

Optimization of the composition and recycling strategy of the culture medium for industrial production of microalgae

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ABSTRACT: This work aimed to optimize the composition and medium recycling strategy of culture medium for industrial cultivation of *Nannochloropsis oceanica*. An initial cultivation study of *N. oceanica* was done in a semi-continuous outdoor culture with medium recycling and three recipes of nutritive media were tested: CNASAM_2 – standard medium, CNASAM_3 – optimized recipe and BIOFAT_2 – industrial-made medium. Two formulations of culture media were also tested: ASAM_2 – with laboratorial sodium chloride (NaCl) and ASAM_2.1 – with purified seawater salt. The medium recycling strategy led to a maximum productivity loss of 50% in comparison to cultures without medium recycling, which achieved up to $0,529\pm 0,085$ g_{D.W.}/(L.day) in steady state. The CNASAM_2 cultures achieved higher productivities and showed less susceptibility to growth limiting factors. The cultures with CNASAM_3 showed significant values of productivity but all resulted in culture death. The cultures with BIOFAT_2 achieved lower values of productivity and average susceptibility to the growth limiting factors. It was not possible to detect negative impact of industrial NaCl source in *N. oceanica* cultivations, and the purified seawater salt was considered an adequate source of NaCl for marine microalgae cultivations. The medium recycling strategy led to the proliferation of biological contaminants which was related with the loss of productivity. In cultivation of *N. oceanica* with medium recycling, the disinfection process of exhaust culture medium with sodium hypochlorite and subsequent neutralization with sodium thiosulfate was also tested. The efficiency of the process was proven and disinfected recycled medium cultures showed tendency to achieve higher productivity in long-term cultivations.

Keywords: *Nannochloropsis oceanica*, industrial production, microalgae, culture medium, culture medium recycling.

INTRODUCTION

Microalgal Technologies, Potential and Applications

Microalgae are microscopic organisms that through photosynthesis are capable of converting solar energy into chemical energy, they are not extremely fastidious microorganisms – can grow rapidly and in a simple and efficient way (binary division) – and live in harsh conditions due to their unicellular or simple multicellular structure [1]. Microalgae can be found almost anywhere on Earth, in freshwater, marine, and hyper-saline environments [2]. They are genetically a very diverse group of organisms with a wide range of physiological and biochemical characteristics, the nutritional requirements of a wide array of microalgal strains are known [3]; thus they naturally produce many different and unusual fats, sugars, bioactive compounds, which raises the commercial and industrial interest in these little organisms [4]. These characteristics justify the currently global blossoming interest in microalgal cultivation [5] and usage in different industries: food, feed, pharmaceutical, cosmetics and biodiesel industry [5], [6].

Microalgae and Biodiesel

Recently, there are two kinds of important issues: environment and energy crisis.

The environmental issue is mainly connected to global warming. It is known that using fossil fuels has caused global warming; they are the largest

contributor of greenhouse gases (GHGs) to the biosphere. Regarding the energy crisis issue, where increasing global petroleum crude oil prices due its shortage of reserves have impacts on industrial and domestic energy situations as well as on local society life [3]. Some microalgae, which are capable to achieve high content of lipids (generally 20 – 50 % dry weight biomass [7]), appear to be a suitable group of oleaginous microorganisms for lipids production and consequently suit as feedstock for the biodiesel industry. Microalgae have an extended potential and could meet almost all of the requirements needed to make a significant contribution to meeting the primary energy demand, while simultaneously providing environmental benefits with a parallel and judicious exploitation of microalgae technology [2].

Nannochloropsis oceanica potential and applications

Nannochloropsis oceanica is a Eustigmatophyceae [8] characterized by small spherical cells, with a diameter range of 2–5 µm. Actually, the major application of *N. oceanica* biomass is as aquaculture feed and dietary supplement in human food. Regarding to the accumulation capacity of eicosapentaenoic acid (C20:5 EPA), *N. oceanica* has been used indirectly (after oil extraction) in human food is due to the accumulation [8]. The principal application of *N. oceanica* which raises the biotechnological and economical interest of this

species of microalgae is the industrial production of biofuel since this microalga can produce very large amounts of biomass and lipids (up to 68% w/w [9]). This species is characterized by very high levels of palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic acid (C20:5 EPA) [10], [11].

Microalgal Cultivation Parameters

The growth and composition of microalgae is influenced by several parameters, such as temperature, carbon source, pH, light intensity, photoperiod and salinity [12]–[14]. To grow *N. oceanica*, several values are identified according to different growth studies and different purposes. Table 1 lists the most common parameters used to grow this microalga.

Table 1 – Typical parameter values used to grow *N. oceanica* in large-scale systems.

Parameter	Value
Temperature (°C)	20 – 26 [9]
Carbon Source	CO ₂ (Autotrophic growth) [12]
pH	6,75 – 9,2 [15]
Salinity (g/L)	15 – 35 [16]
Mixing (vvh)	< 14 [15]

Light requirement is one of the most important parameters to be monitored in microalgal growth through photobioreactors (PBRs). Light is necessary for microalgal photosynthesis, yet should indeed be provided at the appropriate intensity, duration, and wavelength. Excessive or insufficient incident light constrains optimal performance — in terms of biomass or metabolite yields — leading to photoacclimation/ photoadaptation or photoinhibition phenomena [14].

Medium Recycling Strategy

The medium recycling strategy is, basically, a simultaneous process of dilution and recycling of culture medium with extraction/harvesting of the biomass. This strategy becomes more relevant to marine microalgae cultivation since the culture medium contains saltwater (artificial or natural) that has more costs attached. Not only due to reagent costs or labour, the use of saltwater in the cultivation process makes the discharge to a wastewater treatment station impossible. Thus, if the supernatant obtained from the harvest of the biomass is not recirculated to the system, high amounts of nutrients are thrown out. It is possible to re-use those nutrients by using closed systems to recirculate the water and take the most of the nutrients not consumed [17], [18]. Therefore, recirculation strategy allows the decreasing of water-footprint of microalgal production process and at long-term this strategy allows saving considerable production costs.

On the other hand, this strategy frequently leads to a loss of productivity. It is thought that is related with the unfolding of various growth limiting factors: non-consumed nutrients accumulation; production of auto-inhibitory compounds in high cell density cultures (mainly secondary metabolites); cellular debris accumulation (e.g. plasmatic membrane released to culture medium after cellular lysis may induce the aggregate formation and trapping microalgal cells inside); proliferation of nutrient competitive or predator contaminants (fungi, protozoa and bacteria) [12], [14], [19]–[21].

Microalgal Nutrition

There are two kinds of nutrients supplied to grow microalgae in autotrophic conditions: macronutrients and micronutrients. Microalgal growth depends on two macronutrients, nitrogen and phosphorous. Nitrogen is an essential constituent of all structural and functional proteins in the algal cells and accounts for 7%–10% of cell dry weight [12], [14], it is a fundamental element for the formation of proteins and nucleic acids, it is also an integral part of essential molecules such as ATP. Nitrogen is mostly supplied (and “favourite” source) as nitrate (NO₃), but often ammonium (NH₄⁺), urea and free amino acids are also used, with similar growth rates recorded [11], [12], [16], [22].

Phosphorous is essential for growth and many cellular processes such as energy transfer or biosynthesis of nucleic acids. Although algal biomass contains less than 1% P, it is often one of the most important growth limiting factors in algal biotechnology. The preferred form in which it is supplied to algae is as orthophosphate (PO₄³⁻) and its uptake is energy-dependent.

Micronutrients are an important part of the nutritive medium; it is a group of nutrients that have to be added in smaller quantities than the other nutrients. [14] and they are essential for cellular growth since they are incorporated into essential organic molecules, particularly a variety of coenzyme factors that enter into photosynthetic reactions [13]. Deficiencies in trace metals can limit algal growth, whereas excesses or high metal concentrations (above the toxicity threshold) may inhibit growth, impair photosynthesis, deplete antioxidants, and damage the cell membrane [12], [13], [16], [23]. In Table 2 are presented the major micronutrients frequently added to microalgal cultivations and the respective biological functions.

Table 2 – Biological role of major micronutrients [14], [16], [23],

Micronutrient	Biological role
Iron	Nitrogen reduction; Photosynthesis; Respiration; Ribonucleotid reduction;

	Metal ion transport
Magnesium	Photosynthesis – Structural element of chlorophyll Aggregation of ribosomes RNA polymerases, ATPases, protein kinases,
Calcium	Secretion; Glycolysis and gluconeogenesis; Ion transport; Cell division and growth
Zinc	Hydrolases Nucleic-acid polymerases and transcription factors; Metallothionein
Copper	Similar functions to iron (e.g. ion transport)
Cobalt	Structural element of vitamin B12 (possible supplement to the microalgal growth) Photosynthesis – electron transport chain
Manganese	Catalysis of oxygen evolution in the PSII complex Nitrogen reduction
Molybdenum	Nitrogen reduction; Oxidation of aldehydes, purines and sulfites

MATERIALS AND METHODS

Microalgae Growth

The *N. oceanica* cultivation assays occurred between May and September of 2014 in the industrial facilities of A4F – Algafuel, in Pataias. The inoculum was shared with A4F within the scope of the BIOFAT project and the strain was kept isolated and contaminant free in the algae collection of A4F – Algafuel.

The cultivations were done in outdoor conditions in a semi-continuous regime using lab-scale PBRs that aimed to simulate the abiotic and physical constraints and stimuli suffered by the production cultures in the pilot-PBRs. Their capacity is approximately 3,7 L and their constitution resembles to an air-lift type reactor, with a transparent part (approximately 93 % of the total volume of reactor). Throughout the assays the established operating volume for cultivations was 1 L. The uniform mixing was established through a piping in the bottom of the reactor which was the air supply to the system. The air supply was enriched with CO₂, which allowed the real-time pH control, with the proportion of in the air supply being controlled by a valve system. The air intake flow was adjusted to provide a uniform and efficient mixing and prevent a non-excessive shear stress to microalgal cells. Nutrients were supplied based on the amount of nitrate ion in the culture, which was adjusted to 6 mM. The other nutrients were adjusted proportionally to the nitrate ion concentration.

The temperature of the culture system was monitored by collecting specific and programmed samples of the culture and the temperature control was achieved through a thermoregulation system by water spraying. With the temperature rising above 25 °C, there was a constant water flux (controlled by a

valve system) to each reactor wall, cooling them down very quickly.

The inoculum used in the assay 1 and 2 was as concentrated as possibly in order to prevent the photoinhibition of cultures.

The culture media and process water were maintained disinfected with sodium hypochlorite. Before usage, the culture media was neutralized with sodium thiosulfate solution.

All volumes (culture and nutritive medium, process water) were previously sterilized at 121 °C for 40 minutes before being added to the cultures at atmospheric temperature.

Culture Control

To ensure culture stability the following parameters were checked along the assays. Culture cell concentration was controlled through measurement of optical density (OD) at 600 nm using the portable OD meter WPA CO 8000. The OD values were converted afterwards to dry weight (g_{D.W.}/L) using a linear correlation. The cultures productivity (g_{D.W.}/L.day) was calculated through the multiplication of the daily renewal rate (fraction of the total volume removed in a daily basis and afterwards made up to the operating volume of the reactor) by the dry weight of the culture (g_{D.W.}/L). The nitrate ion concentration was measured using a spectrophotometric methodology adapted from [24]. The pH and temperature of cultures was monitored three times per day (except weekends) through culture sample analysis using a portable pH meter HI 98130 – Hanna Combo. Culture salinity was measured twice a week using an optical refractometer Optech K71904. The samples of cultures were observed using a BA 400 – Motic optical microscope in order to analyse the culture welfare and detect any contamination.

Daily Renewal

The renewal process consists in the removal of a fraction of the total culture system volume (which contains water, salts, biomass and nutrients) and the respective volume replacement (with culture medium and nutrients).

The renewal was done in three distinct ways: without recirculation of the exhaust culture medium after biomass removal (i.e. volume replacement by fresh culture medium), with exhaust culture medium recycling after biomass removal by centrifugation (17 minutes at 3500 rpm in the laboratory centrifuge Hermle Z400 K), in this procedure only 90% (v/v) of the exhaust media was replaced and the remaining volume was filled by fresh culture medium. The last procedure of daily renewal contemplated the medium recycling of disinfected exhaust culture medium after

biomass removal. The cultures were disinfected by addition of sodium hypochlorite at a final concentration of 10 ppm and stored in an aseptic environment for 2 hours. After this time the exhaust culture medium was neutralized through a sodium thiosulfate solution (150 g/L) and 90% (v/v) of the exhaust medium was reintroduced in the culture. The remaining volume was respectively filled with fresh medium (and nutrients, if necessary).

Medium Formulations

Throughout this work it was tested three formulations of nutritive medium: CNASAM_2, CNASAM_3 and BIOFAT_2. It was also tested two formulations of culture medium: ASAM_2 and ASAM_2.1.

CNASAM_2 is a result of optimization work done by A4F – Algafuel which had basis in the composition of f/2 medium [25] and the modifications done to the original recipe reflects the needs and excesses verified in marine microalgae in laboratorial cultures. The CNASAM_3 medium is the second result of optimization work done by A4F – Algafuel and the modifications done reflects the needs and excesses verified in *N. oceanica* in laboratorial cultures. The composition of this medium diverges from CNASAM_2 recipe in some elements concentration: Iron (+405%), zinc (– 75%), copper (– 100%) and cobalt (– 100%). It is important to be referred that this medium had never been tested in cultivations under industrial conditions but it was expected that would provide a positive influence in the microalgal growth.

BIOFAT_2 is an outsourced industrial supplied medium which was used in the *N. oceanica* large-scale production. The BIOFAT_2 recipe is basically

the same as CNASAM_2 and theoretically has the same nutrients.

ASAM_2 is the standard medium for marine microalgal cultivation and the recipe is also result of optimization work done by A4F – Algafuel. The difference between ASAM_2 and ASAM_2.1 resides only in the sodium chloride (NaCl) source. The ASAM_2 is prepared with laboratorial NaCl and is intended for laboratory use which represents a costly but very reliable option and the ASAM_2.1 is prepared with marine purified salt which allows a significant cost reduction in the microalgal cultivation process.

RESULTS

Assay 1

This assay was developed from 19th May 2014 to 8th July 2014 in the industrial facilities of A4F – Algafuel. The data was provided by the detailed monitoring of 9 cultures of *N. oceanica* operating in outdoor lab-scale PBRs under fed-batch mode. The cultures were divided in three sets of conditions: Condition 1 (C1) – CNASAM_2 + ASAM_2, Condition 2 (C2) – CNASAM_3 + ASAM_2 and Condition 3 (C3) – BIOFAT_2 + ASAM_2.1. The cultivation process took place in three different stages, the culture growth phase, the daily renewal phase (DR phase) and the daily renewal with culture medium recycling phase (DRMR phase). The culture growth phase started with the inoculation of the reactors when the cultures were diluted and continued until they reach a specific level of concentration that allows the consecutive daily renewal of culture medium. After two weeks on daily renewal regime the cultivation switched to daily renewal with medium recycling.

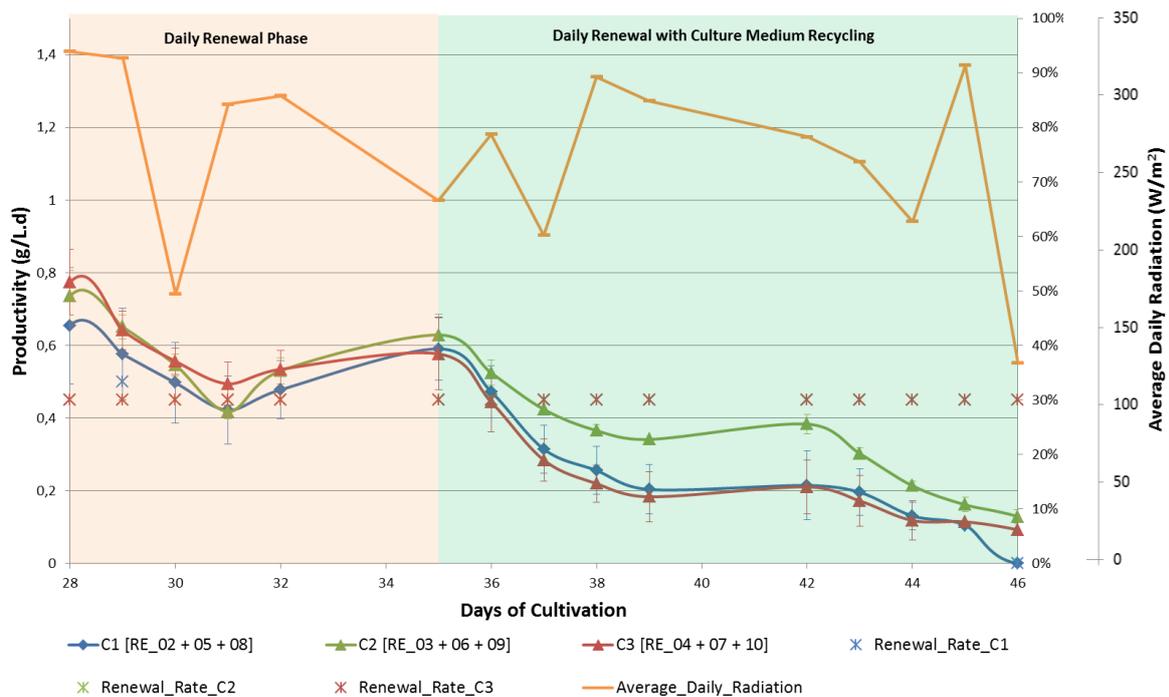


Fig. 1 – Presentation of culture productivity evolution between the 28th day of cultivation (DR phase) and the end of DRMR phase after data treatment. The data was organized in 3 sets of cultures that grown under the same cultivation condition.

The culture growth phase was not performed as planned due to the death of various cultures. This deaths were related with photoacclimation problems after the inoculation (when cultures were less concentrated) which led to the photoinhibition phenomenon. After some culture growth attempts the

source of inoculum switched and were used the renewals of the acclimated cultures to inoculate the reactors.

The DR phase occurred as planned but it was not possible to achieve the steady state of productivity in the first week.

Table 3 – Presentation of average stabilized productivity (at the steady state of production) achieved by the cultures under different conditions and under different cultivation phases. It is also presented the productivity loss between DR and DRMR cultivation phases (Δ Week 1 and Δ Week 2, respectively), and the first and second week of DRMR phase (Δ DRMR phase).

Condition	Daily Renewal Phase	Daily Renewal With Medium Recycling		Productivity Loss		
	Average stabilized productivity (g _{D.W./L.day})	Week 1 – Average stabilized productivity (g _{D.W./L.day})	Week 2 – Average stabilized productivity (g _{D.W./L.day})	Δ Week 1 (DRMR) (%)	Δ Week 2 (DRMR) (%)	Δ DRMR phase (%)
1 – [CNASAM_2 + ASAM_2]	0,466±0,164	0,258±0,104	–	44,6	–	–
2 – [CNASAM_3 + ASAM_2]	0,499±0,048	0,378±0,020	0,169±0,029	24,3	66,2	55,3
3 – [BIOFAT_2 + ASAM_2.1]	0,529±0,085	0,229±0,102	0,109±0,023	57,9	50,2	52,5

All cultures under different cultivation conditions achieve stabilized productivity values in the same range at the daily renewal phase. It is also possible to note that all cultures suffered a considerable stabilized productivity loss when the regime was switched to

daily medium recycling possibly due to accumulation of growth limiting factors such as biological contaminants or non-consumed nutrients.

Despite the productivity loss of C2 cultures between the first and second week of DRMR phase

(55,3 %) these cultures were able to maintain the higher value of stabilized productivity even in the second week of DRMR phase ($0,169 \pm 0,029$ g D.W./L.day). On the other hand, C3 cultures suffered a minor impact (52,5%) of the inherent cultivation strategy in the second week (of DRMR phase) in comparison with the values of first week and also stabilized to a productivity tier close to C2 cultures ($0,169 \pm 0,029$ g D.W./L.day and $0,109 \pm 0,023$ g D.W./L.day, respectively). This approximation has an inherent error, because there were compared the productivity of one healthy C3 culture (RE_07) and three C2 cultures. Regarding this it is possible to note that C2 cultures showed strength, constant and consistent response to the imposed cultivation conditions.

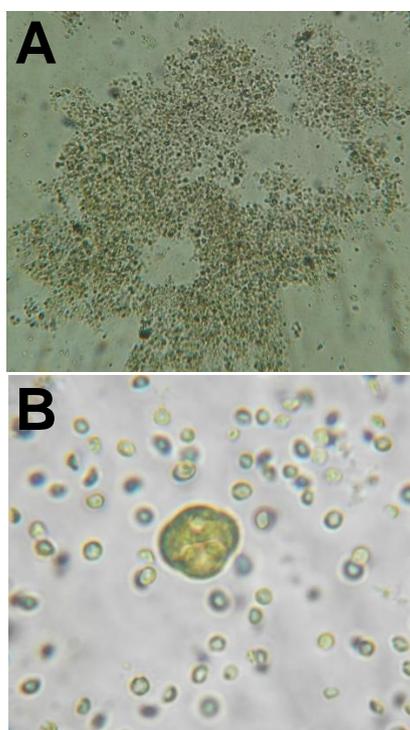


Fig. 2 – Example of typical microscopic observation of a contaminated culture during the assay 1. In the end of the assay all cultures showed a yellow tonality and microscopically showed a large number of biological contaminants (fungi, bacteria and protozoa). A – Photography of RE_02 microscopic observation taken at 06/06/2014 at 100x magnification. B – Photography of RE_02 microscopic observation taken at 06/06/2014 at 400x magnification. The presented small round green cells are *N.oceanica* cells with 2-5 μm of diameter.

In short, and regarding to the presented cultivation information during assay 1 it is possible to consider that the C2 conditions, and specifically, the CNASAM_3 nutritive medium is the most appropriate option for *N.oceanica* cultivation with medium recycling strategy. However, it is far away of the best modus, and some strategy problems, such as proliferation of foreign organisms, needed to be overcome in the

search for the best conditions for industrial cultivation of *N.oceanica*.

Table 4 – Lipid content of the samples collected in the end of assay 1. The operational procedure of this analysis is based on [26] and was adapted during this work.

Sample	Lipid Content [% (DW/DW)]
Inoculum cultures	$19,81 \pm 15,0$
Condition 1	$19,84 \pm 5,4$
Condition 2	$12,37 \pm 9,3$
Condition 3	$14,41 \pm 6,6$

In the assay 1 all cultures presented a very similar lipid content value within the considered error range. It is important to refer that the culture conditions were optimized for culture productivity and not for lipid production (frequently cultivated under induced stress after reaching the stationary phase). Regarding this fact, it is possible to consider these values acceptable and coincident within the literature references which referred that the high productivities cultures generally present a range of 3 - 15 % (w/w) of lipid content [10], [25], [27].

Elemental analysis was performed on samples of culture medium of each condition studied: from the beginning of the assay (inoculum culture), collected in the switching day of DR to DRMR phase (midpoint), and collected at the end of the assay (end point).

Phosphorous, calcium and iron showed an excess in the inoculum cultures that contrast with the lower percentage of variance in the DR cultivation phase. Magnesium also shows a higher concentration in the inoculum culture than in the DR cultures. In a general point of view, the majority of elements such as phosphorous, magnesium, manganese, molybdenum, cobalt and boron show a cumulative evolution between the DR and DRMR phase which may be explained by the medium recycling impact on cultures with the gradual accumulation of non-consumed elements. Calcium shows a gradual decrease in the accumulation during the essay but is excess in the culture medium. Cobalt, zinc, molybdenum and manganese are referred in the literature as growth limiting factors for microalgal cultures when present in not very high concentrations [12], [16], [28].

The former elements are present in all culture medium in excess, especially in DRMR cultures, which may explain the verified high loss of cultures productivity.

Assay 2

The assay was developed from 5th August 2014 to 5th September 2014 (end of the curricular internship) in the industrial facilities of A4F – Algafuel, in Pataias. This assay was developed under the same operational pattern and technology of assay 1 (see Section 4.1.1). The data was also provided by the monitoring of 9 cultures of *N.oceanica* operating in the same outdoor lab-scale PBRs under fed-batch mode.

The goal of this assay was to prove the efficiency of the disinfection process (DP) of the exhaust culture medium and reduce the impact of the accumulation of biological contaminants verified in the assay 1.

The cultures were divided in three sets of conditions: Condition 1 (C1) – CNASAM_3 + ASAM_2.1 without DP, Condition 2 (C2) – CNASAM_3 + ASAM_2.1 with DP and Condition 3 (C3) – BIOFAT_2 + ASAM_2.1 with DP.

It was planned to perform the cultivation process in During the first week of DRMR the C3 cultures started to show a yellow tonality that gradually became more evident during the days of cultivation culminating in the death of one C3 culture in the beginning of the second week of DRMR phase. On the other hand, C1 and C2 cultures showed a healthy colour during the cultivation time.

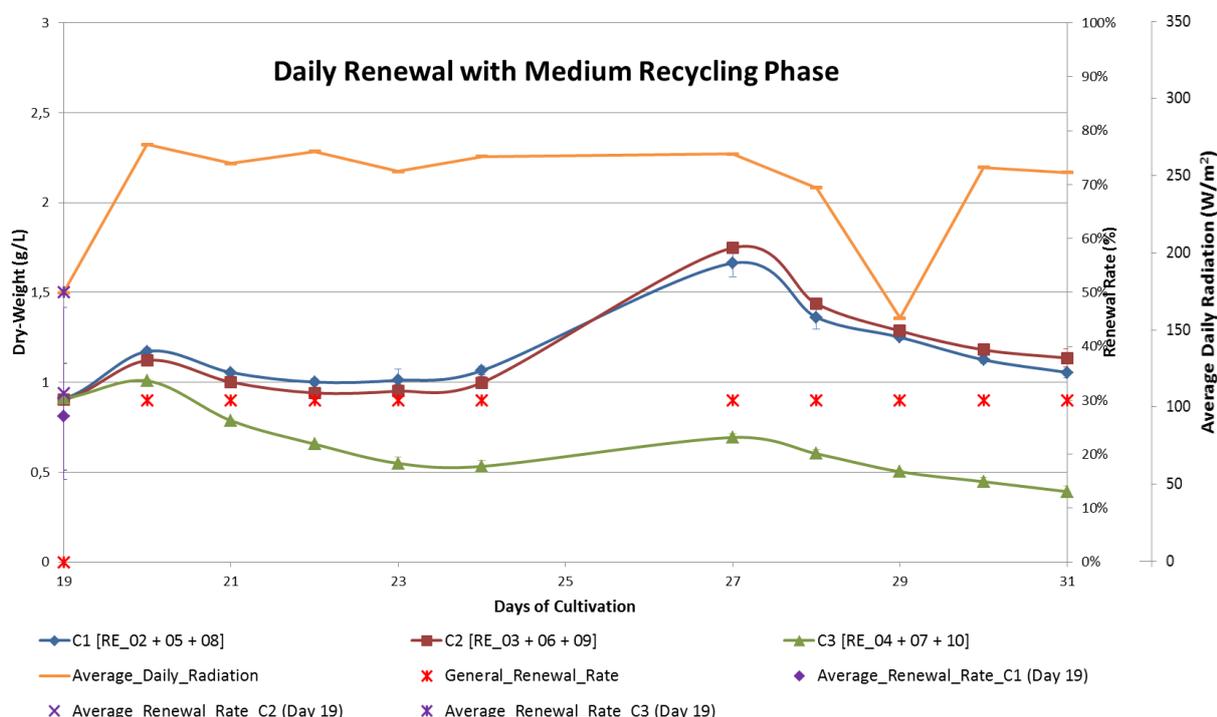


Fig. 3 – Presentation of culture concentration evolution during the daily renewal with medium recycling (DRMR) phase of assay 2. The data was organized in 3 sets of cultures that grown under the same cultivation condition. The culture growth phase was extended due to the various losses of cultures and with the conjugation of the time constraint (approach of the internship end) the culture growth phase was followed by two weeks of DRMR phase.

Attending to the first week of DRMR phase it is possible to observe that C1 and C2 cultures achieved the same average stabilized productivity value (in the steady state of productivity) considering the error range. This result was previously expected because, before the DRMR phase start there was no difference between these set of cultures. Only in the first day of DRMR phase different cultivation conditions started to be applied to each set of cultures with the exhaust medium from C2 cultures being disinfected during two hours and neutralized before being added to the respective cultivation system. On the other hand, the exhaust medium from C1 cultures did not

suffered any additional procedure or treatment (control condition). However, it is possible that C2 culture cells suffered some acclimation process to the new chemical species added to the culture medium, and C2 cultures productivity was possible affected by this fact. Nevertheless, it is only a hypothesis that cannot be quantified or concluded through this analysis.

Attending to the first week average stabilized productivity values, it is possible to see that C3 cultures achieve a significant lower value that probably is related to the nutritive medium impact on *N.oceanica* growth under the considered conditions.

Table 5 – Presentation of average stabilized productivity achieved by the cultures under different conditions during the first and second week of DRMR phase. It is also presented the productivity variance between the first and second week (of DRMR phase).

Condition	Daily Renewal With Medium Recycling		Stabilized Productivity Variance
	Week 1 – Average stabilized productivity (g _{D.W./L.day})	Week 2 – Average stabilized productivity (g _{D.W./L.day})	Δ (%)
1 [CNASAM_3 – without disinfection process]	0,308±0,018	0,343±0,009	10,3
2 [CNASAM_3 – with disinfection process]	0,289±0,018	0,363±0,007	19,8
3 [BIOFAT_2 – with disinfection process]	0,174±0,005	0,134±0,009	– 29,6

As stated in the literature [12], [17], [21], it was expected that all cultures productivity started to decrease in the second week of daily renewal with medium recycling cultivation strategy mainly due to the accumulation of contaminants, auto-inhibitory compounds and toxic levels of non-consumed elements (see Section 1.7) but in this assay, only C3 cultures showed this behaviour, achieving a productivity loss of 29,6%. C1 and C2 cultures showed a 10,3% and 19,8% (respectively) higher average stabilized productivity in the second week of DRMR phase. This fact can be explained by the conjugation of an high and consistent verified solar radiation between the 1st and 8th day of DRMR phase and the absence of significant growth limiting factors that is reflected in the high productivity during the week and also led to great culture recovery (or concentration) during the renewal days-off (7th and 8th day of cultivation).

It was possible to note that C1 and C2 cultures also achieve a similar value of average stabilized productivity (0,343±0,009 g_{D.W./L.day} and 0,363±0,007 g_{D.W./L.day}, respectively) but the C2 value is slightly higher what probably is related with the disinfection process applied to the exhaust medium of this cultures and the inherent contribution for minimizing the accumulation of growth limiting factors in the culture medium. If this cultivation phase was continued for more time, it is possible to consider the hypothesis of C2 cultures achieving a significant higher stabilized productivity value and probably the C1 cultures would show productivity decreasing and culture stress indicators.

Regarding this, it is possible to take two major conclusions: CNASAM_3 (present in C1 and C2 cultures) is the nutritive medium formulation that allowed the higher and consistent *N.oceanica* culture productivity under industrial cultivation conditions; and

the disinfection of exhaust medium process (with the respective neutralization) has proven to be a considerable advance step for improving *N.oceanica* cultures productivity at long term cultivation.

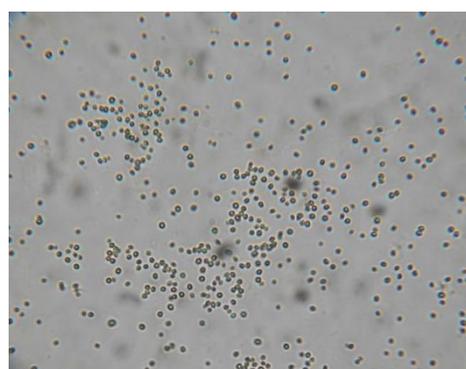


Fig. 4 – Example of typical microscopic observation of cultures during the assay 2. Photography of RE_09 (C2) microscopic observation taken at 03/09/2014 at 100x magnification. The presented small round green cells are *N. oceanica* cells with 2-5 μm of diameter.

During the DRMR phase, C1 cultures also showed a non-significant concentration of biological contaminants but in comparison with C2 and C3 cultures (which tested the disinfection of exhaust medium treatment) it was possible to observe that the number of contaminants (such as fungi and bacteria) was considerably higher. It was also possible to observe that C1 cultures showed a gradually increase of the number of foreign organisms and cellular debris that was directly connected with the number of renewals with medium recycling.

In the end of the assay were collected samples from all culture media and sent to elemental analysis. It was possible to see that calcium, copper, cobalt, zinc manganese and molybdenum were one more time in excess meaning that the respective cellular uptake is lower than the addition rate. It was also observed that phosphorous and iron were in a great deficiency in the

culture media (concentration approximately null). This element deficiency and excess possibly are limiting the cellular growth of microalgae. However, and considering the element analysis data from the assay 1 it was possibly to suggest some adjustments in CNASAM_3 and ASAM_2 formulations (Table 6).

Through the assay 2 element analysis it was tracked down the possible accumulation of sulphur derived from the addition of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$). All cultures show the same level of accumulation (12 – 14 mM) and it was considered that the disinfection process tested through this assay does not have a significant impact on the accumulation of additional chemical species in the culture medium.

Table 6 – Reformulation of CNASAM_3 medium (CNASAM_3.1) conditions and reformulation of ASAM_2 medium (ASAM_2.2) for improved *N.oceanica* cultures productivity under industrial. All values are presented in percentage variance considering the original recipe of each medium.

CNASAM_3.1			ASAM_2.2	
Nutrient	Compound	Adjustment	Compound	Adjustment
Nitrogen	NO_3^-	0%	NaCl	0%
Phosphorous	H_2PO_4^-	+35%	CaCl_2	-60%
Iron	Fe^{3+}	+50%	MS_1	-30%
Magnesium	Mg^{2+}	0%		
Zinc	Zn^{2+}	-50%		
Copper	Cu^{2+}	0%		
Manganese	Mn^{2+}	0%		
Cobalt	Co^{3+}	0%		
Molybdenum	MoO_4^{2-}	+20%		

Assay 3

Through the collected and analysed data from the assay 1 and 2 it was possible to do to this evaluation and formulate another non-independent assay. During the assay 1 cultivation period, A4F – Algafuel defined the ASAM_2.1 medium as standard culture medium for *N.oceanica* cultivations due to operational constraints. In this assay it was evaluated the impact of ASAM_2 and ASAM_2.1 medium in the culture growth and in the possible element contaminations that may result in the excessive accumulation of non-consumed elements in the culture media that may affect the microalgal growth, the cultures productivity and possibly culminate in loss of cultures.

It was analysed the culture growth evolution and the element analysis (for tracking additional accumulation of DP derived chemical species) during assay 1 and 2 it was not possible to relate the ASAM_2.1 medium as source of elements accumulation in the culture medium; it was also not possible to relate this medium with the loss of productivity of *N.oceanica* cultures under the tested cultivation conditions and the purified seawater salt was considered an adequate source of NaCl for marine microalgae cultivations.

Conclusions and Future Work

Throughout this work it was possible to observe that *N. oceanica* cultures after a long period of cultivation and through medium recycling cultivation

strategy show a loss of productivity about 50%. The loss of productivity might be related with the accumulation of growth limiting factors such as nutrients (above the toxicity threshold), proliferation of competitive foreign organisms (bacteria, protozoa and fungi) and accumulation of cellular debris (subsequent from cellular lysis).

It was possible to observe that *N. oceanica* cultures under industrial cultivation conditions are capable to achieve a steady state with the highest productivity of $0,529 \pm 0,085 \text{ g}_{\text{D.W.}} / (\text{L} \cdot \text{day})$ under the renewal without medium recycling strategy and $0,378 \pm 0,020 \text{ g}_{\text{D.W.}} / (\text{L} \cdot \text{day})$ with medium recycling.

During this work it was observed that cultures complemented with CNASAM_3 medium achieved higher productivities and showed less susceptibility to other growth limiting factors, since none of these cultures was lost during the assays. After the end of this work, A4F – Algafuel considered CNASAM_3 as the best choice for *N. oceanica* cultivation and consequently defined this medium as standard nutritive medium for industrial cultivations of *N. oceanica*.

The cultures complemented with CNASAM_2 medium showed significant values of productivity but were the most susceptible to growth limiting factors resulting in all cultures loss in the first assay. The cultures complemented with BIOFAT_2 medium achieved lower values of productivity and average

susceptibility to the accumulation of growth limiting factors.

Attending to the culture medium, it was possible to conclude that the purified seasalt is an adequate source of sodium chloride for marine microalgae cultivations. It was not possible to detect any negative impact of this source in the *N. oceanica* cultivations and in the accumulation of additional chemical species in respective culture supernatants. This conclusion results in a cost-reduction of 70% of the sodium chloride source for marine microalgal cultivation on an industrial scale. On the other hand, it is suggested to perform an independent assay to create an in-depth evaluation of the substitution of the sodium chloride source in the culture medium formulation.

Throughout this work adaptations to the lipid content method were developed in order to make available a regular routine analysis integrated in the production unit. Using this quantification method, it was observed that all cultures achieved low values of lipid content which is expected in high productivity cultures. However, due to time constraints, it was not possible to do a precise analysis of this parameter due to the inherent high values of standard deviation. The implemented method has already provided reliable analysis for the lipid content determination after a few additional steps of optimization.

Through the long-term cultivations under medium recycling strategy it was possible to observe that all cultures presented a relatively high number of biological contaminations (fungi, protozoa, bacteria and other microalgae). This phenomenon is directly related with the medium recycling strategy and it was observed that it may have contributed for the inherent loss of productivity. Concerning this, the disinfection process of the exhaust culture medium was tested through the addition of sodium hypochlorite and subsequent neutralization with sodium thiosulfate solution. The efficiency of the disinfection process was proven and all cultures submitted to this process (and cultivated with medium recycling) showed a significant reduction of the number of biological contaminants present in the culture medium. Additionally, it was proven that these disinfected cultures also showed tendency to achieve higher levels of productivity in long-term cultivations. These results were considered very promising by A4F – Algafuel, who after the end of this work, started adopting this disinfection process in industrial cultivations of *N. oceanica*.

It was not possible to extend the duration of the cultivation in the disinfection of exhaust medium test due to time constraints and, consequently, it was not possible to perform the long-term cultivation test. Regarding this, it should be considered repeating the former test in *N. oceanica* long-term cultivations.

Throughout this work it was possible to relate the photoinhibition as major problem for laboratory-scale outdoor cultivation assays. All cultures showed in many cases difficulties in the photoacclimation process after inoculation to the outdoor lab-scale PBRs. However, it was proven that using higher inoculum concentrations is an efficient solution to overcome the problem since it allows decreasing the radiation incidence per microalgal cell. Another efficient solution developed was the usage of the successfully acclimated cultures as inoculum source from other reactors. It was also provided an additional excess of nutritive medium to the recent and diluted cultures in order to stimulate the cellular growth rate and lead to the cellular growth yield improvement [12], [29], [30].

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