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Study programme: Ecology

Mgr. Antonín Střížek

Glycerolipids and carotenoids in microalgae: the implications in ecophysiology and applied phycology

Glycerolipidy a karotenoidy v mikrořasách: význam v ekofyziologii a aplikované algologii

Doctoral Thesis

Supervisor: doc. RNDr. Linda Nedbalová, Ph.D.

Prague, 2024

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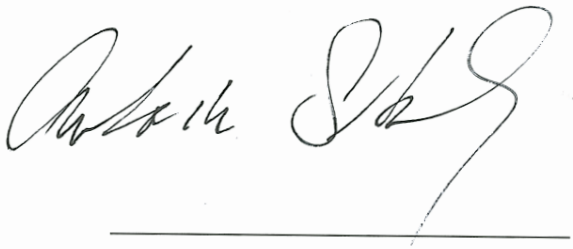

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ABSTRACT

Lipids are basic biomolecules found in all organisms. They have a key function as structural molecules forming cell membranes, and, in the form of fats and oils, energy is also stored. There are a huge number of lipid types that have other functions, for example, in cell signaling, enzyme support, protection against stress, and others. Microscopic algae are the main primary producers in both freshwater and marine ecosystems. Therefore, algal biosynthesis has a fundamental effect on the trophic networks of aquatic ecosystems and ultimately on humans. The environment affects the ecophysiology of algae, which is reflected in their biochemical composition, i.e. in the composition of their lipids. This work is focused on two groups of lipids, namely glycerolipids, which consist of fatty acids, and carotenoids, which are photosynthetic pigments with antioxidant and photoprotective properties. In these groups of lipids, the target substances were further selected, namely polyunsaturated fatty acids and the carotenoid fucoxanthin belonging to xanthophylls. These substances are important not only in the ecophysiology of algae, but they are valuable substances that have positive effects on the human organism and, with the help of optimized cultivation, could be obtained from algae for industrial production under certain conditions.

Although much attention has been paid to this research direction for a long time, there are still many gaps in our knowledge, some of which this Ph.D. thesis tries to fill. Its core is formed by seven scientific publications as well as several outputs from applied research. The work deals with different species and groups of algae with different origins and ecology. In addition to the selected target substances, the unifying element is also the methodological approach, i.e. the implementation of extensive manipulative multi-parametric experiments. In this way, it is possible to monitor the influence of individual biological factors (geographical origin or taxonomic affiliation) or abiotic factors (temperature, light intensity, or composition of the culture medium) on the ecophysiology of selected algae strains manifested at the level of selected target substances.

The greatest attention was paid to the freshwater flagellated alga *Hibberdia magna* (Chrysophyceae), in which we studied in detail its reactions to temperature and lighting with regard to the productivity of biomass and selected valuable substances. Fucoxanthin productivity was optimal under different conditions than biomass and polyunsaturated fatty acid productivity. This work presents the first insight into the biotechnological potential of this poorly explored species, which can serve as a model organism for future studies of photoautotrophic chrysophytes.

In another study, we compared the alga *Hibberdia magna* with the species *Chlorochromonas danica*, which belongs to the same class but differs in its trophic strategy (mixotrophy). The presence of light stimulated the increase in fucoxanthin productivity, the content of which was minimal in the dark. Both species had similar fucoxanthin productivity under optimal conditions, which was comparable to other fucoxanthin-producing strains. Both organisms were positively evaluated as candidates for the multi-target biorefinery concept.

In the third article, we focused on freshwater diatoms, another group that is not so well known in terms of manipulative culture experiments and lipid composition. Based on the incubation of 11 newly isolated strains from different climatic zones (tropical, temperate, polar) at different temperatures, we found clear trends in their fatty acid profiles. Surprisingly, the geographic origin of the strains had a comparable influence as the temperature of cultivation, and taxonomic affiliation was similarly significant. The most sensitive to temperature changes were the polar strains, which were also characterized by the highest average proportion of polyunsaturated fatty acids compared to the temperate and tropical strains.

In another work, we tested an Antarctic strain of the green alga from the genus *Monoraphidium* for the production of polyunsaturated fatty acids on a pilot scale. From the point of view of the year-round use of cultivation capacities in the temperate zone, the cultivation of psychrotolerant strains can be advantageous. The tested strain was isolated from a frozen lake and, in addition to the shifted growth optimum to low temperatures, it was also characterized by low light requirements and increased content of polyunsaturated fatty acids compared to other members of this genus. Cultivation on a thin-layer platform in the winter season proved that this strain is promising.

Other organisms studied in terms of the influence of conditions on lipid composition were five strains from the Haptophyta group. Using reversed-phase high-performance liquid chromatography (NARP-HPLC) with atmospheric pressure chemical ionization (APCI), we characterized the composition of their triacylglycerols at different salinity levels, with an emphasis on 18-carbon polyunsaturated fatty acids. Salinity affected the proportion of saturated and unsaturated fatty acids, as well as their composition in terms of regioisomers.

The other two works were devoted to methodological aspects of algal biotechnology. It involved optimizing the extraction and purification of fucoxanthin from the biomass of the marine diatom *Phaeodactylum tricornutum* using high-performance countercurrent chromatography (HPCCC), which consistently preserved its biological activities. Furthermore, we focused on the comparison of different approaches for the early detection of contamination of algal cultures using the example of a culture of the diatom *Phaeodactylum tricornutum* contaminated with the alga *Chlorella* sp. The use of PCR and qPCR methods proved to be the most reliable, detecting contamination already at a level of around 75 cells/ml, and therefore appear to be the best approach for the early detection of contamination in algal cultures.

Last but not least, the work also includes outputs from applied research. The first of them is the presentation of a functional prototype of the cultivation device, which was developed as part of the doctoral study, and with its help experiments were carried out, which are published as part of the main results of this work. Furthermore, the outputs from the semi-operational cultivations of the algae *Hibberdia magna* and *Phaeodactylum tricornutum* are presented. This work was carried out in cooperation with the Czech company Algamo s.r.o., which deals with the commercial cultivation of microalgae.

ABSTRAKT

Lipidy jsou základní biomolekuly, které se vyskytují ve všech organismech. Mají nezastupitelnou funkci jako strukturální molekuly tvořící buněčné membrány a v podobě tuků a olejů je také ukládána zásobní energie. Lipidů je obrovské množství typů, které mají další funkce například v buněčné signalizaci, podpoře enzymů, ochraně vůči stresům a jiné. Mikroskopické řasy jsou hlavními primárními producenty ve sladkovodních i mořských ekosystémech. Řasová biosyntéza má tedy zásadní vliv na trofické sítě vodních ekosystémů a konečném důsledku i na člověka. Prostředí ovlivňuje ekofyziologii řas, což se projevuje v jejich biochemickém složení, tedy i ve složení jejich lipidů. Tato práce je zaměřena na dvě skupiny lipidů a to glycerolipidy, které se skládají z mastných kyselin, a karotenoidy, což jsou fotosyntetické pigmenty s antioxidačními a fotoprotektivními vlastnostmi. V těchto skupinách lipidů byly dále vybrány cílové látky, a to polynenasycené mastné kyseliny a karotenoid fukoxantin patřící mezi xantofyly. Tyto látky mají význam nejen v ekofyziologii řas, ale jsou to cenné látky, které mají pozitivní účinky na lidský organismus a pomocí optimalizované kultivace by mohly být za určitých podmínek získávány z řas pro průmyslové účely.

Přestože je této problematice dlouhodobě věnována velká pozornost, stále existuje mnoho mezer v našich znalostech, z nichž některé se snaží vyplnit tato doktorská práce. Jejím jádrem je sedm přiložených vědeckých publikací a také několik výstupů z aplikovaného výzkumu. Práce se zabývá různými druhy i skupinami řas s různým původem a ekologií. Jednotícím prvkem je kromě vybraných cílových látek také metodický přístup, tedy provádění rozsáhlých manipulativních multi-parametických experimentů. Tímto způsobem je možné izolovaně sledovat vliv jednotlivých biologických faktorů (geografický původ nebo taxonomická příslušnost) nebo abiotických faktorů (teplota, intenzita osvětlení nebo složení kultivačního média) na ekofyziologii vybraných kmenů řas projevující se na úrovni vybraných cílových látek.

Největší pozornost byla věnována sladkovodní bičíkaté řase *Hibberdia magna* (Chrysophyceae), u které jsme podrobně sledovali její reakce na teplotu a osvětlení s ohledem na produktivitu biomasy a vybraných cenných látek. Produktivita fukoxantinu byla optimální v jiných podmínkách, než byla optimální produktivita biomasy a polynenasycených mastných kyselin. Tato práce představuje první vhled do biotechnologického potenciálu tohoto málo prozkoumaného druhu, který může sloužit jako modelový organismus pro budoucí studie fotoautotrofních zlativek.

V další studii jsme řasu *Hibberdia magna* srovnávali s druhem *Chlorochromonas danica*, který patří do stejné třídy, ale liší se trofickou strategií (mixotrofie). Přítomnost světla stimulovala zvýšenou produktivitu fukoxantinu, jehož obsah byl ve tmě minimální. Oba druhy měly v optimálních podmínkách podobnou produktivitu fukoxantinu, která byla srovnatelná s jinými fukoxantin produkujícími kmeny. Oba organismy byly pozitivně hodnoceny z hlediska biorafinace mikrořasové biomasy s obsahem většího množství cenných látek.

Ve třetí práci jsme se zabývali sladkovodními rozsivkami, což je další skupina, která není z hlediska manipulativních kultivačních experimentů a složení lipidů tolik známá. Na základě inkubace 11 nově vyizolovaných kmenů z různých klimatických zón (tropické, temperátní, polární) v různých teplotách jsme zjistili průkazné trendy projevující se v profilech jejich mastných kyselin. Srovnatelný vliv jako teplota kultivace měl překvapivě geografický původ kmenů, podobně významná byla též taxonomická příslušnost. Nejvíce citlivé na změny teploty byly polární kmeny, které se také vyznačovaly největším průměrným podílem polynenasycených mastných kyselin ve srovnání s temperátními a tropickými kmeny.

V další práci jsme testovali antarktický kmen zelené řasy z rodu *Monoraphidium* pro produkci polynenasycených mastných kyselin v poloprovozním měřítku. Z hlediska celoročního využití kultivačních kapacit v mírném pásu může být kultivace psychrotolerantních kmenů výhodná. Testovaný kmen byl vyizolován ze zamrzlého jezera a kromě posunutého optima růstu do oblasti nízkých teplot se vyznačoval též nízkými nároky na světlo a zvýšeným obsahem polynenasycených mastných kyselin oproti jiným zástupců tohoto rodu. Kultivace na tenkovrstvé plošině v zimním období prokázala, že je tento kmen perspektivní.

Dalšími studovanými organismy z hlediska vlivu podmínek na složení lipidů bylo pět kmenů ze skupiny Haptophyta. Pomocí vysokoúčinné kapalinové chromatografie s reverzní fází (NARP-HPLC) s chemickou ionizací za atmosférického tlaku (APCI) jsme popsali složení jejich triacylgycerolů při různých úrovních salinity s důrazem na polynenasycené mastné kyseliny s 18-ti uhlíkovým řetězcem. Salinita měla vliv na podíl nasycených a nenasycených mastných kyselin a také jejich složení z hlediska regioizomerů.

Další dvě práce se věnovaly metodickým aspektům řasové biotechnologie. Jednalo se o optimalizaci extrakce a purifikace fukoxantinu z biomasy mořské rozsivky *Phaeodactylum tricornutum* pomocí vysokoúčinné protiproudé chromatografie (HPCCC), která prokazatelně zachovala jeho biologické aktivity. Dále jsme se zaměřili na porovnání různých přístupů pro včasnou detekci kontaminace řasových kultur na příkladu kultury rozsivky *Phaeodactylum tricornutum* kontaminované řasou *Chlorella* sp. Jako nejspolehlivější se ukázalo využití metod PCR a qPCR, které odhalily kontaminaci již na úrovni kolem 75 buněk/ml, a jeví se tedy jako nejlepší přístupy pro časné odhalení kontaminací v řasových kultivacích.

V neposlední řadě jsou součástí práce taky výstupy z aplikovaného výzkumu. Prvním z jich je prezentace funkčního prototypu kultivačního zařízení, které bylo vyvinuto v rámci doktorského studia a s jeho pomocí byly prováděny experimenty, které jsou publikovány v rámci hlavních výsledků této práce. Dále jsou prezentovány výstupy z poloprovozních kultivací řas *Hibberdia magna* a *Phaeodactylum tricornutum*, které probíhaly ve spolupráci s českou firmou Algamo s.r.o. Ta se zabývá komerční kultivací mikrořas.

LIST OF SUPPLEMENTS

Chapter 1

Střížek, A., P. Přibyl, M. Lukeš, T. Grivalský, J. Kopecký, T. Galica & P. Hrouzek (2023)

***Hibberdia magna* (Chrysophyceae): a promising freshwater fucoxanthin and polyunsaturated fatty acid producer**

Published: Q1, *Microbial Cell Factories* 22: 73 (doi: 10.1186/s12934-023-02061-x)

Chapter 2

Střížek, A., M. Lukeš, P. Hrouzek, M. Mylenko, J. Lukavský, L. Nedbalová & P. Přibyl

Alternative production of fucoxanthin and PUFAs using *Chlorochromonas danica* and *Hibberdia magna*, unicellular chrysophytes with different trophic modes.

Submitted: *Algal Research*

Chapter 3

Střížek, A., T. Řezanka, E. Hejduková & L. Nedbalová

Adaptation of freshwater diatoms to various climatic zones manifested at the level of fatty acid profiles

Manuscript draft

Chapter 4

Řezanka, T., L. Nedbalová, J. Lukavský, **A**. **Střížek** & K. Sigler (2017)

Pilot cultivation of the green alga *Monoraphidium* sp. producing a high content of polyunsaturated fatty acids in a low-temperature environment.

Published: Q1, *Algal Research* 22: 160–165 (doi: 10.1016/j.algal.2016.12.017)

Chapter 5

Nedbalová, L., **A**. **Střížek**, K. Sigler & T. Řezanka (2016)

Effect of salinity on the fatty acid and triacylglycerol composition of five haptophyte algae from the genera *Coccolithophora*, *Isochrysis* and *Prymnesium* determined by LC-MS/APCI

Published: Q1, *Phytochemistry* 130: 64–76 (doi: 10.1016/j.phytochem.2016.06.001)

Chapter 6

Bárcenas-Pérez, D., **A**. **Střížek**, P. Hrouzek, J. Kopecký, M. Barradas, A. Sierra-Ramirez, P. J. Fernandez-Marcos & J. Cheel (2021)

Production of fucoxanthin from *Phaeodactylum tricornutum* using high performance countercurrent chromatography retaining its FOXO3 nuclear translocation-inducing effect

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Chapter 7

Grivalský, T., **A**. **Střížek**, P. Přibyl, J. Lukavský, R. Čegan, R. Hobza & P. Hrouzek (2021)

Comparison of various approaches to detect algal culture contamination: a case study of *Chlorella* sp. contamination in a *Phaeodactylum tricornutum* culture

Published: Q1, *Applied Microbiology and Biotechnology* 105: 5189–5200 (doi: 10.1007/s00253-021-11396-7)

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(NCK TAČR, TN01000048/03 – V019, https://hdl.handle.net/11104/0337704)

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(NCK TAČR, TN01000048/03 – V011, http://hdl.handle.net/11104/0325385)

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(NCK TAČR, TN01000048/03 – V01, http://hdl.handle.net/11104/0322471)

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(NCK TAČR, TN01000048/03 – V02, http://hdl.handle.net/11104/0322475)

CONTENT

INTRODUCTION

1. Lipids

Lipids are essential building blocks for all life on Earth. They are subcellular components with many fundamental functions. Moreover, they are not only essential for life itself, but also from an anthropogenic perspective, as they are one of the main energy carriers that fuel our modern way of life. Due to their crucial importance, there is an overwhelming background of knowledge about lipids today and of course, many more to be explored in the future. In this chapter, I will focus on a brief overview of lipids essentials, their classification, and description of the major lipid classes with more focus on those important in the context of the whole thesis, while smaller or less important in microalgal context lipid species are overlooked.

1.1. Definition

Despite their great importance, it may be surprising that lipids lack a generally accepted definition. While there is no doubt that core lipid classes such as fatty acids (FAs) esters the classification of some peripheral classes as lipids is subject to the personal preferences of professional biochemists, which can be confusing. However, there are some partial definitions available. The most widespread one is based on lipid solubility. According to this definition, lipids are small, naturally occurring hydrophobic or amphiphilic molecules, typically less than 1 kDa in size, that are soluble in organic solvents and alcohols. However, it is important to note that some lipids, such as certain bile acids, are also soluble in water. On the other hand, not all small molecules that are soluble in organic solvents are lipids, such as organometallic compounds (e.g. trimethylarsine).

Another definition that could be used is based on the biosynthesis of lipids. It is a bit more complicated in the original (Fahy et al. 2005), but in a simplified way it says that lipids are small molecules that are partially or totally formed by the condensation of thioesters (FAs, polyketides, and their derivatives) or by the condensation of isoprene units (terpenes, and terpenoids, or isoprenoids, in other words, and their derivatives). The largest lipid structure database to date, Lipid Maps® (www.lipidmaps.org), is based on this definition, but this definition still has certain drawbacks. First, using this definition requires advanced knowledge of the biochemistry and biosynthetic pathways of the molecule evaluated, and second, the definition is quite broad and includes a variety of molecules that are not usually considered lipids. This is especially true for polyketides, which are a very large and heterogeneous group of compounds with different functions, and solubilities. Of course, it is possible to find other alternative definitions proposed over the years, but it is not necessary to follow this path deeper in this thesis.

1.2. Classification

While there is no universally accepted standard classification, in this context it is not such confusing because each scientific discipline can suit different classification approaches. One straightforward approach is to sort them based on their chemical structure simply. The International Lipid Classification and Nomenclature Committee, a scientific panel sponsored by the already mentioned structure database Lipid Maps®, has selected this classification system. It includes eight major lipid classes, which are further divided into multiple subclasses (Fahy et al. 2009; Liebisch et al. 2020). One potential disadvantage of this classification is its focus on lipids of human or animal origin. From the perspective of phycology or protistology, it may be clearer to have slightly different categories. Hence the classification used by Lipid Maps® was adopted and slightly modified by me in the direction to suit better from a phycology perspective (Table 1). However, classifications based on traits other than chemical structure may be more appropriate in different cases. Lipids can be also classified based on their: a) polarity, as neutral (hydrophilic) or polar (amphiphilic); b) function, such as storage, structural, signaling, protective, light absorption, or as intermediates or precursors; c) complexity (count of the primary product after hydrolysis); d) occurrence in nature and biosynthetic origin. In addition to the lipid classes presented, there are composite molecules such as lipoproteins, proteolipids, or lipopolysaccharides that can be difficult to assign to lipids but still fit the definition to some extent. These examples highlight the challenging nature of categorizing such a diverse range of molecules found in living organisms using simple, man-made definitions.

Main class	Sub-class
Fatty acid - lipids	Free fatty acids
	Glycerolipids
	Waxes
	Oxylipins
Terpenoids (Isoprenoids)	Carotenoids
	Sterols
	Tocopherols
Sphingolipids	
Polyketides	

Table 1
Lipid classification; Based on the chemical structure. Modified from (Fahy et al., 2009).

1.3. Fatty acids & oxylipins

Lipids consisting of FAs are usually considered to be the core lipid group. These acyl lipids are the most abundant, have the greatest molecular diversity, and usually make up the largest lipid mass in the cells and bodies of organisms. There are several thousands of different FAs and FA-originated molecules found in nature. Elementary FAs are saturated carboxylic acids with six or more carbons usually in even numbers. They can vary in terms of the number of carbons (FA length) and saturation, thus the number and position of double bonds, and the associated *cis* or *trans* conformation. FA saturation is an important characteristic that influences the shape and melting temperature of the lipid. This can be illustrated using kitchen oils. Pork lard is composed mostly of saturated 16-carbon palmitic acid (16:0) and 18-carbon monounsaturated oleic acid (18:1n9) and remains solid even at room temperature. Similarly, e.g. coconut fat has a high content of saturated 12-carbon lauric acid (12:0). In contrast, cod liver oil, which is rich in long and highly unsaturated FAs, has a melting point of -5 °C. This nature of FAs is an important parameter commonly used to describe food or feed and it is also a frequent scientific parameter evaluated in parts of this thesis (Suppl. Chapters 1-5).

FAs are identified by their systematic name, although they may also have a trivial name or commonly understood abbreviations. However, the most practical and descriptive way to refer to them is by using alphanumeric codes called lipid numbers. Lipid numbers take the form: *C:DnX* where *C* is a carbon count; *D* is a count of double bonds; and *X* describes the position of the first double bond. It is important to note that an FA molecule has two ends: one with a carboxyl group and the other ending with the last carbon. By convention, this last carbon in the chain is labeled as ω (omega), and the position of the double bonds is determined by distance from the ω carbon (Fig. 1). In the nutritional context the letter *n* in lipid number is substituted by the symbols ω, Ω, or the word 'omega', but they all refer to the description of the position of the first double bond in FA. Since it is common for FA to have more double bonds, they are referred to as monounsaturated (one double bond – MUFA) or polyunsaturated (more than one double bond – PUFA), while FAs without double bond are saturated FAs (SFAs). The coding for PUFAs is deficient in describing the exact position of double bonds. However, the enzymatic apparatus of PUFA synthesis is typically conservative, resulting in a standard interval of 3 carbons between each double bond and their *cis* conformation. If there are conjugated double bonds (Nagao & Yanagita 2005) or other non-standards PUFAs described then the position as well as the *cis* or *trans* conformation of each double bond should be given by numbers, and the systematic name should be used preferably.

In addition to the standard FAs found in virtually all living organisms, many modifications occur. First, only the carbon chain is modified without the addition of any other elements. The carbon chain may contain triple bonds, branching, or cyclic structures, which are typical forms of bacterial FAs but can also arise during food processing. The next diverse group includes FAs with oxygen bonded to

the carbon chain, known as oxylipins. Oxygen can be present in the form of can contain hydroperoxyl, hydroxyl, epoxy, oxo, and/or endoperoxide groups in either linear or cyclic (aromatic) form. Some of them are present mainly in esterified forms, while other core oxylipins remain as solitary molecules. Oxylipins have important functions as signaling molecules, mediators, hormones, and play roles in plant immunity and defense responses. They play a role also in inflammatory reactions, but the detailed functions are not fully understood yet. Long chain PUFAs (LC-PUFAs) are precursors for the biosynthesis of oxylipins. Therefore, cells require an excess of LC-PUFAs to be able to produce oxylipins when needed (Domínguez 2013).

Besides oxygen, may further contain other elements, such as nitrogen (e.g. lipo-amino acids, nitro-FAs, cyanolipids), sulfur, or halogens. Special and little-known types of FA-derived lipids are chlorosulfolipids (FA with -OSO_3 and -Cl) (Bedke & Vanderwal 2011). These are abundant parts of the cell membranes of mixotrophic chrysophytes (Moss et al. 2020), which are widespread unicellular phagocytic flagellates with a distinctive predatory behavior (Suppl. Chapter 2). Although their function is unknown, they are confirmed to be highly toxic. No information is available on their biosynthesis, toxicity mechanism, or self-toxicity defense (Hiltunen et al. 2012). The general hypothesis regarding their function is an anti-predatory effect on grazers or allelopathy (negative effects on competitors). Through numerous culturing experiments in which I fed them with various prey organisms (data not published), different hypotheses came to my mind. These flagellates have a successful life strategy, they occur worldwide in either fresh or salt water, significantly reducing other planktonic microorganisms by grazing. They lack a cell wall and use flagella to directly touch and draw prey cells. So, I hypothesize that these unusual toxic membrane lipids could be involved in the process of phagocytosis, and rather than being toxins, the chlorosulfolipids may serve as venom. To confirm this and describe the poisoning mechanism could be a nice discovery indeed.

1.4. Glycerolipids & other fatty acid esters

1.4.1. Acyl-coenzyme A

FAs are rarely found as individual molecules due to their high reactivity, both specifically and non-specifically. Only oxylipins and some other uncommon FAs derivates, such as the forenamed chlorosulfolipids, are active in an unesterified form. One of the most common esters is FA thiol ester of the coenzyme A (acyl-CoA). CoA is a carrier molecule that is abundant in all organisms playing an important role in the stabilization, transport, biosynthesis, and decomposition of FAs via β-oxidation in mitochondria. However, the occurrence of FAs as acyl-CoA is intermediate. For FAs deposition or structural purposes, they are primarily esterified to the trihydric alcohol glycerol (Fig. 1) to form glycerolipids.

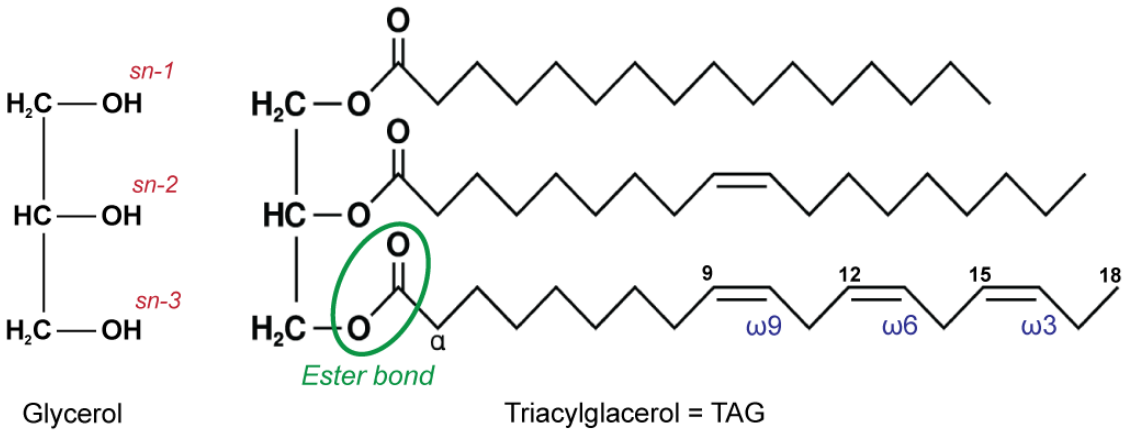


Figure 1
Glycerol molecule with indication of *sn-* positions and sample of triacylglycerol (TAG) molecule. This TAG has bound three different fatty acids (FA) linked via ester bond as is marked in green. The FA at position *sn-1* is saturated FA (C16:0), at position *sn-2* is monounsaturated FA (C18:1n9), and at position *sn-3* is polyunsaturated FA (C18:3n3). The position of the double bonds can be labeled from the side of terminal ω carbon as ω3, 6, 9. Alternatively from the side of carboxyl moiety as Δ9, Δ12, Δ15.

Glycerolipids are a core lipid class and besides their biological functions, they are the most important lipids from an anthropogenic perspective. Their major structural function is to form the cellular bilayer membrane, making them likely participants in the origin of Life, forming such an essential barrier between living and non-living. Glycerolipids have a simple structure consisting of a glycerol backbone (Fig. 1) with FAs bonded by an ester bond and possibly another substituent such as a phosphate moiety (Fig. 2). Glycerol is a trihydric alcohol that can exist solely within cells without reacting with other biomolecules. It is also widely used in pharmaceutical, chemical, food, cosmetic, and other industries. In some groups of algae, it serves as an osmolyte, balancing the osmotic pressure of the ambient environment or preventing freezing. Its advantage over other osmolytes is that it does not contain any limiting elements such as N, P, or metals. Furthermore, glycerol is also a storage molecule that contains chemically fixed energy.

1.4.2. Neutral glycerolipids

Glycerolipids exist in two main forms (Table 2). The first are neutral or simple glycerolipids. Those lipids are completely hydrophobic and consist of only two major components: glycerol and FAs. The most common and important forms of neutral glycerolipids are triacylglycerols (TAGs) or short triglycerides. In this case, all three hydric groups of glycerol are bonded by ester bonds with typically three different FAs. The position of the FAs on the glycerol backbone is labeled *sn-1*, *sn-2*, and *sn-3*. If only one or two FAs are esterified to the glycerol, then they are mono- or diacylglycerols. However,

these variants are usually not present in significant amounts in cells and play a role as intermediates in the synthesis of TAGs or polar glycerolipids. TAGs are chemically inert and stable, have a high energy density and, due to their hydrophobic nature, form compact droplets that do not mix or interfere with other cellular structures. The primary purpose of TAGs in microbial cells as well as in plant and animal tissues is energy storage but also storage of biomolecular precursors, hence TAGs can be converted into structural polar lipids or other FA-based lipids such as oxylipins or waxes. Further, TAGs are the major lipids in adipose tissue, which have important functions in the animal body such as insulation, shock absorption, buoyancy maintenance, or fitness signaling. The FA composition of TAGs is highly variable, reflecting mainly taxonomic groups, but it also differs between tissues, life stages, and environmental conditions, and is influenced by the FA composition of the diet (Ahlgren et al. 2009).

Neutral (simple)	Triacylglycerol			**TAG**
	Diacylglycerol			**DAG**
	Monoacylglycerol			
Polar (complex)	**Glycerophospholipids** (phospholipids)	Phosphatidic acid		**PA**
		Phosphatidylglycerol		**PG**
		Diphosphatidylglycerol (Cardiolipin)		**DPG**
		Phosphatidylserine		**PS**
		Phosphatidylethanolamine		**PE**
		Phosphatidylcholine		**PC**
		Phosphatidylinositol		**PI**
	Glycosyldiacylglycerols (glycolipids)	Galactolipids	Monogalactosyldiacylglycerol	**MGDG**
			Digalactosyldiacylglycerol	**DGDG**
		Sulfono-glyceroglycolipids	Sulfoquinovosyldiacylglycerol	**SQDG**
	Betaine lipids	Diacylglycerohydroxymethyl-N,N,N-trimethyl-β-alanine		**DGTA**
		Diacylglyceryl-N,N,N-trimethylhomoserine		**DGTS**
		Diacylglycerylcarboxy-N-hydroxymethylcholine		**DGCC**

Table 2:
Glycerolipids; Main classes and compound types.

1.4.3. Polar glycerolipids

The second main group of glycerolipids is the polar, in other terms complex glycerolipids. These molecules have only two FAs bonded to the glycerol backbone, and the third position is substituted. Therefore, these lipids are complex because they are composed of three or more primary components (glycerol, FA, and substituent). The substituent moieties that are bonded to the rest of the lipid are polar and thus hydrophilic. This results in the amphiphilic nature of polar glycerolipids. Polar glycerolipids are a fundamental component of cellular membranes, forming a bilayer that orients the hydrophilic head

groups toward the outer aqueous environment (Leonard et al. 2023). While hydrophobic carbon chain tails of FAs are directed inside the bilayer (Fig. 2). Polar glycerolipids may also form a monolayer when creating lipid droplets (Zadoorian et al. 2023). In this scenario, the inner environment of the droplet lacks the second layer and is filled with hydrophilic TAGs and potentially other neutral lipids, such as sterols or lipid-soluble vitamins. Such lipid droplets can be artificially formed using other amphiphilic molecules, typically detergents.

Cellular membranes vary depending on their location and function, (outer membrane, nuclear membrane, mitochondrial membrane, and thylakoid membrane, etc.). The composition of the upper and lower layers of the membrane bilayer varies as well. The primary difference between membranes is the occurrence and proportion of polar lipid substituents. FA composition also varies between membrane types and is further influenced by other factors, particularly temperature, which will be discussed later. The hydrophilic head group of polar glycerolipids is formed by three main types of substituents (Table 2). A) phosphate group forms glycerophospholipids (Fig. 2); B) polar carbohydrate forms glycosyldiacylglycerols; C) betaine moiety linked by an ether bond forms betaine lipids. In addition, other substituents may occur, such as the combination of phosphate and carbohydrate moieties to form glycophospholipids or sulfur-containing taurolipids. However, these unusual substituents are beyond the scope of this thesis.

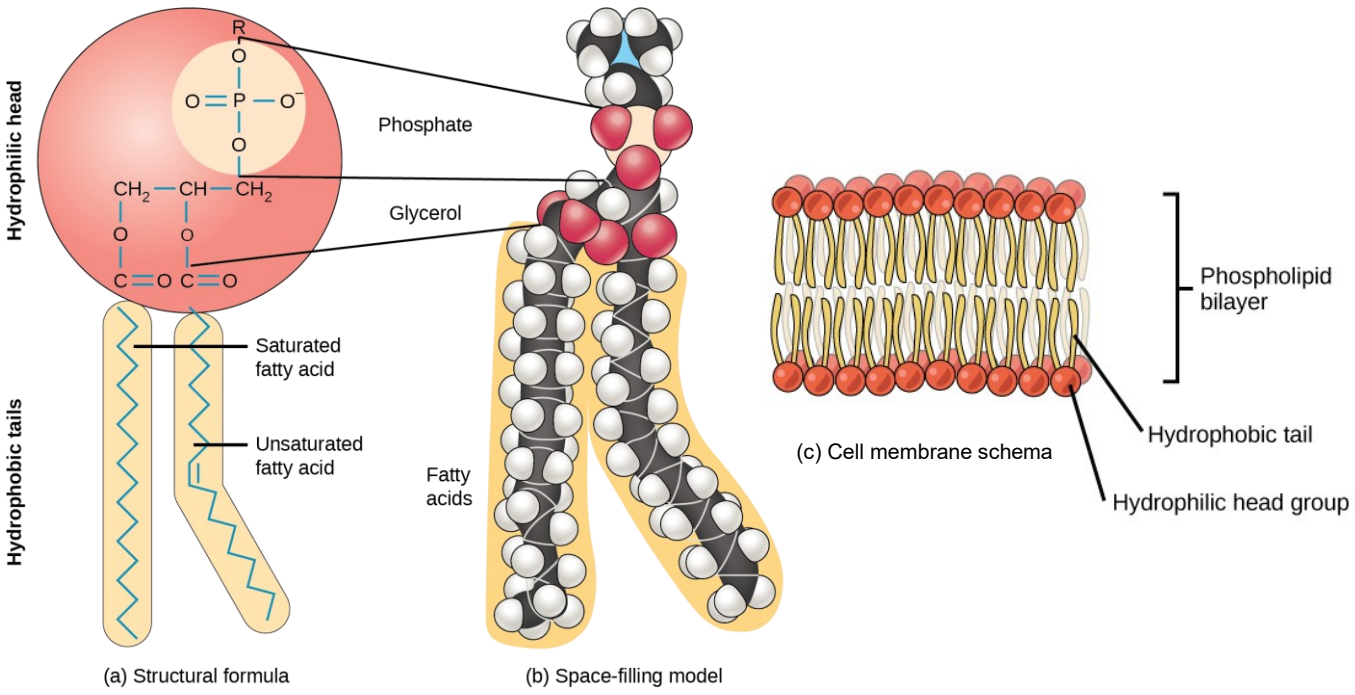


Figure 2
Sample of polar glycerophospholipid (a), its space model (b), and membrane bilayer simplified schema (c). Modified from (Clark et al. 2018).

Glycerophospholipids are the dominant compounds forming the cell membranes in most organisms, although in some protist groups, betaine lipids may replace phospholipids, typically in situations of phosphorus limitation (Bolik et al. 2023). In addition to structural function, they could be involved in cell signaling. They act as precursors of oxylipins and can influence protein functions by forming lipoproteins. The most common types of glycerophospholipids are presented in Table 2. Phosphatidic acid (PA) is the essential intermediate in the biosynthesis of other glycerolipids. However, PA is not abundant in cells due to its instability. To enhance the stability of the membrane phospholipids, they are further linked with another component, such as cyclic polyhydric alcohol inositol (PI), glycerol (PG), or whole other PG forming DPG, amino acid L-serine (PS), or other nitrogen-containing moieties such as choline (PC) or ethanolamine (PE).

Glycosyldiacylglycerols or shortly glyceroglycolipids, are often confused with the more general term glycolipids. However, term glycolipids also include other non-glycerol-containing lipids, such as glycosphingolipids or simple carbohydrate FA esters. Some of these small glycolipids can be found in the membranes of cyanobacterial heterocysts, which are cells specialized in nitrogen fixation. The core glyceroglycolipids have basic structures consisting of a glycerol backbone with two FAs linked by an ester bond at positions *sn-1* and *sn-2*, and a polar heterocyclic carbohydrate moiety bonded in position *sn-3*. Glyceroglycolipids are divided into galactolipids, which contain one (MGDG) or two galactoses (DGDG), and sulfono-glyceroglycolipids, which consist of only one molecular type composed of sulfo-6-deoxyglucose (SQDG). Polar glyceroglycolipids along with PG play a crucial role in photosynthesis as they are a major component of thylakoid membranes. Thylakoids are intracellular systems of vesicles bearing photosynthetic reaction centers and light-harvesting complexes integrated into the membranes. The composition of the thylakoid membranes is highly conservative in evolution and polar glyceroglycolipids are thus abundant in chloroplasts of all photosynthetic organisms, particularly in the photosynthetic tissues. They are not only structural components of the thylakoid membranes, but they also interact with photosynthetic protein complexes and participate in photosynthesis to a certain degree. The FAs esterified in plastid glyceroglycolipids are mostly longer and less saturated, which likely also plays a role in photosynthesis. Additionally, FAs bonded to glyceroglycolipids are more bioavailable for consumers of plants and algae than those in the form of TAGs.

As mentioned above, betaine lipids can replace glycerophospholipids in cell membranes. However, betaine lipids are only found in some eukaryotic protists, fungi, and cyanobacteria. Structurally, they are similar to other polar glycerolipids but differ only in the substituent in the *sn-3* position of the glycerol backbone, which is a polar betaine group linked by an ether bond. The betaine group consists of trimethylated amino acid glycine, thus it is a small nitrate-containing moiety.

1.4.4. Other important fatty acid esters

Waxes are another important type of FA ester. They are synthesized through the esterification of FAs with a monohydric long-chain alcohol. Waxes serve various roles in both plants and animals, such as impregnation, mechanical, light or desiccation protection, and lubrication. They can also function as energy storage. Finally, FA methyl ester is worth mentioning. Although they are not significant from a biological perspective, as they are not natural compounds. FA methyl esters are important from analytical and industrial perspectives. Natural glycerolipids can be extracted and transesterified by methanol, forming methyl esters. These stable molecules are easily stored, transported, and processed, and they are the main component of biofuels.

1.5. Carotenoids, sterols & other terpenoids

Terpenes are among the most abundant and variable organic molecules found in living organisms, with tens of thousands of different molecule types described and probably many more yet to be discovered. They are generally defined as the biosynthetic derivatives of the isoprene moiety (Fig. 3), while terpenoids are the oxygenated derivatives of terpenes. Terpenes can be in linear, cyclic, bicyclic, or polycyclic forms and can be classified according to the number of terpene groups such as monoterpenes (C10), diterpenes (C20), triterpenes (C30), etc. Terpenoids have many essential biological functions and are also important for pharmaceutical use.

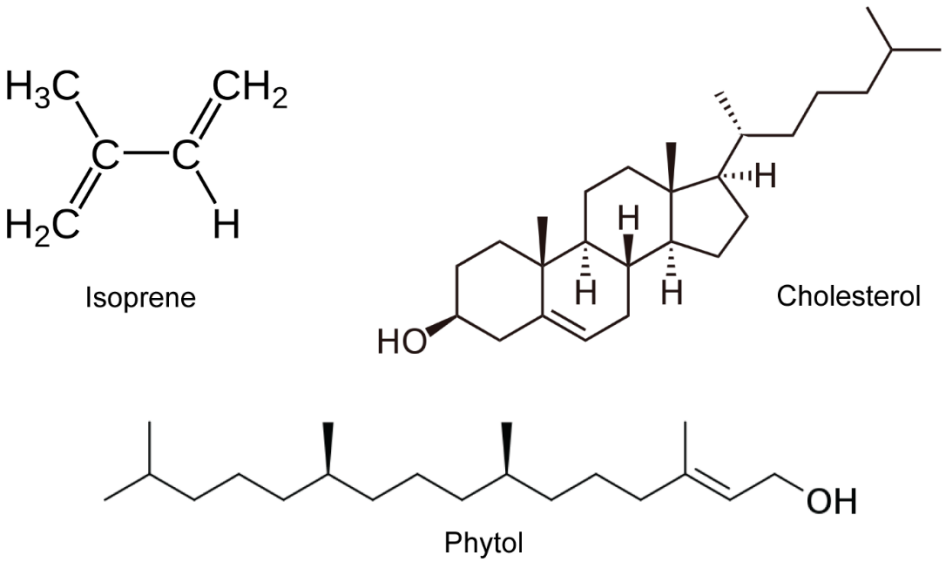


Figure 3
Isoprene molecule model and samples of terpenoid molecules (Cholesterol and Phytol).

1.5.1. Sterols

Perhaps the most important terpenoids are steroids and especially sterols as their derivatives. Sterols are polycyclic terpenoid lipids consisting of four cycles (Fig. 3), which have a fundamental function in all eukaryotes but are mostly absent in bacteria. Sterols are amphiphilic structural biomolecules that, together with the polar glycerolipids described above, contribute to the formation of the cell membrane. First, sterols significantly influence the biophysical properties of membranes, such as their fluidity and permeability, and affect the mobility of fatty acyl chains. Second, they are important precursors of fat-soluble vitamins (vitamin D), steroid hormones (progesterone, testosterone, estradiol, and others), and bile acids. Third, they can modulate the activity of membrane-bound enzymes and fourth, they can serve as secondary messengers (intracellular signaling molecules). In animal tissues, cholesterol is by far the most abundant sterol (zoosterol). In plants and algae, there are several different types of sterols called phytosterols. Phytosterols are more diverse and in addition to the functions mentioned above, they also play a role in plant immunity, resistance, and stress response (Darnet et al. 2021). They also contribute to cell differentiation and proliferation.

1.5.2. Carotenoids

The next important group of terpenoids are carotenoids. They can be divided into two groups: Carotenes, which are hydrocarbons, and Xanthophylls, which contain one or more oxygen-containing moieties. Carotenoids are tetraterpenes consisting of 40 carbons in the basic form. The molecule usually has a linear isoprene chain in the middle and two β-ionone cycles at each end. Linear or monocyclic carotenoids are minor. There are hundreds of different carotenoids that are typical for the specific organism or taxonomic group and can be used as chemotaxonomic markers (Takaichi 2011) (Jeffrey et al. 2011). Among animals, the most important carotenoid is likely β-carotene (Fig. 4), which serves as an essential precursor of retinoids (vitamin A). Retinoids are crucial compounds that cannot be synthesized by animals and are directly involved in vision function (Choi et al. 2021), as well as growth and development, reproduction, and infection resistance. It is further required in the epithelium of the digestive tract, lungs, nervous system, and skin. It also has beneficial functions for the immune system and disease prevention. However, similar positive effects on human health have other carotenoids as well (Eggersdorfer & Wyss 2018; Pereira et al. 2021).

Carotenoids are primarily produced by photosynthetic organisms, although several bacteria and fungi are also capable of producing them (Yabuzaki 2017). Even for protists, carotenoids may have a function related to vision, as they are part of a light-sensitive organelle (stigma) required for phototaxis. However, for photosynthetic organisms, carotenoids are particularly important due to their involvement in the process of photosynthesis as light-harvesting pigments (Egeland 2016). Carotenoids have

absorption spectra that differ from those of chlorophylls allowing them to increase the quantity of photons utilized by photosynthesis. Additionally, carotenoids act as antioxidants and photoprotectants. They not only transfer light energy to photosynthetic reaction centers but also absorb excessive light energy in the case of intense radiation. The carotenoid absorption spectra are controlled by the length of its polyene tail causing specific coloration. Important carotenoids found in algae are described in further chapters.

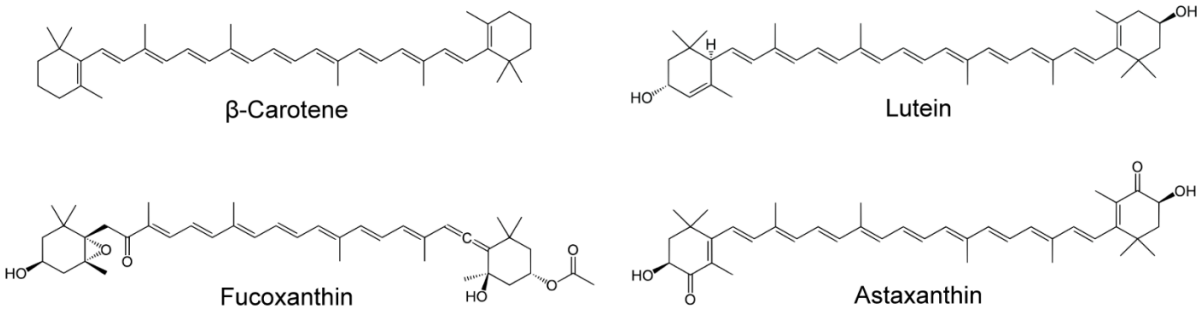


Figure 4
Samples of carotenoids molecules (β-carotene, Lutein, Fucoxanthin, and Astaxanthin).

1.5.3. Other important terpenoids

In addition to the described sterols and carotenoids, there are numerous other terpenoids that have significant biological functions. It is worth briefly mentioning some of these. There are amphiphilic fat-soluble vitamins and antioxidants produced by plants, such as tocopherols and vitamin K. Both compounds are important components of thylakoid membranes in photosynthetic organisms, where they play roles as photo-protectants and antioxidants (tocopherols) or in electron transfer between chlorophyll and the photosynthetic reaction center (vitamins K). Another important group of terpenoids are ubiquinones, also known as Coenzyme Q (CoQ). These are hydrophilic molecules located inside the mitochondrial membrane bilayers and are involved in proton transfer and ATP synthesis by mitochondria ATPase.

1.6. Sphingolipids & polyketides

Lipids are a vastly diverse group of compounds and describing them all could take endless pages. These last two groups of lipids are indeed very important in many biological aspects. However, they are beyond the focus of this thesis. Briefly, sphingolipids are amphiphilic molecules resembling glycerophospholipids, with a polar hydrophilic head and two non-polar hydrophobic tails occurring as part of cell membranes (Mamode Cassim et al. 2020). However, their similarity ends there. The

backbone structure is not glycerol, but a sphingoid base linked via an amide bond to one long-chain FA. Sphingolipids can be modified to form various derivatives, such as Glycosphingolipid, Sphingomyelins, Gangliosides, and others. Sphingolipids play crucial roles, including cellular messengers, part of the immune system, or components of membrane micro-domains called rafts.

Polyketides are again a large and complex molecular class containing small molecules derived from ketone (C=O) and methylene-containing precursors. They exhibit various biological activities, including antibiotic, antifungal, cytostatic, antiparasitic, and natural insecticidal properties. Thanks to that they are important from an agricultural, industrial, and medical perspective. However, their classification as lipids is a matter of debate. According to the above-mentioned structure database Lipid Maps® and their authors (Fahy et al. 2009), all polyketides are considered lipids due to their biosynthetic origin through the condensation of thioesters but biochemically do not resemble typical lipids.

1.7. Lipidomics

Lipidomics is an emerging scientific discipline that focuses on a complete lipid survey of a given tissue or organism at the systems level. The goal of lipidomics is not only the identification and quantification of the lipid classes including individual lipid molecular species in the sample but ideally the exploration of lipid functions and dynamics in the biological system in a broader context and connection with other metabolomic and physiological disciplines (Wenk 2005). From the above exhaustive but still far from complete overview of lipids, it is clear that a lipidomic survey is not simple due to the enormous diversity of forms and functions of lipids, resulting in the detection of up to thousands of molecular species (Hewelt-Belka et al. 2014). It also requires the involvement of several analytical methods, including high-end technical equipment. Two main approaches can be used: "shotgun lipidomics" consists of the direct injection of total lipids into the mass spectrometer or lipids can be separated by high-performance liquid chromatography and later identified by mass spectrometry (Řezanka et al. 2020). Since many types of lipids or their functions are still unknown, it requires many skills, technical abilities, and diligence to accomplish such a task. However, the original view of lipids as mainly structural and energy storage molecules has long since been replaced by new knowledge about the multitude of their biological activities (da Costa et al. 2016). Therefore, the lipidomic approach is a logical outlet that can lead to new innovative solutions for human medicine, more gentle treatment of the environment, and general deepening of human knowledge (Watson 2006).

2. Glycerolipids, fatty acids and carotenoids in microalgae

2.1. Biosynthesis & chemotaxonomy

Microalgae are a polyphyletic group with ecological and physiological rather than taxonomic delimitation. This is a consequence of the multiple parallel events of endosymbiosis that occurred during algal evolution. Various eukaryotic microbial lineages acquired photosynthetic abilities through primary, secondary, or multiple endosymbiosis events leading to the formation of distinct algae groups (Neilson & Durnford 2010). This independent origin of each major algal taxon together with the long evolutionary time causes a certain degree of uniqueness of their physiology and biochemical composition among each other. Their genetic and biochemical profile is a hybrid of the original eukaryotic host and the endosymbiotic cyanobacteria transformed into plastid (Shanshan Wang et al. 2018). However, this is only true for the Archaeplastida kingdom, which are the only photosynthetic organisms that acquired plastid via primary endosymbiosis. Other algal groups exhibit even greater ecological, biochemical, and genetic complexity gained due to endosymbiosis. They originated through several independent secondary endosymbiosis events involving heterotrophic phagotroph and red alga (Stramenopila, Haptophyta, Cryptophyta, and Alveolata (Dinophyta, Apicomplexa)) or green alga (Euglenophyta and Chlorarachniophyta). In addition, dinoflagellates tend to engulf other endosymbionts originating from the already-established secondary endosymbiotic algae, resulting in tertiary endosymbiosis or a switch from their original red algae-derived plastid to a green algae-derived one. Some dinoflagellates have lost their former plastid, while others have retained both secondary and tertiary plastids. Diatoms likely undergo a very complex endosymbiotic evolutionary history (Vancaester et al. 2020). Their genome indicates multiple horizontal genome transfers, likely originating from gained and lost endosymbionts. Recent advances in analytical techniques, including genomics, cultivation, and microscopy, have provided evidence that gene transfers and the presence of other endosymbiotic bacteria within algal cells are not uncommon (Yurchenko et al. 2018). Some of such 'roommates' have predictable functions e.g. nitrogen-fixing cyanobacterial endosymbionts inside marine haptophyte *Braarudosphaera bigelowii* (Hagino et al. 2013), while others, such as observed bacteria inside of one strain of *Cryptomonas* (George et al. 2023), have unknown functions. However, this is only an illustration that the lipid composition of algae can be influenced by the presence of compounds that are not of algal origin, and the term 'algal origin' itself may be misleading.

Plastid plays a fundamental role in photosynthetic organisms, not only due to photosynthesis. It is also a critical biosynthetic hub where the production of basic building blocks is running. In contrast, the genes and synthetic machinery for protein production are usually suppressed in the plastids and rather carried by the nucleus and the ER. For example, enzymes involved in carotenoid synthesis are synthesized by ribosomes in the ER, then labeled and delivered to the chloroplast. However, individual algal lineages differ in these mechanisms due to different plastid origins (Hildebrand et al. 2013), and a

lot remains unknown about the specific synthetic pathways in each algae lineage. Most are probably known from model organisms such as green alga *Chlamydomonas reinhardtii* (Li-Beisson et al. 2015) of course, but model organisms for other taxa are much less explored or even non-established. In supplementary chapters 1 and 2, I have proposed a potential model organism for the class Chrysophyceae. Despite the described divergence in algal evolution, the photosynthesis itself, and related biosynthetic processes represent a much higher level of conservatism. There are clear similarities between different algal lineages in plastid-related processes. Among others, carotenoids, FA, and part of the polar glycerolipids are synthesized in chloroplasts.

Briefly, *de novo* FA synthesis involves the following steps. The initial substrate is acetyl-coenzyme A (CoA), which is a molecule composed of an acetyl moiety (C2) linked to CoA by a thioester bond, as previously described (Chapter 1.4.1. Acyl-coenzyme A) Pyruvate is likely the primary source of acetyl-CoA synthesis, a molecule also involved in other primary metabolic pathways and is a product of glucose glycolysis. Acetyl-CoA is converted to malonyl-CoA by the ATP-dependent enzyme carboxylase. Malonyl-CoA enters the cycle of FA formation, which is controlled by 4 enzymes collectively called FA synthase. Additional malonyl-CoA molecules are gradually added to the cycle and the FA chain is lengthened. When the FA chain typically has 18 carbons (it can also be C16 or C14), the FA is released from the cycle and subsequently moves out of the chloroplast into the cytosol, where it is bound to CoA to form acyl-CoA and continues to the endoplasmic reticulum (ER). Alternatively, the novel FA remains in the chloroplast and, together with the second FA, is esterified to glycerol 3-phosphate (G3P). The substitution of two phosphate moieties of G3P by the two FAs results in the formation of phosphatidic acid (PA). In the plastid, PA serves as a precursor for the biosynthesis of glyceroglycolipids, or phosphatidylglycerol (PG), which are the essential polar lipids forming the thylakoid membranes.

The subsequent modification of FA takes place in the membranes of the ER. FA undergoes a gradual process of elongation and desaturation involving several enzymes called elongases and desaturases (Fig. 5). These enzymes have substrate specificity, so for each type of FA, there is one enzyme that modifies it only in a specific way. The omega 3 double bonds can only be formed if the FA is C18 or shorter. The omega 3 double bonds are then retained in the molecule during the elongation process because the elongation of the FA takes place from the carboxyl end of the FA, i.e. from the side of the glycerol backbone (Nakamura & Li-Beisson 2016; Khozin-Goldberg 2016). This is also true for the omega 6 double bonds. These enzymes that desaturate the omega 3 or omega 6 carbon are not present in vertebrates, so it is essential to obtain a certain amount of these compounds from the diet (Castro et al. 2016).

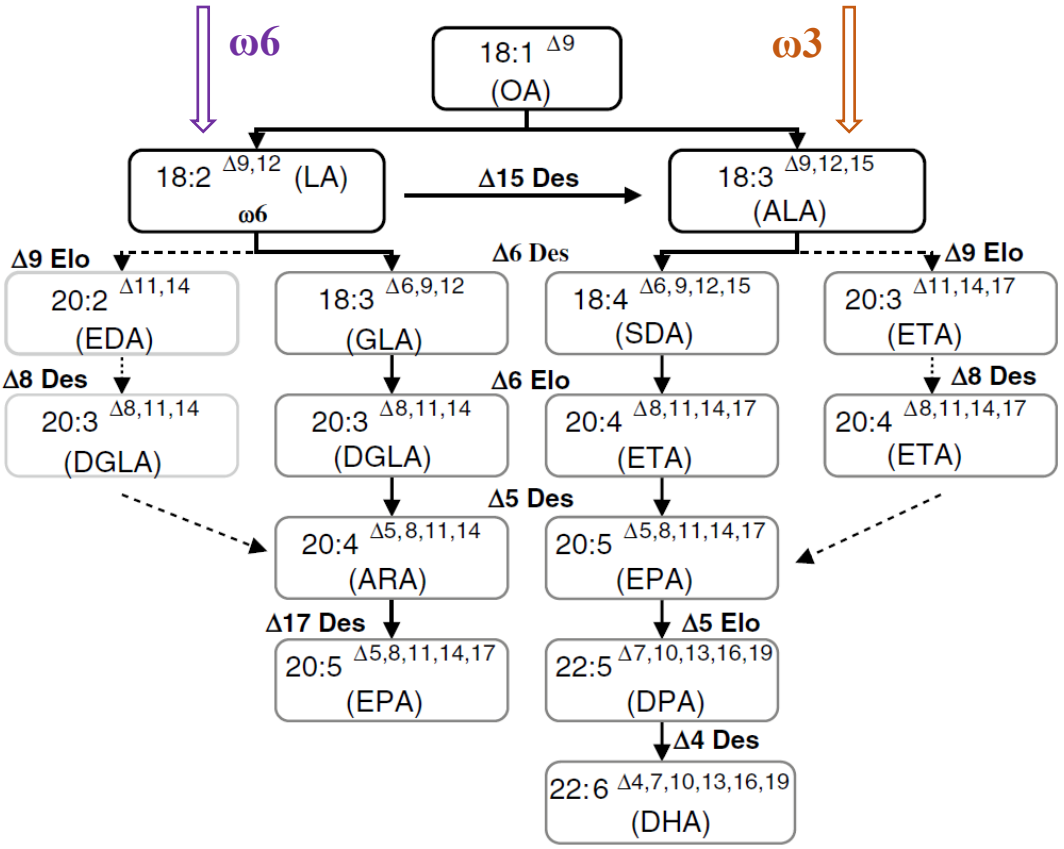


Figure 5

Pathways for the biosynthesis of long chain polyunsaturated fatty acids in microalgae (Khozin-Goldberg 2016). The boxes represent each fatty acid the entering one is oleic acid (18:1n9), arrows represent the ω6 and ω3 sequential pathway with showing the enzymes Elongases (Elo) and Desaturases (Des) involved, Δ number tells the position where the enzyme place a double bond or add 2 carbon elongation.

Carotenoids are also *de novo* synthesized in plastids (Takaichi 2011). Briefly, the primary substrate is again pyruvate, which is converted to the so-called active isoprene, which is isopentenyl diphosphate or its isomer with different positions of the double bond. These are the primary building blocks not only of carotenoids but also of other terpenoids such as sterols and phytol (Fig. 3). The next step is the formation of the linear tetraterpene phytoene (C40). This is formed by the stepwise condensation of eight isopentenyl diphosphates. Subsequent steps lead to desaturation, conjugation, and isomerization of the resulting linear carotene, and finally, cyclization of the linear ends, yielding in carotenes such as β-carotene (Fig. 4). Subsequently, the gradual addition of oxygen to the carotene molecule or other modifications can be catalyzed, resulting in numbers of different xanthophylls (Lohr 2011).

The distinct endosymbiotic evolutionary history of separate algae lineages has a strong impact on their genome, physiology, and biochemical composition. As a result, certain compounds or combinations of compounds are typical for particular lineages and can serve as biomarkers, providing a kind of footprint for certain algae groups. Algal glycerolipids, in combination with carotenoids and/or sterols, are good examples of such compounds (Peltomaa et al. 2023). Significant chemotaxonomic patterns in the FA composition can be observed even at a fine level phylogenetic level among algal genera (Mori et al. 2018). According to the generally accepted hypothesis, primary endosymbiotic cyanobacteria lack the biosynthetic machinery for LC-PUFAs, whereas all algal groups except the majority of green algae are capable of *de novo* LC-PUFA synthesis (Nakamura & Li-Beisson 2016). Therefore, it is expected that this ability was present in the common ancestor of Archaeplastida and was carried by the host eukaryote. However, during evolution, most green algae lost the necessary enzymes for PUFA elongation and they only form C18 PUFA as the longest. As far as I know, there are no hypotheses in the literature about what selection pressures caused this loss. This lack of explanation allows me to make my hypotheses. First, it reduces the metabolic investment that would otherwise be spent on the demanding process of elongation and desaturation to make LC-PUFAs and reduces the number of these enzymes that would otherwise need to be synthesized. Second, the loss of LC-PUFAs could impose limitations on consumers. Most algae consumers are adapted to high doses of LC-PUFAs in their diet, so minimizing the LC-PUFAs content could result in a reduction in predation. In my opinion, the elimination of the requirement for LC-PUFAs is rather beneficial for green algae. However, eliminating them is biologically complicated, which is why other algal groups still produce them. But this only applies to fresh water. In marine environments, the LC-PUFAs are probably more needed. It is also interesting that only freshwater green algae have more parallel lineages adapted to exclusively terrestrial habitats. In fact, plants evolved from green algae, and plants do not produce LC-PUFAs. It is possible that the loss of the requirement for LC-PUFAs may have facilitated the colonization of land by green algae.

2.2. Factors influencing lipid composition

The main factors influencing the qualitative composition of FAs and carotenoids among the major algal lineages are the taxonomic relationships of the original host organism and the origin of its plastid, which cause a significant chemotaxonomic imprint. However, other traits, mostly related to ecophysiology, also affect the lipid composition of algal biomass. The most studied are abiotic factors such as temperature, light intensity, and quality (Suppl. Chapters 1, 3), and the chemical composition of the aquatic environment, either under natural or artificial conditions. Within chemical composition, the type and availability of macronutrients, typically N, P, Fe, S, Mg, and Si, or the presence of organic substrates in the case of mixotrophs, play an important role (Suppl. Chapter 2). Other conditions such

as pH, salinity (Suppl. Chapter 5), or concentrations of dissolved gases such as CO_2 and O_2 are also considered. Stress factors such as toxic metal elements, radioactive isotopes, pesticides, or UV light have also been tested. Biotic factors such as phytohormones, culture density, age, cell cycle phase, adaptations (Suppl. Chapter 3), or interaction with competitors, consumers, association with bacteria, or with bacterial extracts etc. all of the above usually have some effect on the proportion of different classes of glycerolipids (TAGs vs. phospholipids vs. glycolipids vs. betaine lipids), the quantity and quality of carotenoids, and the length, saturation, distribution, and quantity of FAs. There are a tremendous number of research articles, both *in situ* and *in vitro*, that provide very complex results on the effects of these factors on algal ecophysiology and lipid composition but often show results that are difficult to generalize. In this part of the thesis, I will only briefly focus on the most prominent, general, and commonly known effects.

The first is the accumulation of lipids in the form of neutral TAGs in response to nutrient limitation. Limitation by some elemental nutrients, typically nitrogen, results in the storage of excess energy from photosynthesis in lipid droplets filled with TAGs (Roopnarain et al. 2014; Ghafari et al. 2018). These molecules are advantageous because they contain only C, H, and O, which are mostly available, and have a higher energy per mole as well as per volume than other biomolecules. This reaction to limitation is mostly universal among the algae groups. Some of the so-called oleaginous algae are able to store more than 70% of their bio-volume in TAGs alone (He et al. 2015), which usually consist mostly of saturated and monounsaturated C16 and C18 FAs.

The next well-established adaptation is an increase in photosynthetic pigments that are part of light-harvesting complexes, typically carotenoids and chlorophylls under light limitation (Makarov 2012). This response makes obvious sense in the context of increasing light-harvesting efficiency by increasing the amount of light-harvesting complexes (McClure et al. 2018). However, the carotenoid content could also be impacted by stress, leading to the overproduction of so-called secondary carotenoids. The purpose of these secondary carotenoids is not light harvesting, but photoprotection of photosynthetic reaction centers and antioxidant activity. Secondary carotenoids are usually overproduced in situations of abiotic stress, typically due to high radiation and overheating, but also in response to nutrient deficiency.

A very important factor influencing the FA composition (FA profile) of algae is temperature (Xin et al. 2011; Teoh et al. 2013). This influence is related to the physical properties of FAs, specifically their fluidity and melting temperature. To maintain optimal cell membrane function, the correct composition of FAs in polar glycerolipids is required. Ambient temperature affects membrane fluidity and permeability which decreases with decreasing temperature. Longer and more unsaturated FAs have lower melting points, so they become more abundant in membranes as the temperature decreases, preserving their good function. This principle is called homeoviscous adaptation (Sinensky 1974) and

applies not only to algae but also to all microbes and poikilothermic animals. This reaction was also observed in the results of this thesis (Suppl. Chapters 1, 3). Other factors affect the FA profile in algae, e.g. light (Suppl. Chapter 1) or salinity (Suppl. Chapter 5). However, these FA profile shifts in response to such factors are more difficult to generalize and show specific responses that differ among particular organisms. However, a less specific FA profile response can be proposed. A wide range of stressors such as nutrient limitation, excessive light, or other abiotic factors such as salinity, pH, and temperature, if they are outside the optimum for a given alga usually result in physiological constraints. In these situations, the typical response is a reduction in cellular metabolism, a reduction in membranes composed of structures such as plastids, and a suppression of cell division. Such stressed algae begin to accumulate storage compounds such as carbohydrates and TAGs (Roleda et al. 2013). This response leads to changes in the FA profile. Due to a higher proportion of neutral storage TAGs, which have a higher proportion of SFA and/or MUFA, while actively growing and dividing algae have more polar lipids and a higher proportion of PUFAs (Hu & Gao 2006).

2.3. Microalgae lipids in aquatic food webs

Lipids have thousands of forms and many important functions in all organisms (Tietel et al. 2023), and microalgae stay at the bottom of the trophic pyramid as the primary producers of the absolute majority of both freshwater and marine ecosystems. Therefore, from a global perspective, microalgal biomass is of great ecological and social importance. It is estimated that half of the world's primary production is provided by microalgae. Animals and other algae consumers usually lack the ability to *de novo* synthesize C18 omega-3 and omega-6 PUFAs (Monroig & Kabeya 2018), so microalgae have a significant impact not only in the aquatic environment. Aquatic insects, birds, mammals, and other consumers of aquatic biomass transport an important LC-PUFA to terrestrial environments, ensuring the flow of these essential biomolecules through the biosphere.

Microalgae are also producers of other essential lipid compounds such as carotenoids, sterols, vitamins, coenzymes, and a variety of irreplaceable precursors. Most of these compounds can't be synthesized by zooplankton. However, the quality of microalgal biomass as a food varies due to their chemotaxonomy and the impact of environmental conditions. Typically, cyanobacteria, a direct competitor of microalgae, are considered an inadequate food source for zooplankton due to their lack of sterols and LC-PUFAs. Similarly, green algae, which are low in LC-PUFAs, are not considered to be high-quality food sources (Yan et al. 2023). The anthropogenic pollution that causes eutrophication can dramatically alter the composition and/or diversity of the phytoplankton community. Eutrophication in inland waters typically leads to an increase in cyanobacteria and green algae, which can significantly reduce the availability of LC-PUFA in the ecosystem (Galloway & Winder 2015). Increasing temperature is another factor that reduces the total amount of LC-PUFA in the environment (Hixson &

Arts 2016; Strandberg et al. 2022). When the diversity of phytoplankton lineages is reduced, the diversity of essential biomolecules with taxonomic specificity, such as carotenoids, is also reduced. This in turn can have a negative impact on the higher trophic levels.

3. Algal lipids from a human perspective

3.1. Algal lipids as a source

The production of lipids from algae has received much attention over time due to its potential application as a source of biofuels. This is because the composition of TAGs produced by algae can be easily refined into biodiesel (Mata et al. 2010). In addition, algae can grow rapidly while using widely available resources such as sunlight, CO_2, and water, and can accumulate large amounts of TAGs. However, the main challenge is to produce algae TAGs in a feasible manner. Currently, this appears to be not only economically unfeasible but rather impossible. Of course, it is important to remember that the current consumption of conventional fossil fuels is unsustainable and needs to be replaced or significantly reduced as soon as possible. However, a closer look at applied phycology reveals how complex and delicate these cultivation technologies are. Therefore, it is unlikely that microalgal cultivation alone can solve this urgent problem. The solution will likely require a combination of many small changes and improvements. Fuel based on microalgal lipids may have some specific applications in the future as part of this complex solution.

However, the lipids produced by algae have a much higher value than just the value of the energy stored in these molecules. It is clear from the previous chapters that lipids have many more fundamental functions. The vast majority of aquatic food chains depend on these products as well. Understandably, this primary production also significantly impacts human society because it is fundamentally dependent on ecosystem services, i.e., natural processes such as photosynthesis. In addition to providing a natural ecosystem service, microalgae are also used in applied phycology. This field currently focuses on selecting strains with specific characteristics that favor their use for cultivation under artificial conditions. The biomass produced must have a reasonable value to cover the energy and labor costs required for artificial algae cultivation. Therefore, current applied phycology focuses on identifying and optimizing the production of high-value compounds. These compounds must be in demand, acceptable, and offer some competitive advantages over conventional agriculture, fisheries, or the chemical industry, despite their higher price.

Essential omega 3 LC-PUFAs are a typical example of such high-value compounds that can be feasibly produced by microalgae (Chen et al. 2023). Omega 3 LC-PUFAs are products in high demand not only in healthy human nutrition, but also in feed and aquaculture (Yan et al. 2023). Their intake is essential, especially during pregnancy and the first years of life, for good brain development, vision, and

cardiovascular system. However, their intake has a positive impact on health throughout life. Omega 3 LC-PUFA are part of the regular diet because they pass through the trophic chain, but they are usually insufficient, especially in less developed countries with poor diets, which consist mostly of carbohydrates from grains (FAO Food and Agriculture Organization 2010). Fish oils, usually extracted from cod liver, are a typical example of the dietary supplement used as a source of these beneficial omega 3 LC-PUFA. However, this source has several drawbacks: 1) It comes from fishing for wild marine fish, which is a limited resource that is already under high pressure, and many marine ecosystems are damaged by overfishing . The still growing and developing human population may have a higher demand than the oceans will be able to satisfy; 2) The quality of the product may be compromised by pollution of the marine environment; 3) Wild-caught fish cannot obtain bio/organic certification in the EU or USA; 4) Many people, for various reasons, already reject animal-based diets and follow a vegetarian diet. For these reasons, customers would prefer the microalgae-based omega-3 LC-PUFAs even though they are more expensive than fish oils. Similar principles may support the development of applied phycology in the future, and further development of applied phycology may reduce the gap between the cost of traditional and microalgae-based products.

However, the situation with microalgae omega 3 LC-PUFA currently on the market is more complicated. The question is one of efficiency and sustainability and the resulting environmental impact. Evaluating the environmental impact of microalgae omega 3 LC-PUFA production compared to fish does not necessarily end in favor of microalgae. Microalgae production technologies are technically and energetically very demanding in both photoautotrophic and heterotrophic cultivation modes. This is also the logical reason for the high cost of microalgae-based products.

Currently, most vegan omega 3 LC-PUFA supplements contain mostly EPA (20:5n3) and DHA (22:6n3) and usually claim to be derived from microalgae. However, the compositional information usually indicates that the source organism is a strain of *Schizochytrium* sp., which is not an alga, but a member of a lineage of non-photosynthetic protists basal to Stramenopile. However, microalgae can also grow mixotrophically or fully heterotrophically (without light, using only organic molecules as a source of carbon and energy). Heterotrophic growth changes the physiology and biochemical profile of the algae, but in the case of PUFA production, these changes are not necessarily negative. Heterotrophic cultivation in the context of high-value products has certain advantages over autotrophic cultivation. It is much more predictable, scalable, and transferable. When using a high-quality facility and following a precise culture protocol, the risk of failure is minimized. As a result, costs can be more accurately calculated. Autotrophic cultivation under sunlight in a not perfectly closed and sterile environment is prone to failure, and the quality of the biomass produced is much more variable. In addition, in autotrophic cultures, the biomass density is much lower, growth is slower, and the cultivation units require much more surface area, all of which increases the expenses. In fact, the savings

from avoiding the use of organic substrates may be less than the costs associated with these disadvantages of autotrophic culture. Heterotrophic culture, on the other hand, requires a lot of energy to sterilize culture media and bioreactors, and other energy to maintain the conditions required by the cultured organisms. Both autotrophic and heterotrophic cultivation approaches are used today, and both are specialized for different products. However, the production of EPA and DHA by microorganisms is likely carried out mainly heterotrophically by *Schizochytrium sp.* and related organisms of the order Thraustochytrida.

However, PUFA produced by microalgae has another important aspect from a human perspective. This is the use of microalgae as live feed in marine aquaculture (Muller-Feuga et al. 2007). A large part of the fish and seafood on the market is farmed. Many of these farmed species depend on live phytoplankton in their juvenile stages. These live phytoplankton must contain vital components such as PUFAs and other lipids and can be produced directly by the aquaculture farm on site or can be sold and transported. Certain species of phytoplankton, such as *Nannochloropsis oceanica*, have an optimal composition of PUFAs and can be concentrated by centrifugation, transported, and stored for several months if kept refrigerated. Alternatively, such algae can also be dried and sold as non-living biomass for various applications, e.g. as a feed supplement for livestock. However, microalgal biomass is still too expensive to be widely used. The use of live phytoplankton is mostly applied in marine aquaculture (Ma & Hu 2023). Freshwater farmed fish species can reproduce without live feed. However, live plankton supplementation increases growth and reduces the mortality of immature fish. This is the research area where applied phycology can find novel applications, and I would like to participate in this field in my future perspective.

3.2. Algal carotenoids on the market

3.2.1. Fucoxanthin

The carotenoid pigment fucoxanthin (FX) (Fig. 4) is a microalgae-based product that has only recently been established in the market with dietary supplements or products for weight control. Like the other xanthophylls, FX participates in photosynthesis by broadening the wavelengths absorbed by the light-harvesting complexes in the chloroplasts. It absorbs wavelengths around 450 nm, which penetrate the deepest into the water column. It is estimated to be the most abundant carotenoid in the biosphere (Peng et al. 2011) due to its occurrence in the most common marine algal lineages such as diatoms, haptophytes, seaweeds, and some dinoflagellates. FX is also found in chrysophytes and smaller lineages of Stramenopiles such as Raphidophyceae, Pinguiophyceae, and Dictyochophyceae. Basically, it is present in all Stramenopiles except Eustigmatophyceae and Xanthophyceae (Jeffrey et al. 2011).

FX has been found to have a positive effect on its consumers and has many beneficial effects on human health, such as antioxidant, anti-inflammatory, anticancer, anti-obesity, anti-diabetic, hepatoprotective, skin-protective, antiangiogenic, cerebrovascular-protective, bone-protective, vision-protective, and antimalarial activities (Peng et al. 2011; Pajot et al. 2022). Therefore, an increasing demand for its sources can be expected. Unlike some other carotenoids, FX is an algae-specific pigment, as it is not produced by other microorganisms or plants. Furthermore, unlike many other carotenoids, it cannot yet be synthesized artificially, likely due to the presence of an unusual allene bond (conjugated double bonds within three carbons, C=C=C), which likely contributes to its broad bioactivity features (Zigmantas et al. 2004). Seaweeds have traditionally been the primary source of FX in the human diet, but the concentration of FX in seaweed is one or two orders of magnitude lower than it can be in microalgae biomass after certain optimizations. This scarcity of alternatives for FX production presents an opportunity for the development of microalgae-based FX production.

Another advantage of microalgae-based FX production is the existing knowledge of the strains that contain FX and their cultivation techniques. These strains are already used as live feed in marine aquaculture, which means that FX producing microalgae could be established more quickly. One such example is the marine diatom *Phaeodactylum tricornutum*, a microalgae species used primarily in this application. *P. tricornutum* is a unique diatom because of its lack of a siliceous (hydrated silicon dioxide) cell wall called a frustule, which is a characteristic feature of all other known diatoms. *P. tricornutum* was cultivated as part of this thesis (Suppl. Chapters 6, 7). There are many other strains tested in the context (for a review, see Song Wang et al., 2021; Leong et al. 2022), but so far only *P. tricornutum* has succeeded as an industrially used strain for large-scale FX production. Due to the involvement of FX as a direct compartment of light-harvesting complexes, its production is associated with photoautotrophic cultivation. However, a two-step cultivation technique is discussed (Suppl. Chapter 2). The first step employs heterotrophic growth to increase the biomass productivity rate, and the second step involves photoautotrophic cultivation, stimulating FX production and accumulation.

Recently, at least two companies have been known to develop and trade microalgal FX-containing products. These are Algatechnologies Inc. (Israel/USA) with extracts under the trade name FucoVital™ and Microphyt (France) with extracts under the trade names PhaeOptim™ and GamePhyt™. All these products are naturally based on an extract of *P. tricornutum*.

3.2.2. Astaxanthin

Astaxanthin (Fig. 4) is probably the most relevant example of a high-value compound that can be feasibly produced by microalgae on an industrial scale. Several companies produce astaxanthin from microalgae (Ambati et al. 2019), including the Czech company Algamo s.r.o., which collaborated on the applied research project presented in this thesis (Suppl. Appendix II–V). It is produced exclusively by the green alga *Haematococcus pluvialis* on a large scale. However, there is a significant concurrence of other sources. Firstly, astaxanthin can be produced synthetically and such astaxanthin is about ten times cheaper on the world market than the natural astaxanthin from *H. pluvialis*. However, the quality of such artificially produced astaxanthin differs due to various isomers that do not occur in nature. Natural biosynthesis uses specific enzymes to produce a particular astaxanthin isomer. It is claimed that these artificially produced astaxanthin isomers have a much lower bioactivity and lack most of the health benefits of natural astaxanthin (Lu et al. 2021). However, these findings are not supported by very strong evidence and synthetic astaxanthin still provides the characteristic red color that gives salmon flesh and some crustaceans their color. Farmed salmons and shrimps typically obtain their color from synthetic astaxanthin because it is much cheaper. Synthetic astaxanthin is widely used in aquaculture, but it cannot be sold as a supplement for humans. This creates an opportunity for microalgal-based production. However, other organisms synthesize astaxanthin *de novo* (Ambati et al. 2014), with the best-known example being the red yeast *Phaffia rhodozyma* (Luna-Flores et al. 2022). However, the astaxanthin content of these alternative producers is much lower than that of *H. pluvialis*, which is responsible for the vast majority of commercial natural astaxanthin production. The large-scale production of astaxanthin by *H. pluvialis* is a nice example of a two-step microalgae cultivation technique (Grujić et al. 2022). I can personally refer to the facility of the company Algamo s.r.o., which I have visited several times. The first step involves cultivating the motile 'green phase' cells of *H. pluvialis* for cell growth at lower temperatures around 20 °C and lower light intensities of white and red colors. After reaching a biomass density of about 4 g L^{-1}, the cultures are transferred to the second part of the facility. Here, stronger red and blue light is combined with sunlight, and the temperature is increased to around 26 °C. An important step in this process is the change of the culture media. In the first step, the medium is replenished with nutrients, while in the second step, it is deficient in nutrients, mostly nitrogen. The algae are further diluted to a density of about 2 g L^{-1}. In this condition, the algal cells stop dividing, but increase in size, lose motility, and form thick-walled cysts, while most importantly accumulating about 4–5 % of dry biomass with astaxanthin alone. Astaxanthin is considered as one of the most potent natural antioxidant (Hwang et al. 2020) with numerous beneficial effects on human health, including anti-cancer activity, prevention of cardiovascular disease, and support of the immune system (Higuera-Ciapara et al. 2006; Davinelli et al. 2018).

3.2.3. β-carotene

Microalgal production of β-carotene (Fig. 4) is carried out by the halophilic green alga *Dunaliella salina* (Christaki et al. 2013). However, microalgal-based production faces significant competition from chemically synthesized β-carotene, plant products, and genetically engineered heterotrophic microorganisms. The production of β-carotene by *D. salina* represents a much simpler cultivation technique compared to the production of astaxanthin. *D. salina* is cultivated in desiccating, shallow ponds with extremely high salinity, under natural conditions. The technical control is minimal, resembling more field farming than applied phycology. However, the details of these production techniques are beyond the scope of this thesis. β-carotene has many applications in the food and feed industry, as it is a provitamin A and it is also used in cosmetics (Eggersdorfer & Wyss 2018)

3.2.4. Lutein

Lutein (Fig. 4) is an isomer of the xanthophyll zeaxanthin, with distinct biological activities. It possesses general antioxidant and vision-protective properties. In chloroplasts, it participates in the xanthophyll cycle, which converts excess light into heat. Lutein is commonly used in the food and agricultural industries as a colorant for eggs and poultry and is also used in cosmetics. However, like other carotenoids, it is light-sensitive and degrades in the presence of oxygen. Lutein is traditionally extracted from plants, particularly from the flowers of *Tagetes erecta* (marigold). However, it is also commonly found in green algae (Ambati et al. 2019; Fernández-Sevilla et al. 2010). Although it is not a microalgae-based product that is currently established on the market, it is worth mentioning in the context of this thesis. The genus of green algae *Monoraphidium*, which is included in the results of this thesis (Suppl. Chapter 4) showed high concentrations of lutein (data not published). Therefore, our future work should focus on this potential source.

MOTIVATION, AIMS & APPROACHES

Microalgae are a polyphyletic and highly variable group that emerged *via* multiple independent events of endosymbiosis through which they acquired the plastid. They represent a notably diverse range of life strategies and adaptations to various environments. They are widespread in freshwater and marine habitats, but they can also be found in terrestrial ecosystems and extreme habitats such as snowfields or glaciers, hot springs, acidic lakes, or brines. They also occur in mutualistic relationships with corals or as photobionts of lichens. Additionally, they exhibit a diversity of trophic strategies, ranging from strict photoautotrophs to predominantly heterotrophic organisms that use photosynthesis only marginally under certain conditions (Sanders et al. 1990). This ecological and phylogenetical diversity is to some extent reflected in the diversity of their ecophysiological patterns and biochemical composition. Microalgae are the key primary producers in most aquatic ecosystems, converting solar energy into biomass that provides essential building blocks required by higher trophic levels. Lipids are one of such essential compartments that represent many different structure types and functions, including polyunsaturated fatty acids (PUFAs), carotenoids, sterols, vitamins, coenzymes, and the diversity of important biosynthetic precursors (Chen et al. 2023). Algal ecophysiology is closely linked to the production of these compounds, making it a highly relevant research area.

Furthermore, the ecophysiology and biochemical composition of microalgae have gained attention from an applied phycology perspective. Microalgae are capable of growing under artificial conditions as isolated single-species, monoclonal, and sometimes even axenic cultures. This is a fundamental feature for any advanced ecophysiological study, and for biochemical analysis, which usually requires a sufficient amount of biological material. During the pioneer *in vitro* studies of algal growth carried out more than a century ago, researchers observed their ability to grow relatively fast under the optimized condition of phototrophic culture mode. According to the theoretical extrapolation of such growth rates, a tremendous potential for photosynthesis-based microalgae biomass production was postulated (Myerson 1954). However, the feasibility of establishing rapid biomass production as a large-scale industry is still uncertain due to several unresolved complications and limitations (Kenny & Flynn 2017). Recently, attention has shifted from bulk, but relatively cheap commodities, such as biofuels, to fine, high-value, and algal-specific compounds (Borowitzka 2013). This change is due to a more realistic perspective that such products can make microalgae cultivation technology and downstream processes feasible even on a smaller scale. If these technologies prove to be viable, emerging companies may further develop the sector and perhaps achieve the goal postulated decades ago eventually.

Based on the described, the production of PUFAs and fucoxanthin (FX) was selected as the main topic of the thesis. These target compounds have significant relevance both in ecophysiological and applied phycological contexts (Barta et al. 2021; Pajot et al. 2022). They are involved in the fundamental structures and metabolic processes within algal cells and are in demand by the market. A broader background on these compounds as high-value products is presented in the previous section (Chapter 3. Algal lipids from a human perspective).

The basic approach used throughout most of this thesis was to perform multiparametric comparative cultivation experiments focused on the impact of selected parameters on the content and productivity of selected target compounds in microalgae biomass. The parameters monitored include temperature, light intensity, salinity, strain origin, and culture media composition. The monitored parameters as well as the tested organisms differ among the research and are presented separately in seven scientific articles (Supplements). Each of the articles addressed independent research objectives, and the experimental designs were chosen accordingly. Experiments were conducted *in vitro* using established single-strain algal cultures, and various scales, from small volumes of a few milliliters to a semi-production scale of hundreds of liters. However, most experiments were performed in medium volumes between 150 mL and 500 mL. The focus was primarily on lesser studied organisms, which can raise awareness of groups where similar studies are scarce, but in minority cases, the model organisms were also chosen. Algae strains were further selected based on their origin, adaptation, accessibility for experimental cultivation, and growth characteristics. The studied algae belonged to various taxa (Chlorophyta, Bacillariophyta, Chrysophyta, Haptophyta) and different ecological characteristics (polar, tropical, temperate, freshwater, marine, autotrophic, mixotrophic), these selections again depended on the focus of each research article. Within the articles carried out in the context of applied phycology, particular attention was paid to determining the volumetric productivity values of biomass and selected target products.

In summary, the main objective of this thesis was to advance research in microalgal ecophysiology by examining changes in PUFAs and FX content. This was achieved through manipulative cultivation experiments to monitor the effects of biotic and abiotic parameters on various microalgae, determine productivity, identify important parameters, and suggest cultivation optimization. The following results chapter will be divided into three sections: PUFA, FX, and microalgae cultivation optimization, in accordance with the set goals.

RESULTS

1. Polyunsaturated fatty acids

The examination of fatty acid (FA) content with regard to abiotic and biotic factors was one of the main focuses of the thesis. This topic was explored in five attached publications (Suppl. Chapters 1–5), with the first three publications mainly discussing the influence of temperature, lighting, the origin of strains, and their trophic strategy. The first publication focused solely on the freshwater autotrophic flagellate *Hibberdia magna* (Chrysophyceae). In the second publication, the same algal species was used again, but related *Chlorochromonas danica* (Chrysophyceae), which has a different trophic strategy (mixotrophic) was added. The third publication examines a total of 11 strains of freshwater diatoms isolated from various climatic zones. The first and third publications addressed the effect of temperature on the FA profile and demonstrated a strong impact of this factor. The length and unsaturation of FAs and amounts of LC-PUFA increased with lower temperatures in all tested strains. These results align with the described homeoviscous adaptation (introduction chapter 2.2. Factors influencing lipid composition). Additionally, the first publication tested the impact of light intensity on FA content in crossed-gradients of light and temperature, resulting in complex data. In summary, the results indicated that the lighting intensity did not significantly affect FA profiles, but rather impacted the quantity and productivity of the total FA. The second publication evaluated the influence of life strategy (autotroph *vs.* mixotroph) on FA profiles. The results presented that the autotrophic *H. magna* had significantly greater amounts and diversity of PUFAs, particularly omega 3 PUFAs, compared to the mixotrophic *C. danica*. The fourth publication focused on the cultivation of a single strain of the freshwater green alga *Monoraphidium* sp. isolated in the polar region (Antarctica). Cultivation was performed in semi-industrial mode under sunlight during early spring. The algae grew well under these conditions and had a high content of omega 3 PUFAs. When the FA profile was compared with other published data, this polar strain had the highest PUFA content ever reported for this genus. These results were consistent with the third publication that showed that diatoms isolated from polar regions had higher levels of PUFA and LC-PUFA than those isolated from other climatic zones, even when cultured under identical conditions. This shows the adaptation to the climatic conditions that are reflected in the FA profile. The fifth publication examined the effect of different salinities on the FA profiles of five different marine algae belonging to the phylum Haptophyta. The study showed that salinities outside the optimal range resulted in a reduction of the PUFA content at the expense of the saturated fatty acid (SFA) content in the algae.

The presented publications reveal the following brief conclusions regarding the FA content:

- Temperature has a clear effect on FA profiles. At lower temperatures, FAs are longer and more unsaturated (Suppl. Chapters 1, 3).
- Algae from polar regions have higher proportions of PUFA compared to strains from other climatic zones, even when cultured under the same conditions (Suppl. Chapters 3, 4).
- Photoautotrophic algae have longer and more unsaturated FAs compared to mixotrophic algae, likely due to their presence in more abundant thylakoid membranes (Suppl. Chapter 2).
- The effect of light intensity on the amount and profile of FAs is unclear, but higher light increases the productivity of total FAs (Suppl. Chapter 1).
- Algae that are stressed and outside their optimal cultivation conditions have a reduced amount of PUFAs and an increased amount of MUFA and/or SFAs (Suppl. Chapters 1, 5).

2. Fucoxanthin

The FX content with regard to culture conditions was monitored in the first two publications (Suppl. Chapters 1, 2), where the autotrophic *Hibberdia magna* (Chrysophyceae) and the mixotrophic *Chlorochromonas danica* (Chrysophyceae) were used as experimental organisms. Under optimized culture conditions, both organisms exhibited similar FX volumetric productivity values of about 1.1 mg L^{-1} day^{-1}, which are comparable values to those of other FX-producing microalgae (Pajot et al. 2022). *H. magna* had a slightly higher FX concentration per biomass but grew more slowly, while *C. danica* had a relatively low FX concentration but grew more quickly.

FX concentrations per biomass varied with time. In *H. magna*, the amount of FX tended to decrease over time, while in *C. danica*, the opposite was observed. These contrasting responses can be explained by their different ecophysiological strategies. The older culture of *H. magna* likely restricted photosynthesis in the stationary phase, leading to a decrease in FX content from a relatively high concentration, and started to accumulate storage compounds. *C. danica*, on the other hand, had sufficient sources of organic molecular substrates at the beginning of its growth and therefore had a very low concentration of FX. However, after the depletion of these substrates, it increased its photosynthetic activity and concentration of photosynthetic pigments to survive in substrate-limited conditions. Furthermore, the concentration of FX in *H. magna* was found to be strongly influenced by light intensity. Under high light conditions, the FX content was highly limited, despite the absence of any signs of stress on the growth rates. In the low light conditions, the amount of FX was much higher. However, the relationship between light intensity and FX concentration was not linear, but rather exponential. In very low light conditions, the FX concentration was more than ten times higher than in high light conditions for *H. magna*. Unfortunately, the effect of different light intensities on *C. danica* was not evaluated. It

would be interesting to do this in the future, as it can be assumed that the response of *C. danica* would be different due to its different ecophysiological strategy.

Other publications have also worked with organisms containing FX (Suppl. Chapters 3, 5, 6, 7), but its content was not determined in these works as their focus was different. Only in the sixth publication, FX was extracted from the alga *Phaeodactylum tricornutum* biomass. However, the purpose of this extraction was not to examine the influence of cultivation conditions on algal biomass. Instead, the focus was primarily on the effective extraction of FX from the biomass using the multiple injection high performance countercurrent chromatography (HPCCC) extraction method. FX extracted by this method retained its expected biological activities. This was experimentally proven by its application in human osteosarcoma-derived tissue cultures.

3. Microalgal production optimization

3.1. Results of supplemented articles

All attached publications are related to optimizing microalgal cultivation for producing high-value compounds. The first publication clearly demonstrated that the conditions leading to the highest biomass productivity for *H. magna* were not the same as those where the productivity of the target compounds was highest. In this context, the inclusion of a multi-parametric cultivation experiment is recommended to identify the precise conditions where the productivity of the target compounds is optimal. In this research, the target products were both FX and LC-PUFA. The study found that under the conditions of the highest productivity of LC-PUFA, the productivity of FX was minimal. In this case, it is important to evaluate which product is primary and which is a by-product and adjust the cultivation conditions accordingly. Cultivating microalgae that contain multiple high-value compounds can be advantageous because their production can be modulated depending on factors, such as customer demand, external cost fluctuations, or seasonal changes. If the quality of the cultivated biomass is lower because it contains less of the primary target product, it may still have some value due to the content of by-products.

The second publication demonstrated that the productivity of FX is significantly influenced by the physiological state and trophic strategy of the cultured microalgae. This finding can be advantageous for utilizing a two-step cultivation mode. In the first step, the focus is on producing biomass with maximum productivity, regardless of the target compound content. In the second step, the culture conditions are modified to stimulate the accumulation of the target compound. This technique is already used for the production of astaxanthin by *Haematococcus pluvialis*. However, it is not universally applicable due to the significant growth suppression that usually occurs in the second stage (Zhang et al. 2014). The studied alga *C. danica* could be a good candidate for this technique, which has already

been introduced for FX production by marine diatoms *Nitzschia laevis* with great results (X. Lu et al. 2018). *C. danica* increases its photosynthetic pigments, including FX, in response to the unavailability of organic substrate. However, in the presence of organic molecules or prey cells, such as bacteria or other algae, its FX content is low, but biomass production is very rapid.

The third and fourth publications showed the importance of careful strain selection for microalgal biomass production. Not only the phylogenetic relationship but also the adaptation to the natural habitat plays a role in this context. The results of these publications showed that microalgae from polar regions have a higher proportion of PUFAs than those from other regions, which can be advantageous for PUFA production. On the other hand, the third publication also showed that a similar microalgal strain isolated from a tropical region can grow in warmer conditions and even in the moderate temperature of 25 °C better than the polar ones and *vice versa*. This suggests that different strains can be utilized for microalgal production depending on the season, potentially reducing temperature control costs.

The fifth publication showed that microalgae respond to salinity stress by changing their FA profiles. This again can be used in the two-stage cultivation technique and shows that variable stress inducers can be considered. The sixth publication focuses on optimizing the downstream process of FX extraction and purification, rather than the culture conditions optimization. However, downstream processes influence the cultivation technique in a way that cultivation should be optimized to minimize downstream process costs.

The last, seventh publication was again not directly focused on the optimization of cultivation techniques, but on the search for a solution for the early detection of contamination in algal cultures. The study compared different approaches for contamination detection and evaluated their sensitivity. Molecular techniques (PCR, qPCR) were two orders of magnitude more sensitive and faster than the commonly used light microscopy. However, these molecular methods are technically and personally more demanding. Contamination is, of course, one of the main limitations of applied phycology and *in vivo* microbiology in general. However, selecting the right organisms for this application can bring certain solutions. For example, *C. danica* (Suppl. Chapter 2) has demonstrated the ability to resist some contamination due to its strong predatory behavior. It ingests contaminating microorganisms such as bacteria, yeasts, or other algae. Only filamentous microorganisms such as cyanobacteria and fungi remain a problem (personal observation).

3.2. Feasible production of microalgal biomass

The presented results indicate that making microalgae production feasible requires addressing many individual but interrelated aspects in a complex manner. The scientific literature often presents only isolated tasks that are developed only in theoretical connection to the whole microalgae cultivation technology. These tasks typically focus on topics such as bioprospecting for algal strains, comparing or modifying them, optimizing culture media, evaluating the impact of biotic or abiotic factors, searching for optimal culture modes, evaluating productivities, designing cultivation equipment, and downstream processing. However, to implement the novel technology, it is necessary to integrate all these tasks and make them work as a cohesive unit. This task may be better suited for the private sector with the support of academic institutions rather than scientific institutions alone. My motivation is to further support the evolution of this industry from this complex perspective. Some literature claims that large-scale cultivation of microalgae can only be successful with the use of genetically engineered organisms, and significant effort is put into their engineering. There are currently established technologies that can be used without the need for engineered microalgae. Furthermore, genetically modified microorganisms have drawbacks and pose certain risks to the environment. Selecting the appropriate organism is just one aspect of this complex puzzle. Comprehensive culture optimization and choice of proper cultivation techniques are necessary for achieving desired results.

4. Appendices

The last part of this thesis presents the outcomes of applied research. Appendix I presents a cultivation device that was largely constructed by me as the main designer, developer, and constructor in one person. This temperature and light controlled microalgae cultivator can be used to perform a variety of multi-parametric cultivation experiments. This device facilitates this research approach, and its use has yielded the main results of this thesis. I can highly recommend the use of this device to anyone involved in similar research. Similar multi-parametric experiments are sometimes performed in microbial well plates, but they have serious drawbacks. For example, they cannot be mixed by bubbling, so the biomass tends to settle to the bottom. The conditions in the well plates differ significantly from those in the photobioreactors used for larger scale microalgal production. The next disadvantage of well plates is their small volume, which makes it difficult to take samples during cultivation. Additionally, the amount of biomass harvested at the end of the experiment may not be sufficient for further analysis. To address this issue, a cultivation device presented in Appendix I allows for the cultivation of up to 54 individual culture tubes, each with a working volume of 180 ml. Each tube position is independently illuminated and provided with an aeration system for bubbling. The culture tubes are placed in six separate temperature-controlled water baths. Maintaining multiple independent temperatures is

a common workplace issue because laboratory incubators capable of maintaining multiple independent temperatures simultaneously are not widely available.

Appendices II–V present the outcomes of applied research focused on the cultivation of microalgae for FX and LC-PUFA. They are therefore clearly aligned with the content of this thesis. Unfortunately, they are written only in the Czech language. However, I have provided each of the appendices with a short abstract written in English. These outcomes are two verified technologies and two functional samples developed in cooperation with the company Algamo s.r.o., a Czech company dedicated to the cultivation of the microalgae *Haematococcus pluvialis* for the purpose of producing the natural carotenoid astaxanthin. The technologies used microalgae strains of freshwater chrysophyte *Hibberdia magna* (Suppl. Appendices II, III) and the marine diatom *Phaeodactylum tricornutum* (Suppl. Appendices IV, V). The technologies provide a detailed description of how to cultivate these microalgae to obtain biomass that contains the target compounds. The technologies have been verified using the facility operated by Algamo s.r.o. They also include the verification protocols with the assessment of biomass productivity, target compound content, and economic evaluation.

AUTHOR CONTRIBUTION STATEMENTS

I, doc. RNDr. Linda Nedbalová, Ph.D. declare the participation of Mgr. Antonín Střížek in the research and writing of the following publications and applied research outcomes:

Chapter 2

Střížek, A., M. Lukeš, P. Hrouzek, M. Mylenko, J. Lukavský, L. Nedbalová & P. Přibyl: **Alternative production of fucoxanthin and PUFAs using *Chlorochromonas danica* and *Hibberdia magna*, unicellular chrysophytes with different trophic modes**. *submitted to Algal Research*

Antonín Střížek participated in the preparation of the article as follows: Conceptualization; Cultivation of *H. magna*; Experiments handling; Fucoxanthin content analysis; Data analysis; Graphics; Writing & editing; Finalizing.

Chapter 3

Střížek, A., T. Řezanka, E. Hejduková & L. Nedbalová: **Adaptation of freshwater diatoms to various climatic zones manifested at the level of fatty acid** profiles. *manuscript draft*

Antonín Střížek participated in the preparation of the article as follows: Algal strains collection, selection, isolation & maintenance; Conceptualization, preparation & handling of experiments; Writing & editing.

Chapter 4

Řezanka, T., L. Nedbalová, J. Lukavský, A. Střížek & K. Sigler (2017): **Pilot cultivation of the green alga *Monoraphidium* sp. producing a high content of polyunsaturated fatty acids in a low-temperature environment**. *Algal Research* 22: 160–165

Antonín Střížek participated in the preparation of the article as follows: Algal strain maintenance; Preparation & handling of experiments; Editing.

Chapter 5

Nedbalová, L., A. Střížek, K. Sigler & T. Řezanka (2016): **Effect of salinity on the fatty acid and triacylglycerol composition of five haptophyte algae from the genera *Coccolithophora*, *Isochrysis* and *Prymnesium* determined by LC-MS/APCI**. *Phytochemistry* 130: 64–76

Antonín Střížek participated in the preparation of the article as follows: Algal strain maintenance; Cultivation system preparation; Preparation & handling of experiments; Biomass samples preparation.

APPENDIX I

Development and construction of a multi-purpose light and temperature controlled cultivation unit for the multi-parametric experimental study of aquatic microorganisms

Antonín Střížek participated in the preparation of the applied research outcome as follows: Author of the main design idea; Concept development; Selecting, purchasing, and optimizing the required components; Construction; Testing and validations; Improving; Presentation.

Nedbalová

doc. RNDr. Linda Nedbalová, Ph.D.

I, RNDr. Pavel Hrouzek, Ph.D. declare the participation of Mgr. Antonín Střížek in the research and writing of the following publications and applied research outcomes:

Chapter 1

Střížek, A., P. Přibyl, M. Lukeš, T. Grivalský, J. Kopecký, T. Galica & P. Hrouzek (2023): ***Hibberdia magna* (Chrysophyceae): a promising freshwater fucoxanthin and polyunsaturated fatty acid producer**. *Microbial Cell Factories* 22: 73

Antonín Střížek participated in the preparation of the article as follows: Conceptualization; Algal strains selection & maintenance; Experiments handling; Fucoxanthin content analysis; Dry weight & cell count analysis; Data analysis growth curve model selection and application; Graphics; Writing & editing; Finalizing.

Chapter 6

Bárcenas-Pérez, D., **A. Střížek**, P. Hrouzek, J. Kopecký, M. Barradas, A. Sierra-Ramirez, P. J. Fernandez-Marcos & J. Cheel (2021): **Production of fucoxanthin from *Phaeodactylum tricornutum* using high performance countercurrent chromatography retaining its FOXO3 nuclear translocation-inducing effect**. *Marine Drugs* 19: 517

Antonín Střížek participated in the preparation of the article as follows: Algal strains selection and maintenance; Culture media optimization; Large-scale algae cultivation support; Text editing.

Chapter 7

Grivalský, T., **A. Střížek**, P. Přibyl, J. Lukavský, R. Čegan, R. Hobza & P. Hrouzek (2021): **Comparison of various approaches to detect algal culture contamination: a case study of *Chlorella* sp. contamination in a *Phaeodactylum tricornutum* culture**. *Applied Microbiology and Biotechnology* 105: 5189–5200

Antonín Střížek participated in the preparation of the article as follows: Content conceptualization; Algal strain maintenance and experimental material preparation; Results evaluation; Text editing.

APPENDIX II

Production of *Hibberdia magna* algae biomass with a high content of fucoxanthin, polyunsaturated fatty acids, and polysaccharides

Antonín Střížek participated in the preparation of the applied research outcome as follows: Technology designing & writing; Technology verification; Algal strain maintenance & preparation; Culture media optimization; Experimental cultivations; Fucoxanthin content analysis; Results analysis & protocol writing

APPENDIX III

Biomass of alga *Hibberdia magna* containing fucoxanthin, polyunsaturated fatty acids, and polysaccharides

Antonín Střížek participated in the preparation of the applied research outcome as follows: Algal strain maintenance & preparation; Experimental cultivations; Fucoxanthin content analysis; Results analysis & protocol writing

APPENDIX IV

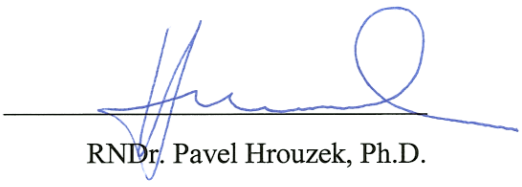
Cultivation of alga *Phaeodactylum tricornutum* for fucoxanthin production under industrial conditions

Antonín Střížek participated in the preparation of the applied research outcome as follows: Technology designing & writing; Algal strain maintenance & preparation of starting cultures; Culture media optimization; Fucoxanthin content analysis; Results analysis & protocol writing

APPENDIX V

Algal biomass with the declared content of high-value compounds

Antonín Střížek participated in the preparation of the applied research outcome as follows: Algal strain maintenance & preparation; Fucoxanthin content analysis; Results analysis & protocol writing



RNDr. Pavel Hrouzek, Ph.D.

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As part of my work at Centre Algatech, I participated in a project to present algal biotechnologies at EXPO 2020.