Universidade do Minho
Escola de Engenharia

Leandro Filipe Feiteira Madureira

Use of agro-industrial by-products for *Pavlova* spp. culture and heterotrophic growth of *Nannochloropsis* spp. as relevant production strategies for oleaginous microalgae

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Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efectuado sob a orientação do
Professor Doutor António Augusto Vicente

e coorientação do
Professor José Filipe Gonçalves Maciel

outubro de 2009

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STATEMENT OF INTEGRITY

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Utilização de subprodutos agroindustriais para o cultivo da microalga *Pavlova* spp. e crescimento heterotrófico de *Nannochloropsis* spp. comos estratégias para a produção de estirpes oleoginosas.

Resumo

Os ácidos gordos polinsaturados estão divididos em dois grupos relevantes em termos nutricionais, omega-6 (n-6) e omega-3 (n-3), e desempenham variadas funções importantes no organismo humano. Contudo, diferentes fatores levaram a um consumo de ácidos gordos polinsaturados n-6 aproximadamente 20 vezes superior ao consumo de ácidos gordos polinsaturados n-3, ressaltando-se o baixo consumo de ácido eicosapentaenóico (EPA) e ácido docosahexaenóico (DHA), que advém de peixes de água salgada, como salmão e cavala, ou de óleos de peixe. Como resultado da crescente demanda por alimentos, associada a uma crescente população mundial, estas fontes de ácidos gordos não são suficientes para atender aos requisitos nutricionais de EPA e DHA. Tornou-se por isso necessário explorar fontes alternativas, como as microalgas. As microalgas marinhas dos géneros *Nannochloropsis* e *Pavlova* são importantes produtores de EPA e DHA, exibindo um elevado conteúdo de ácidos gordos n-3 por biomassa de peso seco.

Apesar das espécies *Nannochloropsis* e *Pavlova* serem atualmente comercializadas a nível global, a produção em larga escala de microalgas ainda não é uma prática generalizada. Uma ampla implementação à escala industrial requer melhorias de forma a reduzir os custos de produção. Para atingir este objetivo, abordagens como (1) aumento da produtividade da biomassa por meio de culturas em condições de heterotrofia ou (2) substituição do meio de cultura sintético por subprodutos agroindustriais, podem ser implementadas. O potencial destas duas estratégias de produção foi avaliado com recurso às seguintes espécies de microalgas marinhas: *Nannochloropsis gaditana*, *Nannochloropsis oceanica*, *Pavlova lutheri*, *Pavlova pinguis* e *Pavlova gyrans*.

Avaliou-se o crescimento heterotrófico das espécies *N. gaditana* e *N. oceanica*, e foi possível determinar que ambas as espécies não apresentam crescimento nestas condições. As microalgas *P. pinguis* e *P. gyrans* cultivadas em fertilizante orgânico obtiveram crescimentos celulares e teor lipídico semelhantes ou significativamente superiores às respetivas culturas controlo (meio de cultura f/2). Verificou-se também que o crescimento celular e o teor lipídico em meio de fertilizante orgânico não estéril são estatisticamente comparáveis aos obtidos com recurso a fertilizante orgânico previamente esterilizado. *P. gyrans* produzidas com fertilizante orgânico

exibiram variação da morfologia das células, resultando em células mais pequenas, alongadas e com motilidade. A utilização de efluentes sintéticos de aquacultura como meios de cultura das espécies *P. pinguis* e *P. gyrans* promoveram um crescimento significativamente inferior ao obtido pelas respetivas culturas controlo (medi de cultura f/2), sugerindo um efeito inibitório de elevadas concentrações de nitrato (16 a 20 mM). O conteúdo lipídico manteve-se inalterado.

De um modo geral, os resultados obtidos revelaram que a substituição de meios de cultura sintéticos por subprodutos agroindustriais é uma abordagem viável para reduzir os custos de produção de espécies de *Pavlova* à escala industrial, contribuindo simultaneamente para uma economia mais sustentável e obtenção de biomassa de microalgas certificada como orgânica.

Palavras chave: Conteúdo lipídico; Efluentes sintéticos de aquacultura; Fertilizante orgânico; Heterotrofia; *Nannochloropsis*; *Pavlova*; Subprodutos agroindustriais; Variação da morfologia das células.

Use of agro-industrial by-products for *Pavlova* spp. culture and heterotrophic growth of *Nannochloropsis* spp. as relevant production strategies for oleaginous microalgae

Abstract

The polyunsaturated essential fatty acids are divided into two relevant groups in human nutrition, omega-6 (n-6 PUFAs) and omega-3 (n-3 PUFAs) and carry on several important roles in the human body. However, several factors resulted in a consumption of n-6 PUFAs approximately 20 times greater than the consumption of n-3 PUFAs, showing a lack of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the dietary requirements. Both come from marine fish, such as salmon and mackerel, or from fish oils. As result of the increasing demand for food associated with a growing global population, these sources are not sufficient to answer the requirements for EPA and DHA, so it became necessary to explore alternative sources such as microalgae. The marine microalgae of the genera *Nannochloropsis* and *Pavlova* are important producers of EPA and DHA, since both exhibited the highest content of these n-3 PUFAS per dry weight biomass.

Despite *Nannochloropsis* and *Pavlova* being currently used on the global food and feed market, the large-scale production of microalgae is not a widespread practice yet. The efficient use and wide implementation at an industrial scale requires improvements to reduce production costs. The main challenges to overcome are related with high nutrient supply and low production efficiency of biomass and high value-added metabolites. The potential of use agro-industrial by products and heterotrophic conditions as production strategies to reduce production costs and enhance microalgal biomass productivity of marine microalgae was evaluated with the following strains: *Nannochloropsis gaditana*, *Nannochloropsis oceanica*, *Pavlova lutheri*, *Pavlova pinguis* and *Pavlova gyrans*.

The heterotrophic growth was evaluated with *N. gaditana* and *N. oceanica*. It was determined that both *Nannochloropsis* strains are not able to grow in heterotrophic conditions. *P. pinguis* and *P. gyrans* achieved similar or significant cell growth and lipid content in the medium composed of organic fertilizer than in synthetic control medium (f/2 medium). Cell growth and lipid content in the non-sterilized organic fertilizer medium were also found to be comparable to those in sterilized organic fertilizer media. *P. gyrans* cultured in organic fertilizer media suffered an interesting cell morphology variation, resulting in small, more elongated and motile cells. The use of synthetic aquaculture effluents as a culture medium to *P. pinguis* and *P. gyrans* cultivation,

promoted an opposite trend, as lower cell growth was determined for both of these strains, suggesting the inhibitory impact of high nitrate concentrations (16 to 20 mM). The lipid content was unaltered.

Overall, our results demonstrate that the replacement of synthetic culture medium by agro-industrial by-products is a viable approach to reduce production costs of *Pavlova* strains at industrial scale, while also contributing to a more sustainable economy and to produce organic microalgal biomass.

Keywords: Agro-industrial by-products; Heterotrophy; Lipid content; Morphological cell change; *Nannochloropsis*; Organic fertilizer; *Pavlova*; Synthetic aquaculture effluents.

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List of Abbreviations

AD - Anaerobic Digestion

ALA - α-Linolenic Acid

ARA - Arachidonic Acid

CFU - Colony Forming Unit

COD - Chemical Oxygen Demand

DHA - Docosahexaenoic Acid

EFSA - European Food Safety Authority

EMP - Embden-Meyerhof Pathway

EPA - Eicosapentaenoic Acid

FAO - Food and Agriculture Organization

GLA - γ-Linolenic Acid

LA - Linoleic Acid

metE - vitamin B_{12}-independent methionine synthase genes

metH - vitamin B_{12}-dependent methionine synthase

MUFAs - Monounsaturated Fatty Acids

n-3 PUFAs - Omega 3 Polyunsaturated Fatty Acids

n-6 PUFAs - Omega 6 Polyunsaturated Fatty Acids

OD - Optic Density

OF - Organic Fertilizer

PBR - Photobioreactor

PIE - Phosphate Intermedian Effluent

PLE - Phosphate Limited Effluent

PPP - Pentose Phosphate Pathway

PPP - Pentose Phosphate Pathway

PRE - Phosphate Rich Effluent

PUFAs - Polyunsaturated Fatty Acids

S/V - Surface-to-volume ratio

SFAs - Saturated Fatty Acids;

TAG - Triacylglycerol

TCA - Tricarboxylic Acid Cycle

1. Introduction

Long-chain polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), show very relevant functions in human metabolism, both being derivatives of marine fish like salmon or from fish oils. However, due to several factors, these sources aren't enough to respond to the growing needs for pure EPA and DHA, and it is necessary to explore alternative sources, such as microalgae. Currently, microalgae-based molecules have been recognized for their market potential but when comparing the production cost for microalgae with other biomass sources, it becomes clear that microalgae are not a cheap source of biomass. This highlights the necessity to optimize productivity and decrease the production costs of microalgae to achieve a more sustainability process.

To optimize the productivity of these microorganisms and its PUFAs content it is necessary to understand several variables, namely cultivation conditions (open or closed systems), growth conditions (autotrophy, mixotrophy or heterotrophy), ratio between nutrients (carbon, nitrogen, phosphorus), temperature, and pH, taking also into consideration the microalgae species. The use of industrial effluents or by-products as a substitutes of culture medium for the growth of microalgae can also be an alternative way to reduce the production costs and contributes to a sustainable food and feed production.

1.1 Essential fatty acids on global-scale

Fatty acids are the major components of triacylglycerols, phospholipids and other complex lipids (cholesterol esters[1], sphingolipids[2], etc.). They are widely dispersed in nature and have a great impact on various cellular properties resulting in altered metabolism, gene expression, hormonal response and bioactive compounds production pathways. They can be classified as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and most of them are provided to the body through diet but can also be synthesized in the human body by other fatty acids or non-lipid precursors such as glucose. Although, there are exceptions – essential fatty acids – that comes exclusively from food[1,3].

All the fats, including SFA, are important to metabolism and other body functions. We must emphasize the importance of essential fatty acids because despite being crucial for the proper organism functioning, they are not naturally produced by itself. Part of these fatty acids are PUFAs and these are divided into two groups: omega-6 (n-6 PUFAs) that includes linoleic acid, arachidonic

acid and γ-linolenic acid and omega-3 (n-3 PUFAs) that includes α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid[3,4] **(Table 1)**.

The European Food Safety Authority (EFSA) has approved health claims on omega-3 fatty acids[5]. They are incorporated in various body parts, namely cell membranes[6] where they play important roles related to flexibility and anti-inflammatory processes of cells[7]. They are essential to the appropriate fetal development and healthy growth[8] and act as precursors of several metabolites (resolvins and protectins)[9], which are considered potent beneficial lipid mediators in prevention or treatment of several diseases[9] such as Alzheimer's and cardiovascular diseases[10]. The DHA is also a key component of all cell membranes and it's abundantly present in the brain and retina[11].

Table 1: Major food sources of the n-3 and n-6 PUFAs.

PUFAs	Name	Abbreviation	Structure	Food Source
Omega-3	α-linolenic acid	ALA	18:3n-3	Walnuts, flaxseed oil and soybean
	Eicosapentaenoic acid	EPA	20:5n-3	Fatty fish (salmon, mackerel, tuna) and fish oils
	Docosahexaenoic acid	DHA	22:6n-3	Fatty fish (salmon, mackerel, tuna), fish oils and algal oils
Omega-6	Linoleic acid	LA	18:2n-6	Corn, safflower, soybean, cottonseed and sunflower oils
	Arachidonic acid	ARA	20:4n-6	Meat, poultry and eggs
	γ-linolenic acid	GLA	18:3n-6	Evening primrose oil, borage oil and black current seed oil

Adapted from International Food Information Council Foundation[12].

In European countries the mean intake of these fatty acids varies according to sex, age group, and dietary habits. Dietary recommendations for EPA and DHA for European adults and children are between 250 and 500 mg/day. However, according to EFSA, children aged between 3 to 13 years, adolescents and adults from both sex, from several countries consume low amounts of omega-3 (between 25 to 100 mg/day)[5].

The lack of n-3 PUFAs, especially EPA and DHA, is a consequence of the following factors:

- **modern large-scale production methods** that reduce the n-3 PUFAs content of vegetables, eggs, meat and fish[13], for example by changing the diet given to livestock, poultry and fish to a different diet that they would consume in nature (e.g. livestock fed with grains instead of grass, which is rich in n-3 and n-6 PUFAs)[14];
- **food processing or food confection,** which may result in a decrease of essential fatty acids or convert them into unhealthy trans-fatty acids;

- **vegetable oils consumption** many of which have higher n-6 PUFAs content. High levels of these fatty acids decrease the conversion of ALA to EPA and DHA[15].

Globalization brought some dilemmas that society has to deal with. The population growth increases the consumption rates of meat and livestock products[16], the intensification of industrialization and reduction of resources and the deficient utilization of nutrients for food and feed production. This reality has major impacts on the environment, which have been already observed[17-19]. Furthermore, the decline of available irrigation water and arable land, along with rising global temperatures that undercut farming systems are a threat for feed and food security[20].

Increased global demand and competition, coupled with reductions in supply as a consequence of climate change, led to price increases and low availability of n-3 PUFAs sources[21]. Nowadays, as a consequence, more than 15 % (and increasing) of the people around the globe suffers from highly deficient calorie intake, and an even bigger percentage of the population experiences micronutrient dietary deficiency[22].

1.2. Marine fish – an important source of EPA and DHA

In general, freshwater fish require LA (n-6 PUFA) or ALA (n-3 PUFA) or both, whereas marine fish require EPA (n-3 PUFA) and/or DHA (n-3 PUFA)[23]. Indeed, marine fish like sardines, mackerel, anchovies, and some salmon species are rich in EPA and DHA, yet they cannot synthesize these fatty acids. EPA and DHA are obtained by marine fish via the consumption of algae and planktons (primary food sources)[24]. As a means to supply the demand for food from a growing global population and to battle the consequences of an intensive wild fishery, aquaculture is going through a phase of fast development[25,26].

According to Food and Agriculture Organization of the United Nations (FAO), in 2016 human consumption of fish from aquaculture provides 47 % (80,03 million tonnes) of the global fish production and it is growing **(Fig. 1)**. It is expected that in the nearly future, aquaculture will become the main industry providing aquatic products[27].

Intensification of aquaculture practice led to serious concerns about environmental pollution as a consequence of aquaculture effluents[26,28] and overfishing of wild fish stocks to be used as fish-feed (in form of fishmeal and fish oil, processing or other waste and whole fish)[27,29].

At last, the presence of pollutants such as heavy metals (e.g. mercury)[30,31] in fish oil can be harmful to consumers and the smell makes it unattractive[32]. Moreover, fish oil is not suitable for vegetarians, which is promoting research on new products, creating new market opportunities.

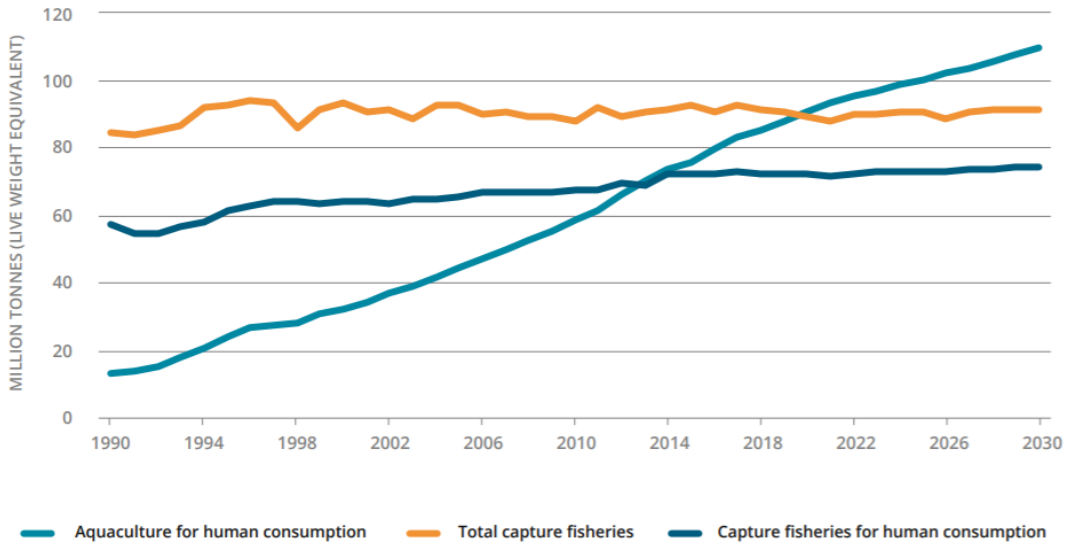


Fig. 1: Global capture fisheries and aquaculture production, 1990–2030 (FAO 2018).

In summary, the need of EPA and DHA consumption, their low availability in food and depletion of fish resources have raised the research of alternative sources of these fatty acids such as bacteria, fungi, microalgae and genetically modified plants[33-36].

1.3. Alternative EPA and DHA sources

There is a variety of alternative EPA and DHA sources such as plants, bacteria, fungi and microalgae that are currently being explored for commercial production. Oil production from oleaginous plants present numerous advantages but they need arable land, have longer growth times and their metabolism don't have the enzymes required for producing EPA and DHA unless they had been genetically modified[36]. The cultivation of transgenic crops in open ecosystems brought public concerns. These, together with regulatory issues, restrict the large-scale production of genetically modified crops[37].

In opposite, the mentioned microorganisms are natural oil producers and its PUFAs composition is similar to those obtained from fish[38,39]. Currently bacteria, fungi and microalgae can accumulate between 10 to 60 % of total lipids as EPA and DHA, depending on the microorganism[40],

instead of fish or fish oil that can contain only 20-30 % of these PUFAs[32]. Microorganisms also have high growth rates, low nutrient requirement, easily controllable culture conditions[41] and a significant amount of natural antioxidants, such as carotenoids and tocopherols. These compounds play an important role in protecting n-3 PUFAs from oxidation and thus raise their storage stability[42,43]. All this information highlights bacteria, fungi and microalgae as alternative sources of EPA and DHA.

Employment of bacterial and fungal systems for n-3 PUFAs production allows the use of several types of bioreactors and it is a profitable process with operations and manutention already very usually performed in other types of biotechnology-based industry (baker's yeast production, beer, yoghurt, among others), resulting in a significant reduction of production costs[44,45]. However, microalgae-based technologies appear more promising, since they are not limited either by low lipid production (bacterial systems) or by long growth periods (fungal systems)[32,40].

Microalgae not only have proved to be the best primary producers of EPA and DHA - presenting superior productivities – but its use has also eliminated some problems normally related to fish oils such as stability[32]. When microalgae are grown under controlled conditions, the composition of the fatty acids shows no seasonal variation[46] and in contrast to terrestrial crops, microalgae can grow in aquatic environments such as the sea, freshwater, and marshlands. They can also be cultivated in wastewater and brackish water, so they avoid competition with agricultural land, utilization of non-organic fertilizers, and habitat destruction[40,47,48]. Unlike fish oil, microalgal n-3 PUFAs extracts are less susceptible to be contaminated by heavy metals[30], since microalgae are the initial EPA and DHA producers in the marine food chain, and their consumption decreases cholesterol levels[49-51].

Microalgae are gaining increasing attention due to their important nutritional value for human health as well as for aquaculture feeding.

1.4. Microalgae

There are around 100,000 different algae species with widely varied characteristics[52]. Microalgae are unicellular or multicellular microorganisms, prokaryotes (cyanobacteria) or eukaryotes (green and red algae and diatoms), which can grow autotrophically using solar energy and CO_2[53]. Many species can also grow heterotrophically in several sources of organic carbon or mixotrophically[54]. They are excellent sources of proteins, carbohydrates, lipids and vitamins[33,55] with many applications in the pharmaceutical and cosmetic industry (antioxidants, pigments, PUFAs),

agriculture (food supplements, dyes) and animal feed (aquaculture, poultry or pig farming). Additionally, they are promising organisms for green chemistry (bioplastics), phytoremediation (wastewater treatment, CO_2 mitigation) and energy production (biodiesel, bioethanol, hydrogen)[52,56,57].

As it was previously stated, adding to the synthesis of several products by microalgae, characteristics like high growth rate, regardless of the time of the year in non-arable land without needing potable water, high photosynthetic efficiency, high lipid production and adaptability to different cultivation conditions contribute to their wide interests and advantages[48].

1.4.1. Growth regimes

In autotrophic growth, the cells capture solar energy and use carbon dioxide as carbon source[58]. Currently, this is the most common growth regime for microalgae cultures, and these can growth in natural or artificially illuminated environments. In terms of production, this regime represents a simple and easy approach for microalgae culture, it results in the synthesis of photosynthetic based-products (e.g. chlorophylls and carotenoids) and it intervenes in CO_2 mitigation of the environment. There are some disadvantages related to this regime: (1) Low light diffusion; (2) Growth dependent on local weather conditions or it is required high investment in infrastructures that allow continuous illumination; (3) Harvest is hard, costly and sometimes it is limited by low cell density values, which results in limited food and pharmaceutical products[59,60].

As an alternative to autotrophic cultures, some microalgae can grow under heterotrophic conditions[61,62], in other words, they can grow in the absence of light and replacing the atmospheric carbon dioxide fixation by dissolved carbon sources in the culture medium. The autotrophic culture medium composition is similar to the heterotrophic culture medium, excepted that in the latter an organic carbon source is added[63].

Heterotrophic growth eliminates the two main disadvantages of autotrophic bioreactors, since that: (1) It allows the use of several types of bioreactors, and consequently, occurs a significant reduction of production costs[45]; (2) It is a profitable process with well-known operations and manutention, usually performed in other types of biotechnology-based industry[44]; (3) It promotes higher biomass density and greater lipid content than in autotrophic growth; (4) Consistent and reproducible biomass productivity[45,64-67].

However, this growth regime has also limitations, but some of which can be easily overcome: (1) Only some microalgae can grow under heterotrophic conditions; (2) There is an increase in costs related to organic carbon source; (3) Contamination and competition with other microorganisms – aseptic conditions and sterile bioreactors, materials (pH, temperature and oxygen probes, and others) and culture medium are required; (4) There is no light-induced metabolites production[66].

The mixotrophic growth is an intermediary condition carried on in light, in which CO_2 and organic carbon are consumed simultaneously. Many species of microalgae can adapt their metabolism to autotrophic and heterotrophic conditions, exchanging between these two growth regimes[68]. The mixotrophic regime is considered a good strategy to obtain high amounts of biomass, high growth rates[69] and photosynthetic metabolites[66].

1.4.2. Industry scale-up of microalgae production systems

Nowadays microalgae are cultivated in a broad range of different cultivation systems placed outdoors or indoors and can be classified as open systems (open ponds) or closed systems (photobioreactors), wherein the most used on a large scale and a commercial basis are open systems. The technology to produce microalgae is still immature. Research and development have been done in recent years, considering the requirement of increasing the production scale with a simultaneous decrease in the production costs, improvement of process strategies and development of new concepts[52].

1.4.2.1. Open systems

Open systems are highly dependent on external factors. They are located at outdoors and are simpler to build and operate. The most common open systems for microalgae production are ponds. These systems are usually between 0.1 and 0.3 m deep, allowing the supply of enough sunlight for photosynthesis. The culture is kept in circulation in a closed channel and the mixing is ensured by a mechanical arm stirring in a circular motion in circular ponds, or more commonly by a paddle wheel in so-called raceway ponds, which also prevent sedimentation **(Fig. 2A).** CO_2 or flue gases containing CO_2 can be sprayed in the culture[52,70,71].

Major weaknesses of open systems are that there is almost no possibility for temperature control, can occurs water loss by evaporation and they are also very susceptible to contamination

by other microalgae strains or undesired species, such as bacteria, fungi or protozoa, leading to a reduction in the productivity and variability in the biomass composition. However, they are systems with low capital and operational cost that represent a high advantage given its industrial implementation[52,72,73].

1.4.2.2. Closed systems

There is a large variety of closed systems used for microalgae production. Tubular systems placed either vertically **(Fig. 2D)** or horizontally **(Fig. 2E)**, polyethylene sleeves or bags **(Fig. 2C)** and flat panels **(Fig. 2B)** are the most commonly used. New insights contribute to making new designs like biodomes and even floating bags on ocean waters, nevertheless, accidents or careless handling may breach the containment of these systems. Closed systems are typically referred to as photobioreactors (PBRs). They can be placed outdoors or inside greenhouses to allow more controlled conditions[52,60,72].

PBRs are suitable for the production of high-value products for Food and Pharmaceutical industries since closed systems can prevent or minimize contamination, offer better control over cultivation conditions and produce higher cell concentration and therefore higher volumetric productivity. At the same time, they prevent evaporation and reduce water use and CO_2 issues due to outgassing, but the installation and operation costs of PBRs are higher than those of open pond systems **(Table 3)**[52,72,73].

Other type of closed system bioreactor, named fermenter-type reactor is more suitable for heterotrophic metabolism because the surface-to-volume (S/V) ratio (i.e. the ratio between the illuminated surface of the reactor and its volume) is quite low, therefore, the efficiency of sunlight capture is poor, which impairs autotrophic growth[72]. This type of reactor allows high control of all factors that directly interfere with microalgae growth, so it was able to reach higher biomass concentrations in little time and produces higher value products[60]. However, the much higher costs of fermenter-type reactors, when compared to the previously mentioned ones, restrains the application of these reactors at an industrial scale.

Another approach at the industrial scale is the sequential use of heterotrophic and autotrophic culture conditions. In the first stage, the inoculum is produced in a fermenter-type reactor, in heterotrophic conditions, and then it is transported to PBRs, where microalgae will grow in autotrophic conditions. This two-stage system demands lower production costs, as it allows to

increase the inoculum concentration, to reduce the autotrophic scale-up stages and to shorten the process of production of light-induced metabolites producing strains[52,60,72-74].

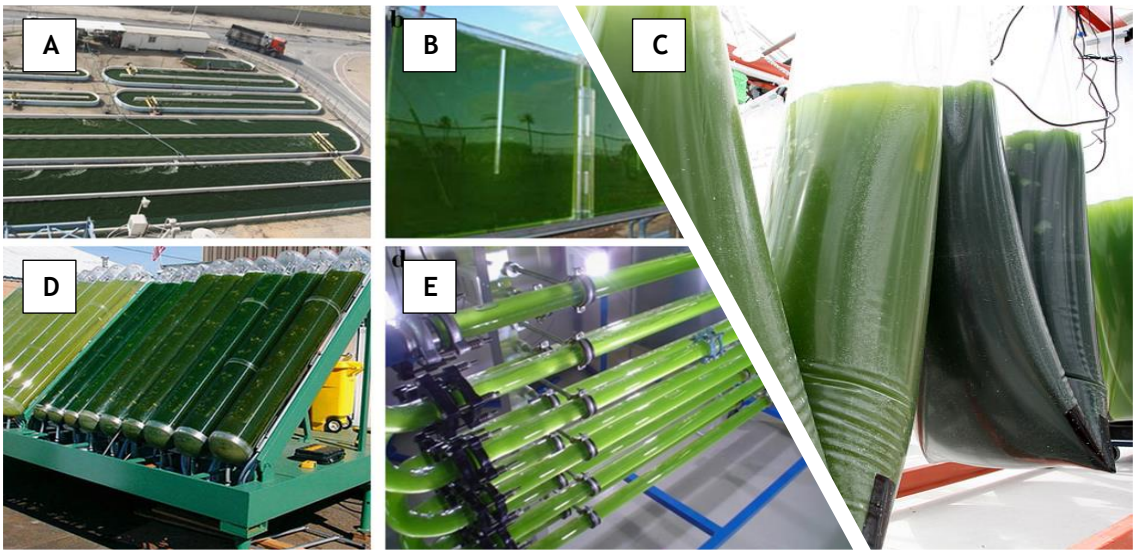


Fig. 2: Open and closed systems to microalgae culture. A - Open pond system; B - Flat panel system; C - Bag-type photobioreactor; D - Vertical tubular system; E - Horizontal tubular system.

1.4.3. Economic overview of microalgae based-products

Similarly, to n-3 PUFAs some microalgae-based molecules have specific advantages over their conventional alternatives, which make their use commercially viable. For instance, synthetic molecules are only available in specific isomers, which are frequently much less effective than natural variants for certain applications, such as in infant formulas, fish pigment enhancers or dietary supplements[75,76]. Unfortunately, these molecules are less competitive than standard synthetic and traditional alternatives **(Table 2 and 3)**, mainly because traditional/synthetic alternatives are lucrative processes with huge know-how in terms of operations.

The current market of microalgae biomass is approximately 20,000 tonnes/year of dry matter[77], with an expected yearly growth of 10 %[78], and the price ranges between 100 and 465 €/kg for human consumption (food market) and 5 to 20 €/kg for animal and fish feed (feed market)[52,76].

Table 2: High-value molecules produced with microalgae used in the food and feed market.

High-value component		Microalgae source	Traditional/synthetic alternatives
Pigments	β-carotene	*Dunaliella salina*	Synthetic form (but only trans-isomers, cis-isomers only come from natural sources)
	Astaxanthin	*Haematococcus pluvialis*	Synthetic form *Phaffia* yeast extract
	Phycocyanin	*Arthrospira* sp. *Porphyridium* sp.	Synthetic pigments
Fatty acids	EPA	*Nannochloropsis* sp. *Phaeodactylum* sp. *Nitzschia* sp.	Fish oils
	DHA	*Schizochrytium* sp. *Cryptocodinium* sp.	

Adapted from Enzing *et al.*[52]

The prices of microalgae based-products, such as PUFAs and astaxanthin, for food market are higher than their alternatives, which are available for 0.04 and 220 €/kg, at least. In the other hand, in feed market, microalgae are more competitive since they are cheaper than fishmeal or fish oil (55 – 500 €/kg). Only soybean, which is, in the EU, the major vegetable feed for animal consumption[79], is available at a lower price, 0.27 €/kg.

Table 3: Economic overview of food and feed markets and alternatives for microalgae products.

	Microalgae product	Value (€/kg)	Alternatives	Value (€/kg)
Food market	Whole microalgae biomass	120	Heterotrophic microorganisms	45
	Astaxanthin	100 – 465	Synthetic/ natural	220 – 2,200
	PUFA	100 – 1,235	Fish-PUFA	0.04
Feed market	Aquaculture	5 – 20	Fishmeal/ fish oil	55 – 500
	Livestock feed		Soybean	0.27
	Feed additives		Botanicals, antibiotics etc	-

- data not found.
Enzing *et al.*[52], van der Voort *et al.*[73] and Sathasivam *et al.*[76]

The optimization of microalgae production systems is crucial to decrease the production costs as a means to turn microalgae into a more inexpensive source, when compared to their substitute products or, at least in a more sustainable source in terms of cost/benefit ratio.

Discussion about better production system is an ongoing concern since none of the systems seems to completely surpass each other **(Table 4)**. Open ponds are simple and, in some cases, require half of the initial investment than closed systems like horizontal tubular PBR[52], but instead, these open systems have lower biomass productivities, due to noncontrolled conditions

and more exposure to contaminations[74]. Besides the fact that closed systems need greater investment, they are recommended for the cultivation of species suitable for human consumption. Undeniably, the production of high-value compounds obligates to strict control of culture variables in pursuit of satisfying the good manufacturing practice requirement for human feed or pharmaceutical products. As a matter of fact, most of the species cultivated for oil production require strict control of the temperature between 20 and 30°C, which is problematical to maintain in open ponds[74,77].

Several studies showed that closed systems have more potential for improvement, being that flat panel PBR is the most convenient system in terms of production costs (3.4 - 5.96 €/kg)[52,74]. These costs cannot compete with traditional/synthetic alternatives, for this reason, it is necessary (1) the improvement on PBR design as an approach to achieve higher light conversion efficiency beyond the one currently attained[77] or (2) the development of new strategies for a more sustainable production, such the employment of agro-industrial by-products as cheap substrates[79-81]. These approaches seem to be the main way to develop cost-effective tubular PBR, about 1.80 €/kg.

Table 4: Production costs of microalgal systems in three main types of production technologies.

		Microalgae production costs (€/kg)				
		Capital costs	**Labour**	**Other variable costs (utilities, culture medium)**	**Total costs for a large plant (100 to 200 ha)**	**Optimal theoretical total costs**
Production technology	**Open ponds**	3.02	0.15	1.57	4.95 - 22.53	0.21 – 0.68
	Horizontal tubular PBR	2.74 - 9.80	0.36 - 0.88	0.92 – 1.65	4.15 – 12.60	-
	Flat panel PBR	2.01	0.35	1.01	3.40 - 5.96	1.80

PBR – Photobioreactor; - data not found.
Adapted from Enzing *et al.*[52] and Ruiz *et al.*[74]

1.5. EPA and DHA microalgae producers

Microalgae of the genus *Chrypthecodinium*, *Nannochloropsis*, *Nitzschia*, *Odontella*, *Schizochrytium*, *Ulkenia* (food market), *Isochrysis*, *Nannochloropsis*, *Pavlova* and *Phaeodactylum* (feed market) are currently on the global market, as the main producers of EPA and DHA[52].

Lipids production is closely linked to microalgal growth stages, functioning as an energy stock and contributing to their adaptation and survival in different or unfavourable environmental conditions, due to its high energy content and flow properties, which are essential for cellular functions[40,82,83]. Marine microalgal strains produce a high percentage of total lipids (up to 30–70 % of dry weight), of which 10 to 30 % are PUFAs[53,84,85].

Omega-3 PUFAs are mostly accumulated in glycolipids and phospholipids, cell membrane constituents, while saturated (SFA) and monounsaturated (MUFA) fatty acids are mainly accumulated in triacylglycerols (TAG)[86-90]. Higher n-3 PUFAs content is observed during the early stationary phase and decrease throughout the stationary phase. However, lipid synthesis and fatty acid profiles are frequently affected by nutrient availability (nitrogen deprivation, phosphate concentration), pH, temperature, carbon supplies and salinity, and indeed, these approaches generally lead to an end of cell growth and promote accumulation of storage lipids, i.e. TAGs, rather than glycolipids or phospholipids, decreasing the percentage of EPA and DHA relative to total fatty acids present in biomass[54,91-95].

Despite these variables lead to lower amounts of referred fatty acids in many species, it has been reported to occur PUFAs partitioning to TAG in a few species, such as *Nannochloropsis oculata*, *Parietochloris incisa*, *Pavlova lutheri*, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* [96,97]. The ability to concentrate n-3 PUFAs in the form of TAGs offer much higher stability and higher bioavailability than those present as free fatty acids or as glycolipids or phospholipids[98]. Additionally, TAGs are easier to isolate from the rest of the cell content since many microalgae can produce amounts of TAGs between 20 % and 50 % of dry cell weight, under stress conditions[99,100]. The pathway by which n-3 PUFAS, such as EPA and DHA, are incorporated into TAG is not fully understood but it seems to be species-specific and growth phase dependent[96,101,102].

This data is described in several studies that contributed to the characterization of EPA and DHA producers currently on market, not even at EPA and DHA content but also at biomass and lipid yields **(Table 5)**. The genus *Nannochloropsis* and *Pavlova* have the highest content of EPA and DHA (% per dry weight biomass) however, the EPA and DHA yield of both genera are lower than the others, as a consequence of its lower biomass productivity. It should be noted that n-3 PUFAs yields are calculated by the ratio between biomass production and EPA and DHA content[103]. *Pavlova* can reach at 33.22 % and 10.50 % of EPA and DHA content, respectively, but its biomass productivity ranges between 0.01 and 0.28 g/L/day. *Nannochloropsis* is more appreciated due to

EPA content, that can reach 8.2 %, but similarly to *Pavlova*, it has low biomass productivity, between 0.14 and 1.43 g/L/day, when compared to other microalgae species **(Table 5)**.

The data mentioned suggests that both microalgae could be the main producers of these n-3 PUFAs if its biomass productivity was enhanced. To achieve this aim, approaches such (1) increase biomass productivity through a hetero-photoautotrophic two-stage cultivation process[52,60,72-74,104,105] (see **Section 1.6**) or, as mentioned before, (2) substitute synthetic culture medium to agro-industrial by-products, in order to reduce production costs, could be applied (see **Section 1.7**).

Table 5: Characterization of microalgae producers of EPA and DHA, currently on food and feed market: DHA, EPA and lipid content, lipids and biomass productivity. Note that the value ranges were obtained by a literature review, with different conditions and regime cultures. A – Autotrophic, M – Mixotrophic, Heterotrophic.

Microalgae	n-3 PUFAs				Lipid content	Lipid productivity	Biomass productivity	Growth regime	Production scale
	EPA %	DHA %	EPA	DHA					
	% dry weight biomass		mg/L		% dry weight biomass	mg/L/day	g/L/day		
***Crypthecodinium* sp.** [53,61,106-111]	0	0.8 - 1.3	0	15.0 – 193.8	20.0 - 51.1	210.0 – 790.0	5.10 - 10.0	A | M | H	250 ml Flask; 5 L Fermenter;
***Isochrysis* sp.** [53,61,112-117]	0 – 0.9	0.2 – 4.7	0 – 8.0	1.7 – 40.0	7.0 – 40.0	37.7 – 180.0	0.04 – 1.6	A | M	250 ml Flask; 100 L Airlift; Turbidostat; PBR; 15 L Carboy;
***Nannochloropsis* sp.** [45,53,61,85,88,116,118-120]	0.6 – 8.2	0 – 0.7	2.0 – 14.0	0 – 2.6	12.0 – 53.0	37.6 – 142.0	0.14 – 1.43	A | M	250 ml Flask; PBR; Turbidostat; Raceway pond;
Nitzschia sp. [53,121-124]	1.1 – 12.6	-	118.07 – 721.9	-	16.0 – 47.0	-	1.03 – 6.75	A | M | H	250 ml Flask; 500 mL Flask; 2.4 L Fermenter; 3.7 L Fermenter;
***Odontella* sp.** [125-128]	0.78 – 3.16	-	-	-	8.0 – 19.7	-	0.4 – 0.64	A	400 mL Cylindrical glass column; 1.2 L Cylindrical glass column;
***Pavlova* sp.** [53,61,116,117,129-133]	0.2 – 12.1	0.3 – 10.5	0.6 – 7.5	0.4 – 2.9	21.47 – 36.0	40.2 – 50.2	0.01 – 0.28	A | M	250 ml Flask; PBR; 2 L Glass flask; 2 L Fermenter; 1 L Flask; 5 L Plastic container; 30 and 300 L Spherical tanks;
***Phaeodactylum* sp.** [53,61,95,116,134-140]	0.2 – 5.5	0.06 – 0.4	1.3 – 130.0	0 – 11.0	18.0 – 57.0	44.8	0.19 – 1.9	A | M | H	250 ml Flask; PBR;
***Schizochytrium* sp.** [61,91,141,142]	1.0 – 1.2	0 – 0.3	6.1 – 9.3	0 – 1.0	-	-	0.02	A | M | H	250 ml Flask;
***Ulkenia* sp.** [143]	5.0*	13.7*	-	-	-	-	-	A	-

- data not found.
* % total fatty acids.

1.5.1. *Nannochloropsis gaditana* and *Nannochloropsis oceanica*

Considering the EPA and DHA content, the species *Nannochloropsis gaditana* and *Nannochloropsis oceanica* have stood out from the others, mainly due to the EPA content (**Table 5**). These species contain values around 4.3 and 4.4 – 5.5 % of dry weight[88] of this PUFA, respectively.

Nannochloropsis is a genus of eukaryotic, unicellular and non-motile marine microalgae belonging to the family Eustigmataceae. It was initially classified by Hibberd[144] and comprises six species: *Nannochloropsis gaditana*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis oceanica*, *Nannochloropsis oculata* and *Nannochloropsis salina*[89]. They exhibit a simple spherical or oval cell, with diameters ranging from 2 to 5 μm[145] and plant-like plastids. These microalgae have a single chloroplast[68] and a lipid droplet, that acts as an energy reservoir, and it can increase in size under stress conditions[146]. They reproduce asexually, i.e., microalgae are divided and originate two daughter cells[147]. *Nannochloropsis* can be found mainly in salt water[148] and this genus grow mostly under autotrophic[33,61,93,118,119,149,150] and mixotrophic conditions[93,118,119,149], however there are indications that evidence the possibility of heterotrophic growth[45,119,120].

Microalgae of the genus *Nannochloropsis* differ from another genus due to the presence of chlorophyll a[151]. The characterization/differentiation of each the six species is only achieved using sequence analyzes of the *rbc*L gene and the 18s rRNA. These analyzes also allowed deducing the phylogenetic proximity of these species[152,153].

There are some studies that infer the possibility of *Nannochloropsis* to be grown in heterotrophic conditions[45,119,120], although significant growth has not been achieved yet. This potential approach could greatly increase the economic value of this genus. As it is known *Nannochloropsis* can grow under photoautotrophic conditions, but large-scale production of EPA will be hindered by its inability to obtain high cell densities in photobioreactors due to light limitation.

As possible solution, the hetero-photoautotrophic two-stage cultivation process will occur in two stages: Stage 1 - Heterotrophic growth eliminates the requirement for light and offers the potential of enhancing inoculum concentration; Stage 2 – Photoautotrophic growth initiate at higher inoculum concentration and thus this will result in higher biomass concentration and productivity at the end of the culture.

1.5.2. *Pavlova lutheri*, *Pavlova gyrans* and *Pavlova pinguis*

Pavlova is a genus of eukaryotic, unicellular and motile marine microalgae belonging to the family Pavlovaceae[154,155]. Butcher isolated and described the first species of the genus *Pavlova* in 1952, *Pavlova gyrans* [156]. Since then, only eight species of Pavlova have been described: *Pavlova ennorea*, *Pavlova granifera*, *Pavlova gyrans*, *Pavlova lutheri*, *Pavlova noctivaga*, *Pavlova pinguis*, *Pavlova virescens* and *Pavlova viridis*. They inhabit littoral, brackish water and sometimes freshwater environments[154] and can grow under autotrophic and mixotrophic conditions[61,116,117,129-133].

These microalgae cells are spherical, solitary (high metabolism)[156] or may form non-motile aggregations, contain chlorophyll a + c and they are characterized by the presence of two golden-brown or yellow-green chloroplasts, organic body scales and a unique filamentous appendage, called haptonema[154]. *P. gyrans* and *P. pinguis* shown asexual reproduction, that it takes place by cell division in either the motile or non-motile phase, while *P. lutheri*, which also shown asexual reproduction, only shown cell division on non-motile phase[157].

The flagellar arrangement arises sub-anteriorly on the ventral side and it is composed by two unequal and heterodynamic flagella, responsible for characteristic swimming movement, and the haptonema, a third appendage used for attachment and food handling. Pavlovophyceae is the only Class in the Phylum Haptophyta, where the two flagella are markedly different in length (posterior flagellum is shorter than anterior flagellum) and the anterior one is covered by knob scales and fine hairs. Haptonema has a variety of lengths and is located between the flagella, moving itself to aggregates nutrient particles and move them to the surface of the cell, where they are phagocytized into a food vacuole[155]. Regardless of these common characteristics between different *Pavlova* species, it was observed characteristics that distinguish them, such as plastids, motile and non-motile stage, cell size and form and capable of pseudopodia **(Table 6)**[154,156-158].

With the exception of *P. lutheri*, there are not many studies about *Pavlova* species. Although *P. lutheri* and *P. gyrans* have been in use as feedstocks in aquaculture for a long time[89,159-161], due to its composition and similar characteristics, like a fragile cell wall composed by small cellulose scales (knob sales)[162]. The cell wall hardness of microalgae is related to nutrient digestibility in fish or other animal studies. Studies have shown that lower cell wall hardness can increase the *in vivo* accessibility and the digestibly of nutrients and natural colorants[163-165].

Recently, Bendif *et al.*, throughout phylogenetic analyses, concluded that *P. gyrans* is more similar to *P. granifera* and *P. pinguis* than to *P. lutheri*, even so, it was considered transfer *P. lutheri* to *Diacronema* genus[154].

Table 6: Detectable characteristics specific of each *Pavlova* species.

Genus		***Pavlova***		
Species		***gyrans***	***pinguis***	***lutheri***
Motile stage		irregular, elongate, compressed strongly metabolic	irregular, elongate, compressed strongly metabolic	compressed with ventral depression; not metabolic
Eyespot		red-orange, located near the flagellar insertion	red, located near the flagellar insertion	red
Non-motile stage		not recorded	homogeneous mucilage, appendages not abbreviated	not recorded
Reproduction		asexual by cell division in non-motile and motile stages	asexual by cell division in non-motile and motile stages	asexual by cell division in non-motile stage
Cel size (µm)		3 - 6	3 - 4	5 - 7
Knob scales cover the cell		+	-	+
Pseudopodia		+	+	np
Plastid	**Characteristics**	1 golden-brown	1 golden-brown	1 yellow-green
	Arrangement of thylakoids	parallel	parallel	parallel
	Pyrenoid	posterior-bulging	posterior-bulging	np

np - not present.
Adapted from Bendif *et al.*[154]

1.6. Heterotrophic metabolism

In general, algae, bacteria, fungi, vascular plants and mammals have the same metabolic pathways for cellular respiration (oxygen, organic substrate consumption, CO_2, energy and water production)[166]. The respiration rate for organic substrate consumption ($mol_{O2}.mol_{c}^{-1}.d^{-1}$) is closely related to cell growth and division, as it increases with the growth rate, and it performs two main functions in microalgae: (a) It is the only energy source available for maintenance and biosynthesis in the absence of light and (b) Provides essential carbon compounds for biosynthesis. It is assumed that the regulation of cellular respiration is controlled by the need for products, supplied by its metabolism, such as energy in the form of ATP and NADH and carbon compounds from the organic substrate[167,168]. Therefore, it is possible to affirm that, independently of the organic carbon source provided, higher growth rates are promoted through high aeration values[169].

Nevertheless, the carbon source(s) also influence directly the growth and respiration rates, since the main molecules present in microalgae (proteins, carbohydrates and lipids) have carbon as their main constituent, while oxygen, hydrogen and nitrogen are present in lower amounts.

It should be noted that different carbon sources result in different biomass/substrate ratios and they affect the synthesis of desired products, i.e. large quantities of desired products are not always related to maximum biomass concentrations. One of the examples that corroborate this, is the culture of *Crypthecodinium cohnii* in ethanol, where it can be observed that less biomass concentration produces higher DHA content in a shorter period of time[109], when compared with the culture of this microalgae in acetic acid[170].

Glucose is the most widely used carbon source for microalgae culture in heterotrophic growth, leading to higher growth and respiration rates[169] when compared with other carbon sources (1 mol of glucose has a high energy amount)[171]. Out of all the pathways used by microorganisms to perform aerobic glycolysis (microalgae cannot metabolize glucose under anaerobic conditions, in the absence of light), only two were demonstrated in microalgae: Embden-Meyerhof Pathway (EMP) and Pentose Phosphate Pathway (PPP)[166]. Both pathways occur in the cell´s cytoplasm, however, under heterotrophic conditions, glucose is mainly metabolized by the PPP. It is important to note that in the growth regime mentioned, there are signs that in plant cells the PPP can also occur in chloroplasts.

The heterotrophic growth with glycerol has also been demonstrated in several microalgae, most of them, in species that inhabit high osmolarity places (oceans and salines)[166]. However, the use of this carbon source may have some limitations: (1) The PPP appears to be inhibited when glycerol is the sole carbon source[172]; (2) Some microalgae species can only assimilate glycerol in the presence of light and without an external CO_2 source[173]. Nevertheless, the species *Nannochloropsis* sp., *Rhodomonas reticulate* and *Cyclotella cryptica*, under mixotrophic conditions and when nitrate (source of nitrogen) is added to the medium, seem to prefer glycerol instead of glucose or acetate[174].

Acetate is one of the most common carbon sources for several microorganisms, including microalgae[175], although high concentrations of acetate may be toxic and inhibitors of growth[174]. It can be oxidized through two metabolic pathways: (a) glyoxylate cycle, which leads to the formation of malate in the glyoxysomes (specialized plastids); (b) TCA cycle (occurs in the mitochondria), with consequent formation of citrate, which in turn, provides carbon and energy compounds in the

form of ATP and NADH. Generally, microalgae with the ability to grow with acetate as a carbon source, perform the glyoxylate cycle, which converts acetyl-CoA to carbon compounds[171,176,177].

Other carbon sources, namely sucrose, lactic acid, lactose and ethanol were tested in heterotrophic microalgae cultures, but they have not shown significant results so far at the cell growth and metabolite levels[178-180].

Generally, heterotrophic conditions promote greater accumulation of lipids in the form of TAG[181]. However, some parameters support the EPA and DHA accumulation: (1) Carbon source[109]; (2) Excess of carbon source promotes fatty acids saturation[174]; (3) Nitrogen source[182]; (4) High growth rates[183]; (5) Temperature below the optimum microalgae growth temperature[95].

As previously mentioned, significant values of heterotrophic growth, in particular, of the species *Nannochloropsis oceanica* and *Nannochloropsis gaditana*, have not been reached so far, which can indicate that both species do not present the metabolic pathways necessary to proliferate in this regime. This information is partially contradicted by the comparison of the genomes *Nannochloropsis oceanica* CCMP1779[153], *Nannochloropsis gaditana* CCMP526[184] and *Nannochloropsis gaditana* B-31[185], where it is possible to observe the existence of all the genes necessary to the central metabolism (glycolysis, PPP, gluconeogenesis, TCA cycle and glyoxylate cycle)[153] in both species, being given special attention to the PPP and the glyoxylate cycle - main metabolic pathways in the heterotrophic regime (**Table 7** and **8**).

Table 7: Genes encoding enzymes present in *N. oceanica* and *N. gaditana*, essential for glyoxylate cycle activity, glycerol metabolism and carbon source carrier proteins. *Match* indicates that there is a homologous sequence that is not described yet. - indicates that the sequence of a gene is not present in the microalgae species.

Glyoxylate cycle	*Nannochloropsis*	
	oceanica	*gaditana*
Malate synthase	6945-mRNA-1	Nga03739.01
	11836-mRNA-1	Nga06726 Nga00084
Isocitrate lyase	1574-mRNA-1	Nga01446
	4636-mRNA-1	Nga05520
Alanine-glyoxylate aminotransferase	9944-mRNA-1	Nga01189
	3460-mRNA-1	Nga05986
Glycine decarboxylase	6047-mRNA-1	Nga02832
	5912-mRNA-1	Nga04200.01
	2342-mRNA-1	-
Citrate synthase	10757-mRNA-1	Nga02449
	10051-mRNA-1	Nga02812
	7201-mRNA-1	Nga05548
Aconitase	9186-mRNA-1	Nga05606

Glycerol metabolism	*Nannochloropsis*	
	oceanica	*gaditana*
Glycerol kinase	8404-mRNA-1	Nga01611
Glycerol-3-phosphate dehydrogenase	1750-mRNA-1	Nga06665.1
	Match	Nga01869
	6334-mRNA-1	Nga04914
	464-mRNA-1	Nga05226
	-	Nga30015
Triose phosphate isomerase	3017-mRNA-1	Nga04865
	3017-mRNA-1	
	10440-mRNA-1	Nga01493.01
	5281-mRNA-1	-
Carrier proteins	***Nannochloropsis***	
	oceanica	***gaditana***
Glucose transporters	1694-mRNA-1	Nga07017
	8883-mRNA-1	Nga02209.01
	8483-mRNA-1	-
Glycerol transporters	11191-mRNA-1	Match
	3520-mRNA-1	Nga30544
Acetate transporters	-	-

Table 8: Genes encoding essential proteins for glycolysis metabolism - EMP and PPP - present in *N. oceanica* and *N. gaditana*. *Match* indicates that there is a homologous sequence that is not described yet. - indicates that the sequence of a gene is not present in the microalgae species.

EMP	*Nannochloropsis*	
	oceanica	***gaditana***
Hexokinase	11432-mRNA-1	Nga05398
	11432-mRNA-1	
Glucose-6-phosphate isomerase	11835-mRNA-1	Nga00072
	2243-mRNA-1	Nga05996.1
Phosphofructokinase	6373-mRNA-1	Nga01845
	10476-mRNA-1	Nga06146.1
	5677-mRNA-1	Nga01843
Aldolase	135-mRNA-1	Nga01632 Nga03625
	567-mRNA-1	Nga02263.01
	7930-mRNA-1	Nga06350
Triose phosphate isomerase	3017-mRNA-1	Nga04865
	3017-mRNA-1	
	10440-mRNA-1	Nga01493.01
	5281-mRNA-1	-
Glyceraldehyde-3-phosphate dehydrogenase	2231-mRNA-1	Nga00153
	4900-mRNA-1	
	3236-mRNA-1	Nga21005
Phosphoglycerokinase	9105-mRNA-1	Nga02421.01
	3273-mRNA-1	Nga05531
	3273-mRNA-1	
	4644-mRNA-1	Match
Phosphoglycerate mutase	7187-mRNA-1	Nga05541
	7012-mRNA-1	-
	1942-mRNA-1	Nga06095
	7187-mRNA-1	Nga05541
	4572-mRNA-1	Nga30366
	214-mRNA-1	Nga02519.01
	214-mRNA-1	Nga02519.01
Enolase	7147-mRNA-1	Nga06360
	7147-mRNA-1	
Pyruvate kinase	497-mRNA-1	-
	5759-mRNA-1	Nga02195.01
	10510-mRNA-1	Match
	8741-mRNA-1	Nga00020
Phosphoenolpyruvate carboxylase	3970-mRNA-1	Nga05921
Carbonic anhydrase	11263-mRNA-1	Nga01240
Lactate dehydrogenase (anaerobic EMP)[166]	11349-mRNA-1	Nga00491
	10879-mRNA-1	Nga06921
PPP	***Nannochloropsis oceanica***	***gaditana***
Glucose-6-phosphate dehydrogenase	640-mRNA-1	Nga00833
	2822-mRNA-1	Nga00601.01
6-phosphogluconate dehydrogenase	734-mRNA-1	Nga05184
	6395-mRNA-1	Nga02587
	2147-mRNA-1	Nga02855
	11127-mRNA-1	Nga06144.1
Ribose 5-phosphate isomerase	5917-mRNA-1	Nga04197.01
L-ribulose-5-phosphate 4-epimerase	2948-mRNA-1	Nga03397.1
	5327-mRNA-1	Nga00779.01
Transketolase	6748-mRNA-1	Nga05755
	852-mRNA-1	Nga01090.01
	667-mRNA-1	Nga01757.01
	7418-mRNA-1	Nga01286
	6166-mRNA-1	Match
RuBisco Small Subunit	10920-mRNA-1	Nga03188
RuBisco Large Subunit	6968-mRNA-1	Nga20613
RuBisco Small Subunit	10920-mRNA-1	Nga03188
RuBisco Large Subunit	4877-mRNA-1	Nga00364
RuBisco Large Subunit	11329-mRNA-1	Nga00769
Phosphoglycerate kinase	3273-mRNA-1	Nga05531
	9105-mRNA-1	Nga02421.01
	3273-mRNA-1	Nga05531
	4644-mRNA-1	Match
Glyceraldehyde-3-phosphate dehydrogenase	2231-mRNA-1	Nga00153
	4900-mRNA-1	Nga01745
	3236-mRNA-1	Nga21005
Phosphoribulokinase	11364-mRNA-1	Match
	9418-mRNA-1	Nga01924
Gluconolactonase	1682-mRNA-1	Nga05464
Sedoheptulose-bisphosphatase	2947-mRNA-1	Nga03392
	4791-mRNA-1	Nga04459
		Nga05055

However, none of the gene sequences mentioned in **Tables 7** and **8**, are reviewed by the UniProtKB database, and the homology between the gene sequences of the two microalgae species is, in most of cases, much greater than 50 %, but less than 100 %. Therefore, it remains unclear whether the expression of the mentioned enzymes occurs in both microalgae species.

It should also be noted that the presence of multiple copies of a gene coding for a particular protein indicates that it may be present in multiple cell compartments, whereas a single copy indicates that the protein activity is restricted to a single cell compartment. This aspect is critical, since some enzymes participate in different metabolic pathways that occur in different cellular compartments, such as aconitase – it act in TCA cycle (mitochondria) and in glyoxylate cycle (glyoxysome)[153].

1.7. Agro-industrial by-products as culture medium

World population is increasing, and it is estimated that it will reach about 9.3 billion people by 2050[186]. This growth is followed by an enhance in the demand for natural resources (food, feed, clean water and energy), materials for clothing and infrastructures as well as the demand for education and health services[187]. The current urbanization will almost multiply two-fold the waste generation to 2.6 billion tonnes per year by 2025, due at most, the enormous production of domestic residues and effluents/by-products derived from industrial processes. Traditional disposal techniques, such as landfills and incineration, result in methane and CO_2 and/or come along with a secondary waste generation (dioxins, furans, acid gases and particulates), which are toxic and can cause serious environmental and health hazards[188].

For those reasons, recycling industrial by-products for their sustainable and resourceful conversion in several value-added products is essential. This circular economy represents a significant shift in socio-economic, agricultural, energy, environment and technical systems, once that grow photosynthetic food is the best way to decrease levels of carbon in atmosphere[189,190]. Therefore, microalgae, can be the basis of this economic concept, since it is rich in proteins, carbohydrates, lipids and vitamins, and can be used in many different areas.

Furthermore, the nutrients supplied in form of chemicals are major contributor towards the production costs of microalgae. Utilization of nutrients from agro-industrial effluents could improve the economics of microalgal biomass generation. The great performance of microalgae for nutrients assimilation has been widely observed in the remediation of agricultural and industrial effluents **(Table 9)**. As it was mentioned before, convert nutrients from effluents or by-products to microalgal biomass could decrease the costs of biomass production and simultaneously simplify their treatment processes. The industry effluents and by-products, besides their content in valuable nutrients, may contain also various other organic and/or inorganic compounds that could contaminate the biomass, turning them unsuitable for feed or food production. Such contaminants include pathogens and antibiotics[79,191,192].

Therefore, many countries implemented legislations for agro-industrial effluents, in order to categorize them according to their origin (animal by-products, food waste etc.), which is correlated to their level of health risk, and indicate regulatory approaches to their further usage or disposal. The chemical oxygen demand (COD) and turbidity are also useful parameters for determining the suitableness for effluents support microalgae growth. High turbidity limits

photosynthesis due to a reduction of light penetration into the culture media, which is caused by material in suspension, like organic matter and suspended or dissolved solids (Total solids)[193,194]. Otherwise, COD is applied to quantifying the total amount of oxidisable pollutants (biodegradable and nonbiodegradable and both dissolved and particulate) found in water, measured by the amount of oxygen required for the oxidation of the organic matter[194,195].

In general, agro-industrial effluents have lower chemical risks than other industrial effluents and are potentially available on a large scale[196,197]. The focus is on aquaculture effluents, due to its already intensification in years to come, and on effluents from anaerobic digestion (AD) technology, such as organic fertilizers. AD is widely used to treat part of the agro-industrial effluents.

It is also important to note that it has been frequently reported that non-axenic cultures are more suitable for large scale cultivation on industrial effluents than unicellular cultures[198,199]. In this symbiotic relationship, photosynthetic microalgae release O_2, which is utilized by aerobic bacteria to consume organic and inorganic compounds and in turn, bacteria provide CO_2 and NH_3 as carbon and nitrogen sources, respectively[200-202].Some bacteria also produce vitamin B_{12} and phytohormones, such as indole-3-acetic acid that are growth-promoting agents that play a regulatory role in microalgae cell division, elongation, chlorophyll and protein metabolism and enhance tolerance to several stresses such as heavy metal toxicity, osmotic, and salt stresses[200,203]. This relationship could turn out in an antagonistic one when microalgae and bacteria compete for nutrients and microalgae exhibit lower specific growth rates than bacteria[67,204], but interestingly, the cultivation of microalgal-bacteria consortia on this effluents commonly results in significantly reduce of bacteria[205-207]. However, it is unclear if such reduction was attained through pathogen death or inactivation.

Table 9: Comparison between Conway medium (synthetic medium) and industrial effluents or by-products composition. Identification and culture conditions of microalgae species that could grow in industrial effluents or by-products. A – Autotrophic growth; M – Mixotrophic growth; H – Heterotrophic growth; 1 – 500 ml Flask; 2 - 5 L Bioreactor; 3 - 2 L Flask; 4 - 1 L Bubble column; 5 - 5 L Bubble column; 6 - 15 L Airlift flat plate; 7 - 1 L Flask; 8 - 250 mL Flask; 9 - 1 L Glass type vessel flask; 10 - 100 mL Flask; 11 - 5 L Cylindrical PBR; 12 - Cylindrical glass rods; 13 - 250 mL Bubble column; 14 – PBR; 15 – 0.5 L PBR; 16 – 50 mL Flask; 17 - Multi-chamber bioreactor; 18 - 2 L Glass bottle; 19 - 1.8 L Chemostat; 20 - 2 L Borosilicate flask; 21 – 1 L PBR; 22 – 15 L PBR.

Synthetic medium (f/2) / Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
f/2 medium	75,000	5,000	3,150	4,360	9.8	6.3	22.0	10.0	180.0	-	-	-	-	-	0.2	0.1	8.0 – 8.5	-	-			
Molasses hydrolysate [110]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	30,000	nd	-	-	-	-	a) *C. cohnii* [110] b) *C. protothecoides* [208] c) *S. platensis* [209] d) *I. galbana* [210]	a) 1 | H b) 2 | H c) 3 | M d) 4, 5 | M	a) 0.37 b) 9.31 c) 0.5 d) 0.40
Sugarcane vinasse [211]	823.7	33.26 - 560.0	44.9	-	0.06	0.17	1.2	-	4.9	23.9	162.4	1.620	3.16	19,864	-	-	4.0 – 4.9	32,000 – 109,700	14,362 [212]	a) *Micractinium* sp.[213] b) *C. biconvexa* [213] c) *C. vulgaris* [212]	a,b) 6 | M c) 7 | M	a) 0.16 – 0.18 b) 0.18 – 0.22 c) 0.07

Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
Palm oil mill effluent [214]	4 – 80	94 – 131	-	-	-	-	-	-	-	180 – 1,400	-	-	-	-	-	-	3.4 – 9.0	15,000	100,0000	a) *P. lutheri* [215] b) *I. galbana* [215] c) *C. sorokiniana* [216] d) *P. tricornutum* [217]	a, b) 8|A c) 9|A d) 10|A	a) 0.10 – 0.13 b) 0.09 – 0.14 c) 0.13 – 0.15 d) +
Brewery effluent [218]	1.86 – 11.16	56.98 – 325.75	-	-	-	-	-	-	-	3.07 – 106.44	-	-	-	-	-	-	3.0 – 12.0 [219]	565 – 7,837	500 – 8,750 [219]	a) *S. obliquus* [220] b) *P. kessleri* [221]	a) 11|A b) 8|M	a) 77.9 – 217.0 b) 0.43 – 0.86
Winery wastewater [222]	22 - 28	44 - 88	-	-	-	-	-	-	-	79 - 147	8 - 293	80 - 486	7 - 27	-	-	-	8.5 – 8.8	154 - 298	-	a) *Leptolyngbya* and *Limnothrix* consortium [223] b) *A. platensis* [224] c) *C. vulgaris* [224] d) *Diplosphaera sp.*[225]	a) 12|A b,c) 13|A d) 8|A	a) 0.02 – 0.03 b) 0.04 – 0.10 c) 0.03 – 0.05 d) 0.08

Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
Olive mill wastewater [226]	-	-	0.45 - 20	-	0.49 – 2.96	-	1.7 – 4.98	-	0.46 - 20	60 - 15	30 - 170	730 – 6,100	30 - 290	16,700 – 81,600	-	-	4.7 – 5.7	16,500 – 190,000	15,000 – 102,500	a) *Scenedesmus* sp.[227] b,c) *C. pyrenoidosa* [228,229] d) *A. platensis* [230]	a) 5 | M b) 14 | M c,d) 15 | M	a) 0.04 b) 0.001 c) 0.12 – 1.04 d) 0.11
Cheese whey [231]	7 - 10	340 - 430	-	-	-	-	-	-	-	200 – 1,760	-	-	-	44,020 – 68,000	-	-	3.9 – 6.5	50,000 – 86,300	5,930 – 70,900	a) *C. vulgaris* [232] b) *S. obliquus* [233]	a) 15 | A; M b) 16 | M; H	a) A: 0.10; M: 0.32 – 0.75 b) M: 0.28; H: 0.21
Brine wash [234-236] **(salinity source)**	<10 - 42,000	<10 - 290	-	-	-	-	-	-	-	<10 – 46.58	3.575	635.0	471.8	148	-	-	6.56 - 7.64	-	-	a) *D. tertiolecta* [236] b) *D. salina* [237] c) *T. tetrathele* [237] d, e) *N. salina* [236,237] f) *P. tricornutum* [234] g) *P. lutheri* [234]	a,e) 8 | A b,c,d)17 | A f,g) 16 | A	a) 0.13 b, c, d, f, g) + e) 0.16

Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
Organic fertilizer [238]	4.2 – 21.6	47 - 365	8.3 – 22.6	-	0.13 – 0.04	-	2.5 – 18.0	-	1.22 – 5.7	330.8 – 495.7	60 - 257	921- 923	386 - 545	3,504 – 6,046	-	-	7.4 – 8.6 [239]	-	-	a, j) *N. salina* [236,240] b) *D. tertiolecta* [236] c) *Scenedesmus* sp.[241] d) *P. lutheri* [242] e) *P. tricornutum* [242] f) *T. obliquus* [243] g) *A. maxima* [243] h) *C. vulgaris* [244] i) *M. inermum* [245]	a,b,f,g) 8|A c) 19|A d, e) 20|A h) 21|M i) 8|A j) 18|A	a) 0.17 b) 0.24 c) 0.58 – 0.67 d) 0.02 e) 0.03 f) 0.06 – 0.07 g) 0.10 - 0.12 h) 0.12 i) 0.16 j) 0.09 – 0.16
# Spent coffee grounds [246]	-	1475.1	118.7	-	32.3	-	15.1	-	40.1	-	1293.3	3549.0	777.4	8.5	-	-	-	-	-	-	-	-

Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
Aquaculture effluent [247]	18.1	2.1	0.069	-	0.0007	-	0.022	-	0.003	18.5	10	65	64	-	-	-	7.0	253	-	a) *S. limacinum* [248] b) *C. sorokiniana* [249] c, i) *S. obliquus* [81,249] d) *A. falcatus* [249] e) *C. calcitrans* [250] f) *N. maculate* [250] g) *T. chuii* [250] h) *C. vulgaris* [250] j) *E. gracilis* + *Selenastrum* [192] k) *T. suecica* [251] l) *D. tertiolecta* [251] m) *I. galbana* [251]	a) 8 | H b,c,d,h,i) 7 | A e, f, g) 22 | A j) 20 | A k,l,m) 5 | A	a) 0.42 – 0.49 b) 0.11 – 0.16 c) 0.09 d) 0.16 – 0.20 e, f, g) 0.001 h) 0.007 i) 0.006 j) 0.02 – 0.09 k) 0.09 l) 0.05 m) 0.02
Cork wash water [252]	8.6	0.9	0.35	-	-	-	-	-	-	212.8	-	-	-	-	-	-	2.29	253.9	529.9	-	-	-

Industrial effluents or by-products	Nutrient solution		Trace metal solution							Other compounds					Vitamins solution		Parameters			Culture conditions		
	Nitrate	Phosphate	Iron	EDTA	Copper	Molybdenum	Zinc	Cobalt	Manganese	Other nitrogen sources or Total nitrogen	Magnesium	Potassium	Calcium	Organic matter	Thiamine (B1)	Cyanocobalamin (B12)	pH	COD	Total solids (TS)	Microalgae specie	Production scale | Growth regime	Biomass productivity
	mg/L																	mg/L				g/L/day
Textile industrial effluent [253]	0.002	1.51	380.4	-	7.05	-	8.61	-	10.5	0.0006	159.72	-	140.28	-	-	-	8.1	51.2	735	a) *C. vulgaris* [253]	a) 1 | M	a) +
Tannery wastewater [254]	49	3.90 – 50.5	-	-	4.90 – 48.1	-	7.4 – 20.02	-	-	-	65.2 – 67.1	-	232.5 – 234.5	9.02 – 9.27	-	-	5.6 – 7.8	872 – 3,980	4528	a,b) *Scenedesmus* sp.[255,256] c) *C. vulgaris* [257] d) *P. pringsheimii* [257]	a) 8 | M b) 7 | M c,d) 1 | M	a) + b) 0.21 c,d) +
Pulp and Paper mill effluents [258]	-	0.30 – 3.90	-	-	-	-	-	-	-	5.0 – 18.7	-	-	-	-	-	-	4.51 – 10.53	-	-	a) *C. reinhardtii* [258] b) *S. obliquus* [258] c) *D. pulchellum* [258] d) *N. oculate* [259]	a, b, c, d) 8 | A	a) 0.01 – 0.07 b) 0.03 – 0.07 c) 0.03 – 0.09 d) +

- data not found.

+ Microalgae was able to grow, but biomass productivity was not found in literature.

[#] Nutrients and parameters are in mg/kg, except pH (adimensional).

1.7.1. Aquaculture effluents

The problems of traditional aquaculture industries are mostly related to water deterioration. Even if the water deterioration does not cause the failure of aquaculture, aquatic animals with diseases or toxins may cause serious food safety problems and negative impacts on human health[260].

The causes and effects of water deterioration are mainly: (a) Excess amount of traditional aquaculture feed (biomass rich in protein and lipid) – feed, that is not fully eaten by aquatic animals, would be converted into soluble nutrients, pushed by bacterial activities, which, in part, contributes to the water eutrophication; (b) Aquatic animals excreta such as nitrogen and phosphorous – aquaculture with high stocking density appears to be more suitable for bacterial reproduction; (c) In closed aquaculture system, eutrophication would accelerate the microbial reproduction and cause harmful algal bloom - algal bloom species, particularly cyanobacteria, could consume oxygen and, simultaneously, produce toxins[261-264]; (d) In an attempt to control diseases of aquatic animal caused by this phenom, antibiotics are used, even known that the antibiotics abuse enhances the presence of residual antibiotics in commercialized aquaculture products and contributes also to aquaculture pathogens becoming antibiotics-resistance[265,266].

The technologies commonly applied for the aquaculture effluents treatment are effective (e.g. aerobic[267] and anaerobic[268] treatment to remove organic carbon and nitrogen and chemical precipitation processes to remove phosphorous[269]). However, the cultivation of microalgae in aquaculture effluents offers the combined advantages of nutrients removal and simultaneously production of algal biomass, which can be used to produce valuable products to aquaculture feed, health products and others.

According to previous studies, microalgae can transform the nitrogen and phosphorus (more concentrated nutrients in aquaculture effluents), CO_2, H_2O and other substances into organic compounds such as protein, carbohydrate, lipid and other ingredients through photosynthesis[81,205,249,270,271].

The employment of microalgae production coupled with aquaculture effluents treatment has numerous advantages and economic benefits: (1) Oxygen production by microalgae decreases the risks of oxygen depletion and reduces the energy consumption of traditional aeration devices; (2) Microalgae growth is able to limit the growth of unfavourable or toxic microorganisms, and consequently, the water replacement frequency of a fish tank or pond would be minimized and the

related cost would be reduced; (3) As the immunity of aquatic animal is enhanced by the microalgae feed, use of antibiotics in aquaculture could be avoided, increasing the safety of aquaculture products and maximizing the market acceptance; (4) The aquaculture effluents could be treated by advanced microalgae biotechnology at low cost; (5) As the harvested biomass is used to partly replace the traditional aquaculture feed, the cost of fish rearing could be controlled; (6) Aquaculture effluents replaces the synthetic microalgae culture medium, and this will decrease the production costs of microalgal production[272].

1.7.2. Digestate (Commercial organic fertilizers)

In general, agro-industrial effluents are permitted to be used for agriculture, in the form at organic fertilizers, after appropriate treatment, such as composting or AD (EU Regulation (EC) No 1069/2009).

AD is a mature biological technology applied as an approach to treat organic effluents, where a consortium of microbes breaks down organic material and produce biogas, a source of renewable energy. This is a very efficient technology when compared to other treatment methods, like aerobic treatments and thus, it is a profit choice for high strength effluents, likewise agro-industrial effluents or by-products. AD can remove until 80 % of organic loads, but the remaining inorganic elements in the feedstock, such as nitrogen (N), phosphorus (P), potassium (K), sulfur (S), magnesium (Mg), copper (Cu), zinc (Zn) and iron (Fe) are mineralized and almost fully preserved in the digestion liquor (digestate)[273,274]. Consequently, the effluents of AD, which are called digestate, are rich in inorganic nutrients, including N and P and some of them are already applied as organic fertilizers for plant production[275].

In the same way, cultivation of microalgae, resorting to an organic fertilizer as culture medium, has gained increased attention because of the high potential of producing useful biomass with the simultaneous reduce of microalgae productions costs.

Furthermore, the microalgae production with an organic fertilizer highly contributes to microalgae by-products differentiation on market, organic products, certified according to European standards. These microalgal organic based-products could be used for human consumption, or animal farming allowing the production of poultry, fish and livestock under organic certification. This objective fits in with the demands of today's society, where the consumption of organic food is an increasing trend.

2. General aims

1st objective: Evaluate the growth of *N. gaditana* and *N. oceanica* in heterotrophic conditions with different organic carbon sources: glucose, glycerol, sodium acetate and ethanol at concentrations of 5, 10, 20 and 40 mM.

2nd objective: Evaluate the growth of *P. lutheri*, *P. pinguis* and *P. gyrans* cultured in organic fertilizer and in synthetic aquaculture effluents (phosphate rich effluent, phosphate intermedian effluent and phosphate limited effluent). Assess the total lipid content and bacterial growth (contaminants) in control and agro-industrial by-products cultures.

3. Materials and methods

3.1. Strains

Nannochloropsis oceanica, *Nannochloropsis gaditana*, *Pavlova lutheri*, *Pavlova pinguis* and *Pavlova gyrans* used in this work were provided by the Algafarm collection. Both *Nannochloropsis* strains were axenic, whereas *Pavlova* strains were non-axenic. All microalgae species were cultured in modified f/2 medium.

3.2. Culture media

The modified f/2 medium was used for the maintenance of the selected marine microalgae species and as control medium. The chemical composition of f/2 medium was as follows: nutrient solution ($NaNO_3$, 75.00 g m^{-3}; $NaH_2PO_4.H_2O$, 5.00 g m^{-3}; $Na_2SiO_3.9H_2O$, 30.00 g m^{-3}), trace metals solution ($Na_2H_2EDTA.2H_2O$, 4.36 g m^{-3}; $FeCl_3.6H_2O$, 3.15 g m^{-3}; $MnCl_2.4H_2O$, 180.00 mg m^{-3}; $ZnSO_4$, 22.0 mg m^{-3}; $CoCl_2.6H_2O$, 10.00 mg m^{-3}; $CuSO_4.5H_2O$, 9.80 mg m^{-3}; $Na_2MoO_4.2H_2O$, 6.30 mg m^{-3}; Na_2SiO_3, 30.00 g m^{-3}), Conway vitamins solution (Thiamin HCl, 200 mg m^{-3}; Cyanocobalamin, 10 mg m^{-3}) and a salinity of 30 g L^{-1}.

The heterotrophic medium was the f/2 medium supplemented with different carbon sources (glucose, sodium acetate, glycerol and ethanol), in concentrations of 5, 10, 20 and 40 mM.

The organic fertilizer was composed by three enriched solutions of macronutrients, micronutrients and iron. The organic media experiments were carried out using the enriched organic macronutrients solution at different concentrations of 0.016 % (N1), 0.040 % (N2), 0.080 (N3), 0.097 (N4) and 0.190 % (N5) (v/v) supplemented with the f/2 medium trace metals and Conway vitamins solution. Then the organic medium experiments were performed adding 0.016 % (N1) of organic macronutrients to the enriched organic micronutrients solution at different concentrations of 0.0002 % ($M1_{N1}$), 0.002 % ($M2_{N1}$), 0.003 % ($M3_{N1}$) and 0.004 % ($M4_{N1}$) (v/v) supplemented with iron and Conway vitamins solution. Lastly, the organic medium experiments were completed with the enriched organic iron solution at concentrations of 1 % (F1) and 0.01 % (F2) (v/v) supplemented only with Conway vitamins solution. All experiments were carried out at a salinity of 30 g L^{-1}.

The chemical composition of synthetic aquaculture effluents had the following base composition (in g L^{-1}): NaCl, 27.00; $MgSO_4.7H_20$, 6.60; $CaCl_2$, 1.50; KNO_3, 1.00; KH_2PO_4, 0.07; $FeCl_3.6H_2O$, 0.014; Na_2EDTA, 0.019 and 1 mL L^{-1} of a microelement solution (containing in mg L^{-1}: $ZnSO_4.7H_20$, 40.00; H_3BO_3, 600.00; $CoCl_2.6H_2O$, 1.50; $CuSO_4.5H_2O$, 40.00; $MnCl_2$, 400.00 and $(NH_4)_6Mo_70_{24}.4H_2O$, 370.00). Taking into account the different compositions of the fish farm effluents, it was added to the previous medium (in mg L^{-1}): (1) Phosphate rich effluent, PRE, (NO_3-N, 136.8; NO_2-N, 2.9; PO_4^{-3}, 6.9); (2) Phosphate intermediate effluent, PIE, (NO_3-N, 175.4; NO_2-N, 2.7; PO_4^{-3}, 3.35) or (3) Phosphate limited effluent, PLE, (NO_3-N, 213.9; NO_2-N, 2.5; PO_4^{-3}, 0.2). These effluents were supplemented with the f/2 medium vitamins solution and the experiment was carried out with a salinity of 30 g L^{-1}.

3.3. Culture conditions

3.3.1. Heterotrophic experiments

N. oceanica and *N. gaditana* were cultivated in an orbital shaker at room temperature using 50 mL Erlenmeyer flasks with a cotton wool plug containing a final volume of 30 mL. The photoautotrophic controls were cultivated in autoclaved f/2 medium with a continuous illumination of 100 µmol photons m^{-2} s^{-1}, using LED technology lighting systems. Heterotrophic experiments were carried out in the f/2 medium supplemented with different carbon sources (glucose, sodium acetate, glycerol and ethanol) in concentrations of 5, 10, 20 and 40 mM. Heterotrophic flasks were covered with aluminium foil to avoid the light exposure. Samples (200 µL) were taken every 2 days, and the absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT.

3.3.2. Organic fertilizer experiment with *P. lutheri*

3.3.2.1. Preliminary experiment with aeration

The effect of the organic macronutrients, in varying concentrations (N1, N2, N3, N4 and N5), on the growth of *P. lutheri* was evaluated at room temperature using glass test tubes with a cotton wool plug containing a final culture volume of 35 mL. The cells were cultivated in autoclaved enriched organic macronutrients solution supplemented with the f/2 medium trace metals and Conway vitamins solutions. The control cultures were cultivated in autoclaved f/2 medium. This experiment was performed with continuous illumination of 100 µmol photons m^{-2} s^{-1}, using

LED technology lighting systems. Samples (600 µL) were taken every 2 days and the absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT.

3.3.2.2. Preliminary experiment without aeration

The effect of organic macronutrients (N1, N2, N3, N4 and N5) and micronutrients ($M2_{N1}$, $M3_{N1}$ and $M4_{N1}$) concentrations on the growth of *P. lutheri* was evaluated in an orbital shaker at room temperature using 50 mL Erlenmeyer flasks with a cotton wool plug containing a final culture volume of 35 mL. The cells were cultivated in (1) autoclaved enriched organic macronutrients solution supplemented with the f/2 medium trace metals and Conway vitamins solutions or in (2) autoclaved enriched organic macronutrients and micronutrients solutions supplemented with Conway vitamins solution and iron. The control cultures were cultivated in autoclaved f/2 medium. This experiment was performed with continuous illumination of 100 µmol photons m^{-2} s^{-1}, using LED technology lighting systems. Samples (600 µL) were taken every 2 days. The absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT and the cells were counted resorting to a Neubauer hemocytometer.

3.3.2.3. Organic fertilizer experiment with *P. pinguis*

The effect of organic macronutrients (N1), micronutrients ($M1_{N1}$, $M2_{N1}$ and $M4_{N1}$) and iron (F1 and F2) concentrations on the growth of *P. pinguis* was evaluated in an orbital shaker at room temperature using 250 mL Erlenmeyer flasks with a cotton wool plug containing a final culture volume of 100 mL. The cells were cultivated in autoclaved enriched organic macronutrients, micronutrients and iron solutions supplemented with the Conway vitamins solution. The control cultures were cultivated in autoclaved f/2 medium. This experiment was performed with continuous illumination of 100 µmol photons m^{-2} s^{-1}, using LED technology lighting systems. Samples (600 µL) were taken every 2 days. The absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT and the cells were counted resorting to a Neubauer hemocytometer.

3.3.2.4. Organic fertilizer experiment with *P. gyrans*

The effect of organic macronutrients (N1), micronutrients ($M1_{N1}$, $M2_{N1}$ and $M4_{N1}$) and iron (F2) concentrations on the growth of *P. gyrans* was evaluated in an orbital shaker at room temperature using 250 mL Erlenmeyer flasks with a cotton wool plug containing a final culture volume of 100 mL. The cells were cultivated in autoclaved enriched organic macronutrients, micronutrients and iron solutions supplemented with the Conway vitamins solution. The control cultures were cultivated in autoclaved f/2 medium. This experiment was performed with continuous illumination of 100 µmol photons m^{-2} s^{-1}, using LED technology lighting systems. Samples (600 µL) were taken every 2 days. The absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT and the cells were counted resorting to a Neubauer hemocytometer.

3.3.2.5. Synthetic aquaculture effluents experiment

The effect of PRE, PIE and PLE on the growth of *P. pinguis* and *P. gyrans* was evaluated in an orbital shaker at room temperature using 250 mL Erlenmeyer flasks with a cotton wool plug containing a final culture volume of 100 mL. The cells were cultivated in autoclaved PRE, PIE and PLE supplemented with the Conway vitamins solution. The control cultures were cultivated in autoclaved f/2 medium. This experiment was performed with continuous illumination of 100 µmol photons m^{-2} s^{-1}, using LED technology lighting systems. Samples (600 µL) were taken every 2 days. The absorbance was read at 540, 680 and 750 nm in a microplate reader Bio-Tek Synergy HT and the cells were counted resorting to a Neubauer hemocytometer.

3.4. Analytical methods

The kinetic parameters, maximum specific growth rate, μ_{max} (d^{-1}), and maximum biomass productivity, P_x (cells mL^{-1} d·1), were used to compare the performance of the different experiments. Along with this data, it was also evaluated the pH variation and the nitrate consumption over the time of culture. We also intended to know if the aquaculture effluents could be more suitable to contaminants growth, such as bacteria, since the species inocula were not axenic.

3.4.1. Measurement of cell growth

Microalgal cell growth was estimated by measuring the absorbance values at 540, 680 and 750 nm using a microplate reader Bio-Tek Synergy HT and by cell counting resorting to a Neubauer hemocytometer. The μ_{max}, and P_X of *P. pinguis* and *P. gyrans* was estimated as follows:

$$\mu_{max} = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1)$$

$$P_X = \frac{X_2 - X_1}{t_2 - t_1} \quad (2)$$

where X_1 and X_2 are number of cells mL^{-1} at time 1 (t_1) and time 2 (t_2) in the exponential growth phase, respectively.

3.4.2. Bacterial viable plate counts

For viable bacterial counting, samples (1 mL) were taken at the initial and end of experiments. 10^{-3}, 10^{-4} and 10^{-5} dilutions were made with peptone salt solution from each culture condition (n=3); 0.1 mL was spread over the surface of the plate count agar plates, in duplicate. The colonies were counted after 3 days of incubation at 27 °C. The number of colonies were estimated as follows:

$$\text{CFU } mL^{-1} = CFU\ x\ \frac{1}{dilution\ factor}\ x\ \frac{1}{plated\ volume} \quad (3)$$

3.4.3. Determination of nitrogen concentration

Nitrate concentration was determined according to Ultraviolet spectrophotometric screening method (4500-NO_3^- B) (APHA, 1995). For this method samples were analyzed using a microplate reader Bio-Tek Synergy HT in the ultraviolet (UV) range at wavelengths of 220 nm and 275 nm. Measurements at both wavelengths are used to correct for possible interference by dissolved organic matter. Dissolved organic matter may be absorbed at 220 nm and 275 nm while NO_3^- is only absorbed at 220 nm. The nitrate concentration was calculated by subtracting the absorbance at 275 nm from the absorbance at 220 nm and then comparing the corrected

absorbance to a calibration curve (**Fig. 3**) developed using standards of known nitrate concentration.

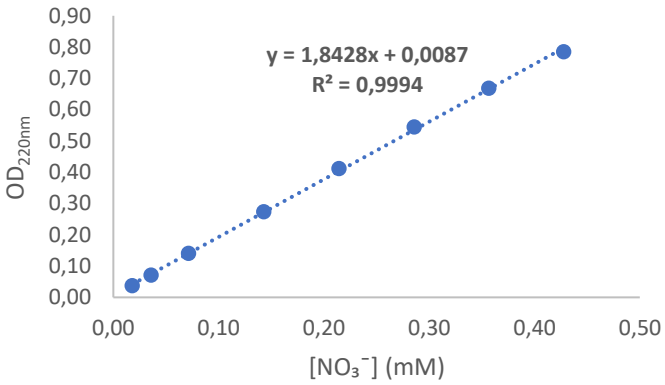


Fig. 3 Nitrate calibration curve.

3.4.4. Lipid content

The microalgae total lipid content was extracted and quantified by resorting to the Bligh and Dyer method[276], with modifications. We started by adding 1 mL of a mixture of solvent chloroform/methanol (2:1, v/v) to 50 g of microalgae powder. This mixture was homogenized, by vortexing for 2 minutes, incubated on a heating block at 30 °C for 30 minutes, and then centrifuged at 358 *g* for 10 minutes. The organic phase (supernatant) was collected to a pre-weighted glass tube and the biomass residue (in the pellet) was re-extracted seven more times with 1mL of solvent (until the solvent had no pigmentation). The resultant organic phase was dried, under a stream of nitrogen gas, the tube was weighted, and the initial extract weight was recorded (initial extract). To remove non-lipid contaminants, the initial extract was re-dissolved in 2 mL of chloroform and 1 mL of methanol. This mixture was vortexed, and 0.75 mL of water were added, followed by additional vortexing for 2 minutes.

To promote phase separation, the mixture was centrifuged during 10 minutes at 358 *g*, the organic (lower) phase was collected to a new pre-weighted tube, and the aqueous phase was re-extracted by adding 2 mL of chloroform. The combined organic phases were then dried under N_2 stream and weighted.

3.4.5. Statistical analysis

Experimental results were given as the average values of three independent experiments and their standard deviation. Statistical analysis of the data was carried out using the software Origin (Pro). Analysis of significance was determined by analysis of variance (ANOVA) and Tukey HSD tests performed at a confidence level of 95 % and p values < 0.05 were considered to be statistically significant.

4. Results and Discussion

4.1. Effects of organic carbon sources on *Nannochloropsis gaditana* and *Nannochloropsis oceanica* growth

We started by assessing the microalgae growth of *N. gaditana* and *N. oceanica* with organic substrates. As it is presented in **Fig. 4**, no significant growth was determined for both *Nannochloropsis* spp. under heterotrophic conditions, which is in accordance with what was previously described in the literature[45,119,120]. Furthermore, when cultured in the presence of varying concentrations of either ethanol or sodium acetate, none of the *Nannochloropsis* spp. was able to growth, indicating the inhibitory effect of these compounds over both species.

N. oceanica presented a slight OD increase when cultured in the presence of 20 and 40 mM of glycerol and 20 mM of glucose (**Fig. 4B**). However, this increment was not as significant when compared with the values determined in photoautotrophic growth conditions (control). Overall, this data demonstrates that *Nannochloropsis* spp. tested in the present work are not able to grow in heterotrophic conditions.

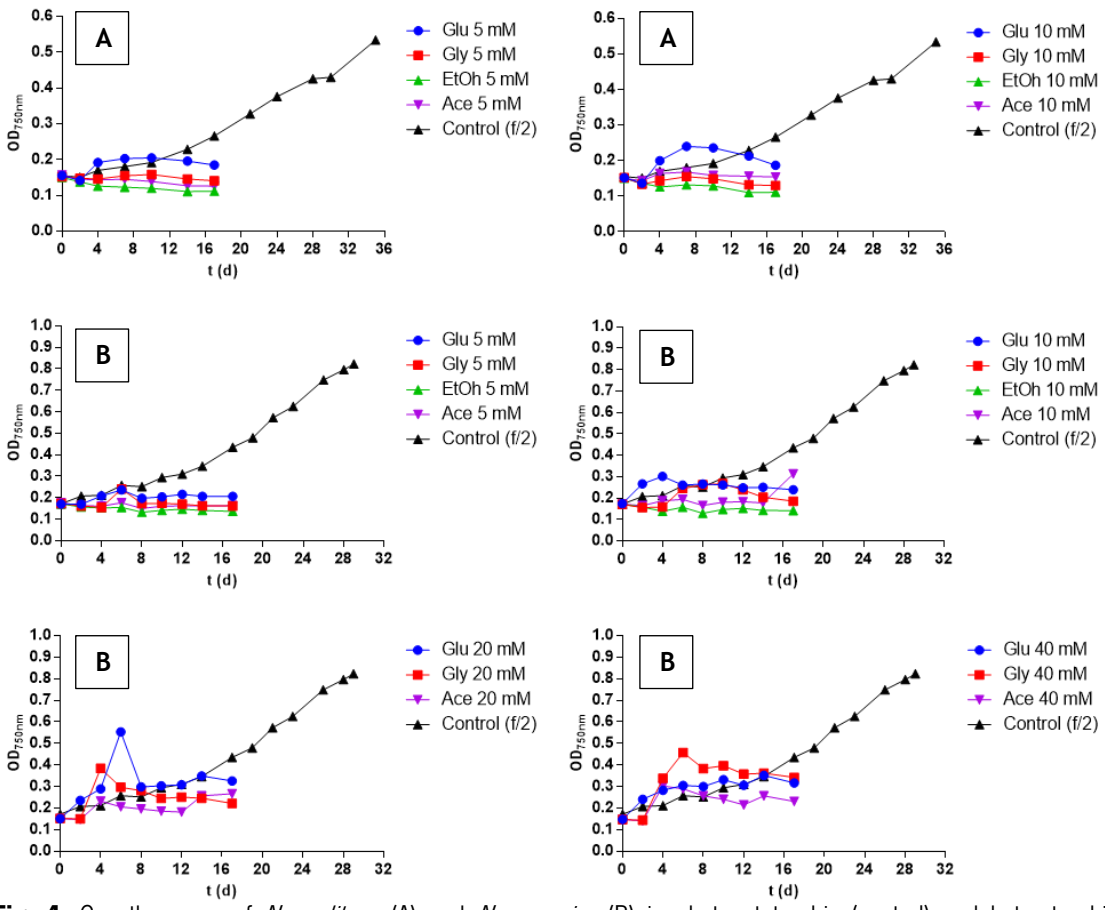


Fig. 4: Growth curves of *N. gaditana* (A) and *N. oceanica* (B) in photoautotrophic (control) and heterotrophic conditions with different organic carbon sources: glucose (Glu), glycerol (Gly), sodium acetate (Ace) and ethanol (EtOH) (*n*=2).

4.2. Effect of organic fertilizer on microalgal growth

4.2.1. Experiment 1: *Pavlova lutheri* – Determination of optimal organic macronutrients concentration

The organic fertilizer was composed by three enriched solutions of either macronutrients, micronutrients or iron, in order to achieve higher or similar growth parameters to the ones determined using synthetic medium (f/2 medium) cultures.

Initially, the effect of five organic macronutrients concentrations, 0.016 % (N1), 0.040 % (N2), 0.080% (N3), 0.097 % (N4) and 0.190 % (N5) (v/v), was evaluated on *P. lutheri* growth. As, in the present work *P. lutheri* proved to be difficult to grow without aeration, the first preliminary experiment occurs with aeration (**Fig. 5**).

The final absorbance values (day 12) indicate that the condition N1 potentiates a similar *P. lutheri* growth, when compared to the control culture, while the conditions N2, N3, N4 and N5 led to a lower microalgal growth.

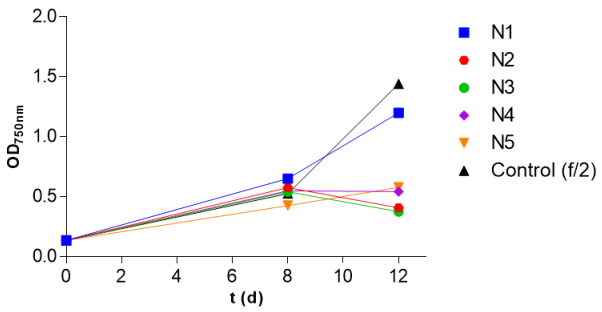


Fig. 5 Growth curves of *P. lutheri* at different organic macronutrients concentrations (N1 to N5) compared to control culture (f/2 medium). **N1–0.016 %**; **N2–0.040 %**; **N3–0.080 %**; **N4–0.097 %**; **N5–0.190 % (v/v)** (*n=2*).

These results were further validated by performing a new preliminary experiment where the same conditions were applied in the absence of aeration. It was also tested three new conditions, in which the microalgae were grown in a mixture of N1 and varying concentrations of the organic micronutrients' solution ($M2_{N1}$, $M3_{N1}$ and $M4_{N1}$) (**Fig. 6**). These three concentrations were selected taking into consideration the control medium (f/2) micronutrients and the boron (B), sulfur (S), calcium (Ca) and manganese (Mn) concentrations. The presence of B is defined, by Carvalho *et al.*, as limiting to *P. lutheri* cell growth, while S is essential to cell survival. These authors also revealed that cultures deprived of Ca or Mn revealed cellular yields significantly below those obtained in media enriched with both micronutrients[131].

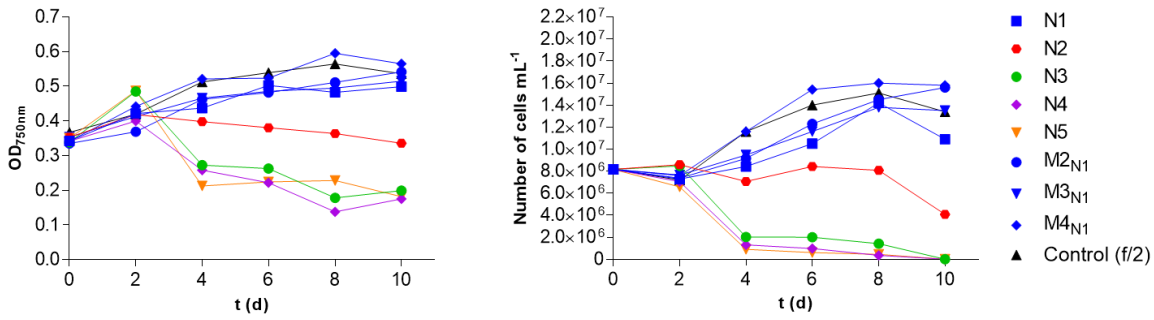


Fig. 6 Growth curves of *P. lutheri* at different organic macro- (N1 to N5) and micronutrients ($M2_{N1}$ to $M4_{N1}$) concentrations compared to control culture (f/2 medium). **N1-0.016 %**; **N2–0.040 %**; **N3–0.80 %**; **N4–0.097 %**; **N5-0.190 %**; **$M2_{N1}$-N1+0.002 %**; **$M3_{N1}$-N1+0.003 %**; **$M4_{N1}$-N1+0.004 % (v/v)** (*n*=2).

Three species of blue-green algae, *N. muscorum*, *A. cylindrica*, *M. aeruginosa* and nitrogen fixing species, *C. parietina*, showed a marked uptake and growth response to boron when nitrate was omitted from the culture medium. In contrast, three species of green algae, *C. pyrenoidosa*, *D. plumose* and *S. tenue* were shown neither to require boron nor to absorb it in appreciable amounts[277]. Fabregas *et al.* also demonstrated that B was non-essential for growth of *H. pluvialis* [278].

S is a macro-element essential to all organisms in the form of sulphur-containing amino acids methionine and cysteine[279]. Ca levels might be related to nitrate reductase activity. Deficiency in Ca seems to reduce nitrate reductase activity, which leads to a decrease of nitrate utilization. As a consequence, photosynthesis is reduced, as such microalgal growth[280]. Mn plays a role in chlorophyll synthesis and O_2/CO_2 utilization in photosynthesis[281].

As seen in the previous experiment, the results showed that the condition N1 potentiates a microalgae growth similar to the control culture and validated that the use of concentrations greater than 0.016 % (N1) of organic macronutrients' solution (conditions N2, N3, N4 and N5) inhibits *P. lutheri* growth (**Table 10**).

Table 10: Average value ± [lower limit-upper limit] (*n*=2) of the effects of different organic macro- and micronutrients concentrations on the growth rate (μ_{max}), biomass productivity (P_x) and maximum cell density of *P. lutheri*.

Condition	μ_{max} (d^{-1})	P_x ($x10^6$ cells mL^{-1} d^{-1})	Max. cell density ($x10^7$ cells mL^{-1})
N1	0.13±[0.11-0.16]	1.44±[1.25-1.64]	1.42±[1.41-1.43]
N2	ng	ng	ng
N3	ng	ng	ng
N4	ng	ng	ng
N5	ng	ng	ng
$M2_{N1}$	0.14±[0.14-0.14]	1.44±[1.29-1.59]	1.56±[1.49-1.64]
$M3_{N1}$	0.11±[0.08-0.14]	1.04±[0.86-1.22]	1.38±[1.37-1.40]
$M4_{N1}$	0.19±[0.14-0.21]	1.94±[1.65-2.24]	1.65±[1.60-1.70]
Control (f/2)	0.17±[0.13-0.20]	1.70±[1.46-1.93]	1.51±[1.44-1.59]

ng – no growth.

This inhibition could be related to the increasing concentrations of N (as ammoniacal and organic forms), P or organic matter (absent in the control medium), mainly constituents of the enriched macronutrients solution (N1<N2<N3<N4<N5). It was known that the concentration and composition of available N, P and organic matter in the culture medium have a significant influence on the microalgae physiological responses.

Carvalho *et al.* revealed that *P. lutheri* cell growth was significantly higher when the culture medium is enriched with nitrogen present as nitrate (NO_3^-) rather than ammonium (NH_4^+) or urea, as this species exhibited $1.61\pm0.47x10^7$, $1.27\pm0.21x10^7$ and $0.81\pm0.03x10^7$ cells mL^{-1} respectively. Regarding the P concentration, this research also contributed to understand that *P. lutheri* showed a statistically similar cell growth in a range between 0.01 and 0.9 mM of P[131].

In the present work, the prepared dilutions of the enriched macronutrients' solution resulted in a range of N and P concentrations in the organic media near to the concentrations available in the control medium used in this project (9 mM of NO_3^- and 0.04 mM of P) and in the culture media used in Carvalho *et al.* experiments (3 mM of NO_3^-, NH_4^+ or urea and 0.01 to 0.90 mM of P)[131]. This data confirms that the N and P concentrations in conditions N2, N3, N4 and N5 are not available at limiting or excess levels, so these nutrients are not associated to the growth inhibition of *P. lutheri*.

Nevertheless, Eustance *et al.* demonstrated that the growth of *Scenedesmus* sp. and *Monoraphidium* sp. on ammonium resulted in a significant decrease of pH during the exponential phase, causing microalgae growth inhibition[282]. The same conclusion was reached by Scherholz and Curtis when both ammonium and nitrate were provided during the culture time of *C. vulgaris* and *C. reinhardtii* [283].

The mentioned studies highlighted the possibility of *P. lutheri* growth inhibition taking place due to increasing NH_4^+ concentrations in organic fertilizer media (N1<N2<N3<N4<N5) and its consumption, which causes a significant decrease in pH. Further work must be done to understand the effects of NH_4^+ consumption by *Pavlova* species when cultured with both NH_4^+ and NO_3^-, prior to accepting this hypothesis.

Based on all mentioned data, it was hypothesized that concentrations of NH_4^+ and/or organic matter higher than 0.016 % (N1) might promote conditions that favour cell growth inhibition. These results demonstrated the existence of an important stoichiometric limitation. As such, 0.016 % (N1) of the enriched macronutrients solution was used in subsequent experiments.

Furthermore, in this experiment, we also determined that replacing inorganic micronutrients to organic ones in conditions $M2_{N1}$, $M3_{N1}$ and $M4_{N1}$ does not change the growth trend of condition N1, since in these conditions *P. lutheri* exhibited growth trends similar to both N1 and control conditions. These organic micronutrients concentrations were found to be suitable for microalgal growth, which suggests that the previously mentioned micronutrients, B, S, Ca and Mn are present at proper concentrations.

4.2.2. Experiment 2: *Pavlova pinguis* – Determination of optimal organic iron concentration

The optimal organic iron concentration was determined through the growth assessment of *P. pinguis* (**Fig. 7**). Making an analogy between the phylogenetic similarities among the selected Pavlova species[154] and the species-specific nutrient requirements, we assumed that the optimal organic macronutrients concentration of *P. pinguis* would be similar to *P. lutheri*, 0.016 % (N1).

Taking into account the iron concentration of the control medium, two concentrations of organic iron were evaluated, 1 % (F1) and 0.010 % (F2) (v/v). The latter was selected since in the first one the medium acquired a dark colour, and it is common knowledge that several microalgae species cannot grow in dark media, since these limit microalgae photosynthetic activity.

Additionally, due to the similar growth previously determined in all organic micronutrients' concentrations ($M2_{N1}$, $M3_{N1}$ and $M4_{N1}$) (**Fig. 6**), it was tested a larger range of organic micronutrients' concentrations ($M1_{N1}$, $M2_{N1}$ and $M4_{N1}$) (**Fig .7**).

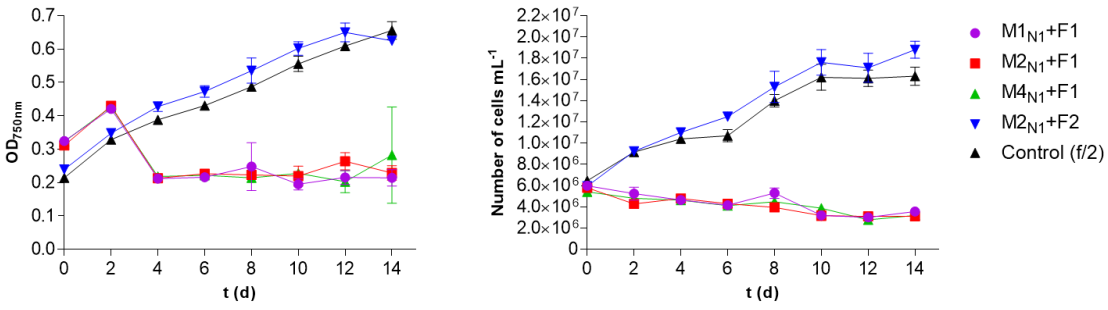


Fig. 7 Growth curves of *P. pinguis* at different organic micronutrients ($M1_{N1}$ to $M4_{N1}$) and iron (F1 and F2) concentrations compared to control culture (f/2 medium). **$M1_{N1}$-N1+0.0002 %**; **$M2_{N1}$-N1+0.002 %**; **$M4_{N1}$-N1+0.004 %**; **F1-1 %**; **F2-0.010 % (v/v)**; bars indicate standard deviation (*n*=3).

We observed that different media with 1 % of organic iron did not promote microalgae cell growth (**Fig. 7**), however *P. pinguis* grew well in medium with 0.010 % of organic iron.

In fact, the condition $M2_{N1}$+F2 showed a statistically higher cell growth in terms of μ_{max}, P_x and maximum cell density (0.15±0.01 d^{-1}, 1.27±0.09x10^6 cells mL^{-1} d^{-1} and 1.93±0.03x10^7 cells mL^{-1}, respectively) when compared to control culture (0.12±0.01 d^{-1}, 0.99±0.04x10^6 cells mL^{-1} d^{-1} and 1.70±0.08x10^7 cells mL^{-1} respectively) ($p<0.05$). As such, 0.010 % (F2) of organic iron was used in subsequent experiments (**Table 11)**.

The microalgal growth inhibition, observed in conditions supplemented with F1, is very likely related to high turbidity and to pH of the culture medium. Addition of 1 % (v/v) of organic iron to culture media not only resulted in darker media but also in a decrease of pH values to 6.1±0.1, whereas culture medium supplemented with 0.010 % (v/v) maintained a low turbidity and a pH value of 8.1 (**Table 14**). Both parameters are similar to control medium.

These results are in agreement to Shah *et al.*, which determined that *P. lutheri* growth and lipid accumulation was optimum at pH 7–9[130].

Table 11: Average value ± standard deviation (n=3) of the effects of different organic macro-, micronutrients and iron concentrations on the growth rate (μ_{max}), biomass productivity (P_x) and maximum cell density of *P. pinguis*. Values in the same column with different superscript letter (a-b) are statistically different at p <0.05 by Tukey's test.

Condition	μ_{max} (d^{-1})	P_x (x10^6 cells mL^{-1} d^{-1})	Max. cell density (x10^7 cells mL^{-1})
$M1_{N1}$+F1	ng	ng	ng
$M2_{N1}$+F1	ng	ng	ng
$M4_{N1}$+F1	ng	ng	ng
$M2_{N1}$+F2	0.15±0.01 [a]	1.27±0.09 [a]	1.93±0.03 [a]
Control (f/2)	0.12±0.01 [b]	0.99±0.04 [b]	1.70±0.08 [b]

ng – no growth.

Iron plays a crucial role in processes that require electron transfer reactions, including photosynthesis and nitrogen assimilation. The iron deficiency on photosynthesis results into a decrease in cellular content of proteins of the photosynthetic apparatus (e.g. D1-protein)[284], chlorophyll *a* and other pigments involved in light harvesting[285]. The functioning of the photosynthetic electron transport chain is also affected by a decrease in iron-sulphur complexes, e.g. ferredoxin. Davey and Geider showed that, as a consequence, photosynthetic efficiency is reduced in iron-limited *C. muelleri* cultures[286]. Iron is also a structural component of the metallo-enzymes that are involved in nitrate uptake, nitrite and nitrate reductase. The activity of nitrate reductase is low in iron-deficient phytoplankton[287,288].

Carvalho *et al.* stated that *P. lutheri* cultures deprived of iron revealed cellular yields significantly below those obtained in iron enriched cultures. Although the condition F2 corresponds to a lower iron concentration than the available in the control medium, several studies have shown that small amounts of iron are sufficient to meet the requirements of this metal. Song *et al.*, van

Oijen *et al.* and Tao *et al.* established that optimal level of iron for microalgal biomass are 0.27, 1.00 and 7.00 µM for *B. braunii* [289], *C. brevis* [290] and *S. acuminatus* [291], respectively.

The reported iron concentrations are as much as one and two orders of magnitude lower those available at condition F2, which suggests that 0.010 % (v/v) of organic iron was enough to supply the iron requirements of *P. pinguis*.

4.2.3. Experiment 3: *Pavlova gyrans* – Determination of optimal organic micronutrients concentration under sterile and non-sterile culture media

As in the previous experiment it was only possible to determine the optimal iron concentration, we had to perform a new experiment to assess the optimal micronutrient concentration. The following experiments was performed resorting to *P. gyrans*. This species was described as phylogenetically more similar to *P. pinguis* than *P. lutheri* [154], so on the already determined macronutrients (N1) and iron (F2) concentrations will be employed in the following experiments.

The previous range of organic micronutrients' concentrations ($M1_{N1}$, $M2_{N1}$ and $M4_{N1}$) was evaluated again in culture media enriched with 0.010 % of iron (F2). In the non-sterile experiment, we selected the $M2_{N1}$+F2 culture medium, which promoted a significant *P. gyrans* cell growth (**Fig. 8**).

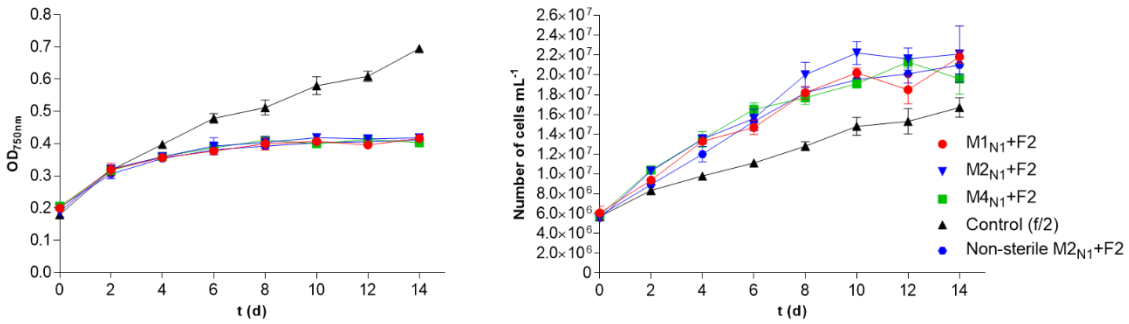


Fig. 8 Growth curves of *P. gyrans* at different organic micronutrients ($M1_{N1}$ to $M4_{N1}$) compared to control culture (f/2 medium). **$M1_{N1}$-N1+0.0002 %**; **$M2_{N1}$-N1+0.002 %**; **$M4_{N1}$-N1+0.004 %**; **F2-0.010 %**; bars indicate standard deviation (n=3).

The growth parameters of each experiment are presented in **Table 12**. The higher values of growth rate (0.22±0.02 d^{-1}), biomass productivity (2.00±0.22x10^6 cells mL^{-1} d^{-1}) and cell density (2.32±0.27x10^7 cells mL^{-1}) were found in experiment $M2_{N1}$+F2. These results are statistically higher

than the results obtained in the control culture (p <0.05), which support the ability of the organic fertilizer replace the inorganic f/2 medium in order to cultivate *P. pinguis* and *P. gyrans*.

Table 12: Average value ± standard deviation (n=3) of the effects of different organic micronutrients concentrations on the growth rate (μ_{max}), biomass productivity (P_x) and maximum cell density of *P. gyrans*. Values in the same column with different superscript letter (a-c) are statistically different at p <0.05 by Tukey's test.

Condition	μ_{max} (d^{-1})	P_x (x10^6 cells mL^{-1} day^{-1})	Max. cell density (x10^7 cells mL^{-1})
$M1_{N1}$+F2	0.20±0.04 ^{a,b}	1.80±0.26 ^{b}	2.18±0.08 ^{b}
$M2_{N1}$+F2	0.22±0.02 ^{b}	2.00±0.22 ^{b}	2.32±0.27 ^{b}
$M4_{N1}$+F2	0,21±0.01 ^{b}	1.91±0.21 ^{b}	2.13±0.08 ^{b}
Non-sterile $M2_{N1}$+F2	0,18±0.02 ^{a,b}	1.56±0.25 ^{a,b}	2.10±0.11 ^{b}
Control (f/2)	0,14±0.02 ^{a}	1.03±0.13 ^{a}	1.70±0.10 ^{a}

The statistical analysis of the results also revealed that the growth parameters of experiment $M2_{N1}$+Fe2 were equivalent to those obtained in the experiments $M1_{N1}$+Fe2, $M4_{N1}$+Fe2 and Non-sterile $M2_{N1}$+Fe2, which showed to us that *P. gyrans* grew well in a micronutrients concentration range between 0.0002 % (M1) and 0.004 % (M4). The results also showed that it is possible produce *P. gyrans* without previous fertilizer medium sterilization, as such the growth parameters obtained in non-sterile $M2_{N1}$+Fe2 condition are statistically equivalent to those obtained in experiment $M2_{N1}$+Fe2 (**Table 12**). This is a great advantage which promotes the application of organic fertilizer as culture medium for microalgal production, in order to achieve a differentiating product on the market and microalgal production on open systems.

Curiously, when we observed the growth curves of *P. gyrans* cultivated on organic fertilizer media (**Fig. 8**) were possible to detected that, in terms of OD_{750nm} evaluation, the control culture curve showed much higher optical densities than those obtained in organic fertilizer media. It appears that the increase of cell number of *P. gyrans* under organic fertilizer conditions is not accompanied by a proportional increase in optical density, opposing to it was observed in *P. lutheri* (**Fig. 5 and 6**) and *P. pinguis* (**Fig. 7**) when cultivated in organic fertilizer media.

Microscopic observations made over the time of culture suggested that *P. gyrans* cultivated in organic fertilizer media was subjected to a change in cell morphology, resulting into smaller cells than the cells growing in control medium.

This morphology variation was evaluated again in the following experiments and discussed in the **Section 4.2.6.**

4.2.4. Experiment 4: *Pavlova gyrans* – Evaluate the influence of vitamins B_1 and B_{12} under sterile and non-sterile culture media

The concept that vitamin B_1 (thiamin) and B_{12} (cobalamin) availability in seawater controls the productivity and structure of eukaryotic phytoplankton communities has been discussed for half a century[131]. The latter is claimed to be essential for algal flagellates and diatoms growth, whereas vitamin B_1 is supposed to act as a stimulant[131].

As a consequence of this discussion and the divergence of results, depending on the marine species studied, in the present work all media were enriched with vitamins B_1 and B_{12}, in order to promote a better cell growth. After the assessment of the optimal concentrations of the organic fertilizer were achieved, we intended to understand the requirement of these vitamins for the growth of *Pavlova* species. To address this, a new experiment was performed resorting to the organic fertilizer medium, $M2_{N1}$+F2 and to the control medium, enriched with or without both or one vitamin (**Fig. 9**).

The growth parameters of each experiment presented in **Table 13**. showed that the higher values of growth rate (0.31±0.02 d^{-1}) and biomass productivity (2.27±0.18x10^6 cells mL^{-1} d^{-1}) were found in experiment $M2_{N1}$+F2 w/ B_1 and B_{12}. These results are statistically higher than the results obtained in the control culture, 0.23±0.03 d^{-1} and 1.43±0.23x10^6 cells mL^{-1} d^{-1}, respectively. Nevertheless, the results obtained in experiment $M2_{N1}$+F2 w/ B_1 and B_{12} were equivalent to those obtained in the experiment $M2_{N1}$+F2 wo/ B_1 and B_{12}, as the values of growth rate and biomass productivity were 0.31±0.02 d^{-1} and 2.22±0.10x10^6 cells mL^{-1} d^{-1}, respectively. In fact, all experiments performed into organic fertilizer media with or without both or one vitamin were statistically equivalent ($p < 0.05$).

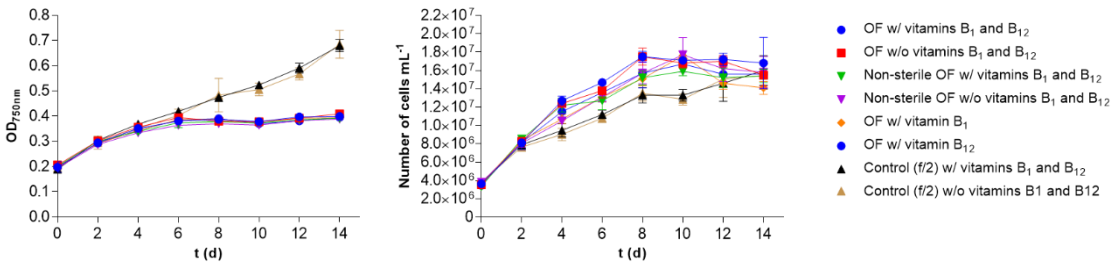


Fig. 9 Growth curves of *P. gyrans* at sterile or non-sterile organic fertilizer ($M2_{N1}$+F2) with or without both or one of the vitamins B_1 and B_{12} compared to control culture (f/2 medium) with vitamin B_1 and B_{12}. **OF-organic fertilizer: $M2_{N1}$-N1+F2; w/-with; w/o-without;** bars indicate standard deviation (n=3).

The combination of these data with the fact that the results obtained in the control culture w/ B_1 and B_{12} were statistically similar to results of the control culture wo/ B_1 and B_{12}, appears to be an evidence that microalgae *P. gyrans* did not require vitamins B_1 and B_{12} enriched media to promote cell growth (**Table 13**). In order to interpret these results, it is required understand the role of vitamin B_1 and B_{12} in algal metabolism.

Table 13: Average value ± standard deviation (n=3) of the effects of vitamins B_1 and B_{12} on the growth rate (μ_{max}), biomass productivity (P_x) and maximum cell density of *P. gyrans*. Values in the same column with different superscript letter (a-c) are statistically different at p <0.05 by Tukey's test.

Condition	μ_{max} (d^{-1})	Max. biomass productivity (P_x) (x10^6 cells mL^{-1} d^{-1})	Max. cell density (x10^7 cells mL^{-1})
Organic fertilizer w/ vitamin B_1 and B_{12}	0.31±0.02 ^{b}	2.27±0.18 ^{c}	1.84±0.21 ^{a}
Organic fertilizer w/o vitamin B_1 and B_{12}	0.31±0.02 ^{b}	2.22±0.10 ^{b,c}	1.76±0.10 ^{a}
Non-sterile organic fertilizer w/ vitamin B_1 and B_{12}	0.32±0.03 ^{b}	2.22±0.24 ^{b,c}	1.61±0.03 ^{a}
Non-sterile organic fertilizer w/o vitamin B_1 and B_{12}	0.25±0.03 ^{a,b}	1.68±0.20 ^{a,b}	1.86±0.16 ^{a}
Organic fertilizer w/ vitamin B_1	0.27±0.02 ^{a,b}	1.77±0.18 ^{a,b,c}	1.76±0.06 ^{a}
Organic fertilizer w/ vitamin B_{12}	0.30±0.04 ^{a,b}	2.01±0.28 ^{b,c}	1.71±0.05 ^{a}
f/2 medium w/ vitamin B_1 and B_{12}	0.23±0.03 ^{a}	1.43±0.23 ^{a}	1.60±0.20 ^{a}
f/2 medium w/o vitamin B_1 and B_{12}	0.22±0.03 ^{a}	1.40±0.21 ^{a}	1.58±0.31 ^{a}

Croft *et al.* did important scientific findings about the role of vitamin B_{12} in algal metabolism. The authors found that vitamin B_{12}-dependent algae contains the gene for the vitamin B_{12}-dependent methionine synthase (*metH*) only, while independent ones contain vitamin B_{12}-independent methionine synthase genes (*metE*) or both[292].

Another aspect was showed by Croft *et al.* related to the supplying of vitamin B_{12} to algae. The vitamin B_{12} biosynthesis is upregulated by bacteria in a mutualistic relationship with microalgae. Bacteria consume the products of microalgal photosynthesis to grow and provide vitamin B_{12} required to microalgae growth[292].

More recently, transcriptomic experiments performed by Nef *et al.*, suggest that none species of Haptophyta phylum encode *metE* nor a pseudogene, which presuppose that these microalgae are in majority B_{12} auxotrophs[293].

This theory is corroborated by Carvalho *et al.*, which demonstrate that *P. lutheri* cells are not viable if vitamin B_{12} was not supplied[131], and partially refuted by Croft *et al.* who found that whereas *P. gyrans* is B_{12} auxotroph, *Pavlova* sp. did not require vitamin B_{12} for growth[292].

Related to vitamin B_1, Gutowska *et al.* found that *Pavlova* sp. cultures cannot grow under B_1-depleted conditions[294]. In opposite, Carvalho *et al.* reported that *P. lutheri* cell growth was

unrelatedly to the amounts of vitamin B_1. However, in latter study none information about bacterial contamination was obtained, since vitamin B_1 is mainly produced by bacteria. If *P. lutheri* culture was contaminated with bacteria, it would be able to grow.

We can conclude that, in this work, *P. gyrans* obtained statistically similar cell growths when cultured in media with or without vitamins B_1 and B_{12}, as result of bacteria presence in non-axenic cultures of *P. gyrans*. Bacteria enables the supplying of both vitamins.

Altogether, the results obtained through *P. lutheri*, *P. pinguis* and *P. gyrans* experiments are in agreement with several studies which showed that organic fertilizers or others AD effluents enable relevant microalgae growth (**Table 9**)[236,240-245].

Additionally, the overall effects of microalgal cultivation and the growth media on the pH of the media can be seen in **Table 14**.

The pH variation is a good measure to assess if autotrophic microalgae cell growth occurred, since photosynthetic growth is accompanied by an increase in pH value. Changes in the pH of the media may be attributed to two main mechanisms: (1) alkalization of the media through the release of hydroxyl ions as a result of microalgal dissolved inorganic carbon uptake (photosynthetic CO_2 assimilation), and (2) acidification of the media through the release of protons as a result of ammonium consumption by microalgae or contaminants, such as bacteria[282]. Therefore, the pH variation may suggest that one of the mechanisms is dominant over the other.

As expected, the pH of the control medium (f/2) increased by ≈1.0 in both *P. pinguis* and *P. gyrans* experiments, suggesting that the effect of photosynthetic CO_2 assimilation may be dominant under these control conditions (values highlighted at grey in the **Table 14**). In opposition, when *P. pinguis* was cultured in conditions $M1_{N1}$+Fe1, $M2_{N1}$+Fe1 and $M3_{N1}$+Fe1, which it was not observed microalgae cell growth, the final pH decreased by ≈2.0, suggesting that the effect of ammonium utilization was dominant. This occurred as a consequence of microalgae cell death due to microalgal cultivation into darker media with pH values of 6.1±0.1 (**Table 14**) (**see Section 4.2.2**). At these conditions, ammonium content of microalgal biomass is released to the medium and favor the bacterial utilization of ammonium.

Interestingly, although *P. pinguins* and *P. gyrans* grew more in the organic fertilizer conditions than in control conditions, the pH variation measured was significantly lower in organic fertilizer conditions (0.0 to 0.4).

Table 14: Value ± standard deviation (*n*=3) of the effect of microalgal cultivation and growth media on pH.

Microalgae strain	Condition	Initial pH	Final pH	ΔpH
	Experiment 2			
	$M1_{N1}$+F1	6.1	4.2±0.1	-1.9±0.1
	$M2_{N1}$+F1	6.1	4.0±0.3	-2.1±0.3
P. pinguis	**$M3_{N1}$+F1**	6.2	4.2±0.1	-1.9±0.1
	$M2_{N1}$+F2	8.1	8.5±0.1	0.4±0.1
	Control (f/2)	8.2	9.3±0.0	1.1±0.0
	Experiment 3			
	$M1_{N1}$+F2	8.3±0.0	8.4±0.1	0.1±0.1
	$M2_{N1}$+F2	8.2±0.0	8.4±0.1	0.2±0.1
	$M4_{N1}$+F2	8.2±0.0	8.3±0.1	0.1±0.1
	Non-sterile $M2_{N1}$+F2	8.3±0.1	8.4±0.1	0.1±0.2
	Control (f/2)	8.6±0.0	9.2±0.1	0.6±0.1
	Experiment 4			
P. gyrans	**OF w/ vitamin B_1 and B_{12}**	8.2±0.0	8.3±0.1	0.1±0.1
	OF w/o vitamin B_1 and B_{12}	8.2±0.0	8.4±0.1	0.2±0.1
	Non-sterile OF w/ vitamin B_1 and B_{12}	8.4±0.0	8.3±0.1	-0.1±0.1
	Non-sterile OF w/o vitamin B_1 and B_{12}	8.4±0.0	8.4±0.0	0.0±0.0
	OF w/ vitamin B_1	8.2±0.0	8.3±0.1	0.1±0.1
	OF w/ vitamin B_{12}	8.2±0.0	8.2±0.1	0.0±0.1
	Control (f/2) w/ vitamin B_1 and B_{12}	8.5±0.0	9.4±0.1	0.9±0.1
	Control (f/2) w/o vitamin B_1 and B_{12}	8.5±0.1	9.3±0.1	0.8±0.2

We propose three hypotheses that could justify these results: (1) the organic fertilizer medium has a buffer capacity, (2) alkalization of the media, throughout photosynthetic CO_2 assimilation, is balanced with acidification of the media as a result of ammonium consumption by *Pavlova* strains or bacteria (non-axenic cultures) or (3) *Pavlova* strains can metabolize organic carbon, available only in the organic fertilizer medium, in mixotrophic conditions, instead of performing photosynthesis.

The first hypothesis was supported by the results obtained by Racharaks *et al.*, which suggested that a high buffer capacity of culture media could minimize the influence of the opposing mechanisms responsible for alkalization or acidification of the media[236].

The second hypothesis was already discussed in **Section 4.2.1**, based on the results obtained by Eustance *et al.*[282] and Scherholz and Curtis[283]. Both studies showed that microalgal growth on media supplemented with ammonium as nitrogen source or both ammonium and nitrate results in a significant decrease in pH during the exponential phase causing microalgae growth inhibition. Additional data about who might be responsible for ammonia consumption (*Pavlova* cells or bacteria) which may be balancing pH variation with nitrate consumption is provided by the following growth assessment of bacteria (contaminants), present in non-axenic cultures used in the present work.

The effects of organic fertilizer on bacterial growth can be seen in **Table 15** and it can be detected no statistically CFU mL^{-1} variations between organic fertilizer and control conditions, except in the following conditions of experiment 3: non-sterile OF w/ and w/o vitamins B_1 and B_{12}, OF w/ vitamin B_1, OF w/o vitamin B_{12} and control (f/2) w/ vitamins B_1 and B_{12} (values highlighted at grey in the **Table 15**) (p <0.05).

In the mentioned conditions, it was observed a significant decrease (around $150x10^4$ to $302x10^4$ CFU mL^{-1}) in bacterial number in the final of the experiment. The results obtained are not sufficient to clarify this data, however this decrease could be related to the higher number of bacteria present at the beginning of experiment 3 compared to the number of bacteria present at the beginning of experiments 1 and 2.

Nevertheless, this data was important to prove that the organic matter available in the organic fertilizer media did not potentiate the growth of bacteria present in the microalgae non-axenic cultures. Moreover, it also suggested that, if it is proved that the low pH variation under organic fertilizer conditions is related to the balanced consumption of ammonia and nitrate, it does not appear to be associated to ammonium uptake by bacteria, since no increase in number of bacteria was observed. Thus, the selected *Pavlova* strains might be responsible for ammonium consumption.

Regarding the third hypothesis, Guihéneuf *et al.*[295,296] and Bashir *et al.*[297] demonstrated that *P. lutheri* cell density and lipid content are significantly enhanced in mixotrophic conditions with acetate (2.07 and 5mM) or 10 mmol of sucrose, respectively, compared to the microalgal phototrophic growth.

According to Racharaks *et al.* the opposing hypotheses 2 and 3 could be correlated in order to explain pH variations of marine microalgae cultivation in an AD effluent (such as an organic fertilizer). The authors suggest that the effect of algal dissolved organic carbon uptake is favoured when ammonium is used as the nitrogen source[236].

Coupled to the requirement of understand the effects of ammonium consumption, further work must be done to prove the organic carbon uptake by *P. pinguis* and *P. gyrans*, then to search the effects of organic matter levels and composition on microalgae cell growth and lipid metabolism.

Table 15: Average value ± standard deviation (n=3) of the effect of organic fertilizer in growth of bacteria (CFU mL^{-1}). Values in the same line with different superscript letter (a-b) are statistically different at p <0.05 by Tukey's test.

Microalgae strain	Condition	x10^4 CFU $mL^{-1}_{initial}$	CFU mL^{-1}_{final}
	Experiment 2		
P. pinguis	**$M2_{N1}$+F2**	25±7 ^{a}	145±83x10^3 ^{a}
	Control (f/2)	25±7 ^{a}	108±56x10^3 ^{a}
	Experiment 3		
	$M1_{N1}$+F2	19±7 ^{a}	33±19x10^4 ^{a}
	$M2_{N1}$+F2	19±7 ^{a}	41±38x10^4 ^{a}
	$M4_{N1}$+F2	19±7 ^{a}	22±3x10^4 ^{a}
	Non-sterile $M2_{N1}$+F2	50±8 ^{a}	36±10x10^4 ^{a}
	Control (f/2)	19±7 ^{a}	110±33x10^3 ^{a}
	Experiment 4		
P. gyrans	**OF w/ vitamin B_1 and B_{12}**	309±48 ^{a}	190±66x10^4 ^{a}
	OF w/o vitamin B_1 and B_{12}	309±48 ^{a}	240±71x10^4 ^{a}
	Non-sterile OF w/ vitamin B_1 and B_{12}	352±25 ^{a}	175±124x10^4 ^{b}
	Non-sterile OF w/o vitamin B_1 and B_{12}	352±25 ^{a}	188±120x10^4 ^{b}
	OF w/ vitamin B_1	309±48 ^{a}	70±30x10^4 ^{b}
	OF w/ vitamin B_{12}	309±48 ^{a}	141±39x10^4 ^{b}
	Control (f/2) w/ vitamin B_1 and B_{12}	309±48 ^{a}	71±26x10^3 ^{b}

4.2.5. Effect of organic fertilizer on microalgal lipid content

The results from the lipid content of *P. gyrans*, cultivated in either the organic fertilizer media or the f/2 medium, in the experiment 3, are shown in **Fig. 10**. The lipid content of this species in conditions $M1_{N1}$+F2 and $M2_{N1}$+F2 were statistically higher than the values obtained in control condition (p<0.05). The highest lipid content was founded in condition $M1_{N1}$+F2 (15.47±1,47 % w/w), which promoted an increase by ≈19 % on lipid content when compared to the control lipid content (11.25±0.98 % w/w). Conditions $M4_{N1}$+F2 and non-sterile $M1_{N1}$+F2 exhibited a lipid content equivalent to $M1_{N1}$+F2, $M2_{N1}$+F2 and control conditions (p<0.05).

The lipid content was as high as 15.47±1.47 % of the dry cell weight when the enriched organic micronutrients solution loading was 0.0002 % v/v ($M1_{N1}$) and appears to slight decreased as the organic micronutrients concentration increased (**Fig. 10**). These results were consistent with those obtained by Cai *et al.*[240] and Wang *et al.*[274] when both cultivated *N. salina* and *Chlorella* sp., respectively, into an AD effluent.

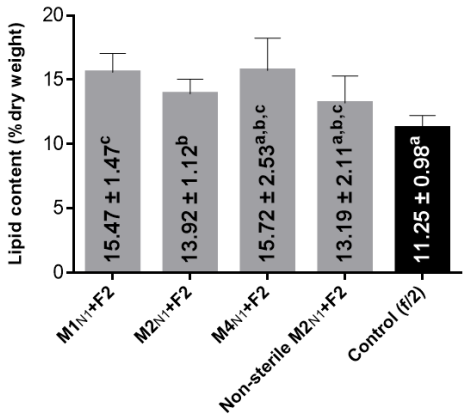


Fig. 10 Average value ± standard deviation (*n*=3) of the effect of organic fertilizer in lipid content of *P. gyrans* compared to control culture (f/2 medium). Values with different superscript letter (a-b) are statistically different at $p<0.05$ by Tukey's test; bars indicate standard deviation.

In addition, the lower N and P concentrations of the condition N1 compared to control condition may have positively impacted the microalgal lipid content. As it was already demonstrated, depleted nutrient medium is a stress condition who results in cell growth cessation and as a consequence, the lipid content (energy store) of some microalgae increases as a survival strategy[129,298-301]. The possible relation between ammonium uptake and mixotrophic metabolism also could contribute to increase the lipid content, since it was recently proved, by Li *et al.*, that presence of ammonium and organic carbon increase the microalgal growth and its lipid content in mixotrophic conditions[302]. The lipid content of Pavlova strains was not only affected by nutrient availability, but also by other factors such as photoperiod, temperature, irradiance, and salinity[90,130,295].

The lipid content of *P. lutheri* is usually in the range of 15-36 of dry cell weight[117,130,297,303]. On the other hand, the lipid content of *P. pinguis* is about 7.6 of dry cell weight[101], which is more in agreement with the present work (11.25±0.98 % to 15.47±1.47 % w/w) (**Fig. 10**), since *P. gyrans* was described as phylogenetically more similar to *P. pinguis* than to *P. lutheri* [154] .

Racharaks *et al.* obtained a lipid content of 30.8±2.2 % and 13.5±0.3 (w/w) when cultured *N. salina* and *D. tertiolecta*, respectively in an AD effluent. These values were equivalent to those obtained in control cultures[236]. Cai *et al.* also determined a lipid content of 35 % of the dry cell weight in *N. salina* cultivation using an AD effluent. The lipid productivity obtained in this study was much higher than for *N. salina* fed with artificial sea water in outdoor ponds[240].

In the present work, the highest lipid content obtained was 15.47±1.47 % (w/w). This value is lower than the previously mentioned in *N. salina* cultures[236,240], however this value is higher

than both obtained from *P. gyrans* cultivated in control medium and *D. tertiolecta* culture in AD effluent[236]. These results enhance even more the ability of the organic fertilizer replace the synthetic culture media.

The present work is the first to evaluate the effect of an organic fertilizer on growth and lipid content of the microalgae *P. gyrans*. We reported a significantly increase of the growth parameters and lipid content compared to those obtained in synthetic f/2 medium (p <0.05).

Nevertheless, further work must be done to assess the effect of this organic fertilizer in fatty acids profile with the main goal of verify whether the increase in lipid content was extended to the n-3 PUFAs, EPA and DHA.

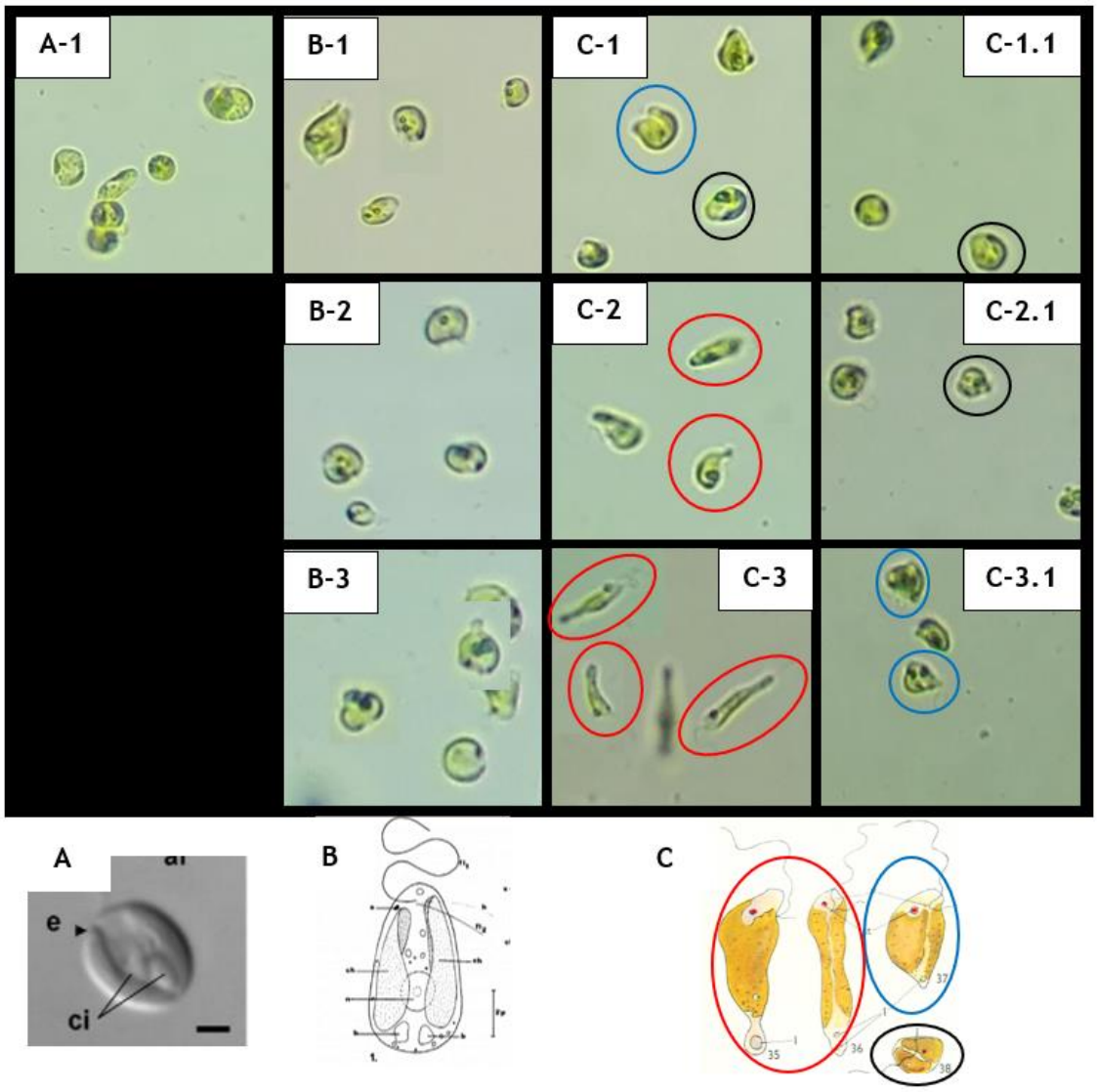
4.2.6. Microscopic observations of *P. lutheri*, *P. pinguis* and *P. gyrans*

P. lutheri cells were described as spherical and not metabolic motile cells. In contrast *P. pinguis* and *P. gyrans* were more irregular, in terms of cells morphology, presenting either elongate, ovoid or amoeboid cells. Both species are strongly metabolic cells[154,156,157].

In the present work, the three *Pavlova* species when cultivated in f/2 medium, commonly used for growth of marine microalgae species, exhibited spherical and non-motile cells at all over the time culture. However, generally, in the very final days of each experiment (stationary phase) these microalgae began to show some motility, which led to us to considerer that these exchanges amongst non-motile and motile cells could be related to growth cease as a result of nutrient-depleted conditions or pH of culture media above 9.

This theory was partially refuted when we replaced f/2 medium by an organic fertilizer in order to growth *P. lutheri* (**Fig. 11A**), *P. pinguis* (**Fig. 11B**) and *P. gyrans* (**Fig. 11C**). We realize that besides obtaining higher cell growth, around the days 4 and 6 of cultivation the microalgae *P. gyrans* exhibited motile cells, in contrast with the same cells growing in f/2 medium. Therefore, we conclude that the microalgae growth cessation is not related to the appearance of motility cells and that organic fertilizer might be more suitable to *Pavlova* cell growth.

Fig. 11 Microscopic observations of *P. lutheri* (A), *P. pinguis* (B) and *P. gyrans* (C) cultivated in organic fertilizer compared to literature observations. *P. gyrans* cultivated in organic fertilizer was also compared with *P. gyrans* cultivated on f/2 medium (1.1 to 3.1). Day 0 (1); Day 4 (2); End of culture (3). Images A[154], B[157] and C[156] were collected from other researches.



Another important and extremely rare modification it was observed in *P. gyrans* cells morphology when cultured in organic fertilizer. This microalga strain completely changed its morphology, around the days 4 and 6 of culture from spherical cells to elongate and apparently smaller cells (**Fig. 11C-2**) compared to cells growing under control conditions (**Fig. 11C-2.1**). *P. lutheri* (**Fig. 11A-1**) and *P. pinguis* (**Fig. 11B-2**) exhibited motile cells at the end of culture and both maintained its morphology, similar to control cells.

We are apparently facing a morphological cell change specific to microalgae *P. gyrans* with higher growth rates. Barely information was described about microalgae morphological changes and the most commonly reports occurs under stress conditions which lead to cell growth cessation. As a consequence, some microalgae metabolites content increase, such as lipids and polyglucans

(stores of metabolic energy) as a survival strategy. This metabolites accumulation generally results in an increase of the cells size[300,304].

Saruwatari *et al.* reported that the haptophyte microalgae *E. huxleyi* also change its size and morphology due to response to temperature and salinity stress conditions. These variations were linked to growth rate decreases, as aforementioned[305].

The present work is the first study to achieve and evaluate how this morphological variation on *P. gyrans* cells efects its growth rates. Nevertheless, the first and only report of achieving this morphological change due to variations in culture conditions was at 1952. Butcher observed that in excess light on the microscope slides *P. gyrans* cells lose its long flagellum and motility. In terms of morphology, the elogates cells become oval or spherical. Butcher suggests that this form might be considered a pathological condition[156].

Our results are in agreement with Butcher observations. The hypothesis that we propose, in which *P. gyrans* could be able to grow in mixotrophic conditions, consuming ammonium and organic carbon, might be able to explain this change, since according to this author light excess slows down the microalgae motility.

As it was previously suggested, ammonium uptake decrease photoshynthetic activity[302], then inhibits this pathological condition, according to Butcher, at light conditions that seem to trigger it in f/2 medium cultures.

Liu *et al.* showed a diferrent perspective, related to the toxicity of copper, lead, and cadmium on the motility of two marine microalgae *I. galbana* and *T. chui*. A significant reduction in motility for I. galbana and T. chui was observed when Cu was present at 31.4 and 1.3 mM, respectively, while for Pb it was 37.8 and 10.9 mM and for Cd it was 121.6 and 37.8 mM, respectively[306]. Both organic fertilizer and f/2 medium used in this work only exhibits Cu, however the Cu concentration in organic fertilizer is much lower than the available in control (f/2) medium. Thus, it is possible that Cu available in f/2 medium could be related to non-motille cells.

Further work must be done in order to evaluate the micronutrients composition and the NO_3^-, NH_4^+ and organic matter content of organic fertilizer, cross micronutrients composition and consumption profiles with morphologic changes and follow cell morphological evolution resorting to a fluorescent stain for detection of intracellular lipid content (Nile red) to understand cell lipid variation.

Overall, our body of work highlighted organic fertilizer, as relevant microalgal culture medium, contributing not only to increasing biomass and lipid productivity of *Pavlova* species but also to obtaining a certified organic biomass.

The results obtained in the present work, require further validation at industrial scale, since the optimal concentrations of the three enriched solutions (macronutrients, micronutrients and iron) might be limiting to cell growth, at industrial scale. Considering that concentrations of NH_4^+ and/or organic matter higher than 0.016 % (N1) or the consumption of NH_4^+ concentration higher than those available in condition N1 might promote conditions that favour cell growth inhibition, a promising approach would be a fed-batch addition of stoichiometrically-balanced concentrations of organic fertilizer[283].

4.3. Evaluation of aquaculture effluent as an effective and low-cost medium

4.3.1. Effect of synthetic aquaculture effluents on growth of *P. pinguis* and *P. gyrans*

The potential of aquaculture effluents replaces the synthetic f/2 medium to microalgae production was evaluated through *P. pinguis* and *P. gyrans* experiments.

We tested three diverse synthetic aquaculture effluents media in order to simulate seasonal variations of the waterbodies. The synthetic effluents had different concentrations of nitrate, nitrite and phosphate, in order to compare the culture growth parameters between each other and synthetic f/2 medium (**Table 16**).

Table 16: Macronutrients characterization of the synthetic aquaculture effluents.

Medium	Theorical concentration (mM)			NO_3^- concentration measured (mM)
	NO_3^-	NO_2^-	PO_4^{3-}	
PRE	13.07	0.21	0.08	16.06±1.22
PIE	15.82	0.19	0.05	17.74±1.71
PLE	18.58	0.19	0.01	20.05±1.53
Control	8.82	-	0.04	8.41±1.27

The cultivation of *P. pinguis* (**Fig. 12-A**) showed biomass productivities of $1.74\pm0.29x10^6$, $1.47\pm0.15x10^6$ and $1.25\pm0.09x10^6$ cells mL^{-1} d^{-1} in PRE, PIE and PLE, respectively. In the same trend, the cultivation of *P. gyrans* (**Fig. 12-B**) showed biomass productivities of $0.77\pm0.16x10^6$, $0.65\pm0.18x10^6$ and $0.43\pm0.20x10^6$ cells mL^{-1} d^{-1} in PRE, PIE and PLE respectively. While the

biomass productivities in f/2 medium were 2.23±0.30x10^{6} cells mL^{-1} d^{-1} for *P. pinguis* and 1.06±0.09x10^{6} cells mL^{-1} d^{-1} for *P. gyrans* (**Table 17**).

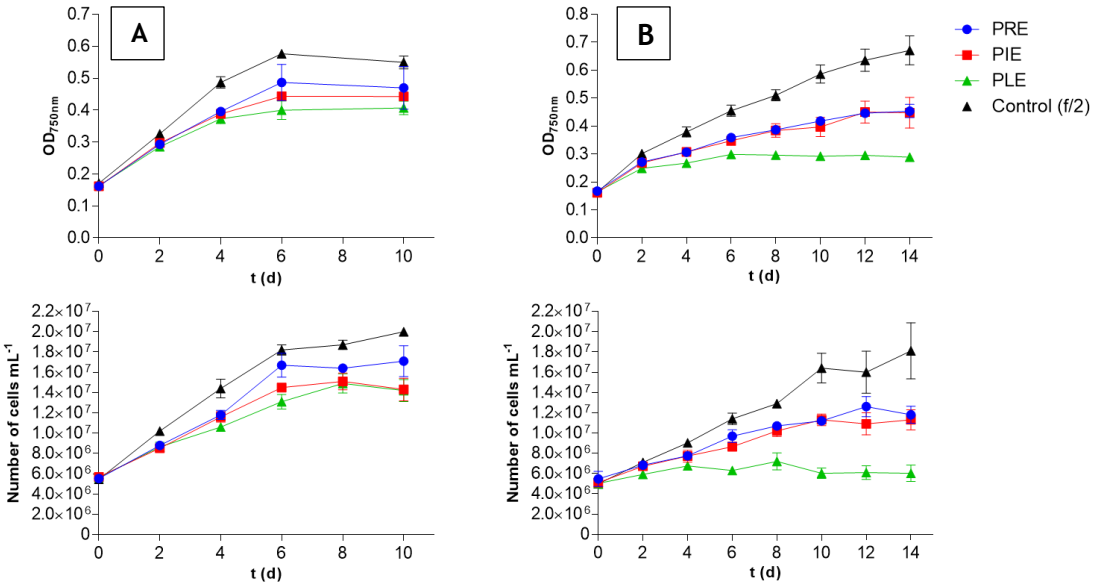


Fig. 12 Growth curves of *P. pinguis* (A) and *P. gyrans* (B) in the synthetic aquaculture effluents compared to control culture (f/2 medium). PRE-Phosphate rich effluent; PIE-Phosphate intermedian effluent; PLE-Phosphate limited effluent; bars indicate standard deviation (n=3).

The biomass productivities of *P. pinguis* when cultured in PRE and *P. gyrans* when cultured in PRE and PIE are statistically equivalent to values obtained in control cultures, **Table 17**. However, in terms of maximum cell density, the results reached by both microalgae species in mentioned aquaculture effluents, are statistically lower than those reached in control cultures (p <0.05).

Contrary to control medium, aquaculture effluents contain some differences, in terms of nitrate and phosphorus concentrations. Whereas nitrate concentrations from PRE, PIE and PLE (16, 18 and 20 mM, respectively) is considerable higher than in control medium, the range of phosphorus concentrations (0.01 to 0.08 mM) are similar to the content available in control medium (0.04 mM).

However, Laws *et al.* described the relationship between growth rate and phosphorus concentration as nonlinear and well defined by the Monod equation, with a half-saturation constant of 2.6 nM. This means that levels of phosphorus above 5.2 nM do not produce variations on growth rate. The determined value is as much as four orders of magnitude lower than the phosphorus concentration available in the aquaculture effluents[307]. Carvalho *et al.* supported this affirmation

showing that phosphorus concentrations in culture media between 0.014 and 0.9 mM do not significantly changed *P. lutheri* cell density.

We imply that variation of phosphorus concentration in aquaculture effluents is not directly linked to cell density decrease. Otherwise, as previously mentioned nitrogen concentrations are higher than those available in control medium. Among the aquaculture efluents used, both *P. pinguis* and *P. gyrans* exhibited higer cell densities in PRE, which contains the lowest nitrate concentration (987.9 mg L^{-1} which corresponds to 16 mM). The reviewed literature, shows that the highest nitrogen concentration in aquaculture efluent already tested was 348.2 mg L^{-1} only[81,192,248-251].

It seems that nitrate concentrations between 16 and 20 mM have a slight limiting effect on the growth of *P. pinguis* and *P. gyrans*. Interestingly, when Taziki *et al.* grew *C. vulgaris* in high concentrations of nitrate and nitrite, they found that at 3000 mgL^{-1}, nitrate appeared to inhibit microalgal growth but not nitrate uptake.

Indeed, *P. pinguis* and *P. gyrans* cultured in PRE and PIE had lower cell densities than in control conditions but mantained comparable nitrate uptakes. Otherwise, when both species were cultured in PLE showed a significantly decrease in cell density and nitrate uptake (**Table 17**).

These findings may suggest that in nitrate excess media (until 18 mM of nitrate) both *Pavlova* species decrease the fraction of nitrate consumed intended to cell gowth metabolism.

Table 17: Average value ± standard deviation (n=3) of the effects of the synthetic aquaculture effluents on the growth rate (μ_{max}), biomass productivity (P_x) and maximum cell density of *P. pinguis* and *P. gyrans*. Values in the same column with different superscript letter (a-c) are statistically different at p <0.05 by Tukey's test.

Microalgae strain	Medium	μ_{max} (d^{-1})	P_x (x10^6 cells mL^{-1} d^{-1})	Max. cell density (x10^7 cells mL^{-1})
P. pinguis	**PRE**	0.19±0.01 ^{a}	1.74±0.29 ^{a,b}	1.79±0.12 ^{b}
	PIE	0.18±0.01 ^{a}	1.47±0.15 ^{a}	1.56±0.03 ^{a}
	PLE	0.16±0.01 ^{a}	1.25±0.09 ^{a}	1.50±0.12 ^{a}
	Control (f/2)	0.24±0.02 ^{b}	2.23±0.30 ^{b}	2.02±0.02 ^{c}
P. gyrans	**PRE**	0.10±0.03 ^{a}	0.77±0.16 ^{a,b}	1.27±0.11 ^{b}
	PIE	0.10±0.02 ^{a}	0.65±0.18 ^{a,b}	1.16±0.10 ^{a,b}
	PLE	0.07±0.04 ^{a}	0.43±0.20 ^{a}	0.75±0.05 ^{a}
	Control (f/2)	0.15±0.02 ^{a}	1.06±0.09 ^{b}	1.84±0.28 ^{c}

4.3.2. Effect of aquaculture effluents on nitrate (NO^{3-}) removal from aquaculture effluents

Through the quantification of NO^{3-} concentration during the time of culture of both microalgae species (**Fig. 13**), we showed that the highest NO_3^- consumptions (38 and 58 %) are associated to lowest nitrogen concentration available in control medium (**Table 18**). These results clearly showed that under nitrogen conditions between 16 and 20mM *P. pinguis* and *P. gyrans* were inhibited.

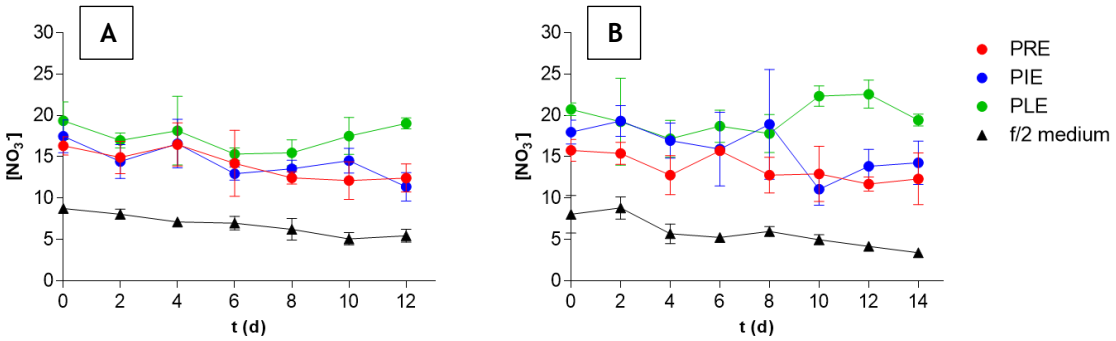


Fig. 13 Effects of *P. pinguis* (A) and *P. gyrans* (B) cultivation at synthetic aquaculture effluents on nitrate (NO_3^-) consumption compared to control culture (f/2 medium). PRE-Phosphate rich effluent; PIE-Phosphate intermedian effluent; PLE-Phosphate limited effluent; bars indicate standard deviation (n=3).

Most of the work carried out with aquaculture effluents resorted to effluents enriched with a N content (4.03 to 34.1 mg/L) much lower than that used in the present work (987.9 to 1544.7 mg/L). Regardless to the P content, the values range is similar amongst different studies (0.42 to 8.82 mg/L). All the reported studies achieved removal efficiencies in the range of 75.4 to 89.2 % for N and 82.7 to 100 % for P contents[81,192,248,249,251], while, in the present work the removal efficiencies for NO_3^- varies between 2 and 58 % (**Table 18**).

Ansari *et al.* cultivated *C. sorikiniana* and *A. falcatus* in an aquaculture effluent supplemented with 200, 400, 600 and 1500 mg/L of $NaNO_3$ and observed that higher biomass productivities and lower lipid content were associated to increasing $NaNO_3$ concentrations. Indeed, cultures grown at aquaculture effluent supplemented with 1500 mg/L of $NaNO_3$ resulted in highest biomass productivities and lowest lipid content (15.05 and 20.50 % w/w) for *C. sorikiniana* and *A. falcatus*, respectively, due to nitrate excess[249]. These authors determined that *C. sorikiniana* and *A. falcatus* were able to grow in nitrate excess conditions.

Table 18: Average value ± standard deviation (*n*=3) of the effects of the synthetic aquaculture effluents on nitrate removal (NO_3^-) by *P. pinguis* and *P. gyrans*.

Condition	% NO_3^- consumption	
	P. pinguis	*P. gyrans*
PRE	24±3	22±4
PIE	35±4	21±4
PLE	2±3	6±1
f/2	38±1	58±3

In addition, photosynthetic activity of *P. gyrans* was also studied in terms of pH variation (**Table 19**). It was not possible to measure the pH values when evaluating *P. pinguis* growth. In the *P. gyrans* experiment, the media PRE and f/2, which promote higher cell growth, were the ones when it was observed major pH variations, such as 0.6±0.1 and 0.7±0.2, respectively. The synthetic aquaculture effluents have no organic compounds, then the cell growth only takes place trough photosynthesis. Therefore, superior pH variations were associated to higher growth rates and biomass productivities.

Table 19: Value ± standard deviation (n=3) of the effects of *P. gyrans* cultivation on pH.

Condition	Initial pH	Final pH	ΔpH
PRE	8.2±0.0	8.8±0.1	0.6±0.1
PIE	8.3±0.1	8.6±0.2	0.3±0.3
PLE	8.3±0.0	8.5±0.1	0.2±0.1
Control (f/2)	8.5±0.1	9.2±0.1	0.7±0.2

Interestingly, the number of bacteria In the *P. gyrans* experiment did not variate in f/2 medium cultures, however increased significantly in aquaculture effluents cultures (**Table 20**). These results suggest that aquaculture effluents composition (high nitrate levels and presence of nitrites) might potentiate bacterial growth instead microalgal growth and require further approval.

Table 20: Average value ± standard deviation (n=3) of the effect of synthetic aquaculture effluents in growth of bacteria (CFU mL^{-1}). The initial CFU mL^{-1} values were determined only on n=3 control condition. Values in the same line with different superscript letter (a-b) are statistically different at p <0.05 by Tukey's test.

Condition	$x10^4$ CFU/$mL_{initial}$	$x10^4$ CFU/mL_{final}
PRE	27±5 ^{a}	95±8 ^{b}
PIE	27±5 ^{a}	132±52 ^{b}
PLE	27±5 ^{a}	93±8 ^{b}
Control (f/2)	27±5 ^{a}	35±11 ^{a}

4.3.3. Effect of aquaculture effluents on microalgal lipid content

The results of lipid content obtained from *P. pinguis* and *P. gyrans*, cultivated in either synthetic aquaculture effluents or f/2 medium, is shown in **Fig. 14**. The lipid content of *P. pinguis* and *P. gyrans* were statistically similar than the content of control cultures (p <0.05).

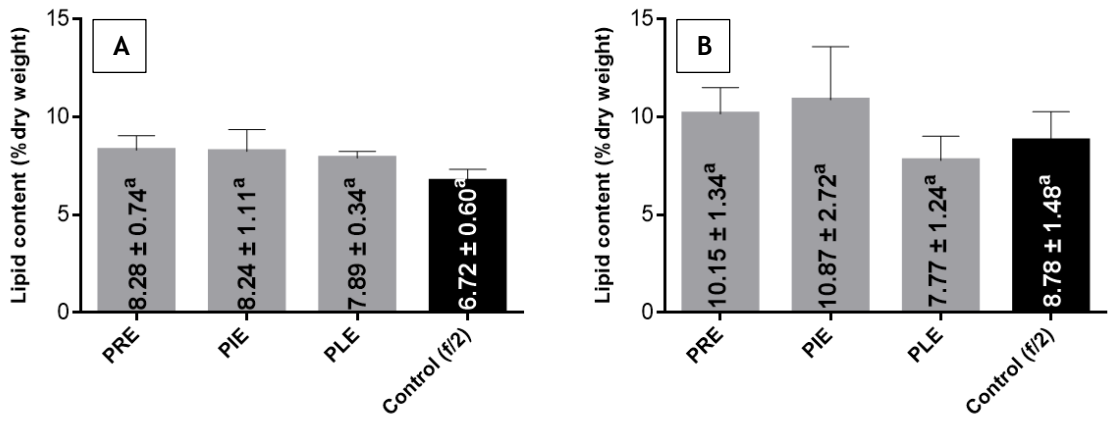


Fig. 14 Effects of *P. pinguis* (A) and *P. gyrans* (B) cultivation at synthetic aquaculture effluents on lipid content compared to control culture (f/2 medium). PRE-Phosphate rich effluent; PIE-Phosphate intermedian effluent; PLE-Phosphate limited effluent; bars indicate standard deviation (n=3).

The lipid content of *P. pinguis* is about 7.6 of dry cell weight[101], which is in agreement with the present work (6.72±0.60 % to 8.28±0.74 % w/w) (**Fig. 14**). Since *P. gyrans* was described as phylogenetically more similar to *P. pinguis* than to *P. lutheri*[54], the obtained values from *P. gyrans* cultures (8.78±1.48 % to 10.87±2.72 % w/w) are also similar to *P. pinguis* lipid content.

Contrarily to Ansari *et al.* study, both *P. pinguis* and *P. gyrans* exhibited a less cell growth when cultivated in nitrate excess conditions and higher nitrate concentrations do not decrease its lipid content.

The present work is the first to evaluate the effect of synthetic aquaculture effluents on growth and lipid content of the microalgae *P. pinguis* and *P. gyrans*. We reported a significantly decrease in terms of maximum cell density and a similar lipid content compared to those obtained in synthetic f/2 medium (p <0.05).

Further work must be done to assess the effect of these synthetic aquaculture effluents in diluted concentrations as form to overtake the reported decrease of maximum cell density and hold potential of aquaculture effluent as microalgal culture medium.

5. Conclusions

We attempted to evaluate the possibility of cultivating *Nannochloropsis* in heterotrophic conditions, and to understand the viability of replacing synthetic culture medium by agro-industrial by-products in *Pavlova* production, namely organic fertilizer and synthetic aquaculture effluents.

The microalgae *N. gaditana* and *N. oceanica* exhibited absent cell growth in heterotrophic conditions, preventing further implementation of these microalgae in a hetero-photoautotrophic two-stage cultivation process. The use of organic fertilizer as a culture medium to *Pavlova* cultivation significantly increased microalgal cell growth and lipid content, when compared to control cultures (f/2 medium). Moreover, we observed that *P. gyrans* cultured in organic fertilizer suffered an interesting phenomenon of cell morphology variation, resulting in smaller, more elongated and motile cells. In contrast, the use of synthetic aquaculture effluents as a culture medium to *Pavlova* cultivation, significant decreases the cell growth, suggesting the inhibitory impact of high nitrate concentrations (16 to 20mM). However, the lipid content was unaltered.

Overall, the presented results highlighted the potential of agro-industrial by-products in industrial scale production of microalgae, as more efficient and cost-effective alternatives when compared to more conventional synthetic culture media. Further research at industrial scale is needed to prove these results.

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