

Integrated Master in Bioengineering
Specialization in Biological Engineering

Optimization of *Nannochloropsis oceanica* cultivation in
pilot-scale raceway ponds

Master's Thesis by

Pedro Cunha

FEUP Supervisor: Professor Doctor Manuel SIMÕES

Allmicroalgae Supervisor: Doctor Joana SILVA

Allmicroalgae Co-supervisor: Doctor Hugo PEREIRA

September 2019

Agradecimentos

Para começar quero agradecer ao professor Manuel Simões e à Joana Laranjeira pela oportunidade que me deram de passar 6 meses incríveis na Algafarm. Como a passagem da faculdade para uma fábrica de produção de microalgas não é nada fácil, quero agradecer a algumas pessoas que me ajudaram muitíssimo no período de adaptação: à Joana Teles por me ter ensinado tudo o que sei sobre *outdoor* e por ter sido sempre o pronto-socorro quando alguma coisa corria mal (sem me dar um único sermão!); ao Nuno por ter sido o coitado que levou comigo durante dois meses a chateá-lo para ter os *raceways* prontos - foi graças a ele que consegui ter os reatores operacionais; ao Miguel por ter sido o gajo que mais me ouviu desde que cheguei à Algafarm, por me ter ensinado tudo o que sei de oficina (e não sabia nada!) e por ter consertado tudo aquilo que eu estragava; ao Humberto, o mais fixe dos operacionais dos turnos, por me ter passado o inóculo para IBCs sempre que precisava (especialmente aos fins-de-semana e feriados!); ao Dinis por me ter dado sempre tudo aquilo que pedia, desde IBCs, a milhares de litros de *Nanno*, a sal, a água de magnésio, enfim, tudo e mais alguma coisa; ao Fabrício por ser o único que ia buscar a empilhadora à Secil sempre que eu lhe pedia; ao Rui Correia principalmente pelo cabo que ligava à estação meteorológica, mas não só (também foi uma das pessoas que levou bastante comigo, se bem que muitas vezes me mandava à fava); ao Quelhas que, apesar de me ter feito a vida negra, me ajudou sempre que precisei dele na Algafarm; e à Soraia, a todos os operacionais (pessoal dos turnos e limpezas) e pessoal do laboratório porque acho que não houve uma única pessoa à qual eu não tenha ido bater à porta para pedir ajuda durante a minha estadia na Algafarm.

No entanto, há uma pessoa que merece um agradecimento especial, o Hugo. O Hugo, apesar de ser um *bully* do pior, foi das pessoas mais importantes para esta tese ter pernas para andar. Foi ele que idealizou o trabalho e foi a ele que liguei centenas de vezes para perguntar tudo e mais alguma coisa sobre microalgas. Nunca sei quando é que ele está a falar a sério e respondo-lhe à letra sempre que me provoca (porque sou do Porto e o pessoal da invicta é assim), mas não podia ter pedido um mentor (vê lá o respeito que eu te tenho, oblá) melhor.

O maior agradecimento de todos vai para o Chico, a Inês, a Joana, a Susana, a Rosângela e a Sara, por terem sido a minha família fora de casa. Passei 6 meses incríveis ao lado desta gente, 24 sobre 24 horas, fins-de-semana e feriados e disfrutei de cada momento. Vivemos aventuras incríveis, fizemos da Algafarm uma segunda casa e da casa 70 o nosso lar. Criámos um grupo tão unido (UNIÃO!) que, por mais incrível que pareça, nem senti falta de casa. Não há palavras que cheguem para agradecer tudo o que fizeram por mim... Mas como uma imagem vale mais do que mil palavras, deixo aqui uma num dos nossos sítios/momentos



sagrados, os almoços no Cantinho.

Quero também deixar aqui uma palavra em especial para a Margarida, o João, o Bernardo e a Mafalda (ah, e para o João, o eremita, como é que me podia esquecer dele!). À Margarida pela cumplicidade; ao João por me ter acompanhado durante a escrita da tese e por tê-la revisto vezes e vezes sem conta; ao Bernardo por ter sido o meu professor de estatística e por fazer uma festa sempre que me cruzava com ele; à Mafalda por ter sempre uma palavra amiga a dizer e ao João eremita por ser ele próprio. Uma palavra também para o Fábio que, apesar de ter chegado mais tarde à AlgaFarm e à 70, se tornou num bom amigo.

Quero também agradecer às pessoas que me acompanharam durante todo o curso: à Carla, à Sílvia e à Paula, as melhores técnicas de laboratório do mundo; ao Silveira, um portista como eu; ao Raio, à Mariana e à Filipa, o grupo da Jamizade. Mas não posso deixar de destacar o Losa, o Maio e a Rita. Nós os quatro formámos o tão conhecido grupo 'A Rita e os Pedros' e foi com eles que passei grande parte da minha vida na faculdade. Uma coisa é certa, se não fossem eles ainda não tinha acabado o curso. Losa, obrigado pelos milhões de exercícios e exames resolvidos que me emprestaste; Rita, obrigado por seres uma chata do pior e Maio, obrigado por teres ensinado a usar o LaTeX e a dar a volta às suas idiossincrasias. Se, há pouco, disse que o maior agradecimento de todos ia para o meu grupinho da AlgaFarm, um agradecimento igualmente grande vai para vocês por estes 5 anos de amizade.

Por fim, agradecer à família por ser o suporte que todos precisamos. Em especial, ao meu velhote (que não faz mais do que a obrigação dele, por ser meu pai) e ao meu irmão, que, apesar de ser mouro, até é um gajo porreiro.

Abstract

Microalgae biotechnology is an emerging area that has been explored to address some of the global problems. Microalgae high protein content makes them a suitable option to face famine and malnutrition in developing countries. High lipid-producing microalgae are a promising eco-friendly alternative to non-renewable fossil fuels. Besides, the wide range of bioactive compounds synthesized by microalgae, especially cyanobacteria, triggered the search for microalgal metabolites with biological activity.

Air-open reactors, particularly raceway ponds, are the most commonly microalgal production systems used at an industrial scale. In this work, two different raceway configurations (a standard design widely used and a modified configuration) were tested, under the same processing conditions. Biomass productivity, biochemical composition of the biomass produced, carbon fixation efficiency and power requirements were evaluated. Water depths of 0.20 and 0.13 m, and culture circulation velocities of 0.30 and 0.15 m s⁻¹ were used.

Standard configuration proved to be the more energetic efficient one, consuming at least 100% less energy than the required by modified configuration, depending the process conditions used. Since the results obtained for all the other assessment criteria were similar, standard raceway is the best option for microalgae cultivation. Thus, this configuration will be used in the scale-up process. Power demands were also the differentiator criteria for the choice of 0.20 m and 0.15 m s⁻¹ as the best process conditions. The energy consumption was 54% and 100% higher with a 0.13 m water depth and a 0.30 m s⁻¹ circulation velocity comparing with a 0.20 m water depth and a 0.15 m s⁻¹ circulation velocity, respectively. Despite the use of outdoor production systems, the quality of the biomass produced was ensured by the fact that the ω -3/ ω -6 and PUFA/SFA ratios were higher than 2 and 1, respectively.

Keywords: Raceway ponds, *Nannochloropsis oceanica*, biomass productivity, fatty acid profile, carbon sequestration, power requirements

Resumo

A biotecnologia de microalgas é uma área emergente que tem sido explorada para dar resposta a algumas das problemáticas globais. Dado serem seres vivos ricos em proteínas, podem ser um meio para combater a fome e malnutrição nos países desenvolvidos. Os biocombustíveis sintetizados a partir de microalgas com um elevado conteúdo lipídico são uma alternativa ecológica e renovável aos combustíveis fósseis. Para além disto, a grande variedade de compostos bioativos produzidos por microalgas, em especial cianobactérias, desencadeou a procura por metabolitos sintetizados por microalgas com atividade biológica.

A grande maioria dos sistemas comerciais de produção de microalgas são sistemas ao ar livre, principalmente *raceways*. Neste trabalho, testaram-se dois *raceways* com diferentes configurações, um com o *design* standard, universalmente utilizado, e outro com um *design* modificado. A sua *performance* foi avaliada com base nos seguintes critérios: produtividade em biomassa, composição bioquímica da biomassa produzida, eficiência de fixação de carbono e consumo de energia. Foram utilizadas lâminas de água de 0,20 e 0,13 m e velocidades de circulação de 0,30 e 0,15 m s⁻¹.

A configuração *standard* provou ser mais eficiente do ponto de vista energético, consumindo, consoante as condições processuais, metade ou menos de metade da energia necessária para operar o *raceway* modificado. Tendo em conta que, para todos os outros critérios, os resultados obtidos foram semelhantes, concluiu-se que a melhor configuração é a *standard*. Logo, o processo de aumento de escala vai ser realizado a partir desta configuração. No que diz respeito ao processo de otimização da lâmina de água e da velocidade de circulação da cultura, também as questões energéticas foram o critério decisivo. A lâmina de água de 0,20 m e a velocidade de 0,15 m s⁻¹ foram as que obtiveram melhores resultados. O consumo energético foi 54% e 100% superior para uma lâmina de água de 0,13 m e uma velocidade de circulação de 0,30 m s⁻¹ em relação às outras duas condições processuais utilizadas. Apesar de terem sido utilizados sistemas de produção de microalgas ao ar livre, a obtenção de rácios ω -3/ ω -6 e ácidos gordos polinsaturados/ácidos gordos saturados superiores a 2 e 1, respetivamente, atesta a qualidade da biomassa produzida.

Palavras-chave: *Raceways*, *Nannochloropsis oceanica*, produtividade em biomassa, perfil de ácidos gordos, fixação de carbono, consumo energético

Statement

I hereby declare, under honour compromise, that this is an original work and that all the non-original contributions were appropriately mentioned with the identification of their source.

Pedro Cardoso e Cunha

September 2019

Contents

List of Figures	iii
List of Tables	iv
1 Introduction	1
1.1 Microalgae	1
1.1.1 General overview	1
1.1.2 Biodiversity	1
1.1.3 Growth dynamics	3
1.1.4 Biochemical composition	4
1.1.4.1 Macro and micronutrients	4
1.1.4.2 Added-value compounds	6
1.1.5 Biotechnological applications	9
1.2 <i>Nannochloropsis</i> spp.	12
1.3 Biomass production	14
1.3.1 Open vs. Closed systems	14
1.3.2 Open production systems	15
1.3.3 Biomass harvesting and processing	16
1.4 Contextualization	17
1.4.1 Secil/Algafarm/Algavalor	17
1.5 Goals	18
1.6 Outline of the work	18
2 Materials and Methods	20
2.1 Raceway ponds	20
2.2 Microalgal strain and culture medium	21
2.3 Setup of the experimental trials	21
2.4 Cultivation process layout	21
2.5 Sampling methodology	22
2.6 Microalgal growth assessment	23
2.7 Photosynthetic efficiency	24
2.8 Biochemical composition	25
2.8.1 Proximal composition	25
2.8.2 Fatty acid profile	25
2.9 Total organic carbon	27

2.10	Stoichiometric equation	27
2.11	Carbon sequestration	27
2.12	Statistical analysis	28
3	Results and Discussion	29
3.1	Comparison of two different raceway's configurations	29
3.1.1	Economic assessment	33
3.2	Water depth optimization	34
3.3	Culture circulation velocity optimization	35
4	Conclusions and Future perspectives	37
	References	38
	Appendices	i
A	Calibration curve	i
B	Growth curves	i
C	Meteorological conditions	iii

List of Figures

1	Taxonomic classification of eukaryotic microalgae. Adapted from Heimann & Huerlimann [16].	2
2	Applications of microalgae biomass. Adapted from Naidoo <i>et al.</i> [39].	7
3	Microscopic view (40 $\times$) of <i>N. oceanica</i> grown in Algafarm's pilot-scale raceway ponds.	13
4	Algafarm, the microalgae industrial production plant in Pataias, Leiria. . . .	18
5	Pilot-scale raceway ponds used in these study. Modified raceway at right and standard raceway at left.	20
6	Pilot-scale raceway ponds schematic representation.	21
7	Layout of the microalgae cultivation process and subsequent biomass analysis.	22
8	Biomass homogenization by cell breakdown.	26
9	FAME analysis procedure.	27
A.10	Calibration curve of optical density at 540 nm as a function of <i>N. oceanica</i> biomass dry weight.	i
B.11	Growth curves of <i>N. oceanica</i> cultivated under process conditions 1. Error bars represent the standard deviation calculated from two replicates.	i
B.12	Growth curves of <i>N. oceanica</i> cultivated under process conditions 2. Error bars represent the standard deviation calculated from two replicates.	ii
B.13	Growth curves of <i>N. oceanica</i> cultivated under process conditions 3. Error bars represent the standard deviation calculated from two replicates.	ii
C.14	Temperature and instant radiation registered on site during first growth trial under process conditions 1.	iii
C.15	Temperature and instant radiation registered on site during second growth trial under process conditions 1.	iv
C.16	Temperature and instant radiation registered on site during first growth trial under process conditions 2.	v
C.17	Temperature and instant radiation registered on site during second growth trial under process conditions 2.	vi
C.18	Temperature and instant radiation registered on site during first growth trial under process conditions 3.	vii
C.19	Temperature and instant radiation registered on site during second growth trial under process conditions 3.	viii

List of Tables

1	Daily sampling methodology.	23
2	Set of processing conditions used.	29
3	Biomass productivities and specific growth rates obtained in the two raceways used. Productivity values are presented in dry weight basis.	30
4	Photosynthetic efficiency of <i>N. oceanica</i> cultures.	31
5	Biomass proximal composition. Values are presented in a dry weight basis. .	32
6	Fatty acid profile of the biomass produced (n.d., not detected).	32
7	CO ₂ requirements for biomass production and CO ₂ fixation efficiency of race- way ponds used.	33
8	Paddlewheel rotation speed and associated energy consumption.	34

1 Introduction

1.1 Microalgae

1.1.1 General overview

Microalgae are a diverse group of microorganisms which combine properties of higher plants with some properties of prokaryotes [1, 2]. They comprise eukaryotic photoautotrophic protists and prokaryotic photosynthetic cyanobacteria (also called blue-green algae) [3]. Microalgae can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure [4]. Their size ranges from few micrometres to few hundred micrometres [5]. They are ubiquitous and can be found in a variety of aquatic and terrestrial environments. Microalgae usually grow in fresh and seawater, whereas several other species in extremely saline environments, e.g. - the Dead Sea in Israel. They can also be found in rich humus soil, desert sands, rocks and snowfields. Microalgae can occur virtually in every type of terrestrial environment, from biotic crusts in hot deserts to Antarctic snow [3]. Although they are generally free-living organisms, some microalgae live in symbiotic association with a variety of other organisms, as in the case of lichens, which are a mutualistic relation between algae and fungi [3, 6].

Microalgae are very important members of the biota as they play an important role in the global carbon and nitrogen cycles. In fact, by using light energy to produce organic matter, algal photosynthesis contributes to around 50% of the total planetary primary production [7, 8]. Regarding nitrogen, heterocyst and non-heterocyst cyanobacteria fix atmospheric nitrogen (N_2) into organic nitrogen and are the major contributors to global biological nitrogen fixation. A single genus, the non-heterocyst marine planktonic cyanobacterium *Trichodesmium*, is responsible for a significant part of the total global nitrogen fixation [9, 10].

1.1.2 Biodiversity

The microalgae term is devoided of taxonomic value [11]. They constitute a polyphyletic and highly diversified group of microorganisms which are currently placed in four kingdoms (Bacteria, Plantae, Chromista and Protozoa) [12] and can be classified according to their pigmentation, chemical nature of the storage products and cell wall constituents and structure [6, 11]. Additional criteria take into consideration the following cytological and morphological characters: occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and a possible connection between the endoplasmic reticulum and the nuclear membrane [6].

Microalgae can be placed in two domains/superkingdoms, Bacteria and Eukaryota. Eu-

karyota domain includes the eukaryotic microalgae and can be divided into four supergroups, Archaeplastida (Glaucophyta, Rhodophyta, Chlorophyta), Chromalveolata (Cryptophyta, Haptophyta, Stramenopiles, Alveolata), Rhizaria (Chlorachniophyta) and Excavata (Euglenophyta), which in turn comprise several phyla/divisions, as can be seen in the Figure 1 [13, 14]. Bacteria domain includes the prokaryotic microalgae and is divided in two phyla/divisions, Cyanophyta and Prochlorophyta [15].

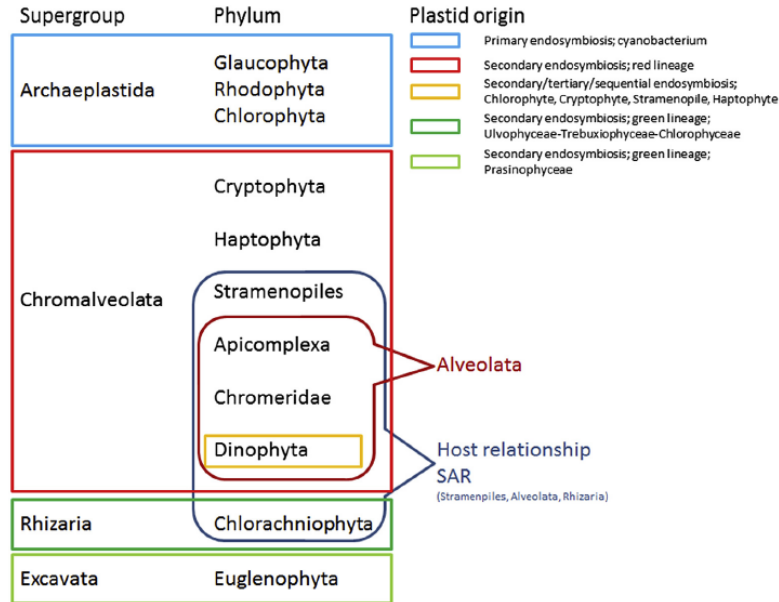


Figure 1. Taxonomic classification of eukaryotic microalgae. Adapted from Heimann & Huerlimann [16].

Division-level classification, as with kingdom-level classification, is tenuous for algae. Classes are better defined and more accepted than divisions, thus taxonomic discussions of algae are usually constrained at the class level [17]. The three most important classes of microalgae in terms of abundance are the diatoms (Bacillariophyceae), the green algae (Chlorophyceae), and the golden-brown algae (Chrysophyceae) [5]. There are still other large groups such as, the brown algae (Phaeophyceae), the red algae (Rhodophyceae) and the dinoflagellates (Pyrrophyceae).

In recent years, major advances have been made in the study of the systematics of algae, especially the microalgae where taxonomies are based on the limited morphological characters available. Molecular analyses are providing new characters well beyond the morphology-based systematics, leading to a much better understanding of what may constitute a species, as well as the phylogenetic and evolutionary relationships between them. In the future, developments in proteomics and metabolomics will also lead to new insights into the speciation of algae [12].

1.1.3 Growth dynamics

Although the pigmentary profile of microalgae makes the photosynthesis the principal metabolic model, these organisms stand out by notable versatility to provide the necessary energy to growth and maintenance [11]. They are capable of different kinds of trophic (nourishment), that can be classified according to the carbon source and the energy source [18]. From the use of the carbon source, one can differentiate two basic types of nutrition: autotrophic and heterotrophic [11]. Autotrophs use carbon dioxide (CO_2) as the carbon source and heterotrophs use an organic compound produced by other organisms [18]. Regarding the energy source used one can classify the organisms as phototrophs and chemotrophs, being the former divided in chemoorganotrophs and chemolithotrophs, if the electron donor is an organic or an inorganic molecule, respectively. In phototrophy, the light energy is the only energy source being converted in chemical energy by the photosynthesis process [11]. On the other hand, in chemotrophy the energy source is an exogenous organic or inorganic molecule.

Most algae are photoautotrophs, that is, they only use energy from light, CO_2 and water to produce organic compounds through oxygenic photosynthesis. However, there are also several algae that can grow in the absence of light using simple organic molecules, such as acetate or glucose, as both a carbon and an energy source. These algae have a heterotrophic metabolism [20].

Among all these metabolisms, two of those are extreme. The first comprise the photosynthetically competent algae which cannot use exogenous organic carbon to stimulate light- and inorganic carbon-saturated growth even if organic carbon can be assimilated by the cells. These organisms are called obligate photolithotrophs. On the other hand, the obligate heterotrophs are organisms which are clearly derived from photosynthetically competent ancestors, but lack photosynthetic capacity and can only grow using exogenous organic carbon as the energy and carbon source [20]. Between these extremes, there are another metabolic process that is common to observe, called mixotrophy. As the name implies, this metabolism is a sort of a mix between the autotrophic and heterotrophic processes. A definite switch between autotrophy and heterotrophy is not manifested and both processes are present, except in total darkness [18]. The respiratory and photosynthetic systems work simultaneously being both organic compounds and CO_2 necessary to the growth [11]. Mixotrophic algae use photosynthesis as the major source of energy, but may also use organic compounds to stimulate the growth [19, 20]. However, it is important to note that mixotrophic organisms assimilate organic compounds as a carbon source, while using inorganic compounds as electrons donors [11].

Auxotrophy is another mode of nutrition used by algae. It is a form of heterotrophy

in which the organisms require only small amounts of essential organic molecules, such as vitamins and amino acids, that cannot be synthesized by the auxotroph organism [19].

There are some photosynthetic microalgae that, in the presence of organic molecules as sugars, organic acids and acetate, are capable of switching to the heterotrophic metabolism in the dark [11]. The substitution of CO₂ supply of the photosynthetic cultures by exogenous carbon sources, allows the increase of biomass concentration and productivity during the night period [11]. Thus, some amino acids and urea have been used as source of carbon and nitrogen to support the microalgal growth in the dark [11]. Microalgae not able to perform this metabolic transition, have a biomass loss overnight due to dark respiration [21]. Biomass-specific dark respiration rates in microalgae are highly species-dependent, varying from 0.01 to 0.6 day⁻¹, and can comprise as much as 30% of the strain's maximum specific growth rate [22].

1.1.4 Biochemical composition

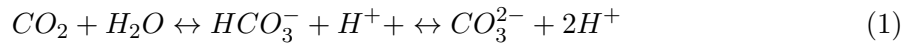
1.1.4.1 Macro and micronutrients

The culture medium suitable to a good growth must provide all the micro and macronutrients demanded by microalgae [11]. The group of macronutrients includes nitrogen (N) and phosphorus (P). In heterotrophic growth, Carbon (C) is also considered a macronutrient. As for micronutrients, sulfur (S), potassium (K), sodium (Na), magnesium (Mg), calcium (Ca) and trace metals like iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo), cobalt (Co), zinc (Zn), boron (B), vanadium (V) and selenium (Se) are the most relevant [11, 18].

The three most important nutrients for autotrophic growth are C, N and P and their supply is essential to algal biotechnology [18]. Inorganic carbon occurs in water in the form of CO₂, HCO₃⁻ and CO₃²⁻ with the relative proportions of each species being mainly dependent on the pH, temperature and salinity [20]. Due to its low diffusion rates from the atmosphere into the culture media, atmospheric CO₂ is not enough to satisfy C-requirements of high yielding autotrophic algal production systems [18]. Thus, external inorganic carbon supply is frequently required.

It is important to note that pH is a very important factor in carbon (C) assimilation by cells. The inorganic carbon speciation is strongly influenced by pH. At pH 6 about 50% of the inorganic carbon is in the form of CO₂ and 50% in the form of bicarbonate, whereas at about pH 9 the inorganic C is about 50% bicarbonate and 50% carbonate with little or no CO₂ [20]. Considering that all algae can assimilate inorganic carbon in the form of CO₂, many can assimilate bicarbonate and none can assimilate carbonate, the carbon fixation by the cells is favoured at lower pHs [20]. However, as the assimilation of inorganic carbon by the cells is pH-dependent, also the pH of the cultures depends on the CO₂ supply into

the culture media. For high photosynthetic activity there is an increase in culture pH that may reach pH values as high as 11 in high algal density production systems [23]. Thus, pH must be lowered and the best way of doing so is through pH static control via direct CO₂ sparging into the culture. This method allows not only to decrease the system pH, but also to supply the carbon source needed for high yield in mass photosynthetic algal cultures, being considered the most convenient method of pH control. This method makes use of the bicarbonate-carbonate buffer system generally present in freshwaters, whose equilibrium is presented in the following equation:



Carbon can also be supplied organically as sugars, acids and alcohols [18].

Nitrogen (N) is the second most important nutrient to microalgae growth. Its content in the biomass can range from 1% to more than 10% [18]. The most common sources of inorganic nitrogen are nitrate (NO₃⁻) and ammonium (NH₄⁺), whereas for the organic nitrogen, urea (CO(NH₂)₂) is the most used example [24]. However, microalgae can also use nitrite as N-source [25]. From among these compounds, urea has the particularity of being hydrolyzed in NH₃ and CO₂ before its N is incorporated into the algal cells, being the two generated compounds able to be utilized to the cellular growth [11, 19]. When the N-source is a combination of these compounds, NH₄⁺ is generally taken up preferentially before NO₃⁻ and the same occurs to NO₃⁻ regarding urea. However, no significant differences in growth rates when these substrates are supplied in separate have been reported [18, 25]. The pH behaviour is a function of the substrate assimilated by the cells. Thus, NO₃⁻ uptake leads to an alkalisation of the medium, whereas NH₄⁺ uptake leads to acidification [25]. These pH changes, as well as the associated changes in alkalinity, will affect the availability of inorganic carbon and potentially other nutrients. Therefore, the availability of NO₃⁻ and NH₄⁺ in the medium and their uptake may affect the productivity of the culture. This is the reason why the supply of nitrogen as NO₃⁻ and NH₄⁺ must be done using a fed-batch addition of stoichiometrically-balanced media, avoiding build-up of these compounds in the culture media [25]. High concentrations of NH₄⁺ (>1 mM) may inhibit the growth and lead to death of the culture, especially at high temperatures [19].

Phosphorus (P) is another essential nutrient for microalgae growth, being integral to various aspects of cellular metabolism. This compound is involved in energy storage, cell signalling and it has an important role in cellular energy transduction. Besides, it is part of cellular structure and genetic material of cells [20, 25]. It is often one of the most growth limiting factors in algal biotechnology, despite algal biomass contains less than 1% of phosphorus. The major form in which algae take up phosphorus is as inorganic phosphate (H₂PO₄⁻ and

HPO_4^{2-}), but many algae can also utilize organic P sources. These algae hydrolyze organic phosphate compounds extracellularly and then take up the inorganic P produced [19]. Phosphate is assimilated through several $\text{Na}^+/\text{H}_2\text{PO}_4^-$ symporters and/or H^+/P_i symporters [25]. Therefore, pH is a parameter that affects P-uptake. Beyond this, the high pH reached by algal ponds during the day leads to phosphorus precipitation by bounding to other ions, such as carbonate and iron, becoming unavailable for algal uptake [18, 26, 27]. Thus, the importance of control the pH is proved once again. However, many algae are able to store excess phosphorus within the cells in polyphosphate bodies during the so-called ‘luxury uptake’ that can be used in shortage periods [18, 28].

The supply of phosphorus also influences the composition of the produced biomass. Internal and external supplies of these element affect specially the lipid content and carbohydrates [29]. Also important is the ratio of N:P in the growth media as this not only determines the potential productivity but it is important in maintaining the dominance of the candidate species in culture [18].

In addition to the above macronutrients, algae also require a range of micronutrients to grow, among which stands out the iron (Fe). This element is involved in nitrate and nitrite assimilation and in the nitrogen fixation by cyanobacteria [30, 31]. It is particularly important in oxygenic photosynthesis to maintain the Fe-rich electron transport chain, as it is present in the two photosynthetic reaction centers (PSI and PSII) and in some cytochromes [32]. In microalgae, the uptake of Fe by the cells is an active transport process. In order to be assimilated by the cells it usually has to be available in a chelated form [19]. Although, microalgae have mechanisms to utilize both free dissolved Fe and Fe bounded to complexes, such as EDTA or citrate [20].

In spite of being essential for algae, when present in high concentrations, iron is potentially toxic for cells and inhibits microalgal growth [19]. In fact, Fe is highly reactive and can produce hydroxyl radicals through the Fenton reaction [33]. On the other hand, the scarcity of iron in the culture media may result in reduced chlorophyll content, a decline in photosynthetic efficiency and reduced growth rates [34].

Many algae also require vitamins, especially vitamin B₁₂ and other B series vitamins [19]. In fact, more than 50% of cultured microalgae species require an exogenous supply of vitamin B₁₂ (cobalamin), over 20% require vitamin B₁ (thiamine) and only about 5% require vitamin B₇ (biotin). However, coincidence or not, most of the species currently cultured on a large-scale, such as *Dunaliella salina*, *Haematococcus pluvialis* and *Chlorella* spp., do not have a vitamin requirement [35].

1.1.4.2 Added-value compounds

Microalgae were first utilized some 2,000 years back by the Chinese to survive famine during drought periods [36, 37]. Nonetheless, the focus in algal biomass came about in the early 1950s due to the rise in world population and the possibility of food shortage, which led to a search for alternative novel protein sources [38]. Species such as *Arthrospira* (*Spirulina*) and *Aphanizomenon* has been utilized for decades as a source of food [38]. However, innovative technologies using microalgae began to emerge recently [37]. In fact, microalgal culture is a new area of biotechnology research and, given the wide range of resources they can provide, its commercial application is virtually untapped, existing only a limited number of revolutionary companies that have persevered to large-scale production of algal biomass and products [36, 38, 39].

The most predominant research that surrounds algae has been targeting the production of biofuels [40]. However, a recent shift in the trend of using microalgae for the production of value-added compounds has been explored [41]. Being a biodiverse resource, microalgae can produce a range of value-added products such as proteins, lipids, carbohydrates, pigments and different compounds (toxins and isotopes) for various product applications (Figure 2) [39, 40]. Of these, carotenoids, phycobiliproteins and polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid must be highlighted.

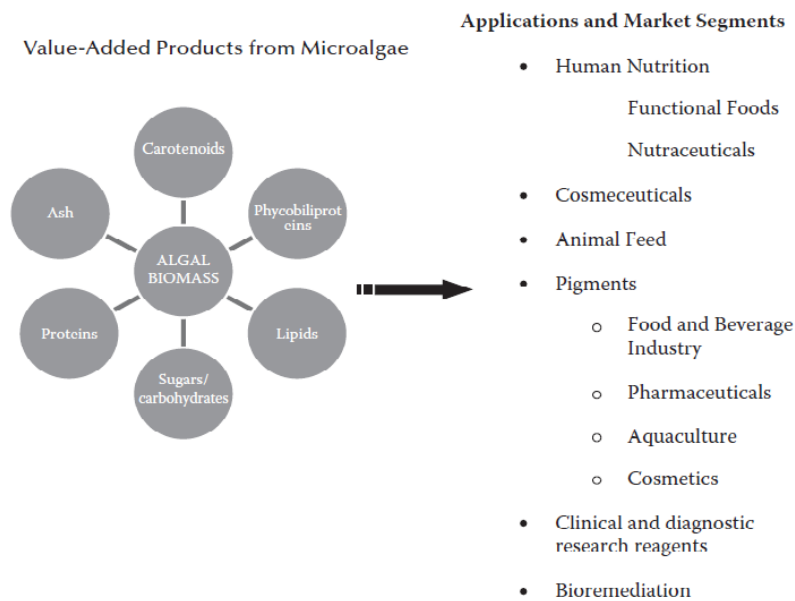


Figure 2. Applications of microalgae biomass. Adapted from Naidoo *et al.* [39].

Carotenoids are natural lipid soluble pigments produced by microalgae which are responsible for red, orange and yellow colorations [42]. These attractive colours make them valuable compounds as natural dyes for food and feed [11]. Other than their colouring properties, carotenoids are frequently incorporated in the formulation of cosmetics and pharmaceuticals given their potential health benefits associated with their strong antioxidant activity, pre-

venting cancer, atherosclerosis, degenerative diseases, obesity, hypertension and aging [43–45]. Since carotenoids cannot be synthesized by animals or humans, they must be absorbed from their diet and metabolized [46]. The two most commonly exploited algal carotenoids are β -carotene and astaxanthin, which are mainly produced by *D. salina* and *H. pluvialis*, respectively [1, 36, 38, 47]. However, there are other carotenoids with great commercial potential such as lutein, zeaxanthin and lycopene [39].

Phycobiliproteins are photosynthetic accessory pigments mainly found in cyanobacteria and red algae [39, 41]. They are deeply coloured (red or blue), water-soluble proteins that can be divided in four main classes based on UV-Visible absorption spectra: phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin [39, 48]. Phycobiliproteins have a broad spectrum of potential applications as natural colouring agents in food, feed and cosmetic industries and as fluorescent agents. They are also used in pharmaceutical industry due to their antioxidant, anti-inflammatory, neuroprotective and hepatoprotective properties. The major sources of phycobiliproteins are *Arthrospira platensis* and *Amphanizomenon flo-aquae* [49, 50].

Polyunsaturated fatty acids (PUFA) are another class of compounds that are known to have positive health benefits, namely brain health and development, prevention of cardiovascular disease, stroke, asthma and rheumatoid arthritis [51, 52]. They also have anti-hypertensive and anti-inflammatory activity and improve lipid serum profiles in terms of decreasing triglyceride levels and increasing HDL [53]. Aside from human health, omega-3 PUFA (ω -3) has significant advantages for growth and development [54].

Among ω -3 PUFA, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are of particular interest, as very long chain polyunsaturated fatty acids are only synthesized to a limited extent by humans [42, 134]. Thus, these fatty acids must be consumed in their diet [55]. However, many argue that it is not imperative to consume both EPA and DHA as the human body efficiently converts EPA to DHA. As a result, the potential new players on the algal market are focusing their efforts on increased production of high-purity EPA [39].

In addition to carotenoids, phycobiliproteins and PUFAs, microalgae produce several other compounds with a wide range of applications. Some proteins, peptides and amino acids present biological function associated to nutritional benefits and human health (hypolipidemic and hypoglycaemic properties, and appetite suppression). As most of the fresh water microalgae species are composed of more than 50% of proteins in dry weight, these molecules can be used as nutraceuticals or included in the formulation of functional foods [11]. Microalgae produce also isotopically labelled compounds due to their ability to incorporate stable isotopes from inexpensive inorganic molecules into high-value isotopic organic chemicals. These stable isotopic compounds can be used in research (metabolic studies) and

medical diagnosis or for the production of bioplastics [36, 38, 47, 56]. In addition, microalgae produce some solar blocking compounds such as mycosporine-like amino acids (MAAs) and scytonemin, whose photoprotective action make them very appealing to cosmetic industry [11].

1.1.5 Biotechnological applications

Due to their abundant composition in proteins (up to 70% of dry weight), vitamins and essential fatty acids, microalgae have been reported as a specially well-suited protein source in developing countries, being used as food for humans in China, Japan, Mexico and Africa [41, 56]. However, the high cost of microalgal biomass compared to other protein sources has limited its widespread application [56]. Nonetheless, the increase in global production of microalgal species such as *Arthrospira* (3,000 t dry weight year⁻¹), *Chlorella* (2,000 t dry weight year⁻¹), *D. salina* (1,200 t dry weight year⁻¹) and *Aphanizomenon flos-aquae* (500 t dry weight year⁻¹) indicates that microalgae have a possibility of becoming an interesting source of food for human population in the years to come [38]. In fact, the production is expected to increase further by 27,552 tons by 2024 with a compound annual growth rate of 5.32% [41].

Chlorella and *Spirulina* have been the most commonly sold food-microalgae and have been widely used in the production of tablets, capsules, and liquids and marketed as a source of vitamins and antioxidants [41, 57, 58]. Despite having health benefits for humans, the biomass obtained from microalgae contain large quantities of nucleic acids which undergoes metabolic degradation to uric acid and might result in gout or kidney stones [59]. However, nutritional studies conducted on humans proved that consumption of algae to a certain extent (20 g/d) had no adverse effects even if the intake was prolonged for longer periods of time [41].

Microalgal biomass has also been used as animal, fish (aquaculture) and poultry feed [41]. In fact, the use of microalgal species as animal feed supplements has grown rapidly in the past few decades, making up about 30% of the algal biomass sold worldwide [38]. The commonly used genera of microalgae for animal feed include *Chlorella*, *Isochrysis*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Tetraselmis*, *Dunaliella*, *Scenedesmus*, *Thalassiosira* and *Skeletonema* [41]. Their biomass, when incorporated into the feed of animals, can positively influence the growth, immune response and gut function [41].

The cosmetic industry can effectively use microalgae in the treatment of skin disorders like aging, tanning and problems related with pigmentation [60]. *Spirulina* spp. and *Chlorella* spp. are the most commonly microalgae employed in cosmetic industries. The extracts obtained from the biomass of these species have been used in the production of creams,

lotions, shampoos and sun protection commodities [58, 61].

Nowadays, there is an urgent need for new drugs to treat disease, in particular, new classes of antibiotics to overcome the growing problem of antibiotic resistance in many bacterial pathogens [62]. As a result, an increase in the production of bioactive compounds from natural sources, e.g. microalgae, has been observed [63]. Some strains of microalgae can produce metabolites with antibiotic activity aimed at killing or inhibiting bacterial growth such as fatty acids, terpenes, carbohydrates, glycolipids, lipoproteins, bromophenols, tannins, polyketides, no ribosomal peptides and alkaloids. As a consequence, in the last decade, the screening and bioprospecting of microalgae and cyanobacteria for antibiotics and other pharmacologically active compounds has received increasing attention [64]. Besides, the production of vaccines from microalgae that can be administrated orally seems to be also an interesting aspect that can be explored on the future [41].

The most commonly employed microalgae genera to produce bioactive compounds, of pharmaceutical interest, include *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Nostoc* [41].

Microalgae can also be used as a sustainable, renewable and eco-friendly feedstock to produce biofuels and bioenergy [56, 65]. Indeed, this area has burgeoned again in the last decade and is again the focus of extensive research and development [56]. There are several aspects that make the microalgae (third-generation feedstock) a more suitable source to produce biofuels than first (crop species) and second-generation feedstocks (grasses and trees, which mainly consist of lignocellulosic biomass) [66]. The production of second-generation biofuels is quite energy-intensive since it involves breaking the complex structure of lignin and converting the crystalline cellulose to amorphous cellulose [67]. In addition, contrarily to crop species, microalgae do not require arable land and fresh water to grow and, as they are not edible, its use as biofuels feedstock do not affect human and animal's food chain [68]. Furthermore, as they possess a simple cellular structure, microalgae have a photosynthetic efficiency of 9-10%, much higher than terrestrial plants, enabling them to have a production of oil per unit area around 30 times greater than of terrestrial plants [69–71]. Besides, microalgae-derived biofuels production may be combined with wastewater treatment and sequestration of CO₂ from flue gasses released by industries [72].

The most common biofuels produced from microalgal biomass are biodiesel, bioethanol and biohydrogen. There are different methods available for converting microalgal biomass into biofuels [11]. These include biochemical conversion, thermochemical conversion, chemical reaction and direct combustion [41]. Regarding microalgal derived biodiesel, its production involves the following steps: cultivation, harvesting, dewatering and microalgae concentration, extraction of lipids from microalgae and separation of triglycerides and free fatty

acids from the crude lipids. The final step is the esterification or transesterification, a chemical reaction process commonly employed for converting lipids into biodiesel [69]. Lipid-rich microalgae have been used for biodiesel production, as the oil content they produce can be very high, up to 80% of dry weight [41, 56].

The production of bioethanol from microalgae can be obtained directly by dark fermentation or from algal metabolites, being the latter option the most widely used [41]. Some carbohydrates-rich microalgae like *Chlamydomonas reinhardtii* and *Chlorella vulgaris* possess high contents of different carbohydrates, such as glycogen, starch, agar and cellulose, which can be easily converted to fermentable sugars [73]. These sugars have to be extracted from the algal cells and need pre-treatment to break the polymer molecules to monomeric forms that can be subjected to microbial fermentation to produce bioethanol [74].

Nevertheless, there are alternatives to the use of lipids and carbohydrates to produce biofuels which consist of the use of the whole biomass as a fuel feedstock [56]. Direct wet conversion processes, like anaerobic digestion and hydrothermal liquefaction, are used to produce biogas and biocrude, that can be directly burned in a boiler or upgraded through hydrotreating into a biofuel. These processes have the advantage of not requiring a high lipid/carbohydrate-producing microalgae, since the whole biomass is processed [75]. However, the economic value of biogas is too low at present, thus anaerobic digestion is not an economically profitable solution for microalgae biomass valorization [75].

The production of biohydrogen from microalgae can be obtained through several processes, namely direct bio-photolysis, indirect bio-photolysis, dark fermentation and photo-fermentation [76, 77]. However, the photobiological production of hydrogen without the involvement of organic feedstocks may be more promising as it is not restricted by a supply of organic substrates that may have other competing uses [78]. Even though H_2 yields from biological processes have been improved through physiological manipulation (mainly sulphur deprivation), the process is far from being scalable and commercially viable [78, 79]. This is partly due to the low yield of the process, a consequence of the strong inhibitory effects of oxygen on the production of hydrogen [78].

Despite having a great potential, none of the biofuels derived from microalgae is economically profitable.

As previously mentioned, another microalgae application is wastewater treatment. They are a particular attractive way of treating wastewaters since they have the ability of assimilating nutrients as organic matter, NO_3^- , PO_4^{3-} , NH_4^+ , CO_2 and heavy metals [80]. Independent of the metabolic pathway adopted in the cultivation, photosynthetic or heterotrophic, the microalgal biomass present mineral substantial composition, that can reach 25% dry weight, presenting elements of commercial importance as nitrogen, phosphorus and potas-

sium. Hence, microalgae biomass is an excellent source of biofertilizers [11]. Furthermore, the photosynthetic cultivation of microalgae is one of the most promising CO₂ mitigation technologies, given the high photosynthetic rates and resistance to high concentrations of CO₂ demonstrated by these microorganisms [81].

Even though microalgae have been studied for over 50 years now, there are still only just a few corporations that are cultivating microalgae on a commercial scale. The high operational and capital costs make the microalgal investment not economically feasible, being the payback longer than expected. Thus, the biorefinery approach is the best choice for obtaining sustainable and economical production [82]. It consists in producing several products from the same raw material for different applications. The approach is to produce high value-added products with residual biomass valorization in the form of a fuel [41]. Genetic engineering of microalgae is another approach that has been considered for improve the yield of valuable products at reduced costs [36].

1.2 *Nannochloropsis* spp.

Nannochloropsis is a picoplanktonic genus of unicellular and nonmobile marine microalgae belonging to Phylum Heterokontophyta, Class Eustigmatophyceae and family Eustigmataceae, which comprise six known species: *Nannochloropsis gaditana*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica*, *Nannochloropsis oculata* and *Nannochloropsis salina* [6, 83]. The cells are small and have a simple morphology with diameters varying from 2 to 8 μm [6, 83]. They have a single mitochondrion, Golgi body and chloroplast [83, 84]. This latter has several clearly visible bands of photosynthetic lamellae, each with three thylakoids per band, and is in close proximity to the nucleus. Indeed, the chloroplast endoplasmic reticulum is continuous with the nuclear envelope [6, 83]. They also have polysaccharide cell walls and a lipid droplet that functions as an energy deposit and that can be augmented in size under stressful conditions [6, 83].

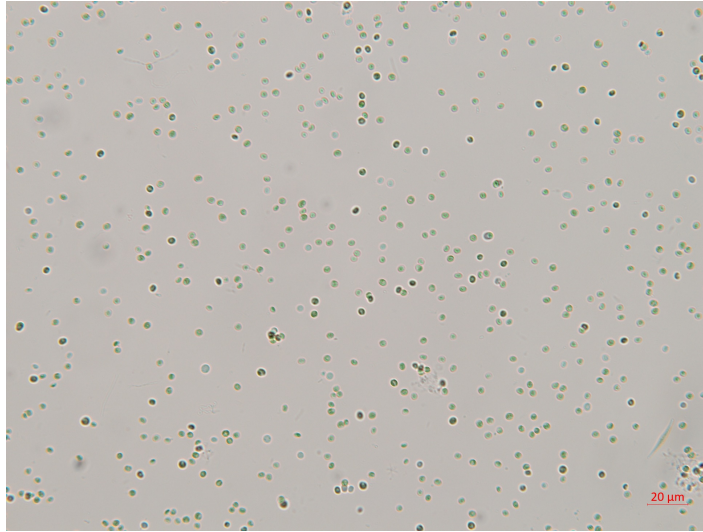


Figure 3. Microscopic view (40 $\times$) of *N. oceanica* grown in Algafarm's pilot-scale raceway ponds.

Nannochloropsis spp. have received ever-increasing research attention, being considered as a potential oleaginous model microalga [83]. This is because of the great photosynthetic efficiency, high lipid productivity, well established genetic toolbox and relatively mature technology for outdoor large-scale cultivation systems [85–87]. Indeed, Doan *et al.* [88] conducted a comprehensive high-throughput screening study and concluded that three *Nannochloropsis* strains out of 96 tested strains were the best feedstock for biodiesel production. In its turn, Ma *et al.* [89] evaluated the potential of nine *Nannochloropsis* strains for biodiesel production and reported that a *N. oceanica* strain was the best one. *N. oceanica* has a lipid content ranging from 49% to 53% of dry weight and a fatty acid distribution mostly composed of long-chain fatty acids with 16, 18 and 20 carbons (C16, C18 and C20, respectively) [89]. It accumulates high quantities of EPA which can range from 2.7 to 5.5% of dry weight [83]. The lipid profile of *Nannochloropsis* spp. can be influenced by many nutritional and environmental factors, such as irradiance, nitrogen concentration and salinity [83]. Various biochemical approaches have been suggested to enhance lipid accumulation in *Nannochloropsis* spp. Cell growth and EPA synthesis are favored by moderate illumination (less than 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), adequate nutrient components (like nitrogen and phosphorus) and lower salinities (such as 25‰). On the other hand, excess light, nutrients starvation and higher salinities (such as 35‰) favor the accumulation of triglycerides (TAGs) [90–93]. In general, *N. oceanica* has specific growth rates ranging from 0.11 to 0.21 d^{-1} [89]. However, these values are reached in small scale laboratory conditions and more conservative values are reported by Baohua *et al.* [94] in indoor raceway ponds (0.05–0.07 d^{-1}). The same author refers a lipid content ranging from 21 to 28% of dry weight and an EPA content varying between 36 and 40% of total fatty acids.

Nowadays, several species of *Nannochloropsis* genus are successfully cultivated autotrophically at large scale by companies such as Solix Biofuels, Aurora Algae, Seambiotic and Proviron [86].

1.3 Biomass production

1.3.1 Open vs. Closed systems

There are two different systems used for microalgae cultivation, open and closed reactors. The major difference between them is that in the open systems the culture is in contact with the atmosphere. Open systems are the most widely applied bioreactors in industrial microalgal processes mostly because of having lower capital and operational costs [95]. Besides, they are easy to construct, operate, maintain and clean after cultivation [96]. However, despite being more economical, open systems have several drawbacks comparatively with closed systems. For instance, they are very susceptible to contaminations making it difficult to maintain monoculture cultivation for long periods [97]. A commonly used procedure to overcome this issue is by operation the open reactor under highly selective culture conditions, only suitable to extremophile microalgae, such as *Dunaliella* and *Spirulina*, which require high salinity and pH to grow, respectively [95]. The use of fast-growing species (*Chlorella*, *Scenedesmus*, *Phaeodactylum*) is also an option, since they can outgrow most of their competitors under optimum growth conditions [19]. In addition, these systems allow only a limited control of the operation conditions, being difficult to maintain optimal growth [11]. Indeed, they are very susceptible to changes in temperature and irradiance due to local weather and climatic conditions [95]. The temperature fluctuation along with inefficient mixing and CO₂ dispersion, light limitations and evaporation losses result in low biomass productivities [98]. Thus, low cell densities are usually obtained, requiring large volumes to be processed during harvesting and increasing the cost of product recovery [95]. Other limitation of open systems is that they require large land areas and the same does not happen with closed systems, since they can be constructed vertically [99].

Closed systems, often called photobioreactors, are more efficient in terms of biomass production than open ponds. They have high photosynthetic efficiencies owing to greater control on environment and growth conditions, lower risk of contamination, minimization of water losses and optimal cultivation area use [100–102]. However, these systems have some problems which include difficulty in scaling-up, biofouling, overheating, oxygen accumulation and cell damage due to shear stress [4].

1.3.2 Open production systems

Microalgae open production systems include natural water bodies, circular ponds, raceway ponds and cascade systems [97]. The first are lakes and dams where the growth of microalgae either occurs naturally or through the addition of nutrients [97]. Such systems have small or no costs of cultivation, although the productivity and product quality (biologically and toxicologically) cannot be assured [103]. Regarding circular ponds, they are simple, round, concrete ponds mixed by a rotating circular arm fixed in the center of the pond, usually used in Japan, Taiwan, Indonesia and Ukraine for *Chlorella* cultivation [97, 103, 104]. Another system used to cultivate microalgae is the thin-layered cascade in which a culture depth of less than 0.01 m is used, reaching cell densities of up to 10 g L⁻¹ [105]. However, the biomass productivity, 25 g m⁻² d⁻¹, is comparable with the one achieved on raceways [103, 106].

A raceway pond is a shallow, oval-shaped, closed-loop flow channel, usually 0.15-0.30 m deep, internally lined with cement, PVC or clay, with mixing and aeration provided through circulation by a rotating paddlewheel [98, 104, 107, 108]. It is a pond style suitable for microalgal growth that optimizes light utilization in a given unit area, in a cost-effective manner, making it the most commonly used design for commercial microalgae production [97, 109]. Theoretically, raceways ponds could be over 5 ha in size, however, a single pond does not usually exceed 0.5 ha [110, 111]. Channel length to width ratio must be 10 or larger, but practical paddlewheel design limits it to a maximum of 20 [110, 111]. If this ratio is too small, the flow in the straight parts of the raceway begins to be affected by the disturbances caused by the bends at the ends of the channel [111]. Baffles are often placed in the bends of the flow channel. They ensure an uniformity of flow throughout the curved bends and minimizes the formation of dead zones, which impair mixing, allow solids to settle and cause unwanted energy losses [112]. In this context, 0.10 m s⁻¹ is the minimum flow velocity required to prevent sedimentation of algal biomass [113]. In practice, a straight channel velocity of at least 0.2 m s⁻¹ is needed to ensure that a velocity of 0.1 m s⁻¹ is achieved in the whole raceway [113]. Regarding flow velocity, 0.3 m s⁻¹ is the most commonly used for biomass production [113]. The surface-to-volume ratio in raceways is always 1/pond depth. Thus, a lower depth increases the surface-to-volume ratio and, consequently, improves the light penetration [111]. Therefore, for exclusively photoautotrophic growth, shallower ponds are more productive than deeper ponds [111]. However, in large ponds the depth cannot be much less than 0.25 m, as a small tilt of the bottom relative to the horizontal causes a large difference in depth in different parts of the pond [111]. Tredici [114] reported that at water depths lower than 0.15 m a severe reduction in flow and turbulence occur in large-scale raceways. This long light

path results in large areal volumes (150 L m^{-2}) and cell concentrations of less than 0.6 g L^{-1} that facilitate contamination [114]. Despite areal productivities of $40 \text{ g m}^{-2} \text{ d}^{-1}$ and higher have been reported in experimental algal ponds, typically, well managed raceway ponds may achieve $20\text{--}25 \text{ g m}^{-2} \text{ d}^{-1}$ for short periods, while long-term productivity in large commercial raceways rarely exceeds $12\text{--}13 \text{ g m}^{-2} \text{ d}^{-1}$ [114]. López *et al.* [115] points out $20 \text{ g m}^{-2} \text{ d}^{-1}$ as the threshold average annual productivity rarely exceeded in large-scale outdoor production. Oxygen build-up in raceway ponds can reduce the rate of photosynthesis and adversely affect the biomass productivity [113, 116]. Values of 300% (or higher) of the level in air saturated water can be reached [117]. CO_2 fixation efficiency is also an important parameter since carbon constitutes about 50% of algal biomass [110]. Between 35 and 70% of the pure CO_2 sparged into a pond is lost to the atmosphere [118]. Indeed, inevitable losses of at least 2 grams CO_2 per gram of biomass produced must normally be considered [110].

1.3.3 Biomass harvesting and processing

Generally, harvesting can be defined as a series of processes for removing water from the microalgal growth culture (solids content $<1\%$) and concentrating it to slurry or paste containing 5% to 25%, or more, total suspended solids (TSS). The large volumes of culture required to process, due to the low density of the cultures and the small size of microalgae, make the harvesting process very energy-intensive. Indeed, it can contribute up to one-third of the biomass production cost, being therefore a major challenge for the commercial-scale production of low-cost products (e.g., biofuels) [119]. The process would only be viable if the biomass harvested was used in the production of high-value products, such as nutraceuticals [120]; otherwise, harvesting cost and energy requirements must be reduced at least by a factor of 2 to assure the economic viability of the process [119].

To date, there has been no specific commercial-scale algal harvesting technique that has been developed, and the approach has been to adapt separation technologies used in wastewater treatment and food processing industries [119]. Conventional processes used to harvest microalgae are gravity sedimentation, centrifugation, filtration, flotation and flocculation.

It is important to carefully analyze several parameters, such as cell morphology, ionic strength of the media, pH, culture density and final downstream processing of harvested biomass, when selecting a suitable harvesting technique. However, so far, no single harvesting technique can be universally applied to algae cultivation systems due to technical and economical limitations. Hence, the use of custom-designed, multi-stage harvesting techniques for algal farms, in which a variety of harvesting technologies are arranged in a specific sequence, is the best way to achieve maximum biomass concentrations with minimum energy usages, that is, highest efficiencies at lowest costs [119]. For example, in an open pond system,

wherein dominant microalgal species can range from small unicellular to large colonial or filamentous species, the first stage of harvesting could be a filtration to remove filamentous and auto-flocculated algae. Next, a flocculation, sedimentation and/or flotation could be applied to produce algal slurries with 1% to 5% solids, which could be subjected to centrifugation to achieve >20% solids [119].

The harvested biomass slurry cannot be used immediately for any purpose, unless it undergoes extensive downstream processing, such as dewatering/drying, extraction and purification of desired biomolecules [121]. It must be quickly processed because it is perishable and should get spoiled in only a few hours in a warm climate [121, 122]. However, the downstream processing involved in the processing of algal biomass is energy intensive, raising the cost of algae-based products and representing a major economic limitation to the production of low-cost commodities (food, feed, fuels) [121, 122]. Hence, it is imperative to develop efficient and cost-effective methods of algal biomass processing [121].

1.4 Contextualization

1.4.1 Secil/Algafarm/Algavalor

Secil is the biggest Portuguese cement company [123]. Cement plants are highly pollutant since they release to the atmosphere large quantities of CO₂, a by-product of cement industry. Indeed, 600 kg of CO₂ are sent to the atmosphere per ton of limestone processed to make cement [124]. Thus, to decrease the greenhouse gases emissions and achieve the European targets, Secil created its own microalgae production plant, Algafarm (Figure 4) [125]. This project came out to perform the sustainability policy established by the company that seeks new applications for employing factory waste, including CO₂ and heat [123]. It is expected to reduce the CO₂ emission in 20-25% in a first phase and reach 40% of CO₂ fixation by 2030 [126]. Even though CO₂ sequestration is the core of the project, Allmicroalgae – Natural Products (the company owned by Secil which explore the microalgae production) is already marketing food and feed products, having food safety, quality, health and environment systems certified [123, 125]. Thereby, this is an excellent example of circular economy, in which, from a pollution problem it was found a biotechnological solution with potential to become another business [124].



Figure 4. Algafarm, the microalgae industrial production plant in Pataias, Leiria.

Algavalor is an ongoing P2020 financed project led by Secil that intend to assess the technoeconomic viability of the production of high-quality microalgae-derived products for food, feed, biofertilizers and cosmetic industries [127]. This master thesis project is involved in subproject 4 of Algavalor project, which aims processes optimization and to prove the sector's sustainability based on the scale-up DEMO in 'new generation' open reactors. The information gathered from the operation of two different pilot-scale raceway ponds will be used in the project and design of the DEMO raceway ponds which, in turn, will hopefully validate the 'new generation' raceway technology allowing the scale-up to industrial-scale reactors.

1.5 Goals

This master thesis project has three main goals: assess the growth of the microalgae *N. oceanica* into two pilot-scale raceway ponds with different design configurations; test different water depths and cultures circulation velocities; assess the carbon fixation efficiency and evaluate the biochemical composition of produced biomass.

1.6 Outline of the work

After the bibliographic revision above, there is a Materials and Methods section where the main differences existing between the raceways used and the cultivation process layout are explained. It is also described the setup of experimental trials and the sampling methodology adopted. Then, the methodology used for growth parameters calculation, photosynthetic and carbon sequestration efficiency determination, and microalgal biochemical composition

assessment are presented. Next, there are a section of results presentation and discussion, followed by the general conclusions that were drawn of this work and future perspectives for further experiments in this field.

2 Materials and Methods

All experiments described in the present work were performed at Algafarm's facilities between February 4th and July 28th of 2019, while the biochemical profile assessment of produced biomass was performed at the MarBiotech group of the Center of Marine Sciences (University of Algarve) between July 29th and August 9th of 2019.

2.1 Raceway ponds

In this project *N. oceanica* was cultivated into two pilot-scale raceway ponds (Figure 5) with different configurations. They both had 15 m long and 2 m wide, having each channel 1 m wide. The channels were separated by a central baffle. There were also baffles on the bends to reduce the pressure losses and maintain the culture velocity as constant as possible. There was a paddlewheel that assured the circulation of the culture, as well as the mixture and aeration. The carbonation of the culture was made through one CO₂ diffuser placed right after the paddlewheel at the bottom of the raceway.



Figure 5. Pilot-scale raceway ponds used in these study. Modified raceway at right and standard raceway at left.

The main differences between the raceways were in the paddlewheel width and the number of baffles. Standard configuration had a full channel width paddlewheel and two sets of three baffles, one in each bend of the bioreactor. On the other hand, the modified configuration had a half channel width paddlewheel and only one set of baffles, placed in the opposite bend of the paddlewheel. Besides, the central baffle of the modified raceway had an 'island' fill on the deflectors bend side (Figure 6).

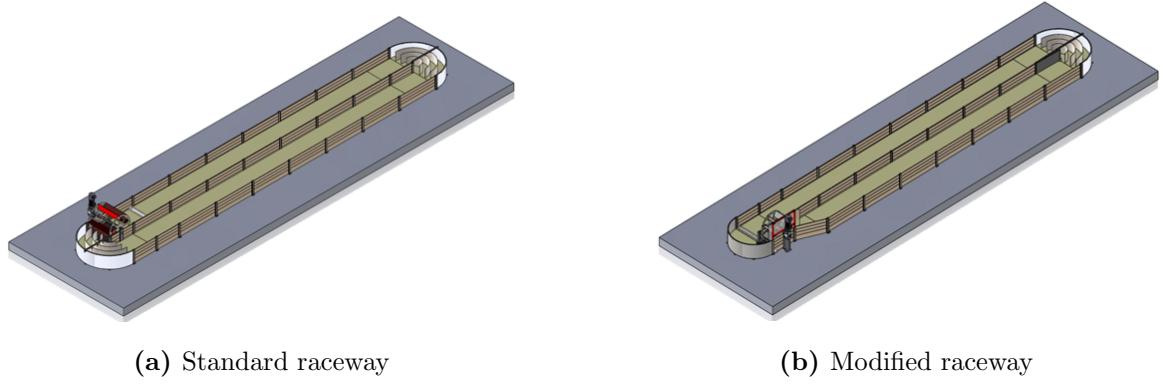


Figure 6. Pilot-scale raceway ponds schematic representation.

2.2 Micralgal strain and culture medium

The microalgae strain used in this work, *N. oceanica*, was obtained from Algafarm’s culture collection. The base medium used for culture growth was MNS medium, which was further supplemented with iron (5 mM). It was also added magnesium water (Necton, Portugal) and sodium chloride (NaCl) (30 g L⁻¹). The water source used was the local water from the adjacent cement factory (Secil - Cibra Pataias).

2.3 Setup of the experimental trials

The first set of experiments was performed to optimize the culture water depth. Two different water depths were tested, 0.20 and 0.13 m, with a culture circulation velocity of 0.30 m s⁻¹. With the culture depth optimized, another trial was done to optimize the culture circulation velocity. Thus, a velocity of 0.15 m s⁻¹ was used with a 0.13 m water column. All the assays were made in duplicate, except the 0.13 m and 0.15 m s⁻¹ trial in modified raceway which was only performed once.

2.4 Cultivation process layout

Each trial had 13 days duration and was performed simultaneously in both raceways. *N. oceanica* was inoculated at a 0.3 g L⁻¹ concentration in MNS medium with a NaCl and nitrate concentrations of 30 g L⁻¹ and 5 mM, respectively. In every experiments fresh inoculum was used, which was provided by Algafarm’s production department. It came from the closed tubular photobioreactors used in the company for the commercial production of microalgae. A microscopic observation of the inoculum was always performed to evaluate the culture integrity both morphologically and microbiologically. Throughout the assay the culture was closely monitored, and a special attention was given to pH control given that the system was not automated. The CO₂ system was manually operated in response to pH fluctuations,

being this parameter kept between 8.0-8.2. Water evaporation and nutrients availability were also monitored through water depth and nitrate concentration variation. The NO_3^- concentration was always kept above 2 mM and water refilled whenever it was necessary. To evaluate if there were any contaminations in the cultures, regular microscopic observations were performed. In the end of the assay the culture was harvested and centrifuged and the resulting biomass and supernatant were biochemically analyzed. A schematic representation of the whole process is shown in Figure 7.

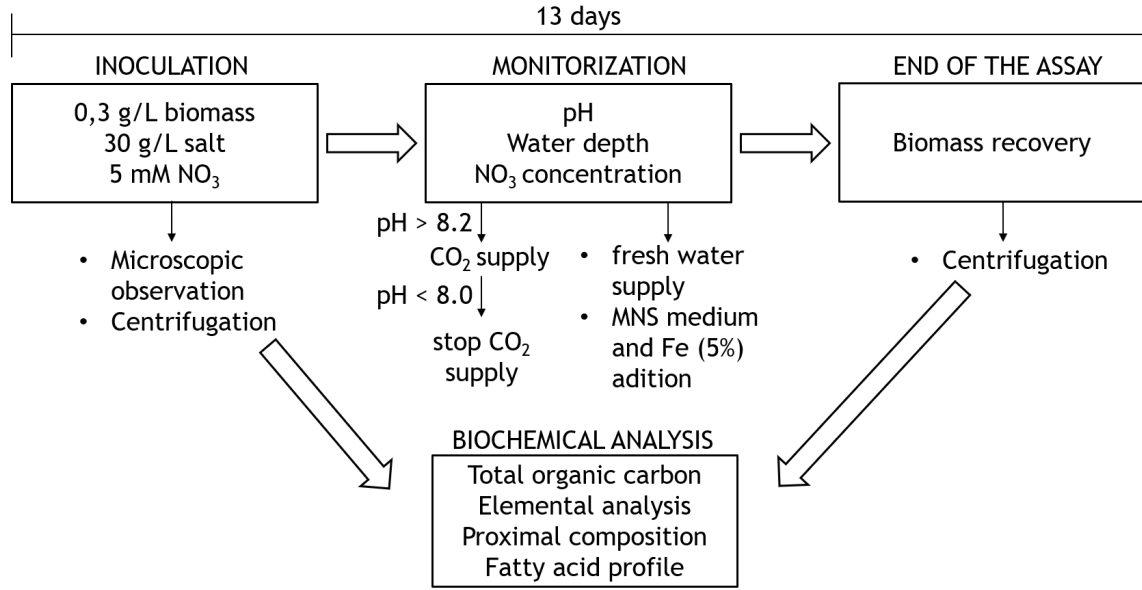


Figure 7. Layout of the microalgae cultivation process and subsequent biomass analysis.

2.5 Sampling methodology

The sampling was done in a daily-basis (Table 1). Biological parameters of the culture, such as optical density (OD) and dry weight (DW), were measured to follow microalgal growth. Operating parameters of the culture were monitored, namely pH, salinity, dissolved oxygen (DO), temperature, nitrate concentration and water depth, to assure that there were no significant deviations from the required culture conditions.

Table 1. Daily sampling methodology.

Biological parameters	9h00	14h00	18h00
Optical density (540 nm)	x		x
Dry weight (g L ⁻¹)	x		x
Physicochemical parameters	9h00	14h00	18h00
pH	x	x	x
Salinity (g L ⁻¹)	x		x
Dissolved oxygen (% saturation)	x	x	x
Culture temperature (°C)	x	x	x
Nitrate concentration (mM)	x		
Water depth	x		x

2.6 Microalgal growth assessment

Optical density was performed at 540 nm using a spectrophotometer (4251/50, Zuzi, Spain). 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 10 mL ammonium formate solution at 35 g L⁻¹, the filter was dried and analyzed in a moisture analyzer (MA 50.R, RADWAG, Poland). The results of these two methods to evaluate the biomass growth were correlated and the calibration curve was plotted and is available at Appendix A.

For nitrate medium analysis, samples were centrifuged for 10 minutes at 2000 g (MiniStar silverline, VWR, Portugal). The pH was registered by a pH meter (Combo pH/Conductivity/TDS Tester, Hanna instruments, Portugal). The temperature and oxygen concentration were measured using a dissolved oxygen sensor (phenomenal OXY 11, VWR, Portugal) coupled to an OX 4100 H meter (VWR, Portugal). The integrity of the cultures was assured by microscopic observations (Primo Star, ZEISS, USA).

Volumetric biomass productivity, P (g L⁻¹ d⁻¹), was determined by the ratio of DW differences in g L⁻¹ (X_1 and X_2) and the time interval (t_1 : time of X_1 sample, and t_2 : time of X_2 sample) (Equation 2).

$$P \text{ (g L}^{-1} \text{ d}^{-1}\text{)} = \frac{X_2 - X_1}{t_2 - t_1} \quad (2)$$

Areal biomass productivity, P_a (g m⁻² d⁻¹) was determined by multiplying the volumetric

biomass productivity by the volume of the raceway, in L (V) and dividing by the surface area of the raceway, in m² (A) (Equation 3).

$$P_a \text{ (g m}^{-2} \text{ d}^{-1}) = \frac{P \times V}{A} \quad (3)$$

Specific growth rate, μ (d⁻¹), was 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 4).

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \quad (4)$$

2.7 Photosynthetic efficiency

Photosynthetic efficiency (PE) was assessed by growing *N. oceanica* in two different raceway ponds operating in parallel. The outside temperature and solar irradiance were measured every 15 min using a meteorological station (WatchDog 2000 series Weather Station).

The higher heating value (HHV) was calculated according to Callejón-Ferre *et al.* [128], as described in Equation 5, where C, H, and N are the percentages of carbon, hydrogen, and nitrogen, respectively, determined by elemental analysis (CHN) using a Vario el III (Vario EL, Elementar Analyser system, Germany), according to the procedure provided by the manufacturer [129].

$$HHV \text{ (kJ g}^{-1}) = -3.393 + 0.507 C - 0.341 H + 0.067 N \quad (5)$$

PE was determined by the ratio between the stored energy of biomass produced and the solar energy that reaches the reactor during growth (Equation 6). The stored energy in the form of biomass is the biomass weight (difference of the final and initial biomass concentration (X_2 and X_1) in g L⁻¹ multiplied by the volume of the reactor in L (V)) and the HHV. The solar energy that reaches the reactor is obtained by the multiplication of radiation, in kW m⁻² (rad), the area occupied by the reactor in m² (A), and the time between solar radiation measurement, in s (t). The total solar energy is the sum of all energy values obtained between the initial and final time (t_2 time of X_2 sample, and t_1 time of X_1 sample) [129].

$$PE = \frac{(X_2 - X_1) \times V \times HHV}{\sum_{t_1}^{t_2} rad \times A \times t} \quad (6)$$

2.8 Biochemical composition

2.8.1 Proximal composition

The ash content was determined by burning 1 g of biomass in a furnace (J. P. Selecta, Sel horn R9-L) at 550°C for 8 hours.

For the lipid determination, a biomass weight ranging 5-10 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 [130]. 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 calculation of the percentage of lipid in the biomass is described in Equation 7. 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.

$$Total\ Lipids = \frac{\frac{(FW - IW) \times Total\ Volume\ of\ Chloroform}{Evaporated\ Volume\ of\ Chloroform}}{Sample\ Weight} \times 100 \quad (7)$$

Total proteins were calculated by multiplying the percentage of nitrogen by 6.25 [131] after CHN analysis [132]. Carbohydrates were determined by difference between the other macromolecules.

2.8.2 Fatty acid profile

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 & Roy [133] as described in Pereira *et al.* [134].

The freeze-dried biomass was weighted (5-10 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 8). 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

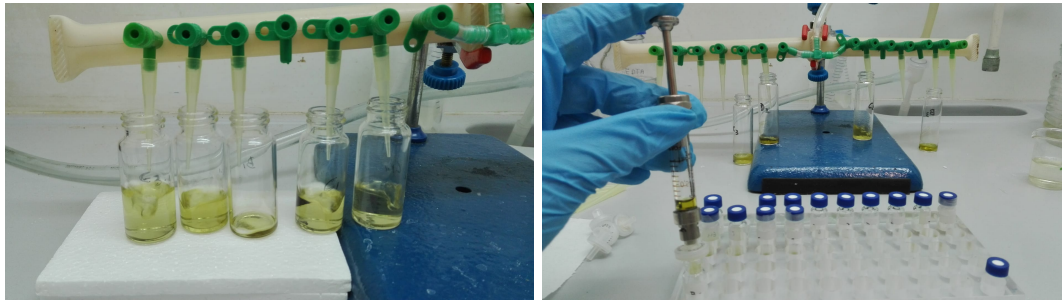
with Teflon to avoid losses). After cooling down in ice, the vessels content was transferred to centrifuge tubes and 1 mL of distilled water and 4 mL of hexane were added. These tubes were vortexed (VV3, VWR, Portugal) at maximum speed in two cycles of 30 seconds and centrifuged for 5 minutes at 2000 g (Heraeus™ Multifuge™ X3, ThermoFisher Scientific, USA). After centrifugation, the supernatant was transferred to new tubes. In the centrifuge tubes, 4 mL of hexane were added and the mixture procedure with the vortex and centrifuge was repeated.



Figure 8. Biomass homogenization by cell breakdown.

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 in 0.22 μm filters (EMD Millipore SLLGC13NL IC Spectra Syringe Filter, Cole-Parmer, USA). A nitrogen gas flow was applied to evaporate the hexane (Figure 9a) 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 9b). The chromatograph was equipped with a 30 m length column with an internal diameter and film 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.



(a) Hexane evaporation.

(b) Vials filling.

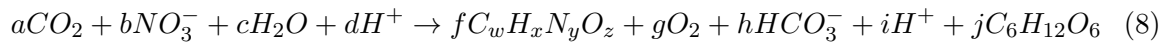
Figure 9. FAME analysis procedure.

2.9 Total organic carbon

To determine the total organic carbon (TOC) dissolved in the culture medium, cultures were centrifuged at 4000 g for 15 min and the supernatant was collected. Thereafter, the supernatant was filtered through a sterile syringe filter (0.2 μm , Whatman) before being injected in a TOC-VCSN-Total Organic Carbon analyzer (Shimadzu) with combustion at 720°C [129]. The method has a measuring range of 0.1 to 4000 mg L^{-1} [135].

2.10 Stoichiometric equation

The stoichiometric equation of *N. oceanica* grown in autotrophy [136] was determined as described in Quelhas *et al.* [129] (Equation 8). It was used to calculate the ratio of CO_2 required per gram of biomass produced (a/f) and the CO_2 fixation efficiency ($\frac{f+j}{a}$).



The extracellular dissolved organic carbon is represented by a sugar (molecule with stoichiometric coefficient j).

2.11 Carbon sequestration

The quantification of sequestered CO_2 carried out in the raceway ponds was determined considering that the CO_2 concentration dissolved in the culture medium at the beginning and end of the experiment were identical if pH, salinity, and temperature remained the same [137]. The volume of CO_2 added to the culture within a given period of time was registered by placing a rotameter in the injection system. The biomass DW was related to the percentage of carbon determined by CHN and the TOC of the corresponding supernatant analysed. The amount of CO_2 sequestered by the microalgae was the mass of carbon accumulated by the

microalgae biomass during growth. The CO₂ released from the raceway was the remaining CO₂ that was injected into the reactor, which was not used for biomass or extracellular organic carbon production. The sequestered CO₂ was defined by the sum of extracellular organic carbon and biomass growth [129].

2.12 Statistical analysis

Results were presented as the mean and the standard deviation of the samples replicates. Data were statistically analyzed using R software (version 3.6.1), through RStudio IDE (version 1.2.1335). Statistical significance was assessed applying a Student *t*-test, considering a significance level of 0.05.

3 Results and Discussion

The results are divided in three main sections, comprising the comparison of two different raceways configuration, the optimization of water depth and culture circulation velocity. Each of these was assessed in terms of growth parameters, biochemical composition of biomass produced, carbon fixation efficiency and operational costs. Capital costs are also briefly discussed at raceways comparison section.

3.1 Comparison of two different raceway's configurations

This section aimed to assess which of the two raceway's configurations used was the best. Thus, raceway configuration was defined as the independent variable. *N. oceanica* growth experiments were made under three different sets of process conditions (Table 2). Growth kinetics were separately evaluated for each set of process conditions (growth curves are plotted in Appendix B).

Table 2. Set of processing conditions used.

Process condition	Water depth (m)	Circulation velocity (m s ⁻¹)
1	0.20	0.30
2	0.13	0.30
3	0.13	0.15

Equivalent global and maximum productivities were obtained in both raceways for process conditions 1 and 2 ($p \geq 0.05$). There were also no statistically significant differences in global and maximum specific growth rates (Table 3).

For process condition 3, a statistical analysis of the results was not possible since only one trial was performed. However, this trial can be compared with the one which simultaneously occurred in standard raceway. The global productivities were very similar in both raceways (5.207 and 5.124 g m⁻² s⁻¹ to modified and standard raceways, respectively) as well as were the global specific growth rates (0.056 and 0.055 d⁻¹ to modified and standard raceways, respectively) and maximum growth rates (0.117 and 0.119 d⁻¹ to modified and standard raceways, respectively).

The range of productivities achieved was two-fold lower than the value (14 g m⁻² d⁻¹) achieved in a demo-scale 530 m² raceway ponds using a *N. oceanica* F&M-M24 strain [138]. de Vree *et al.* [139] indicates an average areal productivity of 9.7 g m⁻² d⁻¹ for the growth of a *Nannochloropsis* sp. CCAP 211/78 in a raceway pond similar to the used in this experimental

work (25.4 m² surface area) with 0.20 m culture depth. In the same study, the productivity ranged from 6.2 to 14 g m⁻² d⁻¹. However, López *et al.* [115] reported a productivity of 6.0±2.5 g m⁻² d⁻¹ (for irradiation levels identical to the ones occurred in the trials of this project), very similar to the global productivities obtained in these master thesis growth assays, for *Nannochloropsis gaditana* cultivated in raceways consisting of 23.5 m long and 1.5 m wide channels, with a culture depth of 0.08 to 0.12 m (depending on the season) and a 0.30 m s⁻¹ circulation velocity. However, the same author reported considerably higher values (24.707 g m⁻² d⁻¹) for trials performed under higher average irradiation. In a similar experiment operating in continuous mode, a productivity of 22.4 g m⁻² d⁻¹ was obtained for *Nannochloropsis gaditana* when grown in 7.2 m² raceway ponds (5.0 m long and 0.6 m wide channels) with a culture depth of 0.11 m and a circulation velocity of 0.30 m s⁻¹ [140]. Besides, 20.37 g m⁻² d⁻¹ was the average areal productivity achieved in a culture of *Nannochloropsis* sp. cultivated in 25,000 L (dimension of 35 m by 3.5 m) outdoor raceway ponds situated in Qatari desert [141]. Even though the different reactor configurations, the global and maximum productivities achieved are comparable with the average annual productivity of 20 g m⁻² d⁻¹ rarely exceeded in large-scale production in outdoor raceways, since the systems are affected by factors that cannot be controlled, e.g. weather conditions [115].

The specific growth rates obtained are lower than the range of previously reported data (0.11-0.21 d⁻¹) [89]. Nevertheless, the values reported by Baohua *et al.* [94] for three different *N. oceanica* strains grown in 8000 L raceway ponds are identical to the ones obtained in this work (0.047±0.001 d⁻¹, 0.066±0.005 d⁻¹ and 0.051±0.003 d⁻¹). It is relevant to note that these trials were performed with the injection of flue gas from coal fired power plants as the carbon source.

Table 3. Biomass productivities and specific growth rates obtained in the two raceways used. Productivity values are presented in dry weight basis.

Raceway configuration / Process condition	Productivity (g m ⁻² d ⁻¹)		Specific growth rate (d ⁻¹)	
	Global	Maximum	Global	Maximum
modified				
1	6.610±0.859	15.282±1.092	0.048±0.004	0.141±0.012
2	7.691±1.176	16.364±2.350	0.069±0.008	0.175±0.001
3	5.207	10.164	0.056	0.117
standard				
1	6.277±0.726	19.007±0.314	0.047±0.003	0.172±0.004
2	7.089±0.879	18.102±2.953	0.065±0.006	0.166±0.008
3	5.375±0.251	11.131±2.563	0.057±0.003	0.130±0.011

Photosynthetic efficiency was statistically different for process condition 1 ($p < 0.05$), being higher in modified raceway (Table 4). However, for process condition 2, the results were not statistically different ($p \geq 0.05$). PE values obtained are comprised in the 0.5-1.5% range of PE reported by de Vree *et al.* [139]. However, these are lower than the 1.2% average PE reported by the same author for a *Nannochloropsis* sp. CCAP 211/78 grown in 4730 L raceway pond (25.4 m² ground area) with a culture depth of 0.20 m and a circulation velocity of 0.25 m s⁻¹.

Table 4. Photosynthetic efficiency of *N. oceanica* cultures.

Process condition / Raceway configuration	Photosynthetic efficiency (%)		
	1	2	3
modified	0.569±0.067	0.747±0.001	0.597
standard	0.532±0.065	0.674±0.023	0.618±0.063

There were no statistical significant differences in proximal composition (Table 5) and fatty acid profile (Table 6) of biomass produced in standard and modified raceways ($p \geq 0.05$), with exception for octadecadienoic acid (C18:2n-6) produced under process condition 2 ($p < 0.05$). Biomass lipid content was lower than the values reported by Das *et al.* [141] (26.0±1.1%) and Baohua *et al.* [94] (21.40±1.53-28.00±1.00%), for *Nannochloropsis* sp. cultivated in outdoor raceway ponds and three different *N. oceanica* strains cultivated in indoor raceway ponds, respectively. On the other hand, the values reported by López *et al.* [115] (12.80±3.54-15.15±4.16%) are similar to the ones achieved in these trials.

As regards fatty acid profile, C16 and C20 FAME are the most prevalent. Ma *et al.* [83] stated that C16, C18 and C20 FAME make the majority of fatty acid profile of *Nannochloropsis* strains, but this does not match this study results, since C18 constitute a very small part of the overall FAME synthesized. EPA content ranged from 26.17±3.32 to 29.45±0.41 of total FAME, which was lower than the values reported by Baohua *et al.* [94] for three different *N. oceanica* strains cultivated in indoor raceways (35.58±0.63%, 38.78±2.49%, 40.13±0.28%). The higher relative content of PUFA and ω -3 in comparison with SFA and ω -6 are interesting indicators of the quality of biomass produced by the *N. oceanica* strain used.

Table 5. Biomass proximal composition. Values are presented in a dry weight basis.

Raceway configuration / Process condition	Proteins (%)	Lipids (%)	Carbohydrates (%)	Ashes (%)
modified				
1	38.9±4.9	13.2±3.0	30.5±3.1	17.4±4.8
2	44.2±2.0	17.4±3.4	28.7±4.1	9.7±1.3
3	36.5	10.9	39.7	12.9
standard				
1	40.4±6.6	14.5±2.0	27.1±3.5	18.0±5.2
2	41.7±2.8	19.0±7.9	25.1±8.9	14.2±1.9
3	39.1±3.0	12.7±3.1	36.2±1.7	12.0±1.6

Table 6. Fatty acid profile of the biomass produced (n.d., not detected).

Raceway configuration	modified			standard		
Process condition	1	2	3	1	2	3
C14:0	1.28±1.28	1.97±1.97	5.71	2.13±1.01	2.71±1.39	3.19±0.37
C16:0	20.53±1.14	24.02±0.60	29.23	19.93±0.54	21.62±2.23	23.46±3.22
C16:1	40.14±0.72	36.06±0.46	36.64	42.06±2.97	34.04±0.12	35.60±1.86
C18:2n-6	n.d.	0.44±0.44	0.58	1.96±1.96	3.37±0.62	1.45±1.45
C20:4n-6	8.61±0.17	9.61±1.14	8.98	8.20±0.06	8.98±0.12	10.13±0.68
C20:5n-3	29.45±0.41	27.90±1.40	18.86	27.85±1.62	29.29±3.24	26.17±3.32
SFA	21.90±0.04	25.99±2.56	34.94	19.93±0.54	24.33±3.62	26.65±3.59
MFA	40.06±0.64	36.06±0.46	36.64	42.06±2.97	34.04±0.12	35.60±1.86
PUFA	38.04±0.59	37.95±2.10	28.42	38.01±3.51	41.63±3.74	37.75±5.46
ω -3	29.42±0.44	27.90±1.40	18.86	27.85±1.62	29.29±3.24	26.17±3.32
ω -6	8.63±0.15	10.05±0.70	9.56	10.16±1.89	12.35±0.49	11.58±2.13
ω -3/ ω -6	3.41±0.01	2.78±0.05	1.97	2.81±0.36	2.37±0.17	2.28±0.13
PUFA/SFA	1.74±0.02	1.48±0.23	0.81	1.91±0.23	1.77±0.42	1.47±0.40

The amount of CO₂ required to produce 1 g of biomass and the CO₂ fixation efficiency were determined through the stoichiometric equation obtained for each trial (Table 7). These are theoretical values that should be compared with the real ones calculated as it is described in Carbon sequestration section of Materials and Methods. However, the CO₂ inflow rate oscillations, due to the integrated CO₂ supply system feeding different reactors, hindered a rigorous quantification of raceways carbonation.

CO₂ consumption/biomass production ratio and photosynthetic efficiency results were

statistically similar in both raceways ($p \geq 0.05$). The ratios obtained are very close to the widely reported ratio of 1.83 g of CO₂ needed to produce 1 g of microalgal biomass [110]. CO₂ fixation efficiency in open-culture systems ranges from 10 to 30% [142]. Despite the higher values obtained in these experiments, the latter are theoretical and may be looked as the best results possible for the systems used. Indeed, such values are similar to the ones achieved (53% and 56%) in closed raceway ponds with enhanced supplied gas flow rate [142]. A lower value (36%) was reported by Putt *et al.* [143] in an open-air raceway pond.

Table 7. CO₂ requirements for biomass production and CO₂ fixation efficiency of raceway ponds used.

Raceway configuration / Process condition	CO ₂ consumption/biomass production (g/g)	CO ₂ fixation efficiency (%)
modified		
1	1.79±0.04	55.94±1.11
2	1.87±0.07	54.97±2.00
3	2.00	50.80
standard		
1	1.87±0.05	54.18±1.33
2	1.92±0.01	52.99±0.54
3	1.84±0.01	54.49±0.39

3.1.1 Economic assessment

Analysing the capital costs, there were not significant differences in the construction costs of both raceways. Standard raceway had a slightly higher cost due to the wider paddlewheel and the use of an extra set of baffles. However, modified raceway consumed at least twice the energy consumed by standard configuration (Table 8). This happened because of the non-uniform velocity profile generated with an half channel width paddlewheel mixing. The typical plug flow of raceways [111] was altered and there was even zones wherein occurred an inversion of flow direction. Thus, an higher rotation frequency were needed to attain a given circulation velocity.

Table 8. Paddlewheel rotation speed and associated energy consumption.

Process condition / Raceway configuration	Energy consumption (W m^{-3})			Mixing speed (rad s^{-1})		
	1	2	3	1	2	3
modified	10.9	20.9	8.4	1.29	1.68	0.59
standard	5.2	8.0	4.0	0.67	0.67	0.31

Given that differences in raceways configuration do not influence biomass productivity, biomass composition and CO_2 fixation efficiency; raceway construction costs differences are insignificant; energy costs are much greater in modified raceway, it can be concluded that standard configuration is the best alternative of design and should be used in the scale-up. Based on these results, from now on only standard raceway performance will be discussed on the following sections.

3.2 Water depth optimization

In this section it will be discussed which of the two water depths used had the best performance. Thus, this parameter was defined as the independent variable. Optimization of culture depth was based on the comparison of trials performed under process conditions 1 and 2.

There were no statistical significant differences in global areal productivities and growth rates obtained ($p \geq 0.05$). These were somewhat unexpected results, since generally areal productivity increases with an increase in pond depth [144]. For example, areal productivity increased 134-200% in algal ponds at a depth of 0.40 m compared to 0.20 m depth ponds [145]. An increase of 10.44 to 14.10 $\text{g m}^{-2} \text{d}^{-1}$ was described for *Nannochloropsis* sp. F&M-M24 grown in raceway ponds. However, the statistical similarity of photosynthetic efficiency obtained for both process conditions, justifies the productivity results. Contrariwise, San Pedro *et al.* [140] and de Vree *et al.* [139] mentioned that large pond depths would lead to light-limited cells and to the creation of large dark zones wherein microalgae respire, reducing the net productivity of the culture.

The quality of biomass produced was similar, since there were not statistical differences in % of lipids and EPA ($p \geq 0.05$), which is the main focus related with *Nannochloropsis* biomass production. PUFA/SFA and ω -3/ ω -6 ratios were also identical for both process conditions ($p \geq 0.05$).

As for CO_2 fixation efficiency and CO_2 consumption/biomass production ratio, the results obtained were also statistical similar ($p \geq 0.05$). However, Weissman *et al.* [110] mention that

greater pond depths results in greater potential carbon storage capacity. He states that water depth, mixing velocity and friction coefficient of the pond bottom determine the depth of the stagnant boundary layer and thus the mass transfer coefficient for CO₂ release through the surface of a raceway pond.

On the other hand, it was verified an 54% increase in energy consumption with process condition 2, as a result of higher paddlewheel rotation speed. A power consumption increase between 0.20 m and 0.10 m water depth trials was reported by Mendoza *et al.* [146]. This author proved that a 0.20 m water column is the optimum value for minimizing power consumption. Chiaramonti *et al.* [147] described an increase in total head losses caused by reduction in cultures depths.

Thus, once more, power consumption was the differentiator factor. The lower energy required with a pond depth of 0.20 m make it is the best option for microalgae cultivation.

3.3 Culture circulation velocity optimization

In this section it will be discussed which of the two culture circulation velocities used had the best performance. Thus, this parameter is defined as the independent variable. It was optimized comparing the results of trials performed under process conditions 2 and 3.

There were no statistical differences in the global biomass productivities, global growth rates and photosynthetic efficiencies obtained ($p \geq 0.05$). These result differ from the one reported by Moazami *et al.* [148] wherein an increase in paddlewheel rotation speed from 1.4 to 2.1 rad s⁻¹ result in a biomass productivity increase. However, Weissman *et al.* [110] reported that different mixing velocities had no apparent effect on productivity.

Regarding the same biomass quality parameters used in the previous section, it stands out that, despite the lipid content and ω -3/ ω -6 ratio were statistically similar ($p \geq 0.05$), the same did not happen to EPA content and PUFA/SFA ratio ($p < 0.05$). Indeed, these two parameters were higher for a culture velocity of 0.30 m s⁻¹.

As for CO₂ fixation efficiency and CO₂ consumption/biomass production ratio, the results did not reveal statistical significant differences ($p \geq 0.05$). However, Weissman *et al.* [110] affirmed that doubling the velocity, triples the CO₂ mass transfer coefficient through raceways surface.

Specific power consumption was two-fold higher with a 0.30 m s⁻¹ circulation velocity. This was because of the two-fold higher paddlewheel rotation speed needed to double the culture circulation velocity. Mendoza *et al.* [146] and Weissman *et al.* [110] also reported an increase in specific power consumption with liquid velocity. The former author refers an exponential relation between these two parameters due to the exponential relation between the liquid velocity and its kinetic energy.

In order to have an accurate decision on the best culture circulation speed, an economic assessment should be done to compare which of two factors (EPA productivity and power consumption) prevail. However, since there was only a difference of about 3% in EPA content and the energy costs were two-fold higher for 0.30 m s^{-1} , it is reasonable to conclude that 0.15 m s^{-1} was the best circulation velocity option.

4 Conclusions and Future perspectives

Microalgae biomass and microalgal derived high-value compounds have increasingly been the focus of the scientific community and industry. Despite having endless of applications, microalgal biotechnology is a quite recent industry, existing only a limited number of revolutionary large-scale commercial production companies. Most of these companies use outdoor raceway ponds to cultivate their microalgae since such systems are the more economical biomass production systems.

A comparison of two different pilot-scale raceway pond configurations and an optimization of the water depth and culture circulation velocity were performed. These were evaluated in terms of the growth dynamics and biochemical composition of the used microalga, CO₂ fixation efficiency and power consumption requirements. An economic assessment of both prototype raceways was also performed.

Power consumption was the differentiating factor since productivities, biomass quality and carbon sequestration were similar in both raceways under the same process conditions. It determined that the standard configuration is the best design and the one that must be adopted in the scale-up process to the DEMO raceway ponds. Besides, power requirements were also the basis for choosing the 0.20 m water depth and the 0.15 m s⁻¹ culture velocity as the best conditions for a cost-effective biomass production. Further studies using flue gases exhausted from the adjacent cement factory needs to be performed in order to evaluate the suitability of such residues as a carbon source for microalgae production.

However, nowadays, the overall cost of the microalgae production process is still to high because of the lower productivities achieved in open ponds and the huge power requirements of harvesting and drying processes. Future approaches should be focused on the raceway design and on the creation of a biosystem similar to the natural growth conditions of microalgae, including natural raw materials and water dynamics optimization. Nevertheless, beyond the improvements needed in the cultivation and in the energetic efficiency of downstream processes, a biorefinery approach is the only viable option to achieve an economical and sustainable production. The use of industrial effluents may also reduce the overall cost, besides contributing to an environmental sustainable circular economy.

References

1. Del Campo, J. A., García-González, M. & Guerrero, M. G. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Applied Microbiology and Biotechnology* **74**, 1163–1174 (2007).
2. Guedes, A. C., Amaro, H. M. & Malcata, F. X. Microalgae as sources of carotenoids. *Marine drugs* **9**, 625–44 (2011).
3. Singh, J. & Saxena, R. C. in *Handbook of Marine Microalgae* (ed Kim, S.-K.) 11–24 (Academic Press, 2015).
4. Mata, T. M., Martins, A. A. & Caetano, N. S. Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy Reviews* **14**, 217–232 (2010).
5. Venkatesan, J. & Manivasagan, P. in *Handbook of Marine Microalgae* (ed Kim, Se-Kwon) 1–9 (Academic Press, 2015).
6. Tomaselli, L. in *Handbook of Microalgal Culture* (ed Richmond, Amos) 1–19 (Blackwell Publishing Ltd, Oxford, UK, 2004).
7. Behrenfeld, M. J. *et al.* Biospheric primary production during an ENSO transition. *Science* **291**, 2594–2597 (2001).
8. Falkowski, P. & Raven, J. *Aquatic photosynthesis* 2nd ed. (eds Falkowski, P. & Raven, J.) 512 (Princeton University Press, 2007).
9. Berman-Frank, I., Lundgren, P. & Falkowski, P. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Research in Microbiology* **154**, 157–164 (2003).
10. Bergman, B., Sandh, G., Lin, S., Larsson, J. & Carpenter, E. J. *Trichodesmium* – a widespread marine cyanobacterium with unusual nitrogen fixation properties. *FEMS Microbiology Reviews* **37**, 286–302 (2013).
11. Siqueira, S. F., Queiroz, M. I., Zepka, L. Q. & Jacob-Lopes, E. in *Microalgal Biotechnology* (ed Jacob-Lopes, Eduardo) (InTech, 2018).
12. Borowitzka, M. A. in *The Physiology of Microalgae* (ed Borowitzka, Michael, Beardall, John and Raven, John) 655–681 (Springer International Publishing, Cham, 2016).
13. Adl, S. M. *et al.* The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *The Journal of Eukaryotic Microbiology* **52**, 399–451 (2005).
14. Ruggiero, M. A. *et al.* A higher level classification of all living organisms. *PLoS One* **10**, e0119248 (2015).

15. *Phycology* second (ed Lee, R.) 645 (Cambridge University Press, 1989).
16. Heimann, K. & Huerlimann, R. Microalgal classification: major classes and genera of commercial microalgal species. *Handbook of Marine Microalgae*, 25–41 (2015).
17. Robert A. Andersen & Ralph A. Lewin. *Classification of algae*
18. Grobbelaar, J. U. in *Handbook of Microalgal Culture* (ed Richmond, Amos) 95–115 (Blackwell Publishing Ltd, Oxford, UK, 2007).
19. Michael Borowitzka. *Algae culture and biotechnology* 2014. (2019).
20. Borowitzka, M. A. Biology of microalgae. *Microalgae in Health and Disease Prevention*, 23–72 (2018).
21. Huesemann, M. *et al.* A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. *Algal Research* **13**, 195–206 (2016).
22. J. Geider, R. & Osborne, B. A. Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth. *New Phytologist* **112**, 327–341 (1989).
23. Richmond, A. & Grobbelaar, J. U. Factors affecting the output rate of *Spirulina platensis* with reference to mass cultivation. *Biomass* **10**, 253–264 (1986).
24. Raven, J. & Giordano, M. in *The Physiology of Microalgae* (ed Borowitzka, Michael, Beardall, John and Raven, John) 143–154 (Springer International Publishing, Cham, 2016).
25. Borowitzka, M. A. in *The Physiology of Microalgae* (ed Borowitzka, Michael, Beardall, John and Raven, John) 601–652 (Springer International Publishing, Cham, 2016).
26. Olsen, L., Öztürk, M., Sakshaug, E. & Johnsen, G. Photosynthesis-induced phosphate precipitation in seawater: ecological implications for phytoplankton. *Marine Ecology Progress Series* **319**, 103–110 (2006).
27. Larsdotter, K., Jansen, J. I. C. & Dalhammar, G. Biologically mediated phosphorus precipitation in wastewater treatment with microalgae. *Environmental Technology* **28**, 953–960 (2007).
28. Powell, N., Shilton, A., Chisti, Y. & Pratt, S. Towards a luxury uptake process via microalgae – defining the polyphosphate dynamics. *Water Research* **43**, 4207–4213 (2009).
29. Borowitzka, M. in *Microalgal Biotechnology* (eds Borowitzka, M. & Borowitzka, L.) 87–257 (Cambridge University Press, 1988).

30. Milligan, A. J. & Harrison, P. J. Effects of non-steady-state iron limitation on nitrogen assimilatory enzymes in the marine diatom *Thalassiosira weissflogii* (bacillariophyceae). *Journal of Phycology* **36**, 78–86 (2000).
31. Berman-Frank, I., Quigg, A., Finkel, Z. V., Irwin, A. J. & Haramaty, L. Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnology and Oceanography* **52**, 2260–2269 (2007).
32. Marchetti, A. & Maldonado, M. T. in *The Physiology of Microalgae* (ed Borowitzka, Michael, Beardall, John and Raven, John) 233–279 (Springer International Publishing, Cham, 2016).
33. Halliwell, B. & Gutteridge, J. M. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS letters* **307**, 108–12 (1992).
34. Behrenfeld, M. J. & Milligan, A. J. Photophysiological expressions of iron stress in phytoplankton. *Annual Review of Marine Science* **5**, 217–246 (2013).
35. Croft, M. T., Warren, M. J. & Smith, A. G. Algae need their vitamins. *Eukaryotic Cell* **5**, 1175–1183 (2006).
36. Milledge, J. J. Commercial application of microalgae other than as biofuels: a brief review. *Reviews in Environmental Science and Bio/Technology* **10**, 31–41 (2011).
37. Rath, B. Commercial and industrial applications of microalgae – a review. *Journal Algal Biomass Utilization* **3**, 89–100 (2012).
38. Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* **101**, 87–96 (2006).
39. Naidoo, T., Zulu, N., Maharajh, D. & Lalloo, R. in *Biotechnological Applications of Microalgae* (ed Bux, Faizal) 137–160 (CRC Press, 2013).
40. Misra, R., Guldhe, A., Singh, P., Rawat, I. & Bux, F. Electrochemical harvesting process for microalgae by using nonsacrificial carbon electrode: a sustainable approach for biodiesel production. *Chemical Engineering Journal* **255**, 327–333 (2014).
41. Bhalamurugan, G. L., Valerie, O. & Mark, L. Valuable bioproducts obtained from microalgal biomass and their commercial applications: a review. *Environmental Engineering Research* **23**, 229–241 (2018).
42. Rosales-Mendoza, S. in *Algae-Based Biopharmaceuticals* (ed Rosales-Mendoza, Sergio) 121–141 (Springer International Publishing, Cham, 2016).
43. Rodrigues, D. B., Menezes, C. R., Mercadante, A. Z., Jacob-Lopes, E. & Zepka, L. Q. Bioactive pigments from microalgae *Phormidium autumnale*. *Food Research International* **77**, 273–279 (2015).

44. Murthy, K. C., Rajesha, J., Swamy, M. M. & Ravishankar, G. Comparative evaluation of hepatoprotective activity of carotenoids of microalgae. *Journal of Medicinal Food* **8**, 523–528 (2005).
45. Sandmann, G. in *Advances in biochemical engineering/biotechnology* (ed Scheper, Thomas) 449–467 (2014).
46. Egeland, E. S. in *The Physiology of Microalgae* (ed Borowitzka, Michael, Beardall, John and Raven, John) 507–563 (Springer International Publishing, Cham, 2016).
47. Pulz, O. & Gross, W. Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* **65**, 635–648 (2004).
48. Sekar, S. & Chandramohan, M. Phycobiliproteins as a commodity: trends in applied research, patents and commercialization. *Journal of Applied Phycology* **20**, 113–136 (2008).
49. Odjadjare, E. C., Mutanda, T. & Olaniran, A. O. Potential biotechnological application of microalgae: a critical review. *Critical Reviews in Biotechnology* **37**, 37–52 (2017).
50. de Jesus Raposo, M. F., de Morais, R. M. S. C. & de Morais, A. M. M. B. Health applications of bioactive compounds from marine microalgae. *Life Sciences* **93**, 479–486 (2013).
51. Ward, O. P. & Singh, A. Omega-3/6 fatty acids: alternative sources of production. *Process Biochemistry* **40**, 3627–3652 (2005).
52. Judé, S. *et al.* Dietary long-chain omega-3 fatty acids of marine origin: a comparison of their protective effects on coronary heart disease and breast cancers. *Progress in Biophysics and Molecular Biology* **90**, 299–325 (2006).
53. La Rovere, M. T. & Christensen, J. H. The autonomic nervous system and cardiovascular disease: role of n-3 PUFAs. *Vascular Pharmacology* **71**, 1–10 (2015).
54. Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A. & Millington, K. J. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics* **17**, 449–459 (2004).
55. Simopoulos, A. P. Essential fatty acids in health and chronic disease. *The American Journal of Clinical Nutrition* **70**, 560s–569s (1999).
56. Borowitzka, M. A. in *The Algae World* (eds Sahoo, D. & Seckbach, J.) 319–338 (Springer, Dordrecht, 2015).
57. Soletto, D., Binaghi, L., Lodi, A., Carvalho, J. & Converti, A. Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. *Aquaculture* **243**, 217–224 (2005).

58. Varfolomeev, S. D. & Wasserman, L. A. Microalgae as source of biofuel, food, fodder, and medicines. *Applied Biochemistry and Microbiology* **47**, 789–807 (2011).
59. García, J. L., de Vicente, M. & Galán, B. Microalgae, old sustainable food and fashion nutraceuticals. *Microbial Biotechnology* **10**, 1017–1024 (2017).
60. Wang, H.-M. D., Chen, C.-C. & Huynh, P. Exploring the potential of using algae in cosmetics. *Bioresource Technology* **184**, 355–362 (2015).
61. Fabrowska, J., Łska, B., Schroeder, G., Messyas, B. & Pikosz, M. in *Marine Algae Extracts* (ed Kim, Se-Kwon and Chojnacka, Katarzyna) 681–706 (Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2015).
62. Hill, R. T. & Fenical, W. Pharmaceuticals from marine natural products: surge or ebb? *Current Opinion in Biotechnology* **21**, 777–779 (2010).
63. Herrero, M., Castro-Puyana, M., Mendiola, J. A. & Ibañez, E. Compressed fluids for the extraction of bioactive compounds. *Trends in Analytical Chemistry* **43**, 67–83 (2013).
64. Martínez-Francés, E. & Escudero-Oñate, C. in *Microalgal Biotechnology* (ed Jacob-Lopes, Eduardo) (InTech, 2018).
65. Khan, M. I., Shin, J. H. & Kim, J. D. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial Cell Factories* **17**, 36 (2018).
66. Stephenson, P. G., Moore, C. M., Terry, M. J., Zubkov, M. V. & Bibby, T. S. Improving photosynthesis for algal biofuels: toward a green revolution. *Trends in Biotechnology* **29**, 615–623 (2011).
67. Lam, M. K. & Lee, K. T. Microalgae biofuels: a critical review of issues, problems and the way forward. *Biotechnology Advances* **30**, 673–690 (2012).
68. Park, H. & Lee, C.-G. Theoretical calculations on the feasibility of microalgal biofuels: utilization of marine resources could help realizing the potential of microalgae. *Biotechnology Journal* **11**, 1461–1470 (2016).
69. Bhaskar Singh, Y. Liu & Yogesh C Sharma. in *Biotechnological Applications of Microalgae* (ed Bux, F.) 99–112 (CRC Press, 2013).
70. Formighieri, C., Franck, F. & Bassi, R. Regulation of the pigment optical density of an algal cell: filling the gap between photosynthetic productivity in the laboratory and in mass culture. *Journal of Biotechnology* **162**, 115–123 (2012).
71. Melis, A. Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. *Plant Science* **177**, 272–280 (2009).

72. Rashid, N., Ur Rehman, M. S., Sadiq, M., Mahmood, T. & Han, J.-I. Current status, issues and developments in microalgae derived biodiesel production. *Renewable and Sustainable Energy Reviews* **40**, 760–778 (2014).
73. Ryohei Ueda, Shin Hirayama, Kiyoshi Sugata & Hiroshi Nakayama. *Process for the production of ethanol from microalgae* 1994.
74. Harun, R., Danquah, M. K. & Forde, G. M. Microalgal biomass as a fermentation feedstock for bioethanol production. *Journal of Chemical Technology & Biotechnology* **85**, n/a–n/a (2009).
75. Randrianarison, G. & Ashraf, M. A. Microalgae: a potential plant for energy production. *Geology, Ecology, and Landscapes* **1**, 104–120 (2017).
76. Shaishav Sharma, Singh Ram Narayan & Satyendra Tripathi. Biohydrogen from algae: fuel of the future. *International Research Journal of Environment Sciences* **2**, 44–47 (2013).
77. Saifuddin, N. & Priatharsini, P. Developments in bio-hydrogen production from algae: a review. *Research Journal of Applied Sciences, Engineering and Technology* **12**, 968–982 (2016).
78. Dubini, A. & Gonzalez-Ballester, D. in *Algae Biotechnology Products and Processes* (eds Bux, F. & Chisti, Y.) 165–193 (Springer International Publishing, 2016).
79. Dasgupta, C. N. *et al.* Recent trends on the development of photobiological processes and photobioreactors for the improvement of hydrogen production. *International Journal of Hydrogen Energy* **35**, 10218–10238 (2010).
80. Dos Santos, A., Severo, I., Queiroz, M., Zepka, L. & Jacob-Lopes, E. in *Industrial Waste: Management, Assessment and Environmental Issues* (ed Barton, Stanley) 41–64 (2016).
81. Jacob-Lopes, E., Scoparo, C. H. G., Lacerda, L. M. C. F. & Franco, T. T. Effect of light cycles (night/day) on CO₂ fixation and biomass production by microalgae in photobioreactors. *Chemical Engineering and Processing: Process Intensification* **48**, 306–310 (2009).
82. Özçimen, D., İnan, B., Koçer, A. T. & Vehapi, M. in *Microalgal Biotechnology* (ed Jacob-Lopes, Eduardo) (InTech, 2018).
83. Ma, X.-N., Chen, T.-P., Yang, B., Liu, J. & Chen, F. Lipid production from *nannochloropsis*. *Marine Drugs* **14**, 61 (2016).

84. Suda, S., Atsumi, M. & Miyashita, H. Taxonomic characterization of a marine *Nannochloropsis* species, *N. oceanica* sp. nov. (eustigmatophyceae). *Phycologia* **41**, 273–279 (2002).
85. Rodolfi, L. *et al.* Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering* **102**, 100–112 (2009).
86. Radakovits, R. *et al.* Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. *Nature Communications* **3**, 686 (2012).
87. Vieler, A. *et al.* Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. *PLoS Genetics* **8** (2012).
88. Doan, T. T. Y., Sivaloganathan, B. & Obbard, J. P. Screening of marine microalgae for biodiesel feedstock. *Biomass and Bioenergy* **35**, 2534–2544 (2011).
89. Ma, Y., Wang, Z., Yu, C., Yin, Y. & Zhou, G. Evaluation of the potential of 9 *Nannochloropsis* strains for biodiesel production. *Bioresource Technology* **167**, 503–509 (2014).
90. Gu, N. *et al.* Effect of salinity on growth, biochemical composition, and lipid productivity of *Nannochloropsis oculata* CS 179. *Engineering in Life Sciences* **12**, 631–637 (2012).
91. Sukenik, A., Carmeli, Y. & Berner, T. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. *Journal of Phycology* **25**, 686–692 (1989).
92. Pagnanelli, F., Altimari, P., Trabucco, F. & Toro, L. Mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata*: interaction between glucose and nitrate. *Journal of Chemical Technology & Biotechnology* **89**, 652–661 (2014).
93. Solovchenko, A. *et al.* Interactive effects of salinity, high light, and nitrogen starvation on fatty acid and carotenoid profiles in *Nannochloropsis oceanica* CCALA 804. *European Journal of Lipid Science and Technology* **116**, 635–644 (2014).
94. Baohua, Z. *et al.* Large-scale biodiesel production using flue gas from coal-fired power plants with *Nannochloropsis* microalgal biomass in open raceway ponds. *Bioresource Technology* **174**, 53–59 (2014).
95. Carvalho, A., Meireles, L. & Malcata, F. Microalgal reactors: a review of enclosed system designs and performances. *Biotechnology Progress* **22**, 1490–1506 (2006).

96. Ugwu, C., Aoyagi, H. & Uchiyama, H. Photobioreactors for mass cultivation of algae. *Bioresource Technology* **99**, 4021–4028 (2008).
97. Griffiths, M. in *Biotechnological Applications of Microalgae* (ed Bux, Fayzal) 51–76 (CRC Press, 2013).
98. Brennan, L. & Owende, P. Biofuels from microalgae - a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14**, 557–577 (2010).
99. Ashok, A., Doriya, K., Rao, D. R. M. & Kumar, D. S. Design of solid state bioreactor for industrial applications: an overview to conventional bioreactors. *Biocatalysis and Agricultural Biotechnology* **9**, 11–18 (2017).
100. Razzak, S. A., Hossain, M. M., Lucky, R. A., Bassi, A. S. & de Lasa, H. Integrated CO₂ capture, wastewater treatment and biofuel production by microalgae culturing - a review. *Renewable and Sustainable Energy Reviews* **27**, 622–653 (2013).
101. Leite, G. B., Abdelaziz, A. E. & Hallenbeck, P. C. Algal biofuels: challenges and opportunities. *Bioresource Technology* **145**, 134–141 (2013).
102. Sierra, E. *et al.* Characterization of a flat plate photobioreactor for the production of microalgae. *Chemical Engineering Journal* **138**, 136–147 (2008).
103. Lee, Y.-K. Microalgal mass culture systems and methods: their limitation and potential. *Journal of Applied Phycology* **13**, 307–315 (2001).
104. Pulz, O. Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology* **57**, 287–293 (2001).
105. Setlik, I., Veladimir, S. & Malek, I. Dual purpose open circulation units for large scale culture of algae in temperate zones. I. Basic design considerations and scheme of a pilot plant. *International Journal of Phycological Research* **1**, 111–164 (1970).
106. Richmond, A., Lichtenberg, E., Stahl, B. & Vonshak, A. Quantitative assessment of the major limitations on productivity of *Spirulina platensis* in open raceways. *Journal of Applied Phycology* **2**, 195–206 (1990).
107. Handler, R. M. *et al.* Evaluation of environmental impacts from microalgae cultivation in open-air raceway ponds: analysis of the prior literature and investigation of wide variance in predicted impacts. *Algal Research* **1**, 83–92 (2012).
108. Sompech, K., Chisti, Y. & Srinophakun, T. Design of raceway ponds for producing microalgae. *Biofuels* **3**, 387–397 (2012).
109. Packer, M. A., Harris, G. C. & Adams, S. L. in *Algae Biotechnology Products and Processes* (eds Bux, F. & Chisti, Y.) 217–247 (2016).

110. Weissman, J. C., Goebel, R. P. & Benemann, J. R. Photobioreactor design: mixing, carbon utilization, and oxygen accumulation. *Biotechnology and Bioengineering* **31**, 336–344 (1988).
111. Chisti, Y. in *Algae Biotechnology Products and Processes* (eds Bux, F. & Chisti, Y.) 21–40 (Springer, Cham, 2016).
112. Chisti, Y. Raceways-based production of algal crude oil. *Green* **3** (2013).
113. Becker, E. W. *Microalgae biotechnology and microbiology* (Cambridge University Press, 1994).
114. Tredici, M. R. in *Handbook of Microalgal Culture* (ed Richmond, A.) 178–214 (Blackwell Publishing Ltd, Oxford, UK, 2007).
115. López, A. R. *et al.* Sustainable cultivation of *Nannochloropsis gaditana* microalgae in outdoor raceways using flue gases for a complete 2-year cycle: a circular economy challenge. *Journal of Applied Phycology* **31**, 1515–1523 (2019).
116. Mendoza, J. *et al.* Oxygen transfer and evolution in microalgal culture in open raceways. *Bioresource Technology* **137**, 188–195 (2013).
117. Moheimani, N. R. & Borowitzka, M. A. Limits to productivity of the alga *Pleurochrysis carterae* (haptophyta) grown in outdoor raceway ponds. *Biotechnology and Bioengineering* **96**, 27–36 (2007).
118. Weissman, J. C., Tillett, D. M. & Goebel, R. P. *Design and operation of an outdoor microalgae test facility*. tech. rep. (Solar Energy Research Institute, 1989).
119. Singh, M., Shukla, R. & Das, K. in *Biotechnological Applications of Microalgae* (ed Bux, F.) 77–88 (CRC Press, 2013).
120. Molina Grima, E., Belarbi, E.-H., Acien Fernández, F., Robles Medina, A. & Chisti, Y. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* **20**, 491–515 (2003).
121. Shahid, A. *et al.* in *Algae Based Polymers, Blends, and Composites* (eds Zia, K., Zuber, M. & Ali, M.) 273–299 (Elsevier, 2017).
122. Grima, E. M., Fernández, F. G. A. & Medina, A. R. in *Handbook of Microalgal Culture* (ed Richmond, A.) 215–252 (Blackwell Publishing Ltd, Oxford, UK, 2004).
123. *Allmicroalgae - Natural Products* 2019. <https://www.allmicroalgae.com/>.
124. Coelho, A. *O que leva uma cimenteira a produzir microalgas?* 2016. <https://www.publico.pt/2016/10/27/ciencia/noticia/o-que-leva-uma-cimenteira-a-produzir-microalgas-1748934>.

125. Pinto, C. *COMPETE 2020 alavanca investimento da Secil na produção de microalgas* 2018. <https://www.compete2020.gov.pt/noticias/detalhe/Proj17608-FermALG-Secil-IDT-NL156-24052018>.
126. *Secil inaugura 16 milhões de investimento na produção de microalgas* 2016. <http://www.jornaldaeconomiadomar.com/secil-inaugura-16-milhoes-investimento-na-producao-microalgas/>.
127. Silva, R. *Investimento de 10,7 milhões para produzir microalgas para alimentação* 2019. <https://www.jornaldeleiria.pt/noticia/investimento-de-107-milhoes-para-produzir-microalgas-para-al-10134>.
128. Callejón-Ferre, A., Velázquez-Martí, B., López-Martínez, J. & Manzano-Agugliaro, F. Greenhouse crop residues: energy potential and models for the prediction of their higher heating value. *Renewable and Sustainable Energy Reviews* **15**, 948–955 (2011).
129. Quelhas, P. M. *et al.* Industrial production of *Phaeodactylum tricornutum* for CO₂ mitigation: biomass productivity and photosynthetic efficiency using photobioreactors of different volumes. *Journal of Applied Phycology* **31**, 2187–2196 (2019).
130. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917 (1959).
131. Marinho, Y. F. *et al.* Effect of the addition of *Chaetoceros calcitrans*, *Navicula* sp. and *Phaeodactylum tricornutum* (diatoms) on phytoplankton composition and growth of *Litopenaeus vannamei* (Boone) postlarvae reared in a biofloc system. *Aquaculture Research* **48**, 4155–4164 (2017).
132. Nunez, M. & Quigg, A. Changes in growth and composition of the marine microalgae *Phaeodactylum tricornutum* and *Nannochloropsis salina* in response to changing sodium bicarbonate concentrations. *Journal of Applied Phycology* **28**, 2123–2138 (2016).
133. Lepage, G. & Roy, C. C. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *Journal of lipid research* **25**, 1391–6 (1984).
134. Pereira, H. *et al.* Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Marine Drugs* **10**, 1920–1935 (2012).
135. Mata, T. M., Melo, A. C., Simões, M. & Caetano, N. S. Parametric study of a brewery effluent treatment by microalgae *Scenedesmus obliquus*. *Bioresource Technology* **107**, 151–158 (2012).

136. Myint, M. T., Ghassemi, A. & Nirmalakhandan, N. A generic stoichiometric equation for microalgae–microorganism nexus by using clarified domestic wastewater as growth medium. *Desalination and Water Treatment* **51**, 6632–6640 (2013).
137. Laws, E. A., Bidigare, R. R. & Popp, B. N. Effect of growth rate and CO₂ concentration on carbon isotopic fractionation by the marine diatom *Phaeodactylum tricornutum*. *Limnology and Oceanography* **42**, 1552–1560 (1997).
138. Natascia, B. *et al.* Cultivation of *Nannochloropsis oceanica* F&M-M24 and *Tetraselmis suecica* F&M-M33 in the two 0.5-ha BIOFAT pilot plants for biofuel production in *AlgaeEurope* (Lisbon, 2016).
139. De Vree, J. H., Bosma, R., Janssen, M., Barbosa, M. J. & Wijffels, R. H. Comparison of four outdoor pilot-scale photobioreactors. *Biotechnology for Biofuels* **8**, 215 (2015).
140. San Pedro, A., González-López, C., Acien, F. & Molina-Grima, E. Outdoor pilot production of *Nannochloropsis gaditana*: influence of culture parameters and lipid production rates in raceway ponds. *Algal Research* **8**, 205–213 (2015).
141. Das, P., Thaher, M. I., Hakim, M. A. Q. M. A. & Al-Jabri, H. M. S. Sustainable production of toxin free marine microalgae biomass as fish feed in large scale open system in the Qatari desert. *Bioresource Technology* **192**, 97–104 (2015).
142. Li, S., Luo, S. & Guo, R. Efficiency of CO₂ fixation by microalgae in a closed raceway pond. *Bioresource Technology* **136**, 267–272 (2013).
143. Putt, R., Singh, M., Chinnasamy, S. & Das, K. An efficient system for carbonation of high-rate algae pond water to enhance CO₂ mass transfer. *Bioresource Technology* **102**, 3240–3245 (2011).
144. Kumar, K., Mishra, S. K., Shrivastav, A., Park, M. S. & Yang, J.-W. Recent trends in the mass cultivation of algae in raceway ponds. *Renewable and Sustainable Energy Reviews* **51**, 875–885 (2015).
145. Sutherland, D. L., Turnbull, M. H. & Craggs, R. J. Increased pond depth improves algal productivity and nutrient removal in wastewater treatment high rate algal ponds. *Water Research* **53**, 271–281 (2014).
146. Mendoza, J. *et al.* Fluid-dynamic characterization of real-scale raceway reactors for microalgae production. *Biomass and Bioenergy* **54**, 267–275 (2013).
147. Chiaramonti, D. *et al.* Review of energy balance in raceway ponds for microalgae cultivation: re-thinking a traditional system is possible. *Applied Energy* **102**, 101–111 (2013).

148. Moazami, N. *et al.* Large-scale biodiesel production using microalgae biomass of *Nannochloropsis*. *Biomass and Bioenergy* **39**, 449–453 (2012).

Appendices

A Calibration curve

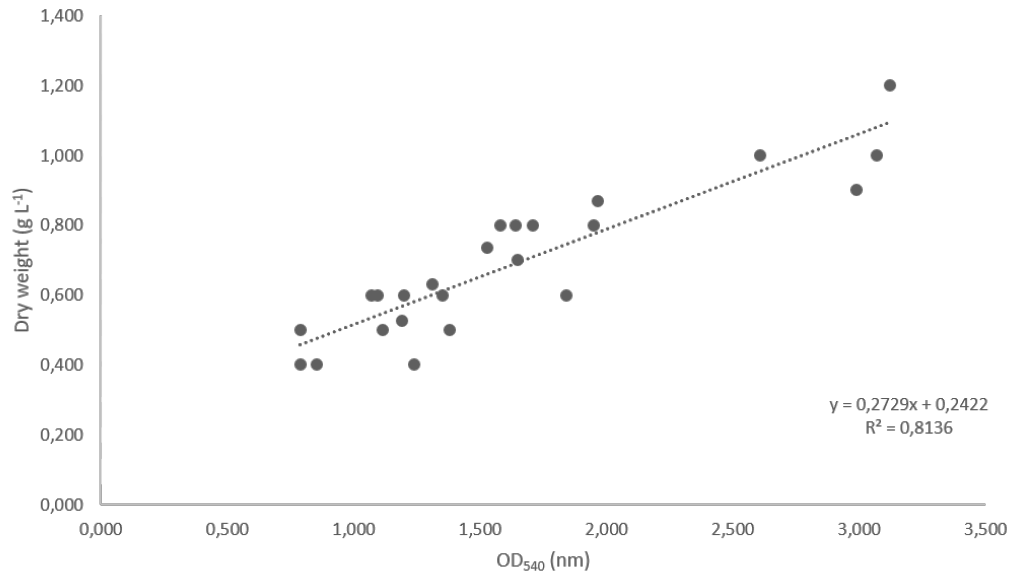


Figure A.10. Calibration curve of optical density at 540 nm as a function of *N. oceanica* biomass dry weight.

B Growth curves

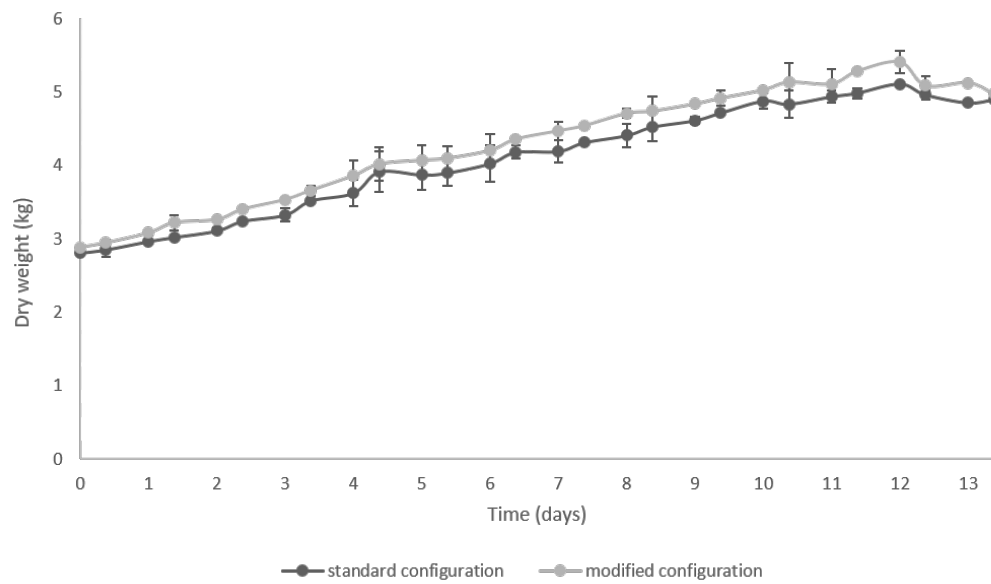


Figure B.11. Growth curves of *N. oceanica* cultivated under process conditions 1. Error bars represent the standard deviation calculated from two replicates.

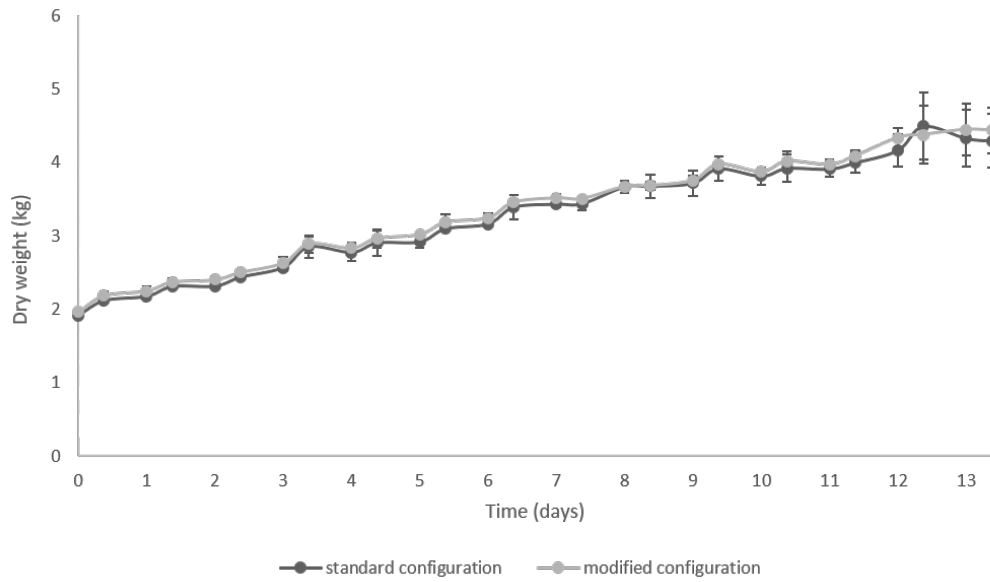


Figure B.12. Growth curves of *N. oceanica* cultivated under process conditions 2. Error bars represent the standard deviation calculated from two replicates.

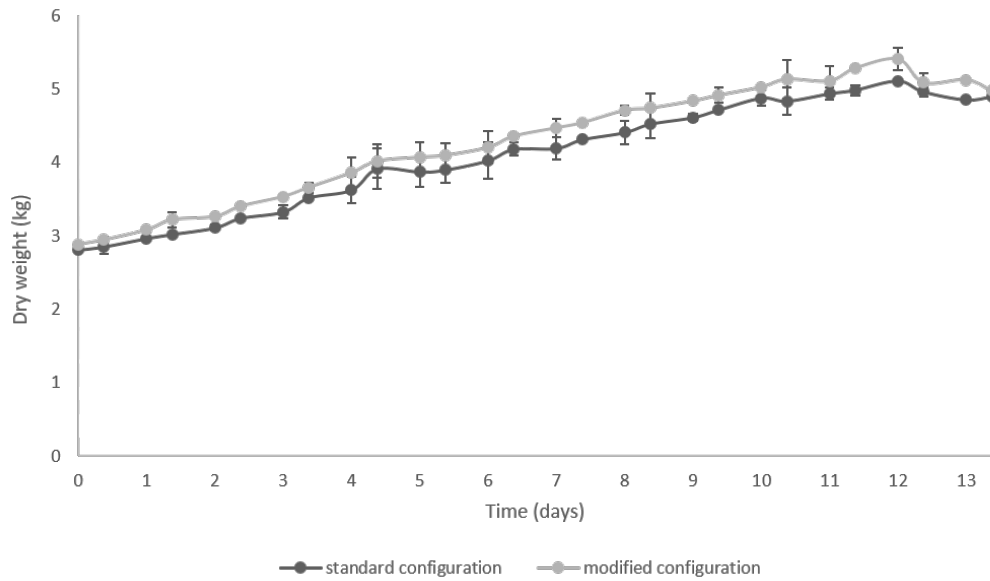


Figure B.13. Growth curves of *N. oceanica* cultivated under process conditions 3. Error bars represent the standard deviation calculated from two replicates.

C Meteorological conditions

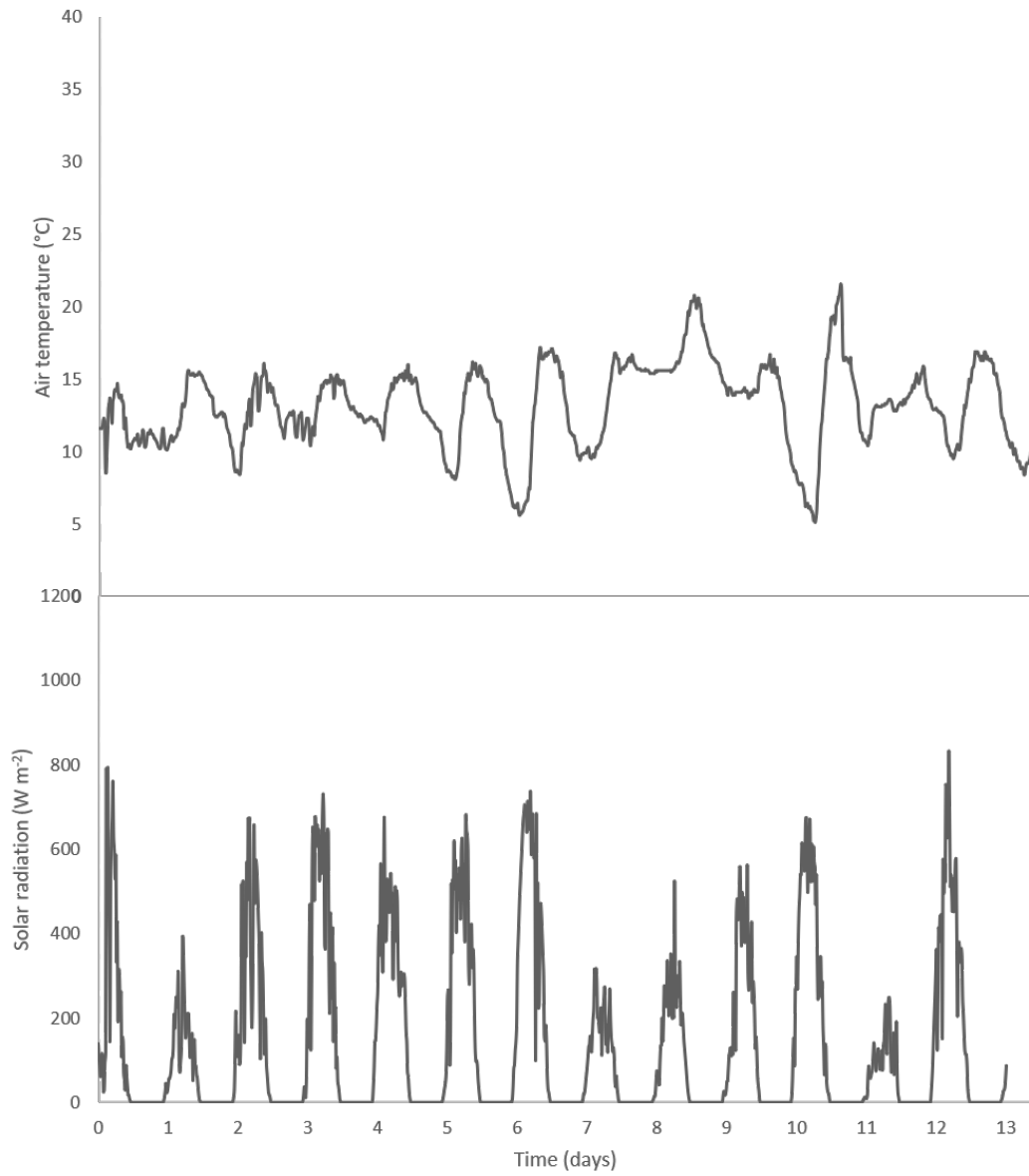


Figure C.14. Temperature and instant radiation registered on site during first growth trial under process conditions 1.

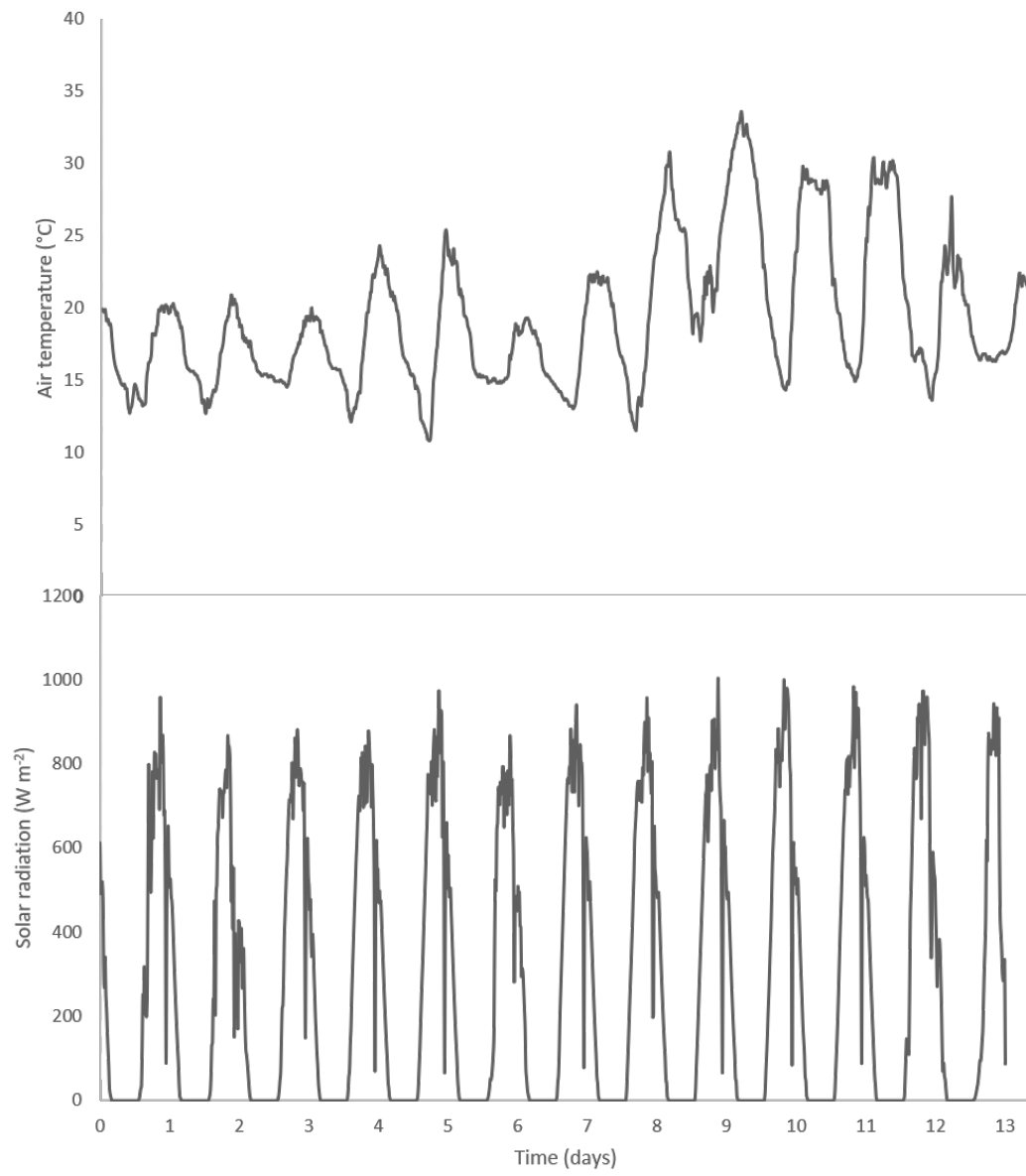


Figure C.15. Temperature and instant radiation registered on site during second growth trial under process conditions 1.

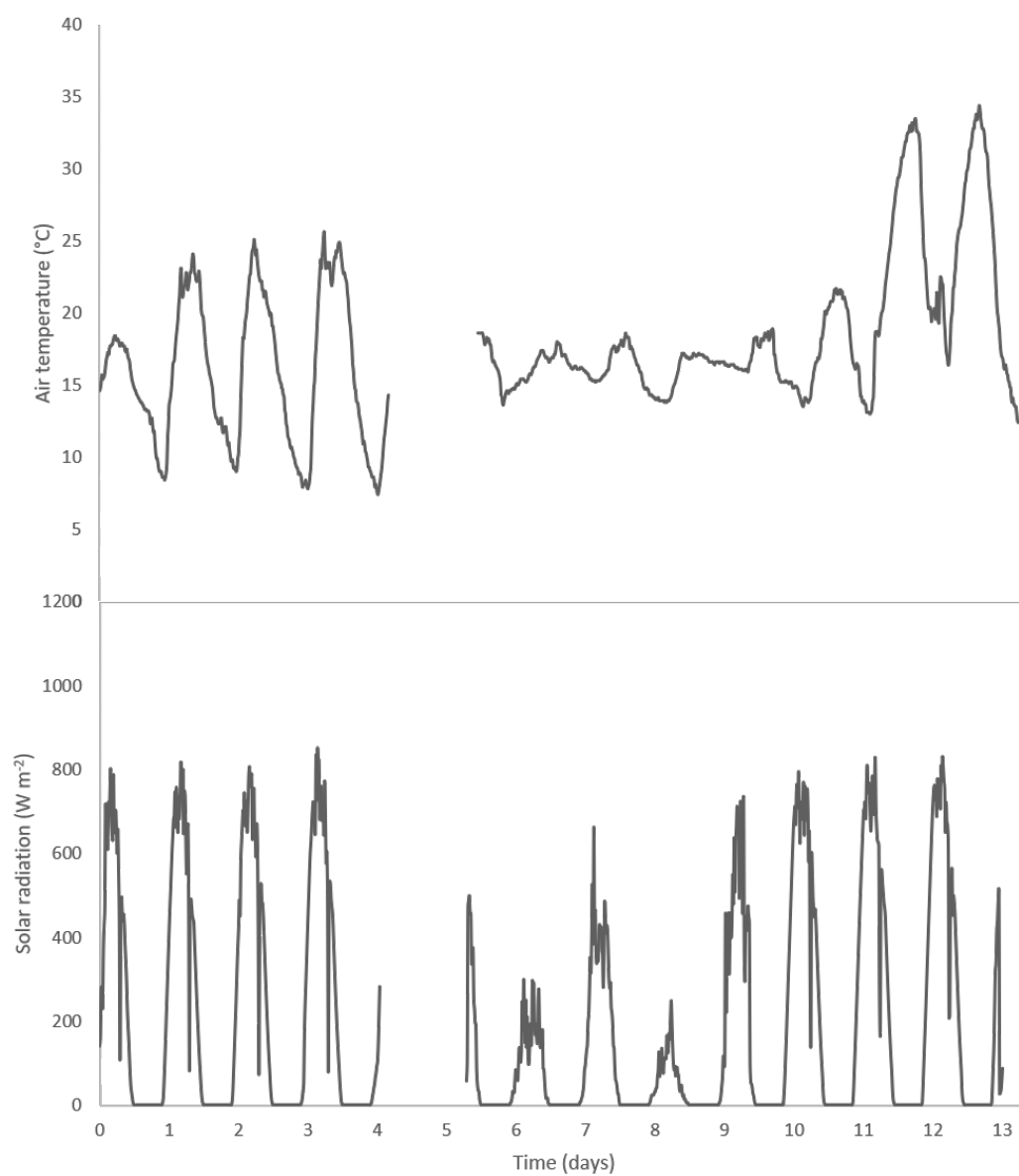


Figure C.16. Temperature and instant radiation registered on site during first growth trial under process conditions 2.

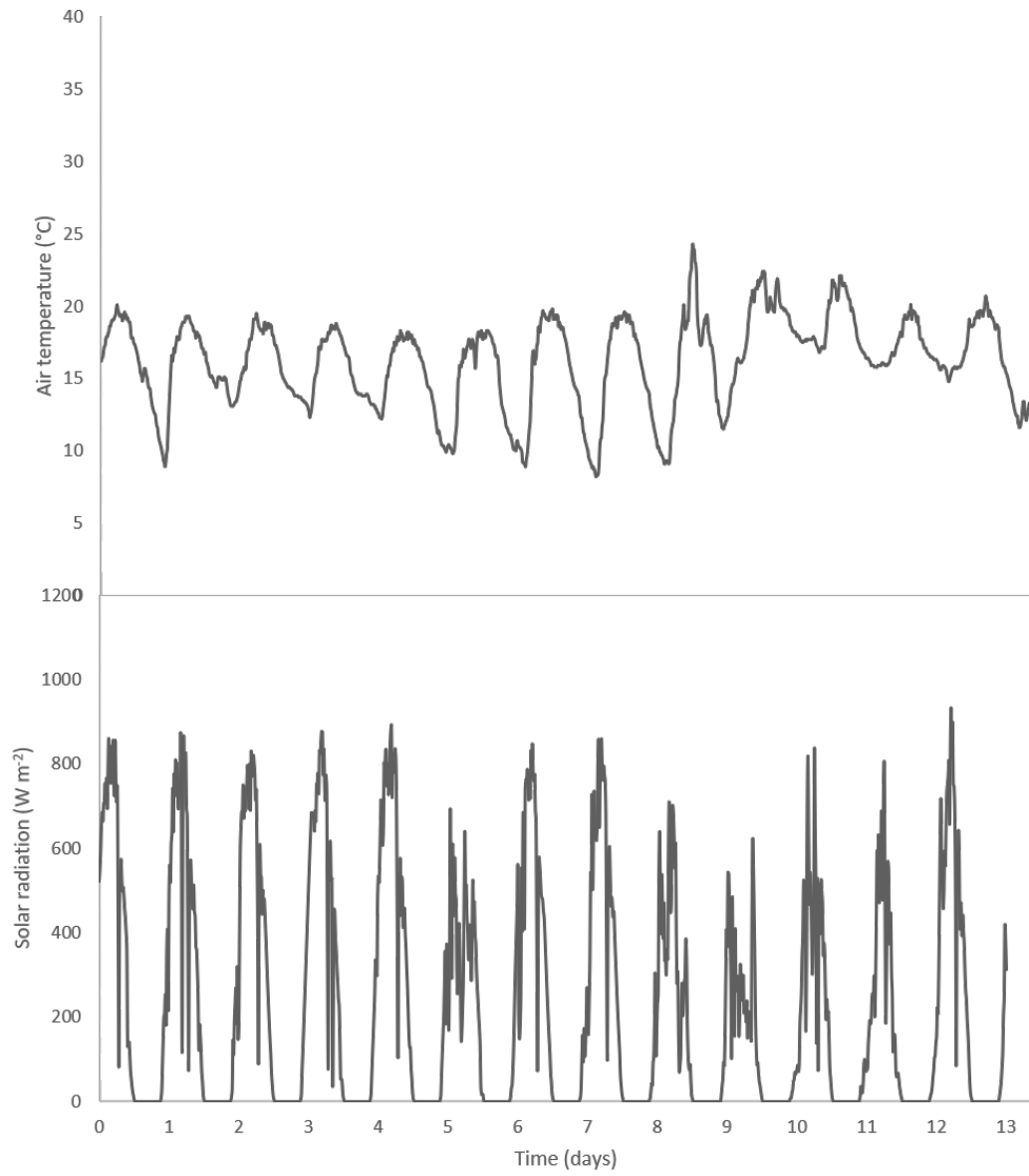


Figure C.17. Temperature and instant radiation registered on site during second growth trial under process conditions 2.

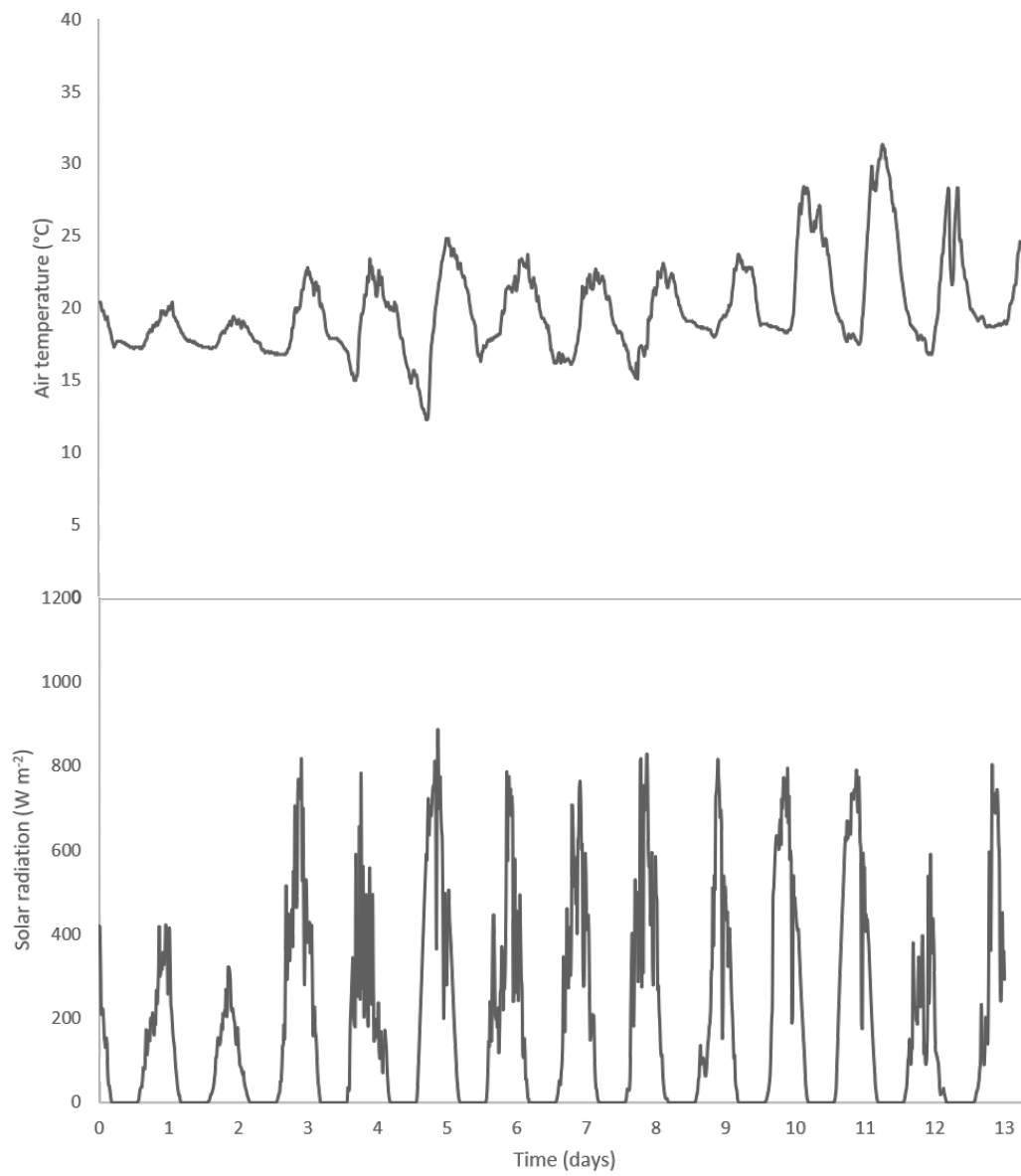


Figure C.18. Temperature and instant radiation registered on site during first growth trial under process conditions 3.

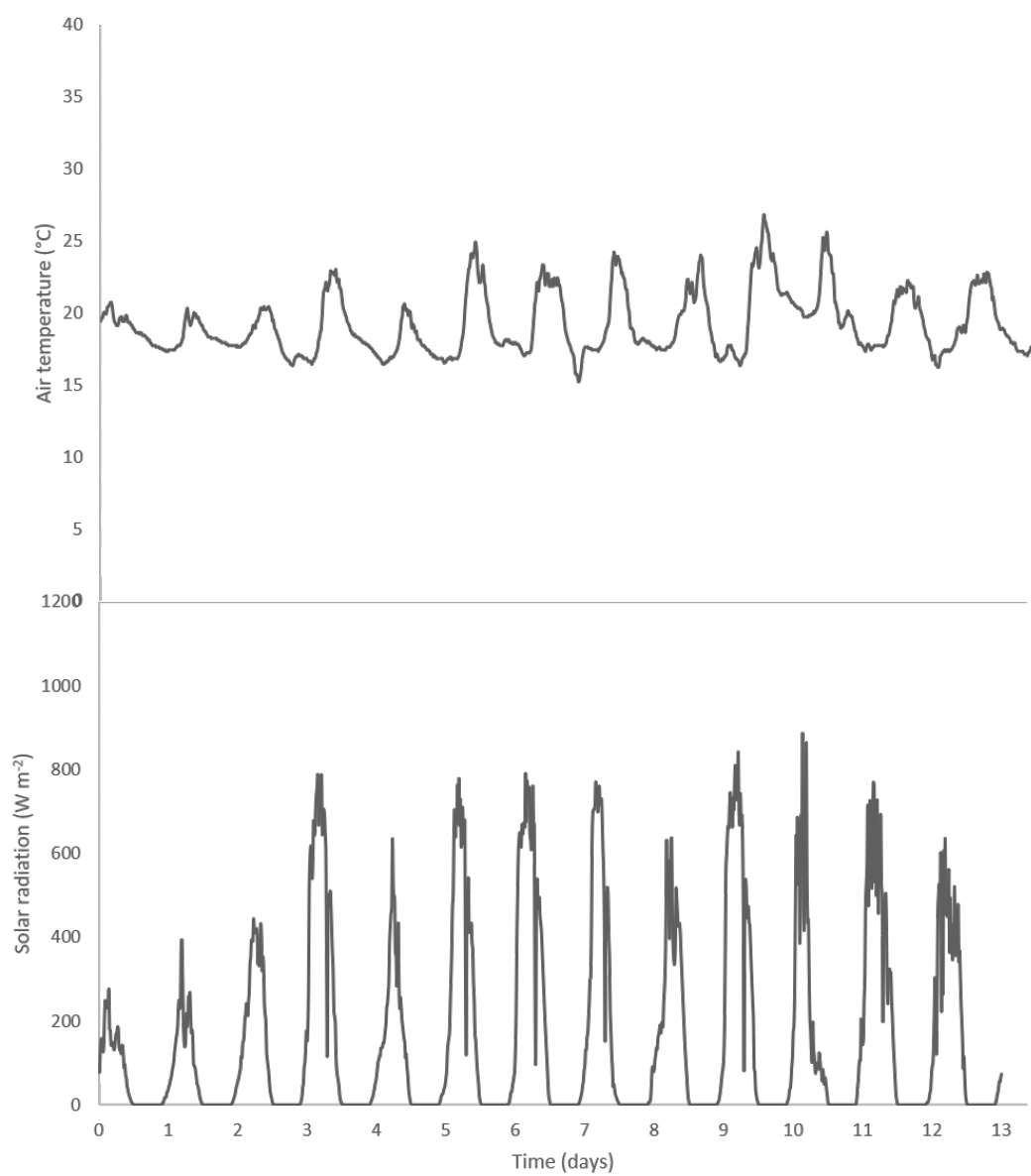


Figure C.19. Temperature and instant radiation registered on site during second growth trial under process conditions 3.