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Faculty of Biosciences, Fisheries and Economics

Phototrophic, heterotrophic and mixotrophic growth in cold-adapted marine microalgae

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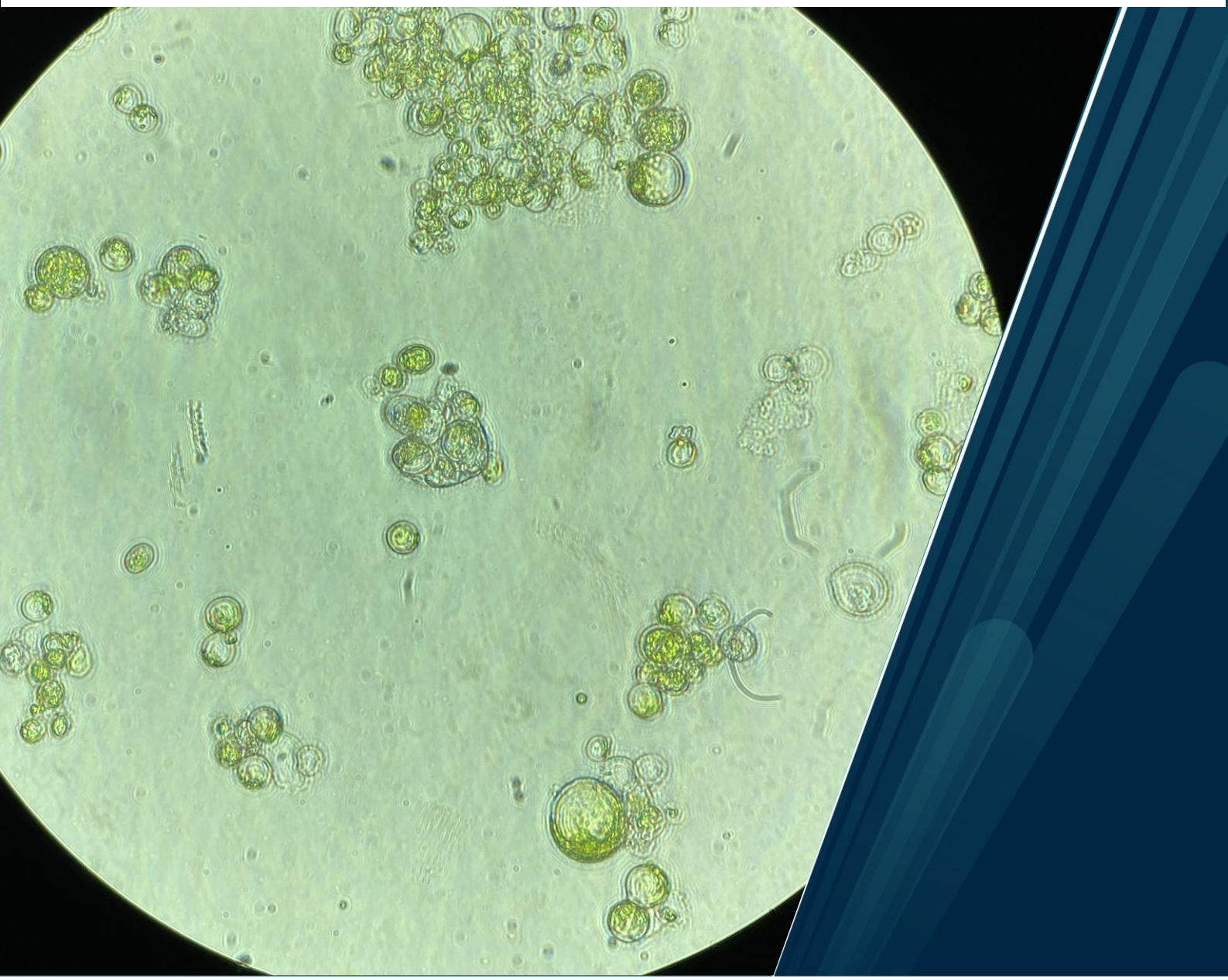

"Life is full of changes … you can fight it or try to make the best of it. And that is all a lot easier if you have got the people who love you to help"

UIT NORGES ARKTISKE UNIVERSITET

Abstract

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Phototrophic, heterotrophic and mixotrophic growth in cold-adapted marine microalgae

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Microalgae are grown in different habitats and conditions, such as high salinity, extreme temperatures, and variable pH. Cold-adapted microalgae may have the capacity of generating high biomass production, which can potentially be produced under cold and light-limited conditions in the Arctic. Most microalgae are obligated photoautotrophs; however, few species have been found living in complete darkness, using heterotrophy as a metabolic path, which allows the algae to obtain energy from organic compounds, such as glucose, glycerol, and acetate. This present thesis investigated the effect of different trophic conditions – phototrophic, heterotrophic and mixotrophic – in the growth performance and the macromolecular composition (protein, carbohydrate, and lipid content) of five different strains of cold-adapted microalgae – *Nannochloropsis oceanica, Dunaliella tertiolecta, Tetraselmis suecica, Chlorella ovalis,* and *Chlorocystis cohnii* –. The results show that only *C. ovalis* and *C. cohnii* were able to grow under heterotrophic conditions. The protein content of all microalgae had the highest production under phototrophic condition, except *N. oceanica*, which had it under mixotrophic condition. However, values of carbohydrates and lipids are only trustful for *C. ovalis*, due to complications in the selected techniques. Therefore, *C. ovalis* had the higher carbohydrate content under the heterotrophic condition while the lipid content was higher in the mixotrophic condition. Thus far, the strains of *N. oceanica, D. tertiolecta*, and *T. suecica* are obligated photoautotrophs in cold environments, while *C. ovalis* and *C. cohnii* are facultative heterotrophs in cold environments.

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Abbreviations

DCW	Dry cell weight
dH_2O	Distilled water
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
GLC	Glucose
HUFA	Highly unsaturated fatty acids
MUFA	Monoinsaturated fatty acid
NIT	Nitrate
OD	Optical density
PTFE	Polytetrafluoroethylene polymer
PUFA	Polyunsaturated fatty acid
Q_X	Biomass productivity for the maximum cell mass produced
SAFA	Saturated fatty acid
μ	Specific growth rate
X	Cell mass produced
X_i	Initial cell mass produced
X_j	Final cell mass produced
$Y_{X/GLC}$	Yield of biomass on glucose
$Y_{X/NIT}$	Yield of biomass on nitrate

Symbols

%	Percentage
°C	Centigrade
µL	Microliter
µm	Micrometer
µmol	Micromol
µmol m^{-2} s^{-1}	Micromol/square meter/second
β	Beta
g	Gram
g/g	Gram/gram
g/L	Gram/liter
h	Hour
L	Liter
m^3	Cubic meter
mg/L	Miligram/liter
mg	Micrometer
mL	Mililiter
min	Minutes
N	Normal
nm	Nanometer
pg	Pico gram
rpm	Revolutions per minute
vvm	Volume of air/Unit of medium/Unit of time

1 Introduction

1.1 Microalgae

Microalgae are a diverse group of photosynthetic microorganisms capable of using CO_2 and light as carbon and energy sources. They live in diverse habitats, such as saline, freshwater, soil, hotsprings, and even in snow. Microalgae can be clasified in green algae (Chlorophyta), brown algae (Phaeophyta), Dinoflagellates (Pyrrophyta), diatoms (Chrysophyta), red algae (Rhodophyta), and Euglenoids (Euglenophyta) (Enamala, et al., 2018). Cyanobacteria (blue-green algae) are also referred as microalgae. It has been estimated that between 200 and 800 thousand species of microalgae exist, considering that of which only approximately 40,000 are known. Nowadays, they are commonly used to generate energy products, such as biodiesel, biofuel, bioethanol, and biomethanol; they are also used in the food, cosmetic, biofertilizer, and bioremediation industries (Wells, et al., 2017).

Microalgae are sources of various bioactive compounds, such as polymers, peptides, fatty acids, carotenoids, toxins and sterols (Venkatesan, et al., 2015). They also have high nutritional value. For example, some species have high-protein content, essential fatty acids, healthy pigments, and antioxidants, among others (Wells, et al., 2017). Besides, microalgae are an extremely diverse collection of organisms with many benefits such as robustness and flexibility in their cultivation conditions as they can grow under different metabolism modes while using simple growth media and can be produced at a large scale (Radmer, 1996; Buono, et al., 2014). Important nutrients for the production of any microalgae species include carbon (e. g. CO_2), nitrate, urea, ammonium, vitamins, phosphorous, nitrogen, iron, manganese, selenium, cobalt, nickel, and zinc (Venkatesan, et al., 2015). They also dependent on light, temperature, nutrient concentration, salinity (for marine species) and pH (Venkatesan, et al., 2015).

Compared to conventional agriculture yields and practices, microalgae have much higher biomass productivities; it can grow in open or closed systems and achieve higher CO_2 fixation, metabolite productivities and growth rates. It also uses less water and land (Raja, et al., 2008).

1.1.1 Polar microalgae

The polar regions are one of the most extreme ecosystems in the planet, which makes it uncommon to be researched for biological sources. However, polar microalgae, are the base of polar food web on those regions. ,Due to the complexity of the environment, polar microalgae have to adapt, not only to the freezing temperatures, but also the solar, osmotic, oxidative, and nutrient stress (Lyon & Mock, 2014).

Polar microalgae must maintain the membrane fluidity at extreme temperature, which is why most of these microalgae have high concentration of polyunsaturated fatty acids (PUFAs) (Lyon & Mock, 2014). The temperature is not the only challenge, the dark adaptation during winter period has the highest importance, studies have shown that Antarctic species have survived from 4 to 9 months in the dark (Peters & Thomas, 1996). Due to this challenge, polar microalgae are able to adapt to the poor light conditions by taking dissolved organic material, such as sugars and starch as carbon and energy sources. Production of polar microalgae may allow to have high PUFA content, which has a great market, which is not yet exploited (Morales-Sánchez, et al., 2020).

1.1.1.1 Polar green microalgae *Nannochloropsis oceanica*

Nannochloropsis oceanica belongs to the division of Ochrophyta, and class of Eustigmatophyceae, which are distinguished for their cytological features, including a reddish pigmented lipid body, a swelling in the flagellum, lamellate vesicles, and plastids without a girdle lamella and lacking continuity with the nuclear envelope (Borowitzka, 2018). This class is known for the straightforward cultivation and high amount of lipids, making them suitable for the production of biofuels, pigments, and long-chain fatty acids (Borowitzka, 2018).

At the moment, only five species of the genus are recognized, one of them is *N. oceanica*, a unicellular, planktonic popular strain that is used for the production of biofuels and fatty acids, which is presented in Figure 1. This specie has a 2-4 µm diameter subspherical shape, containing a yellow-green chloroplast (Borowitzka, 2018). The species is predominantly found in marine environments and freshwater. *N. oceanica* has only been researched in phototrophic and mixotrophic modes of cultivation. However, other species of the genus *Nannochloropsis* have been cultivated heterotrophically.

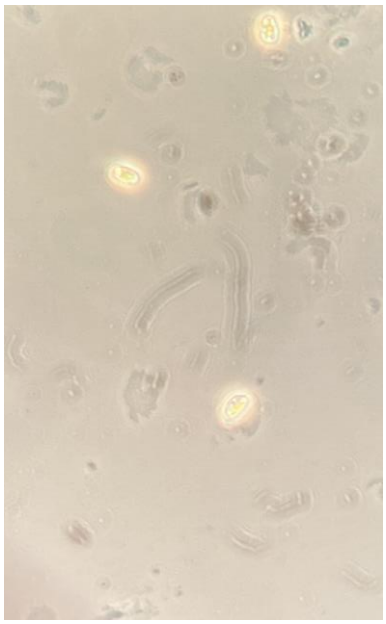

Figure 1. Microscope showing Nannochloropsis oceanica cells grown in f/2 medium and cultivated under phototrophic conditions for 14 days at 6°C. Photo taken by ALTA in a light microscope at 100X magnification.

1.1.1.2 Polar green microalgae *Chlorocystis cohnii*

Chlorocystis cohnii belongs to the division of Chlorophyta, where is common to have yellowish-green or red-green colors due to the presence of carotenoids. They are unicellular, colonial, filamentous, siphonous, and thallus (Matsunaga, et al., 2005). *Chlorocystis'* fits into the class of Ulvophyceae, which are common in benthic marine habitats and are primarily multicellular marine green algae (Leliaert, 2019). The *Chlorocystis* genus has been studied for their lipid and biomass productivities for biodiesel production (Saadaoui, et al., 2018), and it is exclusively found in marine habitats.

C. cohnii (Figure 2) cells have a spherical shape measuring from 16-26 µm in size. These microalgal cells show a bright green color and it is common for these cells to be adhered to piles, which can retain the moisture longer making their grown more favorable (Moore, 1900). The genus *Chlorocystis* and the species *C. cohnii* have only been studied phototrophically.

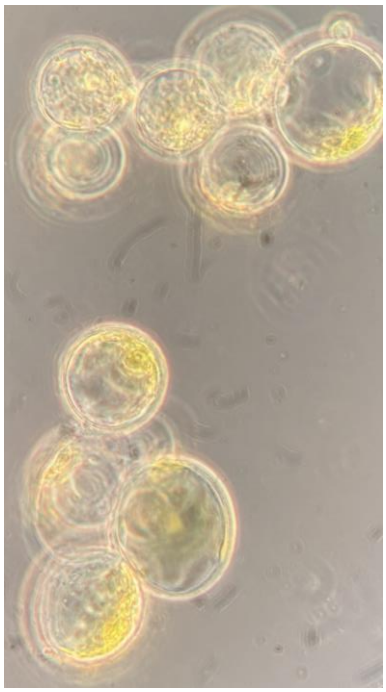

Figure 2. Microscope showing Chlorocystis cohnii cells grown in f/2 medium and cultivated under phototrophic conditions for 14 days at 6°C. Photo taken by ALTA in a light microscope at 100X magnification.

1.1.1.3 Polar green microalgae *Tetraselmis suecica*

Tetraselmis suecica is a green microalgae that belongs to the Chlorophyta division and the Chlorodendrophyceae class, which are distinguished for their lipids that can be used for biofuels (Borowitzka, 2018). This class is known for the unicellular flagellates with 4-8 µm elliptical cells, the flagella are covered by diamond-shaped scales in 24 rows (Borowitzka, 2018).

The genus *Tetraselmis* is well investigated for their lipids for biofuel production, for the feed industry in aquaculture and their easy way to be cultured. *T. suecica* (Figure 3) have been reported to have a total lipid content per dry mass values from 8.5 to 23%, it also has the ability to tolerate a wide range of environmental conditions, such as different temperatures and salinities (Andreaotti, et al., 2019; Borowitzka, 2018). The species are found in marine and freshwater environments. *T. suecica* has been researched in phototrophic, heterotrophic and mixotrophic conditions.

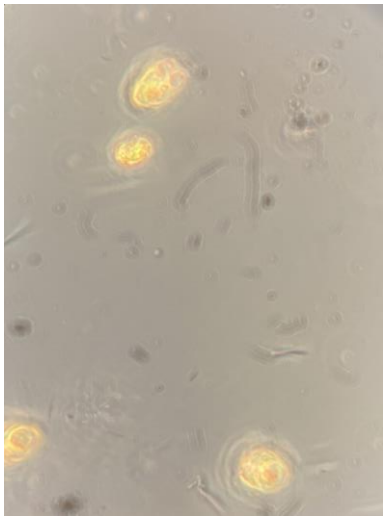

Figure 3. Microscope showing Tetraselmis suecica cells grown in f/2 medium and cultivated under phototrophic conditions for 14 days at 6°C. Photo taken by ALTA in a light microscope at 100X magnification.

1.1.1.4 Polar green microalgae *Dunaliella tertiolecta*

Dunaliella tertiolecta corresponds to the division of Chlorophyta, and class of Chlorophyceae, which are distinguished for their production in the food supplement industry and β-carotene production (Matsunaga, et al., 2005). Normally, the genus *Dunaliella* occur in hypersaline habitats. They are unicellular, biflagellate, uninucleate with a single chloroplast, and they do not contain a cell wall. The cells are 10 µm spherical-shaped. (Borowitzka, 2018). Their production of β-carotene can go up to 14% of dry weight, therefore, they are used in the nutraceutical industry, since the product can be sold as an antioxidant for human health (Borowitzka, 2018).

Dunaliella tertiolecta (Figure 4) have been reported to have an oil yield of 36-42%, it is relatively easy to cultivate, and it is ideal for open cultivation due to its high salinity tolerance that allows sea water to be used. (Chen, et al., 2011). *D. tertiolecta* has an ability to tolerate a wide range of environmental conditions (Andreaotti, et al., 2019). The genus is normally found in marine habitats; however, it can also be found in high salinity environments – salt evaporation ponds and hypersaline lakes –. *Dunaliella* genus has been studied phototrophically, heterotrophically and mixotrophically. However, the species *D. tertiolecta* has only been studied under phototrophic and mixotrophic conditions.

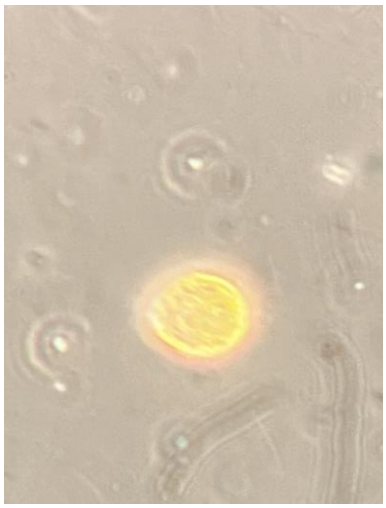

Figure 4. Microscope showing Dunaliella tertiolecta cells grown in f/2 medium and cultivated under phototrophic conditions for 14 days at 6°C. Photo taken by ALTA in a light microscope at 100X magnification.

1.1.1.5 Polar green microalgae *Chlorella ovalis*

Chlorella ovalis corresponds to the division of Chlorophyta, which are known by the green color due to chlorophyll *a* and *b*. *C. ovalis* belongs to the class of Trebouxiophyceae, which are distinguished for being the first ones to be considered for mass cultivation, and the first ones to be commercialized due to their easy cultivation (Borowitzka, 2018). The algae on this class normally are unicellular and coccoid-shaped.

The genus *Chlorella* are 2-10 µm of diameter (Torzillo & Masojídek, 2014), green, nonflagellated, more-or-less spherical algae (Krienitz, 2009); famous for their protein-rich source of food and production of biofuel from carbohydrates and lipids. Nowadays, 44 species of *Chlorella* are recognized (Borowitzka, 2018). Some species of the genus have been researched for their products. For example, some of them have, in DCW basis, up to 60% of proteins, 10-15% of polysaccharides, 12-15% of lipids. Besides, important content of unsaturated fatty acids, carotenoids, vitamins, and minerals have also been found (Torzillo & Masojídek, 2014). The species *C. ovalis* is presented in Figure 5. Several species of *Chlorella* are found mainly in freshwater environments. Some species of *Chlorella* have been investigated under phototrophic, heterotrophic and mixotrophic conditions; however, *C. ovalis* has only been studied under phototrophic conditions.

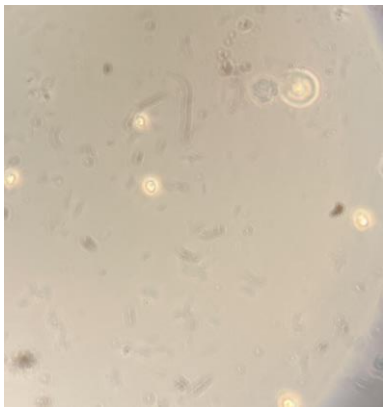

Figure 5. Microscope showing Chlorella ovalis cells grown in f/2 medium and cultivated under phototrophic conditions for 14 days at 6°C. Photo taken by ALTA in a light microscope at 100X magnification.

1.2 Modes of cultivation

Microalgae are by definition phototrophic organisms. But due to the diverse habitats where they can live, microalgae are organisms with high metabolic plasticity (Morales-Sanchez et al. 2020). This means that they are very flexible in terms of the carbon and energy sources that they can use. In this context, we can find microalgae that are obligated phototrophs that can only grow in the presence of light as their energy source. Likewise, there are very few microalgae that can grow in strict darkness switching to a metabolism called heterotrophic by using organic carbon sources. And in the same way, we can find microalgae that can be sustained by both, phototrophic and heterotrophic in a metabolism called mixotrophic, which uses light and organic compounds as carbon and energy sources. The Table 1 summarizes the three most important modes of cultivation available for microalgae. The selection of the mode of cultivation of the microalgae is very important, since it decides the overall productivity, as well as the quantity of macrocomponents (Perez-Garcia & Bashan, 2015).

Table 1. Main characteristics of the three most important modes of cultivation of microalgae.

Mode of cultivation	Energy source	Carbon source
Phototrophic	Light	Inorganic compounds
Heterotrophic	Organic compounds	Organic compounds
Mixotrophic	Light and organic compounds	Inorganic and organic compounds

1.2.1 Phototrophic

Phototrophic conditions are used by microalgae in order to grow, they are based on the use of light (sun/artificial) and CO_2. However, some strains of microalgae do not have an optimal growing in this condition due to, for example, inefficient light supply, which yields diluted cultivations and therefore, low biomass productivity. An important advantage is the low-cost light, carbon dioxide and the use of open cultivations (Perez-Garcia & Bashan, 2015). Some examples of obligated phototrophs are *Volvox carteri*, *Chlamydomonas reinhardtii*, and *Phaeodactylum tricornutum* (Perez-Garcia, et al., 2011).

1.2.2 Heterotrophic

Heterotrophic conditions are used by microalgae in order to grow in darkness, therefore, they obtain the energy from organic compounds, such as glucose, glycerol, and acetate. Some microalgae species have a better cell productivity and lipid content than with phototrophic growth due to the higher energy density in glucose (more carbon molecules) than the ones found in CO_2 (Perez-Garcia & Bashan, 2015; Lowrey & Brooks, 2015; Morales-Sánchez, et al., 2017). One of the advantages for this mode of cultivation are the low costs in infrastructure through simplified bioreactor designs. However, the disadvantages are also considerable, like the additional costs through the organic compounds required to grow, low production of pigments and high value phytochemicals due to dark conditions, high probability of contamination, and the limitation of species that can grow by only using organic compounds. Reported genera include *Chlorella*, *Dunaliella*, *Nannochloropsis*, and *Tetraselmis* (Perez-Garcia & Bashan, 2015; Lowrey & Brooks, 2015).

1.2.3 Mixotrophic

Mixotrophic conditions is a mix between phototrophic and heterotrophic mode of cultivation. The microalgae need light, inorganic and organic compounds, there are several advantages for this type of growing including the presence of pigments and photosynthetic carotenoids, increased growth and resource utilization, higher biomass density and growth rate, and the flexibility to switch between modes (Perez-Garcia & Bashan, 2015; Lowrey & Brooks, 2015). However, there are also disadvantages, like the need of light, organic carbon and O_2 and the limitation of species that can grow in this condition; reported genera having this ability include *Graesiella*, *Dunaliella*, *Chlorella* and *Nannochloropsis* (Perez-Garcia & Bashan, 2015; Lowrey & Brooks, 2015).

1.3 Microalgal macromolecular composition

Microalgae converts solar energy, inorganic, and organic compounds into chemical energy, meaning they mainly produce proteins, carbohydrates, and lipids. But they also produce high-value compounds, such as pigments and vitamins (Barkia, Saari, & Manning, 2019).

1.3.1 Proteins

The structure and metabolism of the microalgae is dependant of the proteins, the biosynthetic building blocks. They are important elements of the membrane and light-harvesting complex, which includes various enzymes that are directly involved in photosynthesis (Barkia, et al., 2019). Microalgal proteins have been known to be an alternative of conventional protein sources, due to the nutrition, high content – 42% to 70% in DCW basis –, and quality – production of all essential amino acids – (Barkia, et al., 2019). However, there are two major disadvantages of protein production from microalgae, the first one is the presence of chlorophyll that can influence the color and taste of microalgae-based products limiting their comercialization; and, the extraction efficiency of the intracellular proteins that can be low due to the rigid cell wall of some strains (Barkia, et al., 2019).

1.3.2 Carbohydrates

Carbohydrates are required for the structure and metabolism of microalgae. They are composed of mono-, oligo-, and polysaccharides, often encountered joint to proteins or lipids. Carbohydrates are very important cellular components because the stability of the cell depends on the complex polysaccharides that compose it. The generation of glucose and starch-like energy storage, the major carbon-containing products of photosynthesis, is another crucial function (Barkia, et al., 2019).

The production of polysaccharides is directly correlated to the type of microalgae, “Cyanophytes are known to accumulate glycogen, although some species synthesize semi-amylopectin. The Chlorophyta synthesize starch in the form of two glucose polymers, amylopectin, and amylose, while Rhodophyta produce a carbohydrate polymer known as floridean starch. Diatoms (Bacillariophycae, Heterokontophyta) produce chrysolaminarin, a linear polymer of β(1,3) and β(1,6) linked glucose units” (Barkia, et al., 2019, p. 5).

Carbohydrate production from microalgae is a suitable option due to the content amount, some studies suggest that diatoms have 30% of their dry weight as (1,3)-β-D-glucan, another case is *T. suecica* which is able to gather between 11% and 47% of its dry weight as starch (Barkia, et al., 2019). The use of these carbohydrates is mainly in the cosmetic industry and also as biofuels like bioethanol (de Jesus Raposo, et al., 2013)

1.3.3 Lipids

Nowadays, research is focusing on the lipids produced by microalgae, specially in the biodiesel, nutraceuticals and infant formulations industries (Qu, et al., 2013). Microalgae contains two important lipid fractions, polar lipids (phospholipids and galactolipids) and neutral lipids (acylglycerols, free fatty acids, and carotenoids). The main fuction of the first ones is to provide structure to the plasma membranes while neutral lipids act as storage energy. (Barkia, et al., 2019).

Fatty acids in microalgae are very common, specially C16 and C18 saturated and unsaturated, also longer carbon-chain lengths, together with various omega fatty acids. The saturated fats are kept in neutral lipid bodies, while the unsaturated ones are kept in the membranes to help with fluidity, therefore, they are related with the polar lipids (Barkia, et al., 2019).

Some studies suggest that the lipid content in microalgae can go from 20% to 50% (DCW basis). Clearly, the lipid productivity depends on the strain of microalga, the modes of cultivation, and temperature, hence values from 1% to 70% have also been documented (Barkia, et al., 2019).

2 Objectives and hypotheses

2.1 Objectives

2.1.1 Main objective

Evaluate the effect of different types of trophic conditions: heterotrophic, mixotrophic and phototrophic in the growth and macromolecular composition of five cold-adapted marine microalgae.

2.1.2 Specific objectives

- Analyze the growth of five cold-adapted marine microalgae under different trophic conditions.
- Establish the growth kinetics and stoichiometric parameters.
- Determine the macromolecular composition - protein, carbohydrate, and lipid content - of five cold-adapted marine microalgae under different trophic conditions.

2.2 Hypotheses

- The five cold-adapted marine microalgae have a higher productivity in mixotrophic conditions compared to phototrophic and heterotrophic conditions.
- The five cold-adapted marine microalga have more macromolecular composition in mixotrophic conditions compared to phototrophic and heterotrophic conditions.
- *Chlorella ovalis* has a higher growing rate in heterotrophic conditions.

3 Materials and Methods

3.1 Workflow

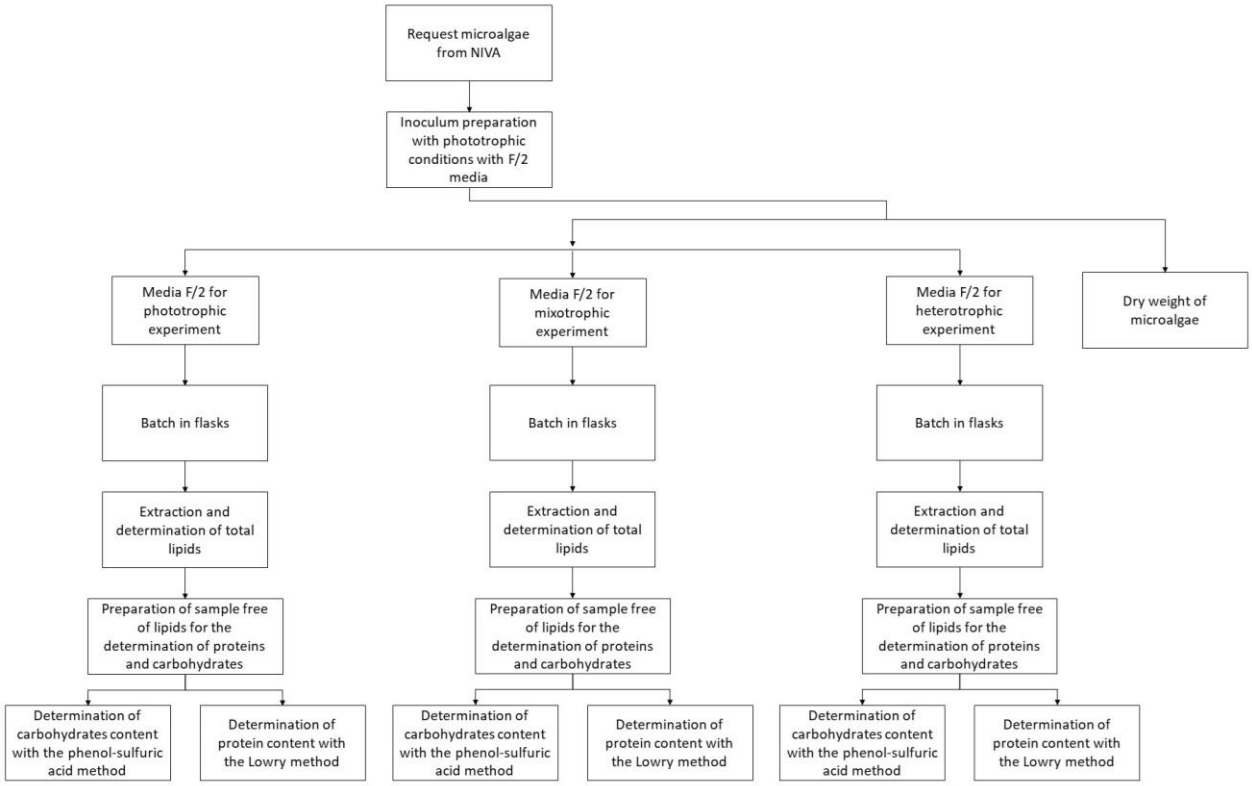

Figure 6. Workflow of the present thesis of phototrophic, heterotrophic and mixotrophic growth in cold-adapted marine microalgae.

3.2 Microalgae

The Table 2 shows the different strains of cold-adapted microalgae studied in the present work.

Table 2. Strains of microalgae used for the experiments of the thesis work of phototrophic, heterotrophic and mixotrophic growth in cold-adapted marine microalgae.

Complete names	Class	Algae bank	Handed state
Nannochloropsis oceanica	Eustigmatophyceae	NIVA	Tube with Z8 media
Tetraselmis suecica	Chlorodendrophyceae	NIVA	Tube with TL30 media
Dunaliella tertiolecta	Chlorophyceae	NIVA	Tube with L1 media
Chlorella ovalis	Trebouxiophyceae	NIVA	Tube with Z8 media
Chlorocystis cohnii	Ulvophyceae	NIVA	Tube with TL30 media

3.3 Cultivation modes

3.3.1 Microorganisms and f/2 medium

Nannochloropsis oceanica (CCAP 2005/1), *Tetraselmis suecica* (CCAP 66/22D), *Dunaliella tertiolecta* (CCMP1320), *Chlorella ovalis* (NIVA-CHL 170) and *Chlorocystis cohnii* (K-0421) were obtained from the NIVA algae bank (Norsk Institutt for Vannforskning). These microalgae were grown in f/2 cultivation medium (Guillard & Ryther, 1962), which contains (per liter of distilled water) 10 mL $NaNO_3$, 10 mL $NaH_2PO_4{\cdot}H_2O$, 10 mL $Na_2SiO_3{\cdot}9H_2O$, 50 mL $MgSO_4{\cdot}7H_2O$, 5 mL KNO_3, 913.5 mL of sea water, trace elements consisting of 0.315 g $FeCl_3{\cdot}6H_2O$, 0.436 g $Na_2EDTA{\cdot}2H_2O$, 100 µL $MnCl_2{\cdot}4H_2O$, 100 µL $ZnSO_4{\cdot}7H_2O$, 100 µL $CoCl_2{\cdot}6H_2O$, 100 µL $CuSO_4{\cdot}5H_2O$, and 100 µL $Na_2MoO_4{\cdot}2H_2O$, and vitamins consisting of 20 mg thiamine·HCl, 100 µL biotin, and 100 µL cyanocobalamin. This medium was autoclaved at 121 C for 115 minutes prior every inoculation.

3.3.2 Inocula preparation

The inoculum for every cultivation test was made in 250 mL shake flasks containing 50 mL of working volume with 10% (v/v) of inoculum. Briefly, to 45 mL of f/2 medium, 5 mL of the strain culture obtained by the algae bank (NIVA) were added to each flask. The flasks were left in a platform shaker for 14 days with LED continuous light at 120 µmol m^{-2} s^{-1}. The inoculum can be observed in the Figure 7.

Figure 7. Microalgae inoculum in phototrophic conditions with f/2 media. (Left to right) D. tertiolecta, C. cohnii, N. oceanica, C. ovalis, and T. suecica.

3.3.3 Cultivation modes set up

In all cultivation modes, f/2 media recipe was prepared following the formula in the section 3.3.1. Additionally, in heterotrophic and mixotrophic cultivations, 3 g/L of D-(+)-Glucose were

added into the f/2 medium. All the cultivation mode experiments were made in in 250 mL shake flasks containing 50 mL of working volume with 10% (v/v) of inoculum. To 45 mL of f/2 medium, 5 mL of the inoculum of each strain was added to each flask. Each cultivation mode was made in at least triplicates. The flasks were left in a platform shaker for 14 days at 6°C. In phototrophic and mixotrophic conditions, the experiments were carried out with LED continuous light at 120 µmol m^{-2} s^{-1}, surrounding them (Figure 8 and Figure 9), and in heterotrophic condition, the containers were covered with aluminum and dark paper to create complete darkness (Figure 10).

Figure 8. Microalgae growth in phototrophic conditions with f/2 media in a platform shaker.

Figure 9. Microalgae growth in mixotrophic conditions with f/2 media in a platform shaker.

Figure 10. Microalgae growth in heterotrophic conditions with f/2 media in a platform shaker.

The microalgae growth curves were created by monitoring the triplicates of cultures every 48 hours and measured by spectrophotometer at 750 nm.

3.3.4 Biomass harvesting and storage

When cells reached stationary phase (that was around 14 days for almost all of them) , the samples were transferred to 50 mL falcon tubes, and centrifugated at 4,000 rpm for 5 minutes at 4°C to remove the supernatant. Then, the microalgae were washed twice with 5 mL of 0.5% ammonium formate to remove salts. After vortexing, the samples were centrifuged at 4,000 rpm for 5 minutes at 4°C and the supernatant was discarded. The pellet was stored at -80°C for further analysis.

3.4 Analytical methods

3.4.1 Growth curves

Glass nitrocellulose microfiber filters of 0.47 µm of diameter were dried in an oven at 105°C for 24 hours (Figure 11). The filters were put in a desiccator and weighted. Dilutions of the five microalgae were made with ammonium formate 0.5% – 1:1, 1:2, 1:5, 1:10, and 1:20 –. A volume of 10 mL of each sample were vacuum filtered, where the product passed through the filter paper and the supernatant was discarded, then twice washed with ammonium formate 0.5%. All filters were dried in an oven at 105°C for 48 hours. After, the filters were put in a desiccator and weighted. The dry weight of the samples was analyzed by weight difference as Equation 1 shows. Each sample was made in duplicates.

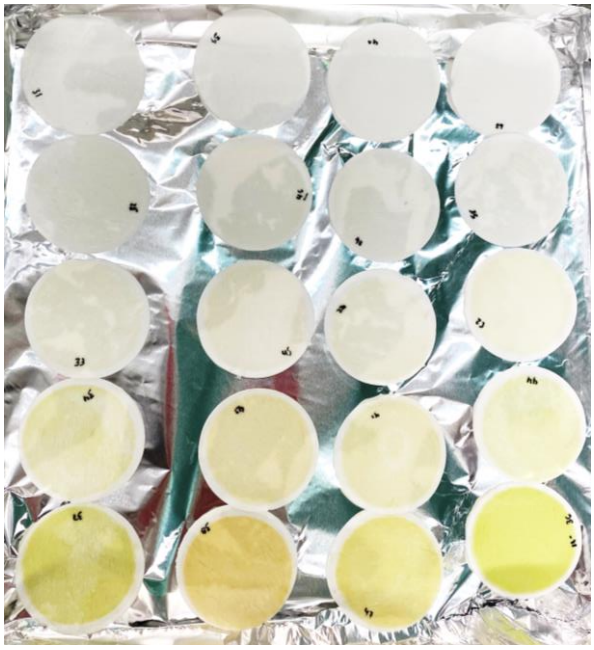

Figure 11. Samples of the microalgae with different dilutions for the DCW work.

$$Dry\ cell\ weight = Dried\ filter\ with\ sample - Dried\ filter$$

Equation 1. Dry weight cell weight formula.

To determine the optical density (OD), dilutions of the five microalgae were made with ammonium formate 0.5% – 1:1, 1:2, 1:5, 1:10, and 1:20 –, and a volume of 200 µL were added into a 96-well microplate in duplicates. The plate was read in a spectrophotometer at 750 nm.

The cell biomass concentration ($g_{DCW}\ L^{-1}$) was correlated by the optical density and dry weight method. A linear regression was plotted using both results of each strain, the result obtained was the equation of the line. The plot and equations of each strain can be found in the Appendix F.

3.4.2 Extraction and determination of total lipids

The frozen microalga samples were centrifuged at 4,000 rpm for 5 minutes at 4°C; 2 mL of solvent of a mix of methanol and chloroform 2:2.5 were added to the samples, then wrapped in aluminum and kept for 48 hours at 5°C. After, the samples with solvent were centrifuged at 4,000 rpm for 5 minutes at 4°C, and the lipids were removed carefully in order to not disturb the pellet and placed in new tubes previously marked.

Another 2 mL of the solvent mix were added to the pellet, mixed, wrapped in aluminum, and kept for 24 hours at 5°C. The samples were centrifuged at 4,000 rpm for 5 minutes at 4°C, the supernatant containing the lipids were removed and placed in the tubes with the previous pool of lipids. Then, 2.5 mL of deionized water were added, centrifuged at 4,000 rpm for 5 minutes at 4°C; the lipids placed in the bottom of bilayer solution were removed (Figure 12) and added to pre-weighted glass tubes. The samples were left to dry at room temperature for 36 hours to determine the total weight of lipids gravimetrically.

The total weight of lipids was measured with the Equation 2.

$$Total\ lipid\ weight\ (g) = Lipids + glass\ weight\ (g) - Empty\ glass\ weight\ (g)$$

Equation 2. Total lipid weight formula.

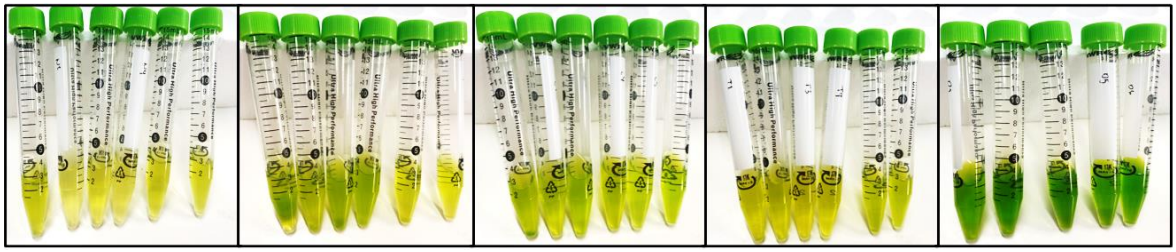

Figure 12. Lipid extraction of (left to right) N. oceanica, C. cohnii, C. ovalis, T. suecica, and D. tertiolecta in mixotrophic cultivation.

3.4.3 Preparation of sample free of lipids for the determination of proteins and carbohydrates

The pellet obtained in section 3.4.2 was divided in two aliquots by resuspending the sample in 1 mL of water, mixing, and separating in two aliquots of 0.5 mL in Eppendorf tubes. Each aliquot was used to extract and quantify the proteins and carbohydrates.

3.4.4 Determination of the protein content

Total protein content was determined by the Lowry method (Lowry, et al., 1951). First, the aliquot obtained in the section 3.4.3 was placed in glass tubes, 3 mL of NaOH 1 N were added; the glass tubes were covered and placed in a water bath at 100°C for 1 hour. A stock of standard protein solution of bovine albumin 0.1% (1 mg/mL), was used to prepare a standard curve by diluting the stock solution with deionized water (Table 3).

Table 3. Standard solution of protein content with bovine albumin 0.1% at 10 different concentrations.

Protein concentration (µg/mL)	Standard solution (µL)	Demineralized water (µL)
0	0	1000
25	25	975
50	50	950
100	100	900
150	150	850
200	200	800
300	300	700
400	400	600
500	500	500
600	600	400

The standard concentrations were treated and read as the samples and the data was used to build a standard curve (Appendix G).

The Lowry method requires the use of 4 different reagents:

- Reagent A: Na_2CO_3 at 2% in NaOH 0.1 N
- Reagent B: mix of 1:1 of two solutions
    - Solution 1: $CuSO_4 \cdot 5H_2O$ at 0.5%
    - Solution 2: potassium sodium tartrate at 1%
- Reagent C: 49 mL of reagent A and 1 mL of reagent B
- Reagent D: reagent Folin 2 N diluted 1:1 with demineralized water

Standard solutions, samples and blank (water) of 20 µL were added to a 96-well plate, then 100 µL of reagent C was added, the solution was left for 10 minutes at room temperature. Then, 10 µL of reagent D were added and left for another 30 minutes at room temperature. After this incubation time, the 96-well plate was read at 590 nm (Figure 13).

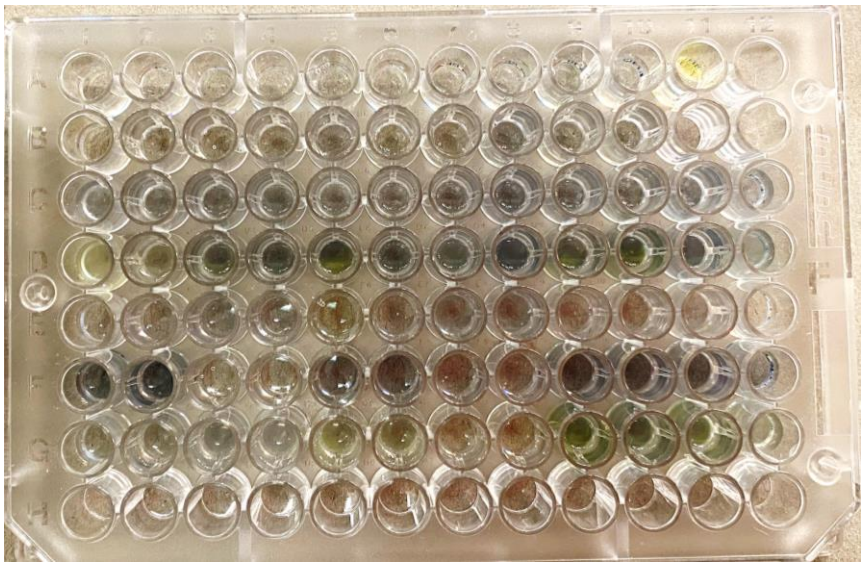

Figure 13. Determination of protein content with the Lowry method in a 96-well plate.

The total concentration of protein was obtained by using the equation obtained in the standard curve (Appendix G).

3.4.5 Determination of the carbohydrate content

Total carbohydrates content was determined by the phenol-sulfuric acid method (Dubois, et al., 1956). First, the aliquot obtained in the 3.4.3 was placed in glass tubes, 3 mL of HCl 2N were added; the glass tubes were covered and placed in a water bath at 100°C for 1 hour. A stock of standard carbohydrates solution of glucose 0.1% (1 mg/mL) was used to prepare a standard curve by diluting the stock solution with deionized water (Table 4).

Table 4. Standard solution of carbohydrates content with glucose 0.1% at 10 different concentrations.

Glucose concentration (µg/mL)	Standard solution (µL)	Demineralized water (µL)
0	0	1000
20	20	980
40	40	960
60	60	940
80	80	920
100	100	900
150	150	850
200	200	800
250	250	750
300	300	700

The standard concentrations were treated and read as the samples and the data was used to build a standard curve (Appendix G).

Microalgae samples were diluted 1:10, 20 µL of standard solutions, samples and blank (water) were added to a 96-well plate, then 20 µL of phenol at 5% was added. Then, 100 µL of H_2SO_4 Were added and left for 30 minutes at room temperature. After this incubation time, the 96-well plate was read at 490 nm (Figure 14).

The total concentration of carbohydrates was obtained by using the equation obtained in the standard curve (Appendix G).

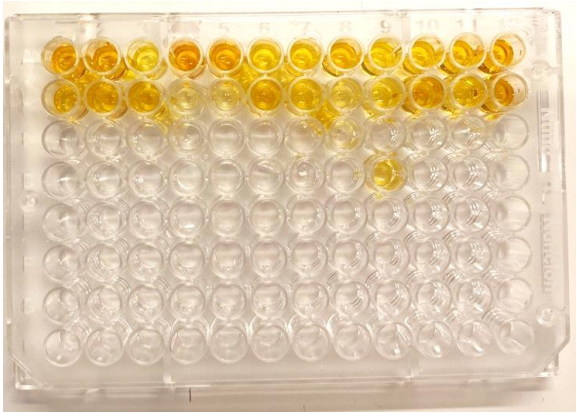

Figure 14. Determination of carbohydrates content with the phenol-sulfuric acid method in a 96-well plate.

3.5 Statistical analysis

For all microalgae in the different cultivations, the normal distribution of data was tested by using the Shapiro-Wilk's test, also the homogeneity of the variance between treatments was examined by using the Brown-Forsythe test. When the tests were positive for normality and homogeneity, then a comparison between the productivity, proteins, carbohydrates and lipids of all microalgae strains and the different modes of cultivation were made by using one-way analysis of variance (ANOVA) and post-hoc Tukey's multiple comparison test. If the results of the test were the opposite, then the data will be analyzed by using the Kruskal-Wallis' test and the Dunn's test. The workflow is illustrated in the Figure 15. Statistical analyses were performed using R software (RStudio Team, 2020) (version 3.6.1) through the RStudio IDE (version 1.2.1335).

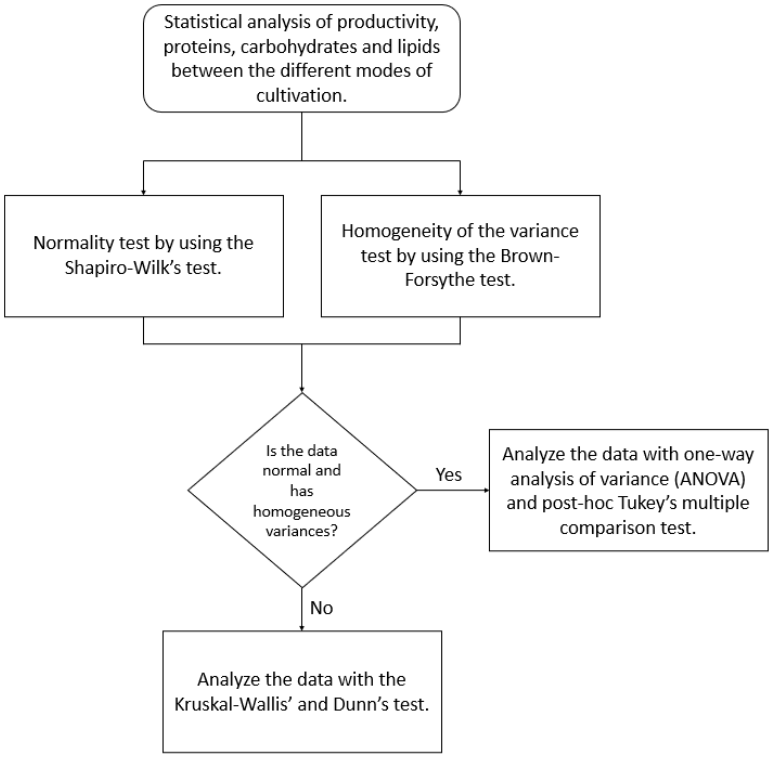

Figure 15. Workflow for the statistical analysis of the data regarding the productivity, proteins, carbohydrates and lipids between the three modes of cultivation.

3.6 Stoichiometric parameter calculations

Four stoichiometric parameters were calculated. The specific growth rates (μ) were determined by fitting the cell number data versus time to exponential regressions for each stage. The cell mass yield for glucose ($Y_{X/GLC}$) was estimated by using Equation 3. The cell mass yield for nitrate ($Y_{X/NIT}$) was estimated by by using Equation 4. Meanwhile, the volumetric productivity of the cell mass and metabolites was determined by using the Equation 5.

$$Y_{\frac{X}{GLC}} = \frac{X_j - X_i}{g_{GLC}}$$

Equation 3. Formula to obtain the cell mass yield for glucose.

$$Y_{X/NIT} = \frac{X_j - X_i}{g_{NIT}}$$

Equation 4. Formula to obtain the cell mass yield for nitrate.

$$Q_X = \frac{X_j - X_i}{Cultivation\ time}$$

Equation 5. Formula to obtain the volumetric productivity of the cell mass and metabolites.

4 Results

4.1 Growth

4.1.1 Nannochloropsis oceanica

The growth of *N. oceanica* cultured phototrophically, heterotrophically and mixotrophically are shown in Figure 16, the results are an average of triplicates. No significant differences in cell mass concentration were found between the phototrophic (3.68 ± 0.46 g_{DCW} L^{-1}) and mixotrophic (4.19 ± 0.03 g_{DCW} L^{-1}) cultivations ($P<0.05$). Cells under heterotrophic conditions did not grow (0 g_{DCW} L^{-1}).

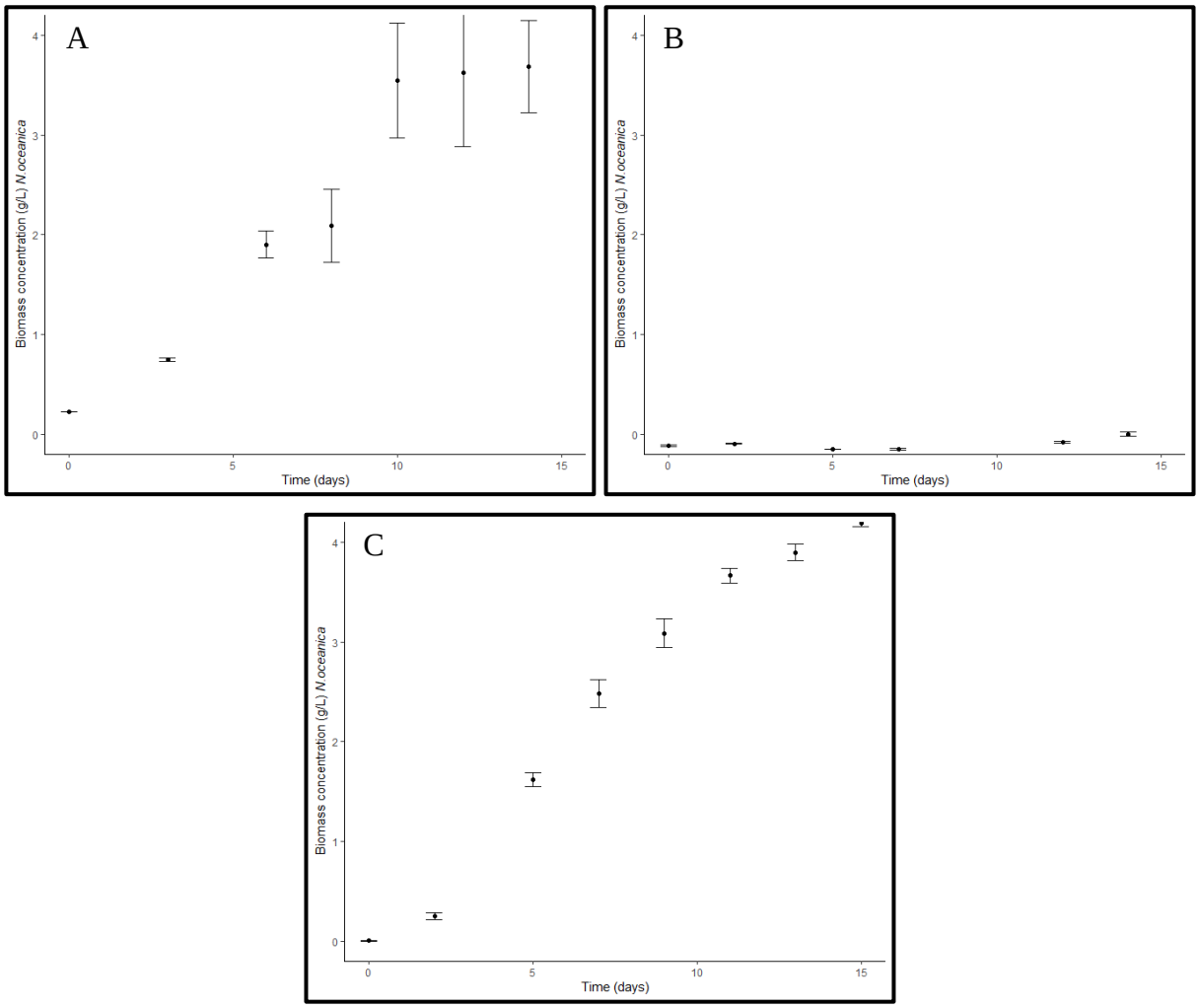

Figure 16. Growth rate of N. oceanica cultured under different trophic conditions (A: phototrophic, B: heterotrophic and C: mixotrophic). Cells were grown in f/2 medium and incubated for 14 days at 6°C, at the different cultivation modes. Results show the average and standard error.

4.1.2 Dunaliella tertiolecta

The growth of *D. tertiolecta* was evaluated under different trophic modes, exhibiting that cell mass concentration was not significantly different between mixotrophic (7.17 ± 0.39 g_{DCW} L^{-1}) and phototrophic (10.72 ± 1.72 g_{DCW} L^{-1}) conditions ($P<0.05$). As shown in Figure 17, cells of *D. tertiolecta* did not grow under heterotrophic conditions (0 g_{DCW} L^{-1}).

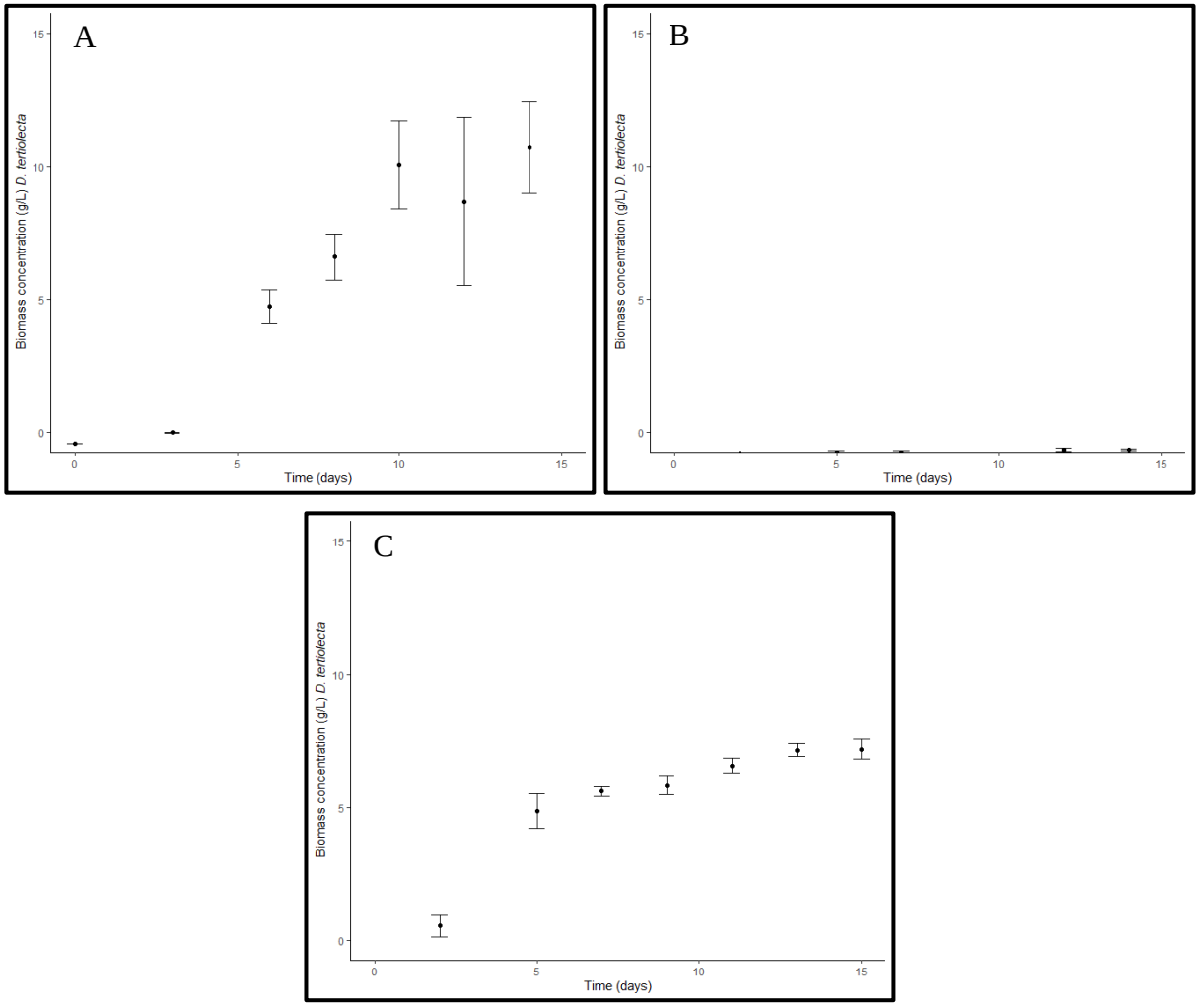

Figure 17. Growth rate of D. tertiolecta cultured under different trophic conditions (A: phototrophic, B: heterotrophic and C: mixotrophic). Cells were grown in f/2 medium and incubated for 14 days at 6°C, at the different cultivation modes. Results show the average and standard error.

4.1.3 Tetraselmis suecica

The cell biomass concentration (average of triplicates) for *T. suecica* in mixotrophic (1.12 ± 0.01 g_{DCW} L^{-1}) and phototrophic culture (1.84 ± 0.05 g_{DCW} L^{-1}) were not significantly different when compared to each other ($P<0.05$). Figure 18, illustrates how the heterotrophic culture did not grow (0 g_{DCW} L^{-1}).

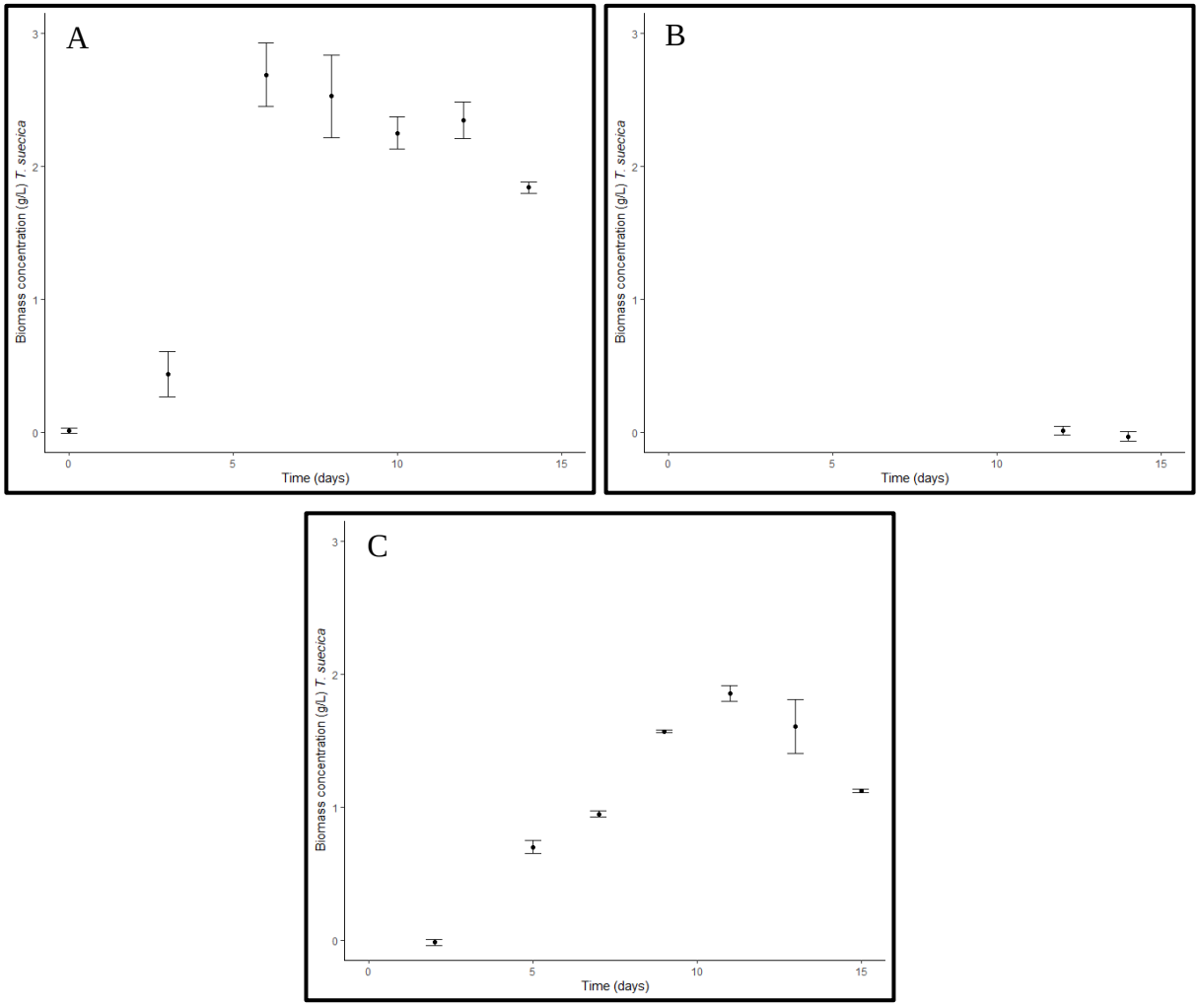

Figure 18. Growth rate of T. suecica cultured under different trophic conditions (A: phototrophic, B: heterotrophic and C: mixotrophic). Cells were grown in f/2 medium and incubated for 14 days at 6°C, at the different cultivation modes. Results show the average and standard error.

4.1.4 Chlorella ovalis

No significant differences were found in *C. ovalis* cell biomass concentration when comparing the three cultivation modes ($P<0.05$) phototrophically (0.59 ± 0.01 g_{DCW} L^{-1}), heterotrophically ($0.47 \pm 0.00 g_{DCW}$ L^{-1}) and mixotrophically (0.54 ± 0.05 g_{DCW} L^{-1}). The results are shown in Figure 19, as an average of triplicates.

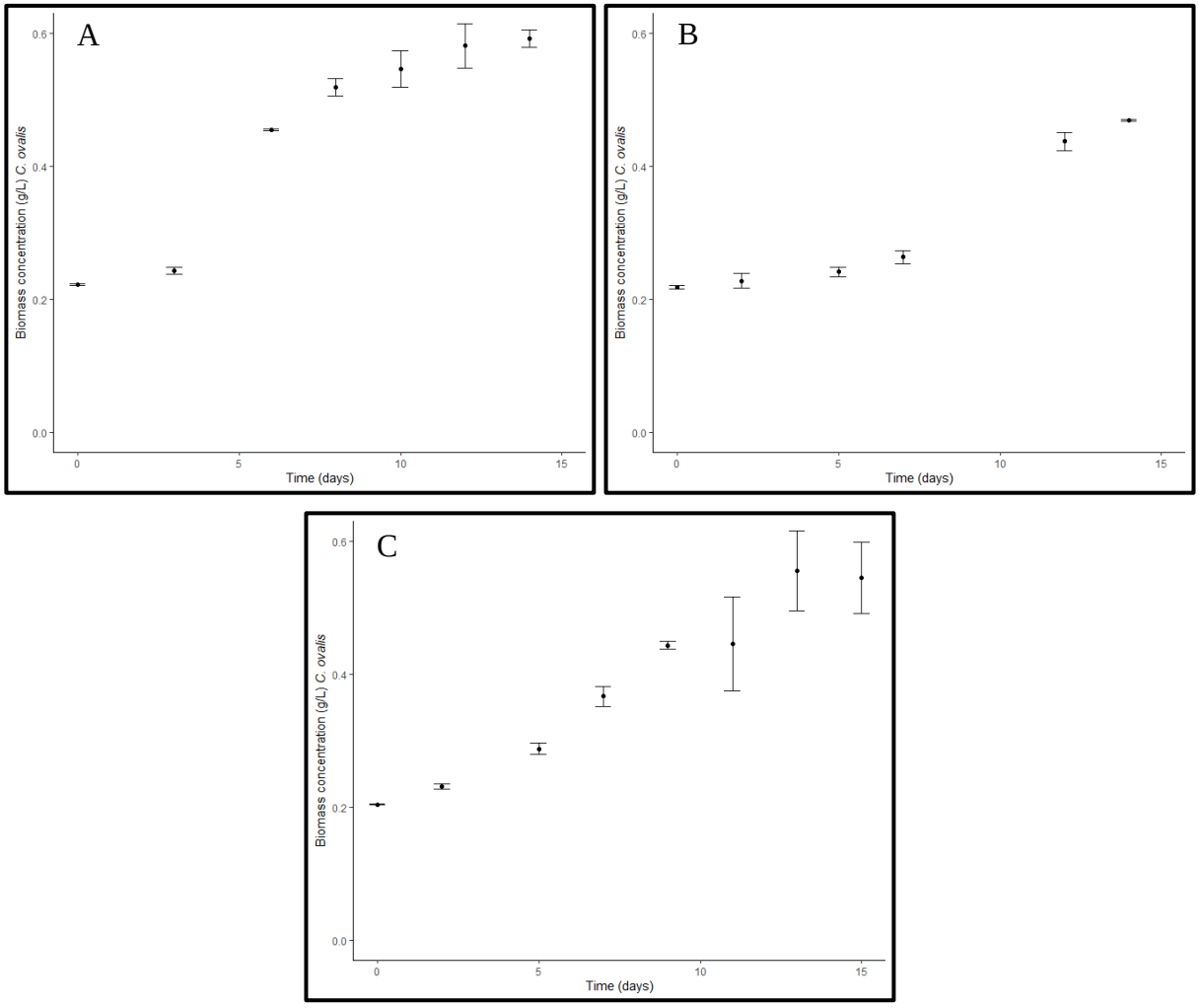

Figure 19. Growth rate of C. ovalis cultured under different trophic conditions (A: phototrophic, B: heterotrophic and C: mixotrophic). Cells were grown in f/2 medium and incubated for 14 days at 6°C, at the different cultivation modes. Results show the average and standard error.

4.1.5 Chlorocystis cohnii

The cell content of *C. cohnii* (Figure 20) was 5.00 ± 0.82, 6.73 ± 1.84 and 0.075 ± 0.02 under phototrophic, mixotrophic and heterotrophic conditions, respectively (average of triplicates). No significant differences were found when comparing the three cultivation modes (P<0.05).

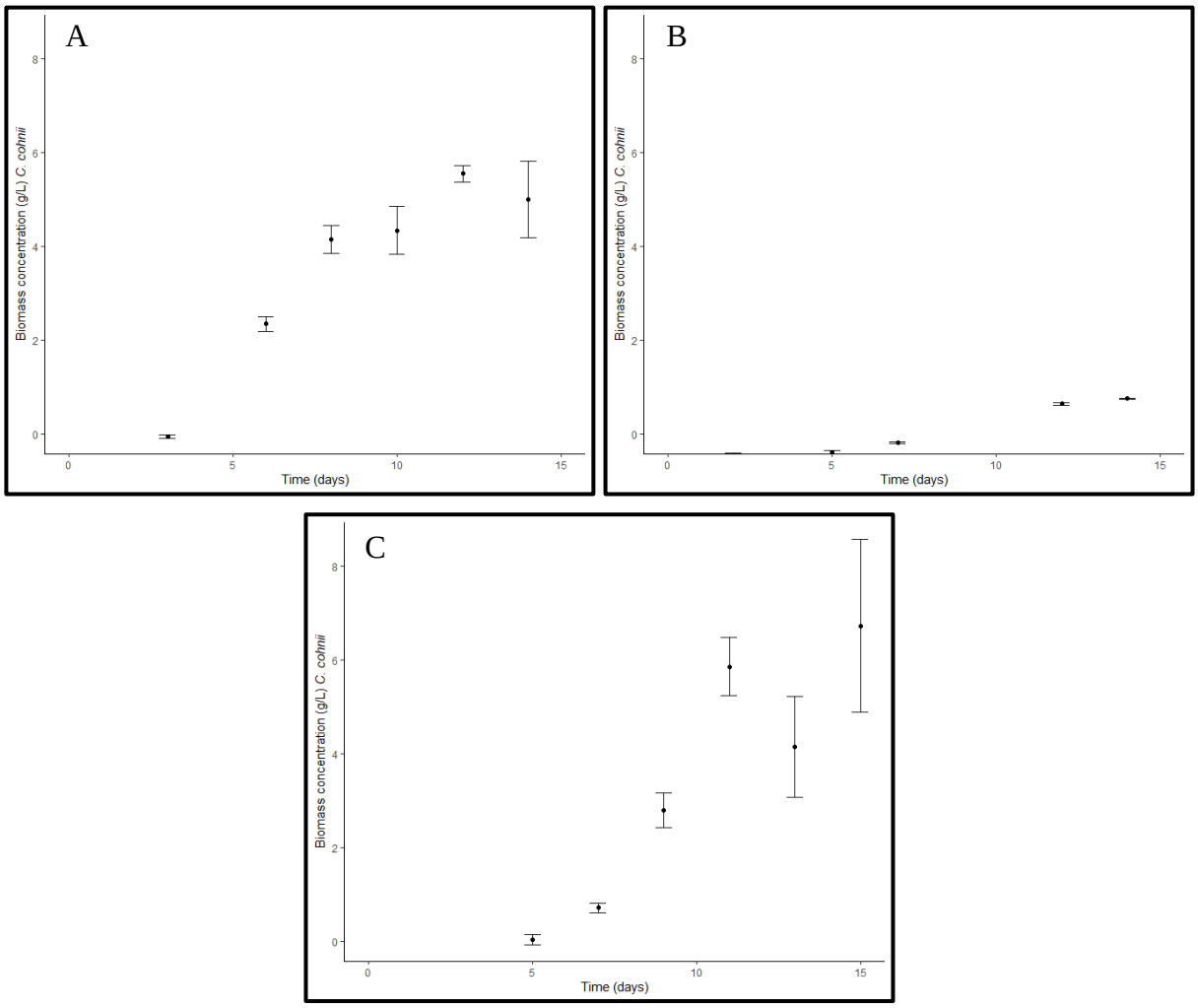

Figure 20. Growth rate of C. cohnii cultured under different trophic conditions (A: phototrophic, B: heterotrophic and C: mixotrophic). Cells were grown in f/2 medium and incubated for 14 days at 6°C, at the different cultivation modes. Results show the average and standard error.

4.2 Characterization

4.2.1 Protein content

The protein content of the five microalgae grown phototrophically, heterotrophically and mixotrophically is presented in Figure 21. The experiments were performed in triplicate, and the results in the figure show the averages and standard error.

The protein content of *N. oceanica* under phototrophic and mixotrophic conditions were the same (0.337 and 0.295 g_{PROT} L^{-1}, respectively, P>0.05). Comparing to cells maintained under heterotrophic conditions (0.062 g_{PROT} L^{-1}), the protein content was 5.4 and 4.8 times higher in mixotrophic and phototrophic conditions, respectively (P<0.05).

The statistical analysis of the protein content of *D. tertiolecta* suggested that the phototrophic and mixotrophic conditions were the same (0.821 g_{PROT} L^{-1}, 0.553 g_{PROT} L^{-1}, respectively, P>0.05). Meanwhile, the protein content under heterotrophic conditions (0.026 g_{PROT} L^{-1}) was statistically different, since it is 31.6 and 21.3 times higher in phototrophic and mixotrophic conditions, respectively (P<0.05).

The highest protein content in *T. suecica* was found under phototrophic (1.531 g_{PROT} L^{-1}) and mixotrophic cultivation (0.780 g_{PROT} L^{-1}), statistical analysis suggests that the values are the not significantly different (P<0.05). Concurrently, the cells maintained under heterotrophic conditions (0.063 g_{PROT} L^{-1}) were significantly different (P<0.05) compared to the phototrophic and mixotrophic conditions, them being 24.3 and 12.4 higher, respectively.

The protein content of *C. ovalis* under phototrophic and mixotrophic conditions were not statistically different (2.557 g_{PROT} L^{-1}, 1.478 g_{PROT} L^{-1}, respectively, P<0.05). As well as mixotrophic and heterotrophic (0.290 g_{PROT} L^{-1}) conditions (P<0.05). However, it was proven that phototrophic and heterotrophic conditions were significantly different (P<0.05), cells maintained under phototrophic conditions were 8.8 times higher than in heterotrophic conditions.

Chlorocystis cohnii cells maintained phototrophically and mixotrophically were the same in protein content (2.957 g_{PROT} L^{-1}, 1.308 g_{PROT} L^{-1}, respectively, P<0.05). Mixotrophic and heterotrophic (0.057 g_{PROT} L^{-1}) conditions were also considered to be the same (P<0.05).

However, while comparing the phototrophic conditions to the heterotrophic conditions, the protein content was 51.9 times higher under phototrophic conditions (P<0.05).

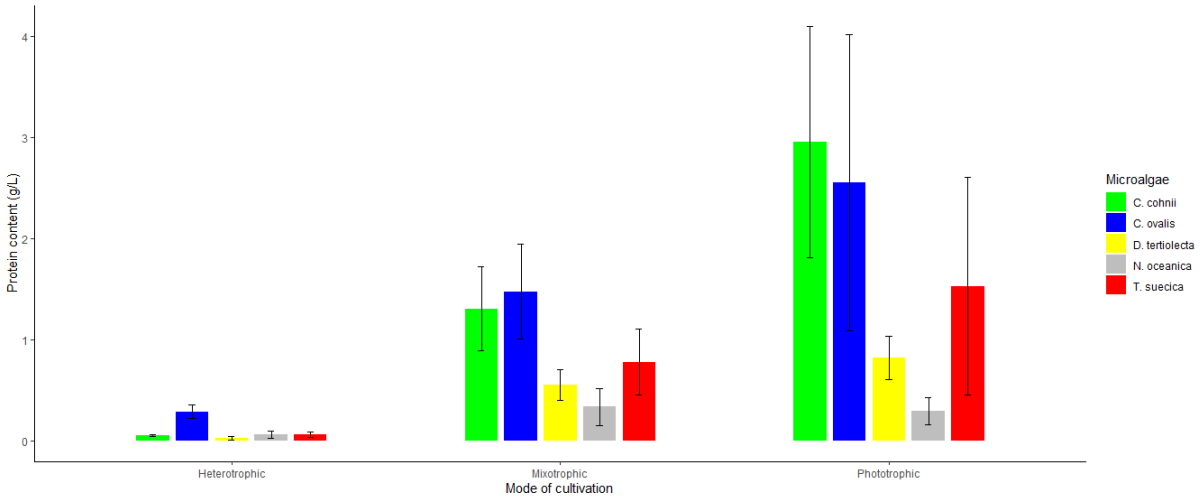

Figure 21. Protein content (g_{PROT} L^{-1}) of all five microalgae in the three different modes of cultivation.

4.2.2 Carbohydrate content

The carbohydrate content of the five microalgae grown phototrophically, heterotrophically and mixotrophically is presented in Figure 22. The experiments were performed in triplicate, and the results in the figure show the averages and standard error.

The carbohydrate content of *N. oceanica* under phototrophic, heterotrophic and mixotrophic conditions were not statistically different (P<0.05). Therefore, all values can be considered the same (0.029 g_{CARB} L^{-1}, 0.011 g_{CARB} L^{-1} and 0.017 g_{CARB} L^{-1}, respectively).

Dunaliella tertiolecta had the same carbohydrate content under phototrophic and mixotrophic conditions (0.179 g_{CARB} L^{-1}, 0.150 g_{CARB} L^{-1}, respectively, P<0.05). As well as mixotrophic and heterotrophic (0.006 g_{CARB} L^{-1}) conditions (P<0.05). However, it was demonstrated that phototrophic and heterotrophic conditions were significantly different (P<0.05), cells maintained under phototrophic conditions were 29.8 times higher than in heterotrophic conditions.

Carbohydrate content in phototrophically cultured cells were not significantly different than in mixotrophically cultured cells (0.118 g_{CARB} L^{-1}, 0.093 g_{CARB} L^{-1}, respectively, P<0.05). Same results were obtained while comparing the mixotrophic and heterotrophic (0.044 g_{CARB} L^{-1})

cultured cells. However, the carbohydrate content was 2.7 times higher in phototrophic conditions compared to heterotrophic conditions ($P<0.05$).

The carbohydrate content of *C. ovalis* in phototrophic and heterotrophic conditions were the same (0.044 g_{CARB} L^{-1}, 0.092 g_{CARB} L^{-1}, respectively, $P<0.05$). The carbohydrate content could not be assessed under mixotrophic conditions due to technical issues.

The highest results of *C. cohnii* were obtained in phototrophic and mixotrophic cultures, since the statistical analysis proved that there was no significant difference between them (0.303 g_{CARB} L^{-1}, 0.128 g_{CARB} L^{-1}, respectively, $P<0.05$). While comparing the mixotrophic and heterotrophic (0.042 g_{CARB} L^{-1}) conditions, the statistical analysis demonstrated that the results can be considered the same ($P<0.05$). Meanwhile, the carbohydrate content was significantly different between the heterotrophic and phototrophic modes ($P<0.05$), phototrophic being 7.2 times higher.

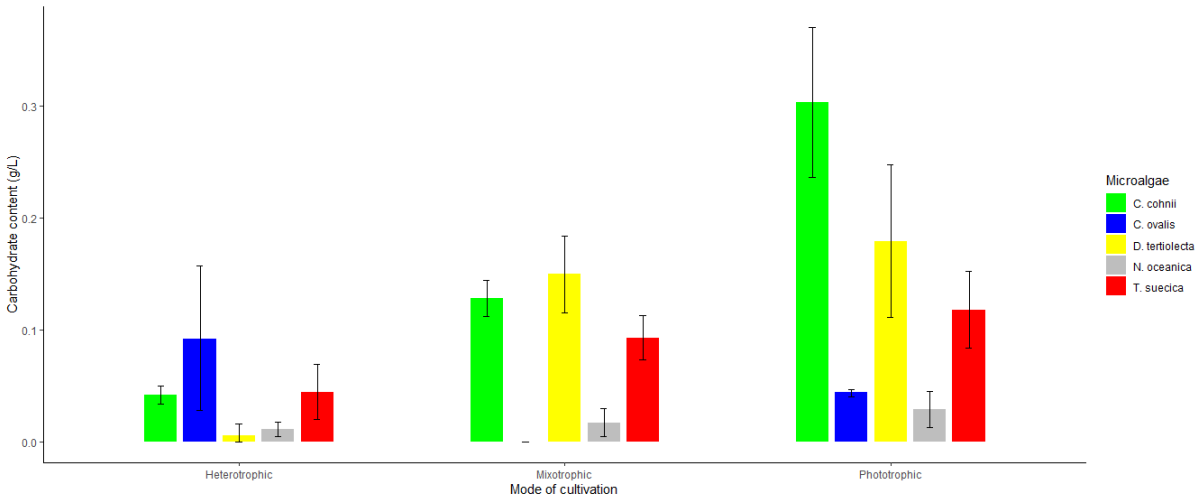

Figure 22. Carbohydrate content (g_{CARB} L^{-1}) of all five microalgae in the three different modes of cultivation.

4.2.3 Lipid content

The lipid content of the five microalgae grown phototrophically, heterotrophically and mixotrophically is presented in Figure 23. The experiments were performed in triplicate, and the results in the figure show the averages and standard error.

The comparison of the lipid content of the marine microalgae *N. oceanica* between phototrophic and heterotrophic conditions were the same (0.055 g_{LIP} L^{-1}, 0.076 g_{LIP} L^{-1}, respectively, $P<0.05$). As well as the comparison between mixotrophic (0.097 g_{LIP} L^{-1}) and heterotrophic

conditions ($P<0.05$). The lipid content was 1.8 times higher under mixotrophic conditions than in the phototrophic conditions ($P<0.05$).

The lipid content of *D. tertiolecta* had significant differences between phototrophic and mixotrophic conditions (0.036 g_{LIP} L^{-1}, 0.090 g_{LIP} L^{-1}, respectively, $P<0.05$), with the mixotrophic condition being 2.5 times higher than the phototrophic one. The comparisons between phototrophic and heterotrophic (0.052 g_{LIP} L^{-1}) conditions were not significantly different ($P<0.05$) and therefore considered to be the same, as well as the comparison between heterotrophic and mixotrophic conditions ($P<0.05$).

Tetraselmis suecica had the same lipid content under phototrophic and mixotrophic conditions (0.037 g_{LIP} L^{-1}, 0.054 g_{LIP} L^{-1}, respectively, $P<0.05$). As well as phototrophic and heterotrophic (0.026 g_{LIP} L^{-1}) conditions ($P<0.05$). However, it was demonstrated that heterotrophic and mixotrophic conditions were significantly different ($P<0.05$), cells maintained under mixotrophic conditions were 2.1 times higher than in heterotrophic conditions.

Chlorella ovalis cells maintained phototrophically and heterotrophically were the same in lipid content (0.078 g_{LIP} L^{-1}, 0.089 g_{LIP} L^{-1}, respectively, $P<0.05$). Mixotrophic (0.166 g_{LIP} L^{-1}) and heterotrophic conditions were also considered to be the same ($P<0.05$). However, while comparing the phototrophic conditions to the mixotrophic conditions, the lipid content was 2.1 times higher under mixotrophic conditions ($P<0.05$).

The statistical analysis of the lipid content of *C. cohnii* proved that the phototrophic and heterotrophic conditions were the same (0.064 g_{LIP} L^{-1}, 0.064 g_{LIP} L^{-1}, respectively, $P<0.05$). Meanwhile, the lipid content under mixotrophic conditions (0.087 g_{LIP} L^{-1}) was statistically different since it is 1.4 times higher than in phototrophic and heterotrophic conditions ($P<0.05$).

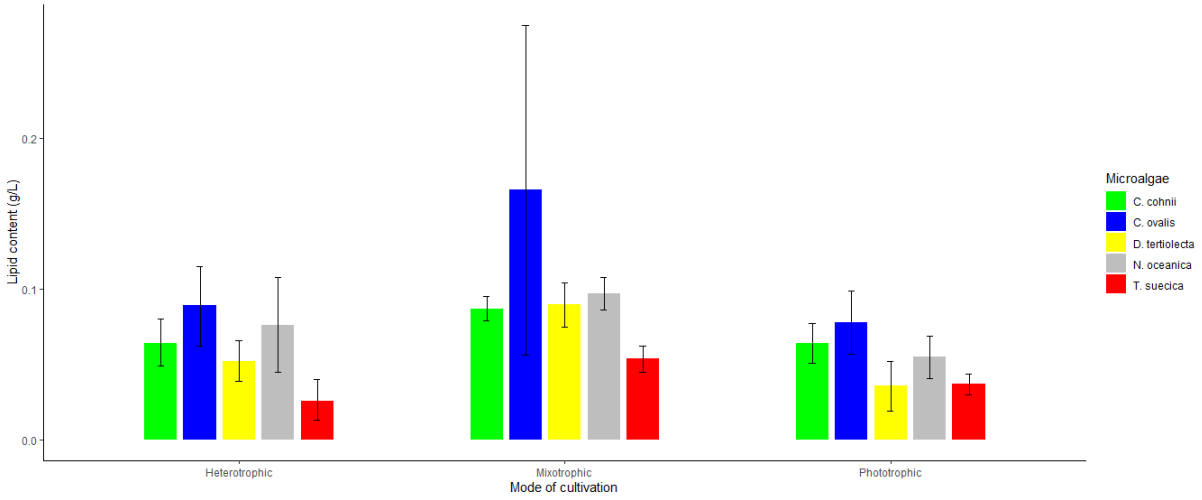

Figure 23. Lipid content (g_{LIP} L^{-1}) of all five microalgae in the three different modes of cultivation.

4.3 Stoichiometric parameter calculations

The three cultivation modes of each microalgae were analyzed separately for 4 stoichiometric parameters – specific growth rate, cell mass yield for glucose, cell mass yield for nitrate and biomass productivity –. Different strains of microalgae did not have growth under heterotrophic conditions, such as *N. oceanica*, *D. tertiolecta* and *T. suecica*. Therefore, they did not have results for specific growth rate, cell mass yield for glucose, cell mass yield for nitrate and biomass productivity. Table 5 shows the complete overview of the resulted parameters for each trophic condition and each strain of microalgae.

Table 5. Stoichiometric parameters for N. oceanica, T. suecica, D. tertiolecta, C. ovalis and C. cohnii under three different modes of cultivation. The parameters were calculated by considering the initial and final values of each cultivation.

	Parameter	*N. oceanica*	*T. suecica*	*D. tertiolecta*	*C. ovalis*	*C. cohnii*
Phototrophic	μ (day^{-1})	0.0854	0.1713	0.0846	0.0415	0.0391
	$Y_{X/NIT}$ (g_{DCW} g_{NIT}^{-1})	2.7962 ± 0.3715	1.4769 ± 0.0411	8.6713 ± 1.3934	0.2990 ± 0.0101	4.0419 ± 0.6625
	Q_X (g_{DCW} L^{-1} day^{-1})	0.2469 ± 0.0328	0.1304 ± 0.0036	0.7655 ± 0.1230	0.0264 ± 0.0009	0.3568 ± 0.0585
Mixotrophic	μ (day^{-1})	0.1455	0.0743	0.0646	0.0301	0.1861
	$Y_{X/GLC}$ (g_{DCW} g_{GLC}^{-1})	0.4644 ± 0.0037	0.1249 ± 0.0011	0.7970 ± 0.0434	0.0378 ± 0.0059	0.7474 ± 0.2042
	$Y_{X/NIT}$ (g_{DCW} g_{NIT}^{-1})	3.3816 ± 0.0267	0.9092 ± 0.0083	5.8033 ± 0.3160	0.2755 ± 0.0432	5.4424 ± 1.4871
	Q_X (g_{DCW} L^{-1} day^{-1})	0.2786 ± 0.0022	0.0749 ± 0.0007	0.4782 ± 0.0260	0.0227 ± 0.0036	0.4485 ± 0.1225
H	μ (day^{-1})	0.0000	0.0000	0.0000	0.0259	0.0346

$Y_{X/GLC}$ (g_{DCW} g_{GLC} $^{-1}$)	0.0011 ± 0.0011	0.0002 ± 0.0004	0.0000 ± 0.0000	0.0280 ± 0.0004	0.0833 ± 0.0018
$Y_{X/NIT}$ (g_{DCW} g_{NIT} $^{-1}$)	0.0081 ± 0.0083	0.0017 ± 0.0029	0.0000 ± 0.0000	0.2039 ± 0.0028	0.6069 ± 0.0132
Q_X (g_{DCW} L^{-1} day^{-1})	0.0007 ± 0.0007	0.0001 ± 0.0003	0.0000 ± 0.0000	0.0180 ± 0.0002	0.0536 ± 0.0012

The biomass productivity – Q_X (g_{DWC} L^{-1} day^{-1}) – of all microalgae was obtained by using the Equation 5. The experiments were performed in triplicate, and the results in the Figure 24 show the averages and standard error. The results of biomass productivity of *N. oceanica*, *D. tertiolecta* and *T. suecia* can be disregarded in heterotrophic conditions, since the microalgae did not grow.

The biomass productivity under phototrophic and mixotrophic cell cultures of *N. oceanica* (0.2469 g_{DWC} L^{-1} day^{-1}, 0.2789 g_{DWC} L^{-1} day^{-1}, respectively), *D. tertiolecta* (0.7655 g_{DWC} L^{-1} day^{-1}, 0.4782 g_{DWC} L^{-1} day^{-1}, respectively) and *T. suecica* (0.1304 g_{DWC} L^{-1} day^{-1}, 0.0749 g_{DWC} L^{-1} day^{-1}, respectively) were not statistically different, meaning the results are considered to be the same ($P<0.05$).

The statistical analysis of the biomass productivity of *C. ovalis* proved that phototrophic condition is 1.5 times higher than the heterotrophic cell cultured (0.0264 g_{DWC} L^{-1} day^{-1}, 0.0180 g_{DWC} L^{-1} day^{-1}, respectively, $P<0.05$). Meanwhile, the comparison between heterotrophic and mixotrophic (0.0227 g_{DWC} L^{-1} day^{-1}), and phototrophic and mixotrophic demonstrated no significant differences, therefore, the values can be considered to be the same ($P<0.05$).

The biomass productivity of *C. cohnii* in phototrophic, heterotrophic and mixotrophic conditions were the same (0.3568 g_{DWC} L^{-1} day^{-1}, 0.0536 g_{DWC} L^{-1} day^{-1} and 0.4485 g_{DWC} L^{-1} day^{-1}, respectively, $P<0.05$).

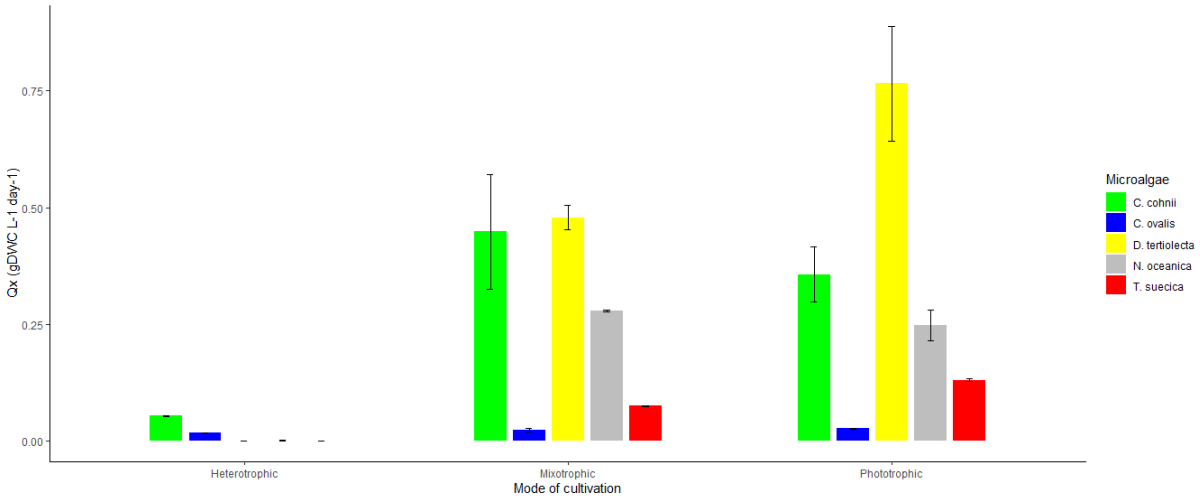

Figure 24. Biomass productivity (g_{DWC} L^{-1} day^{-1}) of all five microalgae in the three different modes of cultivation.

5 Discussion

Nannochloropsis oceanica was studied by Guerra, et al. (2021) where it showed that the cell mass in a phototrophic batch culture was 2.0 g_{DWC} L^{-1}. The difference of results between the prior study and the present thesis might be due to the way they performed the experiments, which was in tubular photobioreactors, with a controlled NO_3 concentration, constant irradiance of 700 µmol m^{-2} s^{-1} at room temperature (24°C), pH of 8.2, and a f/2 medium supplemented with iron. . Jo & Hur (2015) compared the growth of *N. oceanica* between the phototrophic and mixotrophic mode of cultivation; findings in their study did not show any significant differences between them. Cultivation of *Nannochloropsis* sp. has been research by Ma, et al. (2016), where the study showed that poor light penetration may reduce cell growth. Their findings also showed a sucessful growth of *Nannochloropsis* sp. in phototrophic conditions in open raceway ponds (1.0 g_{DWC} L^{-1}) and helical-tubular bioreactors (1.10-3.03 g_{DWC} L^{-1} day^{-1}). Additionally, the same study showed the ability of the *Nannochloropsis* genus to be grown in mixotrophic conditions while using glucose as an organic carbon source (1.0 g_{DWC} L^{-1}). However, the same study reports the unability of *Nannochloropsis* sp. to grow under heterotrophic conditions.

Dunaliella tertiolecta was studied by Santa Moura, et al. (2020); their results showed that the cell mass in a phototrophic batch culture was 0.7 g_{DWC} L^{-1}. The difference between the results in the literature and in the present thesis might be due to the conditions *D. tertiolecta* was cultivated. Santa Moura, et al. (2020) used f/2 medium and ran the experiment in Erlenmeyer flasks at room temperatue, under light intensity of 45 µmol m^{-2} s^{-1}, and continous aeration at a flow rate of 2 L min^{-1}. It was also noticed that in the literature, there are not results regarding the cell concentration in mixotrophic and heterotrophic growth.

Andreotti, et al. (2019) performed an experiment of *T. suecica* in batch culture in phototrophic conditions, which had a cell mass of 0.460 g_{DWC} L^{-1}. Their results are lower than the ones obtained in the present thesis, which might be due to the cultures of *T. suecica* done in bubble column photobioreactors with 150 µmol m^{-2} s^{-1} at a temperature of 23°C, dissolved oxygen of 8.0 mg/L, pH of 8.0 and constant airflow of 2 m^3 h^{-1}.

5.1 Obligated phototrophs and facultative heterotrophs

Most microalgae species are obligated autotrophs, meaning they forcefully need light as an energy source, and inorganic carbon sources in order to grow (Behrens, 2005). A supposition to why some of the microalgae are obligated autotrophs is the insufficient cellular consumption of carbon sources, especially sugars (Chen & Chen, 2006). Therefore, only a few microalgae species are facultative heterotrophs, meaning they can assimilate organic substrates as energy source, and they use organic carbon sources (Table 1).

Chen & Chen (2006) and Behrens (2005) recorded the main characteristics that a microalgae species need to have in order to be functional under heterotrophic cultivation: "(i) faculty of cell division and active metabolisms in absence of light. (ii) ability to grow in culture media with easy-to-sterile organic substrates where energy required for heterotrophic growth must be supplied by oxidation of part of the organic substrate. (iii) ability to adapt to fast environmental changes, and (iv) capacity to resist hydromechanical stress inside the fermentors."

Also, microalgae have two paths to fixatiate carbon, the first one is by using the Calvin-Bensen cycle, which is used during the phototrophic cultivation (photosynthetic growth and fixation of inorganic carbon), and the second one is by absorbing organic carbon in the absence of light, meaning heterotrophic cultivation (Lowrey & Brooks, 2015).

Gladue & Maxey (1994) suggested that some algae are facultative heterotrophs who need very specific conditions with high concentration of organic carbon. Their results of heterotrophic screening of microalgae strains identified *D. tertiolecta*, *T. suecica*, *Nannochloropsis* sp., and *Chlorella* sp. 580 to be positive for growing under heterotrophic conditions. Their cultures were grown at 20-25°C, pH 7.5-8.3 in fermenters. The media used for *D. tertiolecta*, *Nannochloropsis* sp., *T. suecica*, and *Chlorella* sp. 580 contained high levels of organic carbon with complex nutrients added. They created a media that combined inexpensive carbon sources, a mixture of inorganic and complex nitrogen sources. The study suggested that *Chlorella* had a rapid growth under heterotrophic conditions, meanwhile, *Nannochloropsis*, *Dunaliella* and *Tetraselmis* had a slow growth. Behrens (2005) also reported that the genera *Chlorella*, *Dunaliella*, *Nannochloropsis* and *Tetraselmis* were able to grow under heterotrophic conditions.

Results in the present thesis showed an inability of *N. oceanica*, *D. tertiolecta*, and *T. suecica* to grow under heterotrophic cultivation. This might be due to various reasons: (a) the media and nutrients were not suitable for the growth of the microalgae under heterotrophic conditions, (b) the use of a small amount of carbon sources; Gladue & Maxey (1994) used 18 g of glucose, in contrast of the 3 g of glucose used in the present thesis, (c) the low temperature where the microalgae were grown affected their adaptation to heterotrophic function, therefore, they had a very slow growth, which was stopped at 14 days for compatibility purposes, (d) Lee (2001) suggested that heterotrophic cultivation might be unappropriate for microalgae that have been obtained from culture collection centers, since they are usualy isolated and kept under photosynthetic conditions, which might make themunable to assimilate and metabolise organic carbon, derivating in a slow growth. In order to make them adapt easily to the new trophic condition, it is a must to culture the microalgal cells in rich organic materials.

5.2 Macromolecular composition and techniques

5.2.1 Protein content

The total protein content of *N. oceanica* found in Jo & Hur (2015) under phototrophic conditions was 0.134 g/g, while in mixotrophic culture was 0.337 g/g. Jo & Hur (2015) revealed that there was no significant differences between both modes of cultivation. Literature does not reveal studies where protein content was measured for *N. oceanica* in heterotrophic conditions, which might be due to the innability of *Nannochloropsis* sp. to grow under heterotrophic conditions (Ma, et al., 2016).

Mesquita da Silva Gorgonio, et al. (2013) found that the protein content of *D. tertiolecta* under phototrophic conditions was 38.52 % of DCW. Tammam, et al. (2011) cultivated *D. tertiolecta* under photoperiods of 12 h light/12 h dark cycle in phototrophic conditions, with results showing a total of protein content of 15.87 g_{PROT} L^{-1}. Both results in the literature are higher than the ones obtained in the present thesis.

Otero & Fábregas (1997) analyzed the nutritional state of *T. suecica* in phototrophic conditions, finding a total of 55-58% of protein content in them; their results are similar to the ones obtained in the present thesis (56%). Cid, et al. (1992) tested *T. suecica* in mixotrophic culture with different sources of organic carbon, with protein content ranging between 27.5 pg $cell^{-1}$ in phototrophic conditions and 39.73 pg $cell^{-1}$ in mixotrophic cultures with glucose as organic

carbon. Fernández-Reiriz, et al. (1989) reported a total protein content of 41% under phototrophic conditions.

The protein content for *C. ovalis* was unable to be quantified since the values went out of 100%, which might be due to various technical errors. However, several studies give an approximate idea of what the protein content should have been in the experiment. Slocombe, et al. (2013) results showed a protein content of 10.97% during phototrophic conditions. They used a 12 h light/12 h dark periodicity with a light intensity of 50-80 µmol m^{-2} s^{-1}, at 20°C without shaking. Lin´s (2005) results show a general composition of protein of 63.50%.

Saadaoui, et al. (2018) is the only study were the *Chlorocystis* genus has been studied. They used a mofidied f/2 medium containing 0.15 g L^{-1} $NaNO_3$ and 5.6 mg L^{-1} of NaH_2PO_4. They grew the culture at 30°C, pH 8, at 300 rpm in a 12 h light/12 h dark periodicity, and a light intensity of 100 µmol m^{-2} s^{-1}, and 5% CO_2 during the light phase. Their results showed a total protein content of 13.3%, which is lower than the results obtained in the present thesis (54.71%).

The measurement of the protein content was made with the Lowry method, which was successfull in *T. suecica*, since the results obtained in the present thesis match the ones found in literature. However, other microalgae had different protein contents compared to the reported ones in various articles, which can be a result of the different conditions the microalgae cells were cultured in the different studies. The Lowry method was successfully applied in all microalgae, but *C. ovalis*, which was unable to be quantified since the values went out of 100%. Even though the Lowry method is highly sensible and precise (Assemblymade, 2022), the tecnique uses a calibration curve, which can provoke errors as the protein content searched in the samples may not match the protein standard. However, all the standard curves were made without errors, meaning the problem with the *C. ovalis* results is in the samples. The salts contained in the medium might have not been completely removed prior to the use of the Lowry method, creating errors in the samples, (Shen, 2019).

5.2.2 Carbohydrate content

The study performed by Guerra, et al. (2021) is the only study in which carbohydrates have been measured for *N. oceanica* This might be due to the high interest of lipid production instead of other macromolecules (Borowitzka, 2018). Their results showed that phototrophic growth in

batch achieved 37.80% in DCW of carbohydrates. Comparing the results between Guerra, et al. (2021) in phototrophic cultivation and the carbohydrate content in the present thesis, it is clear that the present study had very low values (0.892% in DCW).

Only few studies analyzing the carbohydrate content in *D. tertiolecta* are available at the moment. Mesquita da Silva Gorgonio, et al. (2013) performed an experiment with *D. tertiolecta* in phototrophic conditions, where it was observed that the carbohydrate content was 24.61%, which was the result expected in the present thesis; however, the reality was that the obtained results (1.668%) in the present study are lower compared to Mesquita da Silva Gorgonio, et al. (2013).

Otero & Fábregas (1997) found a content of 13-15% of carbohydrates under phototrophic conditions for *T. suecica*. Cid, et al. (1992) tested *T. suecica* in mixotrophic culture with different sources of organic carbon; carbohydrate content ranged between 6.50 pg $cell^{-1}$ in phototrophic conditions and 48.48 pg $cell^{-1}$ in mixotrophic cultures with glucose as organic carbon. Fernández-Reiriz, et al. (1989) reported a total carbohydrate content in phototrophic conditions of 12%. The results obtained in the present thesis demonstrated a carbohydrate content of 6%.

There is no information regarding the carbohydrate content of *C. ovalis*. However, the *Chlorella* genus was researched by Liu & Hu (2013),in which they found that under phototrophic conditions, the content varies between 10-15% on these microalgae; meanwhile, the results in the present thesis consisted of 8%

Saadaoui, et al. (2018) showed results *of Chlorocystis* sp. of total carbohydrate content of 17%, meanwhile, the results obtained in the present thesis were 5.77% of carbohydrate content.

The colorimetric method used to measure the carbohydrate content was determined by the phenol-sulfuric acid method (Dubois, et al., 1956). This technique depends directly on the dehydration of hydrolyzed saccharides to furfural derivatives during reaction with concentrated sulfuric acid (Quero-Jiménez, et al., 2019). It is widely used due to its sensitivity and simplicity; however, it does require for the acid to be added in a rapid manner to make the reaction. If added incorrectly, not enough heat is generated and errors in the measurement occur (Masuko, et al., 2005), which is probably the reason that the resulting carbohydrate contents were too low

compared to the results found in the literature. It is also suggested that the method used for the analysis of carbohydrate content needs to be adapted to the strain of microalgae, meaning, other methods, such as anthrone, orcinol, or resorcinol could deliver a better result for the microalgae.

5.2.3 Lipid content

The present thesis only explored the lipid content found in the five strains of microalgae, but it did not investigate the type of fatty acids that could be found in them. However, there are several studies where fatty acids were analyzed in each microalgae, which can provide a sense of what could have been found in the present study. For further research, it would be ideal to analyze the types of fatty acids found in the microalgae under the different trophic conditions.

Guerra, et al. (2021) found the lipid content in *N. oceanica* to have a percentage of 22% in phototrophic conditions. Ma, et al. (2016) reported a lipid content of 37-60% in phototrophic conditions. The experiment of Jo & Hur (2015) showed a total lipid content in phototrophic conditions of 0.241-0.251 g/g, while in mixotrophic culture was 0.244-0.295 g/g. It is observable that there was avast difference between the reported results and the present study results.

Jo & Hur (2015) analyzed the types of fatty acids that are present in *N. oceanica* in phototrophic and mixotrophic conditions. In the first type of culture, the most common fatty acids found were C20:5n3, C16:0 and C16:1n7, the less common were C20:2n2, C20:3n6 and C18:3n3. SAFA were the most common with 36.0 µg/mg, followed by PUFA with 29.4 µg/mg, then n3 HUFA with 22.2 µg/mg, and lastly MUFA with 20.5 µg/mg. The mixotrophic culture had C20:5n3, C16:0 and C16:1n7 as the highest amount of fatty acids, the less common were C18:3n6, C18:3n3 and C20:4n6. In Ma, et al. (2016), the most common fatty acids in phototrophic conditions were C16:0, C16:1 and C18:1, the least common were C18:0, C18:2 and C18.3. In Guerra, et al. (2021), the most common fatty acids in phototrophic batch culture in a photobiorreactor were C16:1 and C16:0, while the least amount was from C18:2 ω6, C18:0 and C20:4 ω6; the highest percentage was MUFA, followed by SAFA, then PUFA and lastly PUFA/SAFA.

The study from Mesquita da Silva Gorgonio, et al. (2013) showed a 11.64% result of lipid content while cultivating *D. tertiolecta* in phototrophic conditions. Gouveia & Oliveira (2009) reported a lipid content of 16.7% in phototrophic conditions. Takagi, et al. (2006) found a lipid

content of 60.6-67.8%. Rizwan, et al. (2014) performed an experiment with *D. tertiolecta* while using different sources of organic carbon and different concentrations of CO_2, showing that mixotrophic culture is more favorable than heterotrophic culture. There were vast differences between the results found in the literature and the ones obtained in this study.

Mesquita da Silva Gorgonio, et al. (2013) showed results where SAFA constitutes 33.36% of the fatty acids found in *D. tertiolecta*, MUFA is 54.59% and PUFA is 11.70%. The most prominent fatty acids were 16:0, 18:01 n9, 16:1, and 18:03; the least eminent were 22:0, 20:0, and 22:1. Chen, et al. (2011) reported the fatty acid composition of extracted algae oil from *D. tertiolecta*, the total amount of SAFA was 28.7% and PUFA was 71.3%, the highest amount was found in C18:3 and C16:0, the lowest amount was found in C16:2 and C18:0.

Shah, et al. (2016) results show a total lipid content of 24.43% in *T. suecica* under phototrophic conditions. Otero & Fábregas (1997) reported a total lipid content of 28-30%. Fernández, et al (1989) results revealed a total lipid content of 14.83-16.96%. The results obtained in literature are very different with the ones obtained in the present thesis.

Shah, et al. (2016) results of *T. suecica* showed a total presence of SAFA of 68.74%, with C16:0, C15:0 and C18:0 being the most prominent, and C12:0, C17:0 and C20:0 being the least outstanding. The presence of MUFA of had a total of 12.26%, C18:1 being the highest amount and C16:1 the lowest amount found. The PUFAs found in their study are only 8.88% of the total fatty acids, with C18:3 being the most prominent, C18:2 the least. Soto-León, et al. (2014) results showed a total presence of SAFA of 57.19%, with C16:0 and C18:0 being in the highest concentration. A total presence of MUFA of 16.79% with C18:1 being the most important. The presence of PUFA of 26.02% with C18:2 and C18:3 having the highest amount of them. Penhaul Smith, et al. (2021) results in heterotrophic cultivation show a high presence of C16:0, C18:1w7 and C18:1w9, and a low presence of C15:0, C15:ai, C17:0i, C17:1w7 and 22:6 (n-3). The mixotrophic culture had a high presence of C16:0, C16:0i and C18:1w7, and a low presence of C15:0, C15:ai, C17:0i, C17:1w7. The photoautotrophic culture had a high quantity of C16:0 and C18:1w9, and a low presence of C15:0, C17:0i, C17:1w7, C17:1w8c and 22:6 (n-3).

Liu & Hu (2013) revealed that the *Chlorella* genus may have a total amount of lipids between 12-15% under phototrophic conditions, which is very similar to the result found in the present thesis under the same conditions (16%).The *Chlorella* genus has mainly C16:0, C16:2, C18:1,

C18:2 and C18:3 fatty acids (Liu & Hu, 2013). Meanwhile, Pratoomyot, Srivilas, & Noiraksar (2005) classified the *Chlorella* genus in the Chlorophyceae class, which has a high amount of fatty acids of C18:3 n-3, C16:0 and C18:2n-6. *Chlorella* sp. was found to have a SAFA total of 15.89-21.35%, MUFA of 8.01-14.64% and PUFA of 43.37-47.12%.

Saadaoui, et al. (2018) perfomed an study where it showed lipid content results of *Chlorocystis* sp. with a total percentage of 20. Saadaoui, et al. (2018) also showed the content of fatty acids, where SAFA constituted of 1.8%, MUFA of 90.45% and PUFA 7.74%. C20:1, C18:1n9c, and C24:1 being the main fatty acids found, while C18:1n9t, C20:2, and C22:1 was found in the least quantities.

Most of the microalgae, with *Chlorella* being the exception, had very different results when compared to what was found in the literature.. This might be due to the conditions in which the growth of the cell cultures were performed, and the method used to measure the lipid content, which might have not been the most appropiate for the microalgae, due to the selection of solvents, which is the most critical factor in any lipid extraction tecnique (Kumar Saini, et al., 2021). Different solvents and mixtures of them can be used in further studies to search for the ideal ones that improves the extraction of lipids for each strain of microalgae.Examples of different solvents/mixtures are, ethyl acetate/ethanol, 1-butanol/methanol, chloroform/methanol, butanol/methanol/chloroform, n-hexane/acetone. Another critical part of the lipid content measurement is done previously to the solvent extraction, which is the desintegration of the cell-wall microalgal cells, since the lipids are stuck in the cytoplasm by the cell wall and membrane. It is essential to desintegrate it in order to facilitate the solvent penetration and extraction of lipids and have accurate results, which was missing in the present thesis (Kumar, et al., 2021; Ren, et al., 2017). For further studies it is crucial to test different tecniques in every microalgae, in order to get the most precise results for each of them,as well as performing a cell disruption prior to the lipid extraction, in order to secure that all lipids are drown out and measured.

6 Conclusion

Of all the different strains of microalgae used in the present thesis, *C. cohnii* had the highest volumetric biomass productivity under mixotrophic conditions, while the rest of them had it under phototrophic conditions. *N. oceanica* had a higher protein content under mixotrophic conditions; *C. ovalis* had the highest carbohydrate content under phototrophic conditions and all microalgae had the highest lipid content under heterotrophic conditions. It was also noticeable that the highest growing rate of the *C. ovalis* was found in the phototrophic culture.

In this study, the protein, carbohydrate, and lipid content measurements of the microalgae where different than what was expected according to the literature. This was due to the various reasons, the first one was that the conditions were the microalgae were grown in literature was very different than the one done in the present thesis, which might variate the results of the macromolecules. The second one was that the techniques used in carbohydrate and lipid measurements were not the most suitable ones in the microalgae strains since each strain has different and unique characteristics. Therefore, it is suggested to test different methods in order to find the best one for each microalgae. The third one, the technique used for the measurement of carbohydrate content had different technical errors, such as hydrolysis dilution and the reaction with acid that needed to be added in a rapid manner, which if added incorrectly, not enough hear is generated and errors in the measurement occur. The fourth one, the disruption of the cell microalgal wall was missed in the present thesis, giving errors in the results since the solvents could not extract the lipids properly. Thus far, the strains of *N. oceanica, D. tertiolecta,* and *T. suecica* are obligated photoautotrophs in cold environments, while *C. ovalis* and *C. cohnii* are facultative heterotrophs in cold environments.

For further studies, controlling the environment, such as light intensity, CO2, pH, and temperature are important factors to have more trustworthy data. An important part of the lipid quantification is the destruction of the microalgae cell, which was not achieved correctly due to technical challenges. It is also suggested that the method used for the analysis of carbohydrate content be adapted to the strain of microalgae, meaning, other methods, such as anthrone, orcinol, resorcinol and enzymatic techniques that could deliver a better result for the microalgae.

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Appendixes

Appendix A

Equipment	Producer
96-well plate	Nunclon
Autoclave	GETINGE GROUP
Centrifuge	HERAEUS MEGAFUGE 8R
Centrifuge tubes	VWR
Chamber	HERAGUARD ECO
Desiccator	CSN SIMAX
Eppendorf tubes	Eppendorf AG
Falcon tubes	Corning Incorporated
Filters	GE Healthcare Life Sciences
Glassware	-
Lights	Xing Yuan Electronics Co., LTD
Oven	Thermo Scientific
Pipettes	VWR
Shaker	Edmund Bühler
Spectrophotometer	Molecular Devices VersaMaxTM
Stove/heater	Kervel
Vacuum	NAGENE
Balance	VWR

Appendix B

Chemical	Supplier	Batch number
Ammonium formate	VWR	21B024131
Bovine albumin	Biowest	AO20K7
Chloroform	Supelco Merck	KGaA K52941292102
$CuSO_4$	Not available	Not available
Folin-Ciocalteu's phenol	Supelco Merck	-
D-(+)-Glucose	Sigma	SLCH2444
HCl	Analar NORMAPUR	19CN024108
H_2SO_4	EMSURE	K52311780012
Methanol	Fisher Chemicals	12050479678
Na_2CO_3	Alfa Aesar	10226396
NaOH	Merck KGgA	B1472398732
Phenol	Sigms-Aldrich	SHBL3587
Potassium sodium tartrate	VWR	21CO24108

Appendix C

Component	Stock solution (g/L dH20)	Quantity used	Supplier	Batch number
$NaNO_3$	75.0	1.0 mL	-	-
$NaH_2PO_4 \cdot H_2O$	5.0	1.0 mL	Erling & Morten	338065/1295
$Na_2SiO_3 \cdot 9H_2O$	30.0	1.0 mL	-	-
Trace metals solution	See the trace metals solution components	1.0 mL	-	-
Vitamins solution	See the vitamins solution components	0.5 mL	-	-
$MgSO_4 \cdot 7H_2O$	246.4	5.0 mL	Erling & Morten	334781/1194
KNO_3	0.5	5.0 mL	-	-
NaCl	-	23.4 g 257.2 g	-	-

Trace metals solution

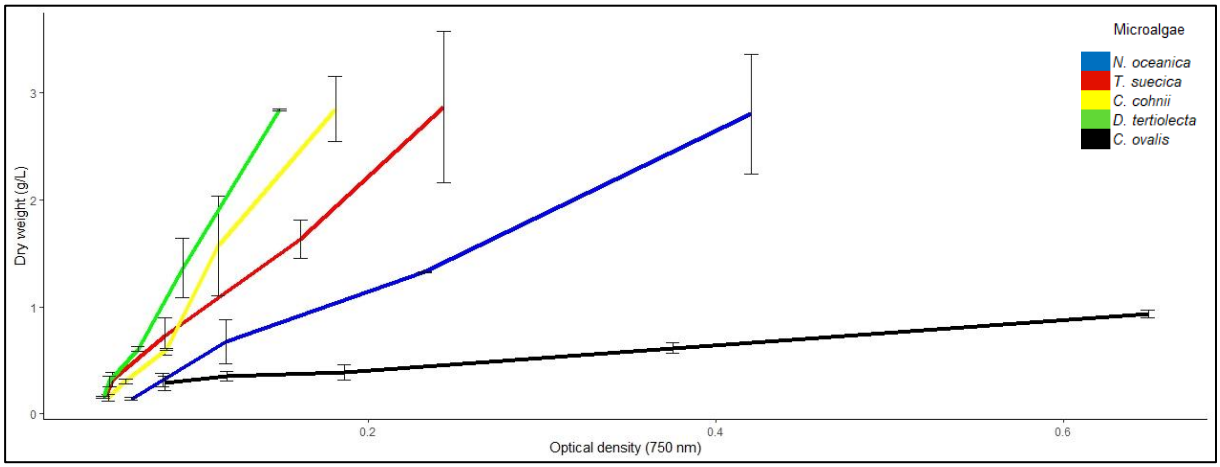
Component	Stock solution (g/L dH20)	Quantity used	Supplier	Batch number
$FeCl \cdot 6H_2O$	-	3.15 g	Alfa Aesar	10227261
$Na_2EDTA \cdot 2H_2O$	-	4.36 g	-	-
$MnCl_2 \cdot 4H_2O$	180.0	1.00 mL	Acros Organics	A0420296
$ZnSO_4 \cdot 7H_2O$	22.0	1.00 mL	Alfa Aesar	10225858
$CoCl_2 \cdot 6H_2O$	10.0	1.00 mL	Alfa Aesar	10225898
$CuSO_4 \cdot 5H_2O$	9.8	1.00 mL	Not available	Not available
$Na_2MoO_4 \cdot 2H_2O$	6.3	1.00 mL	Alfa Aesar	10223684

Vitamins solution

Component	Stock solution (g/L dH20)	Quantity used	Supplier	Batch number
Thiamine·HCl (vitamin B1)	-	200 mg	Acros Organics	AO428392
Biotin (vitamin H)	1	1 mL	Sigma-Aldrich	SLCH1646
Cyanocobalamin	1	1 mL	-	-

Appendix F

Dry cell weight standard curves for *N. oceanica* (blue), *T. suecica* (red), *C. cohnii* (yellow), *D. tertiolecta* (green) and *C. ovalis* (black). The equation of the line is found on the table below.



Microalgae	Equation of the line	R^2
N. oceanica	y=7.2795x-0.2816	0.9959
T. suecica	y=13.411x-0.4364	0.9966
C. cohnii	y=21.371x-0.9952	0.9878
D. tertiolecta	y=26.420x-1.1009	0.9980
C. ovalis	y=1.1315x+0.1955	0.9966

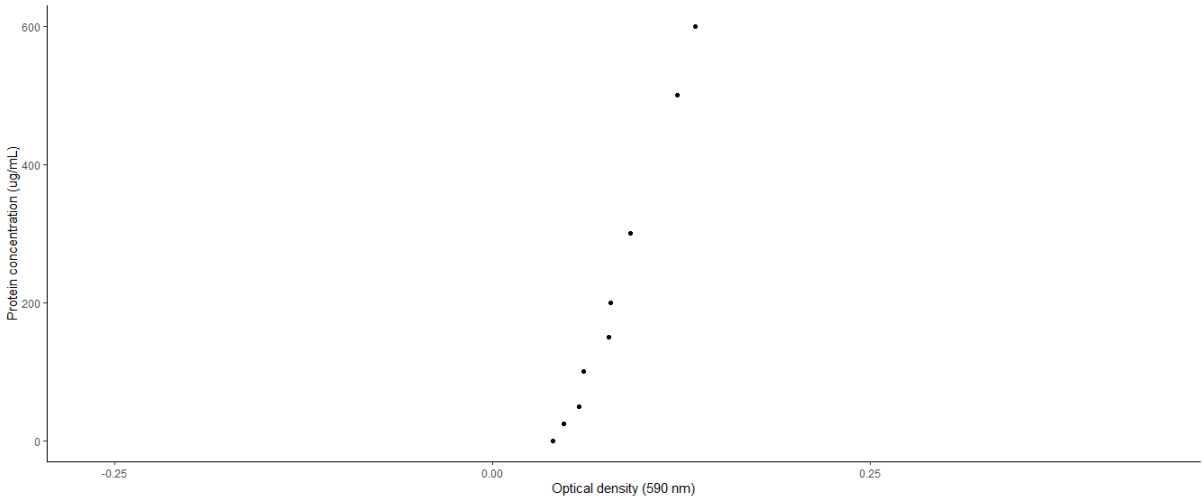
Appendix G

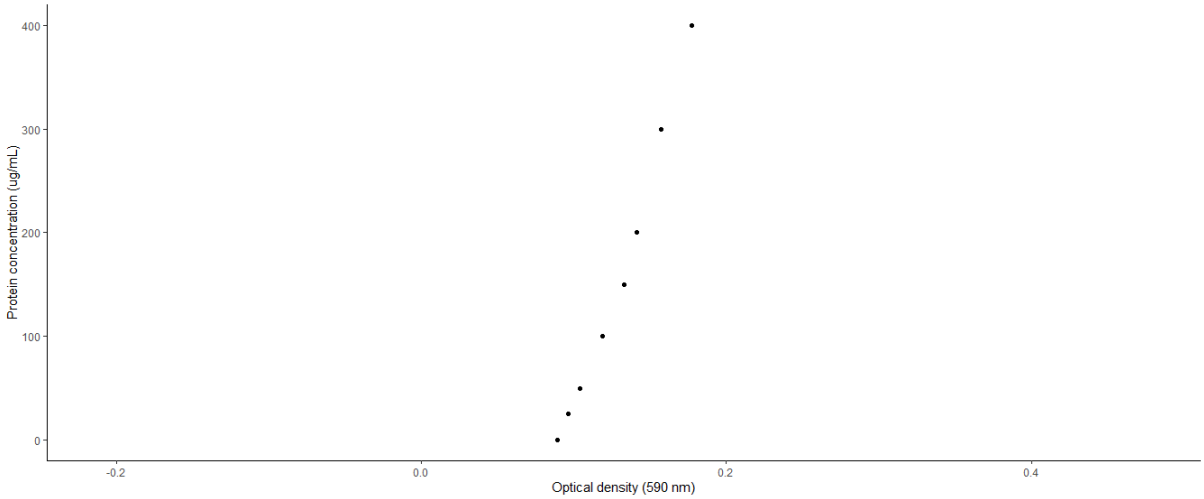
Standard curves for the determination of total proteins and carbohydrates of *N. oceanica*, *T. suecica, C. cohnii, D. tertiolecta* and *C. ovalis*. The graphs and data of each run of modes of cultivation is found below the the table.

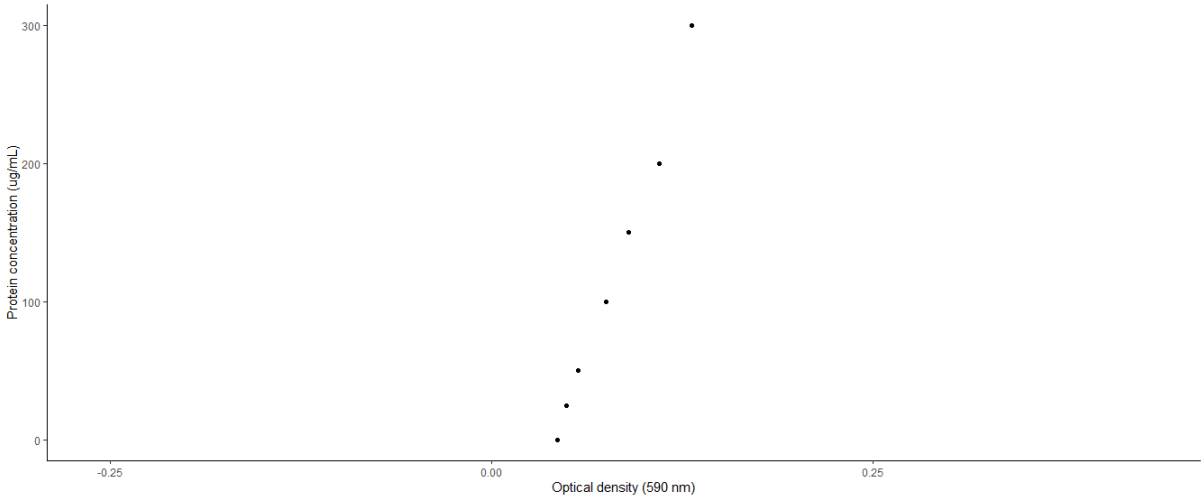
	Mode of cultivation	Equation of the line	R^2
Proteins	Phototrophic	y=6524.1x-297.89	0.9838
	Heterotrophic	y=3234.5x-138.59	0.9915
	Mixotrophic	y=4521.1x-421.06	0.9817
Carbohydrates	Phototrophic	y=855.76x-49.056	0.9903
	Heterotrophic	y=405.06x-20.068	0.9706
	Mixotrophic	y=571.22x-82.497	0.9268

Data obtained from the standard curves for the determination of total protein for the samples with the three different modes of cultivation.

	OD_{590nm}		
Protein concentration	Phototrophic	Mixotrophic	Heterotrophic
0	0.040	0.089	0.043
25	0.047	0.096	0.049
50	0.057	0.104	0.057
100	0.060	0.119	0.075
150	0.077	0.133	0.090
200	0.078	0.141	0.110
300	0.091	0.157	0.131
400	-	0.177	-
500	0.122	-	-
600	0.134	-	-



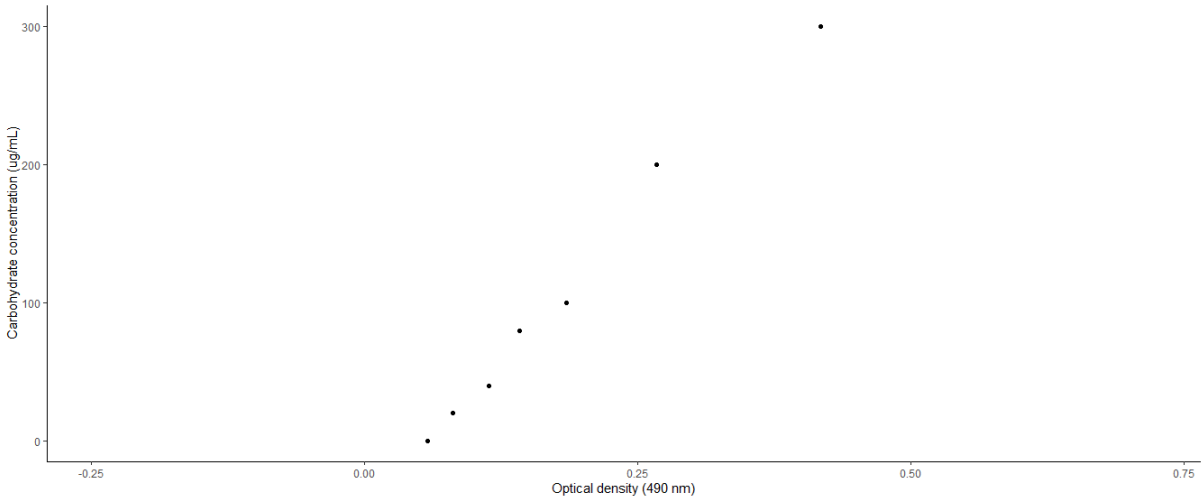


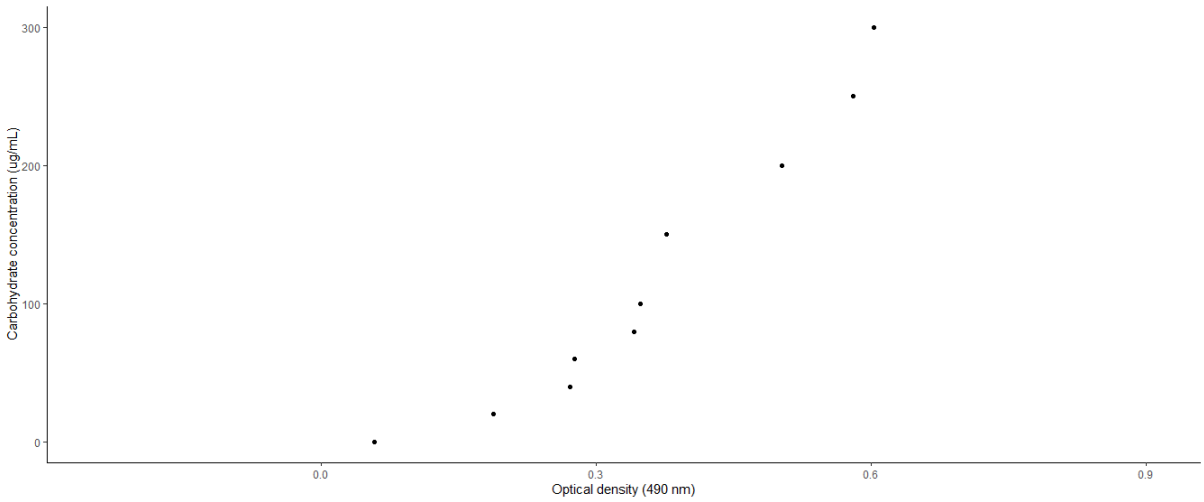


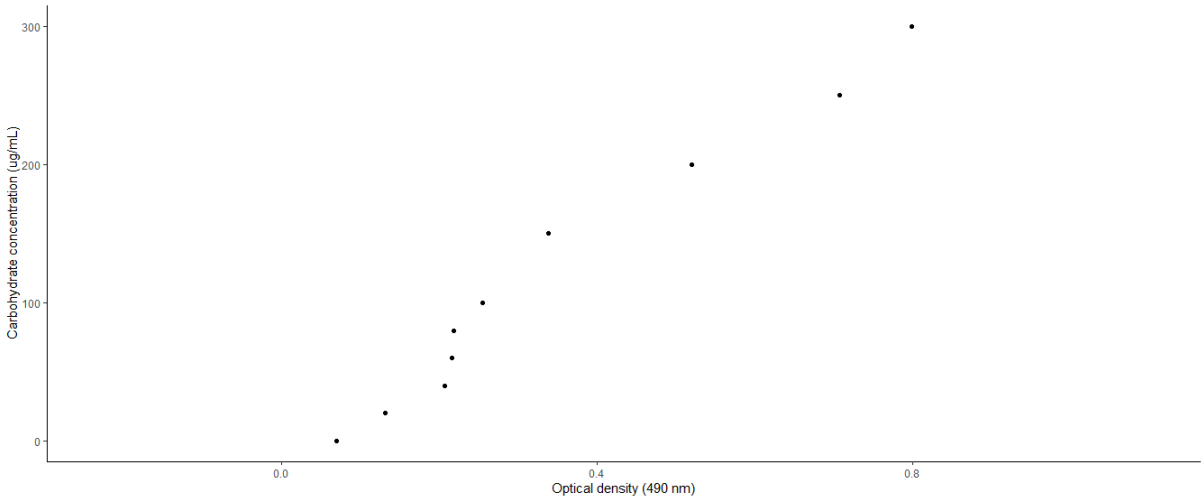
Standard curves for the determination of total proteins of (up to bottom) phototrophic, mixotrophic and heterotrophic cultivation.

Data obtained from the standard curves for the determination of total carbohydrates for the samples with the three different modes of cultivation.

	OD_{490nm}		
Glucose concentration	Phototrophic	Mixotrophic	Heterotrophic
0	0.058	0.168	0.069
20	0.081	0.188	0.131
40	0.114	0.271	0.206
60	-	0.276	0.216
80	0.142	0.341	0.218
100	0.185	0.348	0.254
150	-	0.377	0.338
200	0.268	0.503	0.520
250	-	0.580	0.707
300	0.418	0.603	0.799





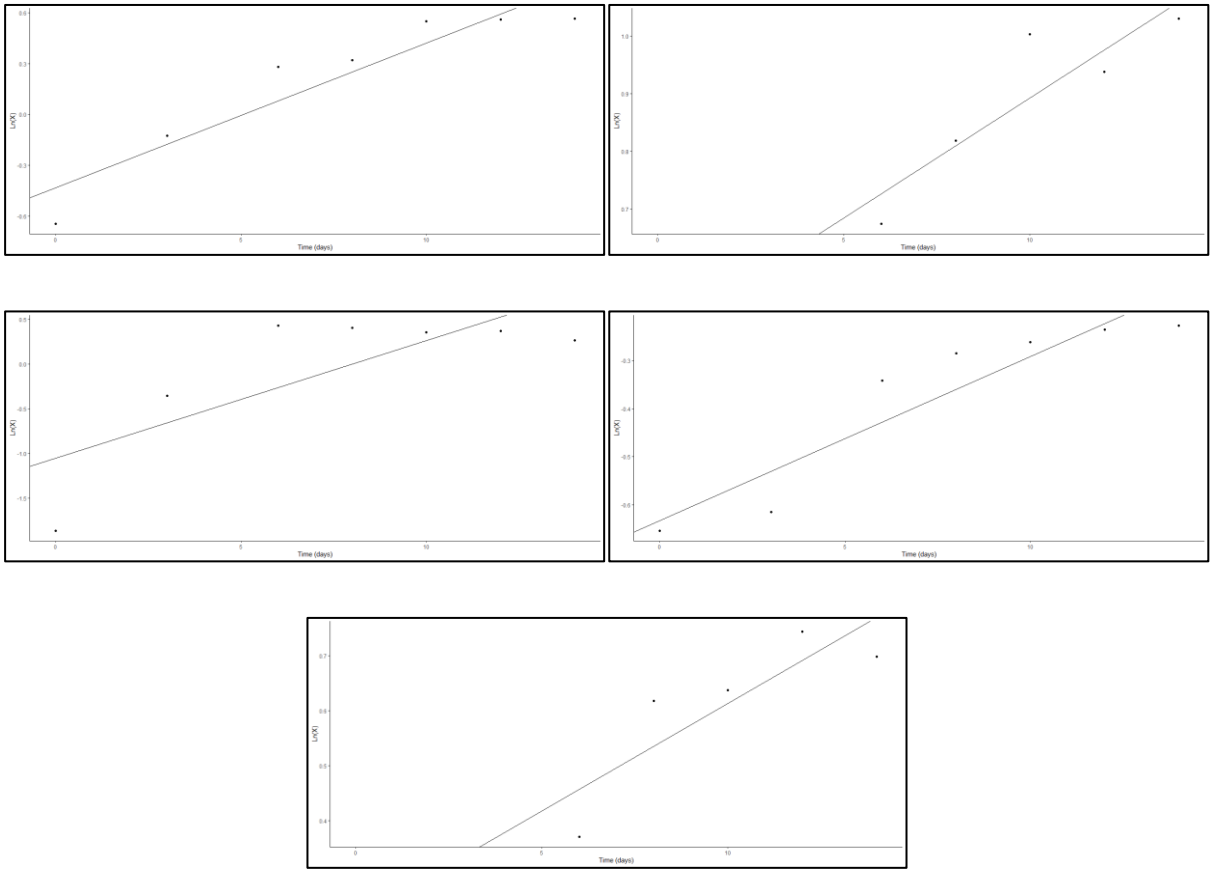


Standard curves for the determination of total carbohydrates of (up to bottom) phototrophic, mixotrophic and heterotrophic cultivation.

Appendix H

The graphs for each microalgae during the phototrophic mode of cultivation, in order to get the equation of the line to calculate the specific growth rate.

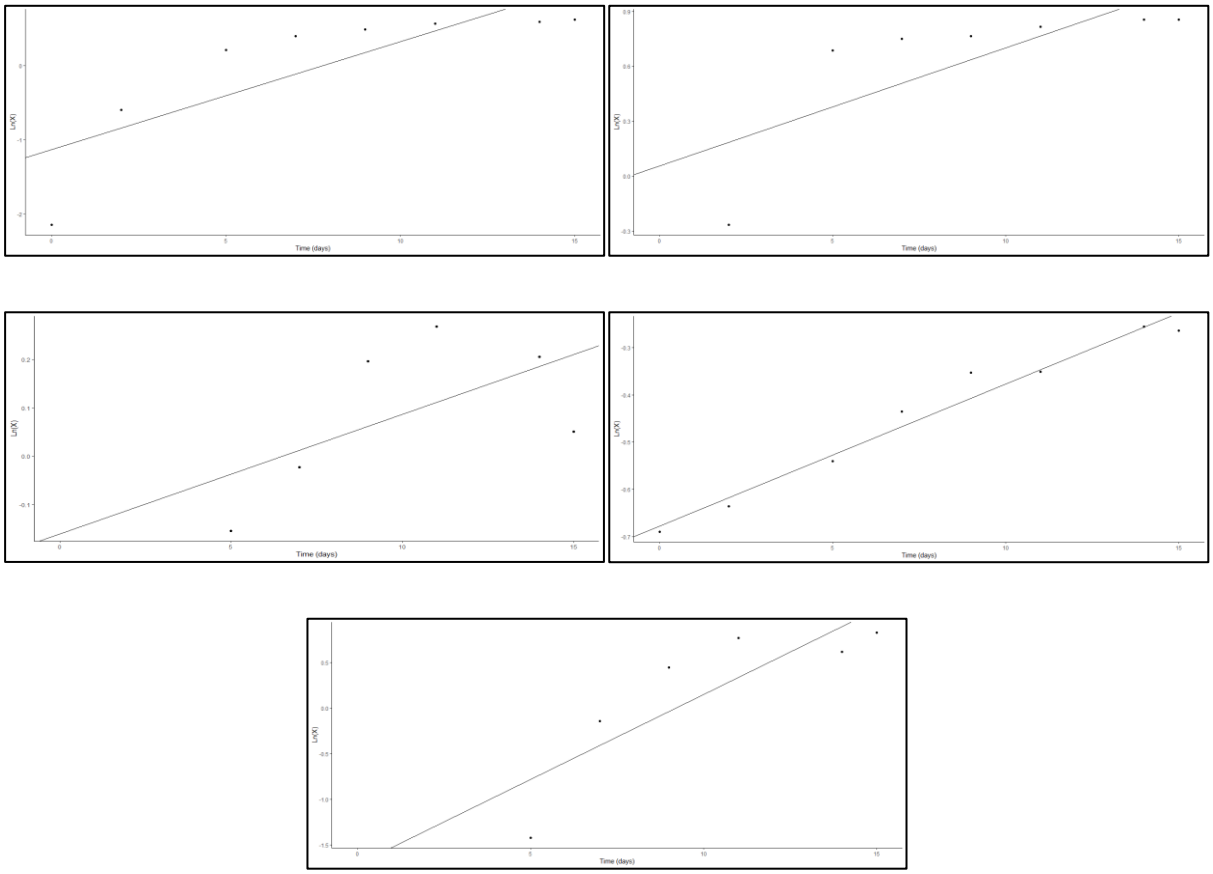
Microalgae	Equation of the line	R^2
N. oceanica	y=0.0854x-0.4323	0.8786
D. tertiolecta	y=0.0415x-0.4773	0.8016
T. suecica	y=0.1713x-1.2253	0.7047
C. ovalis	y=0.0340x-0.6321	0.8645
C. cohnii	y=0.3910x+0.2222	0.7301



Graphs for the equation of the line with the natural logarithm of the biomass of each microalgae in the Y axis and the time in days on X axis. (Left to right, up to bottom) *N. oceanica*, *D. tertiolecta*, *T. suecica*, *C. ovalis*, *C. cohnii*

The graphs for each microalgae during the mixotrophic mode of cultivation, in order to get the equation of the line to calculate the specific growth rate.

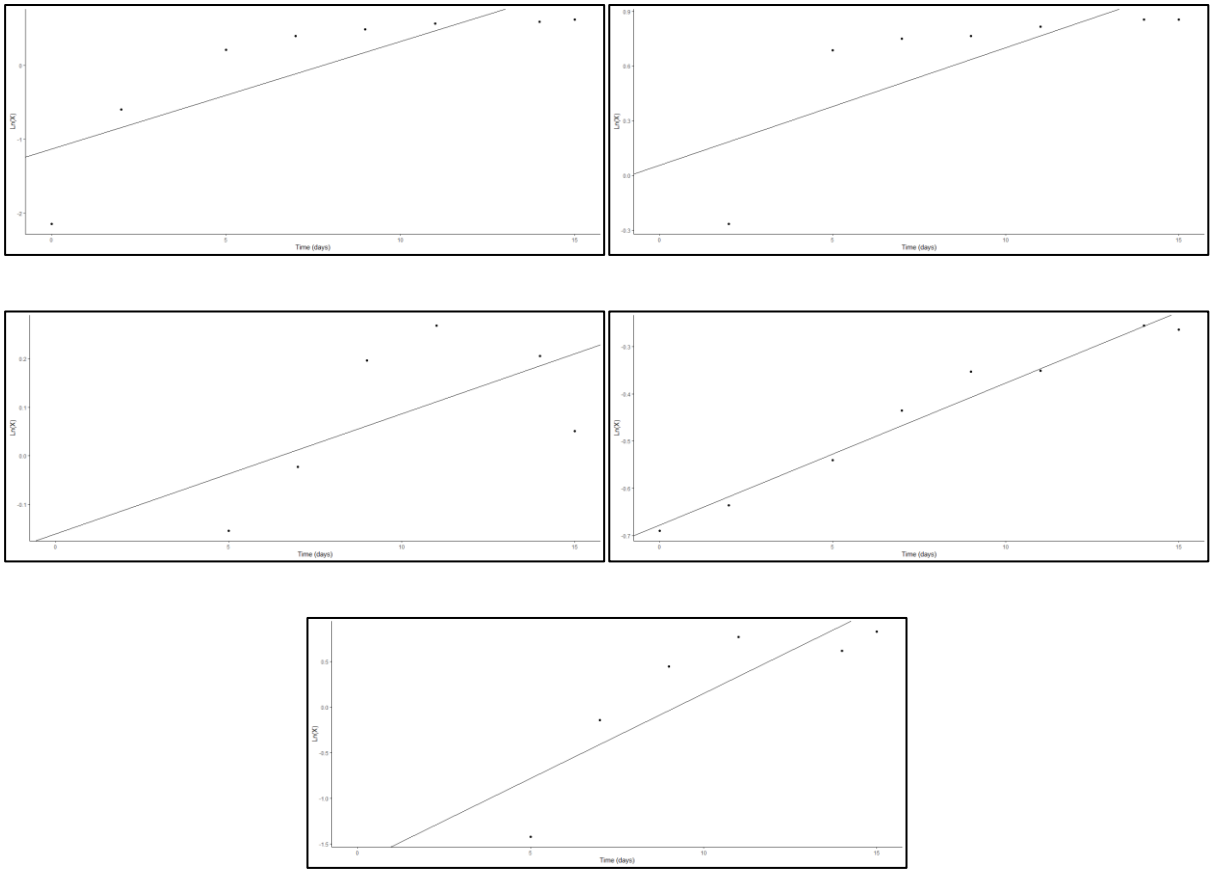
Microalgae	Equation of the line	R^2
N. oceanica	y=0.1455x-1.1297	0.6725
D. tertiolecta	y=0.0646x+0.0553	0.5745
T. suecica	y=0.0743x-0.5224	0.9683
C. ovalis	y=0.0301x-0.6778	0.9688
C. cohnii	y=0.1861x-1.7108	0.7188



Graphs for the equation of the line with the natural logarithm of the biomass of each microalgae in the Y axis and the time in days on X axis. (Left to right, up to bottom) *N. oceanica*, *D. tertiolecta*, *T. suecica, C. ovalis, C. cohnii*

The graphs for each microalgae during the heterotrophic mode of cultivation, in order to get the equation of the line to calculate the specific growth rate.

Microalgae	Equation of the line	R^2
N. oceanica	-	-
D. tertiolecta	-	-
T. suecica	-	-
C. ovalis	y=0.0259x-0.7044	0.9254
C. cohnii	y=0.0346x-0.6090	1.000



Graphs for the equation of the line with the natural logarithm of the biomass of each microalgae in the Y axis and the time in days on X axis. (Left to right, up to bottom) *N. oceanica, D. tertiolecta, T. suecica, C. ovalis, C. cohnii.*

Appendix I

The Shapiro-Wilk's test was used to probe the normality of the data; the null hypothesis of the test is that the population is distributed normally. Therefore, if the P-value is equal to or less than 0.05 the hypothesis of normality is rejected by the Shapiro-Wilk's test. The data of each species was analyzed with its biomass productivity, protein and carbohydrates concentration, and lipids quantity in the three different modes of cultivation.

Results of the Shapiro-Wilk's test of normality on productivity, proteins, carbohydrates and lipids of all microalgae. Values in green highlight show the rejection of the normal data.

	P-value in Shapiro-Wilk's test				
Microalgae	Growth	Biomass productivity	Proteins	Carbohydrates	Lipids
N. oceanica	0.2137	0.0016	0.1213	0.1040	0.1677
D. tertiolecta	0.6323	0.1591	0.0453	0.0267	0.9747
T. suecica	0.7615	0.0500	0.0008	0.8069	0.8674
C. ovalis	0.8168	0.0185	0.0295	0.0150	2.246×10^{-5}
C. cohnii	0.4836	0.1944	0.0242	0.0222	0.8231

The Brown-Forsythe's test was performed in order to test the homogeneity of the variance between the data of each species with its productivity, protein and carbohydrates concentration, and lipids quantity in three different modes of cultivation. The test has the null hypothesis that the variances among the populations are equal. Therefore, if the P-value is less than 0.05, the null hypothesis is rejected, and it is concluded that the variances are not equal among the different populations.

Results of the Bworn-Forsythe's test of homegenity of variances on productivity, proteins, carbohydrates and lipids of all microalgae. Values in green highlight show the acceptance of the homogenity of variances.

Microalgae	Biomass productivity	Proteins	Carbohydrates	Lipids
N. oceanica	0.0049	0.0189	0.1633	0.0372
D. tertiolecta	0.0085	0.0062	0.0215	0.0011
T. suecica	0.0002	0.0428	0.0247	0.0138
C. ovalis	0.0629	0.0125	0.1879	0.1700
C. cohnii	0.0196	0.0009	0.0030	0.0155

One-way ANOVA works with the assumptions of normality, sample independence, and variance equality, the tables above show the p-values of the normality and homogeneity test, where none of the microalgae fulfills both assumptions. Therefore, the Kruskal-Wallis's test was performed since it assumes that the distribution of the population should not be necessarily normal and the variances should not be certainly equal, it also does not assume a normal distribution of the data.

Kruskal-Wallis' test decides whether the population distributions are similar, meaning it probes that there are differences among the groups, but it does not give information regarding which modes of cultivation are different. If the P-value is less than 0.05, it can be concluded that there are significant differences between the modes of cultivation.

Results of the Kurskal-Wallis' test to confirm significant difference between the three types of modes of cultivation on productivity, proteins, carbohydrates and lipids of all microalgae. Values in green highlight show the significant differences between the modes of cultivation.

	P-value in Kruskal-Wallis' test				
Microalgae	Growth	Biomass productivity	Proteins	Carbohydrates	Lipids
N. oceanica	0.3679	0.0273	0.0056	0.1156	0.0288
D. tertiolecta	0.3679	0.0241	0.0039	0.0140	0.0140
T. suecica	0.3679	0.0265	0.0023	0.0135	0.0085
C. ovalis	0.3679	0.0273	0.0063	0.0616	0.0233
C. cohnii	0.3679	0.0509	0.0010	0.0056	0.0091

After performing the Kruskal-Wallis' test and looking at the significant differences between the modes of cultivations, a multiple pairwise comparison – Dunn's test – was realized to determine precisely which conditions are different, if the P-value is less than 0.05, it can be concluded that there are significant differences between the modes of cultivations.

Results of the Dunn's test to confirm significant difference on the type of modes of cultivation on productivity, proteins, carbohydrates and lipids of all microalgae. Values in green highlight show that there is significant difference.

		P-value in Dunn's test				
Micro-algae	Comparison	Growth	Biomass productivity	Proteins	Carbohydrates	Lipids
N. oceanica	Heterotrophic - Mixotrophic	0.4719	0.0219	0.0158	1.0000	0.5645
	Heterotrophic - Phototrophic	1.0000	0.5391	0.0182	1.1992	0.6638
	Mixotrophic - Phototrophic	1.0000	0.5391	1.0000	0.2729	0.0232
D. tertiolecta	Heterotrophic - Mixotrophic	1.0000	0.5172	0.0241	0.0894	0.3125
	Heterotrophic - Phototrophic	0.4719	0.0190	0.0073	0.0307	1.0000
	Mixotrophic - Phototrophic	1.0000	0.5172	1.0000	1.0000	0.0106
T. suecica	Heterotrophic - Mixotrophic	1.0000	0.5337	0.0481	0.1016	0.0101
	Heterotrophic - Phototrophic	0.4719	0.0211	0.0023	0.0232	0.9858
	Mixotrophic - Phototrophic	1.0000	0.5337	0.8415	1.0000	0.0868
C. ovalis	Heterotrophic - Mixotrophic	1.0000	0.5337	0.1365	0.0550	0.1622
	Heterotrophic - Phototrophic	0.4719	0.0211	0.0051	0.8288	1.0000
	Mixotrophic - Phototrophic	1.0000	0.5337	1.0000	0.3135	0.0227
C. cohnii	Heterotrophic - Mixotrophic	0.4719	0.0512	0.1850	0.3067	0.0273
	Heterotrophic - Phototrophic	1.0000	0.3032	0.0006	0.0044	1.0000
	Mixotrophic - Phototrophic	1.0000	1.0000	0.2772	0.7179	0.0167