dehydroporiferasterol suppressed a highly pro-inflammatory cytokine (tumor necrosis factor alpha; TNF-*α*), a pleiotropic cytokine (interleukin (IL)-6), and increased the levels of an anti-inflammatory cytokine (IL-10), showing the anti-inflammatory potential of both sterols and suggesting that phytosterol anti-inflammatory properties might depend on the existence of a synergistic effect of these molecules [91].

Besides anti-inflammatory activity, the ability of phytosterols to cross blood-brain-barrier and act as acetylcholinesterase enzymes inhibitors has sparked the attention of neurodegenerative diseases research [95]. Fagundes et al. [95] studied the neuroprotective potential of *Phormidium autumnale* phytosterol-rich extracts and determined the anti-

cholinergic, antioxidant and anti-inflammatory capacities. In this study, the phytosterol-rich extract demonstrated higher *in vitro* neuroprotective activity than non-enriched extract, exhibiting a moderate-high anticholinergic potential, and showing to be an effective lipoxygenase inhibitor. This was further supported through molecular docking simulation which showed the specificity of stigmasterol interaction with acetylcholinesterase active sites. Moreover, a previous study has found *in vivo* neuromodulatory activity of *D. tertiolecta* – derived phytosterols in selective brain areas of rats [75].

Through Table 1.4 it is possible to visualize that the biological studies with respect to phytosterols were mainly performed *in vitro* presenting some limitations, namely, these types of studies only give partial information on bio-functionality, and lack of systemic factors [96]. However, they provide fast and inexpensive screening of bioactivities, have high sensitivity, and are easy to perform manage and interpret [96]. Moreover, it is crucial to highlight that most of the studies summarized in Table 1.4, are mainly performed in sterol-rich fractions and that future research targeting the potential of the different types of microalgae-derived phytosterols, including their functional activity, and synergistic effects, is crucial for gaining in-depth knowledge of microalgae sterols potential.

Table 1.4. Biological activities studied for microalgae-derived phytosterols in cell culture experiments, and animal models.

Microalga	Extract description	Activity	Assay	Model	Ref.
Phormidium autumnale	Phytosterol-rich fraction	Neuroprotective	*In vitro* *In silico*		[95]
Nannochloropsis oculata	Sterol rich fraction	Anti-inflammatory Anti-cancer	*In vitro*	RAW 264.7 macrophage cells HL-60, A-549, HEP-3B, HCT-116, and SW-480 cancer cells	[92]
Navicula incerta	Isolated stigmasterol	Anti-proliferative	*In vitro*	HepG2 cells	[97]
Dunaliella tertiolecta	Phytosterols Mix of ergosterol, and 7-dehydroporiferasterol Mix of acetylated ergosterol and 7-dehydroporiferasterolergosterol	Immunomodulatory Anti-inflammatory	*In vitro*	Sheep peripheral blood mononuclear cells	[91]
	Phytosterols	Neuromodulatory	*In vivo*	Rats	[75]
Chlorella vulgaris	Isolated sterols	Anti-inflammatory	*In vivo*	ICR mice	[98]

4.3. Strategies for sterol enhancement

To boost phytosterol accumulation in microalgae it is crucial to understand its trigger mechanisms. Table 1.5 summarizes some strategies already employed for induce microalgae phytosterol accumulation. For Haptophyta *P. lutheri* the effects of nutrient-induced changes, salinity, ultraviolet-C (UV-C) radiation, and sampling days have been assessed aiming phytosterol production [66,72]. From these variables, the most effective were UV-C radiation and sampling days [66,72]. Although the UV-C radiation equipment being simple and easy for operation

and maintenance, this physical stressor has a great disadvantage for large scale production which is the significant cell damage connected with UV-C radiation mutagenic factor (as it attacks an organism's deoxyribonucleic acid (DNA) [99–101]. The mutagenic factor also poses a concern since little is known about the stability of modified algal strains or whether they can potentially take any environmental risks [102].

According to Ahmed et al. [66] and Ahmed and Schenck [72], *P. lutheri* did not increased its sterol content when subjected to peroxide hydrogen input, variations in nitrogen and phosphorus concentrations, and changes in salinity. Still previous studies have described salinity-induced changes as an effective tool to induce phytosterol accumulation in microalgae. Francavilla et al. [84] studied the effect of different salinity concentrations for *D. tertiolecta* (Chlorophyta) and *D. salina* (Chlorophyta) using a different approach from the study performed to *P. lutheri* (Haptophyta), and good yields of total sterols were observed at lower salt concentration [84]. Thus, the different observations made in both studies might be derived from: *i)* species-specific differences; and/or *ii)* the different approaches used to impose salinity-induced changes.

Besides growth conditions, the specific growth phase at which microalgal biomass is harvested can influence lipid yields and composition for specific purposes [60,103]. The effect of growth phase on the sterol content of dinoflagellate species (*Prorocentrum donghaiense*, *Prorocentrum minimum*, *Karenia mikimotoi*) in batch cultures was assessed by Chen at. al [104] . In this study, sterol content of two microalgae was responsive to changes in growth phase (from exponential to stationary phase), with the greatest increase being verified for dinosterol (168%) in *P. minimum*, and for brassicasterol (423%) in *K. mikimotoi*. When assessing product accumulation over growth phase, it is also important to take in consideration that there are some growth phases in which the production of stress-associated molecules (*e.g.* carotenoids, lipids) is still balanced with good amounts of growth-associated ones (*e.g.* proteins) [105]. This point is especially important for co-exploitation of microalgae high-value products.

Interactive effects of nutrient and abiotic factors have been reported to enhance the production of phytosterol in some freshwater microalgal species [106,107]. For instance, varying light intensities in both high phosphorus and low phosphorus environments was shown to affect sterol accumulation for Chlorophyta *Scenedesmus quadricauda* and *Chlamydomonas globosa*, and Bacillariophyta *Cyclotella meneghiniana*, with sterol contents increasing with light intensity under high phosphorus [106]. Another strategy applied by Piepho et al. [107] was varying temperature with phosphorus, and silicate supply. For this study *C. meneghiniana* sterol content increased its sterol content from low to high temperature and this was even higher in the high phosphorus treatment. Simultaneous effects of nutrient supply and abiotic factors in microalgae highlight the importance of investigating more than just one environmental factor when inducing the accumulation of a desired product for a field of application.

Table 1.5. Summary of some strategies evaluated for microalgae sterol enhancement.

Microalgae	Variables studied	Total Sterols	Major Sterols	Observations	Ref.
Diacronema lutheri (syn. Pavlova lutheri)	UV-C radiation (50-250 mJ m^{-2})	9.9 – 20.3 mg g^{-1} dw	Poriferasterol	↑TS was found at 100mJ cm^{-2}	[72]
		19.5 – 30.9 mg g^{-1} dw	Clionasterol		
	Hydrogen Peroxide (H_2O_2: 1-500 µM)		4-*α*-methylporiferast-22-enol	No significant increase of TS due to H_2O_2	
	Combined effects of sampling days (2, 4, 6, 12, 14, and 16), and salinity (15, 25, 35, and 45‰)	20.29 – 51.86 mg g^{-1} dw	Methylpavlovol Epicampesterol	Significant differences were observed between sampling days but not for the different salinities	[66]
Dunaliella salina *Dunaliella tertiolecta*	Salinity (0.6, 1.4 and 2.1 M NaCl)	0.89% dw 1.3% dw	7-Dehydroporiferasterol Ergosterol	Good yields of TS were found at lower salt concentrations (0.6M)	[84]
Scenedesmus quadricauda	Combined effects of light intensity (30, 60, 140, 230, and 490 µmol photons m^{-2} s^{-1}), and phosphorus (1 - 50 µM)	8 – 13 µg mg C^{-1}	Fungisterol Chondrillasterol 22-Dihydrochondrillasterol	In the high-P TS increased with light intensity	[106]
Cryptomonas ovata		7 – 8 µg mg C^{-1}	Epibrassicasterol Stigmasterol	No significant changes in TS	
Cycotella meneghiniana SAG 1020-1a		5 – 8 µg mg C^{-1}	24-Methylene-cholesterol 22-Dihydrobrassicasterol		
Chlamydomonas globosa		3 – 4 µg mg C^{-1}	Ergosterol Fungisterol		
Prorocentrum donghaiense	Temperature (15, 20, and 25) N:P supply (10:1, 24:1, and 63:1 molar ratios) Growth phase (exponential and stationary growth phases)	Brassicasterol: 0.03 – 0.12 pg $cell^{-1}$ Dinosterol: 0.15 – 1.54 pg $cell^{-1}$	Brassicasterol Dinosterol	Growth phase changes showed the most pronounced effects, while temperature and nutrient deficiency had moderate effects on sterol contents	[104]
Prorocentrum minimum		Brassicasterol: 0.04 – 0.20 pg $cell^{-1}$ Dinosterol: 0.28 – 1.83 pg $cell^{-1}$			
Karenia mikimotoi		Brassicasterol: 0.07 – 1.56 pg $cell^{-1}$ Dinosterol: 0.20 – 1.30 pg $cell^{-1}$			

Table 1.5. Summary of some strategies evaluated for microalgae sterol enhancement (Continuation).

Microalgae	Variables studied	Total Sterols	Major Sterols	Observations	Ref.
Thalassiosira pseudonana CCMP1335	Rapid cooling (18 ºC to 4 ºC) Salinity (10, 17, 25, 30, 39, 47, 53 and 61 ppt)		24-Methylenecholesta-5,24(24')-dien-3β-ol Fucosterol Isofucosterol Cholesterol		[108]
Phaeodactylum tricornutum CCMP632			24-Methylenecholesta-5,24(24')-dienol Fucosterol Isofucosterol Cholesterol	Shifts its sterol content at a reduced temperature	
Chaetoceros muelleri CCMP1316			Brassicasterol Campesterol Cholesterol	Rapid cooling did not significantly change sterols relative abundance	

5. Carotenoids

Some studies have explored the co-production of carotenoids with lipids [109], and fatty acids [110,111] in microalgae. Carotenoids are lipid soluble compounds synthesized by microalgae and can be divided into carotenes (hydrocarbons), and xanthophylls (oxygenated hydrocarbons) [20,112]. As structural components of light-harvesting complexes, these compounds play key roles within microalgal cells, namely in the protection against excess irradiance, chlorophyll triplets, and reactive oxygen species [113]. Through Figure 1.8 it is possible to visualize that the global market for carotenoids was US$ 1.7 billion in 2020. In microalgae, astaxanthin, *β*-carotene, and lutein are among the key carotenoids with high market potential [20,112,114]. Astaxanthin presents the highest value for the global market size, US$ 663.89 million in 2020, and value projections for 2027, US$ 977.74 million (Fig. 1.8). *Haematococcus pluvialis* and *Dunaliella salina* are the most popular microalgal species exploited for the commercial production of astaxanthin and *β*-carotene, respectively [20]. In *Haematococcus*, astaxanthin is majorly esterified to hexadecanoic acid (C16:0) and unsaturated fatty acids of C18 family (C18:1, C18:2, and C18:3) [112,115].

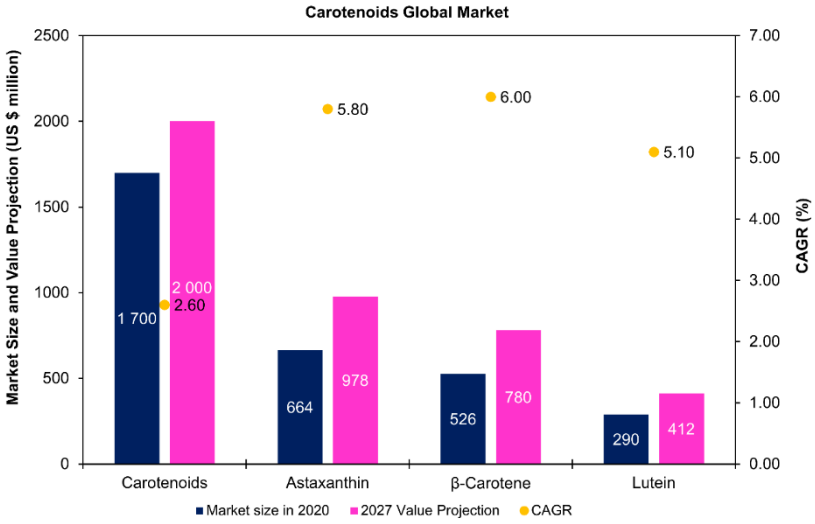

Figure 1.8. Carotenoids global market prospects - market size in 2020, 2027 value projection, and compound annual growth rate (CAGR). The values presented for carotenoids and lutein CAGR correspond to the forecast period of 2020-2027, while for astaxanthin and β-carotene CAGR values correspond to the forecast period of 2021-2027. Data for carotenoids and lutein were collected from StrategyR [19], whereas data for astaxanthin and β-carotene were collected from Global Market Insights [116].

Carotenoids are extensively used in food, feed, nutraceuticals, and cosmetics [117]. The consumption of a diet rich in carotenoids is often associated with positive effects on skin health; cancer, cardiovascular, neuronal, and gastrointestinal protection; and vision and immune system enhancement [117]. As with essential fatty acids and phytosterols, carotenoids cannot be synthesized by humans, which, in turn, must obtain these through their diet [117]. The beneficial effects of carotenoids to human health are thought to be derived from its potent anti-oxidant activity, and the provitamin A activity of some carotenoids which can be converted to retinal (*e.g. α*- and *β*- carotenes) [117].

As can be seen through Fig. 1.7, in microalgae, carotenoids are biosynthesized using the isoprenoid precursor's IPP and DMAPP, which are further condensed to yield geranyl pyrophosphate. This molecule is elongated yielding geranylgeranyl pyrophosphate, which, in turn, can be dimerized to generate phytoene, the precursor of carotenoids [87]. Carotenoid's accumulation often occurs when microalgal is exposed to some stress factors (*e.g.* nitrogen deficiency [118], ultraviolet-A radiation [110], salinity [119,120]) and growth is arrested [20]. Thus, for prompting carotenoids overproduction, for industrial scale, a two-stage cultivation strategy is often applied to firstly obtain higher biomass and then to trigger carotenoids accumulation [20].

6. Lipid characterization

Studies monitoring chemical diversity often screen a specific biological activity, a targeted compound class or individual molecule [121]. This constrains the development of microalgae-based industries once it overshadows the diversity of compounds produced by microalgae [122]. Several methods have been developed to allow a quantitative and simultaneous analysis of many groups of metabolites on complex mixtures by capillary electrophoresis mass spectroscopy (CE-MS), gas chromatography- mass spectroscopy (GC-MS), liquid chromatography-mass spectroscopy (LC-MS), nuclear magnetic resonance spectroscopy (NMR), and Fourier transform ion cyclotron resonance- mass spectroscopy (FTICR-MS) [123,124]. These strategies allow to save time, with respect to laborious isolation and quantification procedures, and give prompt information for complex mixtures constituting a major advantage for microalgal-based industries [124].

Lipidomic comprises the detailed identification and quantification of lipid classes [125]. GC-MS has been widely used for the determination of fatty acids composition of microalgal lipids, which, in turn, are the basis of microalgae lipidomic studies [125]. However, the challenge with microalgal lipidomic studies lies in addressing its vast complexity and chemical heterogeneity [126]. GC-MS methods are recognized by its high detection sensitivity, accuracy and excellent reproducibility, nevertheless sample pretreatment (*e.g.* hydrolysis, derivatization) needs to be performed to samples [127]. A trimethylsilyl 2,2,2-trifluoro-N-trimethylsilylethanimidate (BSTFA) derivatization with GC-MS has been used as a simple, fast, low cost, and powerful tool to gain in-depth knowledge on unknown but relevant lipids [128]. Through this non-target approach, it is possible to analyze simultaneously fatty acids, sterols, monoglycerides, aliphatic alcohols, glycosyl sterols, and other lipid-soluble molecules, like *α*-tocopherols, without prior knowledge of sample composition [129].

Until this stage of algal development, the production of bioenergy from microalgae is still not feasible. Thus, to take advantage of already exploited oleaginous microalgal species, algae farmers are turning their focus towards high-value lipids production for health and food sectors. Nevertheless, the great biodiversity of microalgae remains to be explored, holding back the opportunities that come from the wide diversity of compounds amongst microalgae taxa. Within lipids, the already recognized ω3-PUFA health-promoting properties are turning the focus of microalgal-based industries towards autotrophic ω3-PUFA production. However, more

knowledge on the production strategies to prompt ω3-PUFA accumulation is lacking. Phytosterols are an unexplored lipid resource which could be an opportunity to explore as food additive, as functional food, or dietary supplement. From lipid-soluble compounds carotenoids, are the most extensively used in food, feed, nutraceuticals, and cosmetics. For high-value lipids exploitation, increasing the knowledge on new, simple, and cost-effective techniques to increase the production of these molecules is necessary. Thus, the use of high-throughput methods that allow the identification and quantification of a wide array of lipid components are needed.

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Three marine microalgae species, *Pavlova pinguis* J. C. Green [130], *Hemiselmis* cf. *andersenii* Lane and Archibald [131] and *Chlorella stigmatophora* Butcher [132], were used throughout this doctoral project, their taxonomic description is displayed in Table 1.6.

Table 1.6. Taxonomic information of species *Pavlova pinguis*, *Hemiselmis* cf. *andersenii* and *Chlorella stigmatophora* based on Algaebase [23].

	Pavlova pinguis	***Hemiselmis* cf. *andersenii***	***Chlorella stigmatophora***
Empire	Eukaryota	Eukaryota	Eukaryota
Kingdom	Chromista	Chromista	Plantae
Phylum	Haptophyta	Cryptophyta	Chlorophyta
Class	Pavlovophyceae	Cryptophyceae	Trebouxiophyceae
Order	Pavlovales	Cryptomonadales	Chlorellales
Family	Pavlovaceae	Hemiselmidaceae	Chlorellaceae
Genus	*Pavlova*	*Hemiselmis*	*Chlorella*
Strain ID	RCC 1539	BEA 0118B	RCC 661

RCC – Roscoff Culture Collection; BEA – Spanish Bank of Algae; ID – Identification.

7. General objectives of the thesis

The present work aimed to investigate the potential of the microalgae *Pavlova pinguis* and *Hemiselmis* cf. *andersenii*, as matrices for the bioprospection of phytochemicals. The microalga *Chlorella stigmatophora* was used as a model organism for comparison purposes. For studying the potential of *P. pinguis* and *H.* cf. *andersenii* several parameters were studied namely its growth dynamics, nitrogen uptake, pigments, monosaccharides, and detailed lipid characterization of esterified and non-esterified lipids. Moreover, two different stress-inducement strategies were applied to microalgae with the purpose of assess its robustness and enhancement in high-value lipids. The experimental part of this thesis is divided into five chapters:

a) The second chapter that aimed to evaluate *Pavlova pinguis* lipid composition for further exploitation as natural source of high-value lipids. Thus, *P. pinguis* was grown in a batch cultivation system, and its detailed lipid characterization before and after alkaline hydrolysis through gas chromatography–mass spectrometry (GC–MS) was performed;

b) In the third chapter a comparative analysis of *Hemiselmis* cf. *andersenii* and *Chlorella stigmatophora* strains was performed with the purpose of evaluate *H.* cf. *andersenii* as a potential candidate for high-value lipids production. In this study, it was possible to identify which lipid components may serve as chemotaxonomical markers, as well as novel products for high-value lipids market;

c) The fourth chapter presents novel insights on *P. pinguis* response to nitrogen-induced changes. The main goal of this study was to enhance the production of high-value lipids by *P. pinguis* and understand the mechanisms that lead to the desired product accumulation, without compromising microalgal growth;

d) The fifth chapter aimed to investigate how *H.* cf. *andersenii* and *C. stigmatophora* differently remodeled their growth dynamics, nitrogen uptake, pigments, and especially lipid composition in response to different nitrogen supplementations. For this, the multivariate analysis of microalgae lipid composition revealed to be an effective tool to discriminate species-specific responses;

e) In the sixth were explored the mechanisms that led to carbon allocation and lipid remodeling of *P. pinguis* and *H.* cf. *andersenii*, in the two new promising candidates for high-value lipids production. Its carotenoid, chlorophyll a content, as well as their monosaccharides and lipids profiles were analyzed against several phosphorus regimes.

CHAPTER 2.

Exploring *Pavlova pinguis* chemical diversity: a potentially novel source of high-value compounds

This Chapter is based on the following publication:

Fernandes, T.; Martel, A.; Cordeiro, N. Exploring *Pavlova pinguis* chemical diversity: a potentially novel source of high value compounds. Sci. Rep. 2020, 10, 339. https://doi.org/10.1038/s41598-019-57188-y.

Chapter 2. Exploring *Pavlova pinguis* chemical diversity: a potentially novel source of high-value compounds

Abstract

To uncover the potential of *Pavlova pinguis* J.C. Green as a natural source of value-added compounds, its lipophilic extracts were studied before and after alkaline hydrolysis using gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the lipophilic extracts showed a wide chemical diversity including 72 compounds distributed by fatty acids (29), sterols (14), fatty alcohols (13), and other lipophilic compounds (16). Fatty acids represented the main class of identified compounds presenting tetradecanoic, hexadecanoic, 9-hexadecenoic, and 5,8,11,14,17-eicosapentaenoic acids as its main components. Through the ∑ω6/∑ω3 ratio (0.25) and sterol composition it was possible to observe that *P. pinguis* is a valuable source of ω3 fatty acids and stigmasterol (up to 43% of total sterols). After alkaline hydrolysis, fatty acids and fatty alcohols content increased by 32 and 14% respectively, in contrast to, monoglycerides which decreased by 84%. The long-chain alcohols content enables the exploitation of this microalga as a source of these bioactive compounds. Smaller amounts of sugars and other compounds were also detected. The present study is a valuable reference to the metabolite characterization of *P. pinguis* and shows the potential of this microalga for nutraceutical and pharmaceutical industries.

Keywords: *Pavlova pinguis*, phytochemicals, microalgae, high-value compounds.

1. Introduction

The search for natural products with pharmaceutical and industrial applications has driven the attention of the scientific community towards the marine environment [133,134]. The large spectrum of marine organisms combined with their intrinsic chemical variability make these organisms a huge source from which to isolate new molecules with a broad range of applications [135]. From the marine organisms, microalgae have emerged as versatile cell factories to produce high-value compounds due to their rich biodiversity, growth rate, phenotypic plasticity, and simple nutrient requirements [133].

The richness of microalgal biodiversity is often underestimated in the biotechnological field, being restricted to few species within Chlorophyta and Cyanobacteria that dominate the market [24]. This fact represents a constraint for the full development of microalgae-based industries once it overshadows the diversity of compounds amongst microalgae taxa [136]. Therefore, to exploit the potential of microalgae as versatile cell factories the following challenges are found: microalgal strain selection, cultivation optimization, and downstream biomass extraction [24,137]. These can be overcome through a detailed phytochemical characterization and identification of the high-value components of microalgal extracts [137].

Microalgal cell components have been recognized as precious sources of health-promoting phytochemicals that can prevent and/or improve cardiovascular diseases, hypertension, and arthritis, and act as anti-inflammatory, anticarcinogenic and antitumoral agents [59,84]. Included in the health beneficial phytochemicals synthesized by microalgae are terpenes, sterols, phenolics, polyunsaturated fatty acids (PUFA), vitamins, carbohydrates, proteins among other compounds [129,138].

Although much research has focused on the aquaculture potential of several *Pavlova* species [139–141], only specific algal compounds (*e.g.* fatty acids and sterols) have been analyzed to determine their biological activity, nutritional value and applicability [142]. This target analysis restricts the detection of compounds that are present in low quantities which, in turn, makes difficult the inclusion of unknowns in microalgal extract analysis [143]. From the classes of widespread natural products, the composition of long-chain aliphatic alcohols (LCAA), steryl glycosides, and monoglycerides in microalgae are poorly studied [129].

In the Haptophyta *Pavlova pinguis* J. C. Green [130] only specific classes of compounds have been analyzed to assess its potential as food for larval hatcheries in aquaculture and as ecological biomarkers [85,141,144]. For instance, Milke et al. [139] and Parrish et al. [140] assessed the ability of *P. pinguis* and other *Pavlova* species to sustain postlarval sea scallop growth focusing on its proximate, fatty acid, and sterol composition. In this microalga the complete characterization of lipid components (simple and complex lipids) is still largely unexplored [129,137]. Thus, in the present study the analysis of the lipophilic fraction of *P. pinguis* was performed in order to identify its lipophilic features before and after alkaline hydrolysis through gas chromatography–mass spectrometry (GC–MS) and evaluate its prospects for further improvement in bioactive compounds.

2. Materials and methods

2.1. Growth and culture conditions

The haptophyta *Pavlova pinguis* (RCC 1539) was obtained from the Roscoff Culture Collection (RCC). The microalgal cultures were made by inoculating starter cultures into 1L of sterile f/2 – Si medium with pH adjusted to 7.0 under 70 µmol m^{-2} s^{-1} light intensity with 16:8 h (light: dark cycles) at 25 °C. At the end of the logarithmic phase, the medium was centrifuged for 7 min. at 3720 g and the pellets washed. Microalgae growth was monitored daily with a Neubauer–improved counting chamber (Marienfield–Superior) and a light microscope (Olympus BX41) with a 40x magnification. The specific growth rate was determined as described in Fernandes et al. [145].

2.2. Solvent extraction

The extraction of non-polar phases was made as described by Ma et al. [146], with some modifications. To 0.10g of microalgal freeze-dried biomass an aqueous solution (methanol:water in a 1:1 ratio) and chloroform in 1:1 ratio were added. After homogenization,

the mixture was left stirring for 15 min and centrifuged at 4430 g for 10 min. The organic layer was carefully removed and transferred into pre-weighted tubes. The insoluble residue was washed three times with chloroform and dried in Na_2SO_4 filters. The extracts were evaporated in a nitrogen atmosphere and the amount of extractable substances was gravimetrically quantified and expressed as a percentage by weight of the freeze-dried biomass (dw). The extractable substances are presented as an average of at least three replicates.

2.3. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) with attenuated total reflectance (ATR) was used to identify the major functional groups in the raw microalga and chloroform extracts. FTIR-ATR spectra were collected on a Perkin–Elmer Spectrum Two instrument coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK) scanning over the wavenumber range of 4000 - 650 cm^{-1} at a resolution of 4 cm^{-1} and 36 scans.

2.4. Alkaline hydrolysis

The alkaline hydrolysis was performed in two aliquots of the chloroform extracts to detect molecules in their esterified forms according to Santos et al. [129]. To each extract, 10 mL of 0.5M NaOH in aqueous methanol was added and the mixtures were heated at 100 ºC for 1 h in a nitrogen atmosphere. Then, the samples were allowed to cool, prior to the acidification of the mixtures to pH 2 with 1M HCl. Following this step, the hydrolyzed samples were extracted with dichloromethane. The solvent was evaporated to dryness under nitrogen.

2.5. Gas chromatography–mass spectrometry analysis

Prior to GC–MS analysis, the extracts were silylated accordingly to Santos et al. [129]. Two aliquots of each dried extract (before and after alkaline hydrolysis) and an accurate amount of internal standard (tetracosane, 0.30 and 0.40 mg) were dissolved in 250 µL of pyridine, 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylclorosilane. Then, the mixture was kept at 70 ºC for 30 min to proceed to the conversion of the hydroxyl and/or carboxyl groups into trimethylsilyl (TMS) ethers and/or esters, respectively. TMS were analyzed in a gas chromatographer (Agilent HP 6890) equipped with a mass selective detector (Agilent 5973) and a ValcoBon 17704 capillary column VB1 (30 m × 0.25 mm inner diameter, 0.25 µm film thickness). The chromatographic conditions were as follows: oven initial temperature was 80 ºC for 5 min; increasing 4 ºC min^{-1} until reach the 208 ºC; followed by 2 °C min^{-1} to 260 °C; and 5 °C min^{-1} until reaching the final temperature of 300 °C for 4 min. The temperature of the injector was 250 °C; the transfer line, 290 °C; and the split ratio was 33:1. Helium was used as the carrier gas at a constant flow of 1.0 mL min^{-1}. The identification of the extracted compounds as TMS derivatives was made by comparison of the mass spectra fragmentation to those in the GC-MS spectral library (Wiley-NIST Mass Spectral Library 1999), literature data [147–150] or by injection of standards. For semi-quantitative analysis, GC–MS was calibrated with pure reference compounds (mannose, trans-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5*α*–

chlolestane, cholesterol, stigmasterol, hexadecanoic, and nonadecanoic acids) relative to tetracosane.

2.6. Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined according to Maadane et al. [151] with some modifications. Stock solutions of butylated hydroxytoluene (BHT, 1 mg mL^{-1}) and extract (3 mg mL^{-1}) were prepared in methanol and dimethyl sulfoxide, respectively. The stock solutions were added to 1300 µL of DPPH radical solution (83 µM). Then the absorbance of samples was measured at 520 nm with a UV/Vis spectrometer Lambda 25 (Perkin Elmer), after 30 min in the dark at room temperature. The DPPH scavenging effect was calculated by the Equation 2.1:

$$\text{Scavenging effect } (\%) = \left[1 - \frac{(A_{sample} - A_{sample\ blank})}{A_{control}}\right] \times 100 \qquad \text{Equation 2.1}$$

where A_{sample} is the absorbance of DPPH solution with the sample or standard, $A_{sample\ blank}$ is the absorbance of sample without DPPH, and $A_{control}$ is the absorbance of DPPH solution without sample.

2.7. Statistical analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 24. Differences between treatments were assessed with Student's t-test, *p*-values <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Growth and extraction yield

The overall yield of a specific desired product is dependent on the microalgal growth rate and on the product content [62]. Thus, when exploring the potential of a microalgal strain for further improvement in high-value compounds, the microalgal growth should be considered. The growth rate determined for *P. pinguis* was 0.8 d^{-1} and the maximum cell concentration, reached by this microalga, was 8.46×10^6 cells mL^{-1}, Figure 2.1. According to Steinrucken et al. [25] this microalgal strain can be considered as a high growth rate strain since its growth rate is ≥ 0.7 d^{-1}. The average dry biomass production observed at the end of the batch cultivation was 368 mg L^{-1} achieved in 7 days, this value being close to the dry weight (dw) estimated by Mansour et al. [144] of 390 mg L^{-1}.

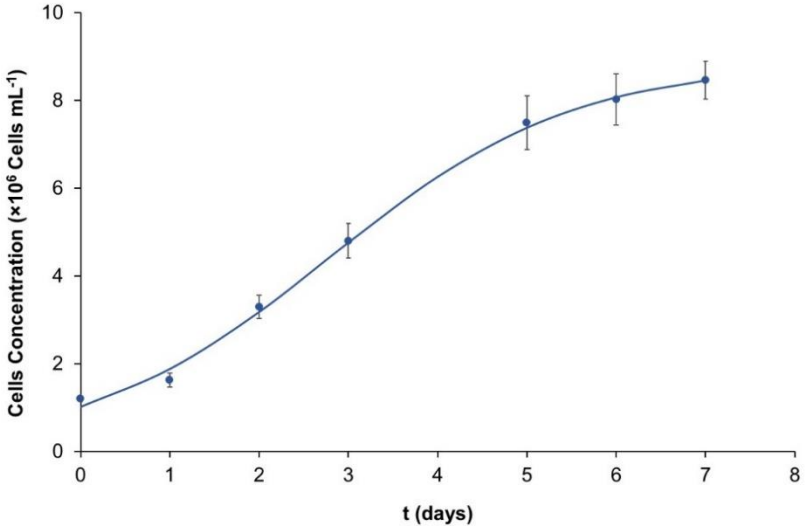

Figure 2.1. Growth curve of *P. pinguis* in f/2 – Si growth medium.

The yields of the chloroform extractable substances, in *P. pinguis*, accounted for 11.92% dw. This yield was higher than that previously reported by Mansour et al. [144] for this microalga cultivated in the fE and GSe growth media and extracted with a modified Bligh and Dyer, in which, the chloroform extracts accounted 7.6% and 3.5% dw, respectively. The total chloroform extractable substances obtained in this study (5.17 pg cell^{-1}) were also higher than that found for other *P. pinguis* strains (2 pg cell^{-1}) grown in f/2 medium and extracted with chloroform through modified Bligh and Dyer [148].

3.2. Fourier transform infrared spectroscopy with attenuated total reflectance

The Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) was employed to *P. pinguis* biomass and chloroform extracts as a first approach to perform a qualitative analysis of the extractable substances of *P. pinguis* (Fig. 2.2). Through the FTIR spectrum of the raw microalga, it is possible to visualize three main regions, that relate to the main macromolecular pools: *i)* the carbohydrate region between 1200 – 900 cm^{-1} (*ν*C–O–C of carbohydrates); *ii)* the protein bands at 1655 cm^{-1} and 1545 cm^{-1} (*ν*C-O of amide II and *δ*N–H of amide I, respectively); *iii)* and the lipid associated peaks at 1740 (*ν*C=O of the ester functional groups) and 3050 – 2800 cm^{-1} [152]. This observation indicates the co-presence of lipids, proteins, and polysaccharides in the microalgal biomass. In the FTIR spectrum of the chloroform extract it is possible to visualize that the signals often attributed to the characteristic functional groups of lipids increased their intensity, namely those in the 3050 – 2800 cm^{-1} region (C-H stretch), which indicates that lipids are the major component of the lipophilic extract. Moreover, the presence of peaks at around 720, 1745, and 3010 cm^{-1}, that are related to the CH_2 rocking, C=O and C-H stretch, respectively, indicates the presence of unsaturated hydrocarbons in the lipophilic extract [153].

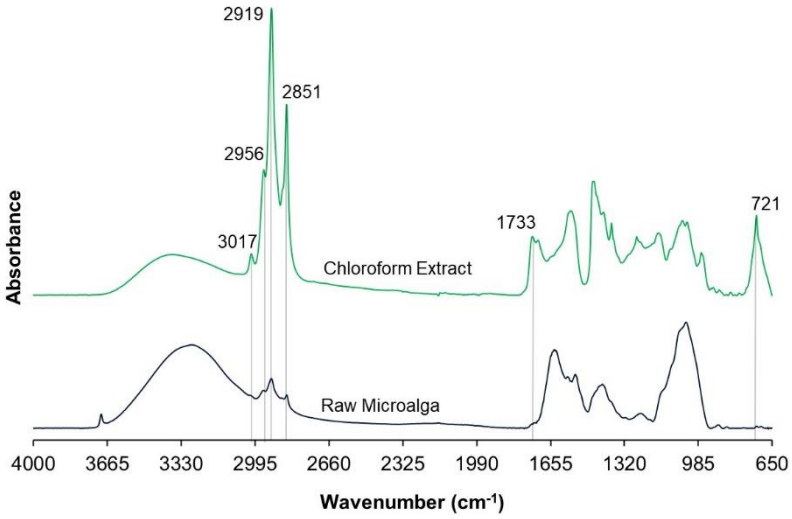

Figure 2.2. FTIR – ATR spectra of the lipophilic extractives and the raw marine microalga *P. pinguis*, the characteristic bands of the lipophilic extracts are highlighted.

3.3. Gas chromatography – mass spectrometry analysis

To screen microalgae for commercial purposes, different aspects have to be covered: the first is to explore the chemical diversity of microalgae; the second is to evaluate the quality of these extracts by searching for known bioactive compounds [121].

The microalgal strain under study presented a wide chemical diversity in the extract: 29 fatty acids, 14 sterols, 13 fatty alcohols, and 16 other compounds. Through Table 2.1 it is possible to observe qualitative and quantitative differences, in the *P. pinguis* chloroform extracts, before and after hydrolysis. Before hydrolysis 71% of the extractable substances were quantified while after hydrolysis this percentage increased to 88%.

Table 2.1. Compounds detected in the lipophilic extracts of *P. pinguis* before (BH) and after (AH) alkaline hydrolysis.

			Content (mg g^{-1} of microalgal dw)		**Content (mg g^{-1} of extract)**	
Nº	**Identified Compounds**	**MW**	**BH**	**AH**	**BH**	**AH**
	Fatty acids		*57.34±1.33^a*	*75.60±4.49^b*	*482.95±26.06^a*	*631.77±23.20^b*
	Saturated		*24.99±0.67^a*	*35.55±2.60^b*	*210.60±12.07^a*	*297.02±14.12^b*
2	Octanoic acid	216	*n.d.*	1.03±0.00	*n.d.*	8.65±0.27
4	Nonanoic acid	230	0.77±0.00^a	1.05±0.01^b	6.49±0.23^a	8.78±0.20^b
6	Decanoic acid	244	*n.d.*	1.03±0.01	*n.d.*	8.62±0.34
13	Dodecanoic acid	272	0.78±0.01^a	1.05±0.01^b	6.54±0.28^a	8.79±0.27^b
17	Tetradecanoic acid	300	7.43±0.41^a	9.43±0.54^b	62.62±5.37^a	78.86±4.03^b
20	Pentadecanoic acid	314	1.06±0.02^a	1.38±0.03^b	8.91±0.45^a	11.53±0.25^b

Table 2.1. Compounds detected in the lipophilic extracts of *P. pinguis* before (BH) and after (AH) alkaline hydrolysis. (Continuation)

			Content (mg g^{-1} of microalgal dw)		Content (mg g^{-1} of extract)	
Nº	**Identified Compounds**	**MW**	**BH**	**AH**	**BH**	**AH**
28	Hexadecanoic acid	328	7.32±0.22[a]	9.97±1.66[b]	61.71±3.62[a]	83.13±11.56[b]
18	Hexadecanoic methyl ester	270	0.85±0.01[a]	1.05±0.01[b]	7.14±0.34[a]	8.79±0.36[b]
30	Heptadecanoic acid[2]	342	2.54±0.01[a]	3.26±0.02[b]	21.37±0.58[a]	27.29±0.71[b]
39	2-Octyl-Cyclopropaneheptanoic acid	354	0.82±0.00[a]	1.09±0.01[b]	6.89±0.23[a]	9.10±0.31[b]
38	Octadecanoic acid	356	1.81±0.03[a]	3.07±0.50[b]	15.24±0.67[a]	25.61±3.40[b]
46	Eicosanoic acid	384	0.79±0.00[a]	1.07±0.01[b]	6.69±0.24[a]	8.93±0.20[b]
53	Docosanoic acid	412	0.83±0.02[a]	1.07±0.01[b]	7.00±0.24[a]	8.94±0.23[b]
	Monounsaturated		*15.20±0.54[a]*	*17.83±1.00[b]*	*128.12±8.53[a]*	*149.07±5.78[b]*
24	9-Hexadecenoic acid[1]	326	10.97±0.39[a]	13.06±0.78[b]	92.46±6.15[a]	109.19±5.00[b]
25	7-Hexadecenoic acid	326	0.92±0.02[a]	1.17±0.01[b]	7.75±0.37[a]	9.79±0.31[b]
37	11-Octadecenoic acid	354	1.27±0.08[a]	1.72±0.16[b]	10.68±0.98[a]	14.33±0.91[b]
36	9-Octadecenoic acid	354	1.25±0.08[a]	1.89±0.08[b]	10.55±0.93[a]	15.76±0.28[b]
44	11-Eicosenoic acid	382	0.79±0.00	*n.d.*	6.68±0.22[a]	*n.d.*
	Polyunsaturated		*16.34±0.29[a]*	*21.09±0.92[b]*	*137.38±5.69[a]*	*176.31±4.50[b]*
22	4,7,10,13-Hexadecatetraenoic acid	322	0.82±0.01[a]	1.08±0.01[b]	6.91±0.27[a]	9.01±0.29[b]
26	Methyl-4,7,10,13-hexadecatetraenoate	262	0.85±0.01[a]	1.05±0.00[b]	6.86±0.27[a]	8.80±0.28[b]
34	9,12-Octadecadienoic acid[1] (LA)	352	1.86±0.03[a]	2.58±0.03[b]	15.65±0.71[a]	21.59±0.81[b]
35	9,12,15-Octadecatrienoic acid (ALA)	350	0.89±0.01[a]	1.18±0.02[b]	7.49±0.30[a]	9.90±0.29[b]
33	6,9,12,15-Octadecatetraenoic acid (SA)	348	2.82±0.09[a]	3.04±0.41[a]	23.80±1.47[a]	25.39±2.67[a]
32	3,6,9,12,15-Octadecapentaenoic acid	346	0.90±0.01[a]	1.18±0.02[b]	7.60±0.30[a]	9.84±0.33[b]
42	5,8,11,14,17-Eicosapentaenoic acid (EPA)	374	3.49±0.09[a]	4.73±0.26[b]	29.43±1.44[a]	39.54±1.51[b]
48	7,10,13,16,19-Docosapentaenoic acid	402	1.59±0.08[a]	2.05±0.08[b]	13.36±0.68[a]	17.14±0.51[b]
49	4,7,10,13,16-Docosapentaenoic acid	402	1.11±0.02[a]	1.53±0.06[b]	9.37±0.32[a]	12.80±0.32[b]
47	4,7,10,13,16,19-Docosahexaenoic acid (DHA)	400	2.01±0.10[a]	2.67±0.15[b]	16.91±0.76[a]	22.30±0.95[b]
	Diacids		*0.81±0.01[a]*	*1.12±0.03[b]*	*6.86±0.27[a]*	*9.37±0.33[b]*
50	Octadecenedioic acid	456	0.81±0.01[a]	1.12±0.03[b]	6.86±0.27[a]	9.37±0.33[b]
	Fatty Alcohols		*9.69±0.26[a]*	*10.90±0.39[b]*	*81.54±0.32[a]*	*91.14±0.90[b]*
1	Octanol	202	*n.d.*	1.20±0.08	*n.d.*	10.05±0.35
7	Undecanol	244	0.63±0.01[a]	0.83±0.00[b]	5.31±0.24[a]	6.93±0.23[b]
12	Dodecanol	258	0.63±0.00[a]	0.83±0.01[b]	5.28±0.18[a]	6.99±0.25[b]

Table 2.1. Compounds detected in the lipophilic extracts of *P. pinguis* before (BH) and after (AH) alkaline hydrolysis. (Continuation)

			Content (mg g^{-1} of microalgal dw)		Content (mg g^{-1} of extract)	
Nº	**Identified Compounds**	**MW**	**BH**	**AH**	**BH**	**AH**
14	Tridecanol	272	0.64±0.01[a]	0.83±0.01[b]	5.36±0.26[a]	6.98±0.25[b]
16	Tetradecanol	286	0.67±0.01[a]	0.88±0.01[b]	5.61±0.26[a]	7.36±0.17[b]
21	Hexadecanol	314	1.50±0.09[a]	1.21±0.08[b]	12.63±0.39[a]	10.07±0.42[b]
31	Octadecanol	342	1.18±0.06[a]	1.17±0.07[a]	9.94±0.16[a]	9.81±0.31[a]
29	Octadece-9-nol	340	1.46±0.12[a]	1.21±0.12[b]	12.29±0.62[a]	10.10±0.72[b]
43	Eicosanol	368	0.69±0.01	*n.d.*	5.77±0.15[a]	*n.d.*
51	Docosanol	396	0.65±0.01[a]	0.85±0.00[b]	5.48±0.13[a]	7.10±0.19[b]
58	Octacosanol	482	0.97±0.02[a]	1.02±0.06[a]	8.13±0.18[a]	8.54±0.25[b]
71	Dotriacontanol	538	0.68±0.00[a]	0.86±0.01[b]	5.73±0.15[a]	7.21±0.21[b]
	Sterols		*14.26±1.04[a]*	*12.79±1.01[a]*	*120.26±11.78[a]*	*106.86±6.16[a]*
57	22-Stigmasten-3-one	412	0.26±0.02	*n.d.*	2.15±0.20[a]	*n.d.*
59	Stigmastane-3,6-dione	428	0.34±0.06[a]	0.19±0.01[b]	2.84±0.58[a]	1.56±0.09[b]
60	24*α*-Methylcholest-5-en-3*β*-ol	472	0.57±0.02[a]	0.56±0.03[a]	4.81±0.29[a]	4.71±0.26[a]
61	24*α*-Ethylcholesta-5,22E-dien-3*β*-ol	484	6.14±0.52[a]	5.07±0.29[b]	51.75±5.69[a]	42.38±1.99[b]
62	24-Ethyl-*δ*(22)-coprostenol	486	0.75±0.10[a]	0.63±0.04[a]	6.31±0.92[a]	5.25±0.31[a]
63	24*α*-Ethylcholest-5-en-3β-ol	486	0.78±0.03[a]	0.90±0.21[a]	6.53±0.31[a]	7.46±1.55[a]
64	4*α*,24-Dimethyl-5*α*-cholestan-3β-ol	488	1.09±0.04[a]	0.95±0.11[a]	9.22±0.57[a]	7.97±0.72[b]
66	4*α*-methyl,24-ethyl-5*α*-cholest-22E-en-3*β*-ol	500	0.77±0.04[a]	0.70±0.10[b]	6.52±0.54[a]	5.82±0.71[a]
68	4*α*-methyl-24-ethyl-5*α*-cholestan-3-ol	502	*n.d.*	0.34±0.03	*n.d.*	2.86±0.23
69	4*α*,24β-dimethyl-5*α*-cholestan-3*β*,4*β*-diol	504*	0.37±0.04[a]	0.32±0.02[a]	3.10±0.44[a]	2.65±0.2[a]
72	4*α*-methyl-24*β*-ethyl-5*α*-cholestan-3β,4*β*-diol	518*	0.71±0.09[a]	0.58±0.05[b]	6.04±0.93[a]	4.88±0.32[a]
67	Unidentified C30 Sterol		1.96±0.12[a]	1.64±0.18[b]	16.48±1.35[a]	13.66±1.12[b]
70	Unidentified C30 Sterol		0.17±0.01[a]	0.21±0.01[b]	1.45±0.08[a]	1.76±0.12[b]
65	Unidentified C30 Sterol		0.36±0.02[a]	0.71±0.07[b]	3.06±0.26[a]	5.90±0.46[b]
	Monoglycerides		*0.27±0.01[a]*	*0.04±0.01[b]*	*2.31±0.12[a]*	*0.37±0.05[b]*
40	2,3-Dihydroxypropyl tridecanoate	432	0.06±0.00[a]	0.04±0.01[b]	0.52±0.05[a]	0.37±0.05[b]
45	2,3-Dihydroxypropyl tetradecanoate	446	0.09±0.01	*n.d.*	0.72±0.05[a]	*n.d.*
52	2,3-Dihydroxypropyl palmitate	474	0.07±0.01	*n.d.*	0.59±0.07[a]	*n.d.*
55	2,3-Dihydroxypropyl stearate	502	0.06±0.01	*n.d.*	0.48±0.07[a]	*n.d.*
	Sugars		*0.17±0.01[a]*	*0.41±0.02[b]*	*1.42±0.10[a]*	*3.64±0.02[b]*
19	Rhamnose	452	*n.d.*	0.21±0.01	*n.d.*	1.85±0.02

Table 2.1. Compounds detected in the lipophilic extracts of P. pinguis before (BH) and after (AH) alkaline hydrolysis. (Continuation)

			Content (mg g^{-1} of microalgal dw)		Content (mg g^{-1} of extract)	
Nº	**Identified Compounds**	**MW**	**BH**	**AH**	**BH**	**AH**
27	Deoxyglucose	452	*n.d.*	0.20±0.01	*n.d.*	1.79±0.00
56	Glucosamine	612	0.17±0.01	*n.d.*	1.42±0.10[a]	*n.d.*
	Others		*2.02±0.02[a]*	*5.60±0.07[b]*	*17.05±0.83[a]*	*46.84±1.29[b]*
3	2,4,6,8-Tetramethyl-1-undecene	210	0.03±0.00	*n.d.*	0.21±0.04	*n.d.*
5	2-Methyltetradecane	212	0.05±0.01	*n.d.*	0.46±0.07	*n.d.*
8	2-Methyl-4-nonadecene	280	0.03±0.00	*n.d.*	0.24±0.04	*n.d.*
10	3-Methyl-4-nonadecene	280	0.04±0.01[a]	0.05±0.01[a]	0.32±0.07[a]	0.41±0.10[a]
9	2,6-bis(1,1-Dimethylethyl)phenol	278	0.99±0.02[a]	1.56±0.08[b]	8.43±0.43[a]	13.02±0.28[b]
15	Methylsuccinic acid	276	0.05±0.01	*n.d.*	0.45±0.04[a]	*n.d.*
11	3-Methoxycinnamic acid	250	*n.d.*	1.18±0.01	*n.d.*	9.88±0.38
41	Dehydroabietic acid	372	0.18±0.02[a]	0.66±0.05[b]	1.49±0.13[a]	5.55±0.49[b]
54	Pinoresinol	502	*n.d.*	1.30±0.03	*n.d.*	10.85±0.55
23	Mannitol	614	0.65±0.00[a]	0.85±0.01[b]	5.46±0.21[a]	7.13±0.29[b]
	Total Identified		*83.75±2.00[a]*	*105.34±5.90[b]*	*705.52±37.96[a]*	*880.41±29.68[b]*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Compounds are numbered by their elution order (see Figure 2.3). All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent TMS derivatives. MW – Molecular weight of compounds after silylation. [1] Contains the iso- and anteiso-isomers; [2] Contains cis and trans isomers; *Identified as the mono-TMS ether; *n.d.* – non detected; dw – dry weight.

Alkaline hydrolysis is often used for the analysis of compounds in their esterified forms [154]. In the chloroform extracts, submitted to alkaline hydrolysis, total fatty acids increased by 32% being the major increase verified for the unsaturated fatty acids (46%). This slight increase might be explained from poor extraction, such that only the smaller lipids will be available for derivatization and analysis, including volatile compounds. Moreover, these observations reveal that 32% of total fatty acids were in complex forms. Additionally, the absence of significant differences (p <0.05) in sterols amounts, in contrast to, sugars and monoglycerides levels, after alkaline hydrolysis, indicates that the major complex forms present in the chloroform extracts of *P. pinguis* where mainly glycolipids and fatty acids esterified with glycerol (mono-, di-, and triglycerides). The chromatogram obtained for the derivatized chloroform extract of *P. pinguis* after alkaline hydrolysis is displayed in Figure 2.3. According to Milke et al. [139] the levels of free fatty acids in *Pavlova* spp., *Chaetoceros muelleri* and *Placopecten magellanicus* samples ranged 0.30 and 3.10%. In the *P. pinguis* studied, fatty acids before hydrolysis accounted 5.71% of microalgal dry weight. These differences might be explained by several factors such as the strain-to-strain variation, the cultivation conditions, the methodology applied.

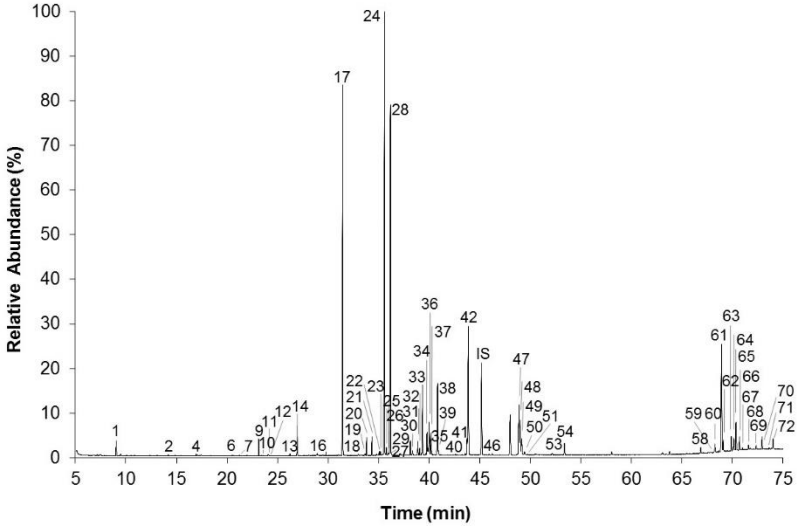

Figure 2.3. Chromatogram of the derivatized *P. pinguis* lipophilic extract after alkaline hydrolysis. Peak identification as in Table 2.1. IS – Internal Standard (Tetracosane, 0.40 mg).

2.1.1. Fatty acids

In Figure 2.4 it are represented the main families identified in the *P. pinguis* chloroform extractable substances before (Fig. 2.4a) and after (Fig. 2.4b) alkaline hydrolysis. Through this figure it is possible to visualize that fatty acids were the main family present in the chloroform extracts accounting up to 72% of the total compounds identified (Fig. 2.4b). The major fatty acids found in *P. pinguis* were 9-hexadecenoic acid (C16:1ω7), tetradecanoic (C14:0), hexadecanoic (C16:0), and 5,8,11,14,17-eicosapentaenoic (C20:5ω3 – EPA) acids which together accounted over 30% of the total identified compounds before and after hydrolysis.

Microalgae are the primary producers of essential fatty acids that cannot be synthesized by humans, which, in turn, must obtain them through their diet [55]. These are 9,12-octadecadienoic (C18:2ω6 - LA) and 9,12,15-octadecatrienoic (C18:3ω3 - ALA) acids which represent the omega-6 LC-PUFA and the omega-3 LC-PUFA, respectively [55]. *P. pinguis* presented a high content of LA (1.86 mg g^{-1} dw) and minor amounts of ALA (0.89 mg g^{-1} dw). As main precursor of the ω3 fatty acid synthesis, the minor amounts of ALA can be explained by the high levels of 6,9,12,15-Octadecatetraenoic acid (SA; 2.82 mg g^{-1} dw), EPA (3.49 mg g^{-1} dw), and 4,7,10,13,16,19-docosahexaenoic (2.01 mg g^{-1} dw) acids [59,155]. These results are consistent with those in literature that point *P. pinguis* as a high value omega-3 LC-PUFA producing strain [142].

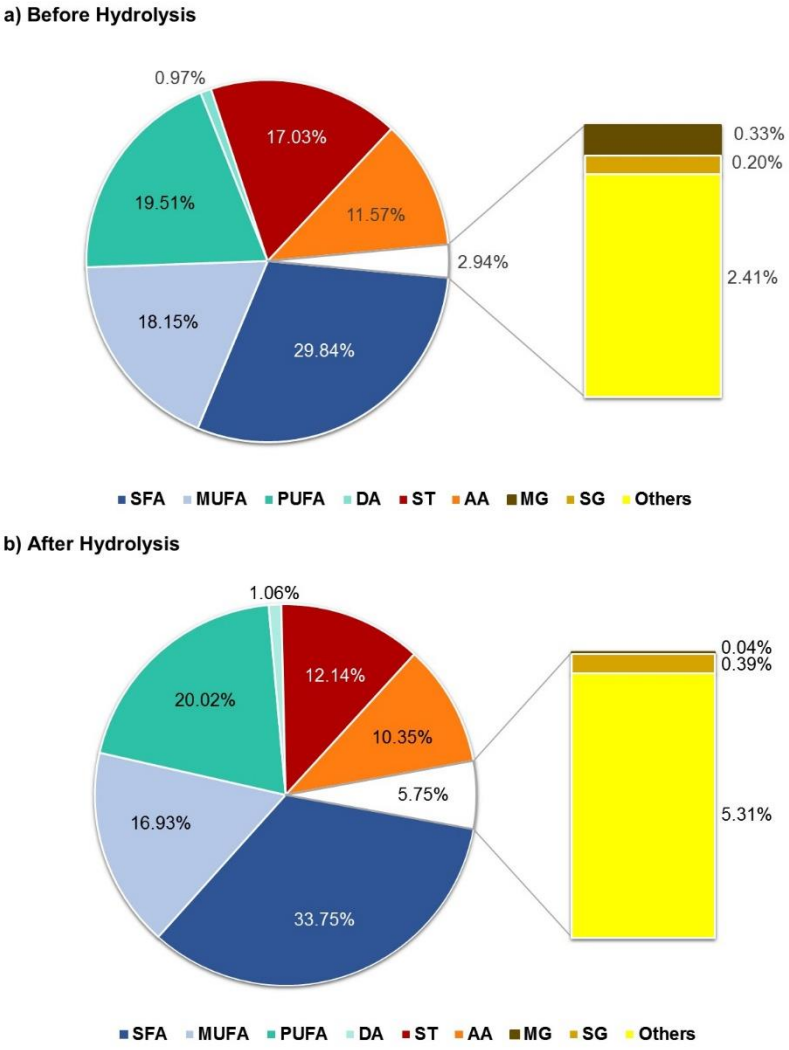

Figure 2.4. Main families identified in *P. pinguis* lipophilic extracts a) before and b) after alkaline hydrolysis, in percentage of the total identified compounds. SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; DA – Diacids; ST – Sterols; AA – Aliphatic alcohols; MG – Monoglycerides; SG – Steryl glycosides.

4,7,10,13,16,19-Docosahexaenoic acid (C22:6ω3 – DHA) is one of the main components of the structural lipids of the brain, whereas EPA display an important role in cardiovascular and immunological health [55]. The government health agencies worldwide recommend a dietary intake of DHA and EPA ranging from 200 and 670 mg d^{-1} [46]. In *P. pinguis*, DHA and EPA accounted a total of 5.50 mg g^{-1} dw, which means that 122 g of dry microalga represents the highest dietary reference value.

The ω6 fatty acids (main precursors of pro-inflammatory mediators) and the ω3 fatty acids (major precursors of anti-inflammatory molecules) compete for the same enzyme sets when metabolized [54]. Therefore, a balanced intake of ∑ω6/∑ω3 fatty acids close to 1:1 is recommended. Western diets are characterized by high levels of ω6 Fatty acids and an unbalanced ∑ω6/∑ω3 fatty acids ratio of 20:1 promoting the pathogenesis of various diseases [55]. This trend might be inverted by decreasing the intake of ω6 rich sources and increasing the

intake of ω3 rich sources [55]. *P. pinguis* presented high amounts of ω3 fatty acids (13.36 mg g^{-1} dw) and a low Σω6/Σω3 fatty acids ratio (1:4) which, in turn, makes it suitable for dietary supply of ω3 fatty acids. This ratio was close to that obtained by Slocombe et al. [142] (1:3) for *P. pinguis*.

2.1.2. Fatty alcohols

Fatty alcohols have been studied for their antibacterial activity and cholesterol-lowering ability [156,157]. The biological properties of these biomolecules are linked with the carbon chain length that is thought to determine their antibacterial activity and mode of action in biological systems [156]. Fatty alcohols accounted up to 12% of the total compounds identified (Fig. 2.4a). The major fatty alcohols found in *P. pinguis* were hexadecanol (C16-OH), octadece-9-nol (C18:1-OH), and octadecanol (C18-OH) which together accounted up to 40% of the total fatty alcohols. The mass fragmentation of C18:1-OH is displayed in Figure 2.5a. Through this figure it is possible to observe three key fragment ions from alcohols: the base peak at m/z 75 $[(CH_3)_2SiOH]^+$, the m/z 325 $[M-15]^+$, and the molecular ion $[M]^+$ at m/z 340.

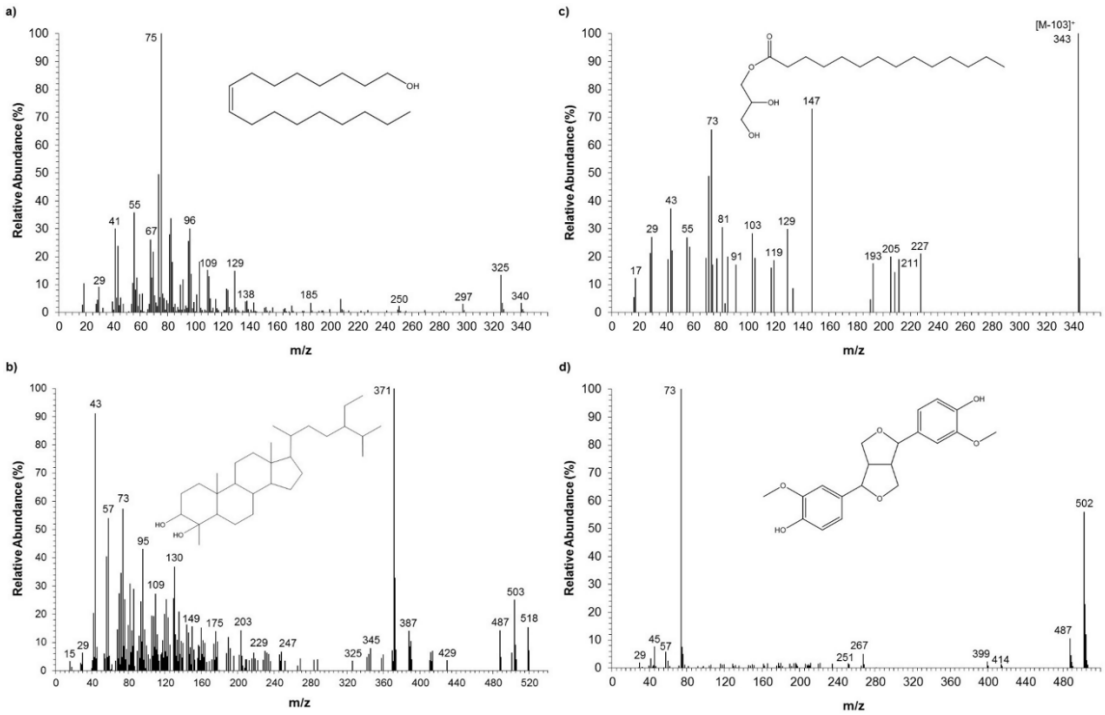

Figure 2.5. Mass spectra of some assigned peaks as trimethylsilyl (TMS) ethers and/or esters: a) Octadece-9-nol (peak 29), b) 2,3-Dihydroxypropyl tetradecanoate (peak 45), c) Pinoresinol (peak 54) and d) 4*α*-methyl-24*β*-ethyl-5*α*-cholestan-3*β*,4*β*-diol as mono TMS ether (peak 72).

In *P. pinguis*, it was identified two very long-chain alcohols: octacosanol (C28-OH) and dotriacontanol (C32-OH). The consumption of 5-20 mg d^{-1} of very long-chain aliphatic alcohols is known to decrease the low-density lipoprotein (LDL) cholesterol [129,157]. Therefore, 3.04-12.15 g of microalgal biomass and 0.36 – 1.44 g of microalgal extract are the quantities needed to fulfill these requirements (Table 2.1). Moreover, alcohols such as undecanol (C11-OH),

dodecanol (C12-OH), and tridecanol (C13-OH), have been pointed in previous studies by their bactericidal activity [156]. After alkaline hydrolysis the fatty alcohols increased by 12%, with the highest increase verified for the C12-OH (32%).

The detected docosanol (C22-OH) is known by its antiproliferative effect of chinese hamster ovary cells K1 (CHO-K1) and human melanoma (CRL-1974TM) cell lines [158]. Investigations concerning the effects of long-chain alcohols are often performed with long-chain alcohols isolated from sugarcane where these compounds make up 0.10-0.30% of its mass [159]. In the present study this class of compounds comprised 0.16 and 0.19% of *P. pinguis* dried biomass, before and after alkaline hydrolysis respectively.

2.1.3. Sterols

Microalgae are recognized for their wide diversity of sterols that are often used for chemotaxonomic and phylogenetic comparisons [84]. This diversity along with the high sterol content make microalgae promising sources of novel sterols with potential novel activities [67].

Sterols accounted up to 17% of the total identified compounds (Fig. 2.4a). The major sterols were 24*α*-ethylcholesta-5,22E-dien-3*β*-ol (stigmasterol; 40 – 43%), 4*α*,24-Dimethyl-5*α*-cholestan-3*β*-ol (7 – 8%), and an unidentified sterol (13 – 14%), which together accounted over 50% of total sterols (Table 2.1). The dominance of stigmasterol across *Pavlova* species namely *P. pinguis* have been reported in studies targeting aquaculture and chemotaxonomy [139,148]. 24*α*-ethylcholest-5-en-3*β*-ol (*β*-sitosterol), 24*α*-methylcholest-5-en-3*β*-ol (campesterol), and stigmasterol - in their non-esterified forms, have been subject to the Food and Drug Admnistration (FDA) health claim for reduced risk of coronary heart disease [67]. *P. pinguis* presented high levels of stigmasterol which alone represented up to 7% of the total identified compounds in the lipophilic profile. Campesterol and *β*-sitosterol were also identified in the *P. pinguis* chloroform extracts accounting 0.57 and 0.78 mg g^{-1} of microalgal biomass and 4.81 and 6.53 mg g^{-1} of extract, respectively (Table 2.1). In contrast to Milke et al. [139] stigmastanol and cholesterol were not detected in *P. pinguis* lipophilic extracts.

Pavlova species are often recognized by their unusual dihydroxylated sterols called Pavlovols [85]. In *P. pinguis* two dihydroxylated sterols were identified, 4*α*-methyl-24*β*-ethyl-5*α*-cholestan-3*β*,4*β*-diol (ethylpavlovol) and 4*α*,24*β*-dimethyl-5*α*-cholestan-3*β*,4*β*-diol (methylpavlovol), and a structurally isomeric form of dinosterol, 4*α*-methyl-24-ethyl-5*α*-cholest-22E-en-3*β*-ol. As with fatty acids, the sterol profiles of microalgae are species-specific and often used as chemotaxonomic markers [85].

Despite in Milke et al. [139] the pavlovols have not been found for *Pavlova* species, the authors recognize that they can constitute the sterol composition of this microalga specie. Moreover, Volkman et al. [148] found the existence of these unusual sterols in the composition of two other *P. pinguis* strains and pointed pavlovols as chemotaxonomic markers of Pavlovales.

Figure 2.5b shows the mass spectrum of the compound identified as ethylpavlovol. Through comparison of the obtained mass spectrum with the one obtained previously by

Volkman et al. [148], it was possible to identify the ethylpavlovol as its mono TMS ether. The assignment was done by the presence of the following mass fragments: m/z 43, 487, 503 and 518, as well as, the base peak at m/z 371 [M-147($C_3H_6O_2$TMS)]$^+$.

Phytosterols are playing a key role in nutraceutic and pharmaceutical industries, as precursors of some bioactive molecules [67,84]. Moreover, it is estimated that the dietary intake of phytosterols is in the range of 150 to 400 mg d^{-1}. *P. pinguis* can contribute to the intake of around 143 mg of free sterols per 100 g of microalgal dry weight. After alkaline hydrolysis it were not verified significant differences (p <0.05) in the amounts of sterols (Table 2.1). This observation indicates that sterols were non-esterified and were as free sterols.

2.1.4. Other compounds

In the classes of monoglycerides, sugars and other components, compositional differences before and after hydrolysis were verified. In Table 2.1 it is possible to observe that after alkaline hydrolysis the monoglycerides: 2,3-Dihydroxypropyl tetradecanoate, 2,3-Dihydroxypropyl palmitate, and 2,3-Dihydroxypropyl stearate, were not detected. Sugars and the other components classes were those who presented the highest increase after alkaline hydrolysis, 3 and 5 times higher respectively. The increase of the sugar content suggests the presence of polar lipids, namely glycolipids incorporating rhamnose and deoxyglucose, in the chloroform extracts of *P. pinguis*. The sulfoquinovosyl diacylglycerols (SQDGs) are one of the most abundant glycolipids found in microalgal cells [125]. SQDGs are constituted by a 6-sulfoquinovose unit, which, in turn, is constituted by a sulphur group attached to the quinovose (6-deoxyglucose) [160]. Moreover, it has been reported that microalgal glycolipids may contain other sugar moieties than galactose such as mannose and rhamnose [161].

The mass fragmentation of 2,3-Dihydroxypropyl tetradecanoate is presented in Fig. 2.5c. Although in the mass spectrum it is not possible to visualize the molecular ion, the presence of the following ions: m/z 73, 103, 147, 205, 343, indicates that this compound is 2,3-dihydroxypropyl tetradecanoate. The fragment m/z 343 corresponds to the mass fragment [M-103 (CH_2OTMS)]$^+$ and the m/z 103, 147, and 205 are associated to the silylated glycerol backbone.

The detection of smaller amounts of 3-methocycinnamic acid and pinoresinol 1.12 and 1.23% of total identified compounds, respectively, was observed after alkaline hydrolysis. According to Klejdus et al. [162] the cinnamic acid derivatives are precursors in the phenyl-propanoid pathway for the synthesis of polyphenols, which indicates that it is possible that this microalgal strain have other phenols that could be extracted by polar solvents. In Fig. 2.5d it is possible to visualize the fragmentation pattern obtained for pinoresinol, namely the base peak m/z 73 [$(CH_3)_3Si$]$^+$, the m/z 487 resultant from the loss of a methyl group [M-15]$^+$, and the molecular ion [M]$^+$ m/z 502. Phenols are natural products that are recognized by their antioxidant, antimicrobial, and antiviral activities [162].

P. pinguis chloroform extracts presented a strong concentration-dependent DPPH radical scavenging activity with a determination coefficient close to 1 (R^2 = 0.99), Figure 2.6. The presence of phenols in the lipophilic fraction after alkaline hydrolysis suggest that this ability might be resultant from polyphenol-associated lipids. The estimated EC_{50} for *P. pinguis* chloroform extracts was of 1057 µg mL^{-1}.

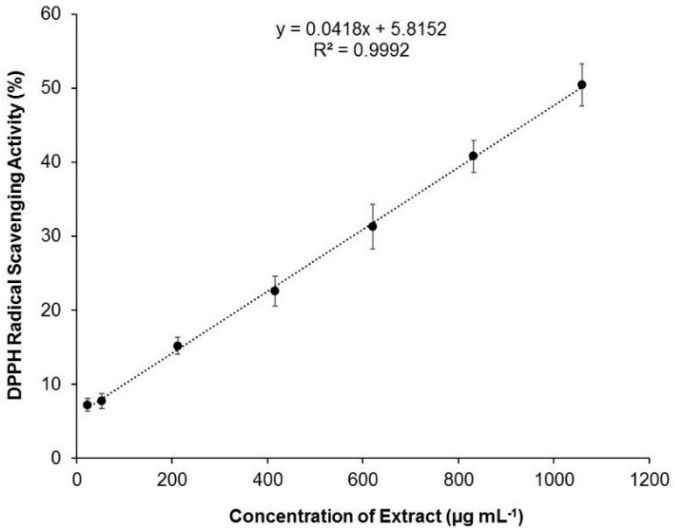

Figure 2.6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%) of *P. pinguis* chloroform extracts (µg mL^{-1}). Butylated hydroxytoluene (BHT) was used as a reference compound and its EC_{50} value is 9.79 μg mL^{-1}.

Besides nutraceutics and pharmaceutics the rich composition verified for *P. pinguis* as for other species of the genus *Pavlova* [163] can also be used in aquaculture for animal consumption as dietetic supply and in food industry as additive and/or nutritional supplements attributing to consumer a higher level of bioactive compounds.

4. Conclusions

The need for naturally derived health-promoting phytochemicals instead of the chemically derived drugs have prompted the search for new sources of natural products. *Pavlova pinguis* presented a manifold range of metabolites which demonstrated its versatility and potential as a source of high-value compounds. The high content of unsaturated fatty acids, long-chain aliphatic alcohols, and stigmasterol, demonstrate the potential of this microalga not only for aquaculture but also for nutraceutics and pharmaceutics uses. To fully exploit the phytochemical features of microalgae for commercial purposes, a non-targeted approach should be taken to uncover whole extract chemical diversity.

CHAPTER 3.

Hemiselmis cf. *andersenii* and *Chlorella stigmatophora* as new sources of high-value compounds: a lipidomic approach

This Chapter is based on the following publication:

Fernandes, T.; Cordeiro, N. *Hemiselmis andersenii* and *Chlorella stigmatophora* as new sources of high-value compounds: a lipidomic approach. J. Phycol. 2020, 56 (6), 1493–1504. https://doi.org/10.1111/jpy.13042.

Chapter 3. *Hemiselmis* cf. *andersenii* and *Chlorella stigmatophora* as new sources of high-value compounds: a lipidomic approach

Abstract

To unlock the potential of *Chlorella stigmatophora* (Trebouxiophyceae, Chlorophyta) and *Hemiselmis* cf. *andersenii* (Cryptophyceae, Cryptophyta) as natural reactors for biotechnological exploitation, their lipophilic extracts were characterized using Fourier Transform Infrared spectroscopy with Attenuated Total Reflectance (FTIR-ATR) and Gas Chromatography-Mass Spectrometry (GC-MS) before and after alkaline hydrolysis. The GC-MS analysis enabled the identification of 62 metabolites, namely fatty acids (27), aliphatic alcohols (17), monoglycerides (7), sterols (4), and other compounds (7). After alkaline hydrolysis, monounsaturated fatty acids increased by as much as 87%, suggesting that the esterified compounds were mainly neutral lipids. *H.* cf. *andersenii* yielded the highest Σω3/Σω6 ratio (7.26), indicating that it is a good source of ω3 fatty acids, in comparison to *C. stigmatophora* (Σω3/Σω6 = 1.24). Both microalgae presented significant amounts of aliphatic alcohols (6.81 – 10.95 mg g^{-1} dw), which are recognized by their cholesterol-lowering properties. The multivariate analysis allowed visualization of the chemical divergence among *H.* cf. *andersenii* lipophilic extracts before and after alkaline hydrolysis, as well as species-specific differences. *C. stigmatophora* showed to be a valuable source of essential fatty acids for nutraceuticals, whereas *H.* cf. *andersenii*, due to its high chemical diversity, seems to be suitable for different fields of application.

Keywords: *Chlorella stigmatophora*, GC-MS, *Hemiselmis* cf. *andersenii*, Lipophilic fraction, Microalgae.

1. Introduction

Microalgae show dual potential as a feedstock for biomass and biofuel production. The advantages that make these organisms sustainable biofactories to synthesize value-added compounds include non-competition for agricultural land, carbon fixation ability, and metabolic plasticity [164]. From the wide biological diversity of microalgae and cyanobacteria, only a few species belonging to *Arthrospira* and *Chlorella* genera dominate the human nutrition market, with an estimated global production of 6600 and 12000 tons of dry matter per year, respectively [165,166].

Among algal metabolites, lipids are of great importance for the biotechnological exploitation of microalgae [167]. For instance, essential fatty acids, such as 9,12-octadecadienoic acid (C18:2ω6) and 9,12,15-octadecatrienoic (C18:3ω3) acids, can be suitable for applications in the food industry as an additive and/or nutritional supplements, while triacylglycerols and saturated fatty acids are suitable for biofuel production [167]. Additionally, given the reduction of global fish stocks and fishes' susceptibility to contamination by pollutants (*e.g.*, heavy metals), microalgae have emerged as sustainable sources of long-chain polyunsaturated fatty acids [168,169]. Besides fatty acids, microalgae-derived lipids also contain aliphatic alcohols,

monoglycerides, and phytosterols which have potential applications in aquaculture feed, food, and pharmaceutical industries [67]. Indeed, phytosterols have been incorporated in food matrices such as low-fat milk, fat-based spreads, and cereal bars due to their efficacy as cholesterol-lowering agents [170].

Cryptophyceae species are used for application in aquaculture industries due to their high polyunsaturated fatty acids and sterol concentrations, but are not widely produced for the purpose of human health [171]. *Hemiselmis* cf. *andersenii* belongs to this taxonomic class and research efforts have been focused on its nucleomorph characterization to study the process of genome evolution [172]. The genus *Chlorella* is recognized as a model oleaginous microalga, *Chlorella stigmatophora* being one of the few marine species that belong to this genus [173]. In both microalgae species, the analysis of the lipophilic fraction is limited to specific families like fatty acids and sterols [142]. The abundance of other important lipophilic classes, such as long-chain aliphatic alcohols and monoglycerides, is unknown.

Lipidomics play a key role in deciphering the potential of microalgae as natural reactors to produce bioactive lipids [125]. This area of metabolomics is responsible for the detailed identification and quantification of the lipid species [125]. In this field of study, GC-MS is often applied due to its low sample requirement and because it is a powerful analytical tool that enables the robust characterization of the total lipid extract [125]. In this sense, GC-MS techniques have been used for the complete study of the lipid extract as a whole [129,174].

The main aim of the present work was to evaluate the potential of *H.* cf. *andersenii* and *C. stigmatophora* strains as potential sources of high-value compounds for further applications in nutraceutical and other related fields. With this purpose, the profiles of the lipophilic extracts from *C. stigmatophora* and *H.* cf. *andersenii* were analyzed using GC-MS. Since the lipophilic fraction can be enriched in molecules in their esterified forms, the study of complex lipids is crucial. Thus, the characterization of the lipophilic extracts was made before and after alkaline hydrolysis.

2. Materials and methods

2.1. Growth and culture conditions

Microalgae were obtained from the Roscoff Culture Collection (RCC) and the Spanish Bank of Algae (BEA). The cultivation of the Chlorophyta *Chlorella stigmatophora* (RCC 661) and the Cryptophyta *Hemiselmis* cf. *andersenii* (BEA 0118B) were performed by inoculating starter cultures in sterile f/2 – Si medium with pH adjusted to 7.0, under 70 µmol m^{-2} s^{-1} light intensity and a 16:8 h (light: dark) cycles, at 25 °C. At the end of the logarithmic phase, cultures were harvested by centrifugation at 3720 g, and pellets were washed twice with an isotonic saline solution (0.09 g NaCl L^{-1}). Cell densities were determined with a Neubauer–improved counting chamber (Marienfield Superior) and a light microscope (Olympus BX41, Tokyo, Japan) with a 40x magnification. The specific growth rate was obtained by the linearization of the following logistic model (Equation 3.1):

$N = K/(1+e^{a-\mu t})$ Equation 3.1

where K (cells mL^{-1}) stands for the carrying capacity, N (cells mL^{-1}) corresponds to the cell concentration in time (t), a is the position of the origin, and μ (d^{-1}) is the specific growth rate [145,175].

Biomass yield (g L^{-1}; in dry weight) was estimated at the end of the batch cultivation by freeze-drying algal samples on pre-weighed flasks. Dry weight (dw) was obtained based on weight difference.

2.2. Extraction of the lipophilic fraction

The lipophilic phase was extracted as reported in Ma et al. [146], with some modifications. Approximately 100 – 150 mg of freeze-dried sample was extracted with an aqueous solution (methanol:water, 1:1) and chloroform in a 1:1 ratio. The mixture was left stirring for 15 min and separated by centrifugation at 4427 g for 10 min. The lipophilic phase was transferred into pre-weighed tubes and evaporated in a nitrogen atmosphere before hydrolysis (BH). The amount of extractable substances was gravimetrically quantified and expressed as a percentage by weight of the freeze-dried biomass (dw).

2.3. Fourier transform infrared spectroscopy with attenuated total reflectance

Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) spectra of microalgal raw freeze-dried biomass and lipophilic extracts BH were collected on a Perkin–Elmer Spectrum Two instrument coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK) scanning over the wavenumber range of 4000 - 650 cm^{-1} at a resolution of 4 cm^{-1} and 36 scans.

2.4. Alkaline hydrolysis and GC-MS analysis

The alkaline hydrolysis was performed in two aliquots according to Santos et al. [129]. To each extract, 10 mL of 0.5M NaOH in 50% aqueous methanol solution was added and the mixture heated at 100 ºC for 1 h in a nitrogen atmosphere. Samples were acidified to pH 2 with 1M HCl and extracted with dichloromethane. The solvent was evaporated to dryness under nitrogen and named AH.

To fully exploit the potential of the lipophilic fraction of *C. stigmatophora* and *H.* cf. *andersenii*, an untargeted approach was implemented. Two aliquots of each dried extract (before – BH and after – AH alkaline hydrolysis) were silylated according to the methodology described by Santos et al. [129]. Briefly, an accurate amount of internal standard (IS; tetracosane 0.20 – 0.30 mg), 250 μL of pyridine, 250 μL of N,O-bis(trimethylsilyl)trifluoroacetamide, and 50 μL of trimethylclorosilane were added to the extracts. The mixtures were then left to react at 70 ºC for 30 min.

The trimethylsilyl (TMS) derivatives were analyzed in a gas chromatograph (Agilent HP 6890, Palo Alto, CA, USA) equipped with a mass selective detector (Agilent 5973) and a

ValcoBon 17704 capillary column VB1 (30 m × 0.25 mm inner diameter, 0.25 µm film thickness). The temperature gradient started with an initial temperature of 80 ºC for 5 min; a linear increase to 208 ºC at 4 ºC min^{-1}, followed by an increase to reach 260 ºC at 2 °C min^{-1}, and a final increase to 300 ºC at 5 °C min^{-1} with 4 min of hold time. The temperature of the injector was 250 °C; the transfer line, 290 °C; and the split ratio was 33:1. Helium was used as the carrier gas at a constant flow of 1.0 mL min^{-1}. The identification of the TMS derivatives was made by comparison of the mass spectra fragmentation to those in the GC-MS spectral library (Wiley-NIST Mass Spectral Library 1999), published data [147,176,177] or by injection of standards. For semi-quantitative analysis, the GC-MS was calibrated with pure reference compounds (mannose, trans-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5*α*-chlolestane, cholesterol, stigmasterol, hexadecanoic acid, and nonadecanoic acid) and the response factor was calculated towards tetracosane (IS).

2.5. Statistical analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 24, IBM Corporation, Armonk, NY, USA. Differences between treatments were assessed with a Student's t-test, and the *p*-values <0.05 were considered statistically significant. Principal component analysis (PCA) was applied to summarize the loadings and record information using a reduced number of principal components. Varimax rotation was selected to represent the planar projection of the loadings and scores for the two principal components.

3. Results and discussion

3.1. Growth and extraction yield

In our study, *Chlorella stigmatophora* displayed the highest growth (Fig. 3.1) with a specific growth rate (µ) of 1.30 d^{-1}, while *Hemiselmis* cf. *andersenii* showed a growth rate of 0.96 d^{-1}. Biomass yields were 0.48 and 0.32 g dw L^{-1} for *C. stigmatophora* and *H.* cf. *andersenii*, respectively. The FTIR-ATR analysis of microalgal freeze-dried biomass displayed different patterns (Fig. 3.2) for the species analyzed with major differences in the regions often linked with polysaccharides (900 - 1200 cm^{-1}) and lipids (1745 and 2850 - 3000 cm^{-1}). Comparing the spectra of raw biomass with the lipophilic extracts before hydrolysis, it was possible to observe specific bands associated with the lipophilic fraction, located at: *i)* 3010 cm^{-1} (*ν*C–H of C=CH– chains); *ii)* 2954-2850 cm^{-1} (*ν*C-H in CH_3 and CH_2 sp^3); *iii)* 1744 cm^{-1} (*ν*C=O in carbonyl groups); *iv)* 1463 cm^{-1} (*δ*CH_2); and *v)* 721 cm^{-1} (*δ*CH_2 rocking) [152,153]. The yields for lipophilic extracts of the marine microalgae strains accounted for 5.76 ± 0.65% and 6.50 ± 0.34% dw in *C. stigmatophora* and *H.* cf. *andersenii*, respectively.

To explore the potential of microalgae as sustainable sources of value-added compounds, it is essential to assess microalgae growth characteristics, biomass yield, and desired product content. The highest specific growth rate verified for *C. stigmatophora* is in accordance with the previous data that showed *Chlorella* as fast-growing strains [171]. In contrast

to *Chlorella*, Cryptophyta are known to grow slowly ($\mu < 0.80\ d^{-1}$), but in suitable conditions, some strains can achieve higher growth rates [171].

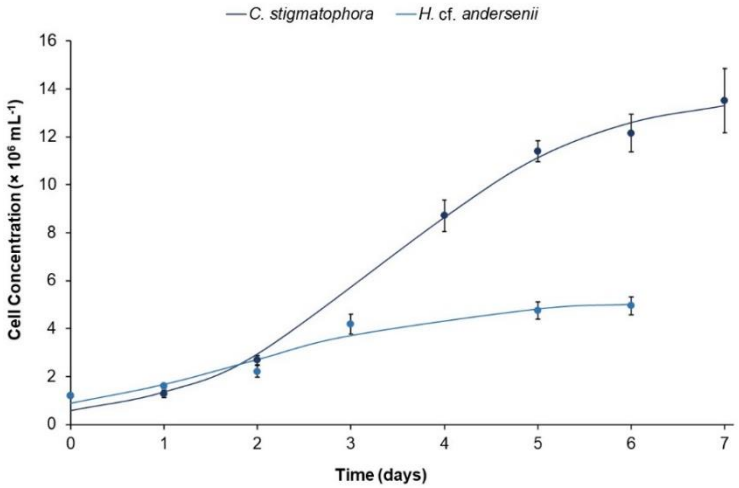

Figure 3.1. Growth curves for *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii*.

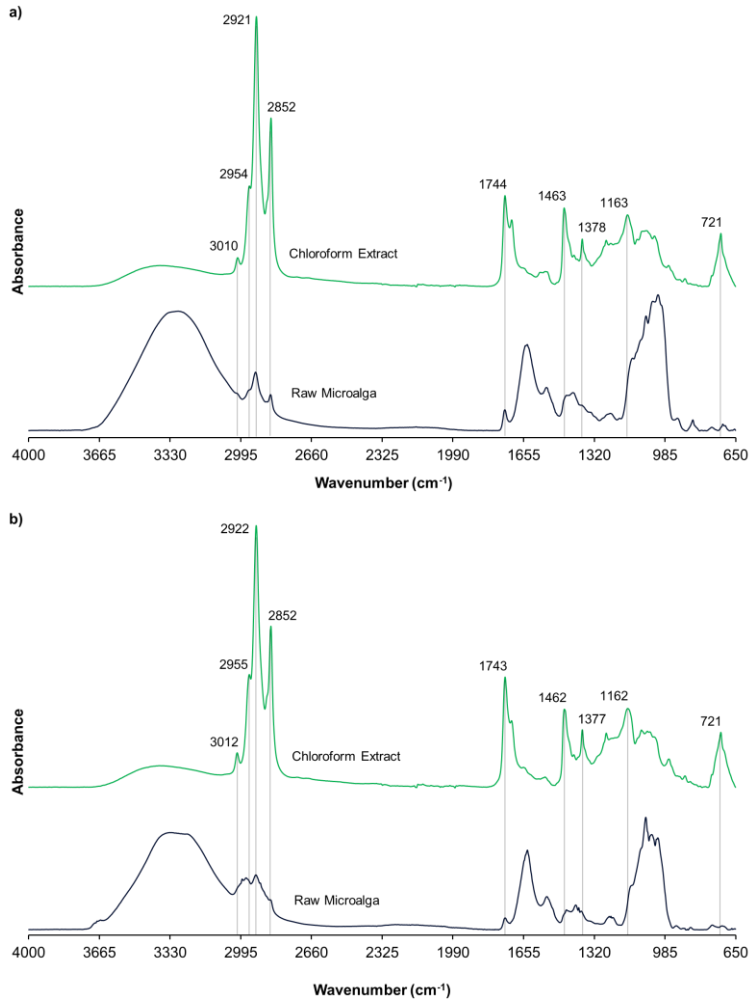

Figure 3.2. Fourier Transform Infrared spectroscopy with Attenuated Total Reflectance (FTIR-ATR) spectra of freeze-dried microalga (raw microalga) and chloroform extract before hydrolysis a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii*.

The nature of microalgae cell covering is known to affect their shear resistance and cellular biomass weight [178]. As a result, the absence of a heavy and rigid cell wall structure in *H.* cf. *andersenii* makes cells lighter and easier to break and process for further commercial purposes than *C. stigmatophora* [171]. This observation explains the fact that the biomass yield verified for *H.* cf. *andersenii* was low, despite its growth rate (estimated from cellular concentration) being high. Biomass values obtained for *C. stigmatophora* were 45% higher than those previously reported by Slocombe et al. [142] that revealed values near to 0.33 g dw L^{-1}.

The amount of lipophilic substances in *C. stigmatophora* was lower than that previously obtained by Slocombe et al. [142]. The low lipophilic extracts' yields might be explained by *Chlorella* strains' growth conditions and the cell physiological status. Although there is no information regarding *Hemiselmis* species lipophilic contents, the values observed in the present study were lower than those determined by Huerlimann et al. [179] for another Cryptophyta (*Rhodomonas* sp.) cultivated in f/2 growth medium, 13% dw.

In the present study, microalgae were scaled up in the same conditions as the inoculum and were collected at the late exponential phase, instead of the stationary phase. The low yields of lipophilic extracts – which in turn influence fatty acids, aliphatic alcohols, sterols, and other compounds contents – might be explained by the assumption that lipids accumulation is often triggered by environmental fluctuations in microalgae growth systems (*e.g.*, nutrient deficiency, light saturation, and growth stage) [62]. Thus, the growth conditions employed were not ideal for eliciting marine microalgae carbon partitioning toward lipid accumulation.

3.2. Lipophilic characterization

The GC-MS metabolite profile (Fig. 3.3) of the lipophilic fraction before (BH) and after (AH) alkaline hydrolysis enabled the characterization of up to 71 and 64% of the total extractable substances from *C. stigmatophora* and *H.* cf. *andersenii*. This holistic approach allowed us to identify and quantify a total of 62 metabolites (Table 3.1). Significant differences (p <0.05) were observed in the metabolites detected for both microalgae before and after alkaline hydrolysis.

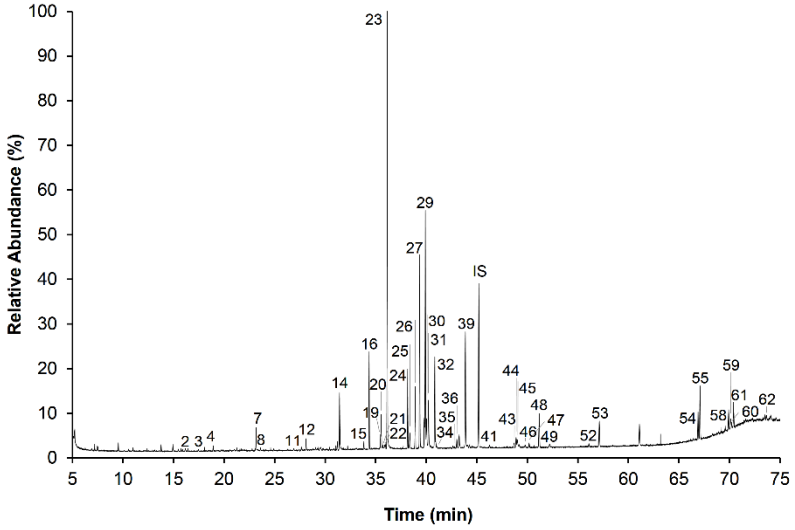

Figure 3.3. Chromatogram example of the derivatized *Hemiselmis* cf. *andersenii* lipophilic extract before alkaline hydrolysis. The peaks are numbered by their elution order and the correspondent identification is displayed in Table 3.1. IS, Internal standard (tetracosane, 0.20 mg)

Table 3.1. Lipophilic profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* before (BH) and after (AH) hydrolysis.

				C. stigmatophora		***H.* cf. *andersenii***	
Nº	**Identified Compounds**	**Formula**	**MW**	**BH**	**AH**	**BH**	**AH**
Fatty acids				*22.22±0.41^a*	*28.62±0.23^b*	*18.30±0.22^a*	*28.10±2.67^b*
	Saturated			*7.34±0.04^a*	*8.34±0.05^b*	*8.17±0.12^a*	*15.18±1.69^b*
3	Nonanoic acid	$C_9H_{18}O_2$	230	0.52±0.00^a	0.52±0.00^a	0.51±0.01^a	0.76±0.02^b
5	Decanoic acid	$C_{10}H_{20}O_2$	244	*n.d.*	*n.d.*	*n.d.*	0.75±0.02
10	Dodecanoic acid	$C_{12}H_{24}O_2$	272	*n.d.*	0.54±0.00	*n.d.*	0.76±0.03
14	Tetradecanoic acid	$C_{14}H_{28}O_2$	300	0.57±0.00^a	0.60±0.00^b	0.72±0.12^a	1.39±0.06^b
15	Pentadecanoic acid	$C_{15}H_{30}O_2$	314	0.53±0.00^a	0.53±0.00^a	0.54±0.01^a	0.81±0.02^b
23	Hexadecanoic acid	$C_{16}H_{32}O_2$	328	3.53±0.03^a	4.50±0.04^b	3.00±0.06^a	5.51±0.86^b
25	Heptadecanoic acid	$C_{17}H_{34}O_2$	342	0.61±0.01^a	0.56±0.00^b	0.65±0.04^a	0.89±0.01^b
32	Octadecanoic acid	$C_{18}H_{36}O_2$	356	1.07±0.02^a	1.09±0.01^a	1.15±0.06^a	2.77±0.88^b
43	10-Hydroxyoctadecanoic acid	$C_{18}H_{36}O_3$	444	*n.d.*	*n.d.*	0.55±0.01^a	n.d.
37	Nonadecanoic acid	$C_{19}H_{38}O_2$	370	*n.d.*	*n.d.*	*n.d.*	0.77±0.02
41	Eicosanoic acid	$C_{20}H_{40}O_2$	384	*n.d.*	*n.d.*	0.52±0.00^a	0.77±0.01^b
49	Docosanoic acid	$C_{22}H_{44}O_2$	412	*n.d.*	*n.d.*	0.55±0.01	n.d.
	Monounsaturated			*6.46±0.14^a*	*9.98±0.14^b*	*3.77±0.12^a*	*7.06±0.12^b*
20	9-Hexadecenoic acid[1]	$C_{16}H_{30}O_2$	326	1.43±0.00^a	1.62±0.01^b	1.13±0.09^a	1.59±0.04^b
21	7-Hexadecenoic acid	$C_{16}H_{30}O_2$	326	0.70±0.01^a	0.79±0.01^b	0.51±0.00^a	0.83±0.01^b

Table 3.1. Lipophilic profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* before (BH) and after (AH) hydrolysis. (Continuation)

				C. stigmatophora		***H.* cf. *andersenii***	
Nº	**Identified Compounds**	**Formula**	**MW**	**BH**	**AH**	**BH**	**AH**
31	11-Octadecenoic acid	$C_{18}H_{34}O_2$	354	0.65±0.01[a]	0.63±0.00[b]	0.87±0.04[a]	1.51±0.17[b]
30	9-Octadecenoic acid	$C_{18}H_{34}O_2$	354	3.68±0.16[a]	6.94±0.03[b]	0.65±0.04[a]	1.29±0.04[b]
33	7-Nonadecenoic acid	$C_{19}H_{36}O_2$	368	*n.d.*	*n.d.*	*n.d.*	0.82±0.03
36	10-Nonadecenoic acid	$C_{19}H_{36}O_2$	368	*n.d.*	*n.d.*	0.60±0.03[a]	1.02±0.03[b]
	Polyunsaturated			8.41±0.24[a]	10.30±0.19[b]	6.35±0.11[a]	7.67±0.10[b]
19	7,12-Hexadienoic acid	$C_{16}H_{28}O_2$	324	*n.d.*	*n.d.*	0.57±0.01[a]	0.80±0.01[b]
17	7,10-Hexadecadienoic acid	$C_{16}H_{28}O_2$	324	1.64±0.03[a]	1.70±0.05[a]	*n.d.*	*n.d.*
18	7,10,13-Hexadecatrienoic acid	$C_{16}H_{26}O_2$	322	1.94±0.02[a]	2.01±0.02[b]	*n.d.*	*n.d.*
22	Methyl-4,7,10,13-hexadecatetraenoate	$C_{17}H_{26}O_2$	262	*n.d.*	n.d.	0.52±0.00	*n.d.*
28	9,12-Octadecadienoic acid (LA)[1]	$C_{18}H_{32}O_2$	352	2.11±0.13[a]	3.21±0.15[b]	0.70±0.02[a]	1.22±0.03[b]
29	9,12,15-Octadecatrienoic acid (ALA)	$C_{18}H_{30}O_2$	350	2.19±0.14[a]	2.67±0.02[b]	2.05±0.05[a]	2.55±0.23[b]
39	5,8,11,14,17-Eicosapentaenoic acid (EPA)	$C_{20}H_{30}O_2$	374	0.53±0.00[a]	0.71±0.07[b]	1.35±0.04[a]	1.86±0.17[b]
45	7,10,13,16,19-Docosapentaenoic acid	$C_{22}H_{34}O_2$	402	*n.d.*	*n.d.*	0.57±0.01	n.d.
44	4,7,10,13,16,19-Docosahexaenoic acid (DHA)	$C_{22}H_{32}O_2$	400	*n.d.*	*n.d.*	0.60±0.01[a]	0.91±0.04
Aliphatic Alcohols				*7.15±0.05[a]*	*6.81±0.02[b]*	*8.19±0.47[a]*	*10.95±0.23[b]*
1	Octanol	$C_8H_{18}O$	202	*n.d.*	*n.d.*	*n.d.*	0.61±0.02
6	Undecanol	$C_{11}H_{24}O$	244	0.43±0.00[a]	0.42±0.00[b]	*n.d.*	0.61±0.02
9	Dodecanol	$C_{12}H_{26}O$	258	*n.d.*	*n.d.*	*n.d.*	0.61±0.02
11	Tridecanol	$C_{13}H_{28}O$	272	0.42±0.00[a]	0.42±0.00[a]	0.42±0.01[a]	0.64±0.02[b]
13	Tetradecanol	$C_{14}H_{30}O$	286	0.47±0.00[a]	0.42±0.00[b]	*n.d.*	0.62±0.03
16	Hexadecanol	$C_{16}H_{34}O$	314	1.04±0.01[a]	0.71±0.01[b]	0.99±0.12[a]	0.98±0.04[a]
26	Octadecanol	$C_{18}H_{38}O$	342	1.48±0.01[a]	0.66±0.00[b]	0.79±0.06[a]	0.87±0.01[a]
24	9-Octadecen-1-ol	$C_{18}H_{36}O$	340	0.81±0.01[a]	0.95±0.01[b]	0.95±0.17[a]	1.00±0.05[a]
40	Eicosanol	$C_{20}H_{40}O$	368	0.58±0.01[a]	0.42±0.00[b]	*n.d.*	*n.d.*
34	11-Eicosen-1-ol	$C_{20}H_{38}O$	366	*n.d.*	*n.d.*	0.44±0.01	*n.d.*
46	Docosanol	$C_{22}H_{44}O$	396	*n.d.*	*n.d.*	0.42±0.00[a]	0.62±0.02[b]
54	Octacosanol	$C_{28}H_{58}O$	482	0.45±0.01[a]	0.49±0.01[b]	0.62±0.04[a]	0.67±0.04[a]
61	Triacontanol	$C_{30}H_{62}O$	510	0.54±0.01[a]	0.45±0.00[b]	0.61±0.06[a]	0.70±0.02[b]
62	Dotriacontanol	$C_{32}H_{66}O$	538	*n.d.*	*n.d.*	0.45±0.01[a]	0.62±0.02[b]
59	Octacosane-1,3-diol	$C_{28}H_{58}O_2$	570	*n.d.*	0.42±0.00[b]	0.49±0.03[a]	0.66±0.02[b]

Table 3.1. Lipophilic profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* before (BH) and after (AH) hydrolysis. (Continuation)

				C. stigmatophora		***H.* cf. *andersenii***	
Nº	**Identified Compounds**	**Formula**	**MW**	**BH**	**AH**	**BH**	**AH**
60	Octacosane-1,2-diol	$C_{28}H_{58}O_2$	570	0.45±0.01[a]	0.43±0.00[b]	0.44±0.01	n.d.
27	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	368	0.48±0.00[a]	1.02±0.01[b]	1.57±0.04[a]	1.74±0.18[a]
Sterols				*0.73±0.01[a]*	*0.62±0.01[b]*	*1.09±0.06[a]*	*0.77±0.07[b]*
55	24*β*-Methylcholesta-5,22E-dien-3*β*-ol	$C_{28}H_{46}O$	470	*n.d.*	*n.d.*	0.76±0.06[a]	0.45±0.04[b]
56	24-Methylcholesta-5,24(28)-dien-3*β*-ol	$C_{28}H_{46}O$	472	0.10±0.01[a]	0.09±0.00[a]	*n.d.*	*n.d.*
57	24*α*-Methylcholest-5-en-3*β*-ol	$C_{28}H_{48}O$	472	0.12±0.01[a]	0.10±0.00[b]	*n.d.*	*n.d.*
58	24*α*-Ethylcholest-5-en-3*β*-ol	$C_{29}H_{50}O$	486	0.51±0.01[a]	0.42±0.01[b]	0.33±0.05[a]	0.32±0.04[a]
Monoglycerides				*n.d.*	*n.d.*	*1.11±0.07[a]*	*0.20±0.03[b]*
35	2,3-Dihydroxypropyl tridecanoate	$C_{16}H_{32}O_4$	432	*n.d.*	*n.d.*	0.04±0.01	*n.d.*
48	2,3-Dihydroxypropyl palmitate	$C_{19}H_{38}O_4$	474	*n.d.*	*n.d.*	0.49±0.01	*n.d.*
47	1,3-Dihydroxy-2-propanyl palmitate	$C_{19}H_{38}O_4$	474	*n.d.*	*n.d.*	0.06±0.01	*n.d.*
53	2,3-Dihydroxypropyl stearate	$C_{21}H_{42}O_4$	502	*n.d.*	*n.d.*	0.47±0.07	*n.d.*
52	1,3-Dihydroxy-2-propanyl stearate	$C_{21}H_{42}O_4$	502	*n.d.*	*n.d.*	0.05±0.01	*n.d.*
42	3-Hexadecoxypropane-1,2-diol	$C_{19}H_{40}O_3$	460	*n.d.*	*n.d.*	n.d.	0.10±0.02
51	3-Octadecoxypropane-1,2-diol	$C_{21}H_{44}O_3$	488	*n.d.*	*n.d.*	n.d.	0.10±0.01
Others				*0.08±0.01[a]*	*0.02±0.00[b]*	*1.89±0.03[a]*	*0.85±0.07[b]*
2	2,4,6,8-Tetramethyl-1-undecene	$C_{15}H_{30}$	210	*n.d.*	*n.d.*	0.11±0.00	*n.d.*
4	2-Methyltetradecane	$C_{15}H_{32}$	212	*n.d.*	*n.d.*	0.06±0.01	*n.d.*
8	3-Methyl-4-nonadecene	$C_{20}H_{40}$	280	*n.d.*	*n.d.*	0.04±0.01[a]	0.43±0.06[b]
7	2,6-bis(1,1-Dimethylethyl)phenol	$C_{14}H_{22}O$	278	0.08±0.01[a]	0.02±0.00[b]	0.22±0.03[a]	0.04±0.01[b]
12	Methylsuccinic acid	$C_5H_8O_4$	276	*n.d.*	*n.d.*	0.09±0.02[a]	0.16±0.00[b]
38	Dehydroabietic acid	$C_{20}H_{28}O_2$	372	*n.d.*	*n.d.*	1.37±0.06[a]	0.11±0.01[b]
50	Pinoresinol	$C_{20}H_{22}O_6$	502	*n.d.*	*n.d.*	n.d.	0.16±0.01
Σω3/Σω6				1.24±0.02[a]	1.10±0.03[b]	7.26±0.14[a]	4.65±0.14[b]
Total identified (mg g^{-1} of microalgal dw)				*30.18±0.39[a]*	*36.07±0.39[b]*	*30.59±0.49[a]*	*41.89±2.68[b]*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Compounds are numbered by their elution order (Fig. 3.3). All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl derivatives. MW, Molecular weight of compounds after silylation. [1] Contains *cis* and *trans* isomers. *n.d*., non detected. *dw*, dry weight. In italic are the main lipophilic classes.

3.2.1. Fatty acids

C. stigmatophora and *H.* cf. *andersenii* fatty acids contained the main set of molecules, with up to 80 and 69% of the total identified compounds, respectively (Fig. 3.4). It was possible to observe compositional differences in the fatty acid profiles of both microalgae strains (Fig. 3.4; Table 3.1). *H.* cf. *andersenii* displayed the highest diversity of fatty acids with 25 detected in comparison to *C. stigmatophora* where only 16 were identified. Monounsaturated fatty acids (MUFA) were the most affected after alkaline hydrolysis, increasing by 54 and 87% in *C. stigmatophora* and *H.* cf. *andersenii*, respectively.

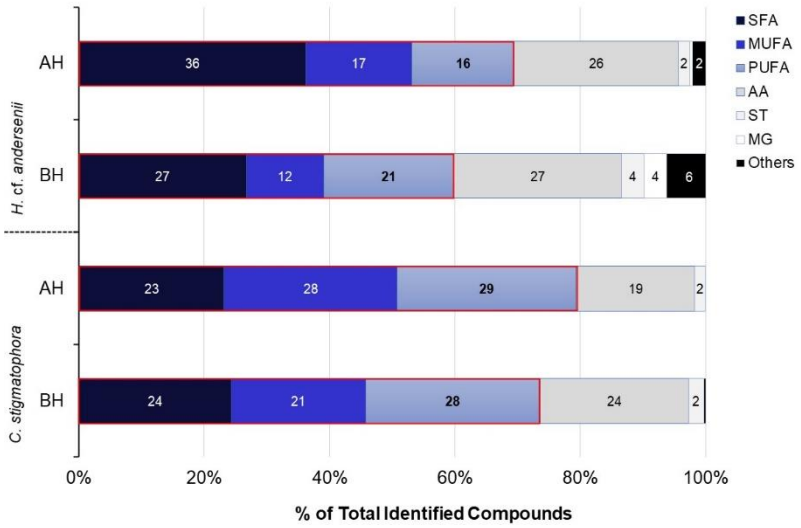

Figure 3.4. Relative abundance of the main classes of compounds identified in the lipophilic extracts of the two microalgae studied before (BH) and after (AH) alkaline hydrolysis. SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; AA – Aliphatic alcohols; ST – Sterols; MG – Monoglycerides. Highlighted boxes are the percentage of total fatty acids.

The results in Table 3.1 shows that hexadecanoic (C16:0), 9-octadecenoic acid (C18:1ω9), 9,12,15-octadecatrienoic (C18:3ω3), and 9,12-octadecadienoic (C18:2ω6) acids were the major fatty acids found in *C. stigmatophora*. In this microalga, C18:1ω9, C18:2ω6, and 5,8,11,14,17-eicosapentaenoic (EPA; C20:5ω3) acids increased more than 34% with alkaline hydrolysis. The four most predominant fatty acids for *H.* cf. *andersenii* were C16:0, C18:3ω3, EPA, and octadecanoic (C18:0) acids. Remarkable increases (>90%) in individual C18:0, C18:1ω9, and tetradecanoic (C14:0) acids levels were observed for *H.* cf. *andersenii* lipophilic extracts after hydrolysis.

Neutral lipids like triacylglycerols are mainly constructed by SFA and MUFA [169]. Therefore, the significant MUFA increase after alkaline hydrolysis might indicate that acylglycerols are mostly constituted by these fatty acids. C18:1ω9 and C18:2ω6 were the main components of the esterified fraction of *C. stigmatophora*, while C14:0, C16:0, C18:1ω9, and C18:2ω6 were the main esterified fatty acids for *H.* cf. *andersenii*.

C. stigmatophora comprised the highest amount of polyunsaturated fatty acids (PUFA) - 10.30 mg g^{-1} dw. The main contributors to this family were the 7,10-hexadecadienoic (C16:2ω6), the 7,10,13-hexadecatrienoic (C16:3ω3), C18:2ω6, and the C18:3ω3 acids. In contrast, *H.* cf. *andersenii* displayed the highest level of saturated fatty acids (SFA; 15.18 mg g^{-1} dw). However, in *H.* cf. *andersenii*, it was possible to observe the presence of 4,7,10,13,16,19-docosahexaenoic acid (DHA; C22:6ω3). In both microalgae, the Σω3/Σω6 ratio was higher than one, with *H.* cf. *andersenii* presenting the highest values when compared to *C. stigmatophora* (7.26 and 1.24, respectively).

In the wide chemical diversity of microalgae, some lipids are ubiquitous, such as C14:0, C16:0, and C18:0 fatty acids, while others are species-specific, being useful as biomarkers [180,181]. For instance, Chlorophyta are recognized to have a predominance of C18:2ω6 and C18:3ω3, whereas Cryptophyta are known for containing long-chain PUFA (EPA and DHA). In a previous study, C16:2ω6 and C16:3ω3 showed to be useful biomarkers for Chlorophyta [180].

PUFA have shown to be effective in preventing or treating several diseases [182]. In the European Union, a remarkable number of health claims have been authorized based on the essentiality of ω3 fatty acids [183]. When declaring that a product is a source of ω3 fatty acids, one of these two requirements must be fulfilled: *i)* must contain 0.3 g of 9,12,15-octadecatrienoic acid per 100 g of product or *ii)* EPA + DHA must be equal or higher than 40 mg per 100 g of product [183]. *C. stigmatophora* extract presented 5.27 g of 9,12,15-octadecatrienoic acid per 100 g of extract and 1.40 g of EPA per 100g of extract after hydrolysis, while *H.* cf. *andersenii* presented 3.85 g of 9,12,15-octadecatrienoic acid per 100 g of extract and 4.24 g of EPA+DHA per 100 g of extract after hydrolysis.

Although both ω3 and ω6 PUFA are essential for human nutrition, a balance between the ingestion of these two series of PUFA is crucial for maintaining health [184]. The western diet favors the consumption of ω6 PUFA leading to an unbalanced Σω3/Σω6 ratio of 1:20 [55]. The ω3 series of fatty acids are known for their anti-inflammatory properties while the ω6 series are known as precursors of pro-inflammatory molecules [55]. Thus, an unbalanced diet incorporating great amounts of ω6 PUFA can have prothrombotic and proinflammatory implications [184]. To achieve a dietary balance of Σω3/Σω6 of 1:1, the consumption of ω3 PUFA rich sources is recommended to prevent and mitigate inflammatory diseases. In the present study, both microalgae presented a Σω3/Σω6 >1, suggesting that they are good sources for supplying a ω3-PUFA-rich diet for aquaculture and nutraceuticals. The greatest Σω3/Σω6 ratio was verified for *H.* cf. *andersenii*.

3.2.2. Aliphatic alcohols

In both *C. stigmatophora* and *H.* cf. *andersenii*, the aliphatic alcohols were the second most abundant class of lipophilic compounds (Fig. 3.4), representing up to 24 and 27% of the total identified compounds, respectively. In Figure 3.5, it is possible to observe that the analyzed microalgae had a great diversity of aliphatic alcohols (a total of 17), with the highest diversity verified in *H.* cf. *andersenii* lipophilic extracts subjected to alkaline hydrolysis being 16 aliphatic alcohols (Fig. 3.5; Table 3.1). Between species, it was possible to observe qualitative and

quantitative variations. General trends include the predominance of hexadecanol (C16-OH), octadecanol (C18-OH), 9-octadecen-1-ol (C18:1-OH), and phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol), which together accounted for more than 40% of the total aliphatic alcohols (Fig. 3.5). Phytol was one of the main aliphatic alcohols affected by the alkaline hydrolysis in *C. stigmatophora*, yielding 2.25 times increase after alkaline hydrolysis. This diterpene alcohol alone accounted for up to 15 and 19% of the total aliphatic alcohols in *C. stigmatophora* and *H.* cf. *andersenii* (Fig. 3.5), respectively.

C10-C20 aliphatic alcohols with C16-OH abundance were previously reported for species belonging to *Chlorella* genus (*Chlorella kessleri*) [181]. In *C. stigmatophora*, the predominant aliphatic alcohols were those with shorter chain lengths (C10-C20). Docosanol (C22-OH) was found in phytoplankton and sediment samples where an algal origin is plausible [181]. This alcohol was only detected in small amounts for *H.* cf. *andersenii* samples.

Phytol comprises the hydrophobic tail of chlorophylls [137,185] which can explain the large contribution of this compound to the hydrolyzed lipid fraction of *C. stigmatophora*. Phytol is presently used as a fragrance constituent, and it has drawn increasing attention from the pharmaceutical and biotechnological fields due to its abundance in biological systems and diverse bioactivities – including cytotoxic, anti-anxiety and anti-inflammatory effects [186].

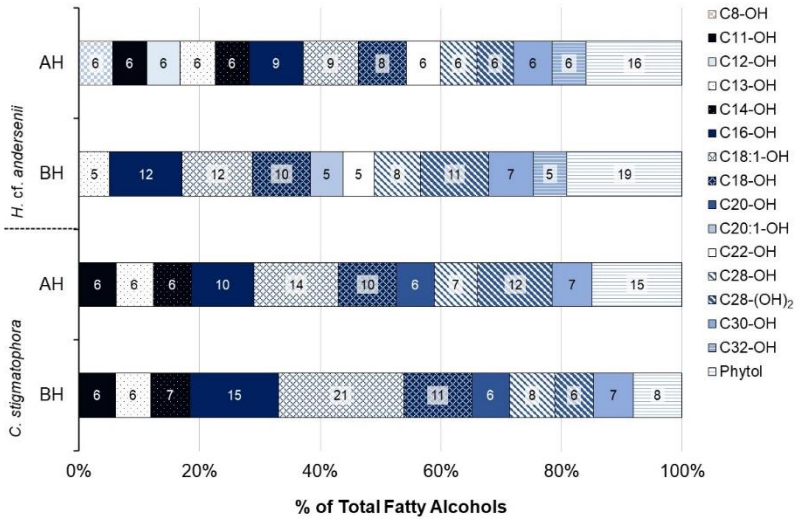

Figure 3.5. Aliphatic alcohols proportion in *C. stigmatophora* and *H.* cf. *andersenii* before (BH) and after (AH) alkaline hydrolysis.

Long-chain aliphatic diols (C28-$(OH)_2$) were detected in the composition of the analyzed microalgae. Both octacosane-1,3-diol and octacosane-1,2-diol accounted for up to 12 and 11% of total aliphatic alcohols identified in *C. stigmatophora* and *H.* cf. *andersenii* (Fig. 3.5), respectively. The mass spectrum of the octacosane-1,3-diol (Fig. 3.6a) presented the characteristic ions of an alkane-1,3-diol: m/z 103 ($TMSOCH_2^+$), 219 ($(CH_3)_3SiOCH_2CH_2(CH_3)_3SiOCH^+$) and [M-117 ($TMSOCH_2CH_2$)]$^+$ [176], while octacosane-1,2-diol (Fig. 3.6b) presented the diagnostic markers at m/z 73, 103, 129, 147, and [M-103 ($TMSOCH_2$)]$^+$, which represents the cleavage between carbons 1 and 2 of the alkane-1,2-diol

moiety. The sum of long-chain aliphatic alcohols (LCAA; C20 to C36), including the two octacosanediols detected, gives a total of up to 2.21 mg g^{-1} dw, in *C. stigmatophora*, and 3.47 mg g^{-1} dw, in *H.* cf. *andersenii*.

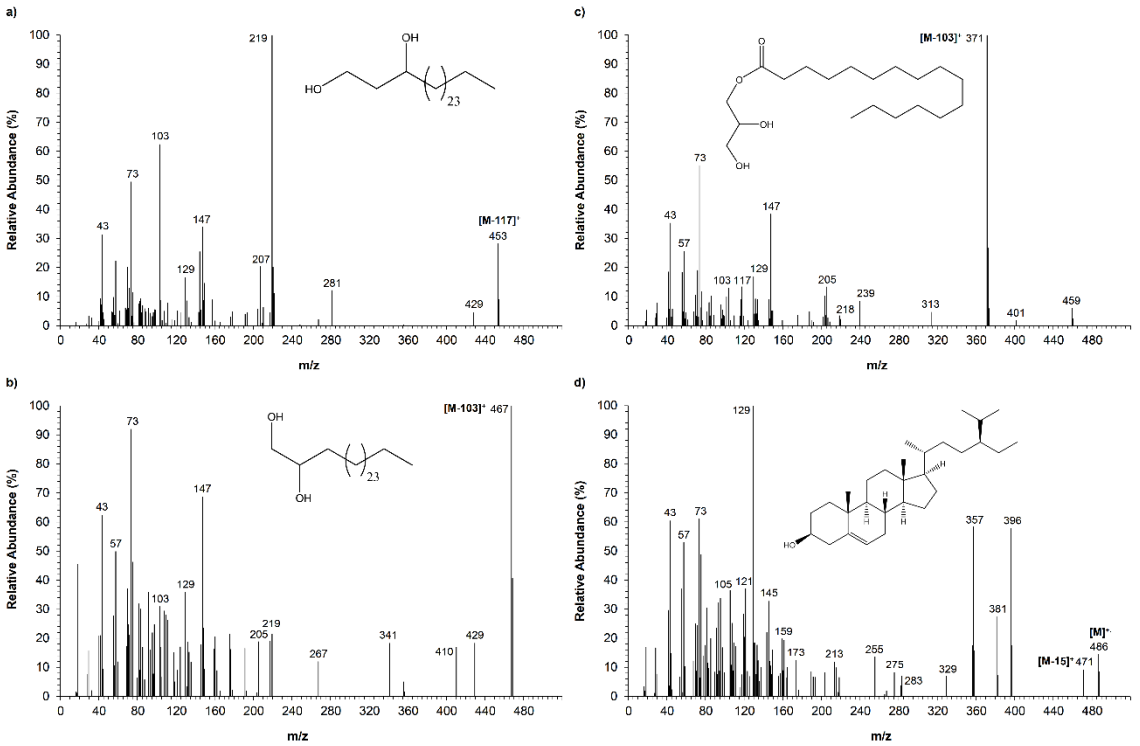

Figure 3.6. Mass spectra for four of the identified compounds: a) octacosane-1,3-diol (peak 59); b) octacosane-1,2-diol (peak 60); c) 2,3-Dihydroxypropyl palmitate (peak 48); and d) *β*-Sitosterol (peak 58), as trimethylsilyl derivatives.

Previous studies have reported the presence of C28-C32 saturated alkyl diols in Cyanobacteria and Ocrophyta (*Nannochloropsis*, *Vischeria punctata*, *Vischeria Helvetica,* and *Eustigmatos vischeri*) [181]. These diols have been suggested to be the building blocks for novel aliphatic biopolymers synthesized by microalgae and cyanobacteria. According to Volkman et al. [181], there is a strong possibility for long-chain alkyl diols to be found in other algal classes, and this observation is corroborated by our results.

Policosanol is a mixture of LCAA that is commercialized as a lipid-lowering supplement [187]. According to Taylor et al. [188], the dietary intake of 20 mg d^{-1} of policosanol is as effective as 100 mg d^{-1} of aspirin in the prevention of thrombosis and cerebral ischemia with the advantage of causing no side effects. Few reports address the presence of LCAA in microalgal biomass [189,190]. In contrast, Fernandes et al. [122] find great amounts of LCAA in a Haptophyta microalga, *Pavlova pinguis*. This finding is in agreement with the LCAA contents found for both *H.* cf. *andersenii* and *C. stigmatophora*, where 20 mg of policosanol is equivalent to 9.09 and 5.76 g of dried biomass, respectively.

3.2.3. Sterols and monoglycerides

Both microalgae strains showed a simple sterol profile dominated by *β*-sitosterol (24*α*-ethylcholest-5-en-3*β*-ol, 68 – 70% of total sterols in *C. stigmatophora*) and by brassicasterol (24*β*-methylcholesta-5,22E-dien-3*β*-ol, 58 – 70% of total sterols in *H.* cf. *andersenii*) (Table 3.1). Through Figure 3.6d it is possible to visualize the mass fragmentation of *β*-sitosterol. The mass fragmentation corresponds to diagnostic peaks at m/z 129 and $[M-129]^+$, which are characteristic of the 3-hydroxy-Δ^5-sterol TMS. The observed peaks at $[M]^+$, $[M-15]^+$, and $[M-90]^+$ are specific of silylated sterols.

Throughout algal classes, there is a predominance of specific sterols. The major sterols in Cryptophyta are C28$\Delta^{5,22}$, for instance brassicasterol, while in Chlorophyta, the prevalent sterols are C28Δ^5 and C29Δ^5 sterols such as campesterol and sitosterol, respectively [180,181]. These observations are in accordance with the results obtained for *H.* cf. *andersenii* and *C. stigmatophora*. The brassicasterol dominance in *Hemiselmis* sterol family is also consistent with that previously reported by Dunstan et al. [191] for other Cryptophyta species.

Previous studies have highlighted the bioactivities of sterols: brassicasterol is recognized for its antiaging and cholesterol-lowering properties, while campesterol is known for its anticancer and antiangiogenic properties [67,171]. From the detected phytosterols, *β*-sitosterol is the most valuable sterol, since it exhibits several bioactive properties like anti-inflammatory, antineoplastic, antipyretic, and immunomodulating activities, and has been subject of FDA health claims [67,187].

In *H.* cf. *andersenii* it was possible to identify 7 monoglycerides: 5 monoacylglycerides and 2 monoalkylglycerides. In the monoacylglycerides group, two stereoisomers designated as 1- and 2-monoacylglycerides were found. The identification of these two isomers was possible by the analysis of the diagnostic markers at m/z 218 and $[M-103]^+$. Therefore, in 1-monoglycerides, the intensity of the peak at m/z 218 was lower than that observed in 2-monoglycerides, and a high-intensity peak at $[M-103]^+$ was observed, in contrast to 2-monoglycerides. In Figure 3.6c, it is possible to visualize the mass spectra obtained for the 2,3-dihydroxypropyl palmitate. The monoglycerides content showed an 82% reduction yield after alkaline hydrolysis, where only 3-hexadecoxypropane-1,2-diol and the 3-octadecoxypropane-1,2-diol were detected.

Monoglycerides are used in the food industry as emulsifiers, stabilizers, and/or thickeners [192]. In living systems, acylglycerols (mono-, di-, and triacylglycerols) are recognized for their storage role and are often composed of C16, C18, and C20 carboxylic acids [193]. In *H.* cf. *andersenii*, esters of glycerol with saturated C16 and C18 fatty acids were detected. In contrast to acylglycerols, alkylglycerols ether bonds are stable to alkali, explaining the detection of these molecules after hydrolysis in *H.* cf. *andersenii* [194].

3.3. Principal component analysis

Principal component analysis (PCA) was performed on the metabolites found in lipophilic extracts of *H.* cf. *andersenii* and *C. stigmatophora* to summarize the metabolites

species information before and after alkaline hydrolysis. Figure 3.7a represents the distribution of the loadings for the species studied before and after hydrolysis in a two components model. The first component (PC1) accounted for 50% of variance, whereas the second (PC2) accounted for 40 %, which together explained 90% of the total variance. The variables strongly associated with positive values of factor 1 were: tetradecanoic acid (14), heptadecanoic acid (25), eicosanoic acid (41), 11-octadecenoic acid (31), 10-nonadecenoic acid (36), 5,8,11,14,17-eicosapentaenoic acid (39), aliphatic alcohols (AA), octacosane-1,3-diol (59), 2,6-bis(1,1-dimethylethyl)phenol (7), and dehydroabietic acid (38). While 10-hydroxyoctadecanoic acid (43), docosanoic acid (49), methyl-4,7,10,13-hexadecatetraenoate (22), 7,10,13,16,19-docosapentaenoic acid (45), eicosanol (40), monoglycerides (MG), 2,3-Dihydroxypropyl palmitate (48), 1,3-Dihydroxy-2-propanyl palmitate (47), 2,3-Dihydroxypropyl stearate (53), 1,3-Dihydroxy-2-propanyl stearate (52), 2-methyltetradecane (4) and methylsuccinic acid (12) were positively correlated to factor 2.

The score plot (Fig. 3.7b) provides evidence for clear chemical divergence in the GC-MS based metabolite profile of *H.* cf. *andersenii* before (BH) and after (AH) hydrolysis. PCA results showed that *C. stigmatophora* samples were richer in PUFA, 9-octadecenoic acid, *β*-sitosterol, and LA. While *H.* cf. *andersenii* BH presented higher levels of sterols, monoglycerides, and DHA, the *H.* cf. *andersenii* AH had higher amounts of the total identified compounds and SFA.

PC1 separated the *C. stigmatophora* samples from *H.* cf. *andersenii*, indicating that the lipophilic profile of both microalgae had unique features that could be further investigated as chemotaxonomic markers. Namely, the compounds 7,12-hexadienoic acid (19), 7,10,13-hexadecatrienoic acid (18), eicosanol (40), 24-methylcholesta-5,24(28)-dien-3*β*-ol (24-methylenecholesterol, 56), and 24*α*-methylcholest-5-en-3*β*-ol (campesterol, 57) that were only present in *C. stigmatophora* samples and the 10-nonadecenoic acid (36), 7,12-hexadienoic acid (19), DHA (44), docosanol (46), and brassicasterol (55) that were only found in *H.* cf. *andersenii* samples.

The sites of cleavage via alkaline hydrolysis (saponification) are the ester bonds of lipids, which result in a carboxylic acid and alcohol that are recovered and derivatized for further GC-MS analysis. This method targets complex lipids - like acylglycerols (mono-, di-, and triacylglycerols), wax, and sterol esters.

The chemical divergence observed in the GC-MS based metabolite profile of *H.* cf. *andersenii* before (BH) and after (AH) hydrolysis is mainly explained by the detection of monoacylglycerols and other metabolites with strong positive correlation with PC2, which were only present in metabolite profiles of *H.* cf. *andersenii* BH. In *H.* cf. *andersenii* the concomitant increase of fatty acids with aliphatic alcohols observed after alkaline hydrolysis suggests that wax esters are abundant in this specie.

Regarding to *C. stigmatophora* only fatty acids significantly increased, as well as phytol after hydrolysis. This might be explained by the fact that lipids are mainly constituted by polar lipids (glyco- and phospholipids) and non-acylglycerol neutral lipids like chlorophylls, which are

more stable to alkaline hydrolysis. This assumption also explains the similarities in the lipophilic metabolite profiles for *C. stigmatophora* before and after alkaline hydrolysis observed in PCA analysis.

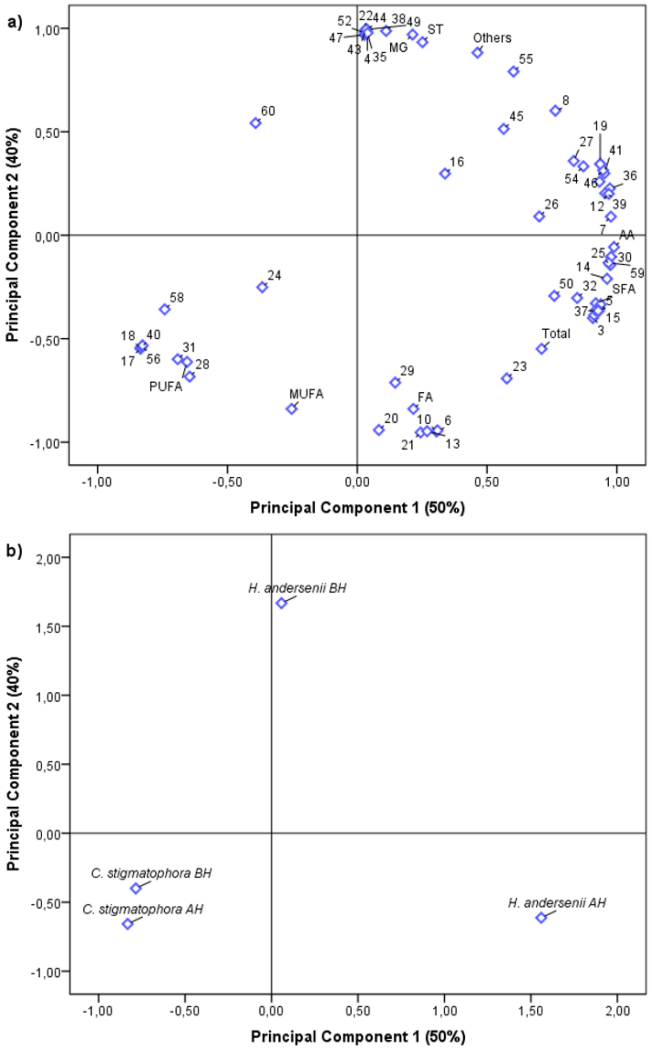

Figure 3.7. Projection (Varimax rotation) of the a) loadings and b) scores of components 1 and 2 obtained in the Principal Component Analysis analysis based on the composition of *C. stigmatophora* and *H.* cf. *andersenii* lipophilic extracts before (BH) and after (AH) alkaline hydrolysis. The corresponding identification of the numbered loadings is displayed in Table 3.1. FA – Fatty acids; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; MG – Monoglycerides; ST – Sterols.

PCA results showed that *C. stigmatophora* samples were richer in PUFA, 9-octadecenoic acid, *β*-sitosterol, and LA, which suggests that this microalga is a good source of essential fatty acids needed to fulfill the dietary requirements of humans and fish. *H.* cf. *andersenii* BH presented higher levels of sterols, monoglycerides, and DHA, and *H.* cf. *andersenii* AH had higher amounts of the total identified compounds and SFA. This indicates that *H.* cf. *andersenii* composition can be suitable not only for nutraceuticals (sterols and DHA) but also for the food industry (monoglycerides) and bioenergy (SFA).

4. Conclusion

Microalgal lipids are often assessed for their biotechnological potential, taking into consideration one specific family, namely fatty acids. This approach constrains microalgae strain selection for further industrial applications. Since FTIR-ATR and GC-MS metabolite profile provides an overall insight into microalgae intracellular metabolites, it can be used to identify components that may serve as chemotaxonomical markers as well as novel natural sources of high-value compounds. The present study contributed to the valorization of *C. stigmatophora* and *H.* cf. *andersenii* as sources of value-added compounds with recognized nutritional benefits. Although the mere presence of these health-promoting phytochemicals may not support its use in a particular application /formulation, their knowledge provides a novel insight for further studies and final product optimization targeting the field of application.

CHAPTER 4.

High-value lipids accumulation by *Pavlova pinguis* as a response to nitrogen-induced changes

This Chapter is based on the following publication:

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Chapter 4. High-value lipids accumulation by *Pavlova pinguis* as a response to nitrogen-induced changes

Abstract

The challenges of exploring the potential of microalgal strains for biotechnological applications include the optimization of their cell growth and chemical composition. To overcome this, it is essential to understand the mechanisms that lead to the accumulation of desired products within microalgal cells. In this study, a gradient of nitrogen concentrations was used for the preparation of *P. pinguis* growth medium. The algal growth dynamics, pigments, nutrient uptake, and detailed lipid composition across treatments were assessed. Increasing the nitrogen level led to higher lipid content (21%), a higher cell uptake rate (0.20 pg N $cell^{-1}$ d^{-1}) and more accumulation of chlorophylls, carotenoids, and high-value lipids. 5,8,11,14,17-Eicosapentaenoic acid, essential fatty acids, phytol and stigmasterol were the key high-value lipids that were positively influenced by higher nitrogen levels. High nitrogen conditions induced an increase of 54% in total sterol content, while low nitrogen conditions resulted in increased proportions of saturated fatty acids (66% more) and decreased proportions of polyunsaturated fatty acids (14% less). The low nitrogen level also led to higher amounts of monoglyceride (1.60 mg g^{-1}; 64% more). The analysis of *P. pinguis* lipids before and after hydrolysis provided an insight into the composition of the esterified lipids across treatments. Nitrogen supplementation was revealed to be an effective strategy for enhancing *P. pinguis* lipid composition, for nutraceutical and pharmaceutical industries (high nitrogen level). A greater understanding of the nitrogen uptake and the use efficiency by *P. pinguis* was reached, showing its potential for further biotechnological applications.

Keywords: *Pavlova pinguis*; nitrogen supply; high-value lipids; biotechnological application

1. Introduction

The search for natural resources that meet the dietary requirements of the growing population without harming the environment is one of the challenges of a bio-based economy [195]. Within such an economy, microalgae are gaining popularity due to their nutrient-rich profiles and sustainability [195]. The main advantages of cultivating these organisms, in a bio-based economy, are avoidance of sludge handling problems (no pesticides and herbicides are used), bio-fixation of carbon dioxide, non-competition for arable lands, ability to readily adapt to changing environmental conditions, and high economic return of algal biomass [175,196].

Microalgal phenotypic plasticity allows the optimization of its chemical composition to target the field of application (*e.g.* for feedstock fertilizers, energy, bioremediation and nutraceutical markets), whilst decreasing the production costs [175]. Nitrogen is one of the key elements that limits the rate of microalgal cell growth and synthesis of essential cellular components [197]. Thus, the manipulation of this nutrient is often used as a simple approach to

increase microalgae productivity for industrial purposes [169,198,199]. Knowing that nutrient uptake and use efficiency is variable among microalgae, it is crucial to assess the effects of nitrogen supplementation in new microalgal strains that show promise for further biotechnological applications.

The algal market is mostly dominated by extremophiles (*Arthrospira* and *Dunaliella*) and freshwater (*Chlorella*) algae [142]. Given the worldwide scarcity of freshwater and the challenges in replicating extreme environmental conditions, it is crucial to search for new, commercially viable strains that can be grown in seawater [142]. *Pavlova* species are recognized sources of nutritionally important products. Within this genus, *Pavlova pinguis* is an underexploited microalgal species [122,200]. This PUFA-producing microalga is often employed in aquaculture as live feed for penaeid shrimp larvae, bivalve mollusc larvae and freshwater prawn larvae [200]. The metabolite profiling of *Pavlova pinguis* J.C. Green showed that this microalgal strain is a potential source of high-value lipids such as polyunsaturated fatty acids (PUFA), long-chain aliphatic alcohols and phytosterols [122]. These lipid sets have recognized therapeutic properties, including antibacterial, antioxidant and anticarcinogenic, as well as cholesterol-lowering properties, making *P. pinguis* suitable for further exploitation in the nutraceutical and pharmaceutical industries [122]. The metabolite characterization of *P. pinguis* in different growth conditions can provide further insights into the regulation of lipid quantity and quality.

Despite the studies performed in *Pavlova* strains as lipid producers, information about the lipid composition of *P. pinguis* under different levels of nitrogen supplementation is scarce, although crucial to this strain valorization [61,163,169]. Although nitrogen abundant conditions are associated with the accumulation of high-value lipids, namely essential fatty acids and long-chain polyunsaturated fatty acids (LC-PUFA), there are few studies addressing the influence of high nitrogen supplementation on the composition of microalgae [61,62]. Thus, to further increase the production of high-value lipids and understand their mechanism of accumulation, the Haptophyta *P. pinguis* J.C. Green was assessed for its growth, nitrogen removal efficiency, lipid accumulation and detailed composition in different nitrogen levels (low, medium and high).

2. Materials and methods

4.1. Growth and culture conditions

The non-axenic haptophyta *Pavlova pinguis* (RCC 1539) was obtained from the Roscoff Culture Collection (RCC). The f/2-Si medium [201,202] was used as the basal formula (Table 4.1), while nitrogen concentration was changed. Three different levels of nitrogen were applied: low nitrogen level (LN) with 140±2 µM $NaNO_3$, medium nitrogen level (MN) with 939±4 µM $NaNO_3$, and high nitrogen level (HN) with 1752±24 µM $NaNO_3$. Starter cultures were inoculated into 1 L of media, and the initial cell concentration was 8.81×10^5 cells mL^{-1}. The following growth conditions were applied: 70 µmol m^{-2} s^{-1} of photon flux, 16:8 h (light: dark) cycles, and a temperature of 25 ± 1 °C. When cultures reached the early-stationary phase, the medium was centrifuged at 3720 *g* for 7min. at room temperature. The obtained pellets were washed twice with a 0.09 g NaCl L^{-1} solution with consecutive centrifugation. The microalgal cell concentration

was monitored daily by measuring the optical density of the microalgal culture at 550 nm (OD_{550}) with an ultraviolet/visible spectrophotometer (UV-6300PC, VWR). A calibration curve (R^2=0.9988) plotting cell concentration (cells mL^{-1}) against absorbance was used to determine cell growth. The cell concentration was estimated by counting cells with a 0.1 mm deep improved Neubauer haematocytometer (Marienfield Superior) in a light microscope (Olympus BX41). The logistic model of Xin et al. [175] was fitted to the observed data using excel add-in solver, in order to describe the microalgal growth.

Table 4.1. Culture medium composition f/2-Si medium used for growing *Pavlova pinguis*.

Component	Concentration in final medium (mol L^{-1})
$NaNO_3$	Variable
$NaH_2PO_4 \cdot H_2O$	3.62×10^{-5}
Na_2SiO_3	1.06×10^{-4}
$FeCl_3 \cdot 6H_2O$	1.17×10^{-5}
$Na_2EDTA \cdot 2H_2O$	1.17×10^{-5}
$MnCl_2 \cdot 4H_2O$	9.10×10^{-7}
$ZnSO_4 \cdot 7H_2O$	7.65×10^{-8}
$CoCl_2 \cdot 6H_2O$	4.20×10^{-8}
$CuSO_4 \cdot 5H_2O$	3.93×10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	2.60×10^{-8}
Thiamine hydrochloride	2.96×10^{-7}
Biotin	2.05×10^{-9}
Cyanocobalamin	3.69×10^{-10}

*$NaNO_3$ concentration in final medium was adjusted to 140µM, 939 µM, and 1752 µM for low nitrogen, medium, and high nitrogen growth media, respectively.

4.2. Nitrogen determination

Nitrogen (N) was determined as nitrate (NO_3^-) using the ultraviolet spectrophotometric screening method previously reported by Wan et al. [203], with some modifications. Briefly, a calibration curve (R^2=0.9999) was drawn using the optical density measured at 220 nm (OD_{220}), plotted against different $NaNO_3$ solution concentrations (0 - 4.07 mg L^{-1}). Prior to NO_3^-- N reading in the ultraviolet/visible spectrophotometer (UV-6300PC, VWR), samples were filtered through 0.45 µm membranes, diluted with deionized water and acidified with 1M HCl.

Nitrogen uptake capacity and cell uptake rate were calculated using Equations 4.1 and 4.2, where N_i is the initial nitrogen concentration (pg L^{-1}), N_f is the final nitrogen concentration (pg L^{-1}), and X is cell concentration (cells L^{-1}) at time t (day - d):

$$\text{Nitrogen uptake capacity (\%)} = \left(\frac{N_i - N_f}{N_i}\right) \times 100 \qquad \text{Equation 4.1}$$

$$\text{Cell uptake rate (pg cell}^{-1}\text{ d}^{-1}\text{)} = \frac{N_i - N_f}{Xt} \qquad \text{Equation 4.2}$$

4.3. Pigment determination

For cellular pigments (chlorophyll *a*, chlorophyll c_1 + c_2, and carotenoids) extraction, 10 mg of freeze-dried biomass was extracted with 80% acetone solution. The samples were vigorously vortexed, ultrasonicated for 90 min., and centrifuged at 4430 *g* for 10 min. The supernatant was transferred to pre-chilled (-20 ºC) tubes, and pellet was continuously washed (with the cold extraction solution) until the supernatant being colorless. The extracted pigments were filtered through 0.45 µm membranes and their absorbance was read in an ultraviolet/visible spectrophotometer (UV-6300PC, VWR). The amount of chlorophyll *a* and total carotenoids was calculated according to Chen and Vaidyanathan [204] by equations 4.3 and 4.4 where Chl *a* is the chlorophyll *a* (µg mL^{-1}), and C_t is the carotenoids (µg mL^{-1}):

$$\text{Chl } a = 12.21A_{663} - 2.81A_{646} \qquad \text{Equation 4.3}$$

$$C_t = \frac{1000A_{470} - 3.27(12.21A_{663} - 2.81A_{646}) - 104(20.13A_{646} - 5.03A_{663})}{198} \qquad \text{Equation 4.4}$$

Chlorophylls c_1 and c_2 determination was performed using the equation described by Jeffrey and Humphrey [205]. Thus, acetone from the supernatant was evaporated on a nitrogen stream and the remaining water was removed by freeze-drying samples. Afterwards, the samples were resuspended on 90% acetone. The amount of chlorophylls c_1 + c_2 (Chl c_1 + c_2; µg mL^{-1}), was calculated by the following equation:

$$\text{Chl } c_1 + c_2 = 24.36A_{630} - 3.73A_{664} \qquad \text{Equation 4.5}$$

4.4. Lipid extraction

Total lipids were estimated from freeze-dried biomass according to Bligh and Dyer [206], with some modifications described by Fernandes et al. [145]. Briefly, to 50 mg of freeze-dried microalgal freeze-dried, 3mL of a methanol: chloroform (2:1 v/v) solution was added. After homogenization, a 400µL of a NaCl saturated solution was added followed by 2mL of chloroform and 2mL of water. Then, organic phase was removed to pre-weighted tubes, and the remaining biomass continuously washed by addition of chloroform. Extracted organic phase, was dried under a nitrogen stream and gravimetric quantification was performed.

4.5. Lipid alkaline hydrolysis

Alkaline hydrolysis was performed on lipid extracts to detect molecules in their esterified forms, according to Fernandes et al. [122]. 0.5M NaOH in aqueous methanol was added to lipid aliquots and the mixtures were heated at 100 ± 1 °C for 1 h under nitrogen atmosphere. Then, the samples were acidified to pH 2 with 1M HCl, extracted with dichloromethane, dried under a nitrogen stream, and preserved at -20 ± 1 °C until further analysis.

4.6. Gas chromatography – mass spectrometry

Prior to GC–MS analysis, silylation using bis(trimethylsilyl)trifluoroacetamide (BSTFA) of lipid extracts before and after alkaline hydrolysis was performed, as described by Santos et al. [129]. TMS derivatives were analyzed in a gas chromatographer (Agilent HP 6890) equipped with a mass selective detector (Agilent 5973) and a ValcoBon 17704 capillary column VB1 (30 m × 0.25 mm inner diameter and 0.25 µm film thickness). The chromatographic experimental conditions were as described in. The identification of the extracted compounds as TMS derivatives was done by comparison of the mass spectra fragmentation to those in Wiley-NIST libraries, mass fragmentation presented in published literature [122], and, where possible, comparison with injected authentic standards. The GC-MS analysis was calibrated with pure reference compounds (mannose, *trans*-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5*α*–chlolestane, cholesterol (cholest-5-en-3*β*-ol), stigmasterol (24*α*-ethylcholesta-5,22*E*-dien-3*β*-ol), hexadecanoic, and nonadecanoic acids), and the response factor was calculated using tetracosane as the internal standard.

The hypocholesterolaemic/hypercholesterolaemic fatty acids ratio (H/H) [207] and health promoting index (HPI) [208], were calculated by the following equations:

$$H/H = (C18:1\omega9+C18:2\omega6+C20:4\omega6+C18:3\omega3+C20:5\omega3+C22:5\omega3+C22:6\omega3)/(C14:0+C16:0) \quad \text{Equation 4.6}$$

$$HPI = \sum UFA/[C12:0+(4\times C14:0)+C16:0] \quad \text{Equation 4.7}$$

In Equation (4.7) UFA stands for unsaturated fatty acids.

4.7. Elemental analysis

For elemental analysis, Truspec 630-200-200 equipment was used. The combustion furnace temperature was set at 1075 ºC and the afterburner temperature at 850 ºC. The detection methods used were the infrared absorption to carbon and the thermal conductivity to nitrogen.

4.8. Statistical analysis

The results are expressed as the mean ± standard deviation (SD) of at least two replicates and reported relative to microalgal dry weight (dw) basis. Statistical analysis of the data was carried out using the software IBM SPSS Statistics 24. Differences between treatments were assessed with Tukey post-hoc analysis, where *p*-values <0.05 were considered statistically significant. Within samples, the differences before and after alkaline hydrolysis were evaluated using the *t*-student test, where *p*-values <0.05 were considered statistically significant. Metaboanalyst 3.0 was used to perform multivariate analysis [209]. Features were median normalized, $\log_2$ transformed and auto scaled before principal component analysis, Pearson's correlation analysis and hierarchical clustering. For hierarchical clustering heat maps, Euclidean clustering distance and the ward clustering method were used.

5. Results and discussion

5.1. Microalgal growth, nitrogen removal, and chemical composition

The plots of *Pavlova pinguis* growth and medium total nitrogen (Fig. 4.1) show that nitrogen concentrations decreased over time, with a concomitant increase in *Pavlova pinguis* cell concentration. The harvest cell concentration of *P. pinguis* in low (LN), medium (MN) and high (HN) nitrogen levels were 3.37×10^6, 1.17×10^7 and 1.17×10^7 cells mL^{-1}, respectively. The similar (p <0.05) cell concentrations observed for the MN and HN treatments could be from at HN conditions *i)* microalgal cell division slows down as light becomes limiting, and/or *ii)* the primary growth limiting nutrient may be a different one from nitrogen. While in LN treatment, nitrogen seems to be the primary growth limiting factor.

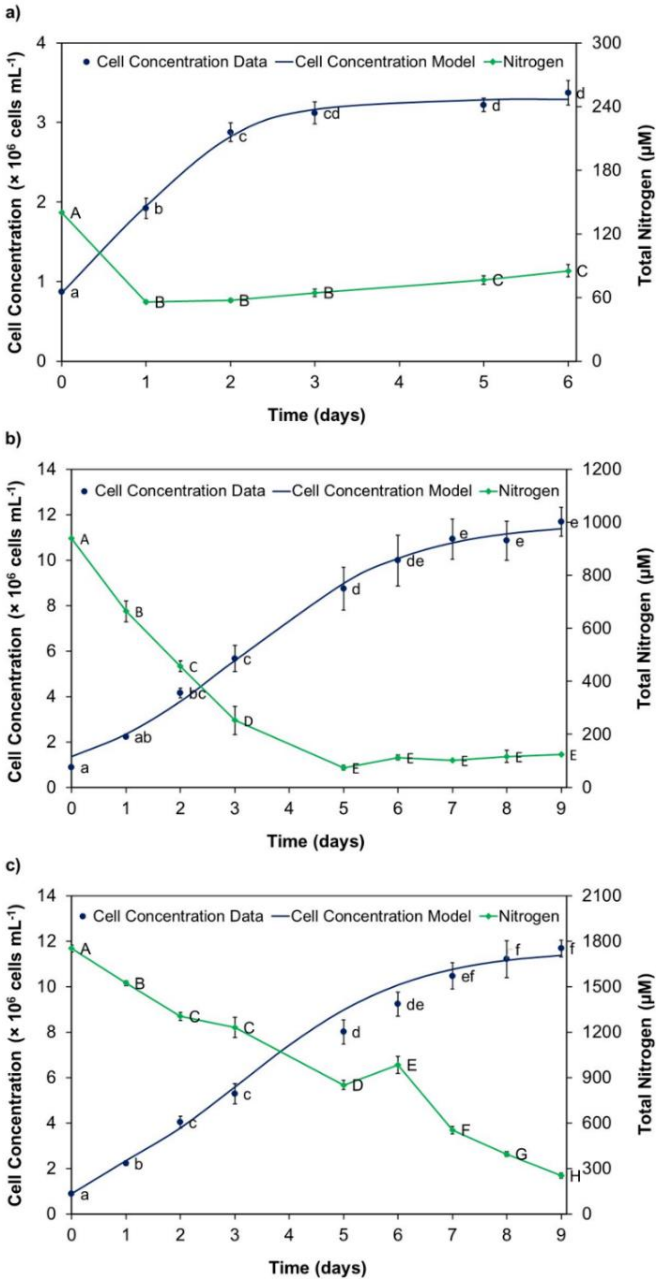

Figure 4.1. Growth curves and total nitrogen assessed as nitrate in *Pavlova pinguis* growth medium throughout the cultivation days. a) low nitrogen level, b) medium nitrogen level, c) high nitrogen level. Data are expressed as mean ± standard deviation (n=3). Differences in cell concentration and total nitrogen

among cultivation days were assessed using analysis of variance followed by Tukey's post-hoc analysis and are represented by different uppercase letters for total nitrogen, and different lowercase letters for cell concentration (p <0.05). Determination coefficients (r^2) for logistic growth models were higher than 0.95.

P. pinguis reached the early-stationary phase at different cultivation times. The cells in the LN treatment showed a gradual increase until day 3, whereas those in both the MN and HN treatments continued to increase until day 7. This observation is corroborated by the statistical treatment, which shows non-significative differences (p <0.05) between cell concentration within treatments from these days onwards. In both LN and MN conditions, total nitrogen reached its residual value and plateau stage before microalgae reached the early-stationary phase. It is known that cell division stops when no nitrogen is available to be assimilated into proteins, needed to maintain growth, thus the former behavior is explained [210]. Although cell concentration showed similar behavior for *P. pinguis* grown in MN and HN conditions, total nitrogen in HN growth conditions did not reach a plateau stage. This can be explained by the luxury storage of nitrogen in *P. pinguis* microalgal cells, which can occur in high nitrogen environments [211].

The effect of nitrogen level on *P. pinguis* nitrogen uptake rate and removal efficiency, intracellular nutrient stoichiometry, lipid content, harvest cell concentration, and pigments were studied (Table 4.2). In HN cultures, the highest cell uptake rates (0.20 pg N $cell^{-1}$ d^{-1}) and nitrogen removal (86%) were obtained. Meanwhile, the lowest values for cell uptake rate (0.04 pg N $cell^{-1}$ d^{-1}) and nitrogen removal (39%) were observed in *P. pinguis* microalgal cells grown in LN conditions. These findings are in accordance with Tantanasarit et al. [212], which reported that the marine diatom *Chaetoceros calcitrans* can efficiently increase the uptake rate of inorganic nutrients at higher nutrient levels, assimilating them for binary fission. This can indicate that nitrogen acquisition by *P. pinguis* is positively regulated by increasing nitrogen levels, an assumption that has already been verified for other microalgae [175,213]. Varying nitrogen removal efficiencies were also observed for a freshwater microalga (*Scenedesmus*) grown in different nitrogen supplementations [175]. In contrast to the present study, nitrogen inputs led to a decrease in nitrogen removal efficiency (from >99 to 45%). This variability was attributed not only to nitrogen supplies but also to the ratio of nitrogen to the other nutrients present in the microalgal growth medium, which is known to limit nutrient acquisition by cells. Moreover, nutrient uptake and use efficiency is microalga species-specific.

The changes in nitrogen uptake capacity reflected on *P. pinguis* intracellular stoichiometry (Table 4.2), significantly affecting the carbon and nitrogen molar ratio (C/N), which decreased by 68% from LN to HN conditions. This trend indicates that when the nitrogen level is high, *P. pinguis* microalgal cells accumulate more nitrogen in their biomass (proteins) and less carbon-rich metabolites (carbohydrates and lipids) [145,214]. The lipid content ranged from 12.56 to 21.25%, with the highest value being obtained in microalgae grown in HN conditions. Thus, the accumulation of carbon-rich metabolites in LN conditions was not from lipids but from carbohydrates, suggesting that this macromolecular pool is the main carbon reservoir for *P. pinguis* microalgal cells under LN conditions.

Table 4.2. Harvest cell density, nitrogen uptake, pigments, and elemental analysis of *Pavlova pinguis* grown under different nitrogen supplementation.

	Low Nitrogen	**Medium Nitrogen**	**High Nitrogen**
Harvest cell density ($\times 10^6$ cells mL^{-1})	3.37±0.15^a	11.69±0.64^b	11.68±0.36^b
Nitrogen uptake ability (%)	39.19±3.45^a	85.35±2.38^b	85.62±1.32^b
Cell uptake rate (pg N $cell^{-1}$ d^{-1})	0.04±0.00^a	0.11±0.01^b	0.20±0.02^c
Chl *a* (mg g^{-1} dw)	0.31±0.01^a	3.91±0.65^b	11.08±0.54^c
Chl c_1+c_2 (mg g^{-1} dw)	0.29±0.05^a	0.92±0.15^b	1.79±0.17^c
C_t (mg g^{-1} dw)	0.05±0.00^a	0.98±0.06^b	2.91±0.15^c
Lipid (% dw)	12.56±0.89^a	15.64±0.36^b	21.25±0.82^c
C (% dw)	12.22±0.77^a	17.57±0.15^b	31.81±0.31^c
N (% dw)	0.67±0.07^a	1.68±0.03^b	5.52±0.22^c
H (% dw)	3.36±0.19^a	3.60±0.62^a	4.78±0.28^b
C/N (mol:mol)	20.86±0.89^a	12.19±0.12^b	6.72±0.22^c

dw: dry weight. Each value is the mean ± standard deviation ($n \geq 2$). Different letters in the same column indicate significant differences ($p < 0.05$), based on analysis of variance followed by Tukey's post hoc analysis. Sulphur was not detected. Chl *a* – Chlorophyll *a*; Chl c_1+c_2 – Chlorophylls c_1+c_2; C_t – total carotenoids; C – Carbon; N – Nitrogen; H – Hydrogen.

Food's rich in chlorophyll and carotenoids are considered to be very nutritious as their consumption provides recognized health benefits [215]. In chlorophylls, the presence of the heme group is beneficial for the production of red blood cells, while carotenoids are known as chemoprotective agents against several diseases like cancer and diabetes [215]. In this study, *P. pinguis* accumulated these pigments at high nitrogen conditions: 11.08 (chlorophyll *a*); 1.79 (chlorophylls c_1 + c_2); and 2.91 (total carotenoids) mg g^{-1} (Table 4.2). Thus, at HN conditions, *P. pinguis* show a great industrial potential for healthcare and cosmetic industries.

5.2. GC-MS metabolite profile

To decipher the metabolically induced changes in lipid composition of *P. pinguis* at the different nitrogen levels applied, *P. pinguis* lipid extracts were characterized using GC-MS. The chromatograms obtained under these conditions are displayed in Figure 4.2, in which the sterol region was amplified. Through these chromatograms, it is possible to visualize differences in the relative abundance of chromatogram peaks, suggesting that changes in the building blocks of lipids occurred with the nitrogen supply.

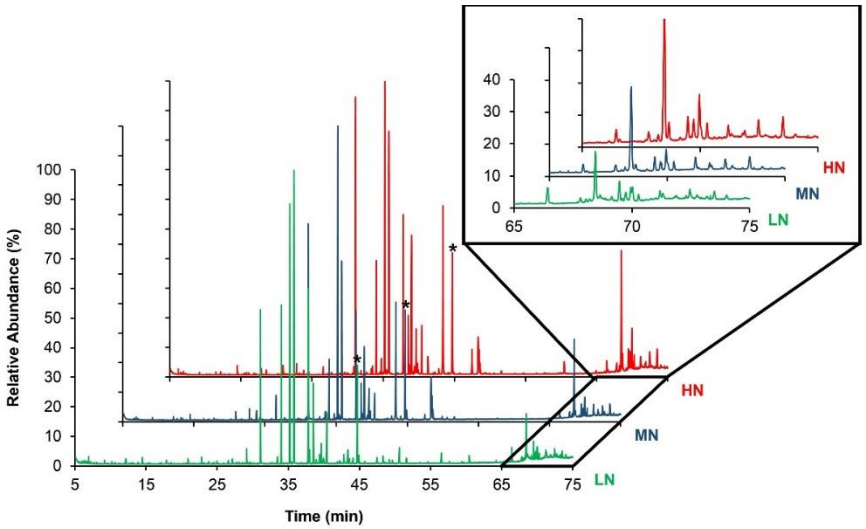

Figure 4.2. Chromatograms obtained for lipid extracts of *Pavlova pinguis* grown in low (LN), medium (MN) and high (HN) nitrogen level, before alkaline hydrolysis. * Internal standard (Tetracosane)

5.2.1. Fatty acids

Fatty acids were the major components of *P. pinguis* extracted lipids, comprising 63 - 76% of the total identified compounds (Table 4.3). The major fatty acids were tetradecanoic (C14:0), hexadecanoic (C16:0), 9-hexadecanoic (C16:1ω7) and 5,8,11,14,17-eicosapentaenoic (EPA – C20:5ω3) acid, which together accounted for more than 30% of the total, under different nitrogen conditions. With an increasing nitrogen level, the quantities and types of fatty acids varied significantly (p <0.05), before hydrolysis (BH; free fatty acids) and after alkaline hydrolysis (AH; esterified fatty acids), Table 4.3. The maximum total fatty acid content was observed in HN conditions (BH: 95.57 mg g^{-1}; AH: 141.62 mg g^{-1}), while the lowest total fatty acid content was verified in LN conditions (BH: 46.82 mg g^{-1}; AH: 71.54 mg g^{-1}).

The LN treatment presented the highest proportion of saturated fatty acids (SFA), 40 – 49% of total identified compounds, and the lowest PUFA proportion, 10 – 11% of total identified compounds. The activity of the enzymes involved in PUFA synthesis is known to be affected by varying nitrogen concentrations [179]. Previous studies have reported that under nutrient limitation, microalgae can change their fatty acid profiles towards higher saturation through the downregulation of fatty acid desaturases and elongases involved in PUFA synthesis [59,216]. These findings are in accordance with the observations made for *P. pinguis* microalgal cells in the present work.

Table 4.3. Lipid profile (mg g^{-1} of dry microalgal weight) of *Pavlova pinguis* grown in three different nitrogen levels before (BH) and after (AH) alkaline hydrolysis.

		Low Nitrogen		Medium Nitrogen		High Nitrogen	
Nº	**Identified Compounds**	**BH**	**AH**	**BH**	**AH**	**BH**	**AH**
	Fatty acids	*46.82±0.69^{aA}*	*71.54±3.04^{b1}*	*91.60±2.72^{aB}*	*115.95±2.17^{b2}*	*95.57±0.30^{aC}*	*141.62±2.24^{b3}*
	Saturated	*29.78±0.48^{aA}*	*46.93±1.88^{b1}*	*39.56±1.44^{aB}*	*55.55±1.10^{b2}*	*42.87±0.24^{aC}*	*64.91±1.88^{b3}*
1	Nonanoic acid	1.04±0.01^{aA}	1.55±0.01^{b1}	1.55±0.00^{aB}	2.01±0.00^{b2}	1.55±0.02^{aB}	2.09±0.01^{b3}
3	Decanoic acid	1.04±0.01^{aA}	1.53±0.00^{b1}	1.55±0.01^{aB}	2.04±0.01^{b2}	1.56±0.01^{aB}	2.09±0.01^{b3}
6	Undecanoic acid	1.03±0.01^{A}	*n.d.*	1.56±0.01^{B}	*n.d.*	1.55±0.02^{B}	*n.d.*
8	Dodecanoic acid	1.04±0.00^{aA}	1.54±0.01^{b1}	1.56±0.01^{aB}	2.06±0.01^{b2}	1.54±0.02^{aB}	2.12±0.02^{b3}
12	Tetradecanoic acid	3.92±0.32^{aA}	7.74±0.49^{b1}	5.73±0.86^{aB}	11.43±0.52^{b2}	7.71±0.18^{aC}	16.96±0.43^{b3}
13	Pentadecanoic acid	1.14±0.01^{aA}	1.72±0.02^{b1}	1.72±0.02^{aB}	2.34±0.02^{b2}	1.68±0.01^{aC}	2.39±0.02^{b3}
21	Hexadecanoic acid	6.15±0.28^{aA}	11.49±0.85^{b1}	5.41±0.55^{aA}	9.12±0.36^{b1}	6.32±0.18^{aB}	10.80±1.40^{b1}
23	Heptadecanoic acid	1.30±0.06^{aA}	1.69±0.03^{b1}	1.79±0.05^{aB}	2.17±0.00^{b2}	1.82±0.03^{aB}	2.26±0.03^{b3}
31	Octadecanoic acid	1.99±0.04^{aA}	3.55±0.43^{b1}	2.30±0.15^{aB}	3.31±0.06^{b1,2}	2.70±0.03^{aC}	4.49±0.43^{b2}
36	Eicosanoic acid	2.20±0.02^{aA}	3.22±0.02^{b1}	3.26±0.01^{aB}	4.19±0.01^{b2}	3.25±0.03^{aB}	4.33±0.02^{b3}
43	Docosanoic acid	2.27±0.03^{aA}	3.27±0.02^{b1}	3.33±0.02^{aB}	4.26±0.02^{b2}	3.33±0.03^{aB}	4.37±0.01^{b3}
45	Tetracosanoic acid	2.19±0.01^{aA}	3.19±0.01^{b1}	3.26±0.01^{aB}	4.17±0.02^{b2}	3.25±0.04^{aB}	4.29±0.00^{b3}
50	Octacosanoic acid	2.20±0.01^{aA}	3.20±0.00^{b1}	3.26±0.01^{aB}	4.17±0.01^{b2}	3.26±0.04^{aB}	4.29±0.00^{b3}
60	Triacontanoic acid	2.27±0.03^{aA}	3.23±0.01^{b1}	3.29±0.01^{aB}	4.28±0.04^{b2}	3.35±0.04^{aC}	4.43±0.09^{b2}
	Monounsaturated	*8.91±0.25^{aA}*	*14.90±1.07^{b1}*	*16.87±1.06^{aB}*	*23.96±0.64^{b2}*	*15.83±0.41^{aB}*	*25.83±0.20^{b3}*
11	9-Tetradecenoic acid	*n.d.*	1.54±0.00^{1}	1.56±0.01^{aA}	2.00±0.01^{b2}	1.57±0.02^{aA}	2.10±0.03^{b3}
18	9-Hexadecenoic acid[1]	5.36±0.25^{aA}	8.07±0.84^{b1}	9.90±0.94^{aB}	15.24±0.56^{b2}	8.92±0.19^{aB}	16.34±0.16^{b2}
19	7-Hexadecenoic acid	1.06±0.00^{aA}	1.55±0.01^{b1}	1.65±0.05^{aB}	2.09±0.01^{b2}	1.62±0.02^{aB}	2.20±0.01^{b3}
30	11-Octadecenoic acid	1.10±0.01^{aA}	1.68±0.04^{b1}	1.74±0.04^{aB}	2.21±0.03^{b2}	1.78±0.04^{aB}	2.49±0.12^{b3}
29	9-Octadecenoic acid	1.39±0.01^{aA}	2.06±0.19^{b1}	2.01±0.29^{aB}	2.42±0.04^{a2}	1.94±0.22^{aB}	2.70±0.07^{b2}
	Polyunsaturated	*8.12±0.06^{aA}*	*9.71±0.09^{b1}*	*35.17±0.52^{aB}*	*36.44±0.43^{b2}*	*36.87±0.42^{aC}*	*50.89±0.24^{b3}*
15	7,12-Hexadienoic acid	*n.d.*	*n.d.*	1.58±0.06^{A}	*n.d.*	1.59±0.01^{aA}	2.10±0.06^{b}
16	7,10-Hexadecadienoic acid	*n.d.*	*n.d.*	1.67±0.13^{aA}	2.03±0.08^{b1}	1.76±0.13^{aA}	2.37±0.18^{b2}

Table 4.3. Lipid profile (mg g^{-1} of dry microalgal weight) of *Pavlova pinguis* grown in three different nitrogen levels before (BH) and after (AH) alkaline hydrolysis. (Continuation)

		Low Nitrogen		Medium Nitrogen		High Nitrogen	
Nº	**Identified Compounds**	**BH**	**AH**	**BH**	**AH**	**BH**	**AH**
17	7,10,13-Hexadecatrienoic acid	*n.d.*	*n.d.*	1.61±0.06^{A}	*n.d.*	1.60±0.07^{aA}	2.17±0.11^{b}
20	Methyl-4,7,10,13-hexadecatetraenoate	*n.d.*	*n.d.*	3.28±0.06^{A}	*n.d.*	3.27±0.04^{aA}	4.31±0.01^{b}
27	9,12-Octadecadienoic acid	1.21±0.01^{aA}	3.22±0.05^{b1}	3.25±0.15^{aB}	4.08±0.02^{b2}	3.23±0.03^{aB}	4.35±0.08^{b3}
28	9,12,15-Octadecatrienoic acid	*n.d.*	*n.d.*	3.28±0.03^{aA}	4.21±0.01^{b1}	3.35±0.03^{aB}	4.45±0.01^{b2}
25	3,6,9,12,15-Octadecapentaenoic acid	*n.d.*	*n.d.*	1.67±0.01^{aA}	2.14±0.01^{b1}	1.71±0.01^{aB}	2.32±0.01^{b2}
32	5,8,11,14,17-Eicosapentaenoic acid	2.43±0.03^{aA}	3.28±0.03^{b1}	6.75±0.55^{aB}	8.47±0.20^{b2}	8.28±0.06^{aC}	12.10±0.06^{b3}
38	7,10,13,16,19-docosapentaenoic acid	2.17±0.01^{A}	*n.d.*	3.91±0.12^{aB}	5.01±0.08^{b1}	4.01±0.08^{aB}	5.48±0.02^{b2}
39	4,7,10,13,16-docosapentaenoic acid	*n.d.*	*n.d.*	3.57±0.03^{aA}	4.56±0.03^{b1}	3.58±0.05^{aA}	4.87±0.02^{b2}
37	4,7,10,13,16,19-Docosahexaenoic acid	2.32±0.02^{aA}	3.21±0.01^{b1}	4.60±0.23^{aB}	5.94±0.17^{b2}	4.48±0.10^{aB}	6.38±0.07^{b3}
	Alcohols	*18.07±1.43^{aA}*	*16.74±0.66^{a1}*	*27.31±1.15^{aB}*	*25.73±0.40^{a2}*	*26.20±0.87^{aB}*	*26.28±0.69^{a2}*
2	Decanol	0.90±0.00^{A}	*n.d.*	1.28±0.01^{B}	*n.d.*	1.34±0.00^{C}	n.d.
4	Undecanol	0.89±0.02^{aA}	1.25±0.01^{b1}	1.33±0.06^{B}	*n.d.*	1.40±0.01^{aB}	1.67±0.02^{b2}
7	Dodecanol	0.86±0.01^{aA}	1.28±0.01^{b1}	1.35±0.09^{aB}	1.65±0.01^{b2}	1.30±0.01^{aB}	1.75±0.08^{b2}
9	Tridecanol	0.87±0.01^{aA}	1.28±0.00^{b1}	1.52±0.27^{aB}	1.65±0.00^{a2}	1.34±0.03^{aB}	1.85±0.07^{b3}
10	Tetradecanol	1.04±0.04^{aA}	1.32±0.03^{b1}	1.41±0.04^{aB}	1.61±0.01^{b2}	1.47±0.02^{aB}	1.70±0.01^{b3}
14	Hexadecanol	2.96±0.48^{aA}	1.95±0.15^{b1}	2.92±0.51^{aA}	2.30±0.04^{a2}	3.06±0.20^{aA}	2.56±0.08^{b2}
22	Octadece-9-nol	3.49±0.53^{aA}	2.29±0.28^{b1}	3.99±0.58^{aA}	3.45±0.10^{a2}	4.17±0.39^{aA}	3.48±0.03^{b2}
24	Octadecanol	2.05±0.24^{aA}	1.88±0.09^{a1}	2.26±0.25^{aA}	2.40±0.04^{a2}	2.33±0.15^{aA}	2.43±0.03^{a2}
33	Eicosanol	0.95±0.04^{A}	*n.d.*	1.32±0.03^{aB}	1.62±0.00^{b1}	1.32±0.01^{aB}	*n.d.*
40	Docosanol	*n.d.*	*n.d.*	1.27±0.01^{a}	1.61±0.01^{b1}	*n.d.*	*n.d.*
47	Hexacosanol	*n.d.*	*n.d.*	1.26±0.00	*n.d.*	*n.d.*	*n.d.*
48	Octacosanol	1.18±0.03^{aA}	1.47±0.02^{b1}	1.51±0.04^{aB}	1.89±0.07^{b2}	1.55±0.01^{aB}	1.90±0.01^{b2}
57	Triacontanol	1.08±0.03^{aA}	1.38±0.02^{b1}	1.40±0.04^{aB}	1.79±0.03^{b2}	*n.d.*	*n.d.*
63	Dotriacontanol	0.89±0.01^{aA}	1.26±0.00^{b1}	1.30±0.03^{aB}	1.65±0.01^{b2}	1.37±0.04^{aC}	1.68±0.01^{b3}

Table 4.3. Lipid profile (mg g^{-1} of dry microalgal weight) of *Pavlova pinguis* grown in three different nitrogen levels before (BH) and after (AH) alkaline hydrolysis. (Continuation)

		Low Nitrogen		Medium Nitrogen		High Nitrogen	
Nº	**Identified Compounds**	**BH**	**AH**	**BH**	**AH**	**BH**	**AH**
26	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.91±0.00^{aA}	1.39±0.06^{b1}	3.21±0.30^{aB}	4.13±0.09^{b2}	5.54±0.13^{aC}	7.25±0.68^{b3}
	Sterols	*6.76±0.41^{aA}*	*6.63±0.55^{a1}*	*11.19±1.18^{aB}*	*11.91±0.95^{a2}*	*17.28±0.13^{aC}*	*18.09±0.33^{b3}*
49	24*α*-Methylcholest-5-en-3*β*-ol	0.31±0.01^{aA}	0.36±0.02^{b1}	0.51±0.04^{aB}	0.60±0.07^{a2}	0.64±0.02^{aC}	0.67±0.12^{a2}
51	24*β*-Ethylcholesta-5,22E-dien-3*β*-ol	0.22±0.02^{aA}	0.23±0.00^{a1}	0.35±0.04^{aB}	0.32±0.01^{a1,2}	0.48±0.03^{aC}	0.38±0.09^{a2}
52	24*α*-Ethylcholesta-5,22E-dien-3*β*-ol	2.01±0.16^{aA}	1.88±0.21^{a1}	4.30±0.63^{aB}	4.39±0.53^{a2}	6.86±0.15^{aC}	6.81±0.25^{a3}
53	24-Ethyl-*δ*(22)-coprostenol	0.35±0.05^{aA}	0.31±0.01^{a1}	0.55±0.06^{aB}	0.55±0.05^{a2}	1.01±0.04^{aC}	1.11±0.03^{b3}
54	24*α*-Ethylcholest-5-en-3*β*-ol	0.77±0.08^{aA}	0.87±0.07^{a1}	0.82±0.10^{aAB}	1.17±0.14^{b2}	0.97±0.12^{aB}	1.20±0.03^{b2}
55	4*α*,24-Dimethyl-5α-cholestan-3*β*-ol	0.54±0.06^{aA}	0.39±0.04^{b1}	0.63±0.08^{aA}	0.68±0.02^{a2}	1.06±0.09^{aB}	1.07±0.02^{a3}
58	4*α*-Methyl,24-ethyl-5*α*-cholest-22E-en-3*β*-ol	0.35±0.02^{aA}	0.36±0.02^{a1}	0.53±0.04^{aB}	0.55±0.04^{a2}	0.82±0.01^{aC}	0.87±0.05^{a3}
59	4*α*-Methyl,24-ethyl-5*α*-cholestan-3-ol	0.40±0.03^{aA}	0.24±0.01^{b1}	0.73±0.04^{aB}	0.36±0.04^{b2}	0.70±0.04^{aB}	0.56±0.02^{b3}
61	4*α*,24*β*-Dimethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	0.27±0.02^{aA}	0.28±0.01^{a1}	0.35±0.01^{aB}	0.41±0.01^{b2}	0.48±0.03^{aC}	0.53±0.01^{b3}
64	4*α*-Methyl-24*β*-ethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	0.48±0.07^{aA}	0.47±0.06^{a1}	0.79±0.11^{aB}	0.84±0.11^{a2}	1.17±0.06^{aC}	1.28±0.05^{b3}
56	Unidentified Sterol	0.58±0.05^{aA}	0.61±0.06^{a1}	1.00±0.11^{aB}	1.07±0.10^{a2}	2.15±0.06^{aC}	2.36±0.06^{b3}
62	Unidentified Sterol	0.49±0.03^{aA}	0.63±0.09^{b1}	0.63±0.05^{aB}	0.95±0.16^{a1,2}	0.93±0.03^{aC}	1.24±0.23^{a2}
	Monoglycerides	*1.60±0.33^{A}*	*n.d.*	*0.77±0.08^{B}*	*n.d.*	*0.18±0.02^{C}*	*n.d.*
35	2,3-Dihydroxypropyl myristate	0.30±0.02^{A}	*n.d.*	0.44±0.06^{B}	*n.d.*	0.09±0.01^{C}	*n.d.*
34	1,3-Dihydroxy-2-propanyl myristate	0.16±0.03^{A}	*n.d.*	0.15±0.02^{A}	*n.d.*	0.05±0.01^{B}	*n.d.*
42	2,3-Dihydroxypropyl palmitate	0.61±0.16^{A}	*n.d.*	0.13±0.01^{B}	*n.d.*	0.04±0.01^{C}	*n.d.*
41	1,3-Dihydroxy-2-propanyl palmitate	0.12±0.01^{A}	*n.d.*	0.04±0.00^{B}	*n.d.*	*n.d.*	*n.d.*
44	2,3-Dihydroxypropyl stearate	0.40±0.11^{A}	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	Others	*1.26±0.05^{A}*	*n.d.*	*0.64±0.02^{B}*	*n.d.*	*0.62±0.03^{aB}*	*0.06±0.00^{b}*
46	2-Phenyl-1,2-propanediol	1.08±0.04^{A}	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

Table 4.3. Lipid profile (mg g^{-1} of dry microalgal weight) of *Pavlova pinguis* grown in three different nitrogen levels before (BH) and after (AH) alkaline hydrolysis. (Continuation)

		Low Nitrogen		Medium Nitrogen		High Nitrogen	
Nº	**Identified Compounds**	**BH**	**AH**	**BH**	**AH**	**BH**	**AH**
5	2,6-bis(1,1-Dimethylethyl)phenol	0.18 ± 0.01^{A}	*n.d.*	0.35 ± 0.01^{B}	*n.d.*	0.34 ± 0.01^{aB}	0.06 ± 0.00^{b}
65	Campesteryl glycoside	*n.d.*	*n.d.*	0.29 ± 0.01^{A}	*n.d.*	0.28 ± 0.04^{aA}	*n.d.*
	Total (mg g^{-1})	*74.94 ± 1.13^{aA}*	*94.91 ± 4.25^{b1}*	*132.32 ± 4.52^{aB}*	*153.59 ± 3.52^{b2}*	*140.53 ± 0.98^{aC}*	*186.05 ± 2.45^{b3}*

Values (means ± SD; n=4) in the same row not sharing a common superscript are significantly different (p <0.05). The significance of the differences before and after alkaline hydrolysis was tested using t-student and is represented by superscript lowercase letters. Differences among treatments using Tukey post-hoc analysis are represented by superscript uppercase letters- before hydrolysis, and by superscript numbers - after hydrolysis. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains *cis* and *trans* isomers. [2]Identified as the mono-TMS ether. *n.d*. – non detected. The compound numbers were attributed accordingly to the elution order.

EPA and 4,7,10,13,16,19-docosahexaenoic acid (DHA – C22:6ω3) have well-established promoting effects on human health (*e.g.* reduce the risk of cardiovascular disease, anti-inflammatory, and neuroprotective) and understanding the conditions that induce the synthesis of these fatty acids by microalgae is essential to further explore the uses of these compounds for the food and pharmaceutical industries. Through Figure 4.3 it is possible to visualize a positive effect of increasing initial nitrogen levels on the sum of EPA and DHA (EPA+DHA) by *P. pinguis*, indicating that a high nitrogen supply is more suitable for high-value lipids accumulation by *P. pinguis*. The highest DHA+EPA was obtained at HN conditions after hydrolysis comprising 18.48 mg g^{-1} dw. The trend verified in the present study for EPA+DHA was also described for *Nannochloropsis* species [62,199], where nutrient-limiting conditions was suggested to reduce the synthesis of LC-PUFA (EPA).

Both hypocholesterolaemic/hypercholesterolaemic fatty acids ratio (H/H) and health-promoting index (HPI) reflect the effect of the fatty acid composition on cardiovascular diseases [208]. For these nutritional indexes, the higher the value the greater the health-benefit. The highest values observed for HH (1.28 – 2.16) and HPI (0.95 – 1.76) were found at MN and HN conditions. Moreover, it is interesting to visualize that these indexes varied significantly (p <0.05) with the cultivation conditions reflecting the chemoplasticity of *P. pinguis* (Fig. 4.3).

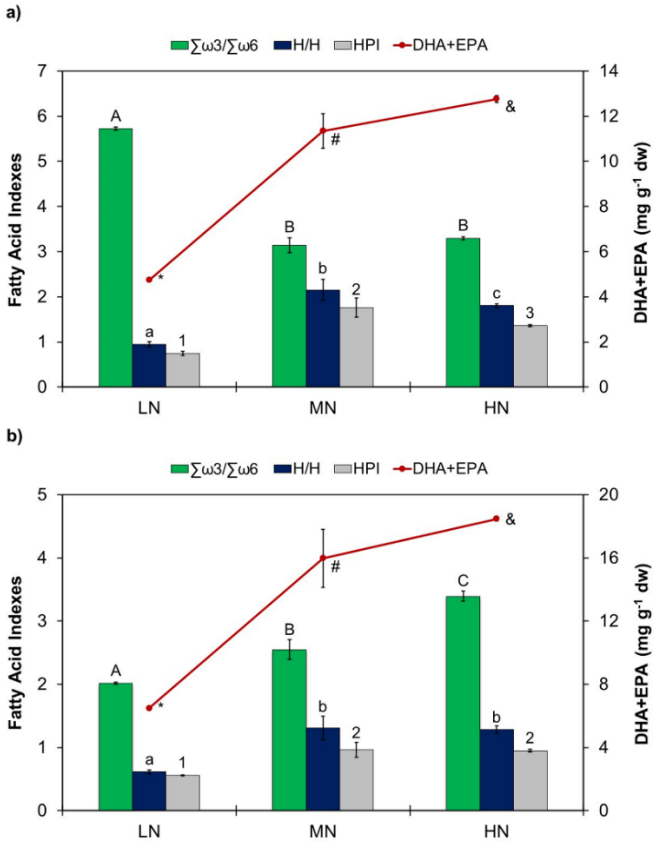

Figure 4.3. Changes in fatty acid nutritional ratios from *Pavlova pinguis* in response to nitrogen a) before hydrolysis and b) after hydrolysis. Values are presented as mean ± standard deviation of 4 replicates. For each variable different symbols represent significant differences among treatments (p <0.05). LN – Low nitrogen; MN – Medium nitrogen; HN – High nitrogen

The forms (free or esterified) in which fatty acids are present in microalgal oils affects their absorption by animals [192]. The highest absorption of nutritionally important fatty acids is reported to occur when they are in their free chemical form [192]. However, due to their lower oxidation stability, fatty acids are often preserved in their esterified forms, comprising the most common and desirable form in edible oils [217].

This research work shows that the alkaline hydrolysis (esterified lipid hydrolysis) of *P. pinguis* affected the fatty acid classes in the following order: monounsaturated fatty acids (MUFA) > SFA > PUFA. The highest MUFA and SFA increase after hydrolysis was observed for the lipid extracts of *P. pinguis* grown in LN conditions (67 and 58%, respectively). Meanwhile, the highest PUFA rise was observed for lipid extracts of *P. pinguis* grown in HN conditions (38%). These observations suggest that the esterified lipids from *P. pinguis* mostly consist of SFA and MUFA, and that this predominance changes in HN conditions, towards PUFA. Guihéneuf and Stengel [169] report that *Pavlova lutheri* responded to LN conditions by increasing the triacylglycerol fatty acid composition in C16:0 and C16:1ω7 and decreasing the composition in EPA. In the present study, the predominant changes in fatty acids occurred for C16:0, C14:0, C18:0 (octadecanoic acid) and C16:1ω7, increasing more than 50% in all treatments after alkaline hydrolysis.

With a low ω3/ω6 being often associated with a pro-inflammatory state, while a great ω3/ω6 fatty acid ratio often attributed to an anti-inflammatory one, a balanced ω3/ω6 fatty acid ratio, close to (1:1), is crucial for the maintenance of normal physiological processes in animals [122]. In western diet, a low ω3/ω6 ratio (1:20) have been observed, and thus the consumption of ω3 rich sources is essential to mitigate this nutritional issue [122]. The trend displayed for ω3/ω6 fatty acid ratio was different after and before hydrolysis among treatments (Fig. 4.3). For this nutritional index, LN presented the highest ω3/ω6 fatty acid ratio (5.72) before hydrolysis (free fatty acids), in contrast to samples analyzed after hydrolysis (free and esterified fatty acids) in which HN presented the highest ω3/ω6 fatty acid ratio (3.39). This observation indicates that esterified composition of *P. pinguis* at LN conditions is richer in ω6 fatty acids than HN conditions. This is an important remark since a higher dietary intake of ω6 fatty acids is often associated with the aggravation of inflammatory processes. Moreover, for all treatments ω3/ω6 fatty acid ratio was higher than 1 showing the potential of this microalga as a rich source of ω3 fatty acids.

5.2.2. Aliphatic alcohols

In different nitrogen levels, *P. pinguis* microalgal cells changed their aliphatic alcohol profile (Table 4.3). Thus, in LN conditions the dominant aliphatic alcohols were octadecen-9-ol and hexadecanol, while in MN and HN conditions they were octadecen-9-ol and phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol). From MN conditions to HN conditions, phytol levels increased by 76%. Knowing that this compound is the main component of chlorophyll's hydrophobic tail, and that chlorophyll synthesis is closely dependent on nitrogen availability, the phytol trend suggests an increase in chlorophyll content in *P. pinguis* in HN conditions [122,218].

This assumption is corroborated by the chlorophylls a and c_1+c_2 content (Table 4.2), which increased by 183 and 95%, respectively, from MN to HN conditions. This observation is in accordance with Beuckels et al. [214], which suggests that in nitrogen-rich environments, microalgal cells trigger the synthesis of chlorophyll molecules, which in nutrient limiting conditions are used to fulfill cell requirements. A previous study performed by Zhu et al. [216] reported a positive correlation between PUFA and chlorophyll synthesis after nitrogen resupply, suggesting the synthesis of structural lipids (polar lipids) in chloroplast membranes. In this study, phytol (26) is positively correlated (R^2 >0.90) with 7,10-hexadecadienoic acid (16), C18:3ω3 (28), EPA (32), and 3,6,9,12,15-octadecapentaenoic acid (25), in the samples before and after hydrolysis (Fig. 4.4).

In contrast to free phytol, which can be readily converted to pristanic acid in the gastrointestinal tract and lymph, phytol linked to chlorophylls cannot be absorbed by humans [219]. Although, at supra-physiological concentrations, this alcohol may trigger the development of pathophysiological states. Phytol and its derivatives have gained interest as potential dietary compounds for cancer prevention [219]. The major dietary source of phytol is the skin of nuts, comprising 1 g of phytol per 100 g of food. In the present study, the highest free phytol content was achieved in *P. pinguis* under HN cultivation conditions, 0.55 g of phytol per 100 g of dried microalgae.

The wide range of biological properties of phytol have also drawn the attention of the pharmaceutical and biotechnological industries [186]. For instance, in cosmetics, phytol is used as a fragrance [186]. Through the hydrolysis of lipids obtained from *P. pinguis,* phytol can be concentrated up to 0.73 g of phytol per 100 g of microalgae. This value is close to other microalgae like *Nannochloropsis* sp. and *Scenesdesmus* sp. analyzed after hydrolysis, comprising 0.72 and 0.74 g of phytol per 100 g of dried biomass [137].

5.2.3. Sterols

A total of twelve sterol compounds were detected in *P. pinguis* subjected to different initial nitrogen levels (Fig. 4.2; Table 4.3). Different nitrogen levels induced significant differences (p <0.05) in sterol content. The dominant sterol was stigmasterol (24*α*-ethylcholesta-5,22*E*-dien-3*β*-ol), which across all treatments accounted for more than 8% of total sterols. *Dunaliella* species are considered promising natural sources of phytosterols due to their high sterol content (0.90 – 1.20%) [72]. In the present study, sterol contents varied between 0.66 and 1.81% of *P. pinguis* biomass (Table 4.3), indicating that this microalga is a potential source for phytosterol for industrial exploitation.

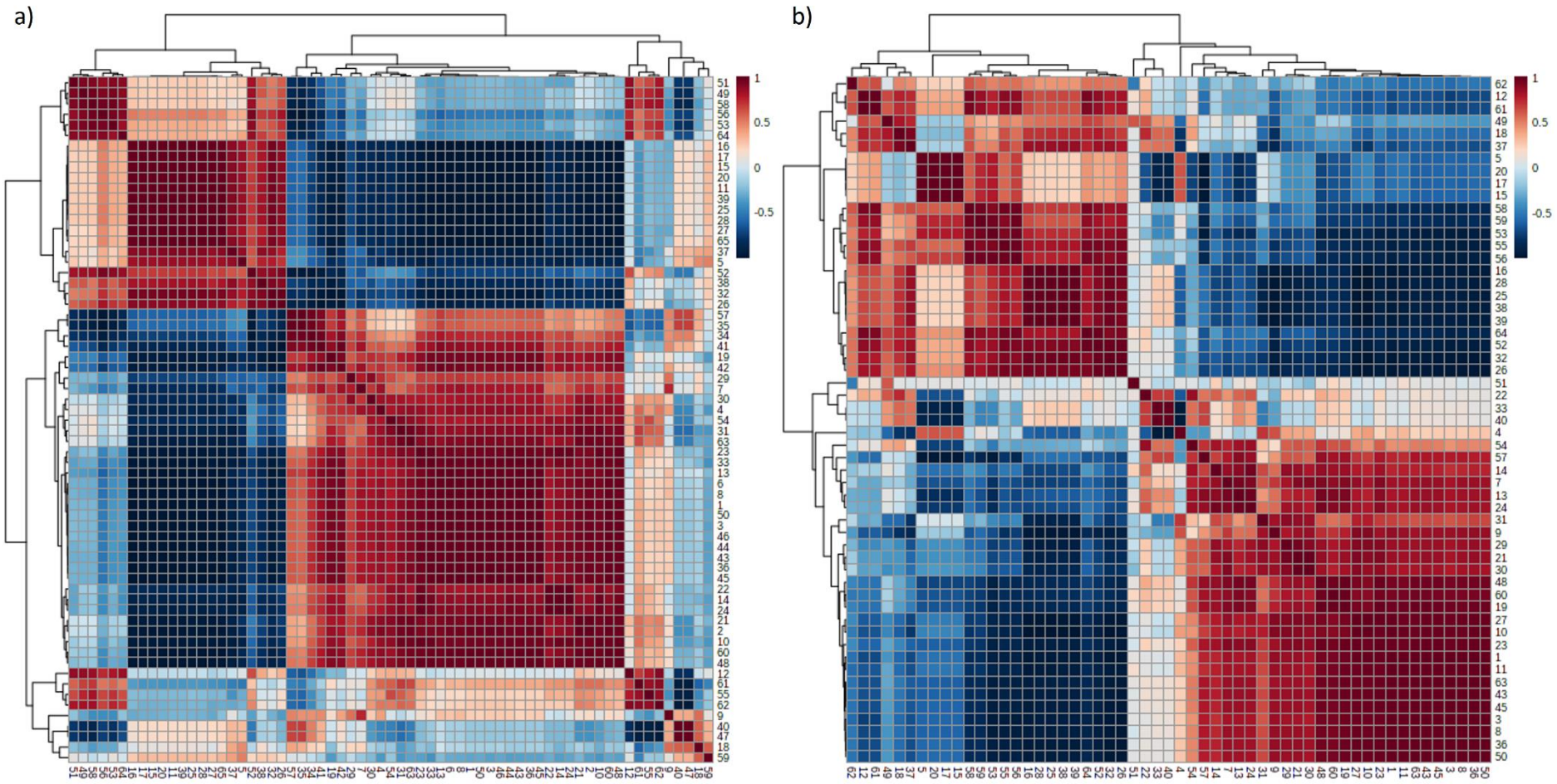

Figure 4.4. Heat map of metabolite-metabolite correlation based on Pearson's linear correlation sampling coefficient (n=4). The description of metabolites can be seen in Table 4.3. Positive correlation is shown in red and negative correlation is displayed in blue. a) before hydrolysis, b) after hydrolysis.

Through the analysis of Table 4.3, it is possible to observe that HN conditions induced an increase, of *P. pinguis* microalgal cell sterol content among treatments, increasing up to 54% from MN to HN. Meanwhile, *P. pinguis* grown in LN conditions had a decrease in sterol content, down to 44% from MN to LN. In opposition to fatty acids, microalgal sterols are known to be more stable in changing environmental conditions [85]. However, differences in phytosterol synthesis have been reported for *Pavlova* species with varying UV-C radiation, salinity and growth phase [66,72,220].

The lowest sterol content values reported in this research work, for *P. pinguis* at LN level, are similar to *Scenedesmus quadricauda*, *Cryptomonas ovata* and *Cyclotella meneghiniana*, where nutrient limitation strategies resulted in a decrease of total sterol content [107]. This seems to indicate that *P. pinguis* responded to LN conditions by down-regulating the enzymes involved in sterol synthesis and/or by reducing sterol incorporation in plasma membranes [107].

The stigmasterol proportion increased from 28 to 38% of the total sterol content as the nitrogen level increased, while *β*-sitosterol (24*α*-ethylcholest-5-en-3*β*-ol) decreased from 13 to 6% of total sterols (Table 4.3). In the phytosterol biosynthetic pathway of *Dunaliella tertioleta*, *β*-sitosterol is the precursor of stigmasterol [221]. Thus, the observed opposite trend might suggest that *P. pinguis* microalgal cells transform *β*-sitosterol to stigmasterol towards high nitrogen levels. Through Table 4.3 it is possible to observe that *P. pinguis* total sterols did not increase with alkaline hydrolysis, which indicates that the detected sterols were mainly free sterols. This finding is in accordance with Volkman [85], which reported that free sterols predominate in microalgae.

Plant phytosterols have been widely used as functional ingredients in food industry due to their low cholesterol abilities [66,72]. From microalgal lipid composition, sterols are an underexploited resource for food industry. Thus, the great amounts of phytosterols verified for *P. pinguis* towards HN conditions should be further studied as a sustainable source of phytosterols for food industry.

5.2.4. Monoglycerides and other compounds

In LN conditions, monoglycerides were positively influenced by increasing nitrogen levels, nearly doubling with respect to MN conditions (Table 4.3). An opposite trend was verified by *P. pinguis* microalgal cells grown in HN conditions, where monoglycerides decreased by 77% (Table 4.3). The monoglycerides before hydrolysis were those with acyl bonds to C16:0, C14:0 and C18:0 (Table 4.3), which are included in the fatty acids where the highest increase after alkaline hydrolysis treatment occurred (Table 4.3). However, small levels of monoglycerides cannot explain the increase in fatty acids after alkaline hydrolysis and indicate that these compounds are present mainly as other acyl-glycerides in *P. pinguis* microalgal cells. For instance, triacylglycerols are cells' energy reservoirs, whose production is enhanced under unfavorable growth conditions [179]. In nutrient limiting conditions, microalgae are known to redirect photosynthetic carbon fixation towards triacylglycerol accumulation [222].

Campesteryl glycoside was detected in *P. pinguis* microalgal cells (0.28 – 0.29 mg g^{-1}) at MN and HN conditions before hydrolysis (Table 4.3). Increasing nitrogen concentration in *P. pinguis* growth medium enhances its production. The observed steryl glycoside was also reported for *Pavlova viridis* in an amount of 0.52 mg g^{-1} [154]. These amphipathic molecules have an enhanced solubility in food products when compared to free phytosterols which, in turn, make them easier to add to food products as functional ingredients to lower blood cholesterol [154].

5.3. Modulating lipids composition by nitrogen supplementation

Principal component analysis (PCA) showed a clear separation between the lipidomes of the LN, MN and HN groups in the scores plot before hydrolysis (Fig. 4.5a). The loadings distribution (Fig. 4.5b) enabled the observation of the metabolites responsible for the discrimination pattern. Therefore, before hydrolysis, principal components described 90% of the variation. PC1 separated the LN from the MN and HN groups, while PC2 separated the MN and HN groups. The first distinction was primarily due to SFA, monoglycerides, some specific alcohols like decanol (2), tetradecanol (10), eicosanol (33), and octacosanol (48), and the fatty acid 9-hexadecenoic acid (18), positively correlated with PC1, while the second was mostly due to sterols (negatively correlated with both PC1 and PC2).

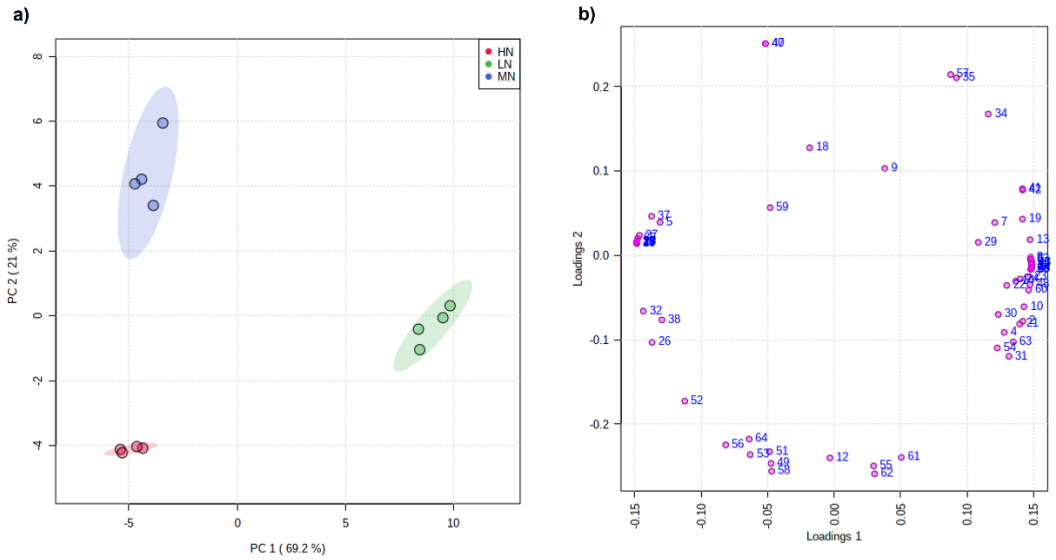

Figure 4.5. Principal component analysis for *Pavlova pinguis* lipid extracts before hydrolysis a) scores plot (each colored dot represents an individual replicate, n=4), and b) loadings plot. The description of the variables can be seen in Table 4.3. LN - low nitrogen level; MN - medium nitrogen level; HN - high nitrogen level

SFA and MUFA are the best ingredients for biodiesel production [223]. The abundance of these compounds is responsible for the key properties that reflect the quality of biofuel [222,223]. Higher levels of SFA and MUFA contribute to a higher cetane number, decreased NO_x emissions, shorter ignition delay time, oxidative stability, and low-temperature fluidity [222,223].

Thus, and according to the multivariate analysis, the best lipid profile for this purpose was recorded for *P. pinguis* cultivated in LN conditions (positively correlated with PC1).

Phytosterols have several industrial applications such as: *i)* pharmaceutical (raw materials for therapeutic steroids), *ii)* nutraceutical (food additive, cholesterol lowering properties and cardiovascular health maintenance), and *iii)* cosmetics (additive in creams and lipsticks) [66,72]. Thus, and according to the multivariate analysis, HN conditions are a useful tool for inducing sterol production in *P. pinguis*.

The variability explained by the principal components after hydrolysis (Fig. 4.6a, 85%) was close to the one obtained before hydrolysis, and so was the clustering pattern of the different groups as compared with the previous PCA. However, the loadings contribution for cluster discrimination was different (Fig. 4.6b). Thus, for PC1 the main loadings contributing positively for LN separation were SFA, 9-tetradecenoic (11), 7-hexadecenoic (19), and 9,12-octadecadienoic acids (27), and the alcohols tetradecanol (10), octacosanol (48) and dotriacontanol (63). Meanwhile, for PC2 they were polyunsaturated C16 fatty acids 7,12-hexadienoic acid (15); 7,10,13-hexadecatrienoic acid (17), and methyl-4,7,10,13-hexadecatetraenoate (20), undecanol (4), and 2,6-bis(1,1-Dimethylethyl)phenol (5).

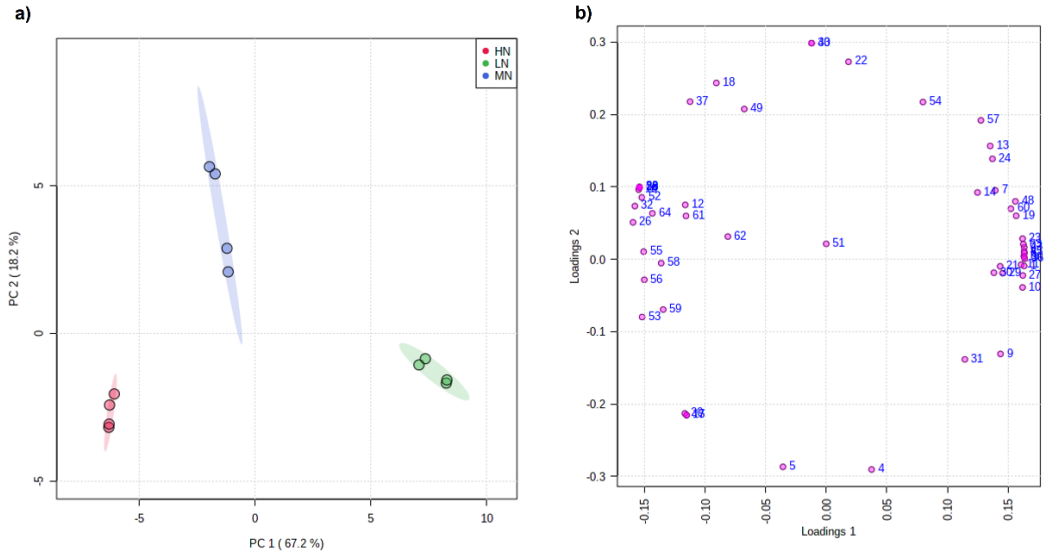

Figure 4.6. Principal component analysis for *Pavlova pinguis* lipid extracts after hydrolysis a) scores plot (each colored dot represents an individual replicate, n=4), and b) loadings plot. The description of the variables can be seen in Table 4.3. LN - low nitrogen level; MN - medium nitrogen level; HN - high nitrogen level

Shen et al. [224] studied the relationship between the characteristic fatty acid and triacylglycerol content in microalgae, concluding that PUFA are often abundant in polar lipids (glycolipids and phospholipids) rather than in triacylglycerols. For instance, C16:4 and C16:3 comprise the bulk of fatty acids in monogalactosyldiacylglycerols for *Chlamydomonas reinhardtii* and the model plant *Arabidopsis thaliana,* respectively [224]. Thus, the correlation of *P. pinguis* cultivated in HN conditions with the polyunsaturated C16 fatty acids after hydrolysis

demonstrated in Fig. 4.5 might be due to higher levels of glycolipids. When supplying dietary PUFA for fish hatcheries, fatty acids in polar lipids are nutritionally more available than those in neutral lipids, making the HN condition suitable for increasing dietary intake for nutraceuticals and aquaculture [192]. Moreover, SFA and MUFA esterified into glycerol-based neutral lipids (like mono-, di-, and triacylglycerols) can be converted to biodiesel [222], which again makes *P. pinguis* grown in the LN treatment the most promising candidate for biodiesel or fuel production.

The heat maps (Fig. 4.7) allowed the visualization of clusters of samples (columns) and features (rows) according to the hierarchical clustering analysis. The data matrix displays the normalized data organized according to similarity. It is possible to observe that the LN group was furthest from the MN group, indicating that nitrogen limitation presented the most considerable perturbation of the lipidome. This group showed opposite trends compared with the other clusters for most metabolites. Moreover, through the heat maps it was possible to observe that *P. pinguis* cultured in both MN and HN conditions had greater levels of nutritionally important fatty acids (PUFA) and sterols.

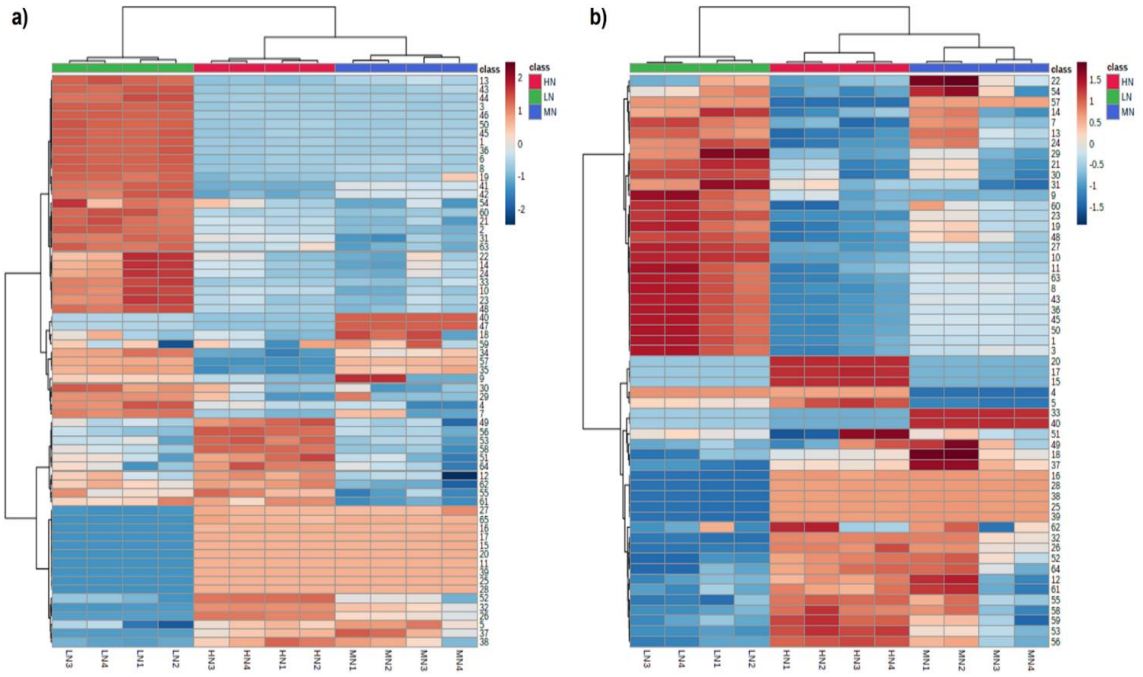

Figure 4.7. Hierarchical clustering analysis heat-maps based on Euclidean clustering distance and the ward clustering method (n=4). The heat-maps reflect the relative levels of metabolites among different treatment groups for *Pavlova pinguis* a) before and b) after alkaline hydrolysis. A description of the features can be seen in Table 4.3. The color scheme is associated with the elevation and reduction on the metabolite level through treatments: dark blue - lowest; dark red - highest. LN - low nitrogen level; MN - medium nitrogen level; HN - high nitrogen level

6. Conclusions

A greater understanding of nitrogen uptake and use efficiency by *Pavlova pinguis* was achieved. Nitrogen supplementation provides not only a simple approach for industrial applications to assess the robustness of promising microalgal strains for large scale production,

but also gives an insight to the processes that lead to lipid accumulation and nitrogen remediation. According to the field of application, different levels of nitrogen are recommended: higher nitrogen levels are recommended for chlorophyll, carotenoid, and high-value lipid accumulation with applications in nutraceutics, pharmaceutics, and cosmetics, while low nitrogen levels should be used for enhancing *P. pinguis* lipid quality for biodiesel. Given the great diversity and versatility found for *P. pinguis* lipid extracts across treatments, more research should focus on the potential of the different types of lipids derived from microalgae, including their functional activity, synergistic effects, and biosynthetic pathways.

CHAPTER 5.

Comparative lipidomic analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* in response to nitrogen-induced changes

This Chapter is based on the following publication:

Fernandes, T.; Ferreira, A.; Cordeiro, N. Comparative lipidomic analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* in response to nitrogen-induced changes. Algal Res. 2021, 58, 102417. https://doi.org/10.1016/j.algal.2021.102417.

Chapter 5. Comparative lipidomic analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* in response to nitrogen-induced changes

Abstract

The current focus of algae biotechnology is the production of high-value lipids, and its improvement by employing abiotic perturbations such as nitrogen-induced changes. In the present study, the growth dynamics, nitrogen uptake, pigments, and lipid composition of *Chlorellla stigmatophora* and *Hemiselmis* cf. *andersenii* were studied, in response to low (LN), medium (MN) and high (HN) nitrogen supplementations. Both microalgae responded to increased nitrogen levels by increasing their nitrogen uptake rate and pigment content. However, for lipid accumulation, *C. stigmatophora* presented a different pattern (LN: 16.56% > MN: 11.51% > HN: 10.95%) to that of *H.* cf. *andersenii* (MN: 15.37% > HN: 13.06% > LN: 6.71%). Untargeted gas chromatography-mass spectrometry analysis allowed the visualization of the biochemical diversity of *C. stigmatophora* and *H.* cf. *andersenii*, as well as differences in lipid regulation upon nitrogen-induced changes among species. For instance, glycosyl sterols were only detected for *C. stigmatophora* samples grown under MN and HN conditions. Moreover, lipid analysis of *H.* cf. *andersenii*, before and after alkaline hydrolysis, suggests that wax esters play a key role in the response of this microalga to high nitrogen levels. The cultivation of *H.* cf. *andersenii* at MN and HN was shown to be ideal for providing a rich source of ω3 and polyunsaturated fatty acids for nutraceutical purposes. The hierarchical cluster analysis showed the differential intra- and interspecific effects of nitrogen on lipid composition. The diverse ways by which both microalgae responded to nitrogen-induced changes highlighted the influence of phylogeny on the carbon flux through metabolic networks, and accumulation.

Keywords: Nitrogen supplementation, *Hemiselmis* cf. *andersenii*, *Chlorella stigmatophora*, lipidome analysis, nutraceutical lipids.

1. Introduction

The active search by consumers for health-conscious foodstuffs has been a driving force for studying the chemical composition of microalgae. According to Bhattacharya and Goswami [225], worldwide small scale algal growth is estimated to generate US$6.5 billion, of which US$2.5 billion comes from the health food segment. However, one of the major limiting factors for the production of microalgae continues to be the low yield of biomass and extracted products [226]. To overcome this challenge, several strategies have been studied. For example, genetic engineering techniques, like introduction of new genes, elimination of non-essential assimilatory pathways, and biochemical engineering approaches, such as optimization of nutrient formulations, study of innovative cultivation techniques - utilization of plant growth regulators [227,228].

Microalgal cells provide a biotechnological platform to modify, control and maximize the production of a targeted compound [229]. When considering algae as potential new food sources, one critical factor is their composition and nutritional content. Among lipids, the nutritional quality of microalgal species is positively associated with high levels of polyunsaturated fatty acids (PUFA), phytosterols and long-chain aliphatic alcohols (LCAA), which, in turn, can be transferred up the food-chain, adding commercial value to feeds and human dietary supplements [122,142,230]. Besides non-acylglycerol neutral lipids (hydrocarbons, free fatty acids, ketones), microalgae comprise polar (phospholipids and glycolipids) and acylglycerol lipids [231]. As with non-acylglycerol lipids, the quality of microalgae esterified lipid fraction is crucial for the selection of cultivation and processing strategies [199].

Microalgal lipid synthesis and accumulation can be enhanced through abiotic perturbations such as nitrogen-induced changes [232]. The main chemical forms by which photosynthetic organisms can acquire nitrogen include nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) [233]. Although the information on the regulation of nitrogen acquisition and assimilation is limited to few microalgal species, previous studies have shown that NO_3^- is involved in the regulation of carbon-rich pools, such as carbohydrates and lipids [218]. In algae, NO_3^- acquisition and assimilation seem to be positively associated with its presence in the surrounding environment [218].

At the current stage of algal development, the production of biofuel from microalgal fatty acids is still a challenge due to high production costs. Therefore, the current focus of algae biotechnology is the production of high-value lipids [17]. Cryptophyta species have been shown to be rich sources of high-value products such as ω3 - PUFA, carotenoids, phycobiliproteins, and sterols [234,235]. Belonging to the Cryptophyta division, *Hemiselmis* species lack heavy cell wall structures made of silica or cellulose, which, in turn, makes these microalgae easier to break down and process for commercial purposes [171,235]. In aquaculture industry, *Hemiselmis* is used as feed for fish hatcheries to improve growth and survival rates [191]. Although previous studies [171,236] have reported Cryptophyta *Hemiselmis* species as promising candidates for the sustainable and profitable production of health-promoting lipids, the modulation of *Hemiselmis* lipid quantity and composition is understudied. In this context, lipidomic studies are essential not only to reveal potential targets that may be of commercial interest, but also to understand lipid regulation in microalgae [167].

The Chlorophyta division contains the *Chlorella* genus, which is well-known to generally possess rigid, recalcitrant cell walls [237]. Species from the C*hlorella* genus are known for their health benefits when consumed as a whole, and represent the first microalgal species to be produced on a large scale [238]. Thus, species from this genus have been extensively studied, comprising models when trying to understand the mechanisms that underlie desired product accumulation [238]. Although the divisions of *H.* cf. *andersenii* and *C. stigmatophora* are rather distinct, the potential of *H.* cf. *andersenii* for high-value compound exploitation was compared to *C. stigmatophora* as one of the few marine *Chlorella* studied for the commercial application of microalgae.

While many studies assessing the impact of nitrogen-limiting conditions have been performed, few information is known about the effect of excess nitrogen supplementation in microalgae [239]. Thereby, the aim of the present study was to determine how two diverse microalgal species remodeled their growth dynamics, nitrogen uptake, pigments, and especially lipid composition in response to three different nitrogen supplementations (low, medium, and high), using gas chromatography-mass spectrometry (GC-MS) untargeted analysis as a robust method for the identification of multiple metabolites.

2. Materials and methods

2.1. Growth and culture conditions

The Cryptophyta *Hemiselmis* cf. *andersenii* (BEA 0118B) was obtained from the Spanish Bank of Algae (BEA), and the seawater Chlorophyta *Chlorella stigmatophora* (RCC 661) was obtained from the Roscoff Culture Collection (RCC). The f/2-Si medium [201,202] (8.82 × 10^{-4} M $NaNO_3$, 3.62 × 10^{-5} M $NaH_2PO_4.H_2O$, 1.06 × 10^{-4} M Na_2SiO_3, 1.17 × 10^{-5} M $FeCl_3.6H_2O$, 1.17 × 10^{-5} M $Na_2EDTA.2H_2O$, 9.10 × 10^{-7} M $MnCl_2.4H_2O$, 7.65 × 10^{-8} M $ZnSO_4.7H_2O$, 4.20 × 10^{-8} M $CoCl_2.6H_2O$, 3.93 × 10^{-8} M $CuSO_4.5H_2O$, 2.60 × 10^{-8} M $Na_2MoO_4.2H_2O$, 2.96 × 10^{-7} M Thiamine.HCl, 2.05 × 10^{-9} M Biotin, 3.69 × 10^{-10} M Cyanocobalamin) was used as the basal formula for the nitrogen manipulations, with an initial pH of 7, measured with a HI 2209 pH meter from Hannah instruments. The microalgae were grown under three different nutrient conditions by tailoring the nitrate concentrations: nitrogen-limited (low nitrogen - LN; 177 ± 11 µM $NaNO_3$), control (medium nitrogen - MN; 989 ± 13 µM $NaNO_3$) and supplemented (high nitrogen - HN; 1768 ± 14 µM $NaNO_3$). For the cultivation of the microalgae, 100mL of exponentially growing microalgae was inoculated in 1 L flasks, giving the following initial cell concentrations: 4.95×10^5 cells mL^{-1} for *H.* cf. *andersenii*, and 7.90×10^5 cells mL^{-1} for *C. stigmatophora*, and grown under 70 µmol m^{-2} s^{-1} light intensity, 16:8 h (light: dark cycles) at 25 °C. In the early stationary phase, the medium was centrifuged for 7 min at 3720 x *g*, the supernatant was discarded, and the pellets were washed twice with an 0.09 g L^{-1} NaCl solution before being freeze-dried in a Labconco freeze dry system at -50 ºC for nearly 3 days and stored at -20 ºC until further analysis. According to Safafar et al. [240] storage of freeze-dried microalgae at low temperature is more effective for increasing storage stability of biomass than oxygen-reduced storage conditions (*e.g.* vacuum packaging). The number of microalgal cells was monitored daily by measuring the optical density of the microalgal culture at 550 nm (OD_{550}) with an ultraviolet/visible spectrophotometer (UV-6300PC, VWR, China), R^2 = 0.9929. The logistic model of Xin et al. [175] was used to describe the microalgal growth and fitted to the data with excel add-in solver.

2.2. Nitrogen determination

Nitrogen (N) was determined as nitrate (NO_3^-) by the ultraviolet spectrophotometric screening method previously reported by Wan et al. [203]. Briefly, a calibration curve (R^2= 0.9999)

was performed using 220 nm optical density (OD_{220}) against nitrogen concentrations (0 - 4.07 mg L^{-1}) from $NaNO_3$ solutions. The rate of N uptake by microalgal cells (Eq. 5.1) was calculated as follows:

$$\text{Cell uptake rate (pg cell}^{-1}\text{ d}^{-1}) = \frac{N_i - N_f}{Xt} \qquad \text{Equation 5.1}$$

Where N_i is the initial nitrogen concentration (pg L^{-1}), N_f is the final nitrogen concentration (pg L^{-1}) and X is the cell concentration (cells L^{-1}) at time t (d).

2.3. Elemental analysis

The elemental analysis of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) was performed on an elemental analyser Truspec 630-200-200, LECO, USA. For elemental analysis, up to 10 mg of microalgal dried biomass were combusted in a furnace at 1075 ºC. Then, the combustion gases were carried out using Helium, and further detected by infrared absorption (C, H, and S) and thermal conductivity (N).

2.4. Chlorophylls and carotenoids determination

Pigment extraction was performed from 10 mg freeze-dried biomass with an 80% acetone solution. Samples were ultrasonicated for 90 min, and centrifuged at 4430 x *g* for 10 min. The supernatant was removed, and the pellet was continuously washed until the supernatant became colorless. The supernatant was passed through a 0.45 µm filter, and its absorbance was read in a spectrophotometer (UV-6300PC, VWR). The pigment content was calculated according to Chen and Vaidyanathan [204] by equations 5.2-5.4, where Chl *a* is the chlorophyll *a* (µg mL^{-1}), Chl *b* is the chlorophyll *b* (µg mL^{-1}), and C_t is the carotenoids (µg mL^{-1}):

$$\text{Chl } a = 12.21A_{663} - 2.81A_{646} \qquad \text{Equation 5.2}$$

$$\text{Chl } b = 20.13A_{646} - 5.03A_{663} \qquad \text{Equation 5.3}$$

$$C_t = \frac{1000A_{470} - 3.27\text{Chl } a - 104\text{Chl } b}{198} \qquad \text{Equation 5.4}$$

Marine *Chlorella stigmatophora* belongs to the Chlorophyta division, which possesses chlorophylls *a* and *b*, whereas *Hemiselmis* cf. *andersenii* is a marine microalga belonging to the Cryptophyta division, which only possesses chlorophylls *a* and c_2 [205,234]. Thus, for Cryptophyta *H. cf. andersenii*, chlorophyll c_2 determination was performed using the equation described by Jeffrey and Humphrey [205]. Thus, acetone from the supernatant was evaporated on a nitrogen stream and the remaining water was removed by freeze-drying samples. Afterwards, the samples were resuspended on 100% acetone. Chl c_2 is the chlorophyll c_2 (µg mL^{-1}), which was calculated by the following equation:

$$\text{Chl } c_2 = 27.09A_{630} - 3.63A_{663} \qquad \text{Equation 5.5}$$

2.5. Lipid extraction

Total lipids were estimated according to Bligh and Dyer [206], with some modifications as described by Fernandes et al. [145]. Briefly, 50-100 mg of lyophilized microalgal biomass was used to estimate total lipids. Lipids were extracted with a methanol:chloroform mixture (2:1; v:v), followed by 400 µL of a saturated solution of KCl and 2 mL of chloroform. After homogenization, 2 mL of distilled water was added. The organic phase was removed, and the remaining biomass was continuously washed until the organic phase became colorless. Then, the organic phase was transferred to pre-weighted tubes and dried under a nitrogen stream. The extracted lipids were left to stabilize in a desiccator prior to gravimetric quantification and stored at -20 ºC until further analysis.

2.6. Alkaline hydrolysis

Alkaline hydrolysis was performed to detect molecules in their esterified forms according to Santos et al. [129]. 0.5M of NaOH in aqueous methanol was added to two lipid aliquots, and the mixtures were heated at 100 ºC for 1 h in a nitrogen atmosphere. Then, the samples were acidified to pH 2 with 1M HCl.

2.7. Gas chromatography–mass spectrometry analysis

Prior to gas chromatography–mass spectrometry (GC-MS) analysis, bis(trimethylsilyl)trifluoroacetamide (BSTFA) silylation of lipid extracts before and after alkaline hydrolysis was performed as described by Santos et al. [129]. Then, trimethylsilyl (TMS) derivatives were analyzed in a gas chromatographer (6890N, Agilent Technologies, China) equipped with a mass selective detector (5973Network, Agilent Technologies, USA) and a ValcoBond capillary column from Vici Valco (30 m × 0.25 mm inner diameter, 0.25 µm film thickness). The chromatographic conditions were those described in Fernandes et al. [236]. Briefly, the oven was set to 80 ºC for 5 min, increasing by 4 ºC min^{-1} until 208 º C, then by 2 °C min^{-1} until 260 °C and by 5 °C min^{-1} until the final temperature of 300 °C, which was maintained for 4 min. The injector temperature was 250 °C, the transfer line temperature was 290 °C, and the split ratio was 33:1. Helium was used as the carrier gas at a rate of 1.0 mL min^{-1}. The identification of the extracted compounds as TMS derivatives was done by comparison of the mass spectra fragmentation with those from the Wiley-NIST library, literature [177,241–243], and when possible, by comparison with standards. For quantitative analysis, GC-MS was calibrated with pure reference compounds (mannose, *trans*-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5*α*-chlolestane, cholesterol, stigmasterol, hexadecanoic, and nonadecanoic acids) relative to tetracosane (internal standard).

The hypocholesterolaemic/hypercholesterolaemic fatty acid ratio (H/H), as in Santos-Silva et al. [207], was calculated as follows (Eq. 5.6):

$$H/H = (C18:1\omega9+C18:2\omega6+C20:4\omega6+C18:3\omega3+C20:5\omega3+C22:5\omega3+C22:6\omega3)/(C14:0+C16:0) \quad \text{Equation 5.6}$$

2.8. Statistical analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 24. Differences between treatments were assessed with one-way analysis of variance (ANOVA) followed by a Tukey post hoc analysis, with p <0.05 considered statistically significant. Metaboanalyst 3.0 was used to perform multivariate analysis and metabolite-metabolite correlation analysis [209]. Prior to the data analysis, variables were median normalized, $\log_2$ transformed and auto scaled. For hierarchical clustering heat maps, the Euclidean clustering distance and ward clustering method were used.

3. Results and discussion

3.1. Culture growth and nitrogen removal assessment

Chlorella stigmatophora and *Hemiselmis* cf. *andersenii* were grown under three different nitrogen conditions and sampled in the early stationary phase. The harvest days were determined as those on which the cell concentration was similar for 2 to 3 consecutive counting days; this was further corroborated by the non-significant differences found for the last cultivation days in both microalgae (p >0.05). Figure 5.1a shows the growth curves obtained for *C. stigmatophora*. Through this figure it is possible to see that the early stationary phase was reached at different days with respect to nitrogen supplementation: day 9 for LN conditions; and day 15 for MN and HN conditions. Meanwhile, in *H.* cf. *andersenii* (Fig. 5.1b), strong effects on microalgal cell growth were also observed, with the early stationary phase being reached at day 6 for LN conditions and day 8 for both MN and HN conditions.

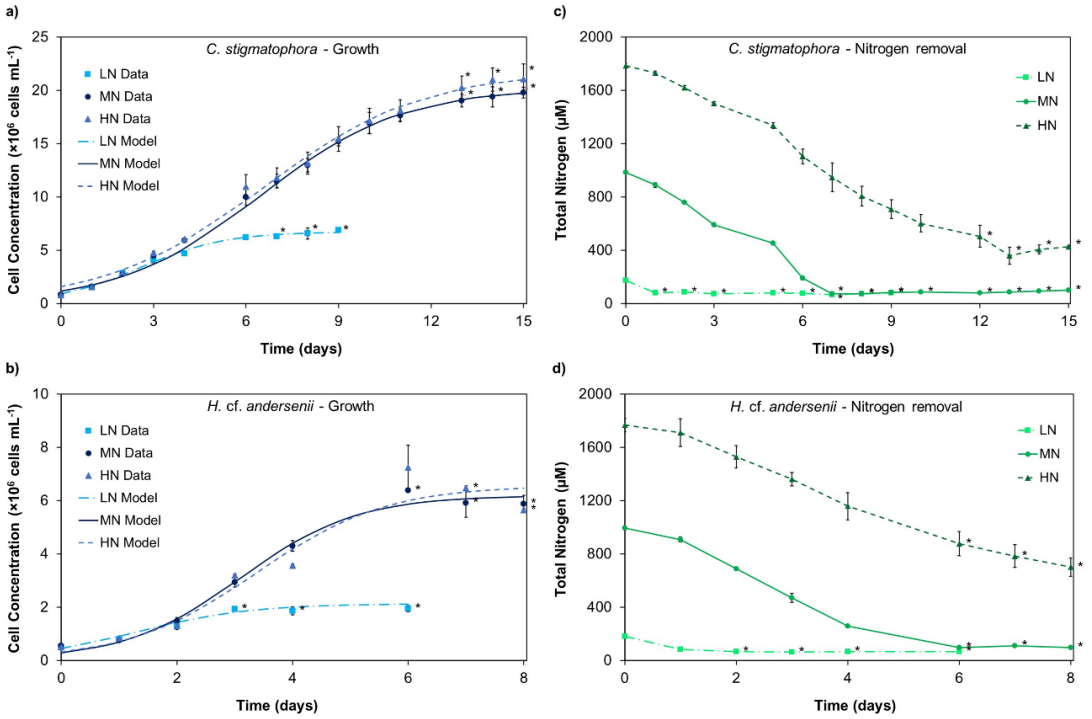

Figure 5.1. Dynamics of growth and nitrogen removal for *Chlorella stigmatophora* (a, c) and *Hemiselmis cf. andersenii* (b, d) under low (LN), medium (MN) and high (HN) nitrogen experimental conditions. Values

are expressed as mean ± standard deviation, n=3 replicates. The determination coefficients (r^2) for the growth models were higher than 0.90. * Values are not significantly different (p >0.05) among cultivation days, within each treatment.

Changes in total nitrogen (Fig. 5.1c and 5.1d) in the growth medium showed that both microalgae grown in LN conditions internalized the available nitrogen by day 1, confirming the initiation of nutrient-depleted conditions. In MN conditions, nitrogen was fully internalized by day 7 and day 6 for *C. stigmatophora* and *H.* cf. *andersenii*, respectively. These observations indicate that *C. stigmatophora* and *H.* cf. *andersenii*, under LN and MN cultivation conditions, continue to utilize the acquired nitrogen to support cell growth and division until they reach the early stationary phase. In both microalgae cultured under HN conditions, the nitrogen has not been fully consumed, which seemed to indicate that light-limitation occurred [244,245]. According to Lee et al. [245] cell concentration of a photosynthetic culture will continue to increase exponentially until all photosynthetically available photons are absorbed. Therefore, to verify if light-limitation did occur half the cells were returned to the conditioned media, and cell concentration was evaluated against time. For this assay, non-significant differences (p >0.05) were found for cell concentration, and the highest variation rate observed for cell concentration was only 5%, indicating that a return to exponential growth did not occur, which, in turn, shows that the assumption of light-limitation was not accurate. Thus, with the increase in initial nitrogen concentrations, a stoichiometric imbalance between nitrogen and other nutrients in the culture medium was created, which may have caused the primary growth limiting nutrient to be a different nutrient (like phosphorus) than nitrogen.

Nitrogen supplementation triggered changes in the elemental stoichiometry of both microalgae (Table 5.1). An interesting observation is that despite *H.* cf. *andersenii* under LN conditions had presented the lowest nitrogen content, this microalga presented the lowest (3.00) carbon to nitrogen ratio (C/N). This observation, along with the carbon content, indicates that LN conditions lead to reduced carbon fixation. In *C. stigmatophora*, the C/N ratio decreased by as much as 61% with nitrogen supplementation indicating that this microalga responded to higher nitrogen supplementation by allocating a larger proportion of carbon into nitrogen-rich pools like protein [145]. Changes in the elemental stoichiometry of microalgae in response to nutrient-induced have also been reported in other microalgae like *Rhodomonas marina*, *Nannochloropsis gaditana*, *Isochrysis* sp., and *Chlorella vulgaris* [145,246].

Under nitrogen-rich conditions, nitrogen is mainly integrated in the key structural components of microalgae, such as proteins and chlorophylls, needed to sustain cell growth [214]. In both microalgae under examination, chlorophylls and carotenoids displayed an increasing trend with nitrogen enrichment (Table 5.1). The observation that the lowest values were observed at LN conditions for both *C. stigmatophora* and *H.* cf. *andersenii* pigments seems to indicate that these microalgae respond to low nitrogen supplementations by triggering the degradation of the photosynthetic machinery, in search of alternative nitrogen sources for protein synthesis to fulfil cell requirements [247].

Table 5.1. Nutrient removal, cell uptake, cell uptake rate, biomass, cell dry weight, lipid content, protein content, and elemental analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* under low, medium, and high nitrogen supplementation.

	C. stigmatophora			***H.* cf. *andersenii***		
	Low	***Medium***	***High***	***Low***	***Medium***	***High***
Cell uptake rate (pg cell^{-1} d^{-1})	0.02±0.00^{a}	0.04±0.00^{b}	0.06±0.00^{c}	0.16±0.02^{A}	0.27±0.01^{B}	0.34±0.02^{C}
DCW (pg cell^{-1})	26.32±3.19^{a}	28.35±3.31^{a}	23.36±1.52^{a}	105.54±2.66^{A}	39.43±3.18^{B}	33.65±3.04^{B}
Lipid (% dw)	16.56±0.75^{a}	11.51±1.26^{b}	10.95±0.52^{b}	6.49±1.22^{A}	15.37±1.38^{B}	13.06±0.52^{C}
Chl *a* (mg g^{-1})	2.01±0.03^{a}	5.20±0.13^{b}	8.01±0.48^{c}	0.34±0.01^{A}	3.31±0.01^{B}	3.10±0.00^{C}
Chl *b* and Chl c_2 (mg g^{-1})*	0.50±0.02^{a}	1.30±0.01^{b}	2.14±0.13^{c}	0.28±0.00^{A}	0.74±0.10^{A}	0.65±0.07^{A}
C_t (mg g^{-1})	0.89±0.02^{a}	2.31±0.05^{b}	3.10±0.22^{c}	0.12±0.00^{A}	1.94±0.01^{B}	0.64±0.00^{C}
C (% dw)	29.70±1.41^{a}	30.11±1.91^{a}	35.98±1.35^{b}	4.18±0.04^{A}	27.29±0.37^{B}	24.24±0.00^{C}
N (% dw)	1.06±0.02^{a}	1.75±0.03^{b}	3.27±0.06^{c}	1.63±0.10^{A}	3.27±0.00^{B}	4.68±0.02^{C}
H (% dw)	4.59±0.21^{a}	4.48±0.21^{a}	4.97±0.23^{a}	2.12±0.13^{A}	4.30±0.11^{B}	3.81±0.06^{C}
C/N (mol/mol)	32.61±1.57^{a}	20.06±0.96^{b}	12.82±0.24^{c}	3.00±0.20^{A}	9.72±0.19^{B}	6.04±0.03^{C}

Values (means ± SD of at least two replicates) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for *H.* cf. *andersenii*. DCW - dry cell weight; Chl *a* - Chlorophyll *a*; Chl *b* - Chlorophyll *b*; Chl c_2 - Chlorophyll c_2; * Chlorophyll *b* was only estimated for *C. stigmatophora,* and chlorophyll c_2 was only estimated for *H.* cf. *andersenii*; C_t - Total carotenoids. Sulphur was not detected for *C. stigmatophora* and *H.* cf. *andersenii* samples regardless treatments applied.

The great biodiversity attributed to microalgae is reflected in the diverse ways by which core metabolic networks operate in microalgal species [248]. *C. stigmatophora* and *H.* cf. *andersenii* showed similar trends with respect to their cell uptake rate, which increased significantly (p <0.05) with growth medium nitrogen concentrations (Table 5.1). For lipid accumulation, the trend displayed by both microalgae was different. For *C. stigmatophora*, the lipid content significantly (p <0.05) decreased from LN (16.56% dw) to MN (11.51% dw) and HN (10.95% dw) treatments by as much as 34%, whereas for *H.* cf. *andersenii*, the lipid content significantly (p <0.05) increased up to 2.74 times with increasing nitrogen concentrations, from LN (6.49% dw) to MN (15.37% dw) and HN (13.06% dw). Phylogeny determines to a large extent the efficiency of various steps involved in the desired product accumulation [248]. Moreover, intracellular organizational differences across evolutionary-distinct classes of microalgae may affect processes, such as photosynthesis, carbon flux through metabolic networks, and accumulation [248]. Thus, the highest C/N ratio (32.61) and lipid content (16.56% dw) observed at LN conditions suggests that *C. stigmatophora* responded to nitrogen-limiting conditions by increasing its intracellular composition in carbon-rich pools like lipids. In contrast, *H.* cf. *andersenii* seemed to have displaced its efforts towards nitrogen storage, evidenced by the lowest C/N ratio (3.00) and lipid content (6.49%) obtained at LN conditions.

3.2. Lipid variability in response to nitrogen supplementation

Knowing that different lipid classes accumulate under specific growth stages, the early stationary phase was selected for the harvest and analysis of microalgal biomass, as it is often considered the best compromise for the simultaneous exploitation of PUFA and other high-value products, such as proteins and pigments [105]. Detailed lipidomic analysis (Fig. 5.2) revealed that *C. stigmatophora* and *H.* cf. *andersenii* exhibited different strategies of lipid metabolism in response to nitrogen supplementation. The lipid profiles of both microalgae revealed the presence of 66 metabolites, including fatty acids, aliphatic alcohols, sterols, monoglycerides, and other compounds (Tables 5.2 and 5.3). Significant differences (p <0.05) were found for the metabolites analysed across treatments. The analysis of *H.* cf. *andersenii* and *C. stigmatophora* samples before and after alkaline hydrolysis showed that *H.* cf. *andersenii* samples were more affected by alkaline hydrolysis than *C. stigmatophora* samples. Alkaline hydrolysis targets the ester bonds of lipids like acylglycerols, wax, and sterols esters [236]. Thus, the small changes verified for *C. stigmatophora* samples after alkaline hydrolysis might be justified by its lipid composition being richer in non-acyl lipids (like alkylglycerols) and lipid soluble compounds (such as carotenoids), which are stable to alkaline hydrolysis [236].

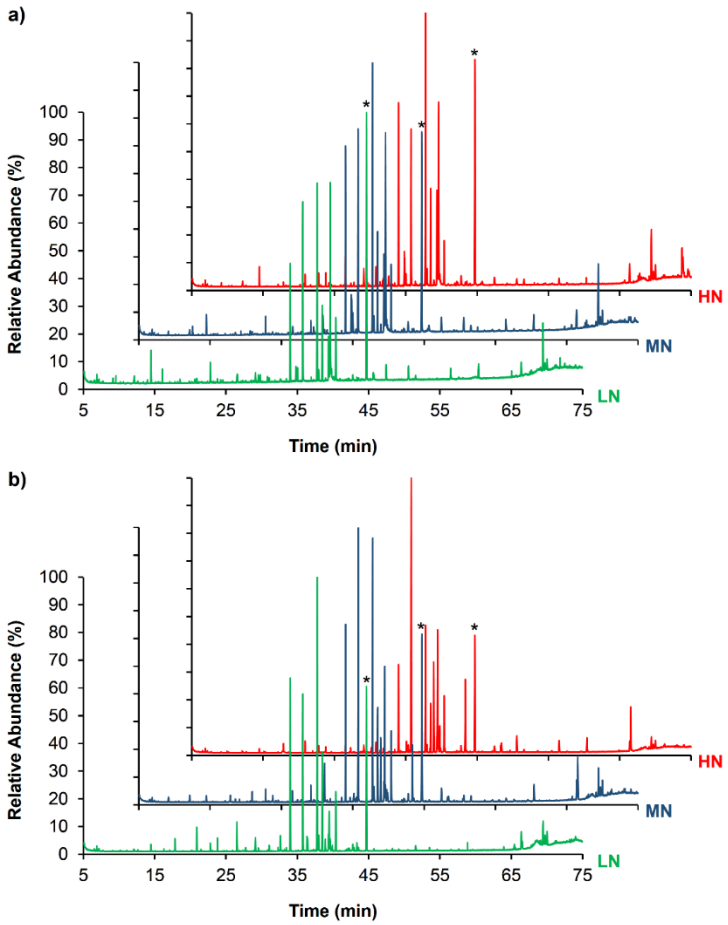

Figure 5.2. Chromatograms of the lipid extracts from a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii* grown in low (LN), medium (MN) and high (HN) nitrogen supply, before alkaline hydrolysis. * Internal standard (tetracosane 0.20 – 0.40 mg).

Table 5.2. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations before alkaline hydrolysis.

		Chlorella stigmatophora			***Hemiselmis* cf. *andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
	Fatty acids	*42.97±1.00^{a}*	*32.38±1.27^{b}*	*31.33±0.96^{b}*	*26.64±0.26^{A}*	*53.92±0.37^{B}*	*40.09±1.15^{C}*
	Saturated	*26.03±0.23a*	*17.75±0.73^{b}*	*17.19±0.43^{b}*	*17.49±0.12^{A}*	*27.68±0.43^{B}*	*20.43±0.64^{C}*
1	Nonanoic acid	1.53±0.01^{a}	1.03±0.01^{b}	1.02±0.01^{b}	1.04±0.00^{A}	1.55±0.00^{B}	1.03±0.01^{C}
3	Decanoic acid	1.53±0.01^{a}	1.03±0.01^{b}	1.01±0.00^{c}	1.04±0.00^{A}	1.55±0.02^{B}	1.02±0.01^{A}
6	Undecanoic acid	1.52±0.01^{a}	1.03±0.02^{b}	1.00±0.00^{c}	*n.d.*	*n.d.*	*n.d.*
8	Dodecanoic acid	1.53±0.01^{a}	1.03±0.01^{b}	1.01±0.00^{c}	1.03±0.00^{A}	1.54±0.01^{B}	1.02±0.01^{A}
11	Tetradecanoic acid	1.55±0.01^{a}	1.08±0.01^{b}	1.05±0.01^{c}	1.10±0.00^{A}	1.97±0.17^{B}	1.22±0.01^{A}
12	Pentadecanoic acid	1.54±0.01^{a}	1.05±0.01^{b}	1.02±0.01^{c}	1.06±0.01^{A}	1.59±0.01^{B}	1.06±0.02^{A}
20	Hexadecanoic acid	3.34±0.17^{a}	2.43±0.47^{b}	2.31±0.25^{b}	2.69±0.07^{A}	5.67±0.19^{B}	5.44±0.34^{B}
22	Heptadecanoic acid	1.71±0.04^{a}	1.18±0.05^{b}	1.16±0.00^{b}	1.28±0.01^{A}	1.80±0.09^{B}	1.23±0.05^{A}
29	Octadecanoic acid	2.36±0.12^{a}	1.51±0.21^{b}	1.44±0.10^{b}	1.82±0.02^{A}	2.45±0.01^{B}	2.02±0.09^{C}
36	Eicosanoic acid	1.64±0.07^{a}	1.11±0.06^{b}	1.04±0.03^{b}	1.06±0.00^{A}	1.57±0.01^{B}	1.05±0.01^{A}
43	Docosanoic acid	1.59±0.01^{a}	1.08±0.01^{b}	1.06±0.01^{c}	1.12±0.01^{A}	1.64±0.04^{B}	1.10±0.03^{A}
45	Tricosanoic acid	1.54±0.00^{a}	1.04±0.00^{b}	1.02±0.01^{c}	1.05±0.01^{A}	1.58±0.02^{B}	1.04±0.01^{A}
49	Tetracosanoic acid	1.54±0.00^{a}	1.05±0.00^{b}	1.02±0.01^{c}	1.05±0.00^{A}	1.57±0.00^{B}	1.05±0.02^{A}
56	Octacosanoic acid	1.54±0.01^{a}	1.04±0.00^{b}	1.01±0.01^{c}	1.07±0.01^{A}	1.59±0.01^{B}	1.07±0.01^{A}
60	Triacontanoic acid	1.55±0.01^{a}	1.04±0.01^{b}	1.02±0.01^{b}	1.07±0.01^{A}	1.61±0.02^{B}	1.08±0.01^{A}
	Monounsaturated	*8.33±0.63^{a}*	*7.52±0.49^{ab}*	*7.20±0.37^{b}*	*4.37±0.03^{A}*	*11.40±0.03^{B}*	*8.06±0.13^{C}*
17	7-Hexadecenoic acid	1.63±0.02^{a}	1.21±0.02^{b}	1.11±0.02^{c}	1.05±0.00^{A}	3.17±0.03^{B}	2.11±0.02^{C}
18	9-Hexadecenoic acid	n.d.	1.03±0.01^{a}	1.01±0.01^{b}	1.03±0.00^{A}	1.58±0.01^{B}	1.12±0.02^{C}
27	9-Octadecenoic acid	3.40±0.54^{a}	3.04±0.46^{a}	2.89±0.28^{a}	1.20±0.01^{A}	1.77±0.04^{B}	1.21±0.03^{A}
28	11-Octadecenoic acid	1.75±0.10^{a}	1.19±0.05^{b}	1.18±0.05^{b}	1.09±0.02^{A}	1.78±0.02^{B}	1.50±0.04^{C}
30	10-Nonadecenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.56±0.01^{A}	1.07±0.02^{B}
32	12-Nonadecenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.56±0.01^{A}	1.05±0.01^{B}
35	11-Eicosenoic acid	1.55±0.01^{a}	1.04±0.01^{b}	1.01±0.01^{c}	*n.d.*	*n.d.*	*n.d.*

Table 5.2. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations before alkaline hydrolysis. (Continuation)

		Chlorella stigmatophora			***Hemiselmis* cf. *andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
	Polyunsaturated	*8.61±0.24^{a}*	*7.12±0.20^{b}*	*6.94±0.17^{b}*	*4.78±0.16^{A}*	*14.84±0.11^{B}*	*11.59±0.39^{C}*
14	C16:2ω6	1.64±0.02^{a}	1.35±0.03^{b}	1.26±0.02^{c}	*n.d.*	*n.d.*	*n.d.*
15	C16:3ω3	1.62±0.01^{a}	1.29±0.01^{b}	1.20±0.02^{c}	*n.d.*	*n.d.*	*n.d.*
16	7,12-Hexadienoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.60±0.02^{A}	1.17±0.02^{B}
19	Methyl-4,7,10,13-hexadecatetraenoate	*n.d.*	*n.d.*	*n.d.*	1.04±0.01^{A}	1.59±0.01^{B}	1.09±0.01^{C}
25	9,12-Octadecadienoic acid	1.95±0.10^{a}	1.82±0.12^{a}	1.86±0.08^{a}	1.14±0.09^{A}	1.89±0.05^{B}	1.28±0.03^{C}
26	9,12,15-Octadecatrienoic acid	1.85±0.14^{a}	1.60±0.06^{b}	1.59±0.03^{b}	1.45±0.08^{A}	3.83±0.05^{B}	3.16±0.16^{C}
33	5,8,11,14,17-Eicosapentaenoic acid	1.55±0.01^{a}	1.06±0.00^{b}	1.03±0.01^{c}	1.14±0.00^{A}	2.65±0.02^{B}	2.51±0.13^{B}
38	4,7,10,13,16,19-Docosahexaenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.62±0.01^{A}	1.16±0.02^{B}
39	7,10,13,16,19-Docosapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.64±0.01^{A}	1.24±0.03^{B}
	Alcohols	*23.84±1.00^{a}*	*17.79±1.21^{b}*	*17.59±0.30^{b}*	*19.91±0.20^{A}*	*27.07±2.53^{B}*	*19.74±1.21^{A}*
2	Decanol	1.24±0.01^{a}	0.85±0.02^{b}	0.82±0.01^{b}	0.95±0.01^{A}	1.34±0.02^{B}	0.96±0.04^{A}
4	Undecanol	1.27±0.01^{a}	0.88±0.04^{b}	0.86±0.02^{b}	1.04±0.02^{A}	1.36±0.02^{B}	0.99±0.03^{C}
7	Dodecanol	1.26±0.01^{a}	0.88±0.04^{b}	0.86±0.04^{b}	0.95±0.02^{A}	1.32±0.02^{B}	0.92±0.02^{A}
9	Tridecanol	1.31±0.01^{a}	0.95±0.04^{b}	0.95±0.06^{b}	1.07±0.06^{A}	1.34±0.05^{B}	0.94±0.02^{C}
10	Tetradecanol	1.35±0.02^{a}	0.92±0.02^{b}	0.92±0.00^{b}	0.99±0.02^{A}	1.46±0.05^{B}	0.98±0.04^{A}
13	Hexadecanol	2.55±0.28^{a}	1.94±0.39^{b}	1.98±0.08^{b}	2.68±0.12^{AB}	3.10±0.66^{A}	2.21±0.25^{B}
21	Octadece-9-nol	3.51±0.47^{a}	2.64±0.65a	2.68±0.09^{a}	3.70±0.09^{A}	4.22±1.09^{A}	3.06±0.40^{A}
23	Octadecanol	2.12±0.18^{a}	1.51±0.23^{b}	1.51±0.04^{b}	1.88±0.05^{AB}	2.33±0.37^{A}	1.67±0.18^{B}
34	Eicosanol	1.29±0.01^{a}	0.87±0.01^{b}	0.86±0.00^{b}	0.90±0.01^{A}	1.32±0.02^{B}	0.88±0.01^{A}
40	Docosanol	1.25±0.00^{a}	0.84±0.00^{b}	0.82±0.01^{c}	0.85±0.00^{A}	1.28±0.01^{B}	0.86±0.01^{A}
50	Hexacosanol	n.d.	0.84±0.00^{a}	0.82±0.00^{b}	*n.d.*	*n.d.*	*n.d.*
52	Octacosanol	1.49±0.05^{a}	1.04±0.06^{b}	1.03±0.01^{b}	1.08±0.02^{A}	1.53±0.12^{B}	1.04±0.03^{A}
58	Octacosane-1,3-diol	1.31±0.01^{a}	0.89±0.01b	0.87±0.00^{b}	0.94±0.01^{A}	1.37±0.06^{B}	0.92±0.01^{A}
59	Triacontanol	1.38±0.03^{a}	0.93±0.02^{b}	0.92±0.00^{b}	1.00±0.02^{A}	1.43±0.08^{B}	0.97±0.03^{A}

Table 5.2. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations before alkaline hydrolysis. (Continuation)

		Chlorella stigmatophora			***Hemiselmis* cf. *andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
61	Dotriacontanol	1.27±0.01^{a}	0.85±0.00^{b}	0.83±0.01^{c}	0.88±0.01^{A}	1.29±0.01^{B}	0.86±0.02^{A}
24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.26±0.01^{a}	0.96±0.12^{b}	0.86±0.01^{b}	0.98±0.01^{A}	2.39±0.03^{B}	2.49±0.18^{A}
	Sterols	*2.15±0.20^{a}*	*1.69±0.28^{b}*	*1.56±0.04^{b}*	*1.40±0.09^{A}*	*2.95±0.39^{B}*	*2.65±0.15*
53	24*β*-Methylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.33±0.02^{A}	1.60±0.08^{B}	1.67±0.09^{B}
54	24-Methylcholesta-5,24(28)-dien-3*β*-ol	0.27±0.01^{a}	0.21±0.01^{b}	0.20±0.00^{b}	*n.d.*	*n.d.*	*n.d.*
55	24*α*-Methylcholest-5-en-3*β*-ol	0.31±0.02^{a}	0.26±0.03^{b}	0.25±0.01^{b}	0.20±0.01^{A}	0.28±0.03^{B}	0.20±0.01^{A}
57	24*α*-Ethylcholest-5-en-3*β*-ol	1.27±0.16^{a}	1.02±0.22^{ab}	0.89±0.03^{b}	0.62±0.06^{A}	0.73±0.25^{A}	0.53±0.05^{A}
64	Unidentified Sterol	0.28±0.03^{a}	0.20±0.02^{b}	0.21±0.01^{b}	0.25±0.01^{A}	0.34±0.04^{B}	0.26±0.02^{A}
	Monoglycerides	*0.79±0.08^{a}*	*0.68±0.07^{b}*	*0.32±0.01^{b}*	*0.14±0.04^{A}*	*0.66±0.04^{B}*	*1.58±0.26^{C}*
31	2,3-Dihydroxypropyl dodecanoate	0.06±0.01^{a}	0.06±0.01^{a}	0.05±0.00^{b}	0.06±0.02^{A}	0.07±0.03^{A}	0.09±0.06^{A}
41	1,3-Dihydroxy-2-propanyl palmitate	0.02±0.01^{a}	0.03±0.00^{b}	*n.d.*	*n.d.*	0.05±0.00^{A}	0.10±0.02^{B}
42	2,3-Dihydroxypropyl palmitate	0.35±0.04^{a}	0.28±0.03^{b}	0.11±0.01^{c}	0.05±0.02^{A}	0.26±0.01^{B}	0.67±0.09^{C}
44	3-Octadecoxypropane-1,2-diol	0.04±0.01^{a}	0.02±0.00^{b}	0.02±0.00^{b}	0.03±0.01^{A}	0.07±0.02^{B}	0.08±0.01^{B}
46	1,3-Dihydroxy-2-propanyl stearate	*n.d.*	0.02±0.00^{a}	0.01±0.00^{b}	*n.d.*	0.07±0.01^{A}	0.09±0.02^{B}
47	2,3-Dihydroxypropyl 9-octadecenoate	*n.d.*	0.02±0.00^{a}	0.03±0.00^{b}	*n.d.*	*n.d.*	*n.d.*
48	2,3-Dihydroxypropyl stearate	0.32±0.03^{a}	0.24±0.04^{b}	0.10±0.01^{c}	*n.d.*	0.15±0.01^{A}	0.55±0.08^{B}
	Others	*0.37±0.04^{a}*	*0.57±0.09^{b}*	*1.51±0.09^{c}*	*0.08±0.05^{A}*	*0.27±0.01^{B}*	*0.18±0.06^{B}*
5	2,6-bis(1,1-Dimethylethyl)phenol	0.34±0.04^{a}	0.19±0.05^{b}	0.17±0.04^{b}	0.08±0.05^{A}	0.27±0.01^{B}	0.18±0.06^{B}
51	*α*-Tocopherol	0.03±0.01^{a}	0.08±0.02^{b}	0.06±0.01^{b}	*n.d.*	*n.d.*	*n.d.*
62	C28:2 Steryl glycoside	*n.d.*	0.10±0.01^{a}	0.55±0.01^{b}	*n.d.*	*n.d.*	*n.d.*
63	C28:3 Steryl glycoside	*n.d.*	0.11±0.01^{a}	0.43±0.04^{b}	*n.d.*	*n.d.*	*n.d.*
65	C28:2 Steryl glycoside	*n.d.*	0.08±0.02^{a}	0.17±0.01^{b}	*n.d.*	*n.d.*	*n.d.*
66	C28:3 Steryl glycoside	*n.d.*	n.d.	0.13±0.02	*n.d.*	*n.d.*	*n.d.*
	Total	*70.12±1.78^{a}*	*53.11±2.86^{b}*	*52.31±0.76^{b}*	*48.17±0.42^{A}*	*84.87±3.25^{B}*	*64.24±2.67^{C}*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for *H.* cf. *andersenii*. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains *cis* and *trans* isomers. *n.d.* - non detected.

Table 5.3. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations after alkaline hydrolysis.

		Chlorella stigmatophora			***Hemiselmis cf. andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
	Fatty acids	*48.01±2.09^{a}*	*42.40±3.61^{b}*	*40.05±1.23^{b}*	*25.87±2.20^{A}*	*85.84±4.40^{B}*	*68.66±4.67^{C}*
	Saturated	*22.31±0.54^{a}*	*14.59±0.82^{b}*	*13.95±0.54^{b}*	*15.35±1.22^{A}*	*41.19±3.27^{B}*	*32.66±1.75^{C}*
1	Nonanoic acid	2.01±0.03^{a}	1.26±0.02^{b}	1.19±0.07^{b}	1.03±0.00^{A}	2.02±0.04^{B}	1.53±0.02^{C}
3	Decanoic acid	2.01±0.03^{a}	*n.d.*	1.19±0.08^{b}	1.01±0.00^{A}	2.02±0.04^{B}	1.52±0.00^{C}
6	Undecanoic acid	2.02±0.02	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
8	Dodecanoic acid	2.04±0.01^{a}	1.27±0.03^{b}	1.21±0.07^{b}	1.01±0.00^{A}	2.06±0.04^{B}	1.54±0.01^{C}
11	Tetradecanoic acid	2.08±0.05^{a}	1.33±0.04^{b}	1.29±0.05^{b}	1.33±0.01^{A}	5.25±0.12^{B}	2.89±0.39^{C}
12	Pentadecanoic acid	2.02±0.03^{a}	1.28±0.03^{b}	1.22±0.08^{b}	1.05±0.01^{A}	2.21±0.05^{B}	1.63±0.03^{C}
20	Hexadecanoic acid	5.37±0.27^{a}	4.95±0.55a	4.82±0.10^{a}	5.16±0.08^{A}	14.41±0.90^{B}	14.05±2.80^{B}
22	Heptadecanoic acid	2.12±0.05^{a}	1.30±0.02^{b}	1.26±0.06^{b}	1.15±0.02^{A}	2.27±0.07^{B}	1.81±0.10^{C}
29	Octadecanoic acid	2.64±0.08^{a}	1.93±0.11^{b}	1.76±0.04^{c}	2.06±0.11^{A}	3.75±0.30^{AB}	3.76±0.01^{B}
36	Eicosanoic acid	n.d.	n.d.	n.d.	1.02±0.01^{A}	2.04±0.04^{B}	1.58±0.04^{B}
43	Docosanoic acid	n.d.	1.25±0.02	n.d.	1.05±0.01^{A}	2.08±0.06^{B}	1.58±0.02^{B}
45	Tricosanoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	n.d.	1.51±0.01
49	Tetracosanoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.04±0.05^{A}	1.52±0.01^{A}
60	Triacontanoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.05±0.03	n.d.
	Monounsaturated	*15.65±1.12^{ab}*	*15.93±1.87^{a}*	*13.38±0.49^{b}*	*5.34±0.59^{A}*	*16.10±0.34^{B}*	*14.93±2.02^{B}*
17	7-Hexadecenoic acid	2.39±0.07^{a}	2.06±0.10^{b}	2.06±0.11^{b}	1.06±0.02^{A}	4.28±0.09^{B}	3.25±0.02^{C}
18	9-Hexadecenoic acid	2.02±0.04^{a}	1.31±0.03^{b}	1.26±0.08^{b}	1.04±0.00^{A}	2.22±0.04^{B}	1.77±0.01^{C}
27	9-Octadecenoic acid	9.09±0.99^{a}	9.82±1.68^{a}	8.60±0.25^{a}	1.29±0.04^{A}	2.62±0.07^{B}	2.82±0.96^{B}

Table 5.3. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations after alkaline hydrolysis. (Continuation)

		Chlorella stigmatophora			***Hemiselmis cf. andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
28	11-Octadecenoic acid	2.16±0.04^{a}	1.48±0.04^{b}	1.46±0.06^{b}	1.19±0.03^{A}	2.88±0.08^{B}	3.91±1.05^{B}
30	10-Nonadecenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.06±0.03^{A}	1.61±0.01^{B}
32	12-Nonadecenoic acid	*n.d.*	*n.d.*	*n.d.*	1.01±0.00^{A}	2.04±0.03^{B}	1.57±0.01^{B}
35	11-Eicosenoic acid	*n.d.*	1.26±0.02	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	Polyunsaturated	*10.04±0.43^{a}*	*11.89±0.94^{b}*	*12.72±0.21b*	*5.18±0.39^{A}*	*28.54±0.88^{B}*	*21.07±0.95^{C}*
14	C16:2ω6	2.34±0.09^{a}	2.35±0.15^{b}	2.75±0.11^{b}	*n.d.*	*n.d.*	*n.d.*
15	C16:3ω3	2.38±0.07^{a}	2.15±0.10^{b}	2.28±0.09^{ab}	*n.d.*	*n.d.*	*n.d.*
16	7,12-Hexadienoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.26±0.05^{A}	1.82±0.04^{B}
19	Methyl-4,7,10,13-hexadecatetraenoate	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.03±0.04^{A}	1.52±0.01^{B}
25	9,12-Octadecadienoic acid	2.82±0.17^{a}	3.53±0.43^{b}	3.97±0.11^{b}	1.28±0.02^{A}	3.26±0.03^{B}	2.74±0.61^{B}
26	9,12,15-Octadecatrienoic acid	2.50±0.11^{a}	2.56±0.23a	2.53±0.04^{a}	2.58±0.23^{A}	9.94±0.46^{B}	6.16±0.13^{C}
33	5,8,11,14,17-Eicosapentaenoic acid	*n.d.*	1.29±0.04^{a}	1.20±0.08^{a}	1.31±0.14^{A}	6.05±0.21^{B}	4.80±0.14^{C}
38	4,7,10,13,16,19-Docosahexaenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.44±0.07^{A}	1.93±0.06^{B}
39	7,10,13,16,19-Docosapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	*n.d.*	2.56±0.07^{A}	2.10±0.05^{B}
	Alcohols	*25.49±0.79^{a}*	*15.25±0.15^{b}*	*13.10±0.31^{c}*	*15.33±0.46^{A}*	*31.95±1.65^{B}*	*27.38±0.45^{C}*
2	Decanol	*n.d.*	1.02±0.01	*n.d.*	*n.d.*	1.63±0.03^{A}	1.23±0.01^{B}
4	Undecanol	1.62±0.02^{a}	1.03±0.00^{b}	*n.d.*	0.82±0.00^{A}	1.63±0.03^{B}	1.26±0.04^{C}
7	Dodecanol	1.63±0.02^{a}	1.02±0.01^{b}	*n.d.*	0.83±0.00^{A}	*n.d.*	1.24±0.01^{B}
9	Tridecanol	1.68±0.01^{a}	1.03±0.00^{b}	0.98±0.06^{b}	0.84±0.00^{A}	1.64±0.02^{B}	1.28±0.05^{C}
10	Tetradecanol	1.64±0.02^{a}	1.03±0.01^{b}	0.97±0.06^{b}	0.84±0.00^{A}	1.65±0.02^{B}	1.25±0.01^{C}
13	Hexadecanol	2.17±0.16^{a}	1.35±0.02^{b}	1.31±0.02^{b}	1.65±0.02^{A}	2.76±0.20^{B}	2.37±0.10^{C}
21	Octadece-9-nol	2.83±0.23^{a}	1.67±0.04^{b}	1.70±0.09^{b}	2.28±0.01^{A}	4.02±0.50^{B}	3.54±0.16^{B}
23	Octadecanol	2.05±0.13^{a}	1.27±0.03^{b}	1.26±0.02^{b}	1.50±0.01^{A}	2.55±0.20^{B}	2.06±0.03^{C}
34	Eicosanol	1.63±0.02^{a}	1.02±0.01^{b}	0.97±0.06^{b}	0.85±0.02^{A}	1.65±0.03^{B}	1.24±0.01^{B}
40	Docosanol	1.62±0.02^{a}	*n.d.*	*n.d.*	0.83±0.00^{A}	1.64±0.02^{B}	1.23±0.00^{C}

Table 5.3. Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations after alkaline hydrolysis. (Continuation)

		Chlorella stigmatophora			***Hemiselmis cf. andersenii***		
Nº	**Identified Compounds**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
50	Hexacosanol	*n.d.*	*n.d.*	0.96±0.06	*n.d.*	*n.d.*	*n.d.*
52	Octacosanol	1.73±0.05^{a}	1.08±0.02^{b}	1.05±0.04^{b}	0.99±0.01^{A}	1.82±0.09^{B}	1.40±0.03^{C}
58	Octacosane-1,3-diol	1.64±0.03^{a}	1.02±0.01^{b}	0.98±0.05^{b}	0.86±0.01^{A}	1.69±0.04^{B}	1.26±0.01^{C}
59	Triacontanol	1.68±0.04^{a}	1.04±0.02^{b}	1.00±0.05^{b}	0.94±0.01^{A}	1.76±0.06^{B}	1.33±0.02^{C}
61	Dotriacontanol	1.63±0.02^{a}	*n.d.*	*n.d.*	0.83±0.01^{A}	1.66±0.03^{B}	1.24±0.02^{C}
24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.92±0.04^{a}	1.67±0.04^{b}	1.91±0.01^{a}	1.48±0.15^{A}	5.86±0.38^{B}	5.46±0.02^{B}
	Sterols	*1.10±0.07^{a}*	*1.19±0.08^{a}*	*1.07±0.08^{a}*	*1.18±0.04^{A}*	*2.54±0.21^{B}*	*2.71±0.24^{B}*
53	24*β*-Methylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.32±0.01^{A}	1.40±0.10^{B}	1.50±0.06^{B}
54	24-Methylcholesta-5,24(28)-dien-3*β*-ol	*n.d.*	0.19±0.00a	0.19±0.01^{a}	*n.d.*	*n.d.*	*n.d.*
55	24α-Methylcholest-5-en-3β-ol	0.30±0.01^{a}	0.21±0.01^{b}	0.23±0.00^{b}	0.18±0.01^{A}	0.33±0.01^{B}	0.26±0.02^{C}
57	24*α*-Ethylcholest-5-en-3*β*-ol	0.80±0.06^{a}	0.63±0.06^{b}	0.66±0.09^{ab}	0.50±0.02^{A}	0.81±0.11^{B}	0.77±0.10^{B}
64	Unidentified Sterol	*n.d.*	0.16±0.00^{b}	*n.d.*	0.18±0.01^{A}	0.30±0.01^{A}	0.23±0.01^{A}
	Monoglycerides	*0.10±0.01^{a}*	*0.05±0.01^{b}*	*0.06±0.01^{b}*	*0.18±0.01^{A}*	*0.62±0.11^{B}*	*0.67±0.02B*
31	2,3-Dihydroxypropyl dodecanoate	0.02±0.00^{a}	0.01±0.00^{b}	*n.d.*	0.05±0.00^{A}	0.06±0.02^{A}	0.05±0.01^{A}
37	3-Hexadecoxypropane-1,2-diol	*n.d.*	*n.d.*	*n.d.*	0.03±0.01^{A}	0.26±0.05^{B}	0.25±0.01^{B}
41	1,3-Dihydroxy-2-propanyl palmitate	*n.d.*	*n.d.*	0.01±0.00	*n.d.*	*n.d.*	*n.d.*
42	2,3-Dihydroxypropyl palmitate	0.01±0.00	*n.d.*	*n.d.*	0.04±0.00^{A}	*n.d.*	0.05±0.01^{A}
44	3-Octadecoxypropane-1,2-diol	0.01±0.00^{a}	0.01±0.00^{b}	*n.d.*	0.06±0.00^{A}	0.30±0.05^{B}	0.33±0.01^{B}
46	1,3-Dihydroxy-2-propanyl stearate	0.03±0.00^{a}	n.d.	*n.d.*	*n.d.*	*n.d.*	*n.d.*
47	2,3-Dihydroxypropyl oleate	0.02±0.00^{a}	0.03±0.01^{a}	0.03±0.01^{a}	*n.d.*	*n.d.*	*n.d.*
48	2,3-Dihydroxypropyl stearate	*n.d.*	tr^{a}	0.01±0.00^{b}	*n.d.*	*n.d.*	*n.d.*
	Others	*n.d.*	*0.04±0.00^{a}*	*0.03±0.00^{a}*	*0.09±0.00^{A}*	*0.15±0.15^{AB}*	*0.36±0.11^{B}*
5	2,6-bis(1,1-Dimethylethyl)phenol	*n.d.*	0.03±0.00^{a}	0.02±0.00^{b}	0.09±0.00^{A}	0.15±0.15^{AB}	0.36±0.11^{B}
51	*α*-Tocopherol	*n.d.*	0.01±0.00^{a}	0.01±0.00^{a}	*n.d.*	*n.d.*	*n.d.*
	Total	*74.69±2.92^{a}*	*58.94±3.83^{b}*	*54.30±1.46^{b}*	*42.65±2.32^{A}*	*121.40±6.39^{B}*	*99.78±5.43^{C}*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for *H.* cf. *andersenii*. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains *cis* and *trans* isomers. *n.d.* - non detected.

Through Figure 5.3, it is possible to observe that the relative abundance of MUFA and PUFA significantly (p <0.05) increased from LN to MN and HN treatments. This increase seems to have been achieved at the expense of SFA, which significantly (p <0.05) decreased from LN to MN and HN treatments, in both microalgae. These results indicate that different nitrogen supplementations lead to membrane remodelling to respond to nitrogen-induced changes in both species studied. The degree of unsaturation of biological membranes affects their flexibility and functionality [184]. Moreover, it is known that membrane fluidity and permeability increase with the unsaturation degree of biological membranes [59]. Comparing these results with the nitrogen uptake, it is possible to observe that microalgal cell uptake in both species increased with the unsaturation degree of fatty acid, corroborating the previous assumption.

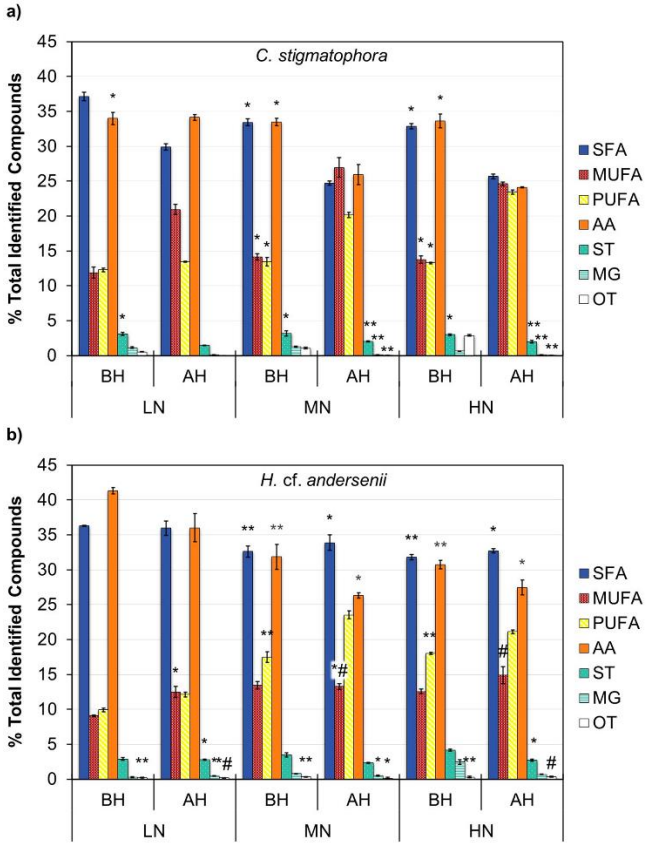

Figure 5.3. Changes in main lipophilic classes before (BH) and after (AH) alkaline hydrolysis in a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii* grown under low (LN), medium (MN) and high (HN) nitrogen experimental conditions. Values are expressed as mean ± standard deviation, n=4 replicates. Values from the same lipid class sharing common symbols (* before hydrolysis, and ** or # after hydrolysis) are not significantly different (p >0.05) among treatments. SFA - Saturated fatty acids; MUFA - Monounsaturated fatty acids; PUFA - Polyunsaturated fatty acids; AA - Aliphatic alcohols; ST - Sterols; MG - Monoglycerides; OT - Other compounds.

C. stigmatophora responded to increasing nitrogen supplementation by decreasing fatty acid levels (Tables 5.2 and 5.3), namely C16:0 and octadecanoic (C18:0) acids, which are products of *de novo* fatty acid synthesis in the chloroplast and comprise the substrates needed

for MUFA, and PUFA biosynthesis [59]. Meanwhile, in *H.* cf. *andersenii*, significant shifts in fatty acid composition caused by increased nitrogen enrichment became visible in higher amounts of hexadecanoic (C16:0), 9-hexadecenoic (C16:1ω7), 9,12,15-octadecatrienoic acid (C18:3ω3; ALA), 5,8,11,14,17-eicosapentaenoic acid (C20:5ω3; EPA), and 4,7,10,13,16,19-docosahexaenoic acid (C22:6ω3; DHA).

Fatty acids were the most affected by alkaline hydrolysis, increasing up to 31 and 71% in *C. stigmatophora* and *H.* cf. *andersenii*, respectively. In *C. stigmatophora* the most affected individual fatty acids by hydrolysis were C16:0, 9-octadecenoic acid (C18:1ω9), C16:2ω6, and 9,12-octadecadienoic (LA; C18:2ω6) acids, with the biggest increase being verified for C18:1ω9, representing a fourfold increase with alkaline hydrolysis. C16:0 and C18:2 fatty acids have been reported in literature as abundant molecular species on the glycolipid's composition of *Chlorella* sp. [125]. Moreover, for other microalgae belonging to Chlorophyta division, like *Chlamydomonas reinhardtii* and *Chlamydomonas nivalis*, C18:1 fatty acids were reported to be major components of glycolipids [125]. Thus, the increase in the mentioned fatty acids after alkaline hydrolysis for *C. stigmatophora* may be because they are associated with glycolipids.

As with *C. stigmatophora*, *H.* cf. *andersenii* presented noticeable changes in fatty acid levels after hydrolysis, namely a great increase in tetradecanoic (C14:0), C16:0, ALA, and EPA contents. These variations were more pronounced with nitrogen enrichment, with C14:0 fatty acid presenting a threefold increase. According to Guschina and Harwood [249], the plastidial glycosylglycerolipids of marine algae may contain very-long chain PUFA like EPA, in addition to ALA and C16:0. As main components of photosynthetic membranes, microalgae naturally possess high amounts of glycolipids when grown in nutrient-repleted conditions [145]. Thus, in both microalgae, the similar behavior of chlorophylls, along with the mentioned fatty acids increase after alkaline hydrolysis with nitrogen enrichment, suggests that these might be structural components of glycolipids.

A higher relative abundance of aliphatic alcohols was found for *H.* cf. *andersenii* cultured under LN conditions (up to 41% of total identified compounds). Most aliphatic alcohols occur in marine organisms as wax esters, which serve several purposes in organisms, such as energy storage and buoyancy generation [250]. Moreover, these compounds have been reported to exist in Cryptophyta, namely in *Chroomonas salina* [251]. In *H.* cf. *andersenii* at MN and HN conditions, alcohols increased by as much as 39%, along with total fatty acids. These results indicate that, towards HN conditions, alcohols are mainly esterified with fatty acids (wax esters). In *C. stigmatophora* aliphatic alcohols presented a similar trend to lipids and fatty acids in response to nitrogen influence. However, after alkaline hydrolysis, a significant increase in total alcohols was not observed, indicating that these compounds are not in wax esters forms, in contrast to *H.* cf. *andersenii*.

3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol (phytol) was found in its lowest amount in *H.* cf. *andersenii* under LN conditions (0.98 mg g^{-1} dw). When comparing samples before and after alkaline hydrolysis, this compound was the most affected by alkaline hydrolysis, increasing up to 145%. According to Yao et al. [137], this aliphatic alcohol can be used as an indicator of

chlorophylls, enabling inspection of the algal cell response when exposed to low nitrogen supplementation [247]. However, in *C. stigmatophora*, the phytol content before and after alkaline hydrolysis did not present the same behavior as chlorophylls. In addition to chlorophylls, phytol is a component of other biological molecules, namely vitamin K, vitamin E, and other tocopherols [252]. As *α*-tocopherol was detected in *C. stigmatophora* (Tables 5.2 and 5.3), this could be a reasonable explanation for the different behavior shown by *C. stigmatophora*. Therefore, these results indicate that phytol is a good indicator of chlorophyll content for *H.* cf. *andersenii* but not for *C. stigmatophora*.

Monoglyceride content displayed a similar trend to lipid content with nitrogen supplementation, showing, once more, that both microalgae differently modified their lipid quantity and quality in response to nitrogen. That is, *H.* cf. *andersenii* monoglyceride content displayed a positive trend with nitrogen supplementation, in contrast to *C. stigmatophora*, which responded to increasing nitrogen supplementation by decreasing monoglyceride levels. According to Zienkiewicz et al. [253], two major pathways of triacylglycerol synthesis have been proposed to function in microalgae: *i)* Kennedy or glycerol phosphate pathway, and *ii)* monoacylglycerol pathway. The observed similar trend of lipids with monoacylglycerides upon treatments suggests that these molecules are important intermediates in anabolic fatty acid ester pathways in the microalgae studied. Thus, at LN conditions *C. stigmatophora* seemed to store carbon as triacylglycerols, in opposition to *H.* cf. *andersenii*.

Glycosyl sterols were only found for *C. stigmatophora* before hydrolysis under MN and HN conditions. These biomolecules have unique amphiphilic properties that make them suitable to be applied in food and pharmaceutical fields [154,241]. Moreover, glycosyl sterols have been reported to exist for other microalgae species belonging to Chlorophyta division, namely *Tetraselmis chui*, *Scenedesmus coastatum,* and *Platymonas helgolandica* [154,254]. Thus, the presence of glycosyl sterols under MN and HN conditions revealed the potential of these conditions for food and pharmaceutical applications.

In *H.* cf. *andersenii*, the increases in 24*α*-Methylcholest-5-en-3*β*-ol and 24*α*-ethylcholest-5-en-3*β*-ol sterols by 30 and 45%, respectively, with alkaline hydrolysis at HN conditions, seem to indicate the presence of sterol esters in its lipid composition. Sterol esters have been reported to occur in other microalgae which, like *H.* cf. *andersenii*, belong to the kingdom Chromista, namely *Isochrysis* aff. *galbana* and *Nannochloropsis salina* [254,255]. Several assumptions have been made with respect to the physiological role of sterol conjugates, like: *i)* sterol conjugates are sterol storage pools which can be depleted when *de novo* synthesis of sterols is not sufficient, or to maintain the organization and fluidity of membranes; *ii)* the esterification of sterols and fatty acids is essential to avoid membrane perturbations from elevated sterols or free fatty acid levels resulting in a critical homeostatic response [254]. Considering this, the possibility of the production of sterol esters by *H.* cf. *andersenii*, at HN conditions, seems to be related with the first assumption, since the highest fatty acid and sterol contents before hydrolysis were found in MN conditions instead of in HN conditions.

3.3. Variations in microalgal nutritional value related to nitrogen supplementation

Figure 5.4 displays the changes in the fatty acid nutritional indexes in free and hydrolysable lipid fractions of the *C. stigmatophora* and *H.* cf. *andersenii* cultured in different nitrogen supplementations. Fatty acid ratios were calculated considering their functional effects. PUFA are one of the key functional ingredients synthesized by microalgae [184]. These essential nutrients play key roles as structural components of cell membranes and cytokine precursors [54,184]. Although these fatty acids have crucial roles in animals' growth and development, PUFA precursors (LA and ALA) can only be obtained through dietary intake, and further elongation and desaturation is not enzymatically efficient [54]. Moreover, as SFA are synthesized by animals, a high PUFA intake is recommended for the diet [184].

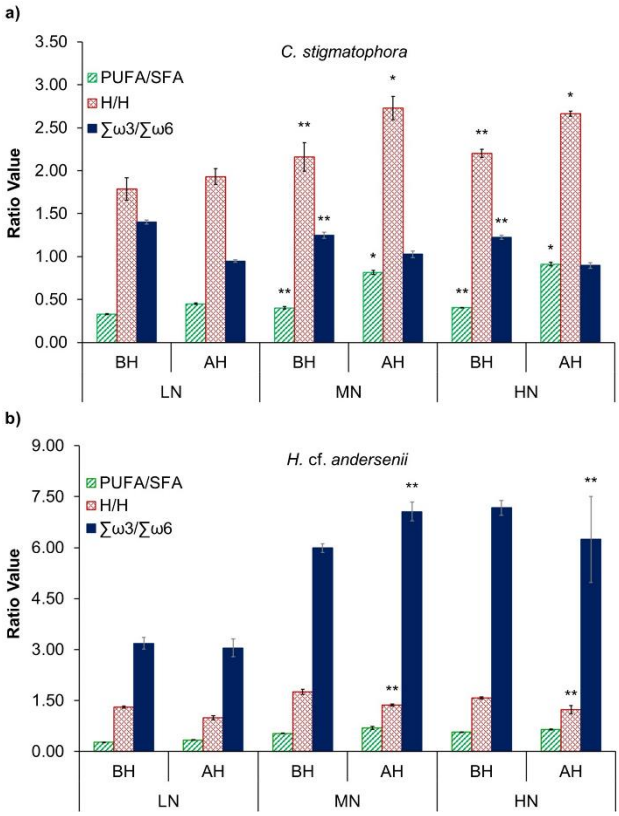

Figure 5.4. Variations in a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii* nutritional values in response to nitrogen-induced changes, n=4 replicates. Values from the same nutritional ratio sharing common symbols (* before hydrolysis, and ** after hydrolysis) are not significantly different (p >0.05) among treatments. H/H - hypocholesterolaemic/hypercholesterolaemic fatty acids ratio; PUFA/SFA - Polyunsaturated to saturated fatty acids ratio; LN - Low nitrogen supplementation; MN - Medium nitrogen supplementation; HN - High nitrogen supplementation; BH - Before hydrolysis; AH - After hydrolysis

When analysing the polyunsaturated to saturated fatty acid ratio (PUFA/SFA) against the different nitrogen supplementations (Fig. 5.4), it was possible to observe that both microalgae responded to increasing nitrogen levels by increasing their PUFA/SFA ratios by around double after hydrolysis. A PUFA/SFA ratio above 0.45 is recommended to induce a decrease in blood cholesterol level [207]. In *C. stigmatophora,* PUFA/SFA ratios over 0.45 were

only achieved in samples cultured in MN and HN conditions after cleavage of fatty acids ester bonds. Knowing that the bioavailability of fatty acids is important for its absorption by animals, and that fatty acids in its free form have increased absorption by humans [256], these results suggest that for the application of *C. stigmatophora* as a source of a healthy PUFA/SFA ratio, a pre-treatment (*e.g.* chemical or enzymatic hydrolysis) of *Chlorella* lipids has to be performed. In *H.* cf. *andersenii* cultured at MN and HN conditions, PUFA/SFA ratios were higher than 0.45, indicating that this microalgal strain is suitable for nutraceutical purposes, in contrast to *H.* cf. *andersenii* cultured in LN conditions.

Although for *C. stigmatophora* the ω3 to ω6 PUFA ratio (Σω3/Σω6) remained rather stable before and after alkaline hydrolysis, in *H.* cf. *andersenii* a positive trend between the Σω3/Σω6 ratio and nitrogen supplementation was observed. In this microalgal species, the Σω3/Σω6 ratio registered more than a twofold increase in response to increased nitrogen levels, suggesting that nitrogen supplementation affected the biosynthesis of PUFA by shifting enzyme specificity towards ω3 fatty acids synthesis instead of ω6 fatty acids [59]. The same trend was displayed for another Cryptophyta (*Rhodomonas marina*) in nutrient replete conditions [59]. The high values observed for ω3 fatty acids make *H.* cf. *andersenii* at high nitrogen supplementation a suitable microalgal strain for ω3 fatty acid supplementation. ω6 predominantly dominates Western diets, with its consumption being 20 times higher than that of ω3 fatty acids [236]. However, as main precursors of pro- and anti- inflammatory molecules (*e.g.* prostaglandins, and leukotrienes), a balanced Σω3/Σω6 ratio close to 1:1 is recommended for the mitigation of inflammatory and chronic diseases [236]. Thus, the cultivation of *H.* cf. *andersenii* at MN and HN conditions is ideal to provide a rich source of ω3 fatty acids for nutraceutical purposes.

When stating that a product is a source of ω3 PUFA, one of the following requirements must be fulfilled: *i)* the product should have 0.3 g of C18:3ω3 per 100 g of product; or *ii)* EPA + DHA must be equal to or higher than 40 mg per 100 g of product [19]. Without hydrolysis (Table 5.2), *H.* cf. *andersenii* samples under MN and HN conditions presented 383 and 316 mg of C18:3ω3 per 100 g of dried algal biomass; and 427 and 367mg of EPA+DHA per 100 g of dried algal biomass, respectively. After alkaline hydrolysis processing (Table 5.3), *H.* cf. *andersenii* presented 994 and 616 mg of C18:3ω3 per 100 g of dried algal biomass; and 849 and 673 mg of EPA+DHA per 100 g of dried algal biomass, at MN and HN conditions, respectively. These observations reinforce the previous assumption that *H.* cf. *andersenii* cultured at MN and HN condition is suitable for nutraceutical purposes. *C. stigmatophora* samples across treatments did not meet the above-mentioned criteria for C18:3ω3 or for EPA+DHA.

Another indicator of the nutritional quality of lipids in food is the hypocholesterolaemic / hypercholesterolaemic fatty acids ratio (H/H). This nutritional index is calculated based on the knowledge of functional effects of individual fatty acids in the cholesterol metabolism, and higher values are considered to be beneficial for human health [207]. H/H values observed for *C. stigmatophora* ranged from 1.79 to 2.73, with the main contributors go the higher H/H values being the high amounts of C18:1ω9, in combination with low levels of C16:0. The major change in H/H index found in *C. stigmatophora* was a significant (p <0.05) increase (41%) observed

among LN and MN conditions after hydrolysis. In the *H.* cf. *andersenii* samples, the H/H ratio varied between 1.00 and 1.76; in this microalgal species, the factor that contributed the most to the highest H/H index was the increase in unsaturated fatty acid content, namely C18:3ω3 and C20:5ω3. Moreover, the greatest increase of H/H value for *H.* cf. *andersenii* was between LN and MN conditions after hydrolysis (37%). Since the hypocholesterolemic fatty acids reduce the low-density lipoproteins (bad cholesterol) [257], samples with the highest H/H values (> 1) may be important as cholesterol-lowering agents. Moreover, the ability to improve this ratio by changing initial nitrogen concentrations comprises an advantage for the biotechnological exploitation of these microalgae.

Through the above-mentioned observations, it was possible to visualize that the way in which *C. stigmatophora* and *H.* cf. *andersenii* respond to nitrogen-induced changes was species-specific and affected their nutritional value. Moreover, the analysis after and before hydrolysis proved to be crucial, since it is a rapid and high throughput methodology that provides information about the desired product bioavailability (non-esterified or esterified), which influences not only the selection of microalgae but also the processing strategies to be used by the industry. For instance, non-esterified fatty acids in fish oils are more efficiently absorbed by humans than their esterified form (triacylglycerol, and ethyl esters) [256].

3.4. Multivariate analysis of lipid profile

Hierarchical cluster analysis (HCA) of microalgal lipid extracts before hydrolysis (Fig. 5.5a) did not reflect the classification of *H.* cf. *andersenii* cultured under different nitrogen supplementations. Instead, *H.* cf. *andersenii* grown in LN conditions (HLN) was farthest positioned, in a different clade closest to *C. stigmatophora* group. This discrimination was promoted by the greater abundance of MUFA, PUFA, C16:0, and phytol observed for *H.* cf. *andersenii* grown in MN (HMN) and HN (HHN) conditions. Moreover, the similarity of *H.* cf. *andersenii* cultured under LN conditions with *C. stigmatophora* samples was related with low values of very-long chain PUFA (EPA, DHA) and high amounts of SFA, which were common features of these samples.

After hydrolysis (Fig. 5.5b), it is noteworthy that *H.* cf. *andersenii* cultured under different nitrogen supplementations are within the same cluster, despite having a distinction among HLN and the other *H.* cf. *andersenii* samples (medium nitrogen – HMN; and high nitrogen – HHN). These observations show once more that *H.* cf. *andersenii* grown in HMN and HHN are chemically more similar than HLN. Conversely, *C. stigmatophora* grown in different nitrogen supplementations (low - CLN; medium - CMN; high - CHN) did not cluster together, with CLN presenting the most distinct metabolic features.

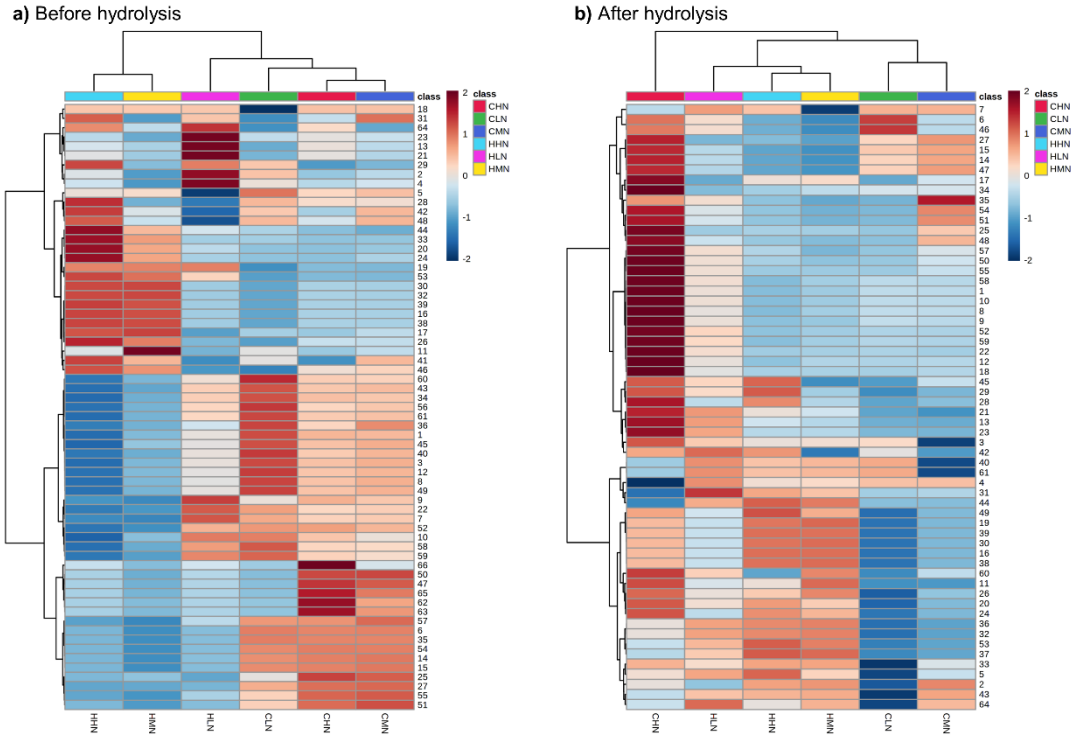

Figure 5.5. Hierarchical clustering analysis heat maps based on Euclidean clustering distance and the ward clustering method, n=4 replicates, for *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown under different nitrogen supplementations a) before and b) after alkaline hydrolysis. Feature descriptions can be seen in Tables 5.2 and 5.3. The heat maps reflect the relative levels of metabolites among different treatment groups, the color scheme is associated with the elevation and reduction in metabolite level through treatments: Dark blue, lowest; Dark red, highest. HLN, HMN, and HHN stands for *H.* cf. *andersenii* grown under low, medium, and high nitrogen supplementations, respectively. CLN, CMN, CHN stands for *C. stigmatophora* grown in low, medium, and high nitrogen supplementations, respectively.

4. Conclusions

Chlorella stigmatophora and *Hemiselmis* cf. *andersenii* growth patterns and nitrogen uptake dynamics suggests that nitrogen was not the growth limiting nutrient under high nitrogen supplementation. Gas chromatography - mass spectrometry untargeted analysis allowed the study of the lipid remodelling of both microalgae in response to nitrogen-induced changes. Microalgae grown under medium and high nitrogen supplementation showed potential for high-value compound exploitation. For instance, glycosyl sterols, suitable to be applied in food and pharmaceutical fields, were only found for *C. stigmatophora* at medium and high nitrogen supplementation, whilst the highest contents of ω3 and polyunsaturated fatty acids were found for *H.* cf. *andersenii* under these conditions. The analysis of chlorophyll and phytol contents show that phytol is a good indicator for the chlorophyll content of *H*. cf. *andersenii* but not for that of *C. stigmatophora*. The diverse ways by which both microalgae modulated their elemental stoichiometry and lipid content in response to the same perturbation highlighted the influence of

phylogeny on the carbon flux, through metabolic networks and accumulation. However, either the use of enzyme inhibitors or genetic manipulation would be necessary to demonstrate the specific changes in metabolism.

CHAPTER 6.

Pavlova pinguis and *Hemiselmis* cf. *andersenii* chemoplasticity as a response to phosphorus-induced changes

This Chapter is based on the following publication:

Fernandes, T.; Cordeiro N. Effects of phosphorus-induced changes on the growth, nitrogen uptake, and biochemical composition of *Pavlova pinguis* and *Hemiselmis* cf. *andersenii*. Under review on J. Appl. Phycol. Manuscript ID: JAPH-D-21-00565.

Chapter 6. *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* chemoplasticity as a response to phosphorus-induced changes

Abstract

The understanding of the phosphorus-induced changes in the biochemical composition of microalgae is of great importance to achieve efficiency in high-value lipids production. To study the chemoplasticity of *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* its growth, carotenoid, chlorophyll *a* content, along with their monosaccharides and lipids profiles were analyzed against several phosphorus (P) regimes: low (LP), medium (MP), and high (HP). For both microalgal cultures increasing initial P concentrations showed a positive effect on biomass productivities. Carbon-rich pools presented significant differences (p <0.05) for *P. pinguis* against P treatments, in contrast to *H.* cf. *andersenii*. Differential responses to P-induced changes in microalgae monosaccharide, and lipid profile were observed. *H.* cf. *andersenii* increased its proportion in galactose (up to 3 times) from LP to HP conditions, whereas *P. pinguis* decreased (up to 20%) its glucose proportion from LP to HP conditions. For *P. pinguis* the lowest amount (13.12 mg g^{-1} dw) of sterols was observed at LP conditions, in contrast to its carotenoid content (4.32 mg g^{-1} dw). P-replete conditions were the most effective in inducing high-value lipids accumulation. Lipid analysis before and after hydrolysis allowed to see which samples would need to be processed to fully exploit its high-value lipids, namely *H* cf *andersenii* at MP and HP conditions. This study demonstrated that P played an important role on carbon allocation and lipid regulation on *P. pinguis* and *H.* cf. *andersenii*, and that P-replete conditions could be useful for optimizing high-value lipids with potential for nutraceutical and pharmaceutical fields.

Keywords: Microalga, *Pavlova pinguis*, *Hemiselmis* cf. *andersenii*, phosphorus, monosaccharides, high-value lipids.

1. Introduction

Increasing concerns regarding consumer safety, environmental sustainability, and regulatory issues over synthetic materials, has turning consumers attention towards natural products [258]. Microalgae are often presented as promising sustainable cell factories for their ability to covert atmospheric carbon dioxide, water, inorganic nutrients, and sunlight to high-value compounds [259]. These organisms are primary producers of essential nutrients that perform vital functions in the human organism and whose dietary intake is mandatory [45]. Improvement of microalgae production for industrial scale often involve the application of several stress-inducement strategies from which nutrient stress is the most employed due to its low cost and easy applicability at both lab and large-scale cultivation [57].

Phosphorus (P) plays a significant role in algal growth and cell division due to its role as essential component of nucleic acids, phospholipids, and phosphorylated sugars [260]. Besides

algal growth, P is involved in metabolic processes like signal transduction, photosynthesis, and energy transfer (in the form of adenosine triphosphate – ATP, and nicotinamide adenine dinucleotide phosphate – $NADP^+$) [260,261]. Due to its fast recycling, P limitation has been observed in microalgae natural environments [262]. Microalgae P requirements are known to vary considerable between species ranging from 0.001 to 0.179 g L^{-1} [260]. This macronutrient can be up taken by microalgae in the form of orthophosphate (most commonly used; PO_4^{3-}) and polyphosphate [261,262]. Moreover, in P replete conditions algae can store excess P in polyphosphate bodies (luxury uptake) [263].

To cope with P limitation, microalgae modulate several metabolic pathways affecting its biomass composition [262]. From the main macromolecular pools, lipids and carbohydrates are especially affected by internal and external P supplies [263]. According to Markou et al. [264] carbohydrate accumulation due to P starvation is reported to occur in diatoms, green algae, and cyanobacteria. Nevertheless, a different response was reported to occur in the Haptophyta *Isochrysis galbana* in which P starvation stimulated lipid accumulation in up to 50% [261].

Phytosterols, polyunsaturated fatty acids (PUFA), and lipid soluble compounds, like carotenoids and tocopherols, are important for a variety of nutraceutical and pharmaceutical purposes, and its market is continuously growing [258]. These compounds are well-known for their health-benefits in humans including neuroprotective, anti-inflammatory, anti-cancer, and antioxidant activities as well as their action as chemoprotective agents in cardiovascular diseases [47,67,265]. Most studies evaluating the effects of P stress on microalgae have focused on the changes of specific classes of lipid families such as fatty acids, and sterols [106,107]. A few studies [266] have also been carried out concerning lipophilic characterization of microalgal species for its unsaponifiable and saponifiable composition, but only studying their response to nitrogen-induced changes.

Although its wide taxonomic diversity and metabolic differences, studies on the effect of P on microalgal lipid profile have been limited to few species most of them belonging to the Chlorophyta phylum [267]. In previous studies the Haptophyta *Pavlova pinguis* [122] and Cryptophyta *H.* cf. *andersenii* [236,266] have demonstrated to be promising candidates for high-value lipids production. However, the mechanisms that led to carbon allocation and lipid remodeling in response to environmental-induced perturbations are poorly explored for these two microalgae. Therefore, to increase knowledge on the chemoplasticity (ability of microalgae to change its chemical composition in response to extrinsic stimuli by reorganizing its metabolome) of Haptophyta *P. pinguis* and Cryptophyta *H.* cf. *andersenii* its carotenoid, chlorophyll a content, as well as their monosaccharides and lipids profiles were analyzed against several phosphorus regimes.

2. Material and methods

2.1. Growth and culture conditions

The marine Haptophyta *Pavlova pinguis* (RCC 1539) was obtained from the Roscoff Culture Collection (RCC), whereas the marine Cryptophyta *Hemiselmis* cf. *andersenii* (BEA

0118B) was obtained from the Spanish Bank of Algae (BEA). The microalgal cultures were started with a 1:10 inoculum from exponentially growing cultures into 1 L of media as in Sun et al. [268] , with initial cell concentrations of 0.54×10^6 cells mL^{-1} for *H.* cf. *andersenii*, and 1.29×10^6 cells mL^{-1} of *P. pinguis*. Microalgal cell cultures were grown on 1L of sterile medium with pH adjusted to 7.0 under 70 µmol m^{-2} s^{-1}, light intensity with 16:8 h (light: dark cycles) at 25°C as in Fernandes et al. [122]. For the phosphorus-induced changes f/2-Si medium [201,202] was used as the basal formula. The different phosphorus regimes were performed by adjusting sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) concentrations to 4 µM (low phosphorus; LP), 36 µM (medium phosphorus; MP), and 72 µM (high phosphorus; HP). The LP concentration was chosen to trigger a reduced phosphorus supply close to phosphorus depletion without compromising biomass production. The HP level was chosen to study the effects of excess phosphorus in a tolerable supplementation in microalgae. At the end of the early stationary phase, microalgae were harvested by centrifugation for 7 min at 3720 *x g* and the pellets washed twice with a 0.09 g L^{-1} NaCl solution. Microalgal growth was monitored daily by measuring the optical density of the microalgal culture at 550 nm (OD550) with an ultraviolet/ visible spectrophotometer (UV-6300PC, VWR, China). A calibration curve (R^2 = 0.9988, *P. pinguis*; R^2= 0.9929, *H.* cf. *andersenii*) plotting cell concentration (cells mL^{-1}) against absorbance was used to determine cell growth. The cell concentration was estimated by counting cells with a 0.1 mm deep improved Neubauer haematocytometer (Marienfield Superior) in a light microscope (Olympus BX41). The logistic model of Xin et al. [175] was used to describe the microalgal growth and fitted to the data with excel add-in solver.

2.2. Pigment determination

Carotenoids and chlorophyll a extraction and determination were performed according to Fernandes et al. [266]. For extraction 10 mg of microalgae dried biomass were used and 7 mL of a cold 80% acetone solution was added. After homogenization, the mixture was put on ultrasounds for 90 min. Then, samples were centrifuged, supernatant transferred to pre-chilled tubes, pellets continuously washed with extraction solution, and supernatant were passed through 0.45 µm filters. For chlorophyll *a* (Chl *a*) and carotenoids (Car) determination through ultraviolet-visible spectrophotometer the following equations used by Chen and Vaidyanathan [204] were employed:

$$\text{Chl } a = 12.21A_{663} - 2.81A_{646} \qquad \text{Equation 6.1}$$

$$\text{Car} = \frac{1000A_{470} - 3.27(12.21A_{663} - 2.81A_{646}) - 104(20.13A_{646} - 5.03A_{663})}{198} \qquad \text{Equation 6.2}$$

2.3. Lipid profile

2.3.1. Lipid Extraction

Microalgal biomass lipid extraction was performed accordingly with a modified Bligh and Dyer [206] described by Fernandes et al. [145]. In brief, to 50 mg of dried microalgal biomass, additions of 3 mL of a methanol:chloroform mixture (2:1;v:v), 400 µL of a saturated solution of

potassium chloride (KCl), 2 mL of chloroform, and 2 mL of distilled water were performed. Then, organic phase was removed and transferred to pre-weighted tubes, and lipids were determined by gravimetric quantification.

2.3.2. Alkaline hydrolysis

Alkaline hydrolysis was performed according to Santos et al. [129]. Thus, to two lipid aliquots 0.5 M of NaOH in aqueous methanol was added and left to react at 100 ºC for 1 h in a nitrogen atmosphere. Acidification to pH 2 was performed with 1 M HCl and the hydrolysis products were further extracted with dichloromethane.

2.3.3. Trimethylsilyl derivatization and GC-MS analysis

Before gas chromatography-mass spectrometry (GC-MS) analysis lipids aliquots before and after alkaline hydrolysis were derivatized to trimethylsilyl (TMS) ethers and/or esters according to Santos et al. [129]. Following the addition of tetracosane (internal standard) to the lipid extracts before and after alkaline hydrolysis, additions of 250 µL of pyridine, 250 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylclorosilane were performed. Then. samples were incubated at 70 °C for 30 min.

GC-MS analysis were performed using a gas chromatographer (6890N, Agilent Technologies, China) equipped with a mass selective detector (5973Network, Agilent Technologies, USA) and a ValcoBond capillary column from Vici Valco (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) using the conditions described by Fernandes et al. [122]. The TMS derivatives were identified by comparing the retention times and mass spectra fragmentation with that obtained through injection of the standards and literature. Semi-quantitative analysis was made through the calculated response factor of each standard towards the internal standard (tetracosane). The standards used were mannose, trans-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5*α*–chlolestane, cholesterol, stigmasterol and hexadecenoic and nonadecanoic acids. Four replicates were performed for each GC-MS analysis being the results presented as the mean value ± standard deviation (SD) expressed in mg g^{-1} of dry biomass weight (DW).

2.4. Monosaccharide profile

2.4.1. Monosaccharide reduction and derivatization

For monosaccharide analysis a two-stage acidic hydrolysis followed by monosaccharides reduction and acetylation were performed according to modified Blakeney et al. [269] and described by Fernandes et al [58]. Briefly, to 10 mg of sample, 400 µL of 72% sulfuric acid were added and left for 3 h at 20 ºC. Next, water was added, and mixtures placed at 100 ºC for 2 h 30 min. At the end of acidic hydrolysis, the internal standard (myo-inositol) was added to the hydrolysate. The reduction of monosaccharides to alditols was performed to 1 mL of hydrolysate by addition of 200 µL of a solution of 25% ammoniac (NH_3) solution and 100 µL of 3 M NH_3 containing 150 mg mL^{-1} of sodium borohydride. The mixtures were incubated at 30 ºC

for 1h, and 50 µL of glacial acetic acid was added twice to the samples with homogenization in between. Alditol acetylation was performed by adding 450 µL of 1-methylimidazole and 3 mL of acetic anhydride to 300 µL of the previous mixture. Then, the solution was incubated at 30 ºC for 30 min. Following water addition (3.75 mL), alditol acetates were extracted with dichloromethane (2.5 mL) and further washed three times with water.

2.4.2. GC-MS analysis

Monosaccharides were analyzed as alditol acetates by gas chromatography (Agilent HP 6890) mass spectrometry (Agilent 5973Network) and a capillary column DB-225 J & W (30 m × 0.25 mm inner diameter, 0.15 µm film thickness) from Agilent. After optimization, the GC-MS conditions were as follows: the inlet temperature was 220 ºC and initial column temperature was 210 °C for 3 min. Then, the column temperature increased at a rate of 1 ºC min^{-1} until reaching 220 ºC and 10 ºC min^{-1} until 230 ºC which was kept for 2 min. The transfer line temperature was 240 °C, the split ratio was 50:1 and Helium was used as the carrier gas with a ramped flow rate of 1.0 mL min^{-1} for 6 min increasing to 1.2 mL min^{-1} and remaining constant until the end of the run. The derivatized monosaccharides were identified by comparing the retention times and mass spectra fragmentation with that obtained through injection of the standards. The monosaccharides quantification was made through the calculated response factor of each standard towards the internal standard (myo-inositol). The standards used were myo-inositol, L(+)arabinose, D(+)xylose, D(+)galactose, D(+)glucose, D(+)mannose, monohydrate D(+)rhamnose, D(+)fucose purchased at Sigma-Aldrich (St. Louis, MO, USA). Four replicates were performed for each GC-MS analysis being the results presented as the mean value ± SD of monosaccharides expressed in mg g^{-1} DW.

2.5. Statistical analysis

Analysis of variance (ANOVA) followed by Tukey post hoc analysis was performed using IBM SPSS Statistics 26 software, *p*-values <0.05 were considered statistically significant. Whereas for principal component analysis (PCA), Pearson correlation, and hierarchical clustering heat maps the Metaboanalyst 5.0 webserver [270] was used. Before PCA, Pearson correlation, and hierarchical clustering heat maps, features were median normalized, log_2 transformed and auto scaled. For hierarchical clustering heat maps, Euclidean clustering distance, and ward clustering method were used.

3. Results and discussion

3.1. Microalgal growth, and chemical composition

Phosphorus is an essential nutrient for the growth and division of microalgal cells [260]. Growth dynamics of *Hemiselmis* cf. *andersenii* (Fig. 6.1a) and *Pavlova pinguis* (Fig.6.1b) were analysed under different initial phosphorus regimes. Through Figure 6.1 it is possible to observe that microalgal growth varied with the different initial phosphorus supplies, with microalgae at LP conditions presenting lower cell harvest density (Table 6.1) and reaching early stationary

phase more quickly than microalgae grown at MP and HP conditions. Biomass productivity determines the prospective of microalgae for efficient high-value compounds. According to Patel et al. [271] higher P loadings are often associated with higher biomass accumulation and productivity. For *H.* cf. *andersenii* and *P. pinguis* cultures significant differences (p <0.05) were observed for biomass productivity across P treatments, with increasing phosphorus concentrations exhibiting a positive effect on biomass productivities, with the greatest values being achieved at HP conditions, 30.05 mg L^{-1} d^{-1} and 43.91 mg L^{-1} d^{-1} respectively. These values are greater than those reported by [142] of 13 mg L^{-1} d^{-1} for another Cryptophyta (*Rhodomonas reticulata*) and 10 mg L^{-1} d^{-1} for *P. pinguis*.

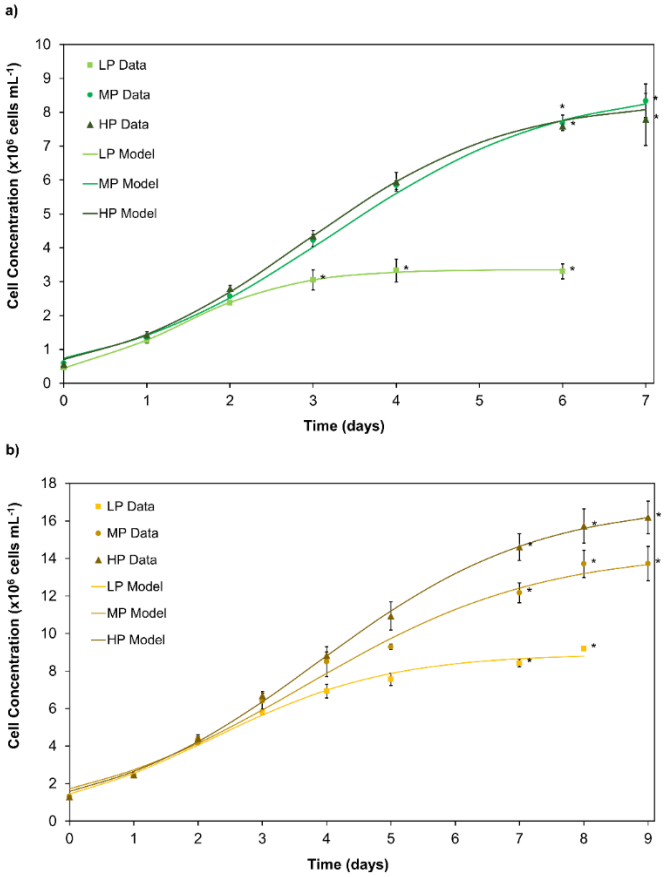

Figure 6.1. Growth curves obtained for **a)** *Hemiselmis* cf. *andersenii* and **b)** *Pavlova pinguis* under low (LP), medium (MP), and high (HP) starting phosphorus concentrations. Values are expressed as mean ± standard deviation, n=3 replicates. The determination coefficients (r^2) for the growth models were higher than 0.90. * Values are not significantly different (p >0.05) among cultivation days, within each treatment.

Table 6.1. Cell harvest density, biomass productivity, lipid, and monosaccharide content of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* under low, medium, high phosphorus supplementation.

	Hemiselmis* cf. *andersenii			***Pavlova pinguis***		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Cell harvest density (x10^6 cells mL^{-1})	3.30 ± 0.22^{a}	8.34 ± 0.50^{b}	7.79 ± 0.77^{b}	9.20 ± 0.02^{A}	13.72 ± 0.91^{B}	16.18 ± 0.87^{B}
Biomass Productivity (mg dw L^{-1} d^{-1})	17.52 ± 1.36^{a}	26.07 ± 4.08^{b}	30.05 ± 0.03^{b}	33.41 ± 0.47^{A}	43.10 ± 3.47^{B}	43.91 ± 3.41^{B}
Lipid (% dw)	12.58 ± 0.64^{a}	20.46 ± 1.09^{b}	17.64 ± 2.3^{b}	23.23 ± 0.93^{AB}	25.11 ± 0.67^{A}	21.87 ± 1.26^{B}
Monosaccharide (mg g^{-1} dw)	132.16 ± 1.21^{a}	240.47 ± 0.74^{b}	193.41 ± 0.61^{c}	180.27 ± 1.40^{A}	156.18 ± 5.27^{B}	139.43 ± 0.78^{C}
Rhamnose	1.24 ± 0.11^{a}	0.97 ± 0.07^{b}	1.10 ± 0.11^{ab}	1.16 ± 0.10^{A}	1.61 ± 0.13^{B}	1.12 ± 0.21^{A}
Arabinose	*n.d.*	*n.d.*	*n.d.*	0.81 ± 0.22^{A}	2.43 ± 0.38^{B}	2.05 ± 0.07^{B}
Xylose	*n.d.*	0.21 ± 0.04^{a}	0.35 ± 0.01^{b}	0.45 ± 0.05^{A}	0.95 ± 0.18^{B}	0.75 ± 0.09^{B}
Mannose	74.04 ± 0.76^{a}	130.24 ± 0.64^{b}	98.97 ± 0.46^{c}	81.83 ± 0.74^{A}	71.13 ± 2.69^{B}	74.70 ± 0.25^{C}
Galactose	0.87 ± 0.06^{a}	3.77 ± 0.13^{b}	3.51 ± 0.12^{c}	4.08 ± 0.12^{A}	6.10 ± 0.16^{B}	4.25 ± 0.08^{A}
Glucose	56.01 ± 0.48^{a}	105.29 ± 0.25^{b}	89.47 ± 0.61^{c}	91.93 ± 0.40^{A}	73.96 ± 1.95^{B}	56.55 ± 0.34^{C}
Lipid/Monosaccharide (%dw/%dw)	0.95 ± 0.05^{a}	0.87 ± 0.01^{a}	0.91 ± 0.12^{a}	1.29 ± 0.05^{A}	1.61 ± 0.05^{B}	1.57 ± 0.10^{B}

Values (means ± SD of at least three replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *H. cf. andersenii*, and by superscript uppercase letters for *P. pinguis*. dw – dry weight.

Carotenoids (photoprotective pigments) to chlorophyll *a* (light-harvesting pigment) ratio (Car/Chl *a*) can serve as indicator of carotenogenesis, and as a marker of physiological stress in microalga under poor environmental conditions [118,262,272]. For this ratio, it was observed significant variations (p <0.05) under different initial phosphorus supplies for both microalgae studied, Figure 6.2. For *H.* cf. *andersenii* LP conditions lead to low carotenoid (0.61 mg g^{-1} dw), Chl *a* (1.72 mg g^{-1} dw), and Car/ Chl *a* (0.36) ratio values, whereas for *P. pinguis* a carotenoid accumulation was observed through an increase of 54% from MN conditions (2.81 mg g^{-1} dw) to LP conditions (4.32 mg g^{-1} dw) and with the highest Car/Chl *a* (0.77) ratio being observed at these conditions. Microalgae-derived carotenoids have a high market potential due to its several biological activities such as antioxidant activity, hypolipidemic, anti-inflammatory and hypotensive [114]. Therefore, the results obtained for carotenoid and Car/Chl *a* ratio indicate that phosphorus limitation constitutes a cost-efficient strategy for carotenoid production by *P. pinguis* for high-value market. A similar behavior to *P. pinguis* was observed for other Haptophyta (*Isochrysis galbana*) in which phosphorus starvation initiated chlorophyll reduction and carotenoid accumulation [260]. The accumulation of carotenoids observed for *P. pinguis* may have been a protective mechanism against photo-oxidative stress prompted by the LP conditions [260].

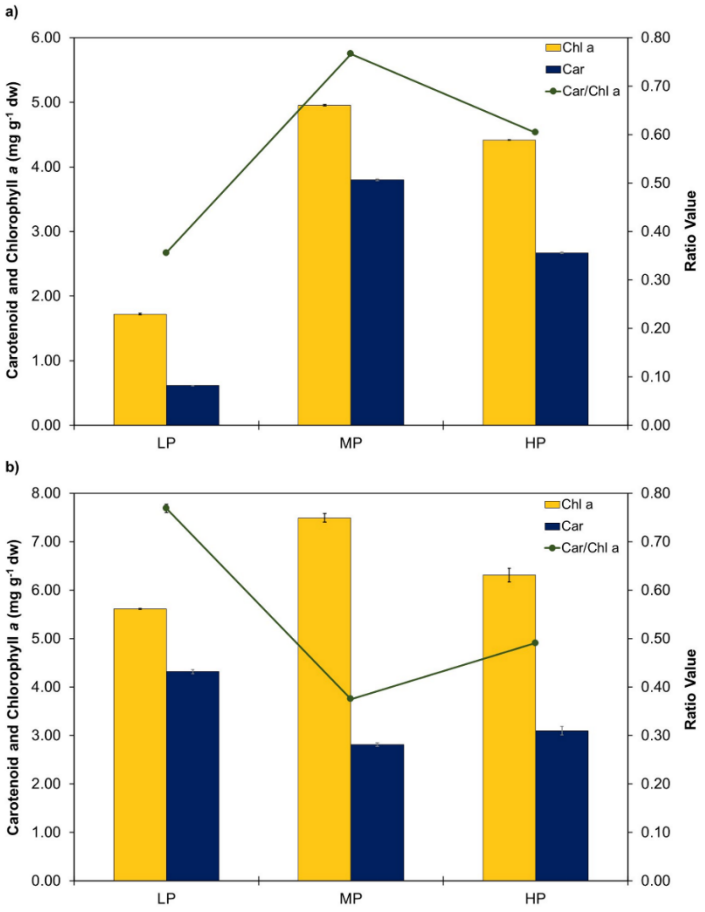

Figure 6.2. Influence of different phosphorus regimes on carotenoid (Car) and chlorophyll *a* (Chl *a*) in **a)** *Hemiselmis* cf. *andersenii* and **b)** *Pavlova pinguis* cultures. LP - Low phosphorus; MP – Medium phosphorus and HP - High phosphorus supplementations. * Values are not significantly different (p >0.05) among treatments.

Microalgae fixed carbon can be distributed among three main macromolecular pools: protein, carbohydrate, and lipid [273]. In general, microalgae use carbohydrate as their primary carbon storage pool switching their carbon partitioning toward energy-rich storage compounds like lipids under poor environmental conditions [273]. However, this partition among carbon-rich pools (carbohydrate and lipid) can be species-specific [145]. For *H.* cf. *andersenii* the lipid/monosaccharide ratio remained stable across treatments with non-significant differences (p >0.05) being observed for this parameter (Table 6.1). In this microalga the lipid/monosaccharide ratio lower than one seem to indicate that carbohydrates are the main carbon-rich pool for this microalga. For *P. pinguis* the lipid/monosaccharide ratio was significantly (p <0.05) affected by initial phosphorus supplies reaching its lowest value (1.26) at LP conditions, suggesting that at these conditions *P. pinguis* displaced its carbon partitioning towards carbohydrate synthesis (Table 6.1). Moreover, an opposite trend between the Car/Chl *a* ratio and the lipid/monosaccharide ratio, suggest that the Car/Chl *a* ratio could be a simple indicator of the carbon partitioning among lipid and carbohydrate.

A convertible relationship between carbohydrate and lipid has been reported to occur in other marine microalgae such as the Haptophyta *Isochrysis* sp., the Cryptophyta *Rhodomonas marina*, and the Ocrophyta *Nannochloropsis gaditana*, as a response to environmental fluctuations [58,145]. In the study performed by Fernandes et al. [145] *Isochrysis* sp., which belong to the same phylum of *P. pinguis*, also accumulated carbohydrates instead of lipid towards low nutrient conditions. Moreover, improvements of carbohydrate production though phosphorus limitation was reported for the *Arthrospira platensis* (Cyanobacteria) [264].

Through Table 6.1 *P. pinguis* displayed the more complex mixture of monosaccharides presenting six identified compounds: rhamnose, arabinose, xylose, mannose, galactose, and glucose. For both *H.* cf. *andersenii* and *P. pinguis* glucose and mannose were the main building-blocks of carbohydrate accounting for 97-98% of total monosaccharides and 93-96% of total monosaccharides, respectively. Looking at the relative abundance of monosaccharides it is possible to observe that *H.* cf. *andersenii* and *P. pinguis* remodeled differently its monosaccharide composition in response to initial phosphorus inputs. Thus, *H.* cf. *andersenii* increased its proportions in galactose (threefold) from LP conditions towards HP conditions (Fig. 6.3a), whereas *P. pinguis* decreased by 20% its glucose relative abundance from LP conditions towards HP conditions (Fig. 6.3b). The main storage product of Haptophyta is chrysolaminaran which is a *β*-1,3-glucan [234]. Thus, the verified lowest lipid/monosaccharide ratio along with the highest monosaccharide content and glucose proportion indicate that *P. pinguis* responded to LP conditions by synthesizing this storage product.

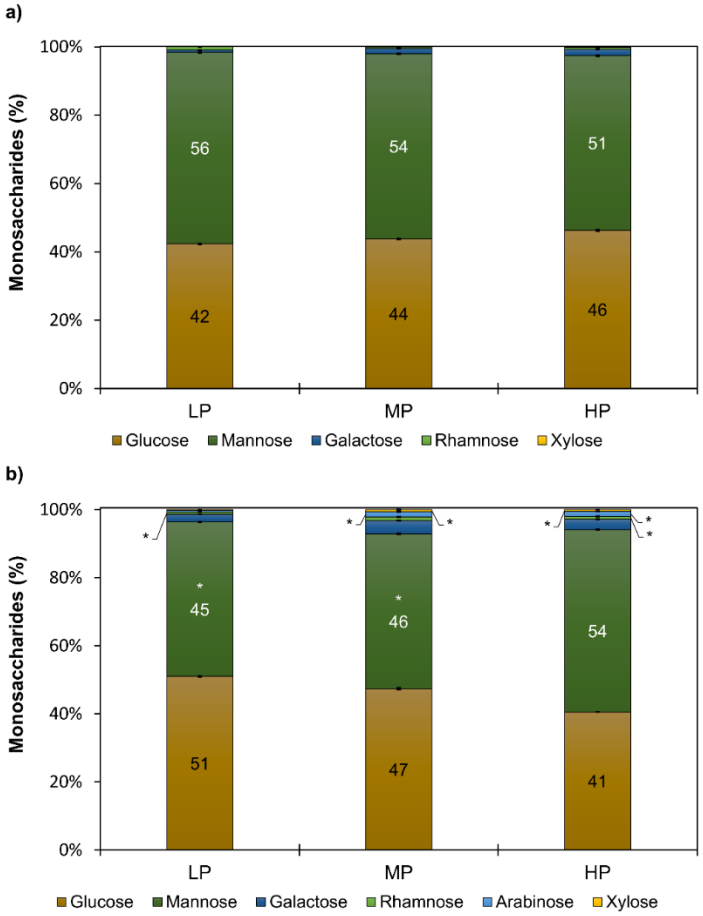

Figure 6.3. Changes in monosaccharides proportions for **a)** *Hemiselmis* cf. *andersenii* and **b)** *Pavlova pinguis* against phosphorus treatments: LP - Low phosphorus; MP – Medium phosphorus and HP - High phosphorus supplementations. * Values are not significantly different (p >0.05) among treatments.

Phosphorus starvation is often referred as an effective strategy to enhance lipid accumulation by microalgae [261,273,274]. Table 6.1 shows that lipid content was significantly (p <0.05) affected by the different phosphorus regimes applied in both microalgae studied. For *H.* cf. *andersenii* lipid levels ranged between 12.58 – 20.48% dw, and for *P. pinguis* lipid amount varied between 21.87 – 25.11% dw. In both microalgae the greatest lipid values were observed at MP conditions, while the lowest were found at LP conditions for *H.* cf. *andersenii* and at HP conditions for *P. pinguis*. The decrease in lipid amounts for microalgae grown in low phosphorus cultures has been reported to occur in other microalgae like *Nannochloris atomus* (Chlorophyta) and *Tetraselmis* sp. (Chlorophyta) [275]. According to Saha et al. [276] most eukaryotic microalgae proportionally accumulate lipids and carotenoids as a response to environmental stresses. In the present study, *H.* cf. *andersenii* showed a similar trend for both lipid and carotenoids as a response to phosphorus-induced changes.

3.2. Lipid remodelling

Several studies [262,267,277,278] have reported the reorganization of microalgal cellular lipids under phosphorus-induced changes. Thus, for study the impact of phosphate-induced changes

on *H.* cf. *andersenii* and *P. pinguis* lipid composition, an untargeted approach analyzing its esterified and unesterified composition was applied. Through Tables 6.2 and 6.3 it is possible to observe that *H.* cf. *andersenii* and *P. pinguis* displayed different patterns of lipid remodeling as a response to phosphorus-induced changes. For all lipid sets analyzed (fatty acids, aliphatic alcohols, sterols, monoglycerides, and other compounds) significant differences (p <0.05) were observed across treatments in both microalgae studied.

Fatty acids are the main lipid compounds studied when assessing the impact of phosphorus-induced changes on microalgae [267]. Previous studies [267,279,280] report that low phosphorus conditions trigger fatty acid accumulation in most microalgae. For *H.* cf. *andersenii* samples total fatty acids increased by increasing initial phosphorus concentrations, which, in turn, lead to a great increase in the main fatty acids hexadecanoic (C16:0), 9,12,15-octadecatrienoic acid (ALA; C18:3ω3), and 5,8,11,14,17-eicosapentaenoic acids (C20:5ω3). For *P. pinguis* a slight decrease of 11% was verified in its total fatty acids' levels in HP samples after hydrolysis.

As building-blocks of lipids, fatty acids were the most affected by alkaline hydrolysis, increasing up to 43% in *H.* cf. *andersenii* at HP conditions and up to 41% in *P.pinguis* at LP conditions. Within fatty acids, polyunsaturated fatty acids (PUFA) presented the highest increase after hydrolysis for *H.* cf. *andersenii* samples (up to 81% at HP conditions), whereas for *P. pinguis* saturated fatty acids (SFA) presented the greatest increase (up to 46% at LP conditions). Since PUFA are often attributed to polar lipids, whereas SFA are mainly enriched in triacyclglycerols [224], this may indicate that *P. pinguis* has a richer composition in neutral lipids (triacylglycerols) than *H.* cf. *andersenii*.

The production of enhanced amounts of nutritionally important fatty acids by microalgae would have added value as an ingredient for various functional food, cosmetics and nutraceutical products [276]. Figure 6.4 shows the effect of phosphorus-induced changes on *H.* cf. *andersenii* (Fig. 6.4a) and *P. pinguis* (Fig. 6.4b) fatty acid dietary ratios before and after alkaline hydrolysis. *H.* cf. *andersenii* presented more variations in fatty acid dietary ratios than *P. pinguis* which seemed to be more stable. For *H.* cf. *andersenii* the ratios more affected by alkaline hydrolysis were the EPA to 4,7,10,13,16,19-docosahexaenoic acid (DHA; C22ω6) and PUFA/SFA ratios, which increased up to 45 and 69% after saponification respectively, indicating that the saponifiable fraction of this microalga is richer in EPA and PUFA. Moreover, the lowest value of PUFA/SFA ratio (BH: 0.48; AH: 0.81) observed at LP conditions shows that *H.* cf. *andersenii* tend to produce SFA in low phosphorus environments. This observation is consistent with a previous study performed for microalga Chlorophyta *Scenedesmus*, in which it was assumed that during phosphorus limitation this microalga tend to produce SFA [273]. *H.* cf. *andersenii* also displayed the highest ∑ω3/∑ω6 ratio (BH: 6.50; AH: 6.78) showing its potential as a rich source of ω3 fatty acids.

Table 6.2. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings before hydrolysis.

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
	Fatty Acids	*33.51±0.92^{a}*	*56.58±0.85^{b}*	*50.72±0.36^{c}*	*94.35±3.44^{A}*	*102.96±1.45^{B}*	*101.78±1.55^{B}*
	Saturated Fatty Acids	*18.06±0.54^{a}*	*28.12±0.36^{b}*	*25.63±0.22^{c}*	*40.72 ± 1.51^{A}*	*43.27±0.42^{B}*	*43.26±1.40^{B}*
1	Nonanoic acid	1.04±0.01^{a}	1.57±0.00^{b}	1.55±0.01^{c}	1.53 ± 0.01^{AB}	1.55±0.01^{A}	1.53±0.00^{B}
3	Decanoic acid	*n.d.*	*n.d.*	*n.d.*	1.54±0.01^{A}	*n.d.*	1.55±0.01^{B}
6	undecanoic acid	*n.d.*	*n.d.*	*n.d.*	1.53±0.01^{A}	1.54±0.02^{A}	1.54±0.01^{A}
8	Dodecanoic acid	*n.d.*	*n.d.*	*n.d.*	1.54±0.01^{A}	1.56±0.02^{A}	1.55±0.00^{A}
12	Tetradecanoic acid	1.12±0.01^{a}	1.95±0.01^{b}	1.72±0.01^{c}	8.01±0.61^{A}	13.57±0.16^{B}	13.03±0.83^{B}
14	Pentadecanoic acid	1.06±0.01^{a}	1.62±0.01^{b}	1.58±0.01^{c}	1.63±0.02^{A}	1.77±0.23^{AB}	1.97±0.03^{B}
23	Hexadecanoic acid	4.03±0.29^{a}	6.88±0.20^{b}	5.21±0.22^{c}	12.63±0.79^{A}	10.68±0.13^{B}	9.79±0.69^{B}
25	Heptadecanoic acid	1.34±0.05^{a}	1.95±0.04^{b}	1.83±0.02^{c}	1.76±0.02^{A}	1.83±0.02^{AB}	1.86±0.06^{B}
33	Octadecanoic acid	1.86±0.04^{a}	2.69±0.05^{b}	2.57±0.04^{c}	2.59±0.03A^{B}	2.66±0.03^{A}	2.51±0.10^{B}
40	Eicosanoic acid	1.07±0.02^{a}	1.60±0.01^{b}	1.57±0.01^{c}	1.56±0.01A^{B}	1.58±0.02^{A}	1.55±0.01^{B}
47	Docosanoic acid	1.16±0.02^{a}	1.70±0.03^{b}	1.65±0.01^{c}	1.6±0.01^{A}	1.61±0.02^{A}	1.61±0.03^{A}
49	Tricosanoic acid	1.06±0.01^{a}	1.59±0.01^{b}	1.56±0.01^{c}	*n.d.*	*n.d.*	*n.d.*
52	Tetracosanoic acid	1.07±0.01^{a}	1.61±0.01^{b}	1.57±0.01^{c}	1.54±0.01^{A}	1.57±0.01^{B}	1.55±0.00^{AB}
59	Octacosanoic acid	1.09±0.03^{a}	1.68±0.01^{b}	1.63±0.04^{b}	1.6±0.05^{A}	1.65±0.13^{A}	1.62±0.05^{A}
71	Triacontanoic acid	1.11±0.04^{a}	1.67±0.03^{b}	1.62±0.03^{b}	1.64±0.03^{A}	1.69±0.09^{A}	1.59±0.01^{A}
77	Dotriacontanoic acid	1.06±0.02^{a}	1.60±0.02^{b}	1.56±0.01^{c}	*n.d.*	*n.d.*	*n.d.*
	Monounsaturated Fatty Acids	*6.72±0.10^{a}*	*10.22±0.06^{b}*	*9.91±0.04^{c}*	*23.19±1.24^{A}*	*25.23±0.31^{B}*	*24.59±0.85^{AB}*
11	9-Tetradecenoic acid	*n.d.*	*n.d.*	*n.d.*	1.54±0.01^{A}	1.58±0.02^{B}	1.57±0.01^{AB}
20	9-hexadecenoic acid	1.08±0.01^{a}	1.61±0.01^{b}	1.59±0.02^{b}	15.82±1.17^{A}	17.93±0.25^{B}	17.44±0.82^{AB}
21	7-Hexadecenoic acid	1.05±0.01^{a}	1.63±0.00^{b}	1.57±0.01^{c}	1.61±0.02^{A}	1.73±0.03^{B}	1.72±0.01^{B}
31	9-Octadecenoic acid	1.30±0.03^{a}	1.95±0.03^{b}	1.79±0.02^{c}	2.42±0.04^{A}	2.14±0.02^{B}	2.06±0.03^{C}
32	11- Octadecenoic acid	1.20±0.03^{a}	1.87±0.02^{b}	1.84±0.01^{b}	1.81±0.03^{A}	1.84±0.04^{A}	1.81±0.0^{A}
34	Nonadecenoic acid	2.10±0.03^{a}	3.16±0.01^{b}	3.11±0.02^{c}	*n.d.*	*n.d.*	*n.d.*
	Polyunsaturated Fatty Acids	8.72±0.29^{a}	18.24±0.45^{b}	15.17±0.16^{c}	30.44±0.87^{A}	34.46±0.87^{B}	33.92±0.78^{B}
22	Methyl-4,7,10,13-hexadecatetraenoate	1.07±0.01^{a}	1.69±0.01^{b}	1.63±0.01^{c}	1.59±0.01^{AB}	1.61±0.02^{A}	1.58±0.01^{B}

Table 6.2. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings before hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
16	C16:4ω3	*n.d.*	*n.d.*	*n.d.*	n.d.	1.56±0.02^{A}	1.55±0.00^{A}
17	C16:2ω6	*n.d.*	*n.d.*	*n.d.*	1.55±0.01^{A}	1.74±0.02^{B}	1.72±0.01^{B}
18	C16:3ω3	*n.d.*	*n.d.*	*n.d.*	1.56±0.01^{A}	1.59±0.02^{B}	1.57±0.00^{A}
19	7,12-Hexadienoic acid	*n.d.*	1.67±0.01^{a}	1.58±0.01^{c}	*n.d.*	*n.d.*	*n.d.*
27	3,6,9,12,15-Octadecapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	1.56±0.01^{A}	1.74±0.03^{B}	1.76±0.02^{B}
29	9,12-Octadecadienoic acid[1]	1.38±0.04^{a}	2.21±0.07^{b}	2.15±0.04^{b}	3.72±0.04^{A}	3.49±0.05^{B}	3.39±0.05^{C}
30	9,12,15-Octadecatrienoic acid	2.55±0.15^{a}	5.24±0.16^{b}	3.49±0.08^{c}	1.71±0.02^{A}	1.73±0.03^{A}	1.69±0.04^{A}
36	5,8,11,14,17-Eicosapentaenoic acid	1.59±0.07^{a}	3.95±0.17^{b}	2.95±0.07^{c}	8.72±0.4^{A}	10.16±0.26^{B}	9.95±0.25^{B}
41	4,7,10,13,16,19-Docosahexaenoic acid	1.06±0.01^{a}	1.74±0.03^{b}	1.68±0.01^{c}	4.69±0.22^{A}	5.21±0.28^{A}	5.17±0.33^{A}
42	7,10,13,16,19-Docosapentaenoic acid	1.07±0.02^{a}	1.75±0.03^{b}	1.69±0.00^{c}	3.46±0.13^{A}	3.26±0.10^{A}	3.29±0.21^{A}
43	4,7,10,13,16-Docosapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	1.89±0.03^{A}	2.35±0.06^{B}	2.26±0.04^{B}
	Aliphatic alcohols	*23.74±1.19^{a}*	*35.14±0.91^{b}*	*30.54±0.62^{c}*	*26.62±1.25^{A}*	*32.35±0.75^{B}*	*33.87±1.76^{B}*
2	Decanol	1.46±0.04^{a}	2.48±0.05^{b}	1.91±0.57^{ab}	1.6±0.13^{A}	1.83±0.03^{B}	1.94±0.08^{B}
4	Undecanol	1.68±0.04^{a}	2.67±0.03^{b}	2.14±0.57^{ab}	1.73±0.18^{A}	2.28±0.06^{B}	2.44±0.10^{B}
7	Dodecanol	1.17±0.03^{a}	1.86±0.04^{b}	1.73±0.19^{b}	1.47±0.06^{A}	1.75±0.04^{B}	1.84±0.06^{B}
9	Tridecanol	1.33±0.07^{a}	2.03±0.04^{b}	1.90±0.16^{b}	1.73±0.05^{A}	2.15±0.06^{B}	2.38±0.15^{C}
10	Tetradecanol	1.06±0.02^{a}	1.59±0.02^{b}	1.49±0.01^{c}	1.45±0.02^{A}	1.50±0.03^{AB}	1.50±0.03^{B}
15	Hexadecanol	3.09±0.31^{a}	4.13±0.21^{b}	3.40±0.23^{a}	3.07±0.25^{A}	3.24±0.34^{A}	3.58±0.44^{A}
24	Octadece-9-nol	4.40±0.54^{a}	5.88±0.39^{b}	4.70±0.41^{a}	4.07±0.38^{A}	4.38±0.50^{A}	4.99±0.72^{A}
26	Octadecanol	2.12±0.17^{a}	2.88±0.12^{b}	2.48±0.14^{c}	2.24±0.14^{A}	2.42±0.18^{A}	2.60±0.24^{A}
28	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.58±0.07^{a}	3.04±0.07^{b}	2.50±0.03^{c}	2.73±0.09^{A}	4.77±0.06^{B}	4.44±0.18^{C}
37	Eicosanol	0.93±0.03^{a}	1.37±0.01^{b}	1.33±0.00^{c}	1.31±0.01^{A}	1.32±0.02^{A}	1.33±0.02^{A}
44	Docosanol	0.86±0.01^{a}	1.30±0.02^{b}	1.28±0.01^{c}	1.26±0.01^{A}	1.29±0.02^{B}	1.27±0.01^{AB}
53	Hexacosanol	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.27±0.01^{A}	1.25±0.00^{A}
56	Octacosanol	1.14±0.06^{a}	1.63±0.04^{b}	1.56±0.04^{b}	1.55±0.05^{A}	1.58±0.05^{A}	1.57±0.07^{A}
66	Octacosane-1,3-diol	0.98±0.03^{a}	1.44±0.03^{b}	1.39±0.02^{c}	*n.d.*	*n.d*	*n.d.*

Table 6.2. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings before hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
68	Tricosanol	1.05±0.04^{a}	1.52±0.04^{b}	1.46±0.03^{b}	1.44±0.03^{A}	1.45±0.03^{A}	1.43±0.05^{A}
74	Dotriacontanol	0.88±0.01^{a}	1.32±0.02^{b}	1.29±0.01^{c}	1.31±0.01^{A}	1.47±0.13^{B}	1.30±0.01^{A}
	Sterols	*1.99±0.21^{a}*	*3.79±0.20^{b}*	*3.18±0.18^{c}*	*14.39±1.13^{A}*	*20.03±0.30^{B}*	*18.02±0.47^{C}*
54	22-Stigmasten-3-one	*n.d.*	*n.d.*	*n.d.*	0.30±0.04^{A}	0.32±0.02^{A}	0.30±0.01^{A}
57	24*β*-Methylcholesta-5,22E-dien-3*β*-ol	0.85±0.08^{a}	2.10±0.18^{b}	1.78±0.09^{c}	*n.d.*	*n.d.*	*n.d.*
58	24*α*-Methylcholest-5-en-3*β*-ol	0.22±0.03^{a}	0.40±0.07^{b}	0.33±0.05^{b}	0.52±0.07^{A}	0.74±0.06^{B}	0.76±0.06^{B}
60	24*β*-Ethylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.44±0.08^{A}	0.41±0.02^{B}	0.58±0.06^{C}
61	24*α*-Ethylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	4.71±0.37^{A}	8.32±0.31^{B}	7.15±0.22^{C}
62	24-Ethyl-*δ*(22)-coprostenol	*n.d.*	*n.d.*	*n.d.*	0.49±0.04^{A}	0.92±0.18^{B}	0.83±0.07^{B}
63	24*α*-Ethylcholest-5-en-3*β*-ol	0.74±0.10^{a}	0.98±0.08^{b}	0.83±0.09^{ab}	0.94±0.18^{A}	1.22±0.04^{A}	1.20±0.26^{A}
64	Stigmasta-3,5-dien-7-one	0.18±0.01^{a}	0.31±0.02^{b}	0.25±0.01^{c}	0.41±0.08^{A}	*n.d.*	0.28±0.03^{B}
65	4*α*,24-Dimethyl-5*α*-cholestan-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.95±0.12^{A}	1.10±0.03^{B}	0.93±0.05^{A}
67	Unidentified Sterol	*n.d.*	*n.d.*	*n.d.*	1.26±0.10^{A}	1.83±0.12^{B}	1.57±0.07^{C}
69	4*α*-methyl,24-ethyl-5*α*-cholest-22E-en-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.80±0.07^{AB}	0.91±0.08^{A}	0.76±0.00^{B}
70	4*α*-methyl,24-ethyl-5*α*-cholestan-3-ol	*n.d.*	*n.d.*	*n.d.*	0.80±0.07^{A}	1.05±0.17^{B}	0.71±0.10^{A}
72	4*α*,24*β*-dimethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	*n.d.*	*n.d.*	*n.d.*	0.41±0.03^{A}	0.53±0.03^{B}	0.45±0.02^{A}
73	Unidentified Sterol	*n.d.*	*n.d.*	*n.d.*	1.26±0.04^{A}	1.26±0.17^{A}	0.98±0.07^{B}
75	4*α*-methyl-24*β*-ethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	*n.d.*	*n.d.*	*n.d.*	1.1±0.23^{A}	1.45±0.17^{A}	1.51±0.37^{A}
	Monoglycerides	*1.48±0.04^{a}*	*1.64±0.18^{a}*	*0.99±0.07^{b}*	*1.45±0.09^{A}*	*1.24±0.07^{B}*	*0.98±0.03^{C}*
38	1,3-Dihydroxy-2-propanyl myristate	*n.d.*	*n.d.*	*n.d.*	0.14±0.01^{A}	0.10±0.00^{B}	0.08±0.01^{C}
39	2,3-Dihydroxypropyl myristate	*n.d.*	*n.d.*	*n.d.*	0.23±0.01^{A}	0.20±0.03^{A}	0.10±0.01^{B}
45	1,3-Dihydroxy-2-propanyl palmitate	0.06±0.00^{a}	0.12±0.02^{b}	0.08±0.01^{c}	0.21±0.01^{A}	0.12±0.01^{B}	0.13±0.01^{B}
46	2,3-Dihydroxypropyl palmitate	0.75±0.02^{a}	0.76±0.06^{a}	0.43±0.04^{b}	0.55±0.03^{A}	0.40±0.04^{B}	0.30±0.05^{C}
48	3-Octadecoxypropane-1,2-diol	0.06±0.01^{a}	0.09±0.02^{b}	0.10±0.01^{b}	0.05±0.01^{A}	0.07±0.02^{A}	0.07±0.00^{A}
50	1,3-Dihydroxy-2-propanyl stearate	0.05±0.01^{a}	0.12±0.01^{b}	0.07±0.00^{a}	0.05±0.01^{A}	0.07±0.01^{B}	0.09±0.02^{B}

Table 6.2. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings before hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
51	2,3-Dihydroxypropyl stearate	0.56 ± 0.02^{a}	0.54 ± 0.07^{a}	0.31 ± 0.02^{b}	0.22 ± 0.02^{A}	0.28 ± 0.03^{B}	0.24 ± 0.02^{AB}
	Others	*0.13 ± 0.01^{a}*	*0.20 ± 0.01^{b}*	*0.23 ± 0.04^{c}*	*0.58 ± 0.04^{A}*	*0.44 ± 0.06^{B}*	*0.31 ± 0.05^{C}*
5	2,6-bis(1,1-Dimethylethyl)phenol	0.13 ± 0.01^{a}	0.18 ± 0.01^{b}	0.19 ± 0.02^{b}	0.14 ± 0.02^{A}	0.16 ± 0.01^{A}	0.14 ± 0.02^{A}
13	Rhamnose	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
55	*α*-Tocopherol	*n.d.*	0.03 ± 0.00^{a}	0.04 ± 0.01^{b}	0.10 ± 0.01^{A}	0.04 ± 0.01^{B}	0.12 ± 0.01^{C}
76	Campestenyl glycoside	*n.d.*	*n.d.*	*n.d.*	0.17 ± 0.03^{A}	0.14 ± 0.03^{A}	0.07 ± 0.02^{B}
78	Campesteryl glycoside	*n.d.*	*n.d.*	*n.d.*	0.17 ± 0.02^{A}	0.08 ± 0.01^{B}	n.d.
	Total	*60.85 ± 2.30^{a}*	*97.35 ± 2.11^{b}*	*85.66 ± 0.27^{c}*	*137.38 ± 1.83^{A}*	*157.02 ± 0.96^{B}*	*154.96 ± 2.08^{B}*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *H.* cf. *andersenii*, and by superscript uppercase letters for *P. pinguis*. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains cis and trans isomers. [2]Identified as the mono-TMS ether. *n.d.* – non detected.

Table 6.3. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings after alkaline hydrolysis.

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
	Fatty Acids	*44.13 ± 0.34^{a}*	*77.42 ± 4.86^{b}*	*72.62 ± 3.52^{b}*	*132.79 ± 3.70^{A}*	*134.16 ± 3.29^{A}*	*119.34 ± 2.12^{B}*
	Saturated Fatty Acids	*18.65 ± 0.11^{a}*	*32.54 ± 1.95^{b}*	*30.92 ± 1.87^{b}*	*59.49 ± 3.56^{A}*	*59.23 ± 1.77^{A}*	*51.69 ± 1.65^{B}*
1	Nonanoic acid	1.54 ± 0.01^{a}	2.05 ± 0.02^{b}	2.02 ± 0.04^{b}	2.01 ± 0.05^{A}	2.03 ± 0.02^{A}	2.02 ± 0.01^{A}
3	Decanoic acid	*n.d.*	*n.d.*	*n.d.*	2.00 ± 0.05^{A}	2.03 ± 0.02^{A}	2.03 ± 0.01^{A}
6	undecanoic acid	*n.d.*	*n.d.*	*n.d.*	2.06 ± 0.00^{A}	*n.d.*	2.01 ± 0.01^{B}
8	Dodecanoic acid	*n.d.*	*n.d.*	*n.d.*	2.02 ± 0.05^{A}	2.04 ± 0.02^{A}	2.03 ± 0.01^{A}
12	Tetradecanoic acid	1.81 ± 0.02^{a}	4.15 ± 0.26^{b}	3.56 ± 0.38^{c}	13.39 ± 0.67^{A}	18.68 ± 0.22^{B}	15.69 ± 0.32^{C}
14	Pentadecanoic acid	1.57 ± 0.00^{a}	2.19 ± 0.04^{b}	2.12 ± 0.01^{c}	2.18 ± 0.06^{A}	2.63 ± 0.02^{B}	2.53 ± 0.02^{C}
23	Hexadecanoic acid	7.56 ± 0.07^{a}	13.69 ± 1.25^{b}	11.03 ± 1.41^{c}	19.11 ± 0.43^{A}	14.65 ± 0.68^{B}	11.75 ± 0.25^{C}
25	Heptadecanoic acid	1.69 ± 0.01^{a}	2.28 ± 0.05^{b}	2.28 ± 0.01^{b}	2.20 ± 0.10^{A}	2.29 ± 0.04^{A}	2.21 ± 0.01^{A}

Table 6.3. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings after alkaline hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
33	Octadecanoic acid	2.96±0.07^{a}	4.05±0.27^{b}	3.79±0.18^{b}	7.00±0.03^{A}	4.37±0.65^{B}	3.61±0.06^{B}
40	Eicosanoic acid	*n.d.*	2.06±0.03^{a}	2.03±0.03^{a}	2.07±0.08^{A}	2.08±0.03^{A}	2.06±0.01^{A}
47	Docosanoic acid	1.54±0.02^{a}	2.07±0.04^{b}	2.07±0.02^{b}	2.09±0.07^{A}	2.14±0.03^{A}	2.11±0.02^{A}
52	Tetracosanoic acid	*n.d.*	*n.d.*	2.01±0.02	2.00±0.05^{A}	2.04±0.02^{A}	*n.d.*
59	Octacosanoic acid	*n.d.*	*n.d.*	*n.d.*	2.01±0.05^{A}	2.04±0.02^{A}	2.03±0.01^{A}
71	Triacontanoic acid	*n.d.*	*n.d.*	*n.d.*	2.11±0.07^{A}	2.21±0.02^{B}	2.11±0.06^{AB}
	Monounsaturated Fatty Acids	*10.30±0.03^{a}*	*14.33±0.42^{b}*	*14.20±0.08^{b}*	*32.02±0.29^{A}*	*33.12±0.65^{B}*	*29.72±0.34^{C}*
11	9-Tetradecenoic acid	*n.d.*	*n.d.*	*n.d.*	2.03±0.02^{A}	2.05±0.02^{A}	2.04±0.01^{A}
20	9-hexadecenoic acid	1.60±0.00^{a}	2.16±0.03^{b}	2.15±0.01^{b}	20.98±0.90^{A}	23.22±0.25^{B}	20.41±0.32^{A}
21	7-Hexadecenoic acid	1.56±0.01^{a}	2.22±0.04^{b}	2.15±0.02^{c}	2.11±0.04^{A}	2.23±0.02^{B}	2.20±0.01^{B}
31	9-Octadecenoic acid	1.99±0.01^{a}	2.75±0.15^{b}	2.65±0.05^{b}	3.92±0.35^{A}	3.06±0.18^{B}	2.72±0.02^{B}
32	11- Octadecenoic acid	1.98±0.02^{a}	2.98±0.14^{b}	3.09±0.02^{b}	2.99±0.49^{A}	2.56±0.19^{AB}	2.35±0.02^{B}
34	Nonadecenoic acid	3.16±0.02^{a}	4.22±0.06^{b}	4.15±0.07^{b}	*n.d.*	*n.d.*	*n.d.*
	Polyunsaturated Fatty Acids	*15.18±0.38^{a}*	*30.56±2.51^{b}*	*27.51±1.73^{b}*	*41.29±0.11^{A}*	*41.82±0.89^{A}*	*37.93±0.28^{B}*
22	Methyl-4,7,10,13-hexadecatetraenoate	1.54±0.01^{a}	2.14±0.04^{b}	2.07±0.01^{c}	2.03±0.03^{A}	2.06±0.03^{A}	2.04±0.01^{A}
16	C16:4ω3	*n.d.*	*n.d.*	*n.d.*	2.04±0.03^{A}	2.05±0.02^{A}	*n.d.*
17	C16:2ω6	*n.d.*	*n.d.*	*n.d.*	2.03±0.04^{A}	2.19±0.02^{B}	2.18±0.01^{B}
18	C16:3ω3	*n.d.*	*n.d.*	*n.d.*	2.05±0.07^{A}	2.12±0.02^{AB}	2.17±0.02^{B}
19	7,12-Hexadienoic acid	*n.d.*	2.35±0.05^{a}	2.26±0.03^{b}	*n.d.*	*n.d.*	*n.d.*
27	3,6,9,12,15-Octadecapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	2.03±0.05^{A}	2.21±0.02^{B}	2.23±0.01^{B}
29	9,12-Octadecadienoic acid[1]	2.52±0.13^{a}	3.62±0.28^{b}	3.59±0.15^{b}	5.01±0.26^{A}	4.56±0.15^{B}	4.33±0.02^{B}
30	9,12,15-Octadecatrienoic acid	5.24±0.24^{a}	10.86±1.15^{b}	8.55±0.83^{c}	2.44±0.03^{A}	2.20±0.04^{B}	2.13±0.01^{C}
36	5,8,11,14,17-Eicosapentaenoic acid	2.70±0.06^{a}	6.72±0.74^{b}	6.19±0.59^{b}	11.29±0.39^{A}	10.90±0.28^{A}	10.05±0.06^{B}
41	4,7,10,13,16,19-Docosahexaenoic acid	1.57±0.02^{a}	2.41±0.20^{b}	2.43±0.11^{b}	5.71±0.09^{A}	6.63±0.20^{B}	6.15±0.12^{C}
42	7,10,13,16,19-Docosapentaenoic acid	1.59±0.02^{a}	2.44±0.17^{b}	2.42±0.13^{b}	4.18±0.03^{A}	3.99±0.09^{B}	3.90±0.03^{B}
43	4,7,10,13,16-Docosapentaenoic acid	*n.d.*	*n.d.*	*n.d.*	2.48±0.02A	2.89±0.05B	2.76±0.03

Table 6.3. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings after alkaline hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
	Aliphatic alcohols	*23.80±1.32^{a}*	*35.71±1.46^{b}*	*35.14±0.30^{b}*	*33.20±1.19^{A}*	*31.29±0.32^{B}*	*31.03±0.14^{B}*
2	Decanol	1.25±0.00^{a}	1.69±0.01^{b}	1.63±0.03^{ab}	1.63±0.04^{A}	1.65±0.02^{A}	1.64±0.01^{A}
4	Undecanol	1.25±0.01^{a}	1.79±0.03^{b}	1.63±0.03^{ab}	1.66±0.03A^{B}	1.69±0.03^{A}	1.64±0.02^{B}
7	Dodecanol	1.25±0.01^{a}	1.75±0.03^{b}	1.63±0.03^{c}	1.64±0.03^{A}	1.66±0.02^{A}	1.64±0.01^{A}
9	Tridecanol	1.27±0.01^{a}	1.93±0.05^{b}	1.66±0.04^{c}	1.72±0.01^{A}	1.78±0.02^{B}	1.69±0.02^{A}
10	Tetradecanol	1.28±0.02^{a}	1.75±0.03^{b}	1.71±0.01^{c}	1.68±0.05^{A}	1.68±0.02^{A}	1.66±0.01^{A}
15	Hexadecanol	2.29±0.01^{a}	3.10±0.19^{b}	3.25±0.09^{b}	3.23±0.25^{A}	2.97±0.03^{A}	2.63±0.03^{B}
24	Octadece-9-nol[1]	3.19±0.06^{a}	4.26±0.26^{b}	4.62±0.12^{c}	6.42±0.38^{A}	4.06±0.07^{B}	3.67±0.04^{B}
26	Octadecanol	1.95±0.03^{a}	2.58±0.11^{b}	2.76±0.02^{c}	2.60±0.17^{A}	2.52±0.03^{AB}	2.37±0.03^{B}
28	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3.19±0.09^{a}	6.45±0.60^{b}	5.80±0.30^{b}	5.65±0.08^{A}	6.20±0.04^{B}	5.50±0.04^{C}
37	Eicosanol	1.28±0.01^{a}	1.69±0.02^{b}	1.66±0.03^{b}	*n.d.*	*n.d.*	1.66±0.01
44	Docosanol	1.25±0.01^{a}	1.66±0.02^{b}	1.64±0.03^{b}	1.63±0.05^{A}	1.65±0.02^{A}	1.64±0.01^{A}
56	Octacosanol	1.41±0.03^{a}	1.86±0.05^{b}	1.93±0.03^{b}	1.88±0.06^{A}	1.92±0.04^{A}	1.85±0.01^{A}
66	Octacosane-1,3-diol	1.27±0.02^{a}	1.70±0.02^{b}	1.71±0.02^{b}	*n.d.*	*n.d.*	*n.d.*
68	Tricosanol	1.35±0.04^{a}	1.80±0.04^{b}	1.84±0.04^{b}	1.80±0.04^{A}	1.83±0.04^{A}	1.78±0.01^{A}
74	Dotriacontanol	1.27±0.01^{a}	1.69±0.01^{b}	1.67±0.03^{b}	1.65±0.04^{A}	1.68±0.02^{A}	1.65±0.02^{A}
	Sterols	*1.83±0.07^{a}*	*3.14±0.36^{b}*	*3.31±0.10^{b}*	*13.12±0.45^{A}*	*16.96±0.97^{B}*	*14.40±0.94^{A}*
54	22-Stigmasten-3-one	*n.d.*	*n.d.*	*n.d.*	0.28±0.01^{A}	0.30±0.01^{AB}	0.31±0.01^{B}
57	24*β*-Methylcholesta-5,22E-dien-3*β*-ol	0.73±0.01^{a}	1.67±0.25^{b}	1.60±0.03^{b}	*n.d.*	*n.d.*	*n.d.*
58	24*α*-Methylcholest-5-en-3*β*-ol	0.24±0.01^{a}	0.31±0.02^{b}	0.33±0.02^{b}	0.61±0.00^{A}	0.74±0.03^{B}	0.68±0.07^{AB}
60	24*β*-Ethylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.33±0.01^{A}	0.35±0.01^{A}	0.37±0.05^{A}
61	24*α*-Ethylcholesta-5,22E-dien-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	4.22±0.18^{A}	6.64±0.46^{B}	5.45±0.38^{C}
62	24-Ethyl-δ(22)-coprostenol	*n.d.*	*n.d.*	*n.d.*	0.53±0.02^{A}	0.71±0.08^{B}	0.62±0.06^{AB}
63	24*α*-Ethylcholest-5-en-3*β*-ol	0.62±0.07^{a}	0.83±0.08^{b}	1.05±0.05^{c}	1.32±0.05^{A}	1.36±0.07^{A}	1.16±0.09^{B}
64	Stigmasta-3,5-dien-7-one	0.24±0.01^{a}	0.33±0.02^{b}	0.34±0.02^{b}	0.41±0.02^{A}	0.40±0.03^{A}	0.37±0.02^{A}
65	4*α*,24-Dimethyl-5*α*-cholestan-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.83±0.03^{AB}	0.92±0.07^{A}	0.76±0.05^{B}

Table 6.3. Lipid profile (mg g^{-1} of dry weight) of *Hemiselmis* cf. *andersenii* and *Pavlova pinguis* grown in different initial phosphorus loadings after alkaline hydrolysis. (Continuation)

Nº	Identified Compounds	*Hemiselmis* cf. *andersenii*			*Pavlova pinguis*		
		Low	Medium	High	Low	Medium	High
67	Unidentified Sterol	*n.d.*	*n.d.*	*n.d.*	1.17±0.04^{A}	1.65±0.08^{B}	1.29±0.05^{A}
69	4*α*-methyl,24-ethyl-5*α*-cholest-22E-en-3*β*-ol	*n.d.*	*n.d.*	*n.d.*	0.78±0.03^{A}	0.79±0.03^{A}	0.66±0.04^{B}
70	4*α*-methyl,24-ethyl-5*α*-cholestan-3-ol	*n.d.*	*n.d.*	*n.d.*	0.39±0.01^{A}	0.44±0.02^{B}	0.41±0.00^{A}
72	4*α*,24*β*-dimethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	*n.d.*	*n.d.*	*n.d.*	0.42±0.01^{A}	0.48±0.02^{B}	0.42±0.01^{A}
73	Unidentified Sterol	*n.d.*	*n.d.*	*n.d.*	1.02±0.21^{A}	1.01±0.07^{A}	1.05±0.12^{A}
75	4*α*-methyl-24*β*-ethyl-5*α*-cholestan-3*β*,4*β*-diol[2]	*n.d.*	*n.d.*	*n.d.*	0.80±0.04^{A}	1.16±0.07^{B}	1.02±0.01^{C}
	Monoglycerides	*0.10±0.01^{a}*	*0.23±0.05^{b}*	*0.31±0.09^{b}*	*0.10±0.01^{A}*	*0.13±0.02^{A}*	*0.11±0.02^{A}*
46	2,3-Dihydroxypropyl palmitate	0.03±0.01^{a}	0.03±0.01^{a}	0.06±0.01^{b}	0.05±0.00^{A}	0.06±0.01^{A}	0.05±0.01^{A}
48	3-Octadecoxypropane-1,2-diol	0.05±0.01^{a}	0.16±0.04^{b}	0.22±0.08^{b}	0.03±0.00^{A}	0.04±0.01^{B}	0.03±0.00^{AB}
51	2,3-Dihydroxypropyl stearate	0.02±0.00^{a}	0.04±0.01^{b}	0.03±0.01ab	0.02±0.01A^{B}	0.03±0.01^{A}	0.02±0.00^{B}
	Others	*0.32±0.01^{a}*	*0.34±0.11^{a}*	*0.51±0.09^{b}*	*1.37±0.14^{A}*	*1.25±0.18^{A}*	*0.97±0.05^{B}*
5	2,6-bis(1,1-Dimethylethyl)phenol	0.02±0.00^{a}	0.12±0.04^{b}	0.04±0.01^{a}	0.31±0.01^{A}	0.14±0.02^{B}	0.11±0.01^{B}
13	Rhamnose	0.30±0.00^{a}	n.d.	0.40±0.01^{b}	0.41±0.01^{A}	0.41±0.01^{A}	0.41±0.01^{A}
35	Dehydroabietic acid	*n.d.*	0.22±0.08^{a}	0.19±0.01^{a}	0.76±0.06^{A}	0.70±0.15^{A}	0.41±0.05^{B}
55	*α*-Tocopherol	*n.d.*	*n.d.*	*n.d.*	0.03±0.01^{A}	*n.d.*	0.04±0.01^{A}
	Total	*70.18±1.07^{a}*	*116.84±6.84^{b}*	*111.89±3.81^{b}*	*180.59±4.69^{A}*	*183.79±4.73^{A}*	*165.85±2.07^{B}*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different (p <0.05). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *H.* cf. *andersenii*, and by superscript uppercase letters for *P. pinguis*. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains cis and trans isomers. [2]Identified as the mono-TMS ether. *n.d.* – non detected.

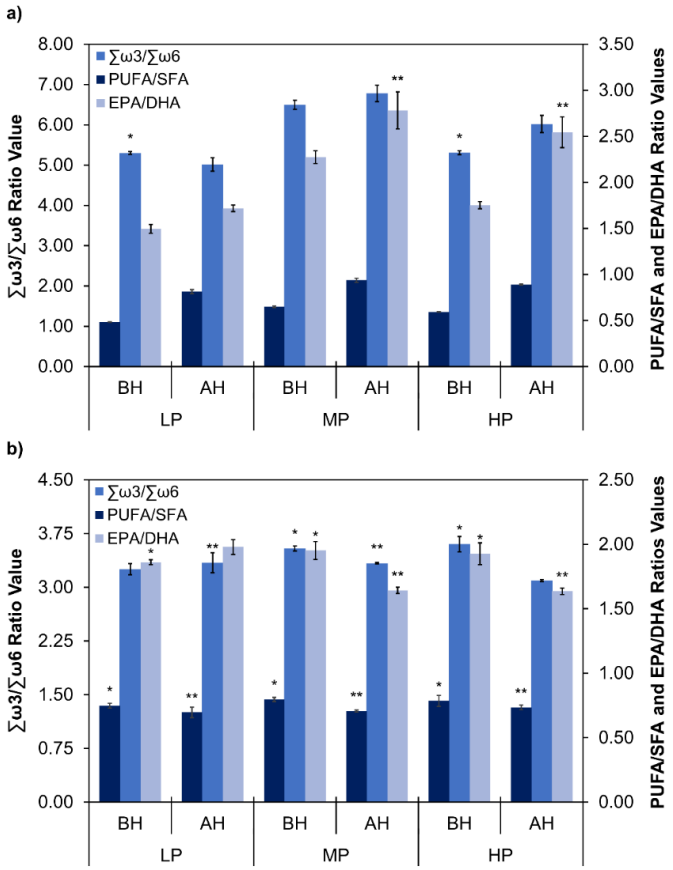

Figure 6.4. Variations in fatty acids nutritional ratios for **a)** *Hemiselmis* cf. *andersenii* and **b)** *Pavlova pinguis* as a response to phosphorus-induced changes (LP - Low phosphorus; MP – Medium phosphorus and HP - High phosphorus supplementations), before (BH) and after (AH) alkaline hydrolysis. Values from the same nutritional ratio sharing common symbols (* before hydrolysis, and ** after hydrolysis) are not significantly different (p >0.05) among treatments.

Decreasing dietary SFA intake and replacing it with PUFA has been mentioned as the main dietary strategy for humans to benefit from PUFA preventive and protective activities against chronic inflammatory, cancer, diabetes, atherogenesis, and Alzheimer's disease [281,282]. Since PUFA differ in their physiological functions, the balance between the ω3 and ω6 fatty acids, along with EPA and DHA should be considered simultaneously [282]. Thus, the ability of *H.* cf. *andersenii* to adjust its PUFA/SFA, ∑ω3/∑ω6, and EPA/DHA constitutes a great advantage for modulating its composition according with the supplementation purposes. Moreover, the study of the nutritional ratios before and after alkaline hydrolysis in both microalgae allow to see which strategies can be better employed to fully exploit microalgae potential for PUFA production.

In both microalgae studied the main aliphatic alcohols were hexadecanol, octadece-9-nol and 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (phytol). Phytol and chlorophyll a content presented a similar trend across phosphorus treatments for *H.* cf. *andersenii* after hydrolysis, with the greatest phytol (6.45 mg g^{-1} dw) content being observed at MP conditions as with chlorophyll a (7.49 mg g^{-1} dw). This result agrees with a previous study performed by Fernandes et al. [266] for this microalga where the positive relation between phytol and chlorophyll a was also

observed. The previous trend was not observed for *P. pinguis* possibly for having in its composition other phytol-derived products such as vitamins K and E, like *Chlorella stigmatophora*. In *H.* cf. *andersenii* samples a very strong positive correlation (> 0.95) was found between phytol and the following fatty acids: tetradecanoic acid (C14:0), C16:0, ALA, .11-octadecenoic acid (C18:1ω7), DHA, and 7,10,13,16,19-docosapentaenoic acid (C22:5ω3). While for *P. pinguis* a strong positive correlation (> 0.90) was found between phytol, EPA and C14:0.

Sterols, carotenoids, and phytol synthesis, initiate with the biosynthesis of the isoprenoids precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [87]. For *P. pinguis* the lowest amount (13.12 mg g^{-1} dw) of sterols was observed at LP conditions, in contrast to its carotenoid (4.32 mg g^{-1} dw). Since phosphorus is an elemental constituent of isoprenoid precursors, the previous observation may indicate that at LP conditions *P. pinguis* promote the accumulation of carotenoids (photoprotective pigments) at the expense of sterols (membrane components). For *H.* cf. *andersenii* the lowest amount (1.83 mg g^{-1} dw) of sterols was also verified at LP conditions. As with the present study, the decrease in sterol content as a response to low phosphorus environments have been reported to occur in other microalgae (Chlorophyta *Scenedesmus quadricauda*, and *Chlamydomonas globosa*, Cryptophyta *Cryptomonas ovata*, Bacillariophyta *Cyclotella meneghiniana*) [106,107].

Marine microalgae have developed efficient strategies to cope with poor phosphorus environments by remodeling membrane lipids in phosphorus-free lipids (such as betaine lipid and triacylglycerols) instead of phospholipids [267,277,278]. For both microalgae the greatest amounts of monoglycerides were verified for the treatments with low phosphorus loadings (LP and MP treatments). This could indicate that at LP conditions both *H.* cf. *andersenii* and *P. pinguis* tend to accumulate neutral lipids such as triacylglycerols. Moreover, campestenyl and campesteryl glycosides were only detected for *P. pinguis* samples (Table 6.2), and the greatest amounts were found at LP conditions, where the highest monosaccharide levels (180.27 mg g^{-1} dw) were observed. As with phospholipids, glycosyl sterols are amphiphilic molecules which can be used by microalgal cells to maintain the organization and fluidity of membranes [266]. Therefore, the previous observations seem to indicate that both microalgae responded to LP conditions by synthesizing non-phosphorus containing lipids to offset the degradation of phospholipids.

3.2.1. Multivariate analysis of lipid composition

Compositional changes were verified for both microalgae submitted to LP, MP, and HP conditions. Thus, to summarize the overall changes in metabolites a principal component analysis (PCA) was performed to lipid data. Through this analysis it is possible to visualize a clear separation between the lipidomic analysis for *H.* cf. andersenii (Fig. 6.5a) and *P. pinguis* (Fig. 6.5c) LP, MP, and HP cultures before and after alkaline hydrolysis. Principal component 1 (PC1) explained 67.4% of variance for *H.* cf. *andersenii* samples and 55.2% of variance for *P. pinguis*. This component clearly separates samples before (left quadrant) and after alkaline

hydrolysis (right quadrant) in both microalgae studied. Principal component 2 (PC2) explained 15.2% of variance for *H.* cf. andersenii and 16.6% for *P. pinguis* and separated samples according with the phosphorus-induced changes. For *H.* cf. *andersenii* samples microalgae grown in LP conditions are distributed in the upper quadrant, while MP and HP conditions are both in the down quadrant. An opposite distribution was verified for *P pinguis* samples with samples at LP conditions being distributed in the down quadrant and the MP and HP treatments being observed at the upper quadrant.

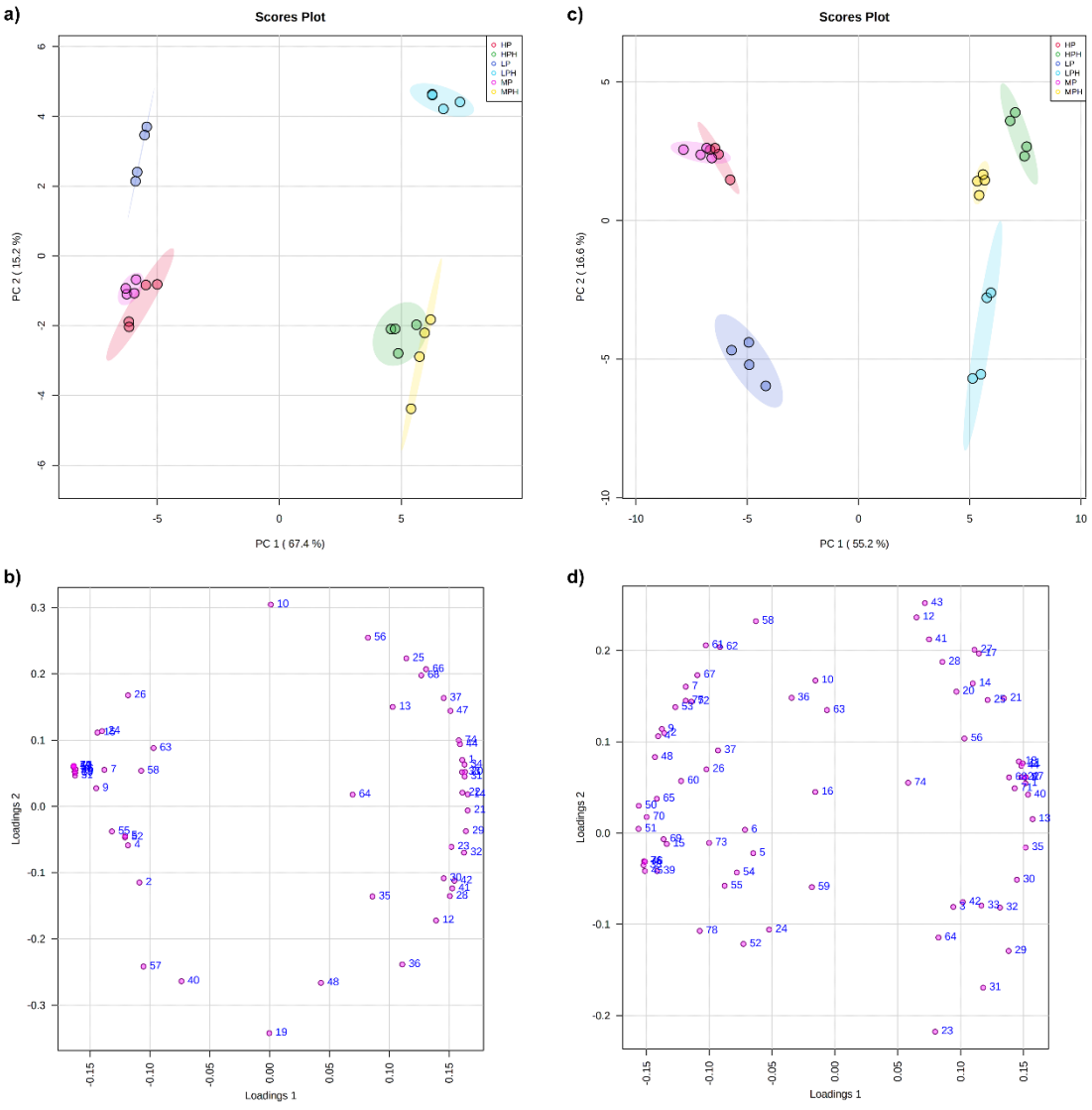

Figure 6.5. Principal component analysis performed for the microalgae studied: **a)** scores plot of *Hemiselmis* cf. *andersenii,* **b)** loadings plot of *Hemiselmis* cf. *andersenii*, **c)** scores plot of *Pavlova pinguis*, **d)** loading plot of *Pavlova pinguis*. HP, MP, and HP stand for *H*. cf. *andersenii* and *P. pinguis* grown under low, medium, and high phosphorus supplementations, respectively, and analyzed before hydrolysis. LPH, MPH, HPH stands for *H.* cf. *andersenii* and *P. pinguis* grown under low, medium, and high phosphorus supplementations, respectively, and analyzed after hydrolysis. Loading's descriptions can be seen in Tables 6.2 and 6.3.

Loadings plots allow to visualize which variables had more influence on samples differentiation. For *H.* cf. *andersenii* (Fig. 6.5b) samples within the loadings most related to MP and HP conditions before hydrolysis were 24*β*-methylcholesta-5,22E-dien-3*β*-ol (brassicasterol; 57), *α*-Tocopherol (55), and C16:2w4 (19) fatty acid, while after hydrolysis phytol (28), and the main fatty acids, C14, essential fatty acids (29, 30), EPA (36), DHA (41) and C16:0 (23) were the greatest contributors for its distribution among PCA scores. The separation of LP cultures to MP and HP conditions was mostly due to monoglycerides (2,3-dihydroxy-2-propanyl myristate (39), 1,3-dihydroxypropyl myristate (38), 2,3-dihidroxy-2-propanyl palmitate (46) and 1,3-dihydroxypropyl palmitate (45)) and the main aliphatic alcohols hexadecanol (15), and octadecene-9-ol (24). After alkaline hydrolysis the monounsaturated fatty acids and long-chain aliphatic alcohols were among the loadings that contributed to its separation.

Cholesterol-lowering and anti-aging activities have been described for Brassicasterol derived from Cryptophyta [235]. To produce this sterol by *H.* cf. *andersenii* phosphorus- replete conditions such those in MP and HP conditions should be used. ALA and 9,12-octadecadienoic acid (LA; C18:2ω6) are two essential fatty acids that are needed for normal growth and development of animals; however, they lack the ability to synthesize these precursors of PUFA (DHA and EPA), which, in turn, makes its dietary intake mandatory. Thus, the rich composition of *H.* cf. *andersenii* saponifiable lipids in essential fatty acids (ALA and LA), and long-chain PUFA (EPA and DHA) at MP and HP cultures, reinforces that phosphorus replete conditions are good to boost *H.* cf. *andersenii* composition in health-promoting lipids.

For *P. pinguis* (Fig. 6.5d) at LP conditions steryl glycosides (76, 78), *α*-tocopherol (55), and four of the seven monoglycerides (2,3-dihidroxy-2-propanyl palmitate (46), 1,3-dihydroxypropyl palmitate (45), 2,3-dihidroxy-2-propanyl stearate (51) and 1,3-dihydroxypropyl stearate (50)) detected for *P. pinguis* were among the loadings that contributed to the separation of this set from the remaining. MP and HP conditions were more related to most sterols, EPA, octadecanol and tetradecanol. After hydrolysis *P. pinguis* at LP conditions were mainly related to saturated and unsaturated C18 fatty acids, hexadecanoic acid and clupadonic acid. While MP and HP conditions exhibited preference towards long-chain alcohols, phytol, DHA, unsaturated C16 fatty acids.

24*α*-Ethylcholesta-5,22E-dien-3*β*-ol (stigmasterol; 61), 24*α*-methylcholest-5-en-3*β*-ol (campesterol; 58), and 24*α*-ethylcholest-5-en-3*β*-ol (sitosterol; 63) have attracted commercial interest in the pharmaceutical, nutraceutical, and functional food industries [283]. These 4-desmethyl sterols have well-established cholesterol-lowering properties, as well as other bioactivities such as anti-cancer and anti-inflammatory [67]. All these three sterols are produced by *P. pinguis* and presented its highest amounts at MP and HP conditions (Table 6.2).

Within lipid-soluble compounds, tocopherols are often referred as an underexploited resource from microalgae [24]. *α*-Tocopherol is specially known for its antioxidant activity and health properties such as prevention of light-induced pathologies, degenerative disorders, cardiovascular diseases, and cancer [284]. In the industrial field this compound is used in the preservation of food, cosmetics and as additive in animal field. Thus, for *α*-tocopherol

production the best conditions in the present study were MP and HP conditions before hydrolysis for *H.* cf. *andersenii*, and LP conditions for *P. pinguis*. Mudimu et al. [265] performed a screen of microalgae strains for *α*-tocopherol content and studied the influence of nitrate reduction on *α*-tocopherol production. In this study, *α*-tocopherol content in microalgae ranged between 20.52 and 1445.66 µg g^{-1} dw, and nutrient reduction lead to an increase in *α*-tocopherol content. In this study, *P. pinguis* presented the highest amount of this molecule accounting 170 µg g^{-1} dw at LP conditions.

For *H.* cf. *andersenii*, hierarchical cluster analysis (Fig. 6.6a) showed the same trends displayed by principal component analysis, with LP treatments showing the deepest lipid remodeling when compared to MP and HP conditions before and after alkaline hydrolysis. In *P. pinguis* (Fig. 6.6b) after hydrolysis LP and MP conditions are closely related according to hierarchical cluster analysis. This trend is different from that displayed by PCA. This may be derived from *P. pinguis* cultured at LP and MP conditions presented the lowest content of unsaponifiable material, with the greatest increase of total identified compounds with alkaline hydrolysis being verified at LP conditions (31%).

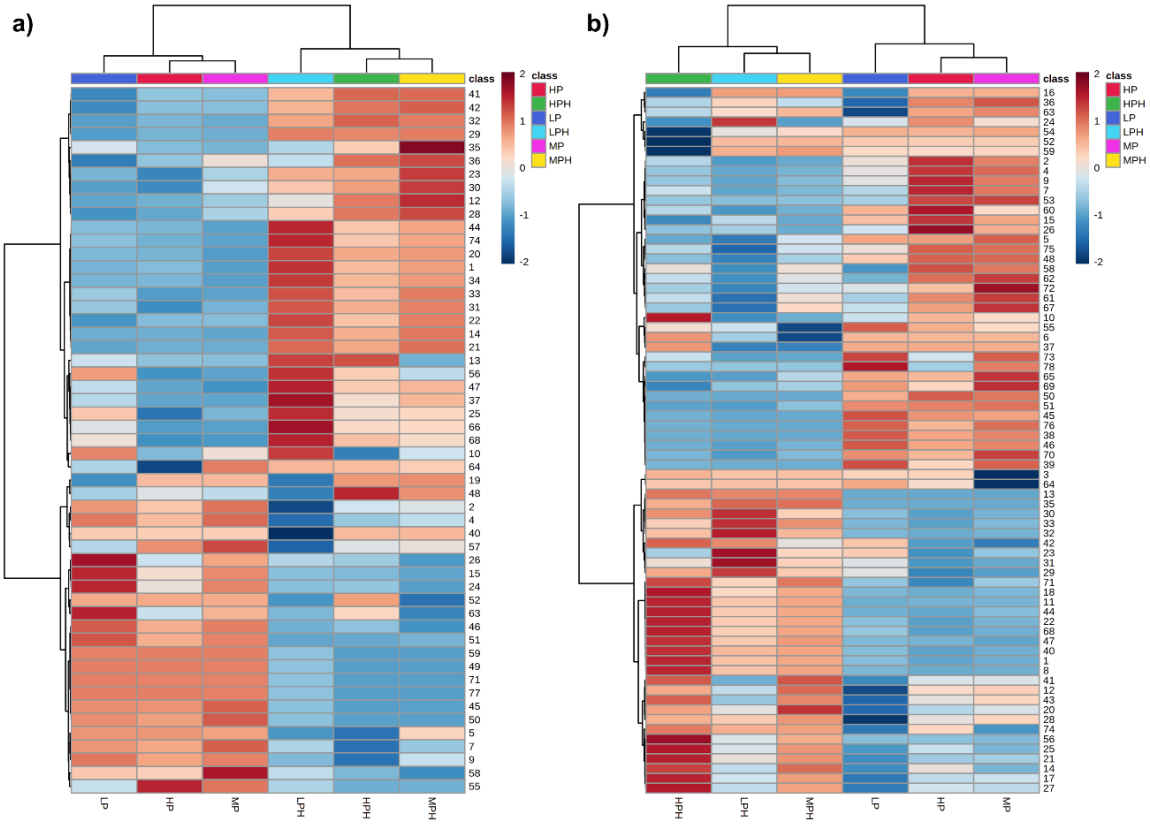

Figure 6.6. Hierarchical clustering analysis heat maps based on Euclidean clustering distance and the ward clustering method, n=4 replicates, for **a)** *Hemiselmis* cf. *andersenii* and **b)** *Pavlova pinguis* grown under different phosphorus supplementations. Feature descriptions can be seen in Tables 6.2 and 6.3. The heat maps reflect the relative levels of metabolites among different treatment groups, the color scheme is associated with the elevation and reduction in metabolite level through treatments: Dark blue, lowest; Dark red, highest. HP, MP, and HP stand for *H.* cf. *andersenii* and *P. pinguis* grown under low,

medium, and high phosphorus supplementations, respectively, and analyzed before hydrolysis. LPH, MPH, HPH stands for for *H.* cf. *andersenii* and *P. pinguis* grown under low, medium, and high phosphorus supplementations, respectively, and analyzed after hydrolysis.

4. Conclusions

This work provided novel insights on the response of marine microalgae to phosphorus-induced changes and its potential as a tool for optimizing high-value lipids production. Increasing initial phosphorus concentrations enhanced *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* biomass productivities, making these conditions suitable for efficient high-value compounds production. Monosaccharide data, along with lipid/monosaccharide ratio indicate that *P. pinguis* responded to low phosphorus conditions by synthesizing a *β*-1,3-glucan, while *H.* cf. *andersenii* seemed to divert its carbon to lipids production regardless of the treatment applied. In *P. pinguis* the Car/Chl *a* could be a simple indicator for determining carbon allocation among carbon-rich pools. In *H.* cf. *andersenii* the co-production of carotenoids and lipids against phosphorus-induced changes, constitutes a major advantage for microalgae-based industries. Phosphorus-induced changes showed to be an effective tool for inducing lipid remodelling in *P. pinguis* and *H.* cf. *andersenii*, with phosphorus-replete conditions being the most effective to induce the accumulation of high-value lipids in both microalgae.

CHAPTER 7.

Conclusions and future perspectives

Chapter 7. Conclusions and future perspectives

Increasing concerns regarding consumer safety, and environmental sustainability has turning consumers attention towards microalgae-derived products. In the biotechnological exploitation of microalgae as natural reactors of high value compounds the following challenges are found: the cultivation of new species, the identification of new molecules, and the optimization of culture conditions to produce the desired phytochemicals. Therefore, the main purpose of this thesis was to investigate the potential of the unexplored microalgae *Pavlova pinguis* and *Hemiselmis* cf. *andersenii* as matrices for the bioprospection of high-value lipids. With this study the main conclusions drawn were:

- The wide range of high-value lipid compounds produced by *Pavlova pinguis* makes this microalga a promising candidate for nutraceutics and pharmaceutics purposes. Meanwhile, *Hemiselmis* cf. *andersenii* was shown to be a rich source of ω3-polyunsaturated fatty acids (PUFA).
- The untargeted analysis of microalgal lipid composition revealed several species-specific metabolites, namely dihydroxylated sterols (*P. pinguis*), and brassicasterol (*H.* cf. *andersenii*), which could be useful as biomarkers.
- Lipid analysis before and after alkaline hydrolysis demonstrated to be important for analyzing the desired product availability (non-esterified or esterified), which influences the selection of microalgae and the processing strategies to be used by the industry.
- Both *P. pinguis* and *H.* cf. *andersenii* presented long-chain aliphatic alcohols in its lipid composition. Sugarcane is often used as source of these compounds which are commercialized as lipid-lowering supplements and are often designated as policosanols. However, few studies address microalgae as natural sustainable sources of long-chain aliphatic alcohols which can be further commercialized.
- In contrast to other microalgae that accumulate lipids in growth-limiting conditions, the greatest amounts of lipids in both *P. pinguis* and *H.* cf. *andersenii* were found at nitrogen-replete conditions without compromising microalgal growth. Which constitutes a major advantage for high-value lipids production by microalgae-based industries.
- In *H.* cf. *andersenii* the strong positive correlation between phytol and chlorophyll show that phytol may be used as a good indicator of the Chlorophyll content. However, this is not applicable to *P. pinguis*.
- For *P. pinguis* the Car/Chl a ratio could be a simple means to assess carbon allocation among carbohydrates and lipid pools. Low phosphorus conditions were shown to be a cost-effective strategy to produce carotenoids, glycosyl sterols, and *α*-tocopherol from *P. pinguis*. Phosphorus-replete conditions lead to increased biomass activities and overall high-value lipids accumulation in *H.* cf. *andersenii* and *P. pinguis*.

- *P. pinguis* and *H.* cf. *andersenii* growth dynamics and chemoplasticity under nutrient stress highlight their ability to readily acclimate to changes in their growth conditions. The similar trends of carotenoids and lipids verified for *H.* cf. *andersenii* regardless the treatment applies, shows that this microalga could be effective for the co-production of carotenoids and lipids.
- The induced changes in microalgae growth media affected the nutritional quality of microalgal lipids.

The current focus of algae and microalgae-based industries is on the production of high-value lipids. Through this thesis, and as a general conclusion, *P. pinguis* and *H.* cf. *andersenii* have shown to be promising candidates for high-value lipids exploitation, having the advantage of lacking heavy cell wall structures, which makes these microalgae easier to break down and process for commercial purposes than *Chlorella* species. In this sense, the diversity observed on the lipid composition of *P. pinguis* and *H.* cf. *andersenii* should be further exploited for nutraceutic and pharmaceutic purposes.

As future perspectives, other experiments are ongoing such as the evaluation of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR), nuclear magnetic resonance spectroscopy (NMR), and thermogravimetric analysis (TGA) as robust tools to perform the simultaneous analysis of complex mixtures of metabolites. Moreover, to support the use of the high-value lipids for pharmaceutical and nutraceutical purposes, the safety for human consumption of both microalgae will be determined through a detailed toxicological analysis and its lipid extracts will be assessed for their biological activities.

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