# Thesis Biobased Chemistry and Technology

## The effect of storage on algae biomass composition

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WAGENINGEN UNIVERSITY
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AlgaePARC

# The effect of storage on algae biomass composition

## based on the model organism

## *Neochloris oleoabundans*

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# Abstract

For biorefinery biomass is often (freeze) dried to maintain the quality of biomass right after harvesting and dewatering. This is not a viable solution for large-scale production, due to the high energy requirements, low capacity and costs of the equipment. Therefore wet storage would be a convenient option. Little research has been done on this field so far. This works aims at the investigation of changes in biomass composition during storage time.

The following research question was formulated: 'How is the biomass composition of algae affected by the storage temperature 4°C vs room temperature and gas conditions ($N_2$ vs air)?'

For 8 weeks, small amounts (3 gram) of fresh algae paste were stored in falcon tubes; half in the fridge (4°C) and half in a dark box at room temperature. Samples were taken in defined frequencies and changes in the main cell component (i.e. total fatty acids, soluble proteins, carbohydrates and starch) were followed. For the fatty acids GC-analysis was applied; all other components were quantified with calorimetric methods.

A steady decrease was found for the protein content with approximately 15% loss in the 8 weeks of storage. Fatty acids in contrast remained rather stable for 4 weeks, before a relative weekly loss of 3% started. After 8 weeks a relative loss of 13% was observed.

As a primary energy reserve for cell maintenance starch reserves were depleted within one week. No major changes in the carbohydrate content were found. However, there are doubts about the reliability of the obtained results. Removing of oxygen by nitrogen-flushing had no significant effects. It is to be discussed if this reflects real findings or if this is due to a failure of complete oxygen removal. For the proteins degradation rates were reduced by 25% when stored at 4°C compared to storage at roomtemperature. Fatty acids were slightly better retained at 4°C. However, neither temperature nor gas condition had a strong impact on the degradation rates. As carbohydrate did not change significantly, no conclusions can be drawn at this point.

If storage is an option, mainly depends on the applications and costs involved. For high value- or food products storage at low temperature is advised, for low value applications such as energy generation and biodiesel biomass deterioration is not a problem. Storage at roomtemperature is thus possible as long as benefits outcompete the losses.

# Preface

This report is the result of my four months Bachelor thesis at the chairgroup Biobased Chemistry & Technology (BCT). Based on the model organism *Neochloris oleoabundans* I analysed how storage affects the biomass composition of algae. This work was done in cooperation with AlgaePARC Wageningen and the chairgroup Bioprocess Engineering (BPE) where part of the experiments were performed.
Without all the help and advices I received, this work would not have been possible. First of all I want to thank my supervisors Ellen Slegers and Rouke Bosma for the good cooperation. Ellen: For your daily supervision, motivation and help with writing! Rouke, our meetings were less frequently, but every time I left the room with new ideas and inspiration. Also a special thanks to Wendy Evers for her help during my work at BPE. Wendy: Lab introduction, trouble solving…In all these cases you gave me a helpful hand! Also I would like to thank Susan Witte and Ruud Verloo for introducing me to the BCT laboratories/ AlgaePARC and Ben van den Broek for his advices on the analytical methods.
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# 1 Introduction

The depletion of natural resources and resulting environmental damages has become an important issue in the last decades. It is generally accepted that only a more sustainable life style can guarantee health and prosperity for humans and the environment on long term. The concept of sustainability comprises several meanings. An easy to remember description is: **PPP - P**eople, **P**lanet & **P**rofit. For one part it describes a responsible handling of our natural reserves such as mineral resources, water, soil and living beings. In addition, it requires efficient and sustainable production processes to ensure competitiveness.

However, many times profit optimization undermines the other two aspects of sustainability (**P**eople, **P**lanet). More focus should be brought into minimizing the impact on humans and environment to go for a true PPP design. One approach to a fossil-independent world is the replacement by renewable sources such as biomass. These are not only considered as 'unlimited', but also have the potential to reduce greenhouse gas emissions as the $CO_2$ released during the burning of bioproducts was incorporated through carbon fixation during growth. This fast recycling of $CO_2$ potentially contributes to greenhouse gas reduction and can be seen as a first step towards becoming $CO_2$ neutral. Sustainability guidelines ask for an optimal use of raw material, which is the focus of this thesis. In contrast to solar or wind energy, biomass has the advantage that new material is created. You can grow a crop specifically for one product, or process the biomass into several products. This last approach is called biorefinery and is defined as 'the sustainable processing of biomass into a spectrum of marketable products (food, feed, fuels, chemicals, heat and power)', which means that it uses all valuable components[1]. Biorefineries thus reduce the amount of waste generated. For many biomass sources biorefinery is essential to make the production of biocommodities economically viable[2].

The green algae *Neochloris oleoabundans*, is one promising algae species for oil production: It is relatively robust, fast growing and under nitrogen depletion up to 56% lipids on dry weight can be accumulated[3]. Also other biomass components such as proteins and pigments can used. As a consequence of the nitrogen shortage growth is restricted, while glucose is taken up in excess. Electrons from the sunlight are stored in the highly reduced fatty acids. These neutral lipids can serve as energy reserves for the nights or periods of starvation when no photosynthesis can be performed[4].

Table 1 gives an impression on how biomass composition of *Neochloris* changes upon nitrogen starvation[5].
Under normal growth conditions (C/N ratio of 17) proteins makes up for the largest part (44%), whereas under nitrogen shortage (C/N 278) an increased lipid production is observed (52%) and protein content is decreased to 14%[5].

**Table 1: *Neochloris* biomass composition[5]**

| | C/N 17 | C/N 278 | Fed-batch |
|---|---|---|---|
| proteins | 43.7 | 14.4 | 11.6 |
| lipids | 24 | 51.7 | 33.7 |
| carbohydrates | 30.9 | 33.3 | 54.2 |
| total | 98.6 | 99.4 | 99.5 |

In general is the use of algae for biorefinery considered a promising approach, as it is a fast and efficient way for biomass production. In contrast to other crops mainly composed of either oil, protein or carbohydrates, algae produce a variety of valuable compounds of industrial interest such as lipids, proteins and pigments. Furthermore algae can be grown on non-arable land, avoiding competition with food crops. Also an extraordinary high growth rate and the fact that they can be grown during the whole year result in a high yield per area. Another advantage is the low water consumption, algae can even contribute to waste water cleaning[6, 7]. Also in this case the **P**rofit in the sustainability description requires optimization of the whole supply chain to allow competiveness. To make commodity production with algae economically feasible, biorefinery is a must[8]. This underlines the need of effective biorefinery techniques to ensure maximal product recoveries and minimal losses to useless by-products.

Although algae are not yet produced at large scale for bulk applications, there are opportunities to develop this process in a sustainable way. Biomass composition determines which products can be obtained from algae biorefineries. The exact biomass composition varies considerably between different algae species and growth conditions[9]. The yield can be maximized when factors of influence, such as light intensities and nutrients are tailored to the culture requirements. Composition control therefore allows maximization of the profit.

For the application choice it will be important to know whether salt or fresh water is used in cultivation. Salt water species have the potential to be used in biorefinery including the production of high value compounds, whereas the fresh water type can be used for waste treatment. In the

latter case biorefinery will rather focus on the production of chemical bulk products. All biomass components should be used[8]. Figure 1 provides an overview of the possible applications from algae biomass. Lipids should be fractionated into lipids for biodiesel, lipids as a feedstock for the chemical industry and ω-3 fatty acids. Proteins and carbohydrates find their application in food products, feed and bulk chemical production. High-value products, such as carotenoids (pigments in general), ω-3-fatty acids and antioxidants such as DHA and EPA contribute to make the production economically viable. The oxygen produced can be used for fish cultivation in form of oxygen enriched gas. In addition, algae can be grown on residual nutrient feedstocks and recycle CO2[8]. Recycling reduces the inputs of nutrients leaving more room for profit, and contributes to reduce the environmental impact.

| | Product | Selling price € /ton |
|---|---|---|
| Biofuel | Biokerosene | 500 |
| | Biochar | 150 |
| Biochemical | Biopolymer | 2,500 |
| | Biolubricant | 2,000 |
| | Biopolymer additivies | 3,000 |
| | Coating | 5,000 |
| | Paint | 10,000 |
| | Bulk Chemical | 1,000 |
| Food/Feed | Protein | 1,000 |
| | Lipids | 950 |
| | Carbohydrates | 750 |
| Food aditives | Poly-unsaturated fatty acids | 75,000 |
| | Functional Protein | 3,000 |
| | Pigments | 1,100,000 |
| Cosmetics | Antioxidants | 30,000 |
| | Glycolipids, Phospholipids | 6,000 |

**Figure 1: Applications of biorefinery products [10]**

By now it was taken for granted that downstream processing has to occur directly after harvesting. However, many of the machineries used for cell disruption and component extraction are constructed for large amounts of biomass. Therefore it would be economically favourable to collect the harvested biomass and store it until enough biomass for one process run has been collected. Another future perspective is to have several algae producing systems at different locations and to further process the harvest in a separate factory. To determine which logistic scenario is most sustainable, it is important to know whether transport and storage affect the quality of biomass. It is expected that after a certain time of storage changes in biomass composition will occur.

These changes will affect the quality of the product. For sure viability of cells and microbial degradation rates will play a role[11-13]. Effective preservation techniques will be needed to ensure high quality standards.

Wet storage can be an option convenient option for short-term storage, however when kept for a longer time period algae has to be dried. Drying plays a crucial role in the stability of valuable compounds (both, in microalgae as well as in food application) as it reduces the moisture content and water activity[14]. Water activity is a measure of the availability of water for reactions occurring in food products leading to quality loss. Drying, and consequently reducing water activity, diminishes the growth of microorganisms and retards enzymatic reactions because of the reduced contact between enzyme and substrate[14]. Some enzymes are also inactivated during the drying process, depending on the time and temperature used[12]. These findings show that drying techniques and process parameters may have an important influence on the properties and stability of dried microalgae during storage, although relatively little research has been done on this topic by now.

On lab scale the commonly applied long-term preservation techniques are freezing and especially freeze-drying. After these treatments biomass composition does not change over time anymore. However, these are very energy consuming processes. Acien et al. calculated that per kg freeze-dried biomass, 4,19kWh is consumed[13]. Scale-up therefore requires a more economic approach. One appealing cheap option would be the storage of algae biomass at low temperatures to slow down degradation processes. This is done with many food products to increase shelf life, for example milk storage. For preservation of algae such low temperatures could also increase the storage time in processing. Possible storage time is very specific and differs considerably between products. A lot of literature on food preservation can be found, however algae cultivation is still in its infancy and nearly no literature on this field is available. Therefore, we can only speculate on how the biomass composition will change during storage.

One common phenomenon occurring during storage of plant materials at low temperatures is chilling damage. Chilling damage is abroad term and it is used with several meanings. In general it is a term for all cell damages occurring during storage at low temperatures, which promote cell death and biomass degradation. Although there are similarities in plants and algae, some differences are expected for algae as these single-celled organisms have a less complex metabolism and cell structure. The various processes in chilling damage are illustrated in Figure 2 and 3.

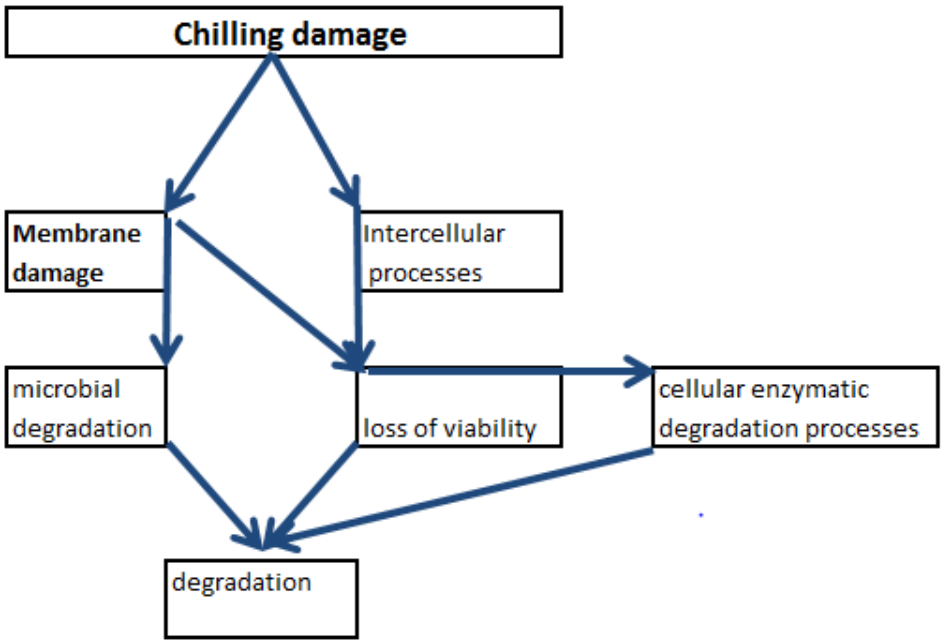



**Figure 2: Processes occurring in chilling damage**

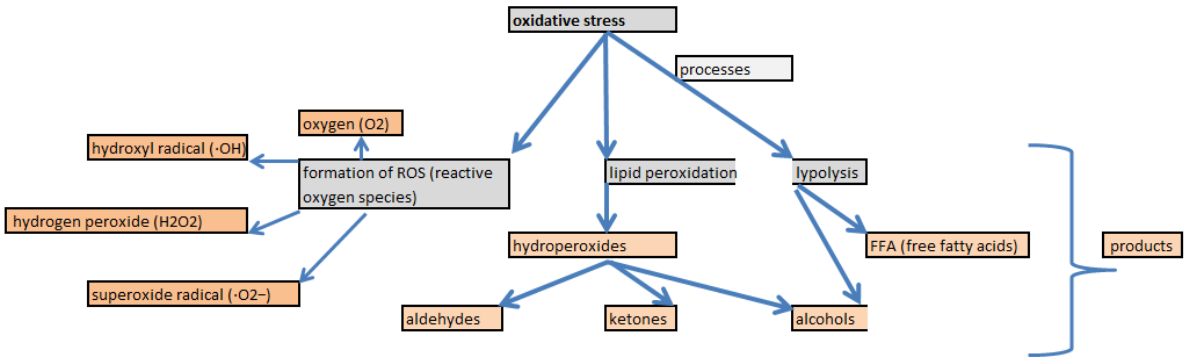



**Figure 3: Oxidative stress processes (shown in blue) & formed products (in orange). A detailed description of each of these processes can be found in Appendix A**

Generally, it is suggested to store algae under oxygen depletion as anaerobic microorganism grow much slower than aerobic ones. Also, low temperatures slow down enzyme kinetics decelerating cell internal enzymatic degradation processes. In addition, storage in the darkness is recommended to prevent photosynthetic activity, which would lead to a decrease in energy reserves in the cell[11].

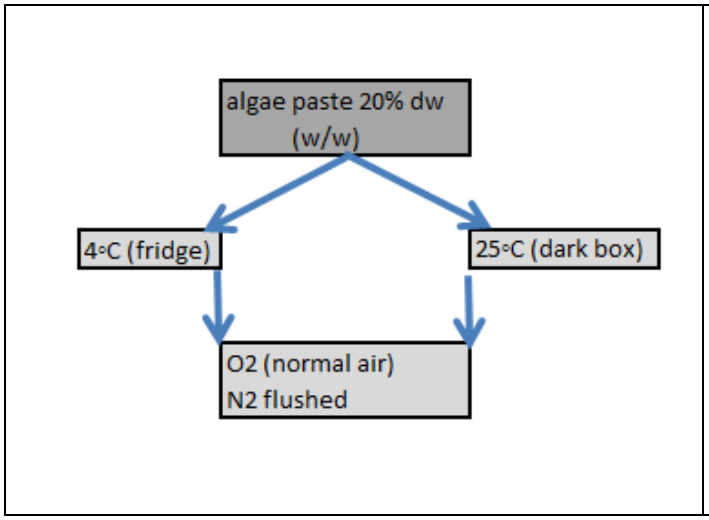
These considerations about two-level degradation (membrane damage and further degradation) and influences of surrounding gas and temperature led to the following research question:
'How is the biomass composition of algae affected by the storage temperature 4°C vs room temperature and gas conditions ($N_2$ vs air)?'

In this work the algae biomass composition is followed for four different storage conditions (two different temperatures and two gas conditions). The focus lies on changes in the three main cell components, i.e. carbohydrates, lipids and proteins during eight weeks of storage.

# 2 Materials and Methods

## 2.1 Experimental Approach

A fixed amount of algae paste was added to tubes (see figure 4). Part of the tubes were flushed with nitrogen (only the headspace) and sealed afterwards, whereas a second group of tubes was closed without removing the oxygen. This serves as control group for investigating the influence of oxygen on spoilage. Half of the tubes was stored in the fridge, the second half was stored at room temperature in a dark box to inhibit photosynthesis saving energy reserves.
In the eight weeks of storage samples were taken in defined frequencies and freeze-dried for further analysis. For more details of the sampling scheme see 'Cultivation and Harvest'.



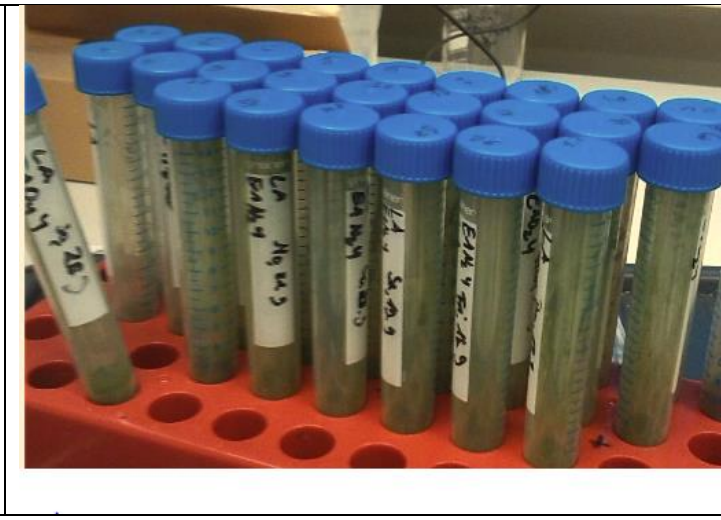



**Figure 4: Comparison of the 4 storage conditions: $N_2$ 4°C; $N_2$ 25°C; $O_2$ 4°C; $O_2$ 25°C**

**Figure 5: Picture of the falcon tubes used for algae storage**

**Figure 6: Picture of the airlift reactor used for cultivation [15]**

## 2.2 Cultivation & Harvesting

On Monday, 14th of September 200g of fresh water *Neochloris oleoabundans* algae paste were obtained from the airlift reactor at AlgaePARC Wageningen. The culture was centrifuged (Evodos10); dry matter determination indicated a w/w ratio of 209 g/l (20.1% DW). Biomass for the second experiment was obtained three weeks later (Monday, 5th of October).

**sampling scheme experiment 1**

| wee | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | total |
|---|---|---|---|---|---|---|---|---|---|
| 25C N2 | 7 | 5 | 3 | 2 | 2 | 2 | | | 21 |
| O2 | 7 | 5 | 2 | 2 | | | | | 17 |
| 4C N2 | 7 | 5 | 3 | 2 | 2 | 2 | 2 | 2 | 25 |
| O2 | 7 | 5 | 3 | 2 | 2 | 2 | | | 21 |
| | | | | | | | | | 84 |

**sampling scheme experiment 2**

| wee | 1 | 2 | 3 | 4 | 5 | 6 | total |
|---|---|---|---|---|---|---|---|
| 25C O2 | 7 | 5 | 3 | 2 | 2 | 2 | 21 |
| 4C O2 | 7 | 5 | 3 | 2 | 2 | 2 | 21 |
| | | | | | | | 42 |

**Figure 7: Sampling scheme first experiment**

**Figure 8: Sampling scheme second experiment**

## 2.3 Sample Preparation

Algae biomass was transferred with a syringe into the falcon tubes. It turned out to be a challenge to avoid algae sticking to the walls (which means drying of the samples and thus shorter life expectancy). Also it is the reason why $N_2$ flushing was difficult to perform as the hose could not be inserted till the bottom of the tubes. Therefore, smaller Eppendorf tubes were chosen for the second experiment. However, viscosity was too high; the tubes were filled till the top with algae making the $N_2$-flush impossible.

Therefore for the second experiment only the two temperatures were compared. Freeze-dried samples were divided over two tubes: Half stayed in the original falcon tubes, half of the content was transferred to Eppendorf tubes. After a few days microbial growth or at least moiety was observed in the Eppendorf tubes. Therefore, the samples of experiment 2 stored in Eppendorf tubes were transferred to falcon tubes after freeze-drying to ensure dry storage.

## 2.4 Analytical Methods

### 2.4.1 Quantification of Proteins - Based on Lowry[1]

In Lowry analysis protein quantification is based on a colorimetric method involving redox reactions between peptide bonds of proteins and copper ions resulting in a blue staining. The blue colour development is based on two independent reactions: The first one called Biuret with copper and the second one involving $Cu^{2+}$ ions forming blue complexes with peptide bonds in alkaline solutions. $Cu^{2+}$ is reduced to $Cu^{+}$ (involving oxidation of aromatic residues of proteins) which in change reduces the yellow Folin–Ciocalteu reagent leading the characteristic blue colour to be measured at 750nm. It has been shown that the blue colour intensity increases in the first 30 to 120 minutes and remains stable for the following two hours[16]. By comparing the absorbance at 750nm to a BSA[2] calibration curve with known concentrations, the protein concentration in the samples is determined. However only soluble proteins can be measured.

A detergent containing lysis buffer is used to lyse cells and separate proteins. The SDS present in the buffer is not only responsible for protein denaturation, but also works as detergent resulting in holes in the cell walls and a porous structure allowing the cell content to permeate out of the cells. In addition, the lysis tubes are beat-beated and incubated for 30 minutes in boiling water to promote protein release. After spinning down the cell debris, a supernatant dilution is prepared which is consequently transferred to the 96-wells before adding reagent A (copper solution) and B (Folin). After an incubation time of 30 minutes at room temperature the absorbance is measured. A more detailed description can be found in appendix C.

### 2.4.2 Quantification of Fatty Acids - Based on Gas Chromatography (GC)

In this experiment fatty acids are isolated in several extraction steps. Cell disruption (freeze-drying, bead beating and sonification) is performed to release fatty acids from the cells before isolating in chloroform. Consequently transesterification with methanol is performed resulting in volatile fatty acid metylesters as only volatile compounds can be analysed on a gas chromatograph (in the following referred to as a GC).

[1] Detergent compatible protein assay
[2] Bovine serum albunin, a standard protein taken as reference

The exact composition of the sample is analysed by separating the fatty acid based on their retention time which differs depending on the structure and length with the shorter molecules emerging first.
It can be distinguished between three main groups: The polar membrane lipids, glyco- and phospholipids and the neutral triglycerides (TAGs).

Integrated peak areas indicate the amounts reaching the detector. Quantification is done by comparing peak areas of the fatty acids to those of an internal standard (C15:0) added before in a known concentration. Taking into account a relative response factor[3], this allows the calculation of the total amount of a specific fatty acid chain in the samples. Taking an internal standard along also allows to correct for possible losses during sample preparation as it can be expected that the decrease will be the same for all fatty acids. As algae do only contain fatty acids with an even number of carbon atoms (C14, C16, etc.), choosing the C15:0 internal standard ensures that overlap of the peaks is avoided and peaks can be identified clearly. A more detailed description of the analysis technique and sample preparation can be found in the protocol in appendix C.

### 2.4.3 Quantification of Carbohydrates – Based on DuBois

The total amount of carbohydrates present in the freeze-dried algae can be measured by hydrolysing the polysaccharides into simple monosaccharides, which give an orange colour upon reacting with phenol and sulphuric acid. The concentration of monosaccharides in the sample can be determined by comparing the absorbance at 485nm to a glucose calibration curve with known concentrations. Not only glucose, but also other small mono-, oligo- and polysaccharides, as well as and their derivatives, including methyl esters with free or potentially free reducing groups give an orange colour upon reaction with phenol[17]. However these sugars give a slightly different response. A more detailed description can be found in appendix C.

[3] The peak area of a certain amount of different fatty acids is not the same for all, so the response factor indicates how a concentration is translated into a peak area

### 2.4.5 Quantification of Starch

Also in this case a colorimetric method is used for quantification. The protocol includes purification with ethanol, beat beating (to ensure starch release) and the use of α-amylase, a specific enzyme converting starch into glucose. Two enzymes present in the GOPOD-reagent are responsible for the pinkish colour development the quantification is based on. By comparing the absorbance of the samples to the glucose standard concentration range, the starch content can be determined. For more detailed information see protocol in the appendix C.

### 2.4.6 Sugar Composition Analysis

With the DuBois method the total amount of sugar is determined. Additionally three of the most abundant sugars were quantified: Glucose, arabinose and galactose.
Two sugar determination kits (K-ARGA 02/15; K-FRGLQR 07/12) were used. A prehydrolysis according to the HPLC protocol was performed before. For more details see appendix C.

# 3 Results

In the following chapter the results for all components are presented: Proteins, fatty acids, carbohydrates, starch content and finally the sugar composition are presented in the mentioned order.

## 3.1 Results Proteins

The soluble protein content of the algae paste during the eight weeks of storage is shown in Figure 9. The effect of the 4 different storage conditions (table 2) was compared[4].

**Table 2: The 4 different storage conditions**

| storage condition | $O_2$ 25°C | $N_2$ 25°C | $O_2$ 4°C | $N_2$ 4°C |
|---|---|---|---|---|

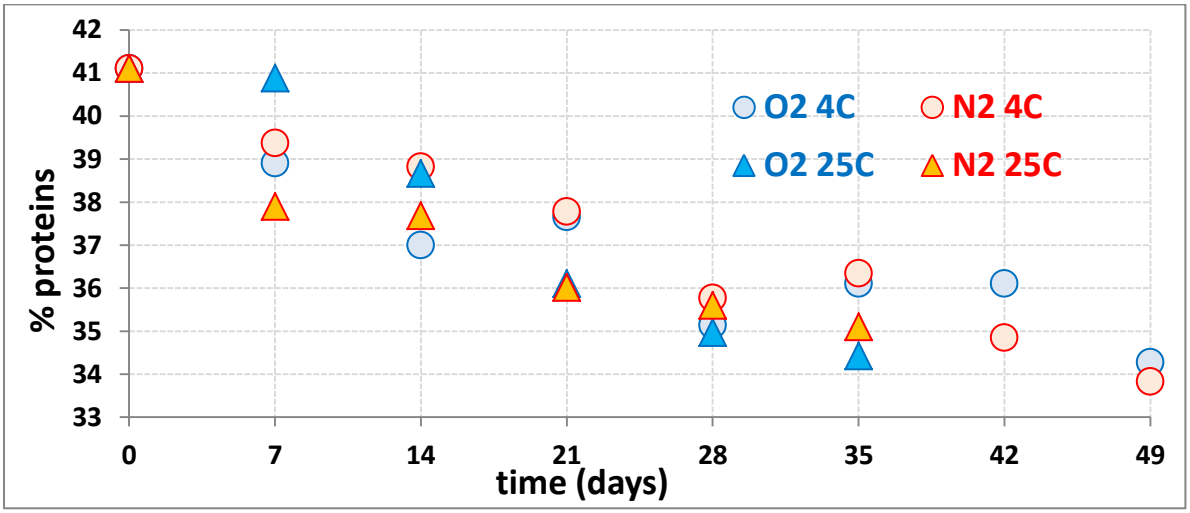



**Figure 9[5]: Development of the protein content during 8 weeks under different temperatures and gas conditions.**

During storage, the protein content showed a steady decrease from 41% to 34-35% from the beginning onwards (see figure 9).The initial protein content was the same for all samples. All measurements are based on single biological samples. In combination with measurement errors probably that is the reason for a few outliers resulting in a rather high variance.

[4] Not all conditions were tested for the same time period. The last oxygen containing samples were taken after 6 weeks. This is the reason why not all graphs show the same amount of measurement points.

[5] As stated in the experimental approach, $N_2$ and $O_2$ at 4°C originate from the first experiment, whereas $O_2$ at 25°C is taken out of the second experiment.

To minimize the effect of measurement errors when visualizing general tendencies, a linear regression analysis was performed (figure 10, table 3). Linearity could be confirmed for the 25°C samples ($R^2$> 0.9), for the 4 °C variance was too high. Based on the slope of the trend lines confidence intervals for the average degradation rates were calculated (see table 4).

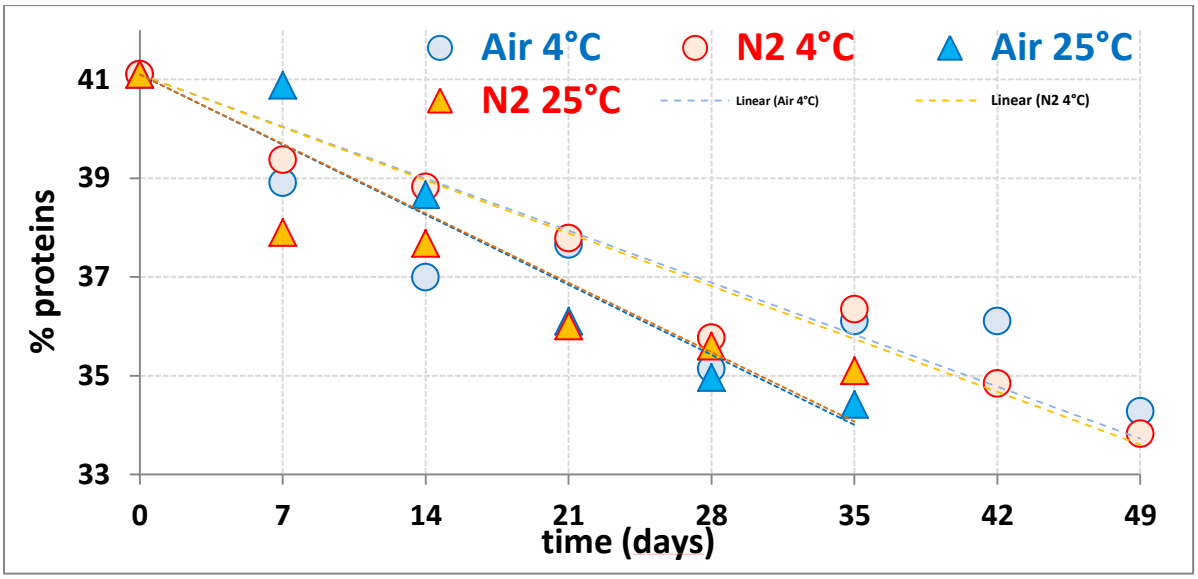



**Figure 10: Degradation at 4°C is reduced by ¼ compared to 25°C. Gas conditions seem to have little impact. Regression lines were drawn for illustration purposes**

Based on the average degradation rates, effects of the storage condition are compared and it was found that higher temperature speed up the degradation process. When stored at room temperature, protein content drops to 34% within six weeks, whereas at a lower temperature (4 °C) eight weeks are needed to obtain a similar decrease (Figure 6). Degradation rate at 4°C is thus about 25% slower compared to 25°C. The surrounding gas (nitrogen or air) in contrast, seems not to have any influence. It is to be discussed if this finding is due to a failure of complete oxygen removal as indicated by the paste observations or if it reflects real biological processes. Table 3, showing the average loss per week, indicates that degradation proceeds the fastest at $N_2$ 25°C, followed by the 4 °C sample with an average loss of 2.6% per week. Strangely, the measurements indicate that degradation is the lowest for *$O_2$ at 25*°C. Confidence intervals were calculated for all degradation rates. In case confidence intervals do not show any overlap, the effect of storage temperature on the degradation rate is proofed. The intervals for nitrogen do not show any overlap. The higher value for *25*°C proof that higher temperature speed up deration processes. Due to the obvious measurement errors, this cannot be shown for air (*$O_2$).* However, when ignoring the gas conditions and treating the temperatures as one group, it can be shown with a 95% confidence interval that temperature does affect degradation processes (see Appendix B)

**Table 3: 5/8 weeks average degradation rates with 95% confidence intervals for all storage conditions**

| storage condition | % loss per week with std | standard deviation[6] | upper boundary | lower boundary |
|---|---|---|---|---|
| **$O_2$ 25°C** | -2.58% | ± 0.4% | -3.4% | -4.3% |
| **$N_2$ 25°C** | -3.8% | ± 0.3% | -2.1% | -3.1% |
| **$O_2$ 4 °C** | -2.6% | ± 0.7% | -2.9% | -4.0% |
| **$N_2$ 4 °C** | -2.6% | ± 0.3% | -1.4% | -3.7% |

## 3.2 Results Fatty Acids

The fatty acid contents is shown in figure 11. The first measurements indicate a percentage of 8.9%. During storage a 15% decrease to approximately 7.5% was observed. For all four conditions the fatty acid content remained stable in the first weeks with a constant decrease starting from week 4 onwards.

Deviation between the single measurements is quite low. In case of the proteins general tendencies were shown by comparing the average losses per week. However, for the 25°C samples no data are available after 4 weeks of storage, when noticeable decrease starts. Therefore regression analysis is not a good option.

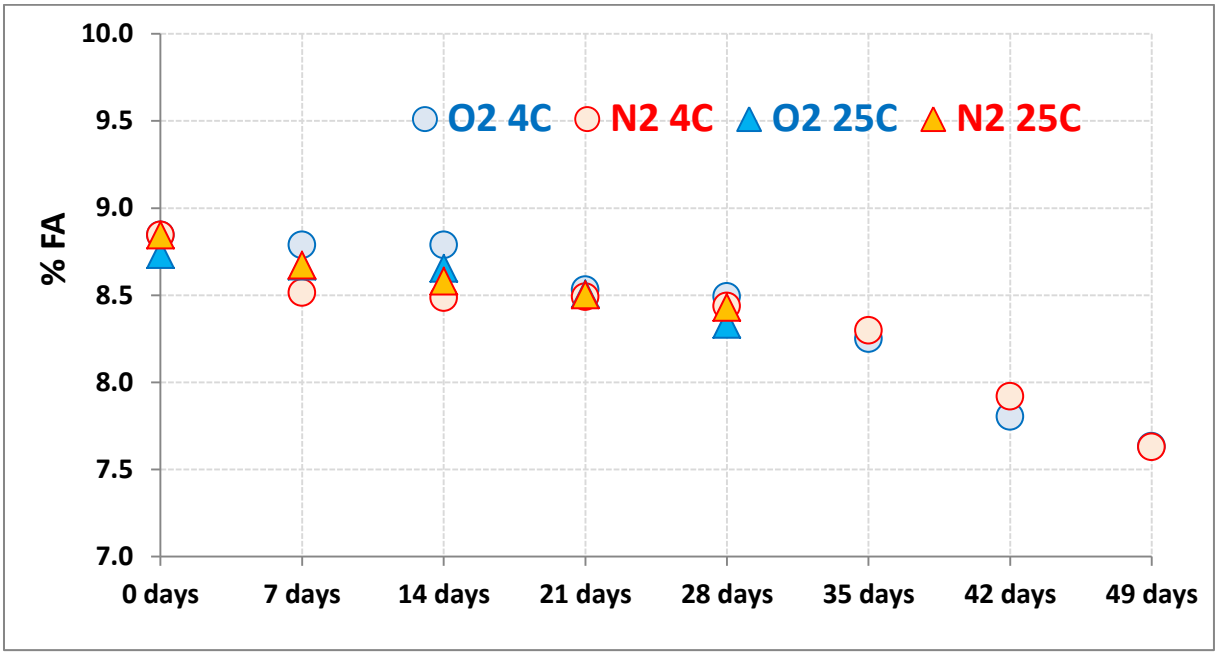



**Figure 11: Development of the total fatty acid content for the 4 different storage conditions**

[6] Fitting of measurements to regression line

The fatty acid pattern is quite consistent between all samples (between technical replicates as well as during storage time), no major changes in fatty acid composition were observed (see figure 12 &13).
Saturated and unsaturated C16 and C18 (50,3%; 47,3% respectively) chains dominate the fatty acid profile, which C16:0 (26.7%) being the most abundant fatty acid. Only small amounts of C14 fatty acids are found.

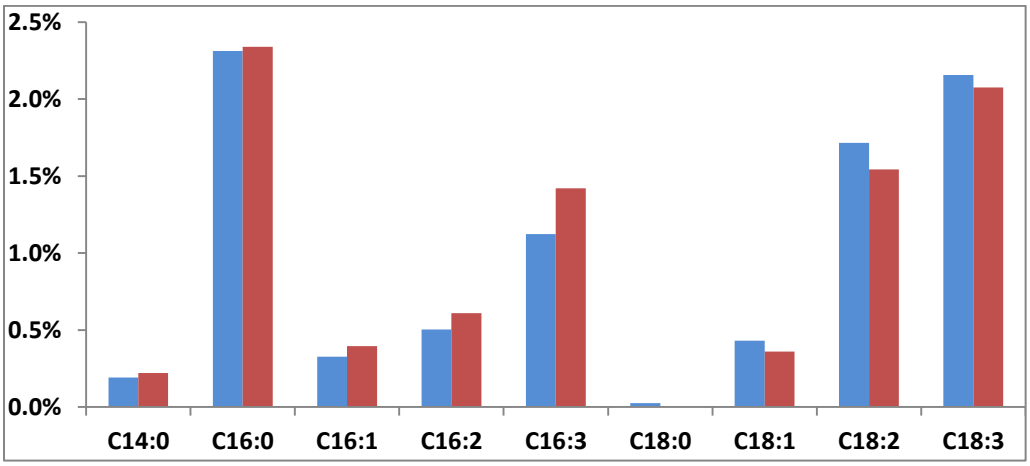



**Figure 12[7]: Changes in fatty acid pattern over time: average of the initial measurements (blue) vs 4°C samples of week 7/8 (red)**

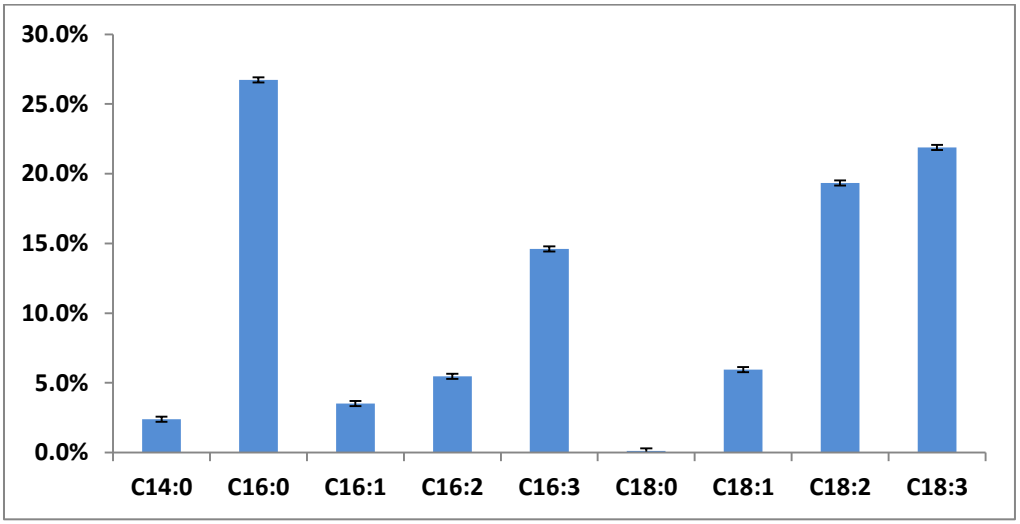



**Figure 13: Relative abundance of different fatty acid with standard deviation[8]**

[7] Initial values reflect the average of 5 day 0 measurements. The end situation was obtained by combining all 4°C samples of week 7 and 8

[8] Percentage of total fatty acid content as an average of all analyzed samples

## 3.3 Results Carbohydrates

Carbohydrate content ranged between 5 and 15%. In general variation between samples is high and no clear tendencies during storage could be detected (for example, table 4). Also technical replicates suggest deviating carbohydrate content. Many adoptions[9] on the protocol were tried, however without significant improvements. Due to the high variance in measurements and doubts about reliability of the findings no conclusion could be drawn regarding carbohydrate content.
Table 4 shows a few representative findings when following the protocol attached in the appendix C.

**Table 4[10]: Carbohydrate content**

| days | 0 | 7 | 14 | 21 | 28 | 35 |
|---|---|---|---|---|---|---|
| **$O_2$ 25°C** | 10.8% | 10.8% | 13.3% | 7.5% | 12.1% | 12.2% |
| **$O_2$ 4°C** | 10.3% | 7.4% | 8.6% | 6.3% | 7.9% | 11.3% |

## 3.4 Results Starch

The results for the starch analysis are shown in table 5. As expected in the first week of storage starch content decreased from approximately 8% to 1.5% of dry weight. This was observed for all samples independent of storage conditions. The first samples (day 0) of the second experiment contain slightly more starch than the ones of the first experiment (8.8% vs 7.4%).

**Table 5: Initial starch content and after one week for the 4 storage conditions**

| Storage conditions | day 0 | day 7 |
|---|---|---|
| **$N_2$ 4°C** | 8.9% | 1.4% |
| **$N_2$ 25°C** | 8.9% | 1.5% |
| **$O_2$ 4°C** | 8.9% | 1.5% |
| **$O_2$ 25°C** | 8.9% | 1.5% |

[9] Different types of pre-hydrolysis, order and speed of adding the reagents
E.g. Without any pre-treatment, with ydrolysis as suggested in the HPLC-protocl, cooking in concentrated sulfuric acid.
Order: Swapping phenol and sulfuric acid; fast adding of the acid
[10] Percentages found for the first 5 weeks of 2. experiment at 4 and 25°C

## 3.5 Results Sugar Composition Analysis

Sugar composition analysis suggests a carbohydrate content of 21.8%. This estimation is based on the measurement of 3 of the 4 most abundant sugars in *Neochloris* (see table 6). The obtained values are twice as high as suggested by the DuBois method, but still below the expectations *for Neochloris*: Around 35% [18]. However, rhamnose, the second most abundant sugar couldn't be measured, therefore lower outcomes are expected.
The outcome for glucose (8.5%) fits with the values obtained for the starch quantification. Also the ratio between galactose and arabinose is similar to the findings of Ben van den Broek, however much lower values where obtained in his case.

**Table 6: Three of the most abundant sugars in *Neochloris* in % of dry weight**

| D-glucose | D-galactose | L-arabinose | SUM |
|---|---|---|---|
| 8.5% | 8.8% | 4.4% | 21.8% |

# 4 Discussion

The need for effective storage conditions as described in the introduction led to the following research question. 'How is the biomass composition of algae affected by the storage temperature 4°C vs room temperature and gas conditions ($N_2$ vs air)?'
Differences in the external appearance (colour, smell, gas development) between the two storage temperatures were obvious. Optical differences between the gas conditions were very little. All tubes showed gas development (giving a sound and strong smell when opening the tubes). That suggests that total oxygen removal did not succeed[11]. Also the paste showed some changes in colour. The samples kept at room temperature developed a brownish colour after one week. In the fridge colour change was delayed starting after approximately three weeks.
Despite of these obvious optical differences, analytical findings indicate very little differences between storage conditions.
In the following part 'Biomass Composition' the analytical findings will be discussed in more detail; followed by a method discussion.

[11] These measurements of the oxygen samples were repeated (in a second experiment) and therefore not included, however due to a lack of alternatives the results for $N_2$ 25°C are based on these measurements.

## 4.1 Biomass Composition

**Table 7: Overview of the main results**

| | initial % | final % | relative loss | time period of loss |
|---|---|---|---|---|
| **proteins** | 41.1 | 34.5 | 16.1% | 5-8 weeks |
| **fatty acids** | 8.8 | 7.6 | 13.6% | 5-8 weeks |
| **carbohydrates** | 10 | 10 | not applicable | |
| **starch** | 9 | 1.5 | 83.3% | 1 week |

All of the in the following described findings are summarized in table 7.
A soluble protein content of 41% was found in *Neochloris oleoabundans*. During storage this content gradually decreased to 34-35% after 5/8 weeks. The fatty acid content at the beginning of storage was 8.8%. During storage an approximately 13% decrease to 7.6% was observed. The fatty acid content remained rather[12] stable in the first weeks for all four conditions, and showed a constant weekly loss of 3% starting from week 4 onwards. Sugar composition analysis suggests a carbohydrate content of 21.8%. This estimation is based on the measurement of 3 of the 4 most abundant sugars in *Neochloris*, i.e. glucose, arabinose, galactose. As expected starch reserves were quickly depleted. Within one week an 80% reduction to 1.5% was observed. Such low starch percentages cannot be seen as relevant for downstream processing, therefore starch content was not followed longer than one week.
For microalgae plenty of literature is available on the changes of lipid content and fatty acid profiles. The experimental results of this work are supported by literature findings, i.e. publications of Montaini, de Winter and Breuer. De Winter and Breuer found similar fatty acid percentages for *Neochloris* 8.8% on dry weight[19, 20]. Also the fatty acid profiles are comparable. De Winter found slightly more C18:2 and C18:3 chains, whereas a higher C16:3 percentage was found for this work[20]. Exact fatty acid composition also depends on the growth conditions, small deviations can therefore be seen as normal[21].
Also literature research confirmed that *Neochloris* does not contain any fatty chains longer than 18 carbon atoms.

[12] Showing an average loss of 1% per week

Montaini et al., who performed a similar experiment with algae storage at *4*°C in the darkness found a stable fatty acid content[11]. However, the experiment was stopped after 2 weeks. For this time period also in this work stable values were observed.

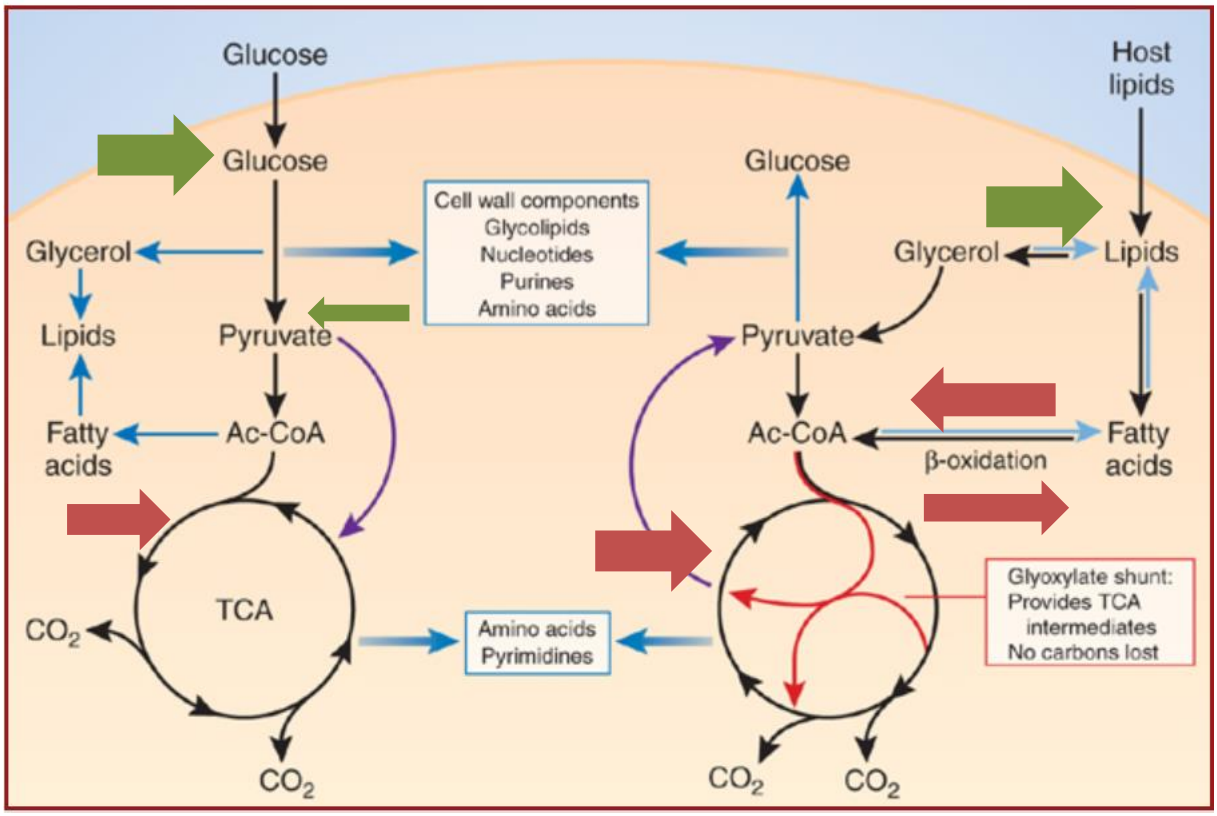
Also for the proteins experimental outcomes are supported by literature findings. Tibbetts et al. measured 30% of proteins for *Neochloris*[18]. De Winter found percentages ranging between 31 and 38% of cell dry weight[20] with the lowest value right after cell division. Comparable to starch, protein content underlies quite large natural fluctuations related to the cell cycle[20]. After cell division protein content is low in comparison to other cell components and will quickly increase in the following hours. The deviation of 7% found by de Winter makes up for more than 20% of the total protein content. However, in this work for the total storage time a relative loss no higher than 15% was observed.
Hardly any literature is available on changes in the total protein content. Only speculations on how proteins are degraded can be made. For sure microbial degradation will play a role. However, also microbes produce biomass. After freeze-drying microbial biomass will also be measured with the Lowry method[21]. The same applies for losses due to porous cell membranes: In aqueous systems proteins would diffuse out of the cells, but as all moiety is removed during freeze-drying, these proteins will be measured as well[21]. It is likely that proteins will be affected by the increased formation of radical oxygen species (ROS, for a more detailed description see appendix A). In living cells permanent maintenance of all cell components is performed. Upon starvation, peptide bonds will slowly be broken down into single amino acids, making an detection with the Lowry method impossible. Another explanation is the conversion of proteins via the citric acid cycle into energy. However, therefore enzymatic degradation into single amino acids and transport to the mitochondrial membranes is needed[22]. The yield of carbohydrates and lipids is much higher[23].
For seaweed the effect of storage has been the objective of many studies. However, as seaweed is usually produced for consumption, in nearly all studies seaweed was frozen or freeze-dried before storage. Analysis therefore rather focusses on changes in the amino acid profile (such as essential amino acids and valuable food components) than on total protein contents. Comparison of the obtained results to seaweed is therefore difficult as the storage conditions are totally different.

No reliable carbohydrate measurements could be obtained. The obtained values for sugar composition analysis are twice as high as suggested by the DuBois method (10%), but still below the expectations for *Neochloris* reported in literature around 35%[18]. However, the second most abundant sugar, rhamnose, couldn't be measured, which could explain the lower outcomes. A rather fast decrease in the sugar content is expected as small sugars (oligosaccharides) serve as primary energy reserves[21]. A more detailed description on the exact degradation mechanisms can be found in the starch section.

Starch accumulation in microalgae is quite well studied as it is closely related to increased TAG accumulation for oil production. Starch is considered a primary energy reserve as it is easily accessible for cell maintenance. Therefore starch is expected to be depleted first. Naturally starch content underlies quite remarkable fluctuations. Over day, when photosynthesis is performed, starch accumulation takes place. It is stored in granules in the chloroplasts to be consumed at night[24]. This phenomenon is commonly known as 'dark respiration'[25]. Compared to literature, the starch percentages found (8%) are rather low. De Winter, who followed the periodic, cell cycle dependent changes in biomass composition found starch percentages ranging between 16 and 25% with the lowest values right after cell division[20].

There are different pathways for the conversion of starch into energy carriers such as ATP. Under growth conditions the upper and lower glycolysis are the most common forms of energy generation[26]. However, under stress conditions such as storage in the darkness when no photosynthesis can be performed, cell metabolism switches to oxidative pathways, i.e. the pentose-phosphate pathway and glycerine degradation [25]. On short term relatively large amounts of energy are released through the oxidation. However, this leads to the increased development of reactive oxygen species (ROS, for a more detailed description see appendix A) leading to severe cell damage[26].

Lipids have a long-term energy storage function[23]. From studies on increased oil production in microalgae upon nitrogen starvation it is well known that starch can be converted into TAGs (neutral lipids) in case the cellular nitrogen content drops below a certain limit[27].
The reverse citric acid cycle pathway including beta-oxidation of lipids into Acetyl-CoA is followed when algae are exposed to stress condition such as starvation and darkness. Figure 14 provides an overview of these processes.




inner membranes of mitochondria

cytosol/ other organelles

**Figure 14: Overview of different metabolic pathways [28]**

TAGs are usually stored in small storage bodies, pyrenoids, present in the chloroplasts of many algae species. Therefore they are rather protected and do not serve as primary energy sources. Transport to the inner mitochondrial membranes is needed before being used for ATP generation (see figure 14).

General results could be supported with literature findings: The order of percentages measured for the different cell components, the fact that starch is depleted first, whereas lipids serve as long-term energy reserves. The effect of storage on lipid and starch composition has been the objective of many studies. For proteins however, the mechanisms leading to protein degradation are still unknown. For carbohydrate content somewhat literature is available.

## 4.2 Analytical Quantification Methods

Experimental findings indicate less degradation than expected based on the visual observations. There are several possible explanations for this phenomenon. First of all, the used quantification methods do not characterise the full biomass composition. Each of them provides a partial estimation on the soluble protein content (not all proteins), fatty acids (instead of total lipids), and starch (1 polysaccharide). In addition, the calorimetric methods used for soluble proteins, starch and especially total carbohydrates are generally not considered as the most precise analytical methods. A few of the limitations and possible solutions are now discussed.

### 4.2.1 Protein Analysis

Accurate alternatives to the calorimetric method for soluble protein determination are the Dumas or Kjeldahl methods. In contrast to Lowry, in these approaches the total amount of proteins is measured. Both, Kjeldahl and Dumas, can be considered precise methods for total nitrogen quantification from which the total protein content can be estimated. However, these methods are not suitable to follow protein degradation during storage time as the total amount of nitrogen stays the same. Therefore, following the amount of soluble proteins during time can be done best using the Lowry that is quantifying the number of peptide bounds present in the samples.

### 4.2.2 Lipid Analysis

GC analysis provides a valuable tool to gain more insight into the lipid and fatty acid composition of biomass. In contrast to calorimetric methods, samples can be prepared at any time and kept for later analysis. This minimizes deviations due to differences in the measurement procedure. A drawback of GC analysis is that extraction and sample preparation are quite time-consuming.

The three main groups accounting for the fatty acids are the polar glycol- and phospholipids as well as the neutral TAGs. In this work, all three classes were quantified together. Polar and neutral lipids can be separated when performing a solid phase extraction (SPE) or thin layer chromatography[10]. In the theory part it was stated that polar membrane lipids are very likely to be affected by lipolysis and especially oxidation and degradation caused by radicals. TAGs in contrast are rather protected in small storage bodies (pyrenoids).

Therefore, it would be interesting to look into the differences in degradation between these two lipid classes and evaluate if chilling damage as described in the theory part can be proofed. Based on literature findings a faster decrease in the membrane phospholipids and glycolipids is expected.

### 4.2.3 Sugar Analysis

Especially in the sugar analysis the results were lower and different than expected from literature. Considering the low obtained carbohydrate percentages, it can be concluded that either not all sugars were hydrolysed or there is a general problem with the DuBois staining.
In addition, the variation between samples and technical replicates was high (18% in average).

For a complete sugar hydrolysis a strong heat development is needed; also to avoid the formation of disturbing side-products which lead to an underestimation of carbohydrates[21]. One of these unwanted side products is probably phenolic-sulfonic[13] acid leading to a decrease in colour intensity for many hexoses and pentoses tested[21, 29]. Switching the order of reagents, starting with the sulfuric acid and adding phenol afterwards is suggested in this paper to overcome this problem[29, 30]. However, in practise no improvements were found.

The DuBois protocol relates colour development to sugar content. However, different sugars do not show the same response as glucose which was taken as standard. So if sugars others than glucose are present in larger amounts, usually this leads to an underestimation of total sugars as the absorbance will be lower. Aminosugars, for example do not show any color formation at all[31]. Figure 15 gives an overview on how outcomes are influenced by the sugar composition.

[13] In situ sulfonation of phenol

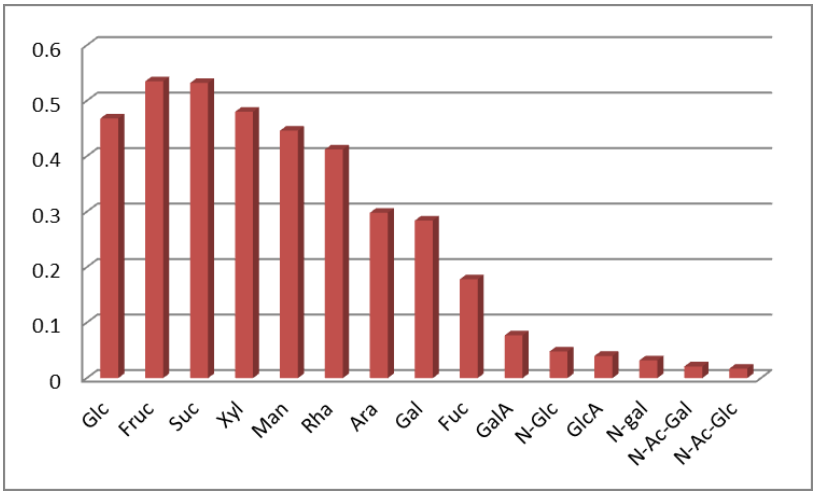



**Figure 15: molar extinction coefficients of different sugars leadings to an deviating response [32]**

An alternative to determining the total amount of sugars would be HPLC analysis in order to quantify the abundance of different sugars. Algae samples have to be hydrolysed before sugars content is determined by anionic HPLC (HPAEC).

In general slightly lower values are found when using HPAEC compared to the DuBois[21]. It is suggested that phenolic compounds naturally present in the samples also contribute in the reactions leading to an overestimation when using the DuBois method.

### 4.2.4 Starch Analysis

The starch content of the *Neochloris* biomass was low compared to literature[20], but still within the expected range when comparing to the total carbohydrate content. Also in this case insufficient extraction can be a limitation, with some starch still being trapped in the cell debris. Therefore it is not measured in the supernatant used for analysis. Also it is possible that not all starch is converted into glucose responsible for the characteristic pinkish colour the absorbance measurement is based on. Enzymatic reactions are quite sensible. Although the standard protocol was followed, conditions might have been not optimal for the enzymatic conversion (α-amyloglucase). A detection failure (colour development based on the GOPOD-reagent) can be excluded as the positive control (a certain amount of starch) showed the expected response.

## 4.3 Understanding Chilling Damage and Expanding to Biorefinery Applications

In this work the major biomass components, i.e. fatty acids, soluble proteins, starch and carbohydrates were followed. However, in biorefinery also aspects others than pure quantification will play a role: Flavour, consistency of the algae paste and especially preservation of the valuable, perhaps unstable compounds. The specific requirements on biomass composition mainly depend on the application. In case of food (algae for consumption) or food additives (i.e. ω-3-fatty acids such as EPA, DHA) the quality demands are high since the products are easily degraded and a bad odour will be problematic. Storage of algae biomass for these applications is therefore not advised. For biodiesel production in contrast, changes in the consistency and composition can be tolerated as long as losses in the lipid content stay within an acceptable range. Therefore, for each specific application an individual recommendation applies.
The extent of chilling damage could not be quantified in this work, because detecting and identifying the specific degradation products is a challenge[33]. After lipolysis (as described in the theory part in Appendix A), free fatty acids can still be turned into methylester.

However, if the length of carbon chains is reduced due to lipid oxidation and the formation of radical species, retention time will be much shorter and the fatty acids present will be considered as background noise in GC analysis. This means it is impossible to distinguish reduction in fatty acids due to degradation from losses as a consequence of inaccurate sample preparation.
There is a possible, although time-consuming solution.
Secondary oxidation products such as aldehydes, ketones and alcohols can be detected on a gas chromatography and be compared to MS-library findings. Spiking of samples with external standards of known composition and concentrations is another method used to identify degradation products.

# 5 Recommendations

De Winter showed that biomass composition is characterized by quite large natural fluctuations caused by the cell cycle including cell division. For protein content a variation (difference between the highest and lowest measurement within 24 hours) of 7% on dry weight was found[20]. This deviation makes up for more than 20% of the total protein content.

During the storage period of 8 weeks however, a difference not higher than 15% difference was measured. The same accounts for the starch content: A difference of 9% (min 16%, max 25%) means that fluctuation comprises maximal half of the total starch reserves. Given this high variance, the amount of biological replicates should therefore be considerably increased. Instead of daily sampling, several tubes of the same condition should be prepared for each measurement on a weekly base. Additionally scale-up is advised in order to obtain results applicable to biorefinery. In this experiment 3 grams of biomass were stored in falcon tubes. However, degradation kinetics will differ when a whole block of algae paste is stored. Differences in the surface area (contact area with air, microbial contamination) for sure will play a role. Also loss of viability as a consequence of drying out is more likely to occur when storing small amounts. Total oxygen removal by nitrogen flushing will not be feasible with large amounts. An interesting option to investigate would be the effect of vacuum storage to minimize microbial contamination.

If I had another 6 months for investigation, I certainly would repeat the experiment on a larger scale, i.e. instead of 3 gram 50 or 100 gram could be stored in plastic bags with a few biological replicates. Instead of nitrogen flushing, I would analyse the effect of vacuum storage on biomass perseveration. Also more sophisticated analytical methods should be tested: HPLC for sugar analysis (as described in the carbohydrate discussion part); also to determine the relative abundance of different sugars. Dumas or Kjeldahl to confirm the results obtained by the Lowry method and to quantify the soluble protein fraction. The term ‘soluble protein fraction’ is somewhat ambiguous as the amount of dissolved proteins strongly depend on the solvent[21]. Therefore a second calorimetric method such as the Bradford method could be included.
For the fatty acids it would be interesting to see if the theory of the chilling damage can be proofed. Neutral storage lipids (TAGs) and polar membrane lipids should be separated. Biomass from a nitrogen starved algae culture with an increased TAG contents should be analysed.

GC analysis combined with mass spectroscopy (GC-MS) can be used for fatty acid isomer identification in order to detect the most valuable compounds.

If I had a whole PhD study (4 years) I would immerge deeper into biorefineries. What are the most valuable compounds (i.e. antioxidants such as EPA, DHA, polyunsaturated ω-3-fatty acids) and how are these compounds preserved during storage? Does the effect of storage on different algae species differ? How is further downstream processing affected by changes is biomass consistency? Can storage eventually even contribute to shorten cell disruption time and are some components already excreted out of the porous cells? Also a detailed analysis of different degradation mechanisms would be interesting. How do the algae maintain themselves, which energy reserves are depleted first? Is there a way to influence this process?

By now, not many studies have been performed on this field, leaving room for further investigation. Based on the results obtained in this work wet storage  can be considered a promising approach, which should be further pursued.

# 6 Conclusion

Biomass composition analysis showed that the impact of different storage conditions on the composition is much smaller than expected based on optical exterior differences, smell and gas development.
In all cases losses occurred after a certain time with the exact starting point and degradation rates depending on the main components. Protein content showed a 15% loss with a steady decrease starting from the beginning onwards. At 4°C the degradation rate was reduced by 25% compared to roomtemperature. Gas conditions did not show significant differences. Fatty acid content in contrast remained rather stable for one month[14], followed by a relative weekly loss of 3%. After 8 weeks a relative loss of 13% was found. The removal of oxygen had a small beneficial effect on the fatty acid conservation. In general the values were slightly higher for $N_2$, although the degradation rates from week 4 onwards were the same for both gas conditions.
Due to doubts on the reliability of the methods, no conclusions could be drawn for the carbohydrates. Measurements indicated a stable carbohydrate content, however as a primary energy reserve a relatively fast decrease is expected.
This fast decrease was shown for the starch content (80% reduction within one week). The reduction of glucose should be visible in the carbohydrate measurements as well. Also the obtained values were about one third of what is stated in literature (10% instead of 30-35%).

Different criteria apply for different applications (food production, biodiesel, feed stock), therefore each application requires an individual recommendation. If storage is an option, mainly depends on the applications and costs involved. For high value- or food products storage at low temperature is advised, for low value applications such energy generation and biodiesel biomass deterioration is not a problem. Storage at roomtemperature is thus possible as long as economic benefits outcompete the losses.

[14] With an average loss of 1% per week

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# 8 Appendices

## 8.1 Appendix A Background Theory

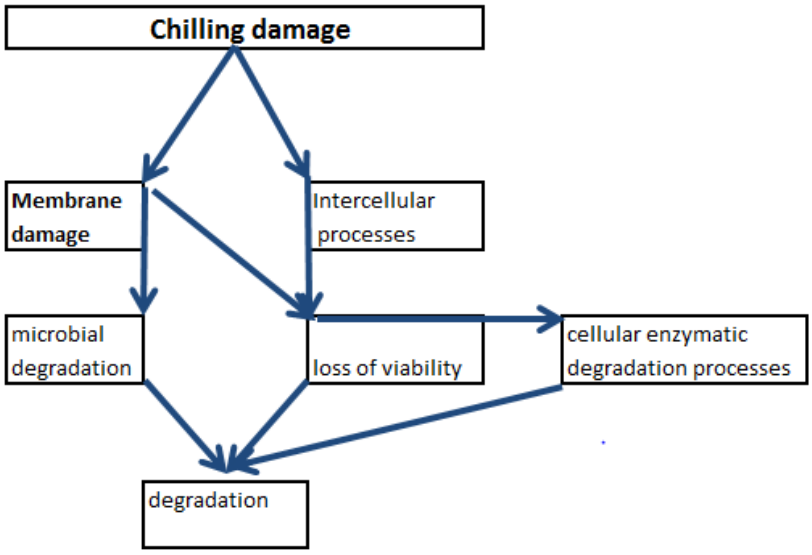


**Figure 2: Processes occurring in chilling damage**

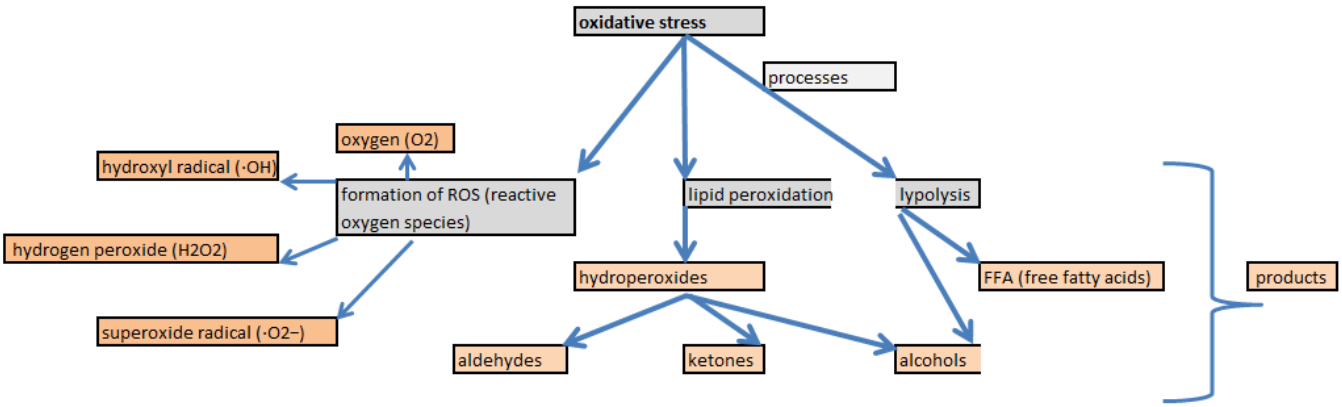


**Figure 3: Oxidative stress processes (shown in blue) & formed products (in orange)**

Membrane lipids in the outer and internal membranes, as well as intercellular compounds are degraded by reactive oxygen species
ROS caused degradation processes will lead to loss of viability of the cells which in turn will promote other intracellular enzymatic degradation processes, contributing to overall degradation of the biomass. Once the cell content is

accessible for microbes, fungi and other contamination sources, changes in composition are expected to proceed much faster. Some cell components such as water soluble proteins, depolymerized pectin or cellulose are released into the medium[34] which makes recovery more challenging. Sugar and proteins will be degraded by microbes. TAGs[15] in contrast are accumulated in so called pyrenoids[16] and will be less affected by microbial degradation.

Oxidative stress (Figure 3) is one of the first processes occurring during storage at suboptimal conditions such as too low or high temperatures, irradiation and wet storage. It is responsible for the increased formation of reactive oxygen species (ROS), lipid peroxidation and decreased membrane functionality[17][36]. ROS are defined as oxygen containing radicals such as hydroxyl radicals, hydrogen peroxides, superoxide and radical single oxygen radicals[37].
In a chain reaction ROS target cellular components leading to degradation processes and exponential increase of free radicals.
Unsaturated fatty acids are preferably targeted due to their double bonds being attacked by ROS. As a result membrane lipids will fall apart, while new radicals are created. This leads to a self-enforcing chain reaction as more and more phospholipids are degraded.

Lipid peroxidation involves two steps: First highly reactive hydrogen species are formed. These are subsequently degraded to secondary oxidative products such as aldehydes ketones and alcohols. Aldehydes are the most prevalent volatile components in microalgae and can give desirable as well as rancid flavours[38]. In case of algae storage it can be desired to remove the odour developing after some time. These aldehydes should therefore be targeted. It can be distinguished between three different mechanisms of lipid peroxidation induction: 1.Auto-oxidation when cell functionality is affected, 2. photo-oxidation through single reactive oxygen species, and 3. enzymatic degradation mainly caused by lipoxygenases (LOX)[38]. In chemical lipid oxidation shorter chain linear aldehydes are often formed by, whereas enzymatic lipid oxidation leads to branched and aromatic aldehydes[39].
In general antioxidants have a positive impact, slowing down the reaction, while high temperatures, light and air supply contribute to lipid peroxidation.
Another widely occurring phenomenon food and microalgae during shelf life is lipolysis. The term describes a hydrolysis of fatty acid esters leading to the release of free fatty acids (FFA) from triglycerides[38].High temperatures and a high water activity promote lipolysis. Also the pH is of influence. In food application this leads to unfavourable flavours[38]. It has been shown that the drying process can affect the extent of lipolysis occurring during dry storage.

[15] TAG: Triacylglycerides are used for biofuel production and can make up to 80% of the total amount of fatty acids 35. Tornabene, *Lipid composition of the nitrogen.* 1983.
[16] Pyrenoids: storage bodies similar to vacuoles located in the chloroplasts
[17] Limited functionality, e.g. loss barrier function, scavenger enzymes are weakened and restricted transport processes along the membranes.

## 8.2 Appendix B: Tables & Graphs

### Protein content in % for all 4 conditions

| days | $O_2$ 25°C | $N_2$ 25°C | $O_2$ 4°C | $N_2$ 4°C |
|---|---|---|---|---|
| **0** | 41.1 | 41.1 | 41.1 | 41.1 |
| **7** | 40.9 | 37.9 | 38.9 | 39.4 |
| **14** | 38.7 | 37.7 | 37.0 | 38.8 |
| **21** | 36.1 | 36.0 | 37.7 | 37.8 |
| **28** | 35.0 | 35.6 | 35.1 | 35.8 |
| **35** | 34.4 | 35.1 | 36.1 | 36.3 |
| **42** | | | 36.1 | 34.8 |
| **49** | | | 34.3 | 33.8 |

### 95%-confidence intervals for the proteins, when ignoring gas conditions. No overlap is found

| **temperature** | lower boundary | upper boundary |
|---|---|---|
| **25°C** | -0.208 | -0.194 |
| **4°C** | -0.168 | -0.136 |

### Fatty acid content in % for all 4 conditions

| days | $O_2$ 25°C | $N_2$ 25°C | $O_2$ 4 °C | $N_2$ 4 °C |
|---|---|---|---|---|
| **0** | 8.7 | 8.8 | 8.8 | 8.8 |
| **7** | 8.7 | 8.7 | 8.8 | 8.5 |
| **14** | 8.7 | 8.6 | 8.8 | 8.5 |
| **21** | 8.5 | 8.5 | 8.5 | 8.5 |
| **28** | 8.3 | 8.4 | 8.5 | 8.4 |
| **35** | | | 8.2 | 8.3 |
| **42** | | | 7.8 | 7.9 |
| **49** | | | 7.6 | 7.6 |

### Fatty acid profile: relative abundance of different chain lengths

| | average relative FA% | total FA% (on DW) |
|---|---|---|
| **C14:0** | 2.39% | 0.21% |
| **C16:0** | 26.73% | 2.33% |
| **C16:1** | 3.51% | 0.31% |
| **C16:2** | 5.46% | 0.48% |
| **C16:3** | 14.61% | 1.27% |
| **C18:0** | 0.12% | 0.01% |
| **C18:1** | 5.94% | 0.52% |
| **C18:2** | 19.34% | 1.68% |
| **C18:3** | 21.89% | 1.90% |

### Fatty acid kinetics: Average weekly loss for two separate intervals: Temperature clearly has an impact, the presence of $O_2$ seems to slightly speed up degradation. After one moths higher losses are observed

| time | $O_2$ 25°C | $O_2$ 4 °C | $N_2$ 25°C | $N_2$ 4 °C |
|---|---|---|---|---|
| **days 0 -28** | -1.13% | -1.09% | -0.95% | -0.95% |
| **days 28-49** | | | -3.44% | -3.19% |
| **loss after 28 days** | 4.57% | 4.68% | 3.98% | 4.60% |
| **loss after 49 days** | | | 14% | 14% |

### Mass balance: All components

| carbohydrates | proteins | fatty acids | ash (literature) | unknown | SUM |
|---|---|---|---|---|---|
| 21% | 41% | 9% | 10-15% | 14 -19% | 100% |

## 8.3 Appendix C: Protocols