Cultivation scale-up of microalgae strains with potential for high added value products: from laboratory to pilot plant

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Abstract

Recently, there has been a global demand for polyunsaturated fatty acids (PUFAs) and microalgae represent a promising alternative to seafish for ω-3 fatty acid production. Therefore, three relatively unstudied microalgae strains - snow microalgae Raphidonema sp., diatom Thalassiosira sp. and dinoflagellate Prorocentrum sp. - were selected for cultivation at pilot scale, due to their high content of PUFAs.

Firstly, the evolution over cultivation time and culture productivities in different pilot-scale systems were determined, namely panel photobioreactors and unilayer and multilayer horizontal tubular photobioreactors. In some systems, different renewal rates with different periodicities were used, allowing the culture to remain in the linear growth phase. Besides that, throughout the cultivation some parameters were explored, such as the dissolved oxygen concentration and the shear stress of the pump frequency. For all the used microalgae species, *Prorocentrum* sp. was the one that produced the highest amount of biomass, originating consequent higher quantities of DHA and EPA.

Secondly, the impact of an alternative culture medium to *Prorocentrum* sp. growth was researched, as well as a silicate's concentration reduction. These tests were made in the Innovation Laboratory of Lisbon, in A4F, aiming to lower the culture and nutritive media's cost. The renewal cycles' productivities were analysed and the elemental composition of both media too. It was concluded that *Prorocentrum* sp. can only be cultivated in the new medium if the lack of some nutrients was compensated, and that the productivity levels of this microalgae aren't lowered by the reduction of silicates.

Key-words: Polyunsaturated fatty acids (PUFAs); Microalgae; Raphidonema sp.; Thalassiosira sp.; Prorocentrum sp.; Pilot scale; Productivity; Culture medium

1. Introduction

Lately, several studies are proving the polyunsaturated fatty acids (PUFAs) vital role in maintaining the health and wellbeing of humans. Besides reducing the risk of some diseases, they also provide them energy and nutrients. (Yakindra Prasad Timilsena, 2017) Currently, the main source of these fatty acids is seafish. However, with imminent environmental threats and overexploited actions increasing exponentially regarding fish stocks, alternative sources of PUFAs have been researched, being microalgae the best option, by presenting the highest productivities.

Besides PUFAs, microalgae are also a source of other highly valuable molecules such as pigments, vitamins, antioxidants, pharmaceuticals and other biologically active compounds. (Stamatia Bellou, 2014) Thus, their cultivation has great potential and its optimization for large scale production is of high importance. (Ling Xu, 2009) (Teresa M. Mata, 2010) Depending on the microalgae strain and its product application, special consideration should be given to the selection of the most adequate cultivation conditions, parameters and systems.

There are several variables influencing microalgae growth. The most important ones comprise abiotic factors such as light, temperature, nutrient concentration, O2, CO2, pH and salinity; biotic factors like pathogens and competition by other microalgae; and, finally, operational factors as shear stress produced by mixing, dilution rate, optical pathlength or harvest frequency. (Quentin Béchet, 2013) Regarding nutrient concentration, in large-scale cultures, nutrients are mostly supplied on the basis of the same media formulae

as used for laboratory cultures, which are generally not optimized and in excess. This is a huge disadvantage since they represent a significant part of the cost of microalgae production.

In the past decades, several photobioreactors (PBRs) have been developed, being divided into two systems: open or closed systems. (Han Ting, 2017) Open systems represent the oldest configurations and around 90% of the global algae production. They are less expensive to build and operate, have a convenient operational method and higher life time period and areal productivities. On the other hand, they are more susceptive to contaminations, they have no control on environmental parameters, and they also occupy more land area. (Han Ting, 2017) (Małgorzata Płaczek, 2017) Regarding closed systems, despite being more expensive, they provide a better control over culture conditions. Therefore, these systems are commonly used in high value-added products production, like therapeutics. (Han Ting, 2017) Although there are already several types of systems developed, only a few can be used for the mass culture, being the most promising ones flat panel PBRs, tubular PBRs and column PBRs. (Qingshan Huang F. J., 2017)

Flat panel PBR resembles a rectangular box formed by two adhesive sheets of transparent or semi-transparent materials. They have been receiving attention due to their low cost and for presenting a large surface area to volume ratio that offers a high efficiency of photosynthesis. However, these systems suffer of an inadequate mixing, their scale-up is not so easy, and, although they are easy to built, the bags are inherently fragile which make their lifespan short. (Qingshan Huang F. J., 2017) (Han Ting, 2017) (Agnieszka Patyna, 2016) (Małgorzata Płaczek, 2017)

The tubular PBRs are constituted by tubes that can be arranged in different patterns and by a gas exchange system that performs CO2 supplementation and acts towards the removal of oxygen originated during photosynthesis. Microalgae are circulated through the tubes by a pump or by impetus resulting from aeration in the airlift systems, which also promotes culture homogenization. Bubbling of air will help to avoid surface biological deposition and thus prevents from photo inhibition. The scale-up of these reactors can be achieved, in a simple way, by installing a new set of tubes to the already existing installation or by increasing the length or diameter of the tubes. (Małgorzata Płaczek, 2017) (Qingshan Huang F. J., 2017) (Agnieszka Patyna, 2016)

Column PBRs are one of the most promising configurations, since they have a cheap design and easy operation. These systems have the disadvantage of a propensity to biofilm formation on the column walls, limiting the light penetration. Besides that, especially when considering outdoor cultivations, they have difficulties in controlling the temperature of the culture and the photoinhibition phenomenon. (Agnieszka Patyna, 2016) (Małgorzata Płaczek, 2017) (Qingshan Huang F. J., 2017)

The cultivation of three microalgae with high content of PUFAs were studied: a freshwater cryophilic microalga, *Raphidonema* sp. that produces EPA, and that is gaining a lot of attention for possibly growing through an annual algae crop rotation (ACR) mode with other algae; a diatom, *Thalassiosira* sp., with a silica cell wall, that produces high amounts of EPA and DHA, (A.S.D. Harris, 1995, 30) (Maxime Suroy B. M., 2014); and a dinoflagellate, *Prorocentrum* sp., that has both EPA and DHA high levels.

2. Materials and Methods

2.1 Biological Material and cultivation conditions

All of the strains used in this work were available from A4F Culture Collection.

Raphidonema sp.

Before the scale-up to the pilot scale unit, the algal strain was grown autotrophically at A4F Innovative Laboratory. Since this microalgae is cryophilic, the cultivation occurred in round glass flasks of 2L volume, adapted with an enclosing cooling system that maintained the temperature controlled below 15 ºC (8-12 ºC). In each flask, a gas distributor in the bottom,

sprinkles air enriched with 0.5% CO_2, ensuring a good mixing of the culture, its aeration and sufficient CO_2 supply. Still in the aeration system, air filters were placed both in the inlet and in the outlet of it, in order to maintain sterile conditions and avoid unwanted cross-contamination. The culture was grown in an autoclaved freshwater and nutritive medium, and maintained ate a pH between 7.5 and 8.5. Considering the light, since the LEDs were located on the surface where the flasks were located. These flasks had a constant illumination under 24 hours, with an approximately photon flux density of 30 $\mu E.m^{-2}.s^{-1}$.

Thalassiosira sp.

This algal strain was grown autotrophically at A4F Innovative Laboratory, under similar conditions like to *Raphidonema* sp..The differences between them reside in cultivation salinity, where this microalga grown at 30-35 $g.L^{-1}$, temperature that was maintained at 25 °C and light, where the flux density was of 100 $\mu E.m^{-2}.s^{-1}$

Prorocentrum sp.

This algal strain was grown under equal conditions to *Thalassiosira* sp.. For both laboratory tests, the culture was grown in smaller flasks, with 1L volume.

2.2 Culture Medium Formulation

The culture medium used for cultivation of the freshwater strain *Raphidonema* sp., consists of chemically disinfected freshwater. Regarding *Thalassiosira* sp. and *Prorocentrum* sp., the culture medium used in the pilot scale photobioreactors is the result of an optimization work for marine microalgae cultivation by A4F consisting of an artificial saltwater with a specific mineral solution (MS).

The nutritive medium used to supplement CW in pilot scale for all the cultures is an industrial medium based on a recipe developed by A4F.

2.3 Cultivation systems

Firstly, the scale-up was done in the laboratory, to volumes up to 5L. When there were multiple 5L flasks with concentrated culture, a step forward to the pilot scale unit was done, where, under batch and/or fed-batch modes, microalgae strains were grown autotrophically. The cultivation was done in gradual steps, starting in flat plane PBRs that then subsequently served as inoculum to the horizontal tubular PBRs. In fewer cases, the culture in a PBR was also the inoculum of another similar system, being transferred through a renewal. In this study there were also indoor and outdoor systems. Therefore, since the average daily radiation data was measured outside the greenhouse through a weather station installed at A4F Experimental Unit, for the indoor systems, a discount of 30% lower had to be done.

The Flat panel system used consists of a disposable plastic bag located between two metal frames that support it. The temperature in these systems was controlled through an intermittent water flux from a coil thermoregulation system and was monitored by taking programmed culture samples three times per day. The homogenization of the culture was established through an air diffuser located in the lower part of the reactor. The regulation of the pH control is done manually by adjusting valves that act in the injection of pulses of pure CO_2.

The horizontal tubular systems are composed by two parts, explicitly one or more continuous transparent tubular loops that receive the light and a tank. This reservoir was where the gas exchanges occurred through the aeration system, and where the temperature control was done, by cooling/heating water that was pumped through a heat exchanger coil. The temperature values were continuously read and monitored online. Through this controlling system, the pH was also followed, and the control was done automatically with CO_2 pulses. The culture was continuously recirculated using a pump. This recirculation along with the aeration in the system, promoted the homogenization of the culture, avoiding its sedimentation and biofilm formation.

2.4 Operational Procedures

In the laboratory tests, the renewals were made in two ways: considering the same volumetric renewal rate or the same initial dry weight value. Although the first one is more practical, the other one becomes more efficient, guaranteeing the same starting point between the control and the test cultures. In order to minimize the differences between the flasks regarding light conditions, all systems were switched between them on a daily basis.

2.5 Analytical methods

Optical Density (OD)

OD measurements were done at a wavelength of 600 nm, using a UV-Vis spectrophotometer (Genesys UV-Vis 10S). Each sample was read in duplicate in plastic cuvettes with 1 cm of path, against fresh culture medium. If the culture samples were too concentrated, a dilution was applied, guaranteeing the linearity of the Beer-Lambert law.

Dry weight (DW)

In the two laboratory tests, the biomass dry weight ($g.L^{-1}$) was calculated as it is shown below, where m_f corresponds to the mass (g) of biomass and filters after filtration; m_i to the mass (g) of filters before filtration and Vol to the volume (L) dried. The culture samples were previously filtrated with the vacuum pump, using pre-weighed filters and then washed with ammonium formate to remove all of the culture medium salts. Subsequently, the filters with culture were heated and dried at 180ºC in the moisture analyzer.

$$DW\ (g.L^{-1}) = \frac{m_f - m_i}{Vol}$$

Determination of the culture volumetric productivity

The volumetric productivity ($g.L^{-1}.day^{-1}$) was calculated according to the equation presented below, where X_t corresponds to the biomass concentration at the time t ($g.L^{-1}$), X_i corresponds to the initial biomass concentration at the time i ($g.L^{-1}$).

$$Culture\ average\ volumetric\ productivity = \frac{X_t\ (g.L^{-1}) - X_i\ (g.L^{-1})}{t_{cultivation,\ i \to t}\ (days)}$$

Determination of the culture photosynthetic productivity

The photosynthetic productivity ($g.m^{-2}.day^{-1}$) of the culture was calculated according to the following equation, where VP corresponds to the culture volumetric productivity ($g.L^{-1}.day^{-1}$).**Erro! A origem da referência não foi encontrada.**

$$Culture\ average\ photosynthetic\ productivity = \frac{VP\ (g.L^{-1}day^{-1})\ \mathrm{Vol}\ (L)}{Photosynthetic\ area\ (m^2)}$$

Determination of the culture normalized productivity

The normalized productivity ($g.m^{-2}.day^{-1}$) of the culture was calculated according to the equation below, where AP corresponds to the culture photosynthetic productivity ($g.L^{-1}.day^{-1}$).

$$Culture\ average\ normalized\ productivity\ (g.MJ^{-1}) = \frac{AP\ (g.m^{-2}day^{-1})}{Average\ Irradiation\ (MJ.m^{-2}day^{-1})}$$

Determination of the culture growth rate

The growth rate (day^{-1}) of the culture was calculated according to the following equation.

$$Culture\ growth\ rate = \mu\ (\mathrm{day}^{-1}) = \frac{\mathrm{VP}\ (\mathrm{g.L^{-1}day^{-1}})}{\mathrm{X_i(g.L^{-1})}}$$

Microscopic Observation and Determination of nitrate ion concentration

A standard procedure including microscopic observations and nitrate concentration measurements was applied. The concentration of nitrate ion was determined using an UV absorption spectrometry method and the control through microscopic observation was done aiming to monitoring the cells and also possible contaminations.

3. Results and Discussion

The experimental work can be divided in two distinct assays: pilot-scale cultivation and laboratory tests.

3.1 Assay 1: Pilot-scale Cultivation

3.1.1 Cultivation of *Raphidonema* sp.

Two identical FP-PBRs were used, having all parameters equals. It was possible to see that lower initial concentrations may lead to photoinhibition phenomenon that may influence the natural course of the culture.

Raphidonema was also cultivated in an outdoor UHT-PBR, where the impact of the aeration was seen, since due to a failure in this system, the dissolved oxygen achieved values above the saturation (243%) inhibiting the cell growth.

In order to compare both systems, the average productivities are summarized in

Table 1. Regarding the outdoor UHT-PBR, it was only used the period after turned on the aeration and, since there was a renewal, the period that followed it was considered as a new cultivation.

Table 1 - Summary of some of the factors that have an impact on productivity. Productivities of the different cultivation systems used.

	UHT-PBR		FP-PBR 1	FP-PBR 2
	Before renewal	After renewal		
Volume (L)	1097	1097	77	230
Average Irradiation ($MJ.m^{-2}.day^{-1}$)	15.19	17.72	16.46	16.46
Average Photosynthetic Area (m^2)	21.16	21.16	1.18	3.53
Surface to volume ratio (m^{-1})	19.29	19.29	15.27	15.37
Volumetric Productivity ($g.L^{-1}.day^{-1}$)	0.08	0.07	0.08	0.14
Photosynthetic Productivity ($g.m^{-2}.day^{-1}$)	4.11	3.77	5.14	8.84
Normalized Productivity ($g.MJ^{-1}$)	0.27	0.21	0.31	0.54

Considering the two cultivations in each system, their average normalized productivities was 0.42 gMJ^{-1}, with a standard deviation of 0.16, for FP-PBR and 0.24 gMJ^{-1}, with a standard deviation of 0.04 for UHT-PBR. These values are illustrated in Figure .

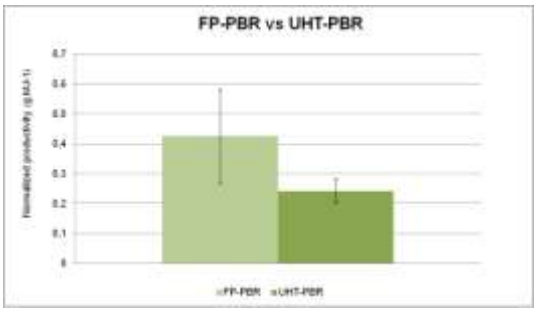

Figure 1 - Normalized productivities of the different systems used for Raphidonema's cultivation, with the average of the two identical FP-PBRs and the two cultivations periods considered in the UHT-PBR, as well as their respective standard deviations.

Photobioreactors are generally installed in order to have the smallest volume for the largest possible area, maximizing the surface to volume ratio, S/V. In general, PBRs exhibit a photosynthetic area that depends on the position of the sun and the shadow that it casts at certain times of the day. Since the UHT-PBR considered was horizontal and only had one layer of tubes, this problem was not as significant. Therefore, it explains the difference in the photosynthetic area between the systems used and the fact that the S/V ratio was higher in that tubular system. In this way, it is safe to assume that among the systems used, UHT-PBR is the one that best exploits the incident energy. Besides that, this system presents other advantages when in comparison with the FP-PBRs, such as the automation of the process, the controlled parameters and the easy scale-up. It is also worth mentioning that the semi-continuous mode, more easily applied in the UHT-PBR than in FP-PBR, is the one that best promotes the wellbeing of the culture, avoiding its stagnation.

Despite that, it is possible to verify that all productivities have better values in the FP-PBRs, although the standard deviation of UHT-PBR is still slightly within the values obtained for FP-PBR's standard deviation. This can be explained by other characteristic constraints of tubular reactors, which can affect the cells, such as the length of the tubes, in this case of 27.2 m, which promotes some dead zones in its interior. In addition, as the culture is recirculated by means of a pump, there is always the disadvantage of it exerting some kind of shear stress to the cells.

Taking all of this into account, considering the low number of systems used, and the fact that this was the first time this species was grown outside the laboratories in an external tubular PBR, t is not possible to draw definitive conclusions from this study. Thus, future optimizations can be made to increase productivity in these two systems, especially in UHT-PBR.

3.1.2 Cultivation of Thalassiosira sp.

Thalassiosira sp. was successfully cultivated in a 1400 L indoor MHT-PBR through different semi-continuous growth periods, in order to understand what would be the best cultivation mode for this strain and to see if the cells could adapt to different renewal rates. In theory, the batch

mode is the one where the growth stops at a given point due to limiting substrate depletion or growth-inhibiting products accumulation, and, thus, the semi-continuous mode is of huge importance when it comes to maintaining cells' proliferation and replacing the substrate, avoiding, through the renewals, the stagnation of the culture. However, analyzing the productivity results throughout this assay, the batch mode periods considered, were the ones that reach the most satisfactory values, probably because when the renewals occur, the culture becomes more fragile. (Bruno D. Fernandes, 2015)

Regarding the 210 L UHT-PBR, the culture has grown well throughout the cultivation period, under a semi continuous mode. Since the cultivation conditions were identical, it was studied the influence of the radiation on the growth of this microalga. As expected, the irradiation tends to increase from February to March, having correspondent higher productivity levels.

Table 2 summarizes the overall characteristics of the systems used.

Table 2 - Summary of some of the factors that have impact in productivity and global productivities achieved, regarding the cultivation of Thalassiosira sp, in a MHT and UHT PBR.

	MHT-PBR	UHT-PBR
Volume (L)	1400	210
Average Irradiation ($MJ.m^{-2}.day^{-1}$)	13.94	13.24
Average Photosynthetic Area (m^2)	32.34	6.91
Volumetric Productivity ($g.L^{-1}.day^{-1}$)	0.07	0.19
Photosynthetic Productivity (g.m-2.day^{-1})	3.20	5.70
Normalized Productivity ($g.MJ^{-1}$)	0.33	0.62

The two systems used are quite similar in terms of operation and in monitoring the parameters. The major differences between both of them are in the number of tube layers and in the type of the deposit. The UHT-PBR, having only one layer of horizontal tubes and a transparent tank, is constantly exposed to incident solar energy, not allowing the cells to move between light and dark zones. In opposite, the MHT-PBR has several layers of tubes, which might shadow each other at certain times of day, and has an opaque dark tank. These differences will consequently affect their productivities.

As expected, UHT-PBR being the one with the highest S/V, 32.90 m^{-1}, in relation to 23.10 m^{-1} of the MHT-PBR, was the one that also had the higher photosynthetic productivity with a value of 5.70 $g.m^{-2}.day^{-1}$ against the 3.20 $g.m^{-2}.day^{-1}$ obtained by the other.

Each renewal cycle of each one of them was considered as a new cultivation, and its productivities are shown in Figure 1.

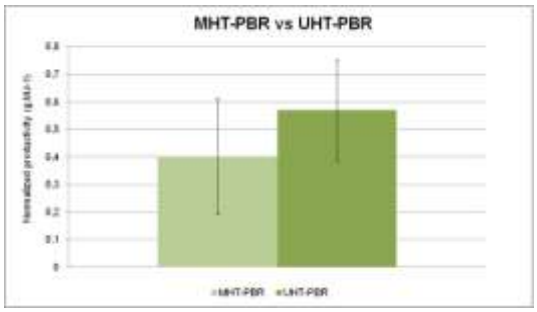

Figure 1 - Normalized productivities of the different systems used. It features an average of each renewal cycle of the UHT-PBR and MHT-PBR cultivation, as well as their respective standard deviations.

In these systems, a perfect compromise between a pump frequency that allows for good homogenization of the culture without otherwise damaging the cells is not always possible. However, even with this in mind, UHT-PBR shows the best results for the cultivation of *Thalassiosira* sp..

In addition, besides the fact that all productivities are higher in the UHT-PBR, it should be noted that its implantation area, 7.68 m^2, is barely above half the area of the MHT-PBR, 14.90 m^2. Since this area is the area that the reactor by itself will occupy without affecting others there are beside it, it is easy to understand that once again the UHT-PBR is more advantageous.

In conclusion, although this study is a good indication that a UHT-PBR, among the systems considered, is the most suitable for the pilot scale cultivation of *Thalassiosira*

sp., was the first time this species was cultivated in this type of reactor. Thus, further studies to confirm this should be done and, at the same time, a continuous optimization of the process as well.

3.1.3 Cultivation of *Prorocentrum* sp

The cultivation of *Prorocentrum* sp. in FP-PBRs occurred in order to obtain a sufficiently concentrated culture that serves as inoculum in the scale up to other bigger systems. Regarding the MHT-PBR, two different systems were used: MHT-PBR 1 with 4m tubes and MHT-PBR-2 with16.5m.

In MHT-PBR 1 the cultivation started during the winter and ended in the summer, so the impact of an increasing irradiation at productivity levels was evaluated. Besides this, at different times, the aeration was turned on and the pump frequency was increased. These two latter factors also enhance the productivity levels.

After 24 days of cultivation in MHT-PBR 2, there was an increase in the frequency of the bioreactor pump that lasted until the end of the cultivation. As an unexpected failure on the temperature control occurred on that day, with the culture reaching 34.3 °C, and as all of these factors occurred right after a renewal, the cells became even more stressed, and the productivity lower.

A 310L UHT-PBR was also studied for the cultivation of Prorocentrum sp. Although the culture has grown well throughout the cultivation time, there were some drawbacks, since this specific microalgae has a high tendency to sediment.

A comparison between the performances of the different PBRs used was made, using their optimized cycles. The average productivities and some of their characteristics summarized in Table 333. **Erro! A origem da referência não foi encontrada.**3 and figure 4 **Erro! A origem da referência não foi encontrada.**shows these productivities and their respective standard deviations, now calculated considering the different renewal cycles in each one of them as new cultivations.

Table 33 - Summary of the average productivity levels achieved in the optimized cycles of the systems that were used for Prorocentrum sp. cultivation.

	MHT 1	MHT 2	UHT	FP
Photosynthetic area (m2)	2.44	11.72	6.91	1.62
Average Irradiation (MJ.m-2day-1)	27.48	29.15	28.79	21.0
Volumetric Productivity (g.L-1day-1)	0.06	0.12	0.08	0.05
Photosynthetic Productivity (g.m-2day-1)	15.30	13.83	3.54	6.05
Normalized Productivity (g.MJ-1)	0.80	0.68	0.18	0.38

Comparing all the tubular systems above, it is possible to conclude that the MHT-PBR 2 and UHT-PBR are the ones that had the better volumetric productivity levels, while MHT-PBR 1 had the highest photosynthetic and normalized ones. The differences verified between both MHT-PBR can be due to the fact that they are positioned side by side and sometimes MHT-PBR 2 shadows MHT-PBR1, not allowing the use of all its photosynthetic area. MHT-PBR 2, with longer tubes, has a larger photosynthetic area and a higher surface to volume ratio compared to MHT-PBR 1. Despite that, although the length of the tubes of MHT-PBR 2 allows for a better photosynthetic area, it may lead to gradients of temperature, pH and nutrient concentration. In fact, MHT-PBR 2 had some problems, especially in relation to the high temperature levels reached. Regarding the UHT-PBR, as previously mentioned, this was the first time that *Prorocentrum* sp. was cultivated in these tubular reactor, and some difficulties were encountered during the growing period. However, of all tubular reactors, this is the one with a higher surface/volume ratio with the potential to achieve higher values of productivity. (Chun-Yen Chen, 2015) This reactor presents photosynthetic productivity values similar to those obtained for this MHT-PBR in the non-optimized cultures, so its optimization will be very advantageous.

In relation to FP-PBRs, their low productivities may be due to the fact that their aeration system is not as effective in blending the culture as in tubular reactors. This difference leads to a worse

homogenization of nutrients and gases, and also to a higher biofilm formation. Despite that, the majority of FP-PBR presents a higher surface to volume ratio, and they are easier to install and are also much cheaper.

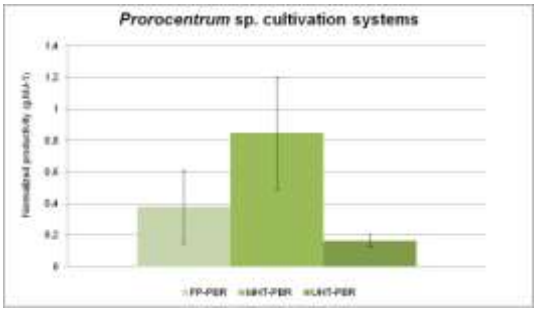

Figure 3 - Normalized productivities of the systems used for *Prorocentrum* sp. cultivation. It features an average of the renewal cycles of the optimized cultivation periods considered in each system, as well as their respective standard deviations.

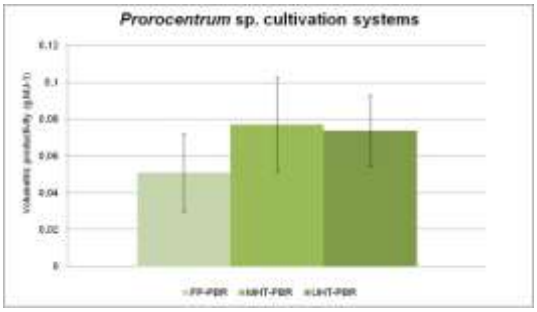

Figure 42 - Volumetric productivities of the systems used for *Prorocentrum* sp. cultivation. It features an average of the renewal cycles of the optimized cultivation periods considered in each system, as well as their respective standard deviations.

Although its standard deviation is slightly elevated, , once again, MHT-PBR is the one with the best productivity levels. In addition, it is confirmed that even though it is not yet an optimized process, UHT-PBR cultivation has quite satisfactory volumetric productivities, presenting great indications of being able to be the most advantageous.

3.1.4 Summary of assay 1

There was no explicit trend in productivities any of the species. *Thalassiosira* sp. had the highest levels of volumetric productivity, despite being very close to Raphidonema sp.'s values, while *Prorocentrum* sp. had the best ones of photosynthetic and normalized productivities.

Besides that, although *Thalassiosira* sp. has the lipidic profile with the most satisfactory values for EPA (mg/g of biomass), taking into account the biomass productivities, the cultivation of *Prorocentrum* sp. was the one that obtained higher photosynthetic and normalized productivities demonstrated , consequently, higher EPA productivities per m^2 and after discounting the radiation factor. Even *Raphidonema* sp., whose EPA composition of 10.28 mg EPA/g biomass is significantly lower than that of *Thalassiosira* sp. of 17.68 mg EPA/g biomass, had higher photosynthetic biomass productivity values and consequently produced higher EPA values. The cultivation of *Prorocentrum* sp. shown to be even more attractive, since it also produces a greater amount of DHA.

3.2 Assay 2: Laboratory tests

The first test was made with the intention of understanding if it was possible to cultivate *Prorocentrum* sp. in a culture medium different from the one usually used at A4F, maintaining the growth and productivities that were previously achieved. This alternative medium, medium A besides being cheaper, it is more easily obtained by A4F. For that purpose, 7 different cycles of renewals were followed.

In the first cycle of renewals, the culture in the medium A (F_1) has grown less than the culture in the control flask (F_0), but recovered in the following two cycles. However, at the end of the 4^{th} cycle, the difference between the two flasks was significantly superior, with a decrease in productivity of about 60% in the test flask. At this point, it was hypothesized whether this was due to a decrease or even a lack in magnesium concentration. Considering this, an initial boost of this micronutrient ($MgSO_4$.7H2O) was added to the culture upon renewal and in the following cycles in a lower and constant concentration. In addition, since no duplicates were made during this test, the same study was started in another flask (F_2), without magnesium. Although F_1 had recovered, once again, it was possible to verify a decrease over time in F_2 productivity.

Through the elemental analysis, it was possible to see that medium A presents a reduction of some of the crucial nutrients for microalgae growth, Table 4, which made this medium by itself incomplete for the cultivation of *Prorocentrum* sp..

Table 44 - Qualitatively nutrient variation through both cultivation conditions used, with the culture medium usually used as reference. Only variations which were equal or superior to 30% were considered relevant.

Element	(%)

Ca	++
Cu	-
Fe	--
K	--
Mg	--
Mn	--
Mo	++++++
P	+++++++
S	+
Zn	+
Pb	++++++
Co	--
As	++++++

The second laboratory test had the aim of realizing if it would be possible to reduce the concentration of silicate of *Prorocentrum* sp. cultivation to half of its value. In terms of resources used during cultivation, this reduction is advantageous not only in costs but also because it promotes a decrease in the agglomerates, boosted by the combination of this micronutrient with magnesium.

Throughout the majority of the cycles there was not much difference in the growth of this microalgae between the test flask (F_3) and the control one (F_0). Thus, the results are a good indication that *Prorocentrum* sp. can be grown over several generations with a reduction of the silicate concentration.

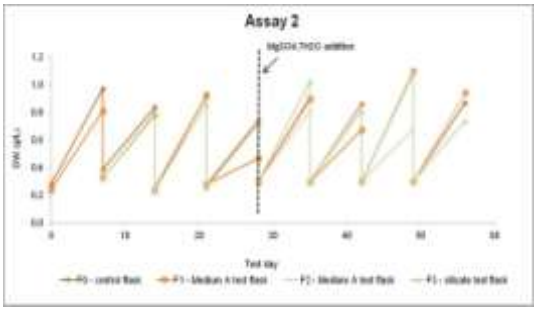

Figure 53 - *Prorocentrum* sp. cultivation under the assay 2. The initial and final dry weight obtained in each renewal cycle is represented. F_0 is the control flask, F_1 the test medium A with addition of $MgSO_4.7H_2O$ in the 4th cycle, F_2 the second medium A test and F_3 the silicate test flask with half of the silicate concentration of F_0.

Table 55 - Productivity levels ($g.day^{-1}$) obtained in each renewal cycle of *Prorocentrum* sp. cultivation under assay 2. F_0 is the control flask, F_1 the test medium A with addition of $MgSO_4.7H_2O$ in the 4th cycle, F_2 the second medium A test and F_3 the silicate test flask with half of the silicate concentration of F_0.

Cycle	Productivity (DW/day)			
	F0 Control	F1 Medium A test	F3 Silicate test	F2 Medium A test
1	0,10	0,08	-	-
2	0,06	0,07	0,07	-
3	0,10	0,10	0,09	-
4	0,07	0,03	0,07	-
5	0,09	0,09	0,10	0,07
6	0,08	0,05	0,07	0,08
7	0,11	0,11	0,11	0,05

4. Conclusions and Future Work

During the study of the several systems, regarding *Raphidonema* sp., from the two FP-PBRs used, one of them had similar values to the UHT-PBR while the other one showed the highest productivities levels of all of them. For the cultivation of *Thalassiosira* sp, it was possible to compare the performance of the UHT-PBR with the MHT-PBR, where the first PBR technology had the best result, due to its higher surface to volume ratio. As for *Prorocentrum* sp, MHT-PBR showed the best results in comparison to UHT-PBR and FP-PBR. However, it should be taken into account that this system was accompanied thought some months, suffering some optimizations during the process, in opposite to UHT-PBR. Since UHT-PBR will allow for a higgest photosynthetic area and a higher surface-to-volume ratio, it becomes a very interesting system. It was also done an analysis of which would be the more suitable species for the production of omega 3 fatty acids. Although *Thalassiosira* sp. has a richer EPA lipidic profile, *Raphidonema* sp. had produced higher amounts of EPA as well as *Prorocentrum* sp. that has showed to have the capacity to produce the highest amounts of that omega-3 fatty acid. In addition to EPA, *Prorocentrum* sp. has also shown to produce the highest amounts of DHA.

During this work, a new culture medium, medium A, was also evaluated for the cultivation of *Prorocentrum* sp. and a possible reduction on the concentration of silicate to half of the value that was established as well. After the analysis of the several renewal cycles and the elemental comparison between medium A and the one that is usually used by A4F, it was

possible to conclude that, due to the lack of some nutrients, the productivities were decreasing after some cycles and the culture started to present more depigmented. A magnesium supplement seemed to be enough for the microalgae to survive and grow in medium A but more optimization studies need to be made, as well as a cost analysis. Regarding the silicate concentration, half of it showed to be an enough quantity for the cultivation of this microalga giving identical productivities along the cultivation period and similar to the ones previously obtained.

Since this study was the first step towards the cultivation of these microalgae strains at a pilot scale, more work is needed to fully understand each one of them. In that way, some research should be made, namely:

- ✓ Continuous optimization of the cultivation of Prorocentrum sp. in UHT-PBR;
- ✓ Compare the usage of equal systems inside and outside the greenhouse, for *Prorocentrum* sp. and *Thalassiosira* sp;
- ✓ Semi continuous cultivation optimization and renewal percentages to work with; try medium recycling;
- ✓ Productivity tests at pilot scale using medium A or a reformulated recipe from this one and compare the results with the ones obtained in this project;
- ✓ Productivity tests at pilot scale using a half reduced silicate concentration and laboratory test using a higher reduction.

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