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Microalgae Analytical Handbook

Contents

Task 2.1. Genetic analysis DNA

Microalgae have the potential to revolutionize biotechnology in several areas including nutrition, pharmaceuticals, cosmeceuticals and biofuels. The biological and chemical diversity of the microalgae has been the source of unique bioactive molecules with the potential for industrial development as pharmaceuticals, cosmetics, nutritional

supplements, molecular probes, enzymes, chemicals, and agrichemicals. Microalgae biomass represents a rich source for discovery in both academic and industrial sectors. This represents the main scope of the *AlgaeCeuticals* project.

i) Microalgae Biomass production technologies. Microalgae are thought to be an attractive alternative to traditional forms of biomass for biofuel production, or to produce high added value compounds to be used for cosmetics or as nutrients due to high productivity, ability to be cultivated on marginal lands, and potential to utilize carbon dioxide (CO_2) from industrial flue gas (Schoepp et al., 2014)
As the most characterized algal species with the largest set of genetic tools and techniques, *Chlamydomonas reinhardtii* is an excellent model organism to understand and improve cosmeceuticals and nutraceutical production in algae. *C. reinhardtii* has led the field in the development of molecular tools for strain selection and engineering for green alga. *C. reinhardtii* is also among the first of the engineered algal species to be studied in commercial settings, which allows academic researchers to begin to understand the challenges of bringing transgenic algae to commercial-scale production (Gimpel et al., 2013 Schoepp et al., 2014).

ii) Microalgae "-omic" resources. Microalgae have the potential to revolutionize biotechnology in several areas including nutrition, aquaculture, pharmaceuticals, and biofuels. Advances in genomics, metagenomics, proteomics, combinatorial biosynthesis, synthetic biology, expression systems, bioinformatics, and the increasing availability of sequenced genomes provide more opportunities than ever in the discovery of novel bioactive compounds and biocatalysts from marine microorganisms. While many bacterial and fungal genomes are already available, not many microalgal genomes have been sequenced to date thus there is an urgent need for their sequence.

Work package 1:

Microalgae cultivation, screening and analysis

Task 1.1. Optimization of growth under various conditions and growth media (INAB, Ecoduna)

Cultivation of algae in CERTH has been successful in collaboration with Eparella GmbH, in respect to growth media protocols for laboratory scale given the fact that different microalgae require different growth media. Various strains have been supplied by Eparella GmbH and the main species are the following: different *Chlamydomonas* sp strains. *Arthrospira platensis*, *Arthrospira maxima, Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella minutissima*, *Haematococcus pluvialis*, *Nannochloropsis limnetica*. The maintenance of the cultures requires monthly change of the media and re-culturing. Cultures are kept in flasks or petri dishes. The photo-bioreactor is used to grow the reference and starting cultures due to the optimal controlled conditions.

Like any other organism, microalgae need a number of components to grow (Carvalho et. al., 2019). Only after population growth can there be a significant rise in biomass production, which includes the creation of energy transporters, proteins, and cell walls.

Nitrogen and phosphorus are necessary components. Additionally, a variety of minerals are required for the growth of microalgae, including Mg, which is essential to produce chlorophyll, potassium, sodium and trace elements, all of which function as co-factors for enzymes and are necessary for osmoregulation.

For the development of the cultivation, the following media recipes were used. To cultivate microalgae, a suitable growth medium is required that provides all the necessary nutrients, trace elements, and environmental conditions to support their growth and reproduction. There are several types of media available for the cultivation of microalgae.

The following tables describe the ingredients used for the media used in the lab.

Table 1. BG11

Stock Name	Components	Stock [$g*l^{-1}$ dH_2O]
1	Citric Acid*H_2O	6
	NH_4 Iron^{III} citrate	6
2	$NaNO_3$	150
3	K_2PO_4	30
4	$MgSO_4$ * 7 H_2O	76
5	$CaCl_2$ * 2 H_2O	32
6	Na_2CO_3	20
7	NaFeEDTA	1
8	H_3BO_3	2,860
	$MnCl_2$ * 4 H_2O	1,810
	$ZnSO_2$ * 7 H_2O	0,222
	Na_2MoO_4 * 2 H_2O	0,390
	$CuSO_4$ * 5 H_2O	0,080
	$Co(NO_3)_2$ * 6 H_2O	0,050
9	Hepes	200

Add the stocks in the given order to 950 ml dH2O. Set pH to 7,5 – 8,0 using NaOH. Fill up to 1000ml and sterilize by autoclaving.

Table 2. BG11 Agar

Component	[$g * l^{-1}$ BG11]
Agar Kobe	10

Sterilize by autoclaving.

Table 3. BG11 Plate Count Agar

Component	[$g * l^{-1}$ BG11]
Plant Count Agar	23,5

Sterilize by autoclaving.

Table 4. Zarrouk I

Stock Name	Components	Stock [g*l^{-1} dH_2O]
NaCl Solution	NaCl	100
NaFe - EDTA Solution	NaFeEDTA	120
K_2SO_4 Solution	K_2SO_4	100
2	$NaNO_3$	150
4	$MgSO_4$ * 7 H_2O	76
5	$CaCl_2$ * 2 H_2O	32
8	H_3BO_3	2,860
	$MnCl_2$ * 4 H_2O	1,810
	$ZnSO_2$ * 7 H_2O	0,222
	Na_2MoO_4 * 2 H_2O	0,390
	$CuSO_4$ * 5 H_2O	0,080
	$Co(NO_3)_2$ * 6 H2O	0,050

Sterilize by autoclaving.
NaCl, NaFe-EDTA, and K_2SO_4 solution are sterilized by autoclaving.

Table 5. Zarrouk II

Component	Quantity Used per Litre Zarrouk II [g]
K_2HPO_4	1
$NaHCO_3$	33,6

Sterilize by autoclaving.

Table 6. Zarrouk Vitamins

Component	[g * l^{-1} dH_2O]
B12 - Cyanocobalamin	0,005
B1 - Thiamin	0,01

Zarrouk Vitamins are filtered sterile, aliquotes into tubes (0,7 ml/aliquot), and stored at -20°C.

Table 7. Final Zarrouk

Component	Quantity used per Litre Zarrouk
Zarrouk I	750
Zarrouk II	250
Zarrouk Vitamins	0,025

Mixing of Zarrouk I, II, and Zarrouk Vitamins is done under sterile conditions! Do not autoclave the final medium!

Table 8. Zarrouk Agar

Components	Amounts make up 1 litre Zarrouk Agar
Agar Kobe	10g
5x Zarrouk I	200 ml
5x Zarrouk II	200 ml
Zarrouk Vitamins	0,25 ml
dH2O	600 ml

Autoclave 600ml 1% Agar Kobe. Afterwards mix it with 200ml sterile 5x Zarrouk I, 200 ml sterile 5x Zarrouk II, and 0,25 ml sterile Zarrouk Vitamins.

Table 9. Zarrouk Plate Count Agar

Components	Amounts make up 1 litre Zarrouk Plate Count Agar
Plate Count Agar	23,5 g
5x Zarrouk I	200 ml
5x Zarrouk II	200 ml
Zarrouk Vitamins	0,25 ml
dH_2O	600 ml

Autoclave 600ml 1% Plate Count Agar. Afterwards mix it with 200ml sterile 5x Zarrouk I, 200 ml sterile 5x Zarrouk II, and 0,25 ml sterile Zarrouk Vitamins.

Table 10. OHM

Stock Name	Components	Stock [g * l^{-1} DH_2O]
KNO3 Solution	KNO3	41
Na2HPO4 Solution	Na2HPO4	30
4	MgSO4 * 7 H2O	76
5	CaCl2 * 2 H2O	32
Trace elements	Fe^{III} Citrate * H2O	2,640
	MnCl2 * 4H2O	0,980
	CR2O3	0,075
	Na2MoO4 * 2 H2O	0,120
	CuSO4 * 5H2O	0,012
	CoCl2 * 6 H2O	0,011
OHM Vitamins	B12 – Cyanocobalamin	0,015
	B1 – Thiamin	0,01752
	B7 - Biotin	0,025
Earth Extract	Earth Extract	-

Sterilize by autoclaving.
Preparation of earth extract: autoclave one fertilizer bag in one liter of RO water for 20 minutes. Let it cool down. Then centrifuge the solution at 4000 rpm for 10 minutes and filter the supernatant with a pleated filter. Autoclave the final solution again for 20 minutes.
*OHM Vitamins are filtered sterile, aliquoted into tubes (0,7 ml/ aliquot), and stored at 20°C. OHM Vitamins are added after autoclaving of the medium under sterile conditions.
**Amount oh OHM Vitamins and earth extract equals 25% of original values.

Task 1.2. Development scale up process

CERTH and Particulla are under close collaboration and due to the secondments performed both have gained experience in algae growth. Under Eparella supervision protocols for the best suitable growth conditions for the overproduction of the desired molecules has been developed in CERTH. The protocol has been adopted from Eparella to produce large scale of algae biomass for the extraction of the biomolecules.

Task 1.3. Develop microalgae specific targeted LC-MS/MS method for mycosporin-like amino acids (FEM)

FEM performed the microalgae mycosporin-like amino acids (MAA) analysis using the microalgal strain *Chlorella,* that is considered to be an efficient MAA producer, after culturing the strain under different conditions for enhancing the production of MAA. Adequate UV stimulation was the most effective factor (Figure 1). Improvement of the

extraction protocol and the analytical procedures (combination of LC with both DAD and MS) have been also been carried out. Purification of MAA has been attempted, but results have been quite poor. Further studies are needed to acquire enough pure compounds for structural studies.

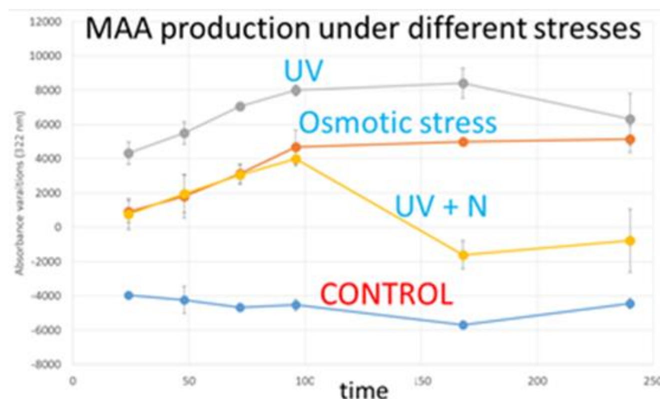


Figure 1. MAA production under different stresses

Mycosporines are a class of water-soluble, low molecular weight compounds that are produced by various organisms, including microalgae, cyanobacteria, fungi, and lichens. Mycosporines are known for their UV-absorbing properties and are believed to protect the producing organisms from UV radiation damage.
Mycosporine-like amino acids (MAAs) are a subclass of mycosporines that are structurally related to amino acids. MAAs have a high absorption coefficient for UV radiation and are therefore believed to provide photoprotection to organisms that produce them. MAAs have been found in a wide range of microalgae, including diatoms, dinoflagellates, green algae, and red algae.

A microalgae-specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method can be developed to analyze the metabolites produced by microalgae. Here is a brief overview of the steps involved in developing an LC-MS/MS method for microalgae:

1. Sample preparation: The LC-MS analysis was carried out on the hydrophilic fraction of the intracellular algal content. In brief, an aliquot of the algal culture was centrifuged andthe pellet suspended in a water/methanol mixture and sonicated. After centrifugation, the clear solution was evaporated to dryness and the residue resuspended in water.
2. The extracted samples were analyzed using a Waters Acquity UPLC system equipped with PDA detector, coupled to a SCIEX 4000 Q TRAP mass spectrometer. Ascentis Express OH5 column (50 x 2.1 mm, 2 µm) was employed as separating column (kept at25 °C).
3. The LC separation was achieved with a 8 minutes binary gradient of solvent A (acetonitrile 1 % in water, containing 10 mM ammonium formate and formic acid) and solvent B (acetonitrile 95 % in water, containing 10 mMammonium formate and formic acid). At time 0 the A:B ratio was 20:80; at 5 min 30:70; at 6 min 70:30; at 8 min 20:80. The flow rate was 0.3 mL/min. The detection

wavelength of the PDA was between 200 and 400 nm. The mass detector was operated in positive ESI mode.

4. An IDA (Information Dependent Acquisition) method was used for the screening of the algal extract. This untargeted method consisted of a initial full scan analysis (survey scan), followed by a set of two dependent analysis:
   1) high resolution scan and
   2) product ion analysis.
   The latter 2 scans were performed on the most intense peaks found during the survey scan. The 3 most intense signals found in the full scan analysis were selected for a more accurate analysis consisting of an enhanced resolution scan (for exact charge and isotopic pattern determination) and a product ion scan (for determining the fragmentation pattern).

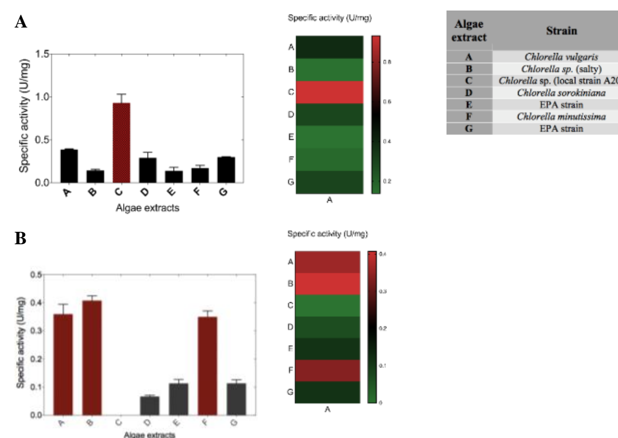
LC-MS analysis of (poly) phenolics from microalgae

Aqueous and hydroalcoholic alcoholic extracts were directly injected after extraction. Ultraperformance liquid chromatography was performed on a Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, an autosampler, and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 µm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C.
Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.4 mL/min. The gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume of both the standard solutions and the samples was 2 µL. After each injection, the needle was rinsed with 600 µL of weak wash solution (water/methanol, 90:10) and 200 µL of strong wash solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.
Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150 °C; desolvation temperature was 500 °C; cone gas flow, 50 L/h; and desolvation gas flow, 800 L/h. Unit resolution was applied to each quadrupole. Flow injections of each individual metabolite were used to optimize the MRM conditions. For most of the metabolites, this was done automatically by the Waters Intellistart software, whereas for some compounds the optimal cone voltages and collision energies were identified during collision-induced dissociation (CID) experiments and manually set. A dwell time of at least 25 ms was applied to each MRM transition. Data processing was performed using the Mass Lynx Target Lynx Application Manager (Waters).

Task 1.4. Microalgae proteases screening and analysis (AUA,)

Agricultural University of Athens (AGR_UNI_ATHENS) has been focused on screening of a wide range of algae strains for protease activity. Among other strains, the

proteolytic activity of *Chlamydomonas* sp, *Arthrospira platensis*, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella minutissima* and *Trachidiscus minutus* was investigated (**Figure 2**). Freeze-dried microalgae samples were obtained from the partner Eparella GmbH and extracted for obtaining free-cell crude extract that was used for protease activity determination. The protocol for protease extraction from microalgae was as follows: 1 g of freeze-dried microalgae sample was resuspended in 7 mL of potassium phosphate buffer. Cells were lysed using ultrasonic (4 cycles of 30 sec each). Subsequently, the samples were centrifuged (for 15 min at 16,000 g) and the supernatants were subjected to solution-based assays for total protein and protease activity analysis. Stopped and continuous spectrophotometric measurements were employed for protease activity determination in microalgae extracts, using different substrates: azocasein, albumin, azogelatin as well as azocollagen.



Algae extract	Strain
A	*Chlorella vulgaris*
B	*Chlorella sp.* (salty)
C	*Chlorella* sp. (local strain A20)
D	*Chlorella sorokiniana*
E	EPA strain
F	*Chlorella minutissima*
G	EPA strain

Figure 2. Protease activity of algae extracts. Column and heat map charts of specific activity with azogelatin (A) and azocasein (B).

The assays conditions (pH, ionic strength, temperature) were optimized, since for the development of a reliable assay protocol these conditions are considered as critical parameters. Azocazein and azogelatin was proven to be the most reliable and effective substrates. The results showed that among all microalgae strains used in the analysis, *Arthrospira platensis* exhibits the higher protease activity and was selected for further study. The kinetic parameters and substrate specificity of the protease was investigated using steady-state kinetic analysis. The enzyme was assayed using as substrate azogelatin. Kinetic analysis of the protease activity was also carried in different pH and in the presence of a range of different metal ions. The results showed that the kinetic parameters fall within the range expected for proteases and are partially dependent on metal ions. In addition, zymography will be used for detecting and characterizing the activity and isoenzymes that can utilize collagen, casein or gelatin as a substrate. The results showed one major activity band using casein or gelatin as substrate (**Figure 3**).

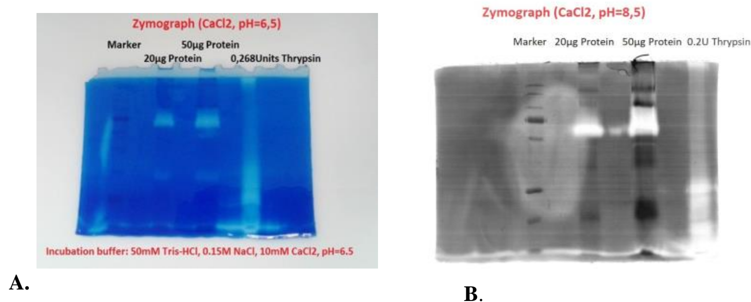


Figure 3. Zymography of *Arthrospira platensis* extract for protease detection using casein (A) and gelatin (B) as substrates.

Extraction and measurement of protease activity from *Chlamydomonas* sp., showed that its specific activity ranges between 0.2-0.5 U/mg. The effect of the concentration of total nitrogen, pH, UV light and temperature on the expression of total protease activity from *Chlamydomonas* sp. was studied. Among all tested condition, nitrogen starvation was established as a condition that strongly enhances the expression of protease activity.

Work package 2

-Omics resources for microalgae

Microalgae are unicellular organisms that have been gaining attention due to their potential for biotechnological applications. The comprehensive study of the genomics, proteomics, metabolomics, and transcriptomics of microalgae, collectively known as 'omics' analysis, is critical for the development of sustainable biotechnology. The integration of omics resources can provide a better understanding of the metabolic pathways of microalgae, their responses to environmental stimuli, and their potential applications in various fields. In this context, the objective of many research projects is to generate genomic resources using metabolomics, transcriptomics, and proteomics analysis, which will provide a comprehensive understanding of the molecular mechanisms of microalgae. These resources can help to develop novel biotechnological applications of microalgae, including biofuel production, wastewater treatment, and high-value compound extraction.

Task 2.1. Genetic analysis DNA –Barcoding (INAB).

Species identification is important as algal industries invest in technology based on the extracted end-product from several algae taxa. DNA barcoding has been successfully used for plant and algae identification. The identification is based on the genetic diversity of genomic regions characterized by their universality across diverse taxa, and their effectiveness in identifying inter-/ intra- species- specific differences. Herein, to identify the EPA-strain from Eparella GmbH we have used primers that amplify the *ITS1-ITS4* region, the Cyanobacterial 16S rRNA region and the ribulose biphosphate carboxylase (*rbcL*) restricted to photosynthetic organism's chloroplast regions

(*rbc*L_A8 and *rbc*L_D2). The *rbc*L region, despite its lower resolution capacity, it is a more suitable region for amplification, sequencing, and alignment, which makes it an ideal DNA barcode for conventional plant phylogenetic studies. More specifically, DNA-barcoding analysis was performed using the aforementioned primers and after sequencing the taxonomic identification of the strains was based on: i) the sequence similarity approach using the GenBank nucleotide database (NCBI) by basic local alignment search tool against (BLAST; setting: blastn, megablast). Correct identification was concluded when the best BLAST hit of the query sequence had over 96% query coverage and identity; ii) neighbour joining clustering method used to demonstrate the represented differences as an unrooted dendrogram using MEGA X (Kumar et al. 2018). Statistical support for each constructed tree was provided by two statistical data analysis as bootstrapping (1000 replications) and pairwise distance model. The identity of the EPA- strain from Eparella GmbH was verified as *Trachydiscus minutus*.

Table 11. Algae samples from cultures and lyophilised biomass

Species	Origin of sample	Material
Chlorella minutissima	Ecoduna/INEB	Liquid culture
Chlorella minutissima	Ecoduna/INEB	Liquid culture
Chlorella minutissima	Ecoduna/INEB	Liquid culture
Chlorella minutissima	Ecoduna/INEB	Liquid culture
Chlorella vulgaris	Ecoduna/INEB	Liquid culture
Nannochloropsis limnetica	Ecoduna/INEB	Liquid culture
Chlorella sorokiniana	Ecoduna/INEB	Liquid culture
Chlorella sorokiniana	Ecoduna/INEB	Liquid culture
Chlorella sorokiniana	Ecoduna/INEB	Liquid culture
Chlorella sorokiniana	Ecoduna/INEB	Liquid culture
Arthrospira maxima	AUA	Lyophilised biomass
Arthrospira platensis	AUA	Lyophilised biomass
Chlorella sorokiniana	AUA	Lyophilised biomass
Chlorella vulgaris	AUA	Lyophilised biomass
Crete	AUA	Lyophilised biomass
Chlamydomonas	Ecoduna/INEB	Liquid culture

The Bar-HRM technology, was used for confirmation of species identification. High Resolution Melting (HRM) analysis is based on the physicochemical properties of DNA by comparing the melting curves of different DNA amplicons and can detect even small DNA variations amongst closely related species. Herein, we used samples from cultures of *Chlorella minutissima*, *Chlorella sorokiniana*, *Chlorella vulgaris*, *Nannochloropsis limnetica*, *Arthrospira maxima*, *Arthrospira platensis*,

Chlamydomonas sp. and a Crete-strain (Table 1). More specifically, we used cultures grown in INEB and cultures from Athens. After isolating the DNA with the CTAB method and NucleoSpin® Plant II kit Genomic DNA from plant [Macherey Nagel Gmbh & Co. KG – Düren, Germany] and checking the quality and quantity we performed Bar-HRM for the molecular identification of the algae species using primers that amplify the *ITS2* and *trnL* regions. All samples were analysed in duplicate. The protocol for the Bar-HRM, follows below:

Table 12. Protocol for *ITS2* and *trnL* analyses

trnL & *ITS2* - protocol	
Buffer 1X	2,5 µl
dNTPs (0,2 mM)	0,5 µl
Forward primer (0,5µM)	1,25 µl
Reverse primer (0,5µM)	1,25 µl
Taq polymerase U/µl	0,2 µl
DNA (40ng)	2 µl
Syto™ 9 Green	0,6 µl
H_2O	16,7 µl

Additionally, samples were sent for sequencing to attain the sequences of the specific amplified regions. Using the Bar-HRM it was possible to distinguish the different algae species with *ITS2* region (Figure 4) having better discrimination ability compared to *trnL* region (Figure 5).

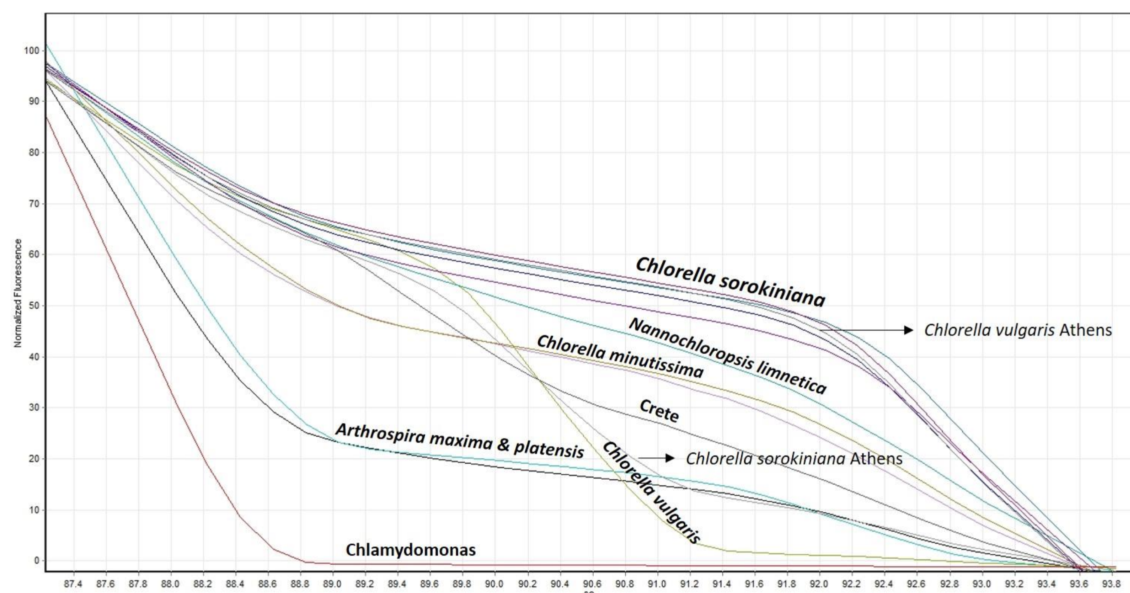


Figure 4. Normalised fluorescence graph of algae species using HRM analysis with the *ITS2* nuclear marker.

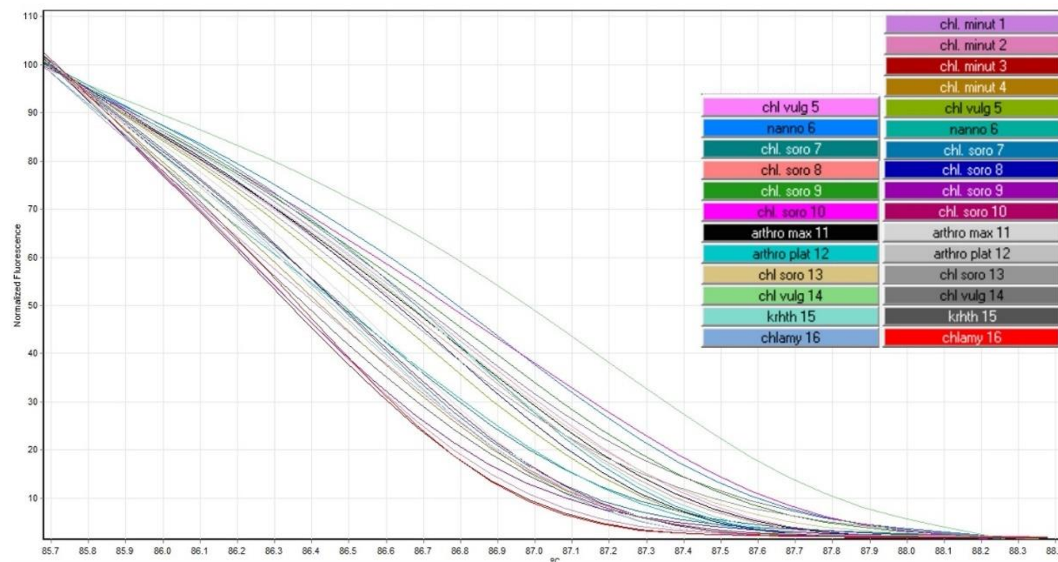


Figure 5. Normalised fluorescence graph of algae species using HRM analysis with the *trnL* chloroplast marker.

CERTH also used several isolates from Eparella GmbH, for their molecular identification based on *ITS2* and *trnL* barcoding regions. The results showed that ITS2 was a more effective barcoding region. Due to lack of reference for many algae groups the analysis was performed with 18S or rbcL, since both rbcL and ITS are broadly used for green algae.

FEM activities have also focused on the preparation and updating of bioinformatic pipelines that have been used in the analysis of high throughput sequencing data obtained within the AlgaeCeuticals consortium. In particular, all the procedures are implemented also in the context of wider active collaborations with other EU projects, i.e. Eco-AlpsWater-EAW (Alpine Space). EAW has, among its objectives, the updating and tuning of metagenomic methods for the determination of many biological elements, including bacteria, cyanobacteria and algae, i.e. key organisms that are the object of study in the AlgaeCeuticals project.

FEM performed the taxonomic determination of the algal strains used in Eparella GmbH for the inocula of bioreactors. The identification has been carried out using a polyphasic approach, based on the integration of genetic analysis, obtained by the amplification and sequencing of the 18S rRNA ribosomal gene, and morphological characterization using light microscopy (40×). Primers were specific for algal eukaryotes. After preliminary quality checking, the sequences were saved in FASTA files. The taxonomic identification of strains was based on: 1) morphological characterization of strains by light microscopy (LM); 2) direct comparison of sequences with those deposited in the International Nucleotide Sequence Database Collaboration (INSDC) using BLAST queries; 3) phylogenetic analyses using NJ (confirmed by ML). Ten sequences belonging to Chlorococcales were identified. Species were attributed to 3 groups, i.e. Scenedesmaceae, Chlorellaceae and Chlamydomonadales.

Task 2.2 Transcriptome analysis (INAB) stress experiments

CERTH standardized the protocols and methods on *Chlamydomonas* sp., regarding the RNA isolation. More specifically, protocols such as the commercially available kits Monarch RNA kit and the TRIzol® method have been used. However, the sufficient quality and yield are crucial especially, in meeting the requirements for downstream NGS applications. The cell wall of the microalga is highly resistant and extraction of nucleic acids from green microalga is hindered by the high concentration of lipids and polysaccharides that co-precipitate with the nucleic acids resulting in low yield and quality. The optimization of high-quality RNA isolation from the microalga especially for next-generation sequencing, was further proceed using the TRIzol method with additional steps of homogenization with liquid nitrogen and/or precipitation with NaCl, and the commercial Spectrum™ Plant Total RNA kit.

Several laboratory culture experiments have been performed along with stress experiments including N_2 starvation, low pH, UV light and low temperature stresses to induce production of high added value biomolecules. Algae have developed unique protection mechanisms to cope with the extreme environments they are exposed to. As a protection mechanism, microalgae accumulate metabolites induced in response to abiotic stress conditions. Microalgal metabolites, such as proteins, enzymes, carbohydrates, lipids and trace nutrients are high-value biomolecules of great commercial importance. However, the underlying mechanisms and the molecular pathways involved in this process are unknown. In the present work, a transcriptomics and proteomics systems-based approach was employed to characterize the metabolic pathways activated under stress in the green alga ***Chlamydomonas sp***. A two-stage cultivation system was employed. Initially, the algae were grown in a small scale photobioreactor under optimum conditions to generate the biomass, and later were exposed to low temperature (4°C), nitrogen (N_2) deficiency, low pH (5.4), and UV light for stimulating the accumulation of metabolites by the cells due to the abiotic stress. During the time course experiment across four time points the differential gene expression of the strain to the different stress conditions was investigated. The experimental set up had 3 replications per treatment per time point. We collected samples for transcriptomics analysis at 8 and 24hours and 8 and 15days that were performed in CERTH. Once the quality and concentration of RNA were confirmed, the RNA samples were processed to generate RNA libraries using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® **(**New England Biolabs, Ipswich, MA). After library preparation, the concentration of the resulting RNA libraries was quantified using a Qubit fluorometer. To enable multiplexing, i7 and i5 indexes were added to the individual RNA libraries. These indexes allow multiple libraries to be pooled together and sequenced simultaneously, with each library's reads later separated based on their respective indexes. To check the size distribution and quality of the prepared RNA libraries, a fragment analyzer system was used. After pooling and cleaning up the samples, the Qubit fluorometer was used again, to determine the quantity of the pooling samples. Next, a qPCR was performed to accurately determine the concentration of the prepared RNA libraries and then we proceeded with the

Illumina sequencing protocol, where the RNA libraries were ready for sequencing using the Illumina platform.

For the proteomics analysis the samples were collected at 8, 24, 8 and 15days and after being pelleted down they were frozen in liquid nitrogen and the biomass was lyophilised. Regarding low temperature treatment we had 3 replications per time point and the sampling for transcriptomics analysis occurred on 3, 6 and 24 hours and 4 days whereas for the proteomics analysis the samples were taken at 3, 6 and 24 hours and 4 days. The collected samples were centrifuged to remove the excess of the culture medium and then were either stored in -80o C for RNA isolation and Transcriptomics analysis or after being pelleted down they were frozen in liquid nitrogen and the biomass was lyophilised for the proteomics analysis.

CERTH has also developed a transcriptomics analysis workflow using reference RNA-seq data of *Chlamydomonas reinhardtii* under diel (exogenous) conditions to identify cycling genes (Figure 6). The reference organism data are available here: ftp://ftp.ensemblgenomes.org/pub/plants/release-42/fasta/chlamydomonas_reinhardtii/ and the data used are available here: https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-62671/.

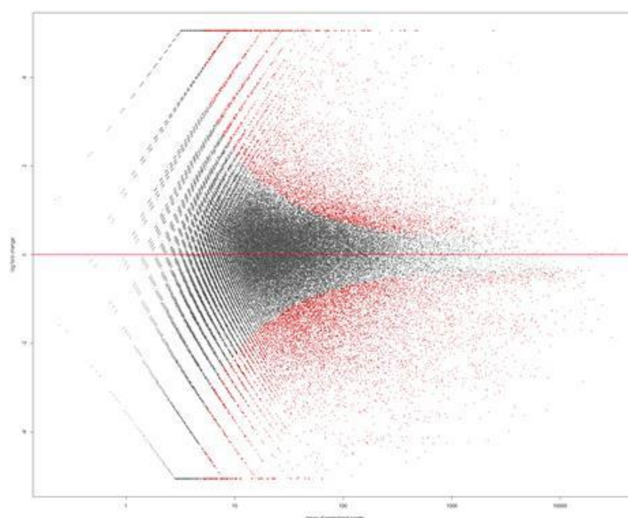


Figure 6. MA plot for visualization and identification of gene expression changes from two different conditions (control vs. treated) in terms of log fold change (M) on Y-axis and log of mean of expression counts of control and treated samples (A) on X-axis.

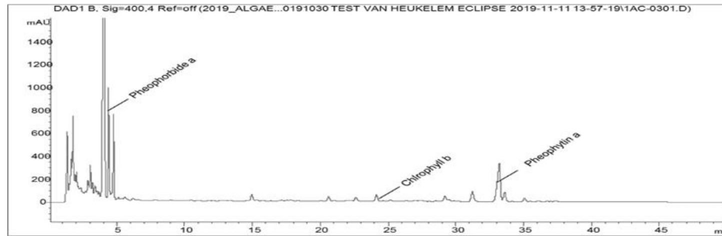
Task 2.3. Metabolomic analysis of microalgae under different culture conditions (FEM).

For the carotenoid profiling in algae samples, FEM used a UPLC-DAD based method. This method was more stable and showed effective separation of the algae carotenoids in a suitable time frame of 50 min per run.

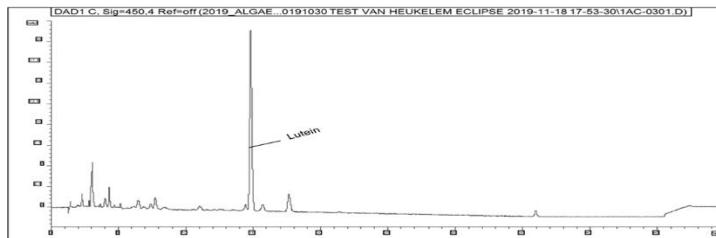
Two different extraction methods were compared: 1) fast extraction with methanol and 2) extended hexane extraction including saponification. Various samples (different cultured algae strains and oils obtained from Eparella GmbH were extracted and screened for carotenoid profiles. Methanol extracts (1) show mainly chlorophylls and related derivatives, while these metabolites were almost absent after saponification

within the hexane method (2). Here the carotenoids became more visible for *Chlorella*, *Nannochloropsis* and Spirulina (Figure 7). Several more common carotenoids could be identified by co-chromatography, yet identification of detected unknown carotenoid-like peaks (predicted from UV spectra) in the extracts is required.

Chlorella

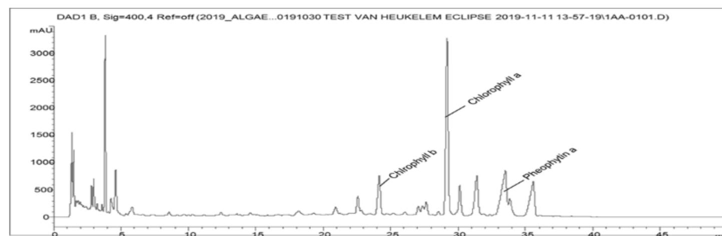


MeOH

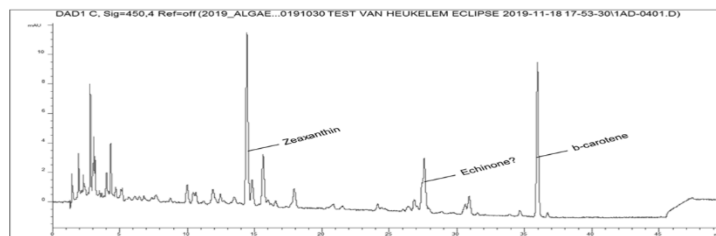


Hexane

Nannochloropsis

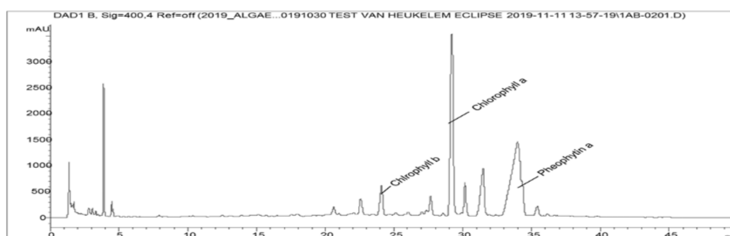


MeOH

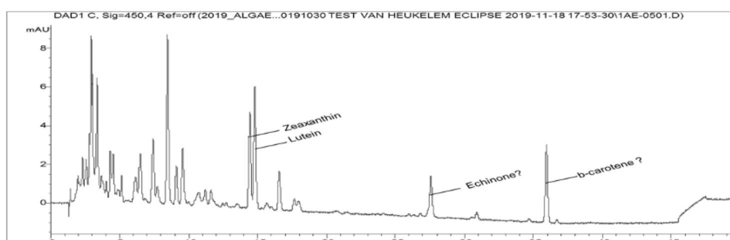


Hexane

Spirulina



MeOH



Hexane

Figure 7. Detection of carotenoids for the *Chlorella* sp., *Nannochloropsis* and SPIRULINA algae using two different extraction methods.

Task 2.4. Algae protein screening and proteomics analysis (IGV Greenfood).

Cell disruption, extraction and fractionation method for total protein separation was achieved. Optimization of a multi-step processes (extraction, separation and partial purification) for total proteins has been performed in order to obtain a sufficiently high yield total protein for developing products with hair and skincare properties. Moreover, a protocol for catalomics analysis based on: (1) solution measurements (stopped and continues assays); and (2) SDS-PAGE for the analysis of total protease activity from microalgae extracts was developed and optimized. The protocol was further validated using extracts from different microalgae species.

Work package 3.

Functional analysis and bioactive properties of microalgae derived products

Examining the functional properties and bioactivity of microalgae-derived products through in vitro enzyme assays and human cell line studies is crucial for identifying bioactive compounds, ensuring product safety, gaining mechanistic insights into their effects, optimizing production processes, and facilitating product development and commercialization. It provides valuable information for the development of new drugs, functional foods, and nutraceuticals, contributes to our understanding of cellular processes and therapeutic targets, and enables the creation of high-quality, standardized products with specific health benefits.

Task 3.1. In vitro enzyme bioassays.

Screening and examination of the bioactivity of selected algae extracts have been carried out using in vitro enzyme assays by AGR_UNI_ATHENS. The work focused on evaluating the inhibition potency of microalgae extracts (*Arthrospira platensis, Chlorella vulgaris, Chlorella sorokiniana, Chlorella minutissima, Chlamydomonas reinhardtii* and *Trachidiscus minutus*) towards enzymes that regulate anti-wrinkle, anti-inflammatory and skin-whitening effects.

For assessing the inhibition potency of microalgae extracts, we employed a range of different assays. The following assays were employed: a) elastase inhibition assay for the assessment of anti-wrinkle, anti-inflammatory and antiaging potential of the extracts, b) tyrosinase inhibition assay for assessing the skin-whitening properties of the extract. Elastase was assayed spectrophotometrically using succinyl-Ala-Ala-Pro-p-nitroanilide as substrate and monitoring the release of p-nitroaniline at pH 8 and at 25°C. The amount of p-nitroaniline was determined by measuring the absorbance at 410 nm. The tyrosinase inhibitory activity was determined by measuring the amount of dopachrome formation at 475 nm using as substrate L-Tyr.

The results showed that the *Trachidiscus minutus* extract displayed high inhibition potency towards elastase (Figure 8), suggesting a potential activity in controlling skin aging and inflammatory process, and was selected for further study. In addition, the total antioxidant capacity of algae extracts was measured based on different assays such as Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC), Folin-Ciocalteu, DPPH and ABTS Radical Scavenging Assays (Figure 9). TAC measurements indicated significant differences among the different strains indicating a large chemical diversity of antioxidants. Among all different strains *Trachidiscus minutus* extract showed the highest total antioxidant capacity. Taken together, these results demonstrate that *Trachidiscus minutus* extract can provide promising extracts rich in antioxidants, anti-aging and skin-whitening ingredients with the potential for use as raw material in cosmetics industry.

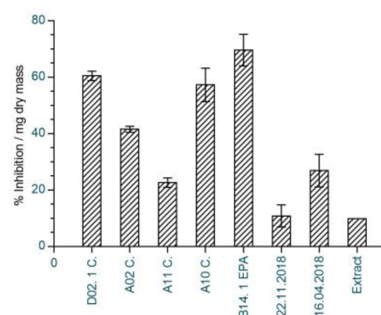
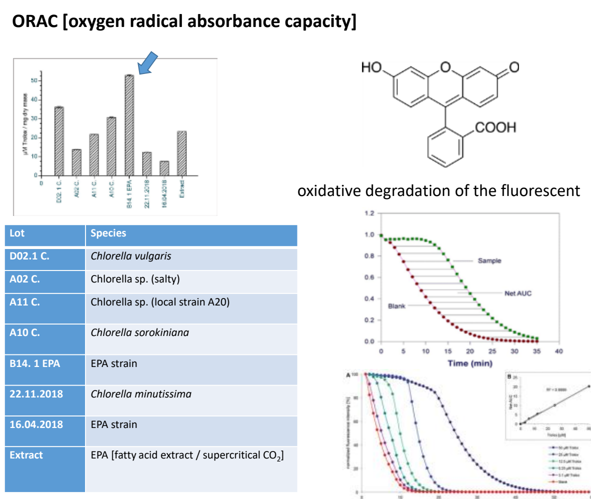


Figure 8. Inhibition potency of algae extracts towards elastase. Where: D02.1 C: *Chlorella vulgaris*; A02 C: *Chlorella sp*. (salty); A11 C: *Chlorella sp*. (A20); A10 C: *Chlorella sorokiniana*; B14. 1 EPA: EPA strain; 22.11.2018: *Chlorella minutissima*; 16.04.2018: EPA strain; Extract EPA: fatty acid extract / supercritical CO_2.



Lot	Species
D02.1 C.	*Chlorella vulgaris*
A02 C.	Chlorella sp. (salty)
A11 C.	Chlorella sp. (local strain A20)
A10 C.	*Chlorella sorokiniana*
B14. 1 EPA	EPA strain
22.11.2018	*Chlorella minutissima*
16.04.2018	EPA strain
Extract	EPA [fatty acid extract / supercritical CO_2]

Figure 9. Total antioxidant capacity of selected microalgae extracts using the ORAC method.

Task 3.2. Transcription effect of microalgae extracts or compounds on skin-related genes using human cell lines.

Bionos Biotech has tested several algae derived extracts and products using *in vitro* assays on human cell lines. The first products examined were Orange Algae and Black algae, provided by Eparella Gmdb. Cell viability and cytotoxic potential was assessed for the different products after treatment on human cell lines in culture (*in vitro*), to

determine non-toxic and effective concentrations to be used for subsequent functional studies (*in vitro* antioxidant and anti-inflammatory capabilities).

Normal Human Dermal Fibroblasts (NHDF) and HaCaT keratinocytes were used. The analytical equipment used, follows below: inverted microscope, laminar flow hood, cell culture incubator (37°C, 5 % CO_2, 90 % relative humidity (RH), Bürker chamber, pipettes, rack and consumables. Regarding the reagents, the following were used: Distilled Water (Braun), specific culture medium and supplements for each cell line (PromoCell), PBS (Gibco), Trypan Blue (Bio-Rad), Trypsin (Sigma Aldrich), Dimethylsulfoxide (DMSO, Sigma Aldrich), MTT reagent [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (Invitrogen). Cell number and viability of the initial cell cultures were determined using Trypan-Blue staining and counting in a Bürker chamber under the microscope. Human cells were cultured overnight at a 10.000 cells/well density in a 96 well plate, in supplemented growth medium. 24 h later, the culture medium was replaced with fresh medium containing the tested sample at 8 different concentrations. After 24 hours of incubation, the medium was removed, and MTT solution was added to each well. Plates were incubated at 37ºC for 3 h. MTT reactive solution was removed, and 100% DMSO was added to each well to solubilize formazan crystals prior to absorbance measurements at 550 nm and 620 nm as reference on a scanning multi-well spectrophotometer. Results indicated that products Orange Algae and Black Algae do not present any significant cytotoxicity for Normal Human Dermal Fibroblasts (NHDF) and HaCaT keratinocytes cells after 24 hours treatment.

To evaluate the antioxidant capacity, the following were used: microscope, laminar flow hood, incubator, refrigerated centrifuge, statistical analysis software, micropipettes, pipettes, propipette, rack, Glomax Discovery Promega Multi-mode detection system, microcentrifuge tubes, vortex, Bürker chamber, heating block, and consumables. Cells were cultured overnight at 10000 cells/well density in a 96 black well plate, in growth media. 24 hours later, medium was replaced by fresh medium containing the tested products at the selected concentrations. Cells were incubated in the presence of products for 24 h more. Then, Fluorometric Intracellular Ros Kit was added to wells following manufacturer's instructions and cells were irradiated with 2.5 J/cm^2 UVA radiation. After irradiation, cells were incubated at 37ºC for a maximum of 2 hours since the addition of ROS reagent. Finally, fluorescence was measured at λ_{ex} = 540/λ_{em} = 570 nm using 5 technical replicates per condition. Results indicated that treatment with Orange Algae and Black Algae significantly protected from ROS accumulation.

Additionally, anti-inflammatory capacity was studied through gene expression, after treatment with LPS (Lipopolysaccharide). For the gene expression the following were used: Inverted microscope, laminar flow hood, cell culture incubator (37°C, 5 % CO_2, 90 % relative humidity (RH), Bürker chamber, pipettes, rack, Nano-Drop spectrophotometer, vortex, Quant studio 5 (Applied Biosystem) Quantitative real-time PCR (ThermoFisher), heating block, thermocycler. Regarding the reagents: Distilled Water (Braun), cell culture medium and supplements, PBS (Gibco), Trypan Blue (Bio-Rad), Trypsin (Sigma Aldrich), LPS (Sigma Aldrich), RNeasy extraction kit (Qiagen), DNAse-I (Qiagen), PrimeScript RT reagent kit (Perfect Real Time, TaKaRa),

oligonucleotides for qPCR amplification TNFα, IL6, IL8 and ACT TaqMan ® qRT-PCR. For the gene expression assay, cells were cultured in supplemented growth medium at a density of 100,000 cells/well in 24-well plates at 37ºC, 5 % CO2. 24 hours later the medium was replaced with fresh medium containing LPS (1 µg/ml) to induce an inflammatory response. After 30 min incubation, the tested products were added at the selected concentrations and incubated for 24 h more at 37ºC, 5 % CO2. After that, cells were collected in lysis buffer to proceed with the RNA extraction. Total RNA was extracted using RNeasy kit (Qiagen) and treated with DNAse-I to remove any contamination from genomic DNA. RNA quality and quantity were checked in a Nano-Drop spectrophotometer, and 1 µg of total RNA was used to synthesize cDNA, using First-strand Synthesis kit (TaKaRa). Finally, quantitative PCR (qPCR) was performed in a real time PCR machine (QuantStudio 5, Applied BioSystem). To perform raw data analysis, we used the 2–ΔΔCt method (Livak & Schmittgen, 2001) to calculate the gene relative expression ratio to non-treated control (C). TNFα, IL6 and IL8 gene expressions were assessed. Actin (ACT) was used as a reference housekeeping gene. Results indicated that treatment with LPS significantly increased *TNFα* expression in HaCaT and NHDF, compared to the untreated control, showing that LPS was activating the inflammatory response through these metabolic pathways, and treatment with Orange and Black Algae significantly reduced *TNFα* expression, showing anti-inflammatory activity (Figure 10).

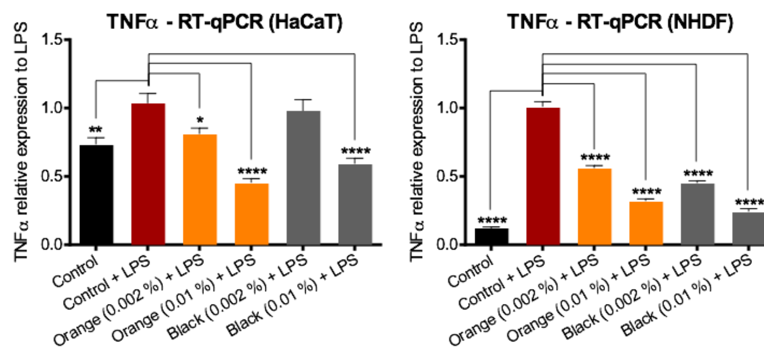


Figure 10. *In vitro* toxicity tests

Task 3.3. In vitro toxicity assessments of microalgae extracts or compounds.

The *in vitro* toxicity assessments of microalgae extracts were performed by Bionos Biotech on two crème formulations provided by Eparella GmbH: Creme 1% Pulver Spirulina and Creme 1% Extract Spirulina (Figure 11).

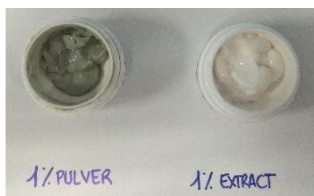


Figure 11. Creme formulations containing on the left: 1% Pulver Spirulina and on the right: 1% Extract Spirulina.

Cell viability was assessed to determine non-toxic and effective concentrations to be used for subsequent functional studies. HaCaT, NHDF and Normal Human Epidermal Melanocytes (NHEM) were used. Results indicated that 1% Pulver and 1% Extract present significant cytotoxicity at high concentrations after 24 hours treatment, meaning that concentrations from 0.03% and

0.01% may be use for subsequent assays, without any relevant decrease in cell viability. After that, the transcription effect of these creme formulations on skin-related genes (*Collagen1* and *Elastin)* was performed in NHDF. Results indicated that 1% Pulver and 1% Extract promote the synthesis of Collagen and Elastin, even thoug h results were not statistically significant for Elastin expression (Figure 12).

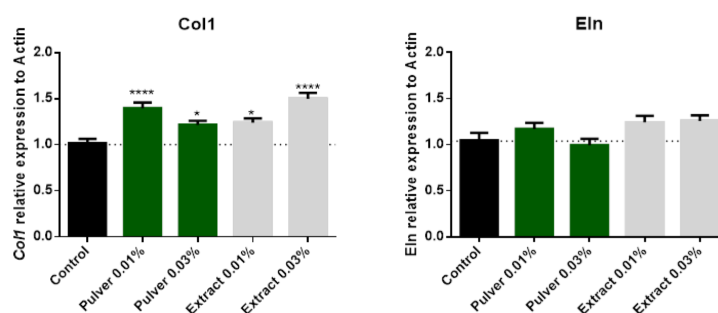


Figure 12. Relative expression of Col1 and Eln genes relative to the reference actin gene.

Additionally, possible tanning or depigmentation effects were evaluated using Melanocytes repeatedly exposed to ultraviolet radiation to mimic sun exposure. Inverted microscope, laminar flow hood, cell culture incubator (37°C, 5 % CO2, 90 % relative humidity (RH), Bürker chamber, pipettes, rack, heating block, plate reader spectrophotometer, UVA irradiator and UVA irradiance sensor were used, as well as distilled Water (Braun), melanocytes cell culture medium and supplements, PBS (Gibco), Trypan Blue (Bio-Rad), Trypsin (Sigma Aldrich) and sodium hydroxide (NaOH, Sigma Aldrich). Cells were seeded in 24-well plates at a density of 2x104 cells/well and incubated overnight at 37 ºC in a CO_2 incubator. Culture medium was then removed and replaced with fresh medium containing the tested products at the selected concentrations and incubated for 24 h in the presence of the product. To mimic sunlight exposure, cells were irradiated with UVA light in 3 cycles of 15 - 20 min over 48 h. The irradiance measurements indicated a total dose of 3.5 J/cm2. After the last cycle, cells were maintained at 37 ºC in a CO_2 incubator for a resting period of 24 h to allow melanin accumulation. Then, the supernatant of the cells was collected, and melanin was quantified by measuring the absorbance at 405 nm. On the other hand, melanin was extracted from attached cells by adding NaOH 1 M, incubating at 60 ºC for 1 h and quantifying melanin levels by measuring the absorbance at 405 nm, as reported elsewhere [Kollias and Bager, 1987; Dwyer et al., 1998; Ou-Yang et al., 2004; Kollias and Bager, 1985]. A MTT assay was performed in parallel under the same conditions to correct melanin quantification fluctuations due to changes in cell viability.
Results indicated that 1% Pulver and 1% Extract significantly decreased melanin levels compared to the irradiated untreated control, displaying whitening and protective effects against UVA-induced melanin synthesis (Figure 13).

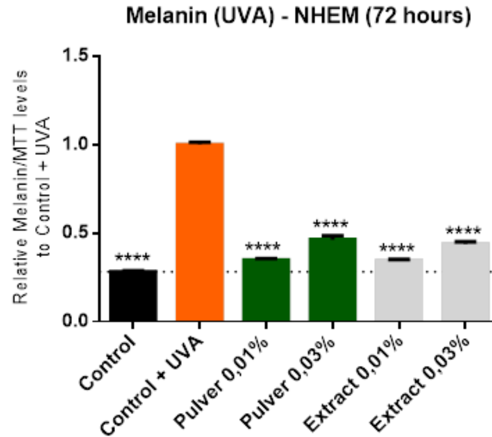


Figure 13. Induction of melanin synthesis. Cell viability quantification through MTT assay after 72 hours. The untreated control was used as reference control.

The cosmetic efficacy of these creme formulations was evaluated *in vivo* after topical treatment in forearms of 15 human volunteers, through quantification of skin hydration using Corneometer®. Results indicated that treatment with 1% Pulver and 1% Extract significantly increased skin hydration after 1 hour, 6 hours and 8 hours of treatment, respectively, compared to basal values (0 hour) (Figure 14).

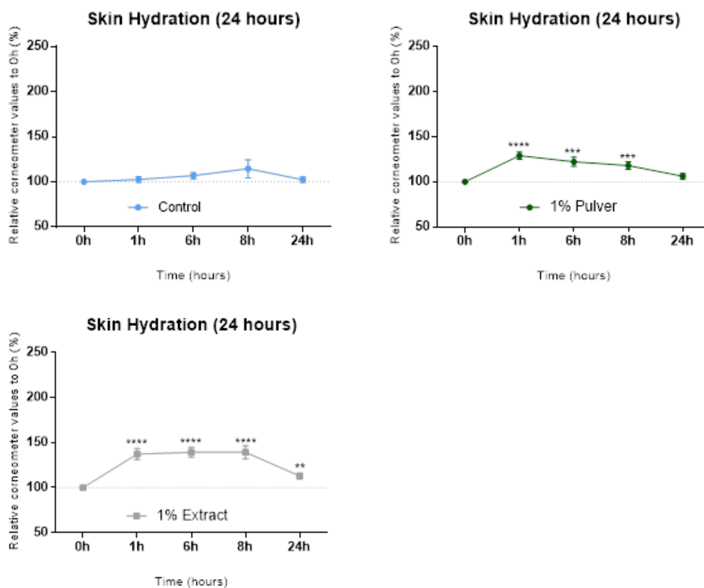


Figure 14. *In vivo* cosmetic efficacy of creme formulations after topical treatment in forearms of 15 human volunteers, through quantification of skin hydration and trans-epidermal water loss (TEWL) using Corneometer®.

An anti-pollution study was conducted, based in the protocol explained in Antioxidant activity – ROS quantification, but cells were pre-treated with Urban Dust instead of UVA radiation. The analytical equipment used, follows below: Inverted microscope, incubator, statistical analysis software, thermoblock, refrigerated centrifuge, micropipettes, pipettes, Glomax Discovery Promega Multi-mode detection system, laminar flow hood, transparent and black cell culture plates. Cells were cultured overnight at a 10.000 cells/well density in a black 96-well plate, in growth media. 24

hours later, the culture media was removed and substituted for new culture medium supplied with the tested products. Urban Dust suspension was sonicated for 30 minutes to avoid aggregation of particles before adding to cell medium. After 24 hours of incubation period, culture medium was replaced by PBS and ROS master mix in all cultured wells for 1 hour, including 2 blank controls (wells without cells for basal signal determination). Non-treated controls with cells were incubated at 37ºC during this time in the dark. Immediately after the treatment, ROS amount was measured in all samples. Fluorescence quantification was measured at λ_{ex}=490/ λ_{em}=525. In parallel, cell viability was quantified through MTT assay, which allowed to normalize ROS levels to the number of live cells to avoid false positive due to Urban Dust-induced cytotoxicity. 6 technical replicates for all the conditions were used.

Work package 4.

Downstream processing and purification strategies

Downstream processing and purification strategies play a vital role in the identification, fractionation, and characterization of high-value added enzymes and polysaccharides derived from microorganisms, including microalgae, for potential applications in cosmetology. These strategies are essential for isolating and purifying specific bioactive compounds from complex mixtures, removing impurities, and obtaining products with high purity, bioactivity, and stability. By effectively separating and characterizing these enzymes and polysaccharides, their unique properties and functions can be elucidated, enabling their formulation into cosmetic products that offer desirable effects such as moisturization, anti-aging, skin rejuvenation, or wound healing. Moreover, downstream processing and purification techniques ensure consistency, scalability, and regulatory compliance, thus providing a solid foundation for the development and commercialization of cosmetically relevant bioactive compounds derived from microorganisms.

Task 4.1. Development of efficient cell disruption, extraction and fractionation method.

AGR_UNI_ATHENS performed the optimization of a multi-step processes (extraction, separation and purification) for total proteins, in order to obtain a sufficiently high yield and low cost to allow a cost-effective scale-up of the entire process, for products with hair and skincare properties. Efficient algae cell disruption (*Arthrospira platensis*, *Chlorella vulgaris* and *Trachidiscus minutus*) and protein extraction was developed based on physical technologies such as pressure homogenization and ultrasounds. The optimal conditions for maximizing the extraction of total protein was investigated. Non-chromatographic methods were employed to provide initial protein purification. These methods included precipitation/fractionation using acid precipitation employing citric acid and polyethyleneglycol (PEG) (Figure 15). The concentration at which soluble protein precipitates varies among different microalgae species and therefore detailed study was performed to analyse the effect of concentration (citric

acid, PEG), pH, temperature and ionic composition of the system on the precipitation of total protein. The results showed the about 30-60% total protein can be extracted under optimal conditions. At each step of the purification procedure, the precipitate was analyzed using SDS PAGE. The acid fractionation technique enables several fold enrichment of the protein in crude extract.

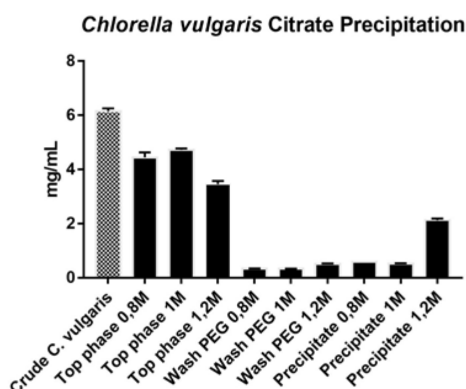


Figure 15. Protein precipitation of *Chlorella vulgaris* D02. 1C by citrate salts.

Task 4.2. Purification of protease from microalgae.

The objective of the work was the identification and characterization of high added value protease, by AGR_UNI_ATHENS, with potential applications in cosmetology. The work was focused on proteases as they represent a kind of enzyme with a potentially interest in the cosmetic industry. Protease activity was extracted by partitioning in ATPS composed by phosphate (Pi) and polyethylene glycol (PEG) (Figure 16, 17). Stopped spectrophotometric measurements were used for measuring protease activity in microalgae extracts using as substrate azogelatine and azocasein. Microalgae extracts (*Arthrospira platensis*) were first prepared as follows: freeze-dried biomass from Eparella GmbH, was resuspended in phosphate buffer in a proportion 0.1 g biomass/mL buffer. Cell lysis was performed using ultrasonics (4 cycles of 30 s each, 20% amplification), followed by centrifugation for 15 min at 16,000 g, 4 ºC. Supernatants were then recovered and were subjected to ATPS. Several factors that affect the partition behaviour of protease activity were investigated, such as molecular weight of PEG, proportion of PEG and Pi in the system, the presence of a metal ion, pH, NaCl and the effect of total protein concentration itself. Protein partition between the two different phases was measured in relation to initial protein input. SDS-PAGE analysis and protease activity measurements were used for evaluating purity and recovery. The method could be used in future studies for the purification of other novel proteases from additional microalgae species. The thermal stability of the protease from the bottom phase of ATPS was assessed by measuring their residual activity after incubation of the protease at different temperatures (4°C, 25 °C and 40°C) in 0.1M Tris-HCl, pH 8.5 for two hundred days. The results showed that the activity of the purified protease was remained essentially stable over the course of the experiment.

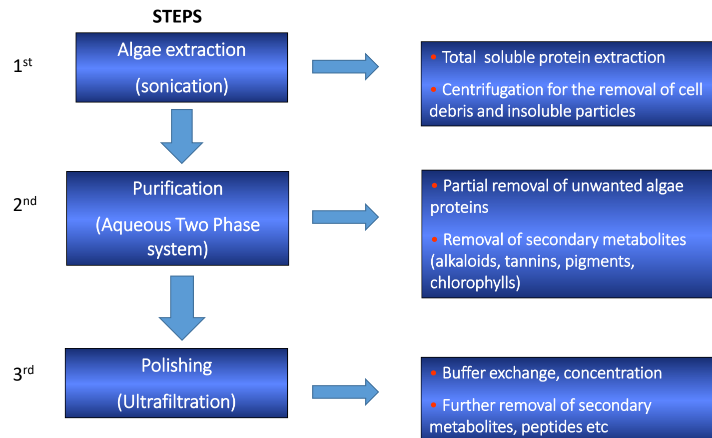


Figure 16. Downstream processing strategy for the purification of protease from microalgae extracts.

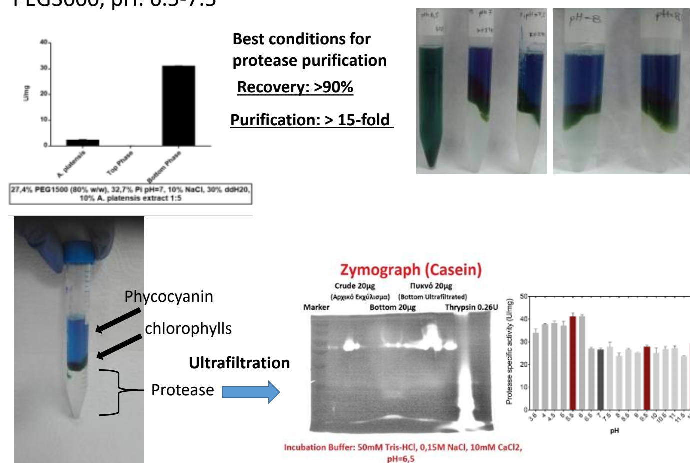


Figure 17. Purification of protease from microalgae extract using an optimized aqueous two-phase system.

Task 4.3. Purification of microalgae protein for skin and hair product development (CTNC, AUA, Fresh Formula).

Cell disruption, extraction and fractionation method for total protein separation was achieved. Optimization of a multi-step processes (extraction, separation and partial purification) for total proteins has been performed in order to obtain a sufficiently high yield total protein for developing products with hair and skincare properties.

Task 4.4. Production, purification and characterization of microalgae and mycosporine-like amino acids (AUA, FEM, CTNC, Fresh Formula).

Two different *Chlorella* strains, one we already used and one from Ecoduna have been used. Cultures, after exposed to UV stress, were extracted in different ways: 1.SOLVENT: water, ethanol, butylene glycol (not in favor of Fresh Formula) and only used as control), 2. RATIO: 1, 5 and 10% biomass, 3. DURATION: 1, 5 and 10 hours extraction time.

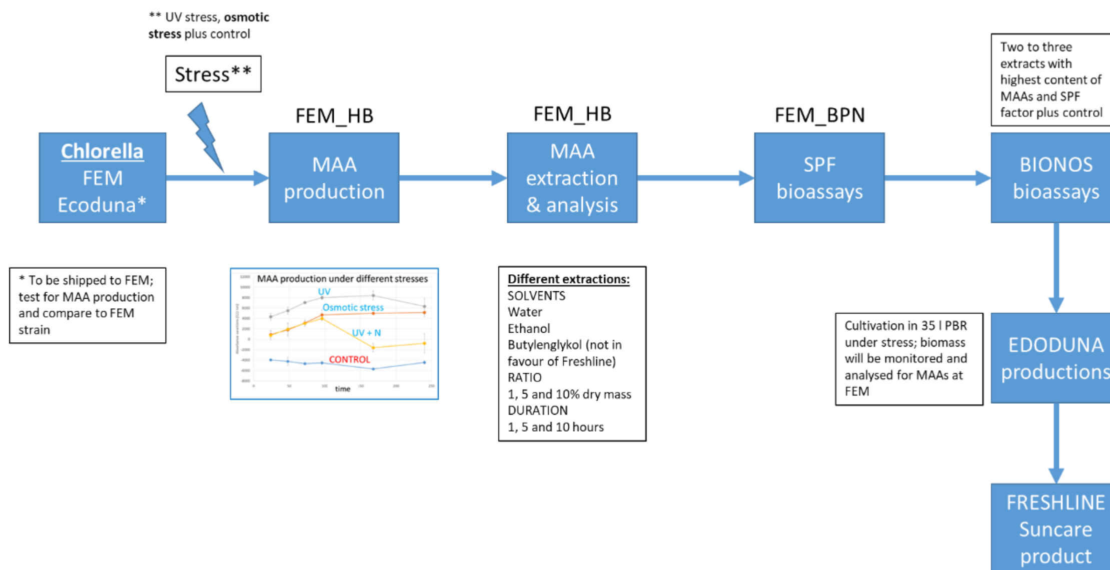


Figure 18. Schematic representation of MAA production

Work package 5

Development of cosmeceuticals and nutraceuticals from microalgae

The development of cosmeceuticals and nutraceuticals from microalgae has gained significant attention in recent years due to the numerous beneficial properties exhibited by these microorganisms. Microalgae are rich sources of bioactive compounds such as carotenoids, polyunsaturated fatty acids (PUFAs), proteins, peptides, and vitamins, which have been found to possess antioxidant, anti-inflammatory, and anti-aging properties. These compounds can be extracted and incorporated into skincare products and dietary supplements, offering a natural and sustainable alternative to synthetic ingredients. The unique biochemical composition of microalgae allows the production of innovative formulations with potential applications in the cosmetic and nutraceutical industries. Additionally, the cultivation of microalgae for these purposes presents an environmentally friendly approach, as they have a high photosynthetic efficiency and can grow in various types of water, including wastewater and brackish water. As research in this field continues to advance, microalgae-based cosmeceuticals and nutraceuticals hold promise as effective and eco-friendly solutions for promoting skin health and overall well-being.

Task 5.1. Encapsulation of bioactive ingredients of microalgae extracts

CTNC has recently used dried algae biomass from *Chlorella* sp., *Spirulina* (*Arthrospira* sp.) and an alga rich in oleoresin, provided by the Eparella GmbH, and developed edible algae spheres or as named Algae Caviar (Figure 19).

Figure 19. Algae Caviar (encapsulated algae and algae extracts as food)

Task 5.2. Monitoring the stability of the encapsulated or free microalgae extracts or compounds (Fresh Formula, CTNC, AUA).

A number of different extraction techniques and trials were conducted at the Particula Group d.o.o. company in Rijeka, Croatia. The company operates a 3rd generation middle size microalgae cultivation plant powered by photo voltaic plant. Different extraction trials were done on a lab scale (SFE, PLE, ultrasonically assisted extraction and osmotic pressure method). The best results were reached via ultrasonically assisted extraction, done in cooperation with the Agricultural University of Athens and CERTH.

The second step of the process was the upscaling of extraction to a larger scale, namely *Chlamydomonas reinhardtii* medium was grown in a 500L photo-bioreactor and 5.000L photo-bioreactor. For such trials it was impossible to conduct ultrasonically assisted extraction, so the decision was made to conduct the osmotic pressure method and the enzyme-assisted extraction, due to large batch and high operational costs of large scale extraction trials. The osmotic pressure technique was considered an ecological and cost-effective way to compete with other extraction methods, because of the availability of sea water from the Adriatic Sea. Seawater quantity of 200L was added for 15 days into the 5.000L batch, and the osmotic pressure disturbed algal cell walls after 10 days. As a result, the extracellular extraction trial was shown to produce 20% more of PUFA and lipids compared to other extraction techniques done in the

laboratory. Such an achievement will result in further research and development on the osmotic pressure technique in Particula Group company in future.

Task 5.3. Formulation of cosmeceuticals and nutraceuticals.

Fresh Formula has developed three different potential algae extract carrier forms: i) a water in oil (W/O) emulsion cream, ii) an oil in water (O/W) emulsion cream and ii) an aqueous gel. These three carrier forms were developed, and pre-stability testing has been successfully completed (centrifuge, freeze-thaw circles). A full product stability procedure was performed in a typical timeframe of three months with the samples being assessed at the end of each month.

Regarding the main stability results of the three different potential algae extract carriers which developed in the past months (a water in oil (W/O) emulsion cream/ an oil in water (O/W) emulsion cream/ an aqueous gel), it was decided to proceed with an oil in water (O/W) emulsion cream and an aqueous gel. Both bases were formulated using 0.1% and 0.5% (w/w) of glycerine-aqueous extracts derived from SPIROULINA and B14.1 EPA. Both extracts were provided to us by the Agricultural University of Athens. For all measurements, a cream and a serum without extracts were used as a control.

The procedure for the preparation of oil/water emulsion was as follows: the oily phase (Cetearyl Alcohol, Sodium Cetearyl Sulfate, Caprylic/Capric Triglyceride) and the aqueous phase (water, glycerine, Polyacrylate Crosspolymer-6, sodium gluconate) were heated separately to 80◦C. Then the oily phase was stirred into the water phase and intensive agitation followed using a Silverson mixer. The system was maintained under constant stirring until it cooled down to 35◦C. Subsequently, phenoxyethanol (1% w/w) and the microalgae extracts (0.1% and 0.5% w/w) were added and the emulsion was uniformly mixed. Regarding the aqueous gel, the preparation procedure was as follows: Polyacrylate Crosspolymer-6 was dispersed in the aqueous phase (water, glycerine and sodium gluconate) at room temperature with intensive agitation using a Silverson mixer. The active ingredients (microalgae extract 0.1% and 0.5% w/w) and the preservative were incorporated into the gel uniformly (Tables 13, 14, 15)

Table 13. Composition of the phases used for the preparation of cosmetic cream formulation.

A. **Water phase**	**% (w/w)**
Water (ddH_2O)	Q.S.
Glycerin	2.0
Polyacrylate Crosspolymer-6	0.5
Sodium Gluconate	0.1
B. **Oil phase**	**% (w/w)**
Cetearyl Alcohol, Sodium Cetearyl Sulfate, Disodium Phosphate, Potassium Phosphate	5.0
Caprylic/ Capric Tryglyceride	10.0
C. **<40oC**	**% (w/w)**
Phenoxyethanol	1.0
*Microalgal Extracts	% (w/w)

*As Microalgal Extracts, 0.1 % w/w and 0.5 % w/w were added in the final preparation.

Table 14. Composition of the water phase used for the preparation of aqueous gel formulation.

Water phase	**% (w/w)**
Water (ddH_2O)	Q.S.
Glycerin	2.0
Polyacrylate Crosspolymer-6	1.0
Sodium Gluconate	0.05
Phenoxyethanol	1.0
*Microalgal Extracts	% (w/w)

*As Microalgal Extracts, 0.1 % w/w and 0.5 % w/w were added in the final preparation.

Table 15. Composition of the phases used for the preparation of cosmetic sunscreen formulation.

Water phase	% (w/w)
Water (ddH_2O)	Q.S.
Glycerin	3.0
Sodium Polyacryloyldimethyl Taurate	0.6
Oil phase	**% (w/w)**
Disodium Cetearyl Sulfosuccinate	0,3
Dibutyl Adipate	9,5
Ethylhexyl Methoxycinnamate	4,3
Diethylamino Hydroxybenzoyl Hexyl Benzoate	3
Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine	1
Sodium Polyacrylate (55-65), Hydrogenated Polydecene (30-40), Trideceth-6 (<5)	1,2
Caprylic/Capric Triglyceride	0,5
D. <40oC	**% (w/w)**
Phenoxyethanol	0,9%
Ethylhexylglycerin	0,1%
Methylene Bis-Benzotriazolyl Tetramethylbutylphenol (nano), Aqua, Decyl Glucoside, Propylene Glycol, Xanthan Gum	3%
Tris-Biphenyl Triazine (nano), Aqua, Decyl Glucoside, Butylene Glycol, Disodium Phosphate, Xanthan Gum	2%
*Microalgal Extracts	% (w/w)

*As Microalgal Extracts, 0.5 % w/w were added in the final preparation.

Regarding the main stability results of the three different potential algae extract carriers, the best options were: the oil in water (O/W) emulsion cream and the

aqueous gel. Both bases were formulated using 0.1% and 0.5% (w/w) of glycerine-aqueous extracts derived from SPIROULINA (Arthrospira sp.) and B14.1 EPA. Both extracts were provided by the Agricultural University of Athens. For all measurements, a cream and a serum without extracts were used as controls.

Food product development is a key point for food industry, from refining an already existing product to developing a completely new one. It is a process with a high risk that often ends in failure. Algae are a complicated matrix that gives the food special colours and flavours. The development of new food products containing algae by CTNC is implemented at laboratory scale, however some of the products are produced at pilot scale. Several recipes have been assessed considering the organoleptic properties (such as colour and flavour) that algae may induce to the food products. Some of the developed food products are: Pate of mussels and melva with algae, Kiwi jam with algae, Fish soup with algae, Onion cream with algae, Omelette with algae, biscuit, bread, lettuce gazpacho, vegetable burger, smoothies with chlorella, chicken – fish and vegetable cream, algae spherifications (spirulina, chlorella, algae oil) and a non-food products: a soap with spirulina (Figure 20)

Figure 20: Developed products with microalgae

The indicatively flowcharts of the developed products follow below:

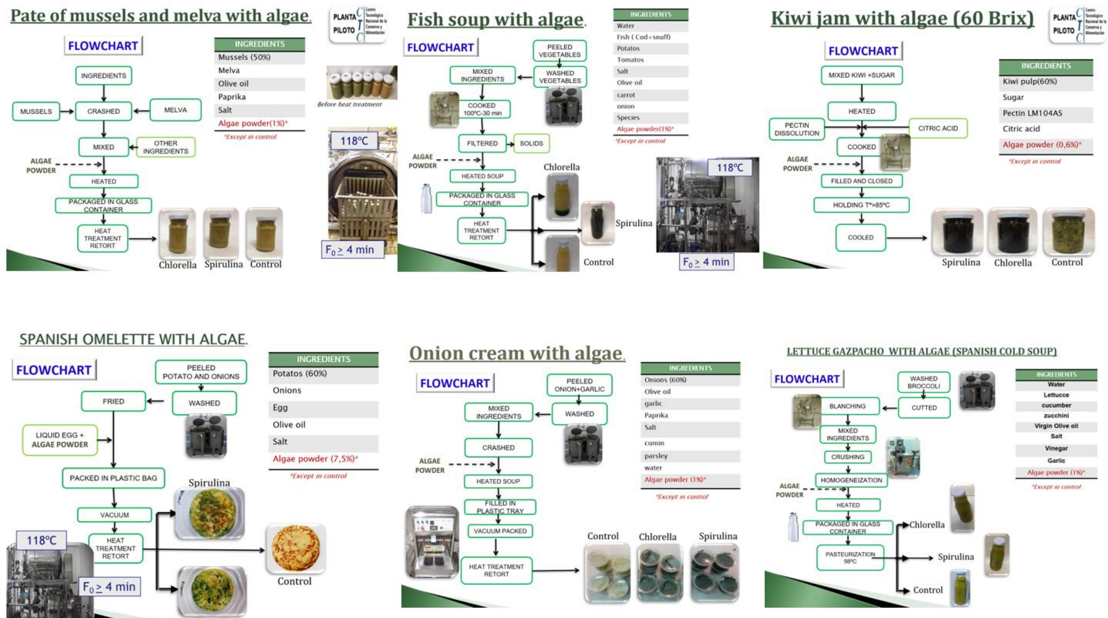


Figure 21: Flowcharts of pate of mussels and melva with algae, fish soup with algae, kiwi jam with algae, Spanish omelette with algae, onion cream with algae and lettuce gazpacho with algae

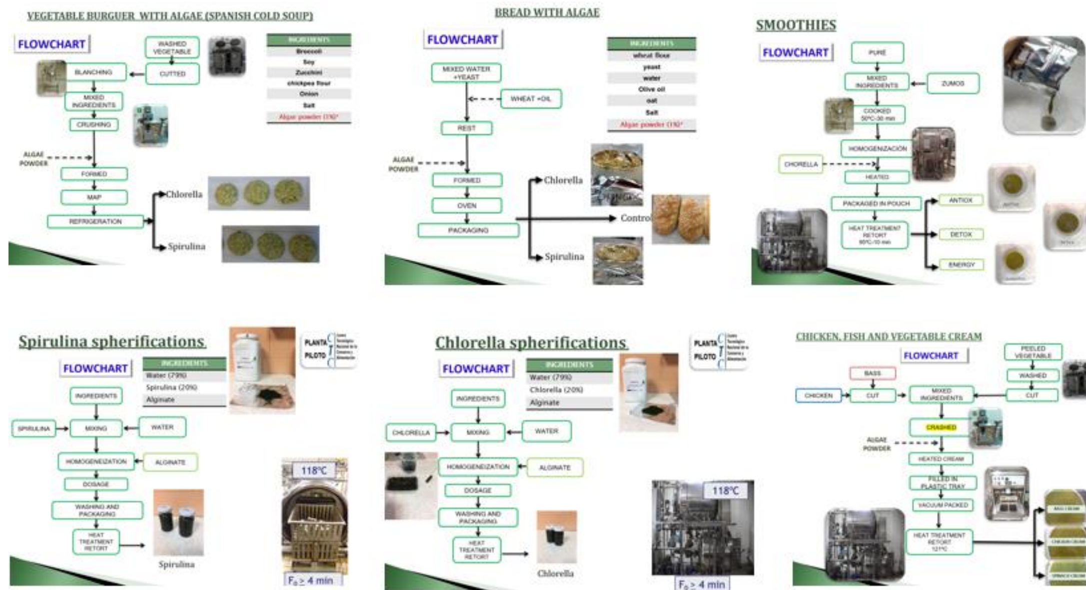


Figure 22: Flowcharts of vegetable burger with algae, bread with algae, smoothies, spirulina and chlorella spherifications, and chicken, fish and vegetable cream

Task 5.4. Quality control of cosmeceuticals.

Fresh Formula evaluated the physical and chemical properties in creme samples, using plastic containers suitable for cosmetics. Approximately 60g of samples were exposed to different storage conditions such as high and low temperatures (50oC, 45oC, 40oC, 5oC), room temperature and daylight (UV) (Figure 23).

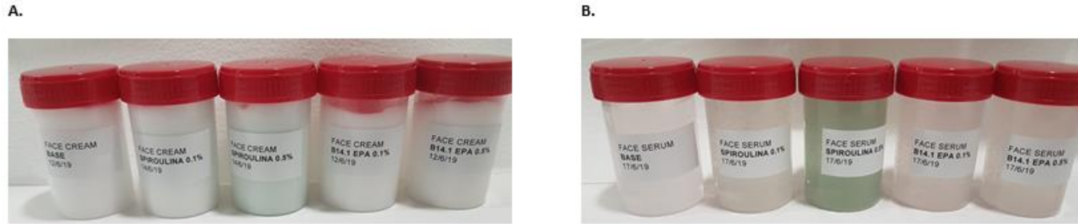


Figure 23: A. 60g of oil in water (O/W) emulsion cream containing 0.1%w/w and 0.5%w/w of microalgae extracts (SPIROULINA and B14.1 EPA) placed in suitable plastic containers**. B.** 60g of aqueous gel containing 0.1%w/w and 0.5%w/w of microalgae extracts (SPIROULINA and B14.1 EPA) placed in suitable plastic containers.packaged in suitable containers

Physical and chemical properties were evaluated in samples packaged in a suitable container at the initial time and after 1st, 2nd and 3rd month of storage using the following methods:

- **pH**: The pH was measured using pH meter Denver instrument UB-S equipped with a glass electrode directly into the samples.
- **Analytical centrifugation** (Only for face cream samples): Samples (5g) were centrifuged at 3000 rpm for 2h (1h, rest for 30min and repeat for 1h) and the eventual phase separation was analyzed in order to assess their stability during ageing.
- **Viscosity**: The viscosity was determined by Brookfield viscometer using S95 at 1,5rpm.
- **Homogeneity**: The samples were evaluated for the homogeneity by their visual appearance and touch affinity assessment.

Regarding the main stability results of a water in oil (W/O) emulsion cream and an aqueous gel which developed in the past months it was decided to proceed with an oil in water (O/W) emulsion as the final algae extract carrier. The aim of this study was to formulate bioactive compounds from microalgae into a sunscreen SPF20 for face in order to boost the photoprotective response. The sunscreen SPF20 base formulated using 0.5% (w/w) of glycerine-aqueous extracts derived from Chlamydomonas reinhardtii. The extract was provided to us by the Agricultural University of Athens. For all measurements, a sunscreen SPF20 base without extract was used as a control.

The procedure for the preparation of oil/water emulsion was as follows: the oily phase with UVA and UVB filters (Disodium Cetearyl Sulfosuccinate, Dibutyl Adipate, Ethylhexyl Methoxycinnamate, Diethylamino Hydroxybenzoyl Hexyl Benzoate, Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine, Sodium Polyacrylate, Hydrogenated Polydecene, Trideceth-6, Caprylic/Capric Triglyceride) and the aqueous phase (water, glycerine, Sodium Polyacryloyldimethyl Taurate, sodium gluconate) were heated separately to 80◦C. Then the oily phase was stirred into the water phase and intensive agitation followed using a Silverson mixer. The system was maintained under constant stirring until it cooled down to 35◦C. Subsequently, the preservative system

(phenoxyethanol 0,9% w/w and Ethylhexylglycerin 0,1% w/w), the nano board spectrum UV filters (Methylene Bis-Benzotriazolyl Tetramethylbutylphenol, Tris-Biphenyl Triazine) and the Chlamydomonas reinhardtii extract (0.5% w/w) were added, and the emulsion was uniformly mixed.

Afterwards, product stability testing was carried out in order to determine any changes that may occur in the characteristics of the subjective samples more rapidly than would be expected in 'normal' conditions of storage. Using plastic containers suitable for cosmetics, approximately 60g of samples were exposed to different storage conditions such as high and low temperatures (50oC, 45oC, 40oC, 5oC), room temperature and daylight (UV) (Figure 24).

A.

Figure 24: A. 60g of oil in water (O/W) emulsion cream containing 0.5%w/w of microalgae extract (Chlamydomonas reinhardtii) placed in suitable plastic containers.
Physical and chemical properties were evaluated in samples packaged in a suitable container at the initial time and after 1st, 2nd and 3rd month of storage using the methods previously described.

CTNC evaluated the thermal processing of peach, apple and grape juices enriched with 1% dehydrated algae at different temperatures. In the processed samples the concentrations of aerobic mesophilic microorganisms, total carotenoids and vitamin C were assessed. Reference samples as a basis to determine the effect of the algal addition to the juice were the same peach, apple and grape juices without the addition of algae. In this context, the sensorial properties the products' shelf-life was assessed, along with several analytical assays such as:

- Microbiological Analysis: Stability tests according to French Standard AFNOR NF V08-401 (7 days 55ºC, 21 days 32ºC)
- Nutritional Analysis: Regulation EU Nº 1169/2011 of the European Parliament on food information provided to consumers

The best results were obtained for the omelette and for the kiwi jam. Future work necessitates the adjustment of the different processes and formulae in the frame of the general normative and the consumers' preferences.

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