Integrated Master in Bioengineering – Biological Engineering

Process optimization for *Nannochloropsis* spp. biomass production

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Dissertation for the Degree of Master in Bioengineering

Performed at Algafarm

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June 25th, 2018

"Knowing is not enough; we must apply.

Willing is not enough; we must do."

Johann Wolfgang von Goethe

Acknowledgments

Despite the cliché, indeed, we never walk alone. There are always teams in which we are included: we are part of working teams, friend groups, a family. For all these groups of persons that surrounded and supported me along this period I would like to send my acknowledgements and highlight their importance for the final shape of this work.

My salutations are extended to both of my supervisors that, in cooperation, gave me opportunity to perform my master thesis project in a company and applied their working experience in this field to overcome the obstacles found along the journey.

A special greeting to Algafarm that besides the warm reception in the facilities, afforded the accommodation and food expenses.

This work was in part funded by projects (i) POCI-01-0145-FEDER-006939 (Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, UID/EQU/00511/2013) - funded by FEDER through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) – and by national funds through FCT - Fundação para a Ciência e a Tecnologia and (ii) NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO-INNOVATION, funded by FEDER - Fundo Europeu de Desenvolvimento Regional, through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and Programa Operacional Regional do Norte (NORTE2020).

Declaration

I declare, under an honour pledge, that this work is original and that all non-original contributions were appropriately referenced with source identification.

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Signature and date

Abstract

Several external conditions are manipulated to stimulate microalgae growth and high-value biomass production. Among the possible attempts, this work was focus on the influence of different scale reactors on *Nannochloropsis* spp. growth kinetics, pioneering experiments with microalgae polycultures, and biomass production using alternative growth media. *Nannochloropsis* spp. strains showed different global productivities at the lowest and highest scale evaluated, however, at the pilot scale the strains were not differentiated by their growth performances. Along the scale-up, the overall decreases on the maximum productivity and maximum specific growth rate suggest that the reactors design had significant influence on these parameter results. The ω-3/ω-6 ratio was higher than 2 for all the *Nannochloropsis* spp. strains and eicosapentaenoic acid (EPA) had the highest percentage in their fatty acid profiles. The biochemical composition of *Nannochloropsis* spp. proved that these microalgae biomass is a promising vegetable source of high-value compounds. Polycultures strategies induced a stressful growth condition that did not enrich the biomass composition. The presence of the shortest and mainly saturated fatty acids in the polycultures biomass, suggests that microalgae pursued a membrane fluidity adaptation, by increasing its stiffness. However, in terms of cell number, the polyculture of *Nannochloropsis* sp. and *Phaeodactylum tricornutum* reached an equilibrium which means that none of the species was inhibited in the mixture. The analysis of the growth efficiency as a function of the substrate consumption revealed that 0013NN and the polyculture of this strain with *P. tricornutum* were the microalgae cultures that used the larger substrate fraction for biomass production. The use of a saline effluent provided positive results for *Nannochloropsis* sp. growth. The higher global productivity and specific growth rate in comparison with the company standard growth medium are promising results for a larger scale application of this effluent for microalgae production. The outcomes of this work indicate that the process optimization for biomass production is dependent on engineering modifications in the reactors design and monitorization of the growth kinetics, as well as the evaluation of the biomass biochemical composition over the cultivation period. Industrial wastewater streams are promising candidates for a cost-effective microalgae production.

Keywords: Alternative growth media, biomass productivity, fatty acid profile, *Nannochloropsis* spp., polycultures, reactor designs

Resumo

Várias condições externas são manipuladas para estimular o crescimento de microalgas e a produção de biomassa de alto valor. De entre as possíveis tentativas, este trabalho teve como foco o estudo da influência de reatores de diferentes escalas na cinética de crescimento de três estirpes de *Nannochloropsis* spp., experiências pioneiras com policulturas de microalgas e produção de biomassa usando meios de crescimento alternativos. As estirpes de *Nannochloropsis* spp. apresentaram diferentes produtividades globais na menor e maior escala avaliadas, no entanto, as estirpes não foram diferenciadas pelas suas performances de crescimento na escala piloto. Ao longo do aumento de escala, as diminuições tanto da produtividade máxima como da taxa específica de crescimento máxima sugerem que o design dos reatores teve influência significativa nos resultados. O rácio ω-3/ω-6 foi superior a 2 para todos as estirpes de *Nannochloropsis* spp., e o ácido eicosapentaenóico (EPA) apresentou a maior percentagem nos seus perfis de ácidos gordos. A composição bioquímica das estirpes de *Nannochloropsis* spp. provou que a biomassa destas microalgas é uma promissora fonte vegetal de compostos de alto valor. As estratégias com policulturas induziram uma condição de stress que não enriqueceu a composição da biomassa. A presença dos ácidos gordos mais curtos e, principalmente, saturados na biomassa das policulturas sugere que as microalgas reagiram a esta condição de crescimento através de uma adaptação da fluidez da membrana, aumentando a sua rigidez. No entanto, em termos de número de células, a policultura de *Nannochloropsis* sp. e *Phaeodactylum tricornutum* atingiu um equilíbrio, o que significa que nenhuma das espécies foi inibida na mistura. A análise da eficiência de crescimento em função do consumo de substrato revelou que a estirpe 0013NN e a policultura desta microalga com *P. tricornutum* foram as culturas de microalgas que utilizaram a maior fração de substrato para a produção de biomassa. O uso do efluente salino proporcionou resultados positivos no crescimento de *Nannochloropsis* sp. A maior produtividade global e a maior taxa específica de crescimento obtidas com este efluente em comparação com o meio de crescimento standard usado pela empresa são resultados auspiciosos para a sua aplicação na produção de microalgas em maiores escalas. Os resultados deste trabalho indicam que a otimização do processo de produção de biomassa é dependente de modificações no design dos reatores e monitoração da cinética de crescimento da microalga, bem como a avaliação da composição bioquímica da biomassa ao longo do período de cultivo. Os efluentes industriais são fortes candidatos para uma produção de microalgas economicamente mais lucrativa.

Palavras-chave: Design de reatores, meio de crescimento alternativo, perfil de ácidos gordos, policulturas, produtividade de biomassa, *Nannochloropsis* spp.

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Nomenclature

C12:0	Dodecanoic acid	Lauric acid
C14:0	Tetradecanoic acid	Myristic acid
C14:1ω-5	Tetradecenoic acid	Myristoleic acid
C15:0	Pentadecanoic acid	Pentadecylic acid
C16:0	Hexadecanoic acid	Palmitic acid
C16:1	Hexadecenoic acid	Palmitoleic acid
C16:2	Hexadecadienoic acid	
C16:3 ω-3	Hexadecatrienoic acid	Hexadecatrienoic acid (HTA)
C16:4	Hexadecatetraenoic acid	
C17:0	Heptadecanoic acid	Margaric acid
C17:1	Heptadecenoic acid	
C18:0	Octadecanoic acid	Stearic acid
C18:1ω-9	Octadecenoic acid	Oleic acid
C18:2 ω-6	Octadecadienoic acid	Linoleic acid (LA)
C18:3 ω-3	Octadecatrienoic acid	α-linolenic acid (ALA)
C18:3 ω-6	Octadecatrienoic acid	ɣ-linolenic acid (GLA)
C18:4 ω-3	Octadecatetraenoic acid	Stearidonic acid (SDA)
C20:1 ω-9	Eicosenoic acid	Gondoic acid
C20:3 ω-6	Eicosatrienoic acid	Dihomo-ɣ-linolenic acid (DGLA)
C20:4 ω-6	Eicosatetraenoic acid	Arachidonic acid (ARA)
C20:5 ω-3	Eicosapentaenoic acid	Eicosapentaenoic acid (EPA)
C22:5 ω-3	Docosapentaenoic acid	Docosapentaenoic acid (DPA)
C22:6 ω-3	Docosahexaenoic acid	Docosahexaenoic acid (DHA)
C24:0	Tetracosanoic acid	Lignoceric acid
FAME	Fatty acid methyl ester	
GC-MS	Gas chromatography-mass spectrometry	
GWP	Green wall panel	
MUFA	Monounsaturated fatty acid	
PBR	Photobioreactor	
PMMA	Polymethyl methacrylate	
PUFA	Polyunsaturated fatty acid	
SFA	Saturated fatty acid	
TAG	Triacylglycerol	

1. Introduction

Algafarm is an industrial-scale microalgae production facility for production of *Chlorella* biomass for food and feed applications (Fonseca et al. 2016). The company integrates the entire production process, from small volume production at laboratory scale to the final product packaging.

The primary objective of the group was to study the CO_2 fixation by microalgae, in order to accomplish the targets proposed by the European Commission on the reduction of greenhouse gases emissions until 2030 (Silva et al. 2017). Owned by Secil and located in Pataias, Leiria (Portugal), Algafarm (Figure 1.1) is the largest European industrial unit of microalgae production in closed cultivation systems.

Figure 1. 1. Industrial facilities of Algafarm.

After the main goal that boosted the company development, Algafarm has been facing different challenges along its evolution. Besides the participation in European projects, the company is also directly linked with clients and consumers that have specific needs and requirements. For instance, one of the most recent on this concern consists on the increase of vitamin B_{12} percentage in the microalgae dried biomass. Growth medium supplementation with cobalt and microalgae growth in biological medium are ongoing experiments in this field.

However, to assure the company successful development, market tendencies must be studied and followed. Moreover, opportunities that can be profitable to the company and allow the recognition of the research and development department are promising. In this context, the present focus and consequent motivation to this work consist in the increase of the production rates of *Nannochloropsis* spp. to competitive values and the enhancement of these microalgae nutritional value.

The internal regulation pathways of microalgae play a pivotal role on the process optimization approaches for biomass production. Both the growth dynamics and the high-value compound synthesis metabolic networks are activated as a function of external factors.

In fact, the low affinity with external carbon sources registered for *Nannochloropsis* spp. justifies the uncommon practise of heterotrophic growth strategies as a rapid and effective method to achieve higher cell densities (Vazhappilly et al. 1998). However, high productivities are commercially attractive given the remarkable high-value composition of this microalgae species biomass, mainly as a consequence of the lipid content and fatty acid profile (Marudhupandi et al. 2016; Ma et al. 2014).

To overcome the limitations of *Nannochloropsis* spp. growth kinetics, photoautotrophic growth experiments with different reactor designs and different cultivating conditions were studied in this work to evaluate the larger scale growth viability. Besides, polyculture systems of saline microalgae, *e.g. Nannochloropsis* sp. and *Phaeodactylum tricornutum*, were performed in order to analyse whether a mutualism between microalgae could lead to a productivity increase and a commercial value enrichment of the final dried biomass.

Associated with significant productivities and its implementation attempts at higher scale reactors are the ever-increasing production costs. These inevitable expenses must be minimized to increase the revenues and to allow a cost-effective upstream. Thus, the influence of alternative growth medium reactants in the growth behaviour of the microalgae was studied by means of adaptions of the standard growth medium used by the company. Given the salinity growth conditions required for the microalgae growth, a synthetic saline water based on a shrimps' cultivating medium and a saline effluent from a codfish treatment company were used. The maximization of *Nannochloropsis* spp. productivity with wastewater is an attractive and sustainable procedure given the resources valorisation and the circular economy established.

Only an optimal balance between all the studied variables, *i.e.* reactor configuration operating at the highest energetic efficiency, symbiotic interdependence of the polyculture system and a cost-effective growth medium allow a fruitful outcome and guarantees a comfortable and favourable position for a market penetration.

The thesis structure is divided in an introductory state of the art assembling the theoretical knowledge about the biological pathways and some of the reported results on microalgae growth under different conditions. Then, the methodology used for the growth parameters calculation and the biochemical composition assessment of microalgae biomass are described, followed by the presentation of the experimental results and discussion. The last sections consist in the general conclusions that were drawn based on the results and future perspectives for further experiments in this field.

2. State of the art review

The present section is constituted by a structured view of the fields of study. A literature survey on these subjects, *i.e. Nannochloropsis* spp. morphology and biochemical composition, cultivation conditions, different reactor designs as innovative attempts to increase biomass productivity, the polyculture systems reported achievements, the market value and future commercial perspectives led to this critical review in the context of the stated research question and related topics.

2.1. Microalgae – Brief introduction to *Nannochloropsis* spp.

Algae are photosynthetic eukaryotes with high atmospheric carbon fixation rate. They assimilate about 50% of the planet's atmospheric carbon, and a main contribution to this percentage is their colonization of the oceans (Field et al. 1998). Generally, algae are classified as microalgae (unicellular organisms) or macroalgae (multicellular organism). These photosynthetic organisms are deprived of typical components present in photosynthetic plants such as roots, leaves and stems (Croft et al. 2006).

Even though the photosynthetic capacity is thought to have been created as a result of an endosymbiotic system of a cyanobacterium-like organism with a non-photosynthetic eukaryote, these organisms are included in particular taxonomic groups depending on the ancestor (Keeling 2004).

In 1981, Hibberd characterized *Nannochloropsis* as a marine unicellular microalgae genus, belonging to the Phylum Heterokontophyta, Class Eustigmatophyceae, Order Eustigmatales and Family Eustigmataceae (Hibberd 1981). This microalgae genus that comprises 6 different species was the focus of the present work. *Nannochloropsis gaditana*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica*, *Nannochloropsis oculata* and *Nannochloropsis salina* are the currently identified species. The classification of *Nannochloropsis* species is performed by a sequence analysis of the ribosomal DNA and the 18S ribosomal RNA (Suda et al. 2002; Starkenburg et al. 2014).

2.1.1. Morphological structure and biochemical composition

With cell sizes between 2 and 8 µm, *Nannochloropsis* spp. morphologic structure is equivalent to plant cells (Figure 2.1.1.1) (Ma et al. 2016). It is essentially composed by a nucleus, chloroplast, mitochondria, and lipid droplet. These organelles are surrounded by a mechanically resistant bilayer cell wall consisting in an inner cellulosic wall and covered with a hydrophobic algaenan layer (Scholz et al. 2014).

Among the different species of *Nannochloropsis* spp. genus, and neglecting the consequences of different cultivation periods and growth conditions, an average total lipid percentage ranging from 23 to 58% and a fatty acid profile were defined for these microalgae (Ma et al. 2014; Hodgson et al. 1991). Under optimum growth conditions, the lipid content associated to membranes structures is the result of esterification reactions having the fatty acids intracellularly formed as reactants (Ma et al. 2016). Though, when lipid production is forced, neutral lipids in the form of triacylglycerol (TAG) are synthetized, and values can reach 60% of the dried biomass (Ma et al. 2014).

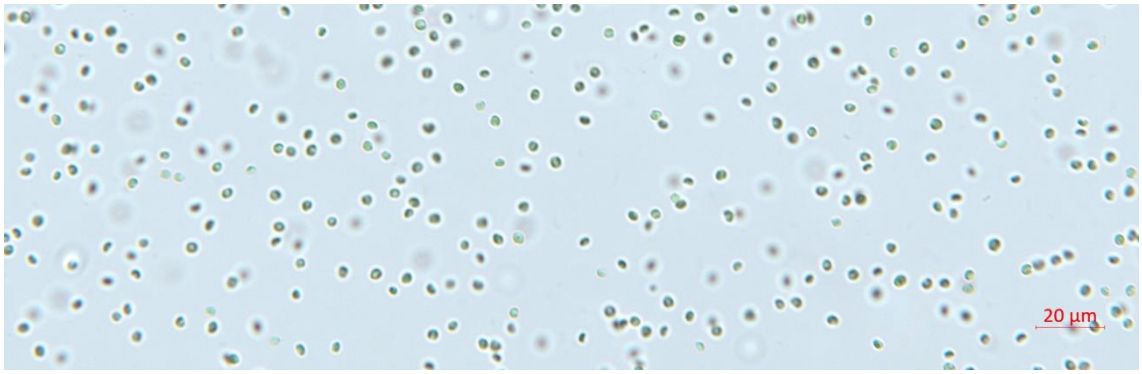

Figure 2.1.1. 1. Microscopic observation of *Nannochloropsis* sp. cells.

Regarding the other biomolecules and the inorganic content, standard compositions for these microalgae biomass have been published (Rebolloso-Fuentes et al. 2001). Carbohydrates and proteins dry weight ratios of 37.6 and 28.8%, respectively, and mineral composition containing about 972 mg of calcium, 659 mg of sodium, 533 mg of potassium, 316 mg of magnesium, 136 mg of iron, 103 mg of zinc, and 35 mg of copper in a 100 g dry weight biomass base are some of the reported results.

These values and compounds profile outstand among the microalgae diversity, which places *Nannochloropsis* spp. in a predictable close relation with high commercial value markets (Griffiths et al. 2009). However, to optimize the production of high-value products for further extraction from the organism, the underlying mechanisms that rule the lipid and fatty acids synthesis must be studied.

A complete description of the biosynthetic pathway used for lipids production by *Nannochloropsis* spp. is still to be reported. Often, these microalgae internal metabolism for lipid production is assumed similar to *Chlamydomonas* (Banerjee et al. 2017). For the microalgae, lipids have structural and energy storage functions while polyunsaturated fatty acids (PUFAs) are usually associated with the neutralization of reactive oxygen species by its oxidation after interaction with the unsaturated bond of PUFAs (Mühlroth et al. 2013). Table 2.1.1.1 summaries the average

percentage ranges of each fatty acid in comparison with the total amount of fatty acids that are commonly present in *Nannochloropsis* spp. biomass (Ma et al. 2016).

Table 2.1.1. 1. Typical fatty acid profile (% range of total fatty acids) of *Nannochloropsis* spp. (Ma et al. 2016)

C14:0	C16:0	C16:1	C16:2	C18:0	C18:1	C18:2	C18:3	C20:1	C20:4	C20:5
2.1	21.8	22.7	3.0	0.6	4.2	0.7	0.1	1.5	2.5	2.9
4.5	45.9	30.2	3.1	3.3	28.5	4.7	1.6		21.8	33.7

In this fatty acid profile are included long chain fatty acids with a minimum number of 14 carbons and a maximum number of 20 carbons. Within this group, saturated and unsaturated fatty acids are differentiated by the number of double bonds present in the hydrocarbon backbone (0 and 1 or more, respectively). Besides, polyunsaturated fatty acids are distinguished in accordance with the position of the carbon that establishes the first double bond (the carbon number count is initiated from the hydrocarbon backbone towards the carboxylic group). For instance, C20:5 is an omega-3 fatty acid, since the first unsaturated double bonds is in the third carbon of the backbone. This fatty acid named eicosapentaenoic acid (EPA, C20:5 ω-3) totalizes 5 unsaturated double bonds over its 20 carbons structure.

EPA has been one of the compounds produced by *Nannochloropsis* spp. with ever-increasing commercial interest (Hoffmann et al. 2010). As a consequence, different microalgae screenings and growth medium optimization have been tried to increase EPA production rate (Abirami et al. 2017).

A sequence of crucial and coordinated steps is essential for a successful fatty acid biosynthesis (Figure 2.1.1.2) (Manuelle et al. 2009). The first requirement is the presence of acetyl-CoA and reduced cofactors, *e.g.* NADPH. Acetyl-CoA can be obtained by the oxidation of pyruvate in a process mediated by pyruvate dehydrogenase. Another alternative is the conversion of pyruvate to acetaldehyde by pyruvate decarboxylase and subsequent oxidation of acetaldehyde to acetyl-CoA by acetaldehyde dehydrogenase.

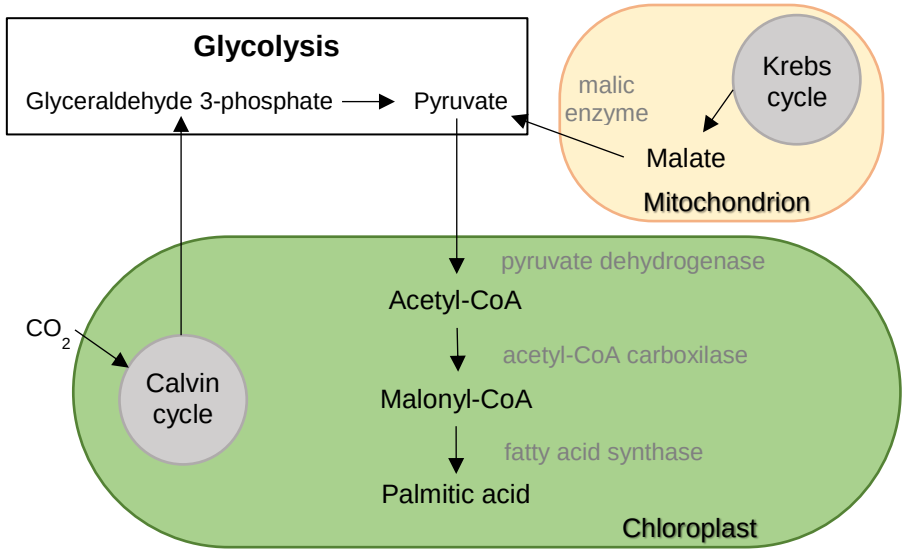

Figure 2.1.1. 2. Schematic representation of the essential intracellular reactions for fatty acid synthesis (adapted from Ma et al. 2016).

This acetyl-CoA precursor is the final product of glycolysis and it can be further synthetized by the glycolysis intermediated glyceraldehyde 3-phosphate that is formed in the Calvin cycle, by the fixation of atmospheric CO_2 during photosynthesis. Another pyruvate source is the oxidation of malate (intermediate in Krebs cycle) by malic enzyme, generating NADPH. This group of acetyl-CoA formation reactions and associated coenzymes reduction are essential for the following steps of fatty acid synthesis that occurs in chloroplast.

The second main step is the production of malonyl-CoA, by means of a carboxylation reaction on acetyl-CoA catalysed via acetyl-CoA carboxilase. Then, the final step is the malonyl-CoA elongation through the intervention of fatty acid synthase, forming the long chain saturated fatty acid with 16 carbons, *i.e.* hexadecanoic acid (palmitic acid, C16:0) and by the addition of two more carbons synthetizing an 18 carbons fatty acid, *i.e.* octadecanoic acid (stearic acid, C18:0), in the endoplasmic reticulum. The size of the fatty acids is defined by the number of extension cycles performed by elongase, and the presence of double bonds is managed by specific desaturases that both changes the conformation of saturated to monounsaturated fatty acids and mono-saturated to polyunsaturated fatty acids (Figure 2.1.1.3). Metabolic engineering techniques have been applied to increase the percentage of unsaturation in long chain fatty acids (Kaye et al. 2015).

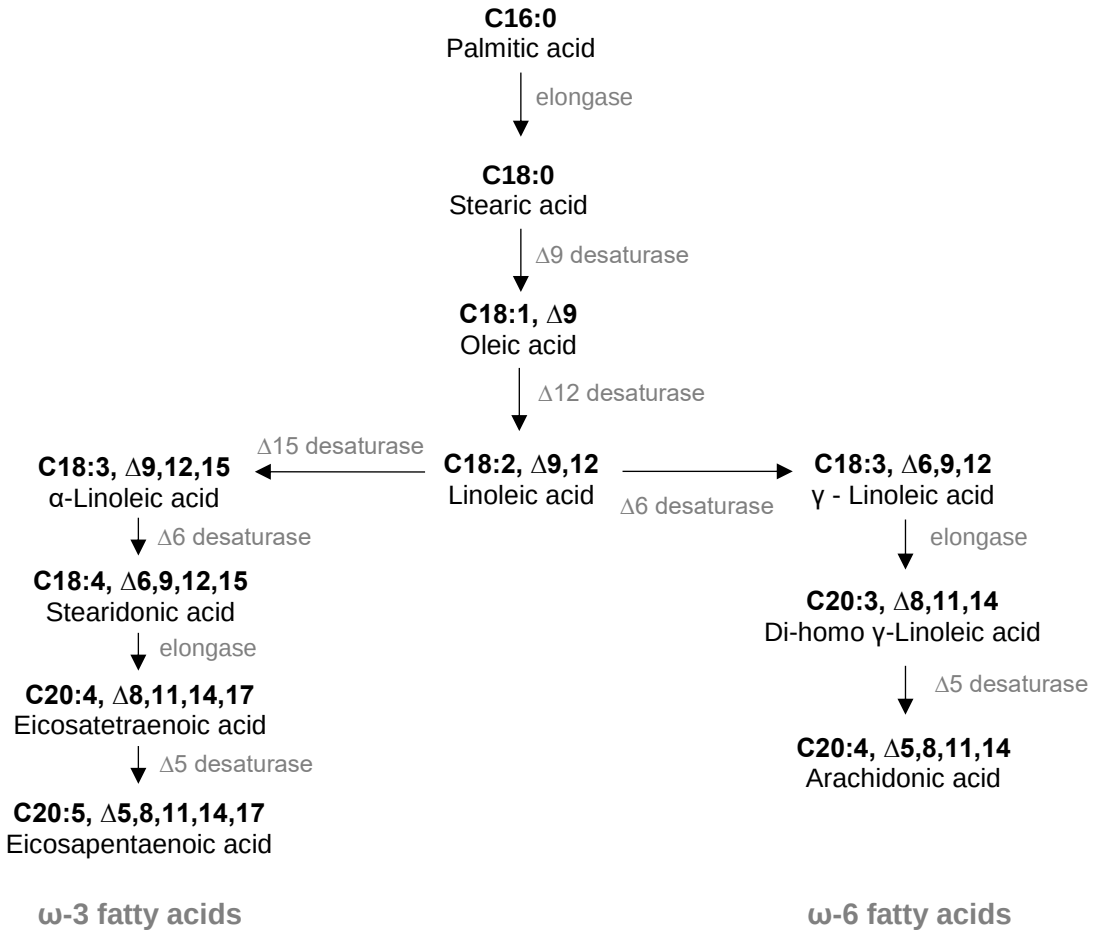

Figure 2.1.1. 3. Fatty acids biosynthesis flowchart mediated by elongase and desaturase (adapted from Ma et al. 2016).

Many authors have been discussing whether the amount of NADPH formed in the oxidation reactions associated with the acetyl-CoA formation would be sufficient for the following catabolic reactions in the process, and what would be the most rate limiting step on the entire procedure. Some conclusions have been drawn on these topics, suggesting that NADPH source is not restricted to the coenzymes formed in the fatty acids synthesis process (Ratledge 2014), and that acetyl-CoA carboxylase overexpression is related with higher amount of fatty acids synthetized, however different external factors have influence on the gene expression regulation such as the growth phase, leading to different fatty acid accumulations (Huerlimann et al. 2014).

Kennedy pathway or glycerol phosphate pathway has been reported as the most biochemically used pathway for microalgae TAGs biosynthesis (Liu et al. 2016). Chemically, it is an acylation chain reaction mediated by transferases and phosphorylation performed by phosphatases. In detail, glycerol 3-phosphate is acylated by glycerol-3-phosphate acyltransferase, forming lysophosphatidic acid. The second acylation occurs via lysophosphatidic acid acyltransferase, converting lysophosphatidic acid to phosphatidic acid. Then, phosphatidate phosphatase removes the phosphate group from phosphatidic acid, producing diacylglycerol. A

final acylation step in diacylglycerol leads to the formation of TAG, via diacylglycerol acyltransferase.

In opposition to the intrinsic approach of extracting industrial amounts of fatty acids and lipids from *Nannochloropsis* spp. biomass, pigments are commodity-like products. Carotenoids are the most abundant pigment type in *Nannochloropsis* spp. biomass (Nobre et al. 2012). Some of the reported pigments include zeaxanthin, canthaxanthin and astaxanthin (Lubi et al. 2000; Ambati et al. 2018). Even though some strategies have been focused on the intracellular pigments content increase, more studies are needed to guarantee an efficient extraction method (Kumar et al. 2013).

2.2. Biomass productivity and high-value compounds accumulation

A wide network of variables can be manipulated to display a desired effect on microalgae (Pal et al. 2011). These effects, *i.e.* organism's feedbacks to a certain set of conditions, aim at either boost the biomass productivity or high-value compounds synthesis. In other words, the management of microalgae growth is limited by two biological boundaries: a set of optimum growth conditions or stressful conditions to activate the self-defence mechanisms of the microalgae. Frequently, PUFAs content increases under optimum growth conditions whereas lipid content is mainly formed under critical growth conditions (Schüler et al. 2017).

Therefore, in one-stage batch operating reactors, the growth strategy must be defined based on the trade-off between high biomass production and high-value compounds content. However, to have significant amounts of high-value biomass, typically two stages growth strategies are performed (Su et al. 2011).

In the following subdivisions some reported examples on the influence of different variables on *Nannochloropsis* spp. growth and biomass biochemical content are provided. Among the several combinations of factors that have been set for *Nannochloropsis* spp. growth evaluation, the average results for the specific growth rate varies between 0.11 and 0.21 per day, biomass productivity from 1.10 g to 3.03 $gL^{-1}d^{-1}$ in tubular photobioreactors and a dry weight biomass concentration around 1 gL^{-1} was obtained in raceways open ponds reactors (Ma et al. 2016).

2.2.1. Light dependency and reactors design

Photosynthetic growth of microalgae requires light as membrane photoreceptors activator for the intracellular anabolic pathways (Banerjee et al 2017). Regardless the light intensities variations over the day, the natural sunlight is the most common and less expensive light source for microalgae growth (Chisti 2007). Consequently, photoautotrophic regimes are often performed for microalgae growth.

However, low cell densities cultures may undergo photoinhibition under high light levels (Chen et al. 2012). Microalgae growth in a culture medium supplemented with external carbon sources and deprived of photons is an alternative to this light inhibition event. Given the low affinity with the most common external carbon sources, *e.g.* glucose and acetate, the efficiency of this heterotrophic regime is poorly reported for *Nannochloropsis* spp. (Marudhupandi et al. 2016; Vazhappilly et al. 1998).

Photoinhibition is a stressful condition that also occurs under high cell densities. To overcome the lack of a homogenic light availability, microalgae culture medium is fortified with external organic carbon – mixotrophic regime. It has been reported that low initial concentration ranges of the carbon source improves *Nannochloropsis* spp. growth productivity (Cheirsilp et al. 2012; Pagnanelli et al. 2014).

In parallel with the previous mentioned cultivating strategy to outdo the photoinhibition caused by high cells densities, the theme of light availability and penetration in the culture has been motivating the construction of advanced reactors designs (Chen et al. 2011; Verma et al. 2018). Microalgae are cultivated in open or closed reactors, *e.g.* raceway ponds and flat-panel photobioreactor, respectively (Crowe et al. 2012; Cheng-Wu et al. 2001). Raceway ponds are a low-tech and cost-effective engineering systems for microalgae growth, however, the culture is more vulnerable to contaminations given the exposed interface with the air (Sun et al. 2018). Conversely, flat-panel photobioreactors are mechanically more robust and the reactor thickness is optimized to guarantee a continuous light beam through both panels (Richmond et al. 2001; Quinn et al. 2012). Biomass productivity reach higher values under the controlled conditions assured by this reactor design (Safafar et al. 2016). A study on the energy life-cycle analysis of microalgae biomass production has revealed that these two reactors designs are economically feasibility, as opposed to tubular photobioreactors (Jorquera et al. 2010).

2.2.2. External factors and consequent influence on growth kinetics

The light dependency in photoautotrophic growths and its photoinhibition associated outcome are commonly used to optimize the lipid content production. Indeed, high levels of irradiance have a negative effect on the light receptors, *i.e.* antenna system and photosynthetic reaction center II, leading to lower photosynthetic efficiencies and carbon fixation (Li et al. 2009). As a defence from high light densities, an increase in TAGs content and a simultaneously decrease in polyunsaturated fatty acids production were registered (Solovchenko et al. 2014). Based on these consequences, the sunlight utilization system of microalgae has been a recent matter of research. One of the latest attempts in this field is the application of different fluorescent dyes as a mean to increase the absorption spectrum of *Nannochloropsis* spp. and therefore to maximize

the yield of the natural sunlight use for lipid production (Sung et al. 2018). Artificial lights with different wavelengths have also been applied in microalgae growth, resulting in an increase of saturated fatty acids content when microalgae were grown under red light in comparison with blue and white light (Kim et al. 2014). Another study has revealed that the combined used of red and blue light optimized the production of EPA present in glycolipids and phospholipids (Chen et al. 2015).

Nutrients concentration in the growth medium also dictates the microalgae growth dynamics. Nitrogen starvation is widely accepted as the most efficient condition to promote TAGs accumulation in microalgae (Paes et al. 2016). Under this condition, *Nannochloropsis* spp. reduce their photosynthetic rate and focus in the lipid biosynthesis metabolism (Solovchenko et al. 2014). Some reported results indicate a two-times fold increase in lipid accumulation due to a three fourth decrease in the nitrate concentration (Converti et al. 2009). On the other hand, EPA and biomass production are dependent on nitrogen-replete growth media.

As marine microalgae, *Nannochloropsis* spp. are affected by the salt concentration in the medium. Salinity ranging between 10 to 35 gL^{-1} have been reported as effective for microalgae growth (Gu et al. 2012). Within this range, 25 gL^{-1} led to a higher EPA productivity while a more extreme concentration (35 gL^{-1}) triggered neutral lipid formation, over a cultivating period of 19 days. Even though the fatty acid profile changes, the 16 carbons fatty acids content was the highest over all the different salinities tried. In the same study, a relation between the growth period and the biomass productivity at different salinities were also evaluated. For the first 10 days of cultivation, biomass productivity was significantly higher at the lowest salinity (25 gL^{-1}) and during the last five days of the experiment period, the maximum biomass productivities were obtained at the highest salinity (35 gL^{-1}). This halophilic characteristic of these microalgae allows contamination reduction by salinity adjustments (Bartley et al. 2013).

In addition, it has been reported that growth rate and lipid productivity reach maximum values after 16 cultivating days, meaning that the cultivation period has a limiting effect on biomass production and lipid synthesis (Hu et al. 2015). Besides, temperature is an important factor since microalgae adjust their membrane fluidity in response to this parameter oscillations. Higher content of unsaturated fatty acids is found under low temperatures whereas saturated fatty acids are dominant under high temperatures (Wagenen et al. 2012). Different CO_2 aeration rates have also been experimented in semi-continuous *Nannochloropsis* spp. growth, demonstrating that the biomass and lipid content was maximized under a 2 % CO_2 aeration concomitantly with a daily growth medium renovation (Chiu et al. 2009).

Frequently, more than one external factor is combined to induce an intensive change in microalgae behaviour. Synergetic effects on lipid productivity were achieved by associating light

and nutrient stressful conditions (Yeesang et al. 2011). Moreover, the co-action of extreme salinities and high light intensities in *Nannochloropsis* spp. cultures increased the fatty acids content (Li et al. 2009). The improvements are unquestionable, however, theoretical explanations for the advantageous effects on the integration of several external factors in microalgae growth remain unclarified (Yeesang et al. 2011).

2.2.3. Saline microalgae polycultures

The symbiotic effects due to the mixture of different microalgae strains have been considered for many applications (Richard et al. 2012). For instance, microalgae polycultures have been applied in aquaculture systems as a feed product as well as in wastewater streams, *e.g.* brewery and bovine effluent for nutrient recovery (Neori 2011; Davis et al. 2015).

A relation between the species biodiversity that are part of the integrated system and the final polyculture stability and productivity was established (Corcoran et al. 2012). This evidence provides a culture advantage to outgrow possible system contaminants. Though, the equilibrium between the species present in microalgae polyculture systems and consequent benefits may not be achieved. Indeed, external factors variations are likely to modify the growth dynamics and composition of each specie (Kent et al. 2015). For example, the same ammonium concentration and light intensity condition has been reported as a good strategy for higher lipid production by *Phaeodactylum tricornutum* while no improvements were registered for *Nannochloropsis* spp. (Huete-Ortega et al. 2018). Excreted metabolites with inhibitory nature are another variable that has an essential interference in the mutualism relation in polycultures. A significant increase of inhibitory activity in *Nannochloropsis* spp. growth medium filtrates over the days of cultivation has been reported (Richmond 2004). This mesh of variables has significant effects on the final biochemical properties and composition of the polyculture biomass (Kent et al. 2015).

A polyculture with two microalgae genera was tested in the present work. *Nannochloropsis* sp. culture was mixed with the saline microalgae specie *Phaeodactylum tricornutum*.

Four different morphologies are described for this diatom that is mainly cultivated under photoautotrophic conditions (Marinho et al. 2017). Heterotrophic and mixotrophic growth strategies have been reported as biomass productivity stimulator (García et al. 2006; Morais et al. 2009).

One of the motivations for the addition of this species in a polycultures system is associated with the carotenoids, vitamins, and antioxidants content (Gügi et al. 2015). Though, the main purpose of this mixture was the attractive lipid percentage and ω-3 and ω-6 fatty acid profile (Hamilton et al. 2016). The list is formed by eicosapentaenoic, octadecatetraenoic (SDA, C18:4 ω-3), octadecatrienoic (ALA, C18:3 ω-3), docosahexaenoic (DHA, C22:6 ω-3), docosapentaenoic

(DPA, C22:5 ω-3) omega-3 fatty acids and octadecadienoic (LA, C18:2 ω-6), octadecatrienoic (GLA, C18:3 ω-6), eicosatetraenoic (ARA, C20:4 ω-6) and eicosatrienoic (DGLA, C20:3 ω-6) omega-6 fatty acids (Zhao et al. 2014; Hamilton et al. 2016). The characteristic fatty acid profile of *Nannochloropsis* spp. is similar to some of the synthetized fatty acids by *Phaeodactylum tricornutum*. However, the polyculture biomass includes a broader fatty acid range.

Phaeodactylum tricornutum synthetizes, based on a sequence of fatty acids elongation and desaturation enzymatic reactions, one of the most well-known and high-value fatty acids: DHA (Mühlroth et al. 2013). This production procedure is different from the one undertaken by animals that follow the Sprecher pathway for DHA synthesis.

Modifications in the growth medium conditions influence this diatom metabolic pathways, leading to an overaccumulation of some compounds. For instance, the presence of glycerol and low temperatures favour PUFAs production (García et al. 2006; Sharma et al. 2012). Besides, depending on the nitrogen source provided, *i.e.* urea and ammonia, EPA production is improved or inhibited, respectively (Yongmanitchai et al. 1991; Wen et al. 2003).

2.2.4. Bioremediation and alternative culture media

Most of the nutrient requirements for microalgae growth are present in wastewater standard composition (Ma et al. 2016). A circular economy approach could be established by growing microalgae in the effluents of several companies. Given the high nutrient content of typical waste, the bioremediation requires preliminary dilutions to circumvent growth inhibition (Cai et al. 2013). Different studies have reported a successful use of pre-anaerobically digested pulp and paper sludge, municipal wastewater, and pesticides industry effluents to produce EPA enriched biomass (Polishchuk et al. 2015; Mitra et al. 2016). Targeting a process optimization, the growth dependence on light intensity and temperature of *Nannochloropsis* spp. biomass cultivated in municipal effluents have been tested in low scale reactors (Sheets et al. 2014).

Medium recirculation has also been reported as an eco-friendly process to reduce *Nannochloropsis* spp. biomass production costs (González-López et al. 2013). To prevent toxic metabolisms accumulation, ozonation has been described as the most efficient medium sterilization method. Nevertheless, *Nannochloropsis* spp. cells cultivated in recycled medium tend to aggregate (Zhang et al. 2016). This biological configuration is commonly a response to a stress condition, that has been associated with the presence of autoinhibitory substances and cell wall remains (Rodolfi et al. 2003).

Some experiments on the *Nannochloropsis* spp. biomass production optimization have reported the use of deep-see water, desalinisation concentrate from desalination plants, standard media supplemented with commercial fertilizers and palm oil mill effluent as potential alternative

culture media (Chen et al. 2018; Matos et al. 2015; Camacho-Rodríguez et al. 2013; Hadiyanto et al. 2017).

2.3. Biomass applications and commercial value

Microalgae benefits are initiated in the biomass growing phase and are extended until the final dried biomass product. During the growth phase, CO_2, nitrogen and phosphates are bioaccumulated (Ma et al. 2016). Due to this mitigation, greenhouse gases emission is reduced, and wastewater streams are biologically treated.

In addition, gathering the aforementioned biochemical characteristics, microalgae have naturally been suggested as a potential source of bioactive compounds to be used in the food and pharmaceutical industries (Tang et al. 2011; Ibañez et al. 2013). High lipid percentages in the final biomass have been the priority for microalgae application as biodiesel, however, a large monetary and technologic input is required to convert this idea in an economically feasible process (Mata et al. 2010).

Another high-value compounds present in the microalgae structure are the fatty acids, namely, the ω-3 and ω-6 series. *Nannochloropsis* spp. have been distinguished by their significative content of both ω-3 and ω-6 fatty acids from a vegetable source (Krienitz et al. 2006), one of the food trends in 2018. These bioactive substances have been reported as anti-inflammatory and proinflammatory agents, respectively (Wen et al. 2003). The ratio between both unsaturated fatty acids has nutritional impacts in human health. Western diets tend to be poor in ω-3 fatty acids, leading to significative ω-6/ω-3 ratios (around 15/1), which are associated with autoimmune diseases and cancer (Simopoulos 2008). The inverse, *i.e.* low ω-6/ω-3 ratios, reduce the probability of several chronic diseases.

Besides, more examples of human health benefits associated with fatty acids, specifically ω-3 EPA and DHA, comprise positive effects on vision, reduction of elderly related diseases *e.g.* Alzheimer disease, coronary heart disease prevention, and brain development (Martins et al. 2013; Lai 2015). This last proactive function of ω-3 PUFAs, mainly associated with DHA, targets the neurologic and cognitive development of early life foetus, by a maternal seafood diet supplementation (Hibbeln et al. 2007).

Indeed, human intake of these nutritional valuable compounds is mainly by means of seafood and fish. Over the food chain, since the primary producers up to the secondary consumers, *i.e.* from microalgae to humans, compounds are bioaccumulated (Wen et al. 2003). A direct human consumption of microalgae biomass requires a product assessment and quality certification by a food security entity. *Chlorella vulgaris* and *Spirulina* are examples of microalgae that have been recognized by the European Food Safety Authority (EFSA) as a food ingredient (Kent et al. 2015).

Hitherto, no toxicity or negative side effects have been reported in the safety evaluation of *Nannochloropsis* spp. biomass in rats (Kagan et al. 2015).

The usual application of *Nannochloropsis* spp. biomass is the rotifers feeding in aquaculture companies (Rchibeque et al. 2009). Lately, these companies have been facing the difficult challenge of producing high quantities of seafood in aquaculture systems to satisfy the growing market of consumers (Gladue et al. 1994). The inherent limitation is the production of large scale microalgae biomass feeds, that even being reported as fulfilling the biochemical aquaculture requirements and produced under the most economic photobioreactors designs, it is becoming scarce to satisfy this market needs (Camacho-Rodríguez et al. 2015; Norsker et al. 2011). Here, a market opportunity seems to be open and microalgae polycultures may play a central role in this field. However, microalgae polycultures advantages and nutritional applications are still to be demonstrated (Kent et al. 2015).

3. Materials and methods

The growth experiments were performed at Algafarm facilities within the period of 5th of February and 20th of June of the present year. Both the research and innovation department as well as the production department of the company were used for the growth assessment, however, only the biomass harvested at lower scale (from the 50 L reactors) was further analysed in terms of the biochemical composition. This last analysis was made at the University of Algarve, in cooperation with the MarBiotech group of the Centre of Marine Sciences, since the 21st to 30th of May.

In order to have comparable results, the growth conditions of the cultures were kept constant throughout the different experiments. Besides, the same reactors geographic locations used for each assay were maintained for all the experiments of the project. Therefore, the results variability associated with the different available light intensity according to the reactor position was minimized.

The next subsections describe the reactors design and the general procedure that was applied to evaluate the growth and to analyse the biochemical composition.

3.1. Microalgae strains

Four microalgae strains from the culture collection of Algafarm were used in this study. Within this group of microalgae, two genera are present: three strains of *Nannochloropsis* spp. and *Phaeodactylum tricornutum*. In accordance with the industrial confidentiality, the strains are hereby differentiated by a numeric code. Thus, 0011NN, 0013NN and 0039NN refer to the *Nannochloropsis* spp. strains, and 0018PA stands for *P. tricornutum*. This nomenclature will be preserved along the work.

3.2. Reactors setup

The smallest scale reactor setup with a volumetric capacity of 2 L was a closed system connected to an air feeding by means of sterilized pipes to guarantee the culture aeration through 0.2 µm air filter (Midisart® 2000 PTFE, Sartorius, Germany). The same filters were used at the air outlet. Light was continuously provided by LED lamps with a luminous flux of 50 W (6332 Lm) (PROTEK SECOM, Industrial Flood Light, Spain). The distance between the photons source and the reactors was kept roughly constant to assure similar intensity for the reactors over the experiments period. The temperature at this scale was controlled at about 23 ˚C. The same reactor configuration and conditions were applied for the 5 L reactors.

At the pilot scale, 50 L reactors were inoculated with roughly 10 L of the lower scale initial inoculum. The reactor air feeding system was kept. However, this reactor design was modified, being this reactor defined as a flat panel (FP), usually known as "Green Wall Panel" (GWP). In this outdoor reactor, natural light was the source of photons and the temperature was regulated by water spray on the reactor surfaces to keep it under 25 ˚C.

The next scale of growth was a 1000 L GWP reactor that was inoculated with around 100 L. The reactor design was similar as the previously described, however, it was built-up inside a greenhouse that allowed a growth under more stable and controlled conditions (Figure 3.2.1. A). Besides the natural light source, LED lamps were used to provided photons overnight. The temperature was regulated by means of a mobile roof that shifted its coordinates as a response to temperature deviations. For instance, to decrease the temperature, the roof position was changed to increase the shadow area inside the greenhouse.

The 10500 L industrial scale reactor (Figure 3.2.1. B) was inoculated with 2000 L from two GWP reactors. This outdoor reactor design was made of polymethyl methacrylate (PMMA) tubes with 56 mm of inner diameter and a total length of 24 m. The flow rate inside the pipes was 0.8 ms^{-1}. The thermoregulation system applied was the same as for the 50 L GWP reactor. Given the design, this reactor was classified as a photobioreactor (PBR).

Figure 3.2. 1. Flat-panel reactor and tubular photobioreactor designs used for *Nannochloropsis* spp. scale-up: (A) 1000 L GWP reactors inside the greenhouse and (B) industrial scale PBR with10500 L.

Regardless the scale, the pH of each experiment was maintained within the range of 7-8 (FU20, Yokogawa, Japan and Combo pH/Conductivity/TDS Tester, Hanna instruments, Portugal). The pH fluctuations were stabilized by a negative feedback response mediated by CO_2 pulses into the air feeding system.

3.3. Growth conditions and cultures scale-up

The smallest operating scale was the laboratory scale, which was used to grow the initial inoculum of each strain. Periodically, the initial inoculum was renovated with fresh medium to keep the growth stage of the microalgae and to avoid biomass losses out of contamination and consequent cells death. Besides this inoculum source function, this scale was also used to perform the alternative growth media experiments and to glimpse the ideal scale up approaches with preliminary experiments on the polyculture systems.

With exception for the alternative growth media experiments, the standard growth medium used for all the experiments was the Guillard's F2 culture medium adapted to the local water and fortified with nitrate to reach an initial concentration of 15 mM.

In addition to the temperature and pH condition described in the previous section, the salinity of the culture was settled at 25 gL^{-1} given the growth efficiency registered in previously reported studies (Gu et al. 2012). The microalgae growth kinetics were analysed until the stationary phase, accounting for about 18 days.

3.3.1. *Nannochloropsis* spp. strains scale-up

The growth of the three different *Nannochloropsis* spp. strains was evaluated in the 50 L and 1000 L GWP reactors and in the 10500 L PBRs. A triplicate of the 50 L reactors assay and a duplicate of the 1000 L and 10500 L reactors were performed for each strain.

3.3.2. Polycultures preparation

A preliminary screening of a range of percentages of each strain in the final mixture was performed at 2 L and 5 L scale. Details on the percentages applied are discriminated in Appendices (section i, Table i.1-2). Based on the growth performance, only the optimum ratio of each strain applied in the polyculture systems was scaled-up to a 50 L reactor. The monoalgal growth of *P. tricornutum* was also studied at that scale. All these experiments were repeated three times.

3.3.3. Alternative growth media

This set of experiments was initially made in 2 L reactors and then scaled-up to 50 L reactors. Two different growth media were used to evaluate the growth of 0011NN in alternative nutrients sources media. The growth media compositions are listed in section ii, in Appendices (Table ii.1-2). One of the medium formulations is used as shrimps cultivating medium in aquaculture (mentioned as synthetic saline water) (Centro de Produções Técnicas 2018) and the other is a saline effluent of a codfish processing company (Lugrade, Coimbra).

As an attempt to evaluate the cost-effectiveness of the alternative growth media, the price of each component required for the synthetic saline water growth medium preparation was inquired to different chemical products selling companies. These include Quimidroga Portugal, JMGS – José Manuel Gomes dos Santos and VWR. The last did not provide any feedback. The economic analysis does not include the saline effluent since this wastewater price was not negotiated with Lugrade. Therefore, the economic evaluation for the synthetic saline water was initially performed considering the average price payed by the company to a set of components required for this medium preparation, and the missing prices were completed having the online selling prices of Sigma-Aldrich as an overestimated reference (Sigma-Aldrich 2018a, 2018c, 2018b). Then, these results were compared with the selling prices for the components provided by Quimidroga and JMGS.

As a note, since Quimidroga does not sell one of the required components, its price was considered similar to JMGS selling price. For a comparative analysis with the standard medium currently used by the company, the underestimated cost of the specific components used for this medium preparation is 0.005 $€L^{-1}$.

3.4. Microalgae growth analysis

For the microalgae growth measurement, samples were taken and analysed periodically over the days of run. At the lowest and at the higher scales the samples were taken 2 to 3 times per week. For the 50 L reactors, the sampling was daily performed.

The biomass content was analysed by spectrophotometric analysis (4251/50, Zuzi, Spain) at 750, 600, and 540 nm. Besides, the biomass concentration was estimated by dry weight measurements. In this procedure, 10 mL of culture were filtrated through a 0.7 µm glass fiber filter (VWR, Portugal), by means of a laboratory vacuum pump (N86 LABOPORT, KNF, USA). After being washed with an ammonium formate solution at 37 gL^{-1}, the filter was dried in a moisture analyser (MA 50.R, RADWAG, Poland). The results of these two methods to evaluate the biomass growth were correlated and the calibration curves for each strain and polycultures are plotted in Appendices (section iii, Figure iii.1-6).

Besides the biomass concentration, samples were centrifuged for 10 minutes at 2000 g (MiniStar silverline, VWR, Portugal) for analysis of the medium content in nitrate over time (Clescerl et al. 1999) (Figure iv.1, section iv, in Appendices). The temperature and pH were registered by means of a pH meter (Combo pH/Conductivity/TDS Tester, Hanna instruments, Portugal). The integrity of the cultures was assured by microscopic observations (Primo Star, ZEISS, USA) and cell counting with Neubauer chambers (double net ruling with clamps, Hirschmann, Germany) was used to count the cells and to calculate the concentration of each strain in the

polyculture of the two saline microalgae genera. This methodology is dependent on the number of cells counted in the 4 squares of the chamber and the dilution used (Equation 3.4.1). Due to the square volume, 0.1 µL (0.1 cm×0.1 cm×0.01 cm), the equation is adjusted with a 10000 factor to provide the biomass concentration as a ratio of the cells number and the volume in millilitres.

$$Concentration\ (cell/mL) = \frac{Number\ of\ cells \times 10000}{Number\ of\ squares \times Dilution} \quad \text{Equation 3.4.1}$$

Besides the description of the punctual microalgae growth conditions, differences in the biomass concentration over time allow the calculation of growth parameters and the evaluation of growth dynamics. Mathematical formulas for the specific growth rate, volumetric productivity and substrate to biomass conversion yield are following explained.

The productivity, P ($mgL^{-1}d^{-1}$), quantifies the biomass increment in a determined period. This parameter can be expressed as an overall productivity (considering the initial and final stages) or as a maximum productivity (the highest productivity value calculated based on two consecutive sampling points). The formula is expressed in Equation 3.4.2, where X_1 and X_2 (gL^{-1}) are the biomass concentration at the initial time, t_1 (days), and the final time, t_2 (days), respectively.

$$P = \frac{X_2 - X_1}{t_2 - t_1} \quad \text{Equation 3.4.2}$$

The equation for the specific growth rate, µ (d^{-1}), calculation is a result of a mass balance for the biomass in the reactor (Equation 3.4.3). After being solved, this equation is expressed as a ratio between the difference of the final and initial logarithmic biomass concentrations (X_2 and X_1, respectively) and the associated time range (t_2 and t_1, respectively) (Equation 3.4.4).

$$\mu X = \frac{dX}{dt} \quad \text{Equation 3.4.3}$$

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \quad \text{Equation 3.4.4}$$

The conversion yield of substrate in biomass is a factor that expresses the amount of biomass formed by a determined substrate consumption. Thus, this dimensionless parameter is a ratio between the biomass and the substrate concentration within a certain period. In Equation 3.4.5, S_1 and S_2 (gL^{-1}) are the substrate concentrations at the initial and final time, respectively.

$$Y_{X/S} = \frac{X_2 - X_1}{S_1 - S_2} \quad \text{Equation 3.4.5}$$

3.5. Biochemical composition

The biochemical analysis of the biomass was focus on the determination of the lipid content and the fatty acid profile. These parameters were measured on the biomass that was harvested from the 50 L reactors that were used for the three *Nannochloropsis* spp. strains scale-up and for the polycultures experiments. Before the treatments, the biomass was freeze-dried.

3.5.1. Fatty acid profile

The fatty acids were determined by its reduction with a methyl group, to the final structure of fatty acids methyl esters (FAMEs). This protocol was adapted from Lepage et al. 1984 as described in Pereira et al. 2012.

The freeze-dried biomass was weighted (10-20 mg) (PA214C, OHAUS, Australia) in derivatization vessels and homogenized by the Ultra Turrax (IKA T-25 ULTRA-TURRAX, Cole-Parmer, USA) with 1.5 mL of a methanol/acetyl chloride solution (20:1, v/v), for 90 seconds (Figure 3.5.1.1 A). Then, 1 mL of hexane was added to the vessels and a heat treatment (SUB Aqua 12 Plus, Grant Equipments, USA) at 70˚C for 60 minutes was applied (vessels were sealed with Teflon to avoid losses). After cooling down in ice, the vessels content was transferred to centrifuge tubes and 1 mL of distilled water and 4 mL of hexane were added. These tubes were vortexed (VV3, VWR, Portugal) at maximum speed in two cycles of 30 seconds and centrifuged for 5 minutes at 2000 g (Heraeus™ Multifuge™ X3, ThermoFisher Scientific, USA). After centrifugation, the supernatant was transferred to new tubes (Figure 3.5.1.1. B). In the centrifuge tubes, 4 mL of hexane were added and the mixture procedure with the vortex and centrifuge was repeated.

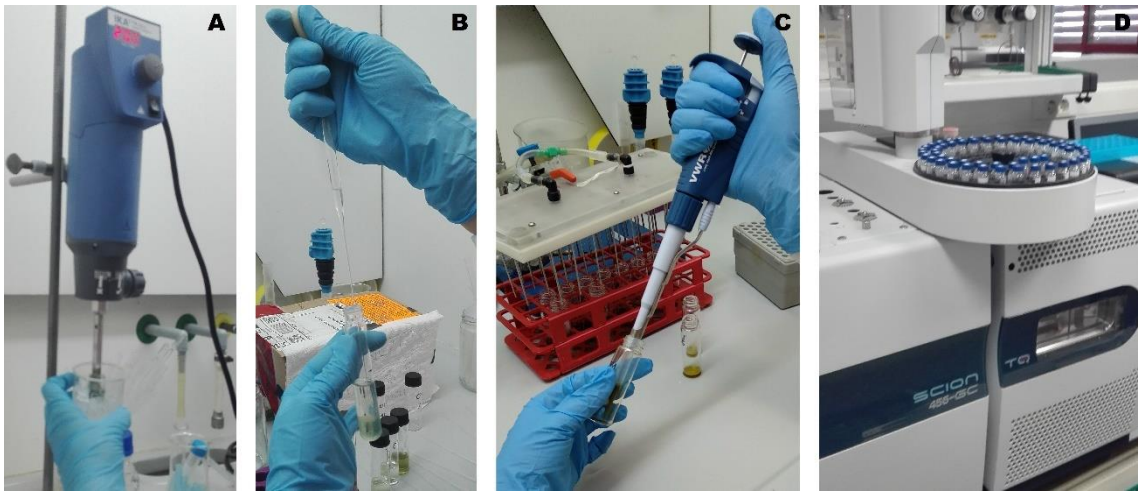

Figure 3.5.1. 1. Sequence of steps and equipment used for fatty acids determination: (A) biomass homogenization with Ultra Turrax; (B) extraction of hexane fraction after centrifugation; (C) hexane evaporation under a nitrogen gas flow (back) and resuspension of the dry matter in chromatography-grade hexane; (D) gas chromatography-mass spectrometry equipment with autosampler.

After the second supernatant removal, this sequence of extraction steps was repeated once again. Then, anhydrous sodium sulphate (Na_2SO_4) was added to the collected supernatant fraction, and the solution was filtered by 0.22 µm filters (EMD Millipore SLLGC13NL IC Spectra Syringe Filter, Cole-Parmer, USA).

A nitrogen gas flow was applied to evaporate the hexane and the dried fraction was immediately resuspended in 0.5 mL gas chromatography-grade hexane and transferred to vials for gas chromatography-mass spectrometry analysis (GC-MS) (SCION 456-GC, Bruker, USA) (Figure 3.5.1.1. C-D).

The chromatograph was equipped with a 30 m length column with an internal diameter and film thickens of 0.25 mm and 0.25 µm, respectively (ZB-5MS, Phenomenex, USA). Helium was the carrier gas. The identification of the FAME present in the samples was made by a comparison of the results with a standard solution containing a mixture of 37 different FAMEs (Supelco® 37 Component FAME Mix, Sigma-Aldrich, Portugal). A calibration curve of the chromatogram peak area and the concentration of each compound was established. These curves were applied for the quantification of the FAMEs present in samples, and in case a FAME that was not identified with the standard solution was present in the sample, the calibration curve of the most structurally similar compound was applied. After being identified and quantified, the FAME profile was expressed as a relative percentage of the total fatty acids content present in the biomass.

3.5.2. Lipid content

For the lipid determination, a biomass weight ranging 20-30 mg was solubilized in 0.8 mL of distilled water. A mixture of methanol (2 mL), chloroform (2 mL) and distilled water (1 mL) was used to extract the lipid fraction from the biomass (Bligh et al. 1959). The mass transference phenomenon from the biomass to the solvent was optimized by the homogenization of the biomass with an Ultra Turrax, for 120 seconds. After centrifugation for 10 minutes at 2500 g, the chloroform fraction was separated, and 0.7 mL of this fraction was pipetted to a pre-weighted tube. A heat treatment at 60 ˚C (QBD2, Grant Instruments, England) to evaporate all the chloroform was performed and after 3 hours in the desiccator, the final weight of the tube was measured in the precision balance (M5P, Sartorius, Germany).

The mathematical procedure for the calculation of the percentage of lipid in the biomass is described in Equation 3.4.6.

$$\%Total\ Lipids = \frac{\frac{(FW-IW)\times Total\ Volume\ of\ Chloroform}{Evaporated\ Volume\ of\ Chloroform}}{Sample\ Weight} \times 100 \qquad \text{Equation 3.4.6}$$

The percentage of total lipids (% Total Lipids) is a function of the lipid weight (mg), *i.e.* difference between the final and initial weight of the tube (FW and IW, respectively), extracted from the initial Sample Weight (mg), considering the Total Volume of Chloroform (2 mL) used in the extraction steps and the Evaporated Volume of Chloroform (0.7 mL) during the heat treatment.

3.6. Statistical treatment

Results were presented as the mean and the standard deviation of the samples replicates. These data were statistically analysed using IBM SPSS Statistics 25. Statistical significance was assessed applying *t*-test and ANOVA, depending on the number of samples to be compared (two and more than two, respectively). Equal variances were assumed for the statistical analysis. The statistical One-Way ANOVA test was used with the Tukey's range test as a multiple pairwise comparable method. Two-Way ANOVA was used to evaluate the combined influence of two independent variables on a dependent variable. A significance level of 0.05 was considered.

4. Results and discussion

The results are divided in accordance to the three main project goals, comprising the scale-up and comparison of three different *Nannochloropsis* spp. strains, attempts to increase the productivity by means of polycultures and the study of alternative growth media. Growth analysis was performed for all the experiments and the lipid content and fatty acid profile was assessed for the biomass harvested from the 50 L GWP reactors.

4.1. Scale-up and comparison of the three different *Nannochloropsis* spp. strains

The growth kinetics of the three *Nannochloropsis* spp. strains were separately evaluated for each reactor scale. Thus, strains were defined as independent variables.

At the 50 L GWP reactors scale and under the same growth conditions, equivalent specific growth rates and maximum productivities were obtained for all the strains ($p \geq 0.05$). *Nannochloropsis* spp. growth curves are plotted in Figure 4.1.1 and the kinetic parameters described in Table 4.1.1.

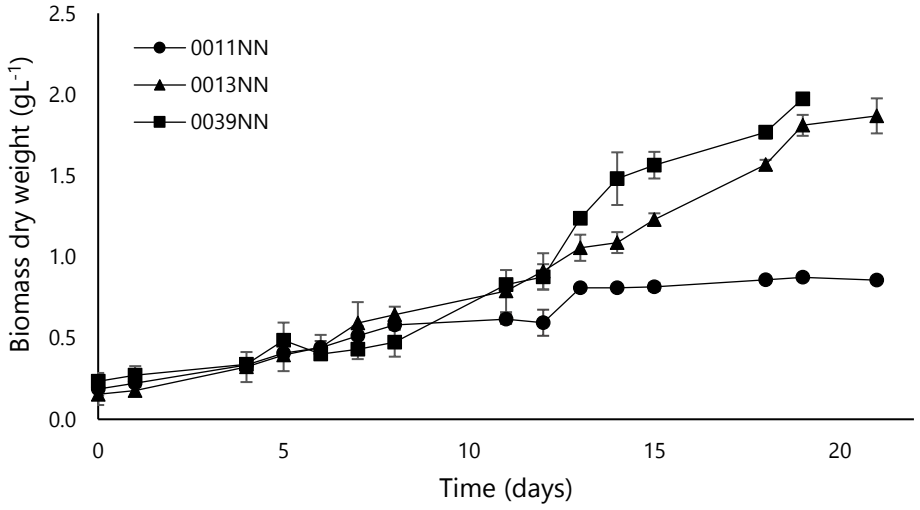

Figure 4.1. 1. Growth curve of the three different *Nannochloropsis* spp. strains in 50 L GWP reactors.

Global productivities among the strains were statistically different. This growth parameter registered the lower value for 0011NN (0.036±0.007 $gL^{-1}d^{-1}$) and the highest for 0039NN (0.095±0.010 $gL^{-1}d^{-1}$). Besides the statistical difference between these two strains ($p<0.05$), no significant variances were registered for the other pairwise comparison of the strains.

Despite the different reactor volumes, these results are slightly lower in comparison to the previously reported results obtained for *Nannochloropsis* spp. growth in a 500 L flat-panel photobioreactor during the summer (0.18 $gL^{-1}d^{-1}$) (Cheng-Wu et al. 2001) and more than two-fold lower in comparison to a 200 L optimized flat-panel photobioreactor (0.24 $gL^{-1}d^{-1}$) (Richmond et

al. 2001). The specific growth rates obtained are within the range of previously reported data (0.07 to 0.21 d^{-1}) (Ma et al. 2014).

Table 4.1. 1. Productivities and specific growth rates of the three different *Nannochloropsis* spp. strains in 50 L GWP reactors.

Microalgae strains	Productivity ($gL^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
0011NN	0.036±0.007	0.186±0.097	0.076±0.015	0.367±0.087
0013NN	0.084±0.010	0.419±0.055	0.131±0.015	0.438±0.126
0039NN	0.095±0.010	0.276±0.034	0.116±0.004	0.239±0.040

These three strains were grown at higher scale reactors in order to evaluate the influence of larger scales and different reactor designs in these microalgae growth dynamics. The growth parameters in the 1000 L greenhouse GWP reactors and in the 10500 L tubular photobioreactors are presented in Table 4.1.2.

At the pilot scale, no statistical differences were obtained among the strains evaluated ($p \geq 0.05$). However, the scale-up led to a notable overall decrease in the maximum productivity ($p < 0.05$). Besides this steep decrease in the maximum productivity, which means that the slope of the exponential growth phase was attenuated, the global productivities results are lower than the published for open raceways ponds with the same reactor volume capacity (Sheets et al. 2014).

Table 4.1. 2. Productivities and specific growth rates of the three different *Nannochloropsis* spp. strains at pilot and industrial scale reactors.

Reactor scale / Microalgae strains	Productivity ($gL^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
1000 L GWP				
0011NN	0.057±0.008	0.093±0.005	0.102±0.013	0.240±0.009
0013NN	0.060±0.004	0.103±0.012	0.096±0.013	0.263±0.046
0039NN	0.091±0.006	0.132±0.009	0.118±0.004	0.246±0.008
10500 L PBR				
0011NN	0.061±0.005	0.124±0.013	0.095±0.011	0.166±0.034
0013NN	0.032±0.001	0.112±0.043	0.135±0.042	0.251±0.017
0039NN	0.073±0.003	0.100±0.003	0.088±0.005	0.138±0.020

The industrial scale resulted in a significant global productivity decrease for 0013NN microalgae. Under these tubular photobioreactor design, the three strains showed comparable results for the maximum and global specific growth rates and maximum productivity ($p \geq 0.05$). The transition from GWP reactors to the tubular photobioreactors led to a significant overall decrease in the maximum growth rates of the three *Nannochloropsis* spp. strains. Hence, this

means that the adaptation phase (lag phase) in this reactor was longer than in the smaller pilot scale reactor. This was likely to be the reason for the low global productivity values obtained in comparison with the reported global productivities of 0.15 and 0.16 $gL^{-1}d^{-1}$, and maximum productivity of 0.37 $gL^{-1}d^{-1}$ for two different *Nannochloropsis* spp. strains grown in outdoor photobioreactors (Quinn et al. 2012).

A statistical assessment on microalgae growth parameters including the two independent variables in this study was performed to analyse the influence of the reactors scale combined with the different microalgae strains. The outcome revealed that the strain, reactor, and the combined strain and reactor interaction have significant effect on the global productivity results ($p<0.05$). The reactor scale was the only independent variable that substantially influenced the maximum productivities and the maximum specific growth rates obtained in the growth of these microalgae ($p<0.05$). Neither the strain, the reactor, nor the combined effects of both caused significant differences on the global specific growth rates of the strains ($p\geq0.05$). These results are of fundamental importance for the large-scale production of *Nannochloropsis* spp. strains. According to these results, adaptations in the reactor designs are the key-factor for a biomass production process optimization.

The biochemical analysis outcome on the lipid content and the fatty acid profile for each *Nannochloropsis* spp. are shown in Table 4.1.3. Strains were not distinguished concerning the lipid content ($p\geq0.05$), which is inside the commonly described range of lipid percentages under non-stressful growth conditions (Ma et al. 2016). The higher standard deviation listed for the total lipids quantification of 0011NN was a result of a short-photoinhibition period occurred in one reactor replicate, leading to an over average lipid synthesis.

The fatty acid profile obtained for the three *Nannochloropsis* spp. strains contains the already published list of fatty acids present in this genus (Abirami et al. 2017). The relative content of palmitic, palmitoleic, and eicosapentaenoic acid are the dominant in all strains as reported by Baumgardt et al. (2016). Similar percentages of C12:0, C15:0, C16:1, C17:1 and C20:3 ω-6 were found in the biomass of all strains ($p\geq0.05$). Among the significant differences registered for the other fatty acids, the EPA content on the 0039NN strain must be highlighted. This high-value compound reached a promising percentage, comparable with the 44.2% of total fatty acids reported by Safafar et al. (2016) for a *Nannochloropsis* sp. growth in a 4000 L flat-plate reactor. Besides, as a nutritional vegetable source, both the highest percentage of polyunsaturated in comparison with saturated fatty acids as well as the higher relative content of ω-3 in contrast with ω-6 (ω-3/ω-6>1) are interesting indicators (Martins et al. 2013; Mühlroth et al. 2013).

Table 4.1. 3. Total lipids percentage and fatty acid profile of the three different *Nannochloropsis* spp. strains biomass cultivated in 50 L GWP reactors (n.d. not detected).

Total lipids and fatty acid profile	Percentage of lipids and fatty acids		
	0011NN	0013NN	0039NN
Total Lipids	27.01±5.49	25.43±0.06	22.88±3.80
C12:0	1.20±0.07	0.84±0.11	0.96±0.06
C14:0	7.42±0.20	5.13±0.20	5.31±0.46
C14:1 ω-5	0.95±0.07	0.63±0.07	0.79±0.02
C15:0	0.87±0.04	1.08±0.27	n.d.
C16:0	20.86±1.39	15.51±0.71	13.79±1.69
C16:1	22.94±0.59	22.36±1.06	23.88±0.20
C16:2	n.d.	0.70±0.08	1.27±0.19
C17:0	0.72±0.08	n.d.	n.d.
C17:1	0.94±0.10	0.91±0.13	0.78±0.06
C18:0	1.47±0.14	n.d.	n.d.
C18:1 ω-9	5.71±1.01	3.95±0.40	2.58±0.26
C18:2 ω-6	4.31±0.44	3.49±0.31	2.6±0.05
C20:3 ω-6	1.32±0.11	1.32±0.27	1.07±0.10
C20:4 ω-6	6.92±0.23	11.53±0.32	9.50±0.46
C20:5 ω-3	23.37±1.46	30.60±1.92	35.36±2.28
SFA	32.57±1.31	22.60±0.64	20.07±2.07
MUFA	30.62±1.00	27.91±0.65	28.06±0.35
PUFA	35.95±2.12	47.71±1.65	49.91±2.87
ω-3	23.37±1.46	30.60±1.92	35.36±2.28
ω-6	8.25±0.24	12.87±0.34	10.58±0.42
ω-3/ω-6	2.83	2.38	3.34
PUFA/SFA	1.10	2.11	2.49

4.2. Polycultures systems

In order to increase both the biomass productivity and the nutritional benefits of *Nannochloropsis* spp. biomass, polycultures of the three different microalgae strains of the *Nannochloropsis* genus and a mixture of one *Nannochloropsis* sp. strain with *Phaeodactylum tricornutum* were tested in 50 L GWP reactors (Figure 4.2.1).

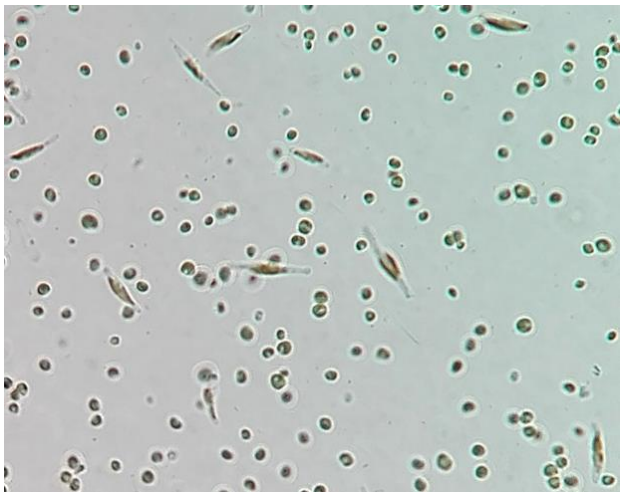

Figure 4.2. 1. Microscope observation (400x amplified) of cells disposition in the 0013NN+0018PA polyculture.

Based on the preliminary study on the ideal percentage of each strain to be used in the polycultures (section i, in Appendices), an equal dry weight percentage of each strain of *Nannochloropsis* spp. and a 40% of *P. tricornutum* (0018PA) and 60% of one *Nannochloropsis* sp. strain (0013NN) ratios were applied.

P. tricornutum was also cultivated at this scale to compare the growth dynamic and biochemical composition differences between the monoalgal and polyculture systems. In the following table (Table 4.2.1) the growth parameters obtained for the monoalgal growth of 0018PA in 50 L GWP reactors are described.

Table 4.2. 1. Productivities and specific growth rates of *Phaeodactylum tricornutum* in 50 L GWP reactors.

Microalgae strain	Productivity ($gL^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
0018PA	0.012 ± 0.002	0.023 ± 0.004	0.116 ± 0.001	0.312 ± 0.086

At the same scale, the biochemical composition on the lipid content and fatty acid profile of 0018PA culture biomass was also evaluated. These results are discriminated in Table 4.2.2.

Table 4.2. 2. Total lipids percentage and fatty acid profile of *Phaeodactylum tricornutum* cultivated in 50 L GWP reactors.

Total lipids and fatty acid profile	Percentage of lipids and fatty acids
Total Lipids	17.77 ± 0.58
C14:0	5.76 ± 0.07
C16:0	9.66 ± 0.43
C16:1	20.72 ± 0.52
C16:3 ω-3	11.25 ± 0.24
C16:4	3.86 ± 0.14
C18:0	0.88 ± 0.14
C18:1 ω-9	1.06 ± 0.16
C18:2 ω-6	3.23 ± 0.29
C18:3 ω-3	2.14 ± 0.16
C20:3 ω-6	1.21 ± 0.08
C20:4 ω-6	2.28 ± 0.46
C20:5 ω-3	32.69 ± 0.43
C22:6 ω-3	1.98 ± 0.17
C24:0	2.36 ± 0.13
SFA	18.69 ± 0.43
MUFA	21.79 ± 0.54
PUFA	58.72 ± 0.36
ω-3	36.82 ± 0.51
ω-6	6.77 ± 0.64
ω-3/ω-6	5.44
PUFA/SFA	3.14

The growth curves of both polycultures in the 50 L GWP reactors are plotted in Figure 4.2.2. The average initial dry weight biomass was 0.17 and 0.07 gL^{-1} for the 0011NN+0013NN+0039NN and 0013NN+0018PA mixtures, respectively. The comparison of the growth parameters of these two independent polycultures (Table 4.2.3) revealed that the higher global and maximum productivities obtained for the 0013NN+0018PA culture and the higher global and maximum specific growth rates obtained for the 0011NN+0013NN+0039NN culture are not statistically different ($p \geq 0.05$).

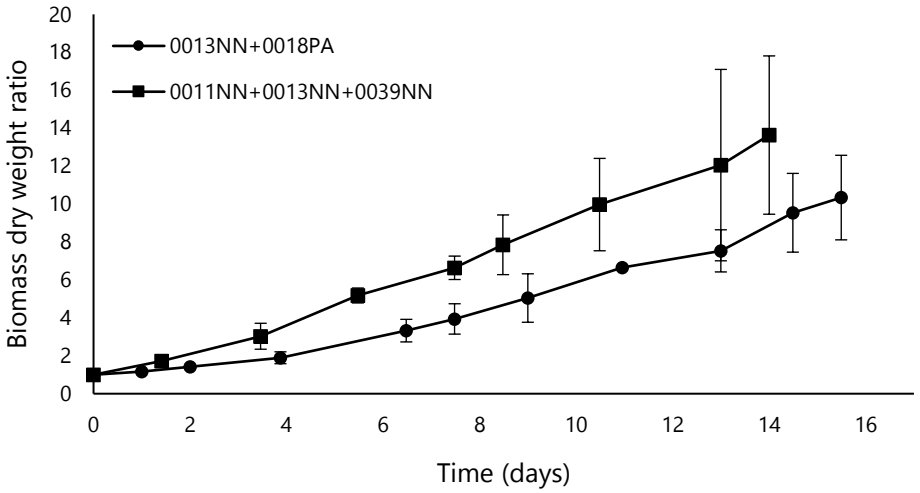

Figure 4.2. 2. Growth curves of the mixture of the three *Nannochloropsis* spp. strains and the polyculture of the two saline microalgae genera strains in 50 L GWP reactors.

Comparing the monoalgal and mixture growth performances, no significant differences were registered neither for the polyculture of *Nannochloropsis* spp. strains in comparison with the results for each strain growth or the 0013NN+0018PA polyculture in comparison with the monoalgal growth of 0013NN ($p \geq 0.05$). However, the maximum productivity of this polyculture was significantly higher when compared to the monoalgal growth of 0018PA (0.023±0.004 $gL^{-1}d^{-1}$) ($p < 0.05$).

Table 4.2. 3. Productivities and specific growth rates of the polyculture systems in 50 L GWP reactors.

Polyculture systems	Productivity ($gL^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
0011NN+0013NN+0039NN	0.067±0.017	0.193±0.022	0.150±0.036	0.378±0.071
0013NN+0018PA	0.091±0.032	0.238±0.030	0.137±0.021	0.346±0.047

Regarding the biochemical analysis (Table 4.2.4), the polyculture of the three *Nannochloropsis* spp. strains showed the highest lipid content (24.03±3.33% of total dry biomass). This value was significantly higher than the value obtained for the two microalgae genera polyculture ($p < 0.05$), however, it was similar to the values obtained in the monoalgal growth of each strain, indicating that lipid biosynthesis was not triggered by the microalgae mixture. For the other polyculture, the lipid content was lower than the value calculated for monoalgal growth of 0013NN ($p < 0.05$), and comparable with the value for the 0018PA monoalgal growth ($p \geq 0.05$).

Table 4.2. 4. Total lipids percentage and fatty acid profile of the polycultures biomass cultivated in 50 L GWP reactors.

Total lipids and fatty acid profile	Percentage of lipids and fatty acids	
	0011NN+0013NN+0039NN	0013NN+0018PA
Total Lipids	24.03±3.33	18.99±0.92
C14:0	27.55±0.14	27.99±0.16
C16:0	52.16±0.11	50.84±2.24
C16:1	20.29±0.21	20.96±2.55
SFA	79.71±0.21	78.85±2.55
MUFA	20.29±0.21	20.96±2.55

The fatty acid profile of both polycultures was shortened in comparison with the fatty acid profile of the biomass corresponding to the monoalgal growth of each strain (Table 4.1.3 and Table 4.2.2). Only three fatty acids were clearly separated and identified in the GC-MS chromatogram of these polycultures: myristic, palmitic and palmitoleic acid. The amount of the 16-carbon saturated and monounsaturated fatty acids was equal between the mixtures, and the 14-carbon saturated fatty acid was significantly higher in the 0013NN+0018PA polyculture (p<0.05). Given the reduced number of fatty acids, the individual percentage of each compound in the total fatty acid content was amplified. Thus, the relative percentage of the saturated fatty acids in the polycultures was significantly higher comparing with the values calculated for the monoalgal growth of each strain (p<0.05). However, the monounsaturated fatty acid content in the mixture of the *Nannochloropsis* spp. strains was lower in comparison with the monoalgal growth results. In opposition, the percentage of this fatty acid was similar for both the two different saline genera polyculture and the monoalgal growth of the participant strains in this mixture (p≥0.05).

The increased fatty acids saturation level and the absence of long-chain PUFAs suggest that the co-presence of different strains in the same reactor was perceived by the microalgae as a stressful condition. As a response, the fatty acid profile indicates that the activated defence metabolisms of the microalgae pursue an adaptation of the membrane fluidity, enhancing this mechanic barrier stiffness.

The microscopic evaluation of the cells integrity and cells number over the growth period for the 0013NN+0018PA polycultures showed that an equilibrium between the two species was reached (Figure 4.2.3), in opposition to an outgrow of one strain in detriment of the other. This result suggests that *Phaeodactylum tricornutum* growth dynamics were not repressed by the inhibitory metabolites released to the growth medium by *Nannochloropsis* spp. strains over the culture days as reported by Richmond (2004).

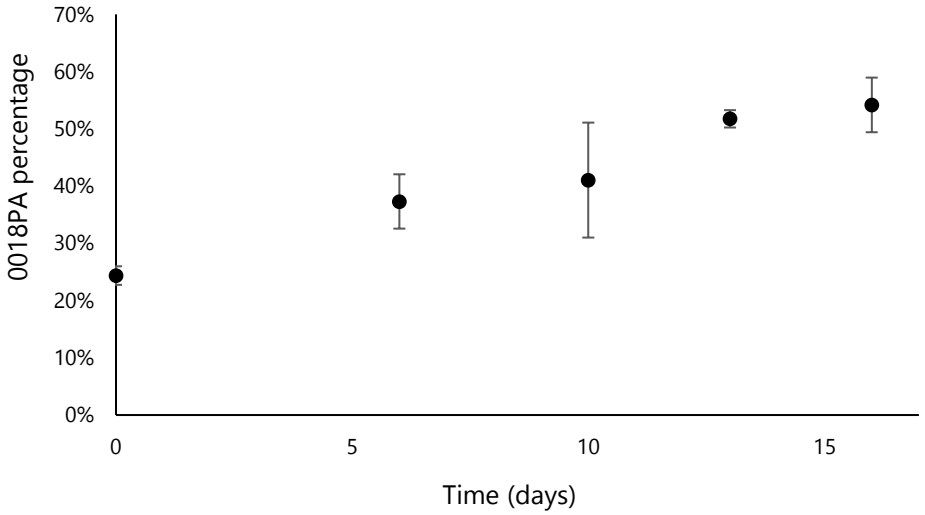

Figure 4.2. 3. 0018PA percentage (0018PA/(0013NN+0018PA)) in the polyculture system over time.

Besides, Figure 4.2.3 elucidates the different cells sizes of both species, by correlating the initial *P. tricornutum* biomass dry weight ratio of 2/5 (*i.e.* 40% of *P. tricornutum* dry biomass) with an initial *P. tricornutum* cells number ratio of about 1/4 (*i.e.* 25% of *P. tricornutum* cells). The difference between the dry weight and the cells number ratios indicates that the cell size of *Nannochloropsis* sp. was 1.6 times smaller than *P. tricornutum* cells.

4.3. Evaluation of the conversion factor of substrate to biomass

The conversion yield was assessed considering the initial and final concentrations of biomass and substrate during the growth period (Table 4.3.1). Even though the inaccuracies associated with the quantification methods of these two parameters, this yield factor calculated for the 50 L GWP reactors allowed an understanding on the most efficient microalgae system.

Table 4.3. 1. Global substrate to biomass conversion yields of the different microalgae systems in 50 L GWP reactors.

Microalgae cultures	$Y_{X/S}$
0011NN	1.99±0.06
0013NN	3.52±0.17
0039NN	2.57±0.22
0011NN+0013NN+0039NN	1.38±0.02
0013NN+0018PA	2.86±0.06

These results indicate that the system that consumes less substrate to grow biomass was the monoalgal culture of 0013NN. The two genera microalgae polyculture was the second more efficient system with a conversion factor comparable with the yield calculated for the monoalgal growth of 0013NN and 0039NN ($p \geq 0.05$). The *Nannochloropsis* spp. strains mixture showed a

significantly low conversion yield, only comparable with the monoalgal growth of 0011NN ($p \geq 0.05$). The less efficient system with significant statistical differences in comparison with the other cultures was the monoalgal growth of *Phaeodactylum tricornutum* (0.34±0.11) ($p<0.05$).

The last group of results described means that the *Nannochloropsis* spp. strains polyculture, the monoalgal cultures of 0011NN, and 0018PA spent a larger fraction of the available substrate in cell maintenance pathways and to synthetize intracellular products. The cultures with the highest conversion yields are the most growth efficient by using the highest percentage of substrate for biomass growth. Thus, for large scale biomass productions, these cultures are the most profitable in term of substrate demand.

4.4. Alternative growth media

The process optimization for *Nannochloropsis* spp. biomass production was tested using 0011NN as a model for the growth assays performed with the alternative synthetic medium composition and the saline effluent.

At the laboratory scale, the growth of this *Nannochloropsis* sp. strain in alternative growth media was positively accomplished (Figure 4.4.1), in comparison with the standard medium currently used by the company. The average initial biomass dry weight of 0011NN was 0.56, 0.54 and 0.36 gL^{-1} for the standard medium, synthetic saline water and saline effluent assays, respectively.

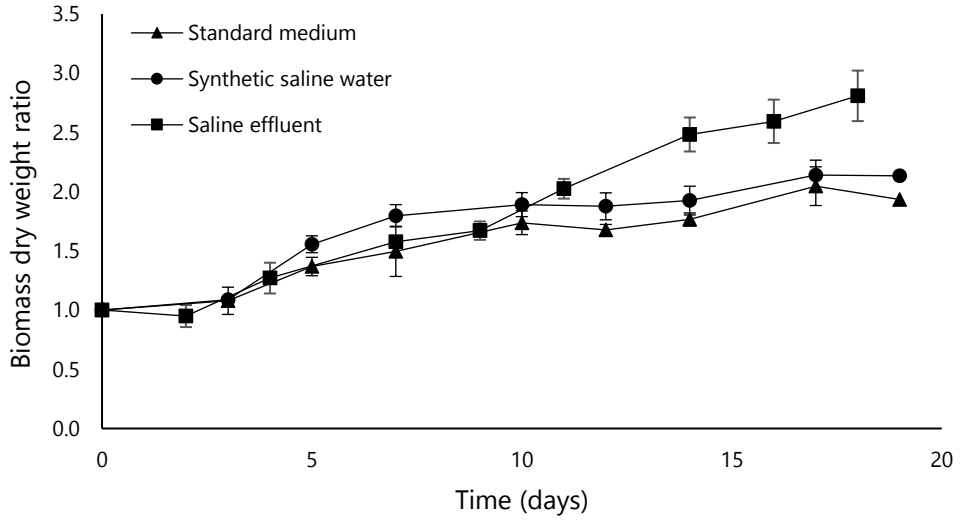

Figure 4.4. 1. Growth curves of 0011NN strain cultivated with alternative growth media in 2 L reactors.

The highest global biomass productivity was obtained using the saline effluent from a codfish company (Table 4.4.1). This value was significantly higher than the average value of this parameter when standard medium was used ($p<0.05$).

The global specific growth rate calculated for the 0011NN growth with the saline effluent was significantly higher than the same parameter for the synthetic saline water and for the medium currently used in the company ($p<0.05$). Regarding the maximum values of these growth parameters, the synthetic saline water enhanced the maximum growth productivity, while the maximum specific growth rates results were not statistically dependent on the growth medium used ($p\geq0.05$).

Table 4.4. 1. Productivity and specific growth rate of 0011NN cultivated with alternative growth media in 2 L reactors.

Growth media	Productivity ($gL^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Global	Maximum	Global	Maximum
Company standard	0.028±0.001	0.088±0.004	0.035±0.002	0.116±0.024
Synthetic saline water	0.032±0.002	0.125±0.019	0.040±0.002	0.177±0.024
Saline effluent	0.036±0.004	0.076±0.009	0.057±0.004	0.144±0.031

Some of the reported data on the global *Nannochloropsis* spp. biomass productivity in alternative growth media experiments have shown that this parameter reached higher values (0.092 $gL^{-1}d^{-1}$) when these microalgae were cultivated in anaerobic digestion effluent, at the same scale (Cai et al. 2013). Besides, a global productivity of 0.028 $gL^{-1}d^{-1}$ was obtained by Mitra et al. (2016) cultivating microalgae with pesticides industry wastewater at laboratory scale (0.5 L reactors), and Chen et al. (2018) reported a biomass productivity of 0.155 $gL^{-1}d^{-1}$ in a 5 L reactor using deep-see water. However, similar results in comparison with the synthetic saline water and saline effluent used in this work were published in a 10 L culture of *Nannochloropsis* spp. grown with palm oil mill effluent (0.034 $gL^{-1}d^{-1}$) (Hadiyanto et al. 2017).

These data suggest that there are several opportunities for further improvements on the use of alternative growth media for biomass production still poorly studied.

The scale-up of both the synthetic saline water and the saline effluent alternative growth media for these microalgae growth was tried in 50 L GWP reactors. The growth dynamic of 0011NN at this scale was not fully understood given the issues found in the reactor operation. In the growth with synthetic saline water, the air feeding was stopped as a result of the salt precipitation and clogs formation. For the culture grown with the saline effluent, the aeration led to foam formation and consequent culture volume losses.

4.4.1. Economic evaluation

The results reported for the different studies on the use of industrial wastewaters streams to grow microalgae are mainly focused on the environmental perspective, *i.e.* nutrients removal, and on the biomass productivities optimization using these alternative growth media (Hadiyanto et al.

2017). However, few reports mention the economic evaluation by correlating the biomass selling price with this product synthesis cost. Among the different parameters that influence the production cost, such us the reactor model and mixing, microalgae have been reported as a profitable feedstock for fuel and high-value compounds extraction at an overall production cost of 0.68 € per kilogram (Norsker et al. 2011).

Regardless the different cost factors involved, in this work, the economic evaluation was exclusively determined for the synthetic saline water growth medium. Nevertheless, all the values described are underestimated since the economic evaluation was deprived of the common components that are used in the company standard growth medium composition and in this alternative growth medium, *e.g.* salt. Thus, the values presented in Table 4.4.1.1 quantify the cost of the components per final reactor volume required for the synthetic saline water preparation.

Table 4.4.1. 1. Reactants cost from different suppliers for the synthetic saline water growth medium preparation.

Company	Cost (€L^{-1})
Algafarm + Sigma-Aldrich	0.154
Quimidroga	0.010
JMGS	0.290

These results indicate that the currently used growth medium was the less expensive (0.005 €L^{-1}). Besides, even with the lowest cost for the synthetic saline water medium preparation (two-fold higher than the standard medium), the ratio between the growth medium preparation cost and the biomass productivity was not profitable for this alternative growth medium. The company growth medium cost-effectiveness was 1.7 times higher in comparison with the cheapest price for the synthetic saline water preparation.

Given the biomass productivity result obtained for the saline effluent, growth medium costs lower than 0.007 €L^{-1} are more cost-effective than the currently option used in the company. Although the technical problems registered in the 50 L GWP reactors, and despite the cost-effectiveness ratios were calculated based on growth results at the laboratory scale, these productivities data and growth medium prices comparison open optimistic insights for the use of wastewater streams as an attempt for profitable larger-scale production of *Nannochloropsis* spp. biomass.

5. Conclusions

The environmental perspective, the high-value composition, the food trends, and the commercial value are the main reasons for the ever-increasing industrial production of microalgae. Regardless the motivation to be part of this market, the production of this biomass product is understudied. Thus, several approaches to gather the optimum conditions that stimulate the maximum efficiency of the biological metabolisms network of microalgae are of fundamental importance to guarantee that the production process is optimized up to the highest cost-effective ratio. Besides the growth conditions, the biomass composition of the microalgae strains is another parameter that must be included in this profitability evaluation.

The comparison of the three different *Nannochloropsis* spp. strains in terms of growth dynamics and biochemical composition, the microalgae polycultures to reach competitive biomass productivities and enrich the biomass composition, and the alternative media compositions were the attempts studied to optimize the variables that are included in the process optimization for microalgae biomass production.

The three *Nannochloropsis* spp. strains are solid candidates for the new era of nutritional value compounds from vegetable sources. Given the high ratio of unsaturated ω-3/ω-6 fatty acids and the significant amount of EPA present in these microalgae biomass, the raw materials for innovative pharmaceutical formulas to benefit human health are found.

From an industrial perspective, the process for these microalgae biomass production needs improvements to meet higher growth parameters values. Since no significant advances were achieved neither on growth or the biochemical content with the polyculture systems and given the discrepancy of these microalgae biomass productivities compared to previously reported works, the optimization strategies must include engineering approaches in the reactors design.

More wastewater streams and alternative growth media must be used for microalgae growth, not exclusively motivated by the cost-effective biomass production but mainly as a mean to decrease the pollutants content present in several industries wastewater streams. The potential of this circular economy benefits both the industrial and environmental views.

6. Future Recommendations

In an environmental perspective, besides the contaminants reduction from wastewater steams, the growth dynamic of *Nannochloropsis* spp. strains should be further studied, comprising the evaluation of atmospheric CO_2 mitigation at industrial scales. The growth parameters must also be assessed regarding the ratio of biomass formed by the light photons available to evaluate the photosynthetic efficiency. Further process optimization strategies should include growth medium recirculation to decrease the nutrients and water waste. Besides the growth parameters optimization, the overall process could be optimized concerning the biomass harvesting procedure. Instead of filtration by membranes, the pressure drop and pumps costs could be decreased if sedimentation or flocculation techniques were applied.

The following works must be focus on the biochemical content of *Nannochloropsis* spp. strains biomass over the growth period, in order to identify fatty acids and lipid content patterns. This tracking should be applied for the polyculture systems and for the growth on alternative growth media. The fatty acid content should also be evaluated as a function of the cultivation season as a mean to study if significant differences are registered in the unsaturation content under lower temperatures.

Besides the biochemical analysis, the growth dynamics of these microalgae monoalgal and mixture cultures should be followed and described by means of a mathematical growth model. This information is crucial to strive higher scale production of polycultures and microalgae growths in alternative growth media.

Since the nutritional value of microalgae is of major concern for the commercial market and it is a brand-new diet tendency, faster growth kinetics and compatible organic carbon sources should be evaluated to perform heterotrophic growth regimes with *Nannochloropsis* spp. towards higher quantity of high-value compounds in these microalgae biomass. Aiming the same end, metabolic engineering strategies may also be studied. With higher control and culture manipulation knowledge, *Nannochloropsis* spp. market could be extended to the pigments content.

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Appendices

This section contains supplementary information about preliminary screening experiments and the description of both growth medium compositions. The correlation curves of the spectrophotometry results and biomass dry weight are also described. The same parametric calibration for the absorbance as a function of the nitrate concentration is included in this section.

i. Preliminary assessment to microalgae polyculture growth

Before the scale-up of the polycultures to higher reactors volumetric capacities, an initial screening was performed for the mixture of the three different *Nannochloropsis* spp. strains and for the mixture of the two saline microalgae genera. In Table i.1 and i.2 are described the initial dry weight percentages of each strain for the different ratios evaluated.

Table i. 1. *Nannochloropsis* spp. strains ratios for a preliminary assessment of the growth performance.

Strain	Dry weight ratio (%)			
0011NN	33.3	50	25	25
0013NN	33.3	25	50	25
0039NN	33.3	25	25	50

Table i. 2. 0013NN and 0018PA ratios for a preliminary assessment of the growth performance.

Strain	Dry weight ratio (%)				
0018PA	10	20	30	40	50
0013NN	90	80	70	60	50

The outcome of these preliminary results showed that the optimum ratio for the higher scale implementation of the polycultures was the equal dry weight percentage of the three *Nannochloropsis* spp. strains for this genus polyculture and the 40% of *Phaeodactylum tricornutum* and 60% of *Nannochloropsis* sp. for the two microalgae genera polyculture.

ii. Alternative growth media formulations

The composition of the different growth media that were used to evaluate the growth of *Nannochloropsis* spp. is described in Table ii.1 and ii.2. Microwave Plasma Atomic Emission Spectroscopy (4200 MP-AES, Agilent, USA) was used to obtain the elementary composition of the saline effluent from a codfish processing company. Despite the different concentrations, both formulations guaranteed the presence of the essential nutrients associated with microalgae growth.

Table ii. 1. Synthetic shrimps cultivating growth medium preparation for a 50 L reactor.

Reactants	Weight (g)
NaCl	1380
$MgSO_4.7H_2O$	672
$CaCl_2.2H_2O$	70
KCl	30
$NaHCO_3$	10
KBr	1.35

Table ii. 2. Elementary composition of the saline effluent obtained from a codfish processing company.

Elements	Concentration (mgL^{-1})
Ba	0.006 ± 0.001
Ca	129.008 ± 0.935
Cu	0.313 ± 0.001
Fe	6.595 ± 0.092
K	5105.898 ± 76.440
Mg	438.154 ± 0.120
Mn	0.567 ± 0.028
Mo	44.196 ± 0.708
Na	79456.167 ± 2949.398
Sb	46.315 ± 0.931
Se	3.464 ± 0.360
Sn	44.404 ± 0.856

iii. Calibration curves of optical density and biomass dry weight

The dry weight of each monoalgal strain and polyculture studied was correlated with the spectrophotometric cell density analysis in order to establish an indirect method for biomass quantification. Thus, by means of the calibration curve, the optical density of a culture was expressed as biomass concentration. The following figures (Figures iii.1-6) represent the calibration curves that were used for the different strains and mixtures.

As a note, it was considered the same calibration curve for a specific strain that was grown both in the standard growth medium of the company or in the alternative growth media. Besides, the cell physiology and morphology were assumed constant over the sequence of experiments performed (this parameter was periodically analysed by means of microscopic observation).

Three different wavelengths were used for the optical measurements. Within this scanning range, the absorbance recorded at 600 nm was defined as the correlation parameter. The optical

density of the calibration curve for *Phaeodactylum tricornutum* was already established and it was measured with Genesys 10s UV-Vis spectrophotometer at 540 nm (Thermo Fisher Scientific, USA).

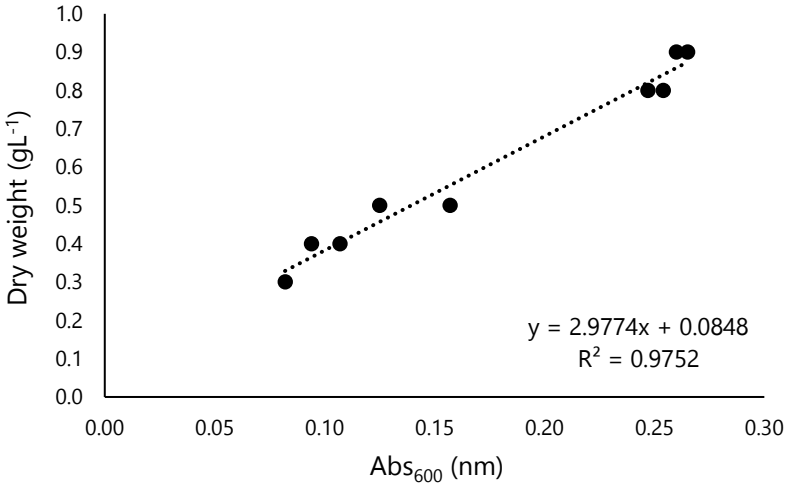

Figure iii. 1. Calibration curve of optical density at 600 nm as a function of the 0011NN biomass dry weight.

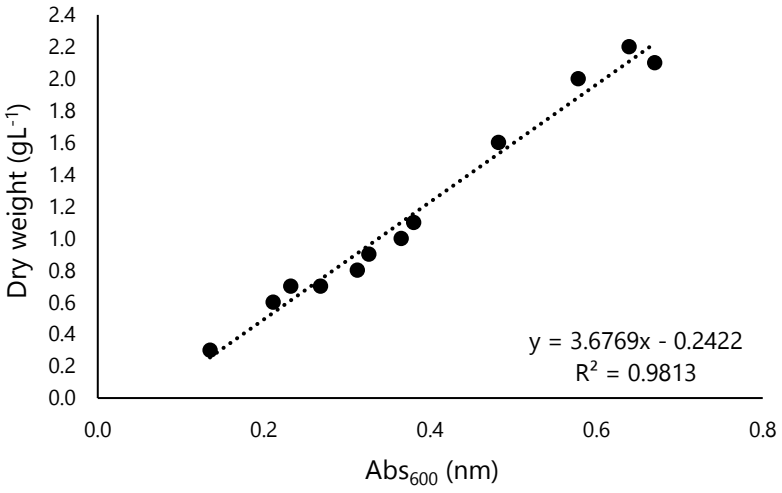

Figure iii. 2. Calibration curve of optical density at 600 nm as a function of the 0013NN biomass dry weight.

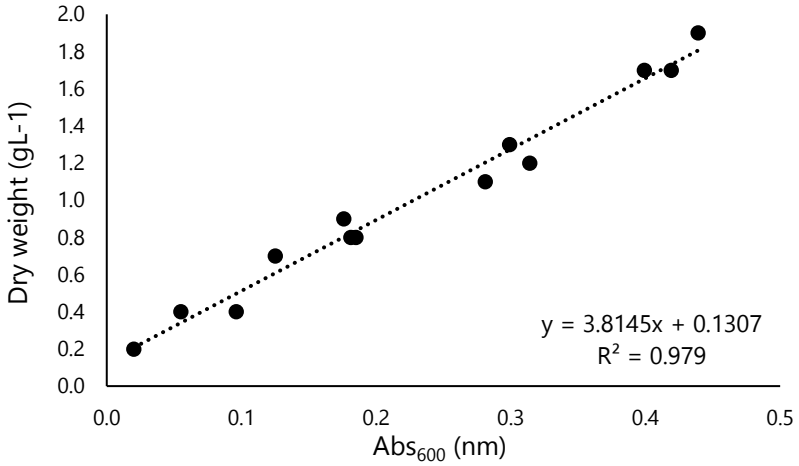

Figure iii. 3. Calibration curve of optical density at 600 nm as a function of the 0039NN biomass dry weight.

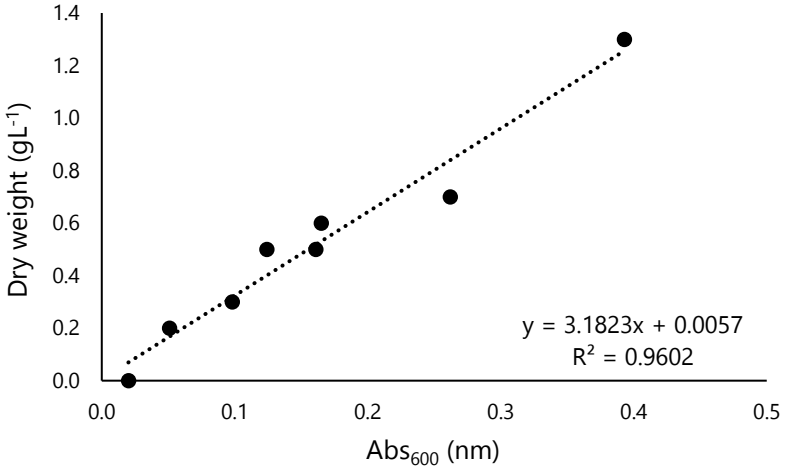

Figure iii. 4. Calibration curve of optical density at 600 nm as a function of the 0011NN+0013NN+0039NN polyculture biomass dry weight.

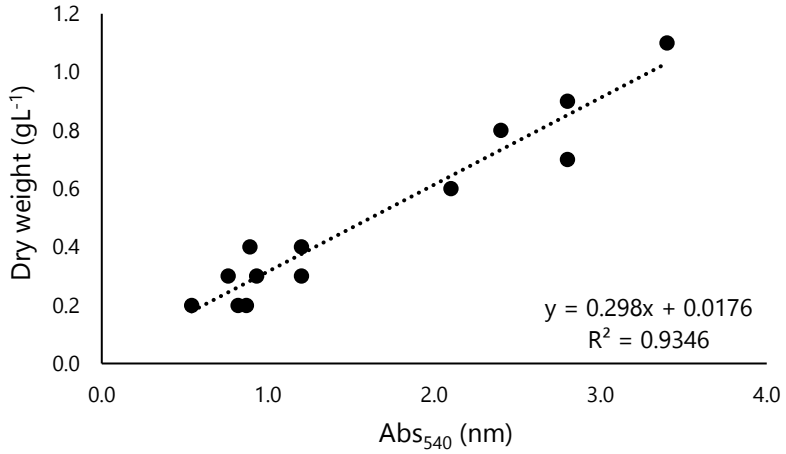

Figure iii. 5. Calibration curve of optical density at 540 nm as a function of the 0018PA biomass dry weight.

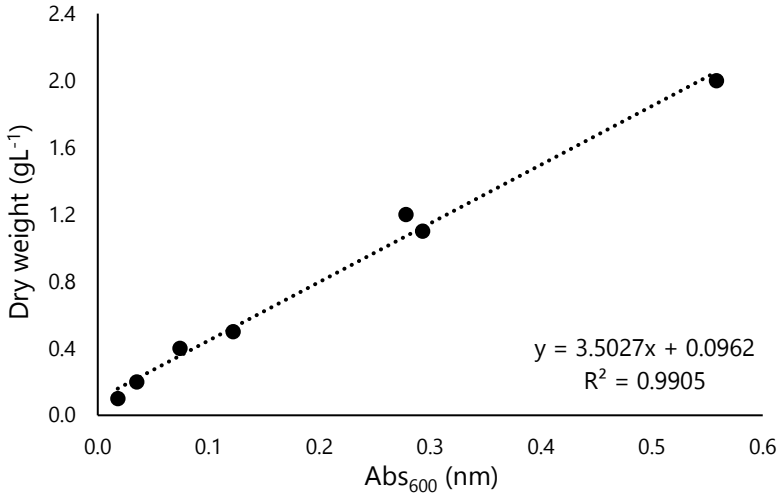

Figure iii. 6. Calibration curve of optical density at 600 nm as a function of the 0013NN+0018PA polyculture biomass dry weight.

iv. Calibration curve for nitrate concentration estimation

The calibration curve used for the nitrate quantification was experimentally determined by spectrophotometric measurement of the absorbance of dilution series of a standard solution (Figure iv.1). The correlated absorbance is a difference of the absorbance measured at 220 nm and two times the absorbance at 275 nm (Clescerl et al. 1999).

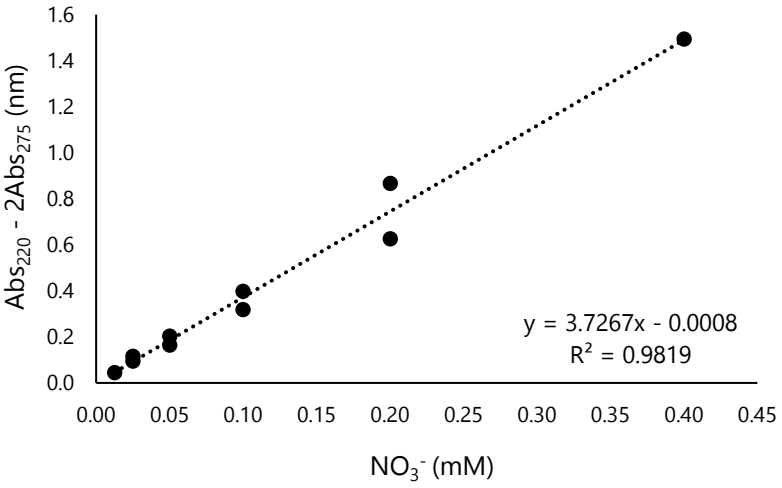

Figure iv. 1. Calibration curve of the absorbance as a function of $NaNO_3$ concentration.