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LIPOLYTIC STABILITY DURING WET STORAGE OF AUTOTROPHIC MICROALGAE

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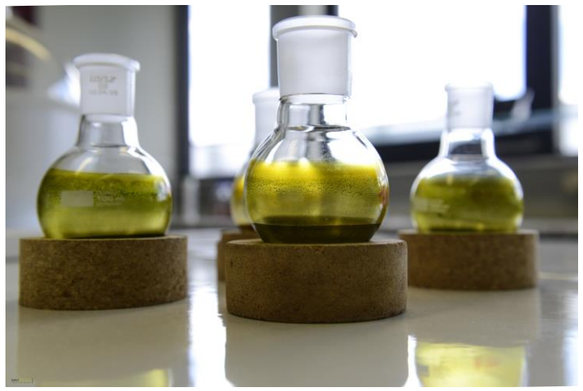
Lipolytic stability during wet storage of autotrophic microalgae



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ABSTRACT

During the last decades, an emerging interest in microalgae has been observed in different industries. Especially the lipid fraction of the microalgal cells is seen as a promising source for biodiesel, as well as for several high value applications (e.g. food, feed, cosmetics).

After harvesting the microalgae, the moisture content in the resulting biomass is still high, which makes that several degradation reactions can occur. Lipolysis, during which the fatty acids are released from their lipid backbone, possibly poses the biggest problem during this wet biomass stage. The formed free fatty acids (FFA) are detrimental for lipid quality, both for biodiesel and high value applications. Therefore, lipolytic reactions in wet biomass have to be reduced to a minimum.

Despite the importance of lipolysis for several applications, this issue is often underestimated in the microalgae industry and it has not been investigated thoroughly before. Therefore, the aim of this PhD research was to determine the factors influencing lipolytic stability during wet storage (Chapters 3-5) and how to improve the stability by different strategies (Chapters 6-7).

In **Chapters 3 and 4**, the lipolytic stability during wet storage was investigated for both *T-Isochrysis lutea* and *Nannochloropsis oculata*, two species with a promising lipid composition for both high value and biodiesel applications, but with a completely different cell (wall) structure. In *T-Isochrysis*, the FFA content was already very high immediately after harvesting and increased very rapidly during subsequent wet storage at 20°C and 4°C. On the other hand, *Nannochloropsis* biomass showed no FFA formation during the first days of wet storage under the same conditions. This difference in lipolytic stability was hypothesized to be caused by the difference in cell (wall) structure between these species. *T-Isochrysis* contains no or only a very thin cell wall, while *Nannochloropsis* possesses a tough cell wall consisting of cellulose and algaenan. Probably, the cells of *T-Isochrysis* were already ruptured during harvesting, causing the lipolytic enzymes to come into contact with intracellular lipids. *Nannochloropsis* cells were probably still intact after harvesting, which made that lipolysis did not start immediately. This hypothesis was tested by disrupting the *Nannochloropsis* cells with a high pressure homogenization (HPH) treatment. The cell disruption indeed induced a rapid FFA formation, which reinforced the

hypothesis that the integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage.

In **Chapter 5,** the influence of different HPH conditions (pressure and number of passes) on *Nannochloropsis oculata* biomass was studied more in detail. The number of passes was found to be the most important factor determining the FFA content in the biomass. The least intensive treatment (400 bar – 1 pass) was not able to improve the lipid extraction efficiency by hexane/isopropanol extraction, but caused a substantial increase of the FFA content when compared to the intact biomass. Apparently, a minor degree of cell disruption is thus sufficient to induce lipolytic reactions. Since the biomass in this experiment was not stored after the HPH treatment, the huge differences in FFA content between the different HPH conditions are formed during the short time period between disruption and freezing. Lipolysis is thus a very rapid process starting immediately from the moment the cell integrity is lost. This can pose several problems and makes research on strategies to diminish lipolytic reactions necessary.

Therefore, Chapters 6 and 7 propose two strategies to improve lipolytic stability during wet storage of *T-Isochrysis lutea* biomass, which normally experiences a very rapid increase of the FFA content after harvesting.

In **Chapter 6,** a heat treatment optimized for *T-Isochrysis lutea* biomass (8 minutes at 85°C) was found to inactivate lipolytic enzymes, as the FFA content remained constant during 7 days of wet storage at 4°C. Additionally, the heat treatment also had a positive effect on the retention of carotenoids and chlorophylls, which could improve color retention in the biomass during wet storage. No negative effects of the heat treatment on oxidative stability, ω-3 content, phenolic compounds or pigment content and composition were observed. It could thus be concluded that applying a heat treatment on the biomass is a promising method to stop lipolytic reactions during wet storage.

In **Chapter 7,** an alternative method was proposed to inhibit lipolytic enzymes in *T-Isochrysis lutea*. Harvesting by $FeCl_3$ flocculation had already been shown in the past to be an efficient method to pre-concentrate the biomass before centrifugation, lowering the costs related to harvesting. During this study, it was observed that the addition of ferric ions as a flocculant simultaneously also caused a lower amount of FFA formed during wet storage, as compared to the biomass that was harvested by

centrifugation. Especially when flocculating at the optimized pH (pH 8), the FFA content after 7 days of storage at 4°C was significantly lower than in the centrifuged biomass. This harvesting protocol also had no impact on oxidative stability, ω-3 content or pigment content and composition.

It could be concluded that lipolytic reactions during wet storage of microalgae can pose serious problems for lipid quality. If the integrity of the cells is lost, lipolysis is induced very rapidly. This PhD shows however two possible strategies to improve the lipolytic stability: inactivation of the lipolytic enzymes by a heat treatment, or $FeCl_3$ flocculation for simultaneous harvesting and inhibition of the lipolytic enzymes.

SAMENVATTING

Gedurende de laatste decennia wordt een steeds stijgende interesse in microalgen waargenomen in verschillende sectoren. Voornamelijk de lipidenfractie van microalgencellen wordt gezien als een veelbelovende bron voor de productie van biodiesel, maar ook voor verschillende hoogwaardige toepassingen (bijv. voeding, voeder, cosmetica).

Eén van de belangrijkste uitdagingen tijdens het productieproces van microalgen is het hoge vochtgehalte in de biomassa na het oogsten, wat ertoe leidt dat verschillende afbraakreacties kunnen optreden. Lipolyse, waarbij de vetzuren worden afgesplitst van de lipidenstructuur, kan mogelijk een groot probleem vormen tijdens de opslag van deze natte biomassa. De gevormde vrije vetzuren (FFA) zijn nadelig voor de kwaliteit van de lipiden, zowel voor biodiesel als voor hoogwaardige toepassingen. Daarom moeten lipolytische reacties in natte biomassa tot een minimum beperkt worden.

Ondanks het belang van lipolyse voor verschillende toepassingen, wordt dit probleem in de microalgenindustrie vaak onderschat en werd tot nu toe geen grondig onderzoek hiernaar verricht. Daarom was het doel van dit doctoraatsonderzoek om enerzijds de factoren te onderzoeken die een invloed hebben op de lipolytische stabiliteit tijdens natte bewaring (Hoofdstukken 3 – 5) en om anderzijds te onderzoeken hoe deze stabiliteit kan verhoogd worden door gebruik van verschillende strategieën (Hoofdstuk 6 – 7).

In **Hoofdstukken 3 en 4** werd de lipolytische stabiliteit tijdens natte bewaring onderzocht voor twee species, *T-Isochrysis lutea* en *Nannochloropsis oculata*. Beide species hebben een veelbelovende lipidensamenstelling voor zowel hoogwaardige als biodiesel-toepassingen, maar bezitten een compleet verschillende cel(wand)structuur. In *T-Isochrysis* biomassa was het FFA-gehalte reeds zeer hoog direct na het oogsten en steeg nog zeer snel verder tijdens daaropvolgende bewaring bij 20°C en 4°C. In *Nannochloropsis* biomassa daarentegen werd geen vorming van FFA waargenomen tijdens de eerste dagen van natte bewaring onder dezelfde omstandigheden. De hypothese werd daarom gesteld dat dit verschil in lipolytische stabiliteit veroorzaakt wordt door het verschil in cel(wand)structuur tussen beide species. *T-Isochrysis* heeft namelijk geen of slechts een zeer dunne celwand, terwijl *Nannochloropsis* een sterke celwand

bezit bestaande uit cellulose en algaenan. Waarschijnlijk worden de *T-Isochrysis* cellen reeds beschadigd tijdens het oogsten, waardoor de lipolytische enzymen in contact komen met de intracellulaire lipiden. *Nannochloropsis* cellen zijn waarschijnlijk nog intact na het oogsten, waardoor lipolyse niet direct van start gaat. Deze hypothese werd getest door de *Nannochloropsis* biomassa te disrupteren door middel van een hogedruk homogenisatie (HPH) behandeling. De celdisruptie induceerde inderdaad een zeer snelle vorming van FFA, wat de hypothese versterkt dat de integriteit van de microalgencel een zeer belangrijke rol speelt in de lipolytische stabiliteit tijdens natte bewaring.

In **Hoofdstuk 5** werd de invloed van verschillende HPH condities (druk en aantal passages) op *Nannochloropsis* biomassa meer in detail bestudeerd. Het aantal passages werd bepaald als de factor met de meeste invloed op het FFA-gehalte in de biomassa. De minst zware HPH behandeling (400 bar – 1 passage) was niet in staat om de lipidenextractie-efficiëntie met hexaan/isopropanol substantieel te verhogen, maar veroorzaakte wel een duidelijk verschil in FFA-gehalte vergeleken met de intacte biomassa. Blijkbaar is dus een minieme hoeveelheid celdisruptie al voldoende om lipolytische reacties te induceren. Aangezien de biomassa in dit experiment niet bewaard werd na de HPH-behandeling, zijn de grote verschillen in FFA-gehalte tussen de verschillende HPH-condities reeds gevormd tijdens de korte tijdsperiode tussen het disrupteren en het invriezen. Lipolyse is dus een zeer snel proces dat direct optreedt vanaf het moment dat de celintegriteit verloren gaat.

In Hoofdstukken 6 en 7 werden daarom twee mogelijk strategieën voorgesteld om de lipolytische stabiliteit tijdens natte bewaring van *T-Isochrysis lutea* biomassa te verbeteren. In dit species wordt namelijk normaal een zeer snelle stijging van het FFA-gehalte waargenomen na het oogsten.

In **Hoofdstuk 6** werd een hittebehandeling geoptimaliseerd voor *T-Isochrysis lutea* (8 minuten bij 85°C). Deze hittebehandeling was in staat om de lipolytische enzymen te inactiveren, aangezien het FFA-gehalte constant bleef tijdens natte bewaring van de biomassa gedurende 7 dagen bij 4°C. Daarenboven had de hittebehandeling ook een positief effect op het behoud van carotenoïden en chlorofyl, wat het behoud van kleur tijdens natte bewaring van de biomassa zou kunnen bevorderen. Er werden geen

negatieve effecten waargenomen op de oxidatieve stabiliteit, het ω-3-gehalte, de hoeveelheid fenolische componenten en pigmenten. Daaruit kon dus geconcludeerd worden dat het toepassen van een hittebehandeling op de biomassa een veelbelovende methode is om lipolytische reacties tijdens natte bewaring te stoppen.

In **Hoofdstuk 7** werd een alternatieve methode voorgesteld om lipolytische enzymen in *T-Isochrysis lutea* te inhiberen. Oogsten door middel van $FeCl_3$ flocculatie werd in het verleden reeds voorgesteld als een efficiënte methode om de biomassa te preconcentreren voorafgaand aan centrifugatie, wat ook de kosten voor centrifugatie vermindert. Tijdens deze studie werd waargenomen dat de Fe(III)-ionen toegevoegd als flocculant tegelijk ook zorgden voor minder vorming van FFA tijdens natte bewaring. Vooral wanneer geflocculeerd werd bij een geoptimaliseerde pH (pH 8) was het FFA-gehalte na 7 dagen bij 4°C significant lager dan in de gecentrifugeerde biomassa. Het oogstprotocol met $FeCl_3$ flocculatie had ook geen negatieve invloed op de oxidatieve stabiliteit, het ω-3-gehalte en de hoeveelheid pigmenten.

Er kan dus geconcludeerd worden dat lipolytische reacties tijdens natte bewaring van microalgen ernstige problemen kunnen veroorzaken voor de kwaliteit van de lipiden. Wanneer de integriteit van de cel verloren is, wordt lipolyse namelijk al zeer snel geïnduceerd. Dit doctoraatsonderzoek geeft echter ook twee mogelijke remediërende technieken om de lipolytische stabiliteit te verhogen: inactivatie van de lipolytische enzymen door een hittebehandeling, of $FeCl_3$ flocculatie om tegelijk te oogsten en de lipolytische reacties te verminderen.

LIST OF ABBREVIATIONS

AFDW	Ash free dry weight
CM	Chloroform / methanol
DAG	Diacylglycerol
DGDG	Digalactosyl diacylglycerol
DGMG	Digalactosyl monoacylglycerol
DGTS	Diacylglycerol-O-(N,N,N-trimethyl)-homoserine
DHA	Docosahexaenoic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
HI	Hexane / isopropanol
HPH	High pressure homogenization
LC-PUFA	Long chain polyunsaturated fatty acid
MAG	Monoacylglycerol
MGDG	Monogalactosyl diacylglycerol
MGMG	Monogalactosyl monoacylglycerol
MGTS	Monoacylglycerol-O-(N,N,N-trimethyl)-homoserine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PV	Peroxide value
SQDG	Sulfoquinovosyl diacylglycerol
SQMG	Sulfoquinovosyl monoacylglycerol
TAG	Triacylglycerol

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CONTEXT AND AIM

During the last decades, research on microalgae and microalgal lipids in particular has evolved considerably. At first, industries were mainly interested in upscaling the production process to produce alternative fuels, as a consequence of the energy crisis from 2000 till 2008. Microalgal biofuels can have several advantages over the current biofuels from palm, rapeseed or soybean oil. First, they can be produced on non-arable land and are thus not in competition with food production [1, 2]. In addition, microalgae have a higher oil content and a higher areal biomass productivity compared to terrestrial crops [1, 3].

Later, several sectors became interested in other bioproducts produced by microalgae, for applications in for example food, feed, pharmaceuticals and cosmetics [4]. Products of interest are unique bioproducts that are not produced by terrestrial plants such as some omega-3 fatty acids and pigments (e.g. phycocyanin, astaxanthin) [5, 6]. Especially interesting for these industries is the presence of high amounts of long chain omega-3 polyunsaturated fatty acids (ω-3 LC-PUFA), especially eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) [7] in some microalgae. These fatty acids have been proven to have beneficial effects on human and animal health [8, 9]. Currently, ω-3 LC-PUFA are taken in by consuming fish, but this source is decreasing as wild-harvest fish stocks are decreasing and the demand for fish is increasing because of the increasing global population. Therefore, alternative sources of ω-3 LC-PUFA, such as several microalgae, are necessary.

Although microalgae often contain high amounts of lipids (until 60% of the biomass, depending on the species and the cultivation conditions) [1], the production of microalgal oil brings several challenges, which makes that research on microalgae is often still on laboratory scale. One of these challenges is the high water content in the biomass, which is still around 75% after harvesting (dewatering) [2, 10]. This implies that several stability problems can occur, e.g. bacterial growth, enzymatic degradation reactions, lipid oxidation. In food industries, these reactions have been demonstrated to have a higher reaction rate at a higher water activity (which is a measure for the amount of available water) [11, 12]. Currently used oil seeds (e.g. linseeds,

cocoa beans, sunflower seeds) often have a much lower water activity and thus the abovementioned problems occur only to a lesser extent. However, in products used for the production of fruit or pulp oils (e.g. oil palm, olive), in which the oil is derived from the pulp instead of the seeds, the water activity is also higher. Consequently, more enzymatic reactions such as lipolysis are initiated by damage of the cells, which makes that more treatments (e.g. heat treatments) have to be applied to stabilize the oil [13, 14].

Although it can be expected that comparable stability problems also arise in wet microalgal biomass, this problem is often underestimated and thus research on this topic is only very limited. However, it is often necessary for practical reasons to store the wet biomass for some time period before drying is performed. In addition, a lot of studies even investigate methods to circumvent the energy and cost intensive drying step, which leads to even more extended period of wet biomass storage. There is for example a lot of research about wet extraction during which lipid extraction is performed from the wet biomass paste and consequently the drying step is omitted [15, 16]. Furthermore, it has also been investigated whether the lipid extraction could be combined with transesterification (i.e. the conversion of fatty acids from the lipids to methyl esters) for the production of biodiesel [17, 18]. Although a stage of wet biomass is thus often used in practice, it is not investigated thoroughly before whether this has consequences for lipid quality.

In literature, there are a few indications that lipolysis, which involves the release of fatty acids from the lipid backbone to produce free fatty acids (FFA), is one of the major problems during wet storage. A first indication is the common presence of high amounts of FFA in harvested microalgae [19-21], which is not the case in living, healthy cells, as FFA are cytotoxic for the cells [1, 22]. This suggests that lipolytic reactions occur during downstream processing of the biomass. Furthermore, there are a few studies that demonstrate a rapid increase of FFA during wet storage of microalgal biomass before drying [19, 23, 24]. The formed FFA have a negative impact on lipid quality for several applications. For high value applications, especially the negative effect of FFA on the flavor is an important disadvantage. In addition, FFA can promote oxidation reactions, which in turn negatively impact flavor and nutritional value [11, 25]. During biodiesel production, FFA cause a decrease of the efficiency of transesterification and an increased need for downstream processing [26, 27].

Despite the importance for several applications, lipolytic processes during storage of microalgae have not been thoroughly investigated before. Therefore, the aim of this PhD study was to investigate the factors influencing lipolytic stability during wet storage of microalgal biomass and different ways to improve this lipolytic stability.

Chapter 1 gives an introduction on the general structure of microalgal cells and the composition of their lipid fraction. Additionally, the different steps in the production of microalgal oil, from cultivation and harvesting to extraction, are briefly explained. In the last part, the different applications of microalgal lipids are described. **Chapter 2** focusses on the main degradation reactions of lipids. Both lipid oxidation and lipolysis are explained, together with the factors influencing the reaction rate and the effects of the reactions on different applications. An overview of the current literature on lipolytic stability during wet storage of microalgae is also given.

In the experimental section, schematically presented in Figure 1, the temperature, time and species dependence of lipolysis were investigated (**Chapter 3 and 4**). Related to this species dependence, also the influence of cell integrity and cell disruption was studied. The effect of cell disruption by High Pressure Homogenization (HPH), which was observed in Chapter 4, was elaborated in **Chapter 5** by studying the impact of different HPH conditions (pressure and number of passes) on the FFA content. As lipolysis was found to occur very rapidly, it was also studied which strategies could be developed to improve lipolytic stability and enable longer wet storage. **Chapter 6** investigates the possibility of inactivating the lipolytic enzymes by a heat treatment, while **Chapter 7** focuses on a method to harvest the microalgae by $FeCl_3$ flocculation which simultaneously improves the lipolytic stability by inhibition of lipolytic reactions. At last, the final conclusions are discussed in **Chapter 8**, together with some future prospects concerning this topic.

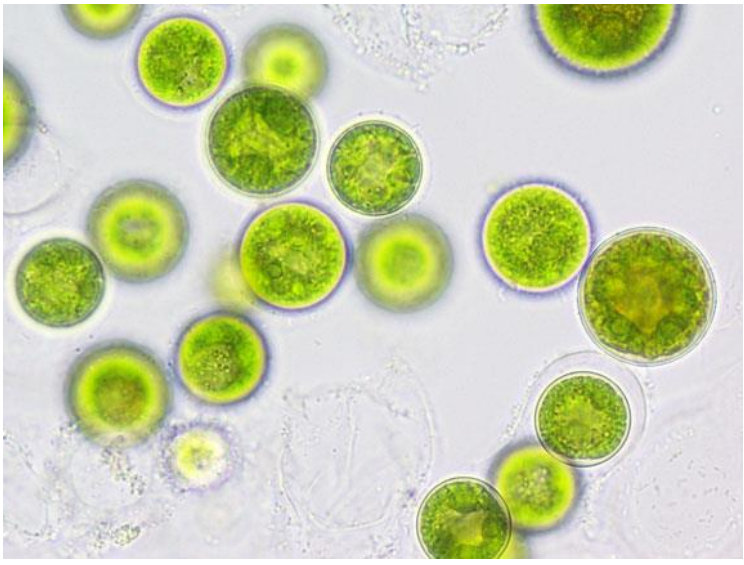
Throughout this PhD, two microalgal species were studied, *T-Isochrysis lutea* and *Nannochloropsis oculata*. Both species contain substantial amounts of lipids and especially also ω-3 LC-PUFA, enabling their application in high value applications, as well as in biodiesel, both industries where lipolysis has disadvantageous effects on product quality. However, the two species strongly differ in cell structure and composition, which makes it possible to study the effect thereof.

Part I: Factors influencing lipolytic stability during wet storage		
	Species	*Influence of*
Chapter 3	 *T-isochrysis lutea*	Time and temperature
Chapter 4	 *T-isochrysis lutea*  *Nannochloropsis oculata*	Time and temperature Species Cell disruption (HPH)
Chapter 5	 *Nannochloropsis oculata*	HPH conditions (pressure, number of passes)
Part II: Strategies to improve lipolytic stability		
	Species	*Strategy*
Chapter 6	 *T-isochrysis lutea*	Inactivation of lipolytic enzymes by a heat treatment
Chapter 7	 *T-isochrysis lutea*	Simultaneous harvesting and inhibition of lipolytic reactions by $FeCl_3$ flocculation

Figure 1: Outline of the experimental part of this PhD dissertation

Chapter 1:

Microalgae as producers of lipids for different applications



CHAPTER 1 MICROALGAE AS PRODUCERS OF LIPIDS FOR DIFFERENT APPLICATIONS

1.1. What are microalgae?

Microalgae represent a genetically diverse group of microorganisms, living in a variety of habitats, from fresh water and sea water to more extreme environments like humus soil, desert sand and rocks [28]. There are 200.000 to several million species, including both eukaryotic microalgae, e.g. diatoms (Bacillariophyta), green algae (Chlorophyta), red algae (Rhodophyta) and dinoflagellates (Dinophyta), and prokaryotic microalgae, i.e. cyanobacteria [29, 30]. Microalgae can have cell sizes from 2 to 50 µm and can have several shapes and forms, such as elongated, filamentous or spherical [10, 28]. Most microalgae are free living and unicellular, but also associations with other organisms and colonial species occur [28].

Depending on the needs of microalgae for their cellular processes, microalgal growth is either obligate photoautotrophic, obligate heterotrophic or sometimes mixotrophic. To grow photoautotrophically, only an inorganic carbon source (mostly CO_2), nutrients (nitrogen, phosphorus and trace metals) and light are necessary to perform photosynthesis [10, 31]. Heterotrophic species on the other hand use organic carbon to produce energy for cell growth. Species growing mixotrophically are mainly growing photoautotrophically, but need also organic compounds as a carbon source. A subtype of mixotrophy is called amphitrophy, which means that these species can switch between both processes, depending on the organic compounds and light sources available [28, 30, 32].

During this PhD, the focus is on eukaryotic microalgae that are growing photoautotrophically. These species have already been demonstrated to be a good source of ω-3 LC-PUFA, as well as several other health promoting compounds such as carotenoids and phytosterols, which makes them interesting for food applications [7]. Several antioxidants present in the biomass provide a better oxidative stability of the obtained oil [33]. In addition, as CO_2 is taken from the environment and no other organic carbon source has to be provided, growth of photoautotrophic microalgae is also more sustainable than heterotrophic microalgae [7]. When mentioning microalgae further in this manuscript, photoautotrophically growing species are meant.

1.2. Structure of the eukaryotic microalgal cell

The structure of eukaryotic microalgal cells is similar to that of other eukaryotes, but some characteristics are also similar to cellular organelles of higher plants [28]. Several organelles are located in the cytosol and all perform specific functions, e.g. the nucleus, which contains the genetic material and the chloroplast, which performs photosynthesis.

Lipid bodies and **starch granules** respectively contain lipids and polysaccharides and are especially formed under stress conditions [28, 34]. Lipid bodies consist of a neutral lipid core surrounded by a monolayer of polar lipids and proteins. This protein fraction (1-5% of lipid droplet weight) includes both structural proteins and enzymes. Lipid bodies can also contain some minor components such as carotenoids and sterol esters [1, 35, 36]. Next to their function for storage of energy reserves, lipid bodies also have their role in lipid synthesis and degradation, vesicle trafficking, lipid secretion and lipid-based signaling [35].

The cells are surrounded by a cell membrane and a cell wall. The **cell membrane** functions as a selective barrier, controlling the passage of compounds in and out of the cell. It is composed of lipids, mainly phospholipids and other polar lipids, and some proteins [28]. The detailed structure and composition of the **cell wall** strongly depends on the species, but also on the cultivation conditions [34, 37]. Beacham et al. (2014)[38] for example found a thickening of the cell wall in *Nannochloropsis* in response to osmotic stress in saline conditions. For most of the species, the exact structure of the cell wall is not well known. However, the cell wall of *Nannochloropsis* cells has been studied and has been found to consist of two polymeric layers, of which the outer layer is algaenan-based, while the inner layer is cellulose-based (Figure 1.1). Algaenan in these *Nannochloropsis* cells is proposed to consist of straight-chain, saturated, aliphatic compounds (approximately C30) that are connected to each other by different ether bonds. Struts connect the inner layer to the cell membrane and some unknown extensions are bound to the outer layer [39]. The cell wall of red microalgae (Rhodophyta) is mainly composed of cellulose or xylan and mucilages (agar or carrageenan) [40], while most diatoms (Bacillariophyta) have a cell wall composed of amorphous silica (SiO_2), proteins, long-chain polyamines and polysaccharides (mainly glucoronomannan) [41]. Other

species are lacking a cell wall or have only a thin layer consisting of mannose (e.g. *Isochrysis sp.*) [42, 43].

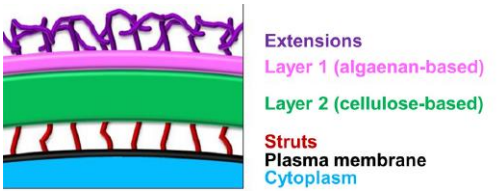


Figure 1.1: Structure of the cell wall of *Nannochloropsis* [39]

1.3. Microalgal lipids

Oleaginous microalgae are traditionally defined as microalgae containing more than 20% lipids [1]. Examples are *Porphyridium*, *Dunaliella*, *Isochrysis*, *Nannochloropsis*, *Tetraselmis*, *Phaeodactylum* and *Chlorella*, which have a lipid content until 60% of their dry weight (Table 1.1). The actual lipid content however strongly depends on cultivation conditions such as temperature, nutrient concentrations and pH [1]. The highest lipid content can be achieved by applying low N concentrations in the growth medium [44-46].

Microalgal lipids comprise a range of lipid classes, varying from acylglycerols, phospholipids, glycolipids, free fatty acids, hydrocarbons and several lipophilic pigments. All of these lipid classes have different characteristics such as polarity and solubility, but also a different location and function within the microalgal cell [1, 47]. In this thesis, lipids are divided into two groups: saponifiable lipids, which contain fatty acids, and unsaponifiable lipids lacking fatty acids and consisting of other chemical structures. The structures of the different compounds belonging to both groups are demonstrated and explained in the next sections.

Table 1.1: Lipid content (% dry weight) in some frequently used microalgal species (based on Mata et al., 2010 [32])

Microalgal species	*Lipid content (% dry weight)*
Chlorella vulgaris	5-58
Dunaliella salina	6-25
Isochrysis galbana	7-40
Nannochloropsis sp.	12-53
Phaeodactylum tricornutum	18 -57
Porphyridium cruentum	9-19
Scenedesmus obliquus	11-55
Spirulina platensis	4-17
Tetraselmis suecica	9-23

1.3.1. Saponifiable lipids

Saponifiable lipids include acylglycerols, phospholipids, glycolipids, sphingolipids, sulfolipids and betaine lipids and consist of one or more fatty acids esterified to a head group. The structure of these fatty acids is explained in 1.3.1.1, while the chemical structure of the saponifiable lipid classes containing these fatty acids is explained in 1.3.1.2.

1.3.1.1. *Fatty acids*

Fatty acids are monocarboxylic acids containing an aliphatic chain of 4 to 24 carbon atoms, which is either completely saturated or unsaturated with one or more double bonds. Apart from the difference in chain length, also the position and configuration of the double bonds are varying. Fatty acids can be named systematically as carboxylic acids or by their trivial name. Although the position of the double bonds normally is numbered counting from the carboxyl end in the systematic name, the numbering from the methyl end of the fatty acids is also often used. In the latter case, it is written as n-x or ω-x, where x is the number of the first double bond counting from the methyl end. If there is more than one double bond, it is implied that the double bonds are separated by a methylene group [48].

Table 1.2 gives an overview of the structure and names of the most prevalent fatty acids in microalgae [47, 48]. Especially interesting is the presence of long chain omega-3 polyunsaturated fatty acids (ω-3 LC-PUFA), e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as these are not present in higher plants [47] and possess several health promoting functions [8, 9] (further described in 1.5.2.1).

The fatty acid composition of microalgal lipids can be altered by the amount of nutrients, the temperature and the light intensity during cultivation. Also the growth phase and aging of the culture can influence the fatty acid composition [47]. For example, the amount of PUFA increases with decreasing temperature, to maintain the membrane fluidity [49].

Table 1.2: Nomenclature of the most common fatty acid in microalgae (based on Scrimgeour and Harwood, 2007 [48] and Hu et al., 2008 [47])

Structure	Systematic name	Trivial name	Shorthand name	Ω
$CH_3(CH_2)_{12}COOH$	Tetradecanoic acid	Myristic acid	C14:0	
$CH_3(CH_2)_{14}COOH$	Hexadecanoic acid	Palmitic acid	C16:0	
$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	Z-9-hexadecenoic acid	Palmitoleic acid	C16:1	
$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Z-9-hexadecenoic acid	Oleic acid	C18:1	
$CH_3(CH_2)_3(CH_2CH=CH)_2(CH_2)_7COOH$	Z,Z-9,12-octadecadienoic acid	Linoleic acid	C18:2	ω-6
$CH_3(CH_2CH=CH)_3(CH_2)_7COOH$	Z,Z,Z-9,12,15-octadecatrienoic acid	α-linolenic acid	C18:3	ω-3
$CH_3(CH_2CH=CH)_4(CH_2)_4COOH$	Z,Z,Z,Z-6,9,12,15-octadecatetraenoic acid	Stearidonic acid	C18:4	ω-3
$CH_3(CH_2)_3(CH_2CH=CH)_4(CH_2)_3COOH$	Z,Z,Z,Z-5,8,11,14-eicosatetraenoic acid	Arachidonic acid	C20:4	ω-6
$CH_3(CH_2CH=CH)_5(CH_2)_3COOH$	Z,Z,Z,Z,Z-5,8,11,14,17-eicosapentaenoic acid	Eicosapentaenoic acid (EPA)	C20:5	ω-3
$CH_3(CH_2CH=CH)_6(CH_2)_2COOH$	Z,Z,Z,Z,Z,Z-4,7,10,13,16,19-docosahexaenoic acid	Docosahexaenoic acid (DHA)	C22:6	ω-3

1.3.1.2. *Chemical structure of saponifiable lipid classes*

Figure 1.2 shows the chemical structure of the most common saponifiable lipid classes in microalgae [1, 29, 50]. **Acylglycerols** comprise triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG), which contain respectively three, two or one fatty acid chain esterified to a glycerol backbone. Hydrolysis at the ester position of these acylglycerols releases **free fatty acids (FFA)** from the backbone.

Phospholipids consist of glycerol, esterified with two fatty acid chains, with the third position of glycerol bound to a phosphate-containing head group. The major phospholipids in microalgae are phosphatidylcholine (PC), phosphatidylglycerol (PG), phophatidylethanolamine (PE) and phosphatidylinositol (PI) [1, 50]. Lyso-derivatives, in which one of the fatty acid chains has been hydrolyzed from the glycerol backbone, are also found in small amounts and are usually indications of lipid hydrolysis [48].

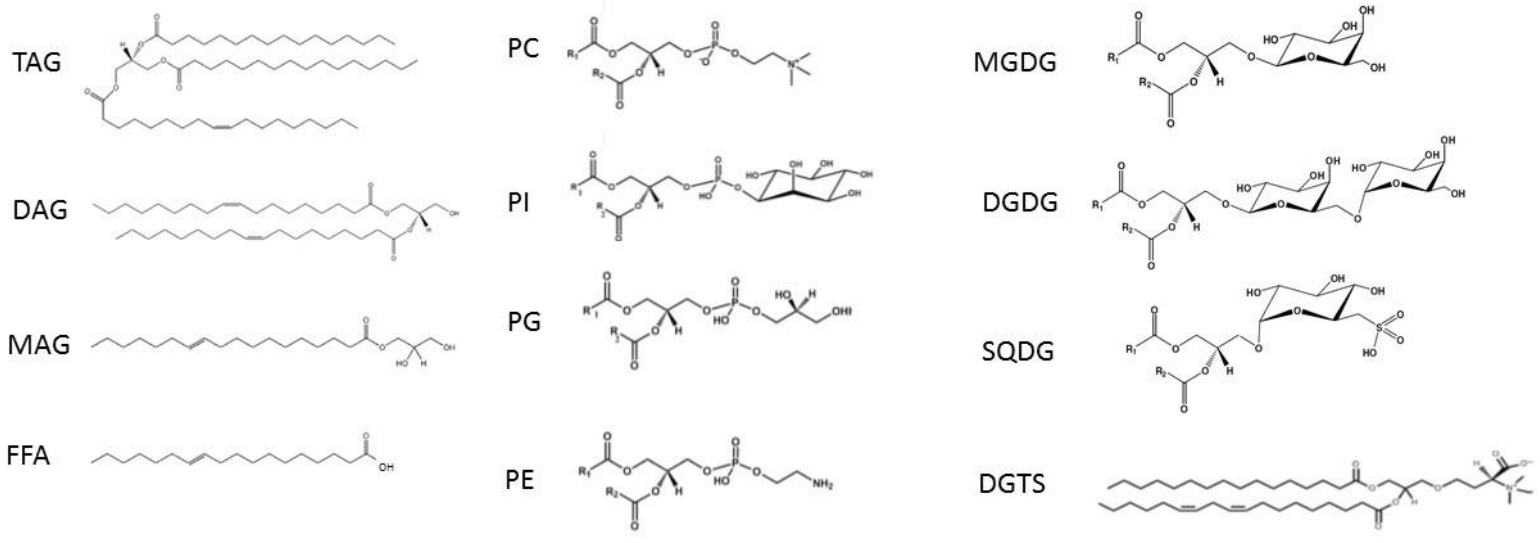


Figure 1.2: Overview of the chemical structures of the most common lipid classes in microalgae: TAG (triacylglycerol), DAG (diacylglycerol), MAG (monoacylglycerol), FFA (free fatty acid), PC (phosphatidylcholine), PI (phosphatidylinositol), PG (phosphatidylglycerol), PE (phosphatidylethanolamine), MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiacylglycerol), SQDG (sulfoquinovosyldiacylglycerol) and DGTS (1,2-diacylglycerol-O-4-(N,N,N-trimethyl)homoserine (adapted from Khozin-Goldberg, 2016 [50] and Dong et al., 2016 [1]).

Glycolipids also consist of a glycerol backbone in which two positions are esterified with fatty acids. The third position is linked to one to four sugars, usually galactose molecules [48]. In microalgae, mostly monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), respectively with one and two galactose units, occur. Also **sulfolipids**, mainly sulfoquinovosyldiacylglycerol (SQDG), are predominant in several microalgae. In this lipid class a sulphonic acid is linked to the sugar molecule [48]. Many microalgae also contain **betaine lipids**, mostly 1,2-diacylglycerol-O-4-(N,N,N-trimethyl)homoserine (DGTS) [50].

Acylglycerols are considered to be neutral lipids, while the addition of other head groups in phospholipids, glycolipids, sulfolipids and betaine lipids gives them a more polar character and makes that they are considered as polar lipids. The relative composition of these lipid classes strongly depends on the species and the cultivation conditions. However, microalgae contain a relatively high amount of polar lipids (mainly phospholipids and glycolipids) between 17 and 90% of the total lipid content [1].

1.3.1.3. *Function and location of saponifiable lipids in the microalgal cell*

When growing in optimal circumstances, microalgae mainly synthesize fatty acids for incorporation in membrane lipids (cell membrane and endoplasmic membranes). These are mainly glycolipids, sulfolipids, phospholipids and in many microalgae also betaine lipids [50, 51]. Glycolipids, sulfolipids and PG are located in chloroplast membranes and have a role in photosynthesis, while PC, PE and betaine lipids are located in the endoplasmic reticulum and the lipid body membrane and have their function in the biosynthesis of PUFA and the changes in membrane lipids in response to environmental condition changes [35, 52].

When conditions turn unfavorable (e.g. in stationary growth phase, when nutrients are depleted) or when certain stress factors are present (e.g. unfavorable light intensity or salinity), microalgal cells change their biosynthetic pathways and start synthesizing TAG to store them as an energy reserve in lipid bodies. The energy reserves built up under stress conditions are used to rebuild membrane lipids under favorable conditions, as this process is much faster than de novo synthesis of fatty acids [35, 37, 47, 53-55]. Next to changes in the total lipid content and lipid classes, stress conditions

can also induce changes in the fatty acid composition. For example, when the temperature is too low, microalgae produce more unsaturated fatty acids, to adapt the fluidity of the membranes [56].

FFA are typically bound to acyl carrier protein (ACP) during metabolic processes and transport of fatty acids within a living cell [57]. The presence of unbound FFA in high concentrations can have a cytotoxic effect, as this can lead to loss of membrane integrity and leakage of compounds from the cells [58, 59]. In addition, FFA inhibit electron transfer in photosynthetic systems and can also be oxidized to other toxic degradation products [1, 22]. The presence of FFA at high levels thus indicates the occurrence of lipolysis, a process that is further described in Chapter 2.

1.3.2. Unsaponifiable lipids

Next to the saponifiable lipids, microalgae can also contain substantial amounts of unsaponifiable lipids, which do not contain ester bonds with fatty acids. Examples are several lipophilic pigments (mainly carotenoids and chlorophylls), sterols and some very long chain alkenones and alkenoates found in specific microalgae species.

Carotenoids are yellow, orange and red pigments with a C40 backbone consisting of isoprene units [60]. Carotenoids are divided into two classes: carotenes, which only contain hydrogen and carbon atoms (e.g. β-carotene), and xanthophylls, which contain one or more oxygen atoms in the form of keto- or hydroxyl groups and therefore have a more hydrophilic character (e.g. violaxanthin, astaxanthin, fucoxanthin, lutein) [60, 61]. Figure 1.3 shows the chemical structure of the most prevalent carotenoids in microalgae, mostly found in ester form [60]. The carotenoid composition depends strongly on the taxonomic class to which a microalgal species belongs [62].

Carotenoids have strong antioxidant properties, which enables them to protect against reactive radicals and to retard lipid oxidation. They also improve the integrity of membranes. Primary carotenoids such as lutein are located in the chloroplast, attached to the thylakoid membrane, and play an essential role in photosynthesis. They absorb light and quench excessive energy in the photosynthetic metabolism. Secondary carotenoids (e.g. astaxanthin, canthaxanthin) are accumulated in lipid droplets in the microalgal cell and form a protective layer in stress conditions [60, 63].

Chlorophylls are green pigments located in the chloroplast [62]. The chemical structure consists of a porphyrin (tetrapyrrole) ring, a central magnesium atom, side chains and a phytol tail. The porphyrin ring is bound to protein layers, while the phytol tail extends into the lipid layers [26]. Depending on the taxonomic class to which the microalgal species belongs, different forms of chlorophylls, of which the structure is given in Figure 1.4, are present (a, b, d, c1, c2, c3) [26, 62, 64].

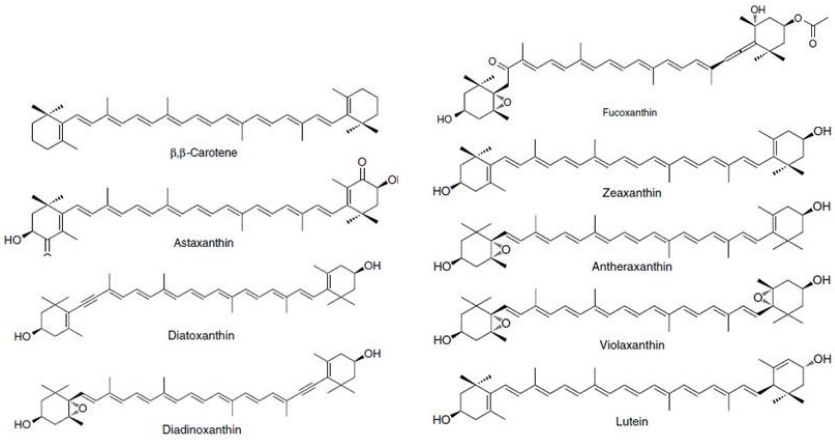


Figure 1.3: Chemical structure of the most common carotenoids in microalgae (adapted from Egeland, 2016 [65])

Next to carotenoids and chlorophylls, also **sterols** are found in the lipid fraction of microalgal biomass. Examples are cholesterol, brassicasterol, campesterol, stigmasterol, fucosterol and sitosterol [66]. The composition and concentration of phytosterols in microalgae strongly depends on the species and the applied cultivation conditions, but percentages of 0.4 to 2.6 % of dry weight can be achieved by the highest producers (*Pavlova*, *Tetraselmis* and *Nannochloropsis*) [67].

Some haptophyta species (e.g. *Isochrysis galbana* and *Emiliania huxleyi*) also contain another type of neutral lipids, polyunsaturated long-chain (C37-C39) **alkenones, alkenoates and alkenes**. They are synthesized in chloroplasts and then transported to lipid bodies, where they function as energy reserves [68, 69].

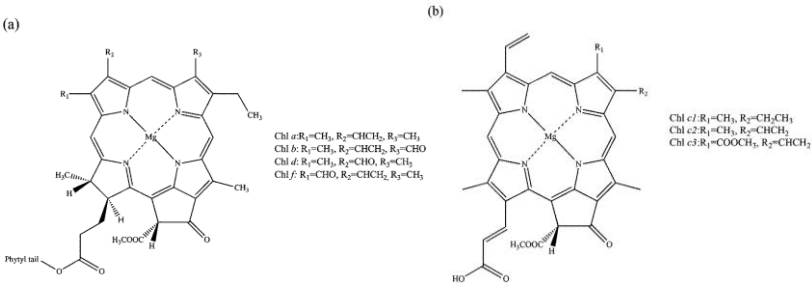


Figure 1.4: Structure of (a) chlorophyll a, b and d, (b) chlorophyll c1, c2 and c3 [64]

1.4. Production of microalgal biomass and microalgal oil

The production of microalgal biomass and the oil derived from this biomass consists of several steps, which are presented in Figure 1.5 and further explained in the following sections.

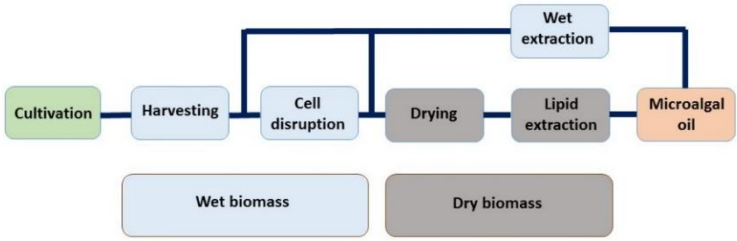


Figure 1.5: General steps during the production process of microalgal oil

1.4.1. Cultivation

Photoautotrophic microalgae cultivation on large scale can be conducted either in raceway ponds or in photobioreactors, each having advantages and disadvantages. **Raceway ponds** are open systems in which mixing and circulation are produced by a paddle wheel (Figure 1.6A). This method is most commonly used and has the lowest building and operating costs. However, light and carbon dioxide are mixed and dispersed less efficiently. In addition, this method reaches lower biomass productivities and is prone to contamination and seasonal variations [2, 3, 27, 70]. **Photobioreactors** (Figure 1.6B) on the other hand, are closed systems and exist in a wide range of designs such as tubular columns, tubular plates and flat plates. These systems are more cost intensive but allow to control parameters (pH,

temperature, CO_2 concentration) in an effective way, which results in a higher productivity. In addition, the risk of contamination in these systems is also reduced [2, 3, 70].

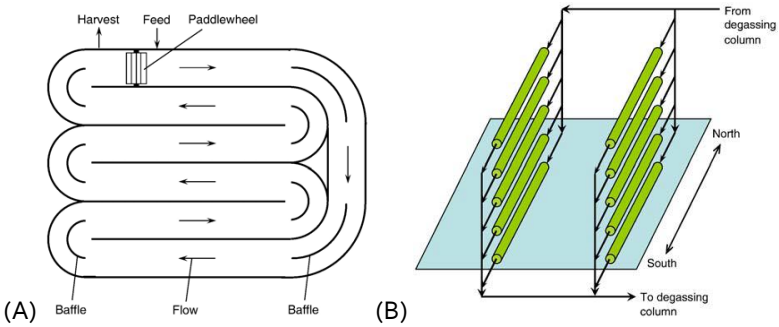


Figure 1.6: Microalgae cultivation in an open pond system (A) and photobioreactor (B) [3]

1.4.2. Harvesting

The small dimensions of the microalgal cells (2 to 50 µm) and the very low density of the microalgae in the culture medium (0.5 to 5 g/L) both make harvesting a costly and challenging step in the production process [34]. Different harvesting techniques are possible, but no universal ideal technique exists. The choice of a harvesting technique rather depends on the microalgal species, the cell density and the type of application [10, 18, 71]. It is often advantageous to conduct harvesting in two steps. The primary or bulk harvesting concentrates the biomass to 2-7% dry weight (e.g. flocculation, sedimentation, flotation), while in the secondary or thickening step the biomass is further concentrated to a dry weight up to 15-25% (e.g. centrifugation, filtration) [2, 10, 34, 72].

Centrifugation, used as a single-step harvesting method, is the most common harvesting technique using centrifugal forces to accelerate the sedimentation rate of microalgal cells. Many different designs of centrifuges are possible, with varying centrifugal forces [10]. Advantages of this technique are the high recovery rate and the possibility to apply on all species. Moreover, the resulting biomass is free of chemicals or flocculants [10]. However, especially for species with smaller dimensions (<10 µm), high centrifugal forces are necessary, which makes it a very energy consuming technique [29, 34]. Furthermore, the gravitational and shear forces can possibly damage the cell structure [10, 70].

Filtration uses a semi-permeable membrane as a barrier for the separation of the microalgal cells and the liquid of the suspension. The pore size of the membrane is chosen smaller than the cell size, so that the cells are retained on the membrane, while the water, salt and other soluble compounds are passing the membrane. The configuration can either be dead-end or tangential, respectively with the direction of the flow perpendicular or tangential to the membrane surface. This technique is an efficient way of harvesting, with resulting microalgal pastes with up to 15% dry weight, which is lower than the dry weight concentrations that can be obtained by centrifugation. Filtration is thus often followed by further concentration by centrifugation. As is the case for centrifugation as well, there are no chemicals present in the resulting biomass. However, membrane fouling can occur due to blocking of the pores and formation of a cake layer [10].

To diminish the volume to be processed by secondary concentration, a pre-concentration step can be used. This also decreases the costs substantially. Examples are sedimentation, flocculation and flotation [10, 34].

Sedimentation uses the gravitational settling of microalgal cells in the suspension. Advantages of this technique are the low operating costs, low energy demand and the integrity of the cells remaining intact. However, due to the negative cell surface (due to acidic groups) and the small size of many microalgae, sedimentation is very time consuming. In addition, the settling tanks need a relatively high land area [10, 73].

Flocculation involves aggregation of several cells into larger flocs for settling [31]. As microalgal cells have a negatively charged surface, they are normally stable in solution and are not easily flocculating [18, 72]. Flocculation can be induced by different mechanisms. Neutralization of the negative charges on the cell surface by positively charged flocculants induces the physical interaction between cells, thereby increasing the tendency to sediment [10, 18]. Next to this, also bridging can occur, in which polymers are used to adsorb to the cell surface of different cells, thereby binding these cells together [10, 72]. A last mechanism is sweeping flocculation, during which the cells are entrapped within a precipitate of a mineral [72].

Several different flocculation types have been developed, each with their advantages and disadvantages. **Chemical flocculation** involves the addition of inorganic (e.g. aluminium sulfate, ferric chloride) or organic (e.g.

polyacrylamide, chitosan) compounds. Floc formation is induced by charge neutralization and/or sweeping in the case of inorganic flocculants and by bridging in the case of organic polymers. The main disadvantage of this technique is the dependence of the flocculation efficiency on many factors such as pH, cell characteristics, water salinity, dose and biomass concentration. Some of these variables are changing during algal cultivation, which complicates this process [10]. **Autoflocculation** is induced by raising the pH to above pH 9, which causes precipitation of calcium and magnesium salts of the culture medium [18, 73]. Sometimes autoflocculation is also induced by prolonged cultivation under limited CO_2 conditions, which causes a pH increase [10, 31, 73]. **Electroflocculation** uses electrodes such as iron or aluminium, which release cations that induce flocculation [10]. Recently, also techniques using non sacrificial electrodes, which do not form metal hydroxides, have been developed to overcome the main limitations of depletion of the metallic electrodes and the accompanying contamination of the microalgal biomass, which are associated with sacrificial electrodes [74].

During **flotation**, microalgae attach to air bubbles and are carried from the bottom of the suspension to the liquid surface, where they are removed [10, 31]. This technique depends on several factors. First, the particle size determines the probability of collision with air bubbles and the likelihood of particles moving to the surface. Smaller particles are more likely to float upwards, but decrease the probability of collisions between air bubbles and microalgal cells. Secondly, this technique requires hydrophobic cells to ensure good attachment to air bubbles. This can possibly be improved by the addition of surfactants, which improve particle separation and increase the probability of adhesion between cells and air bubbles. These factors make optimization of this technique relatively difficult. In addition, also the energy requirements are high [10].

1.4.3. Drying

In most cases, the wet biomass obtained by harvesting is dried to produce a powder which is more stable and more practical in use. The choice of a drying technique depends on the scale of production and the intended application of the end product [75]. For high value products, spray-drying and freeze-drying are often used. Spray-drying is very efficient and fast, but implies high operating costs. In addition, it could rupture intact cells and possibly involves degradation of heat sensitive compounds due to the high temperatures [23, 75].

Freeze-drying has the advantage of being more gentle, as it does not involve elevated temperatures and thus causes less or no degradation of thermolabile compounds [23, 71, 76]. However, an expensive equipment is necessary and it is accompanied by a high energy consumption [23]. Another possible technique is the use of microwaves, which has been reported in many studies for their ability to improve extraction by disrupting the cells [77-80]. As microwave drying is known to cause a more effective heat distribution in the sample, it could possibly also be used for drying of microalgae [81].

As Life Cycle Assessment (LCA) studies have demonstrated that the drying step accounts for a high percentage of the total energy consumption [82, 83], a lot of studies are focusing on avoiding this step by extracting lipids directly from the wet biomass or by direct transesterification reactions in wet biomass [1, 15, 84, 85]. Although the yields of these processes are found to be negatively influenced by the moisture content [1, 15, 84, 85], there are some studies that show promising results with comparable yields as by using dry extraction [15, 16]. However, it is not yet known whether the lipids in this wet biomass remain stable. This aspect is explained more deeply in Chapter 2.

1.4.4. Lipid extraction

Extraction of lipids from microalgal biomass is most efficiently performed on dry biomass by the traditionally used **solvent extraction** methods (e.g. chloroform/methanol extraction) [86]. However, these toxic solvents can only be used for analytical purposes. Therefore, some food grade alternatives are often used, e.g. hexane, isopropanol, ethanol. The lipid extraction efficiency is however substantially lower [87-89].

Also a few solvent-free extraction methods are developed, although also with lower extraction efficiencies [88]. **Supercritical extraction** (usually supercritical CO_2) for example uses an increased temperature and pressure to bring a fluid in the supercritical region, so that it behaves both like a liquid and a gas [86, 90]. However, this method requires high installation and energy costs and a more polar co-solvent (e.g. ethanol) is often necessary to extract more polar lipids [90].

1.4.5. Cell disruption

Lipids are encapsulated in the cells, mostly in lipid bodies and membranes, which hampers their extraction, especially in species with a recalcitrant cell wall, e.g. *Nannochloropsis* [38, 91, 92]. To improve the lipid extraction efficiency, several **cell disruption** methods can be used to liberate the lipids from the cellular matrix [1, 93]. Many cell disruption methods have been studied, each with their advantages and disadvantages. The choice of a cell disruption method is also dependent on the structure and strength of the microalgal cell wall, the stability of the extracted product and the production cost [75, 80]. Cell disruption methods can be classified in two major groups, being mechanical (e.g. high pressure homogenization, ultrasonication, bead milling) and non-mechanical methods (e.g. acid or alkali treatment, osmotic shock, enzymatic cell disruption) [75, 91, 92, 94-96].

Although **mechanical disruption** generally leads to high yields, it also possibly damages cell compounds by the input of shear forces, electrical pulses, waves or heat [95]. **Bead milling** disrupts microalgal cells by collision and friction with solid beads moving at high velocities. It is a very effective technique in disrupting the cells very rapidly [97, 98], but requires a high energy input and generates heat that may degrade target compounds [18, 80, 95]. Another very efficient cell disruption method is **high pressure homogenization (HPH)**, during which the microalgal cell suspension is compressed through a narrow orifice between a valve and valve seat under high pressure, after which the cells are released in a low pressure chamber. Collisions of the cells with the valve or impact ring, turbulence and shear induced by the high pressure and the pressure drop are causing cell lysis [80, 91, 92, 95]. However, the samples also heat up during each HPH pass (about 2.5°C per 10 MPa per pass) [99]. Although this technique is very efficient and easily scalable, it also requires a high energy input and high costs [95, 100, 101]. **Ultrasonication** involves ultrasound waves in a liquid, causing cavitation and shear forces that disrupt the cells [18, 95]. Generally, the extraction yields are much lower than obtained by HPH or bead milling [80, 94, 102]. The efficiency of the disruption is also very dependent on the viscosity and the temperature of the suspension. At higher cell concentrations (and thus higher viscosities), this technique becomes less efficient. Also heating of the medium by the ultrasound waves has a negative impact on efficiency, thus necessitating constant cooling. All these factors make ultrasonication less applicable, especially on larger scales [18, 80, 95].

Pulsed electric field (PEF) applies an electric field to induce an electrical potential across the cell wall, which causes the formation of pores in cell membrane and/or cell wall [80, 95]. Although several studies showed this to be a promising technique [103, 104], it also causes a high temperature increase [80] and the efficiency depends on medium conductivity and cell wall characteristics [95].

Non-mechanical methods use chemical or biological compounds that interact with cell wall or cell membrane and thereby improve passage of extracted compounds to the medium. The advantage of these methods is the much lower energy consumption compared to mechanical methods [18, 95]. However, the efficiency is strongly dependent on the microalgal cell wall composition and structure and the resulting biomass is possibly contaminated [75, 105]. **Chemical treatments**, mostly by strong acids such as sulphuric acid (H_2SO_4), are often used as these are very effective and cheap. However, the acid can possibly also damage the target compounds [18, 95]. **Enzymatic treatments** at the contrary operate under much milder conditions, as these are using enzymes (e.g. pectinase, cellulase, hemicellulase) that hydrolyze cell wall compounds. The hydrolytic enzymes often degrade a certain specific linkage in the cell wall and consequently do not degrade target compounds [18]. However, large-scale implementation of enzymatic treatments is relatively difficult because of the species-dependent approach, for which the cell wall composition has to be determined first. In addition, the reactions are relatively slow, the costs of enzyme production high and the reuse of the enzymes difficult [1, 95]. Creating an **osmotic shock** by a decrease or increase of the salt concentration in the medium, can possibly also cause cell disruption. When the salt concentration in the medium is much higher than inside the cell (hyper-osmotic stress), fluids from inside the cells diffuse outwards, resulting in shrinking and damaging of the cell. Also when the salt concentration in the medium is much lower than inside the cell (hypo-osmotic stress), the cell is damaged because of the absorption of fluids, causing swelling and eventually bursting of the cell [18]. An osmotic shock can be achieved in marine microalgae by transferring the cells from the salt cultivation medium to a medium with low salt concentration [1].This is a cheap and simple process, but yields high amounts of salt wastewater and is not very efficient for all microalgal species [18, 95].

1.5. Commercial and potential applications of microalgae

As this PhD focusses on microalgal lipids and their stability during storage, this overview of current and potential applications of microalgae only considers applications of lipids and lipophilic compounds present in the lipid fraction. Other compounds and applications, e.g. bioethanol, phycocyanin, carbohydrates, are left out of consideration.

1.5.1. Benefits and drawbacks related to the use of microalgal lipids

Production of lipids from microalgae has several advantages compared to plant oils, e.g. the higher photosynthetic efficiency, the high lipid contents (up to 60% of dry weight) and the higher growth rate of microalgae compared to terrestrial plants [1, 3]. This makes the oil production per unit surface up to 200 times higher than for plant oils [3, 45]. In addition, the production can be performed on non-arable land, as such eliminating competition for land with food production [1, 3, 45]. The main disadvantage is the high production cost [70], mainly for harvesting of the biomass from the very diluted cultures with small sized microalgae [6, 26, 34, 45].

1.5.2. High value applications

Currently, most of the microalgal biomass is produced for high value applications, using some compounds of the biomass for their health benefits, color enhancement, … (further explained in 1.5.2.1 - 1.5.2.3).

Most of the microalgal biomass on the market is sold as **dietary supplements and nutraceuticals** in different forms such as powders, capsules, tablets [34, 106, 107]. Also in the **cosmetics** market, especially in skin care products, some products consist of microalgal biomass or compounds extracted from it [106, 108, 109].

About 30% of the current global production of microalgae is used as a **feed** ingredient for different types of animals, ranging from fish in aquaculture to pets and farm animals [110]. Apart from improving the health of the animals [110], microalgae can also be used to enrich food with ω-3 LC-PUFA (e.g. ω-3 enriched eggs) [107, 111, 112].

For use in the **food** sector, mixing the whole biomass with food products could be an option. However, only minor amounts of powder can be used because of the strong green color and the fishy taste and odor [34]. In addition, the amount of interesting compounds that can be converted to a form that can be absorbed from the intestine (bioaccessibility) can possibly be very limited [113, 114]. Especially when using species with a tough cell wall bioaccessibility can be very low, but can be improved by applying cell disruption [114, 115]. Nevertheless, research is ongoing about addition of microalgae in among others pasta products [116] and biscuits [117]. Often however, the interesting high value compounds (e.g. pigments, fatty acids) are first extracted from the biomass and then added to food products [5]. It has to be stressed that for microalgae or extracts from microalgae incorporated in food, only a few microalgal species (e.g. *Spirulina*, *Chlorella*, *Dunaliella*, *Tetraselmis)* are allowed in Europe at this moment, because of the restrictions of the Novel Food Regulation [107]. There are also promising applications for the use of microalgae in **pharmaceuticals** as mentioned for example by Pulz and Gross (2004) [34, 118].

In the following sections, the different high value compounds in the microalgal lipid fraction are discussed.

1.5.2.1. *ω-3 LC-PUFA*

The use of microalgae in high value applications is in many cases related to the presence of **ω-3 LC-PUFA**, particularly EPA and DHA, from which it has been demonstrated that they possess several health promoting functions [8, 9]. An increased intake of EPA and DHA is associated with a reduced risk of cardiovascular morbidity and mortality [119, 120]. In addition, they can have beneficial effects against inflammation [121, 122], may have a protective role against dementia [123, 124], may have benefits for patients suffering from depression [125, 126] and can help in cancer therapies [127, 128]. In the human body, ω-3 LC-PUFA are produced from α-linolenic acid (ALA), which can not be produced by humans and thus has to be taken in from food [129]. However, this conversion is very inefficient [130, 131] and thus the dietary intake of ω-3 LC-PUFA is essential. Therefore, the recommended daily intake by EFSA [132] and FAO/WHO [129] is set at 250 mg – 500 mg.

EPA and DHA are present in high amounts in oily fish (e.g. salmon, herring) and other marine products as krill and squid, but inhabitants of most

countries do not reach the recommended intake [133]. The consumption of fish rich in EPA and DHA is therefore highly encouraged. This will however cause future problems because of the global decline in wild-harvest fish stocks. With the increasing world population, the demand for fish will even increase more. In addition, the consumption of high amounts of fish is also related with risks of intake of contaminants such as mercury. The use of ω-3 LC-PUFA in supplements and functional foods is therefore a rising business, but also for these products the origin is usually fish. Other alternative sources of ω-3 LC-PUFA are thus necessary. Microalgae, as the primary producers of ω-3 LC-PUFA in the food chain, are one of the possible alternatives [7, 134]. Microalgal genera producing high amounts of EPA are for example *Nannochloropsis*, *Pavlova*, *Porphyridium* and *Phaeodactylum*, while *Isochrysis* species produce high amounts of DHA [45].

1.5.2.2. *Lipophilic pigments*

Other interesting compounds in the lipid fraction of microalgae are the lipophilic **pigments**. **Chlorophylls** are present in all types of photoautotrophic microalgae and give a green color, which can be used for coloring food and beverages [6]. Next to its coloring function, certain derivatives of chlorophyll (chlorophyllin) are also reported to exhibit anti-cancer activity [135]. Chlorophyll is known as a sensitizer which forms singlet oxygen during photo-oxidation [25, 136, 137], although it can have an antioxidant effect during autoxidation (in the dark) [137, 138].

Also **carotenoids** are often used as colorants in the food industry [6]. In addition, these compounds have antioxidant and/or anti-inflammatory characteristics and are therefore also used in dietary supplements, aquaculture, pharmaceutical and cosmetic products [66, 107, 139, 140]. Especially astaxanthin is a potent antioxidant, which also has other benefits in the prevention and treatment of several diseases such as inflammation and cardiovascular diseases [26, 61]. In current applications, it is extracted from *Haematococcus pluvialis* [6]. Beta-carotene, mostly sourced from *Dunaliella salina,* is also known as pro-vitamin A and is therefore used in vitamin supplements [6, 34]. Carotenoids are often extracted together with microalgal lipids, which can improve the nutritional value and the oxidative stability of the extracted oil [141, 33].

1.5.2.3. *Sterols*

A last lipid class used for its health promoting function are the phytosterols (e.g. β-sitosterol, fucosterol, stigmasterol). It has been demonstrated that the consumption of these phytosterols is related to a reduction of the cholesterol concentration in blood and consequently the prevention of cardiovascular diseases [67]. Therefore, there has been a growing interest in these phytosterols for use in functional food and pharmaceutical products [67, 142]. The sterol content and composition is strongly dependent on the species and the cultivation conditions (light intensity, temperature, growth stage [67].

1.5.3. Biofuels

As a consequence of the global concern about greenhouse gases, together with an increasing demand for alternatives for fossil fuel, scientists have been investigating several alternative energy sources. Different types of biomass have been proposed as possible sources for conversion to liquid or gas fuels [143]. Nowadays, biofuel is produced mainly from palm oil, rapeseed oil or soybean oil. However, this requires substantial amounts of arable land, leading to competition with the production of food oils. This is not the case for microalgal biofuel, which can be produced on non-arable land [2, 45, 71, 144]. Microalgal biomass can be used to produce a variety of biofuel types such as biodiesel, bioethanol, biomethane and biohydrogen [6, 70, 86]. Biodiesel is however the only one that is produced specifically from the lipid fraction, which is the focus of this PhD.

Biodiesel is formed by a transesterification reaction of saponifiable lipids with methanol to form fatty acid methyl esters (FAME) and glycerol, a reaction which is most often catalyzed by an alkali catalyst (e.g. KOH, NaOH) [145] (Figure 1.7).

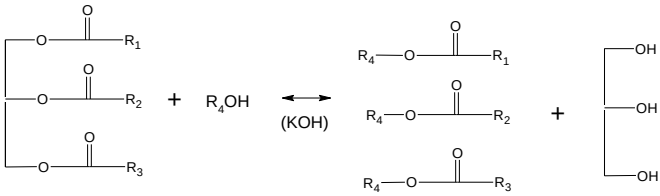


Figure 1.7: Production of biodiesel by transesterification of triacylglycerols with alcohols via alkaline catalysts (based on Atadashi et al., 2013 [145])

The quality of the oil and the degree of downstream processing that is required, is dependent on the **lipid degradation** reactions occurring in the biomass and/or the oil. The consequences of oxidation and lipolysis for biodiesel production are further explained respectively in 2.1.3 and 2.2.3.

Also the **fatty acid composition** of the microalgal lipids plays an important role in the properties of the fuel. Saturated fatty acids result in biodiesel with a good oxidative stability, but worse properties in cold environments [29, 47], in which solid crystals can be formed, which cease the flow [146]. Conversely, lipids with high PUFA contents possess better cold-flow characteristics, but are more sensitive to oxidation, which gives several storage problems, described in detail in 2.1.3 [29, 47, 54, 147]. In addition, unsaturated fatty acids increase the risk of polymerization in the oil [29]. It should also be noted that a significant part of the lipid fraction in microalgae consists of unsaponifiable lipids (e.g. pigments), which are unusable for biodiesel production [54].

1.5.4. Biorefinery

As algae cultivation and downstream processing are time and energy intensive processes, research is shifting from a one-product focus to a multiple-product focus, in which the potential of all microalgal compounds is exploited in a **biorefinery** system. This leads to several products, both fuels and high value products. This concept is comparable with the traditional petroleum refinery, but with other raw materials and technologies used. It has to be taken into account that the process is mild enough to maintain the functionality of all constituents [10, 34, 148, 149].

Chapter 2:

Lipid stability during storage of wet microalgal biomass

Partly based on: Balduyck, L., Goiris, K., Bruneel, C., Muylaert, K., Foubert, I. (2015). Stability of valuable components during wet and dry storage. In S.-K. Kim (Ed), Handbook of marine microalgae. Biotechnology advances. London, UK: Elsevier.

CHAPTER 2 LIPID STABILITY DURING STORAGE OF WET MICROALGAL BIOMASS

During downstream processing, microalgal biomass often needs to be stored in the form of a wet microalgal paste with a dry weight of 5-25% ('wet storage') [71]. The wet storage phase usually takes place before drying for practical reasons, but often it is not meant to be a 'storage' phase. As the drying step accounts for a high percentage of the total energy consumption [82, 83], a lot of studies are focusing on avoiding this step by extracting lipids directly from the wet biomass or by direct transesterification reactions in wet biomass [1, 15, 84, 85]. Avoiding the drying step implies however a longer term during which the biomass is residing as a wet paste, as can also be seen on the scheme in Figure 1.5.

During wet 'storage', wet extraction or wet direct transesterification, the lipid content and/or the composition of the lipid fraction can be altered by several degradation reactions. Although the quality implications of wet storage have hardly been studied, it is already known from food applications that both enzymatic reactions, oxidation reactions and reactions caused by micro-organisms are diminished by drying and thereby lowering the water activity [11, 12]. In addition, when drying is performed by applying heat, the drying process can possibly inactivate degradation enzymes, depending on the thermostability of the enzymes and the time-temperature combination applied [12]. Therefore, it can be expected that also in microalgal biomass, the lipid stability is influenced by the water activity. Lipid degradation reactions are thus expected to occur mainly during stages of wet biomass.

In this chapter, the two most problematic lipid degradation reactions, oxidation (section 2.1) and lipolysis (section 2.2), are explained. Also the factors determining the reaction rate and the effects of these reactions on lipid quality and their implications for the different applications of microalgal lipids are discussed. As the focus of this PhD is on lipolytic stability during wet storage, section 2.3 gives an overview of what is already known about this topic.

2.1. Oxidative stability

2.1.1. What is oxidation?

Oxidation is a deterioration process of lipids, initiated either by free radicals (autoxidation) or by singlet oxygen (photo-oxidation). Furthermore, the reaction can also be enzymatically catalyzed by lipoxygenases [136, 150].

During autoxidation, the **primary oxidation** proceeds as a free radical chain reaction in different phases: initiation, propagation and termination (Figure 2.1) [25]. During the initiation step, a lipid radical ($L^{\bullet}$) is formed by abstraction of a hydrogen atom under the influence of an initiator, which can be for example a preformed free radical, metal ions or heat. During propagation, lipid radicals are converted to lipid peroxyl radicals ($LOO^{\bullet}$) and further to lipid hydroperoxides (LOOH). During this last reaction, also a lipid radical is formed, which reacts with oxygen again and propagates the reaction. In the termination step, radicals react with each other, leading to different non-radical products [25, 136, 137, 150, 151].

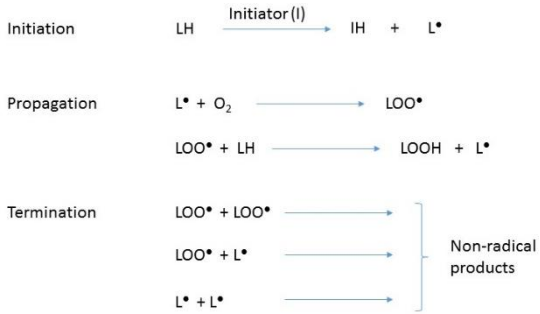


Figure 2.1: Main reactions during primary oxidation of lipid autoxidation (based on Yanishlieva-Maslarova, 2001 [25])

Photo-oxidation is initiated by light and leads to the formation of a lipid radical, which induces a similar chain mechanism as in autoxidation (Type I), or reactive singlet oxygen ($^1O_2$) (Type II), which is promoted in the presence of a sensitizer (e.g. chlorophyll) [137, 150].

During **secondary oxidation**, the hydroperoxides formed during primary oxidation are decomposed by different reactions (e.g. dehydration, cyclization, rearrangement, chain cleavage) to form a variety of secondary products: monomeric products, with the same chain length as the original

lipids, polymeric compounds, with a higher molecular weight, and volatile products, with a shorter chain (aldehydes, ketones, alcohols) [136].

2.1.2. Factors influencing oxidation during storage

The **fatty acid composition** of the lipids plays a major role in the reaction rate of oxidation. Since initiation during autoxidation involves abstraction of a hydrogen atom from a lipid, this step is favored at the methylene position between two double bonds, typically present in polyunsaturated fatty acids. The radical formed in this reaction is delocalized across three atoms, which makes it much more stable. Polyunsaturated fatty acids such as ω-3 fatty acids are thus oxidized faster than saturated fatty acids [137, 150].

The **moisture content** also has an influence on the oxidation rate. In food contexts, the water activity (a_w) is often used instead of moisture content, as it is a measure of the availability of water for several reactions and therefore a better predictor of stability in food [12]. Although lipid oxidation decreases with decreasing a_w until approximately a_w = 0.2, it strongly increases beneath this value, probably because the lipids are more exposed to air without a monolayer of water around them [11, 12].

Lipid oxidation is promoted by the presence of **light** (especially during photo-oxidation), high **oxygen concentrations** and high **temperatures**. Therefore, storage in the dark, under minimized exposure to air and at lower temperatures is recommended to achieve a better oxidative stability [11, 25, 137]. **Metal ions** (e.g. copper, iron) also accelerate oxidation reactions, as they catalyze the decomposition of hydroperoxides produced during primary oxidation, which leads in most of the cases to the formation of radicals. Furthermore, metals can also react directly with lipids to form lipid radicals or they can produce reactive oxygen species, both mechanisms leading to acceleration of lipid oxidation [137]. Even minor traces of metal ions can therefore strongly promote oxidation reactions [25, 150]. **FFA**, released from their lipid backbone, also have a pro-oxidative effect, as they form a complex with hydroperoxides through a hydrogen bond, leading to a faster decomposition of the hydroperoxides [25].

Oxidation can be retarded or inhibited by the presence of **antioxidants**, both endogenous antioxidants (e.g. carotenoids, tocopherols, phenolic compounds) and additives (e.g. butylated hydroxytoluene, BHT) [25, 136, 150, 152].

Antioxidants act by different mechanisms. First, primary antioxidants, also known as chain-breaking antioxidants (e.g. phenolic compounds), react directly with free radicals to form more stable, non-radical products. Secondary antioxidants inhibit lipid oxidation by several other mechanisms, for example singlet oxygen quenching, oxygen scavenging and chelation of metals [151]. On the contrary, the presence of **photosensitizers** (e.g. chlorophyll), can accelerate lipid oxidation[137].

In the case of **enzymatic oxidation** by lipoxygenases, the reaction rate is also determined by the minimum, optimum and maximum working temperature and pH of the lipoxygenase enzymes, which is strongly species dependent. Lipoxygenases only catalyze the oxidation of polyunsaturated fatty acids [11,150].

2.1.3. Effects of oxidation on product quality

Oxidation reactions have several negative effects on lipid quality in microalgae, for both biodiesel and high value purposes. However, photoautotrophic microalgae contain several types of endogenous antioxidants (e.g. carotenoids, phenolic compounds), which can strongly improve oxidative stability of the biomass or the oil [33, 153].

Especially secondary oxidation products, which are in many cases volatiles, can cause off-flavors in **food**. Volatile aldehydes are the most prevalent oxidation products in microalgae and can give desirable as well as rancid flavors. Shorter chain linear aldehydes are often formed by chemical lipid oxidation, while branched and aromatic aldehydes are typically the result of enzymatic oxidation [154]. Oxidation of ω-3 LC-PUFA and several vitamins can also result in a reduction of the nutritional value. Finally, some oxidation products (e.g. 4-hydroxy-nonenal) are possibly toxic. For these reasons, oxidation must be reduced to a minimum in food applications [150, 155]. In most cases, improving oxidative stability in food is achieved by adapting storage conditions (minimizing exposure to light and air, removal of oxygen, storage at lower temperatures) or the addition of antioxidants [25].

As well as for food applications, also for applications in **feed**, oxidation negatively impacts the flavor, which can have a negative impact on feed intake and nutritional value. The growth performance and animal health are also influenced by the extent of oxidation in the feed. More oxidation can

cause a lower energy and nutritional efficiency, which increases the cost of animal production [156]. In aquaculture, the ω-3 LC-PUFA supplemented in fish feed are essential for many marine fish species. Oxidation of lipids in the feed causes the formation of toxic compounds and a decrease of the nutritional value, especially a decrease in ω-3 fatty acids, which leads to a reduced survival and growth of the larvae and a lower ω-3 content in the fish [157].

In **biodiesel** applications, oxidation can cause a reduced fuel quality by polymerization reactions between hydroperoxides and other radicals, which increases the viscosity [158-161]. In addition, the formation of high molecular weight insoluble sediments and gums causes the formation of deposits in tanks, fuel systems and filters [161, 162].

2.2. Lipolytic stability

2.2.1. What is lipolysis?

Lipolysis is a **hydrolytic reaction** during which the fatty acid chains are released from the lipid backbone in the form of FFA (e.g. hydrolysis of TAG in Figure 2.2). The reaction is mostly catalyzed by lipolytic enzymes (lipases). Chemical hydrolysis is also a possibility, but an alkaline or acid catalyst (e.g. KOH, HCl) is necessary to catalyze the reaction, together with a prolonged period of a few hours during which the temperature is increased (refluxing) [136, 163]. As it is assumed that these conditions do not occur inside the microalgal cells during the experiments of this PhD, this literature overview focuses on enzymatic lipolysis.

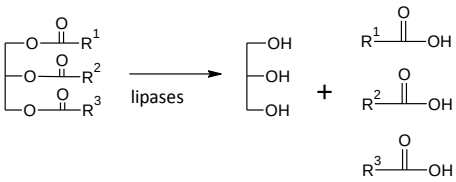


Figure 2.2: Lipolytic degradation of triacylglycerols to glycerol and free fatty acids.

Lipolytic enzymes or **lipases** are produced by several groups of micro-organisms and belong to the group of carboxylesterases. Different types of lipolytic enzymes occur in nature, with a specificity for a certain lipid type and/or a specific site of hydrolysis. As such, lipases can be divided in three groups. The first group consists of 'real' lipases or TAG lipases, which catalyze the release of a FFA from TAG and also the further breakdown of the formed

DAG to MAG and eventually to glycerol (Figure 2.3A). The second group are the phospholipases, which can act on several ester bonds present in phospholipids and are therefore divided in phospholipase A1, A2, C and D (Figure 2.3B). The last group are the glycolipases, which are hydrolyzing glycolipids (Figure 2.3C) [11, 164, 165].

The presence of endogenous lipolytic enzymes in **microalgae** has been demonstrated by isolating and characterizing enzymes from among others *Spirulina platensis* [166], *Chatonella marina* [167], *Botryococcus sudeticus* [168] and *Nannochloropsis oceanica* [169]. Also several genetic studies elucidated the presence of genes encoding for carboxylesterase, thioesterase and/or lipase-like enzymes in *Isochrysis galbana* [170-172], *Phaeodactylum tricornutum* [173] and *Fistulifera solaris* [174]. Mostly based on the knowledge of the alga *Chlamydomonas*, it has been described that lipases are situated at different sites in the microalgal cell, depending on their specificity [165]. TAG lipases are active at the surface of the lipid bodies, while phospholipases and glycolipases act on membrane lipids in the endoplasmic reticulum or chloroplast [165]. In the oleaginous diatom *Fistulifera solaris*, it was found that the majority of the lipases was localized in the endoplasmic reticulum, close to the lipid bodies [174].

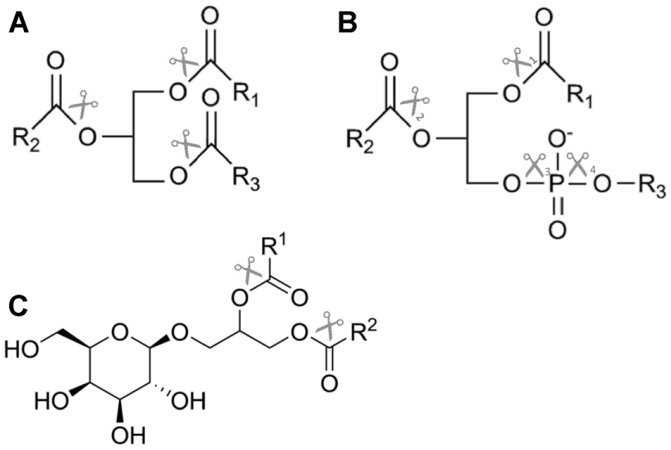


Figure 2.3: Sites of potential hydrolysis by (A) TAG lipases, (B) phospholipases (type A1 (1), A2 (2), C (3) and D (4)) and (C) glycolipases [164].

2.2.2. Factors influencing lipolysis during storage

The reaction rate of enzymatic lipolysis in microalgae is dependent on **pH** and **temperature**, both with a minimum, optimum and maximum working value. Above the maximum temperature, enzymes are denaturated, implying that they lose their conformational structure and thereby also their activity. The optimal temperature and pH of lipases is situated in a broad range because of the diversity of the enzymes and the microalgal species, being respectively between 25 and 45°C and between pH 6.5 and 9 [19, 166, 167, 175-178]. On the contrary, Savvidou et al. (2016) [169] found very thermostable lipases in *Nannochloropsis oceanica*, which became even more active when treated at 100°C for 60 minutes.

As mentioned already before, lowering the **water activity** decreases the mobility of enzymes and substrates and thus decreases the reaction rate of lipolysis [11, 12]. Therefore, dried biomass is expected to have a much better lipolytic stability than wet biomass.

Lipase catalyzed reactions typically are also **substrate** specific and thus degrade a specific group of lipids (e.g. TAG lipases, phospholipases). Studies on (micro)algae revealed different substrate specificities. The specific degradation of polar lipids by phospholipases or glycolipases was reported by several biochemical studies characterizing the enzymes in *Thalassiosira rotula* [179,180], *Gracilaria vermiculophylla* [176], *Chattonella marina* [167], *Cladosiphon okamuranus* [177], *Dunaliella salina* [178], *Gracilaria chilensis* [181] and *Monochrysis lutheri* [182]. On the contrary, genetic studies revealed the presence of TAG lipases in *Phaeodactylum tricornutum* [173]. Also a storage experiment of wet *Scenedesmus* paste revealed the specific breakdown of TAG [19].

The presence of **cations** (e.g. Hg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}) has been demonstrated to exert an inhibitory effect on lipolytic enzymes, as has been shown in several bacteria and fungi [183-185] and some microalgae [166, 168]. Ca^{2+} ions on the other hand sometimes have been found to increase lipolytic activity, also in microalgae (e.g. in *Spirulina* [166] and *Dunaliella* [178]). This can be explained by the reaction between free fatty acids and Ca^{2+} ions to insoluble calcium salts [11]. These reactions cause the free fatty acids to be removed from the interface, thereby avoiding the inverse reaction of resynthesis of esterified fatty acids [186].

2.2.3. Effects of lipolysis on product quality

Lipolytic reactions result in the production of FFA, which can give problematic effects for the different applications of microalgae.

Firstly, FFA can cause rancid off-flavors, which are not desired in **food** applications [11]. The odor and taste threshold values at which FFA are experienced as rancid however vary strongly upon the fatty acids, with shorter chain fatty acids (<14 carbon atoms) having most impact on flavor [11]. Furthermore, FFA can promote oxidation reactions, with the accompanying stability problems described in 2.1.3 (e.g. decrease of the nutritional value) [25, 187-189]. Therefore, the maximum amount of FFA in refined, edible oils is set at 0.05% [190]. In the **cosmetic and pharmaceutical industry**, the quality has to be equal or superior to that in edible oils [191]. Research on the impact of the FFA content in **feed** for pets and farm animals, as well as for fish in aquaculture has to the best of our knowledge not been performed. However, as FFA promote oxidation, which results in loss of nutritional value and the formation of toxic compounds (as described in 2.1.3), it would be expected to have an important role. FFA can be removed from the oils by precipitation as a soap after addition of alkali (chemical deacidification) or by vacuum distillation (physical deacidification). However, chemical deacidification causes losses of lipids in the soap fraction. Also physical deacidification has several disadvantages. As it uses high temperatures, carotenoids and tocopherols are destroyed and isomers and polymers of lipids are possibly formed [192].

In microalgal oils used for **biodiesel** production, problems can possibly arise when the oil contains more than 0.5% FFA, as these can react in a saponification reaction with the alkali catalyst. This leads to a reduced efficiency because part of the catalysts reacts to soaps and is thus not available for transesterification. In addition, saponification causes an increase of the viscosity and the formation of gels, which complicates subsequent downstream processing [26, 27, 193-195]. Therefore, **acid** (e.g. H_2SO_4, HCl) or **enzymatic catalysts** (lipases) are often used in microalgal oils containing a lot of FFA, because these reactions are not dependent on the FFA content of the feedstock [18]. However, biodiesel production by acid catalysts leads to much lower reaction rates than by alkali catalysts [18, 26]. It also necessitates very high catalyst concentrations and causes corrosion [18, 195]. The use of enzymatic catalysts enables milder conditions, but

it has also several drawbacks. A complex and expensive purification of the enzymes is necessary. In addition, rapid inactivation of the enzymes by methanol, the substrate for transesterification, can possibly occur [196]. Optimization of the reaction conditions also makes that a lot of research is still necessary before commercialization [195]. When using alkali catalysts for high FFA oil, a possible solution is the addition of an extra **pre-treatment** step, in which the FFA are esterified to alkyl esters in the presence of an acid catalyst. This extra step, together with the removal of the acid catalyst after this step by centrifugation, however increases the costs. In addition, water is formed during esterification, which is also disadvantageous for the following alkali transesterification [27, 195, 197]. Research is therefore ongoing about heterogeneous catalysts, which circumvent the removal of acid catalyst after the pre-treatment step (e.g. Veillette et al., 2017 [198]). Some studies also focus on the simultaneous catalysis of both transesterification of esterified fatty acids and esterification of FFA by using heterogenous catalysts [199, 200] or (mixtures of) enzymes [201]. Another alternative is the catalyst-free method using **supercritical methanol**. This method is not negatively influenced by a high FFA content [202], but uses a high temperature and pressure, which rises the energy costs and makes application on industrial scale more difficult [195].

2.3. Lipolytic stability during wet storage of microalgal biomass

In living, healthy cells (before harvesting), FFA are only detected in trace amounts, as higher concentrations are cytotoxic (as was described in 1.3.1.2). The high concentrations of FFA that are often observed in microalgae [19-21] are thus indicators of lipolytic reactions that occurred during downstream processing (harvesting, cell disruption, drying and wet storage) or during analytical procedures [22].

There are a few indications from literature that FFA are mainly formed in the wet biomass. Firstly, it was shown by Ryckebosch et al. (2011)[23] that after two days of wet storage of centrifuged *Phaeodactylum tricornutum* paste at 4°C, high amounts of FFA were produced, which was not the case when the paste was freeze-dried or spray-dried immediately after harvesting. As a consequence, the lipid content also decreased significantly during this wet storage stage. Chen et al. (2012)[19] also observed serious stability problems during wet storage of *Scenedesmus* paste (75% moisture). During 4 days of

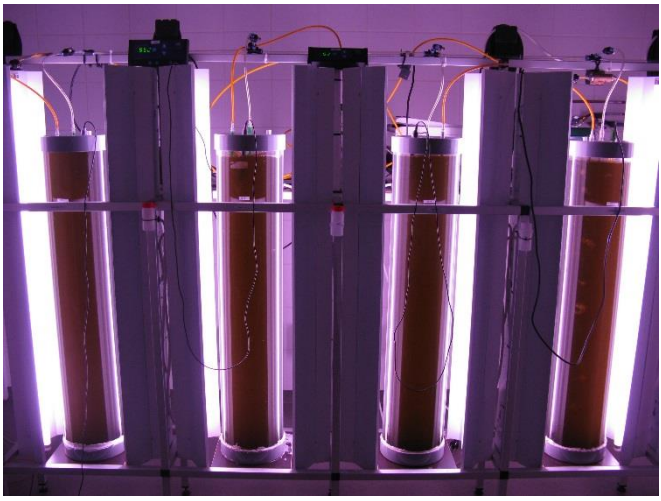
wet storage at 4°C, the FFA content (expressed as percentage of the oil) increased from trace proportions to 62% FFA. At higher storage temperatures, this increase occurred even faster, with 70.3% FFA in the oil after 1 day at 25°C and 66.8% after 1 day at 37°C. From these studies, it can be concluded that also short term wet storage can have detrimental effects on lipid quality in microalgal biomass.

Pernet and Tremblay (2003) [24] optimized an analytical procedure for lipid extraction and thereby investigated storage during 6 weeks of *Chaetoceros gracilis* biomass, harvested on GF/C filters and subsequently dissolved in dichloromethane. They observed that the FFA content after 6 weeks was significantly higher than at the beginning and after 1 week. They found also an effect of the cell disruption treatments prior to lipid extraction. The FFA production was higher after two consecutive disruption processes (ultrasonication and grinding) than after one of these disruption processes. They explained this by the enhanced contact between the hydrolytic enzymes and substrates within the cell if the degree of cell disruption increases.

The effect of cell disruption on lipolysis could possibly also be part of a defense mechanism against grazers, similar to the one that was already described for some diatoms. By causing physical damage to *Thalassiosira rotula* cells by ultrasonication, phospholipase A2 [179] or glycolipase [180] enzymes were activated, as seen by cleavage of added substrates to fluorescent products. It was suggested that the enzymes were already present in the intact cells, but physically separated from their substrates. By rupturing the cells, decompartmentalization occurred and caused contact between the lipids and the lipolytic enzymes [179, 180]. Also in the red algae *Gracilaria chilensis* [181] and *Chondrus crispus* [203], the activity of glycolipases and phospholipases was triggered by cell disruption. The defense mechanism starts with the release of fatty acids, mainly PUFA, from the lipid backbone. These free fatty acids are rapidly oxidized by the action of lipoxygenases or hydroperoxide lyases to form oxylipins (e.g. aldehydes), especially formed from PUFA, which are toxic to grazers (e.g. *Thamnocephalus platyurus*) [181, 203, 204]. An individual cell will not survive by executing this mechanism, but can improve survival of the population by diminishing growth of the grazers [179,-204].

Chapter 3:

Lipolysis in *T-Isochrysis lutea* during wet storage at different temperatures



Adapted from: Balduyck, L., Bijttebier, S., Bruneel, C., Jacobs, G., Voorspoels, S., Van Durme, J., Muylaert, K., Foubert, I. (2016). Lipolysis in T-Isochrysis lutea during wet storage at different temperatures. Algal Research, 18: 281-287.

CHAPTER 3 LIPOLYSIS IN *T-ISOCHRYSIS LUTEA* DURING WET STORAGE AT DIFFERENT TEMPERATURES

3.1. Introduction

From the literature study in Chapters 1 and 2, it is known that lipolysis, during which fatty acids are released from the lipid backbone, possibly occurs during wet storage of microalgal biomass [19, 23, 24]. Although the formed free fatty acids (FFA) are known to have a negative impact on oil quality for both biodiesel and high value applications [11, 25, 26], as was described in 2.2.3, this problem has not been thoroughly investigated before.

The aim of this chapter was to understand the time and temperature dependence of lipolysis and the accompanying FFA formation and accumulation during both short and longer term storage of wet microalgal biomass. Therefore, wet biomass was stored at three different temperatures (20°C, 4°C and -20°C) for 21 days, while the extent of lipolysis was followed by measuring the FFA content and total lipid content after different time intervals. As microalgae contain different lipid classes (e.g. triacylglycerols, phospholipids, glycolipids), it was also investigated whether lipolysis affects all lipid classes to the same degree. Therefore, a detailed separation in lipid classes by ultrahigh-performance liquid chromatography – accurate mass mass spectrometry (UHPLC-amMS) was executed at each time point during storage. For this experiment, *T-Isochrysis lutea* was selected, as this species contains high amounts of lipids, including ω-3 LC-PUFA, and is consequently a promising source of lipids for both biodiesel and high value applications [46].

3.2. Materials and methods

3.2.1. Cultivation and harvesting

T-Isochrysis lutea, formerly named *Isochrysis sp.* (CCAP 927/14; Culture Collection of Algae and Protozoa, Oban, United Kingdom), was cultured in Wright's Cryptophyte (WC) medium [205] to which artificial sea salt (Homarsel, Zoutman Industries, Roeselare, Belgium) was added in a concentration of 30 g/L. The cultivation was carried out in 30 L photobioreactors (column bioreactors with diameter 20 cm) incubated at controlled temperature (20°C). The reactors were irradiated with daylight fluorescent tubes (3000 to 3600 lux at the surface of the reactor) in a cycle of 16 light hours and 8 dark

hours and were aerated with filtered air. During cultivation, the pH was controlled at 8.5 (±0.2) by addition of CO_2 using a pH stat system. Growth of the microalgae was monitored by measuring the optical density at 750 nm.

Harvesting was carried out in the early stationary phase after 10 days of cultivation using a lamella centrifuge (4000 *g*). To remove the biomass paste from the centrifuge and to homogenize it, the paste was resuspended in a small amount of demineralized water. Subsequently, further concentration by centrifugation (9500 *g*, Sorvall RC-5B, Du Pont Instruments) was conducted.

3.2.2. Wet storage of *T-Isochrysis* paste

After homogenizing by stirring, the harvested wet microalgal paste (7.8 ± 0.5 % dry matter as determined by drying the wet paste to constant weight in an oven at 105°C) was divided in 50 mL falcon tubes and stored in the dark at 3 different temperatures (20°C, 4°C and -20°C) during a period of 3 weeks. Samples were collected at 8 different time points (0, 0.5, 1, 2, 3, 7, 14 and 21 days) and immediately freeze-dried and stored at -80°C until analyses. Preliminary experiments have shown that microalgal biomass remains stable in these conditions (detailed results not shown).

3.2.3. Analyses

3.2.3.1. *Total lipid extraction*

Total lipids of all collected samples were extracted with chloroform/methanol (1:1 v/v) according to the method described in Ryckebosch et al. (2012)[206] and determined gravimetrically. Briefly, 4 mL methanol, 2 mL chloroform and 0.4 mL water were added to 100 mg of lyophilized microalgae. In case of extraction for FFA determination (described in 3.2.3.2), 5 mg of lauric acid (C12:0) (Nu-Check Prep, Elysian, USA), dissolved in chloroform, was added as an internal standard. After homogenizing by a vortex stirrer, 2 mL chloroform and 2 mL water were added. The samples were homogenized again and subsequently centrifuged at 750 *g* for 10 minutes. The upper (water) layer was removed, while the lower layer was transferred to another tube. The remaining pellet was re-extracted with 4 mL of chloroform/methanol (1:1) and centrifuged again at 750 *g* for 10 minutes. The supernatant was transferred to the same tube as before while the pellet was extracted again according to the same procedure. The

combined solvent layers were filtered through a sodium sulfate layer to remove the remaining water. The lipid content was measured gravimetrically after evaporating the solvents. All extractions were performed in triplicate.

3.2.3.2. *FFA content*

The FFA content was determined by derivatization of the FFA in the lipid extract to diethylamide derivatives according to Ryckebosch et al. (2011) [23], based on Kangani et al. (2008) [207]. Therefore, 10 mg of the extracted lipids was dissolved in 1 mL dichloromethane. After addition of 10 µl diisopropylethylamine and 30 µl diethylamine (Sigma-Aldrich, Bornem, Belgium), the solution was cooled to 0°C. 20 µl of bis(2-methoxyethyl)aminosulfur trifluoride (Sigma-Aldrich, Bornem, Belgium) was added dropwise. After homogenizing by a vortex stirrer, the mixture was kept at 0°C for 5 minutes, subsequently warmed to room temperature and kept there for 15 minutes. Water (2 mL) and hexane (4 mL) were added, after which the solution was homogenized by a vortex stirrer and centrifuged at 750 *g* for 10 minutes.

The upper layer, containing the amide derivatives, was analyzed by gas chromatography with cold on-column injection and flame ionization detector (FID) (Trace GC, Interscience, Louvain-la-Neuve, Belgium). An EC Wax column (length 30 m, ID 0.32 mm, film 0.25 µm; Grace, Lokeren, Belgium) was used with the following time-temperature program: 100 to 160 °C at 20 °C/min, 160 to 240 °C at 4°C/min, 240 °C for 27 min. Peak areas were calculated with Chromcard 2.5 for Windows software. The areas of the peaks were summed and compared with the area of the internal standard (C12:0) to calculate the total FFA content. Determination of the FFA content was performed in triplicate on each sample.

3.2.3.3. *Separation in lipid classes by UHPLC-amMS*

UHPLC- amMS analysis

To investigate the distribution of the lipid classes, the lipid extracts (obtained in 3.2.3.1) of all collected samples were analyzed with ultrahigh-performance liquid chromatography – accurate mass mass spectrometry (UHPLC-amMS).

A standard stock solution of trans-β-apo-8'-carotenal (Sigma-Aldrich, Bornem, Belgium) was prepared at a concentration of approximately 3000 µg/mL in dichloromethane, to which 0.1% BHT was added (Sigma-Aldrich, Bornem, Belgium) and was used as an internal standard.

After evaporating the solvent from the lipid extracts, approximately 4 mg of the dry extract was redissolved in 0.5 mL chloroform/methanol (1:1) to which the internal standard (*trans*-β-apo-8'-carotenal at a concentration of approximately 15 µg/mL) was added. The samples were injected with a CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) on a Waters Acquity UPLC BEH Shield RP C18 column (2.1 mm x 100 mm, 1.8 µm; Waters, Milford, MA). Analytes were thermostatically (50°C) eluted at a flow rate of 0.5 mL/min with an Accela™ quaternary solvent manager and a 'Hot Pocket' column oven (Thermo Fisher Scientific, Bremen, Germany). The mobile phase solvents consisted of water + 20 mM ammonium acetate (A), methanol + 20 mM ammonium acetate (B) and ethyl acetate (C). The gradient was set as follows (min/%A/%B): 0.00/50/50, 20.00/0/100, 25.00/0/20, 27.00/0/20, 28.00/50/50, 30.00/50/50. The LC-system was hyphenated to an orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Bremen, Germany). Full MS scan data were acquired in HESI positive and negative mode within one analysis with a mass-to-charge range (*m/z*-range) of 90-1350 and resolving power set at 70,000 Full Width at Half Maximum (FWHM). Data were also recorded using selective ion fragmentation (data dependent MS2 or ddMS2) in positive and negative mode (one analysis per mode) to obtain additional structural information (resolving power set at 17,500 FWHM, collision energy 30 V, isolation window: 4 *m/z*). Each sample was analyzed in duplicate.

Data handling

A hybrid quadrupole-orbital trap MS-analyser was used for lipid identification. This enables selective ion fragmentation, a functionality that contributes significantly to compound identification by generating clean product ion spectra. Selective ion fragmentation is particularly useful for associating product ions with precursor ions during coelution of multiple lipids, as is often the case in complex extracts. The most abundant precursor ions were selected for lipid quantification. The total area per lipid class was obtained by summation of all areas of different components (e.g. different

fatty acyl chain) within this lipid class. This area was corrected both for the internal standard (*trans*-β-apo-8'-carotenal) and for the sample weight. Because no response factors were available for all lipid classes, no relative composition of the lipid fraction could be calculated. Instead, arbitrary relative areas were calculated by dividing the corrected areas by the sum of all corrected areas within a sample. Within each lipid class, the highest value was set to 100, thus obtaining relative abundances varying from 0 to 100 in each lipid class. These relative areas do not represent percentages within a lipid sample, but the evolution of the amount of each lipid class during the storage period.

3.2.3.4. *Analysis of volatile compounds in wet biomass by Headspace-Solid Phase Microextraction – Gas Chromatography – Mass Spectrometry (HS-SPME-GC-MS)*

To investigate the presence of alcohols, the wet samples were analyzed by GC-MS. A fully automated sample preparation unit (MultiPurpose Sampler® or MPS®, GERSTEL®, Mülheim an der Rur, Germany), combined with a 6890/5973 GC–MS system (Agilent Technologies®, Palo Alto, CA) was used for compound separation and identification. This method is based on a previous study [208]. Briefly, about 100 mg of the wet biomass was hermetically sealed in 20 mL vials and incubated for 30 minutes at 60°C using agitation. Extraction was done using a 75 µm CAR/PDMS fiber type for 30 minutes. The loaded SPME-fiber was thermally extracted in the GC-injector. Helium was used as a carrier gas (1 mL/min). Injector and transfer lines were maintained at 250°C and 280°C, respectively. The total ion current (70 eV) was recorded in the mass range from 40 to 230 amu (scan mode) and a run time of 52 min. For GC–MS profiling, a ZB-WAXplus column, 30 m x 0.25 mm I.D., 0.25 µm film thickness (Phenomenex®, Utrecht, Netherlands) was used and programmed: 40 °C (5 min) to 250 °C at 5 °C/min, held for 5 min. Identification of volatiles was performed by comparison with the mass spectra of the Wiley® 275 library.

3.2.4. Kinetic model to describe FFA formation during wet storage

The evolution of the FFA content during wet storage was characterized by a rapid, exponential increase after which a plateau was reached, during which

the FFA content remained constant. Therefore, a fractional conversion kinetic model, described by following equation

$X_t = X_f + (X_o - X_f) \exp(-kt)$

was fitted to the data. X_t represents the FFA content (% of total lipids) at time t (days of storage) and the parameters X_f, X_o and k are the FFA content at the plateau (% of total lipids), the FFA content at the beginning of the storage period (% of total lipids) and the reaction rate (d^{-1}) respectively.

The parameters k, X_o and X_f were estimated simultaneously using the statistical software Sigmaplot, in which a non-linear regression was applied. The quality of the fit was determined by means of the adjusted coefficient of determination (R^2 adj).

3.3. Results and discussion

3.3.1. Evolution of lipid content during wet storage

The total lipid content (on dry basis) was measured in the start biomass (before storage, i.e. day 0) and in all collected biomass samples that were stored at three storage temperatures (20°C, 4°C and -20°C) for three weeks. The start biomass of *T-Isochrysis* had a total lipid content of 31.3 ± 0.8 %. This is in agreement with values found in literature for *Isochrysis sp.*, varying between 18 and 34% depending on cultivation conditions such as medium, growth phase and light intensity [51, 209]. No clear increase or decrease of the lipid content as function of time was observed for any of the storage temperatures (Table 3.1).

Table 3.1: Evolution of the total lipid content on dry basis (g/100 g biomass) during storage of wet biomass of *T-Isochrysis lutea* at 20°C, 4°C and -20°C.

Storage time	Total lipid content (g/100 g biomass)		
	20°C	4°C	-20°C
Immediately after harvesting	31.3 ± 0.8	31.3 ± 0.8	31.3 ± 0.8
12 hours	29.5 ± 0.4	29.8 ± 1.1	32.0 ± 0.9
1 day	30.2 ± 0.4	29.3 ± 1.6	30.8 ± 0.3
2 days	27.1 ± 1.7	29.9 ± 1.3	31.0 ± 0.6
3 days	29.2 ± 2.1	30.0 ± 1.0	31.2 ± 1.0
7 days	28.1 ± 1.0	27.8 ± 0.9	29.3 ± 0.4
2 weeks	29.6 ± 0.6	30.2 ± 1.1	31.5 ± 1.1
3 weeks	31.9 ± 1.6	32.0 ± 1.5	27.7 ± 1.9

3.3.2. Evolution of FFA content during wet storage

The FFA content in the lipid fraction of all samples was determined and the results are presented in Figure 3.1. The curves connecting the measurement points represent the kinetic model that was fitted to the data and of which the parameters are given in Table 3.2.

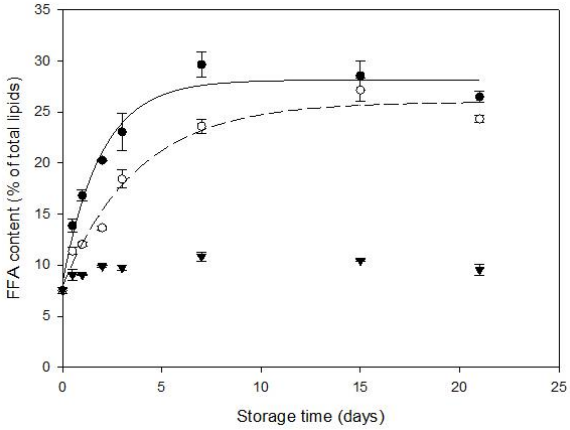


Figure 3.1: Evolution of the FFA content (% of total lipids) during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).The curves connecting the measurement points represent the kinetic model that was fitted to the data and of which the parameters are given in Table 3.2.

Table 3.2: Overview of the kinetic parameters (estimate ± standard error) and accompanying adjusted R^2 by modelling the evolution of the FFA content during wet storage of *T-Isochrysis lutea* as described in section 3.2.4.

	X_f (% of total lipids)	X_0 (% of total lipids)	k (d^{-1})	R^2 adj
Storage at 20°C	28.1 ± 0.9	8.3 ± 1.2	0.52 ± 0.09	0.965
Storage at 4°C	26.0 ± 1.1	7.9 ± 1.1	0.27 ± 0.06	0.961

3.3.2.1. *Temperature effects*

During storage at -20°C, almost no lipolysis was observed. Although the FFA content was somewhat higher after one day of storage than in the start biomass, the FFA content remained approximately stable during 3 weeks of storage. Consequently, the model fitted to the data of storage at -20°C

showed a bad fit, with an adjusted R^2 of 0.764. The observed parameters and the fitted model are thus not realistic and therefore not shown.

When biomass was stored at 4°C, the FFA content increased very rapidly during the first days of storage. In the biomass stored at 20°C, this increase occurred even faster. This demonstrates a strong temperature dependence of lipolytic reactions. These results are also in agreement with the study of Chen et al. (2012) [19], in which the increase in FFA content during 1 day of wet storage of *Scenedesmus* sp. was highest between 25°C and 37°C. When stored at 4°C, a significant increase was also observed, but to a lesser extent, while there were no FFA formed during storage at -20°C and -80°C. Ryckebosch et al. (2011) [23] also found a substantial increase in FFA content during 2 days of wet storage of *Phaeodactylum tricornutum* biomass at 4°C.

The differences in reaction rates can also be observed by comparing the rate constant k, estimated by the kinetic model (Table 3.2). The rate constant at 20°C is 0.52 d^{-1}, while the rate constant at 4°C is significantly lower, 0.27 d^{-1}. The end value at the plateau (X_f) and the initial value (X_0) are, on the other hand, not significantly different between storage at 20°C and 4°C. The model fits the measurement points well, which is also reflected in a very high adjusted R^2 (>0.96). The rate constants at 20°C and 4°C can be used to calculate the activation energy (E_a) of the reaction using the following equation

$$E_a = \frac{RT_1T_2}{T_1 - T_2} \ln\frac{k_1}{k_2}$$

in which R is the universal gas constant (8.314 J/K.mol), T_1 and T_2 the two temperatures installed and k_1 and k_2 the reaction rates as calculated by the model. The activation energy of this enzymatic reaction was calculated to be 27.6 kJ/mol, which is within the broad range of values found in literature for lipolytic enzymes in other organisms (14 to 33 kJ/mol in bacteria and yeasts) [210-212].

3.3.2.2. *Origin of hydrolytic enzymes*

As the cultivation of *T-Isochrysis lutea* was not conducted axenically, an additional experiment was performed to ensure that the observed lipolytic reactions were caused by endogenous enzymes and not by bacterial lipases. Therefore, a mixture of 5 antibiotics (250 µg mL-1 ampicillin, 50 µg mL-1

gentamycin, 100 µg mL-1 kanamycin, 500 µg mL-1 neomycin, 50 µg mL-1 streptomycin) was added to the wet biomass of *T-Isochrysis lutea* after harvesting and subsequently stored at 4°C during 2 weeks. This method was adopted from Cho et al. (2002) [213], in which it was shown that this antibiotic mixture inhibited bacterial growth in a contaminated culture of *Isochrysis galbana*. The FFA content was followed at different time intervals and compared to the control biomass, to which no antibiotics were added. At all four investigated time points, there was no significant difference between the FFA content in the biomass treated with antibiotics and the control biomass (detailed results not shown). This indicates that the lipases causing lipolysis in the biomass are from microalgal and not from bacterial origin. When bacteria would have been the source of the lipases, the enzymatic activity would have been higher in the control biomass, in which bacteria are active and growing, while in the biomass with antibiotics, only the bacterial lipases present at the beginning of the storage period could have led to some activity. It could thus be concluded that the lipolysis observed in *T-Isochrysis* originated from endogenous hydrolytic enzymes present in microalgal biomass.

3.3.2.3. *Lipolysis induction mechanism*

A very high FFA content (7.5 ± 0.3 % of total lipids) was already observed in the biomass immediately after harvesting. To clarify whether these FFA were already present in the biomass prior to harvesting or were formed during harvesting, this value was compared to the FFA content of a smaller sample of *T-Isochrysis* biomass that could be harvested more rapidly and subsequently freeze-dried immediately. In this sample, the FFA content was 2.50 ± 0.04 % of total lipids, which is substantially lower, but still not negligible. However, it is unlikely that high amounts of FFA are present in intact, living cells. FFA are possibly interfering with enzyme functions and inhibit electron transfer. Moreover, the higher sensitivity of FFA to oxidation causes the production of degradation products that are toxic for cells [22]. Therefore, it was hypothesized that the FFA observed in freshly harvested biomass were produced during and immediately after harvesting. The shorter harvesting protocol caused a lower FFA content in the start biomass but was not able to completely avoid lipolysis.

The rapid formation of FFA in *T-Isochrysis* biomass may be caused by an innate defense mechanism against grazers, which was already described in

2.3. It is suggested that in *T-Isochrysis* cells, which have a weak cell wall consisting of mannose [42] or even do not have a cell wall [43], this mechanism initiated by lipase enzymes starts very rapidly, activated by the rupture of the cells during harvesting. This could possibly explain the high amounts of FFA in the start biomass and the very rapid increase during the first hours or days of wet storage.

3.3.2.4. *Possible causes for the level-off effect*

A remarkable observation in Figure 3.1 is the level-off effect that was observed in the FFA content starting after 7 days of wet storage at 20°C and after 2 weeks at 4°C. Several hypotheses can possibly explain this effect.

First, it is possible that conditions in the harvested, microalgal paste are evolving towards unfavorable for enzymatic reactions. The most important factors determining enzymatic reactions are temperature and pH. Temperature was kept constant during storage, and thus could not explain changes in lipolysis rate. The pH was followed during the storage experiment and was found to decrease from 6.0 to 5.2 when storing at 20°C. As in literature rather high pH optima, varying between 6.5 and 9.0, are reported for lipases in microalgae [166, 167], this pH decrease could possibly affect the enzymatic activity. Therefore, an additional experiment was conducted in which the pH of the microalgal paste was set at 4.0 ± 0.1 immediately after harvest to make the pH even more unfavorable for lipolytic enzymes. The pH was kept at this value during 2 weeks of storage at 4°C. Figure 3.2 compares the obtained results with a sample in which the pH was not adapted. The parameters of the fitted kinetic model are given in Table 3.3.

It can be observed that lipolysis was not inhibited by lowering the pH. On the contrary, the process was found to occur faster, as can be seen from the higher reaction rate k (2.0 ± 1.2 d^{-1}) compared to the control biomass (0.40 ± 0.09 d^{-1}). However, because of the high standard deviation of the FFA content at the second time point of the biomass stored at pH 4, also the estimate of the reaction rate k had a high error. Consequently, the difference in reaction rate was not significant. The plateau value that was reached in both cases was not significantly different. It could thus be concluded that the low pH did not negatively influence lipolytic reactions and was not the cause of the level-off of the FFA content in the lipid fraction.

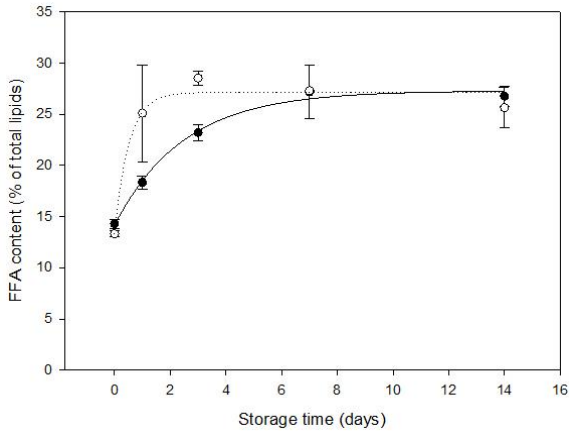


Figure 3.2: Evolution of FFA content (% of total lipids) during storage of wet microalgal paste of *T-Isochrysis lutea* at 4°C, both without adapting the pH (control, ●) and with holding the pH at a lower value (pH 4.0, ○). The curves connecting the measurement points represent the kinetic model that was fitted to the data and of which the parameters are given in Table 3.3.

Table 3.3: Overview of the kinetic parameters (estimate ± standard error) and accompanying adjusted R^2 by modelling the evolution of the FFA content during wet storage of *T-Isochrysis lutea* as described in section 3.2.4.

	X_f (% of total lipids)	X_0 (% of total lipids)	k (d^{-1})	R^2 adj
Control (without adapting the pH)	27.3 ± 0.5	14.2 ± 0.6	0.40 ± 0.06	0.988
pH 4	27.1 ± 0.9	13.3 ± 1.5	2.0 ± 0.9	0.942

A second possible explanation for the stabilization of the FFA content is the lack of substrate. Possibly, the lipase enzymes present in *T-Isochrysis* act specifically on some lipid classes, causing termination of the process when these specific lipid classes are exhausted. To investigate this hypothesis, a separation in lipid classes was conducted on all collected samples. Figure 3.3 presents the evolution of all lipid classes during wet storage of *T-Isochrysis* at 20°C. During storage at 4°C, almost the same trends were observed, although occurring at a lower rate, while at -20°C, no substantial changes were observed. This confirms the (from Figure 3.1) expected temperature dependence of the degradation reactions.

The neutral lipid class consisted mainly of FFA, TAG, DAG and MAG, for which the evolution during storage at 20°C is presented in Figure 3.3A. The evolution of the FFA showed the same trend as was obtained previously by the method described in 3.2.3.2 (Figure 3.1). All other neutral lipids showed a decreasing trend during wet storage. TAG were thus converted to DAG, followed by conversion to MAG and eventually to glycerol, in each step accompanied by the release of FFA. This process started immediately after harvest. TAG, DAG as well as MAG followed the same evolution, decreasing to a value that is not equal to zero. However, because no response factors were available, the remaining percentage of these lipid classes in the lipid fraction after 3 weeks of storage could not be calculated.

In the glycolipid class (Figure 3.3B), the following compounds were found: MGMG (monogalactosyl monoacylglycerol), MGDG (monogalactosyl diacylglycerol), DGMG (digalactosyl monoacylglycerol) and DGDG (digalactosyl diacylglycerol). MGDG and DGDG can be converted to respectively MGMG and DGMG by release of FFA and can be further degraded to galactosylglycerols, also accompanied by the release of FFA. It can be observed from Figure 3.3B that all glycolipids were degraded very rapidly during storage at 20°C and disappeared after only three days of wet storage. Phospholipids (mainly phosphatidylglycerol and phosphatidylinositol) were only found in very small amounts in the lipid samples, as peak areas in LC-MS analyses were very small. Nevertheless, a clear evolution could be observed during storage (Figure 3.3C), again being a very fast decrease during storage. The same observation could be made for the other polar lipids in the samples, which were DGTS (diacylglycerol-O-(N,N,N-trimethyl)-homoserine), MGTS (monoacylglycerol-O-(N,N,N-trimethyl)-homoserine), SQDG (sulfoquinovosyl diacylglycerol) and SQMG (sulfoquinovosyl monoacylglycerol). DGTS and SQDG are degraded to respectively MGTS and SQMG, which can then be further degraded by loss of the last fatty acyl chain, in each step accompanied by the release of a FFA. It could thus be concluded that all polar lipids were broken down very rapidly by a degradation process that started immediately after harvesting and which resulted in the absence of polar lipids after 3 days of wet storage at 20°C.

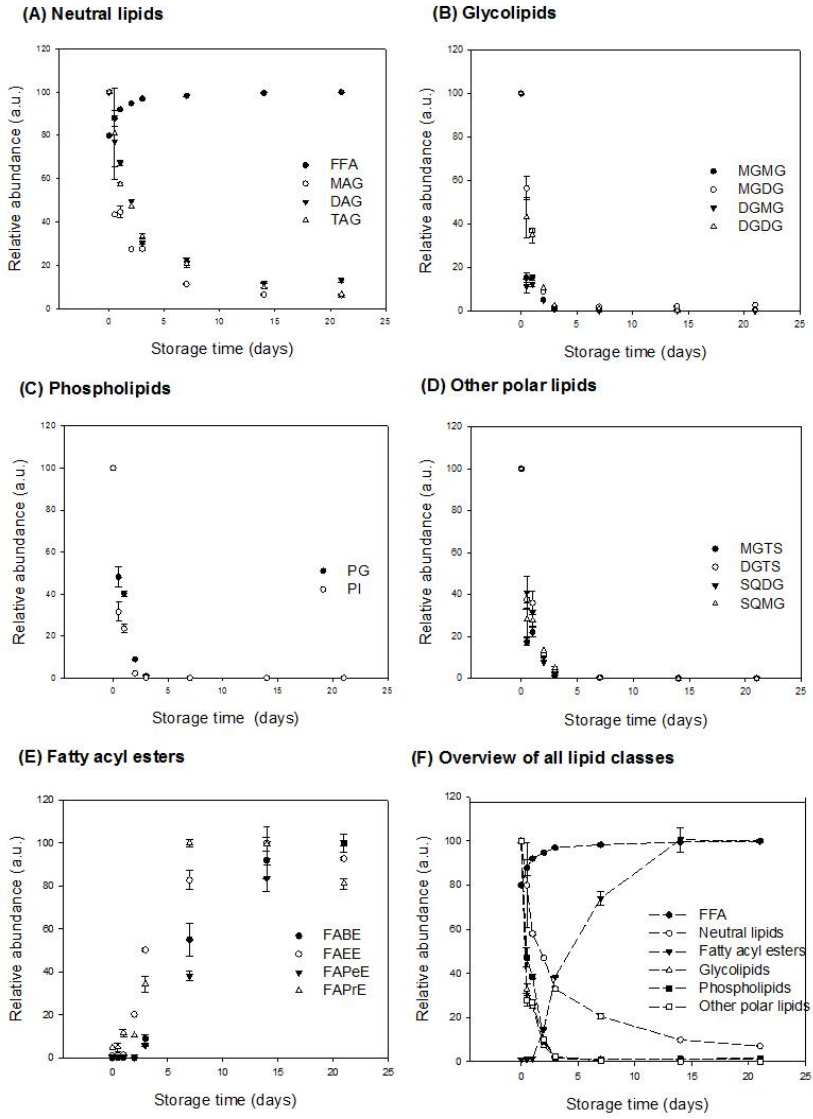


Figure 3.3: Evolution of different lipid classes during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C. Amounts are represented as relative abundances (arbitrary units).

A remarkable observation is the increasing amount of fatty acyl esters, mainly ethyl-, propyl-, butyl- and pentylesters present in the lipid fraction, as presented in Figure 3.3E. Fatty acyl esters were probably formed by an esterification reaction between free fatty acids, released during degradation of lipids, and alcohols. As storage was conducted in the dark and no oxygen supply was present, alcohols were possibly formed during dark fermentation reactions [214]. To confirm the presence of these alcohols, the volatile components of all wet biomass samples (before freeze-drying) were analyzed by HS-SPME-GC-MS. Via this method, the presence of ethanol, propanol and pentanol could be confirmed and the amounts were found to increase during storage at 20°C and 4°C. During storage at -20°C, none of these alcohols were found. The increasing amounts of ethanol even completely matched with the increasing amounts of the corresponding ester (Figure 3.4). These findings confirm the hypothesis that the formation of fatty acyl esters is associated with the presence of alcohols. Possibly, the reaction between alcohols and free fatty acids is an enzymatic reaction catalyzed by the lipase enzymes, as external lipases are already used in several applications to convert free fatty acids to fatty acyl esters, for example in enzymatic transesterification during biodiesel production [194].

Figure 3.3F presents an overview of the trends in the amounts of the different lipid classes. For this figure, the different lipid compounds within one class are taken together, because they followed the same evolution, as could be seen in Figure 3.3A-E. In general, it can be seen that all lipid classes, and not one lipid class specifically, were degraded from the beginning of the storage experiment, immediately after harvest. This implies the presence of non-specific hydrolyzing enzymes, possibly rather esterases than lipases. The polar lipids seem to disappear faster than the neutral lipids, but this may be explained by the lower amounts of polar lipids present in the beginning of the storage experiment. After 7 days of storage at 20°C, the FFA content started to level off, while the neutral lipids were not yet at the minimum value. It could thus be concluded that substrate limitation could not be the reason of the level-off effect. It is hypothesized that the decline in the rate of FFA formation over time is due to a dynamic balance between hydrolysis of esterified fatty acids and re-esterification of free fatty acids with alcohols.

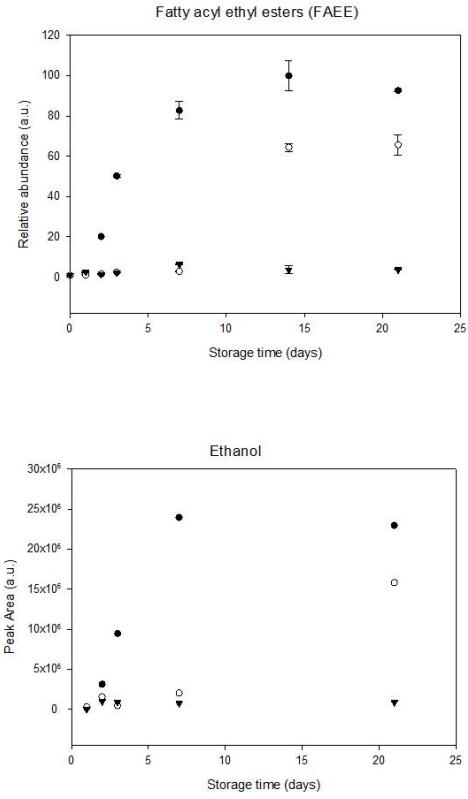


Figure 3.4: Evolution of fatty acyl ethyl esters (FAEE), determined by UHPLC- amMS) compared to the evolution of the corresponding ethanol, determined by HS-SPME-GC-MS, during 3 weeks of storage of wet biomass (8% dry matter) of *T-Isochrysis lutea* at 20°C (●), 4°C (○) and -20°C (▼).

3.3.3. Implications for lipid applications

The results of this study will have important implications for the different applications of microalgal lipids. The fast increase in FFA during storage at 20°C and 4°C makes storage of wet microalgal paste of *T-Isochrysis* at 20°C or 4°C undesirable. Even short term storage at these temperatures will result in high amounts of FFA (7 to 30% of total lipids). To completely avoid lipolysis problems, wet biomass should be stored at -20°C or further processed (dried) immediately. But even then, a high amount of FFA (7.5% of the lipids) is already present in *T-Isochrysis* due to hydrolysis processes going on during harvesting. This high percentage already exceeds the maximum value for biodiesel production (0.5% FFA) [194] and also the provided value for virgin,

edible oils (0.2% FFA) [190, 215] and is consequently problematic for these applications. In current applications with high FFA oils, refining is conducted either by soap formation with alkali materials, or by a pretreatment with acid or alkali catalyzed transesterification processes, in order to strongly reduce the FFA percentage. However, this results in yield losses and much higher production costs [216] and should thus better be avoided. Further research should reveal the microalgal species dependence of lipolysis processes and possible strategies to diminish lipolysis during wet storage.

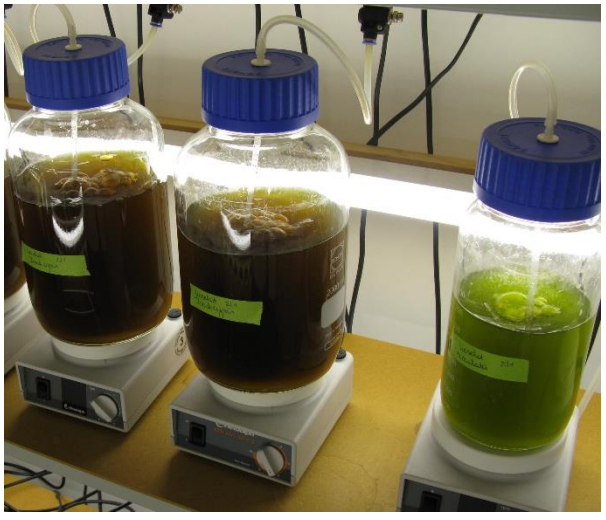
3.4. Conclusions

In this chapter, it was observed that FFA were formed by endogenous enzymes during and/or immediately after harvesting of *T-Isochrysis lutea*. The FFA content increased rapidly during post-harvest wet storage at 20°C and 4°C, while almost no lipolysis was observed at -20°C. However, the FFA content levelled off after several days of storage, probably because FFA reacted with alcohols to form fatty acyl esters. It could thus be concluded that even short term wet storage of *T-Isochrysis lutea* biomass at 20°C or 4°C is detrimental for lipid quality and should be avoided to obtain biomass of good quality for food and fuel applications.

It was hypothesized that the *T-Isochrysis lutea* cells, which have only a weak cell structure, were already ruptured during harvesting, which induced the lipolytic reactions. This would imply that the lipolytic stability during wet storage is determined by the cell integrity after harvesting. This hypothesis will be tested in Chapter 4.

Chapter 4:

Integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage



Adapted from: Balduyck, L., Stock, T., Bijttebier, S., Bruneel, C., Jacobs, G., Voorspoels, S., Muylaert, K., Foubert, I. (2017). Integrity of the microalgal cell wall plays a major role in the lipolytic stability during wet storage. Algal Research, 25: 516–524.

CHAPTER 4 INTEGRITY OF THE MICROALGAL CELL PLAYS A MAJOR ROLE IN THE LIPOLYTIC STABILITY DURING WET STORAGE

4.1. Introduction

In Chapter 3, it was already shown that wet storage of *T-Isochrysis lutea* biomass has a detrimental effect on lipolytic stability, as a very rapid increase of the FFA content was observed during storage at 20°C and 4°C. It was suggested that the low lipolytic stability in *T-Isochrysis* was due to the weak cell structure of this microalgal species. The cell (either the cell wall, cell membrane and/or organelle membranes) was possibly already disrupted during the harvesting process, leading to an increased contact between enzymes and substrates and possibly to the activation of lipase enzymes, as was already observed in some studies on diatoms [179, 180, 217]. The cell strength and structure were hypothesized to play a major role in this cell integrity after harvesting. As this characteristic strongly differs among species, the lipolytic stability would thus be expected to be species dependent.

The aim of this chapter was therefore to verify the hypothesis that the lipolytic stability during wet storage is determined by the cell integrity after harvesting and thus also by the microalgal species. Therefore, two species strongly differing in cell (wall) structure, namely *T-Isochrysis lutea* and *Nannochloropsis oculata,* were selected. In contrast to the weak cell wall of *T-Isochrysis*, consisting of mannose [42, 43], the tough cell wall of *Nannochloropsis* consists of cellulose and algaenan [39].

Firstly, the storage time and temperature dependence of lipolysis in *Nannochloropsis* was studied and compared with the observations made on *T-Isochrysis lutea* in Chapter 3. The observed big difference in lipid stability between these two species reinforced the hypothesis that the cell integrity after harvesting plays a major role in the lipolytic stability. Therefore, the tough cell wall of *Nannochloropsis* was disrupted by high pressure homogenization (HPH). Subsequently, both the disrupted and non-disrupted biomass were stored as a wet paste at 4°C for 4 weeks to investigate the influence of cell disruption on lipid stability. This should elucidate the role of cell integrity in lipid stability during wet storage.

4.2. Materials and methods

4.2.1. Cultivation and harvesting

Cultivation of *T-Isochrysis lutea*, formerly named *Isochrysis sp.* (CCAP 927/14; Culture Collection of Algae and Protozoa, Oban, United Kingdom), and *Nannochloropsis oculata* (SAG 38.85; University of Goettingen, Germany) was performed in 30 L photobioreactors as described in 3.2.1.

Harvesting was carried out in the early stationary phase using a lamella centrifuge (4000 *g*). To remove the biomass paste from the centrifuge and homogenize it, the paste was resuspended in a small amount of demineralized water. Subsequently, further concentration by centrifugation (9500 *g*, Sorvall RC-5B, Du Pont Instruments) was conducted. Cultivation and harvesting was performed once for each part of the experiment (the storage experiment described in 4.2.2.1 was performed separately from the experiment described in 4.2.2.2).

4.2.2. Wet storage of microalgal paste

4.2.2.1. *Influence of storage temperature on lipid stability during wet storage of Nannochloropsis*

The harvested, wet microalgal paste of *Nannochloropsis* (approximately 5% dry matter) was stored in falcon tubes in the dark at 20°C, 4°C and -20°C. After different time intervals (0, 0.5, 1, 2, 3, 7, 14, 21 and 28 days), the microalgal paste was freeze-dried and subsequently stored at -80°C until analyses.

4.2.2.2. *Influence of cell disruption on lipid stability during wet storage of Nannochloropsis*

The harvested, wet microalgal paste of *Nannochloropsis* (approximately 5% dry matter) was stored at 4°C for one day (for practical reasons). Thereafter, a part of this batch was disrupted by **HPH** (4 passes at 1000 bar, Panda 2k high pressure homogenizer, GEA Niro Soavi, Parma, Italy). These conditions have been shown to be sufficient to disrupt the cell wall of *Nannochloropsis* [218]. Although cooling at 4°C was conducted by a cryowaterbath (Haake, Karlsruhe, Germany) during passes through the HPH system, the sample had warmed up during the treatment and was

subsequently cooled in an ice bath. The other part of the batch was used as a control and was not treated by HPH.

The disrupted and non-disrupted microalgal paste was stored in falcon tubes in the dark at 4°C, during a period of 4 weeks (the start of the storage experiment was set immediately after the disruption, being one day after harvesting). Samples were collected at 6 different time points (0, 1, 3, 7, 14 and 28 days), immediately freeze-dried and subsequently stored at -80°C until analyses.

The stability of the disrupted and non-disrupted *Nannochloropsis* paste was compared to that of (non-disrupted) *T-Isochrysis* paste (approximately 4% dry matter) by storing under the same conditions and during the same time periods.

4.2.3. Total lipid extraction

The total lipid content (on dry basis) of all collected samples was determined gravimetrically by extraction with chloroform/methanol (1:1 v/v) according to the method described in 3.2.3.1. All extractions were done in triplicate.

4.2.4. FFA content

The FFA content in the lipid fraction was determined as described in 3.2.3.2. Before extraction, 5 mg of lauric acid (C12:0) (Nu-Check Prep, Elysian, USA), dissolved in chloroform, was added as an internal standard. Determination of the FFA content was performed in triplicate on each sample.

4.2.5. Degree of cell disruption

The degree of cell disruption obtained by HPH was determined in two ways. Next to a visual comparison by means of **microscopy**, the **extraction efficiency with hexane/isopropanol** (3:2) was determined as the ratio of the extraction yield with hexane/isopropanol (HI) compared to the extraction yield with chloroform/methanol (CM). The CM extraction (described in 3.2.3.1) has previously been demonstrated to extract the total amount of lipids, while HI (3:2) is not able to penetrate tough, intact microalgal cell walls and consequently functions less efficiently [88, 206, 218]. Therefore, the degree to which a HI extraction is able to extract lipids is a measure for the degree of cell disruption [218].

The **HI extraction** was conducted as described in Ryckebosch et al. (2013) [33], without the disruption step during extraction. Briefly, 6 mL of HI (3:2) was added to 100 mg of dry, microalgal biomass. After homogenizing by vortex stirring, the samples were centrifuged for 10 minutes at 750 *g*. The upper layer was transferred to a weighed flask. These extraction steps were repeated 4 times. The extracted lipids were transferred to the same weighed flask after each step. The amount of lipids was determined gravimetrically after evaporating the solvents. The HI extraction efficiency was determined in triplicate in each sample.

4.2.6. Separation in lipid classes by UHPLC-amMS

To determine if any preferential breakdown of certain lipid classes occurred, the CM lipid extracts of all collected samples were analyzed with ultrahigh-performance liquid chromatography– accurate mass mass spectrometry (UHPLC-amMS) as described in 3.2.3.3. Each sample was analyzed in duplicate.

The total area per lipid class was obtained by summation of all areas of different components (e.g. different fatty acyl chain) within this lipid class. This area was corrected both for the internal standard (*trans*-β-apo-8'-carotenal) and sample weight. Relative abundances were calculated by setting the highest value within each lipid class at 100, thus obtaining relative abundances varying from 0 to 100 in each lipid class. These relative abundances do not represent percentages within a lipid sample, but the evolution of the amount of each lipid class during the storage period. All polar lipids with one esterified fatty acid are taken together, as well as the polar lipids with two esterified fatty acids, as these followed almost the same evolution during storage.

4.3. Results and discussion

4.3.1. Influence of storage temperature on lipid stability during wet storage of *Nannochloropsis*

The lipid content (on dry basis) and the FFA content in the lipid extract was determined both in the start biomass (before storage, i.e. day 0) and in the collected samples after 0.5, 1, 2, 3, 7, 14, 21 and 28 days of wet storage at 20°C, 4°C and -20°C. The start biomass of *Nannochloropsis* contained 36.3 ± 0.5 % of lipids. This is in agreement with other studies, showing

Nannochloropsis species containing 21 to 38% lipids in the biomass [21, 51]. No clear decreasing or increasing trend of the **total lipid content** was observed during 4 weeks of wet storage of *Nannochloropsis* biomass at none of the storage temperatures (detailed results not shown).

The evolution of the **FFA content** during wet storage is presented in Figure 4.1. An increase of the FFA content was observed during wet storage at 20°C and 4°C, starting respectively after 3 days and 7 days. During storage at -20°C, no lipolysis was observed. This temperature dependence was also found in Chapter 3 during wet storage of *T-Isochrysis lutea* in the same conditions. However, in *T-Isochrysis*, a very rapid increase of the FFA content was observed at 4°C and 20°C, starting immediately after harvest. This is in contrast with the induction period that was seen in *Nannochloropsis*, during which no lipolysis was observed. It is hypothesized that this difference is caused by the difference in cell structure and strength between these two species [39, 42]. Possibly, the cells of *T-Isochrysis* are already damaged during harvesting, while this is not the case in *Nannochloropsis*. Rupture of the cells can induce more rapid degradation reactions by an increased contact between enzymes and substrates. Moreover, induction of lipolysis by disruption of the cell was already observed in some studies on diatoms and red algae. In these studies, the produced FFA were further degraded by lipoxygenases and hydroperoxide lyases, reaction mechanisms that were attributed to a defense mechanism against grazers [179-181, 217]. Possibly, a comparable process is initiated by disruption during harvesting in *T-Isochrysis*, while in *Nannochloropsis*, this process only starts after several days, when cell components begin to degrade as a result of cell death induced mechanisms, caused by several possible forms of stress (e.g. senescence, darkness, oxidative stress) [219]. This could possibly explain the induction period observed during wet storage of *Nannochloropsis* biomass. To confirm this hypothesis, the influence of cell disruption immediately after harvest on lipid stability during wet storage was investigated for *Nannochloropsis* and compared with (non-disrupted) *T-Isochrysis* stored in the same conditions. The results of this experiment are described in 4.3.2.

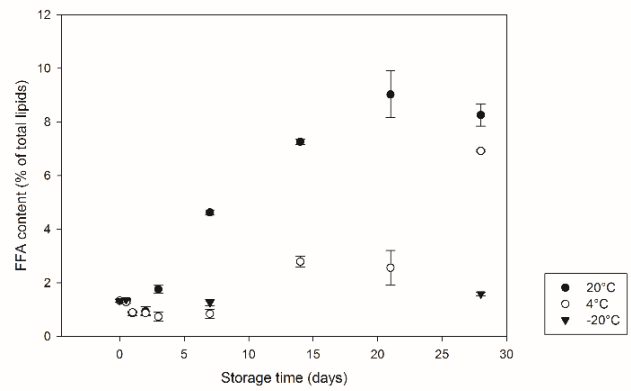


Figure 4.1: Evolution of the FFA content (expressed as % of the total lipid fraction) in *Nannochloropsis* during wet storage at 20°C, 4°C and -20°C.

4.3.2. Influence of cell disruption on lipid stability during wet storage of *Nannochloropsis*

4.3.2.1. *Degree of cell disruption after HPH cell disruption*

Before starting the storage experiment, it was verified whether the HPH treatment achieved its goal and thus disrupted the microalgal cells of *Nannochloropsis* in a sufficient way. The degree of cell disruption was determined by two methods. Firstly, microscopic observations were conducted on both the control sample and the disrupted sample and revealed a strong effect of HPH treatment. In disrupted *Nannochloropsis*, both intact cells and aggregates of cell debris were seen, whereas only intact cells were observed in non-disrupted *Nannochloropsis*. To confirm the visual observations, the HI extraction efficiency was calculated as a measure of the degree of cell disruption. As the HI extraction efficiency strongly increased after the HPH treatment (from 17.3 ± 1.0 % to 80.7 ± 2.8 %), the visual observations were indeed confirmed. An extraction efficiency of 80.7% after disruption suggests that HI (3:2) was not able to extract the total lipid fraction even in disrupted *Nannochloropsis*. Possibly, not all cells were disrupted by the HPH treatment, as suggested by the intact cells observed by microscopy. From these results, it could however be concluded that a substantial degree of cell disruption, sufficient for the aim of this experiment, was obtained. It should be noted that the cell wall composition and strength is also dependent on cultivation conditions, as was seen by an increase of the cell wall thickness of *Nannochloropsis* caused by lowering the salinity of the

cultivation medium [38]. Variation of these conditions thus possibly implies other degrees of cell disruption after HPH.

4.3.2.2. *Evolution of lipid content during wet storage*

The total lipid content, as extracted by CM (1:1), was determined in the start biomass of *Nannochloropsis* and *T-Isochrysis* and in all collected samples during storage. The start biomass of non-disrupted and disrupted *Nannochloropsis* contained respectively 35.4 ± 0.2 % and 33.1 ± 1.1 % lipids, while this was 33.9 ± 0.8 % lipids in the non-disrupted start biomass of *T-Isochrysis*. No clear decreasing or increasing evolution of the total lipid content was observed during wet storage for any of the samples (detailed results not shown).

4.3.2.3. *Evolution of FFA content during wet storage*

In Figure 4.2, the FFA content is presented as a function of the storage time at 4°C, both for the disrupted and non-disrupted samples of *Nannochloropsis* (A) and the non-disrupted samples of *T-Isochrysis* (B).

In non-disrupted ***Nannochloropsis***, the same trend as earlier observed in Figure 4.1 was seen (Figure 4.2A). The FFA content remained almost constant during 7 days of wet storage at 4°C, whereafter the FFA content started to increase. However, in disrupted *Nannochloropsis*, a rapid increase of the FFA content during the first days of wet storage was observed. Moreover, the start biomass already contained a significantly higher FFA content in disrupted biomass as compared with the non-disrupted biomass (respectively 6.7 ± 0.6 % and 0.47 ± 0.05 % of total lipids). This implies that lipolysis already started during and immediately after the HPH treatment and was promoted during storage compared to non-disrupted biomass. Moreover, it was observed that the increase of the FFA content in non-disrupted biomass after 7 days of storage was correlated with the increase of HI extraction efficiency after this period (Figure 4.2C) and thus with the loss of cell integrity. These observations suggest that lipolysis induction is related to the disruption of the microalgal cell and possibly to a defense mechanism against grazers that comes into effect when the cells are damaged and enzymes come into contact with their substrates, as was described in section 2.3 [179].

In non-disrupted ***T-Isochrysis***, the FFA content was already high in the start biomass immediately after harvesting (11.4 ± 0.6% total lipids) and increased very rapidly after harvest (19.2 ± 1.5% of total lipids after one day) (Figure 4.2B). Also the HI extraction efficiency was already high in the start biomass and was not increasing during further storage (Figure 4.2D), again suggesting (partial) rupture of the cell during the harvesting procedure. The FFA content flattened to a constant value of approximately 25% of total lipids. This is in agreement with the observations made in Chapter 3 under the same storage conditions and was explained by the formation of fatty acyl esters resulting from an esterification reaction between an alcohol and a free fatty acid. The levelling off was also observed in disrupted *Nannochloropsis* after 7 days of wet storage at 4°C (Figure 4.2A) and possibly originates from the same type of esterification reaction.

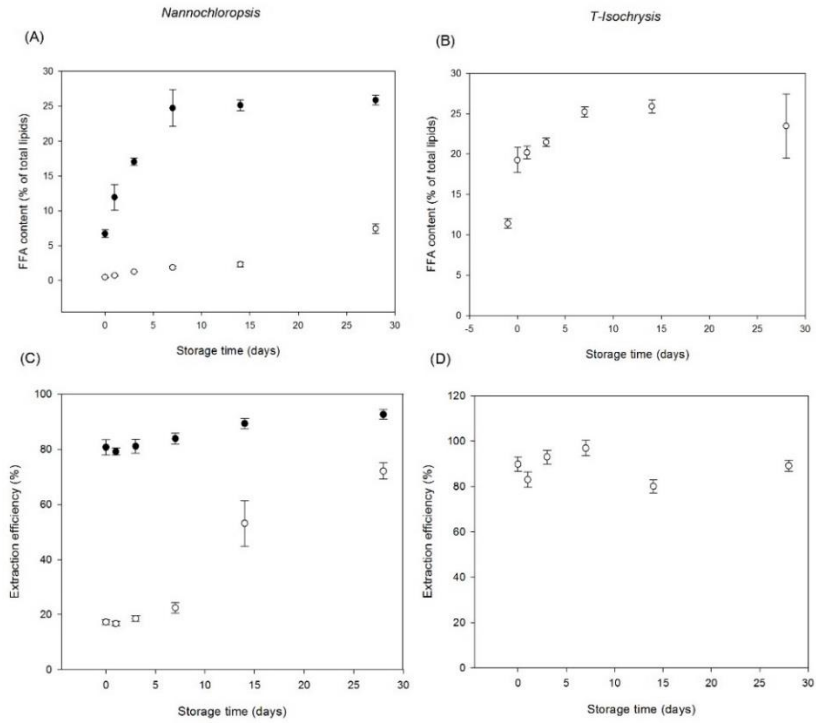


Figure 4.2: Evolution of the FFA content (expressed as % of the total lipid fraction) and the HI extraction efficiency (the efficiency of lipid extraction with HI compared to the total lipid content extracted by CM) in *Nannochloropsis* (A, C) and *T-Isochrysis* (B, D) biomass during wet storage at 4°C, either after a control treatment without disruption (○) or after a HPH cell disruption treatment (●).

4.3.2.4. *Evolution of lipid classes during wet storage*

The evolution of the different lipid classes was followed during the storage period, to determine whether certain lipid classes were broken down preferentially. The results of the lipid classes in disrupted and non-disrupted *Nannochloropsis* and in non-disrupted *T-Isochrysis* are shown respectively in Figure 4.3 and Figure 4.4.

The main **lipid classes** in the *Nannochloropsis* **start biomass** were triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), FFA, diacylglycerol-O-(N,N,N-trimethyl) homoserine (DGTS), monoacylglycerol-O-(N,N,N-trimethyl) homoserine (MGTS), phosphatidyl choline (PC), monogalactosyl diacylglycerols (MGDG) and sulfoquinovosyl diacylglycerol (SQDG). This is almost in accordance with the findings in Yao et al. (2015) [21], who found especially TAG, FFA, DGDG, MGDG, SQDG and PC. In *T-Isochrysis*, almost the same lipid classes were occurring, although phospholipids were less abundant. Next to the traditional neutral lipids (TAG, DAG, MAG), also other highly abundant compounds, tentatively identified as alkenones, were detected in all *T-Isochrysis* samples. Predominant structures were C37:2, C37:3, C37:4, C38:3 and C38:2 (the first number indicates the amount of carbon atoms and the second number represents the amount of double bonds). Also structures resembling C37:2 and C37:3 alkenoates were found, but could not be identified with certainty. Both alkenones and alkenoates have already been shown to be a substantial part of the neutral lipid fraction of several *Isochrysis* species by among others Eltgroth et al. (2005) [68] and O'Neil et al. (2012) [69].

Both in *Nannochloropsis* (Figure 4.3A) and in *T-Isochrysis* (Figure 4.4A), the **FFA trend** confirmed the results obtained by gas chromatography (Figure 4.2A,B). In *Nannochloropsis*, a rapid increase in FFA was observed during and immediately after the HPH treatment until a constant level was reached after approximately 7 days of storage. The non-disrupted batch retained a constant, low FFA value, until lipolysis started after 7 days of storage. In *T-Isochrysis*, the FFA content increased rapidly until also a constant level was reached after 7 days of storage.

In *Nannochloropsis*, big differences in abundance of FFA (Figure 4.3A), MAG (Figure 4.3B), DAG (Figure 4.3C) and polar lipids with one esterified fatty acid chain (Figure 4.3E) were observed between the disrupted and non-disrupted

samples at the start of the storage period. These compounds, originating from hydrolysis of the different ester bonds in (polar) lipids [164], are thus formed **during and immediately after HPH disruption.** However, the TAG and polar lipids with two fatty acids were not decreasing during HPH, although this was expected by the observed increase of the previously mentioned lipid classes. Probably these compounds were present in much higher amounts from the beginning, thereby causing no detectable differences by a small decrease during storage.

During the **first days of wet storage at 4°C**, a lot of changes were already seen in the disrupted *Nannochloropsis* biomass and in the *T-Isochrysis* biomass. In ***Nannochloropsis***, TAG and DAG abundances remained constant, whereas FFA increased and MAG and polar lipids decreased. It is thus hypothesized that the FFA formed during storage are originating mostly from the decomposition of polar lipids and only to a lesser extent from TAG. Possibly, the enzymes catalyzing the breakdown of lipids are preferentially degrading polar lipids, and are thus phospholipases or glycolipases.

In contrast to the specific breakdown of polar lipids in *Nannochloropsis*, a non-specific degradation of all lipid classes (except alkenones) was observed in ***T-Isochrysis*** biomass (Figure 4.4), immediately from the beginning of the wet storage period. As alkenones do not contain ester bonds, these cannot be degraded by lipase or esterase enzymes. After 4 weeks of storage, only neutral lipids are remaining, being almost all alkenones and a small amount of other neutral lipids (TAG, DAG and MAG).

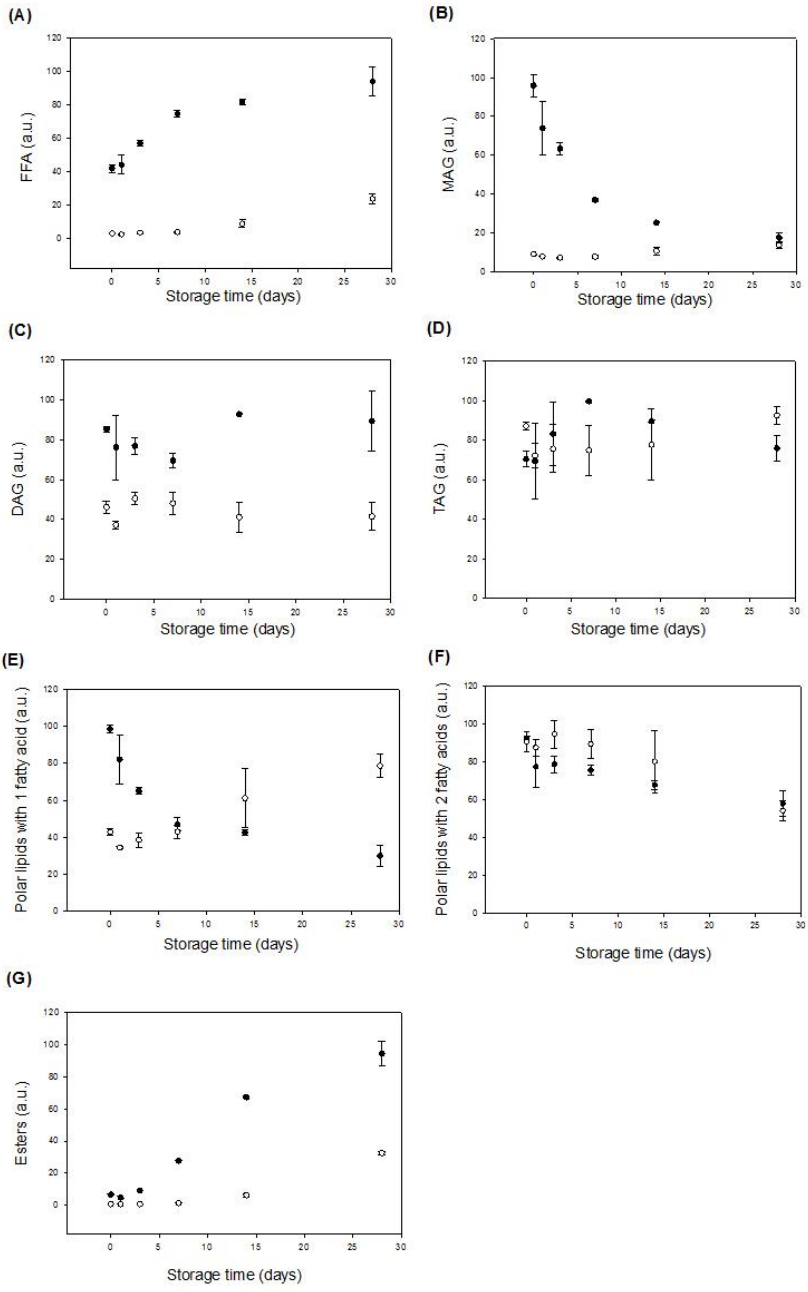


Figure 4.3: Evolution of the different lipid classes (expressed in arbitrary units) during storage of wet *Nannochloropsis* biomass at 4°C, either after a control treatment without disruption (○) or after a HPH cell disruption treatment (●).

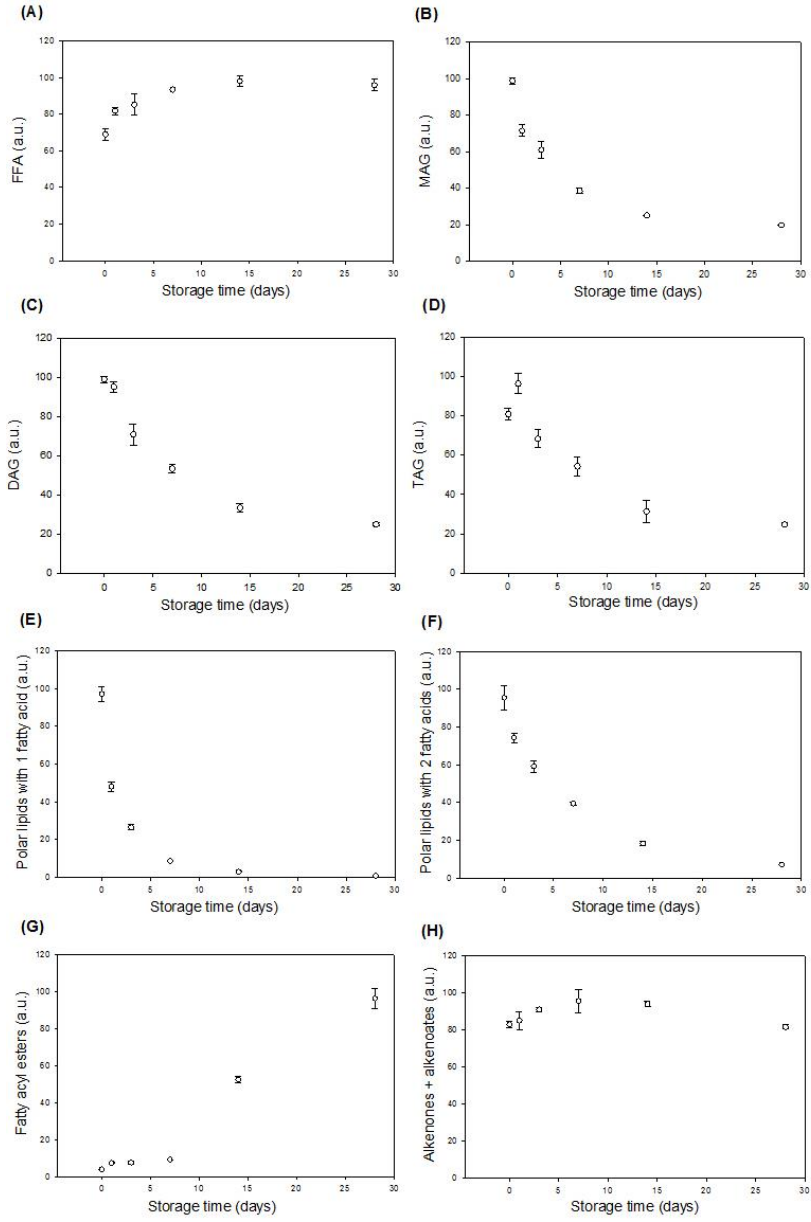


Figure 4.4: Evolution of the different lipid classes (expressed in arbitrary units) during storage of wet *T-Isochrysis* biomass at 4°C without pretreatment.

Different hypotheses are proposed to explain the difference between the specific degradation of polar lipids in *Nannochloropsis* and the non-specific degradation of lipids in *T-Isochrysis*. First, the enzymes present in both species are possibly strongly differing in structure and characteristics. The presence of specific phospholipases and/or glycolipases was already proposed in literature in *Thalassiosira rotula* [179,180], in *Chattonella marina* [167] and in *Dunaliella salina* [178], while in *Phaeodactylum tricornutum*, TAG lipases were found [173]. A storage experiment of wet *Scenedesmus* sp. biomass also showed a specific breakdown of TAG [19]. In *Isochrysis galbana* on the contrary, several genetic studies have demonstrated the presence of genes encoding for carboxylesterase and/or lipase-like enzymes, which are more esterases than specific lipases [170, 220]. The specificity of lipolytic enzymes can thus strongly differ between algae species and could also differ between *Nannochloropsis* and *T-Isochrysis*. Secondly, the localization of the enzymes in the cell could possibly play a role in the different specificity of the lipolytic reactions. Lipolytic enzymes can be located at different sites in the cell (e.g. endoplasmic reticulum, surface of lipid bodies), which implies a different specificity [165, 174] (as was described in 2.2.1). Consequently, rupture at a different organelle within the microalgal cell could induce the activation of different enzymes.

In a **second phase of storage**, starting approximately after 7 days of wet storage at 4°C, it was observed that the FFA content levelled off in disrupted *Nannochloropsis* and in *T-Isochrysis*. This could be explained by the increase in fatty acyl esters, probably formed by an esterification reaction between alcohols and free fatty acids. These were mainly ethyl esters in both species, but also butyl and pentyl esters in *Nannochloropsis*, and pentyl and propyl esters in *T-Isochrysis* (Figure 4.3G and Figure 4.4G). This is in accordance with the results observed during wet storage of *T-Isochrysis* in Chapter 3. In non-disrupted *Nannochloropsis* biomass, lipolysis started after approximately 7 days of wet storage. Neutral lipids were not changing, whereas polar lipids with 2 fatty acid chains were broken down to form polar lipids with one fatty acid chain and FFA. This confirms the preferential degradation of polar lipids. In addition, fatty acyl esters were formed, although to a lesser extent than in disrupted biomass.

4.3.3. Implications for lipid applications of microalgae

From these results, it could be concluded that the start of the lipolytic process is related to the moment at which the microalgal cell is damaged. In *T-Isochrysis*, the harvesting process is possibly already detrimental for the integrity of the cell wall, cell membrane and/or organelle membranes, resulting in an increased contact between lipids and lipolytic enzymes and thus an immediate, rapid increase of the FFA content and breakdown of lipids. In *Nannochloropsis*, the cell remains intact during the harvesting process, as could be seen from the very low HI extraction efficiency. Consequently, the FFA content does not increase during the first days of storage and only starts to increase at the moment the cells are degrading after 7 days of wet storage, possibly as a consequence of cell death induced mechanisms, caused by several forms of stress (e.g. senescence, darkness, oxidative stress) [219]. By disrupting the cell with HPH treatment, the lipolytic process is induced and starts immediately during and after the HPH treatment.

These findings have important implications for the existing applications of microalgal biomass and microalgal lipids in particular. So far, the problem of lipolysis during wet storage was often underestimated and the causes not well understood and studied. By now, it can be concluded that the lipolytic stability of wet microalgal biomass is determined by the cell integrity after harvesting, which is dependent on the strength of the cells (e.g. cell wall strength and composition, membrane composition). Both species with a tough and a weak cell wall pose stability problems by production of high amounts of FFA, which are detrimental for both food applications (e.g. off-flavors, promoting oxidation) [11] and for biodiesel production (e.g. downstream processing problems) [194]. Microalgal species with a weak cell wall (e.g. *T-Isochrysis*) are possibly already damaged during harvesting, which makes wet storage even for a few hours detrimental for lipid quality. Species with a tougher cell wall (e.g. *Nannochloropsis*) remain intact during the harvesting process and may be stored for a few days at 4°C without changes in lipid composition. However, to extract sufficient amounts of lipids, a cell disruption pretreatment is often necessary [91]. In this study, it was shown that this treatment leads to comparable stability problems as in species with a weak cell wall, with very high amounts of FFA formed during and immediately after the disruption. This stage of wet storage after cell disruption (either by harvesting or by cell disruption pretreatment) should

thus be avoided as much as possible, in order to minimize the amount of FFA formed in this stage. Further research should reveal whether pretreatments are possible to avoid the formation of FFA.

4.4. Conclusion

It could be concluded from this chapter that the integrity of the microalgal cell after harvesting, which is dependent on the strength of the cells (e.g. cell wall strength and composition, membrane composition), plays a major role in the lipolytic stability during wet storage of microalgae. Microalgae with a weak cell structure (e.g. *T-Isochrysis*) are possibly already disrupted during the harvesting process, resulting in a very poor lipolytic stability during even short-term wet storage. Microalgae with a stronger cell structure (e.g. *Nannochloropsis*) possess a better lipolytic stability during wet storage. However, if cell disruption is performed to improve the lipid extraction efficiency, a very fast increase of the FFA content is also observed.

Chapter 5:

Influence of high pressure homogenization on free fatty acid formation in *Nannochloropsis* sp.

Adapted from: Balduyck, L., Bruneel, C., Goiris, K., Dejonghe, C., Foubert, I. (2018). Influence of high pressure homogenization on free fatty acid formation in Nannochloropsis sp. European Journal of Lipid Science and Technology.

CHAPTER 5 INFLUENCE OF HIGH PRESSURE HOMOGENIZATION ON FREE FATTY ACID FORMATION IN *NANNOCHLOROPSIS* SP.

5.1. Introduction

In Chapter 4, it was demonstrated that the lipolytic stability during wet storage of *Nannochloropsis oculata* was strongly dependent on the integrity of the cells. Without any cell disruption pretreatment, no lipolysis was observed during the first days of wet storage (dependent also on storage temperature). On the contrary, after cell disruption by high pressure homogenization (HPH), FFA were formed very rapidly immediately after disruption.

HPH is one of the most efficient cell disruption methods to efficiently rupture the cell walls of *Nannochloropsis* species, resulting in increased release of pigments and proteins [101, 221, 222] and an improved lipid extraction efficiency [100, 218, 223-225]. The lipid yield has already been shown to increase with increasing pressure and number of passes [100, 225-228]. However, it has not been investigated before which effect these HPH conditions (pressure, number of passes) have on the FFA formation during and immediately after the HPH treatment.

Therefore, the aim of this chapter was to investigate the impact of HPH pressure and number of passes on the FFA content of *Nannochloropsis* biomass. To do so, the biomass was subjected to different HPH treatments using a three-level full factorial design with pressure and number of passes as independent variables. Their FFA content was subsequently measured. To investigate the relation between FFA formation and cell integrity, the lipid extraction efficiency was also analyzed as a measure of the degree of cell disruption. Next to this, it was also checked whether the different HPH treatments had an influence on the relative fatty acid profile, which would be an indication of selective extraction or degradation of certain fatty acids during HPH.

5.2. Materials and methods

5.2.1. Production of algal samples

Wet microalgal paste (10.6 % dry weight) of *Nannochloropsis* sp. (CCAP 211/78) was sourced from an indoor cultivation system without applying any stress conditions at Proviron Industries nv (Hemiksem, Belgium). The biomass was disrupted by HPH (Stansted Fluid Power, Pressure Cell Homogenizer, UK) one day after harvesting. Cooling at 4°C was conducted by a cryowaterbath during passes through the HPH system. A part of the batch was used as a control and was thus not disrupted. Before analyses, both the disrupted and non-disrupted part of the *Nannochloropsis* batch were freeze-dried (HOF GT200, HOF Sonderanlagenbau, Lohra, Germany) and subsequently stored at -80°C.

5.2.2. Experimental design

The influence of the HPH cell disruption process on the formation of FFA was investigated using a **three-level full factorial design** (3^2), in which two factors were varied: the pressure during HPH and the number of passes through the system. The pressure was set at 400, 700 or 1000 bar, while 1, 2 or 3 passes were used. By this method, 9 different disrupted samples were obtained, which were analyzed for their FFA content and their extraction efficiency and fatty acid profile by hexane/isopropanol extraction.

5.2.3. Total lipid extraction (CM extraction)

The total lipid content of all samples was determined gravimetrically by extraction with chloroform/methanol (1:1) (CM) as described in 3.2.3.1. All extractions were conducted in triplicate.

5.2.4. Hexane/isopropanol extraction (HI)

The lipid yield by extraction with hexane/isopropanol (3:2) (HI) was determined as described in 4.2.5. The HI extraction was performed in quadruplicate on each sample.

Although the extraction efficiency by HI extraction is typically lower than by CM extraction, it has been shown to give the highest extraction efficiency among the non-halogenated and food grade solvents [88]. HI (3:2) is not able

to penetrate tough, intact cell walls and the extraction yield with this solvent can thus be used as a measure of the integrity of the cell wall [88, 218]. Therefore, the lipid extraction efficiency (%) was calculated as the ratio of the HI (3:2) extraction yield relative to the CM (1:1) extraction yield (which is the total lipid content).

5.2.5. FFA content

The FFA content was determined as described in 3.2.3.2. Before extraction, 5 mg of tridecanoic acid (C13:0) was added as an internal standard. Determination of the FFA content was performed in triplicate on each extract obtained by CM extraction.

5.2.6. Fatty acid profile and ω-3 content

The fatty acid profile was determined by derivatization of the fatty acids in the lipid extract to fatty acid methyl esters (FAME) and subsequent analysis by gas chromatography as described in Ryckebosch et al. (2012) [206].

Before CM or HI extraction, 5 mg of tridecanoic acid (C13:0) was added as an internal standard. To 5 mg of the extracted lipids, dissolved in 1 mL toluene, 2 mL of 1% sulfuric acid in methanol was added. FAME were formed during incubation overnight at 50°C. After cooling to room temperature, 5 mL of 5% NaCl was added to improve phase separation. Subsequently, the FAME were extracted with 3 mL hexane and diluted to a concentration of 0.5 mg/mL for chromatographic analysis. The FAME were separated by gas chromatography with cold on-column injection and detected by flame ionization detector (GC-FID) (Trace GC Ultra, Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium). An EC Wax column (length: 30 m, ID 0.32 mm, film: 0.25 µm) (Grace, Lokeren, Belgium) was used and the time–temperature program installed was 70–180 °C (10 °C/min), 180–235 °C (4 °C/min), 235 °C (4.75 min). Identification of the fatty acids was conducted using a standard mixture containing 35 different FAME (Nu-Check Prep, Elysian, USA). Peak areas were quantified with the software Chromcard (Interscience, Louvain-la-Neuve, Belgium).

The HI extraction followed by FAME analysis was performed in triplicate on each sample. On the non-disrupted biomass, also the CM extract was analyzed in triplicate.

5.2.7. Statistical analysis

The statistical software JMP Pro was used to fit a model in which the influence of the factors used in the full factorial design (HPH pressure and number of passes) on the FFA content was studied. Therefore, an analysis of variance (ANOVA) table of the second order model with quadratic and interaction effects of the factors was set up and the significant factors were determined ($p<0.05$). The levels of the variables HPH pressure and number of passes were rescaled to the interval [−1,1].

5.3. Results and discussion

5.3.1. Influence of HPH conditions on the FFA content

The **FFA content** in the non-disrupted starting biomass was already relatively high at 2.55 ± 0.13 g/100 g biomass, which corresponds with 13.2 ± 0.4 % of the total lipid fraction, as extracted with CM (1:1). It is hypothesized that these FFA originate from lipolysis occurring during and immediately after harvest. Before the HPH treatment, the biomass was stored at 2°C for one day for practical reasons, during which some lipolysis reactions could have occurred.

As shown in Figure 5.1, a substantial effect of the HPH treatment on the total FFA content in the biomass (expressed as g/100 g biomass) was seen when comparing the non-disrupted sample with the least heavily treated HPH sample (400 bar – 1 pass), and this effect became more profound when the number of passes was increased. This is in accordance with the findings of Chapter 4, in which also an increase in FFA content was observed after applying 4 passes at 1000 bar on *Nannochloropsis oculata*. It was hypothesized that lipolysis reactions are triggered by the damage to the cell or some parts of the cells caused by the HPH treatment.

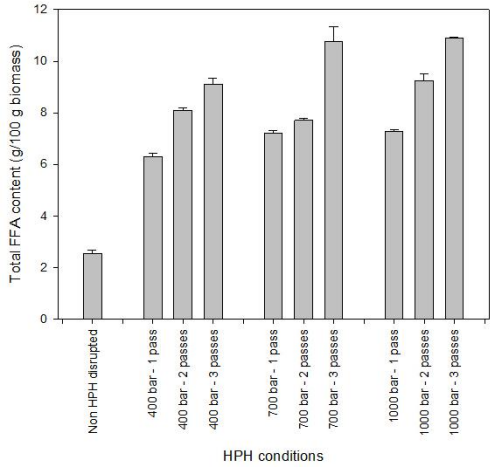


Figure 5.1: Total FFA content in the biomass (g/100 g biomass) of *Nannochloropsis* sp. without cell disruption (non HPH disrupted) or with a HPH treatment with different pressure and number of passes applied.

To investigate the factors determining FFA formation by lipolysis during HPH more thoroughly, a second order model with factors 'HPH pressure' and 'number of passes' was fitted to the results of the total FFA content. The ANOVA table of this model is shown in Table 5.1. Determination of the total FFA content was conducted in triplicate. With the removal of one outlier, this resulted in 26 observations and thus 25 degrees of freedom. The R^2 and adjusted R^2 of this model are 0.935 and 0.919 respectively, which indicates that the model is not predicting the FFA content perfectly. The lack of fit test is also significant (p-value <0.0001), which means that the model lacks one or more factors determining the total FFA content in the biomass.

Table 5.1: Analysis of variance (ANOVA) table of the total FFA content (g/100 g biomass) of *Nannochloropsis* sp. biomass in a second order model with factors 'HPH pressure' and 'number of passes'.

Source		df	Sum of squares	Mean Square	F ratio	p-value (Prob > F)
Model		5	53.5	10.71	58.0	<0.0001
Error		20	3.7	0.18		
	Lack of fit	3	3.0	1.01	25.5	<0.0001
	Pure Error	17	0.7	0.04		
Total		25	57.3			

Table 5.2 shows the parameter estimates with the corresponding p-values of this model. The number of passes appears to be the most important factor determining the total FFA content, followed by the HPH pressure. The quadratic effects of both factors and the interaction effect between the factors were not significant in the model. The major effect of the number of passes on the amount of FFA formed during HPH can either be explained by a higher degree of disruption of the cells, or by the secondary effect of the increased temperature during consecutive HPH passes. Although cooling during the passes was applied, the samples still heated up, which could have an impact on the degree of damage to the cells and on the activity of the lipolytic enzymes. It has also been described in literature that a temperature increase of about 2.5°C per 10 MPa (100 bar) occurs during a HPH treatment [99], which would mean 25°C per cycle of 1000 bar. This is however in the case that no cooling was conducted. Possibly, the temperature after 3 passes is more favorable for lipolytic enzymes than after 1 or 2 passes, causing a higher FFA content. The effect of pressure on the FFA content after HPH could possibly also be explained by a higher degree of disruption by applying a higher pressure, which is also seen in section 5.3.2.

Table 5.2: Parameter estimates for the total FFA content (g/100 g biomass) of *Nannochloropsis* sp. biomass in a second order model with factors 'HPH pressure' and 'number of passes'.

Term	Estimate ± std error	t ratio	p-value (Prob > |t|)
Intercept	8.35 ± 0.19	44.37	<0.0001
Pressure	0.66 ± 0.10	6.48	<0.0001
Passes	1.63 ± 0.10	15.61	<0.0001
Pressure*Pressure	-0.009 ± 0.183	-0.05	0.9621
Pressure*Passes	0.20 ± 0.12	1.65	0.1146
Passes*Passes	0.22± 0.18	1.23	0.2317

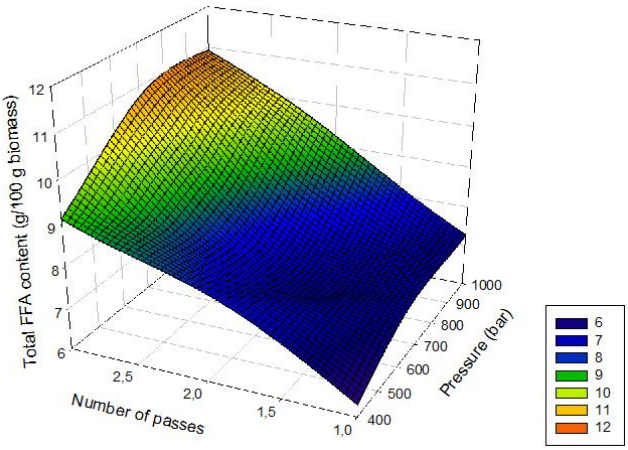


Figure 5.2: Surface plot of the FFA content (g/100 g biomass) of *Nannochloropsis* sp. biomass in function of pressure and number of passes used during the HPH treatment.

5.3.2. Relation between FFA formation and cell integrity

To understand the relation between FFA formation and cell integrity, the lipid extraction efficiency by HI extraction was analyzed as a measure of the degree of cell disruption (Figure 5.3). The substantial effect of applying 1 pass at 400 bar on the total FFA content that was observed (Figure 5.1), is in contrast with the slight effect of this treatment on the lipid extraction efficiency by HI extraction. Thus, it seems that even minimal damage to the cells, which did not result in an increased lipid extraction yield, was sufficient to induce lipolytic reactions. It is hypothesized that the disruption step causes damage to certain parts of the *Nannochloropsis* cells (e.g. cell membrane), which enables the lipolytic enzymes to come into contact with their substrates, thus accelerating the FFA production during and immediately after the HPH treatment.

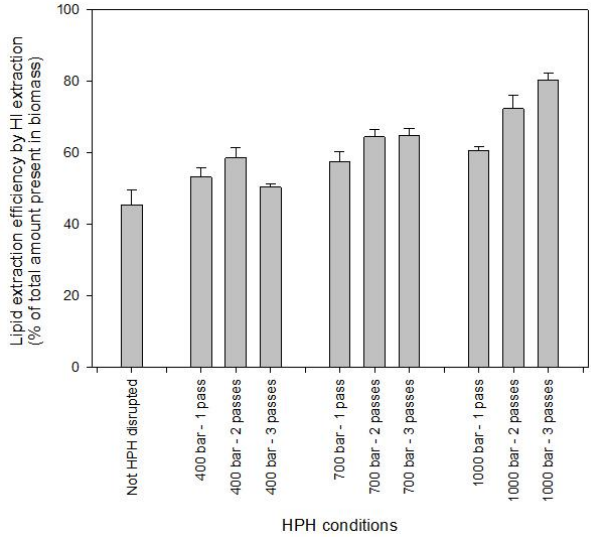


Figure 5.3: Lipid extraction efficiency (expressed as % of the total amount of lipids present in the biomass) in *Nannochloropsis* sp. biomass as obtained by HI lipid extraction without cell disruption (not HPH disrupted) or with a HPH treatment with different pressure and number of passes applied.

5.3.3. Influence of HPH conditions on fatty acid composition

All analyzed extracts (CM extract of the start biomass and HI extracts of all treatments) showed the same relative **fatty acid profile** (expressed as a percentage of the total amount of FAME). The main fatty acids were myristic acid (C14:0, 7.5 ± 0.5 %), palmitic acid (C16:0, 33.1 ± 1.5 %), palmitoleic acid (C16:1, 33.2 ± 1.0 %), oleic acid (C18:1, 6.4 ± 1.3 %), linoleic acid (C18:2, 2.6 ± 0.9 %), arachidonic acid (C20:4n-6, 5.1 ± 0.2 g/100 %) and eicosapentaenoic acid (EPA, C20:5, 10.1 ± 0.8 %).

The yield of the ω-3 fatty acid **EPA** extracted by HI (expressed in g/100 g biomass) increased with an increasing HPH pressure and number of passes. The highest yields were observed in the samples homogenized at 1000 bar for 2 or 3 passes (respectively 0.79 ± 0.01 and 0.90 ± 0.01 g EPA /100 g biomass), which were also the conditions that provided the highest lipid extraction efficiency (Figure 5.3). A good correlation between lipid and EPA yield by HI extraction (R^2 = 0.9278) was found, confirming that the HPH treatment does not induce selective extraction of certain fatty acids. This also explains the constant relative fatty acid profile in all samples. These

observations also imply that EPA was not degraded during the HPH treatment (e.g. by oxidation). Lipolytic reactions have no impact on the fatty acid profile, as FFA are also derivatized to FAME.

5.3.4. Implications of this study for the use of HPH for cell disruption

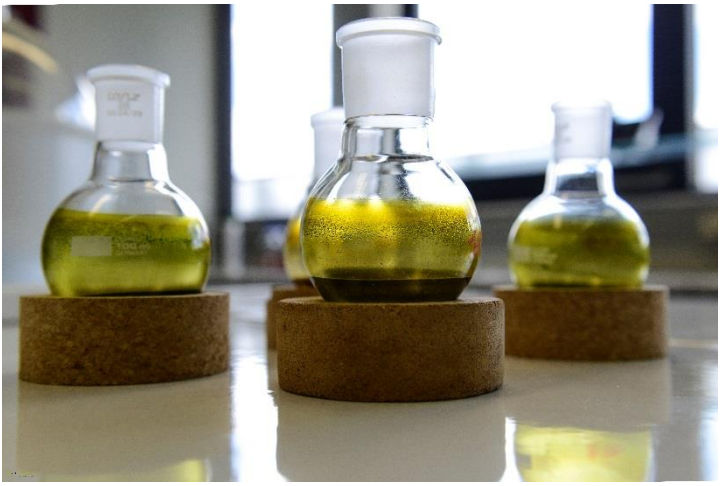
The results of this study have important implications for the application of HPH treatments on microalgae with the aim to improve the extraction efficiency. To improve the lipid extraction efficiency of *Nannochloropsis* cells, HPH treatments are very useful, as the extraction yield by HI extraction was almost doubled by applying 3 passes at 1000 bar. However, HPH treatments also induce the formation of FFA, even when the rupture of the cells is minimal. To obtain biomass with minimal amounts of FFA, the number of passes and the HPH pressure should be minimized. However, to maximize the lipid extraction yield, multiple passes and a higher pressure are necessary and thus a compromise should be made between a high lipid yield and a low FFA content. Alternatively, more research in this field could possibly reveal ways to avoid the formation of FFA during wet storage, especially in disrupted biomass.

5.4. Conclusion

The FFA content in the HPH disrupted *Nannochloropsis* biomass varied from 6.3 to 10.9 g/100 g biomass (depending on the HPH conditions applied), which was a substantial increase compared to the FFA content in the non-disrupted biomass (2.5 g/100 g biomass). The FFA formation was mostly influenced by the number of passes applied, which was probably caused by the increased temperature during the treatment. The HPH pressure was also a significant factor determining the FFA formation. The big difference between the FFA content in the disrupted and non-disrupted biomass is in contrast with the only slight increase of the HI lipid extraction efficiency. This indicates that minor damage to the cell is sufficient to induce lipolytic reactions. When using HPH treatments to improve the extraction efficiency, a compromise should thus be made between a higher extraction efficiency and a lower FFA content. Therefore, in the following chapters, different strategies to avoid FFA formation are investigated.

Chapter 6:

Inhibition of lipolytic reactions during wet storage of *T-Isochrysis lutea* biomass by a heat treatment



CHAPTER 6 INHIBITION OF LIPOLYTIC REACTIONS DURING WET STORAGE OF *T-ISOCHRYSIS LUTEA* BIOMASS BY A HEAT TREATMENT

6.1. Introduction

The previous chapters (3-5) indicated that lipolysis during wet storage of microalgal biomass is an enzymatic reaction caused by lipases. This has also been shown in literature by isolating and characterizing enzymes from among others *Spirulina platensis* [166], *Chatonella marina* [167], *Botryococcus sudeticus* [168] and *Nannochloropsis oceanica* [169]. Also several genetic studies elucidated the presence of genes encoding for carboxylesterase and/or lipase-like enzymes in *Isochrysis galbana* [170-172] and *Phaeodactylum tricornutum* [173]. Most of the studies have reported a temperature optimum between 25 and 45°C and a pH optimum between 6.5 and 9. In contrast, Savvidou et al. (2016) [169] found very thermostable lipases in *Nannochloropsis oceanica*, which became even more active when treated at 100°C for 60 minutes.

One of the possible methods to avoid extensive FFA formation during wet storage, cell disruption or wet extraction is to inactivate the lipolytic enzymes in an early stage by a heat treatment. A few studies already used a boiling water treatment of the wet biomass of *Isochrysis galbana* and *T-Isochrysis lutea* [172], *Skeletonema costatum* [22] and *Pseudo-nitzschia* [229] to lower the FFA content in the biomass. Also in other research fields struggling with lipolysis problems, heat treatments are commonly used. Rice bran, oat and wheat germ also possess high lipase activities and experience a limited shelf life with accompanying off-flavors, decrease of nutritional value and unusability for applications, unless a heat treatment is applied [164, 230-232]. However, heat treatments can promote lipid oxidation due to the higher temperatures to which the lipids are exposed [232]. In addition, heat-sensitive antioxidants broken down during the heat treatment can also cause a lowered oxidative stability during prolonged storage [232, 233]. Oxidation reactions can also have a negative impact on flavor and on the nutritional value due to a loss of among others the health promoting polyunsaturated fatty acids [150].

The substrate specificity, temperature and pH optimum and consequently also the time-temperature combination of the heat treatment necessary for

denaturation of the enzymes depends strongly on the source of the lipases [234]. This makes an optimization specific for microalgae and a differentiation between microalgal species necessary. Working at temperatures below the inactivation temperature could possibly activate the lipolytic enzymes instead of inactivating them, which would cause even higher FFA contents.

In this chapter, a heat treatment was optimized to enable wet storage of *T-Isochrysis lutea* for a longer term (7 days) without FFA formation. A full factorial design (3^2) with varying time-temperature combinations was used to determine the minimum treatment required for inactivation of the lipolytic enzymes. In a follow-up experiment, two adequate treatments were selected to verify whether other quality attributes remained unchanged by the treatment. Therefore, the two treatments were compared to the control (no heat treatment) in terms of ω-3 fatty acids, oxidative stability and content of pigments (carotenoids and chlorophylls) and phenolic compounds.

6.2. Materials and methods

6.2.1. Cultivation

Cultivation of *T-Isochrysis lutea*, formerly named *Isochrysis sp.* (CCAP 927/14; Culture Collection of Algae and Protozoa, Oban, United Kingdom) was performed in pilot scale (30 L) photobioreactors as described in 3.2.1. Cultivation and harvesting was performed separately for the two experiments (described in 6.2.3 and 6.2.4).

6.2.2. Optimization of the centrifugation protocol

In Chapters 3 and 4, it was observed that the FFA content in *T-Isochrysis lutea* biomass was already very high immediately after harvesting. As it could be concluded from Chapter 4 that the major trigger of lipolysis was the loss of cell integrity, it was hypothesized that this high FFA content was caused by rupture during the harvesting protocol. To obtain a biomass with a low FFA content, it was important to reduce the lipolysis that already occurred during harvesting.

Harvesting was carried out using a lamella centrifuge (4000 *g*). To remove the biomass paste from the centrifuge and to homogenize the paste, it was

necessary to resuspend the paste in a small amount of liquid. In Chapters 3 and 4, the biomass was resuspended in demineralized water. As *T-Isochrysis* biomass is cultivated in salt medium, a possible cause for cell disruption during harvesting could be an osmotic shock due to the transfer from salt cultivation medium to demineralized water.

This hypothesis was tested in a preliminary experiment. After cultivation and centrifugation, a part of the biomass was resuspended in demineralized water, while the other part was resuspended in salt cultivation medium. The wet biomass was stored at 4°C during 7 days. At different time intervals, the samples were frozen and freeze-dried. On the dried biomass, the FFA content was determined.

6.2.3. Influence of different heat treatments on FFA production during wet storage

In a first experiment, the microalgal paste, harvested by the protocol optimized in 6.2.2, was homogenized by stirring and divided in glass tubes (10 mL). The dry weight concentration before the heat treatment was approximately 7%. These tubes were put in a water bath at a certain temperature for a certain time period. The temperature and time were varied according to a full factorial (3^2) design, with temperatures 65°C, 80°C and 95°C and time intervals 3, 8 and 13 minutes. After the heat treatment, the tubes were brought immediately into an ice bath. The control sample was not subjected to a heat treatment and was kept at room temperature during the treatment of the other samples. To concentrate the samples to a lower volume, they were centrifuged once more at 1700 g for 10 minutes, after which the supernatant was decanted. Each treatment was performed three times, so that one sample could be frozen (at –80°C) and freeze-dried immediately (T_0), while the other samples were stored for 2 days (T_1) and 7 days (T_2) at 4°C before freezing and freeze-drying to monitor the FFA production during wet storage. Analyses of the total lipid content and the FFA content were conducted on the dried biomass.

6.2.4. Influence of selected time - temperature treatments on quality attributes

For the second part of the experiment, the procedure described in 6.2.3 was repeated for a control sample (which was not heat treated) and for two heat

treatments selected based on the results of the experiment described in 6.2.3: 8 minutes at 80°C (treatment 1, TRT1) and 3 minutes at 95°C (treatment 2, TRT2). The actual temperature in the biomass paste during and immediately after the treatment was followed by a thermocouple (Figure 6.1). Each treatment was performed twice, so that one sample was immediately frozen at −80°C and subsequently freeze-dried (T_0), while the other sample was stored for 7 days at 4°C before freezing and freeze-drying (T_2). Analysis of the total lipid content, FFA content, fatty acid profile, oxidative stability, pigment content and phenolic content were conducted on the dried biomass.

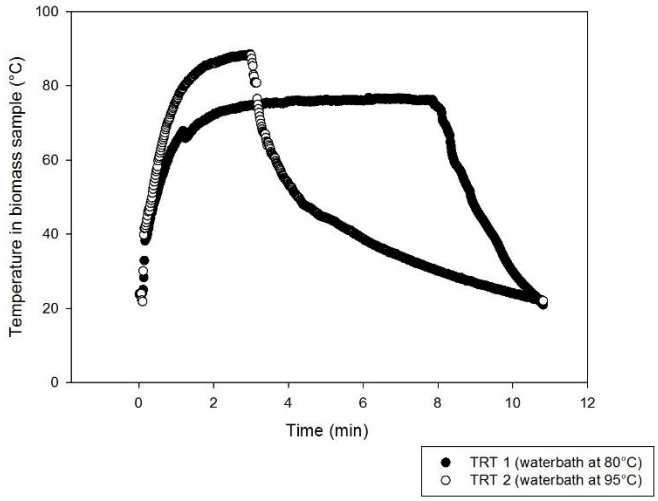


Figure 6.1: Actual temperature in *T-Isochrysis lutea* wet biomass (°C) during heat treatment at 80°C for 8 min (TRT 1) and at 95°C for 3 min (TRT 2) and subsequent cooling in an ice bath.

6.2.5. Analyses

6.2.5.1. *Total lipid content*

The total lipid content (on dry basis) of the biomass samples was determined by chloroform/methanol (1:1) extraction as described in 3.2.3.1. All extractions were conducted in triplicate.

6.2.5.2. *FFA content*

The FFA content in the total lipid fraction was determined as described in 3.2.3.2. This analysis was performed in triplicate on each sample.

6.2.5.3. *Fatty acid profile and ω-3 content*

The fatty acid profile was determined by methylesterification of the lipids in the total lipid fraction according to the method described in 5.2.6. This analysis was performed in triplicate on each sample.

6.2.5.4. *Oxidative stability*

The oxidative stability of the microalgal lipids was analyzed by determining the peroxide value (PV) as a measure of the amount of primary lipid oxidation products (hydroperoxides). The PV was determined by the spectrophotometric ferrous oxidation – xylenol orange (FOX) method as described in Ryckebosch et al. (2013) [33]. This spectrophotometric method measures the amount of ferrous ions (Fe^{2+}) oxidized to ferric ions (Fe^{3+}) by peroxides in the samples. The ferric ions form a blue-purple complex with xylenol orange, of which the absorbance is measured at 560 nm. This analysis was performed in quadruplicate on each sample.

6.2.5.5. *Pigment content and composition*

Carotenoids and chlorophylls in the microalgal biomass samples were extracted with acetone/methanol (7:3). Therefore, 5 mL of acetone/methanol (7:3) was added to 25 mg of freeze-dried biomass. After homogenizing by vortex stirring, the mixture was centrifuged for 10 minutes at 750 *g*. The supernatant was transferred to another tube, while the remaining pellet was extracted three more times in the same way. The collected supernatants were diluted and filtered for HPLC analysis. Extraction was conducted in duplicate on each sample.

The pigment composition of each extract was analyzed by HPLC with a photodiode array detector (PAD) (Alliance, Waters, Zellik, Belgium) set at 436 nm. A Nova-Pak C18 column (4 µm, 3.9x150 mm) with a C18 guard column, in a column oven set at 30°C, was used for separation. The mobile phases used were methanol/ammonium acetate buffer (pH 7.2, 0.5M) (8:2) (solvent A), acetonitrile/milliQ water (9:1) (solvent B) and ethylacetate (solvent C). The following gradient was used: 100% A to 100% B (0 – 3 min), 100% B to 40% B and 60% C (3 – 14 min), isocratic at 40% B and 60% C (14 – 23 min), 40% B and 60% C to 100% B (23 – 25 min), 100% B to 100% A (25 – 26 min). Standards obtained from Sigma-Aldrich (Bornem, Belgium) (chlorophyll a, pheophytin a, fucoxanthin, beta-carotene) and from DHI lab

products (Horsholm, Denmark) (chlorophyll C2, violaxanthin, diadinoxanthin, antheraxanthin, diatoxanthin, lutein, zeaxanthin) were used for identification. External calibration curves were used for quantification of the pigments in the samples. Each extract was injected twice.

6.2.5.6. *Phenolic content*

The phenolic content was determined by the spectrophotometric method described in Goiris et al. (2012) [153]. First, an extract of phenolic compounds was prepared by mixing 100 mg of microalgal biomass with 2 mL of ethanol/water (3:1). After centrifuging at 4500 g for 10 minutes, the supernatant was transferred to another tube, while the pellet was extracted a second time by the same method. The pooled extracts were diluted to obtain values according to the external standard curve. Next, 200 µL of the diluted extract was mixed with 1.5 mL Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 minutes. Subsequently, 1.5 mL of sodium bicarbonate solution (60 g/L) was added and incubated for 90 minutes at room temperature. Subsequently, the absorbance at 750 nm was measured. Each biomass sample was analyzed in duplicate. The total phenolic content was calibrated against a standard curve of gallic acid (25 to 150 mg/L) and expressed as mg gallic acid equivalents (GAE) per g biomass.

6.2.6. Statistical analysis

For the first part of the experiment, involving the factorial design, a response surface model in the time and temperature of the heat treatment was fitted to the data using the weighted least squares option in the statistical software JMP Pro. The model involved linear effects, quadratic effects and the two-way interaction effects. As recommended in the literature on response surface methodology [235, 236], the levels of the variables time and temperature were rescaled to the interval [−1,1]. The inverse variances of the responses, representing the error in the measurement, determined by the analyses in triplicate, were used as weights in the weighed least squares analysis.

Separate response surface models were fitted for the FFA content at T_0, T_1 and T_2. When analyzing the T_0 responses, the values of the samples treated at 65°C – 13 minutes and 80°C – 3 minutes were not included, as something went wrong during freeze-drying. This resulted in abnormal FFA contents. The best fitting models for T_0, T_1 and T_2, obtained after discarding non-

significant model terms, were used to set up contour plots showing the joint effects of time and temperature on the FFA content.

For the second part of the experiment, Analysis of Variance (ANOVA) was performed to detect significant differences between the different treatments or between T_0 and T_2. As the interaction between the treatment and the storage time was found to be significant in this model, the effect of both factors was determined separately. The effect of the treatment within one time point was determined by performing a post-hoc Tukey test with significance level 0.05. Significant differences are indicated with different letters on the graphs. To determine the effect of storage time within one treatment, a student's T-test with significance level 0.05 was performed. Significant effects of time are indicated with different numbers on the graphs.

An important remark to the interpretation of this response surface model is that the actual temperature in the sample is dynamic. A treatment of 3 minutes at 80°C means that the sample tube has been submerged in a water bath at 80°C for 3 minutes and does not mean that the sample itself has been heated at 80°C for 3 minutes. In reality, the sample temperature increases during the treatment until a maximum value that is somewhat below the set water bath temperature (as can be seen in Figure 6.1). This implies that the time and temperature indicated in this chapter will be more correct if longer treatment times are applied. This can possibly limit the correct interpretation of this model.

6.3. Results and discussion

6.3.1. Optimization of the centrifugation protocol

Figure 6.2 presents the lipolytic stability of *T-Isochrysis lutea* biomass during wet storage at 4°C, both when resuspended in demineralized water and resuspended in cultivation medium after centrifugation. It can be observed that the evolution of the FFA content during storage followed the same trend as observed in Chapter 3 and 4 when resuspended in demineralized water. However, when resuspension was performed in cultivation medium, an osmotic shock was avoided and the biomass remained stable for a few hours. After 1 day of wet storage, a rapid increase of the FFA content is also observed and the lipolytic stability is still much worse than in *Nannochloropsis*

oculata (observed in chapter 4). Thus, the problem is delayed for a few hours but not avoided. However, by retaining the FFA concentration at a low value for a few hours, it allows to treat the biomass during this time period and to retain a low FFA content in the biomass.

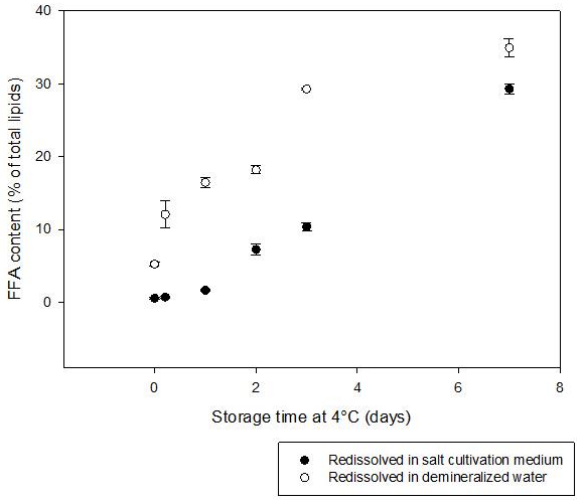


Figure 6.2: Influence of the type of water used for resuspending the *T-Isochrysis lutea* biomass after centrifugation on the lipolytic stability during post-harvest storage at 4°C.

6.3.2. Influence of different heat treatments on FFA production and lipid content during wet storage

The *T-Isochrysis lutea* biomass after a heat treatment at 65°C, 80°C or 95°C for 3, 8 or 13 minutes was compared to the non-treated control biomass. The FFA content and the total lipid content (expressed as percentage of the biomass) at T_0 (immediately after the treatment), T_1 (after 2 days of wet storage at 4°C) and T_2 (after 7 days of wet storage at 4°C) are displayed in Figure 6.3. The results of the analyses of the samples treated at 65°C – 13 minutes and 80°C – 3 minutes for T_0 are not shown, as something went wrong during freeze-drying and abnormal values resulted.

6.3.2.1. *FFA content*

At T_0, the FFA content was already higher in biomass treated at 65°C compared to the control biomass, which remained at room temperature during the treatments. The elevated temperatures during a treatment at 65°C thus favored the activity of the lipolytic enzymes. Further wet storage

at 4°C of the samples treated at 65°C resulted in a rapid increase of the FFA content. A temperature of 65°C is thus not sufficient to inactivate the enzymes completely.

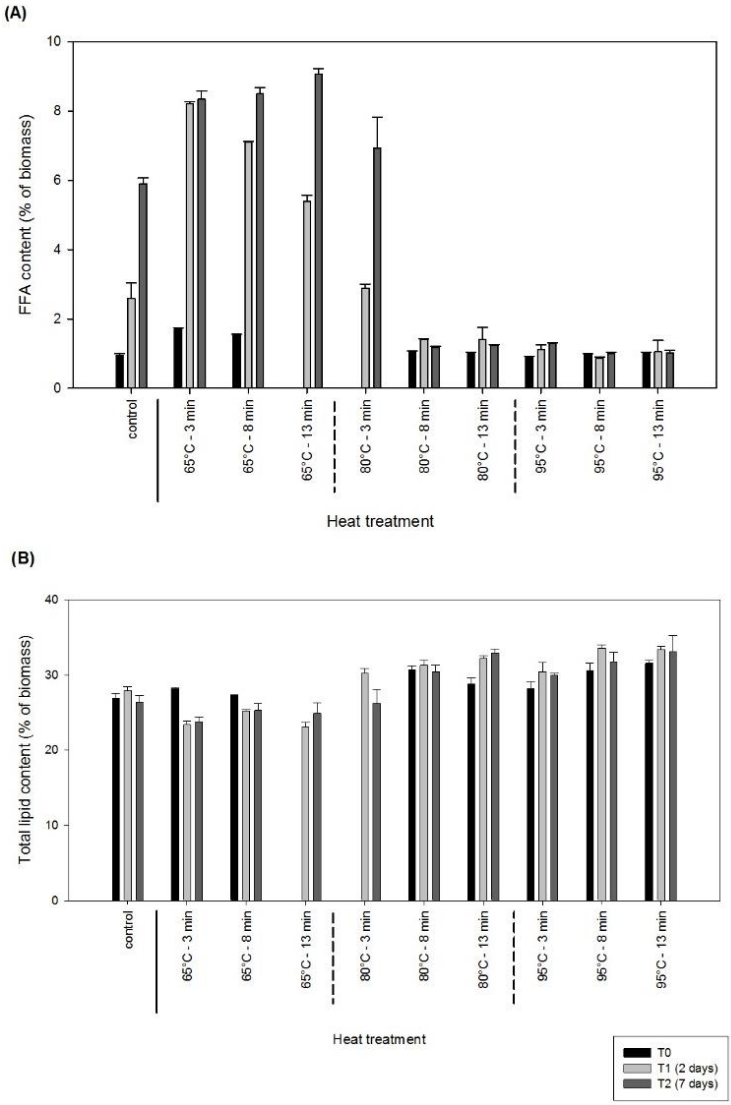


Figure 6.3: FFA content (A) and total lipid content (B) (expressed as percentage of the biomass) of *T-Isochrysis lutea* samples that were not heat treated (control) or heat treated (temperature and time of the treatment indicated) after harvesting and freeze-dried immediately after the heat treatment (T_0) or stored at 4°C for 2 days (T_1) or 7 days (T_2) before freeze-drying.

When applying heat treatments at higher temperatures (80°C and 95°C), the FFA content at T_0 was not higher than the control biomass. Apparently, the biomass reached rapidly a temperature that inactivates the lipolytic enzymes, which was not the case at 65°C. However, during wet storage, the biomass treated 3 min at 80°C still experienced an increase of the FFA content, comparable with the increase of FFA in the control biomass. The other treatments gave no differences between the FFA contents at T_0, T_1 and T_2 and are thus sufficient to completely inactivate the lipolytic enzymes. A sufficient heat treatment (minimum 8 min at 80°C) thus enables to stop lipolytic reactions and to store the treated biomass as a wet paste during at least 7 days at 4°C without FFA formation.

The effect of time and temperature of the heat treatment on the FFA content at T_0, T_1 and T_2 was studied in more detail using a response surface model in treatment time and temperature. Non-significant terms were omitted from the three models. The parameter estimates for the significant model terms are presented in Table 6.1. The coefficient of determination (R^2) of these models was 0.9981, 0.9999 and 0.9999 for the T_0, T_1 and T_2 data, respectively. To facilitate the interpretation of the significant model terms and the effect sizes, the final models are visualized by means of contour plots in Figure 6.4.

At T_0, the main effects of temperature and time, the quadratic effect of temperature and the interaction effect of time and temperature are significant for the FFA content. The lower the time and temperature of the treatment (within the range of 65 to 95°C), the more active the lipolytic enzymes after the heat treatment, leading to elevated FFA contents immediately after the heat treatment. The quadratic effect of temperature is significant because the negative effect of temperature on the FFA content diminishes at higher temperatures. The contour plot for T_0 shows that increasing the temperature beyond 80°C does not improve the FFA content at T_0 anymore, as the lipolytic enzymes are inactivated above this temperature anyway. The duration of the heat treatment only plays a major role when the temperature is below 75°C.

At T_1 and T_2, the duration of the heat treatment even has a smaller influence, especially at T_2, where none of the model terms involving the variable time is significant. This means that the temperature of the heat treatment is the dominating factor, i.e., the factor with the largest impact on the inactivation

of the enzymes, given a minimum treatment time of 3 minutes. The quadratic effect of temperature at T_1 and T_2 is similar to that at T_0.

Table 6.1: Estimated effects for the significant terms in the response surface models for the FFA content in the *T-Isochrysis lutea* biomass at T_0, T_1 and T_2. Non-significant parameters were removed from the model.

Term	Estimate	Std Error	t ratio	Prob > |t|
T_0				
Intercept	1.072	0.004	259.4	<0.0001
Temp	−0.287	0.008	−35.2	<0.0001
Time	−0.053	0.010	−5.1	0.0071
Temp*Temp	0.184	0.009	20.2	<0.0001
Temp*Time	0.104	0.013	8.2	0.0012
T_1				
Intercept	1.364	0.057	23.8	<0.0001
Temp	−3.147	0.072	−43.4	<0.0001
Time	−1.082	0.159	−6.8	0.0003
Temp*Temp	2.601	0.093	28.0	<0.0001
T_2				
Intercept	1.193	0.114	10.5	<0.0001
Temp	−3.766	0.093	−40.6	<0.0001
Temp*Temp	3.679	0.147	25.1	<0.0001

The fact that, at T_2, the effect of treatment time was insignificant, is at odds with the clear difference in FFA formation observed between a treatment of 80°C – 3 minutes and 80°C – 8 minutes shown in Figure 6.3A. Possibly, this can be explained by the fact that the temperature in the sample after 3 minutes was slightly lower than after 8 minutes, as the treatment temperature (80°C) is the temperature of the water bath and not the temperature inside the sample (which is given in Figure 6.1 for two different treatments). The temperature in the sample for the treatment time of 3 minutes was possibly just smaller than the inactivation temperature of the enzymes, which may explain the difference in FFA content between 3 minutes and 8 minutes at 80°C. This measurement at 80°C – 3 minutes was also identified as an outlier in the statistical analysis of the T_2 data. However,

as removing that data point did not noticeably affect the look of the response surface model for T_2, we decided to report the results for the full data set for T_2.

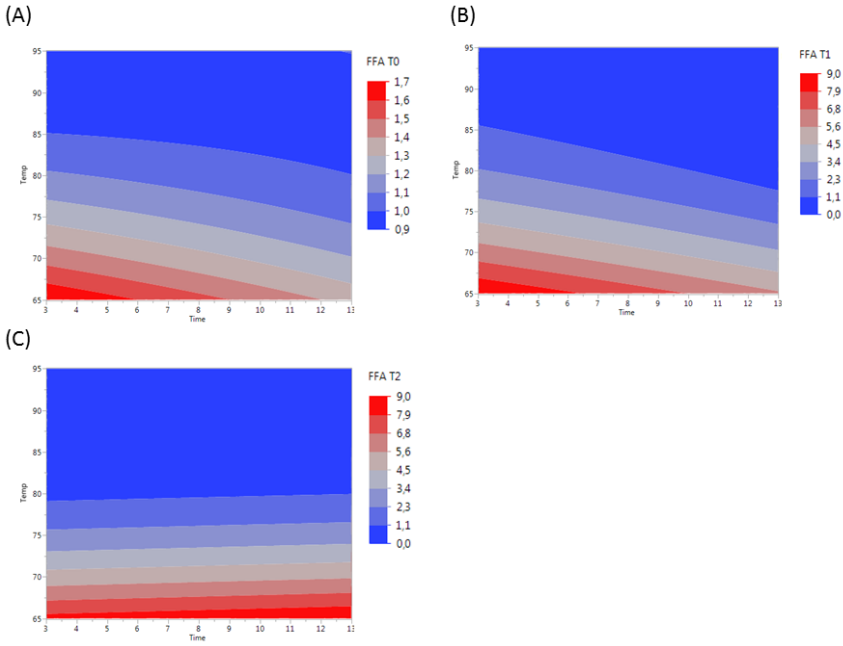


Figure 6.4: Contour plots showing the FFA content (expressed as percentage of the biomass) in *T-Isochrysis lutea* samples at T_0 (A), T_1 (B) and T_2 (C) as a function of time and temperature of the heat treatment.

These findings are in accordance with the optimum and inactivating temperature of (micro)algal lipases found in literature. Demir and Tükel (2010) [166] found a maximal enzymatic activity at 45°C in *Spirulina platensis*, with an activity range between 37 and 60°C. They found no activity at 70°C. Bilinski et al. (1968) [182] found a higher optimum temperature of 60°C in *Monochrysis lutheri*, with inactivation of the enzymes at 80°C. Illijas et al. (2008) [176] and Terasaki and Itabashi (2002) [167] found rather low optimal temperatures (between 25 and 37°C) respectively in *Gracilaria vermiculophylla* and *Chatonella marina*. Only Savvidou et al. (2016) [169] found stimulation of the lipolytic activity at very high temperatures (100°C) in *Nannochloropsis oceanica*. Except for this last study, these findings from literature confirm the observations made in this study, namely an optimum

temperature of the lipolytic enzymes in *T-Isochrysis lutea* between room temperature and 65°C and inactivation of these enzymes from 80°C.

6.3.2.2. *Lipid content*

The lipid content (Figure 6.3B) at T_0 was observed to be lower in biomass samples treated at 65°C and in the control biomass than in the biomass that experienced a more intense treatment (at 80°C or 95°C). This can possibly be explained by the release of intracellular compounds (e.g. proteins, carbohydrates) into the surrounding medium during the heat treatment. These compounds are then discarded together with the supernatant after the centrifugation step that followed the heat treatment, which causes a higher lipid content. Possibly, the least intensively treated biomass is damaged to a lesser extent, leading to less release of intracellular compounds and consequently a lower percentage of lipids in the cells.

It was also observed that in the biomass samples in which the FFA content increased strongly, the lipid content decreased over the same time (Figure 6.3). For example, the increase of the FFA content from T_0 to T_1 in the samples treated at 65°C was accompanied by a decrease of the lipid content when comparing these samples at T_0 and T_1. Also the increase of the FFA content from T_1 to T_2 in the sample treated for 3 min at 80°C was concomitant with as a decrease of the lipid content in this sample. As lipolysis causes release of FFA from the head groups of lipids (both neutral, phospho- and glycolipids), these head groups may consequently dissolve in the aqueous layer during the extraction procedure, leading to a decrease of the gravimetrically determined lipid content. A similar effect was observed by Ryckebosch et al. (2011) [23] in *Phaeodactylum tricornutum*. As approximately one third of the lipids in *T-Isochrysis lutea* are polar lipids (phospholipids and glycolipids) [33], in which the head group is more extensive, this can account for a significant part of the weight. The correlation between the lipid content and the FFA content at T_1 and T_2 were calculated to be 0.932 and 0.942, respectively. As a result, these two parameters are very strongly correlated.

6.3.3. Influence of selected heat treatments on quality attributes

In a second experiment of this study, two heat treatments were selected during which the enzymatic activity of the lipolytic enzymes was stopped: a longer time at a slightly milder temperature (8 min at 80°C, TRT1) and a short time at a very high temperature (3 min at 95°C, TRT2). These two treatments were compared with the control treatment (without heat treatment, CONTR) to investigate the influence of the heat treatments on the quality attributes of the biomass (oxidative stability, carotenoid composition and quantity, phenolic content and ω-3 fatty acid content).

The **total lipid content** (Figure 6.5A) and the **FFA content** (Figure 6.5B) showed the same observations as in the first part of this study. Extensive lipolysis was observed in the control biomass during wet storage at 4°C for 1 week, causing a significant increase of the FFA content (from 0.24 ± 0.01 % to 6.19 ± 0.19 % of the biomass) and a slightly significant decrease of the total lipid content (from 26.9 ± 0.5 % to 24.3 ± 0.7 % of the biomass). In the heat treated samples, only a slight increase of the FFA content was observed and no significant changes in the total lipid content, demonstrating again the usefulness of these treatments to prevent lipolytic reactions during wet storage at 4°C. Slight differences between the first and second part of this study are possibly due to small differences during cultivation, as two different batches were used.

The relative **fatty acid composition** (expressed as percentage of total FAME) and consequently also the relative amount of ω-3 fatty acids (Figure 6.5C) did not change by the heat treatments nor by the lipolytic reactions observed in the control biomass. The main fatty acids in the lipid fraction were myristic acid (C14:0, 16.8 ± 0.5%), palmitic acid (C16:0, 7.9 ± 0.1%), palmitoleic acid (C16:1, 10.9 ± 1.1%), oleic acid (C18:1, 8.4 ± 0.6%), linoleic acid (C18:2, 9.8 ± 0.2%), α-linolenic acid (C18:3n-3, 13.4 ± 0.1%), stearidonic acid (C18:4n-3, 13.7 ± 0.5%) and docosahexaenoic acid (C22:6n-3, 10.8 ± 0.4%). Lipolysis does not lead to changes in FAME composition, as the released FFA also end up in the lipid extract. Oxidation at the contrary could induce preferential degradation of the ω-3 fatty acids, which is not the case here. It could thus be concluded that the ω-3 content experienced no disadvantageous effect of the heat treatment.

The **peroxide value**, as a measure of primary lipid oxidation, was analyzed during wet storage. The increase of the peroxide value from T_0 to T_2 is shown in Figure 6.5D for each of the treatments. It was found that the peroxide value increased during wet storage, both in the control biomass and the heat treated biomass, as seen by the positive values. However, there was no significant difference between the control sample and the heat treated samples. The oxidative stability for short term (1 week) storage at 4°C is consequently not diminished by the heat treatment. More extensive studies are however necessary to reveal whether this is also the case during longer term storage. Oxidation of fatty acids can either be enzymatically catalyzed or initiated by free radicals (autoxidation) or singlet oxygen (photo-oxidation) [136]. The equal extent of oxidation in all samples points out that oxidation is either not enzyme catalyzed or the lipoxygenases are more heat stable than the lipases and not inactivated by the heat treatment applied. When the lipoxygenases would be inactivated during the heat treatment, a higher activity would be expected in the non-treated biomass, which would give a higher peroxide value. Oxidation is normally promoted by high temperatures and the presence of light and can be delayed by the presence of antioxidants [150, 158]. Possibly, the heat treatments used here are not intense or long enough to promote oxidation reactions and/or to degrade the present antioxidants.

T-Isochrysis biomass has been demonstrated to possess a good antioxidative activity, which has been attributed to the presence of **phenolic compounds** and several **carotenoids** [153]. These antioxidants are known to delay the onset of lipid oxidation by scavenging lipid-derived radicals, quenching singlet oxygen (in the case of beta-carotene) and/or chelating metals [61, 153, 237]. In addition, this antioxidant activity can be useful in protecting humans against several disorders (e.g. atherosclerosis, inflammatory diseases, diabetes) [141, 237]. It is thus of major importance to know whether these compounds remain stable during the proposed heat treatments. Therefore, the influence of the heat treatments on the amount of both antioxidant groups was investigated in this study. The results are presented in Figure 6.5E (phenolic compounds) and Figure 6.6 (carotenoids and chlorophylls).

The **phenolic content** (Figure 6.5E) in *T-Isochrysis lutea* was around 6.5 to 7 mg Gallic Acid Equivalents (GAE) per g biomass, which lies in between the values found by Goiris et al. (2012) [153] (2.67 ± 0.22 mg GAE/g biomass in *T-Isochrysis* and 4.57 ± 0.18 mg GAE/g biomass in *Isochrysis* sp.) and by

Maadane et al. (2015) [238](13.4 ± 0.16 mg GAE/g biomass). The phenolic content was only significantly different in the control sample at T2. This sudden difference compared to the other samples could not be explained and is possibly due to a random error. It can thus be concluded that phenolic compounds present in *T-Isochrysis lutea* biomass are not sensitive to the proposed heat treatments as they are present in both the control and the heat treated biomass in equal amounts.

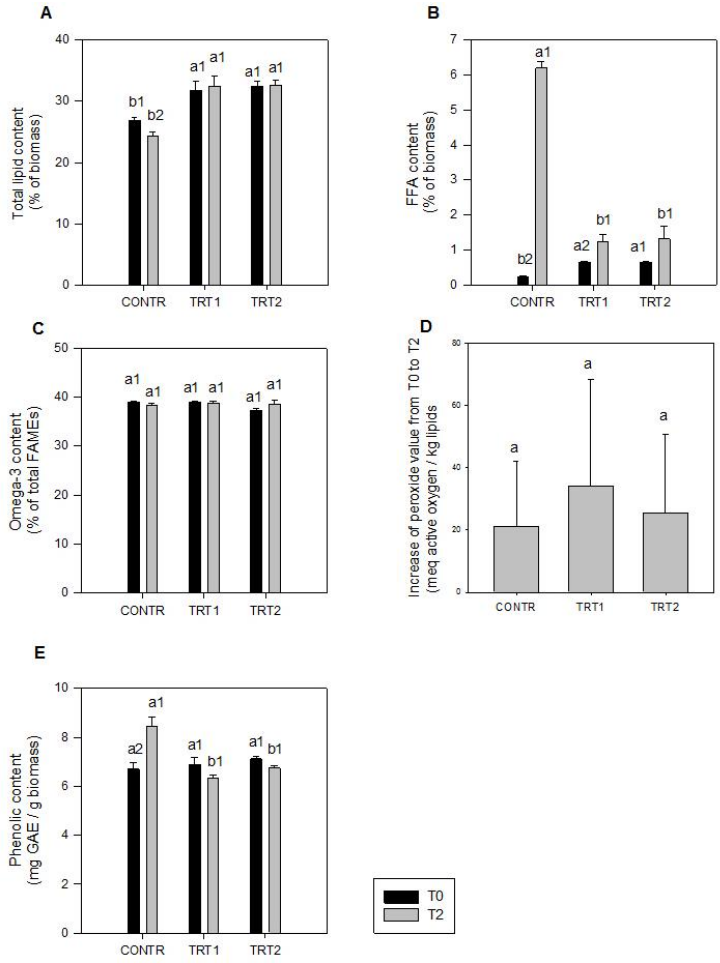


Figure 6.5: Comparison of the quality attributes in *T-Isochrysis lutea* samples that were not heat treated (CONTR), treated at 80°C for 8 min (TRT1) or treated at 95°C for 3 min (TRT2) after harvesting and consequently freeze-dried immediately (T_0) or stored at 4°C for 7 days before freeze-drying (T_2). Significant differences between treatments within a time point are indicated with different letters (a-b), while significant differences between time points within a treatment are indicated with different numbers (1-2).

Seven different **pigments** were identified in the *T-Isochrysis lutea* samples: beta-carotene, fucoxanthin, diadinoxanthin, diatoxanthin, chlorophyll a, pheophytin a and chlorophyll c2 (Figure 6.6). This approximately corresponds with the pigments observed in other studies on *Isochrysis galbana* [239] and *T-Isochrysis lutea* [240]. It was observed that the amounts of beta-carotene, fucoxanthin and chlorophyll c2 were only slightly decreasing during wet storage at 4°C. However, the amount of these compounds was significantly lower in the control biomass than in the heat treated biomass. Two possible explanations for this observation can be proposed. First, although the extraction procedure was optimized thoroughly before this experiment, it is possible that the extraction efficiency is improved by the heat treatment. Damage to the cells can be a consequence of this treatment, which could improve extraction of pigments. However, this is not likely, because *T-Isochrysis lutea* has no or only a very thin cell wall, which is not likely to limit the extraction of pigments from inside the cells. Secondly, it is possible that the heat treatment causes the release of carotenoids and chlorophylls from complexes with proteins [241], enabling their extraction only in the heat treated samples. Finally, the higher lipid content in the heat treated samples can possibly be caused by the loss of some organic compounds in the supernatant during centrifugation following the heat treatment, as was already mentioned as an explanation for the differences in lipid content (6.3.2.2).

It was observed that the diadinoxanthin content decreased during wet storage of the control biomass, while at the same time the diatoxanthin content increased. This was not the case in the heat treated biomass, in which the diadinoxanthin and diatoxanthin content remained constant during wet storage. This can be explained by the enzymatic conversion of diadinoxanthin to diatoxanthin in the control biomass [241], a reaction playing a role in the protection against damage due to reactive oxygen species (ROS) produced during stress conditions (e.g. light stress, heat, senescence). Diatoxanthin has a longer conjugated structure and is therefore a better quencher of ROS compared to diadinoxanthin [242] (chemical structures given in Figure 1.3). The enzyme causing the conversion of diadinoxanthin to diatoxanthin was probably inactivated by the heat treatment. Possibly, some enzymatic activity during the warming-up phase of the heat treatment (before inactivation of the enzyme) caused the slightly higher amount of diatoxanthin in the heat treated samples than in the control sample at T_0.

The same observation could be made for the conversion of chlorophyll a to pheophytin a. Breakdown of chlorophyll a to pheophytin a was observed during storage in the control biomass, while no changes in both compounds were seen in the heat treated samples. This implies that the conversion of chlorophyll a to pheophytin a in the control biomass is catalyzed by enzymes which are inactivated by the heat treatments. The conversion from chlorophyll a to pheophytin a, which includes the removal of the magnesium atom from the chlorophyll a molecule, is also accompanied by a color change from blue-green to olive green. Consequently, the heat treatment also improves color retention in the microalgal biomass. The chlorophyll a content at T_0 was not differing between the treatments, which means that the heat treatment is not strong enough to induce heat related degradation of chlorophyll a.

It can be concluded that some pigments (chlorophyll a, diadinoxanthin) remained stable during wet storage after the heat treatment, while this was not the case in biomass that was not heat treated. For none of the observed pigments there was a decrease as a result of the heat treatment. Overall, the heat treatment thus has no negative influence on the pigment content of the biomass. On the contrary, it stabilizes the amount of some pigments and helps to preserve the color.

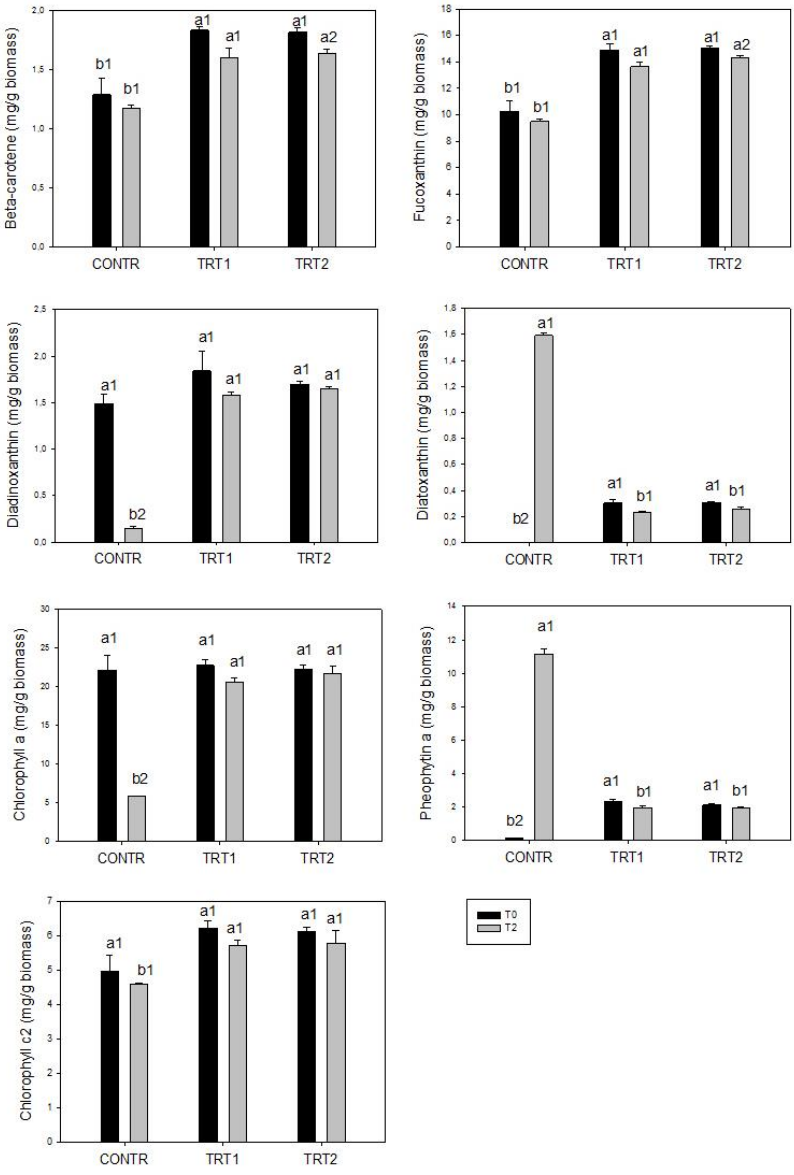


Figure 6.6: Comparison of the carotenoid content in *T-Isochrysis lutea* samples that were not heat treated (CONTR), treated at 80°C for 8 min (TRT1) or treated at 95°C for 3 min (TRT2) after harvesting and consequently freeze-dried immediately (T_0) or stored at 4°C for 7 days before freeze-drying (T_2). Significant differences between treatments within a time point are indicated with different letters (a-b), while significant differences between time points within a treatment are indicated with different numbers (1-2).

6.4. Conclusion

The results of this chapter show that the lipolytic stability during wet storage of *T-Isochrysis lutea* biomass can be strongly improved by avoiding the osmotic shock that occurs when the harvested paste is resuspended in demineralized water. However, the wet biomass still only remains stable for a few hours and lipolysis remains a problems during wet storage.

It was demonstrated in this chapter that a heat treatment of the wet biomass paste before storage can be a solution for these lipolytic stability problems during wet storage. In microalgae which are ruptured during or shortly after harvesting or which have to be disrupted to enable extraction of interesting compounds, lipolysis normally starts very rapidly. By applying a heat treatment before or immediately after the step in which rupture occurs, the formation of substantial and problematic amounts of FFA can be avoided. Also before wet extractions, a heat treatment could be useful to inhibit lipolytic reactions during the extraction procedure. The time-temperature combination of the treatment is however possibly species dependent and should be optimized separately for each microalgal species that is used. This study also showed that the heat treatment has no negative effects on oxidative stability, ω-3, pigment and phenolic content.

Chapter 7:

$FeCl_3$ flocculation for simultaneous harvesting and inhibition of lipolytic reactions in *T-Isochrysis lutea*

CHAPTER 7 $FECL_3$ FLOCCULATION FOR SIMULTANEOUS HARVESTING AND INHIBITION OF LIPOLYTIC REACTIONS IN *T-ISOCHRYSIS LUTEA*

7.1. Introduction

It has been demonstrated in Chapters 3 and 4 that the FFA content in *T-Isochrysis lutea* biomass increases very rapidly during post-harvest wet storage. One possible strategy to improve lipolytic stability during wet storage was investigated in Chapter 6. The harvested biomass was heat-treated for 8 minutes at 80°C, which allowed to inactivate the lipolytic enzymes. Consequently, the wet biomass remained stable for 7 days at 4°C. However, such a heat treatment also implies a high energy consumption, which can pose problems for low value applications (e.g. biodiesel production). Although the application of a heat treatment is very common in the food industry, it has not been implemented in the algae industry. Therefore, the aim of this chapter is to investigate whether the harvesting procedure can be adapted so that the wet biomass also remains stable for a longer time without a heat treatment.

Although the choice of a harvesting technique is strongly dependent on the microalgal species, the cell density and the type of application that is desired, centrifugation is most widely used [10, 18, 71]. It gives a high recovery rate, is applicable on all microalgal species and the resulting biomass is free of contaminants related to harvesting [10]. However, it is a very energy intensive technique to harvest and dewater microalgae from approximately 0.5 g/L to 25% dry weight in a single step [10, 29, 34]. Therefore, a primary or bulk harvesting technique can be used to pre-concentrate the biomass, e.g. flocculation, flotation [10, 34]. This reduces the volume to be centrifuged in the secondary harvesting step and thereby also reduces the energy consumption needed for harvesting [2, 10, 34]. In addition, flocculation does not rely on the use of high shear forces [10] and for the subsequent centrifugation, lower centrifugal forces can be applied because of the larger sizes of the cell aggregates compared to single cells. This two-step harvesting technique is thus milder and could possibly have an additional positive effect on the cell integrity and consequently on the lipolytic stability during wet storage.

The most commonly used flocculant, $FeCl_3$, could moreover reduce enzyme activity, as the inhibitory effect of several cations such as Fe^{2+} and Fe^{3+} on lipolytic enzymes has been described in literature [166, 243, 244]. The use of $FeCl_3$ flocculation as a pre-concentration technique could thus possibly combine the positive effect of reducing the harvesting costs and improving the lipolytic stability at the same time. However, besides these possible positive effects, the presence of cations could also have a negative impact on oxidative stability [25, 150], which also has to be taken into account.

In this chapter, harvesting using flocculation was compared to centrifugation in terms of FFA formation during post-harvest wet storage of *T-Isochrysis lutea* biomass. The flocculation type ($FeCl_3$ flocculation vs. pH induced flocculation), the flocculant dose and the pH were adapted in order to obtain a harvesting protocol that does not lead to a rapid FFA formation during subsequent wet storage. In addition, it was investigated whether this optimal harvesting protocol was also able to preserve oxidative stability and fatty acid and pigment composition.

7.2. Materials and methods

7.2.1. Cultivation

Cultivation of *T-Isochrysis lutea*, formerly named *Isochrysis sp.* (CCAP 927/14; Culture Collection of Algae and Protozoa, Oban, United Kingdom) was performed in pilot scale (30 L) photobioreactors as described in 3.2.1. For each experiment of this study (described in 7.2.2.1 - 7.2.2.5) , a separate 30 L batch was cultivated.

7.2.2. Harvesting and storage

7.2.2.1. *Determination of optimal flocculation conditions*

In a preliminary test, the optimal flocculation type and flocculant dose were determined. First, harvesting with ferric chloride ($FeCl_3$) was compared to pH induced flocculation. A dose-response curve for both flocculation methods was obtained by conducting small scale jar tests as described in Lama et al. (2016) [245]. In brief, 100 mL plastic beakers were filled with equal amounts (100 mL) of microalgal suspension and stirred by using magnetic stirrers. After addition of the flocculant, the solution was stirred for 10 minutes at 350 rpm, followed by 20 minutes at 250 rpm and 30 minutes of settling. Thereafter, the optical density (OD) of the supernatant was determined spectrophotometrically at 750 nm. The flocculation efficiency η was calculated as follows

$$\eta = \frac{OD_i - OD_f}{OD_i} \; x \; 100$$

where OD_i is the initial OD before flocculation and OD_f the final OD of the supernatant after flocculation. The concentration factor (CF) was calculated by dividing the volume of the culture that was flocculated by the volume of the precipitate that was obtained. This is a measure of the remaining water content in the precipitate.

The $FeCl_3$ dose was varied between 0 and 120 mg/L microalgal suspension by adding different volumes of a stock solution of $FeCl_3$. $6H_2O$ (10 g $FeCl_3$/L; Chem Lab, Zedelgem, Belgium). For pH induced flocculation, different amounts of a NaOH stock solution

(0.5 M) were added, with the highest concentration of NaOH (400 mg NaOH/L suspension) leading to pH 10.4.

7.2.2.2. *Scale-up of $FeCl_3$ flocculation*

After determining the optimal dose for efficient flocculation on small scale (100 mL beakers), the results were translated to bigger scale (1L bottles) conditions (adapting dose and stirring speed), for use in the following experiments.

It has to be taken into account that mixing is less efficient in larger volumes. Therefore, the stirring speed was increased from 350 rpm to 480 rpm in the first stirring phase (duration of 10 minutes), to improve contact between the cells and the flocculant in the suspension. The stirring speed in the second stirring phase, in which the flocs are enlarged, was lowered from 250 rpm to 200 rpm (duration of 20 minutes). A settling time of 30 minutes was also used.

A dose of 100 mg $FeCl_3$/L suspension, which was optimal on a small scale, yielded a flocculation efficiency below 90% (around 87%). Therefore, the dose was increased to 120 mg $FeCl_3$/L suspension, which gave a very good flocculation efficiency of 93%. The concentration factor (CF) was around 22, which is rather low compared to the values around 50 obtained by Lama et al. (2016)[245] on the same species but on a smaller scale. These optimized conditions were then used for the following experiments.

7.2.2.3. *Influence of centrifugation and $FeCl_3$ flocculation on lipolytic stability during post-harvest wet storage*

In a first set-up, the lipolytic stability during wet storage of *T-Isochrysis lutea* biomass both after centrifugation and after $FeCl_3$ flocculation was compared. **Centrifugation (C)** was conducted using a lamella centrifuge (4000 *g*). The biomass paste was removed from the centrifuge and homogenized by resuspending in a small amount of salt cultivation medium. **$FeCl_3$ flocculation (F)** was performed as described in 7.2.2.2. The flocculation efficiency was calculated as described in 7.2.2.1. After discarding the supernatant, the remaining precipitate of all bottles was pooled. Both during centrifugation and flocculation, the pH was measured but not adjusted. The addition of 120 mg $FeCl_3$/ L suspension decreased the pH to 3.5, while during centrifugation the pH was around 8.

After homogenizing both the centrifuged and flocculated paste by stirring, the biomass was divided in 50 mL falcon tubes, which were centrifuged at 420 *g* (flocculated paste) or 750 *g* (centrifuged paste) for further concentration. Afterwards, all supernatants were discarded, resulting in a wet biomass paste (19% dry weight in centrifuged biomass, 10% dry weight in flocculated biomass). One part of the biomass was frozen at -80°C and

freeze-dried immediately (T_0), whereas the other parts were stored as a wet biomass paste at 4°C for 1 day (T_1), 3 days (T_2) or 7 days (T_3) before freezing and freeze-drying. Determination of the total lipid content and FFA content was conducted in triplicate on all dried biomass samples.

7.2.2.4. *Influence of pH and presence of ferric ions on lipolytic stability*

To explain the difference in lipolytic stability between centrifuged and flocculated biomass found in the first set-up (7.2.2.3), different harvesting conditions were examined in a following set-up. Five different harvesting conditions were examined: flocculation at pH 4 (F-pH4), flocculation at pH 8 (F-pH8), centrifugation without pH adaptation (C), centrifugation at pH 4 by addition of HCl (C-pH4-HCl) and centrifugation at pH 4 by addition of $FeCl_3$ (C-pH4-$FeCl_3$).

Flocculation was carried out as described in 7.2.2.2, except for the addition of NaOH (0.5 M) until pH 4 (F-pH4) or pH 8 (F-pH8) during the first stirring step. Centrifugation (C) was carried out as described in 7.2.2.3. C-pH4-HCl and C-pH4-$FeCl_3$ implied the addition of HCl 0.5 M (C-pH4-HCl) or $FeCl_3$ (120 mg/ L suspension) and NaOH 0.5 M (C-pH4-$FeCl_3$) to obtain pH 4 before the suspension was centrifuged in the lamella centrifuge. To avoid $FeCl_3$ flocculation in C-pH4-$FeCl_3$, the suspension was stirred only very gently to mix without inducing floc formation. Centrifugation with the addition of $FeCl_3$ and NaOH 0.5 M until pH 8 was also tested, but this caused flocculation, even when stirring only very gently. This condition was consequently not investigated further.

The biomass paste obtained after each harvesting protocol was divided into a part that was frozen and freeze-dried immediately (T_0) and a part that was first stored at 4°C for 7 days (T_1) before freezing and freeze-drying. Determination of the FFA content was conducted in triplicate on all dried biomass samples.

7.2.2.5. *Influence of $FeCl_3$ addition on lipid quality*

To investigate the influence of the harvesting technique on the lipid quality, the best harvesting technique for lipolytic stability (F-pH8) was compared to the standard centrifugation protocol (C). The obtained biomass paste after each harvesting protocol was divided into a part that was frozen and freeze-dried immediately (T_0) and a part that was first stored at 4°C for 2 days (T_1) or 7 days (T_2) before freezing and freeze-drying. Determination of the total lipid content and FFA content was conducted in duplicate on the dried biomass. In addition, also the oxidative stability, the fatty acid composition and the pigment composition were followed during wet storage (analyses in duplicate). To summarize all tested harvesting techniques and conditions in 7.2.2.3 - 7.2.2.5, an overview is given in Table 7.1.

Table 7.1: Overview of the tested harvesting conditions and post-harvest wet storage times on *T-Isochrysis lutea* biomass

Condition	Type of harvesting	pH	Substances added	Time intervals of wet storage
Influence of centrifugation and $FeCl_3$ flocculation on lipolytic stability during post-harvest wet storage (7.2.2.3)				
C	Centrifugation	8	/	0-1-3-7 days
F	Flocculation	3.5	$FeCl_3$	0-1-3-7 days
Influence of pH and presence of ferric ions on lipolytic stability (7.2.2.4)				
F-pH4	Flocculation	4	$FeCl_3$ + NaOH	0-7 days
F-pH8	Flocculation	8	$FeCl_3$ + NaOH	0-7 days
C	Centrifugation	8	/	0-7 days
C-pH4-HCl	Centrifugation	4	HCl	0-7 days
C-pH4-$FeCl_3$	Centrifugation	4	$FeCl_3$ + NaOH	0-7 days
Influence of $FeCl_3$ addition on oil quality (7.2.2.5)				
F-pH8	Flocculation	8	$FeCl_3$ + NaOH	0-2-7 days
C	Centrifugation	8	/	0-2-7 days

7.2.3. Analyses

7.2.3.1. Determination of dry weight and ash free dry weight

Next to the dry weight (DW), it was also necessary to determine the ash free dry weight (AFDW). During flocculation, metal ions of the medium and of the flocculant (in the case of $FeCl_3$ flocculation) can be transferred to the precipitate and consequently end up in the freeze-dried biomass. This can lead to misinterpretation of for example the lipid content [246]. The AFDW only counts the organic part of the freeze-dried powder and thus corrects for the presence of metal salts.

Both DW and AFDW were determined gravimetrically. A few milligrams of wet biomass paste was weighed accurately in a pre-weighed aluminum dish. The dish with biomass paste was weighed again after one hour in an oven at 105°C, which gave the DW, and after two more hours at 450°C, which gave the AFDW.

7.2.3.2. Total lipid content

The total lipid content (on dry basis) of the biomass samples was determined by chloroform/methanol (1:1) extraction as described in 3.2.3.1. The weighed biomass was corrected for AFDW to exclude contaminants (e.g. metal ions) present in the freeze-dried biomass.

7.2.3.3. FFA content

The FFA content in the total lipid fraction was determined as described in 3.2.3.2. Before extraction, 5 mg of lauric acid (C12:0) (Nu-Check Prep, Elysian, USA) was added as an internal standard.

7.2.3.4. Fatty acid profile and ω-3 content

The fatty acid profile was determined by methylesterification of the lipids in the total lipid fraction (7.2.3.2) as described in 5.2.6.

7.2.3.5. Oxidative stability

The oxidative stability of the microalgal lipids was analyzed by determining the peroxide value (PV) as a measure of the amount of primary lipid oxidation products (hydroperoxides) as described in 6.2.5.4.

7.2.3.6. Pigment content and composition

Carotenoids and chlorophylls in the microalgal biomass samples were extracted with acetone/methanol (7:3) and analyzed by HPLC as described in 6.2.5.5. The biomass weighed for the extraction of carotenoids and chlorophylls was corrected for the AFDW.

7.2.3.7. Determination of the Fe content

Sample preparation

The freeze-dried biomass samples from the experiment described in 7.2.2.4 (samples acquired at T_0) and the oil extracted by CM extraction from the samples of the experiment described in 7.2.2.5 (samples acquired at T_0) were both analyzed for their Fe content. Their exact mass was determined by accurately weighing a small amount of biomass (approximately 50 mg) or oil (approximately 10 mg) in a pyrex tube. The extraction solvent was removed by flushing with nitrogen.

Both freeze-dried biomass and oil samples were acidified and dissolved in 16% HNO_3 (J.T. Baker, 69-70%) containing 4% H_2O_2 (Merck, 30%) and treated using microwave-assisted digestion for 35 min (Ethos UP, Milestone) in order to partition the analytes from the sample matrix in solution.

ICP-OES analysis

The ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometry) system is housed in a thermostatically regulated room (22 ◦C) and consisted of a Perkin Elmer type Optima 8300 system equipped with the Syngistix software (version 2.0). The

analyte Fe was measured using emissions at 238 nm. Fe calibration standards were prepared by serially diluting the Fe standard solution in 1.6% HNO_3, resulting in concentrations of 5, 10, 50, 100, 500, 1000, 5000, 10000 ng/ml. Spike tests of 10-50 ng/ml Fe were carried out on the lower concentrations (12-103 ng/ml). The recoveries ranged between 92 and 102% (98.7 ± 2.7%).

7.2.4. Statistical analysis

To determine significant differences between the different tested harvesting conditions and time points, the statistical software JMP Pro 12 was used. Analysis of Variance (ANOVA) was performed to detect significant differences between the different harvesting conditions or between the different time points. As the interaction between the harvesting condition and the storage time was found to be significant in this model, the effect of both factors was determined separately. The effect of the harvesting condition within one time point and the effect of storage time within one harvesting condition was determined by performing a post-hoc Tukey test with significance level 0.05 or a student's T-test with significance level 0.05 (in the case of comparing only two harvesting conditions or time points).

7.3. Results and discussion

7.3.1. Determination of optimal flocculation conditions

Before performing the storage experiments after flocculation and centrifugation, the flocculation procedure was optimized for *T-Isochrysis lutea*. Lama et al. (2016)[245] reported that for this particular species chitosan flocculation was inefficient. Therefore, only flocculation with $FeCl_3$ and pH induced flocculation were investigated. Figure 7.1 shows the dose-response curves of the small scale experiments.

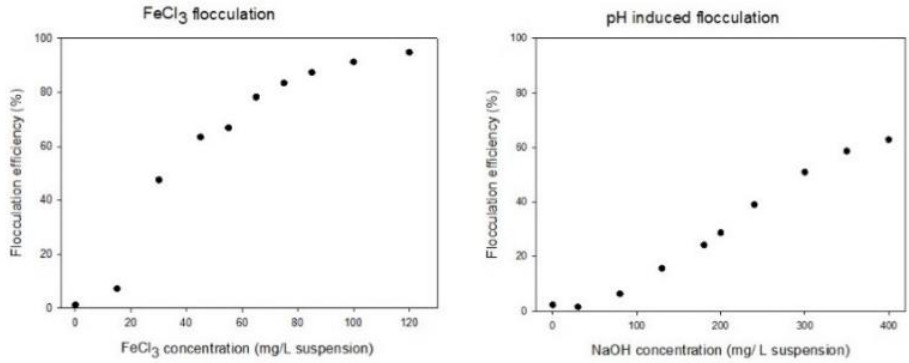


Figure 7.1: Effect of $FeCl_3$ and NaOH dose on the flocculation efficiency (%) of *T-Isochrysis lutea*.

Flocculation with $FeCl_3$ was very efficient from a dose of 100 mg/L suspension upwards, with a flocculation efficiency of more than 90%. On the contrary, pH induced flocculation reached a maximum efficiency of around 60%. These results are in agreement with the results obtained by Lama et al. (2016)[245] for *T-Isochrysis lutea*. Flocculation with $FeCl_3$ at a dose of 100 mg/L suspension was thus considered as the best condition for further use. For further use of this flocculation method in the following experiments, upscaling was conducted. The volume, dose and stirring speed were adapted as described in 7.2.2.2.

7.3.2. Influence of centrifugation and $FeCl_3$ flocculation on lipolytic stability during post-harvest wet storage

After cultivation, a part of the *T-Isochrysis lutea* biomass was harvested according to the flocculation protocol optimized in 7.2.2.2. The other part of the batch was centrifuged by a lamella centrifuge. The lipid content and FFA content were followed during post-harvest wet storage (Figure 7.2).

Figure 7.2 shows that, despite the correction for AFDW (7.2.3.1), the **lipid content** in the centrifuged biomass is significantly higher than in the flocculated biomass. This difference remains constant during wet storage and was already observed by Vandamme et al. (2018)[246] for the marine diatom *Phaeodactylum tricornutum.* It was hypothesized that this difference is caused by a difference in the amount of extracellular organic compounds that ends up in the biomass after harvesting. While the use of AFDW corrects for inorganic compounds (e.g. salts, minerals) present in the biomass, it does not correct for organic matter that is transferred to the precipitate. This leads to a seemingly lower lipid content in the flocculated biomass than in the centrifuged biomass.

A fraction of organic compounds that may possibly explain the difference in lipid content is the algal extracellular organic matter (AOM) which is produced by microalgae. AOM is composed mainly of polysaccharides, with a minor fraction of nucleic acids, lipids and small molecules [247]. This AOM is removed by centrifugation and subsequent resuspension of the biomass in fresh medium [247, 248]. On the contrary, it can interact with the flocculants during flocculation [248]. Consequently, it ends up in the flocs and thus also in the dried biomass. The AOM, but possibly also other organic compounds, can explain the observed difference in lipid content. Further research is still necessary to confirm this hypothesis.

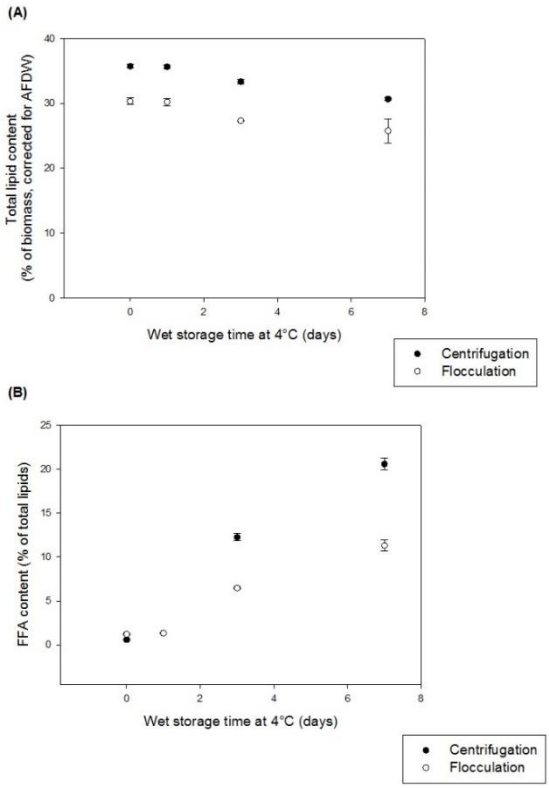


Figure 7.2: Influence of wet storage time at 4°C and harvesting technique on (A) total lipid content of the *T-Isochrysis lutea* biomass, corrected for AFDW and (B) FFA content (expressed as a percentage of the total lipids).

The lipid content also decreased during storage, in centrifuged biomass as well as in flocculated biomass. This may be explained by the lipolytic reactions occurring during storage, which cause the release of fatty acids (as seen by an increase of the FFA content in Figure 7.2B) from different lipid head groups. The more polar head groups are consequently lost during the extraction protocol, as they are dissolved in the aqueous supernatant that is discarded. This leads to a lower amount of gravimetrically determined lipids. The head groups of polar lipids (phospholipids and glycolipids), which comprise approximately one third of the lipids in *T-Isochrysis lutea* [33], can account for a significant part of the weight. This effect was also observed by Ryckebosch et al. (2011)[23] in *Phaeodactylum tricornutum* and in Chapter 6 of this PhD in *T-Isochrysis lutea.*

The **FFA content** was initially relatively low (less than 1.5 % of total lipids) in both the centrifuged and flocculated biomass and remained low after one day of wet storage. However, after 3 days of wet storage at 4°C, the FFA content had already increased to 6.5% of total lipids in flocculated biomass and 12.3 % of total lipids in centrifuged

biomass. The increase of the FFA content continued during further storage to 11.3% FFA in the flocculated biomass and 20.6% in the centrifuged biomass after 7 days.

Several hypotheses can be proposed to explain the significant difference in FFA content between the two harvesting techniques. First, flocculation is a much milder technique, which does not involve high shear forces. Possibly, the cells are damaged to a lesser extent after flocculation than after centrifugation, leading to more intact cells and a better lipolytic stability after flocculation. However, if a real cell disruption had occurred during harvesting, the effect would be expected to be visible immediately after harvesting by a sudden increase of the FFA content, like it was the case in Chapter 4 after cell disruption of *Nannochloropsis* biomass by HPH and in Chapter 3 and 4 after an osmotic shock by resuspending *T-Isochrysis* biomass in demineralized water. Possibly, only a very small degree of damage to the cell was caused by centrifugation, leading to an effect of this damage during a later stage of wet storage.

A second possible explanation is the difference in pH between the flocculated and centrifuged wet biomass paste. The pH of the biomass in the photobioreactor just before centrifugation was around 8, while the pH during $FeCl_3$ flocculation was around 3.5. Possibly, the very low pH during flocculation is not within the activity range of the lipolytic enzymes, as was suggested by a reduced enzyme activity at this pH observed by Bilinski et al. (1968)[182] in *Monochrysis lutheri*, Cho et al. (1985) [178] in *Dunaliella salina*, Demir et al. (2010) [166] in *Spirulina platensis*, Savvidou et al. (2016) [169] in *Nannochloropsis oceanica* and Yong et al. (2016) [168] in *Botryococcus sudeticus*. However, the pH dependence of the lipolytic enzymes is very species dependent and may not be generalized to *T-Isochrysis lutea* in this case .

A last possible explanation is the inhibition of lipolytic enzymes by the added ferric ions, which have shown to inhibit the lipase activity in among others *Penicillium notatum* [184] and *Aspergillus terreus* [185]. To verify if one of these last two hypotheses is feasible, an additional experiment was conducted (7.3.3).

7.3.3. Influence of pH and presence of ferric ions on lipolytic stability

Two possible inhibitory effects of flocculation on lipolytic activity were proposed in 7.3.2: inhibition by an unfavorable pH or inhibition by the presence of ferric ions. To examine the effect of pH, flocculation and centrifugation both at pH 4 and pH 8 were conducted (F-pH4, F-pH8, C, C-pH4-HCl). Additionally, also the effect of the addition of $FeCl_3$ before centrifugation (C-pH4-$FeCl_3$) was tested. This lowered the pH like in C-pH4-HCl, but also introduced ferric ions, which could possibly also have an effect on lipolytic activity. Figure 7.3 presents the FFA content immediately after harvesting (T_0) and after 7 days of wet storage at 4°C (T_1).

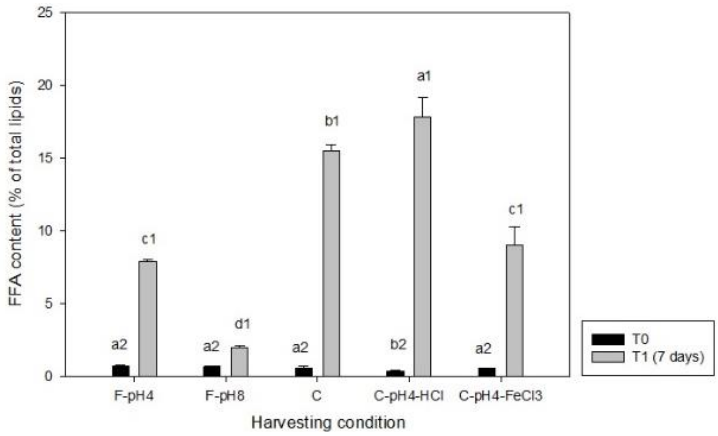


Figure 7.3: Influence of harvesting conditions (pH, presence of ferric ions) on the FFA content (expressed as percentage of the total lipids) of *T-Isochrysis lutea* biomass immediately after harvesting and after storage for 7 days at 4°C. Significant differences between harvesting conditions within a time point are indicated with different letters (a-c), while significant differences between time points within a harvesting condition are indicated with different numbers (1-2).

Figure 7.3 shows that the FFA content at T_0 was almost identical after all harvesting conditions. This indicates that the microalgal cells remained intact during harvesting and that no lipolytic reactions were triggered by damage to the cells. At T_1, after 7 days of wet storage at 4°C, significant differences between the different conditions were observed (values vary between 2% and 18% of total lipids). When comparing F-pH4 and C, which correspond with the flocculation and centrifugation conditions used in the previous experiment (7.3.2), the same difference was observed, being a significantly better lipolytic stability in the flocculated than in the centrifuged biomass. Flocculation at pH 8 (F-pH8) instead of pH 4 even further improved the lipolytic stability during wet storage. After one week of wet storage at 4°C, only 2.0 % FFA were found.

When comparing C and C-pH4-HCl, only a small though significant difference in FFA content could be observed by the change in pH (respectively 15.5 and 17.8 % FFA of total lipids), while the effect of pH during flocculation was clearer. Furthermore, the addition of $FeCl_3$ (C-pH4-$FeCl_3$) significantly decreased the lipolytic activity compared to the centrifuged biomass without $FeCl_3$ (C and C-pH4-HCl). The FFA content after one week of wet storage was not significantly different from the flocculated biomass with the same $FeCl_3$ concentration and the same pH (F-pH4). From these results, it is suggested that the presence of $FeCl_3$ and not the low pH is the explanation for the better lipolytic stability after flocculation than after centrifugation. The influence of metal ions on lipase enzymes, both inhibitory and activating effects, has been described in literature before. Bivalent cations such as Mg^{2+} and Ca^{2+} mostly activate lipases from seeds [243] or bacteria [244, 249, 250], whereas Fe^{2+} and Fe^{3+} have often been found to exhibit an inhibitory action on lipases from *Penicillium notatum* [184], *Aspergillus terreus* [185], *Pseudomonas*

fluorescens [250] and different seed lipases [243]. However, the exact mechanism of this inhibition is not yet known. In addition, the effect strongly depends on the lipase characteristics specific for a certain species [244], which are to the best of our knowledge not yet known for *T-Isochrysis lutea*. Although literature suggests that the Fe ions have a direct inhibitory effect on the lipolytic enzymes, it should be investigated in future experiments whether the Fe concentration and pH do not have a more indirect effect on integrity and thereby also on lipolytic stability.

The difference between F-pH4 and F-pH8 shows that the inhibition effect of ferric ions on lipolytic activity is strongly dependent on the pH. This can possibly be explained by a different speciation of Fe(III) at pH 4 compared to pH 8 [251, 252]. At pH 8, Fe(III) occurs mainly as $Fe(OH)_3$, while at pH 4, mainly $Fe(OH)_2^+$ and $Fe(OH)^{2+}$ occur [253]. A change in speciation can induce a difference in availability for lipase inhibition, as well as a different flocculation mechanism.

First, $Fe(OH)_2^+$ and $Fe(OH)^{2+}$ present at pH 4 can possibly bind to negatively charged cell wall compounds and thus be unavailable for inhibition of the lipases enzymes once the cell integrity is lost and lipase enzymes are released from the cell. The neutral $Fe(OH)_3$ cannot undergo ionic interactions with the cell wall, which makes that it is more easily used for lipase inhibition.

Second, it has been demonstrated by Dong et al. (2015) [252] that a different flocculation mechanism occurs at alkaline and acidic pH. At a low pH, mainly charge neutralization occurs, which leads to bigger, but looser and weaker flocs. On the other hand, at alkaline pH, mostly sweeping flocculation occurs, which causes more rapid formation of stronger, but smaller flocs. As the interactions between the cells and Fe(III) species are different between these two mechanisms, this could also have an impact on the amount of Fe ions in the precipitate after flocculation, as well as on the availability of Fe(III) for lipase inhibition.

To evaluate the amount of Fe present in the biomass, ICP-OES analyses were performed on the samples at T_0 (Figure 7.4). A significant difference between the flocculated samples (F-pH4, F-pH8) on the one hand and the centrifuged samples (C,C-pH4-HCl,C-pH4-$FeCl_3$) on the other hand was observed. In the flocculated samples, the Fe ions added during flocculation are caught in the precipitate, which causes a higher Fe content in the freeze-dried biomass. By using centrifugation without the addition of $FeCl_3$ (C, C-pH4-HCl), only some Fe ions from the cultivation medium can possibly end up in the precipitate, which leads to a very low Fe content in the biomass. When $FeCl_3$ was added before centrifugation (C-pH4-$FeCl_3$), the Fe content in the biomass was significantly higher than in the other centrifuged samples, but significantly lower than in the flocculated samples. This could be explained by some interactions between the Fe ions

and the cells or by uptake of Fe ions into the cells. Another explanation could be that a minor extent of flocculation occurred, which could not be seen by the naked eye. This could also cause some Fe ions to be caught in the precipitate.

When comparing F-pH4 and C-pH4-$FeCl_3$, harvested at the same pH and with the same $FeCl_3$ concentration, the FFA content at T_1 was not significantly different, while the Fe content was strongly different. Possibly, only a small amount of Fe is sufficient to induce inhibition of lipases to a certain extent. A higher Fe concentration in F-pH4 compared to C-pH4-$FeCl_3$ could thus not improve the lipolytic stability, as the maximum lipase inhibition was already reached with the Fe content in C-pH4-$FeCl_3$. However, increasing the pH from pH 4 (F-pH4) to pH 8 (F-pH8) was found to improve lipolytic stability during wet storage. Although the Fe content was significantly higher in F-pH8 than F-pH4, this was probably not the only reason for the much lower FFA content in F-pH8 than in F-pH4. It was thus hypothesized that the main reason for the difference in FFA content between these conditions is the different speciation of iron, causing a difference in availability or efficiency for lipase inhibition.

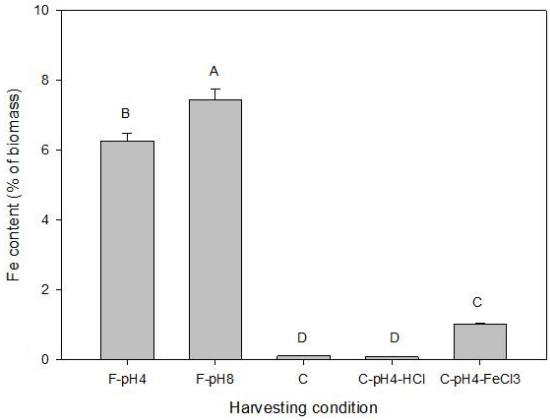


Figure 7.4: Influence of harvesting condition on the Fe content in the *T-Isochrysis lutea* biomass at T_0 (expressed as % of the biomass). Significant differences between harvesting conditions are indicated with different letters (A-D).

7.3.4. Influence of $FeCl_3$ addition on lipid quality

During this additional set-up, F-pH8 (which gave the best lipolytic stability) and C (which is mostly used as a standard harvesting technique) were repeated to investigate the possible negative impact of the addition of $FeCl_3$ on lipid quality. Figure 7.5 shows the total lipid content, FFA content, ω-3 content and peroxide value, while Figure 7.6 shows the carotenoid and chlorophyll content during post-harvest wet storage (after 0, 2 and 7 days at 4°C) both after F-pH8 and C as a harvesting technique.

The **lipid content** (Figure 7.5A) was significantly lower in the flocculated biomass than in the centrifuged biomass. This confirms the observation in the first experiment (Figure 7.2A), where the difference was hypothesized to be caused by a different amount of organic matter in flocculated compared to centrifuged biomass. The decrease of the total lipid content, which was observed in Figure 7.2, was not seen in the flocculated biomass (F-pH8), where also the increase of the FFA content was small. In the centrifuged biomass, a small but significant decrease was observed, which can be caused by lipolytic reactions in this biomass. Also the differences in **FFA content** (Figure 7.5B) between F-pH8 and C, as well as during wet storage, confirmed the observations made before. The lipolytic stability was again improved by using $FeCl_3$ flocculation.

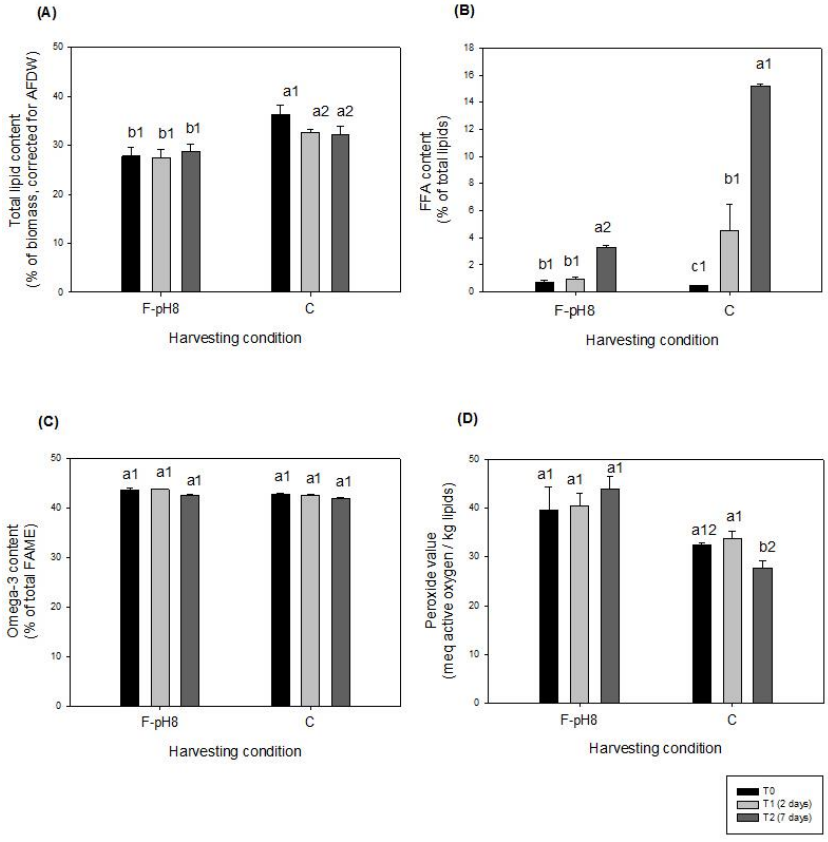


Figure 7.5: Influence of harvesting condition and post-harvest wet storage time at 4°C on (A) Total lipid content, (B) FFA content, (C) ω-3 content and (D) Peroxide value of *T-Isochrysis lutea* biomass. Significant differences between harvesting conditions within a time point are indicated with different letters (a-b), while significant differences between time points within a harvesting condition are indicated with different numbers (1-2).

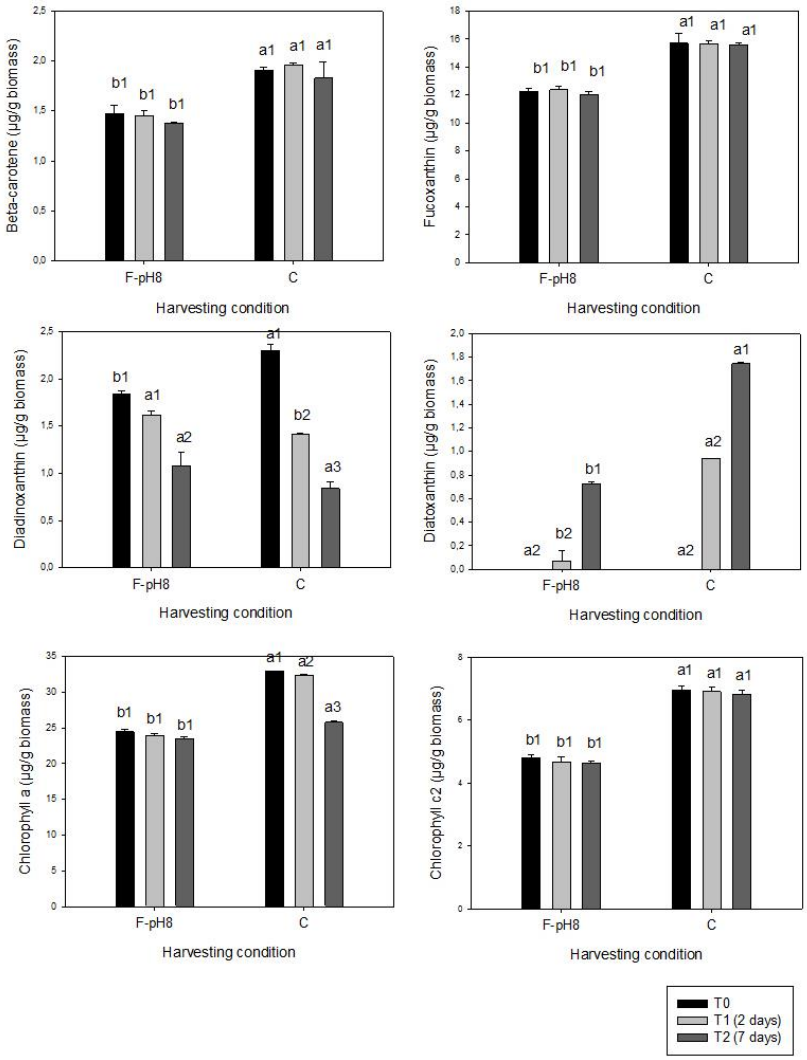


Figure 7.6: Influence of harvesting condition and post-harvest wet storage time at 4°C on the carotenoid and chlorophyll content in the *T-Isochrysis lutea* biomass (corrected for AFDW). Significant differences between harvesting conditions within a time point are indicated with different letters (a-b), while significant differences between time points within a harvesting condition are indicated with different numbers (1-3).

Lipolysis occurring during wet storage (as seen in Figure 7.5B) was, as expected, not reflected in any change of the relative **fatty acid composition**, as the fatty acids, released from the backbone, also end up in the lipid extract. Main fatty acids found were myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3n-3), stearidonic acid (C18:4n-3) and docosahexaenoic acid (DHA,C22:6n-3), which corresponds with the fatty acid

composition determined in Chapter 6. The amount of **ω-3 fatty acids** (mainly α-linolenic acid, stearidonic acid and DHA) consequently remained also constant during wet storage (Figure 7.5C). The **peroxide value** (Figure 7.5D) experienced no clear decrease or increase during storage. This implies that no oxidation occurred during one week of wet storage at 4°C. To determine differences in oxidation rate on longer term or during storage of dried biomass, more experiments should be performed.

Figure 7.6 shows the pigment composition of all samples and the evolution of their concentration during wet storage. It can be observed that all samples contained beta-carotene, fucoxanthin, diadinoxanthin, chlorophyll a and chlorophyll c2 at T_0. For all pigments, the concentration was significantly higher in the centrifuged than in the flocculated samples. As the pigment concentrations are expressed as a mass percentage of the biomass, this difference can possibly again be attributed to a difference in the amount of organic matter. The higher amount of organic matter in the flocculated biomass can cause a seemingly lower concentration of pigments. The concentration of beta-carotene, fucoxanthin and chlorophyll c2 did not significantly change during storage. Chlorophyll a was degraded during wet storage in the centrifuged biomass, like it was also the case in Chapter 6 in the centrifuged, non-heat treated biomass (Figure 6.6). Apparently, harvesting by $FeCl_3$ flocculation caused this degradation reaction not to occur. Also the conversion of diadinoxanthin to diatoxanthin, which was already observed in Chapter 6, occurred to a lesser extent in the flocculated biomass than in the centrifuged biomass. As both the degradation of chlorophyll a and the conversion of diadinoxanthin to diatoxanthin are due to enzymatic reactions [241], the lower reaction rate in the flocculated biomass is possibly caused by inhibition of the enzymes by ferric ions, like it was the case for the lipases.

In addition to the benefits of $FeCl_3$ flocculation as a harvesting technique, a possible drawback is the contamination of biomass with iron, which may limit some high value applications. However, it was demonstrated by ICP-OES analyses that most of the iron is not extracted in the lipid fraction, as the Fe content in the CM extract of F-pH8 and C are both lower than 0.01% of the oil. The oil from F-pH8 contained a slightly higher Fe content (0.0094% or 94 mg/kg oil) than the oil from C (0.0074% or 74 mg/kg oil). Both values are far above the maximum value in virgin, vegetable oils, being 5 mg/kg [215]. However, as both samples contain these high amounts of iron, the iron is probably not originating from the flocculant, but rather from the cultivation medium. The high Fe content is thus possibly inherently present due to the cultivation and harvesting conditions and techniques used at our lab.

7.4. Conclusion

The results of this chapter imply that $FeCl_3$ flocculation is a promising pre-concentration technique for harvesting of *T-Isochrysis lutea* biomass. First, because of the high flocculation efficiency (93% or more) and a concentration factor of 22, this technique strongly decreases the volume that has to be centrifuged afterwards. Consequently, the costs associated with harvesting could be strongly reduced. Secondly, the use of $FeCl_3$ as a flocculant and the associated addition of ferric ions improves post-harvest lipolytic stability, especially when the pH is adapted to pH 8. This allows longer term post-harvest storage of the wet biomass. After one week of wet storage at 4°C, only 2-4% FFA were found in the total lipid fraction, whereas this was around 15% of total lipids in centrifuged biomass without addition of $FeCl_3$. The harvesting method using $FeCl_3$ flocculation did not imply any quality changes in the extracted lipids, as the fatty acid composition, pigment composition and oxidative stability did not change. On the contrary, the conversion reactions of diadinoxanthin to diatoxanthin and the degradation of chlorophyll a were even diminished during wet storage following flocculation.

Chapter 8:

General conclusions and perspectives

CHAPTER 8 GENERAL CONCLUSIONS AND PERSPECTIVES

One of main challenges during downstream processing of microalgal biomass is the high moisture content. After harvesting, a wet biomass with a dry weight up to 25% is obtained [34]. The high moisture content during this stage makes that several degradation reactions can occur. One of the greatest challenges could be the occurrence of enzymatic lipolytic reactions, which cause the release of free fatty acids (FFA) from the lipid backbone. For high value applications, these FFA have a negative impact on the flavor and promote lipid oxidation, which in turn leads to a decrease of the nutritional value and additional off-flavors [11]. In addition, FFA are also disadvantageous during the production of biodiesel, where they react with the alkali catalyst to produce soaps. This leads to a decreased biodiesel yield and a more complicated downstream processing [26, 27, 194].

Although high FFA contents in microalgae have often been encountered [19-21], this problem is often underestimated in industry and not well understood. There are even a lot of studies investigating methods to avoid the expensive drying step (e.g. wet extraction, direct transesterification) [15, 16], which even prolongs the wet storage period. A few studies indicated the occurrence of lipolytic reactions during wet storage [19, 23, 24], but the factors determining the reaction rate, the triggers activating the reaction and the solutions to improve the lipolytic stability were not studied before.

Therefore, the aim of this PhD was on the one hand to determine the factors influencing the lipolytic stability during wet storage and on the other hand to investigate different strategies to improve the stability. In the next paragraphs, the conclusions about these two aspects are summarized respectively in sections 8.1 and 8.2. At the end of each section, some future research possibilities are suggested.

8.1. Factors influencing lipolytic stability during wet storage

8.1.1. Time and temperature dependence

The time and temperature dependence of lipolysis during wet storage was investigated both for *T-Isochrysis lutea* (Chapter 3) and *Nannochloropsis oculata* (Chapter 4), two species containing substantial amounts of lipids (with high ω-3 LC-PUFA concentrations) but strongly differing in cell structure and composition. The three temperatures at which the wet biomass was stored, were chosen at relevant temperatures for practical applications: -20°C (temperature in normal freezers), 4°C (temperature in refrigerators) and 20°C (room temperature). For both species, it was found that no lipolytic reactions occurred at -20°C, while an increase of the FFA content was seen during wet storage at 4°C and 20°C. The reactions were observed to be temperature dependent, as the increase of the FFA content was faster at a higher temperature.

8.1.2. Species dependence

Although lipolytic reactions were observed both in *T-Isochrysis* and *Nannochloropsis*, there was a huge difference between these species in the evolution of the FFA content during wet storage. Figure 8.1 compares the FFA content during wet storage of *T-Isochrysis* (Chapter 3) and *Nannochloropsis* (Chapter 4) at 4°C (A) and 20°C (B). It can clearly be observed that the lipolytic stability of these two species is completely different. In *T-Isochrysis*, the FFA content at the beginning of wet storage was already very high (7.5 ± 0.3 % of total lipids) and increased very rapidly during storage at 4°C and 20°C (leading to an FFA content of around 28% of total lipids after 7 and 14 days respectively). In *Nannochloropsis*, the FFA content started at a much lower value, 1.34 ± 0.06 % of total lipids and remained at this value for several days (7 days at 4°C and 3 days at 20°C). The highest observed value after 21 days at 20°C was still only 9.0 ± 0.9 % of total lipids. It should be denoted however that the integrity of *T-Isochrysis* cells was possibly lost during harvesting due to an osmotic shock. The difference in lipolytic stability between *T-Isochrysis* and *Nannochloropsis* was thus hypothesized to be caused by a difference in cell (wall) structure and strength, an aspect that is further elaborated on in section 8.1.3.

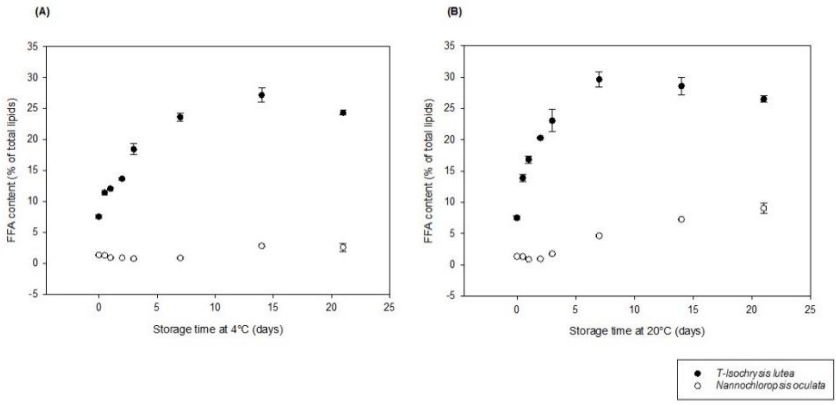


Figure 8.1: Evolution of the FFA content in *T-Isochrysis lutea* (●) and *Nannochloropsis oculata* (○) during wet storage at 4°C (A) and 20°C (B).

Not only the extent of lipolysis was different. Also the lipid classes that were degraded and the specificity of the reaction were species dependent. In *T-Isochrysis*, all saponifiable lipid classes were degraded at the same time, while in *Nannochloropsis*, especially polar lipids were degraded. Both species however showed a flattening of the FFA content after several days of storage. This was related to the formation of fatty acyl esters, which are formed by an esterification reaction between FFA and alcohols.

8.1.3. The role of cell integrity

While *T-Isochrysis* has only a very thin cell wall, consisting of mannose [42], *Nannochloropsis* possesses a tough cell wall containing cellulose and algaenan [39]. Possibly, the *T-Isochrysis* cells are already ruptured during harvesting, while the *Nannochloropsis* cells are still intact. This cell disruption could cause the enzymes to come into contact with their substrates and induce some defense mechanisms against grazers, which has already been observed in several diatoms and red algae [179-181, 203, 217]. Also in food industry, there are examples of products with a restricted shelf life from the moment the cell integrity is broken, e.g. rice bran [230] and oil palm [13, 14].

Several observations throughout this PhD support the abovementioned hypothesis. First, in Chapter 4, the lipolytic stability of high pressure homogenization (HPH) disrupted *Nannochloropsis* biomass was compared to intact, non-disrupted biomass. It was clearly observed that lipolysis in the disrupted biomass started very rapidly during or immediately after the HPH

treatment, while the intact biomass remained stable for several days. Additionally, the increase of the FFA content in the non-disrupted biomass (starting after 7 days of wet storage at 4°C), was also related to a loss of cell integrity, as demonstrated by an increase of the hexane/isopropanol (HI) extraction efficiency.

The influence of a HPH treatment on the lipolytic stability of *Nannochloropsis* biomass was further investigated in Chapter 5. In this chapter, the impact of different HPH conditions (varying HPH pressure and number of passes) was determined. It was observed that the least severe HPH condition (400 bar – 1 pass) was not able to improve the HI lipid extraction efficiency, but did result in a substantial increase of the FFA content (from 2.55 ± 0.13 to 6.30 ± 0.11 g/100 g biomass). Apparently, a minor degree of damage to the cell is thus sufficient to induce lipolytic reactions. As these samples were not stored after the HPH treatment, the lipolytic reactions started already during and immediately after cell disruption. A short wet storage stage is thus sufficient to induce quality problems in ruptured biomass.

Also in *T-Isochrysis*, the lipolytic stability is influenced by the cell integrity. In Chapters 3 and 4, the biomass already contained high amounts of FFA at the beginning of the storage period. This led to the hypothesis that the cells were already ruptured during the harvesting protocol. During the experiments of Chapters 3 and 4, the biomass paste was after centrifugation resuspended in a small amount of demineralized water (for homogenization purposes). Possibly, the cells were damaged by an osmotic shock occurring on transfer from the salt cultivation medium to the demineralized water. In a preliminary experiment in Chapter 6, it was tested whether post-harvest wet storage of *T-Isochrysis* could indeed be improved by resuspending the biomass paste in cultivation medium instead of demineralized water, thereby avoiding an osmotic shock. The results showed that the start of lipolysis was indeed delayed by several hours by resuspending in cultivation medium. This phenomenon was not observed in *Nannochloropsis* biomass, although resuspending in demineralized water was also performed in Chapter 4, probably because of the strength of the cell walls of this species, which is more resistant to osmotic changes.

8.1.4. Future perspectives

Although several major conclusions could be drawn from this PhD, a few hypotheses are still unconfirmed, leading to the need for further experiments.

In Chapter 3, it was hypothesized that the levelling off of the FFA content was caused by a balance between hydrolysis and esterification, both catalyzed by the same lipolytic enzymes. At a certain FFA concentration, product inhibition occurs and the FFA are esterified with alcohols to form fatty acyl esters. This hypothesis could be tested by removing the FFA (e.g. by complexation with calcium ions). If the hypothesis is correct, further hydrolysis of substrates that are still present should then occur.

In Chapter 4, it was demonstrated that the integrity of the microalgal cells determined the lipolytic stability during post-harvest wet storage. It was hypothesized that by rupturing the cells, the lipolytic enzymes come into contact with their substrates and thereby cause a very rapid formation of FFA. However, it is not elucidated yet which parts of the cell (cell membrane, cell wall, organelle membranes) exactly need to be damaged to induce lipolytic reactions. In addition, it also not known yet whether the different specificity of the lipolytic enzymes in *Nannochloropsis* and *T-Isochrysis* is caused by different characteristics of the enzymes or by a different localization in the microalgal cell. Possibly, the visualization of the lipolytic enzymes, for example by fluorescent substrates [179, 254, 255], could give more insight into these unanswered questions. Extraction of the enzymes prior to their exposure to different substrates could give more information about their substrate specificity.

To investigate the relationship between lipolysis and cell integrity in detail, it would also be useful to develop a better method to determine the degree of cell disruption. The methods used nowadays all have their limitations. Cell counting by microscopy or flow cytometry uses the selective coloring of the damaged or the non-damaged cells by a dye [256]. However, damaged cells and/or cell debris often assemble in flocs [257], which makes the counting more difficult. Especially after using flocculation as a harvesting method, these methods are not convenient. The presence of several pigments in the algae also makes that some methods are not applicable to microalgae. Indirect methods, which use the release of proteins or lipids as a measure of the

degree of cell disruption (as was used during this PhD), also have their limitations. In Chapter 5, it was observed that the FFA content increased substantially by applying a HPH treatment, while the lipid extraction efficiency increased only slightly. It is thus not a good measure for the degree of damage to the cell inducing lipolysis during wet storage.

In Chapter 5, it was hypothesized that the effect of the number of passes on the extent of lipolysis was in fact an effect of temperature increase due to the HPH treatment. The enzymes would thus be favored by the temperature increase, which causes a higher activity and thus a higher FFA formation. This could be verified by following up the temperature in the biomass during the treatment, and comparing this with the optimum temperature of the enzymes, which could be determined by extracting the enzymes and measuring the enzyme activity at different temperatures.

In general, detailed biochemical studies are necessary to give more insight into the exact lipolysis mechanism, the reaction pathways occurring in the cells and their relation to the environment. Possibly, also cultivation conditions (e.g. medium composition, pH evolution, growth stage, diurnal variations) have an influence on the FFA formation during wet storage. This was already seen during this PhD, as the use of the same harvesting method but different batches of the same species led to slight changes in FFA concentrations. Possibly, the formation and/or activation of lipases and thus the extent of lipolytic reactions during wet storage are also related to the lipid metabolism in the cell in response to for example the growth stage or the presence of stress conditions. Deprivation of the N content in the medium could possibly induce downregulation of TAG lipases, as more TAG are built up in lipid bodies in these conditions [258]. On the contrary, other stress conditions may lead to a higher expression of genes encoding for lipolytic enzymes [165].

8.2. How to avoid lipolysis during wet storage?

During this PhD study, it was clearly demonstrated that lipolysis during post-harvest wet storage poses a huge problem, especially in damaged or disrupted cells.

Up to now, high amounts of FFA are removed from oils (e.g. edible oils, oils used for biodiesel production) after extraction, mostly by saponification and

subsequent removal of the soap precipitate or by vacuum distillation [14]. However, this leads to substantial losses of lipids, especially when high amounts of FFA have to be removed. It would thus be more interesting to avoid lipolysis and to strongly reduce the FFA content in the biomass. This would reduce losses during refining of the oil. Additionally, when using the whole microalgal biomass, refining cannot be applied.

From the first part of this PhD, a few precautions to take into account during harvesting and post-harvest handling of microalgal biomass can be proposed, in order to minimize FFA formation. These are described in section 8.2.1. However, none of these methods is widely applicable and without any risk of problematic FFA formation. Therefore, in the second part of this PhD, two alternative strategies were proposed: inactivation of the lipolytic enzymes by a heat treatment (Chapter 6) and inhibition of lipolytic reactions by the addition of $FeCl_3$, which at the same time also pre-concentrates the biomass by flocculation (Chapter 7). These are explained and compared for their future industrial applicability in section 8.2.2.

8.2.1. Precautions during harvesting and post-harvest handling to reduce the extent of lipolysis

From Chapters 3 and 4, it is clear that lipolysis can be stopped at a temperature of -20°C. **Storage in a freezer** could thus be an adequate method to store biomass before drying. However, sometimes lipolysis already occurred before freezing was possible (during harvesting or cell disruption). Further lipolysis is then stopped during storage in a freezer, but the FFA content remains too high. It is also important to take into account that the enzymes are not denaturated. When thawing the biomass, the enzymes can again cause lipolysis, especially when cell integrity is lost.

It is also very important to **maintain the integrity of the cells**, as was demonstrated in Chapter 4. As soon as the integrity of the cells is lost, lipolysis is induced and the FFA content increases very rapidly. Some microalgal species remain intact during harvesting and can therefore be stored for a few hours/days depending on the characteristics of the species. In other cases however, the cells are disrupted during harvesting or a cell disruption treatment is necessary to improve extraction of the interesting compounds from the cells and thus cell rupture or damage cannot be avoided.

Drying strongly reduces the water activity and thereby also decreases the enzymatic activity in the microalgal biomass [11, 12]. Especially hydrolases, the enzyme class to which lipases belong, need water molecules for their reactions and consequently their activity is very dependent on the presence of water [11]. However, for longer term dry storage, the lipolytic stability is dependent on the drying technique used. **Freeze-drying** is seen as a very adequate method to maintain heat-sensitive compounds in microalgal biomass [12]. However, enzymes and thus also lipases are not inactivated during the drying step. Consequently, long term storage of the dried biomass can possibly lead to an increase of the FFA content [23]. **Spray-drying** or other techniques involving high temperatures (e.g. hot air drying) can possibly inactivate lipases (depending on the temperature used), but it has to be taken into account that heat-sensitive compounds (e.g. some carotenoids) can then be degraded as well [23].

8.2.2. Strategies to improve lipolytic stability

8.2.2.1. *Heat treatment to inactivate lipolytic enzymes*

In food applications where enzymatic lipolysis is an issue for lipid quality (e.g. rice bran, wheat germ, fruit oils), a heat treatment is often used to inactivate the degradation enzymes [14, 230-232]. Therefore, Chapter 6 investigated whether this practice can be used in microalgae processing as well.

It was found that, when applying a heat treatment of at least 8 minutes at 80°C, lipolysis was indeed stopped in *T-Isochrysis lutea* biomass. During a wet storage of 7 days at 4°C, no FFA were formed and the FFA content remained at around 1% of the biomass (3.5% of total lipids). This was a substantial improvement compared to the 5.9% in the control biomass (22% of total lipids). Next to the positive effect on avoiding lipolysis, the heat treatment also had a positive effect on the retention of some pigments. Chlorophyll a and diadinoxanthin were not enzymatically converted during storage after a heat treatment, while this was the case without a heat treatment. This could possibly improve color retention during wet storage. Importantly, there were no negative effects of the heat treatment on oxidative stability, ω-3 content and phenolic content. It could thus be concluded that the application of a heat treatment prior to wet storage is a promising technique to avoid FFA formation.

8.2.2.2. *Simultaneous flocculation and inhibition of lipolytic enzymes by the addition of $FeCl_3$*

In Chapter 7, an alternative method was found to inhibit enzymatic activity during wet storage of *T-Isochrysis lutea* biomass. When $FeCl_3$ flocculation was used to pre-concentrate the biomass to reduce the volume to be centrifuged, the lipolytic stability during subsequent wet storage was also strongly improved. The best results were obtained when using an optimized dose (120 mg $FeCl_3$/L suspension) and pH 8. This led to an FFA content of only 2% of total lipids after one week of wet storage at 4°C, compared to 15% of total lipids by harvesting using the standard centrifugation protocol. Further experiments suggested that the presence of ferric ions caused inhibition of lipolytic reactions, especially at pH 8. Up to now, it is however not sure whether this inhibition is due to a direct inhibitory effect of ferric ions on the lipolytic enzymes, or due to an indirect effect of the addition of $FeCl_3$ on cell integrity. This could be tested in a future experiment, in which the cells are ruptured by an osmotic shock (by resuspending in demineralized water) and simultaneously exposed to ferric ions.

The adapted harvesting protocol using pre-concentration by $FeCl_3$ flocculation at pH 8, followed by further concentration using centrifugation, had no negative impact on fatty acid profile (and ω-3 content), oxidative stability or pigment composition and content.

By using this method, the stabilization of the wet biomass of *T-Isochrysis lutea* can thus be combined with a pre-concentration step, which reduces the costs for further processing at the same time. It allows for short and medium term (until 7 days) wet storage at 4°C without the formation of substantial amounts of FFA. The term during which wet biomass can be stored is thus prolonged from a few hours until a few days, which simplifies downstream processing of the biomass (e.g. longer term storage before drying, possibility for wet extraction, no or less refining after extraction necessary).

8.2.2.3. *Future perspectives*

This PhD showed two possible strategies to improve the lipolytic stability during wet storage of microalgae. However, a lot of research and optimization is still necessary before implementation in industry is possible. Table 8.1 compares the two proposed strategies on different levels: species

dependence, contamination and quality of the product, practical feasibility and economical costs related to their implementation.

Table 8.1: Comparison of the two proposed strategies to improve lipolytic stability during wet storage of microalgae

	Heat treatment	**$FeCl_3$ flocculation**
Species dependence	Yes, time-temperature combination necessary for inactivation of lipolytic enzymes	Yes, possibility of $FeCl_3$ flocculation + inhibition of lipolytic reactions by Fe ions
Contamination of the product	No contamination	Contamination of biomass with Fe Extracted oil: only slightly higher Fe content than oil from centrifuged biomass
Quality of the product	• Enzymes denaturated: good lipolytic stability after drying assured • Peroxide value, pigment content and composition, phenolic content, fatty acid composition: not changed by treatment • Better color retention during storage	• Enzymes not denaturated: possibly lipolytic reactions during longer term storage, after removal of iron or after drying • Peroxide value, pigment content and composition, fatty acid composition: not changed by treatment • Same color retention during storage • Uncertainty about promotion of lipid oxidation by iron ions during longer term storage
Practical feasibility	Not applied in algae industry yet but frequently applied in other industries Possibilities: batch treatment or continuous (e.g. plate heat exchanger, cfr. pasteurization of milk)	Not applied in algae industry yet but already used in other industries (e.g. water purification) Possibilities: combination of stirring and settling tanks
Economical	Extra cost during downstream processing	Cost of implementation of flocculation, but lower centrifugation costs by reducing the volume

One of the main drawbacks of both proposed strategies is the **species dependent** approach which is needed. During this PhD, the focus was on *T-Isochrysis lutea*, a species with a lot of potential applications but with a very limited lipolytic stability. For this species, the two proposed methods have been demonstrated to be applicable. It can be expected that a heat treatment to inactivate the lipolytic enzymes can be extrapolated to other

species as well. However, the time-temperature combination has to be optimized for the specific type of enzymes in a certain species. $FeCl_3$ flocculation has already been tested on several species and has been shown to be widely applicable [245]. However, the enzyme inhibition that was possibly caused by Fe ions has not been demonstrated in all enzymes and all species. Although this has already been described in literature for several bacteria [183], fungi [184, 185] and microalgae [166], it still has to be investigated whether this is a widespread mechanism. More fundamental biochemical research on microalgal lipases could reveal useful information on their characteristics. This background information could strongly simplify the optimization and implementation of these strategies on industrial scale.

Further research could investigate the lipolytic and oxidative stability during longer term wet storage after applying the different strategies. Also the influence of a cell disruption step on the efficiency of these techniques is not known yet. The effect of a heat treatment or the addition of $FeCl_3$ could possibly be different when applied on disrupted biomass. In addition, investigating the influence of these methods on the stability of dried biomass could give interesting information. There could be a difference in activity of the lipolytic enzymes in the dried biomass, as applying a heat treatment denatures the enzymes, while the flocculation method possibly only inhibits the activity, which can be reversible. Also the oxidative stability is possibly influenced by the use of these strategies, as the presence of Fe ions in the flocculated biomass can possibly accelerate oxidation reactions during prolonged dried storage.

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CURRICULUM VITAE

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Education

Current education

PhD researcher in Bioscience Engineering

Microbial and Molecular Systems, Lab Food & Lipids, KU Leuven Kulak, Kortrijk, Belgium

Topic: Lipolytic stability during wet storage of microalgal biomass

Promotors: Prof. dr. ir. Imogen Foubert, Prof. dr. Koenraad Muylaert

Qualifications

2013	Master in Bioscience Engineering KU Leuven, Belgium *Thesis project: The influence of the structure of pectin on the in vitro bioavailability of carotenoids in emulsions (Laboratory for Food Technology, KU Leuven, Promotor: prof. M. Hendrickx)*
2011	Bachelor in Bioscience Engineering KU Leuven, Belgium
2008	High school degree, science-mathematics Sint-Aloysiuscollege, Menen, Belgium

Additional courses and workshops

2017	Liquid chromatography – mass spectrometry (LC-MS) Avans+, Breda, The Netherlands

Work experience

Research experience

2013- present	PhD project
	Laboratory Food & Lipids
	KU Leuven Kulak, Belgium

Participated at several international conferences with posters and lectures (see list of publications)

Winner of the poster prize at the Euro Fed Lipid Congress (Gent, 18-21 September 2016)

Educational experience

2013-2017	Practical courses Organic Chemistry (2nd bachelor Chemistry and Bioscience Engineering)
2014-2018	Supervisor of bachelor projects (2nd bachelor Bioscience Engineering)
2014-2016	Practical sessions 'Practicum Synthese & Karakterisatie' (2nd bachelor Chemistry)
2014-2015	Supervisor master thesis Thomas Stock (2nd master Bioscience Engineering)
2014	Supervisor internship Cédrick Veryser (1st master Chemistry)

Skills

Languages

Dutch	Native speaker
English	Very good
French	Good

PC

MS Office (Word, Excel, Powerpoint)

Sigmaplot, JMP

Other skills

Chromatography (GC, HPLC)

LIST OF PUBLICATIONS

Articles in internationally reviewed academic journals

Balduyck, L., Dejonghe, C., Goos, P., Jooken, E., Muylaert, K., Foubert, I. Inhibition of lipolytic reactions during wet storage of *T-Isochrysis lutea* biomass by a heat treatment. *Algal research* (under review).

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Foubert I., Bruneel C., Ryckebosch E., Lemahieu C., Balduyck L., Gheysen L., Matton V., Dejonghe C., Muylaert K. (2017). Autotrophic microalgae as an alternative source of omega-3 oils: from green water to health conscious consumer. Algae Biorefineries for Europe. Brussels, 17-18 October 2017.

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