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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: NutMedium_1 – standard medium, NutMedium_2 – optimized recipe and NutMedium_3 – industrial-made medium. Two formulations of culture media were also tested: CultMedium_1 – with laboratorial sodium chloride (NaCl) and CultMedium_2 – 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±0,085 $g_{D.W.}$ /(L.day) in steady state. The NutMedium_2 cultures achieved higher productivities and showed less susceptibility to growth limiting factors. The cultures with NutMedium_1 showed significant values of productivity but all resulted in culture death. The cultures with NutMedium_3 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.

Resumo

Neste trabalho objectivou-se a optimização da composição e estratégia de recirculação do meio de cultura para produção industrial de *Nannochloropsis oceanica*.

Num primeiro cultivo de *N. oceanica* em exterior e em regime semi-contínuo com recirculação de meio de cultura testaram-se 3 formulações de meio nutritivo: MeioNut_1 – Meio standard, MeioNut_2 – formulação optimizada e MeioNut_3 – formulação industrial. Também foram testadas 2 formulações de meio de cultura: MeioCult_1 – com cloreto de sódio laboratorial (NaCl) e MeioCult_2 – com sal marinho purificado.

A recirculação de meio levou a uma quebra de produtividade máxima de 50% em comparação com culturas sem recirculação, que atingiram até 0,529±0,085 g $_{P.S.}$ /(L.dia) em estado estacionário. As culturas com MeioNut_2 atingiram produtividades mais altas e mostraram menos susceptibilidade a factores inibitórios de crescimento. As culturas com MeioNut_1 obtiveram valores razoáveis de produtividade mas acabaram por entrar em fase de morte. As culturas com MeioNut_3 atingiram produtividade inferior e susceptibilidade razoável a factores inibitórios de crescimento.

Não se detectou impactos negativos provenientes da fonte de NaCl em cultivos de *N. oceanica*, e considerou-se o sal purificado como uma fonte adequada de NaCl para cultivo de microalgas marinhas.

A recirculação de meio levou à proliferação de contaminantes biológicos que está relacionada com a quebra de produtividade. Foi testado um processo de desinfecção de meio recirculado com adição de hipoclorito de sódio e respectiva neutralização com tiossulfato de sódio em cultivos de *N. oceanica*. O processo mostrou-se eficaz, visto que se observou tendência para as culturas com este tratamento atingirem produtividade mais elevada a longo prazo.

Palavras-Chave: *Nannochloropsis oceanica*, produção industrial, microalgas, meio de cultura, recirculação de meio de cultura.

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Abbreviation List

Abbreviation	Meaning
C1	Cultivation condition 1
C2	Cultivation condition 2
C3	Cultivation condition 3
DR	Daily Renewal
DRMR	Daily Renewal with Medium Recycling
DMW	Demineralized Water
DP	Disinfection Process
DW	Dry Weight
EPA	Eicosapentaenoic Acid
MS	Mineral Solution
NCC n	Nitrate Concentration Consumption at day n
OD	Optical Density
PBR	Photobioreactor
PUFA	Polyunsaturated Fatty Acid
PW	Process Water
RE	Reactor
RR	Renewal Rate

1 Literature Review

1.1 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].

Microalgae cultivation is a modern biotechnology but it has begun a long time ago. The first well achieved isolation and Beijerinck obtained the first unialgal cultures in 1890 with *Chlorella vulgaris*. Mass cultivation of microalgae really began to be a focus of research after 1948 at Stanford (USA), Essen (Germany) and Tokyo (Japan). Commercial large-scale culture of microalgae commenced in the early 1960's in Japan with the culture of Chlorella. In the next 40 years, and gradually, more and more entrepreneur projects began to appear with facilities producing and harvesting many species of microalgae established all over the world (Mexico, USA, Australia, Japan, Thailand, India, Israel) [6]. This fast growth and expansion of microalgae cultivation technology led to a better knowing of the product itself and actually there are about fifty species of microalgae utilized in biotechnology [5]. These "traditional species" potential converge in some industrial and biotechnological applications illustrated in the following table (Table 1.1).

Table 1.1 - Industrial applications of microalgae cultivation. Adapted from [5] and [7].

Application	Brief Description
Food Industry	Microalgae biomass is used as source of protein, polyunsaturated fatty acids – PUFAs (e. g. omega-3 and omega 6), vitamins (e. g. A and B12) and other **food and dietary supplements** in **human nutrition**.
Feed Industry	Some photosynthetic accessory pigments (phycobiliproteins) and carotenoids produced by microalgae are used as **natural colouring agents** in **animal** production as **feed**. Regarding to microalgae nutritive content, they are used as feed to early stages of fish growth in aquaculture.
Pharmaceutical Industry	Phycobiliproteins and some stable isotope biochemicals (e.g. Selenium) produced by microalgae are used as labels for antibodies and receptors (**immunolabeling**); gastrointestinal or breath **diagnosis tests**, respectively.

Table 1.1 - Industrial applications of microalgae cultivation. Adapted from [5] and [7].

Application	Brief Description
Agricultural Industry	Microalgae biomass is used as **biofertilizers** and **soil conditioners** due to their bioavailable nutrient content.
Wastewater treatment	Microalgae could be used in municipal **wastewater treatment** and **industrial effluents** due to their ability to metabolize sewage rapidly.
Carbon Dioxide Sequestration	Microalgae cultivation is a potential **contribute solution** for **carbon dioxide accumulation problem** in the atmosphere due to their photosynthetic metabolism.
Cosmetic Industry	Microalgae extracts are rich in proteins, vitamins, and minerals, which are incorporated as **active ingredients** into **cosmetic products**. They also contribute with the synthesis of phycobiliproteins, polysaccharides and PUFAs which have an appealing potential to cosmetic formulators.
Biodiesel Industry	Microalgae are responsible for the production of a range of lipids, with contents varying from 1% to 70% of dry weight, and reaching up to 90%. This microalgal **high content of lipids** could be the answer to avoid the competition with human food for the actual source of biodiesel.

All of this potential is generated by exploitation of a diverse range of metabolites with various bioactivities that are yet to be fully exploited. In present days, these metabolites and other microalgae derived compounds (Table 1.2) promote and lead the continuous investment and growth of microalgae technology and market [5].

Table 1.2 – Biotechnological applications of products from microalgae. Adapted from [7]

P	Application	Microalgal Producers
Polyunsaturated fatty acids (PUFA)	Nutritional supplements, aquaculture feed	*Pavlova, Nannochloropsis sp., Monodus, Phaeodactylum*
Eicosapentaenoic acid (EPA)	Infant formula, nutritional supplements, aquaculture feed	*Crypthecodinium & Schizochytrium*
Docohexaenoic acid (DHA)	Nutritional supplements	*Spirulina* *Porphyridium*
Phycobiliproteins **Phycocyanin**	Natural dye for health food and cosmetics (lipsticks and eyeliners) antioxidant	*Spirulina platensis*
Phycoerythrin	Fluorescent agent, tool for biomedical research, diagnostic tool	*Red algae (e.g. Porphyridium cruentum)*
Carotenoids **β-carotene**	Food colourant; antioxidant; cancer-preventive properties	*Dunaliella salina*
Astaxanthin	Pigment for salmon, antioxidant	*Haematococcus pluvialis*

Table 1.2 – Biotechnological applications of products from microalgae. Adapted from [7]

P	Application	Microalgal Producers
Mycosporine-like amino acids (MAA)	UV-screening agent ; sunscreen	*Aphanizomenon flos-aquae*
Polysaccharides	Viscosifiers, lubricants and flocculants for industrial applications; antiviral agent	*Porphyridium cruentum*
Lipids – triglycerides and hydrocarbons	Biofuels	*Chlorella protothecoides* *Botryococcus braunii* *Nannochloropsis sp.*

1.2 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.

With the increase in anthropogenic GHG emissions, mainly due to large-scale use of fossil fuels for transport, electricity and thermal energy generation, it has become increasingly important to develop abatement techniques and adopt policies in order to minimize impacts of global warming. To meet this target, a selection of a range of effective technologies, including chemical and biological CO_2 mitigation possibilities, has been a focus of research [3].

To resolve the worldwide energy shortage crisis, the experts agreed that the solution passes through a development of a new type of biofuel. It needs to be created conditions for a technically and economically viable biofuel resource; to be as competitive as petroleum fuels and it is production cost inferior as well. The production requires low to no additional land use; that enables air quality improvement (e.g. CO_2 sequestration) and requires minimal potable water use [8].

Biodiesel appears to be an attractive energy resource for several reasons. First, biodiesel is a renewable resource of energy that could be sustainably supplied. It is understood that the petroleum reserves are to be depleted in less than 50 years at the present rate of consumption [9]. Second, biodiesel appears to have several favourable environmental properties resulting in no net increased release of carbon dioxide and very low sulphur content [10].

Biodiesel is made from biomass oils, mostly from vegetable oils. The biodiesel made from vegetable oils is a first generation biofuel and has attached important issues of social, ethical and economic nature. The production of this type of biodiesel requires a large area of land to oil crops grows; oil crop derived biodiesel compromise the production of food and other products derived from crops; and the oil crops have low growth rate [11].

In order to solve these first generation biodiesel problems, seeking lipid-rich biological materials to produce biodiesel effectively has attracted much renewed interest [11]. Oleaginous microorganisms such as microalgae have been suggested as potential candidates for biodiesel production because of a number of advantages [3]:

- Microalgae are considered to be a very efficient biological system for harvesting solar energy for the production of organic compounds and they are the most efficient biological producer of oil on the planet [12];
- Microalgae have a simple biological structure and short growth cycles [3];
- Microalgae grow in liquid medium which can be handled easily [12];
- Microalgae can be grown in variable climates and non-arable land including marginal areas unsuitable for agricultural purposes (e.g. desert and seashore lands) [12];
- Many species of algae can be induced or easily modified to produce particularly high concentrations of chosen, commercially valuable compounds, such as proteins, carbohydrates, lipids and pigments [3];
- Microalgae farming achieve high CO_2 fixation and O_2 production [12];
- The farming of microalgae can be grown in non-potable water (e.g. sea or brackish water) or even wastewater (providing additional treatment) [12];
- Microalgae biomass production uses far less water than traditional crops [12];
- Microalgal biomass production systems can easily be adapted to various levels of operational or technological skills [3];
- Microalgae production is not seasonal and can be harvested daily [12].

Some microalgae, which are capable to achieve high content of lipids (generally 20 – 50 % dry weight biomass [13]), appear to be a suitable group of oleaginous microorganisms for lipids production and consequently suit as feedstock for the biodiesel industry. In Table 1.3 some species of microalgae and their lipid content are presented. [14]

Table 1.3 – Lipid content of different microalgal species and potential feedstocks for biodiesel production. Adapted from [14].

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)
Ankistrodesmus sp.	24.0–31.0
Botryococcus braunii	25.0–75.0
Chaetoceros muelleri	33.6
Chlorella emersonii	25.0–63.0
Chlorella protothecoides	14.6–57.8
Chlorella vulgaris	5.0–58.0
Chlorella sp.	10.0–48.0
Chlorella	18.0–57.0
Crypthecodinium cohnii	20.0–51.1
Dunaliella salina	6.0–25.0
Dunaliella tertiolecta	16.7–71.0
Dunaliella sp.	17.5–67.0
Ellipsoidion sp.	27.4
Isochrysis galbana	7.0–40.0

Table 1.3 – Lipid content of different microalgal species and potential feedstocks for biodiesel production. Adapted from [14].

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)
Monallanthus salina	20.0–22.0
Nannochloris sp.	20.0–56.0
Nannochloropsis oculata.	22.7–29.7
Nannochloropsis sp.	12.0–68.0
Neochloris oleoabundans	29.0–65.0
Nitzschia sp.	16.0–47.0
Pavlova salina	30.9
Pavlova lutheri	35.5
Phaeodactylum tricornutum	18.0–57.0
Porphyridium cruentum	9.0–18.8/60.7
Scenedesmus obliquus	11.0–55.0
Skeletonema sp.	13.3–31.8
Skeletonema costatum	13.5–51.3

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].

1.3 *Nannochloropsis oceanica*, a phycological view, potential and applications

The *Nannochloropsis oceanica* Suda & Miyashita, 2002, is a Eustigmatophyceae [15]. The phylogenetic classification of *N. Oceanica* is expressed in the following table (Table 1.4) [16].

Table 1.4 - Phylogenetic classification of N. oceanica [16].

Category	Classification
Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Eustigmatophyceae
Order	Eustigmatales
Family	Monodopsidaceae
Genus	*Nannochloropsis* D.J. Hibbert (1981)
Species	*Nannochloropsis oceanica* Suda & Miyashita (2002)

Small spherical cells, with a diameter range of 2–5 µm, characterize the species of the genus *Nannochloropsis*. They are green cells with a simple morphology and typically live in saltwater habitats. [15] *N. oceanica* has a very simple ultrastructure that consists of a nucleus, a single mitochondrion, a single Golgi body and a chloroplast; they have a simple cell wall and usually a single carbohydrate storage ‘grain’, usually called papilla [17] as can be seen in Fig. 1.1.

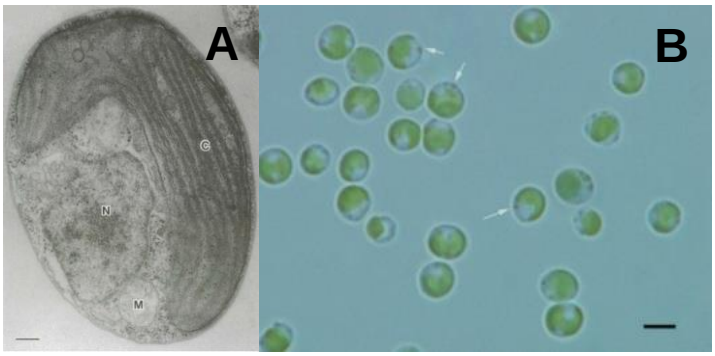

Fig. 1.1 – **1 -** *N.oceanica* cell image achieved with transmission electron microscopy (TEM). C - chloroplast; M - mitochondrion; N - nucleus. Scale bar = 0.2 µm. Adapted from [15]. **2 –** Bright field optical microscopic image showing general cell morphology of *N. oceanica*. The arrows indicate the papilla structure in the cell walls. Scale bar - 5 µm. Adapted from [17].

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 [15]. 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 (see section 1.1 and 1.2). This species is characterized by very high levels of palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic acid (C20:5 EPA) (The fatty acid content and percentage of total fatty acid of dry weight of *Nannochloropsis oceanica* is presented in Appendix 7.1) [17], [18]. The lipid production of *N. oceanica* is not an acquired fact in cultivation processes since cultures with high photosynthetic conversion yield and consequently with a high biomass productivity only are capable to achieve about 3 - 15 % (w/w) of lipid content [19]. Several studies have shown that the quantity of lipids within the cell can vary as a result of changes in growth conditions (temperature and light intensity) or nutrient media characteristics [20]. Regarding to this fact, the *N.oceanica* cultivation with lipid production focus has to be done in a two-phase cultivation process (a nutrient sufficient phase to produce the inoculum followed by stress induced phase to boost lipid synthesis) the crude lipid content of *Nannochloropsis* species biomass have an high increase and the culture attained a much higher lipid productivity compared with a nutrient sufficient single-phase process [21]. It is also stated that under nitrogen-starvation conditions, many *Nannochloropsis* species can accumulate oil exceeding 60% of its biomass on a dry weight basis which makes the nitrogen starvation the most widely used stress inducing method to promote the lipid production on *Nannochloropsis* species cultivation [19].

Nannochloropsis oceanica has a wide potential for commercial production of pigments and lipids, which integrated in the new concept of biorefinery, should create a sustainable production process of added-value compounds (see section 1.1 and 1.2) [22]. A very intense exploitation of the *N. oceanica* biorefinery concept should lead to both economic and environmental benefits in the future [17].

Microalgae of the genus *Nannochloropsis* are capable of accumulating triacylglycerols (TAGs) when exposed to nutrient limitation (in particular, nitrogen [N]) and are therefore considered promising organisms for biodiesel production.[19]

1.4 Microalgal Cultivation Parameters Influence and Control

In Nature, the microalgae growth does not wonder about kinetics, being the growth rate just the enough one for species survival. Microalgae are adapted to scavenge their environments for resources, to store them, or increase their efficiency in resource utilization. In general for biomass growth (consisting of 40–50% carbon) microalgae depend on a sufficient supply of a carbon source and light to carry out photosynthesis [14]. As said, the multiplication rate is highly dependent on environmental conditions, which are not constant in time being dependent of several factors. On the other hand, in artificial microalgae cultivation the goal is to favor the growth rate as much as possible or to push the metabolic route to follow one direction, if a particular metabolite has to be obtained. Looking at microalgae as living-cell factories for value-added compounds production, a deeper control is necessary to achieve the productivity objectives and this can only be done in photobioreactors [23].

Microalgae may assume many types of metabolisms (e.g. autotrophic, heterotrophic, mixotrophic, photoheterotrophic) and are capable of a metabolic shift as a response to changes in reasonably well defined **growth phases** which can be recognized (Fig. 1.2): (1) **lag phase**, representing the phase where microalgae are adapting to the conditions of cultivation; (2) **exponential growth phase**, representing the maximum growth rate under the specific conditions; (3) **linear growth phase**; (4) **stationary growth phase,** which means that, in this phase, the growth rate is equal to death rate; (5) **decline or death phase**. The opposite pattern of the dashed curve (Fig. 1.2) indicates the nutrients depletion during the stationary phase and onwards. [14].

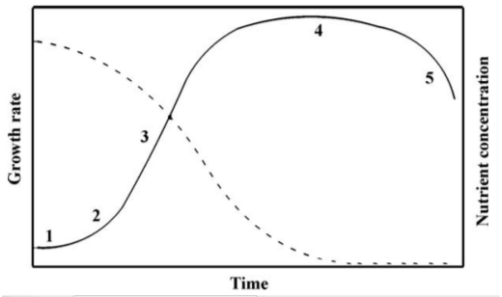

Fig. 1.2- Schematic representation of algae growth rate in batch culture (solid line) [14].

The number of variables that affect microalgae growth rate is huge, with direct, indirect and cross-effect influences. The most important ones are: abiotic factors such as light (quality, quantity), temperature, nutrient concentration, O_2, CO_2, pH, salinity, and toxic chemicals; biotic factors such as pathogens (bacteria, fungi, viruses and protozoan predators) and competition by other algae; operational factors such as shear produced by mixing, dilution rate, depth (or optical pathlength) or harvest frequency. For that reason the optimization of the growth rate is always a difficult task for each species [23], in other way this task can be simpler if all factors are organized by their influence in the growth.

1.4.1 Light

In order to enhance microalgal growth in photobioreactors (PBRs), light requirement is one of the most important parameters to be addressed. 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 [24].

The photoacclimation/photoadaptation process in algae leads to changes in cell properties according to the availability of light and an increase in photosynthetic efficiency. Adaptation can occur through multiple mechanisms such as changes in types and quantities of pigments, growth rate, dark respiration rate or the availability of essential fatty acids [25]. Morphological photoacclimation is accompanied by changes in cell volume and the number and density of thylakoid membranes. Algae overcome light limitation by desaturation of chloroplast membranes [26]. Light intensity increase above saturating limits causes photoinhibition. This is due to the disruption of the chloroplast lamellae caused by high light intensity and inactivation of enzymes involved in carbon dioxide fixation [27].

Photosynthesis (and the corresponding production of biomass) depends indeed on the photonic flux—as graphically represented in Fig. 1.3.

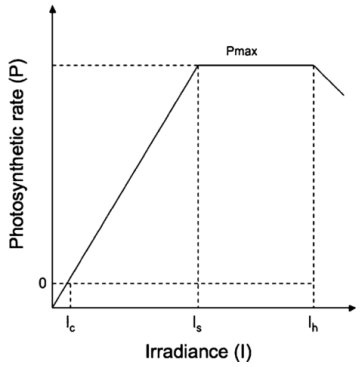

Fig. 1.3 - Typical photosynthesis (P) vs irradiance (I) curve for micro- algal cells (the various symbols are explained in the main text). Adapted from [27]

Three main areas can be distinguished in Fig. 1.3: (**1**) a light-limited region, in which the photonic input rate is fully applied in photosynthesis, so photosynthetic yield rises with increasing irradiance; such a region is delimited by the light intensity at which microalgal cells begin to grow (i.e., the compensation light intensity, Ic), and the light intensity at which no further increase in growth occurs upon increasing light intensity (i.e., the light saturation, Is); (**2**) a light-saturation area, in which the photosynthetic processing capacity of the culture attains its maximum value, and the excessive photonic flux provided to the culture is dissipated as heat or fluorescence; and (**3**) a photoinhibition region, in which increases in light intensity become injurious—and are expressed initially by a decrease in growth rate, eventually attaining photo-damage and even leading to culture death, after a point denoted as photoinhibition (Ih) [27].

The temperature of the medium also influences light intensity requirements for optimal growth: microalgae do in fact tolerate higher irradiances at temperatures closer to their optimum growth temperature [28].

1.4.2 Temperature

Temperature is the most important limiting factor, after light, for culturing algae in both closed and open outdoor systems. The temperature effects for many microalgae species in the laboratory are well documented, but the magnitude of temperature effects in the annual biomass production outdoors is not yet sufficiently acknowledged. Many microalgae can easily tolerate temperatures up to 15 ºC lower than their optimal, but exceeding the optimum temperature by only 2–4 ºC, reduces protein synthesis and results in decreased growth rates and consequently may result in the total culture loss [14]. Also, overheating problems may occur in closed culture systems during some hot days, where the temperature inside the reactor may reach 55 ºC. In this case evaporative water cooling systems may be economically used to decrease the temperature to around 20–26 ºC [14]. The optimum growth temperature of *Nannochloropsis oculata.* (a very similar species of *N. oceanica*) were estimated as 21 ºC [29].

The effect of temperatures lower than the optimal set on cultures is not so drastic but may influence the biochemical composition as consequence, e.g. the fluidity in the microalgal cell membrane may decrease. Cells then compensate by increasing levels of unsaturated fatty acids to increase fluidity. However, it also makes the membranes more susceptible to damage by free radicals. Along with greater fluidity, increased levels of unsaturated fatty acids tend to enhance the stability of the cellular membranes (particularly the thylakoid membrane). This, in turn, protects the photosynthetic machinery from photoinhibition at low temperatures [30].

1.4.3 pH and Carbon Supply

Carbon is the dominant element in organic matter [31] and is one of the other major nutrients that must be supplied to microalgal cells (typical dry weight content of carbohydrates in algae range from 20% to 40% of total cell mass). It is essential for photosynthesis and hence algal growth and reproduction. Carbon fixed by the microalgae can end up in three destinations; it will either be used: (a) for respiration; (b) as an energy backup source; or, (c) as a raw material in the formation of additional cells [24]. Reduced carbon fixation rate implies a reduction in microalgal growth rate. Microalgae require an inorganic carbon source to perform photosynthesis. Depending on the species, carbon can be utilized in the form of CO_2, carbonate, or bicarbonate for autotrophic growth and in form of organic substrate (e. g. acetate or glucose) for heterotrophic growth [24]. For high rates of autotrophic production, supply of CO_2 and HCO_3^- is most important. Contrary to land plants, atmospheric CO_2 cannot satisfy the carbon requirements of high yielding autotrophic algal production systems. The bicarbonate–carbonate buffer system can provide CO_2 for photosynthesis, when injected in the culture system [32]. CO_2 in water may be present in any of these forms depending upon pH, temperature and nutrient content:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

These reactions imply that during photosynthetic CO_2 fixation, OH^- accumulates in the growth solution leading to a gradual rise in pH; frequently pH reaches values of 11 in high algal density production systems where no additional CO_2 has been supplied [32].

Changing pH in media may limit algal growth via metabolic inhibition and consequently change the biochemical profile of the biomass. With an increase in pH, carbonate increases while molecular CO_2 and bicarbonate decrease, limiting the availability of carbon from CO_2, which, in turn, suppresses algal growth for most species [33]. The high value of pH may lead to aggregate formation in *Nannochloropsis* cultures. This phenomenon can be explained by a reduced stability of the algal suspension caused by the alkalinity-induced variation of the cell surface charge. If pH is lowered in the course of few hours, aggregates break up and cells again disperse in the medium [34]. If not, when pH decreases, the culture can alter nutrient uptake or induce metal toxicity and thus affect algal growth [24].

pH-static control via CO_2 sparging into the culture medium is the best and most convenient method of pH control and at the same time supplying CO_2 for high yield in mass algal cultures [32]. There are two possible ways to supply inorganic carbon to microalgae culture: (1) CO_2 can be introduced at low concentrations in the culture by air (0,03%) or, more efficiently, (2) through an aeration system with enriched air [29]. The ideal proportions of CO_2 in the enriched air supply has been studied and the maximal biomass and lipid productivity in a *Nannochloropsis* culture were achieved with 2% CO_2 aeration [35].

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7, though there are species that dwell in more acid/basic environments [36]. The pH range for mass-cultivation of *Nannochloropsis* is 6.75 – 9.2 [29]. The value of pH that should be aimed in a *Nannochloropsis* culture is 8.4 [29]. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH [36].

1.4.4 Salinity

Salinity refers primarily to sodium chloride concentration and is another important factor that alters the biochemical composition of algal cells and each microalgal species has a different optimum salinity range. Theoretically, the higher marine microalgae biomass productivity should be achieved at concentrations closer to their marine habitat [32], and exposing algae to lower or higher salinity levels than their natural (or adapted) levels can change growth rate and alter composition [24] due to osmotic stress felt by the cells, but in a general way, microalgae species can tolerate a reasonably wide range of salinity [36].

In *Nannochloropsis* cultivation under high salinity concentration leads to an increase in the algae lipid content and in EPA proportions, thus cell concentration decreases under influence of high salinity values[37].

In mass-cultivation processes, salinity is an important cultivation parameter to be measured and predicted, not only due to economic factors (e.g. salt cost) and constant aim to maximum biomass

yields, but also because of the constant evaporation of water in culture media that takes place, mainly, in open cultivation systems [37].

1.4.5 Mixing

Mixing is another important growth parameter since it homogenizes the cells distribution, heat, metabolites, and facilitates transfer of gases. Also, a certain degree of turbulence, especially in large-scale production, is desirable in order to promote the fast circulation of microalgae cells from the dark to the light zone of the reactor. On the other hand, high liquid velocities and degrees of turbulence (due to mechanical mixing or air bubbles mixing) can damage microalgae due to shear stress [38]. Studies developed in *Nannochloropsis* cultures show that aeration rate above 14.7 vvh does not lead to further biomass yield improvement certainly because of the stress subjected to the algae [29]. The optimum level of turbulence (above which cell death occurs) is strain dependent and should be investigated in order to avoid decline in productivity [14].

1.5 Microalgae Industrial Cultivation Systems

The microalgae industrial cultivation systems are divided in two main categories, open and closed cultivation systems, and there are just a few types of systems which are technically and economically feasible for large-scale production of algae biomass. All of the systems have different design characteristics and serve different purposes. Different cultivation systems (with different capacity) allow different scales of biomass production. On other hand, the large-scale production process of microalgae is a result of the integration of a variety of bioreactors (with different characteristics). The technical viability of each system is influenced by intrinsic properties of the selected algae strain used, as well as climatic conditions and costs of land. [39] [40]

1.5.1 Open Cultivation Systems

Algae cultivation in open pond production systems has been used since the 1950s [39]. These systems can be categorized into natural waters (lakes, lagoons, and ponds) and artificial ponds or containers. Raceway ponds (Fig. 1.4) are the most commonly used artificial system [39]. They are typically made of a closed loop, elongated rectangular with rounded ends recirculation channels (Fig. 1.4), generally between 0.2 and 0.5 m deep, with mixing and circulation required to stabilize algae growth and productivity. Raceway ponds are usually built in concrete, but compacted earth-lined ponds with white plastic have also been used. In a continuous production cycle, algae broth and nutrients are introduced in front of the paddlewheel and circulated through the loop to the harvest extraction point. The paddlewheel is in continuous operation to prevent sedimentation. The microalgae's CO_2 requirement can be satisfied from the surface air, but submerged aerators may be installed to enhance CO_2 absorption [40].

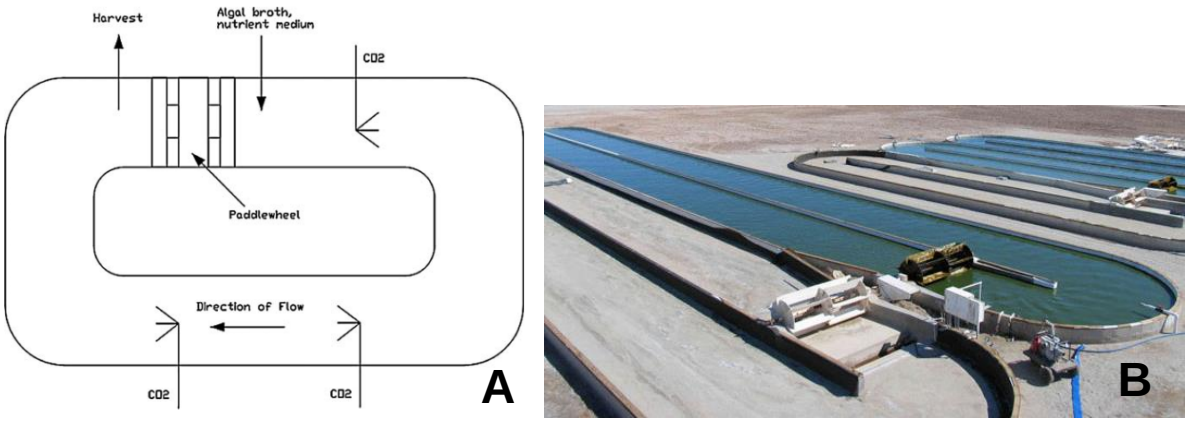

Fig. 1.4 – A – Plan view of a raceway pond. Algae broth is introduced after the paddlewheel, and completes a cycle while being mechanically aerated with CO_2. It is harvested before the paddlewheel to start the cycle again (adapted from [12]). **B –** Large-scale raceway pond. Located in California, USA, this raceway has a capacity of approximately 150 m^3 of culture [41].

Raceway ponds are not normally expensive to build and operate; they are durable and have a large production capacity. However ponds use a lot of energy to homogenize nutrients and the water level cannot be kept much lower than 15 cm (or 150 L/m^2) for the microalgae to receive enough solar energy to grow. Generally ponds are very susceptive to weather conditions, not allowing control of water temperature, evaporation and lighting. Also, they may produce large quantities of microalgae, but occupy more extensive land area and are highly susceptible to contaminations from other microalgae or bacteria [32]; consequently, these pollution and contamination risks associated with open pond systems, for the most part, preclude their use for the preparation of high-value products for use in the pharmaceutical and cosmetics industry [8].

1.5.2 Closed Cultivation Systems

The most commonly used large-scale closed microalgae cultivation systems are the photobioreactors (PBRs). Thus, a photobioreactor is a reactor in which phototrophs (microbial, algal or plant cells) are grown or used to carry out a photobiological reaction. Although this definition may apply to both closed and open-culture systems, for the purpose of this work and in common sense terms, this definition is limited to the former ones (closed cultivation systems) [14].

Microalgae production based on closed system technology is designed to overcome some of the major problems associated with the described open pond production systems. In this type of system the growth of microalgae is restricted and maintained inside of a transparent surface [8].

Closed cultivation systems are flexible systems that can be optimized according to the biological and physiological characteristics of the algal species being cultivated, allowing one to cultivate algal species that cannot be grown in open ponds. On a closed system, direct exchange of gases and contaminants (e.g. microorganisms, dust) between the cultivated cells and atmosphere are limited or not allowed by the reactor's walls. Also, a great proportion of light does not impinge directly on the culture surface but has to cross the transparent reactor walls [14].

The closed cultivation based reactors can be divided in three types of reactors; Tubular PBRs, Flat-plate PBRs, and Column PBRs. The first ones consist of an array of straight glass or plastic tubes (as shown on Fig. 1.5 - A), this tubular array can be aligned horizontally, vertically, inclined or as a helix [8] and the tubes are generally 0.1 m or less in diameter [32]. The flat-plate PBRs (Fig. 1.5 - B) are made of transparent materials for maximum solar energy capture, and a thin layer of dense culture flows across the flat plate, which allows radiation absorbance in the first few millimeters thickness and a high efficiency photosynthesis process [40]. The last type of PBRs, the Column PBRs (Fig. 1.5 - C) are basically vertical columns which are aerated from the bottom, and illuminated through transparent walls, or internally [42].

Fig. 1.5 - Presentation of the types of closed microalgae cultivation systems: A - Tubular PBR [43]; B - Flat-panel PBR [44]; C - Column PBR [45].

The main advantages and limitations of the closed cultivation systems are summarized in the following (Table 1.5).

Table 1.5 - Main advantages and limitations of closed cultivation systems. Adapted from [8].

Closed system	Advantages	Limitations
Tubular PBR	Large illumination surface area Relatively cheap Good biomass productivities	Some degree of wall growth Fouling Requires large land space Gradients of pH, dissolved oxygen and CO_2 along the tubes
Flat-plate PBR	High biomass productivities Easy to disinfect Low oxygen build-up Good light path Large illumination surface area Suitable for outdoor cultures	Difficult scale-up Difficult temperature control Small degree of hydrodynamic stress Some degree of wall growth
Column PBR	Compact High mass transfer Low energy consumption Good mixing with low shear stress Easy to sterilise Reduced photoinhibition and photo-oxidation	Small illumination area Expensive compared to open ponds Shear stress Sophisticated construction

The largest closed cultivation systems and the most suitable for outdoor mass cultures are tubular PBRs, but as seen, they have some limitations. Therefore, they cannot be scaled up indefinitely; hence, large-scale production plants are based on integration of multiple reactor units [8].

1.5.3 Comparison of Cultivation Systems, Open *vs* Closed Systems

The comparison of performances achieved by PBRs and open ponds may not be easy, as the evaluation depends on several factors, among which the algal species cultivated and the method adopted to compute productivity. Presented in Table 1.6 is the comparison between some performances and parameters that can be achieved by the most efficient open and closed large-scale cultivation systems for microalgae, raceway pond and tubular PBR, respectively [14].

Table 1.6- Comparison between open and closed large-scale cultivation system of microalgae. Adapted from [14].

Parameter/Performance	Closed systems	Open systems
Contamination control	Easy	Difficult
Contamination risk	Reduced	High
Sterility	Achievable	None
Process control	Easy	Difficult
Species control	Easy	Difficult
Mixing	Uniform	Very poor
Space required	A matter of productivity	A matter of productivity
Volume Ratio/Area	High (20 – 200 m^{-1})	Low (5 – 10 m^{-1})
Investment	High	Low
Operation costs	High	Low
Light utilization efficiency	High	Low
Temperature control	Uniform temperature	Difficult
Productivity	High	Low
Water losses (includes evaporation)	Depends on cooling design	High
Hydrodynamic stress on algae	Medium-low	Very low
Gas transfer control	High	Low
CO_2 losses	Depends on pH control system	Depends on pH control system
O_2 inhibition	Probable	Inexistent
Biomass concentration	High (approx. 2-8 g/L)	Low (approx. 0.1- 1 g/L)
Scale-up	Difficult	Difficult

As stated by Richmond [32], despite closed systems offer no advantage in terms of areal productivity (productivity per unit of ground area occupied by the reactor [14]), they largely surpass ponds in terms of volumetric productivity (productivity per unit reactor volume [14]), thus productivity can be 8 times higher in closed systems, and cell concentration can be about 16 times higher in closed systems. In conclusion, PBR and open ponds should not be viewed as competing, but complementing technologies.

1.6 Microalgae Nutrition

Before any explanation about microalgae nutrition, it is important to express two important designations: culture medium and nutritive medium; used in this thesis. The nutritive medium is a concentrated set of nutrients that supply the growth of the microalgal cells whereas the culture medium is the liquid medium where microalgae grow, and it is made with the goal of reproducing the conditions of their natural habitat. It contains all the minerals, clean/disinfected water, and nutrients from the nutritive medium. In nature, microalgae do not achieve a continuous and high productivity needed to guarantee the sustainability of a large-scale microalgal cultivation process. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the water [32], [36]. The composition of culture medium take an important role on the growth of microalgae since it is the only supply of elements that cells incorporate in their composition and allows them to grow [32]. The optimization of the recipe/composition of culture medium takes importance in the improvement of microalgal biotechnology since it could provide higher biomass yields, while costs in reagents are reduced and the feasibility of large-scale processes is improved [32], [33].

The ideal culture medium is an accurate mixture of elements and minerals with a particular and specific proportion that allows the continuous growth of microalgae with the correspondent and directly proportional uptake of nutrients [24]. This proportional recipe requires a large number of experimental tests until all nutrients are completely proportional to the growth rate of biomass, since the nutrient requirement is dependent to the metabolism demand of each microalgal species [46] and culture conditions are traditionally optimized by the one-at-a-time strategy, i.e. varying one factor while keeping all others constant [29]. On other hand, the presence and availability of elements in the culture medium is not synonymous of maximum yield of biomass. The excess of nutrient availability, or excess of concentration in the culture medium, carries a negative effect on microalgae; the nutrient becomes an inhibitory factor on the cellular growth when it exceeds a given range of concentration in the culture medium. When a nutrient is not available, the cellular growth could be also affected and may lead to culture collapse [24], [32], [36], [46].

Whereas the use of *Nannochloropsis* as aquaculture feed is a reality, its industrial exploitation as a source of EPA cannot yet compete with fish oil due to the high cost of *Nannochloropsis* biomass production. A considerable running cost in this process is represented by the preparation of the growth medium [34], thus an improvement in this part of the industrial process design should result in an important step towards viability of large-scale cultivation of *Nannochloropsis* to biodiesel production.

1.6.1 Macronutrients

Nitrogen, phosphate and phosphorous are three important macronutrients for growth and metabolism of algal cells. Nitrogen is a fundamental element for the formation of proteins and nucleic acids. Being an integral part of essential molecules such as ATP, the energy carrier in cells, phosphate is another very important nutrient. Phosphate is part of the backbone of DNA and RNA, which are essential macromolecules for all living cells. Phosphorous is also a key component of phospholipids. It is not unusual for algae to become nutrient-limited (i.e., nitrogen- and phosphorous-

limited) in the natural environment. Limitation of these key nutrients shifts the metabolic pathways of the organism. [24]

Nitrogen is an essential constituent of all structural and functional proteins in the algal cells and accounts for 7%–10% of cell dry weight [24], [32], 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, the energy carrier in cells [24]. Inorganic nitrogen taken up by algae is rapidly assimilated into biochemically active compounds and recycled within cells to meet changing physiological needs [24]. Major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids and triglycerides [18], [24], [32], [36], [47], [48] with a concomitant reduction in protein content. This, in turn, results in a higher lipid/protein ratio [24], [48] at the expense of growth rate [24], [32], [36], [48]. Therefore, attempts to increase lipid concentration via nitrogen limitation must be carefully evaluated to ensure high lipid productivity [18], [24], [35], [48]. As showed above, limitation of this nutrient shifts the metabolic pathway of the organism, thus it has to be always in an excess concentration in the culture medium [32].

Nitrogen is mostly supplied (and "favourite" source) as nitrate (NO_3), but often ammonium (NH_4^+), urea and free amino acids are also used, with similar growth rates recorded [18], [32], [36], [48]. Urea and free amino acids are an organic source of nitrogen that raises the possibility of contamination by other microorganisms [32]. Ammonium present in concentrations greater than 25 mM are often reported to be toxic for phytoplankton, so concentrations should be kept at a low level [36], [48].

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 (PO_4^{3-}) and its uptake is energy-dependent. Most microalgae are capable of producing cell surface phosphatases, which allow them to utilize this and other forms of organic phosphate as a source of phosphorous. When phosphorous source is limited, microalgal cells activate the same type of mechanisms as for nitrogen limitation, and its concentration in culture medium is also a growth-limiting factor [24], [32], [36].

1.6.2 Micronutrients

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. It contemplates minerals such as iron (Fe), magnesium (Mg), calcium (Ca) and some trace metals. These trace metals are zinc (Zn), copper (Cu), cobalt (Co), nickel (Ni), manganese (Mn), molybdenum (Mo), chromium (Cr) and vanadium (V) [32]. Trace metals are metals present in cells in extremely small quantities (<4 ppm) [24] 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 [46]. 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 [32], [36], [46], [49].

As the aqueous concentration of trace metals is not an indicator of the bioavailability of metals, trace metal availability to algae is highly dependent on speciation (free ion and chelated concentration). It is thought that molecules that complex with metals (chelators) influence the availability of these elements. Chelators act as trace metal buffers, maintaining constant concentrations of free ionic metal. It is the free ionic metal, not the chelated metal, which influences microalgae, either as a nutrient or as a toxin. Without proper chelation, some metals (such as Cu) are often present at toxic concentrations, and others (such as Fe) tend to precipitate and become unavailable to phytoplankton. In the natural seawaters there are some organic molecules that act as chelators (e.g. citrate), but the most widely used chelator in culture media additions is EDTA. This chelator has to be added according to specific proportions because high concentrations have occasionally been reported to be toxic for microalgae [32], [36], [46].

Some descriptions regarding mineral nutrition, their role and importance in microalgae cell system and are presented in the next table (Table 1.7).

Table 1.7 – Principal function in microalgal metabolism of the micro-nutrients.

Micronutrient	Biological role
Iron	• Nitrogen reduction; • Photosynthesis – Electron transportation; • Respiration; • Ribonucleotid reduction; • Metal ion transport [24], [36], [49].
Magnesium	• Photosynthesis – Structural element of chlorophyll [49]. • Aggregation of ribosomes [50] • Enzyme function – RNA polymerases, ATPases, protein kinases, phosphatases, glutathione synthase, and carboxylases [50].
Calcium	• Secretion; • Glycolysis and gluconeogenesis; • Ion transport; • Cell division and growth [49].
Zinc	• Hydrolases – Metabolism of sugars, proteins and nucleic acids; • Nucleic-acid polymerases and transcription factors; • Metallothionein (located in Golgi complex) – Structural element [49], [51].
Copper	• Similar functions to iron (e.g. ion transport) [24], [49].
Cobalt	• Structural element of vitamin B12 (possible supplement to the microalgal growth) [32], [49]; • Photosynthesis – electron transport chain [52].
Nickel	• Component of hydrolases and hydrogenases [49].
Manganese	• Involved in catalysis of oxygen evolution in the PSII complex (photosynthetic reaction center) [49], [51], [53]; • Involved in nitrogen reduction [54]; • Precursors for aromatic amino acids [54].

Table 1.7 – Principal function in microalgal metabolism of the micro-nutrients.

Micronutrient	Biological role
Molybdenum	• Nitrogen reduction; • Oxidation of aldehydes, purines and sulfite [49].
Chromium	• Functions unknown – it is known that both trace metals have influence in microalgal cell growth [24], [49], [55], [56].
Vanadium	
Strontium	

1.6.3 Natural Seawater and Artificial Seawater

The focus of this work is a marine microalga, *Nannochloropsis oceanica,* thus a seawater equivalent composition is needed to adjust the culture medium. Media for the culture of marine phytoplankton consist of a seawater base (natural or artificial) which may be supplemented by various substances essential for microalgal growth, including nutrients, trace metals and chelators and vitamins, soil. The salinity of the seawater base should first be checked (30–35% for marine phytoplankton), and any necessary adjustments (addition of fresh water/evaporation) made before addition of enrichments [36].

In the beginning of marine microalgae cultivation, when the work to understand the growth of microalgae began, the usage of natural seawater were preferred instead of artificial seawater [57], [58] This could be considered a good choice if large quantities are required (collecting cost has to be considered), and if a good source is really available, or if open ocean species are being cultured in the laboratory [46]. Natural seawater can be collected near shore, but its salinity and quality is often quite variable and unpredictable, particularly in temperate and Polar regions (due to anthropogenic pollution, toxic metabolites released by algal blooms in coastal waters). Artificial seawater, made by mixing various salts with deionized water, has the advantage of being entirely defined from the chemical point of view, but it is very laborious to prepare, and often does not support satisfactory algal growth. Trace contaminants in the salts used are at rather high concentrations in artificial seawater because a large quantity of salt must be added to achieve the salinity of full strength seawater [36]. The major advantage of artificial seawater to this work, in its usage for nutrient limitation studies, is that one can control the amount of the limiting nutrient because there is little or none in the artificial seawater, unlike natural seawater. Similarly, one can precisely control the nutrient ratios. All these facts make the option of artificial seawater the most attractive [46].

Several artificial media have been developed over the last 90 years; enriched seawater, artificial water (ESAW) is among the more popular recipes. ESAW has the advantage of an ionic balance that is somewhat closer to that of normal seawater. The original paper compared the growth of 83 strains of microalgae in natural seawater (ESNW) versus ESAW and determined that 23% grew more poorly in the artificial water. Since 1980, however, the composition of ESAW, as used by the original authors, has changed considerably. Recent tests where eight species that had been shown to grow better in ESNW than in ESAW were selected and compared again, using the currently used recipe. For all but one species, growth rate and final yield was no different between the media but in

one case was slightly higher in ESAW and no differences in cell morphology or volume were found in any case [59]; which makes the artificial seawater a reliable choice to apply in this work.

1.6.4 Marine Microalgae Nutritive Medium

It is hard to compare the extended number of recipes for marine microalgae nutritive medium available in the literature. Many modifications result from a desire to increase the flexibility of a medium (i.e., creating multiple nutrient stocks so that individual macronutrient and micronutrient concentrations may be manipulated) or to reduce the number of stock solutions (such as macronutrients, micronutrients and vitamins solutions) necessary in cases where various different media are being used in a single laboratory. In many cases, minor modifications to the original recipes have been made (e.g., changing a nitrogen source or adding a single trace metal) and an entirely new name has been given to the medium with this minor modification. In other cases, fairly extensive modifications have been made, yet a medium name has been retained or simply designated as modified.

It is not possible to find a pattern in the different nutritive medium due to the different goal and utilization of the extended and different modifications suffered by the nutritive medium (e.g. typically, in the case of media intended for aquaculture, macronutrients are in great excess in comparison with natural concentrations). There are substantial differences in the different counter-ion used to deliver the nutrient to the culture medium (e.g. nitrate is present in nutritive medium recipe linked to different monovalent cations, e.g. potassium and sodium). Iron is used in different forms too; it could be present in the nutritive medium as a divalent cation (Fe^{2+}) or as trivalent cation (Fe^{3+}). The most widely used culture medium (which contains the nutrients) for marine microalgae photoautotrophic cultivation is f/2 and ESAW (Table 1.1) and they have not only evolved much since their recipes were published [32], [36], [46], [59].

Table 1.8 - Recipes of the most used nutritive medium recipes for marine microalgae cultivation. Concentration values adjusted proportionally to 6 mM of nitrate concentration. Adapted from [46].

	Nutrient	Compound	f/2	ESAW
Macronutrients	Nitrogen	NaNO3 (mM)	6,000	6,000
	Phosphorous	$NaH_2PO_4.H_2O$ (mM)	0,246	0,262
	Silicon	$Na_2SiO_3.9H_2O$ (mM)	0,721	1,158
Micronutrients	Iron	$FeCl_3.6H_2O$ (mM)	0,116	0,072
	Copper	$CuSO_4.5H_2O$ (µM)	0,267	0,000
	Zinc	$ZnSO_4.7H_2O$ (µM)	0,520	0,278
	Cobalt	$CoCl_2.7H_2O$ (µM)	0,286	0,622
	Manganese	$MnCl_2.4H_2O$ (µM)	6,190	26,448
	Molybdenum	$Na_2MoO_4.2H_2O$ (nM)	17,687	66,885
	Nickel	$NiCl_2.6H_2O$ (nM)	0,000	68,525
	Selenium	Na_2SeO_3 (nM)	0,000	10,929
	Chelator	Na_2EDTA (mM)	0,116	0,162
Vitamins	Vitamin B1	Thiamine (µM)	2,014	3,235
	Vitamin H	Biotin (nM)	13,946	44,699
	Vitamin B12	Cyanocobalamin (nM)	2,510	16,175

1.7 The Recirculation Strategy

The recirculation strategy is, basically, a simultaneous process of dilution and recycling of culture medium with extraction/harvesting of the biomass. It has been showed that at a specific dilution rate the biomass productivity increases [18], [60], [61], hence the recycling of culture medium has some interest for microalgae commercial/large-scale production as it cuts costs and prevents environmental contamination [60], [61] so, theoretically, the recirculation of culture medium provides a win-win situation for microalgae large-scale production and it is a pass towards the pursuit of an ideal and sustainable microalgae production process. 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 labor, 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 [60], [61]. Therefore, recirculation strategy allows the decreasing of water-footprint of microalgal production process and at long-term this strategy allows saving considerable production costs.

The next figures (Fig. 1.6 and Fig. 1.7) show the flow sheets of a microalgae cultivation process with recirculation and without recirculation of the culture medium (commonly named as renewal), respectively.

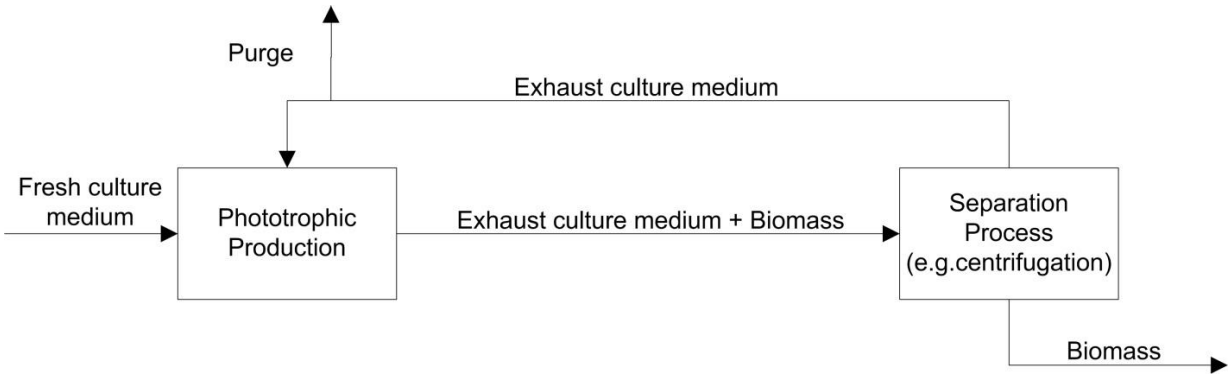

Fig. 1.6 - Representation of microalgal production process operating under recirculation strategy. Arrows represent the materials flux and the boxes represent industrial processes.

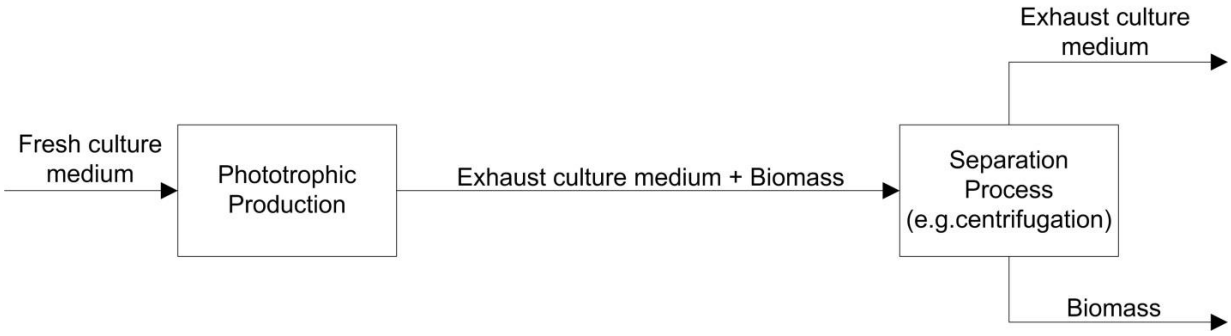

Fig. 1.7 – Representation of microalgal production process operating under renewal strategy (without recirculation of culture medium). Arrows represent the materials flux and the boxes represent industrial processes.

Unfortunately the real impact of recirculation strategy of microalgae cultivation does not yet coincide with theoretical expectations and the production of microalgae at large-scale with medium recycling has not yet been implemented extensively. The life-cycle impacts of large-scale microalgae productions are being debated; specially the impact on water usage, i.e., the water consumption per hectare of land used for algal feedstock production but the life-cycle of water when using seawater and recycling the harvested water to produce microalgae in aquaculture has not been established yet [60], [61]. There are some issues in this strategy of cultivation, mainly due to scale-up problems. With the scale-up to industrial cultivation the number of variables rises and it is difficult to control, manipulate and adjust all the cultivation parameters. The major bottleneck of the recirculation strategy is the accumulation of inhibitory factors for microalgal growth, which lead to a decrease on cell growth rate and possible to culture loss [34]. One of the possible explanations of this phenomenon is the accumulation of non-consumed nutrients to levels of concentration that may inhibit the microalgal growth. This is lack of a properly culture and nutritive medium optimization and their nonspecific character to the microalgae species [24], [32], [34], [57], [58].

On the other hand, not only the nutrients accumulation is responsible to loss of productivity in cultures under medium recycling cultivation; there are other phenomena, such as production of auto-inhibitory compounds in high cell density cultures, mainly secondary metabolites which need to be continuously removed from the culture medium to obtain maximum efficiency of light conversion. It is also stated that cellular debris accumulation, more specifically, the plasmatic membrane released to culture medium after cellular lysis, may induce the aggregate formation and trapping microalgal cells inside which may lead to culture productivity loss and possibly culminate in the culture loss. The proliferation of contaminants, such as fungi, protozoa and bacteria, is also stated as a major growth limiting factor for cultures under medium recycling cultivation strategy. Some of the contaminants may be nutrient competitors to microalgae (e. g. some species of bacteria) and other may be microalgal predators (e.g. some species of fungi and protozoa) [24], [32], [34], [57], [58].

Different microalgal species is equivalent to diverse metabolism which is equivalent to distinctive nutrient needs. In spite of the great efforts and many researches done along the past 100 years there is much more work to do and further advances in the microalgae nutrition to be made [24], [32], [34], [57], [58]..

2 Framework and goals

This work aimed to optimize the industrial cultivation of the microalgae *Nannochloropsis oceanica* with focus in their potential application as source of biodiesel production due to their capacity to synthetize and accumulate a great proportion in weight of fatty acids. The work was developed in the industrial facilities of A4F-Algafuel, the coordinator of the BIOfuel From Algae Technologies (BIOFAT) project.

BIOFAT is a microalgae-to-biofuel European Commission's Seventh Framework Program (FP7) demonstration project that integrates the entire value chain of algae process from optimized growth, starch and oil accumulation, to downstream processing (biorefinery) including biofuel production. The BIOFAT approach integrates and scales up complementary technologies and skills from each partner into a global multidisciplinary project and aims to demonstrate that biofuels made from microalgae can offer energy efficiency, economic viability and environmental sustainability.

The first phase of the project contemplates the process optimization in two pilot scale facilities, each one-half hectare in size, located in Italy and Portugal. The two pilots represent a strategic added value for the following industrial phase, and they will enable a full feasibility study of algae biofuels in the EU.

With the strain selected, (*N. oceanica*) in accordance with environmental conditions and sustainability criteria this phase of the project and was developed under two major scientific and technological emerging challenges [62]:

- Optimization of the culture conditions, adoption of strategies to limit contaminations and abiotic stresses , allowing high oil/starch accumulation:
- Technology integration and optimization, combining photobioreactors (Green Wall Panels and tubular reactors) and raceway ponds, as well as automated harvesting.

These biotechnological challenges led to the two major goals of this thesis work, the improvement of the *N. oceanica* culture medium and the optimization of the recirculation strategy. Regarding to this great challenge, this work aimed to a set of minor but not less important goals:

- Optimize the culture medium recipe for improved productivity;
- Optimize the culture medium recipe for medium recycling;
- Optimize the recirculation strategy;
- Analyse and evaluate the media recipes impact in lipid production;
- Evaluate the substitution of sodium chloride supply impact in productivity;
- Development of complementary operational procedures.

This thesis work is divided in three main parts. The first one refers to literature review which support and serve as framework to the work developed. The first part of the work also contemplates

the material and methods needed to handle and understand the test essays. In the first place, was made a primary approach to the technology and methods developed by A4F – Algafuel S.A. (initially in the Lisbon Laboratory and afterwards in the A4F's industrial facilities in Pataias). With this theoretical and practical background, it was possible to develop proper adaptations to the available technologies, conditions and cultivation systems in order to reproduce the industrial environment and stimuli in the microalgal cultivation. This background also served as basis and impetus for essay design.

The second phase of this work consists in the development and analysis of the practical essays that took place during the curricular internship. The last part of the work includes the major conclusions, suggestions and future perspectives for the continuous challenge of developing a more economic and environmentally sustainable microalgal production process.

3 Materials and Methods

3.1 Reagents

Table 3.1 – List of reagents used in this work and their respective supplier.

Use	Reagent	Supplier
Artificial Seawater Recipe	Sodium chloride 99,0% (w/w)	CFL
	Mineral solution A	Necton S.A.
	Purified sea salt	SaliSal
	Calcium chloride 95,0% (w/w)	Panreac
Nutritive Medium Recipe	Sodium nitrate 97,0% (w/w)	Synthesia
	Copper sulphate pentahydrate 98,0%(w/w)	VWR
	Disodium EDTA 98,0% (w/w)	
	Sodium molybdate dihydrate 95,5% (w/w)	
	Manganese chloride tetrahydrate 98,0% (w/w)	Scharlau
	Ferric chloride (III) hexahydrate 97,0% (w/w)	
	Potassium dihydrogen phosphate 98,0% (w/w)	Panreac
	Zinc sulphate heptahydrate 98,0% (w/w)	
	Strontium chloride dehydrate 99,0% (w/w)	Chem-Lab NV
	Mineral solution B	Hubel
Lipid Quantification Method	Sodium hydroxide	Eka
	Acetic acid glacial	Carlo Erba
	Triethanolamine	Scharlau
	Methanol	
	Chloroform	
	Phosphate buffer solution	Calbiochem
	Copper (II) nitrate hemi(pentahydrate)	Acros organics
Disinfection Process	Sodium hypochlorite 13% (v/v)	Quicel
	Hydrochloric acid 37% (v/v)	Quicel
	Sodium thiosulfate 97% (w/w)	VWR

3.2 Equipment

Table 3.2 - Presentation of the equipment used and their respective model and manufacturer.

Equipment	Model – Manufacturer
Autoclave	Uniclave 88 – AJC
Precision Balance (± 0,2 g)	NV 4101 - Ohaus
Centrifuge	Z400 k – Hermle
Spectrophotometer UV – Vis (± 0,005 A)	Genesys 10S UV-Vis – Thermo Scientific
Portable conductivity and pH-meter	HI 98130 – Hanna Combo
Optical Microscope	BA 400 – Motic
Portable Refractometer	K71904 – Optech
Portable Optical Density Meter	CO 8000 – WPA

3.3 Biological Material

The selected microalgae for this work, was *Nannochloropsis oceanica* (Suda & Miyashita, 2002). 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 inoculum culture was maintained in stationary growth phase in an A4F's artificial seawater medium (ASAM_2) and complemented with the nutritive complement medium (CNASAM_2).

3.4 Cultivation System

The cultivation systems used in this work, as the name indicates, were lab-scale PBRs (Fig 1.8), and were developed 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) allowing the photons to pass through the wall. The uniform mixing was established through a piping in the bottom of the reactor which was the air supply to the system. The bottom location of the air piping prevents the biomass deposition and accumulation and provides a uniform and controlled mixing rate to all parts of the reactor with air bubbles (see section 1.4.5). The air supply was enriched, previously to the entry of the air in the system, with carbon dioxide which allowed the real-time pH control (see section 1.4.3), with the proportion of CO_2 in the air supply being controlled by a valve system. The air intake flow was also monitored and adjusted in order to provide a uniform and efficient mixing and guaranty, in a parallel form, a non-excessive shear stress to microalgal cells.

The top of the system was covered by a removable PVC piece (Fig. 3.1), acting like a barrier to outdoor environment and preventing biological contaminations; and allowing the gas flux through a chimney with autoclaved cotton. The cotton acted like a filter and was changed whenever necessary. The removal of this piece allowed additions to the cultivation system. In the bottom end of the lab-scale PBRs there was a valve system which allowed the culture collection operations.

The temperature of the system was monitored by taking specific and programmed samples of the culture and the temperature control was achieved through a thermoregulation system by water spraying. With the temperature rising there was a constant water flux (controlled by a valve system) to each reactor wall, cooling them down very quickly.

The photoinhibition phenomenon (see section 1.4.1) was predicted and prevented with the use of a very dense net (shade net) which covered part of the transparent wall of the reactor. This action took place only when diluted cultures were in production in order to avoid the excess of photons incidence in microalgal cells. This procedure took place only when necessary due to the limitation impact in the cellular growth.

All the systems of cultivation were chemically disinfected with a muriatic acid solution and with a sodium hypochlorite solution and subsequently neutralized to minimize the risk of biological and chemical contamination and guaranty the results reproducibility.

In the first part of the internship it was verified that all available lab-scale PBRs were in need of several maintenance activities. After replacement of all consumable parts and even building some additional units, it was possible to have a full working set of 14 PBRs. Furthermore, maintenance to the aeration and thermoregulation systems was also performed.

Throughout this work it was also developed the manual of operational procedures for cultivations in this system of cultivation (Appendix 7.4).

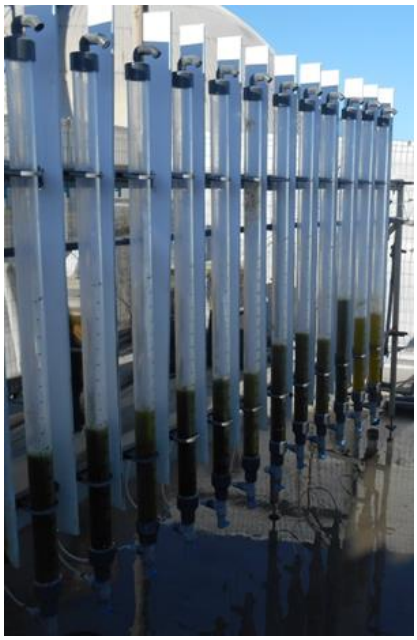

Fig. 3.1– Laboratory-scale photobioreactors used in this work; the lab-scale PBRs were in production conditions.

3.5 Solutions

In order to achieve a detailed monitoring of microalgal cultures there were use three different solutions with various purposes. The process water (PW) was the industrial facility source of water for all cultivations. This water originates from a borehole and is submitted to pre-filtering, disinfection with hypochlorite and finally line filtering down to 1 micron before being fed to cultivation systems. Neutralization of excess free chlorine with thiosulfate may be required. The referred solutions are presented in the next table (Table 3.3).

Table 3.3 – Solutions used in the monitoring analyses of the culture and disinfection processes.

Solution	Preparation	Purpose
Hydrochloric Acid – 1 M	Dilution of hydrochloridric Acid 37% (v/v) in demineralized water (DMW)	Nitrate ion concentration determination analysis
Sodium Chloride – 30 g/L	Sodium chloride salt was dissolved in DMW to achieve the referred concentration	All analyses for which a direct dilution of the culture was needed
Sodium hypochlorite – 50 ppm	Dilution of sodium hypochlorite 13% (v/v) in the respective liquid phase	Disinfection (cultivation system parts and culture medium storage)
Hydrochloric Acid – 1 M	Dilution of hydrochloridric acid 37% (v/v) in process water (PW)	Disinfection of cultivation system parts
Sodium Thiosulfate – 150 g/L	Sodium thiosulfate salt was diluted in PW to achieve the referred concentration and autoclaved	Neutralization of solutions with sodium hypochlorite

3.6 Culture Medium Formulation

The culture medium recipe (Table 3.4) is intended for marine microalgal cultivation and is the result of an optimization work by A4F – Algafuel. In this work two culture medium recipes were tested. The difference between them resides only in the sodium chloride source, one is intended for laboratory use (ASAM_2) which is a costly but very reliable option, and the other source is from an industrial supply (ASAM_2.1) which allows a significant cost reduction in the microalgal cultivation process.

The culture media were prepared by dissolving the salts and adding the mineral solution A (MS_A) into process water (PW) in two lots of 60 L each, according to the information provided by the "Operational Procedure for Artificial Saltwater Preparation", property of A4F – Algafuel. The MS_A is a very specific solution (source is non-publicly available) and very rich source of minerals and nutrients. In this solution there are some major nutrients such as magnesium. After the component adding, the salinity was adjusted to a final concentration of 30 g/L with PW. Despite the various recommendations for microalgal cultivation salinity, this level of salinity was defined by the BIOFAT project consortium for the cultivation of this strain.

The culture media were maintained disinfected with a sodium hypochlorite 50 ppm concentration. Before usage, the culture media were neutralized with sodium thiosulfate solution (150 g/L) and autoclaved at 121 ºC for 40 minutes.

Table 3.4 – Culture medium recipe.

Reagent	Final concentration
NaCl	431 mM
$CaCl_2$	9,97 mM
MS_A	15 mL/(L of culture medium solution)

3.7 Nutritive Medium Formulation

Three different nutritive media were tested in this work, CNASAM_2, CNASAM_3 and BIOFAT_2. CNASAM_2 was the medium used to maintain the *N.oceanica* cultures isolated and provide inoculum to larger-scale production. This medium is a result of previous research by A4F – Algafuel and was used as nutritive complement of ASAM_2 culture medium. 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. The only difference between them is the usage of different salts in the preparation due to large-scale problems of affordable raw materials, homogenization and precipitate formation. This nutritive medium is complemented the ASAM_2.1 culture medium in order to reproduce the large-scale microalgal production conditions implemented in the industrial facilities of A4F – Algafuel at the time of this work

and test the influence in the microalgal growth of the replacement of laboratorial sodium chloride by an industrial supply of purified sea salt.

CNASAM_3 is also a previous result of optimization work done by A4F – Algafuel. This medium recipe had never been tested in industrial scale cultures of *N. oceanica* and it was expected to provide a positive influence in the microalgal growth. The influence of these three nutritive media in *N. oceanica* cultures under industrial conditions was one of the major questions to be answered within this work.

Vitamins are referred in literature as significant push factor for improved microalgal cultures productivity in many cases. Despite the high productivity results achieved in laboratory-scale tests, when the scale-up is done the whole scenario changes. The vitamin consumption rises significantly which compromises the economic viability of production process. The high vitamin consumption by a large-scale culture is not affordable in comparison with the productivity difference achieved by cultures with vitamin supply [24], [36], [46], [56]. Regarding the goals of this work, to simulate the industrial cultivation conditions, vitamin supply was not performed.

3.7.1 CNASAM_2

The preparation of this nutritive medium was done according to the protocol of nutritive medium preparation, property of A4F – Algafuel. It has been prepared in 1 L lots and stored after sterilization in autoclave for 40 minutes at 121ºC. As previously explained this medium is a result of A4F – Algafuel nutrition research, and its composition is based in the previously stated f/2 medium (see Section 1.6.4 – Table 1.8). In order to clarify this recipe and protect the intellectual property of A4F – Algafuel it will be presented the difference between the CNASAM_2 and f/2 medium through ranges of percentage variance (Table 3.5).

Table 3.5 – Comparison between the concentration variance of f/2 and CNASAM_2 nutritive medium.

Element	Δ (%)
Phosphorous	+ [5 to 70]
Iron	- [30 to 99]
Zinc	- [15 to 90]
Manganese	- [15 to 90]
Molybdenum	+ [15 to 180]
Copper	- [15 to 90]
Cobalt	- [15 to 90]
Silicon	- [80 to 100]

3.7.2 CNASAM_3

This nutritive medium was prepared through an adaptation process of the protocol of nutritive medium preparation, property of A4F – Algafuel. It has been prepared in 1 L lots and stored after sterilization in autoclave for 40 minutes at 121ºC. This nutritive medium is an improvement of CNASAM_2 and reflects the needs and excesses verified in *N. oceanica* cultures in laboratorial

cultures. As previously stated, this medium has never been tested in actual cultures, however it is expected that it should result in higher productivity. The modifications made are presented in Table 3.6 and are expressed in terms of percentage variance between CNASAM_2 and CNASAM_3 theoretical concentration.

Table 3.6 – Comparison between the concentration variance of CNASAM_2 and CNASAM_3 nutritive medium.

Element	Δ (%)
Phosphorous	0
Iron	+ 405
Zinc	- 75
Manganese	0
Molybdenum	0
Copper	- 100
Cobalt	- 100
Silicon	0

3.7.3 BIOFAT_2

This nutritive medium, as referred above, is an outsourced industrial made CNASAM_2 medium which has not suffered any modification or preparation after the supplier delivery. It has been stored in 1 L lots after sterilization in autoclave for 40 minutes at 121ºC.

3.8 Methods and Operational Procedures

3.8.1 Inoculation

Within this work, the inoculation is the act of transfer the culture from the cultivation chamber, in the laboratory facilities to the lab-scale PBRs, located outdoors, where microalgae will grow and be tested. From a scientific point of view, this procedure consists in the initial set-point of growth conditions where all conditions must be identical in order to guarantee the reliability of the results.

The inoculum culture was maintained in the controlled environment of the cultivation chamber, inside the laboratory, in flasks with 5 or 6 L of capacity, at a temperature of 20-30 ºC, with direct aeration system with CO2 enrichment (similar to the system described in section 3.4) and with continuous artificial illumination. Periodical renewals of the cultures prevented them to enter in the stationary growth phase. The cultures grew on ASAM_2 culture medium and have been complemented with the CNASAM_2 nutritive medium. The total volume of inoculum was previously homogenized before inoculation.

The great challenge of inoculation operation is the maintenance of the aseptic environment that microalgal cells grew during the transfer to the outdoor reactors. To provide those conditions, the

lab-scale-PBRs were previously disinfected and the respective air flow rate was also adjusted. The biological material was transported in sterilized *Schott* flasks.

In the beginning of each assay, all the reactors were inoculated with direct transfer of laboratory culture, i.e. the inoculated volume corresponded to the total volume of culture during the tests. The differentiated growth conditions started to be applied after inoculation and the wash-out of the previous culture medium was guaranteed by periodical renewal operations in the first part of the assays.

3.8.2 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). In the test assays this procedure was done daily and with a constant renewal rate (rate of volume removed from the culture system – RR) of 30% (v/v).

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 direct recirculation of the culture medium after biomass removal (i.e. with exhaust culture medium recycling), and with recirculation of disinfected culture medium after biomass removal (i.e. with previous treatment of recycled culture medium).

3.8.2.1 Renewal without recirculation – daily procedure

1. The aeration system was turned off and the culture volume was registered. A sample of each culture was taken (20 mL) for culture parameters analyses.
2. The remaining volume of the rate of renewal was removed and the aeration system turned on.
3. There were determined the evaporated water volume, and done the needed analysis (e.g. determination of nitrate ion concentration, determination of optical density of cultures)
4. The volume handling procedures took place in the aseptic environment of cultivation lab-chamber bench (properly disinfected with ethanol at 96%, and close to the burner flame), and all of the volumes added to a *Florence* flask (properly identified, sterilized and capped) in accordance with a specific order.
5. The fresh culture medium (sterilized) volume correspondent to the renewal rate was added.
6. The evaporated volume was replaced by PW (sterilized) correspondent addition.
7. The nutritive medium (sterilized) was added in accordance to the intended nitrate ion concentration.
8. All of the *Florence* flasks were transported to near of the lab-scale PBRs.
9. The exterior of reactor chimneys and top of reactor walls were sprayed with a 0,04% (v/v) sodium hypochlorite solution before being carefully opened for addition of the flasks content.
10. If it was necessary the thermoregulation system was turned back on.

3.8.2.2 Renewal with direct recirculation – daily procedure

For this daily procedure, it was followed the previously described operational steps for renewal without recirculation (Section 3.8.2.1) from 1 to 4.

5. The culture medium (in this procedure, collected carefully to special plastic flasks) was centrifuged by 17 minutes at 3500 rpm in the laboratory centrifuge Hermle Z400 K, to separate the biomass.
6. The fresh culture medium (sterilized) volume correspondent to 10% of the renewal rate was added.
7. The evaporated volume was replaced by PW (sterilized) correspondent addition.
8. The exhaust culture medium volume correspondent to 90% of the renewal rate was added.
9. The nutritive medium (sterilized) was added in accordance to the intended nitrate ion concentration.
10. All of the *Florence* flasks were transported to near of the lab-scale PBRs.
11. The exterior of reactor chimneys and top of reactor walls were sprayed with a 0,04% (v/v) sodium hypochlorite solution before being carefully opened for addition of the flasks content.

3.8.2.3 Renewal with recirculation of disinfected medium – daily procedure

As previously stated for the renewal with direct recirculation, this operational procedure also followed the previously described steps of renewal without recirculation from 1 to 4 (see Section 3.8.2.1).

5. The culture medium (in this procedure, collected carefully to special plastic flasks) was centrifuged by 17 minutes at 3500 rpm in the laboratory centrifuge Hermle Z400 K, to separate the biomass.
6. The exhaust culture medium volume correspondent to 90% of the renewal rate was added.
7. There was added 17 µL of sodium hypochlorite 13% (v/v) (correspondent to 10 ppm of total concentration).
8. After 2 hours of disinfection, the exhaust culture medium was neutralized by addition of 39 µL of thiosulfate solution at 150 g/L.
9. The fresh culture medium volume (sterilized) correspondent to 10% of the renewal rate was added.
10. The evaporated volume was replaced by PW (sterilized) correspondent addition.
11. The nutritive medium (sterilized) was added in accordance to the intended nitrate ion concentration.
12. All of the *Florence* flasks were transported to near of the lab-scale PBRs.
13. The exterior of reactor chimneys and top of reactor walls were sprayed with a 0,04% (v/v) sodium hypochlorite solution before being carefully opened for addition of the flasks content.

3.8.3 Harvesting of culture medium for analysis

This operational procedure was applied when it was necessary to harvest culture medium for external analysis. The culture medium harvesting took place in three different phases of each essay: in the inoculation phase, in the day of changing the daily regime of renewal (e.g. from renewal without recirculation to renewal with recirculation), and in the end day of each essay (i.e. samples from the beginning, the mid and the end of the essay).

During the inoculation phase, the inoculum excess was transferred to specific plastic flasks. These flasks were directly centrifuged in the Hermle Z400 k by 17 minutes at 3500 rpm to separate the biomass. Afterwards the culture medium was carefully transferred to a sample plastic flask (properly identified) in order to prevent the biomass mixing. The culture medium was stored at -18 ºC and sent for external analysis.

In the changing of daily regime of renewal phase of the essays and in end of the essay, the renewal rate was removed from the cultivation system and the same procedure was followed.

3.8.4 Biomass collecting for analysis

This operational procedure was applied when it was necessary to harvest the biomass for complementary analysis, the lipid content. The biomass harvesting took place in the same three different phases of the first essay: in the begging, the mid and the end of the essay. After centrifugation and culture medium harvest, there was only the biomass left (in the centrifuge specific flasks). The biomass were re-suspended in NaCl solution (30 g/L) and transferred to properly identified *falcon* tubes of 50 mL. The biomass were centrifuged and re-suspended another two times (total of 3 cycles of centrifugation - washing) in order to provide a complete washing of salts present in the biomass. Afterwards the biomass was stored in a chest-freezer at -15 ºC until be analysed.

3.9 Analytical methods

3.9.1 Determination of culture concentration

3.9.1.1 Determination of Optical Density (OD)

The culture concentration analysis has been done by measurement of optical density of culture samples at wavelength of 600 nm (with the portable OD meter WPA CO 8000). The optical density values had to be lower than 1, for the linearity between optical density and cellular density be respected and reliable results could be achieved. Therefore, the samples with values higher than 1 were diluted with a 1:15 or 1:30 ratio using a NaCl solution (30 g/L). The OD values were determined daily (except weekends) during this work.

3.9.1.2 Calibration curve of cellular concentration

The optical density of *N. oceanica* cultures is correlated to cellular concentration and also to dry weight (DW) in previous research work done by A4F – Algafuel. The DW refers to weigh of biomass (after being completely dried) per liter of culture (presented in this work as g $_{D.W.}$/L. The calibration curve (Appendix 7.2) was obtained from a various number of analyses and different measurement methods with production cultures with an OD range between 5 and 30.

3.9.1.3 Culture productivity

The daily culture productivity (Equation 1) was obtained through the multiplication of the renewal rate (RR) by the daily culture concentration (C) and is presented as g $_{D.W.}$/(L . day).

$$Prod\ (g\ dw/L.day)\ = RR\ \times C\ (g\ dw/L) \quad (1)$$

3.9.1.4 Interpolation of culture concentration

This calculation (Equation 2) was needed to obtain the culture concentration when the OD analysis could not be done.

$$C_x = C_{x-1} + \frac{C_B - C_A}{N} \quad (2)$$

The x represents the day when culture concentration data is missing; x-1 represents the previous day of the missing culture concentration data; N represents the number of days with culture concentration data missing; A and B represent the lower and upper limits of the data gap, respectively.

3.9.2 Determination of nitrate ion concentration

3.9.2.1 Determination of nitrate ion concentration

The concentration of nitrate ion was determined through an ultraviolet absorption spectrometry method which was an adaptation from [63]. The culture sample was centrifuged and diluted (in NaCl 30 g/L solution) in order to achieve absorbance values (at wavelength of 220 nm) lower than 1 and the linearity between nitrate ion concentration and absorbance be respected. The cultures were diluted in an acidic environment, with the hydrochloric acid 1 M solution addition to achieve a 3% (v/v) proportion. The absorbance of this preparation was measured at 220 and 275 nm of wavelength. The nitrate concentration was correlated to absorbance by calibration curve (Appendix 7.3) which allows obtaining results as ion concentration. The calibration curve was achieved by previous work of A4F – Algafuel, and was done with KNO_3 standard solutions diluted with NaCl 30 g/L solution, also in an acidic environment.

3.9.2.2 Determination of nitrate ion consumption

The determination of nitrate consumption at day n (NCC_n) (Equation 3) is based in the difference between the nitrate concentration adjustment made in the day before of n day (NCA_{n-1}) and the nitrate concentration measured in the day n (NC_n).

$$NCC_n(mM/day) = NCA_{n-1}\ (mM) - NC_n\ (mM) \quad (3)$$

3.9.2.3 Interpolation of nitrate ion consumption

This calculus (Equation 4) was needed to obtain the nitrate ion consumption in an interval of days (N) when the nitrate concentration measurement wasn´t done (NCC_x). The interruption interval is lower limited by the A day and upper limited by the B day. It was assumed that the nitrate consumption remained constant during this interval.

$$NCC_x\ (mM/day) = \frac{NCA_A(mM) + NCA\ (mM) - NC_B\ (mM)}{N} \quad (4)$$

The NCA_A represents the nitrate concentration adjustment made in the day before of the interruption (A day); the NCA represents the concentration added during the days of the interval, and the NC_B represents the nitrate concentration measured in the day B.

3.9.3 Control of cultures salinity

The salinity was measured by a portable refractometer Optech K71904 which allowed the analysis of the light refraction index of a culture sample after centrifugation. If the salinity reached values higher than 39 g/L, PW was added to the culture. On the other hand, if the salinity was below 27 g/L, the addition of PW volume was replaced by addition of culture medium volume.

3.9.4 Microscopic control of cultures

Build-up in the number of foreign organisms in the culture should be regarded as a warning signal, usually indicating that the cultured species have most probably come under stress. Regarding to this fact, the microscopic control of the cultures was done to search for possible contaminants and monitor the cells welfare. One drop of each culture sample was observed under the optical microscope Motic BA 400 in two distinct phases of observation. First the culture was observed through the lower capacity objectives (5x, 10x and 20x) in order to search for contaminants of great proportions (like protozoa and fungi), the presence of microalgal cells in agglomerates, and possible microscopic debris. Afterwards, the density of pigmentation in the microalgal cells, and the shape of their cytoplasmic membrane were observed (low density of pigments and cytoplasmic membrane with irregular shape are indicators of cell stress) in the higher capacity microscopic objective (40x). It was also possible to search for contaminants of lower proportions, such as bacteria and other microalgae through the microscopic observation under this objective. All magnifications should consider an additional 10x scale due to the ocular piece of the microscope.

3.9.5 Control pH and temperature of cultures

The tight control of pH and temperature of cultures was very important due to their influence in microalgal growth. The pH and temperature of cultures was monitored three times per day (except weekends) through culture sample analysis. The pH and temperature of the recently taken culture samples were analysed with the portable pH meter Hanna Combo HI 98130. If the temperature was higher than 25ºC the thermoregulation system was manually activated. As referred in the section 1.4.3, the ideal pH range for microalgal cultivation is between 7,0 and 9,0 values, and if the pH value is above that range, the CO_2 supply valve was slightly opened. Therefore, if the pH value of the cultures was below the ideal range, the CO_2 supply valve was slightly closed.

3.9.6 Elemental Analysis

The elemental analyses were done by an external supplier. This laboratory analysed the samples through the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique. It allows the determination of metallic and non-metallic elements concentration down to 10^{-3} ppm.

3.9.7 Lipid quantification method

The lipid content of microalgal biomass was quantified through UV-visible spectrometry after saponification. The method used was adapted from [64] method by A4F – Algafuel in previous work. The lipids in microalgae were quantified by measuring the fatty acids. After saponification and fatty acids extraction with an organic solvent, the fatty acids reacted with triethanolamine–copper salts. Afterwards, the ternary complex (triethanolamine–copper-fatty acids) was detected at 260 nm using a UV–visible spectrometer. This method is very sensitive since it allows the analysis of low levels of lipids in the range of nanomoles from as little as 1 mL of culture sample.

More adaptations to this method were developed in this work. The adaptations were done to minimize the measurement process time and to simplify the procedures protocol in order to become a regular and routine analysis integrated in the production process. A protocol was also developed (Appendix 7.5) but it couldn´t be validated during the work time; the validation process was done by A4F – Algafuel after the end of this work.

3.9.8 Calculation of percentage variance

The percentage variance (Δ (%)) between the value in analysis (*X*) and the reference value (X_{ref}) was calculated through the following equation (Equation 5):

$$\Delta(\%) = \frac{X - X_{ref}}{X_{ref}} \quad (5)$$

3.9.9 Calculus of mean and standard deviation

The mean of a sample of values ($\overline{X}$), is determined through the Equation 6. The value of each element of the sample was represented by x_i, and the number of elements of the sample is represented by N.

$$\bar{X} = \frac{\sum_{i=0}^{n} x_i}{N} \quad (6)$$

The standard deviation (SD) measures the amount of dispersion from the average and was determined through the Equation 7. The value of each element of the sample was represented by x_i, the mean was represented by $\overline{X}$, and N represent the number of elements present in the sample.

$$SD = \sqrt{\frac{\sum_{i=0}^{n}(x_i - \overline{X})^2}{(N-1)}} \quad (7)$$

4 Results

The experimental phase can be divided in two distinct assays that provided the information needed to achieve the goals of this work. In the first assay was possible to conclude about the best choice of nutritive medium for *N.oceanica* cultivation through the analysis of different cultivation strategies and nutritive media (Section 3.7) growth impact. The second assay had focus in the recirculation strategy testing the impact of different supernatant treatment strategies in microalgal cultivation. Additionally, with the gathered data, there was possible to evaluate the impact of different supply of sodium chloride and formulate another assay (Assay 3 – Section 4).

4.1 Assay 1 – Analysis of nutritive media impact in industrial *Nannochloropsis oceanica* cultivation

4.1.1 Cultivation conditions

This assay was developed from 19th May 2014 to 8th July 2014 in the industrial facilities of A4F – Algafuel, in Pataias. 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 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. In the daily renewal phase the culture were renewed (with fresh medium) in a renewal rate of 30% (v/v), which aimed to prevent the washout, improve the productivity of the cultures and simulate the industrial harvesting process. After two weeks on daily renewal regime the cultivation switched to daily renewal with recirculation of culture medium. The renewal rate remained constant in the recirculation phase but occurred through a different strategy (with medium recycling). The addition of fresh medium only represented 10% (v/v) of the renewed volume and the remaining volume was filled by the exhaust culture medium (after biomass collecting).

The set of cultivation conditions is represented in the Table 4.1.

Table 4.1 - Cultivation conditions for assay 1.

Condition	1			2			3		
Reactor	RE_02	RE_05	RE_08	RE_03	RE_06	RE_09	RE_04	RE_07	RE_10
Nutritive Medium	CNASAM_2			CNASAM_3			BIOFAT_2		
Culture Medium	ASAM_2						ASAM_2.1		
Culture Volume	1 L								
Light Source	Natural Light								
Temperature	Atmospheric with thermoregulation system (Set-point at 25ºC)								
Pressure	Atmospheric								
Carbon Source	Air enriched with CO_2								
Salinity	30 g/L								
Nutrient set-point	6 mM of nitrate								
Renewal Rate	30% (v/v)								
Recirculation Rate	10% (v/v) of fresh medium and 90% (v/v) of exhaust culture medium (after biomass collecting)								
Minimum Concentration for Renewal	0.8 g/L								

4.1.2 Evolution of culture concentration during the assay

The evolution of each culture in terms of concentration (g $_{D.W.}$/L) during the assay is represented in two different figures (Fig. 4.1 and Fig. 4.2). The first figure presents the evolution of each culture concentration during the culture growth phase and the former figure presents the evolution of cultures concentration during DR, DRMR phase and an additional phase, the culture stabilization phase (the goal of this cultivation phase will be explained later in this work). In these figures are also presented the cultures renewal rate (in the first secondary vertical axis) and the average daily radiation (in the second secondary vertical axis) during the assay. The average daily radiation data was gathered through a weather station installed in the industrial facilities of A4F – Algafuel, in Pataias, and is presented in terms of $W/m^{2.}$

Through Fig. 4.1 and Fig. 4.2 analysis it is possible to see that, in many occasions, the concentration drop drastically during the assay. This abrupt fall of concentration symbolize the death of the cultures and happened frequently in different cultures and in different stages of cultivation. Despite of the great effort for maintaining all cultivation conditions constant there were always some stimuli variation in outdoor cultivations. This inherent stimuli variation makes it difficult to understand and justify not only the culture deaths but the data itself. Therefore, it is important to understand the reasons that led to culture death, unrelated to the assay itself, and treat only the representative points of the sample. The data of cultures on death phase should not be considered in the data treatment since they extend the error range and may compromise the assay results. The first phase of cultivation (culture growth phase) is represented in the Fig. 4.1.

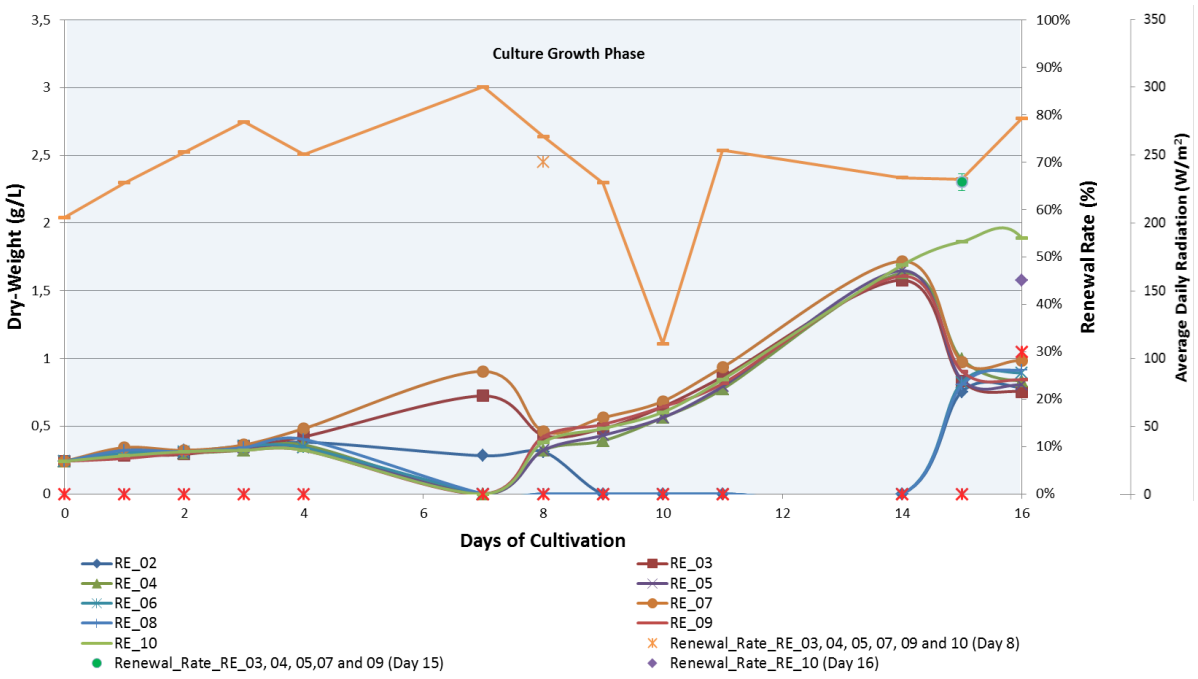

Fig. 4.1 – Presentation of culture concentration evolution during the culture growth phase.

In the 4th day of cultivation, the majority of the cultures in the reactors (RE) 04, 05, 06, 09 and 10 started to show a yellow colour, known as stress indicator. After the 5th day of cultivation this colour was extended to RE_02 and 08 and the cultures concentration started to fall and not consuming nutrients. The number of microalgal cells in agglomerates with microscopic debris and contaminants started to rise with the stress indicator appearance as well. This is typical photoinhibition behaviour of cultures (see Section 1.4.1), a legitimate reason for culture death. It was thought that the true reason for photoinhibition was the combined stimuli of low inoculation concentration (0,21 g $_{D.W.}$/L), and consequently the high light intensity per cell, with the inoculation source.

The laboratory cultures grow under artificial light with lower intensity radiation than the outdoor light (natural) and this fact may lead to difficulties in the cultures photoacclimation process. In order to minimize this factor influence, it was decided that the next inoculations should be carried out with the renewed volume of cultures under production, named as acclimated cultures. On the other hand, the inoculation source must have a lower or equal concentration of culture medium ions than the destination culture, so as not to inadvertently change the actual culture medium of the destination culture in a way that only several days of culture renewal would reduce the concentration of an excess element back to its defined set-point. This rule must be applied to maintain the reproducibility of results. The inoculations are presented in the presentation of the culture growth figures through specific renewal rates.

The re-inoculation of lost cultures took place at day 8 of cultivation, through 60% (v/v) of RE_03 and RE_07. Each of the RE_05 and RE_09 were inoculated with 30% (v/v) of culture from

RE_03. On other hand, each of the RE_04 and 10 were inoculated with 30% (v/v) of culture from RE_07.

The re-inoculation of RE_02, 06 and 08 cultures took place in the 15th day of cultivation. Each of the RE_02 and RE_06 were inoculated with 33% (v/v) of RE_05 culture volume. The RE_06 was inoculated with a total of 35% (v/v) of culture, 17,5% (v/v) from RE_03 culture and other 17,5% (v/v) from RE_09 culture. Additionally, there has been done a concentration adjust in all cultures except RE_10, aiming for 0,55 g $_{D.W.}$/L of final concentration. The concentration adjust of RE_10 was done in the 16th day of cultivation.

The following figure (Fig. 4.2) shows the cultures concentration evolution in the final part of the assay.

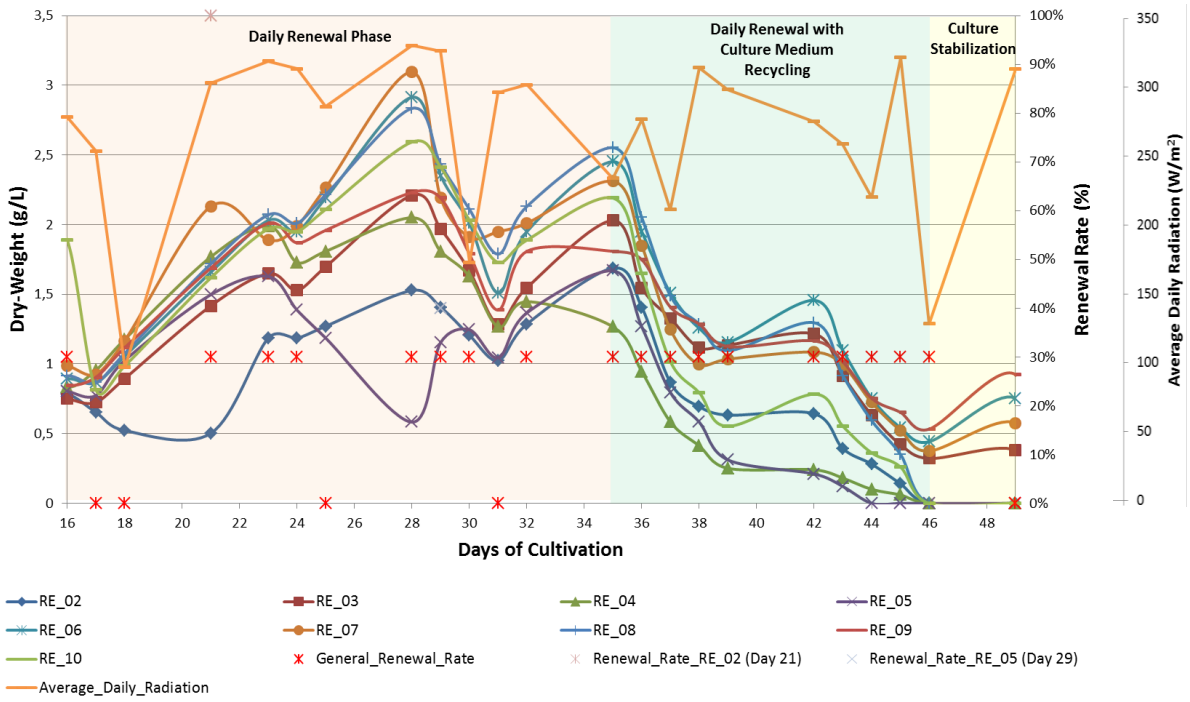

Fig. 4.2 – Presentation of culture concentration evolution from the 16th day of cultivation to the end of essay.

In the 18th of cultivation it is possible to see the fall of RE_02 concentration. This culture started to show the same symptoms that occurred on the 4th day of cultivation (yellow colour, no consumption of nutrients and rise of contaminants and microalgal cells in agglomerates). It was thought that possibly, the culture suffered photoinhibition after the culture dilution in the 15th day of cultivation. The culture was re-inoculated in the 21st day of cultivation with both 30% (v/v) of RE_05 and RE_08 culture volume.

In the 23rd day of cultivation, the RE_05 culture started to show the same yellow tonality in spite of the high culture concentration (1,63 g $_{D.W.}$/L) and it is possible to see the abrupt fall of concentration in the following days, signalling the culture death. The microscope observation of the culture also revealed the accumulation of contaminants such as fungi, protozoa and other non-

identified microalgae since the 18th day of cultivation which often represent the unfolding of many possible growth limitations in cultures [32]. Regarding these facts it is not possible to point out a specific reason for the culture death. The re-inoculation of the RE_05 culture took place at day 28 with the renewed volume of RE_02 and RE_08 cultures.

Through Fig. 4.2 analysis is possible to see that between the 16th and the 35th day of cultivation the renewals did not happened daily so it is not truly a daily renewal phase which justifies the various peaks noted. The main goal of the daily renewal procedure was to simulate industrial growth conditions while the daily renewal symbolizes the industrial process of biomass harvesting. In an industrial microalgal production facility the daily harvesting promotes the culture production stabilization and the achievement of a steady state where the biomass productivity is equivalent to the renewal rate. The non-regularly renewal verified in the first week of DR phase led to variation in the evolution of culture concentration and consequently did not allowed the achievement of the referred steady state. This problem was promptly resolved and be justified with some lack of operational experience that led to operational problems, such as lack of material preparation and culture medium sterilization which made impossible to renewal the cultures.

In the second week (from 28th to 34th day) it is possible to note the daily renewal gap in 31st day. This gap was deliberate and can be justified with the abrupt decreasing of all cultures concentration possibly due to the low radiation intensity peak in the 30th day which may have not allowed the proper microalgal growth after the renewal step. It was thought that the general concentration fall would culminate to photoinhibition and culture loss. With the loss of various cultures the whole assay would be compromised so the daily renewal did not took place at this day. It was also considered that this action would promote the culture growth and strength. In the following day (day 32) the cultures recovered and were renewed as planned. For data analysis, it was considered a constant and continuous growth in all cultures between the 30th and 32nd day of cultivation and further information will be explained later.

It is thought that the decreasing of cultures concentration during the daily renewal with culture medium recycling phase (day 35 to 46) is directly related with the nutritive media composition and it will be explained later in Section 4.1.7 with the aid of elemental analyses results.

For better understanding the relation between the microalgal growth and the nutritive medium impact the following figure (Fig. 4.3) represents the evolution of cultures concentration, organized and treated by growth condition during the DR, DRMR and stabilization growth phases after data treatment (removal of death phase data and day 32 culture concentration estimation).

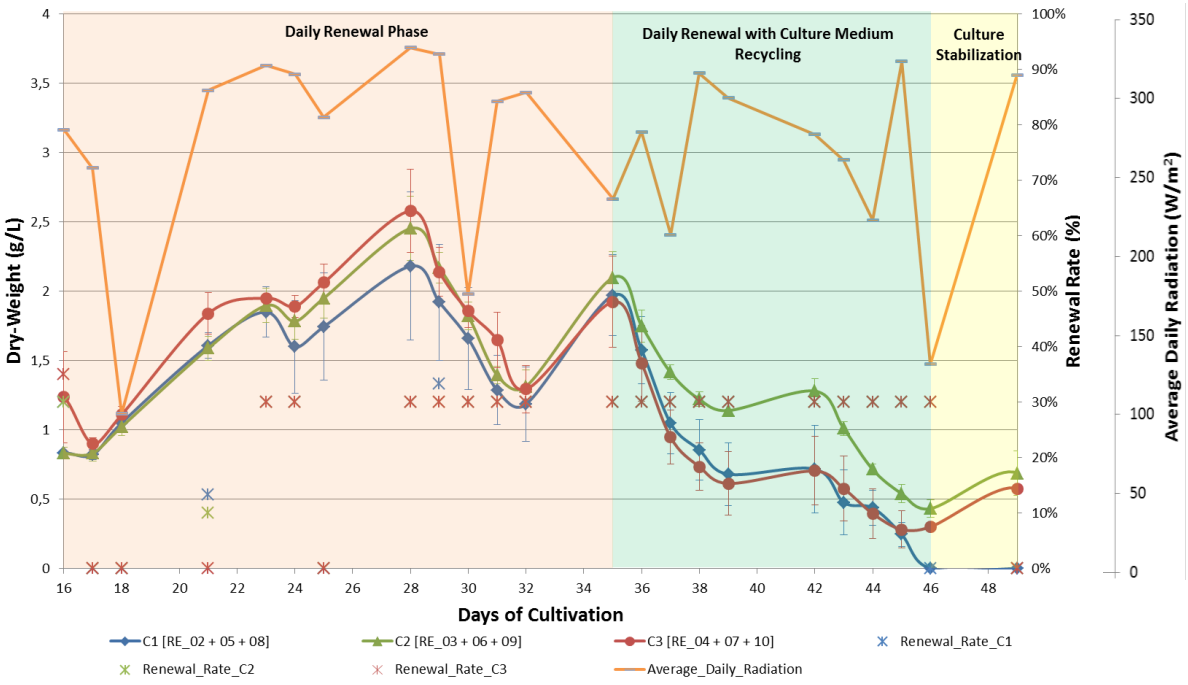

Fig. 4.3 – Presentation of culture concentration evolution from the 16th day of cultivation to the end of essay after the death phase data removal. The data was organized in 3 sets of cultures that grown under the same cultivation condition.

In the Fig. 4.3 it is possible to note the high values of standard deviation represented by error bars. These high values can be justified by the different concentration of cultures of the same set of triplicate results (cultured under the same growth conditions). The different concentration of cultures from the same condition is related with the cultures different stimuli response and possibly entering in death phase. Through a Fig. 4.3 in-depth analysis it can be seen that after data treatment it is not possible to observe the gap day in the second week of DR phase. It was considered a constant and continuous growth in all cultures between the 30th and 32nd day of cultivation, which allowed the calculation and estimation of cultures concentration at day 32 if the cultures were been renewed at day 31 of cultivation.

From a general point of view, through Fig. 4.3 it is possible to see a very similar culture growth evolution in all conditions under DR growth phase. This non-differentiated behaviour can be connected to the culture concentration variation and to the several culture medium renewals in this phase of cultivation. The renewal of culture medium worked as mask for microalgal cells welfare by folding possible growth limitations to the cultures in production. In each renewal step the microalgal cultures growth is promoted by continuously removing a fraction of soluble growth limiting factors and competitive biological contaminants (in minor proportions than the cultured microalgae).

In the DRMR phase it is possible to note the differentiated behaviour of cultures under different conditions. The condition 2 cultures (C2) achieved higher concentrations in all days of cultivation and the condition 1 and 3 (C1 and C3) cultures showed a similar evolution of concentration until the 45th day. Through a simultaneous analysis of Fig. 4.2 and Fig. 4.3 it is possible to note that all

C1 cultures have been lost (RE_05 at 44th day, and RE_02 and 08 at 45th day). It can be seen that only one C3 culture reached the end of this cultivation phase (death of RE_04 and 10 cultures at day 45). It is also possible to note that the condition that achieved higher concentrations (C2) also did not loss any culture.

In the end of this cultivation phase all of the remaining cultures showed stress indicators, they all showed a yellow tonality in a macroscopic point of view and a large number of biological contaminants (microalgal cells trapped in large agglomerates, fungi, bacteria and protozoa) in a microscopic point of view. This phenomenon was previously stated in *Nannochloropsis* cultivation with medium recycling studies [34], [61] and it was thought that can be directly related to medium recycling impact on cultures (see section 1.7). All cultures were renewed 30% (v/v) with fresh medium at 46th day and the culture evolution has been analysed in the following days to point out this phenomenon (culture stabilization phase). As can be seen in Fig. 4.3 all cultures recovered, showed a dark green colour and the contaminant concentration decreased significantly at day 49 of cultivation. It was concluded that after various days of medium recycling, the cultures were pushed to the limits and the growth limiting factors were unfolded.

4.1.3 Evolution of culture productivity during the assay

The evolution of cultures daily productivity (g $_{D.W.}$/L.day) is presented in Fig. 4.4.

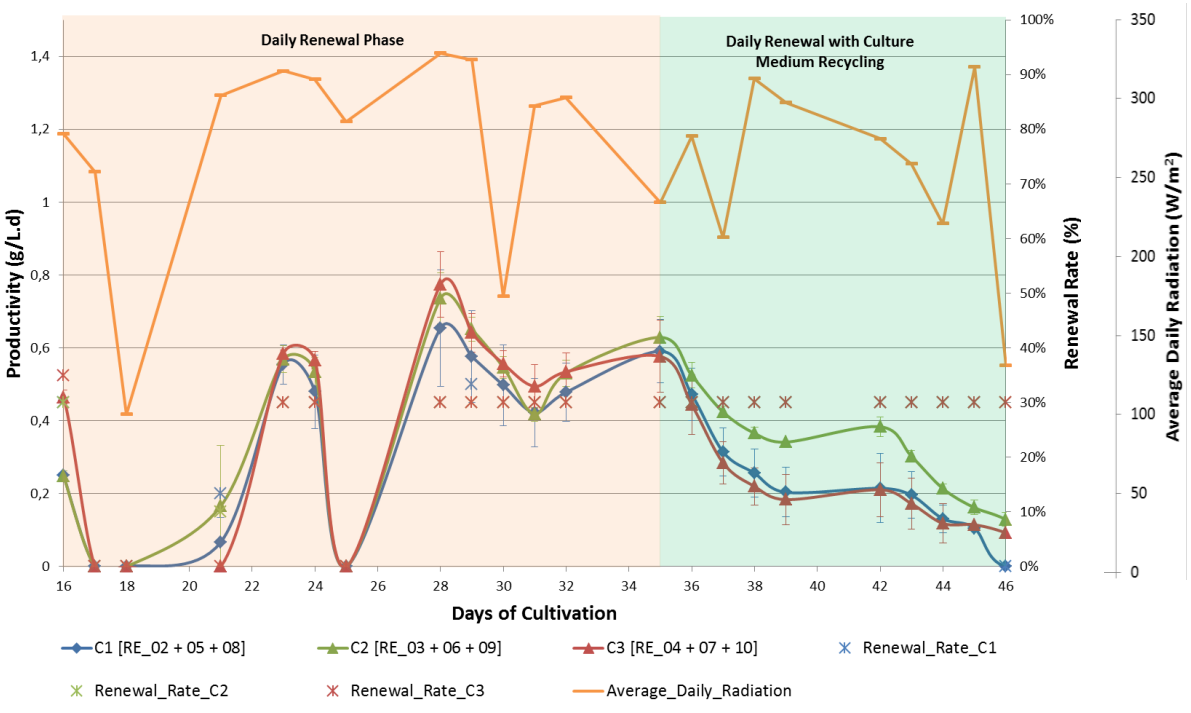

Fig. 4.4 – Presentation of culture productivity evolution between the 16th day of cultivation and the end of DRMR phase after data treatment. The data was organized in 3 sets of cultures that grown under the same cultivation condition.

Through the Fig. 4.4 is possible to note that the evolution of culture productivity is similar to the evolution of culture concentration presented in the Fig. 4.3. This fact is justified by the equation involved in the productivity calculation (Equation 1 – Section 3.9.1.3) with the difference in days of

absence of culture renewal, that the culture daily productivity is null in contrast with the culture concentration.

Attending to the daily renewal phase, it is possible to see that after consecutive renewals the cultures reached a steady state of productivity. In this phase the cultures productivity is equal or superior to the removed culture fraction through the renewal. When cultures could not achieve these values of productivity suffer wash-out. As can be seen in Fig. 4.4 this phenomenon occurred with all C1 cultures after 2 weeks under daily renewal with medium recycling and possibly has happened to all cultures in the second week of daily renewal if the daily renewal has not been interrupted in the 31st day of cultivation. Therefore this interruption had some repercussions in the concentration evolution and not allowed the achievement of the referred equilibrium phase. Regarding to this data importance for this work, culture concentration at day 32 if the renewal had been done at day 31 was estimated allowing the consecutive estimation of the equilibrium achieved in the second week of DR phase.

Regarding this variation in cultures growth evolution during the DR phase, it is not possible to do a precise evaluation of cultures productivity under the different conditions. However, we can conclude that all conditions allow a similarly productivity in cultures under daily renewal phase and all cultures stabilize to close range of productivity after 4 days of daily renewal.

As previously stated in section 4.1.2 and referred in the section 1.7, the medium recycling have a negative growth impact on microalgal cultures, acting like an unfolding agent for growth limiting growth factors and primarily led to a loss of productivity and possible culminate in culture loss This culture growth impact may be justified by a number of reasons such as non-consumed nutrient accumulation (see section 4.1.4), auto-inhibitory microalgal compounds accumulation, accumulation of cellular debris and plasmatic membranes as stated by [32], [34]. Through Fig. 4.4 it can be noticed the general loss of productivity of all cultures between the DR and DRMR phase. It can be also noticed that cultures achieve a lower stabilization tier of productivity at the second week of medium recycling (day 42 to 46) what can be justified by the referred cumulative and negative impact of medium recycling on microalgal cultivation.

Through a brief Fig. 4.4 analysis (and noticed in section 4.1.2) the C1 cultures and two (of three) C3 cultures (RE_04 and 10) suffered a high productivity loss under the medium recirculation phase (more noticed at the second week of this regime) and were washed-out. Regarding to these facts, it is possible to consider that C1 cultures presented the worst productivity behaviour during the assay.

It is also possible to note in Fig. 4.4 that C2 cultures achieved higher values of productivity in almost all days of daily renewal with medium recycling phase and none of these cultures suffered washout.

C1 cultures also achieve a very close productivity values to C3 cultures in the DRMR phase. On the other hand, it is possible to see that at day 45 the C3 cultures concentration diverges from the

C2 cultures, with C3 cultures achieving a productivity increase whereas C1 cultures had a concentration decrease and by the 46th day all C1 cultures were death.

In order to provide more accurate conclusions about the nutrition influence in *N.oceanica* cultures and create a properly comparison between all assay conditions it is important to focus on the stabilization of cultures during the DR and DRMR growth phase. Through the Fig. 4.4 analysis it is possible to note that all cultures stabilize to a common tier of productivity after 3 days of consecutive daily renewal (with or without medium recycling).

The Table 4.2 presents the information about the average productivity of all cultures at the referred stabilization period during the daily renewal phase and daily renewal with medium recycling phase (average stabilized productivity). The average stabilized productivity calculation considers the productivity values of the cultures after 3, 4 and 5 consecutive renewals, which provide a reasonable approximation to the cultures productivity at this stabilization tier if the consecutive renewals were extended for more time. In the Table 4.2 is also presented the loss of culture productivity between the stabilization tiers achieved in the two weeks of DRMR phase and the week of DR phase (Δ Week 1 and Week 2 DRMR, respectively) and between the first and second week of DRMR phase which represents the difference of average productivity between the two weeks of DRMR phase (Δ DRMR phase). However, it is important to understand that the cultures were only submitted to one week of truly DR and two weeks of DRMR phase which includes some error in the comparison between the productivity stabilization tier of second week of DRMR and the stage achieved in the first week of DR phase.

This information simplifies the cultivation data and allows the quantification of the differences between cultures productivity. It is also important to be referred that for C1 cultures average productivity can be considered only the data from the first week of DRMR due to culture lost in the following week.

Table 4.2 – Presentation of average stabilized productivity 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).

	Daily Renewal Phase	Daily Renewal With Medium Recycling		Productivity Loss		
Condition	**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

Through the Table 4.2 analysis it is possible to realize that all cultures under different cultivation conditions achieve stabilized productivity values in the same range at the daily renewal phase. As stated before in this work (see Fig. 4.4 analysis), all cultures showed the same productivity evolution during the referred phase which makes these values an expected result. It is also possible to note that all cultures suffered a considerable stabilized productivity loss when the regime was switched to daily medium recycling but the C2 cultures also achieve the lower productivity loss (24,3 %). However, it is possible to see that the second week of DRMR had a much more significant impact in the productivity of all cultures. As stated before, it is thought that this fact is directly related with the progressive accumulation of limiting growth factors in the culture along the days of cultivation with medium recycling. This phenomenon led to an irreversible productivity loss of all C1 cultures and two C3 cultures during the referred cultivation period and to a great loss of productivity in all C2 cultures.

Despite the higher productivity loss of the former 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±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±0,029 g $_{D.W.}$/L.day and 0,109±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.

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.*

4.1.4 Cultures lipid content analysis

The lipid content of the cultures was analysed in the end of the assay: samples from the remaining cultures biomass were collected at the end of daily renewal with recirculation cultivation phase and the inoculum sample was also collected at the inoculation day (day 0 of inoculation). The C1 sample (RE_08) was collected before the day of culture death (45th day of cultivation) due to possible results interferences in a death culture analysis. The harvesting of samples followed the operations explained in section 3.8.4, and the lipid quantification method followed was also explained in section 3.9.7. It is also important to refer that this analysis was done in the first attempts of method implementation what may have contributed for the error accumulation. As stated in the section 3.9.7 this method suffered several modifications during the curricular internship and the developed operational procedures (Appendix 7.5) protocol was validated by A4F – Algafuel after the end of the internship.

The lipid content (% DW/DW) data of cultures biomass was organized by condition (except the inoculum culture) and the referred information is presented in the Table 4.3.

Table 4.3 – Presentation of lipid content of biomass samples collected in the end of the assay and from the inoculum cultures at day 0 of cultivation.

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

Through a brief analysis of Table 4.3 it is possible to see that all cultures present a very similar lipid content value within the considered error range. Before the discussion of the presented values 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 (see section 1.3).

With the condition 1, 2 and 3 lipid content analysis, it is also possible to note that, as cited by literature, the lipid content values are proportional with the level of stress showed by the cultures at the harvesting day (the culture level of stress was quantified with the combined micro and macroscopic analysis and determination of nutrient consumption; the cultures level of stress were also stated at section 4.1.2 and 4.1.3). At 45th day the C1 culture (RE_08) was considered in death phase, C2 cultures at 46th day of cultivation showed some evidence of stress, and at the same day, the C3 culture (RE_07) showed more evidence of stress than C2 cultures.

The determined lipid content of inoculum culture has also interference that is reflected in the high value of standard deviation. As referred in section 3.3, the inoculum cultures were maintained at stationary phase in the laboratory room and consequently not achieving a high growth rate (see section 1.4) which may have contributed for the lipid accumulation, nevertheless it is not possible to conclude about the veracity of this theory due to high value of standard deviation.

Regarding this fact, if were consider the assay goal of achieving high productivity cultures under medium recycling strategy, and if we consider valid the proportional relation between lipid content and culture stress, it is possible to say that the developed method could provide a reliable analysis for the lipid content determination after few steps of optimization.

4.1.5 Macroscopic observation of cultures

The Fig. 4.5 is a picture of the cultivation system taken at 16/06/2014 (28th day of cultivation of assay 1). At this point, the cultures were at the daily renewal phase of cultivation and it is possible to

see the contrast between the typical healthy and concentrated *N.oceanica* cultures showing a dark green colour (RE_02, 03, 04, 06, 07, 08, 09 and 10) and an typical unhealthy culture (RE_05) showing yellow colour. As can be seen at section 4.1.2, this culture was at dead phase.

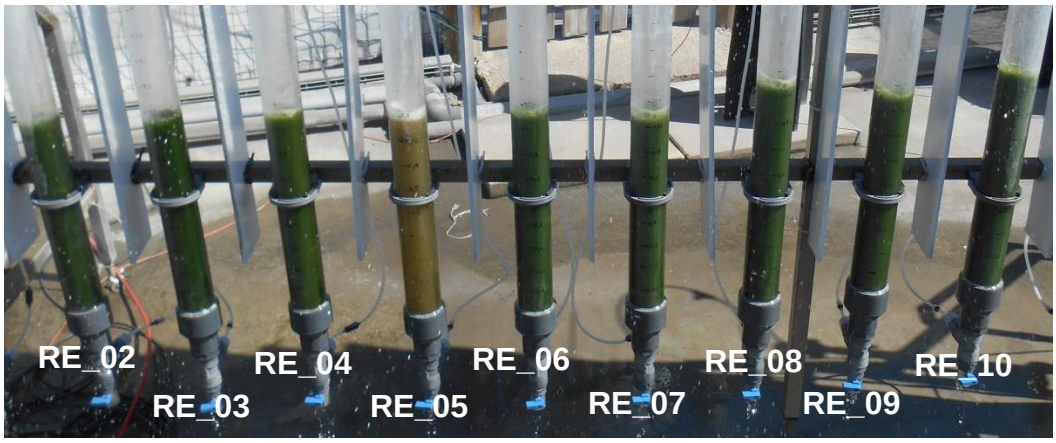

Fig. 4.5 – Presentation of cultures macroscopic observation at day 28 of cultivation of assay 1.

4.1.6 Microscopic observation of cultures

The Fig. 4.6 shows two pictures taken at the microscopic observation of RE_02 culture at 06/06/2014 (18th day of cultivation). These pictures show a typical contaminated culture.

In the centre Fig. 4.6 – A is possible to see a large number of microalgal cells trapped in a agglomerate of great proportions. The Fig. 4.6 – B (at the centre) shows a contamination by other non-identified species of microalgae.

During the assay, all cultures were very similar from a general microscopic point of view showing some normal appearance of fungi and bacteria contaminations. On the other hand, C1 cultures frequently showed an unusual and larger number of contaminants as can be seen in the Fig. 4.6. A possible reason that may justify the agglomerate formation is the intracellular compound release and plasmatic membranes accumulation after cellular lysis [34].

It was also noticed that the number of foreign organisms in cultures was directly proportional to the culture handling operations and additions (involving the opening of the reactors) and also to the days of cultivation.

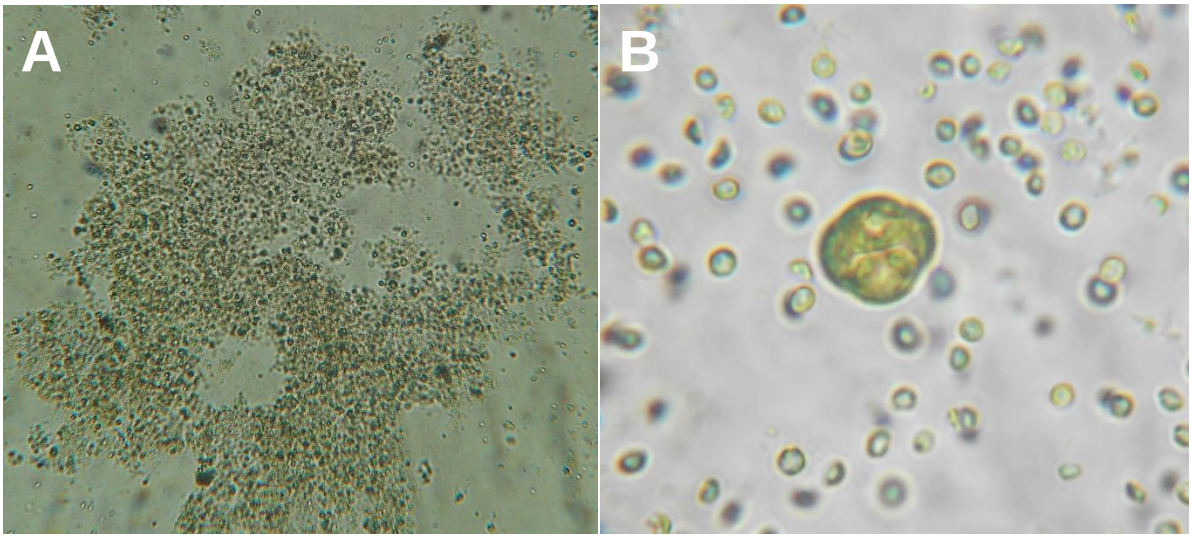

Fig. 4.6 – Example of typical microscopic observation of a contaminated culture during the assay 1.
A – Photography of RE_02 microscopic observation taken at 06/06/2014 at 100x magnification.
B – Photography of RE_02 microscopic observation taken at 06/06/2014 at 400x magnification. The presented small round green cells are *N.oceanica* cells with 2-5 µm of diameter.

4.1.7 Presentation of cultures supernatant element analysis

The main goal of cultures supernatant element analysis was to relate the element composition of the production culture media with the evolution of cultures growth (and productivity) in the different cultivation stages of the assay, and analyse the nutritive and culture medium impact on *N.oceanica* growth. This analysis should create information for, after all assays analysis, formulate an optimized recipe for nutritive medium. The operational procedures followed for this analysis were previously explained at section 3. In order to achieve a reliable analysis, the element concentration given by supernatant analysis was compared with the theoretical element concentration. The theoretical concentration of supernatants was calculated considering the element analysis of all culture medium components (see section 3.6) analysis and the nutritive medium theoretical concentration. On the other hand, the theoretical concentration of the various nutritive media was compared with the nutritive media element analysis. This comparison had an important role to this analysis allowing searching for errors and interferences that may occurred in the nutritive medium preparation (weighing errors or presence of element contaminations in the reagents). Through this nutritive medium quality control, was possible to note a divergent concentration of the majority of elements in the BIOFAT_2 medium. It is also important to recall the fact that this medium is an industrial supplied medium. This fact may lead to some non-expected variance in the C3 cultures supernatant. CNASAM_2 and CNASAM_3 samples analysis also revealed a high deviation from the theoretical concentration which may occurred due to precipitation during the long period of transportation.

It is also important to note that the results will be expressed in terms of percentage variance of elements between the theoretical concentration and the supernatant concentration. These results have an inherent double character; i.e. an element with 100% of accumulation represent that the

analysed concentration is twice as much than the theoretical concentration. In contrast, an element with -100% of accumulation represent that the analysed concentration is null when compared with the theoretical concentration, which has a much more significant impact.

The supernatant of cultures (the culture medium) was collected in three different cultivation stages: It was collected samples from the inoculum cultures at day 0 of cultivation; from all production cultures at day 35 day of cultivation (switching day from DR to DRMR phase); from the remaining cultures in production at the end of the daily renewal with medium recycling cultivation phase (46th day of cultivation). As occurred for the biomass sample collection (see section 4.1.4) the supernatant sample from remaining C1 culture in production (RE_08) was collected at day 45 of cultivation.

As stated in section 3.9.6 this analysis was made by an external entity, and all samples were stored after collected in the freezer. There was a problem in the transportation of samples and the transportation lasted 1 month instead of the predicted 2-3 days. This problem was oblivious to A4F – Algafuel, and the samples were kept under environmental temperatures in a warehouse for 1 month, which for sure contributed for sample deterioration. Regarding this issue, the deterioration stage of all samples and the possible interference presence not allow an in-depth supernatant analysis. Therefore, it was done a qualitative supernatant analysis and not a quantitative analysis as predicted.

4.1.7.1 Presentation of supernatant element analysis by cultivation condition

This analysis allows the presentation of the element concentration evolution in cultures under different cultivation conditions during the assay. In a general point of view, all cultures showed a very high accumulation of zinc and copper elements, these high values do not allow a properly graphical analysis. Regarding this fact, these two element analysis will be presented in separate figures.

The evolution of element concentration in the C1 culture media during the three stages of cultivation is presented in the Fig. 4.7.

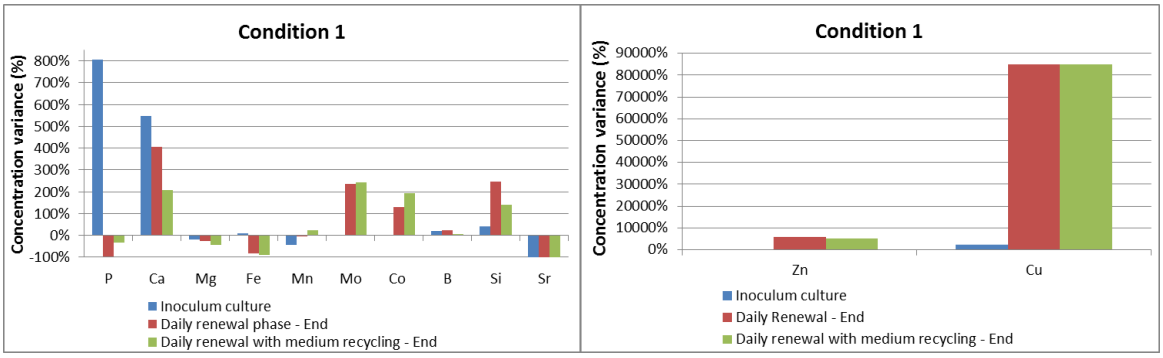

Fig. 4.7 – Presentation of percentage of variance between the theoretical and the analysed concentration of the principal elements in condition 1 cultures during the assay.

Before analysis it is important to recall that the inoculum culture had grown in presence of the same culture and nutritive medium than condition 1 cultures. Regarding this fact it is predicted a cumulative evolution of concentration during this assay. This means that elements that show an excess concentration in inoculum culture should become gradually higher and in contrast, elements

that show deficiency in the inoculum cultures should also show the gradual rise of the deficiency during the assay.

Attending to the Fig. 4.7 it is possible to see that phosphorous and iron, which were in excess concentrations in the inoculum culture showed deficiency in the DR and DRMR cultivation phase. This fact may be justified with the inoculum growth phase since the inoculum cultures were maintained at stationary growth phase in the laboratory room before inoculation. The cellular growth of this culture was lower than the growth achieved in the outdoor cultures and consequently the demand for the major macronutrients may be lower as well (see section 1.4 – Fig. 1.2). This fact could also be justified by the common operational procedure of providing excess of nutrients to the laboratorial inoculum cultures minimizing the culture handling and additions (involving the flask opening) and consequently preventing the proliferation of biological contaminants. Calcium and magnesium were also in higher concentrations in the inoculum culture than the production cultures at the two analysed stages, which can be justified by the former reasons described. Strontium concentration was always in deficiency during the essay which probably means that the cellular consumption is much higher than it addition. Through the Fig. 4.7 analysis it is possible to see that manganese concentration at first place (in inoculum cultures) was in deficiency and had a gradual rise through the DR phase and culminate in excess in cultures at DRMR phase.

Attending to the magnesium concentration variance it is possible to note progressive deficiency along the stages of cultivation, in the inoculum this element was in a non-significant deficiency and this deficiency increased proportionally in the DR phase and in the DRMR phase.

Through a Fig. 4.7 analysis it is possible to note that silicon (which have a residual value of theoretical concentration) was always in excess during the assay, which means that this element may not be essential for *N.oceanica* cultivation as expected. Silicon is referred in the literature as essential element only for diatoms growth needed for formation of a silicon capsular structure [32].

It is possible to note that some analysed elements (molybdenum and cobalt) had a cumulative concentration during the assay and possible meaning that they have been added continuously in excess. Attending to zinc and copper analysis it is possible to note that copper is in a relative low excess proportion in the inoculum culture and gradually accumulate in the culture medium. Regarding this, copper may have been added in great excess during the cultivation. Zinc is also present in great proportions in the culture medium during DR and DRMR phase. These element accumulations may be important to understand the evolution of C1 cultures growth during the essay. These high concentrations in the culture medium may represent a limiting growth factor for C1 cultures especially under DRMR phase. It has been stated that the presence of copper, zinc, molybdenum and cobalt is essential to microalgal growth but in concentrations above the toxic threshold act like a limiting factor for green algae growth, leading in some cases to culture death [51], [65], [66], [67].

The element concentration in the C2 culture media during the three stages of cultivation is presented in the Fig. 4.8.

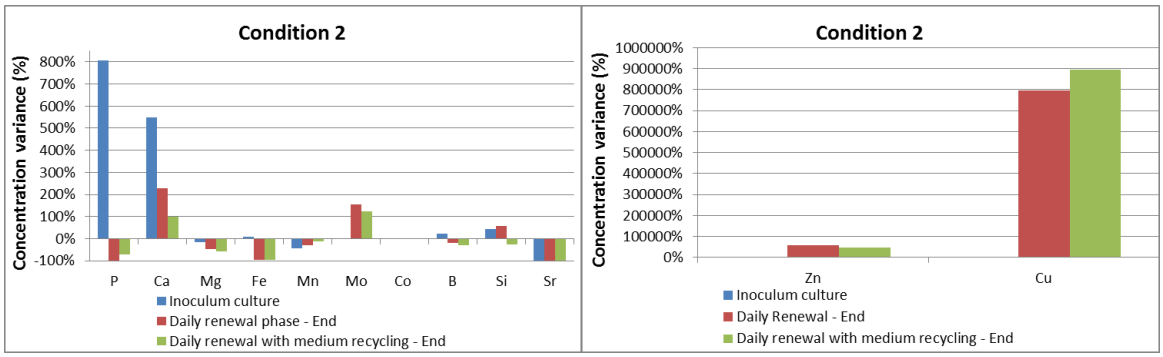

Fig. 4.8 – Presentation of percentage of variance between the theoretical and the analysed concentration of the principal elements in condition 2 cultures during the assay.

The concentration evolution of the majority of the elements is close to C1 cultures previously described. It is possible to see that elements such as phosphorous, calcium, magnesium and iron show a decrease of concentration (or deficiency increase) along the stages of cultivation. This fact may be justified by the higher cellular growth rate achieved by cultures and consequent rise of nutrient demand in the DR and DRMR cultivation phases.

It is possible to note that all element concentrations (except phosphorous, manganese and strontium) are lower in the DRMR cultivation phase than the DR phase. This fact may be justified by the medium recycling impact on cultures with the gradual accumulation of non-consumed elements. In cultures grown under daily renewal strategy a constant fraction of culture medium is constantly removed which may fold the unbalanced recipe of nutritive medium.

The noticed phosphorous increase of concentration in the DRMR phase may be justified by the medium recycling strategy that increases the holding time of elements in the culture medium providing more time for the cellular element uptake. In cultures grown under daily renewal strategy a constant fraction of culture medium is constantly removed and promptly filled with fresh medium which may lead to a decrease of the referred element holding time in the culture medium and fold the unbalanced recipe of nutritive medium. Strontium concentration is always in deficiency which may indicate that the consumption rate is higher than the addition rate.

As verified in the previous analysis, zinc and copper concentration also follow the same evolution than C1 cultures. On the other hand, it is possible to see that copper achieve a very high value of accumulation in cultures under DR and DRMR phases. It is also important to remember (see section 3.7) that CNASAM_3 do not include copper in the recipe, and this fact is reflected in the residual theoretical copper concentration. Therefore, the high levels of copper accumulation showed in the Fig. 1.1.1.1.1 do not reflect high concentration on C2 culture. Cobalt analysis is not present in the Fig. 4.8 due to the same type of problem verified for copper. Cobalt theoretical concentration in C2 cultures is null which makes the determination of percentage variance impossible. The element analysis revealed an accumulation of this element in the supernatant during all cultivation phases, but in comparison with C1 and C3 supernatants, it is possible to note that cobalt is present in less minor proportions and far from the toxicity threshold. Zinc concentration in C2 cultures achieve the same

level of accumulation that in C1 cultures which may led to a limitation in growth of microalgal cultures [66] [51].

The following figure (Fig. 4.9) presents the accumulation or deficiency of elements in C3 cultures supernatant during all stages of the assay.

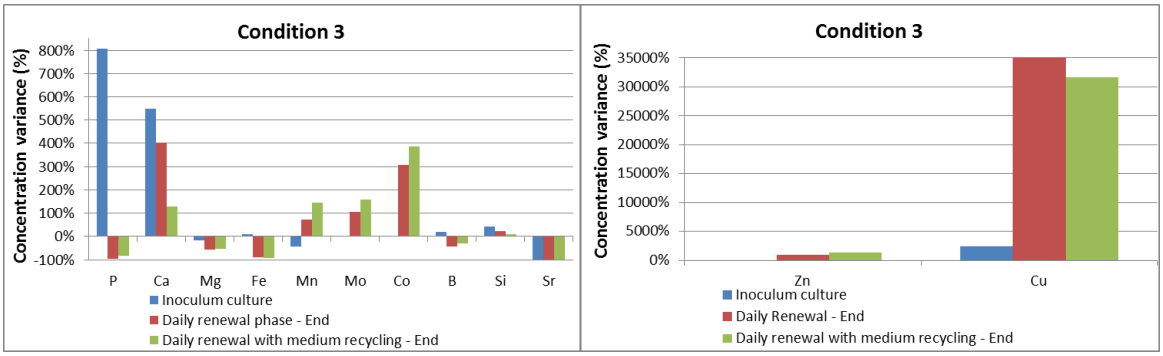

Fig. 4.9 – Presentation of percentage of variance between the theoretical and the analysed concentration of the principal elements in condition 3 cultures during the assay.

Through a brief Fig. 4.9 analysis it is possible to see some similarity between some of the elements present in C3 cultures and condition 1 and 2 cultures. Phosphorous, calcium and iron show 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, molybdenum and manganese are referred in the literature as growth limiting factors for microalgal cultures when present in not very high concentrations [32], [36]. The former elements are present in C3 culture medium in excess, especially in DRMR cultures, which may explain the high loss of C3 cultures productivity verified in the section 4.1.3.

As verified for C1 and C2 cultures, zinc and copper show it is possible to note a high accumulation in C3 culture media through Fig. 4.9. It is possible to note that copper achieve the lower level of accumulation that in C1 cultures possibly due to error in the industrial preparation of the nutritive medium (BIOFAT_2). On the other hand, zinc show the same accumulation pattern in C3 cultures as verified in C1 cultures, which might be a limiting factor for microalgal growth [24], [51], [65], [66] and the respective concentration should be decreased in a further nutritive medium recipe.

4.1.7.2 Presentation of supernatant element analysis by cultivation phase

The main goal of this analysis is to find possible coincident concentration evolution of some elements and create a first input for possible new nutritive medium recipe

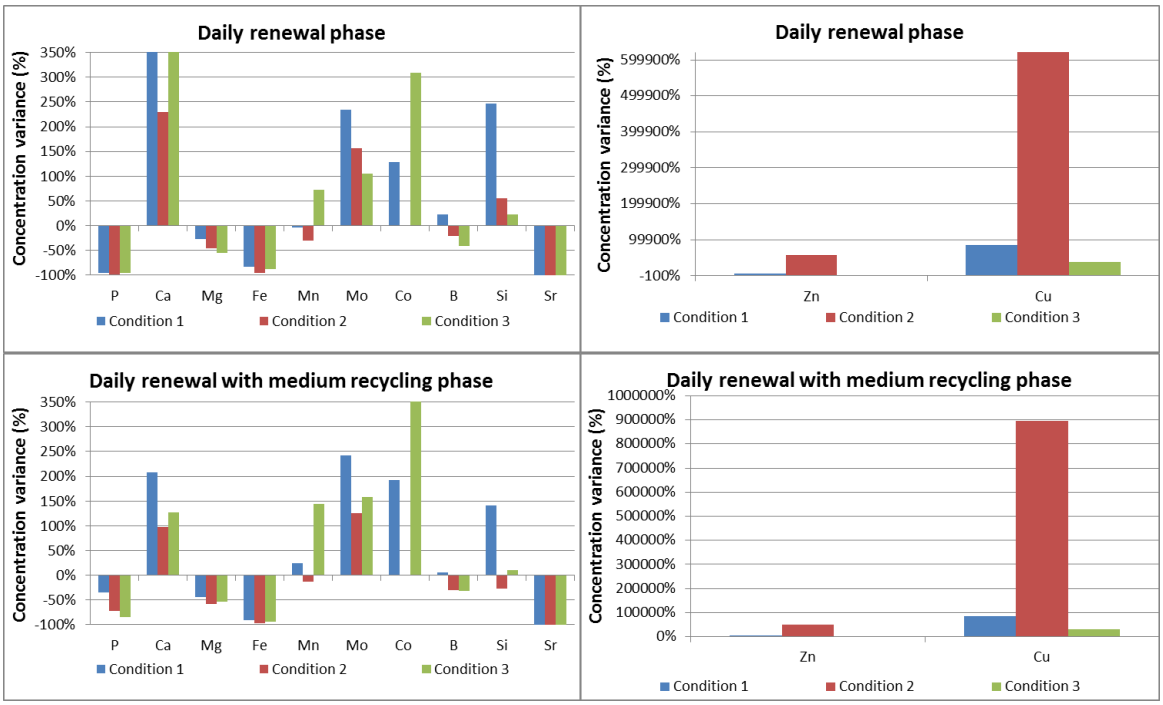

Fig. 4.10 – Presentation of the evolution of percentage of variance between the theoretical and the analysed concentration of the principal elements in the different cultivation conditions during the assay.

Through the Fig. 4.10 analysis it is possible to see that phosphorous, magnesium, and strontium are in deficiency in the supernatant of cultures under the three different cultivation conditions. It is stated in literature [32] that at high growth rates the microalgae possibly become phosphorous-limited. This fact could be justified by the general decrease of the phosphorous concentration deficiency in cultures under DRMR cultivation phase (with stated lower growth rates – see section 4.1.2). On the other hand the phosphorous proportion in the nutritive medium has been received special attention through the several nutritive medium optimization work done and the ideal Nitrogen – Phosphorous ratio that should be present in the culture medium has already been stated [32]. This proportion is related with the growth rate and has already been optimized in previous work of A4F- Algafuel. It is stated on literature that phosphorous limitation may lead to accumulation of lipids [48] and this analysed and general phosphorous deficiency in all cultures may led to the verified high content of lipids (if the cultures were considered as productivity optimized cultures – see Section 4.1.4). Regarding these facts, the phosphorous deficiency may not be adjusted for the high growth rates achieved during the assay, and should be carefully adjusted (increased) after further tests.

Iron concentration is also in deficiency in all culture media and is added in higher proportions in the C2 cultures through CNASAM_3 (see section 3.7). The iron deficiency has been maintained constant in all cultures under DR and DRMR phases. The C1 and C3 cultures show the same iron deficiency and the C2 cultures showed a higher iron concentration deficiency. The general iron deficiency could be explained by previous research conclusions [47], which refers that high consumption of iron by cells is directly related with high level of nitrates (nitrogen source) present in the culture medium. During this assay the nitrates where always present in excess (concentration set-point of 6 mM, see section 4.1.1), preventing the nitrogen starvation of cells which may have led to the

decrease of cultures productivity. Regarding these facts it should be considered the increase of iron concentration in the next step of nutritive medium optimization.

Magnesium is another element that has been shown to be in the same level of deficiency in all of the culture media, which also have the same theoretical concentration. It is possible to see an increase in the magnesium demand in all cultures under DRMR phase, what possible be justified by the magnesium source, since this element is not added to the culture system through the nutritive medium. The source of magnesium is the mineral solution 1 added in the preparation in the culture medium (see section 3.6) which is only represent 10% (v/v) of the daily renewal volume in the DRMR phase. Magnesium deficiency is also referred in the literature as a limiting growth factor that interrupt the cell division which may led to loss of cultures [67]. Regarding this it is possible to consider a magnesium concentration reinforcement in future medium optimization research for microalgal cultivation under medium recycling strategy.

Calcium concentration is very similar in the theoretical concentration of all cultures under different cultivation conditions and it is also possible to see the same level of accumulation in all cultures under the two phases of cultivation through the Fig. 1.4 analysis. The calcium supplied concentration should be decreased in future medium optimization work.

Through the Fig. 4.10 analysis it is possible to see that manganese is added to the culture media in the right proportions in C1 and C2 cultures and in these cases the inherent concentration adjusted may not be needed. In C3 cultures analysis it is possible to see that manganese achieve higher percentage accumulation which may be justified by possible errors in nutritive medium preparation. On the other hand, these accumulated concentrations are below the stated toxic threshold [51]. In the Fig. 4.10 analysis it is possible to note the similar accumulation of molybdenum and manganese. These concentrations only showed to be above the toxic threshold [51] in the C1 cultures, what probably means that molybdenum concentration is properly adjusted in the CNASAM_3.

Boron and silicon are not added specifically through the nutritive medium or other component of the culture medium, and have a residual theoretical concentration which has been reflected in the Fig. 4.10 analysis, which showed a non-concerning accumulation or deficiency percentage in the analysed supernatants.

Strontium element importance for microalgae is not fully understood (see section 1.6.2) and is also not added specifically through the nutritive medium or other component of the culture medium and theoretically has a residual concentration. Through the Fig. 4.10 analysis it is possible to see that this element is continuously consumed during the assay. Regarding this it is possible to consider the addition of a strontium supply in further optimization tests.

As verified in the previous analysis (see section 4.1.7.1), cobalt, zinc and copper show a great accumulation percentage in C1 and C3 cultures under the two cultivation phases. It is also important to remember (see section 3.7) that CNASAM_3 do not include copper in the recipe, and this fact is

reflected in the inherent residual theoretical copper concentration in C2 cultures. Therefore, the high levels of copper accumulation showed in the Fig. 4.10 do not truly reflect high concentration on C2 culture. Cobalt analysis is not present in the Fig. 4.10 cultures analysis due to the same type of problem verified for copper. Cobalt theoretical concentration in C2 cultures is null which makes the determination of percentage variance impossible. The element analysis revealed a non-concerning accumulation of this element in the C2 cultures supernatant during all cultivation phases in contrast with the accumulated concentration of zinc that is present in great proportions in the C2 culture media.

Zinc theoretical concentration is very similar in all cultures under different conditions and, through the Fig. 4.10 analysis it is possible to see that zinc is also present in the cultures supernatant at great proportions. These trace metals (zinc, copper and cobalt) concentrations may be above the toxicity threshold and may inhibit growth, impair photosynthesis, deplete antioxidants, and damage the cell membrane [24], [51] .

After assay 2 result analysis and considering these results it will be presented (in Section 4.2.6) the suggestion for a new formulation of nutritive medium for *N.oceanica* cultivation under medium recycling strategy.

4.2 Assay 2 – Analysis of different medium recycling strategies impact in industrial *N.oceanica* cultivation

The main goal of this assay was directly connected with the results achieved in the first assay. It was possible to note a positive influence of CNASAM_3 nutritive medium on *N. oceanica* cultivation under industrial conditions and this nutritive medium was chosen (by A4F – Algafuel) as the best formulation of nutritive medium for *N.oceanica* cultivations. Concerning this choice, during this assay the implemented industrial nutritive medium formulation (BIOFAT_2) was tested against the nutritive medium formulation which showed better, strong and consistent results during the assay 1 (CNASAM_3), which will allow to distinguish the formulation for industrial *N.oceanica* cultivation that provide the higher culture productivity. During the assay 1 it was also possible to note the continuous and gradually crescent problem of biological contaminants proliferation along the cultivation, which culminate in a critical proportion of foreign organisms by the end of the daily renewal with medium recycling phase. It was thought that this limiting growth factor needed to be overcome and take a step forward in order to improve culture productivity. It was considered that the solution would be a disinfection treatment of the exhaust medium before the re-addition to the cultures.

After a brief analysis and discussion of the several number of options available to overcome this problem, evaluating the inherent economic impact on production costs and analysing the consequent treatment process feasibility, it was considered the best and prompt solution would be exhaust medium treatment through sodium hypochlorite disinfection (at final concentration of 10 ppm) and subsequent neutralization through the addition of sodium thiosulfate solution (150 g/L).

4.2.1 Cultivation conditions

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 average daily radiation was also analysed and the data was gathered through the weather station located in the A4F – Algafuel industrial facilities, in Pataias. The initial design of assay 2 aimed to reproduce the industrial cultivation method, with three different stages of growth: the culture growth phase, a phase with daily renewal of culture medium and finally a phase with daily renewal with culture medium recycling. In the ideal culture behaviour, the culture growth phase would last 1 week for allowing the following week of consecutive culture medium renewal and consequent dilution of cultures. In the end of this growth phase, the daily renewal strategy would switch to medium recycling strategy with supernatant disinfection method (for condition 2 and 3 set of cultures) and this stage would last 2 weeks. Despite the efforts it was not possible to put the cultures under these three cultivation stages and was necessary to re-design the cultivation method (further information will be presented in Section 4.2.2).

The daily renewal rate remained constant in all essay and represented 30% (v/v) of the total culture volume. The renewals in the medium recycling stage consist in 10% (v/v) of fresh culture

medium and 90% (v/v) of exhaust medium after biomass removal. In accordance with the main goal of this assay, the supernatants of two sets of cultures were disinfected for 2 hours and then added to the respective culture after neutralization. In order to guarantee the reproducibility of the results the supernatant of the set of cultures that was not disinfected (control cultures) was also not immediately added to the respective cultures and was kept in aseptic conditions for 2 hours.

After assay 1 started, A4F – Algafuel defined the ASAM_2.1 medium as standard culture medium for large-scale *N.oceanica* cultivations due to operational constraints (the impact of ASAM_2.1 medium will be discussed at section 4.3). Regarding this fact, all cultures were cultivated with this culture medium in this assay.

Table 4.4 – Presentation of cultivation conditions for assay 2.

Condition	1			2			3		
Reactor	RE_02	RE_05	RE_08	RE_03	RE_06	RE_09	RE_04	RE_07	RE_10
Nutritive Medium	CNASAM_2			CNASAM_3			BIOFAT_2		
Culture Medium	ASAM_2.1								
Culture Volume	1 L								
Light Source	Natural Light								
Temperature	Atmospheric with thermoregulation system (Set-point at 25ºC)								
Pressure	Atmospheric								
Carbon Source	Air enriched with CO_2								
Salinity	30 g/L								
Nutrient Adjustment	6 mM of nitrate								
Renewal Rate	30% (v/v)								
Recirculation Rate	10% (v/v) of fresh medium and 90% (v/v) of exhaust culture medium (after biomass collecting)								
Supernatant Treatment	–			**Disinfection** by 2 hours at 10 ppm of sodium hypochlorite; posterior **neutralization** with sodium thiosulfate solution (150 g/L)					
Minimum Concentration for Renewal	0.8 g/L								

4.2.2 Evolution of culture concentration during the assay

As previously referred (see Section 4.2.1), the cultivation not followed the programed three growth stages. As will be explained through the Fig. 4.11 analysis there was some scale-up constraints and the cultures entered in many occasions in death phase before achieving the needed high levels of concentration. The concentration of cultures only started to gradually rise and follow the intended growth culture phase after the second inoculation attempt and after some strategy adjustments. These two culture grow attempts lasted for 2 weeks which compromised the scientific feasibility of the assay (with the end date of the curricular internship approach – 5th September 2014) and turned impossible to follow the three programmed stages of cultivation. Regarding this, the assay

2 was divided in four distinct cultivation phases: the first culture grow attempt (from day 0 to day 2 of cultivation), the second culture grow attempt (between the 2nd and 13th day of cultivation), culture growth phase (which lasted from the 13th to the 19th day), and the daily renewal with medium recycling phase (between the 19th and the 31th day of cultivation).

The evolution of culture concentration during the essay will be presented in two separate parts: the two grow attempts (represented in Fig. 4.11), and the culture growth and daily renewal with medium recycling phases (represented in Fig. 4.12). In these figures are also presented the cultures renewal rate (in the first secondary vertical axis) and the average daily radiation (in the second secondary vertical axis) during the assay. The average daily radiation is presented in terms of W/m^2.

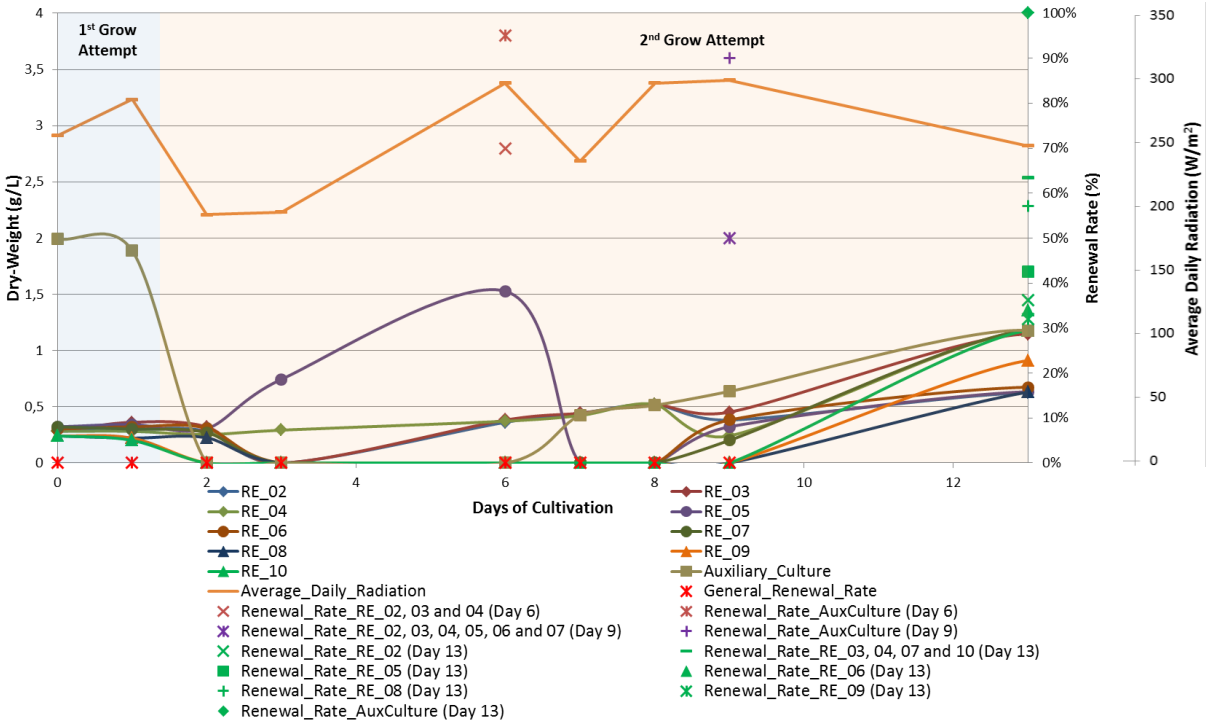

Fig. 4.11 – Presentation of culture concentration evolution during the first and second grow attempt.

The first inoculation took place at place at day 0 of cultivation and occurred by direct transfer of available laboratory cultures with 0,28 g $_{D.W.}$/L of cellular concentration (after proper homogenisation). The auxiliary culture was inoculated at the same day by direct PBR culture transfer. At this time the role of this culture was only for additional pH and culture control. Right at the beginning of day 2 of cultivation the RE_07, 08, 09 and 10 showed a yellow colour and microscopically all cells showed a low pigment density and irregular shape of the plasmatic membrane. In the evening of day 2, it was observed that these culture characteristics were extended to all assay cultures under production, which made possible to consider that the cultures suffered photoinhibition (see section 1.4 and 4.1.2). This phenomenon also occurred in the beginning of assay 1 under the same conditions (low concentration and high daily radiation) and it was also thought that all cultures had problems in the photoacclimation process and adaptation to the outdoor conditions (high radiation intensity), which

led to all cultures death. Through the acquired experience along this work, it was figured out that the next step would be re-inoculation of all cultures with a more concentrated laboratory culture. Due to laboratory culture stock constraints, it was not possible to perform this procedure and considering the time limitation (approach of the curricular internship end) it was also not possible to wait for the growth of laboratory cultures.

At this time, it was also thought that this phenomenon was the possible result of an inadequate adjustment of the nutrients added through the nutritive medium. It was thought that the cellular uptake of a non-identified nutrient was higher than the addition rate. This situation was limiting the cellular growth which led to the photoinhibition and culminated in cultures death. It was then thought that providing an additional excess of nutrients (nutritive medium) would replete the nutrient deficiency and simultaneously stimulate the cellular growth rate and lead to the cellular growth yield improvement. This nutritive medium concentration excess was provided temporarily (switching of nitrate set-point concentration to 12 mM) to recent inoculated (and diluted) cultures and was gradually decreased until the end of the growth phase, when the nitrate concentration come back to the predicted set-point concentration (6 mM). It is also important to refer that this procedure is only economically and technically feasible for lab-scale cultures and should not be applied to the large-scale cultures.

It was decided to test two simultaneous solutions with the re-inoculation of RE_04 and 05 at day 3 of cultivation. The RE_04 was re-inoculated through the direct transfer of laboratory culture (achieving the concentration of 0,28 g $_{D.W.}$/L) and the RE_05 was re-inoculated through the direct transfer of 20% (v/v) of operational volume of culture from a Green-Wall flat panel PBR in production (and make up the volume with fresh culture medium). The final concentration of RE_05 (0,74 g $_{D.W.}$/L) was more than twice the concentration of RE_04 after the inoculation (0,28 g $_{D.W.}$/L). The growth of each culture would be analysed in the following days and after that would be decided which culture would serve as inoculum source for the other reactors. It is important to note that these two cultures were complemented with the nutritive medium which has the lower content of elements (CNASAM_3) to not favour any cultivation condition and guarantee the reproducibility of the results (the explanation of this inoculation procedure and followed rules were previously referred at section 4.1.2).

At the 6th day of cultivation it is possible to note through the Fig. 4.11 that RE_05 culture was by far, more concentrated and with a healthier appearance than RE_04 that already showed photoinhibition evidences. At this day RE_04 showed a yellow colour, the concentration remained almost constant since the inoculation day (it is possible to see this concentration evolution through the Fig. 4.11) and microscopically the majority of cells showed a low pigment density and irregular shape of plasmatic membrane. Regarding these facts, the RE_04 culture was considered a dead culture and the cultivation system was cleaned and disinfected. During the same day, RE_02, 03 and 04 were inoculated with 300 mL of RE_05 culture through specific renewal rates, all culture volumes make up to 1L with fresh medium and the RE_05 switched position to auxiliary culture after inoculation (as can be seen through Fig. 4.11). From this cultivation point forward, the role of the auxiliary culture has also switched and started serving as backup culture for possible re-inoculations needed and preventing the wait for another inoculum growth. It is important to be referred that, after this day, all cultures were

complemented with the specific nutritive medium in agreement with the planned cultivation conditions (see Section 4.2.1). It is also important to be referred that all re-inoculations are presented in Fig. 4.11, Fig. 4.12 and Fig. 4.13 through specific renewal rates.

At day 9 of cultivation it was thought that each culture in production achieved enough concentration for re-inoculation of another reactor. The RE_05, 06 and 07 were re-inoculated through the 50% (v/v) renewal of RE_02, 03 and 04, respectively.

At 13th day of cultivation the graphical presentation of culture concentration evolution (Fig. 4.11) does not represent what truly occurred and may induce in error. At this day both RE_04 and 07 cultures showed a yellow colour and the microscopic evidences of photoinhibition (as stated previously) and consequently considered dead cultures. In this day, the auxiliary culture re-inoculated the RE_04 and 10 through a renewed volume of 33% (v/v) and switched position for RE_07. Considering this day the last of 2nd grow attempt phase, it was carried out the simultaneous procedure of concentration adjust and re-inoculation of RE_08 and 09. The concentration adjust was achieved through specific renewal rates and the inherent target was the concentration of most diluted culture (RE_04, 07 and 10 – 0,44 g $_{D.W.}$/L). RE_08 and RE_09 were re-inoculated with 25% (v/v) of RE_02 and 05 culture, and RE_03 and 06 cultures, respectively. Additionally, the auxiliary culture was also re-inoculated with the excess of culture volume originating from the concentration adjust process.

The culture evolution of concentration during the culture growth phase and daily renewal with medium recycling phase (DMRM) is presented in the Fig. 4.12.

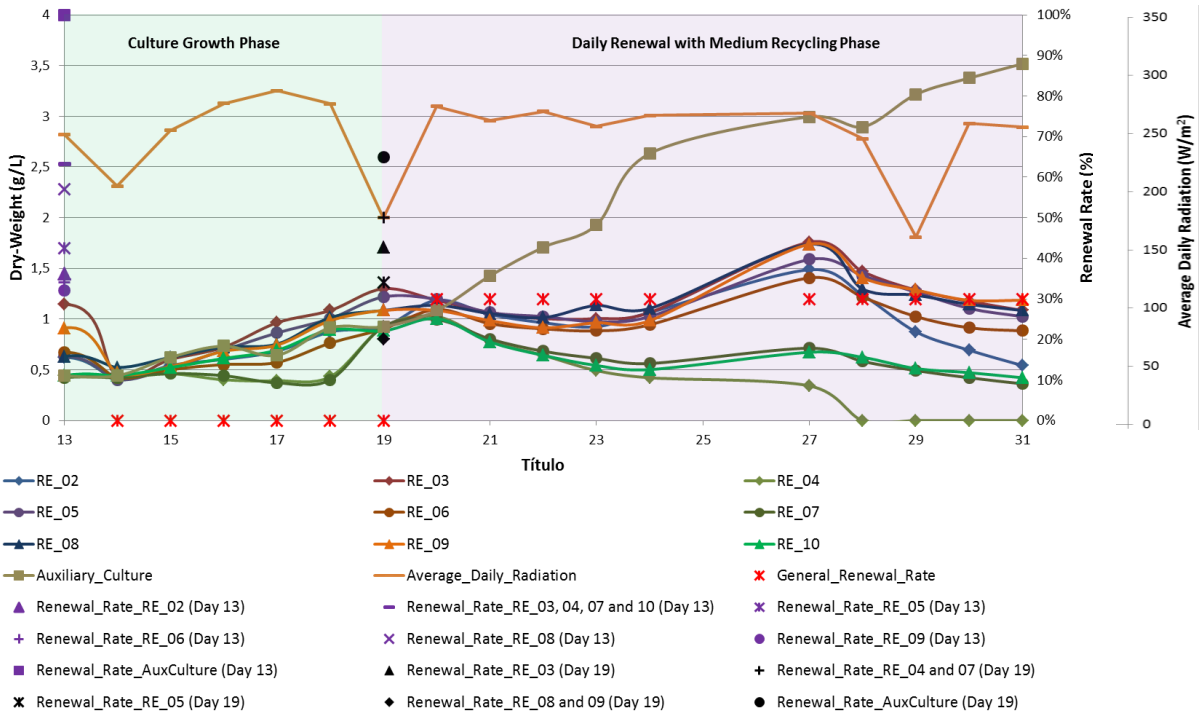

Fig. 4.12 – Presentation of culture concentration evolution between the culture growth phase and the end of the assay.

Through a brief Fig. 4.12 analysis it is possible to note that from the 15th to 18th day of cultivation, RE_04 culture concentration do not followed the general behaviour of all cultures and had a slightly decrease during these days. At 15th day of cultivation the culture started to show a yellow tonality that become gradually more evident along the days of cultivation. The number of foreign organisms (as bacteria and fungi) and cellular debris also started to increase along with the decrease of cellular pigmentation from the 15th day, and by the 18th day of cultivation, RE_04 culture showed a evident yellow colour and the culture was considered death. It was not possible to conclude about this culture death since any other culture (under the same conditions and with close values of concentration) showed these evidences but it was thought that this death was possibly related with nutrition factors (and the possible accumulation of some element above the toxicity threshold).

After RE_04 culture being considered death, the system of cultivation was cleaned and disinfected. In the 19th day of cultivation the RE_04 was re-inoculated through 63% (v/v) renewal of auxiliary culture as can be seen through the abrupt increase of RE_04 concentration presented in Fig. 4.12. In the same figure, it is possible to see that at 20th day of cultivation (start of DRMR phase) the concentration of all cultures is coincident. This intended fact is justified by the concentration adjust done in the day before (19th day) to start the DMRM phase with standardized culture concentrations.

Attending to culture concentration evolution during the DMRM phase presented in Fig. 4.12 it is possible to note that C3 cultures (RE_04, 07 and 10) showed a lower concentration and RE_04 culture was the culture that achieved the lower values of concentration and showed a slightly yellow tonality. It is also possible to note that, after one week of daily medium recycling, RE_04 culture did not followed the general concentration recovery behaviour during the days without renewal (26th and 27th day). During this first week of DRMR phase the referred culture also showed some non-healthy evidences: macroscopically the yellow tonality of the culture become more evident along the days, microscopically the cell pigmentation density become lower and the number of foreign organisms (particularly fungi and bacteria) increased gradually along the days, along these days it was also possible to note a lower (roughly null) nutrient consumption. At day 27 the RE_04 culture was considered death.

During the DRMR phase it is possible to see that C1 and C2 cultures show a very similar concentration evolution achieving a stabilization of concentration at the fourth consecutive medium recycling procedure (day 23), and showed a slightly increase of concentration in the next day even with renewal (day 24).

In the second week of DRMR phase it is possible to see that C3 cultures continued to achieve lower values of concentration than the other cultures which are were also lower than the achieved values in first week of daily medium recycling. Regarding this, it is thought that this fact is directly related with the nutritive media composition and it will be explained later in Section 4.2.7 with the aid of elemental analyses results.

It is also possible to see that RE_02 and RE_06 cultures showed a different concentration evolution than the respective cultures under the same conditions. During this week (27th to 31st day of

cultivation) it is possible to see that RE_02 culture had the higher and continuous fall of concentration which can be justified by inherent culture growth rate being lower than the applied renewal rate. It was observed that this culture also showed microscopic evidences of culture stress presenting a relative high number of foreign organisms (fungi and bacteria), cellular debris, and some microalgal agglomerates of small proportions in the last day of assay. These facts are referred in the literature [34] as typical medium recycling effects which in general, become more evident in the second week under this cultivation strategy. It is also possible to consider this fact as direct impact of the cultivation conditions.

Despite the lower concentration evolution, RE_06 culture did not show a continuous fall of concentration and microscopic evidences of culture stress during last week of the assay. This behaviour can be explained by the smaller RE_06 increase of concentration during the two days without renewal with medium recycling (25th and 26th days of cultivation).

After identifying possible reasons that led to culture lost it was possible to remove the data of cultures on death phase and treat only the representative points of the sample. As was previously referred in section 4.1.2, this type of data extends the error range and may compromise the assay results. Regarding this fact, and to provide a clarified presentation of the relation between the microalgal growth and the cultivation conditions impact the following figure (Fig. 4.13) represents the evolution of cultures concentration, organized and treated by growth condition during the daily renewal with medium recycling growth phase (DRMR phase).

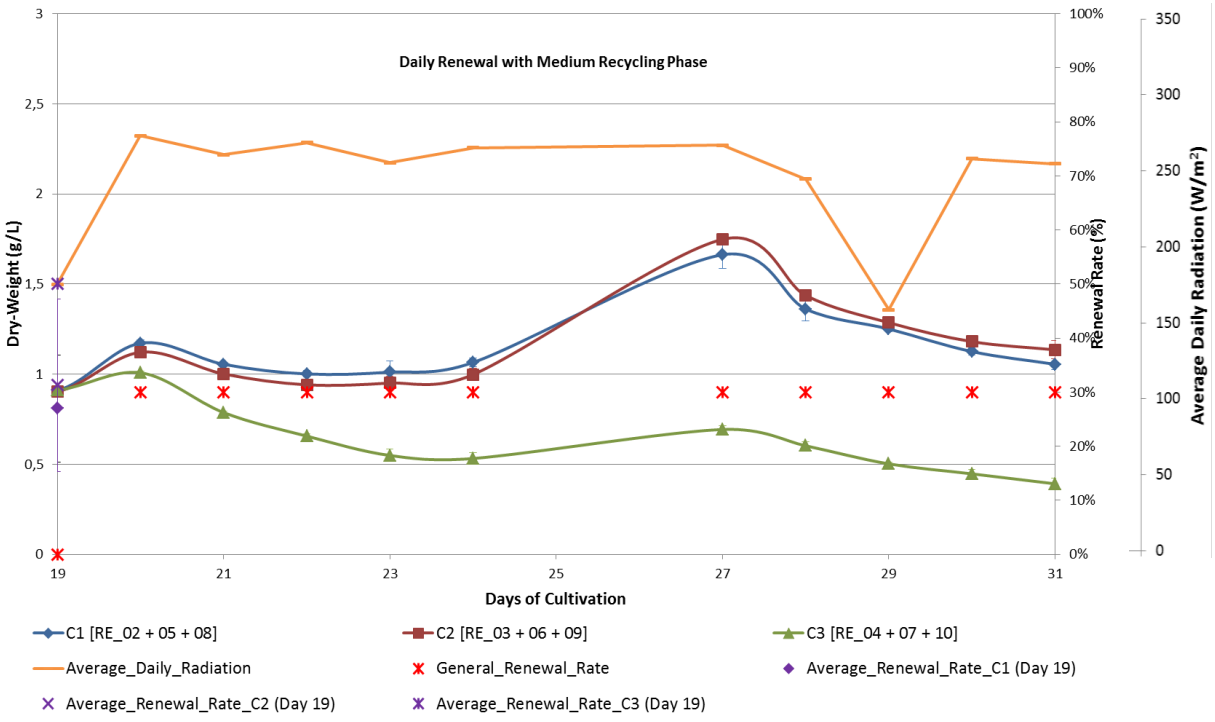

Fig. 4.13 – Presentation of culture concentration evolution during the daily renewal with medium recycling (DRMR) phase after data treatment. The data was organized in 3 sets of cultures that grown under the same cultivation condition.

At first sight, it is possible to observe the contrast between at all points considered in the culture concentration evolution (Fig. 4.12) and the equivalent assay 1 analysis (see section 4.1.2 – Fig. 4.3). More specifically, it is possible to observe less culture concentration variation which reflects the significant lower error (represented through error bars) achieved in all points considered in this analysis. It is thought that this is a direct consequence of operational procedures improvement during the present work that aimed to minimize the stimuli variance and the risk of biological contaminations. These improvements contributed to minimize the variation of culture growth behaviour (e.g. doing the culture concentration adjustment/standardization before the start of a different growth stage). Other factor that may have contributed for this fact was possibly the duration of cultivation, that was inferior in the assay 2 (31 days) than the assay 1 (49 days). It is also important to recall that the inferior duration of assay 2 was only due to the curricular internship deadline and was primarily planned to be extended for the same duration of assay 1.

Through a brief Fig. 4.13 analysis it is possible to notice that since the second day of DRMR phase C3 cultures presented a divergent concentration evolution until the end of the essay. During all days of this period, C3 cultures also achieved significant lower culture concentration values and frequently showed some stress indicators, specifically the cultures presented a yellow tonality. Concerning this fact it is expected that these cultures will present lower productivity values as well (see section 4.2.3). It is also important to recall that one C3 culture did not recovered from the first week of consecutive renewals with medium recycling and by the end of the assay there were only two C3 remaining cultures. It is though that this fact is directly related with the nutritive medium impact.

Attending to C1 and C2 concentration evolution presented in the Fig. 4.13, it is possible to note that both cultures presented similar growth behaviour during this phase. It is also possible to see that at some days of cultivation both cultures achieved concentration values within the same range of error. However, during the first week of DRMR phase, C1 cultures showed a slightly higher concentration values which can be explained through the possible negative acclimation behaviour of C2 cells in response of the simultaneous stimuli provided by the medium recycling strategy and the addition of chemical species through the exhaust medium disinfection treatment (and consecutive neutralization).

Through the Fig. 4.13 analysis it is also possible to note that, in the medium recycling days-off (day 25 and 26), C2 cultures presented a better recovery of concentration and by the 27th day, these cultures achieved slightly higher values of concentration. These specific values (day 27) are non-significant, but it is the start of the tendency of achieving higher values (that is also possible to see through the Fig. 4.13 analysis) during the second week of DRMR phase. On the other hand, some of these values are coincident with the values achieved by C1 cultures, specifically at day 28 and 29. With this graphical analysis it is not possible to create more in-depth conclusions about these two cultivation conditions (C1 and C2) impact in *N.oceanica* growth, but it is possible to note the tendency of C2 conditions providing higher culture concentrations if this regime was continued for more time. This tendency could be explained by the exhaust medium disinfection treatment contribution for limiting the negative growth impact of foreign organism proliferation and possibly degradation of auto-inhibitory compounds (mainly proteins and cellular debris) released to the culture medium after cellular

lysis. Further information about this hypothesis will be explained later in Section 4.2.5, with the aid of microscopic observation of cultures during the assay.

4.2.3 Evolution of culture productivity during the assay

It was only considered as significant the data which allow a representative analysis of the different conditions impact in the *N.oceanica* growth. Regarding this, it will be only presented the data from DRMR growth phase. This data is organized by evolution of cultures daily productivity (g $_{D.W.}$/L.day) and is presented in Fig. 4.14.

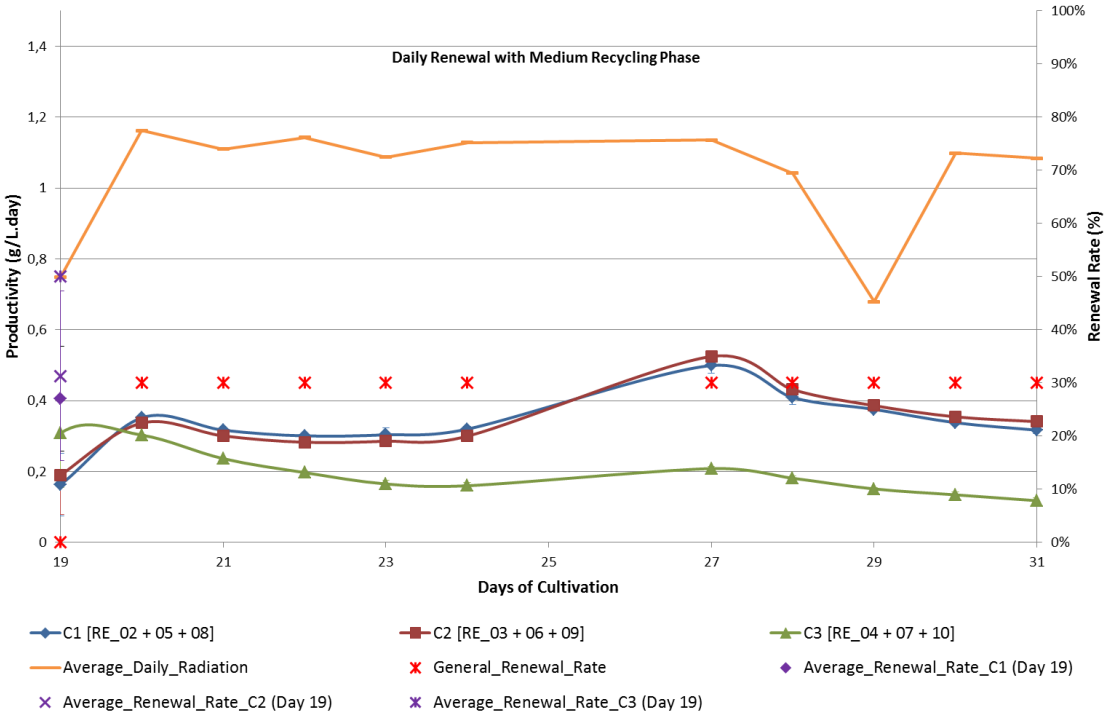

Fig. 4.14 – Presentation of culture productivity evolution during the daily renewal with medium recycling phase after the death phase data treatment. The data was organized in 3 sets of cultures that grown under the same cultivation condition.

Through the Fig. 4.14 is possible to note that the evolution of culture productivity is similar to the evolution of culture concentration presented in the Fig. 4.13 which can be justified by the equation involved in the productivity calculation (Equation 1 – Section 3.9.1.3).

As previously stated in Section 4.1.3 and can be seen through Fig. 4.14, after consecutive renewals, the cultures achieve a stabilization tier where the productivity is equal or slightly superior to the removed culture fraction through the renewal. This stabilization tier takes great importance to this work because it represents and mimics the harvesting process of industrial microalgal cultivation. This tier is also the analysis basis that allows the ultimate comparison between the tested cultivation conditions.

Through a brief Fig. 4.14 analysis it is possible to notice that C3 cultures only achieve the highest productivity in the first day of DRMR phase what can be justified by the higher renewal rate

applied to these cultures in the concentration adjustment process. As expected and previously noticed in Section 4.2.2 , in the remaining days of cultivation, C3 cultures showed a divergent productivity evolution achieving lower values of productivity. As previously referred, all C3 cultures frequently showed a yellow tonality which is considered a culture stress indicator. It was thought that this phenomenon is directly related to nutrition factors and specifically to the nutritive medium influence in the culture growth and will be discussed later in Section 4.2.6 with the aid of element analysis.

Continuing the productivity analysis of C3 cultures, and considering the achievement of the stabilization tier, it is possible to observe that these cultures also achieve the lowest stabilized productivity tiers and also need more time to achieve them. In the first and second week of DRMR phase these cultures achieve this tier at least, one day after the other cultures achievement.

It is also possible to observe that cultures need more time to achieve stabilization tier in the second week of DRMR than the first week. This fact may be explained by the high and constant solar radiation verified in the first week that contrasts with some variation verified in the second week. As previously referred (See section 1.4) the radiation is the major limiting growth factor for microalgal cultivation under autotrophic regime. Regarding this, it is possible to note the fall of average solar radiation verified in the day 29 that probably not allowed the properly microalgal growth and consequently not allowed the achievement of the stabilization tier.

Through Fig. 4.14 analysis it is possible to see that C1 (control condition) and C2 cultures achieve a very similar and consistent productivity evolution during the DRMR phase that is possibly related with the improvement of operational procedures and consequent minimization of stimuli variation. This fact is also reflected in the low variation of productivity values between the cultures under the same cultivation allowing the achievement of very low error range (see Section 4.2.2). Despite the similar productivity evolution between C1 and C2 cultures it is possible to note that C1 cultures achieve slightly higher values of productivity during the first week that can be related to the acclimation of cells from C2 cultures to addition of new chemical species used in the exhaust medium treatment (see Section 4.2.2). On the other hand, in the second week, it is possible to note that C2 cultures achieve slightly higher values of productivity in the majority of days (day 29 and 30 the achieved productivity values by C1 and C2 cultures are coincident in the considered error range) and it is possible to consider that C2 cultures show tendency of achieving higher productivity values if this phase would be continued for more time. This fact was previously discussed at Section 4.2.2 and could be related with the minimized medium recycling impact due to the disinfection process of culture media (minimization of biological contaminants and degradation of auto-inhibitory compounds).

Adapting the same analysis perspective of assay 1 (see Section 4.1.3), it is important to focus on the stabilization tiers achieved by cultures under different cultivation conditions and relate these conditions with the higher stabilized productivity achieved during this assay. Through the Fig. 4.14 analysis (and considering the assay 1 results) it is possible to see that cultures achieve this common equilibrium stage after 3 consecutive renewals so the calculation of the cultures productivity at this tier

considers the mean value of the daily productivity after 3, 4 and 5 renewals (average stabilized productivity).

The Table 4.5 present the information about the average stabilized productivity achieved by cultures under different cultivation conditions in the two weeks of DRMR phase. This table also presents the comparison between the average stabilized culture achieved in the first and second week. The former comparison will be presented in terms of percentage variance (Δ (%))

Table 4.5 – 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).

	Daily Renewal With Medium Recycling		Stabilized Productivity Variance
Condition	**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

Through a brief Table 4.5 analysis, specifically the first week of DRMR phase data, it is possible to observe that C1 and C2 cultures achieved the same average stabilized productivity value considering the error range. This result was previously expected because, if we recall the cultivation conditions description presented in the Section 4.2.1, it is possible to observe that before the DRMR phase start there was no difference between these set of cultures. Only in the 20th cultivation day the 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 and as was referred previously, 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. The impact of disinfection process will be discussed later in section 4.2.6 with aid of element 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.

As stated in the literature [32], [34], [61], 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 solar radiation between the 20th and 28th cultivation days 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 (27 and 28 day of cultivation).

Through the Table 4.5 analysis it is 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 (see Section 4.2.5). It is also important to consider that one C1 culture started to show significant evidences of cellular stress related with the medium recycling impact (see Section 4.2.2) during the second week of DRMR phase whereas none of C2 cultures showed them which gives strength to the former hypothesis.

Regarding these facts, 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.

4.2.4 Macroscopic observation of cultures

The Fig. 4.15 is a picture of the cultivation system taken at 27/08/2014 (22nd cultivation day of assay 1). At this point, the cultures were at the daily renewal phase with medium recycling phase of cultivation (DRMR phase) and it is possible to see the contrast between the typical healthy and concentrated *N.oceanica* cultures showing a dark green colour (RE_02, 03, 05, 06, 08, 09) and the typical cultures showing macroscopic evidence of cellular stress, specifically the previously referred yellow tonality of cultures (RE_04, 07 and 10). As can be seen at section 4.2.2, the C3 cultures started to show this yellow tonality in the start of DRMR phase that gradually become more evident and intense during the cultivation days of the assay (RE_04 was considered lost culture in the 28th cultivation day).

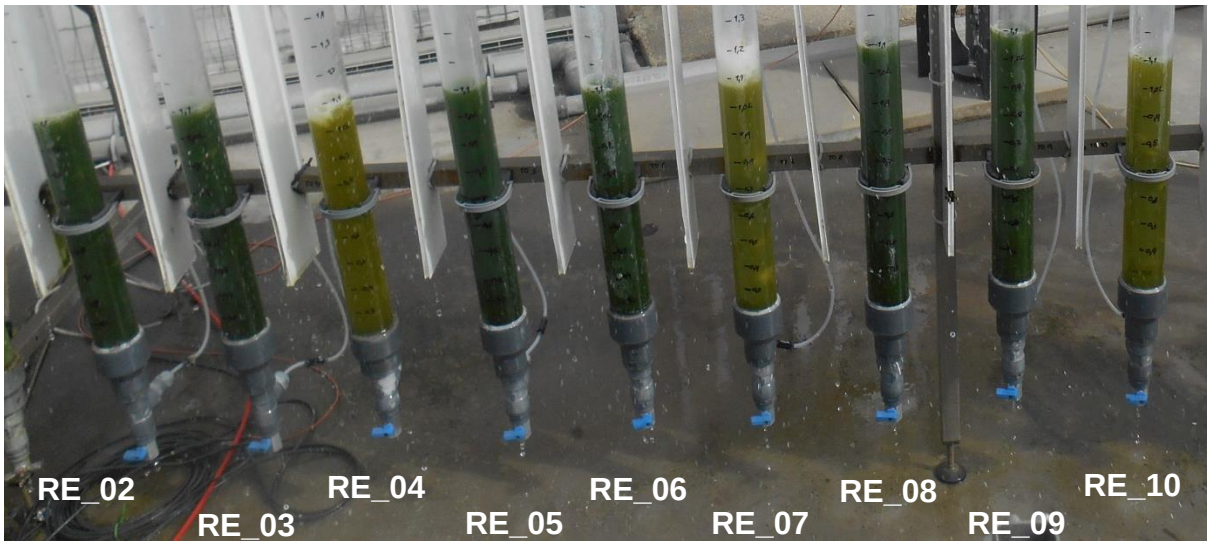

Fig. 4.15 – Presentation of cultures macroscopic observation at day 22 of cultivation of assay 2.

4.2.5 Macroscopic observation of cultures

The Fig. 4.16 shows two pictures taken at the microscopic observation of RE_09 culture at 03/09/2014 (29th day of cultivation). These pictures show a typical healthy culture.

Through Fig. 4.16 it is possible to see that cells show high pigmentation density (derived from chlorophyll) and a round linear shape of plasmatic membrane, it is also possible to note the absence of foreign organisms and cell agglomerates.

During the assay, all cultures were very similar from a general microscopic point of view showing a very small number of minor proportions contaminants (as fungi or bacteria) and in some occasions some cellular debris derived from the cellular lysis. On the other hand, with the start of DRMR cultivation phase also started some differentiation from a microscopic point of view. Along this phase it was also noticed that C3 cultures productivity gradually decreased and has been accompanied by the gradual decreasing of cell pigment density and by the increasing of plasmatic membrane roughness. In these situations it was not observed a significant increase of foreign organisms but, however, it was observed the increase of cellular debris prevenient from the cellular lysis. It is also important to refer that the exhaust medium of this cultures was disinfected during this phase, which probably justifies the non-appearance of significant biological contaminants. Regarding this, it was thought that the unhealthy microscopic state of C3 cultures was directly related with the nutritive medium impact and possibly lack or excess (above the toxicity threshold) of nutrients.

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.

Through the periodically microscopic observation of cultures, and as can be seen through Fig. 4.16 it was possible to note that all C2 cultures did not show the presence of significant biological contaminants and also not shown the accumulation of contaminants during the DRMR phase.

Regarding these facts, it was possible to consider the disinfection method in minimizing the impact of medium recycling strategy, and particularly in minimizing the appearance and proliferation of biological contaminants in the culture medium.

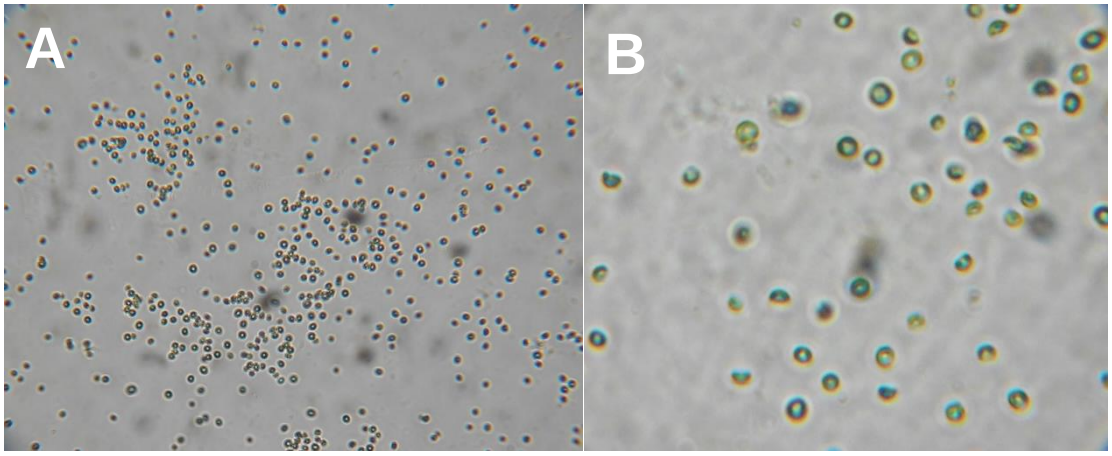

Fig. 4.16 – Example of typical microscopic observation of cultures during the assay 2. **A** – Photography of RE_09 microscopic observation taken at 03/09/2014 at 100x magnification. **B** – Photography of RE_09 microscopic observation taken at 03/09/2014 at 200x magnification. The presented small round green cells are *N.oceanica* cells with 2-5 µm of diameter.

4.2.6 Presentation of cultures supernatant element analysis

As previous done in the assay 1 analysis (see Section 4.1.7), the supernatant of cultures was collected in the last day of the assay (31st cultivation day) and sent to an external entity for element analysis. This type analysis provides information about the concentration of the principal elements present in the different culture medium that, after comparison with the respective theoretical concentration allow the calculation of concentration percentage of variance.

It is important to recall that theoretical concentration of supernatants was calculated considering the element analysis of all culture medium components (see section 3.6) analysis and the nutritive medium theoretical concentration acting like a quality control analysis. With this analysis it was possible to observe that CNASAM_3 showed non-significant deviations from the theoretical concentration. On the other hand, BIOFAT_2, industrial supplied medium, showed high deviations from the respective theoretical concentration, which could be explained by weighing errors, presence of element contaminations in the reagents and formation of precipitates occurred in the preparation process.

It is also important to recall that the results will be also expressed in terms of percentage variance of elements and the double character of this analysis. As previously explained (see Section 4.1.7), an element with 100% of accumulation means that the analysed concentration is twice as much as the theoretical concentration. In contrast, an element with -100% of accumulation represent that the

analysed concentration is null when compared with the theoretical concentration, which has a much more significant impact.

The main goal of this analysis is to find possible coincident exceeded or deprived concentration of some elements and after interconnection with the culture growth behaviour create the second input for possible optimized nutritive medium recipe for medium recycling cultivation. This recipe would contain the input data from the first and second assay.

In a general point of view, all cultures showed a very high accumulation of zinc and copper elements, these high values do not allow a properly graphical analysis. Regarding this fact, these two element analysis will be presented in separate figures.The concentration variance of the major elements in the all culture media during the assay 2 is presented in the Fig. 4.17 and Fig. 4.18.

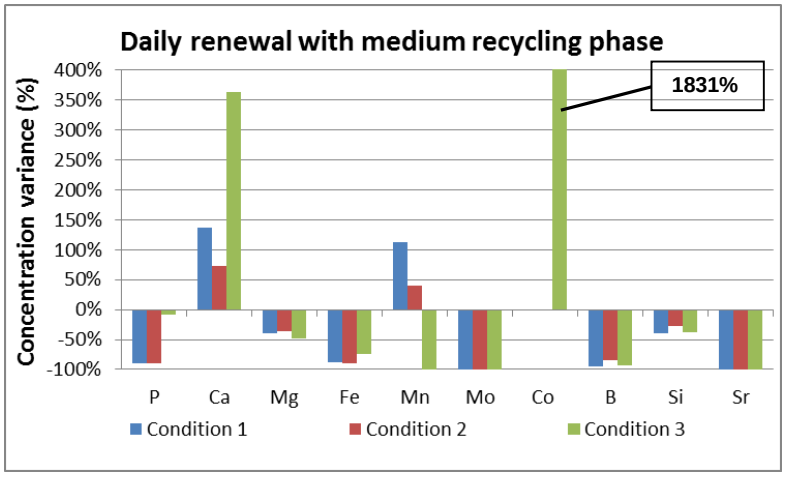

Fig. 4.17 – Presentation of the evolution of percentage of variance between the theoretical and the analysed concentration of the principal elements in the different cultivation conditions during the DRMR growth phase.

Before analysis it is important to recall that C1 and C2 cultures grown in the same culture medium and were complemented with the same formulation of nutritive medium (CNASAM_3) so it is expected a similar concentration variance of all analysed elements.

Attending to the phosphorous analysis, it is possible to notice the great deficiency of this element in the C1 and C2 cultures contrasting with the minor but no less significant phosphorous deficiency verified in C3 cultures. This fact can be explained by the previously stated relation between the cellular growth rate and phosphorous uptake. It is stated in literature [32] that at high growth rates the microalgae possibly become phosphorous-limited. This theory is coincident with the observed culture growth during the assay (see Section 4.2.2 and Section 4.2.3). C1 and C2 cultures which achieved the higher (and similar) growth rate during the assay, also present the higher phosphorous deficiency in this analysis. On the other hand, C3 cultures achieved a low growth rate during the assay and also showed a smaller phosphorous deficiency. However, the nitrogen/phosphorous has been object of several studies [32], [36], [56], and was achieved a well-defined relation between these nutrients. It is also stated that, for high productivity cultures the phosphorous should be supplied in small proportions. It is stated that these conditions favour an improved photosynthetic yield [47].

Regarding this, it is possible to consider the small increase of phosphorous concentration in a development of new nutritive medium formulation and test the respective culture growth impact.

Through a brief Fig. 4.17 analysis it is possible to observe that calcium is present in excess in all cultures but this excess is significantly higher in C3 cultures. Theoretically, the major source of calcium is present in the culture medium and not in the nutritive medium (see Section 3.6 and 3.7). Additionally, if we consider the medium recycling strategy procedure, only 10% (v/v) of the total recycled volume is composed by fresh medium it is possible to consider that the calcium excess verified in the C3 cultures may be related with some reagent element contamination involved in the industrial medium preparation. Nevertheless, it is possible to consider a small decrease of calcium concentration in the culture medium recipe (see Section 3.6) if the microalgal cultivation strategy will involve medium recycling.

Attending to Fig. 4.17, it is possible to see that all cultures showed a similar concentration deficiency of magnesium. Considering, that the magnesium source is present in the culture medium and all cultures presented the same magnesium theoretical concentration, the observed magnesium deficiency means that microalgal magnesium uptake is higher than the supplier rate. However, the element analysis showed that this element is present in the culture medium in great proportions, so it is possible to consider this magnesium deficiency as an non-significant value.

Through the Fig. 4.17 it is possible to note that iron concentration achieve a critical concentration deficiency in all cultures. The often reported phenomenon of iron precipitates formation in microalgal cultures, due to changes of the culture conditions [67] can be a legitimate reason that led to the verified iron concentration deficiency. This significant deficiency can be also justified by the high cellular uptake of iron. It has been reported on literature that high consumption of iron by microalgal cells is often related with the high levels of nitrates (nitrogen source) present in the culture medium. If it is considered that during this assay the nitrates where always present in excess (concentration set-point of 6 mM, see section 4.2.1), this referred hypothesis could be a legitimate reason for the verified iron deficiency. Regarding this, it is possible to include a significant increase of iron concentration in the next recipe for nutritive medium formulation.

Attending to the manganese concentration variance, it is possible to note a smaller excess present in the C1 and C2 cultures supernatant and a critical deficiency present in C3 cultures. The verified excess present in C1 and C2 cultures can be considered non-significant because manganese is supplied to the culture medium in small proportions. The element analysis also showed the manganese presence on the referred cultures in levels of concentration far away from the toxicity threshold [51]. Regarding this it is possible to consider that manganese has been supplied to the culture medium in the right proportions. On the other hand, the manganese concentration present in C3 cultures supernatant is null, which indicates that inherent cellular consumption is way far higher than the addition rate. It is referred in the literature that, micronutrients, such as manganese are very important for microalgal growth and, when present in small quantities, promote the culture growth. However, it is reported that when are in absence or in highly excess (above the toxicity threshold) they

represent a limiting growth factor and possibly lead to the culture lost [24], [32], [51], [56]. Regarding this fact, the high deficiency of manganese may have contributed for the lower C3 cultures productivity achieved during this assay (see Section 4.2.2).

Through the Fig. 4.17 analysis it is possible to observe the common and critical values of molybdenum concentration deficiency in all cultures supernatant. As previously stated the absence of micronutrients, such as molybdenum, can act like a growth limiting factor and promote the decrease of culture productivity. These values symbolize that the cellular consumption of molybdenum was significantly higher than the addition rate of the respective element. This growth limiting factor may have contributed for C3 cultures achieving lower productivities during the assay. Regarding this, it is possible to consider an increase of molybdenum concentration in the next step of nutritive medium recipe optimization.

It is possible to observe that cobalt analysis is not present Fig. 4.17 in the C1 and C2 cultures analysis. Cobalt theoretical concentration in C1 and C2 cultures is null which makes the respective determination of percentage variance impossible. However, the element analysis revealed a non-concerning accumulation of this element in these cultures supernatant during the assay. On the other hand, it was possible to note a significant cobalt concentration excess present in the C3 cultures supernatant. The presence of this micronutrient in high concentration has been reported in literature as a inhibitory growth factor for green algae. It is thought that this element have a inhibitory action in the photosynthesis which often lead to a productivity decrease and, in some cases can culminate in the culture lost [32], [36], [52]. Regarding this, it is possible to consider the inherent impact of the high concentration of cobalt on C3 cultures during the assay, and probably contributed for the achievement of lower productivities (verified in Section 4.2.3).

As previously, stated in the assay 1 element analysis (see Section 4.1.7), boron, silicon and strontium are not added specifically through the nutritive medium or other component of the culture medium, and all have a residual theoretical concentration which is reflected in the Fig. 4.17 analysis, which showed a non-concerning (and similar) deficiency percentage in the analysed supernatants. The boron and strontium importance for microalgae is not fully understood (see Section 1.6.2), and previous studies [32] showed that silicon is only relevant for diatoms cultivation, which allow concluding the non-relevance of this elements in *N. oceanica* cultivation.

In the following figure (Fig. 4.18) is presented the concentration variance of zinc and copper elements in the all culture media during the assay 2.

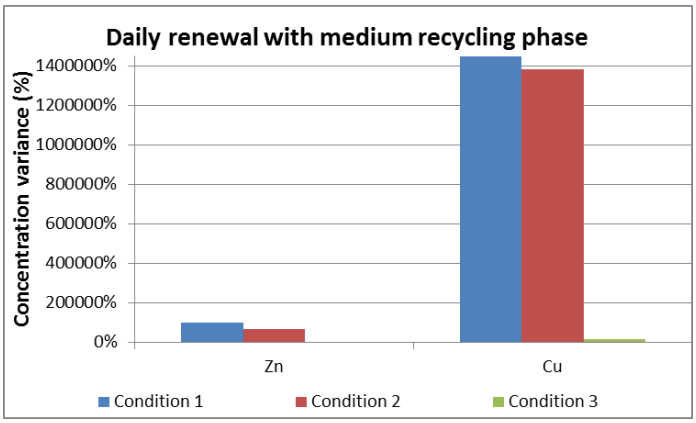

Fig. 4.18 – Presentation of the evolution of percentage of variance between the theoretical and the analysed concentration of zinc and copper elements in the different cultivation conditions during the DRMR growth phase.

Before the Fig. 4.18 is important to recall that copper is not specifically supplied to C1 and C2 cultures through the nutritive medium or other component of the culture medium (see Section 3.6 and 3.7), and have a residual theoretical concentration. Therefore, the high levels of copper accumulation showed in the Fig. 4.18 do not reflect high concentration on the referred cultures. Despite the addition of this element through the C3 nutritive medium (BIOFAT_2), this analysis showed non-significant accumulation of copper in the referred culture medium (lower than the toxicity threshold [68]). This analysis also shows that besides that, the addition rate is higher than the cellular copper uptake during the assay. Concerning this, it is possible to consider that copper should not be present in the nutritive medium composition. It is also important to refer that the C1 and C2 cultures (complemented with CNASAM_3) copper supply resides in the mineral solution 1 (MS_1). This solution is added in the preparation of the culture medium (see Section 3.6).

Attending to the zinc analysis presented in the Fig. 4.18 it is possible to note that this element achieve high accumulation percentage in the C1 and C2 culture medium. As previously, stated (see Section 4.1.7), zinc concentration is a limiting growth factor for microalgae when present above the toxicity threshold [51]. Through the element analysis it was possible to observe that this element was present in the referred culture media in concerning high concentrations. It was also not possible to prove this hypothesis due to the high productivity verified in C1 and C2 cultures in Section 4.2.3, but it should be considered a significant decrease of zinc concentration in a new recipe of nutritive medium.

Regarding this analysis, it was possible to consider that C1 and C2 cultures show less concerning accumulation or deficiency of elements in the respective culture medium, which give strength to the culture growth data analysed in sections 4.2.1 and 4.2.3 and possibly justifies the higher verified culture productivities. On the other hand, it was possible to relate the lower C3 culture productivities achieve during the essay (see Section 4.2.3) are possibly justified by the significant unbalance of nutrients verified through this analysis. In sum, it is possible to consider CNASAM_3 (present in C1 and C2 cultures – see Section 4.2.1) as the best balanced nutritive which achieved the higher productivities of *N.oceanica* cultures under industrial cultivation conditions.

Through this analysis it was not possible to detect some negative impact of the disinfection method applied to the exhaust medium of C2 cultures in the element accumulation or deficiency. It was verified that the C1 and C2 culture medium showed a very similar concentration variance. Regarding this, it is possible to consider that the tested disinfection method did not influence the nutrient uptake of *N.oceanica* cells under the considered cultivation conditions (further information will be presented later in this section).

After analysing the element analysis of all cultures from assay 1 and 2 (present in Section 4.1.7 and 4.2.6, respectively), it was possible to collect information about the needs and excesses of elements that culminate in the presentation of an optimized nutritive and culture medium recipe for *N.oceanica* cultivation. These new medium recipes should allow the improvement of *N.oceanica* cultures productivity under industrial cultivation conditions. Regarding this, in the following table (Table 4.6) it is presented the recipe for the reformulation of CNASAM_3 (CNASAM_3.1) and ASAM_2 (ASAM_2.2) medium. It is important to note that the adjustments are present in terms of percentage variance using the original CNASAM_3 recipe for comparison.

Table 4.6 – Reformulation of CNASAM_3 medium (**CNASAM_3.1**) for improved *N.oceanica* cultures under industrial conditions (with renewal without or with medium recycling); and reformulation of ASAM_2 medium (**ASAM_2.2**) for improved N.oceanica cultures productivity under industrial conditions (with renewal without or with medium recycling). 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%		

It is important to be referred that these element concentration modifications should be done separately, i.e. each modification should be analysed in specific cultivation tests. This procedure should allow the evaluation of cultures response to this specific stimuli, allow the better understanding of elements uptake by microalgal cells and the possible inherent impact of the deficiency or excess of elements in the culture medium.

To evaluate the accumulation of chemical species in the cultures supernatants and consequently the effect of the disinfection process it is legitimate to observe the elements present in the molecules added daily to disinfect the exhaust media. The disinfection active compound is the sodium hypochlorite (NaClO) and the respective neutralization active compound is the sodium thiosulfate ($Na_2S_2O_3$). It is not possible to follow the accumulation of elements such as Na or Cl. The

sodium (Na) and chlorine (Cl) are present in a great variety of compounds that are continuously added to the culture medium as well (e.g. sodium chloride is added to adjust the salinity of all culture media up to 30 g/L concentration). Besides the supplementation of some sulphates (added in the formulation of nutritive medium), sulphur (S) is added on a daily basis through sodium thiosulfate during the DRMR cultivation phase. In the Fig. 4.19 the detected concentration of sulphur in the cultures supernatants is presented.

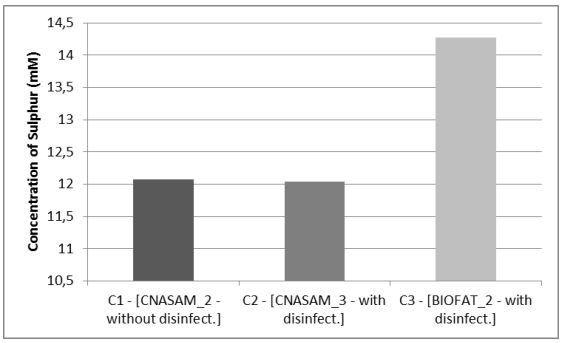

Fig. 4.19 – Presentation of the sulphur concentration detected through the element analysis of supernatants of cultures grown in the different cultivation conditions.

Through the Fig. 4.19 it is possible to see that the sulphur concentration is present in all supernatants in the same tier of 12 – 14 mM. This similar range of concentration possibly verified for all supernatants means that the sodium thiosulfate daily addition during the DRMR does not have influence in accumulation of elements, particularly in the sulphur element. It is also important to refer that sulphur in thiosulfate is added through a solution (see Section 3.1) at 150 g/L of sodium thiosulfate which is daily added to the exhaust culture media in a proportion of approximately 57 µL of sodium thiosulfate solution per Litre of exhaust medium. It is also important to consider that the sulphur is present in the mineral solution 1 at great proportions which is added to the culture medium formulation in a proportion 1,5 % (v/v), the culture medium volume is also stored at 50 ppm concentration of sodium hypochlorite and it is neutralized through the addition of thiosulfate solution (150 g/L) before the respective sterilization and usage (see Section 3.6). Regarding this, it is possible to consider 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.

On the other hand, it should be considered to repeat this assay under the predicted conditions (see Section 4.2.1) in order to do a more accurate analysis of the impact of the exhaust medium disinfection process in long-term cultivations of *N.oceanica*.

4.3 Assay 3 – Evaluation of the impact of different supply of sodium chloride in industrial Nannochloropsis oceanica cultivation

As previously stated (see Section 4), 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. As previously referred, this medium has the same formulation as ASAM_2 and the only difference is present in the sodium chloride (NaCl) supply (see Section 3.6). The ASAM_2 contains a very pure sodium chloride source, intended for laboratory use which is a costly but very reliable option, on the other hand ASAM_2.1 has an industrial sodium chloride source and it is formulated with a purified seawater sodium chloride. The former option represents a significant cost reduction to the production process but may have some draw-backs such as element contaminations that may result in the excessive accumulation of non-consumed elements in the culture media. As previously referred these elements contaminations, when present in concentrations above the toxicity threshold, may affect the microalgal growth, the cultures productivity and possibly culminate in loss of cultures.

Concerning this it is important to evaluate the impact of this new source of sodium chloride through the analysis of the culture growth and productivity throughout the two assays. It is also important to evaluate if it is possible to relate the verified accumulation of elements in the analysed supernatants with this source of sodium chloride.

In order to clarify the analysis basis it will be presented in the following table (Table 4.7) the assay 1 and 2 cultivation conditions. Additional information about the assay 1 and 2 conditions was previously expressed in Section 4.1.1 and 4.2.1.

Table 4.7 – Presentation of cultivation conditions of assay 1 and 2.

Condition	1			2			3		
Assay 1									
Reactor	RE_02	RE_05	RE_08	RE_03	RE_06	RE_09	RE_04	RE_07	RE_10
Nutritive Medium	CNASAM_2			CNASAM_3			BIOFAT_2		
Culture Medium	ASAM_2						ASAM_2.1		
Assay 2									
Reactor	RE_02	RE_05	RE_08	RE_03	RE_06	RE_09	RE_04	RE_07	RE_10
Light Source	CNASAM_3						BIOFAT_2		
Culture Medium	ASAM_2.1								

Through the Table 4.7 it is possible to observe that in the assay 1 there were cultivated 2 set of cultures with ASAM_2 (C1 and C2 cultures) and one set with ASAM_2.1 (C3 cultures). During the assay 2, the cultures were cultivated only through one culture medium formulation (ASAM_2.1).

4.3.1 Analysis of the culture growth evolution during assay 1 and 2

Through the analysis of cultures concentration evolution during the **assay 1** (see Section 4.2.2) it was observed that the all cultures showed a similar growth behaviour in the DR phase and during the DRMR phase the cultures that showed the best concentration evolution were the C2 [CNASAM_3 + ASAM_2] cultures (see Section 4.2.2). The C1 cultures [CNASAM_2 + ASAM_2] were all lost in the second week of DRMR phase due to possible nutritive factors and the C3 cultures [BIOFAT_2 + ASAM_2.1] showed lower levels of concentration during this phase but by the end achieved approximately the same level of concentration than C2 cultures. The analysis of cultures productivity during the same period (see Section 4.2.3) have focus in the productivity achieved by the cultures at steady state (average stabilized productivity) after consecutive renewals without and with medium recycling (DR and DRMR phase, respectively). This information is presented in Table 4.8.

Table 4.8 – Presentation of average stabilized productivity values during assay 1.

	Daily Renewal Phase	Daily Renewal With Medium Recycling	
Condition	**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)**
1 – [CNASAM_2 + ASAM_2]	0,466±0,164	0,258±0,102	–
2 – [CNASAM_3 + ASAM_2]	0,499±0,048	0,378±0,020	0,169±0,029
3 – [BIOFAT_2 + ASAM_2.1]	0,529±0,085	0,229±0,104	0,109±0,023

Through the analysis of Table 4.8 it is possible to note that as expected the average stabilized productivities of ASAM_2 and ASAM_2.1 cultures are coincident or very similar if it is considered the error range in the different growth phases. During the DR phase all cultures achieve the same value within the error range. The cultures that show more differentiation in the steady state achieved in the first week of DRMR phase are C2 cultures. Despite this cultures were grown ASAM_2 it is not possible to relate this difference with the inherent culture medium they have grown due to the fact that C1 [ASAM_2] have grown in the same culture medium and showed a coincident value with C3 cultures that have grown in ASAM_2.1 medium. It is also possible to see that C2 and C3 cultures achieve an approach of the average stabilized productivity in the second week of DRMR phase which gives strength to the former hypothesis.

It is important to focus on the same values of average stabilized productivity achieved by all cultures during the assay 2 in order to compare the productivity achieved by *N.oceanica* cultures that have grown exclusively with ASAM_2.1 medium. It is also important to refer that during the assay 2 the cultivation did not contemplate the DR phase and it is only possible to compare the data from the DRMR phase. This information is presented in Table 4.9.

Table 4.9 – Presentation of average stabilized productivity values during assay 2.

	Daily Renewal With Medium Recycling Phase	
Condition	**Week 1 – Average stabilized productivity ($g_{D.W./}L.day$)**	**Week 2 – Average stabilized productivity (g D.W./L.day)**
1 [CNASAM_3 + ASAM_2.1– without DP]	0,308±0,018	0,343±0,009
2 [CNASAM_3 + ASAM_2.1– with DP]	0,289±0,018	0,363±0,007
3 [BIOFAT_2 + ASAM_2.1 – with DP]	0,174±0,005	0,134±0,009

Through the Table 4.9 it is possible to see that C1 and C2 cultures, both grown with ASAM_2.1 are capable to achieve higher values of productivity than the assay 1 cultures in the second week of DRMR. It is very important to note that this data should not be directly compared with assay 1 data since these cultures grown under different cultivation conditions and the major growth limiting factor, the solar radiation, was also different during this assay. Regarding this the comparisons done in this part of the work are only qualitative. However it is possible to note observed that the cultures that shown lower values of productivity, C3 cultures, and achieve the same level of values than the cultures in the assay 1 that were under the same nutritive conditions (BIOFAT_2 + ASAM_2.1). The C1 cultures were complemented with CNASAM_3 and did not have the possible interference of the disinfection process. Considering this it is possible to do a comparison between the productivity of C2 cultures during the assay 1 (CNASAM_3 + ASAM_2) and the productivity of C1 cultures during the assay 2 (CNASAM_3 + ASAM_2.1). Observing these values it is possible to confirm that both cultures are part of the cultures that achieve the higher productivities and it is possible to observe that, theoretically, this cultures only differ in the NaCl supply of culture medium. Regarding this, considering the data from assay 1 and 2 it is not possible to verify a negative impact of the new supply of NaCl [ASAM_2.1] on *N.oceanica* cultures growth in comparison with the laboratorial NaCl supply [ASAM_2].

On the other hand, to do a more in-depth analysis and to obtain more accurate results this test should be repeated but under different conditions. There have to be a control condition that represents cultures grown with ASAM_2 medium and another condition that must be another set of cultures grown with ASAM_2.1. It is important to consider that these two sets of cultures must be cultivated simultaneously and must be complemented with the same nutritive medium.

4.3.2 Element analyses of supernatants from cultures in assay 1 and 2

Considering the supplier quality control analysis it is possible that the industrial NaCl contains residual concentrations (less than 2 ppm) of lead (Pb), copper (Cu), calcium (Ca) and magnesium (Mg). Through the element analysis of supernatants from cultures in assay 1 and 2 with special attention to these elements it could be discussed the possible accumulation of these elements.

Attending to the Pb analysis it was observed that all analysed supernatants had the same level of accumulation during assay 1 and 2. The values of Pb concentration detected were all residual and close to the limit of detection of the element analysis (10^{-3} ppm) which excludes the NaCl industrial supply as source of Pb accumulation.

Considering the element analysis of supernatants from cultures in assay 1 and 2 (see Section 4.1.7 and 4.1.6, respectively) for Cu, Ca and Mg it is possible to observe that these elements showed high levels of accumulation in all supernatants. In the case of Cu, it was possible to observe that in spite of the high levels of accumulation the detected values were all above the referred toxicity threshold. Attending to the concentration variance of Mg, it was possible to notice that despite the general excess verified in the supernatants this element was also at great proportions in the culture media. It is also important to recall that the major source of this element is the mineral solution 1, which has highly concentrations of this element (see Section 3.6) and makes difficult to relate the residual concentration present in NaCl salt as a possible source of Mg accumulation in the culture media. The case of Ca is similar to Mg, Ca is added individually and in high concentrations (9,97 mM) through the formulation of the culture medium. Despite the high accumulations of this element verified through the element analysis of supernatants it is not possible to directly relate the calcium concentration present in purified seasalt (NaCl) as a contribution for accumulation of this element in the culture medium.

Regarding all these facts it is not possible to relate the ASAM_2.1 medium as source of elements accumulation in the culture medium and it is also not possible to relate this medium with the loss of productivity of *N.oceanica* cultures under the tested cultivation conditions.

5 Conclusions

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±0,085 g $_{D.W.}$ /(L.day) under the renewal without medium recycling strategy and 0,378±0,020 g $_{D.W.}$ /(L.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 [20], [32], [47].

Through this work it was also possible to consider the laboratory-scale PBRs as legitimate solution for reproducing the industrial cultivation conditions in a laboratorial scale and in some way prevent the possible scale-up problems after adapting the optimization conclusions achieved in lab-scale PBRs cultivation tests. However, it is possible to consider some modifications to the present technology in these cultivation systems in order to favour the reproducibility of the achieved results. It should be considered the implementation of a real time pH measurement system. This system would allow minimizing the opening of reactors for collection of samples which results in minimizing the risk of biological contaminations. On the other hand, this technology drawback was overcome through the maintenance of an additional culture under the same cultivation conditions as assay cultures. Through this solution it was possible to collect culture samples to control pH and temperature without affecting the assay cultures. This acclimated culture was also used as back-up for re-inoculation procedures during the assays.

Through the elemental analysis it was possible to observe the accumulation or excess of nutrients in the cultures supernatants and perform the reformulation of the nutritive and culture medium recipe. The modifications done to the element concentration should be tested separately to create an accurate evaluation of the impact of these concentrations on *N. oceanica* cultures and also guarantee that none of the elements are added in excess or in the deficiency. After these individual tests the new formulations are able to be correctly applied to the industrial scale cultivations.

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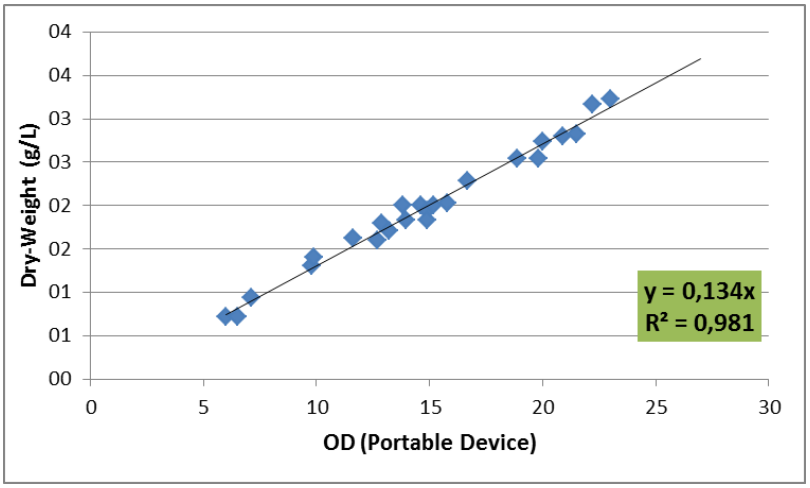
7 Appendix

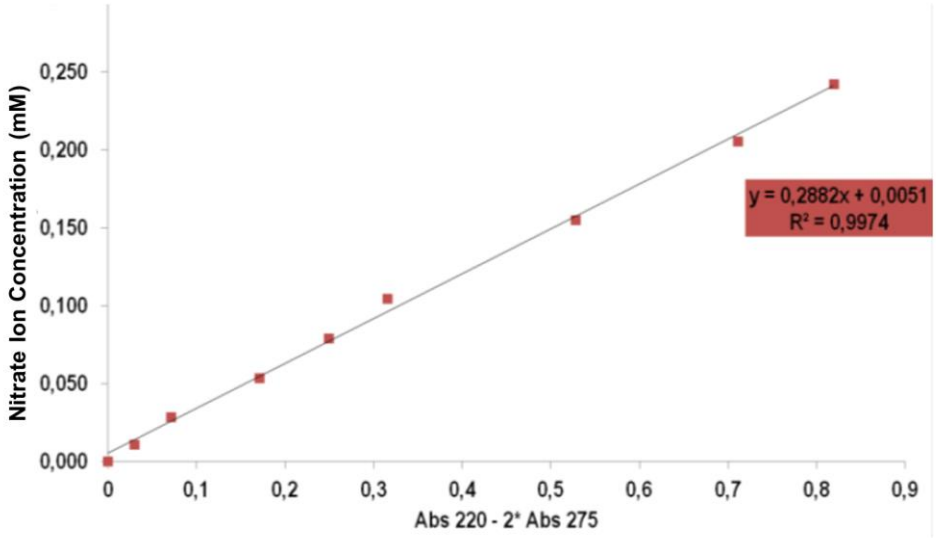
7.1 Fatty acid content and percentage of total fatty acid of dry weight of *Nannochloropsis oceanica* [17]

Fatty acid	Fatty acid distribution (percent of total by mass)
C6:0	0.15
C8:0	0.17
C10:0	0.34
C12:0	0.32
C14:1 (cis-9)	0.10
C14:0	5.36
C15:0	0.65
C16:1 (cis-9)	27.51
C16:0	33.76
C17:0	0.35
C18:2 (cis-9, 12) (omega 6)	1.35
C18:1 (cis-9) (omega 9)	8.87
C18:0	0.83
C20:4 (all cis-5, 8, 11, 14) (omega 6)	0.95
C20:5 (EPA) (all cis-5, 8, 11, 14, 17) (omega 3)	18.38
C20:3 (cis-8, 11, 14) (omega 6)	0.33
C21:0	0.58
Total	1

7.2 Relation between OD measured with the portable optical density meter (CO 8000 – WPA) and the culture concentration in dry-weight (g D.W./L)

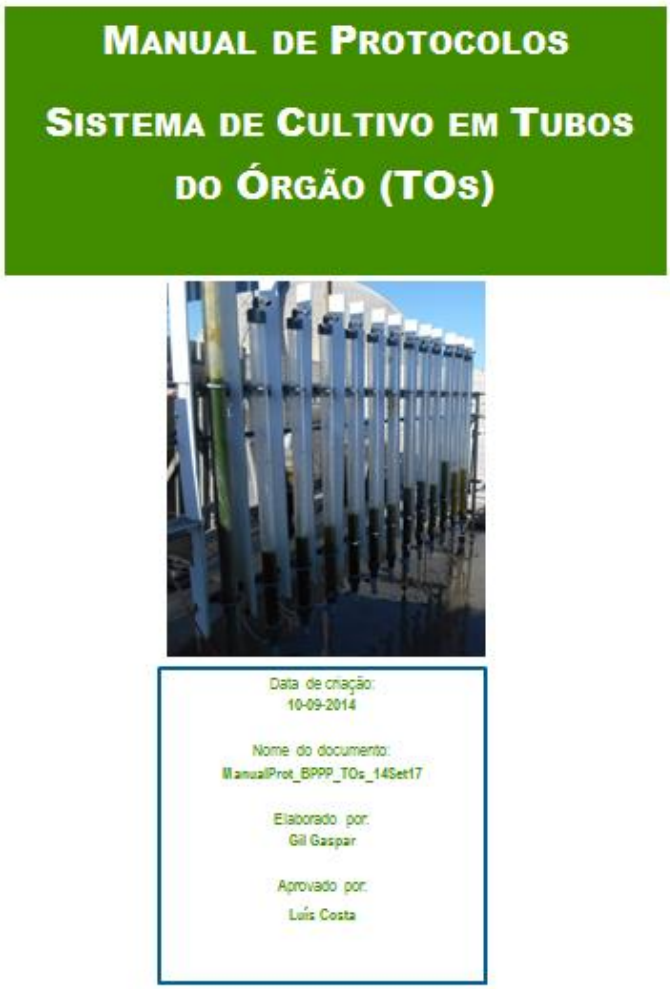


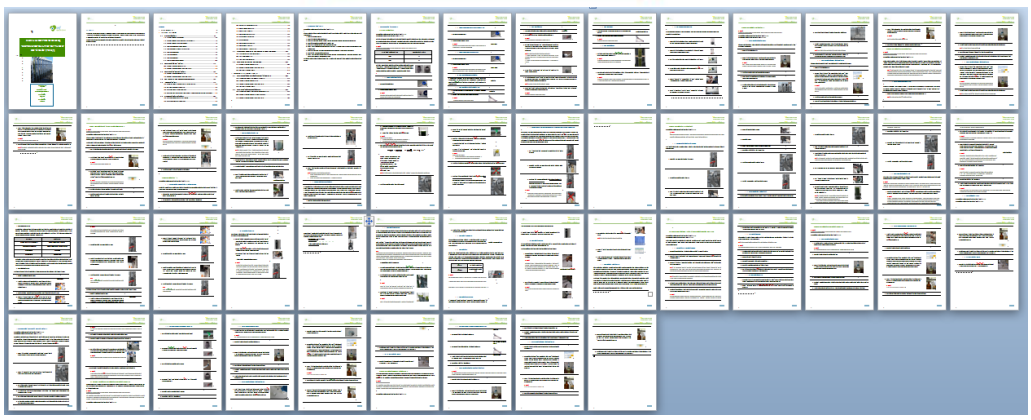
7.3 Calibration curve for determination of nitrate ion concentration



7.4 Manual of Operational Procedures for Laboratory-Scale PBRs cultivation

This document was developed during the curricular internship but it is property of A4F – Algafuel. For confidentiality reasons it would be only presented the first page and the layout of the document.





7.5 Manual of operational procedures for determination of lipid content of *Nannochloropsis oceanica*

As occurred for the former manual of procedures, only the document layout of the manual for determination of lipid content of *Nannochloropsis oceanica* is presented.