Training in the Scale-Up Process and Microalgal Biomass Production with Subsequent Evaluation of Different Extraction Methods to Obtain High-Value Commercial Bioactive Fractions

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Resumo

A crescente procura por novos métodos de produção de energia renovável, assim como de moléculas bioativas com efeito terapêutico e outros compostos bioativos de valor acrescentado, tem alavancado o desenvolvimento de várias áreas de estudo. Alguns dos mais promissores e prolíficos envolvem o uso de micro-organismos, nomeadamente da química, biotecnologia e engenharia. Sua alta taxa de crescimento, rápida produção de biomassa, alto rendimento, facilidade de cultivo, uso e ocupação de terreno e baixo consumo de água, bem como a geração de coprodutos valiosos são alguns dos fatores que as tornam uma alternativa atraente face aos métodos de produção tradicionais. No entanto, as técnicas atuais para o uso de microalgas à escala industrial enfrentam certos problemas que as impedem de competir com os métodos de produção tradicionais, nomeadamente a falta de métodos eficientes de colheita e extração. Os métodos tradicionais de extração têm algumas desvantagens relacionadas com a dificuldade de aumento de escala para a produção industrial, assim como o elevado custo de produção e manutenção de equipamentos e baixa eficiência energética. Além disso, alguns desses métodos exigem o uso de solventes orgânicos, geralmente prejudiciais, caros e tóxicos para o meio ambiente. O uso de um Homogeneizador de Alta Pressão (em inglês HPH) e a ressurgente tecnologia de Aquecimento Óhmico são métodos promissores para a extração de compostos valiosos de biomassa de microalgas. Para testar a eficácia desses dois métodos, quatro espécies de microalgas, incluindo *Nannochloropsis oceanica*, *Nannochloropsis limnetica*, *Tetradesmus obliquus* e *Phaeodactylum tricornutum*, foram cultivadas em fotobiorreatores, em painéis em ambientes industriais, até a colheita. A biomassa resultante foi então processada por meio do uso de HPH, e sua eficácia foi avaliada. Duas espécies marinhas de microalgas foram selecionadas para testar a eficiência de extração do Aquecimento Óhmico, usando tanto seus lotes de cultura quanto os extratos HPH respetivos, o que também permitiu a avaliação de um processo de extração composto por 2 métodos: HPH e Aquecimento Óhmico.

Palavras-chave: Biomassa, Microalgas, Métodos de Extração, HPH, Aquecimento Óhmico

Abstract

The growing search for new methods of renewable production of energy, as well as of therapeutic bioactive molecules and other rare bioactive compounds has proliferated the development of many new avenues of study, namely in chemistry, biotechnology and engineering. Some of the most promising and prolific of these are those involving the use of microorganism, namely microalgae. Their high growth-rate, rapid biomass production, high yield, ease of cultivation, small land and water footprint and generation of valuable co-products are only some of the points that make it an attractive alternative to traditional production methods. However, current techniques for the use of microalgae in industrial production face some shortcomings that prevent them from out-competing them, namely the lack of efficient harvesting and extraction methods. Traditional extraction methods tend to suffer from issues with the scale-up of the technique for industrial production, high energy costs and low energetic efficiency. Additionally, when these methods necessitate the use of organic solvents, which are typically harmful, costly, and toxic to the environment. The use of a High Pressure Homogenizer (HPH), and the re-emerging Ohmic Heating technology, are promising methods for the extraction of valuable compounds in microalgae.

To tests the efficacy of these two methods, four species of microalgae, including *Nannochloropsis oceanica*, *Nannochloropsis limnetica*, *Tetradesmus obliquus* e *Phaeodactylum tricornutum*, were cultivated on industrial-scale flat-panels photobioreactors. The resultant biomass was then processed by HPH, and its extraction efficacy was evaluated. Two marine species of microalgae were selected for testing the extraction efficiency of Ohmic Heating, using both their culture batches and respective HPH extracts, which also allowed for the evaluation of an extraction process consisting of 2 methods: HPH and Ohmic Heating.

Keywords: Biomass, Microalgae Extraction Methods, HPH, Ohmic Heating

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Abbreviation Index

CH	CONVENCIONAL HEATING
DW	DRY WEIGHT
FAME	FATTY ACID METHYL ESTER
GC	GAS CHROMATOGRAPHY
HPH	HIGH PRESSURE HOMOGENIZER
IH	INDUCTIVE HEATING
NB+®	NUTRIBLOOM PLUS®
OD	OPTICAL DENSITY
ΩH	OHMIC HEATING
PUFA	POLYUNSATURATED FATTY ACID
R&D	RESEARCH AND DEVELOPMENT
TAG	TRIACYLGLYCEROL
TLC	THIN LAYER CHROMATOGRAPHY
UV	ULTRA-VIOLET

1. Introduction

In an age marked by a need for rapid industrialization and heightened environmental awareness, the search for sustainable and renewable sources of production has become imperative. Within this scenario of innovation and climate transition, microalgae emerge as a possible bridge between ecological preservation and economic viability.[1,2]

Microalgae are microscopic unicellular organisms, ubiquitous in diverse aquatic environments and distinguished by their proficiency in converting carbon dioxide and sunlight into a wide array of valuable compounds. This unique combination of photosynthetic prowess and biochemical diversity renders them exceptionally versatile agents in the realm of renewable production.[2,3] Furthermore, their remarkable growth rates and capacity to thrive in a wide range of conditions underscore their potential to revolutionize numerous industries, ranging from biofuels and pharmaceuticals to food and cosmetics. A growing toolbox of genetic manipulation only further potentiates the versatility and complexity of what they can produce.[4]

The biotechnology industry, founded on the principles of harnessing biological systems for economic, industrial, and medical applications, considers microalgae a precious ally. These microorganisms are capable of producing an impressive spectrum of bioactive compounds, encompassing lipids, proteins, pigments, vitamins, and antioxidants, among others.[2,4] Furthermore, microalgae can be cultivated in almost any type of water, including wastewater, bypassing the need to compete for arable land to produce biomass. However, the biotechnological application of microalgae at industrial scale has faced some obstacles. One of the most critical challenges lies in mastering extraction methods. The low efficacy of traditional extraction techniques has hindered the cost-effectiveness and environmental sustainability of microalgae-based industries. This inefficiency, in turn, has restrained microalgae from emerging as active competitors to traditional industrial processes.[5]

1.1. Nannochloropsis oceanica and *Nannochloropsis limnetica*

Nannochloropsis is a genus of unicellular, nonmotile marine microalgae classified within the Phylum Heterokontophyta, the Class Eustigmatophyceae, and the family Eustigmataceae. In this project, the two distinct species of *Nannochloropsis, N. oceanica* and *Nannochloropsis limnetica*, were selected for cultivation. *N. oceanica* thrives primarily in brackish and marine environments, being frequently found in temperate and tropical coastal regions, while *N. limnetica* inhabits freshwater environments. These microalgae exhibit a straightforward cellular morphology, with diameters ranging from 2 to 8 µm and, notably, store energy in the form of lipid droplets, which can undergo expansion during adverse environmental conditions, and contain only Chlorophyll a (Fig. 1).[6] Microalgae of the *Nannochloropsis genus* have several properties that highlight them as essential contributors to the biofuels landscape. Their remarkable resilience and adaptability to diverse ecological niches makes them attractive candidates for large-scale cultivation, where highly concentrated cultures, necessary to achieve worthwhile productivity of biomass, subject the microalgae to various environmental stresses. Their ability to yield lipid-rich biomass, namely triacylglycerols (TAG's), which are used to produce biodiesel by secondary chemical processes through lipid transesterification, yielding fatty acid methyl esters (FAMEs), is extremely attractive for the production of biofuels.[2,7] Additionally, microalgae from the *Nannochloropsis* genus are known for their high content of valuable Polyunsaturated fatty acids (PUFAs), polyphenols, carotenoids and vitamins, which have diverse nutritional and therapeutical applications.[8]

1.2. Tetradesmus obliquus

Tetradesmus obliquus, formerly known as *Scenedesmus obliquus*, is a freshwater species of quadriflagellated ovoid microalgae from the phylum Chlorophyta, the class Chlorophyceae, and the family Scenedesmaceae. Typically, these microalgae measure about 2-10 µm in length, and often forms filamentous colonies, typically four cells long. *Tetradesmus obliquus* possess both Chlorophyll *a* and Chlorophyll *b* (Fig. 1), which have various therapeutical applications, and high FAME yields, making it suitable for biofuel production.[9]

In addition to biofuel production, *Tetradesmus obliquus* has applications in wastewater treatment, as it can remove excess nutrients and heavy metals from contaminated water sources.[7] This makes it a valuable tool for environmental remediation efforts, whilst also opening up the possibility to utilize this ability for the production of valuable compounds and biofuel from wastewater-based cultures.

1.3. Phaeodactylum tricornutum

Phaeodactylum tricornutum are marine microalgae that belong to the diatom class, a subgroup of microalgae distinguished by their intricate siliceous frustules which encapsulate their cells, inhabiting a wide range of aquatic environments, from coastal regions to open oceans. Its distribution is ubiquitous, being found in both temperate and polar regions. This adaptability to diverse habitats showcases its resilience and ability to thrive in varying environmental conditions, making it suitable for large scale cultivation. Their frustules are composed of two overlapping silica valves, which not only provide protection to the cell but also contribute to its unique appearance under the microscope, making it easily identifiable, and hold promise for the industrial production of biosilica.[10]

The biochemical composition of *P. tricornutum* shows a variety of essential components that hold significant promise for various applications. *P. tricornutum* is renowned for its fucoxanthin content, a carotenoid pigment. This compound exhibits antioxidant properties and has garnered attention for its potential in the pharmaceutical and nutraceutical industries. Lipids, including fatty acids and triglycerides, which have applications in biofuel production, are also present in substantial quantities. *P. tricornutum* houses an array of bioactive compounds, including PUFAs and other metabolites with diverse applications.[11] It is an abundant source of essential nutrients, making it invaluable in applications such as aquaculture, where it contributes to the growth and enrichment of feedstock. The unique nutritional attributes of *P. tricornutum* align it with the growing demand for sustainable and nutrient-rich sources within these industries. Its adaptability, coupled with its contributions to aquaculture and bioactive compound production, underlines its significance within the biotechnology and renewable production landscape.

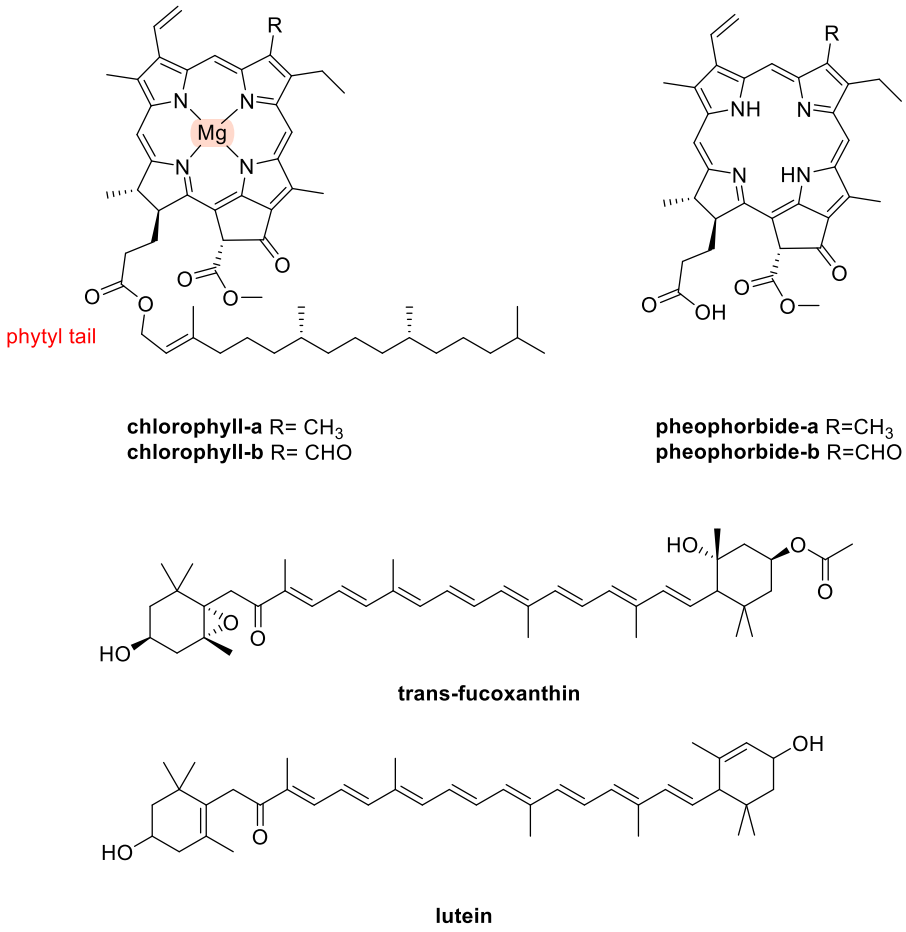

Fig. 1 - Molecular structure of natural pigments found in microalgae

1.4. High Pressure Homogenizer

In the realm of microalgae extraction, where the efficient release of valuable intracellular components is paramount, the choice of extraction method plays a pivotal role. Traditional extraction techniques often fall short in terms of efficiency, presenting challenges to the commercial viability of microalgae-derived products. However, innovative technologies such as High Pressure Homogenization (HPH) have stepped in to address these shortcomings.

HPH is an extraction method that capitalizes on the power of high pressure to disrupt microalgal cells and efficiently release their intracellular content. This approach stands out for its precise control over the homogenization process, setting it apart from traditional methods like mechanical disruption or solvent-based extraction. It operates by

subjecting microalgae cells to extreme pressure conditions, inducing shear forces and cavitation, which, in turn, leads to the rupture of cell walls and the release of the valuable intracellular components. A basic overview of the process may be seen in Fig. 2.HPH's ability to efficiently rupture cell walls and release intracellular components results in higher yields and reduced damage to the extracted materials when compared to traditional techniques. Comparatively, when juxtaposed with traditional extraction methods, HPH offers distinct advantages. Traditional methods are often characterized by lower efficiency and the risk of thermal degradation of sensitive compounds. Solvent-based methods can introduce environmental concerns due to the use of large volumes of solvents and their subsequent disposal.

Another point of consideration is the potential for scaling up HPH technology. The ability to efficiently process large volumes of microalgae biomass is a critical factor for the commercial viability of microalgae-derived products. In this regard, HPH offers the advantage of scalability, making it suitable for applications that require both small-scale and large-scale processing.

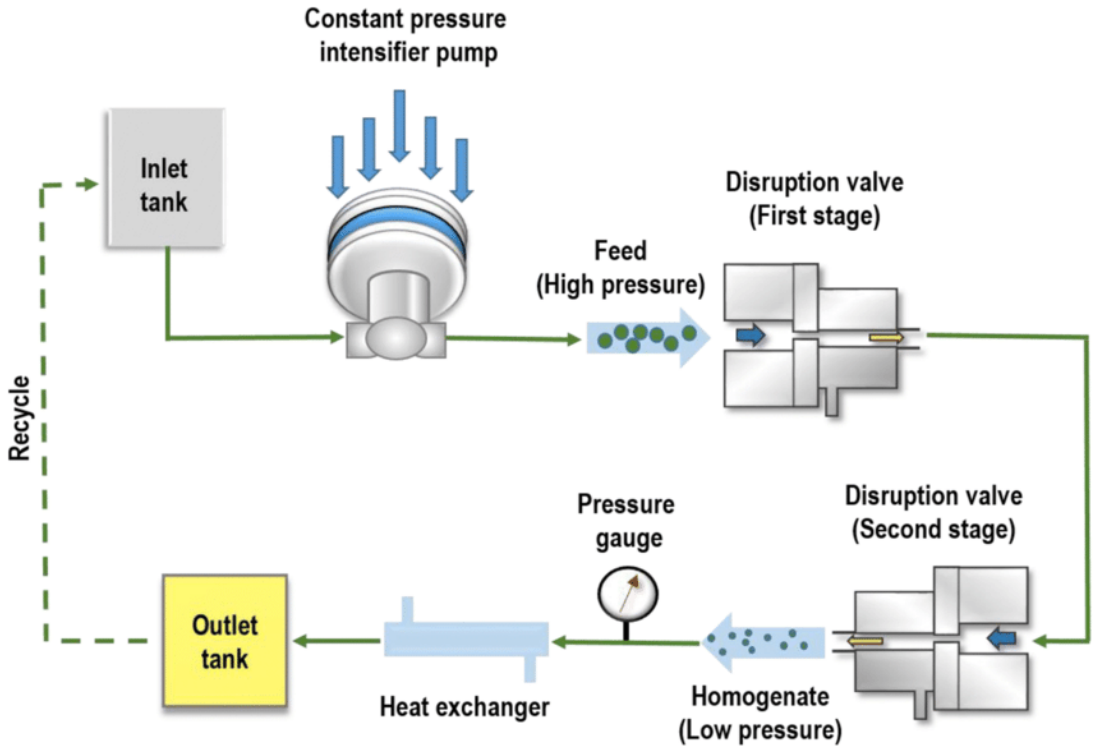

Fig. 2 - Simplified diagram of the homogenization process of a sample by the High Pressure Homogenizer, which was employed as an extraction method for microalgae cultures during the NECTON.S.A internship. The High Pressure Homogenizer model used during said internship was a GEA Lab Homogenizer PandaPLUS 2000 unit.

1.5. Ohmic Heating

Heating is a widely used method for food processing and preservation. In recent years, new advanced heating methods have emerged, offering higher energy efficiency when compared to conventional heating techniques. Ohmic Heating (ΩH), also known as direct Joule heating, is a thermal processing method primarily used in the food industry. Its application in chemical synthesis and extraction of natural compounds has shown great potential, with recent developments taking place in Portugal.[12,13,14]

Heating technologies have made significant strides in recent years with the advent of new methodologies like ΩH. Dielectric heating, which includes microwave radiation (MW), radiofrequency heating, and inductive heating (IH), is more energy-efficient compared to conventional heating (CH) methods. This efficiency is attributed to the internal generation of heat within the material being heated. The initial descriptions of processes using ΩH date back to the late 19th century when pasteurization of milk was achieved by pumping the fluid between two metallic plates (electrodes) subjected to a potential difference. However, this application was discouraged due to high processing costs, and other applications were abandoned due to the lack of electrodes made from inert materials.[15]

At that time, other applications of ΩH in the food industry emerged, including cooking Frankfurter-type sandwiches and blanching potatoes. In the following years, the technology virtually disappeared due to process control limitations and the improper use of electrodes made from non-inert materials. Concerns arose regarding potential side effects and even lethality to consumers due to the passage of electrical current through food. However, in the past two decades, the technique has experienced a resurgence, thanks to the development of new materials used in electrode construction and the design of more efficient and versatile reactors. ΩH has emerged as an advanced heating method with applications in the food, chemical, pharmaceutical, agrochemical industries, and for sterilization purposes. At present, there are numerous applications for ΩH, including blanching, evaporation, dehydration, fermentation, extraction, sterilization, and pasteurization.

In 2013, the first study on the application of ΩH to chemical synthesis, particularly organic synthesis, was published. Researchers from the QOPNA Research Unit of the

Department of Chemistry at the University of Aveiro (QOPNA/UA), in collaboration with researchers from the Faculty of Sciences of the University of Porto (CIQUP and UCIBIO/REQUIMTE) and the University of Minho (CEB/UM), designed, constructed, and patented the first ΩH reactor for laboratory-scale chemical synthesis.[16]

Principles of ΩH, also known as Joule heating, resistance heating, electroheating, or electroconductive heating, is defined as a process in which the material (or reactant mixture) serving as an electrical resistance is heated by the direct flow of alternating electric current. Electrical energy is dissipated directly due to Joule heating within the medium, resulting in very high energy efficiency. This leads to rapid and uniform heating (homogeneous temperature) and a significant increase in molecular dynamics within the reactant mixture. As the system's temperature rises, electrical conductivity also increases due to the accelerated movement of molecules and charged species. ΩH distinguishes itself from other heating methods as it involves the use of electrodes in contact with the material being heated, in contrast to heating with MW (microwave radiation at a frequency of 2.45 GHz) and IH (inductive heating using magnetic nanoparticles) where electrodes are absent. The applied frequency is variable and lower than radio and microwave frequencies, with the sinusoidal waveform being most common (Fig. 3). For practical reasons, frequencies between 50 and 60 Hz are commonly used in industry. The applied frequency and voltage waveform affect the efficiency and quality of ΩH processes due to the typical frequency dependence of impedance). Higher electrical conductivity is achieved with lower frequencies, especially with a sinusoidal waveform.

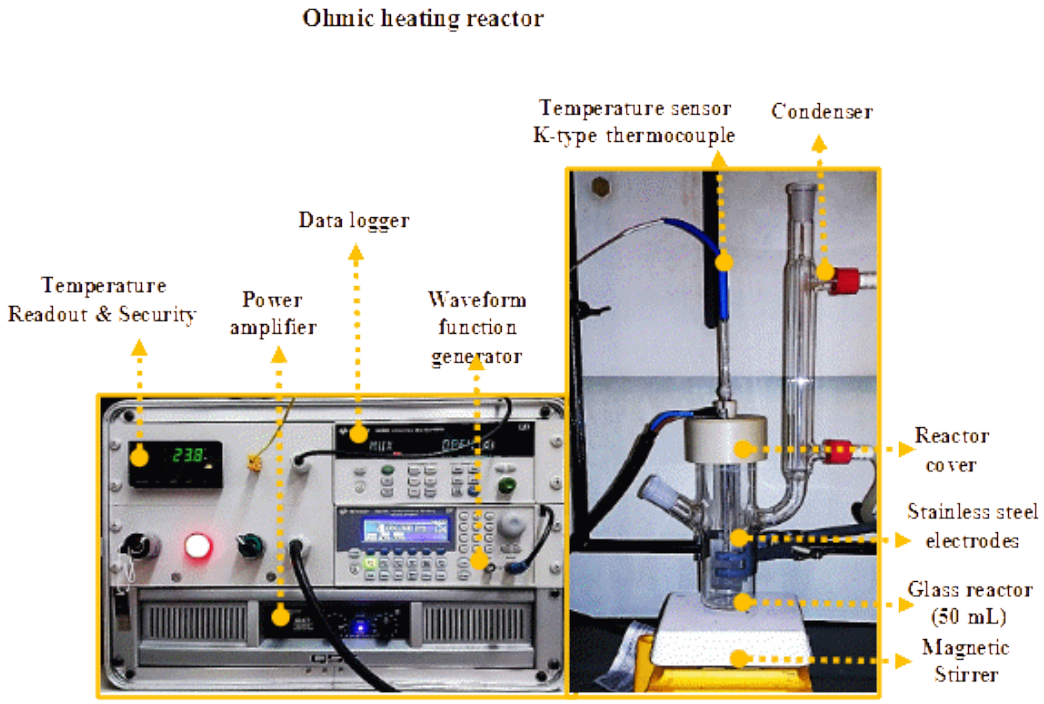

Fig. 3 - Ohmic heating reactor (Portuguese Patent No. 105908, 2011-09-27). The reactor description: VAC power supply; waveform function generator; heating reactor cell (50 mL glass vessel) containing one pair of stainless steel (316) rectangular electrodes (40x25x1 mm length/width/thickness) located at a fixed distance (23 mm) and in contact with the solution; temperature sensor (K-type thermocouple); data logger system; PC computer; data acquisition and control software package. (adapted from reference [17])

2. Proposed Objectives

Aiming to value microalgae biomass, the main objectives proposed for this project are:

1. Produce biomass from four species of microalgae, including *N. oceanica*, *N. limnetica*, *T. obliquus* and *P. tricornutum*;
2. Assessment of biomass produced through microbiological and cell rupture methods;
3. Evaluate the ohmic heating extraction method, with regard to the extraction efficiency and energy efficiency of the method.

3. Experimental Part

3.1. Techniques Developed during the NECTON.S.A. Internship

During my internship at NECTON, the primary focus was on developing essential techniques to cultivate microalgae cultures. These techniques encompassed several key aspects, including microalgae culture inoculation, scaling up of cultures to volumes as large as 800L using flat-panel systems, as seen in Fig. 4, and continuous monitoring of culture conditions and biomass density. This monitoring involved regular Optical Density (OD) readings, pH measurements, and nitrate content analysis. To master this technique, I participated in NECTON's daily culture routines, initially under supervision and later independently until the conclusion of my internship.

Additionally, great emphasis was placed on maintaining a sterile laboratory environment to prevent culture contamination and ensuring strict adherence to safety protocols. As all procedures developed during the internship were applied to every microalgae species cultivated at NECTON S.A., I will first present the general protocols, followed by a detailed discussion of specific procedures.

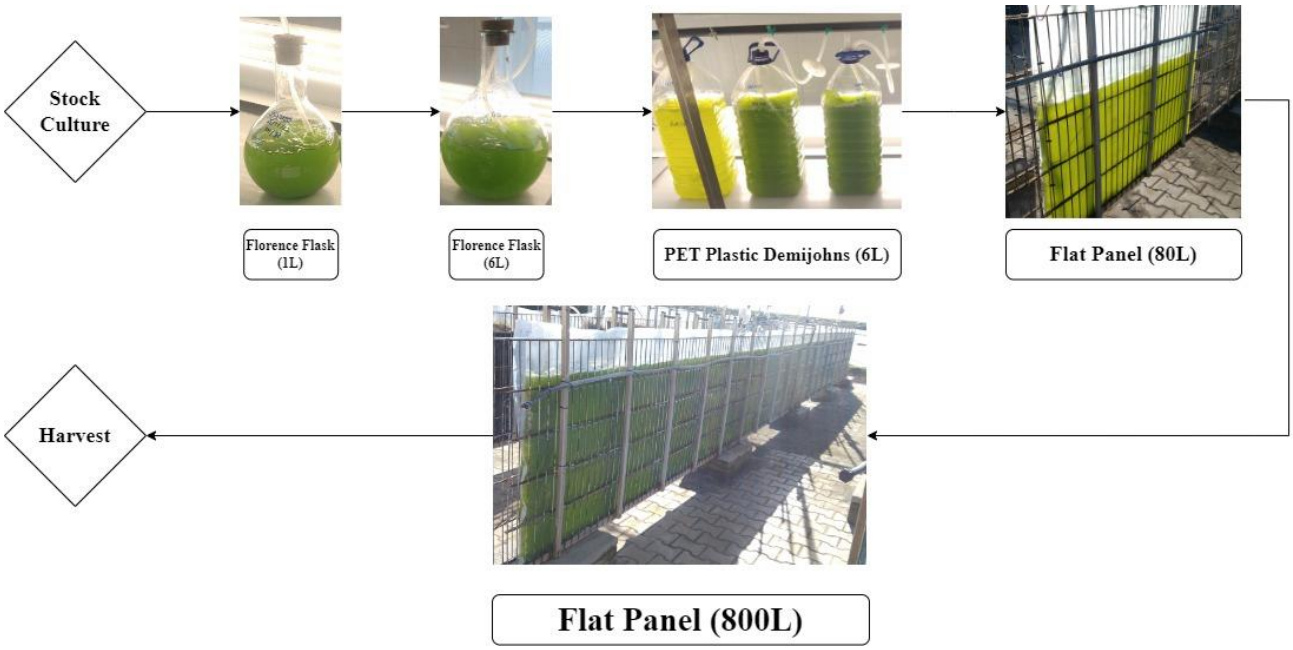

Fig. 4 - Diagram demonstrating the microalgae scale-up process employed during the NECTON internship. Here, the cultures of *Tetradesmus obliquus* were used to exemplify the various stages of the process.

3.1.1. Culture Monitoring

a) OD Readings

Culture growth was assessed trough OD readings, which served as a crucial method to estimate the concentration of microalgae within the culture by combining this data with dry weight measurements. Microscopic examination was used to screen for contaminant microorganisms, including other microalgae species that could compete with the primary culture.

To perform OD readings, a sample of the culture was collected at the beginning of day into a 50-mL Falcon tube, as per Fig. 5, and appropriately diluted. The degree of dilution was determined case by case based on the culture's density, ensuring that absorbance readings met the Lambert-Beer Law, allowing for its correlation with other measurable data. Samples were homogenized before being transferred to plastic cuvettes. Another plastic cuvette was filled with sea or freshwater, according to the algae, to serve as a blank. Duplicate samples were read to ensure the measurement's reproducibility. The absorbance of the samples was read at 750nm, as it was deemed a good wavelength due to falling outside of the visible spectrum.

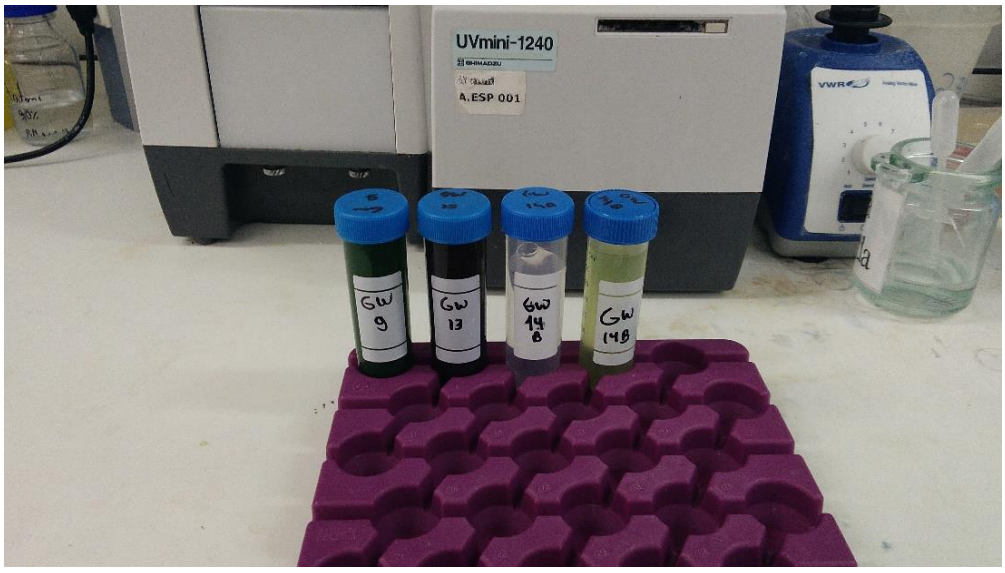

Fig. 5 - Samples from flat panel cultures, two which, by the codes of GW9 and GW13, being the cultures of Nannocloropsis limnetica (GW9) and Phaeodactylum tricornutum (GW13) created during the internship.

b) Dry Weight Analysis

To correlate microalgae biomass with OD readings, dry weight analysis was performed on samples from the microalgae cultures, as seen in Fig. 6. This analysis allowed for the modelling of each microalgae culture's growth and provided insights into their growth rates when combined with OD readings. In turn, these provided insights into the culture's productivity, as expressed in Equation 1 and Equation 2.

$$Productivity = \frac{DW_i - DW_{i-1}}{T_i}$$

Equation 1 - Equation used to determine the productivity of the culture in the time between where two samples were taken. Dry Weight (DW), expressed in g/L, was obtained from its OD reading results, whilst the time between samples (T_i), expressed in hours, was based on the difference between the recorded time and date of sample collection.

$$Average\ Productivity = \frac{\sum(DW_i - DW_{i-1})}{\sum T_i}$$

Equation 2 - Equation that was used to determine the average productivity of a culture system.

To begin, paper filters were prepared by oven-drying overnight at 30˚C and weighed before the analysis. Samples of 10mL were then filtered in triplicate, retaining only its solid contents on the filter surface. If the sample was highly concentrated and failed to drain properly, the process was restarted with a lower volume. Conversely, if the sample was too diluted and the filter appeared very light in color, additional volume from the sample was processed using the same filters. For saltwater microalgae cultures, an equal volume of a 0.1M ammonium hydroxide solution was used to wash out the remaining salt, which could skew the biomass weight results. The total sample volume used on the procedure was recorded for later calculation of biomass concentration. Afterward, the filters were dried in a hot air oven at 30˚C for three days. After drying, the filters were weighed again, and the difference, corresponding to the biomass dry weight, was recorded. This procedure was conducted in parallel with OD readings.

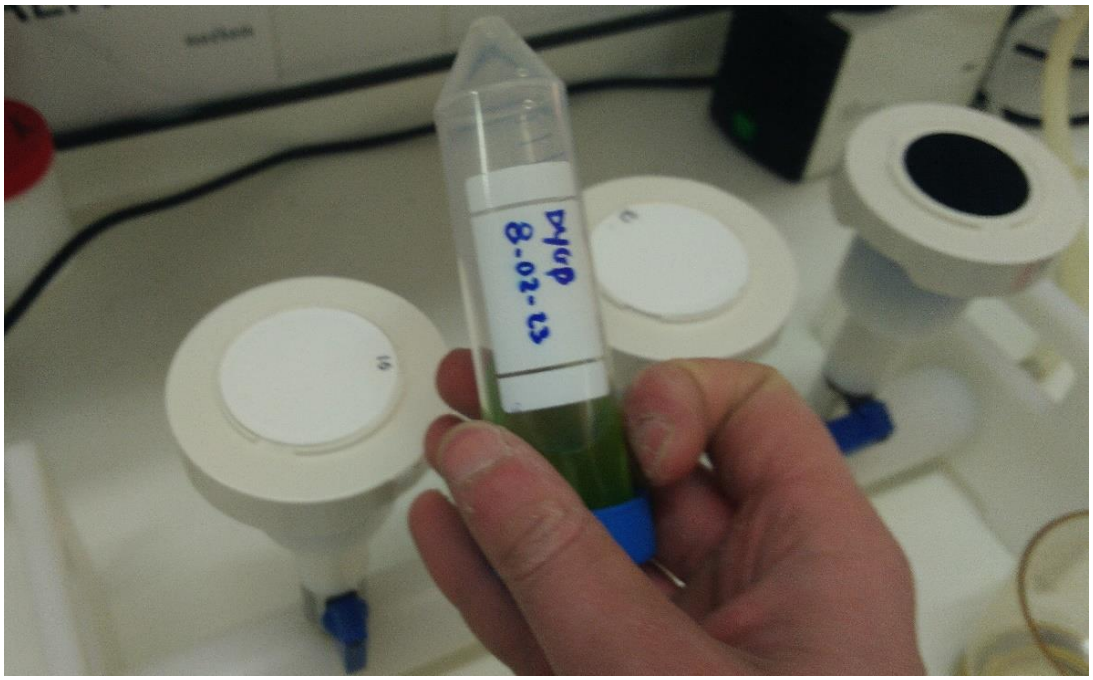

Fig. 6 - Suction filtration unit with the cup-sections detached and two filters fit within the Buchner funnels, with a sample to undergo analysis.

c) Nitrate Content Readings

Nitrate concentration was used to assess nutrient consumption within the cultures, determining whether additional nutrient medium was required and when, according to Baird et al. 2017. During my internship at NECTON, I was actively involved in the daily nitrate analysis, similarly to my involvement in OD readings, as both were performed concurrently using the spectrophotometer. While nitrate readings could not directly provide nutrient content, they reliably indicated nutrient sufficiency and facilitated timely nutrient replenishment.

To perform nitrate content readings, a 50-mL sample was collected and centrifuged for 10 minutes at 4000 rpm. Nitrate content cannot be directly measured by the spectrophotometer due to interference from microalgae. These will coalesce into a pellet after centrifugation due to the cell's comparatively higher density. The dilution process begins by pipetting 9,8 mL of distilled water and 100 µL of a 0.1M HCl solution into 15-mL Falcon tubes. Next, 100 µL of the sample was added to each Falcon tube, in duplicate. The proportions of distilled water and sample volume were adjusted to ensure readings fell within the Lambert-Beer Law range of 0.2 to 0.8 absorbance units

(A.U.). The Falcon tubes are homogenized before a portion is transferred to a quartz cuvette for spectrophotometer analysis. A blank is prepared with only distilled water and 0.1M HCl solution. Afterward, the absorbance of the samples was read at 220 nm and 275 nm.

3.1.2. Microalgae Selection

Microalgae inoculates were prepared from stock cultures provided by NECTON. The inoculation procedure was meticulously standardized, with slight adjustments made to accommodate both saltwater and freshwater microalgae cultures. Over the course of the internship, a diverse array of four microalgae species were cultivated. These species included *Nannochloropsis oceanica*, *Nannochloropsis limnetica*, *Tetradesmus obliquus*, and *Phaeodactylum tricornutum*. This selection was made with a comprehensive rationale based on the following criteria:

A. Comprehensive Representation: The selected cultures encompass both freshwater and saltwater species, allowing for a complete and holistic understanding of microalgae cultivation techniques. The species also served as representatives of three different filum: Chlorophyta (*Tetradesmus*), Eustigmatophyta (*Nannochloropsis*), and Phaeophyta (*Phaeodactylum*). Additionally, this would permit screening of the influence of the source of microalgae on the efficacy of the extraction methods slated for testing.
B. Notorious Resilience: The cellular resilience of these four microalgae species, especially those of *Nannochloropsis oceanica* and *Phaeodactylum tricornutum*, would further demonstrate the robustness of the extraction methods under examination.
C. Structural Parity: The inclusion of the two *Nannochloropsis* microalgae species allows for the comparison of very similar cell wall structures adapted to different environments, with *Nannochloropsis limnetica* inhabiting freshwater environments, and *Nannochloropsis oceanica* inhabiting saltwater environments.
D. Nutritional Profile: These species boast a rich nutritional profile, characterized by the presence of substances such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), both omega-3 fatty acids, and fucoxanthin, which are highly desirable compounds. Demonstrating the efficacy of the extraction

methods on these compounds would serve to further highlight them as valuable extraction methods.

E. Alignment with NECTON's Production Plan: The selection of these microalgae species was congruent with NECTON's strategic production plan, ensuring the internship would not interfere with the company's goals.
F. Seasonal Adaptability: Given the internship would be undertaken during the winter season (from December to late February), it was imperative that the chosen species could be acclimated to thrive in this specific climatic context. All four selected species were known to be adapted for growth during the winter season.

The cultivation process involved scaling up the microalgae cultures until a sufficiently high concentration was achieved, as inferred from OD measurements. The specific threshold for sufficient concentration was evaluated on a case-by-case basis, considering the unique characteristics of each culture. Upon reaching this critical point, a plan for inoculating an 80-L flat panel was drafted.

3.1.3. Scale-up in the Laboratory

To maintain aseptic conditions and minimize contamination risks during inoculation and scale-up procedures, the sterilization steps implemented in the company were employed during the scale-up procedure:

- All equipment and materials used in the laboratory were autoclaved.
- Inoculum handling was conducted in a devoted Inoculation Room within NECTON S.A. R&D facilities. All the materials within the work area were handled within 20 cm of a flame.

The inoculum to be used for scale-up was topped up with freshwater to compensate for evaporation. A sample was also collected and examined under the microscope to confirm its suitability as an inoculate. Once suitability was ascertained, a pre-determined volume (dependent on the concentration of the inoculation culture) of the inoculum was transferred to a second container with Nutribloom Plus® (NB+), a specialized nutrition medium produced by NECTON, in either freshwater or saltwater, depending on the specific microalgae species to be inoculated. The new culture was connected to an air supply supplemented with CO_2.

3.1.4. Outdoor Cultivation in Flat Panels

Two different sizes of flat panels were used: an 80-L model and an 800-L system. The inoculation procedure was performed in one of two different ways depending on the model of flat panel. However, the first steps of the process were the same in every case, and the general assembly of the body of the flat panel was very similar for both the 80-L and 800-L models. All materials that meet with interior of the Flat Panel were disinfected beforehand with ethanol.

a) Assembly Procedure for the Flat Panel

A flat plastic bag was carefully unfurled along the space between the support grates. Sufficient aerators, coupled to filters and a CO_2 diffusor, were inserted in the plastic bag. The turbulence provided by the air supply is necessary to prevent culture stagnation and the formation of biofilm. It is also integral to the homogenization of the culture and the diffusion of nutritional medium added to the flat panel. The flat panel is filled with either ultra-filtered freshwater or seawater, depending on the type of microalgae that was to be inoculated. The water was chlorinated and, afterwards, neutralized with thiosulfate. A tap was inserted into the side of the flat panel for easier sampling.

b) Outdoor Microalgae Cultivation

Contaminations were prevented by capitalizing on the microalgae's exponential growth phase, enabling it to rapidly expand its biomass and outcompete the contaminant. The 800L flat panel cultures were cultivated until reaching a biomass concentration that was deemed suitable for harvesting and subsequent processing into paste. For this, the cultures were centrifuged in industrial centrifuges, with the end-product being constituted by a highly concentrated microalgae paste. This paste was divided into 500 g or 1 kg bags, sealed, and stored at -20˚C. The paste plaques were placed in storage until its dispatch to FCUP. Notably, a fraction of the culture was reserved for High-Pressure Homogenization (HPH) extraction, with the unused portion meticulously sealed and returned to storage. The last microalgae culture to be inoculated into a flat panel was the *N. oceanica* culture. Due to being short on time, this culture as only

taken to the 80L flat panel scale, The *N. oceanica* paste used in the HPH extraction and sent to FCUP to be used in testing the Ohmic Heating extraction methods was generously provided by NECTON S.A. from industrial production stock.

3.1.5. High Pressure Homogenizer Extraction Procedure

Cultures were prepared, in triplicate, from a plaque of each microalgae culture created during the internship. The stored paste was thawed and weighted to make up 28 g of biomass DW. Once this has been done, the paste is transferred into a 1 L beaker and topped up to 300 mL of volume with distilled water. The culture is briefly stirred with a spatula. This process is repeated until three culture-bearing beakers have been obtained. These are then placed unto magnetic stirrers, where they are left to homogenize for at least 30 minutes. After being properly homogenized, the culture within a beaker is transferred into a 1000 mL graduated cylinder. There, its volume is topped up to 350mL, resulting in a culture with a concentration of 80g/L.

Each culture was run through the HPH three times, with duplicate samples containing 20 mg of DW being taken for analysis at the end of every run. The samples were analyzed to obtain the chlorophyll, carotenoid and pheophytin content of the microalgae. An OD reading of the samples was also performed.
Freshwater was used to adjust the pressure of the HPH. Two valves were used to regulate the pressure exerted within the HPH, one regulating the size of the opening and the other the strength exerted by the mobile piston. Firstly, the leftmost valve was slowly rotated to build up the pressure up to 50 bar. Then, the rightmost valve was used to elevate the pressure until it reached 700 bar. When pressure stabilized, the culture was carefully loaded into the HPH, for a total of three runs. Duplicate 2-mL samples were collected before and after each HPH run and stored in the fridge until analysis. If the sample wasn’t to be used within the few following days, it was instead frozen.
The HPH will tend to heat up after prolonged use due to the friction produced by the mobile piston within it. This will also cause the culture to heat up as it passes through it, which can cause degradation of the extracted compounds. To remedy this, the temperature at the inlet and outlet of the HPH was monitored with a thermometer, and the culture sample kept as cool as possible by resting it in an ice bath before and after its processing. The processed culture was kept and sealed in plastic bags at the end of

the procedure. It was then stored with the plaques of the same microalgae culture at -20˚C.

a) HPH Extract Pigment Analysis Procedure

The samples obtained in the previous section were diluted with 38 mL of freshwater (1:20 dilution). These samples were homogenized, and, from them, 10 mL were separated into two 15-mL Falcon tubes, with 5 mL destined for OD reading, and the remaining 5 mL meant for the pigment analysis.

The 5 mL samples destined for pigment analysis were first sent to centrifuge for 10 minutes at 4000 rpm. Once the samples were centrifuged, the supernatant was discarded and 4 mL of acetone at 90% concentration were added to the pellet. The samples were then homogenized with the vortex for 2 minutes, and sent to be centrifuged once more, for 10 minutes at 4000 rpm. The resulting supernatant was collected into a new 15-mL Falcon. These were taken and homogenized before a portion of each was transferred to quartz cuvettes. Another quartz cuvette was filled with acetone at 90% to serve as a blank. Finally, the cuvettes were placed in the spectrophotometer, and their absorbance at 750 nm, 664 nm, 647 nm, 630 nm, 510 nm and 480 nm was read.

For the pheophytin analysis, the samples in the cuvettes were collected back into the tubes, and 3 mL of each sample was pipetted to new 15-mL Falcon tubes, to which 100 µL of hydrochloric acid at 0,1M as added. The acidified samples were vortexed for 20 seconds and left to rest for 70 seconds, upon which a portion was transferred to quartz cuvettes and placed back in the spectrophotometer. Their absorbance at 750nm and 665nm was then read.

3.2. Techniques Developed in FCUP

The work developed at FCUP was focused on testing the efficacy of Ohmic Heating technology as an extraction method for microalgae products. This involved adapting various procedures for use in analyzing the contents of the extraction and drafting an extraction plan, seen in Fig. 7. These methods were developed after determining the general make-up of each microalgae species trough thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Originally, all the four cultivated microalgae species would be used to test the ohmic heating as an extraction method and would also be run through microwave extraction to compare between these two methods. However, time constraints forced us to reduce the methods tested to just the ohmic heating method, using only two of the microalgae species. The microalgae cultures selected for this purpose were *N. oceanica* and *P. tricornutum*, as they were both saltwater species and, so, the results would be comparable. The presence of silica frustules in *P. tricornutum* cells posed an intriguing variable, as it was unknown what effect, if any, it would have on the ohmic heating extraction method.

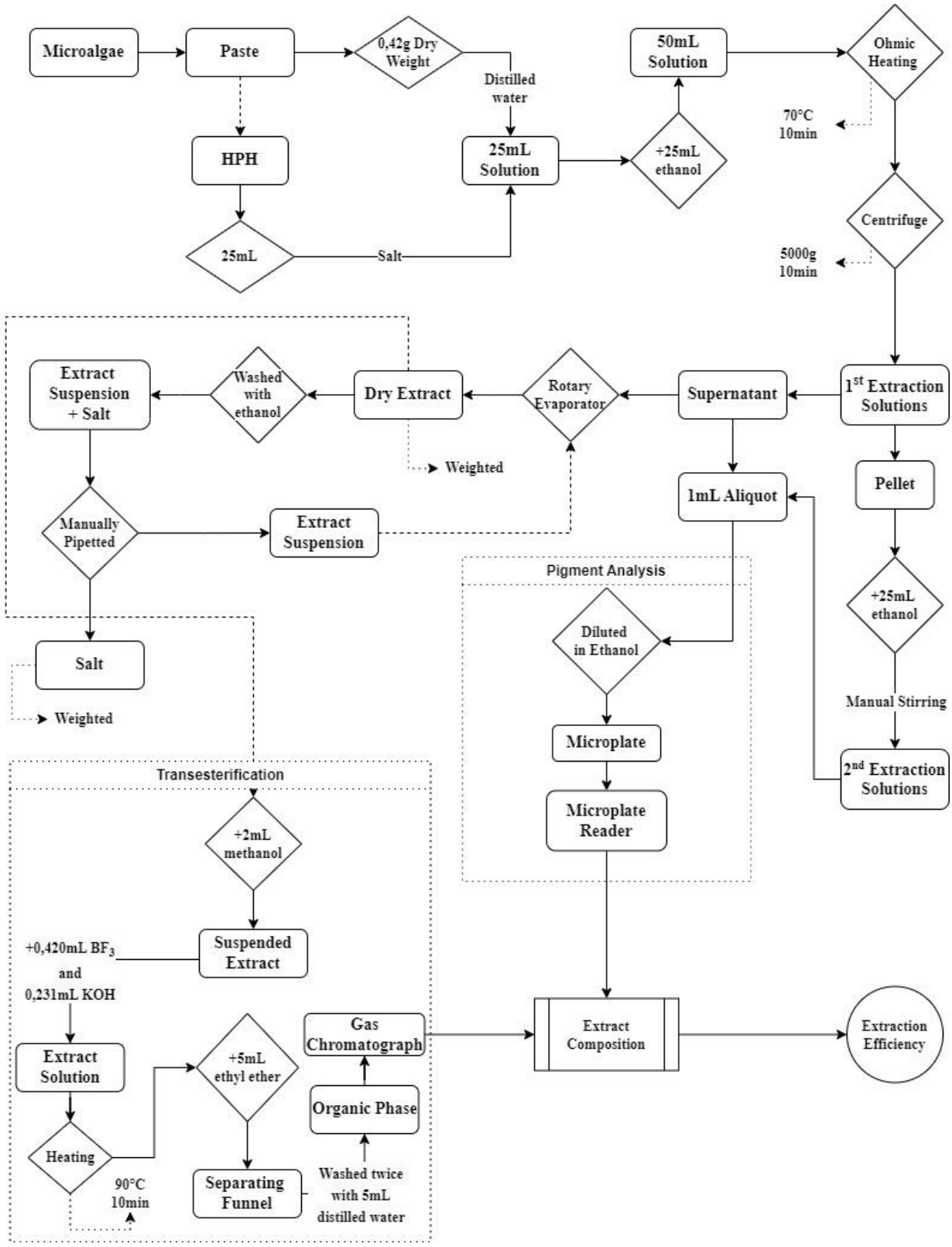

Fig. 7 - General procedure for analysing the efficacy of ohmic heating as an extraction method.

3.2.1. Ohmic Heating Extraction

For the experiments conducted in Ohmic Heating (ΩH), a prototype of the ohmic reactor was employed (Portuguese Patent No. 105908, 2011-09-27), comprising the following elements: an AC power supply; a signal generator; a heating reactor cell (50 mL glass container) containing a pair of rectangular stainless steel electrodes (316) (40 mm in length x 25 mm in width; 1 mm thick) positioned at a fixed distance (23 mm); a temperature sensor (type K thermocouple); the data logger system; a computer for data acquisition with control software. This system is identical to the one previously described in the literature.[16] The AC voltage (VAC) ranges from 5 to 100 VAC, with a typical operating frequency of 25 kHz. The reactor, instrumentation, data acquisition, and control collectively constitute the ΩH system, enabling real-time monitoring of parameters such as temperature (T), AC current intensity (IAC), AC voltage (VAC), and the electrical impedance of the reactor. Additionally, this system allows for the control of applied frequency and generated power.

The solution of culture within the heating cell must contain ionizable elements to allow the formation of electric currents and ohmic resistance which permit the process to take place. Although this is not a problem for the solutions created from the microalgae paste, as saltwater, which easily contains sufficient electrolytes in the form of NaCl, can be used in place of freshwater to top-up the paste to its intended concentration. However, the HPH extract was created using freshwater and diluted to a specific concentration, and as such, cannot make use of saltwater to achieve this effect. Instead, an amount of NaCl salt is added to the HPH solution such that its final concentration is the same as what is found in saltwater. To determine the amount of salt necessary to achieve this, various samples of different concentrations of salt were prepared, and their conductivity was measured, to build a correlation between the salinity and the conductivity of a sample.

a) Materials used in the Procedure

- Assembled Ohmic reactor (as previously described);
- UT230B-EU Power Socket;
- Volumetric Pipette (25mL);
- Ethanol (99,98% concentration);
- Centrifuge Tubes (50mL);

- Centrifuge;
- Precision Scale;
- Round Bottom Evaporating Flask (100mL);
- Rotary Evaporator;
- DURAN round glass flask (250mL).

b) ΩH Extraction Procedure

Before beginning the procedure, the paste or HPH extract had to be retrieved from storage, a freezing arc at -30°C, and left to thaw until it was sufficiently malleable. In the case of the HPH solution, the entire volume of the extract had to be thawed so it could be homogenized before being retrieved. Once this is done, 25mL of the HPH solution were transferred into the heating cell, followed by 25mL of absolute ethanol, using a volumetric pipette. Then, 0.4492 g of NaCl was added to the mixture.
For the paste, it would be thawed to the point where it was possible to retrieve a portion of the plaque with a spatula. The paste was then taken to a precision scale and weighted until it reached the target mass. This mass varied slightly according to the biomass concentration of the paste and of the equivalent HPH solution (Equation 3).
The cup bearing the paste was retrieved and 25mL of saltwater was added to it using the volumetric pipette. Because the HPH extract would have the lower concentration among the two, and it would be impractical to concentrate it, the concentration of the paste had to be made to approximate it through dilution. The paste was mixed until it was completely dissolved in the saltwater volume, after which it was pipetted unto the heating cell, alongside 25mL of ethanol, using the volumetric pipette.

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$$Target\ Mass\ of\ Paste(g) = \frac{(HPH_{biomass\ concentration}(g/L) * 0{,}025mL)}{Culture\ Biomass \in 1gofPaste(g)}$$

Equation 3: General equation to determine target mass of paste to weight to obtain a 25mL solution with an equivalent biomass concentration to that of the HPH solution.

Both the paste and the HPH solution processes resulted in a 50mL solution within the reactor the cell. A small magnet was added to the cell and the lid, bearing the

electrodes and the temperature sensor, enclosed it. The procedure would then begin, as dictated in the following steps:

A. The ΩH Reactor was set to the desired parameters (600mV; 25KHz; amplification channel 2) and activated;

B. Whilst monitoring the temperature, the reactor was left to run until a temperature nearing 70°C was reported from the cell, upon which the amplification channel had to be manually regulated to slow down the heating;

C. Once the temperature reached 70°C, the output of the power socket was recorded. The temperature of the culture had to be maintained as close as possible to 70°C by manually regulating the amplification channel until the end of the run;

D. After 10 minutes of heating, the output of the power socket was once again recorded, and the reactor quickly deactivated. The reactor automatically saved its output data as part of its deactivation process;

E. After cooling to room temperature, the content of the ohmic reactor was transferred to centrifuge tubes. In this case, 25mL were pipetted into each of the prepared centrifuge tubes. This was done despite the tubes' 50 mL capacity to avoid the pressure build-up reaching a point where it could cause the tubes to rupture in the centrifuge;

F. These steps were repeated until all of the runs for that experiment (3, including analytical copies) had been completed. The centrifuge tubes containing the extract were then centrifuged at 5000 rmp for 10 minutes. During this time, the rotary evaporator was activated, and its water bath set to 60°C;

G. A 1mL aliquot was taken of the extract from one in each pair of centrifuge tubes and transferred to a 10-mL vial, which was enveloped in tin foil and stored in a fridge for analysis;

H. The contents of each pair of centrifuge tubes were transferred to a round-bottom evaporation flask, and the solvent was subsequently evaporated in the rotary evaporator at reduced pressure not exceeding 50°C until the extract was completely dry;

I. The dry extract was washed with ethanol until completely suspended, after which it was transferred to a new flask. The salt mixed within the extract would not be suspended, allowing the transference of the extract to a new flask to also serve as a purification step. The complete step was repeated a total of five times to ensure as much of the extract is collected. A sonificator was used to ease the process;

J. The flask containing the salt as weighted in a precision scale to determine the quantity of salt recovered.

K. The suspended extract was dried in the rotary evaporator. Once dry, the extract was scrapped from the flask and weighted.

c) Second Extraction

Although much of the extract is concentrated in the supernatant obtained after centrifuging the Ohmic extraction sample, a portion of it is retained within the pellet. Thus, a second extraction step becomes necessary to recover the totality of the extract, a simple procedure consisting of transferring 25mL of ethanol into each centrifuge tube containing a pellet, and manually suspending it with vigorous shaking. The suspended extract is then transferred into a flask and dried in the rotary evaporator, weighted and stored.

3.2.2. Thin Layer Chromatography Methodology

The thin layer chromatography (TLC) allowed for the separation of the components extracted through ohmic heating processing of the microalgae through the use of a solvent. The thin-layer chromatography was performed in plates of silica gel 60 F254, from Merck and the solvent was determined by testing differing proportions of hexane and acetone, such as 1:1, 2:1 and 3:2.

If convenient, the TLC can be observed under Ultraviolet light to screen for fluorescent pigments. Although TLC didn’t allow us to determine the exact composition of the extracts, the presence of some pigments could be verified.

3.2.3. Lipidic Content Determination

The lipid content of the extract was analysed using gas chromatography. Since lipids are not naturally volatile, the samples underwent transesterification to convert their lipidic content into esters, which can be analysed by a Gas Chromatograph. The resulting data was compared with known patterns to identify the lipidic components of the microalgae extracts.

a) Materials used in the Procedure

- Pasteur Pipette;
- KOH Solution (0,5M);
- BF_3-Methanol Solution;
- Ethyl Ether Solution;
- Hydrolysis tubes;
- Heating plate;
- Centrifuge;
- Graduated pipette (2mL);
- Graduated pipette (5mL);
- Separating Funnel;
- Hotte.

b) Transesterification Procedure

Transesterification is a chemical reaction in which an ester is transformed into a different ester by the exchange of its alkoxy group with another alcohol. It is a key reaction in biodiesel production, where lipids, specifically triglycerides, are converted into Fatty Acid Methyl Esters (FAMEs).
In the reaction drafted for this procedure (Equation 4), boron trifluoride (BF_3), dissolved in methanol, serves as a catalyst. It complexes with methanol to form a reactive species that acts as a Lewis acid catalyst, which facilitates the activation of the ester bonds in the triglycerides, promoting the transesterification reaction, which concludes

with the formation of methyl esters and glycerol. The ethyl ether serves as the solvent for the reaction. As BF_3 is a highly reactive substance, the entire procedure must be carried out in an hotte.

$$Triglyceride + 3\ Methanol \xrightarrow{BF_3\ as\ Catalyst} 3\ Methyl\ Ester + Glycerol$$

Equation 4 – Reaction of Transesterification

Before beginning the procedure, the dry extract is retrieved from storage, of which 200mg are weighted and transferred to a hydrolysis tube. The hydrolysis tube containing the extract is taken to the hotte, alongside containers with the KOH and BF_3 solutions. A heating plaque is activated to heat up a water bath to 90°.

A. Once the water bath has reached a temperature of 90°C, 1mL of the KOH solution and 2mL of the BF_3 solution are added to the hydrolysis tube, after which the lid is quickly closed. The tubes are secured with clamps and submerged in the water bath, where they are left to react for 10 minutes;
B. Afterwards the hydrolysis tubes are taken out and placed into ice bath to cool;
C. Then 5mL of ethyl ether is added to each of the tubes;
D. The contents of a hydrolysis tube are transferred to a separation funnel. Adding 5mL of distilled water, the funnel is gently shaken to mix the contents. Then, the organic phase was removed and washed again with 5 mL of distilled water.
E. Once organic phase has been fully collected, a microspatula of anhydrous sodium sulphate is added to the organic phase and the mixture is gently mixed. The anhydrous sodium sulphate will absorb the water content that may have seeped into the organic phase, ensuring its drying;
F. The organic phase is collected into a transference flask with the aid of a Pasteur pipette.
G. When ready, a 1µL aliquot of an organic phases is injected into the Gas Chromatograph to be read. This step is repeated for all samples of organic phases obtained during the procedure.

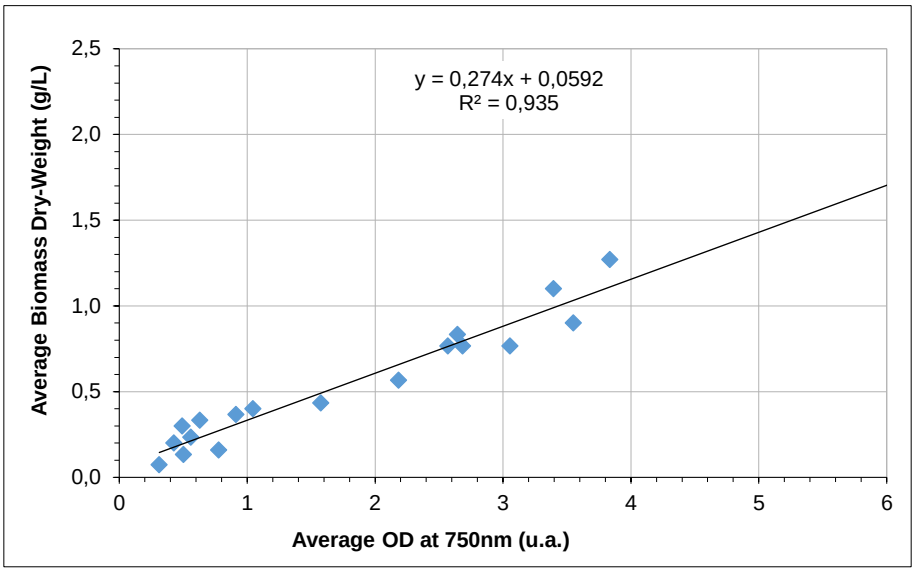
3.2.4. Pigment Content Determination

To determine the pigment content of sample, a part of their respective aliquot was transferred to an Eppendorf tube and diluted in 99.98% ethanol, in duplicate. The proportions of ethanol and sample volumes were adjusted to ensure readings fell within the Lambert-Beer Law range of 0.2 to 0.8 absorbance units (A.U.). The diluted duplicates were then transferred to a microplate, with their respective coordinates, assembled from the values given to each column and line, as were depicted in the microplate, were noted down. Once all the aliquots were in their respective microplate pits, it was inserted into a microplate reader, which was then programmed with the coordinates of the aliquot-bearing pits, allowing them to be read at 664 nm, 649 nm and 470 nm. The data obtained from the reading was then treated with the equations described in Ferreira-Santos et al. 2021 to determine the concentration of chlorophyll a, chlorophyll b and carotenoids of each sample.

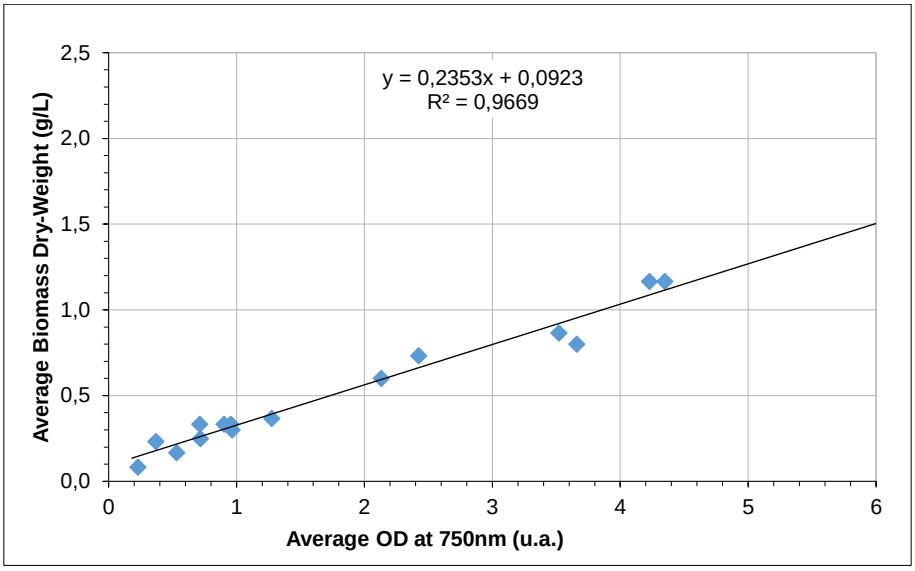
4. Results and Discussion

4.1. Microalgae Cultivation

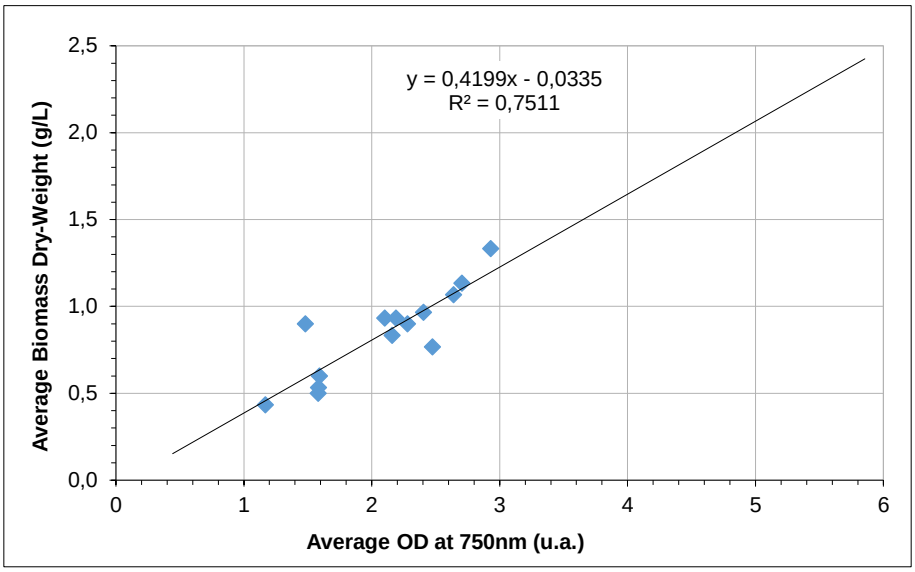
During the course of my internship at NECTON S.A, I conducted an extensive investigation into the cultivation of various microalgae species. It is noteworthy that the *N. oceanica* culture, as previously mentioned, was not subjected to the same monitoring techniques employed for the other cultures due to time constraints. To ensure rigorous control and assessment, a comprehensive set of analytical methods was applied. These techniques encompassed daily monitoring through OD measurements, microscopic examination for contaminant screening, determination of dry weight, and assessment of nitrate content. An essential aspect of this research involved establishing empirical relationships between the OD readings and the corresponding dry weight measurements of microalgae cultures. Linear regression analyses were performed, leading to the derivation of predictive equations that facilitated the estimation of biomass based on OD readings, as demonstrated in Graph 1, Graph 2 and Graph 3.



Graph 1 – Correlation between OD at 750nm and biomass concentration in (g/L) for the *T. obliquus* culture.

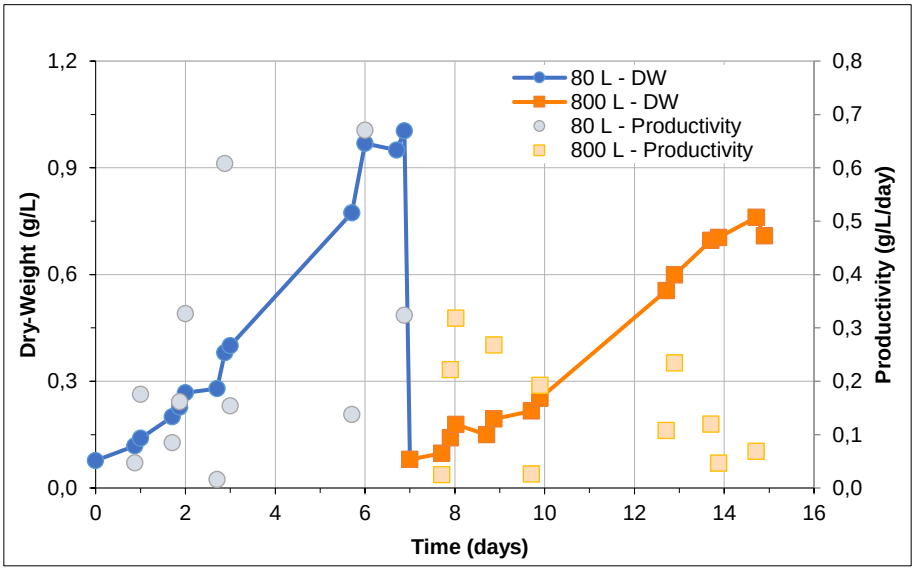


Graph 2 – Correlation between OD at 750nm and biomass concentration in (g/L) for the *N. limnetica* culture.

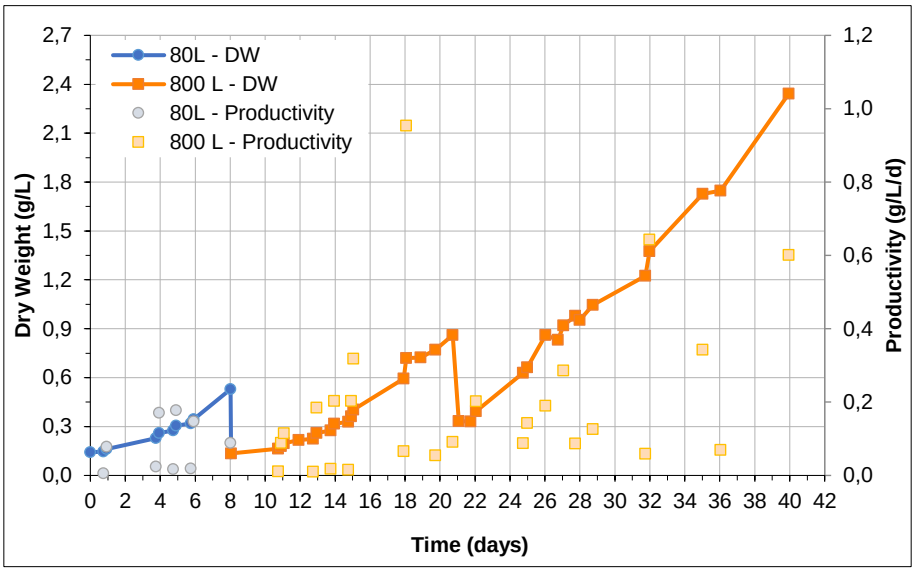


Graph 3 – Correlation between OD at 750nm and biomass concentration in (g/L) for the *P. tricornutum* culture.

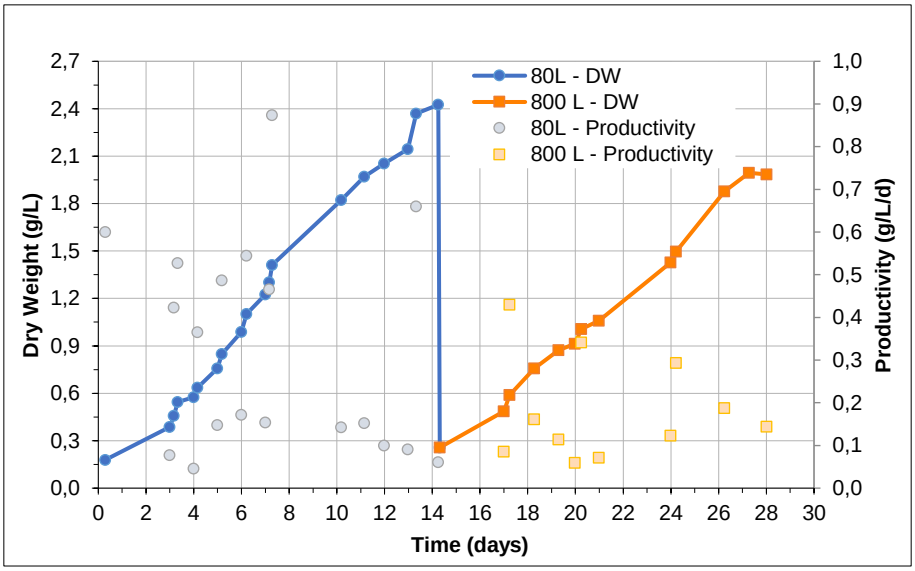
In tandem with the OD data collected throughout the cultivation of flat panel cultures, we charted the growth of each microalgae culture in biomass dry weight per liter, as illustrated in Graph 4, Graph 5 and Graph 6. The assessment of daily productivity, which, in turn, permitted the calculation of the average productivity of each microalgae was also accomplished.



Graph 4 – *T. obliquus*. growth (in biomass dry weight; g/L) and productivity (in g/L/d) along its cultivation in an 80L and an 800L flat panel system. Data corresponding to the 80L flat panel is colored blue, whilst that pertaining to the 800L system is colored orange.



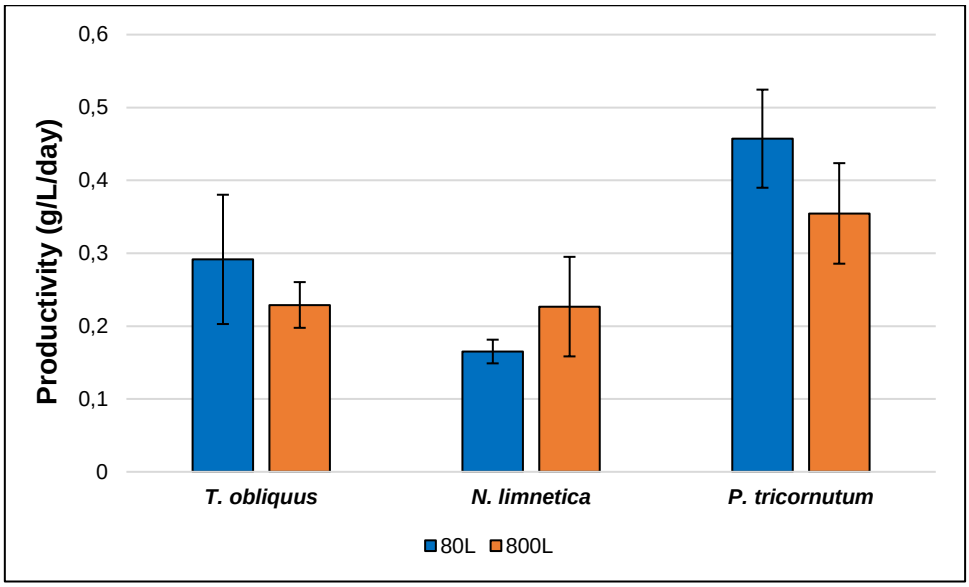
Graph 5 – *N. limnetica* growth (in biomass dry weight; g/L) and productivity (in g/L/d) along its cultivation in an 80L and an 800L flat panel system. Data corresponding to the 80L flat panel is colored blue, whilst that pertaining to the 800L system is colored orange.



Graph 6 – *P. tricornutum* growth (in biomass dry weight; g/L) and productivity (in g/L/d) along its cultivation in an 80L and an 800L flat panel system. Data corresponding to the 80L flat panel is colored blue, whilst that pertaining to the 800L system is colored orange.

In all three microalgae cultures, there is an expected sudden dip in dry-weight concentration due to the time of scale-up from 80L flat panels to the 800L system.

Visually, the average daily productivity of the 80L flat panels appeared to outperform that of the 800L system for the *T. obliquus* and *P. tricornutum* cultures, while the inverse was true for the *N. limnetica* culture. This observation was substantiated through the application of Equation 2 to the dry-weight data, as depicted in Graph 7.



Graph 7 – Average Productivity at 13:00 GMT of the microalgae species cultivated in 80L and 800L flat panel cultures during the NECTON S.A. internship.

Apart from the *N. limentica* culture, the 80L system consistently exhibited higher productivity than the 800L system, with the *P. tricornutum* culture being the most productive of the three microalgae species. The lower productivity of the 80L flat panel *N. limnetica* culture can be attributed to its lower initial biomass concentration, which was a result of time constraints. The initial OD readings for the 80L *N. limnetica* culture indicated that it was perilously close to the minimum viable biomass level established by NECTON. An OD reading below this threshold typically meant that the microalgae culture would not be able to reach critical mass, preventing its establishment in the culture medium. It is reasonable to assume that the *N. limnetica* culture would have followed a similar performance pattern to the *T. obliquus* and *P. tricornutum* cultures had it started with a higher biomass concentration. Additionally, as can be seen in Graph 5, the *N. limnetica* culture experienced a significant drop in dry-weight concentration when grown in the 800-L flat panel. This coincided with the renewal of

the 800L flat panel culture and were attributed to the presence of a contaminating microorganism, shown in Fig. 8, which was discovered during routine microscopic inspections a few days after its scale-up to the 800L flat panel system.

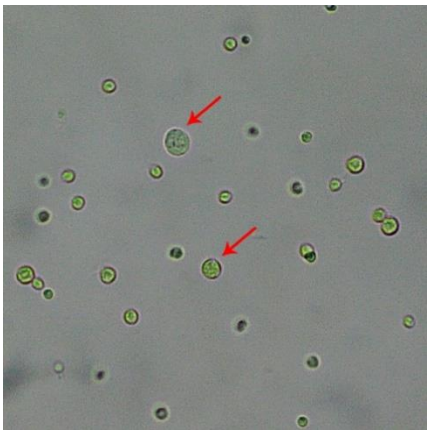


Fig. 8 - Unidentified contaminant microorganisms in a sample from the *N. limnetica* flat panel culture. Picture taken trough microscope lens, amplified 400 times.

In response, a comprehensive remedial plan was devised for the *N. limnetica* culture. A new 800L flat panel system was rapidly assembled, and approximately 250L of culture from the contaminated flat panel was inoculated into the new system. The contaminated flat panel underwent thorough disinfection and subsequent disassembly. Given the relatively lower concentration of the contaminant microorganism compared to that of the *N. limnetica* culture, the strategy focused on transferring the culture into a pristine flat panel with fresh nutrient medium. The goal was to capitalize on the microalgae's exponential growth phase, enabling it to rapidly expand its biomass and outcompete the contaminant. This intervention proved successful. It is reasonable to assume that the low initial biomass concentration of the *N. limnetica* culture, both when the 80L panel was inoculated and when the first 800L system was initiated, contributed to the contamination issue.

Furthermore, by selecting the highest data points from each flat panel culture in the graphical representations, we calculated the total biomass produced by each culture, as presented in Table 1. This analysis clearly indicated that, despite initial setbacks, the *N. limnetica* culture yielded the highest biomass among the three microalgae species. This finding aligned with the fact that it exhibited the highest productivity in the

800L system, which, owing to its larger volume, accounted for the majority of the culture's total biomass. Any observed fluctuations in dry-weight concentration in the graphs were primarily attributed to variations in OD readings, which could be traced to issues like sample homogenization or inherent observational errors. Importantly, these fluctuations did not lead to a decrease in the total biomass of the system and could thus be disregarded when quantifying biomass quantities.

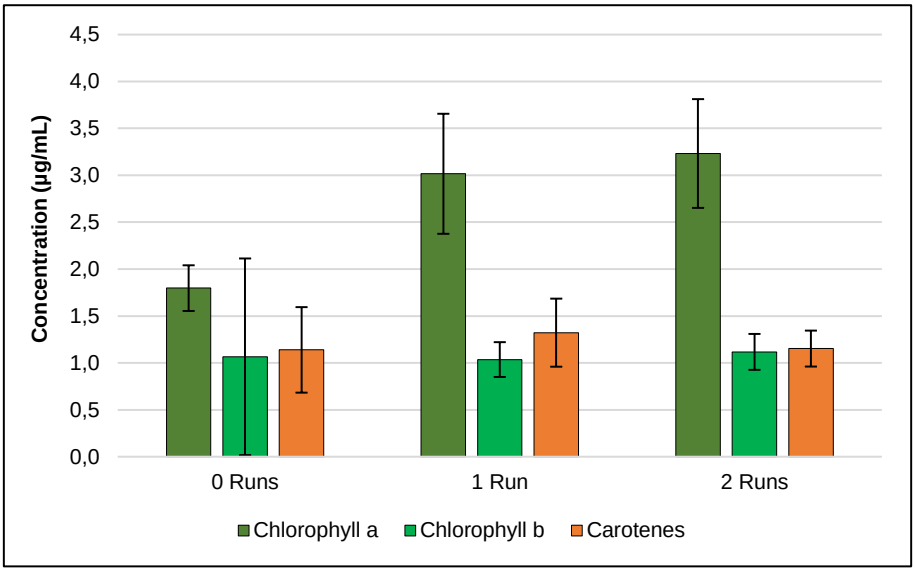
Table 1 - Total biomass produced by each microalgae culture

Microalgae Species	Biomass Yield (g)		Total	Culture Duration (days)	Productivity (g/day)
	80-L System	800-L System			
T. obliquus	74	528	602	15	40
N. limnetica	31	1831	1862	40	46
P. tricornutum	180	1393	1573	28	56

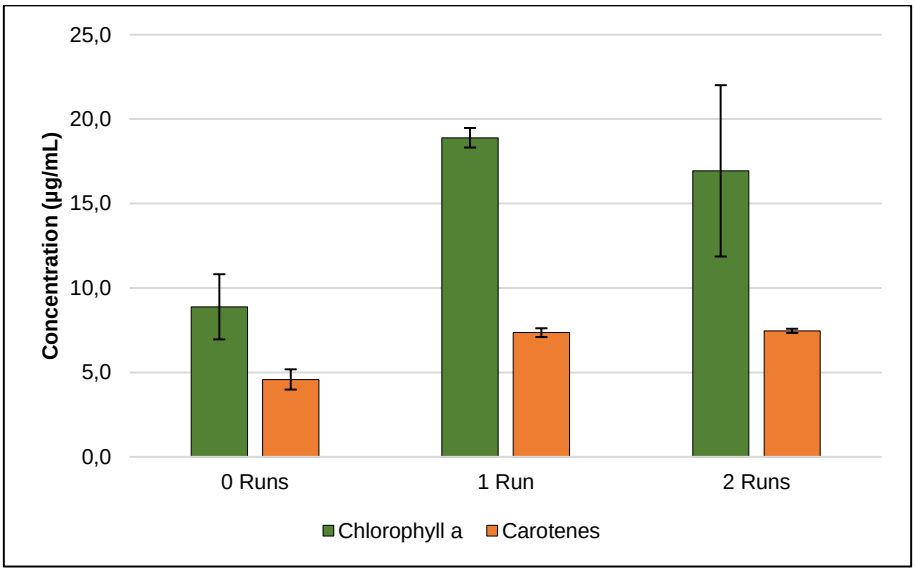
4.2. High Pressure Homogenizer Extraction

The absorbance data from the HPH-treated samples were used to determine the concentration of specific pigments, including chlorophyll and carotenes. To quantify these compounds, equations established in accordance with the standard methodologies outlined by Baird et al. (2017) and Jeffrey & Humphrey (1975) were applied. The analysis involved three distinct categories of samples: those that did not undergo the HPH extraction process, those subjected to a single run of the HPH procedure and those processed through two consecutive HPH cycles. The HPH extraction procedure subjected the samples to 600 bar of pressure. These datasets are presented in Graph 8, Graph 9, Graph 10 and Graph 11. It is important to note that only those compounds that were capable of being suspended in the solvent, owing to the lysis of their host cells, were measured in the analysis. Therefore, an increase in their concentration is a direct reflection of enhanced cell lysis, thereby serving as an indicator

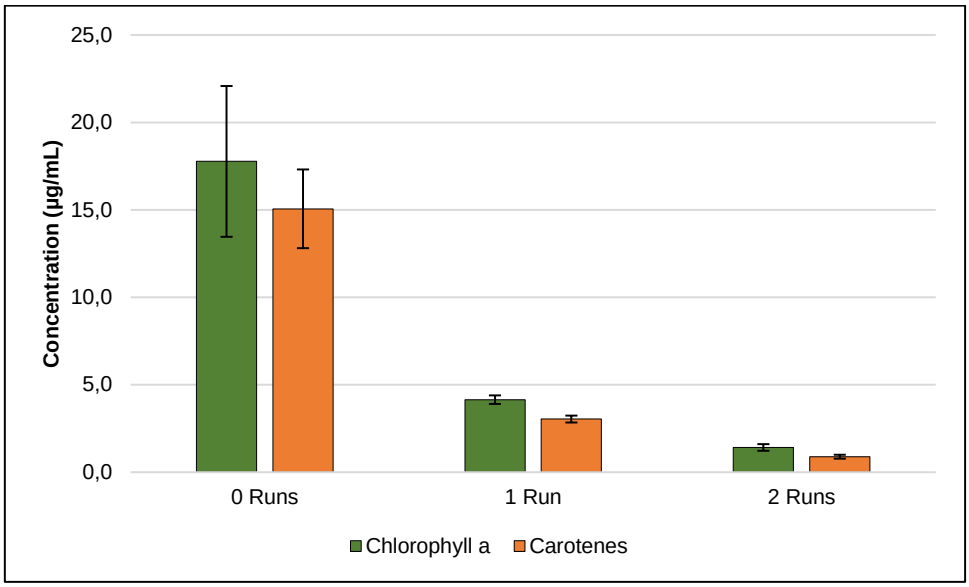
of the efficacy of the HPH extraction method. Furthermore, it enables a comparative assessment of the extraction efficiency across the four microalgae species.



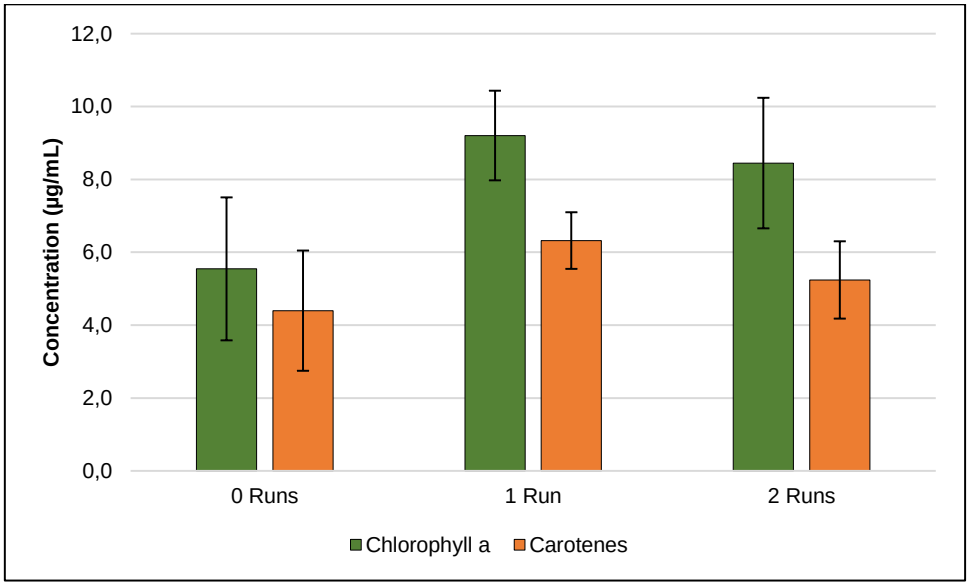
Graph 8 – Concentration of compounds of the *T. obliquus* samples extracted trough the HPH method.



Graph 9 – Concentration of compounds of the *N. limnetica* samples extracted trough the HPH method.



Graph 10 – Concentration of compounds of the *P. tricornutum* samples extracted trough the HPH method.



Graph 11 – Concentration of compounds of the *N. oceanica* samples extracted trough the HPH method.

Among the various chlorophyll species, Chlorophyll b was only identified in the *T. obliquus* sample, which also presented a lower pigment concentration compared to the other samples. Interestingly, despite the known presence of this pigment in *P. tricornutum*, no Chlorophyll *c* was detected in its samples. The data obtained from the *P. tricornutum* samples deviated from the anticipated trends in multiple ways.

For the remaining three microalgae species, a clear increase in compound concentration was observed after a single HPH extraction cycle, suggesting the effectiveness of this extraction method. However, a second run through the HPH extraction system showed either little increase, or even a slight decrease, in compound concentration, implying that multiple cycles through the HPH process do not yield additional benefits.

Notably, all samples exhibited a certain availability of pigments even before undergoing HPH extraction. This is likely attributable to the preparation of these samples from concentrated microalgae paste, which had been previously subjected to freezing. This freezing process likely induced the formation of ice crystals within the culture, causing the rupture of a portion of the microalgae cells and subsequent release of compounds.

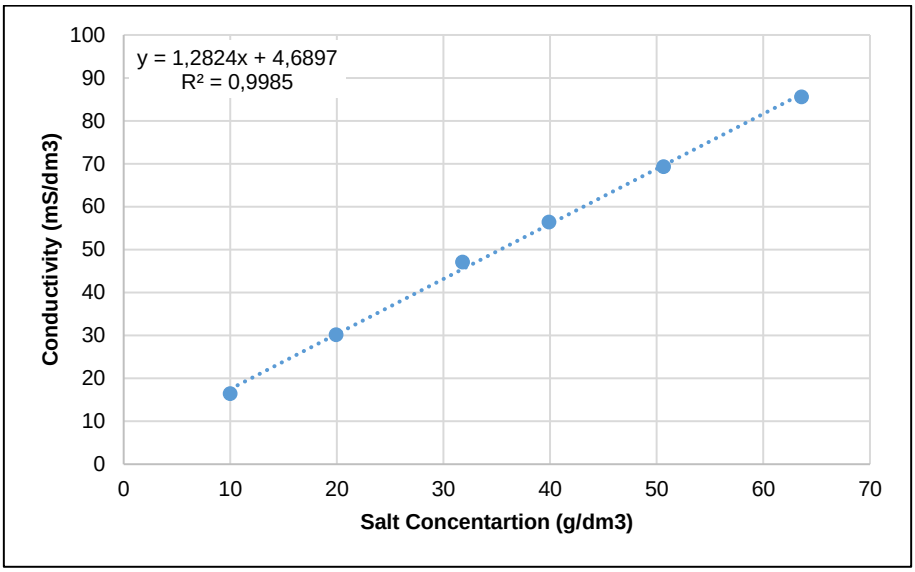
In contrast, the despite the known presence of this pigment in *P. tricornutum* samples demonstrated a consistent decrease in compound concentration with each successive HPH extraction cycle. Due to time constraints and limitations within the scope of the NECTON S.A. internship, the reasons for this unexpected trend were not investigated in detail. However, it may be speculated that the despite the known presence of this pigment in *P. tricornutum* sample exhibited the highest compound concentration among those not subjected to HPH extraction, indicating that the primary source of cell lysis may have been the formation of ice crystals. It is plausible that the unique structural characteristics of despite the known presence of this pigment in *P. tricornutum*, specifically the presence of a silica frustule, could have amplified the lysing effect of ice crystal formation. Furthermore, processing despite the known presence of this pigment in *P. tricornutum* through the HPH extraction method may have resulted in the release of pigments from previously lysed cells, subsequently entrapping them in the supernatant during the initial centrifugation step of the analysis. These compounds were subsequently discarded, preventing their detection during the analysis process. However, even if this was the case, the pigments would have been seriously degraded to explain such a large drop in concentration.

4.3. Ohmic Extraction

The ΩH extraction process relies on the presence of ionizable elements within the heating cell, allowing the formation of electric currents and ohmic resistance, which are essential for the thermal process to occur. In this process the solution, which serves as an electrical resistor, is heated by passing electricity through it (electrodes are in contact with the medium). Thermal energy generation is due to the motion of the charged species in solution as result of the high frequency (25 kHz) AC electric current. Thus, electrical energy is dissipated into heat with high efficiency, which results in a high-speed heating rate and allows for rapid and uniform heating.

The use of sea water in the ΩH reduces the resistance of the solution. When using high concentrations of NaCl, conductivity increases, and heating becomes very rapid. However, it does not promote polarization of the substances present in the solution, reducing resistance and lowering the voltage value. Therefore, the concentration of NaCl may have an influence on the extraction process, both in terms of the extraction efficiency of the components and the energy efficiency of the process.

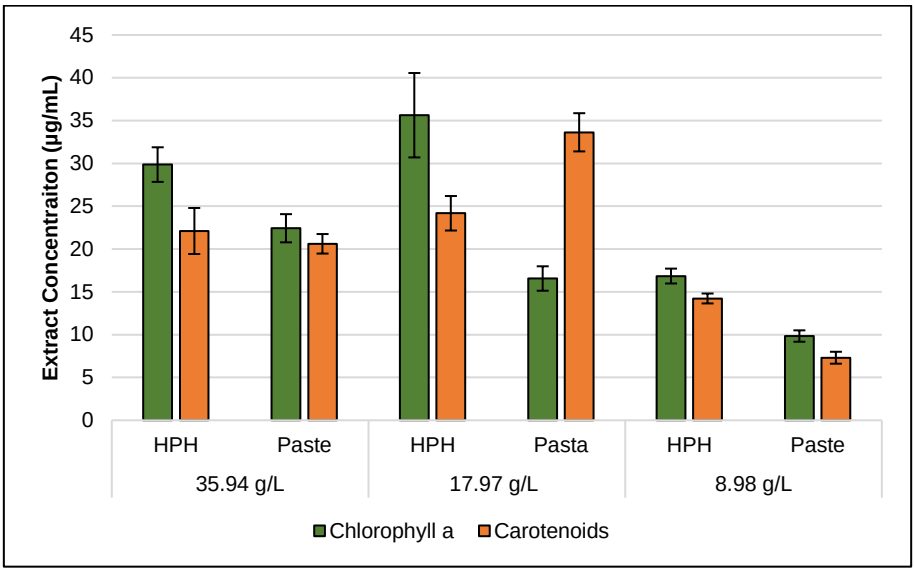
While this requirement is readily met in solutions derived from microalgae paste, as they inherently contain saltwater with sufficient ionic conductivity, a challenge arises when working with HPH extracts, which are initially created using freshwater and then diluted to a specific concentration. To address this, a specific amount of *NaCl* is introduced into the HPH solution to achieve the same salinity that is found in saltwater. To determine the precise quantity of salt needed for this purpose, a series of samples with varying *NaCl* concentrations were prepared, and their conductivity was measured to establish a correlation between sample salinity and expected conductivity, as illustrated in Graph 12. The conductivities of saltwater and the HPH solution were also measured, and the salinity of each was calculated using the previously determined correlation. Consequently, the amount of *NaCl* required to be added to the HPH solution was calculated by subtracting its salinity from that of saltwater and then multiplying the result by the expected solution volume of 50 mL, resulting in 0.8984 g of *NaCl*.



Graph 12 – *Relation between conductivity and concentration of NaCl in different aqueous (1:1 water:etanol) solutions.*

Finally, considering the advantages of using saline solutions, and taking into account results already reported in the literature that show high extraction efficiency of natural pigments with ethanol, it was decided that a mixture of saltwater/ethanol (1:1) would be used as an extraction solvent for ohmic heating experiments. Within this mixture of solvents, a good ionic conductivity to the medium is guaranteed, provided by the saline fraction, while the ethanolic fraction guarantees efficient extraction of the main natural pigments. Furthermore, this mixture of solvents guarantees greater safety in carrying out the ohmic heating process when compared to the use of pure ethanol, as ethanol has a low ignition point and is therefore easily flammable. On the other hand, we are using more ecological solvents, thus promoting the environmental sustainability of the method.

A series of optimization runs were conducted using the ohmic reactor, employing both paste and HPH solution from the *P. tricornutum* culture and using different concentrations of salt: 35.94 g/L, 17.97 g/L and 8.98 g/L. All experiments were carried out using ΩH at 70°C, for 10 min. The results, depicted in Graph 13, demonstrated that a salt concentration equivalent to half that of saltwater yielded the highest extraction efficiency, as measured by the ratio of extract DW to the biomass DW used in the ΩH extraction run , which translates into a higher concentration of chlorophyll a in HPH and carotenoid pigments in paste, in accordance with the results obtained in the spectroscopic method.



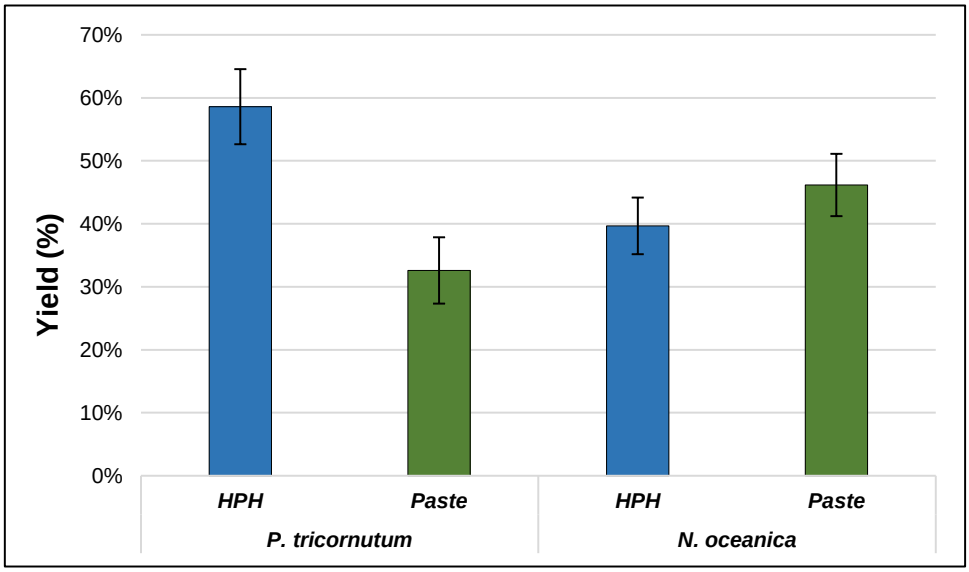
Graph 13 – Evaluation of salinity effect on the extraction process for the *P. tricornutum* samples, on experiments carried out under ohmic heating at 70ºC, for 10 min.

Additionally, energetic efficiency was assessed by correlating the extract DW with the energy consumption recorded by the power socket.

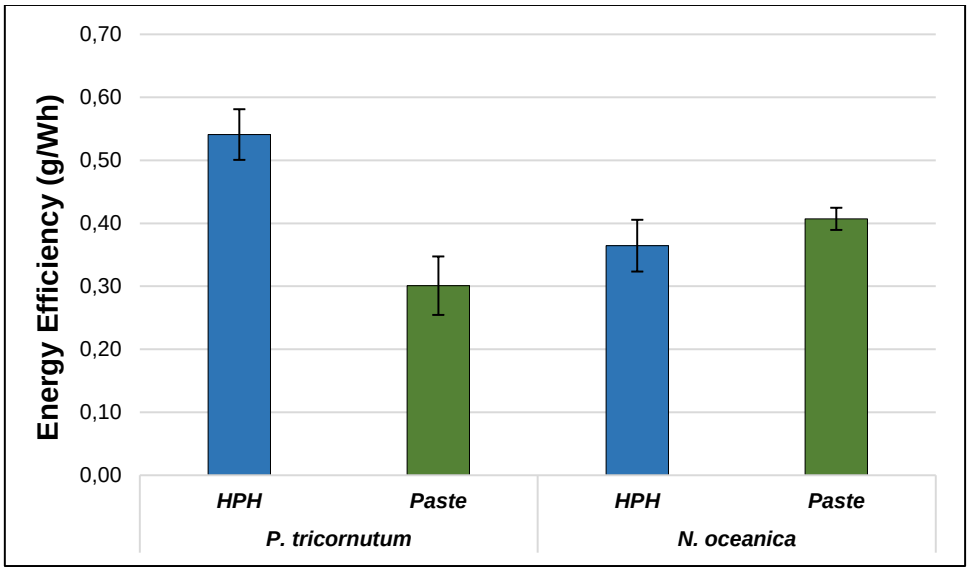
In practice, to carry out the experiments on HPH, 25 mL of HPH and 25 mL of ethanol were measured, whereas for the experiments on the paste, the corresponding amount of paste was weighed, to which 25 mL of saline solution and 25 mL of ethanol were added, from the *P. tricornutum* and *N. oceanica* cultures.

The results, showcased in Graph 14 and Graph 15, indicated significantly greater efficiency of the ΩH extraction process on the HPH sample from *P. tricornutum* in comparison to the paste sample. However, for the *N. oceanica* culture, the yield and efficiency of the ΩH method were comparable. Interestingly, the optimization trials unveiled that prolonged ΩH extraction runs led to reduced yields, particularly concerning Chlorophyll a, as shown in Graph 16. This decline in yield could be attributed to the potential degradation of extracted compounds due to the high temperatures (70˚C) applied during the process. Alternatively, the conditions within the heating cell may facilitate the aggregation of extracted compounds after extended exposure, causing them to precipitate after centrifugation, resulting in their loss in the pellet. Regrettably, owing to time constraints, the optimization of the ΩH extraction method to the processing of the selected microalgae could only be conducted for the *P. tricornutum* samples.

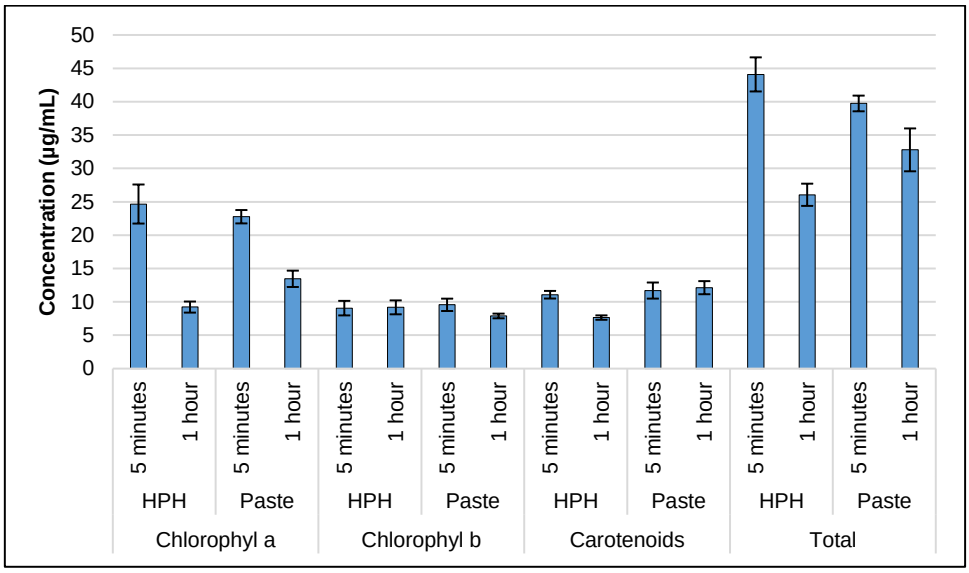
Consequently, it was not possible to confirm whether this effect is more pronounced in the *N. oceanica* samples, potentially explaining the lower efficiency of ΩH for compound extraction in its HPH extract runs.



Graph 14 – Total extraction yield, expressed in % of extract DW to biomass DW, of the different ohmic heating experiments carried out at 70 °C, for 10 min, of extraction for *P. tricornutum* and *N. oceanica* cultures.



Graph 15 – Energy efficiency, expressed in g/Wh, of the different ohmic heating experiments carried out at 70 °C, for 10 min of extraction for *P. tricornutum* and *N. oceanica* cultures.



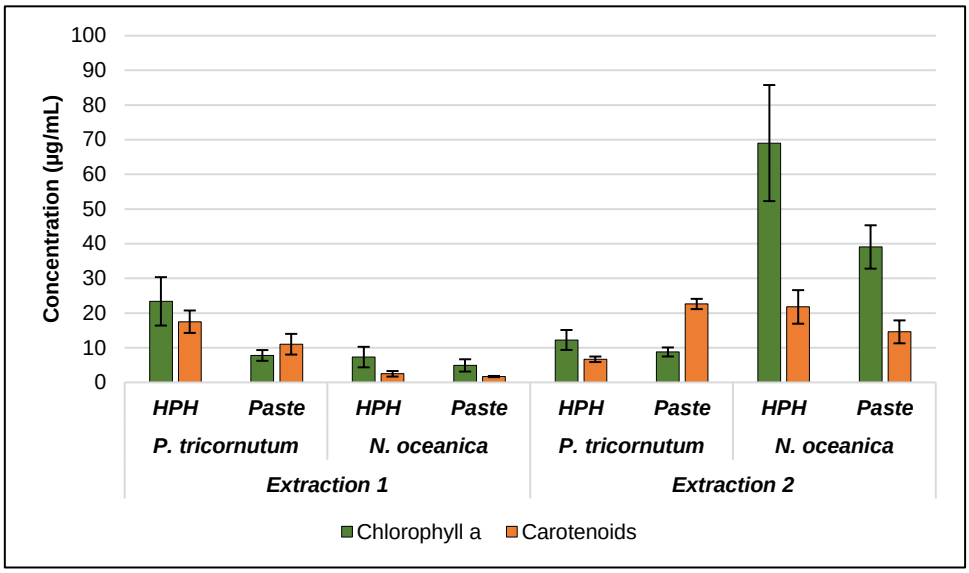
Graph 16 – Concentration of different pigments (chlorophyl a, chlorophyll b and carotenoids) obtained from different ohmic heating experiments carried out at 70 °C, for 5 and 60 min, of extraction for *P. tricornutum* culture.

To verify whether any of the compounds remained within the pellet, an additional extraction step was introduced, which involved the vigorous resuspension of the pellet in a 99.8% ethanol solution. Aliquots were taken of the pellet suspensions. These, together with the aliquots collected during the first extraction step, were meticulously diluted, and subsequently analysed using a microplate reader. The results, presented in Graph 17, provided compelling evidence that a substantial portion of the *N. oceanica* extract had indeed precipitated and could be recovered with this additional step.

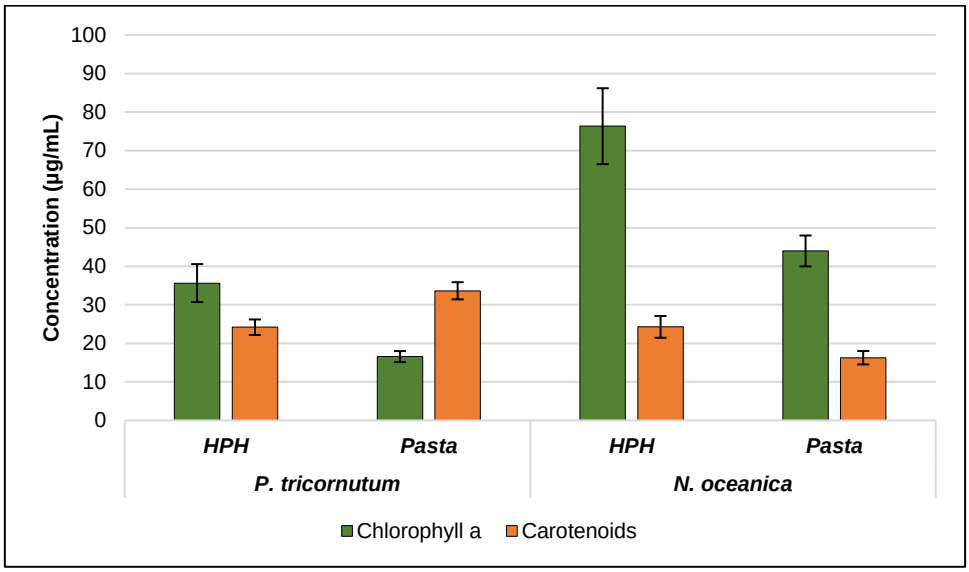
Contrary to the initial findings, the data from the secondary extraction step indicated that *N. oceanica* exhibited the highest overall yield of pigments of the two microalgae species, shown in Graph 18. This observation underlines the significance of the secondary extraction process.

It is also interesting to note that the *P. tricornutum* paste extracts bear a higher proportion of carotenoids when compared to those of its HPH counterpart, which is even more pronounced in the contents of their second extraction steps. This phenomenon suggests that the ΩH extraction method, particularly when complemented by a secondary extraction step, might exhibit some degree of specificity for the extraction of carotenoids.

Furthermore, the fact that the carotenoid content in the *N. oceanica* paste and HPH extracts remains comparable, despite substantial differences in their respective chlorophyll concentrations, raises the possibility that this difference may primarily be attributed to the influence of the HPH extraction. This preliminary evidence tentatively supports the notion that the ΩH extraction method is indeed effective in the selective extraction of carotenoids.



Graph 17 – Concentration of different pigments (chlorophyl II a and carotenoids) obtained from the first (ΩH) and second (resuspension) extractions for *P. tricornutum* and *N. oceanica* cultures.



Graph 18 – Concentration of different pigments (chlorophyl *a* and carotenoids) obtained from the first (ΩH) extraction for *P. tricornutum* and *N. oceanica* cultures.

4.4. Thin Layer Chromatography

The Thin-Layer Chromatography (TLC) technique served as a valuable tool for the separation of components obtained through the ohmic heating processing of microalgae using a suitable solvent. To optimize the separation, a systematic approach was taken to identify the most effective solvent by experimenting with various proportions of hexane and acetone, both common choices in TLC procedures. The proportions tested included a 1:1 ratio, a 2:1 ratio, and a 3:2 ratio. These tests were conducted in duplicate, as depicted in Fig. 9, with the 3:2 ratio displaying superior clarity and an improved ability to separate the different components. While TLC did not provide precise compositional information regarding the extracts, it did confirm the presence of certain pigments. The technique's simplicity, sensitivity, and rapid separation capabilities facilitated on-the-spot insights into the composition of the extracts. This not only validated hypotheses but also guided the refinement of experimental methodologies. TLC proved especially useful for expedited comparisons between microalgae extracts, encompassing those derived from the same culture under varying ohmic heating conditions and those from distinct cultures subjected to identical treatment parameters.

Notably, TLC successfully detected the presence of a xanthophyll species within the composition of the *P. tricornutum* extract.

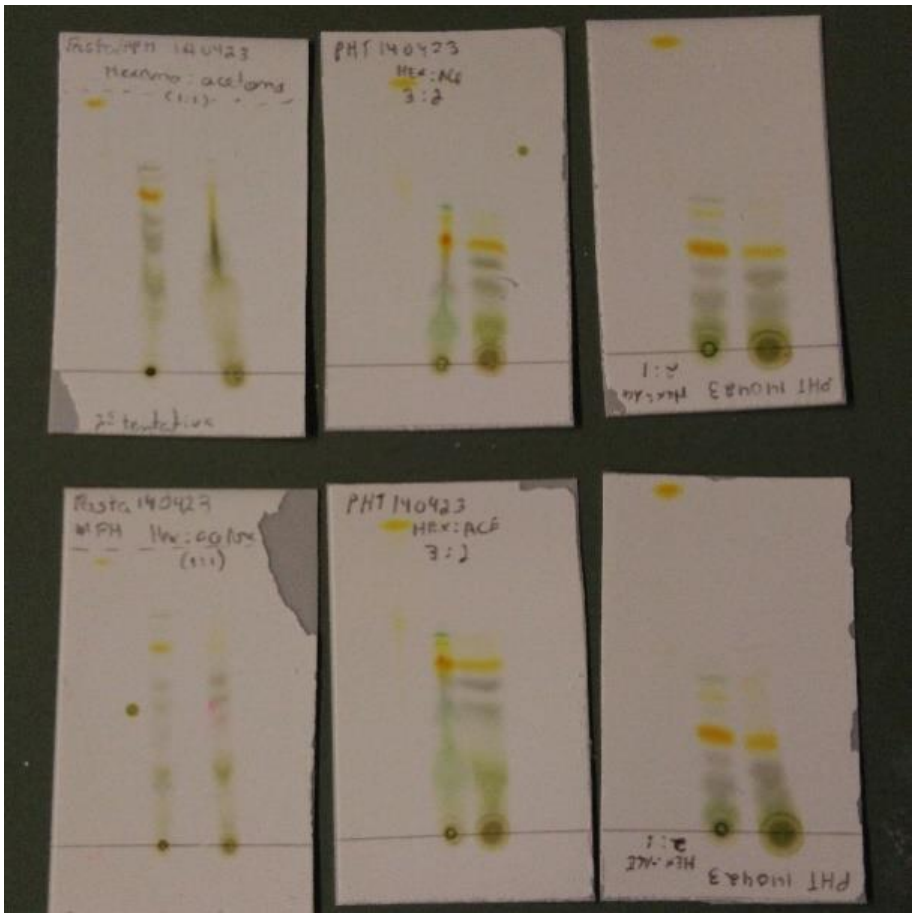


Fig. 9 - *TLC analysis of a P. tricornutum extract, using different proportions of hexane:acetone as solvent, and their respective analytical duplicates. The TLC employing the mixture of hexane:acetone 3:2 is in the middle, 1:1 in the left side and 2:1 in the right side*

4.5. Lipidic Content

Aliquots were taken from the *P. tricornutum* and *N. oceanica* extracts that underwent the transesterification procedure, for a total of four aliquots, and read through the GC. The obtained data was compared with a standard Fatty Acid Methyl Ester (FAMES) pattern reading, as seen in Fig. 10, Fig. 11, Fig. 12 and Fig. 13, allowing for the identification of the various methyl ester derivatives.

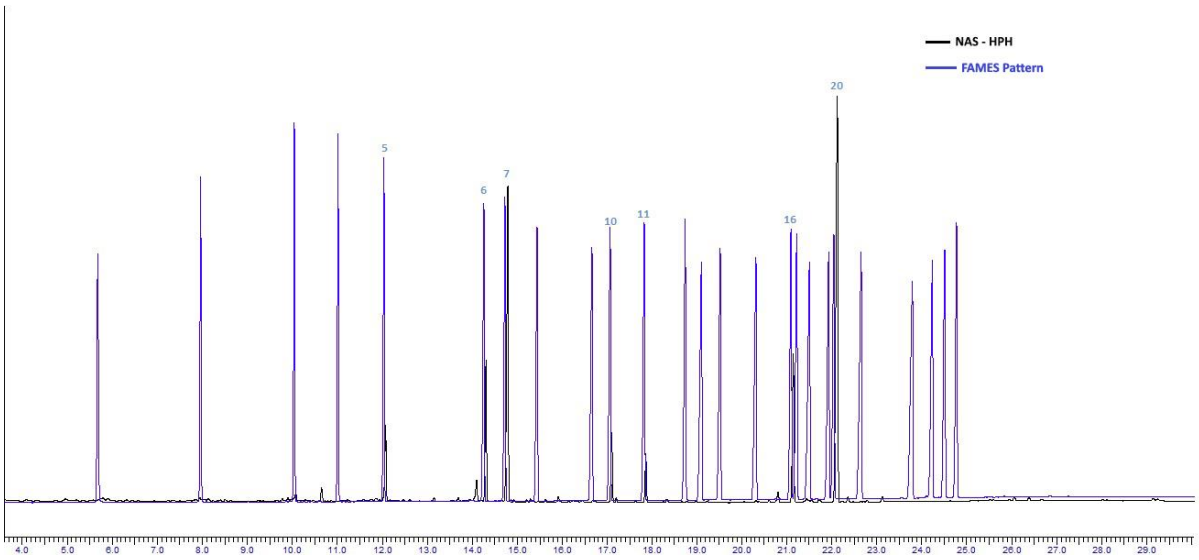


Fig. 10 - *N. oceanica HPH sample's GC reading results, colored black, overlapped under the standard FAMES pattern, in blue.*

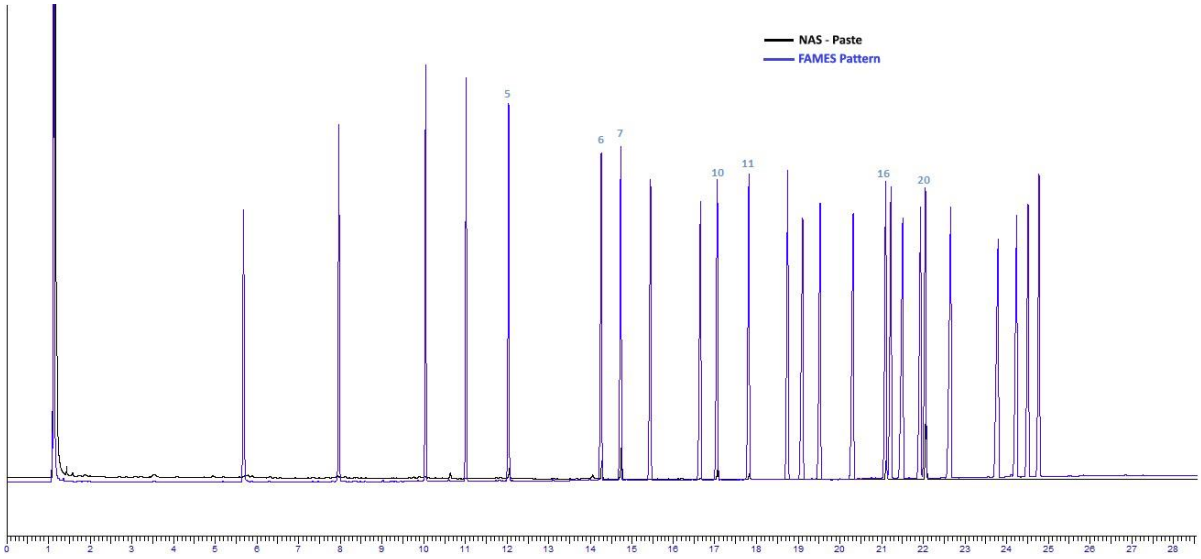


Fig. 11 - *N. oceanica* Paste sample's GC reading results, colored black, overlapped under the standard FAMES pattern, in blue.

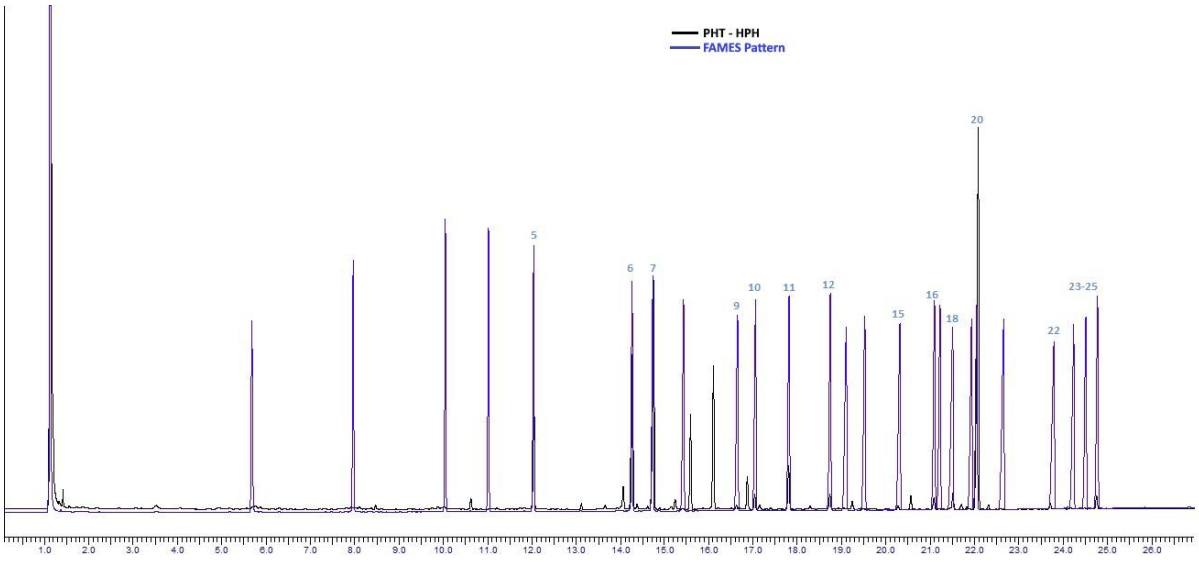


Fig. 12 - *P. tricornutum* HPH sample's GC reading results, colored black, overlapped under the standard FAMES pattern, in blue.

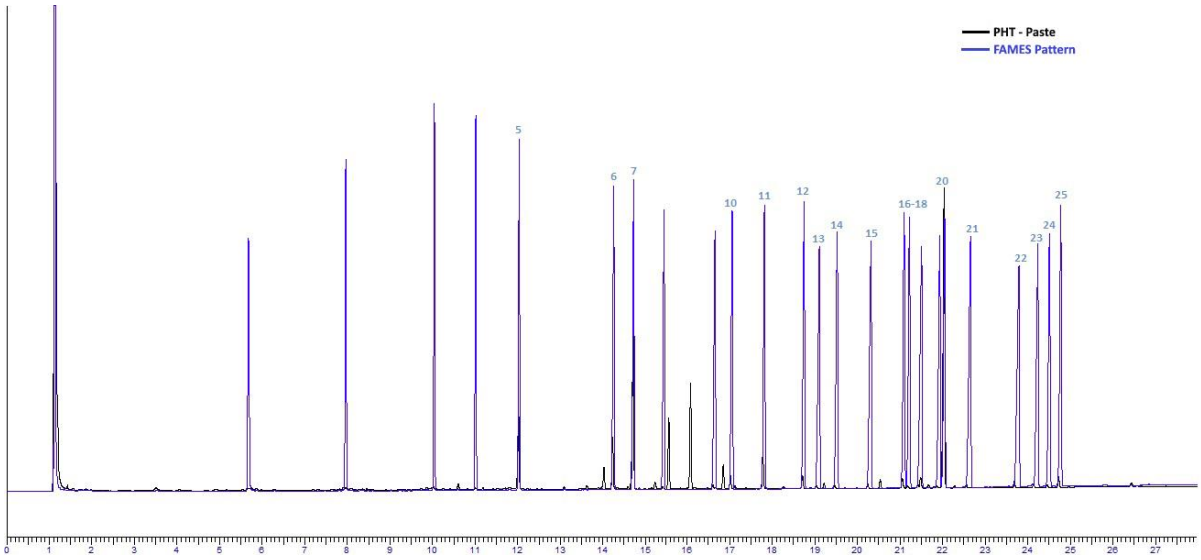


Fig. 13 - *P. tricornutum* Paste sample's GC reading results, colored black, overlapped under the standard FAMES pattern, in blue.

The composition and relative abundance of each fatty acid within the samples could then be determined, as presented on Table 2. Comparing the relative absorbance between HPH and Paste samples from *N. oceanica* and *P. tricornutum* microalgae cultures, it becomes evident that HPH samples contain a relatively higher content of methyl palmitoleate [$CH_3(CH_2)_5CH=CH(CH_2)_7COOCH_3$] for both cultures. In general, the

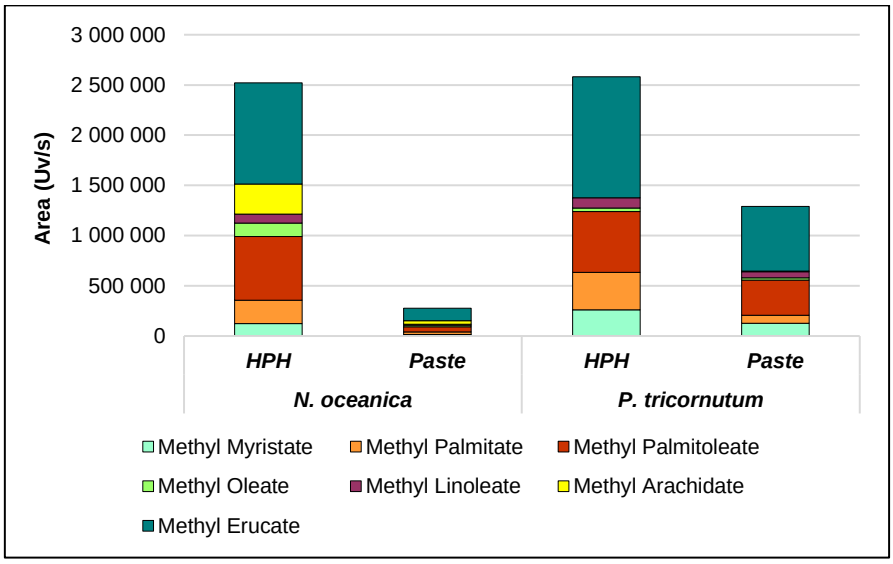
remaining lipidic content does not suffer significant alteration between HPH and Paste. However, for *P. tricornutum* microalgae, the average of methyl palmitate [$CH_3(CH_2)_{14}CO_2CH_3$] in Paste is 5.58% while in HPH is 13.55%, representing a significant increase in this lipid. On the other hand, for *N. oceanica* microalgae, methyl palmitate is found in 9.22% and 9.82%, for HPH and Paste, respectively, meaning that in this case there is no significant difference between samples with and without HPH treatment in terms of relative fatty acid composition. The fact that the overall quantity of fatty acids is lower in the Paste samples for both microalgae was expected. However, the drastic decrease in fatty acids from the *N. oceanica* HPH sample to the *N. oceanica* Paste sample was not. No concrete explanation can be found for this discrepancy. Judging from previous results and the lipidic content data from *P. tricornutum*, it is unlikely that ΩH extraction was ineffective at extracting *N. oceanica* lipidic content. A more likely explanation would be human error during the procedure.

Table 2 - Methyl ester species detected in the GC reading of each sample

Methyl Ester Species	*N. Oceanica* HPH		*N. Oceanica* Paste		*P. tricornutum* HPH		*P. tricornutum* Paste	
	Area (Uv/s)	**% of Total Fatty Acids**	**Area (Uv/s)**	**% of Total Fatty Acids**	**Area (Uv/s)**	**% of Total Fatty Acids**	**Area (Uv/s)**	**% of Total Fatty Acids**
Methyl Myristate	124 574	4,94	13 957	5,05	261 004	9,48	126 879	8,79
Methyl Palmitate	232 494	9,22	27 154	9,82	373 031	13,55	80 485	5,58
Methyl Palmitoleate	635 113	25,18	52 390	18,94	605 417	22,00	349 769	24,23
Methyl Stearate	-	-	-	-	6 885	0,25	-	-
Methyl Oleate	132 553	5,26	15 543	5,62	34 352	1,25	24 042	1,67
Methyl Linoleate	87 998	3,49	9 421	3,41	103 651	3,77	58 677	4,06
Methyl Liholenate	-	-	-	-	34 619	1,26	24 024	1,66
Methyl Arachidate	301 369	11,95	33 855	12,24	-	-	5 210	0,36

Methyl 11-Eicosenoate	-	-	-	-	-	-	4 203	0,29
Methyl Heneicosanoate	-	-	-	-	8 318	0,30	15 949	1,10
Methyl Arachidonate	-	-	-	-	28 111	1,02	18 197	1,26
Methyl 11-14-17-Eicosatrienoate	-	-	-	-	-	-	4 597	0,32
Methyl Eicosapentaenoate	-	-	-	-	36 756	1,34	28 293	1,96
Methyl Erucate	1 007 815	39,96	124 274	44,93	1 204 363	43,76	644 333	44,63
Methyl Tricosanoate	-	-	-	-	-	-	6 121	0,42
Methyl Docosapentaenoate	-	-	-	-	14 345	0,52	11 819	0,82
Methyl Lignocerate	-	-	-	-	3 374	0,12	5 963	0,41
Methyl Docosahexaenoate	-	-	-	-	3 771	0,14	5 221	0,36
Methyl Nervonate	-	-	-	-	34 247	1,24	21 366	1,48
Undetermined	-	-	-	-	-	-	8 513	0,59
Total	2 521 916	100%	276 593	100%	2 752 243	100%	1 443 661	100%

To simplify the analysis, fatty acids detected in trace amounts, which was defined as representing less than 1% of fatty acid content in both microalgae species, were discarded, with the remaining being presented in Graph 19.



Graph 19 – Fatty acid composition of the *N. oceanica* and *P. tricornutum* samples analysed by GC.

5. Conclusion

The scale-up process of microalgae cultures is a crucial factor in maximizing productivity and viability in large-scale microalgae cultivation. During the scale-up of microalgae cultures, as well as during their cultivation in outdoor photobioreactors, it's essential to maintain stringent quality control, monitor contamination risks, and continually optimize processes to maximize productivity. For this, monitoring trough a combined method of OD readings and DW analysis, as well as nitrate content analysis and microscope observation of samples, was essential to the process, allowing for timely risk assessment and evaluation of culture condition.

Individually, HPH and ΩH extraction are effective methods for the extraction of high value compounds. The HPH extraction method necessitates the processing of saltwater microalgae cultures to remove ionizing elements and dilute them to a biomass concentration that it is capable of handling, however, adaption of the method to an industrial scale seems feasible and may alleviate these limiting factors. The ΩH extraction method, on the other hand, appears able to process marine microalgae cultures directly, without needing prior treatment, opening very interesting possibilities for in-*situ*, large-scale extraction processes. The ability to use green solvents and its inherent energy efficiency, stability, and control over the extraction process further highlight its potential. However, a secondary extraction step is necessary to get the most out of the extraction process. Freshwater cultures would also require the addition of a certain amount of ionizable elements to be able to be processed through the ΩH reactor. The presence of nearby seawater, as is the case of some of biorefinery plants, could alleviate this, at the cost of diluting the culture before processing, making the overall extraction slower. This, however, could also contribute to cell lysis, further enhancing the extraction process. The ΩH extraction method seems to have some specificity for the extraction of carotenoids, however, long runs must be avoided to prevent degradation of reactive compounds. It is possible that multiple short runs, interspaced with centrifuging to remove the extracted compounds from the cultures, could greatly alleviate this. The untapped potential of this method should not go understated.

Although the combination of HPH and ΩH may seem anachronistic due to the requirements of each method, with HPH necessitating the absence of ionizable elements, and ΩH requiring them, it highlights the potential of compound extraction

procedures. The problems of combining these two methods were easily solved at a laboratory scale, but this may not be the case at an industrial level. In this particular case, it is possible that processing of HPH extracts trough the ΩH method resulted in a greater degradation of the compounds extracted during the HPH procedure, however, this requires longer run times to be noticeable. Additionally, the yields were overhaul larger when these methods were used in tandem, especially in their lipidic content.

6. References

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