Reached Deliverable

Workpackage: WP2

Activity Title: Processed microalgae for shipment strategy and feasibility

Deliverable/Output: O 3.1

Deadline: 31/03/2021

Report of the reached deliverable (max. 2 pages):

1. General introduction-

The key species involved for this output is *Dunaliella salina*, *Pavlova lutheri, Rhodomonas salina and Nannochloropsis sp.* These species are managed by various partners, which are-

- **PP9** in charge of *Dunaliella salina,*
- **PP4** (HZ) in charge of *Rhodomonas salina,* and
- **PP8** (ULille) for *Pavlova lutheri.*
- **PP1** (ILVO) for *Nannochloropsis* sp and they are involved in processing the biomass for the taste panel and also for analysing certain biochemical parameters of the algal biomass after receiving it from the respective partners.

Each partner worked on the topics of this output (listed below) either by testing the parameters on a small-scale with their respective laboratory equipment(s) or by sending it to other partners (like VITO, PP2) in large volumes mostly for the collection of biomass and also for procuring specific details, focussing on the needful points for the outputs and the deliverables of the project.

2. Objectives of the output

O3.1. Processed microalgae for shipment

- Harvested biomass will be washed to remove salt.
- Pre-treatment cell-rupture strategies will be applied to facilitate in food processing
- Methods for stabilizing biomass before shipment such as spray- or freeze-drying; the addition of anti-oxidants; adjustment of pH value will be tested.
- Effects on biomass yield and biochemical markers will be evaluated.
- Data will be collated in synthesis report, newsletter and stakeholder event (WP6).

****Lead:** PP8, **Partners:** PP1, PP7, PP4, PP9, PP10 - OP: All

3. Detailed contribution from each partner

3.1. Contribution of PP9 (UG)

Species: Dunaliella salina

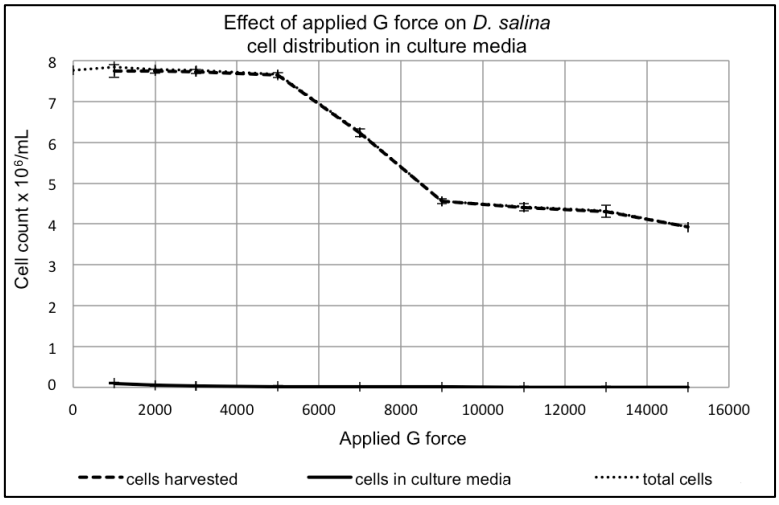
Dunaliella salina cells unusually for members of the Chlorophyta, have no cell wall; they are instead bound by a lipid membrane which is enriched (55% of total lipid) in free sterols of ergosterol and 7-dehydroporiferasterol [1]. Ergosterol is by contrast common in fungal cell membranes and protects against mechanical and oxidative stress. Ergosterol might have been selected by the pressure induced by drying/wetting cycles occurring in fungal habitats [2]; the same may be true for *Dunaliella* cells.

References-
[1] Thomas C. Peeler, Martha B. Stephenson, Kregg J. Einspahr, Guy A. Thompson. Plant Physiology Mar 1989, 89 (3) 970-976; DOI: 10.1104/pp.89.3.970 Lipid Characterization of an Enriched Plasma Membrane Fraction of Dunaliella salina Grown in Media of Varying Salinity.
[2] Dupont, S., Lemetais, G., Ferreira, T., Cayot, P., Gervais, P. and Beney, L. (2012), ERGOSTEROL BIOSYNTHESIS: A FUNGAL PATHWAY FOR LIFE ON LAND? Evolution, 66: 2961 -2968. https://doi.org/10.1111/j.1558-5646.2012.01667.x

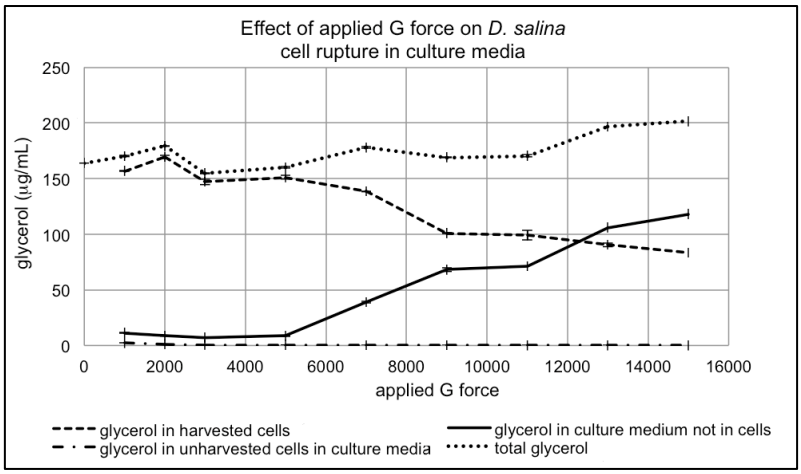
- **The effect of centrifugal force on *Dunaliella* cells**

Algal culture broth with 1.5 ml volume was centrifuged using a bench top micro-centrifuge at room temperature. Centrifugation was carried out at different speeds (1000, 2000, 3000, 5000, 7000, 9000, 11000, 13000, and 15000 g) for 10 min. The amounts of glycerol in the harvested cell pellet and centrifugation supernatant were both determined.

Results show a centrifugal G force lower than 5000 G caused little cell disruption, while a G force higher than 9000 G led to ~40% loss of the intact cells and a reduction in the glycerol yield from the recovered algal pellets.



Effect of centrifugal G forces on the distribution of intact cells of *D. salina* CCAP 19/30.

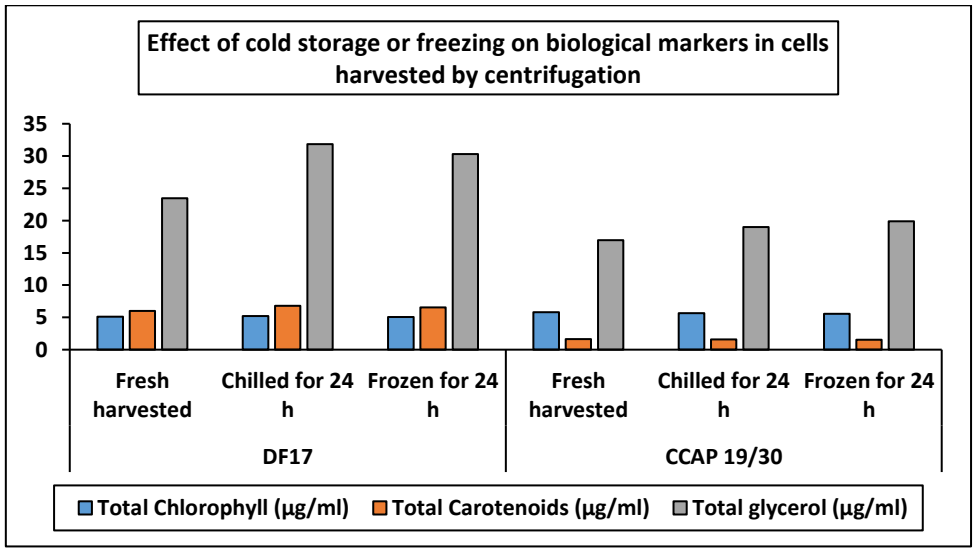


Effect of centrifugal G forces on the glycerol leakage of ruptured *D. salina* cells CCAP 19/30.

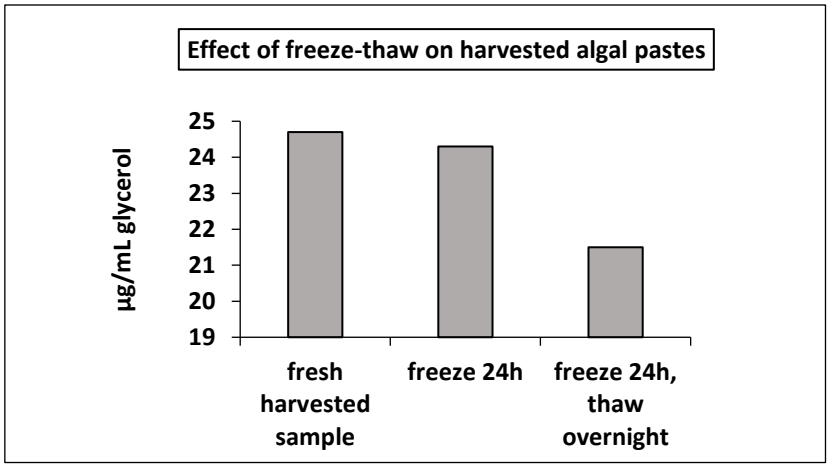
- **Evidence for biomass losses from bacteria in populations of *Dunaliella* cells**

Dunaliella cells were harvested using a laboratory bench top centrifuge at 3000 g for 10 min. Harvested cell pellets were analysed either immediately or kept at 4ºC or -20ºC and analysed after 24 hours for chlorophyll, carotenoids and glycerol content.

Cells kept chilled at 4ºC for 24h or frozen then thawed and immediately analysed were almost the same as fresh harvested samples as judged by chlorophyll, carotenoids and glycerol markers.



However, the glycerol amount decreased significantly in frozen cells that were thawed and then left chilled over-night. Microscopic analyses of algal pastes showed that bacteria were not disrupted after freeze-thaw treatment whereas *Dunaliella* cell pastes were extensively disrupted. The loss of glycerol is likely due to the presence of bacteria that consumed some glycerol.



These data indicate that *Dunaliella* cells can be collected at the centrifuge but will be disrupted, depending on the applied centrifugal force. They can be stabilised by freezing or by drying to minimise losses due to the action of bacteria, which remain intact.

The use of natural antimicrobial agents suitable for food preservation has not been tested but could also provide a means of stabilising liquid samples against biomass losses.

- **Stabilising biomass before shipment by freeze-drying followed by storage at 4°C, -20°C or -80°C**
  1. **Storage of lyophilised powder at 4°C**

In laboratory experiments we tested the use of freeze-drying techniques to preserve harvested cells on carotenoid, chlorophyll and glycerol contents. Unwashed frozen cells were taken from the -20ºC freezer, thawed and rapidly freeze-dried and the resultant lyophilised powders were stored under Argon gas in a sealed desiccator in the dark at 2-4ºC for 6 weeks.

Table 1: Storage at 2-4ºC for 6 weeks of lyophilised powder of *Dunaliella*: Effect on 3 biological markers

	Time 0 (frozen)	Time 6 weeks	% loss
	% AFDW	% AFDW	
Carotenoid	1.94	0.00045	99.98
Chlorophyll	0.39	0.00047	99.88
Glycerol	10.053 ± 2.3	12.061 ± 2.8	0

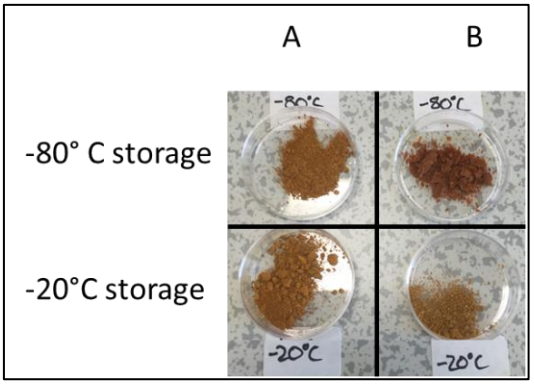
After 6 weeks carotenoids and chlorophylls were degraded almost entirely. However, glycerol content was relatively high and remained constant. Visual observation of the powder before analysis confirmed that the orange-coloured powder had turned a pale green colour following storage.

The most likely explanation for the rapid deterioration of carotenoids and chlorophyll in the unwashed freeze-dried samples may be due to the presence of glycerol in the powders which may hinder complete removal of water containing dissolved oxygen. This will in turn result in oxidative damage.

2. Storage of lyophilised powders at -20°C and -80°C

Two samples were tested that were washed and collected by flocculation using chitosan and acid to remove salt and glycerol, then neutralised. The samples were split into two vacuum bags, sealed and stored at either -20°C or -80°C. After 6 months they were re-examined visually, and by HPLC analysis of the carotenoids.

Algal powders that were washed and collected by flocculation then dried were much more stable stored at -20°C or -80°C. However, powders were slightly lighter after storage at -20°C compared to -80°C suggesting some deterioration (see below).



HPLC analysis of powders after 6 months of storage confirmed that carotenoid deterioration was evident even at -20°C compared to -80 °C. Also, the ratio of peak areas for *9-cis* β-carotene to *all trans* β-carotene was reduced compared to the original ratios. *9-cis* β-carotene appeared to be more unstable than *all trans* β-carotene.

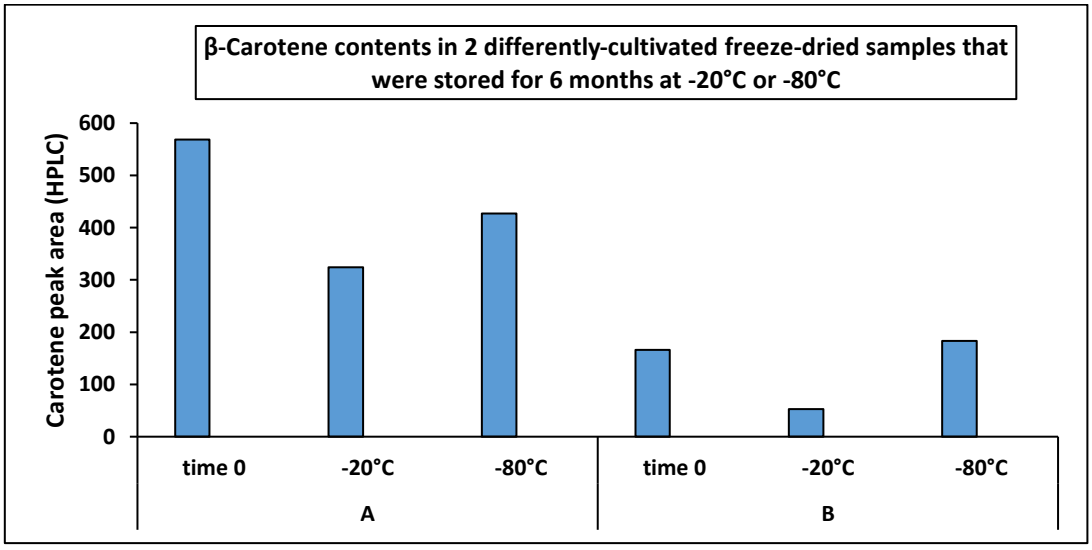


Table 2: Storage of lyophilised powder of *Dunaliella* at -20° C or -80 ° C for 6 months: Effect on 9-cis/all-trans β-carotene ratio

Sample		Ratio all-trans: 9- cis β-carotene
(A)	Time 0	0.91
	-20°C	0.364

	-80°C	0.423
(B)	Time 0	2.035
	-20°C	0.457
	-80°C	1.201

- **Conclusions from PP9-**
  1. Dunaliella cells can be readily ruptured by centrifugation or by osmotic shock treatment.
  2. Cultures are normally non-axenic and co-exist with halotolerant heterotrophic bacteria, which do not appear to be as readily disrupted, since their presence caused significant loss of sample.
  3. It is not possible to store unwashed freeze-dried *Dunaliella salina* powder, even under an inert atmosphere, at 2-4 ^{o} C, without sample deterioration.
  4. The presence of glycerol in unwashed samples may hinder complete removal of bound water containing oxygen.
  5. Harvested washed *Dunaliella* cells can be stored as either freeze-dried or spray-dried powders at -80 ^{o} C successfully in an evacuated sealed plastic bag held in the dark for some months, as judged by comparison of carotenoid profiles, and to some extent at -20 ^{o} C.

  Around 50 L of fresh culture has now been shipped to test dewatering using MAF technology. Cells will be assessed for level of intactness under the microscope and by monitoring the glycerol content

3.2. Contribution of PP8 (U-Lille)

Species: *Pavlova lutheri*

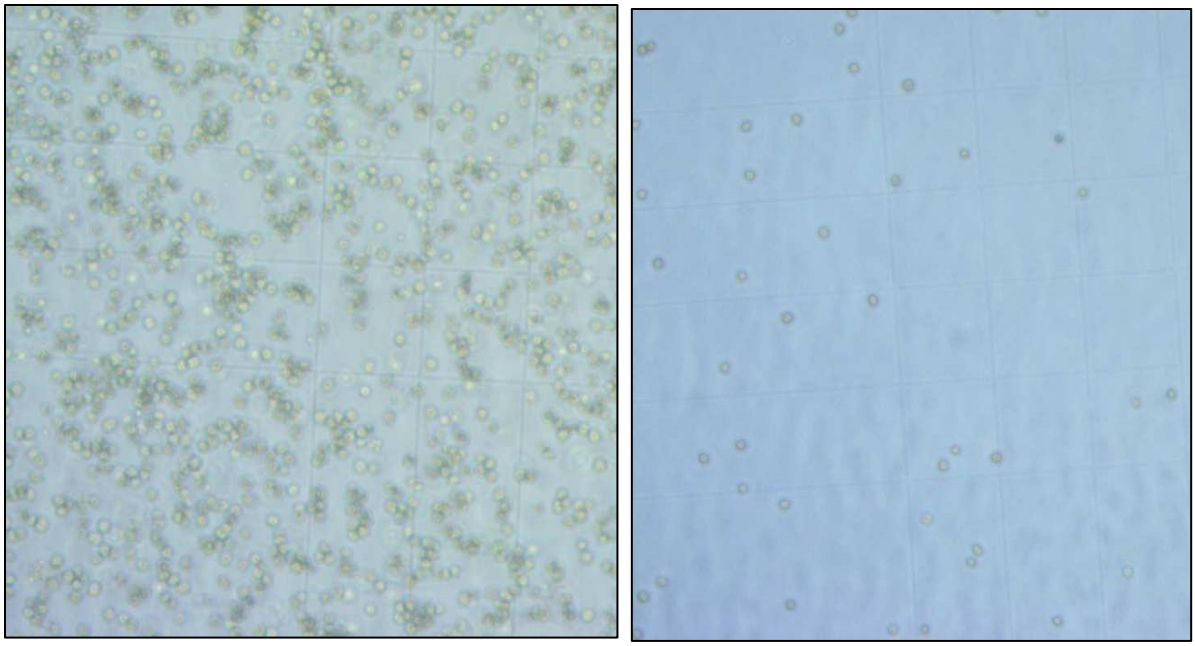
Pavlova lutheri, is a lipid rich microalga found in the marine environment, a phytoflagellate, and a common member of the Pavlovophyceae (Haptophyta), often used as a food source for aquatic feeders and are widely cultured in laboratories.

- **Cell rupture after centrifugation -**

Pavlova in our pilots is generally cultured in different batches ranging from 2L to 6L flasks up to 300L of bioreactor columns. PP8 sent two batches of *Pavlova* to VITO once in October and in December 2020 with an approximate volume of 400L on an average. Before the yield of biomass, the cells of Pavlova were washed by using the MAF system in VITO. Cells were monitored by VITO and their report on cell rupture is awaiting.

On a laboratory scale PP8 centrifuged (3000g for 20 mins) a small volume of *Pavlova* (500ml, 8 million cells/ml) on the 7th day of the growth phase (log phase) to observe the cell rupture when centrifuged. The cells were carefully observed under an inverted microscope (Olympus, IX 71, Tokyo, Japan) along with the supernatant for approximately quantifying the loss of intact cells.

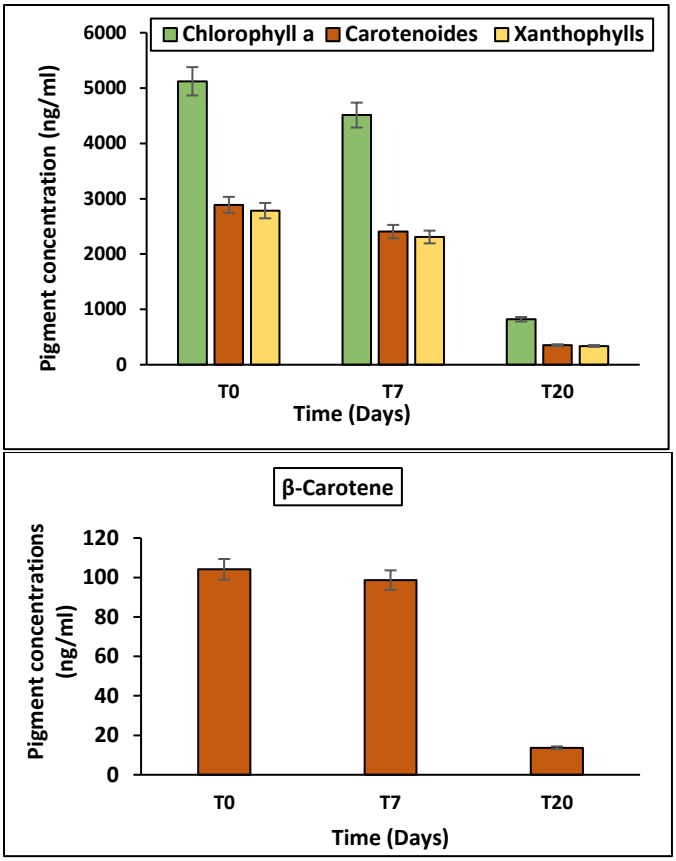
A Pavlova cells after centrifugation **B Supernatant after centrifugation**



Conclusion: Visually the cells appeared intact and on an average of three replicates approximately less than 0.1 million cells/ml lost the intactness after centrifugation given the initial cell density was 8 million cells/ml. Higher G forces will be tested on a laboratory scale before transferring to VITO.

- **Pigment profiles of the biomass stored in -20 º C after a month**

Pigment analyses were carried out for the samples collected at different phases of growth for *Pavlova* and stored in -20 º C for 4 weeks. The samples were directly thawed and subjected to freeze drying after which a profile of pigments for each sample was analysed with HPLC.



Pigment profiles of *Pavlova lutheri* at different intervals of time

Conclusion: Chlorophyll a, carotenoids, xanthophyll and β- Carotene showed gradual decrease mainly towards the end of the algal growth period (T20). The storage at -20 °C showed lower pigment concentration when compared to the samples analysed immediately on T0. Our samples were sent to VITO during the log phase (T5) by directly harvesting from the bio-reactors targeting a high profile of all the pigment concentrations for acquiring a good quality of the biomass. The biomass collected in VITO was later sent to ILVO for the taste panel. However, comparison between other storage temperature and drying methods before harvesting the biomass is on process and shall be accomplished by examining 4 °C and -80 °C storage for varying period of time to have comparative results. After this, VITO shall be coming with their MAF system to our pilot for a third round of biomass collection and further these samples will be sent to ILVO for the taste panel (probably by late 2021).

3.3. Contribution from PP4 (HZ)

Species : *Rhodomonas salina*

PP4 is based on the use of the MAF system of VITO (PP2) that can wash the cells after harvesting. PP4 assumes that VITO was taking samples during the harvesting tests to check the

disruption of the cells. VITO has no other drying method except for freeze-drying. The biomass provided to ILVO (PP1) is always freeze dried.

Moreover, we think that the adjustment of pH during the harvesting with the MAF system is not possible. The biomass that we have produced under different pH settings is according to the pH regulation in the reactor and not in the harvesting unit. So, the effect of pH on biomass yield and biochemical markers, such as pigments, volatiles, free amino acids and nucleotides will be examined, but the pH will be regulated in the reactor and not in the MAF. At the HZ we are going to run two 250 L reactors from March until July, one in pH 7, which is optimal for the growth of *Rhodomonas*, and one in pH 8.5, which is optimal for the phycoerythrin and umami taste. VITO is going to visit our facilities with her MAF systems and harvest the biomass of the reactors. This biomass will be sent to ILVO after washing in VITO's facilities. In VITO they are going to use this biomass in their taste panel. The taste panel is going to evaluate if the equivalent umami concentration that we have estimated from the free amino acid and the nucleotides can be identified in the taste.

3.4. Contribution from PP1 (ILVO)

- **Pre-treatment cell-rupture strategies will be applied to facilitate in food processing:**

High-pressure homogenization (HPH) as cell-rupture strategy on *Nannochloropsis* (big scale) was used to evaluate the effect on taste and odor.

Shortly, 15L of fresh *Nannochloropsis* paste (10% DW) was disrupted using the high pressure homogenisator (GEA Panther Model NS3006L) in the food pilot Melle (ILVO).

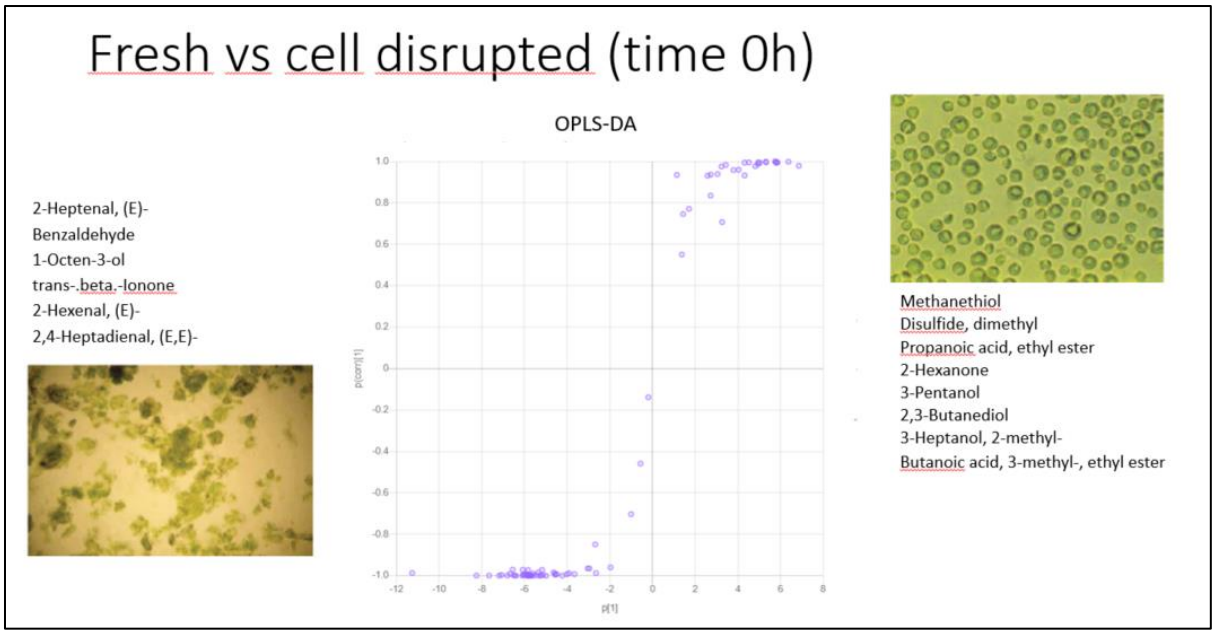
The temperature of the algae paste in the suction vessel was around ± 2°C. The paste in the suction vessel was flushed continuously with nitrogen to prevent the paste form (surface) oxidation reactions. The total pressure during the homogenization is 1200 barg (1st stage pressure: 1100barg and 2nd stage pressure: 100 barg).The paste was fed with a velocity of 100L/h. The homogenized product was passed four times through homogenizer. During the homogenization the temperature was monitored and reaches maximum values of 35°C (which is 15-20°C more than the starting temperature). After homogenization the cell disrupted algae paste was immediately cooled through a double jacket tube (spiral after the homogenizing

valves) with ice water of 4°C (end product reaches max temperature of 17°C). The homogenized and cooled product was collected in 20L vessels that was cooled with ice water and continuously flushed with nitrogen. To prevent bacterial contamination the high pressure homogenization was disinfected with K-500 solution prior use and rinsed afterwards.

After the HPH, volatiles on disrupted and fresh algae paste were determined.

Specific volatiles were detected immediately in the disrupted *Nannochloropsis* paste. Oxidation products of polyunsaturated fatty acids (PUFA) were detected forming important aromatics such as 2, 4-heptadienal, 1-octen-3-ol and 2-hexenal. Those aromatics give the disrupted *Nannochloropsis* freshly cut grass, rancid, fatty and fishy odor. In the non-disrupted *Nannochloropsis* paste other aromatics were dominant such as 2-hexanone, 2, 3-butanediol, disulfide, dimethyl which give a green/fruity, sweet, citrus, vegetable and pleasant odor.

Additionally, disrupted paste shows higher amount of breakdown products of carotenoids (e.g. beta-ionone (floral, woody odor)) and proteins (e.g. benzaldehyde (bitter almond)).



Conclusion: Disruption of microalgae cells has major effect on the volatiles (which is an indication for the flavor of microalgae). The analysis is ongoing and shall be reported soon. The disrupted and non-disrupted *Nannochloropsis* paste will be evaluated by the expert panel.

- **Methods for stabilising biomass before shipment such as spray- or freeze-drying:**

The different methods of drying like spray, oven and freeze-drying techniques on *Nannochloropsis* (big scale) was used to evaluate the effect on taste and odor.

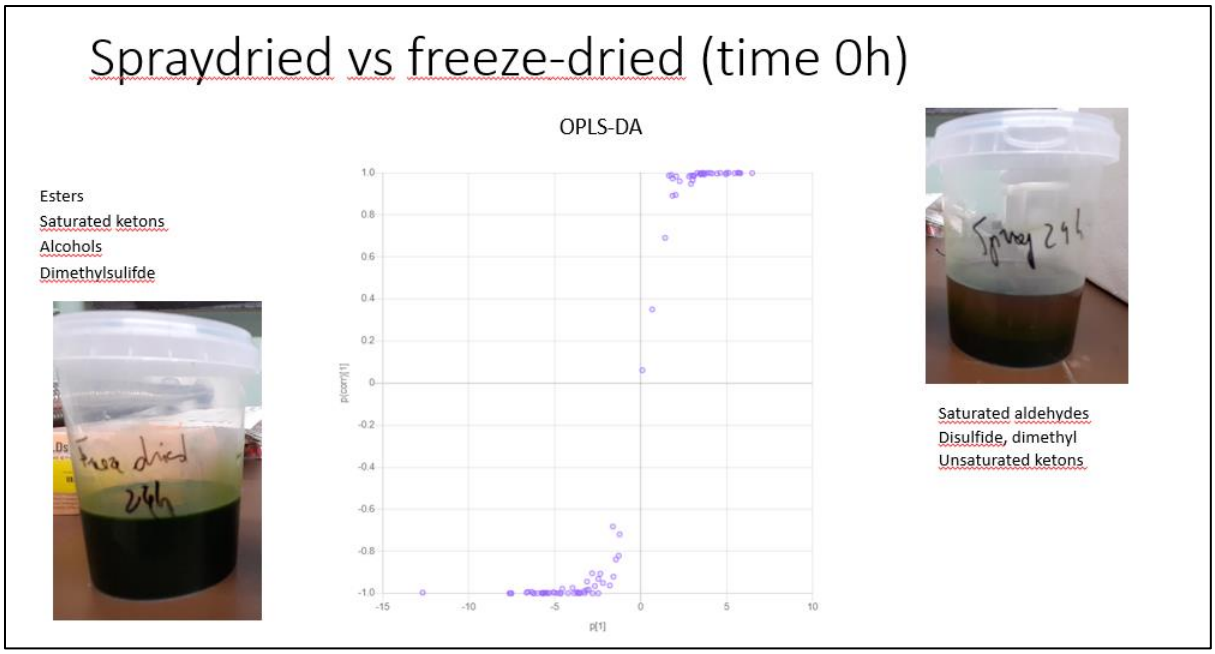
Shortly, 30L of fresh *Nannochloropsis* paste (10% DW) was spray dried in the food pilot (anhydro SPX). Inlet temperature range between 120°C ± 5°C and was fed with a velocity of 48.6 L/h. The paste was preheated to 70°C. Dried samples were immediately stored at -20°C in vacuum sealed bags and protected from light to avoid oxidation. To prevent bacterial contamination the high pressure homogenization was disinfected with K-500 solution prior use and rinsed afterwards.

One liter of fresh *Nannochloropsis* paste (10% DW) was frozen at -20°C and freeze-dried in the food pilot (Epsilon 2-10 D LSC). Dried samples were immediately stored at -20°C in vacuum sealed bags and protected from light to avoid oxidation.

Specific groups of volatiles were characteristic for freeze-dried *Nannochloropsis* such as esters, saturated ketones, alcohols which give a more fruity, citrus and fresh odor. Spray-dried *Nannochloropsis* cells contained more saturated aldehydes and unsaturated ketones indicating that oxidation of PUFA already started giving a more grassy and fatty odor.

Additionally, spray dried *Nannochloropsis* shows higher amount of breakdown products of carotenoids (e.g. beta-ionone, alpha-Cyclocitral) and proteins (e.g. benzaldehyde).

Conclusion: Drying of microalgae has major effect on the volatiles (which is an indication for the flavor of microalgae). The analysis is ongoing and shall be reported soon. The freeze-dried and spray dried *Nannochloropsis* paste will be evaluated by the expert panel.



Addition of anti-oxidants and antibiotics was tested to understand the volatile formation of *Nannochloropsis*:

Shortly, disrupted *Nannochloropsis* paste was treated with antibiotics and antioxidants. Untreated paste was used as control. Volatiles were measured during a 24h storage test at room temperature in the absence of light.

When treated with **antibiotics (Ampicillin and neomycin mix):**

- ➔ Formation of spoilage compounds (alkyl sulfides and esters) stops
- ➔ Methylated short chain carbonyls and alcohols stops (e.g. propanal, 2-methyl; 1-butanol,3-methyl)
- ➔ Production of some ketones stops (e.g. 3-pentanone,3-hexanone,3-octanone,…)

When treated with **antioxidants (BHT):**

- ➔ Production of lipid oxidation products stops (1-penten-3-ol, 1-hexen-3-ol, 1-octen-3-ol, 3-pentanone, 3-hexanone, 2-Pentenal (E), 2-Hexenal (E), 2-Heptenal (E), 2-Octenal (E),…)
- ➔ Production in oxidation products of carotenoids stops (beta-ionone, beta-cyclocitral)

Conclusion

- ➔ Bacterial activity causes formation of spoilage volatiles and degradation of existing volatiles

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➔ The addition of antioxidant limits the formation of new volatiles generated by lipid oxidation and carotenoid oxidation.

4. General conclusion-

To fulfil the objectives for this output, all the partners have worked systematically and step-wise to reach certain scientific conclusions regarding taste, texture, nutritional values, etc. Some of the analyses are ongoing and the needful results shall be reported soon. Partners are analysing critical parameters keeping in mind the objectives of the project either by utilising their own laboratory set up or by collaborating and exchanging samples, instruments and ideas with other partners involved in this project. All the partners have been responsive and willing to co-operate in order to create a network with valuable exchanges that are important for the purpose of the project and even further.