MASTER THESIS

Optimisation of nutrient composition and process water recycling of *Nannochloropsis limnetica* cultures

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Abstract

In recent years the interest upon algae cultivation has been increasing due to the broad spectrum of applications, which reaches from food and feed additives to energy production. However, algae cultivation requires high water and nutrient amounts. For this reason, this work aims on the recirculation of process water, which can reduce the great demand for water and nutrients. In this thesis, the culture medium composition for the cultivation of *Nannochloropsis limnetica* was optimised and the performance of this organism when cultivated in recirculated process water was evaluated. *N. limnetica* was cultivated in different cultivation media, whereas the best suitable medium was then used to cultivate this organism in a flat plate reactor. The biomass was then harvested by two different methods (cross-flow filtration, centrifugation). The process water with replenished nutrients was recirculated to the reactor to start a new cultivation cycle. Growth was monitored by measuring the OD_{436} and calculating the growth rate. Biomass (lipid and pigment content) and process water (organic matter, inorganic nutrients, elemental composition) were analysed after each harvest. Of the tested media, ATCC was best suitable for cultivating *N. limnetica*. The experiments showed that it is possible to cultivate *N. limnetica* in recirculated process water without negatively affecting growth or lipid content. However, the pigment content of the biomass decreased and organic matter as well as nutrients accumulated in the medium. Centrifugation was shown to be most effective for harvesting *N. limnetica*. The recirculation of process water is promising but some identified factors indicate that the culture was under stress. The water demand can be reduced significantly by implementing process water recirculation into an algae cultivation process.

Keywords: Microalgae, *N. limnetica*, cultivation media, process water recirculation, flat plate reactor

Kurzzusammenfassung

In den letzten Jahren stieg das Interesse an Algenkultivierung aufgrund des breiten Anwendungsspektrums, das sich von Nahrungs- und Futterzusätzen bis zur Energieproduktion erstreckt. Algenkultivierung erfordert jedoch hohe Mengen an Wasser und Nährstoffen. Daher zielt diese Arbeit auf die Rückführung von Prozesswasser ab, die den hohen Wasser- und Nährstoffbedarf reduzieren kann. In dieser Arbeit wurde die Medienkomposition für die Kultivierung von *Nannochloropsis limnetica* optimiert und das Verhalten des in rückgeführtem Prozesswasser kultivierten Organismus bewertet. *N. limnetica* wurde in verschieden Nährmedien kultiviert wobei das geeignetste Medium genutzt wurde, um den Organismus in einem Flachplattenreaktor zu kultivieren. Die Biomasse wurde mittels zwei verschiedener Methoden (Querstromfiltration, Zentrifugation) geerntet. Das Prozesswasser mit aufgefüllten Nährstoffen wurde in den Reaktor rückgeführt, um einen neuen Kultivierungszyklus zu starten. Das Wachstum wurde durch OD_{436}-Messung und Berechnung der Wachstumsrate kontrolliert. Die Biomasse (Lipid- und Pigmentanteil) und das Prozesswasser (organisches Material, anorganische Nährstoffe, elementare Zusammensetzung) wurden nach jeder Ernte analysiert. Von den getesteten Medien war ATCC am besten für die Kultivierung von *N. limnetica* geeignet. Die Versuche zeigten, dass eine Kultivierung von *N. limnetica* in rückgeführtem Prozesswasser ohne negative Effekte auf das Wachstum oder den Lipidanteil möglich ist. Der Pigmentanteil an der Biomasse verringerte sich jedoch und organisches Material und Nährstoffe akkumulierten im Medium. Es zeigte sich, dass Zentrifugation am effektivsten für die Ernte von *N. limnetica* ist. Die Rückführung von Prozesswasser ist vielversprechend, aber manche der identifizierten Faktoren deuten darauf hin, dass die Kultur unter Stress stand. Der Wasserbedarf kann signifikant reduziert werden, wenn Prozesswasserrückführung in einen Algenkultivierungsprozess implementiert wird.

Schlagwörter: Mikroalgen, *N. limnetica*, Nährmedien, Prozesswasserrückführung, Flachplattenreaktor

List of abbreviations

aq	Aqueous
ATCC	American Type Culture Collection
ATP	Adenosintriphosphate
BBM	Bold's Basal Medium
$CaCl_2$	Calcium chloride
CCALA	Culture Collection of Autotrophic Organisms
Chl_a	Chlorophyll a
CM	Solution chloroform:methanol 2:1
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
$FeCl_3$	Iron(III) chloride
H_2CO_3	Carbonic acid
H_2SO_4	Sulphuric acid
HCO_3^-	Bicarbonate
IC	Ion chromatography
ICP	Inductively coupled plasma
K_2HPO_4	Dipotassium phosphate
LED	Light-emitting diode
µ	Growth rate
M	Molar (mol/L)
$MgSO_4$	Magnesium sulphate
MQ-H_2O	Milli-Q water
N. limnetica	*Nannochloropsis limnetica*
N. limnetica (CCALA)	*Nannochloropsis limnetica* obtained from CCALA
N. limnetica (ecoduna)	*Nannochloropsis limnetica* obtained from ecoduna
Na_2CO_3	Sodium carbonate
Na_2SiO_3	Sodium silicate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NDIR	Nondispersive infrared sensor
NH_4^+	Ammonium
NO_2^-	Nitrite

NO_3^-	Nitrate
NO_x	Nitrogen oxides
NPOC	Non-purgeable organic carbon
OD	Optical density
OH^-	Hydroxide
pH	Negative decadic logarithm of the molar concentration of dissolved hydronium ions in aquatic solution
PVC	Polyvinylchloride
R^2	Coefficient of determination
RNA	Ribonucleic acid
RO-H_2O	Reverse osmosis water
rRNA	Ribosomal ribonucleic acid
Rubisco	Ribulose-1,5-bisphosphat-carboxylase/-oxygenase
SAG	Sammlung von Algenkulturen Göttingen (The Culture Collection of Algae at Goettingen University)
SO_2	Sulfur dioxide
TOC	Total organic carbon
TS	Total solids
UTEX	Culture Collection of Algae at The University of Texas at Austin
UV	Ultraviolet
WC	Wright's cryptophyte (culture medium)

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1 Introduction

The applications of algae are diverse; thus, interest in their cultivation has been increasing in recent years. However, there are some notable drawbacks as algae have a big water and nutrient demand. Besides adapting conditions such as light, temperature and pH, the composition of cultivation media should be optimised in order to avoid excess nutrients. Moreover, recirculating the process water after harvesting the biomass is a promising approach to save both fresh water and nutrients. The step of harvesting the biomass is another critical factor as it usually is the most energy-intense step in algae cultivation. Therefore, it is crucial to find a suitable method for each specific application as various methods are available, each with its own advantages and drawbacks. This thesis deals with the mentioned problems and aims to help develop a cultivation with decreased water and nutrient demand.

1.1 Algae cultivation

Photosynthetic organisms, i.e. microalgae and cyanobacteria, use solar energy to convert CO_2 and inorganic nutrients into proteins, carbohydrates, lipids and other valuable compounds (Liu et al., 2016). They are promising organisms for producing commercially valuable compounds such as fatty acids, pigments and proteins (Liu et al., 2016; Noda et al., 2017). Algae are able to produce long-chain polyunsaturated fatty acids that most animals cannot produce (Krienitz and Wirth, 2006). Their potential fields of application range from food and pharma industry to fish feed in aquaculture (Liu et al., 2016; Morocho-Jácome et al., 2016). The most important organisms that are used as food supplements in human nutrition are *Arthrospira* sp. (cyanobacterium) and *Chlorella* sp. (green alga) (Liu et al., 2016). Moreover, pigments such as carotenoids (astaxanthin) are produced from microalgae on an industrial scale whereas pharmaceutical products using these pigments are still in development (Liu et al., 2016). CO_2 emission from industrial processes can be used to cultivate microalgae and it is possible to cultivate these organisms on non-arable land (DECHEMA-Fachgruppe "Algenbiotechnologie", 2016; Loftus and Johnson, 2017). The areal productivity is higher than with other oil-based crops (Loftus and Johnson, 2017). But there are also problems associated with microalgae cultivation. Energy, nutrient and water demand are big (Markou et al., 2014). Furthermore, even though the production of algae is usually not causing a direct food vs. fuel debate when used as biodiesel, they are fed with more nutrients than are needed for the production of plants (Markou et al., 2014).

1.1.1 *Nannochloropsis limnetica*

In this thesis, the cultivation of *Nannochloropsis limnetica* was investigated. Therefore, this organism is described in more detail.

The genus *Nannochloropsis* belongs to the class Eustigmatophyceae, which are morphologically simple eukaryotic cells (Fietz et al., 2005; Noda et al., 2017). *Nannochloropsis* sp. cells are very small; usually, they are smaller than 3 µm (Fawley and Fawley, 2007; Krienitz et al., 2000; Krienitz and Wirth, 2006). Thus, they are classified as autotrophic picoplankton (Krienitz et al., 2000; Krienitz and Wirth, 2006). Due to the small size of picoplankton their volume-to-surface ratio is very effective, which makes them highly productive in various environments (Krienitz and Wirth, 2006).

Natural habitat

Nannochloropsis sp. mostly occur in marine environments, especially near coasts (Fawley and Fawley, 2007; Krienitz et al., 2000; Krienitz and Wirth, 2006). *Nannochloropsis limnetica* is the only freshwater species of its genus and was first isolated from a hypertrophic lake in Germany by Krienitz et al. (2000). Large populations of *N. limnetica* have especially been found in cold-weather periods, which indicates a low temperature optimum, possibly between 10-15 °C (Fawley and Fawley, 2007; Fietz et al., 2005). A pH of 8.3 ± 0.1 and a concentration of 4.6 mg N/L are parameters that were found in lakes with high *N. limnetica* populations (Krienitz et al., 2000) .

Differences in some genetic characteristics have been found between *N. limnetica* that were isolated from different locations. This means that they belong to the same species but are different ecotypes that have adapted to their environment. This could imply that, for example, they have different growth rates or vitamin demand. (Fietz et al., 2005)

Biomass composition and application

The pigment composition of *N. limnetica* is similar to that of other species of *Nannochloropsis*. It primarily contains various xantophylls, chlorophyll$_a$ and β-carotene. (Krienitz et al., 2000).

The cells also contain valuable fatty acids in amounts up to 350 µg/mg C. The composition is similar to that of marine *Nannochloropsis* species but *N. limnetica* contains higher amounts of fatty acids. Eicosapentaenoic acid (EPA), an ω-3 fatty acid, oleic and linoleic acid are especially relevant products. (Krienitz et al., 2000) However, fatty acid content and composition are strongly dependent on culture conditions, e.g. on light intensity, temperature and growth rate (Freire et al., 2016). Fatty acid content can reach up to 70 % under nitrogen deficiency and therefore low biomass production (Noda et al., 2017).

Nannochloropsis sp. are used as feed organisms for aquaculture due to their rapid reproduction and unique fatty acid content and composition, especially because of their high content of EPA (Fawley and Fawley, 2007; Krienitz and Wirth, 2006). The fatty acids are transferred to rotifers and then to fish larvae (Freire et al., 2016). For the same reasons, the organism is interesting for food web studies and biotechnology (Krienitz and Wirth, 2006). Little research has been conducted about the application potential of *N. limnetica*. Nevertheless, as they have a similar

fatty acid pattern, it can be assumed that this freshwater strain can be applied in aquaculture the same way as marine *Nannochloropsis* sp. It would have the advantages of growing in freshwater instead of seawater (no production of artificial seawater in regions without access to the sea, lower risk of corrosion of equipment).

1.1.2 Algae cultivation systems

There are various types of bioreactors for algae cultivation. They can be categorised into open systems and closed photobioreactors. Closed systems provide a better control of cultivation conditions, productivity is increased, and contaminations are prevented. They are usually used for lower-volume, higher-quality products (Lizzul and Allen, 2017). Open systems are generally applied when there is no danger of contamination because of extreme cultivation conditions or when contamination would not be a problem (Lizzul and Allen, 2017). The most critical factor in algae cultivation is the availability of light. For this reason, different types and designs of photobioreactors have been developed to increase their efficiency. (Ugwu et al., 2008)
In the following, some types of algae cultivation systems will be introduced briefly.

Open ponds

Open ponds (Figure 1) are easy to construct, to operate and to clean, which results in low production and operating costs. They can also be scaled up without major challenges. Nevertheless, they have some drawbacks. Light energy is utilised very poorly due to high layer thickness in open ponds. To improve this, the ponds need to be very shallow, which in return would lead to larger land area requirements. Moreover, a high amount of water evaporates in open ponds, which are subject to contamination. Therefore, it only makes sense to use this system for the cultivation of organisms that can grow under conditions that would inhibit contamination (extreme salinity or pH). (Singh and Sharma, 2012; Ugwu et al., 2008)

Figure 1: Open pond. (AlgaeForBiofuels, 2010)

Until today, open ponds have been the most popular system for mass cultivation of microalgae (Lizzul and Allen, 2017).

Tubular photobioreactors

Tubular photobioreactors, which consist of glass or plastic tubes, are an example of a closed algae cultivation system. Horizontal and vertical systems are available. An advantage of tubular

systems is the large illumination surface area. Moreover, mixing through air displacement leads to high mass transfer and low shear stress. However, it is challenging to scale up this type of photobioreactor. If the diameter is increased for the scale-up, the illumination surface to volume ratio decreases. Nevertheless, CO_2 and O_2 gradients will form if the columns exceed a certain length. Another drawback is that tubular systems are not easy to clean and subject to biofouling. One way to overcome this is introducing plastic beads into the tubes, which is especially used in horizontal systems (Figure 2, left). In horizontal tubes, a gas exchange system introduces CO_2 at the entrance of the columns. Vertical systems have a gas sparger on the bottom of the tubes that introduces little air bubbles and CO_2 and provides good mixing. (Singh and Sharma, 2012; Ting et al., 2017; Ugwu et al., 2008)

Bubble columns are an example of vertical tubular photobioreactors (Figure 2, right). These systems are easy to build from cheap materials such as PVC and there are no internal parts, so the production and maintenance costs are low. They are commonly used at a scale of 5 to 50 L in aquaculture or as inoculation vessels for larger photobioreactors. (Lizzul and Allen, 2017)

There are, however, exceptions: One of the world's biggest photobioreactors, namely ecoduna's production plant (eparella GmbH), consists of 43,000 glass columns with an overall length of 230 km. (ecoduna, 2018)

Figure 2: Left: Horizontal tubular photobioreactor with plastic beads. Reactor was built in the course of the project "Integrated Sustainable Algae" in Olhao, Portugal. © Meixner. Right: Large scale vertical tubular photobioreactors, eparella GmbH production plant. (ecoduna, 2018)

Flat plate reactors

Flat plate reactors (Figure 3) are solely made of transparent materials to provide maximum utilisation of solar light. Their cuboidal architecture guarantees high photosynthetic efficiencies. The surface area to volume ratio is high and the light path is minimal. This leads to high photosynthetic efficiencies as there is only little self-shading of the culture and less absorption by

the medium. This is a major advantage compared to other types of photobioreactors. Moreover, producing this reactor type is easy and cheap. Difficulties in scale-up and temperature control are some of the drawbacks. Moreover, insufficient mixing may lead to wall growth. (Lizzul and Allen, 2017; Singh and Sharma, 2012; Ugwu et al., 2008)

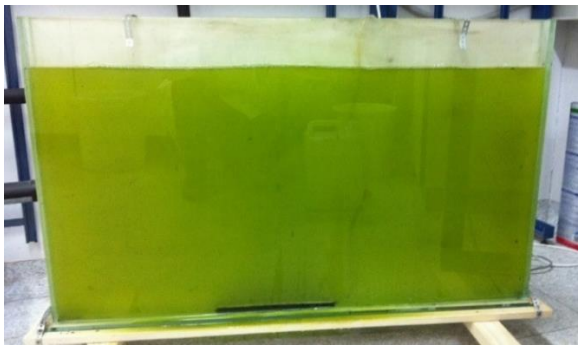

Figure 3: Laboratory-scale flat plate reactor (20 L working volume).

1.1.3 Cultivation conditions in photobioreactors

Light

As algae perform photosynthesis, quantity and quality of light are the most important parameters during their cultivation. If too little light is provided, growth will be limited. Nevertheless, if the amount of provided light is too high, photoinhibition and cell death might be the consequences. Not only the amount of light is important; the wavelengths must be suitable as well (Lizzul and Allen, 2017). Artificial light is used at laboratory scale, but at industrial scale solar light or a combination of both are also in use (Ugwu et al., 2008). The kind of illumination that is used depends on the product and the reactor type. In open ponds, natural light is often the only light source. This could become problematic as during some periods of the day, illumination is insufficient. Seasonal change can also alter biomass composition. Nevertheless, energy savings are immense compared with the use of artificial light, which explains why natural sunlight is still often used in industry. When a light beam travels through a culture, it is attenuated by the medium. Moreover, chlorophyll that is present in algal cells absorbs light in the course of photosynthesis. For these two reasons, light intensity quickly decreases with culture depth. Therefore, a short light path is required. This is provided by a flat plate reactor, as pointed out above. Light can be used even more efficiently if a plate reactor is illuminated from two sides. Efficient mixing will provide all cells with enough light by circulating them to the highly illuminated surface. (Behrens, 2005; Lizzul and Allen, 2017; Zhang, 2011)

Temperature

It is crucial to control the temperature in a photobioreactor. Heat is produced during algae cultivation because the efficiency of photosynthesis to convert light energy into chemical energy is low. Therefore, cooling of reactors might be necessary. (Behrens, 2005)
Some bioreactors can be tempered easily by placing them in a tempered room. But this is only possible at laboratory scale. For large-scale purposes, a double-walled reactor with a heating and cooling circuit might be a suitable option. Another way of preventing the photobioreactor from overheating is to install water sprinklers that spray water onto the reactor surface when a certain temperature maximum is reached. (Carlozzi and Torzillo, 1996; Ugwu et al., 2008)

pH

The optimum pH is different for each algal species. Most species have their optimum between 6.5 and 10. Under photoautotrophic conditions, CO_2 is utilised by algae for photosynthesis and decreases the pH to a big extent, which is described in more detail below (section 1.1.4). In the course of photosynthesis, OH^- ions are produced during the intracellular conversion of HCO_3^- to CO_2. This leads to an increase of the pH up to 11 where CO_2 is mainly available in the form of CO_3^{2-}. This cannot be utilised by most microalgae and growth will decrease. Thus, they have developed mechanisms such as calcification to cope with high pH. Moreover, to regulate their intracellular pH, algae can excrete or take up H^+ ions. In order to provide good growth conditions, the pH should be controlled by adding acid or CO_2 to the medium. (Markou et al., 2014)

1.1.4 Nutrient requirements

Besides providing the right light, temperature and pH conditions, it is vital to supply microalgae with nutrients in the right amounts. Deficiency in one element will cause growth reduction, according to Liebig's law of the minimum. Nevertheless, microalgae might adjust their nutrient uptake and requirements to their availability to a certain extent. They are able to live with deficiencies and to store excess quantities. These phenomena can be utilised in biotechnology to produce e.g. high amounts of fatty acids. Nutrients not only need to be provided sufficiently but also in a bioavailable chemical form and at the right ratio. The ratio of C:N:P should approximately be 106:16:1 (Redfield ratio; elemental composition of phytoplankton). Changing this ratio can lead to a, potentially desired, change in the composition of the algal cell. Most cultivation media are not optimised and nutrients are most often present in excess. This leads to higher cultivation costs as nutrients account for a high part of the expenses. Therefore, media recipes have to be adapted to the needs of the specific cultivation. (Borowitzka, 2016; Markou et al., 2014)

Carbon

Carbon is part of all organic compounds and the main biomass element. Algae are able to convert inorganic into organic carbon and mainly take it up in the form of CO_2. As mentioned above, the availability of CO_2 strongly depends on the pH. It is mainly present in the form of HCO_3^- between pH 6.5 and 10. At lower values there is more H_2CO_3 + CO_2. At higher values CO_3^{2-} dominates. This equilibrium is visualised in Figure 4. Microalgae are able to import CO_2 as well as HCO_3^-. Carbonic anhydrase converts HCO_3^- to CO_2, Rubisco translates CO_2 to organic carbon in the Calvin cycle. CO_2 can be applied to algal cultures by pumping in air or concentrated CO_2 or by addition of bicarbonate salts. As a low-cost alternative, flue gas can be used but care has to be taken as it might contain contaminations, e.g. NO_x and SO_2, that possibly inhibit growth. (Cheah et al., 2015; Chi et al., 2011; Markou et al., 2014)

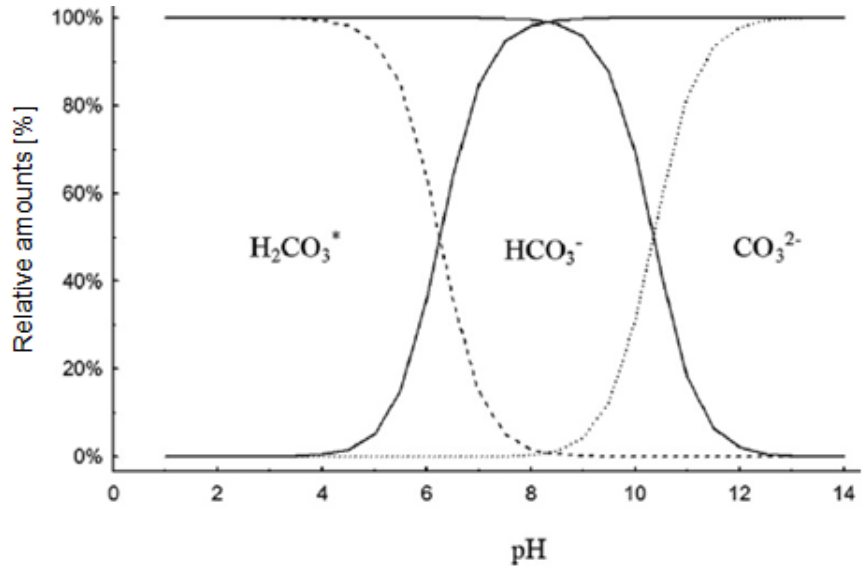

Figure 4: Relative amounts of inorganic carbon species as a function of pH of the solution. H_2CO_3* = $CO_{2(aq)}$ + H_2CO_3. Modified after Markou and Georgakakis (2011).

Nitrogen

Nitrogen is one of the most abundant elements in photosynthetic cells. It is an essential component of catalysts and intermediates of primary metabolism, part of amino acids (thus proteins), DNA, RNA, ATP, vitamins, and chlorophylls. NO_3^- acts as signal for the activation of genes that are needed to acquire and assimilate it and also regulates other pathways. As algae have a high protein content compared to terrestrial plants, their nitrogen requirement is considerable (Markou et al., 2014). Nitrogen is mostly available in the forms of NO_3^-, NH_4^+ and NO_2^-. Many different forms of combined nitrogen can be used by algae, yet only NH_4^+ can be used by all of them. Usually it is supplied as urea, nitrate or ammonium. (Raven and Giordano, 2016)

Phosphorus

Phosphorus is an important cell compound and has functions in the metabolism, cell structure, and storing energy and genetic information. It is present in DNA, RNA, ATP, and phospholipids. There is a considerable demand for phosphorus especially in rRNA. In natural environments, phosphorus often is a limiting nutrient. It is usually applied to cultures in the form of phosphate salts, which are derived from non-renewable stocks. Algae take up phosphorus in the form of orthophosphate via symporters. Therefore, pH affects the uptake. At increased pH, polyvalent cations might precipitate with phosphate, which leads to a reduced availability. (Dyhrman, 2016; Markou et al., 2014)

Potassium

Besides nitrogen and phosphorus, potassium is one of the three primary macronutrients for biomass production. It is an activator for enzymes that are involved in photosynthesis and respiration. Furthermore, it affects protein and carbohydrate synthesis and regulates osmotic potential. At low concentration, it is taken up actively whereas at high concentration, algae take up potassium passively. Potassium is added to cultivation media in the form of various salts. (Markou et al., 2014)

Sulphur

Sulphur is important for the production of the amino acids cysteine and methionine; it is a cofactor and part of vitamins and the cell wall. Usually, it is acquired as sulphate in the chloroplast. Sulphur assimilation is a reductive process: Sulphate is the most oxidised form but sulphur is used in the lowest oxidation state. Thus, high redox energy is needed. When sulphur becomes limiting, algal growth is influenced in several ways. Photosynthesis will be reduced and light and dark reactions will be altered. Moreover, the production of some proteins, especially Rubisco, will decrease. Sulphur is usually provided in the form of $MgSO_4$, which simultaneously delivers magnesium. (Giordano and Prioretti, 2016; Markou et al., 2014)

Magnesium

Magnesium is important for ATP reactions in carbon fixation and is an activator for enzymes. Most importantly, it is a constituent of chlorophyll. Magnesium availability might affect cell multiplication. (Markou et al., 2014)

Trace elements

It is not yet fully known which trace elements are essential for microalgae. However, those involved in redox reactions, acid-base catalysis, structure, energy and genetic information storage are crucial. The most important trace element might be iron. Other examples are

manganese, zinc, copper, molybdenum, cobalt, vanadium, nickel, and cadmium. (Markou et al., 2014; Quigg, 2016)

Iron is a significant part of all microalgae media. It is utilised in photosynthetic organisms to maintain the electron-transport-chain and is involved in other enzymatic processes such as oxygen metabolism and synthesis of biomolecules. Iron is often supplied as ferric EDTA. At too high concentrations, iron might cause cell death. Therefore, care should be taken considering the amount of iron. This should especially be taken into account if medium is recycled. In this case, monitoring of iron accumulation is essential. (Borowitzka, 2016; Markou et al., 2014)

Vitamins

Vitamin requirements vary between species. 50 % of algae require vitamin B12 as cofactor for methionine synthase. 20 % require thiamine and 5 % require biotin. However, many species do not require vitamins at all. (Borowitzka, 2016)

1.1.5 Water demand

In all the different cultivation systems, a lot of water is needed as algae live in aqueous habitats. Water buffers temperature to a certain extent through evaporation and the nutrients needed by algae are dissolved in water. The amount of water per kg biomass strongly depends on the cultivation system. To exemplify; in open systems, a typical cell density is 0.5 g/L. In this case 2,000 L of water are needed to produce 1 kg of dry biomass. This is comparable to some plants that consume high amounts of water, for instance rice and soybeans. In order to make algae cultivation a sustainable process, this immense water consumption must be reduced. 5 to 10 L of water are converted to oxygen during photosynthesis for each kg of dry biomass and a major part of the water is lost due to evaporation. Moreover, during downstream processing, some water is lost. The remaining water (process water) that is only needed to keep the algae in suspension has to be treated before it is disposed as wastewater. This makes the process even less sustainable. Theoretically, the remaining process water can be used again for another cultivation, which would reduce the water demand as well as the need to treat the process water in a waste water treatment plant. In the following, the problems and potentials of recirculating process water will be dealt with. (Depraetere et al., 2015; Farooq et al., 2015; Markou et al., 2014; Morocho-Jácome et al., 2016; Rodolfi et al., 2003)

1.2 Harvesting methods

Harvesting of microalgae is a challenging process and one of the major limiting factors in microalgae production. This is due to low cell densities, even in highly optimised systems, small cell size, and the similar density as that of culture medium. A variety of harvesting methods exists and is in use as there is no universally applicable procedure. (Milledge and Heaven, 2013)

Methods can be divided into physical (e.g. centrifugation) and chemical (e.g. flocculation) ones (Kim et al., 2015). Borowitzka (2016) and Farooq et al. (2015) state that the harvesting method can affect the performance of recirculation. For example, centrifugation can lead to cell breakage, which would release inhibitory organic matter; flocculants can leave toxic aluminium behind. Various harvesting methods will be presented in the following paragraphs.

1.2.1 Sedimentation and flotation

In sedimentation, gravitational forces separate particles or liquids of different densities. This low-cost process can be very slow but the speed varies between species. Sedimentation is not widely used as sole harvesting method but it has potential as a pre-concentration step.
Air bubbles are added to the culture suspension in flotation to make the algal cells float on the surface. These air bubbles can be produced by various methods. Flotation is not very effective except in combination with flocculation. (Milledge and Heaven, 2013)

1.2.2 Flocculation

Flocculation can happen naturally ("autoflocculation") in algae cultures, for example as response to environmental stress. This is not reliable; however, it can also be induced by chemicals, a change in pH or application of electric current, which is more controllable. The flocs then settle or, if air bubbles are introduced, float more quickly due to their increased size. This method can be applied to large quantities and a wide range of microalgae. If inorganic chemicals are used as flocculants, the culture medium might be modified in a way that makes it non-recyclable. Bioflocculants (macropolymers produced by microorganisms; e.g. proteoglycans) are much more suitable as they are environmentally friendly and do not prohibit medium recirculation. (Chen et al., 2017; Farooq et al., 2015; Loftus and Johnson, 2017; Milledge and Heaven, 2013)

1.2.3 Centrifugation

Centrifugation works according to the same principle as sedimentation but force is applied instead of only using gravity. It is a rapid and easy method and suitable for most species. Nevertheless, it requires a lot of energy and is therefore costly, which makes it hardly suitable for industrial applications. Energy-efficiency can be improved by pre-concentrating the culture suspension. Most commonly, disk stack centrifuges are used. (Kim et al., 2015; Loftus and Johnson, 2017; Milledge and Heaven, 2013)

1.2.4 Membrane filtration

In contrast to other methods, the cells are not damaged when membrane filtration is used for harvesting. Filtration techniques are especially suitable for large cells; often, microfiltration can be used. The smaller the pores, the more energy it takes to filter the cells through; for this reason, membrane filtration is not suitable for cells with a diameter below 10 µm. Generally,

energy consumption is low but when membrane fouling occurs, cross-flow velocity and therewith throughput is reduced. Cross-flow filtration is widely used as it decreases fouling due to a tangential flow of the suspension. (Kim et al., 2014; Kim et al., 2015; Milledge and Heaven, 2013)

1.3 Recirculation of process water

As outlined above, a lot of nutrients and water are needed for algae cultivation. Therefore, the process is expensive and hardly economic, especially for low value products. The recirculation of process water is a proposed approach to overcome this problem. This way, nutrient as well as water and wastewater treatment costs might be reduced. (Farooq et al., 2015; Loftus and Johnson, 2017; Zhang et al., 2016)

Alongside these improvements, some challenges might also be faced when reusing process water. After harvesting algae, parts of unused nutrients and organic matter such as cell debris and extracellular substances remain in the process water. The nature of the extracellular organic matter depends on the algae strain, culture conditions, the growth phase at the time point of harvesting, the harvesting process itself, and accompanying microbes (Loftus and Johnson, 2017). The concentration of extracellular substances is lower in the exponential phase than in the stationary phase because in the stationary phase, the cells are stressed and lyse (Loftus and Johnson, 2017; Zhang et al., 2016). The organic matter might include substances that directly inhibit algal growth, for example fatty acids. A large part of this organic matter consists of polysaccharides, which influence the rheological properties of the cultivation media. (Depraetere et al., 2015)

Altered viscosity could lead to difficulties in harvesting and could influence nutrient availability as well as cell aggregation (Loftus and Johnson, 2017). Moreover, extracellular products could also lead to contamination as they may act as organic carbon source for heterotrophic organisms (Farooq et al., 2015). If accumulating organic matter makes recycling medium difficult, it can be removed before the process water is recirculated, e.g. by sterilisation by ozonation or UV irradiation (González-López et al., 2013). However, this would again increase the costs of the process. Supporting this hypothesis, Depraetere et al. (2015) showed that the growth rate of the cyanobacterium *Arthrospira platensis* declined after four cycles of recirculation compared to cultivation in fresh medium. Additionally, the study states that proteins, phycocyanin and total chlorophyll in the biomass were lower whereas total sugars were higher, suggesting that a certain number of recirculations stresses the organism.

When culture medium is recirculated, nutrients have to be added again after each cycle. Ideally, only the nutrients that were used by the algae are replenished (Markou et al., 2014). Otherwise, counter ions of ammonium, sulphate, carbonate etc. in salts that were added as nutrients will accumulate, which may inhibit algal growth. (Borowitzka, 2016)

Despite these theoretical problems, studies at laboratory scale have shown that there is no inhibiting effect when medium is recycled (Borowitzka, 2016; Farooq et al., 2015; Fret et al.,

2017; Markou et al., 2014). It was even proposed by Borowitzka (2016) that sometimes, growth in used medium could possibly be enhanced due to products released by algal cultures. However, many studies that successfully recycled culture medium without negative effects only recycled the medium once or twice (Farooq et al., 2015), which is too little for a feasible process.

2 Aims and objectives

Different algae cultivation systems exist, but in all of them a big amount of water and a big number of nutrients are necessary to cultivate algae. This problem may be solved by recirculating spent process water, which is described in literature, but problems have been observed and the results are dependent on the strain used.

The aim of this thesis was to optimise the nutrient and fresh water effort for cultivating *N. limnetica* and to evaluate possible effects of process water recirculation on its growth and biomass composition.

At first, the nutrient compositions of various potential cultivation media were compared theoretically. As a second step, the growth of *N. limnetica* in chosen media was compared and the most suitable medium was selected. For this purpose, 24-well plates were used and later on replaced by shaking flasks. Finally, recirculation experiments were conducted in a flat-plate photobioreactor with a working volume of 20 L using the cultivation medium selected before. Moreover, two harvesting methods – cross-flow ultrafiltration and centrifugation – were compared. These methodologies are expected to help develop an algae cultivation process with reduced water and nutrient consumption, and decreased waste water production.

3 Materials and methods

3.1 Chemicals and equipment

Most chemicals and equipment were used repeatedly in the experiments. Thus, they are listed alphabetically once below and are referred to in the respective methods.

3.1.1 Chemicals

- **Acetone** $(CH_3)_2CO$; ≥ 98 %; *Bernd Kraft GmbH*
- **Aluminium potassium sulphate dodecahydrate** $AlK(SO_4)_2 \cdot 12\ H_2O$; *Merck KGaA*
- **Ammonium phosphate dibasic** $(NH_4)_2HPO_4$; *Sigma-Aldrich*
- **Biotin** $C_{10}H_{16}N_2O_3S$; ≥ 99 %; *Sigma-Aldrich*
- **Boric acid** H_3BO_3; ≥ 99.8 %; *Sigma-Aldrich*
- **Calcium chloride dihydrate** $CaCl_2 \cdot 2\ H_2O$; *VWR Chemicals*
- **Calcium nitrate tetrahydrate** $Ca(NO_3)_2 \cdot 4\ H_2O$; 95-100 %; *Sigma-Aldrich*
- **Calcium sulphate dihydrate** $CaSO_4 \cdot 2\ H_2O$; 98 %; *Sigma-Aldrich*
- **Carbon dioxide** CO_2; 2 kg; *Dennerle*
- **Chloroform** $CHCl_3$; *Chem-Lab*
- **Chromium (III) potassium sulphate dodecahydrate** $CrKO_8S_2 \cdot 12\ H_2O$; ≥ 98.5 %; *Sigma-Aldrich*
- **Citric acid monohydrate** $C_6H_8O_7 \cdot H_2O$; ≥ 99 %; *Sigma-Aldrich*
- **Cobalt(II) nitrate hexahydrate** $Co(NO_3)_2 \cdot 6\ H_2O$; *Sigma-Aldrich*
- **Copper(II) sulphate pentahydrate** $CuSO_4 \cdot 5\ H_2O$; ≥ 99.0%; *Sigma-Aldrich*
- **di-Potassium hydrogen phosphate** K_2HPO_4; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **EDTA disodium salt** $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$; ≥ 99 %; *Sigma-Aldrich*
- **Ethanol euro denaturated** C_2H_6O; 99 %; *VWR Chemicals*
- **Hydrochloric acid** HCl; 37 %; *Carl Roth GmbH + Co KG*
- **Iron(II) sulphate** $FeSO_4$ exsiccated; *Riedel-de Haën AG*
- **Iron(III) chloride hexahydrate** $FeCl_3 \cdot 6\ H_2O$; ≥ 98 %; *Fluka*
- **Iron(III) nitrate nonahydrate** $Fe(NO_3)_3 \cdot 9\ H_2O$; ≥ 98 %; *Fluka*
- **Kjeltabs** CT; Thompson & Capper Ltd
- **Magnesium sulphate heptahydrate** $MgSO_4 \cdot 7\ H_2O$; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **Manganese sulphate monohydrate** $MnSO_4 \cdot H_2O$; *J.T.Baker®*
- **Methanol** CH_4O; *Chem-Lab*
- **Nickel(II) sulphate hexahydrate** $NiSO_4 \cdot 6\ H_2O$; *Sigma-Aldrich*
- **Oxygen** O_2; ≥ 99.995 %; *Messer Austria GmbH*
- **Potassium chloride** KCl; ≥ 99.5 %; *Carl Roth GmbH + Co KG*

- **Potassium dihydrogen phosphate** KH_2PO_4; ≥ 98 %; *Carl Roth GmbH + Co KG*
- **Potassium hydrogen phthalate** $C_8H_5KO_4$; ≥ 99.95 %; *Sigma-Aldrich*
- **Potassium hydroxide** KOH; ≥ 95 %; *Carl Roth GmbH + Co KG*
- **Potassium iodide** KI; ≥ 99 %; *Sigma-Aldrich*
- **Potassium nitrate** KNO_3; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **Sodium carbonate** Na_2CO_3; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **Sodium chloride** NaCl; ≥ 99,8 %; *Carl Roth GmbH + Co KG*
- **Sodium hydrogen carbonate** $NaHCO_3$; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **Sodium hydroxide** NaOH; 50 %; *Donauchem GmbH*
- **Sodium metasilicate nonahydrate** $Na_2SiO_3 \cdot 9\ H_2O$; ≥ 98 %; *Sigma-Aldrich*
- **Sodium molybdate dehydrate** $Na_2MoO_4 \cdot 2\ H_2O$; ≥ 95.5 %; *Carl Roth GmbH + Co KG*
- **Sodium nitrate** $NaNO_3$; ≥ 99 %; *Carl Roth GmbH + Co KG*
- **Sulphuric acid** H_2SO_4; 95-97 %; *Bernd Kraft GmbH*
- **Thiamine hydrochloride** Vitamin B1 $C_{12}H_{17}ClN_4OS \cdot HCl$; *Sigma-Aldrich*
- **Tris(hydroxymethyl)aminomethane** $NH_2C(CH_2OH)_3$; ≥ 98 %; *Sigma-Aldrich*
- **Vitamin B12** $C_{63}H_{88}CoN_{14}O_{14}P$; ≥ 99.8 %; *Sigma-Aldrich*
- **Zinc sulphate heptahydrate** $ZnSO_4 \cdot 7\ H_2O$; *Sigma-Aldrich*

3.1.2 Devices

- **Analytical balance** DE 150K5N; max 150 kg; d = 5 g; *Kern & Sohn GmbH*
- **Analytical balance** PLS 1200-3A; max 1200 g; d = 0.001 g; *Kern & Sohn GmbH*
- **Analytical balance** SBA 41; max 320 g; d = 0.001 g; *Scaltec*
- **Analytical balance** MSX (SD EE); max 60/120/220 g; min 1 mg; e = 1 mg; d = 0.01/0.02/0.05 mg; *Sartorius Lab Instruments GmbH & Co. KG*
- **Autoclave** MultiControl 12L/18L; *Certoclav Sterilizer GmbH*
- **Autoclave** VE-120; *Systec GmbH*
- **AutoKjeldahl Unit** K-370; *Büchi Labortechnik AG*
- **Bench balance** FCB 12K1; max 12100 g; d = 0.05 g; *Kern & Sohn GmbH*
- **Centrifuge** 5415 D; *Eppendorf AG*
- **Centrifuge** 5920 R; *Eppendorf AG*
- **Centrifuge rotor** F45-24-11; max. 24x3.75 g, max. 13200 rpm; *Eppendorf AG*
- **Centrifuge rotor** S-4x1000; max. load 4x2.1 kg, max. 3700 rpm; *Eppendorf AG*
- **Centrifuge rotor**; Fiberlite™ F10-4 x 1000 LEX; *Thermo Fisher Scientific Inc.*
- **Centrifuge** Sorvall Lynx 4000; *Thermo Fisher Scientific Inc.*
- **Conductivity detector** Dionex; *Thermo Fisher Scientific Inc.*
- **Digestion Automat** K-438; *Büchi Labortechnik AG*
- **Digital Time Relay**; Model No. 7955; *Trumeter Co. Ltd.*

- **Drying oven** kelvitron® t; *Heraeus Instruments*
- **Fluorescent lamp** LF80 36W Natural Daylight 1SL/25; *Pila*
- **Fluorescent lamp** LF80 18W Natural Daylight 1SL/25; *Pila*
- **Lamina flow hood**; LaminAir® HB 2472; *Heraeus Instruments*
- **Light microscope** BX43; *Olympus*
- **Luxmeter** testo 545; *testo*
- **Orbital shaker**; Standard 5000; *VWR*
- **Peristaltic pump**; 505 U; *Watson Marlow*
- **pH-meter** pH 330i; WTW Wissenschaftlich-Technische Werkstätten GmbH
- **Plate reader** Infinite M200 PRO; *Tecan Trading AG*
- **Shaker** Multitron Pro; *Infors AG*
- **Spectrophotometer** DR 3900; *Hach Company*
- **Switch Mode DIN Rail Panel Mount Power Supply**, 120W, 12V dc/ 10A; Stock no. 282-518; *RS Components Ltd.*
- **Total Organic Carbon Analyzer** (with TOC-$V_{CPH/CPN}$ & TOC-Control V Version 2 Software); *Shimadzu Corporation*
- **Ultrasonic Cleaner** Branson 5800; 2.5 gallon, 40 kHzLipids; *Branson*

3.1.3 Equipment

- **24-well cell culture plate**; Cellstar ®, sterile, with lid, Cat.-No. 662160; *greiner bio-one*
- **Anion exchange column** Dionex IonPac AG14A; *Thermo Fisher Scientific Inc.*
- **Disposable cuvettes**; semi-micro 300-900 nm, Order no. 634-0678; *VWR Chemicals*
- **Disposable syringe filters** Chromafil ® Xtra PA 45/25, polyamide, pore size 0.45 µm, Ø 25 mm, inlet Luer-Lock, outlet Luer; *Macherey-Nagel GmbH & Co. KG*
- **Filter unit**; Aqua 30/0.45 CA, 0.45 µm, Cat. No. 10 462 655; *GE Healthcare*
- **Glass-microfibre discs**; MGC grade, Ø 47 mm; Order no. FT-3-1103-047; *Sartorius Lab Instruments GmbH & Co. KG*
- **Glass syringe** #1002; 2.5 mL; *Hamilton Company*
- **Hollow Fiber Cartridge**; Serial # 99062207513, Model # UFP-750-E-4MA, Surface Area 420 cm^2, 750.000 NMWC; *GE Healthcare*
- **LED stripes** 8 mm Flex LED Strip x120 White 6000K 12V, Chromatic Series; Mfr. Part No. F5-55-35-1-120-F8-20-FP; *PowerLED*
- **LED stripes** 8 mm Flex LED Strip x120 White 2500K 12V, Chromatic Series; Mfr. Part No. B5-11-35-1-120-F8-20-FP; *PowerLED*
- **pH paper** pH 0-14; HC556639; *Merck KGaA*
- **Syringe needles**; Sterican® Tief-Intramuskulär G 21 x 3 1/8" / ø 0,80 x 80 mm, grün, 4665465; *B. Braun Melsungen AG*

- **Syringe**; Norm-Ject® 5 mL (6 mL), 4050.000V0; *Henke-Sass Wolf GmbH*

3.2 Algae strain

In all experiments, the organism *Nannochloropsis limnetica* (*N. limnetica*) was used. The first experiments were carried out with an *N. limnetica* strain provided by the project partner ecoduna (Figure 5, left). Ecoduna obtained their strain from the Culture Collection of Algae at the University of Göttingen, Germany (SAG). According to the strain collections, this strain (SAG 18.99) is identical to *N. limnetica* CCALA 864 (CCALA, 2018). Nevertheless, it could not be ruled out that the strain provided by ecoduna had changed during their cultivation. For this reason, an additional *N. limnetica* strain (*Nannochloropsis limnetica* KRIENITZ 1998/3, also called *Nannochloropsis limnetica* Krienitz, Hepperle, Stich et Weiler – CCALA 864) was ordered at the Culture Collection of Autotrophic Organisms (CCALA) (Figure 5, right). In this thesis, the strain obtained from ecoduna will be referred to as "*N. limnetica* (ecoduna)" and the one obtained from CCALA as "*N. limnetica* (CCALA)". Both strains were used in the experiments. The first experiments were carried out with both strains whereas for the later experiments, only *N. limnetica* (CCALA) was used. The reason for using both strains in parallel was to examine whether the results are comparable and can be adopted to the other strain. It is indicated below which strain was used for which experiment.

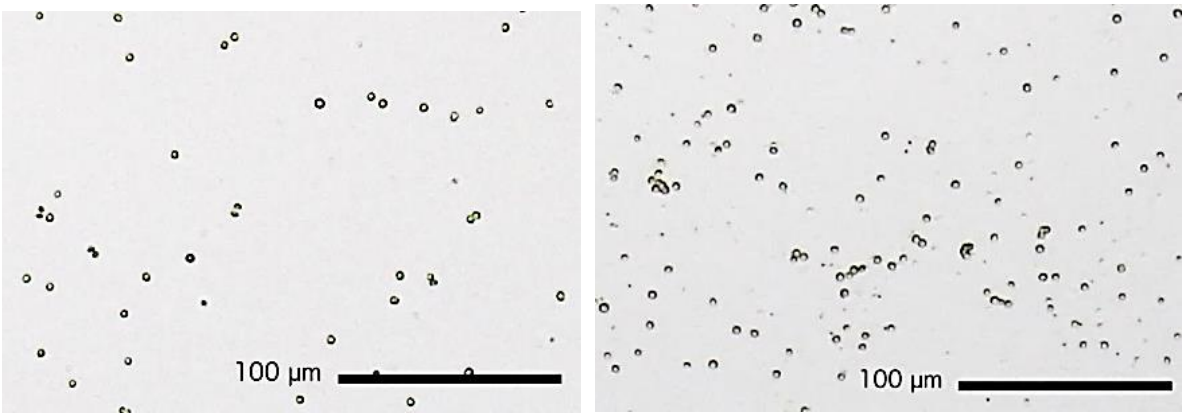

Figure 5: Microscopic picture of *N. limnetica*. Left: *N. limnetica* (ecoduna). Right: *N. limnetica* (CCALA).

3.3 Media preparation

The following media (recipes provided below) were prepared and used in the conducted experiments described below: ATCC, BBM, BG-I, Bourelly, MIII, Waris, WC. Compared to the original media recipe, the trace element solutions and vitamin solutions were slightly modified, as for all media the trace element solution of BG-I was used; 1 mL trace element solution was used for 1 L medium. Moreover, 1 L vitamin solution was prepared. The composition was developed based on various media recipes that suggested the use of vitamins. As the pH-value of the WC-

medium was above the desired value, the pH was adjusted with 1 M NaOH to 8 ± 0.2. In some of the herein described experiments, both versions of WC-medium – with and without pH adaption – were used. For the experiments (sections 3.5.2 and 3.5.3), each medium was prepared as described and vitamin solution was added to one part of each.

3.3.1 Media recipes

The trace element and vitamin solution as well as the cultivation media were prepared for the experiments after the following recipes.

Trace element solution (IFA-Tulln)

Dissolve the following substances in 1 L RO-water and autoclave.

Component		Concentration [mM]
H_3BO_3	0.509 g	8.232
$CuSO_4.5H_2O$	0.150 g	0.601
KI	0.181 g	1.090
$FeCl_3.6H_2O$	0.293 g	1.084
$MnSO_4.H_2O$	0.296 g	1.751
$Na_2MoO_4.2H_2O$	0.082 g	0.339
$NiSO_4.6H_2O$	0.275 g	1.046
$Co(NO_3)_2.6H_2O$	0.100 g	0.344
$ZnSO_4.7H_2O$	0.490 g	1.704
$KAl(SO_4)_2.12H_2O$	0.395 g	0.832
$KCr(SO_4)_2.12H_2O$	0.470 g	0.941

Vitamin solution

Dissolve the following substances in 1 L RO-water.

Component		Concentration [µM]
Cyanocobalamin	0.020 g	14.756
Thiamine HCl	0.100 g	296.499
Biotin	0.005 g	20.466

Filter-sterilise.

ATCC medium – concentrate (IFA-Tulln)

Combine solutions 1, 2 and 3. Add 1 mL trace element solution. The result is a 40-fold concentrate of medium. Dilute 1:10 to receive a 4-fold concentrate that can be used for cultivation. Autoclave.

Solution 1

Dissolve the following in 100 mL RO-water.

Component		Concentration [mM]
$FeCl_3.6\ H_2O$	0.040 g	0.148

Solution 2

Dissolve the following in 400 mL RO-water.

Component		Concentration [mM]
$Na_2SiO_3.9\ H_2O$	0.360 g	1.267

Solution 3

Dissolve the following substances in 500 mL RO-water.

Component		Concentration [mM]
$NaNO_3$	3.100 g	36.47
K_2HPO_4	0.240 g	1.378
$MgSO_4.7\ H_2O$	0.470 g	1.907
$CaCl_2.2\ H_2O$	0.225 g	1.531
Na_2CO_3	0.125 g	1.179
Citric acid	0.015 g	0.156
Na_2EDTA	0.005 g	0.015

BBM medium (Andersen et al., 2005)

Into 900 mL of RO-water add 10 mL of the first 6 stock solutions. Add 1 mL of the other solutions. Add 1 mL of trace element solution. Fill up to 1 L. Autoclave. The final pH should be 6.6.

Component	Stock solution [g/L]	Quantity used (to 1 L)
$NaNO_3$	25.00	10 mL
$CaCl_2.2H_2O$	2.50	10 mL
$MgSO_4.7H_2O$	7.50	10 mL
K_2HPO_4	7.50	10 mL
KH_2PO_4	17.50	10 mL
NaCl	2.50	10 mL
EDTA solution	see following recipe	1 mL
Acidified iron solution	see following recipe	1 mL
H_3BO_3	11.42	1 mL

EDTA solution

Component	Quantity used (to 1 L)
EDTA	50.00 g
KOH	31.00 g

Acidified Iron Solution

Component	Quantity used (to 100 mL)
$FeSO_4.7H_2O$	0.498 g
H_2SO_4 (96%)	0.1 mL

BG-I medium (IFA-Tulln)

Dissolve the following substances in 500 mL RO-water.

Component		Concentration [mM]
$NaNO_3$	1.2 g	14.119
$MgSO_4.7\ H_2O$	0.075 g	0.304
$CaCl_2.2\ H_2O$	0.036 g	0.245
$Fe(NO_3)_3.9\ H_2O$	0.010 g	0.025

Add 500 mL RO-water and dissolve the following substances.

Component		Concentration [mM]
Na_2CO_3	0.020	0.189
K_2HPO_4	0.040	0.230

Add 1 mL trace element solution. Autoclave. The final pH should be between 7.2 and 8.

Bourelly medium (Krienitz and Wirth, 2006)

Prepare stock solutions. Add indicated amounts to RO-water and bring to a final volume of 1 L. Add 1 mL trace element solution. Autoclave.

Component	Stock solution [g/100 mL]	Quantity used (to 1 L)	Final concentration [mg/L]
KNO_3	10	2 mL	200
K_2HPO_4	1	4 mL	40
$MgSO_4 \cdot 7H_2O$	1	3 mL	30
$Ca(NO_3)_2$	1	3 mL	30
$NaHCO_3$	1.68	10 mL	168
Fe-EDTA-solution	see following recipe	0.5 mL	

Fe-EDTA-solution

Component	Quantity used (to 100 mL)	Final concentration [mg/L]
$FeSO_4 \cdot 7H_2O$	0.7	3.5
EDTA (Titriplex III)	0.9	4.5

MIII medium (Körner and Nicklisch, 2002)

Dissolve the following substances in 1 L of RO-H_2O. Add 37 µL of 37 % HCl. Add 1 mL trace element solution.

Component		Concentration [mM]
$FeCl_3.6H_2O$	0.003 g	0.01
$CaSO_4.2H_2O$	0.086 g	0.50
$MgSO_4.7H_2O$	0.062 g	0.25
$NaHCO_3$	0.168 g	2.00
$NaNO_3$	0.042 g	0.50
$CaCl_2.2H_2O$	0.074 g	0.50
KCl	0.007 g	0.10
KH_2PO_4	0.007 g	0.05
$Na_2SiO_3.9H_2O$	0.114 g	0.40
EDTA solution	1 mL	

EDTA solution

Dissolve the following in 100 mL RO-water.

Component		Concentration [mM] (in final medium)
$Na_2EDTA.2H_2O$	0.74 g	0.02

Waris medium (UTEX, 2018)

To 900 mL of RO-water, add each of the components in the order specified while stirring continuously. Add 1 mL trace element solution. Adjust the pH to 6.0. Bring the total volume to 1 L with RO-water. Autoclave.

Component		Concentration [mM]
KNO_3	0.100	0.99
$MgSO_4.7H_2O$	0.020 g	0.08
$(NH_4)_2HPO_4$	0.020 g	0.15
$CaSO_4.2H_2O$	0.060 g	0.35
EDTA solution	1 mL	

EDTA solution

Dissolve the following in 100 mL RO-water.

Component		Concentration [mM] (in final medium)
$Na_2EDTA.2H_2O$	0.447 g	0.012

WC medium (Andersen et al., 2005)

Dissolve the following substances in 1 L of RO-H_2O. Add 1 mL trace element solution. Autoclave.

Component		Concentration [mM]
$NaHCO_3$	0.0126 g	0.15
K_2HPO_4	0.0087 g	0.05
Tris	0.5003 g	4.13
$NaNO_3$	0.0850 g	1.00
$CaCl_2.2\ H_2O$	0.0368 g	0.25
$MgSO_4.7\ H_2O$	0.0370 g	0.15
$Na_2SiO_3.9\ H_2O$	0.0284 g	0.10

3.4 Analyses

In this chapter, all analyses that were carried out are described. In the respective sections it is indicated which analyses were performed in the specific experiments.

3.4.1 Optical density (OD)

The optical density (OD) was measured using a spectrophotometer. The desired wavelengths (given in the specific experiments, section 3.5) were selected. The blank (culture medium) was subtracted from the OD values of the samples. In all experiments that were carried out in 24-well plates, the OD was measured directly in the plate with a plate reader.

3.4.2 Biomass analyses

Biomass concentration - total solids (TS)

For determining the biomass concentration of the algal suspension, filters (glass micro-fibre discs) were dried at 105 °C, cooled to room temperature in a desiccator and weighed to obtain the tare weight (m_{tare}). For each sample, a filter was put onto a Büchner funnel and a certain volume of sample (V) was filtered through using a vacuum pump. Then, the filters holding the samples were dried at 105 °C overnight, cooled in a desiccator and weighed again ($m_{filter+sample}$). The weight of the total solids was obtained according to Equation 1.

$$TS = \frac{m_{filter+sample} - m_{tare}}{V}$$

Equation 1

TS ..... Total solids [mg/mL]

$m_{filter + sample}$..... Mass of filter with sample [mg]

m_{tare} ..... Tare weight of filter [mg]

V ..... Volume of sample [mL]

Lipid content

The lipid content of the algal biomass was determined based on the slightly modified method after Chu et al. (2013), described in the following paragraph. Samples were analysed in double determinations.

Glass vials were dried at 60 °C and stored in a desiccator prior to usage. Approximately 20 mg ($m_{biomass}$) of freeze-dried biomass were weighed into a dried glass vial and 2.25 mL of chloroform:methanol (2:1) solution (solution CM) were added. Then, the vial was wrapped in tin foil to avoid light reactions. The vial was incubated overnight at 30 °C and 150 rpm. After 20 hours, 1.5 mL of solution CM were added and the vial was incubated under the same conditions for another 2 hours. Subsequently, the sample was put into an ultrasonic bath with ice for 30 minutes to break the algal cell walls. The vial was centrifuged for 5 minutes at 3,700 rpm (21,194 g) (centrifuge 5920 R, Eppendorf AG). The supernatant was filtered, through a filter with a pore size of 0.45 µm, into another dried glass vial. 1 mL of solution CM was added to the pellet that was then resuspended. When the solids had settled, the supernatant was again filtered and transferred to the second vial, which already contained the supernatant. 1 mL methanol and 1.8 mL 0.1 % NaCl were added to the supernatant and homogenised. It was left overnight at room temperature in the dark to allow a clear phase separation. The bottom phase containing the lipids was transferred to a dried and weighed (m_{empty}) glass vial (final container) using a glass Pasteur pipette. To the vial containing the upper phase, 0.5 mL chloroform were added. The mixture was then homogenised. The vial was again left overnight for phase separation at room temperature in the dark. The bottom phase was again transferred to the final container. Beginning with the chloroform addition, the procedure was repeated three times in total. The vial containing the combined chloroform phases was put into the drying oven for 6 days until all liquid had evaporated. The vial was put in a desiccator to cool and then weighed (m_{full}). The lipid content was calculated according to Equation 2.

$$Lipids = \frac{m_{full} - m_{empty}}{m_{biomass}} * 100$$

Equation 2

Lipids ..... Lipid content [% of dry weight]

m_{full} ..... Mass of vial + lipids [mg]

m_{empty} ..... Mass of empty vial [mg]

$m_{biomass}$ ..... Mass of freeze-dried biomass [mg]

Pigment content

Pigments were extracted with acetone as solvent; all analyses were done in double determinations.

Approximately 20 mg of freeze-dried biomass were transferred into a dry glass centrifuge tube, and then 10 mL of acetone solution (90 %) were added. The tube was incubated at 4 °C for 16 hours. After the extraction process, the tube was centrifuged at 4 °C and 2,500 rpm (14,320 g) (centrifuge 5920 R, Eppendorf AG) for 10 minutes. The supernatant was transferred to a new vial. After blanking the photometer with 90 % acetone, the OD_{480}, OD_{647} and OD_{664} of the supernatant were measured. The concentrations of $chlorophyll_a$ and $chlorophyll_b$ were calculated according to Equation 3 and Equation 4 (Humphrey and Jeffrey, 1997). Equation 5 (Strickland and Parsons, 1972) was used to calculate the total carotenoid concentration.

$$Chl_a \left(\frac{\mu g}{mL}\right) = 11.93 * OD_{664} - 1.93 * OD_{647}$$

Equation 3

$$Chl_b \left(\frac{\mu g}{mL}\right) = -5.5 * +20.36 * OD_{647}$$

Equation 4

$$Total\ carotenoids \left(\frac{\mu g}{mL}\right) = 4 * OD_{480}$$

Equation 5

3.4.3 Process water analyses

Non-purgeable organic carbon (NPOC)

The non-purgeable organic carbon (NPOC) of process water samples was measured to evaluate the content of organic compounds and to draw conclusions concerning their effects during recirculation. Samples were analysed in triple determinations via TOC-V Total Organic Carbon Analyzer, controlled by the software TOC-$V_{CPH/CPN}$ & TOC-Control V Version 2.

As standard for calibrating the TOC device, two potassium hydrogen phthalate standard solutions were prepared using MQ-H_2O (2.125 g/L and 0.2125 g/L corresponding to 1,000 mg/L and 100 mg/L carbon, respectively) were used. The device was calibrated in the range of 2 - 200 mg/L. MQ-H_2O was used as blank. The samples were filtered (0.45 µm, AQUA 30/0.45 CA) and measured using the "NPOC" (non-purgeable organic carbon) method. The samples were first acidified with hydrochloric acid and then purged with O_2. During this procedure, the inorganic carbon was converted into CO_2 and then removed. The remaining non-purgeable organic carbon was burned through catalytic oxidation and the formed CO_2 was measured by a nondispersive infrared (NDIR) sensor. This value was converted automatically into mg NPOC/L by the software.

Nutrient analysis

To determine which nutrients had been consumed in which amounts during the algae cultivation, chloride, nitrate, phosphate, and sulphate content of the process water samples were analysed using ion chromatography (IC). IC analyses were performed in triple determinations.

In the IC system an anion exchange column (Dionex IonPac AG14A) and a conductivity detector were used. MQ-H_2O was used for preparing the eluent (8 mM Na_2CO_3 and 1 mM $NaHCO_3$) and regenerant (31.5 mM H_2SO_4). The pump was set at a flow rate of 1.8 mL/min for the regenerant while the flow rate of the eluent was set at 1 mL/min.

The samples were diluted 1:5 (one part sample, four parts eluent) and filtered directly into the vials through 0.45 µm filters. The filtered samples were put into the IC system and analysed. The chromatograms were evaluated automatically by the Software Chromeleon; the concentration of the substances was obtained in mg/L.

An elemental analysis using inductively coupled plasma (ICP) was carried out. The principle of this method is as follows: A gas stream (usually argon) flows through a conduction coil producing a magnetic field. This interaction, i.e. inductive coupling, leads to the formation of plasma and creates heat (6000 – 10000 K). A fine aerosol of the liquid sample is introduced to this ICP torch and heated by the plasma, which leads to excitation of the present atoms. This excitation energy is detected (mainly mass spectrometry and optical emission spectrometry detectors are used) and converted into the concentration of the respective elements by computation. (Thompson and Walsh, 1989)

Mass spectrometry (Al, B, Co, Cr, Cu, Mn, Mo, Ni) and optical emission spectrometry (Ca, Fe, K, Mg, Na, P, S, Zn) detectors were used. A team at the laboratory of BIOENERGY2020+ in Graz conducted this analysis.

3.4.4 Comparison of different methods for growth evaluation

Aggregates, usually formed by *N. limnetica*, as well as pigments might affect OD measurements but just slightly affect biomass concentrations (Griffiths et al., 2011). For this reason, OD measurement at different wavelengths (see below) and biomass concentrations (TS) were compared in two experiments.

To determine the wavelengths most suitable for demonstrating the growth of *N. limnetica*, an absorbance spectrum of the inoculum was recorded using a plate reader. The wavelengths showing the highest absorbance were selected for further experiments. This first step was only conducted with *N. limnetica* (ecoduna).

For the next step – the comparison of OD and biomass concentration (TS) – a 2-L flask with 1 L working volume was inoculated with *N. limnetica* (CCALA) and aerated through a sterile filter with ambient air (see Figure 6). The culture was incubated at approximately 23 °C (room temperature not adjusted), 2,500 Lux (fluorescent lamp 36 W) and a day/night cycle of 16:8 hours. After 2 weeks, different dilutions of the culture suspension using ATCC medium were prepared (see Table 1). OD_{436}, OD_{485}, OD_{682} and TS of all dilutions were measured. To evaluate the various methods, a linear regression was calculated using MS Excel. Additionally, a 2-L flask with 1 L working volume was inoculated with *N. limnetica* (ecoduna) and aerated through a sterile filter with ambient air. OD_{436} and TS of this cultivation were measured continuously over 7 days. The ratio of OD/TS was calculated for all time points to obtain the OD normalised to the TS. This normalised OD was plotted against the time and the plot was evaluated. These plots as well as the obtained regressions were used to compare the methods of measuring algal growth.

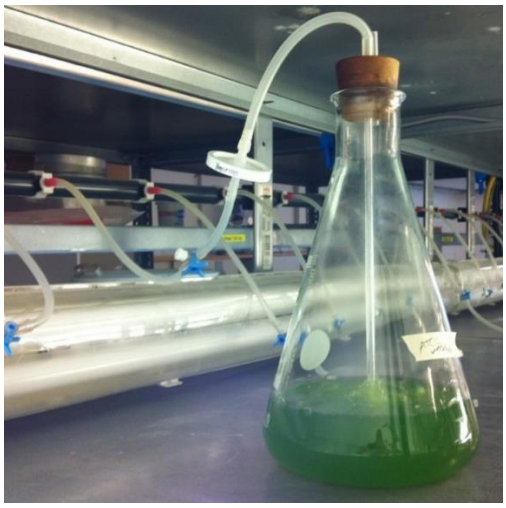

Figure 6: Setup of 2-L flask aerated with ambient air.

Table 1: Dilution series of *N. limnetica* (CCALA) suspension for evaluating different methods determining algal growth.

No.	Cell suspension [mL]	ATCC [mL]	Dilution factor	Cell suspension [%]
1	50	0	1 x	100
2	45	5	1.11 x	90
3	40	10	1.25 x	80
4	35	15	1.43 x	70
5	30	20	1.67 x	60
6	20	30	2.5 x	40
7	10	40	5 x	20
8	0	50	/	0

3.5 Experimental design

An overview of the working schedule and different steps which were carried out to optimise culture conditions and evaluate process water recirculation in *N. limnetica* cultures, is given in Figure 7; these steps are described in more detail below.

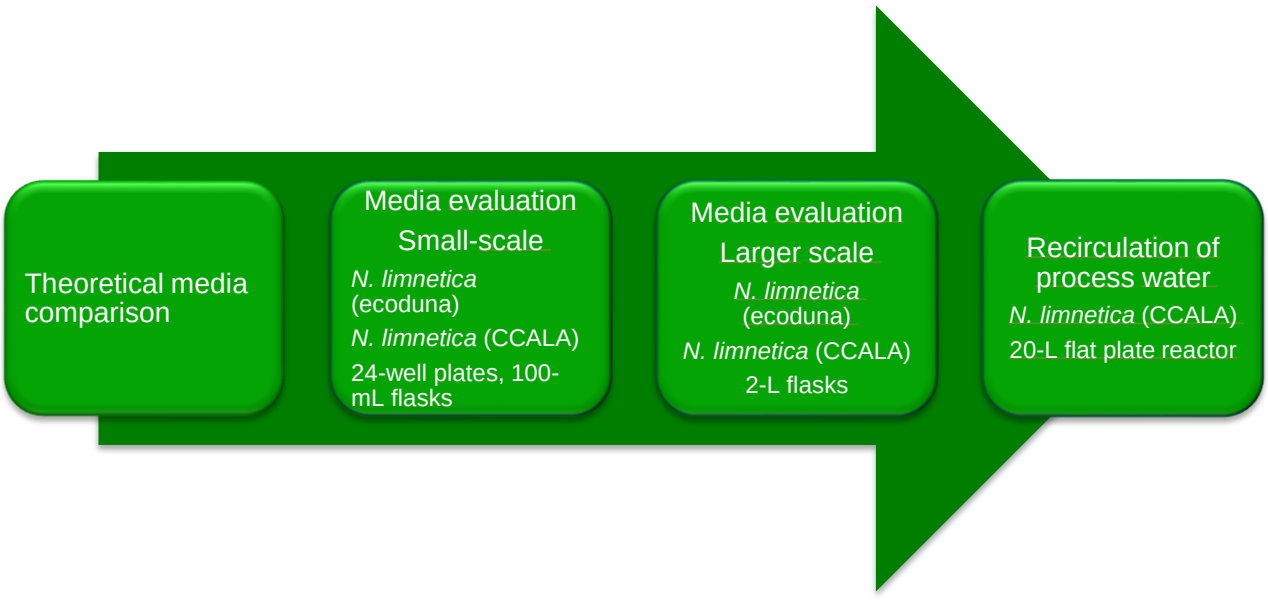

Figure 7: Working schedule.

3.5.1 Theoretical comparison of different cultivation media

The first step was to find suitable culture media. To meet this aim, cultivation media that had already been successfully used for cultivating *N. limnetica* as well as cultivation media suggested by culture collections were chosen. Media that did not seem suitable for reproducible experiments, e.g. such containing soil extracts, were excluded. A list of all considered media, including sources and reasons for the elimination of specific media in further steps, is provided in section 4.2.

After selecting the media, the differences in their nutrient compositions on an elemental basis were evaluated, which was done separately for macro elements, trace elements, and vitamins. Subsequently, the elemental compositions of the media were compared to avoid testing similar setups. Based on this evaluation, media were selected for small-scale cultivation experiments in 24-well plates.

3.5.2 Growth evaluation and selection of most promising media

Cultivation in 24-well plates

The main aim of experiments carried out in 24-well plates was to compare the growth of *N. limnetica* in different media to limit the number of possible cultivation media. For these experiments, both the strain provided by ecoduna and the one ordered at CCALA were used as inoculum. Both strains were incubated in ATCC medium at 3,000 Lux (fluorescent lamp 18 W), approximately 23 °C (room temperature not adjusted) and a day/night cycle of 16:8 hours for 20 days. The procedure of the first experiment (*N. limnetica* (ecoduna)) is described in the following paragraphs; deviations in the repetition with *N. limnetica* (CCALA) are pointed out.

To determine the density of inoculum needed for starting the experiment with an OD between 0.1 and 0.2, a dilution series was prepared. The different dilutions were measured at OD_{485} and OD_{750} in a plate reader. Wavelengths in the range of 485 nm and 750 nm are often reported in literature (Fietz et al., 2005; Fret et al., 2017; Griffiths et al., 2011; Owens et al., 1987; Patel et al., 2012; Rodolfi et al., 2009; Whittle and Casselton, 1975). Furthermore, the wavelength λ = 485 nm was chosen due to previous experiments carried out at the institute (IFA-Tulln). The inoculum was diluted 1-fold, 2-fold, 4-fold, 8-fold, 16-fold and 20-fold (see Figure 8). The experiments were conducted in double determination.

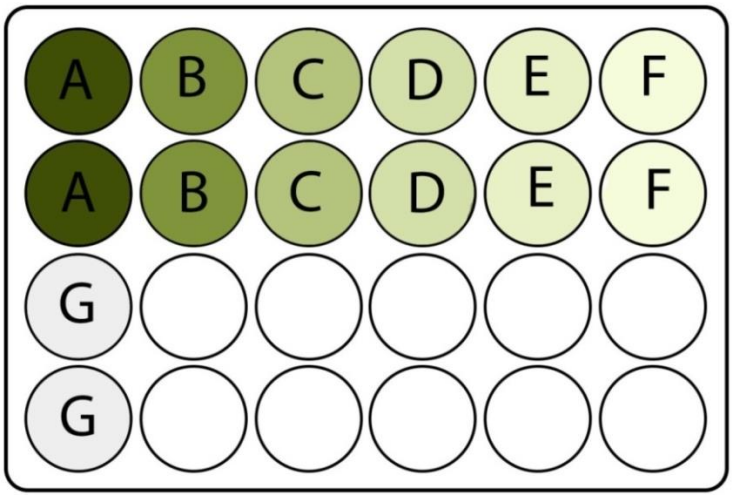

Figure 8: Dilutions of *N. limnetica* in 24-well plate for the determination of inoculation density; A: 2 mL inoculum (no dilution), B: 1 mL inoculum + 1 mL ATCC medium (2-fold dilution), C: 0.5 mL inoculum + 1.5 mL ATCC medium (4-fold dilution), D: 0.25 mL inoculum + 1.75 mL ATCC medium (8-fold dilution), E: 0.125 mL inoculum + 1.875 mL ATCC medium (16-fold dilution), F: 0.1 mL inoculum + 1.9 mL ATCC medium (20-fold dilution), G: 2 mL ATCC medium

To find the most suitable medium for cultivating *N. limnetica*, the growth of both strains (*N. limnetica* (ecoduna) and (CCALA)) in the previously selected media (ATCC, BBM, BG-I, Bourelly, MIII, Waris, WC and WC with pH adjusted to 8) was evaluated. The strains were cultivated in 24-well plates (2 mL working volume); each of the selected media was once used with and once without vitamin addition. To avoid effects caused by nutrients present in the inoculum (grown in ATCC), the inoculum was washed before transferring it to the new medium. Therefore, 2 mL inoculum were transferred to a 2 mL Eppendorf vial and centrifuged for 3 minutes at 8,000 rpm (5,939 g, centrifuge 5415 D; Eppendorf AG). The supernatant was removed using a pipette, then 1 mL NaCl solution (0.9 %) was added and the pellet resuspended. The suspended biomass was centrifuged again for 4 minutes at 8,000 rpm and the supernatant was removed. Then, 2 mL of the respective medium were added and the biomass was again resuspended.

The cultivation was carried out in 6-fold determinations (one row was used for each medium, see Figure 10). In each well, 0.25 mL inoculum were transferred to 1.75 mL of the respective medium. The scheme of the inoculation process described above is shown in Figure 9. The experiment with *N. limnetica* (CCALA) was only performed with the media without vitamins and WC medium was not adjusted to pH 8. Moreover, there were only 3-fold instead of 6-fold determinations. 200 µL of inoculum were added to 1.8 mL medium in each well.

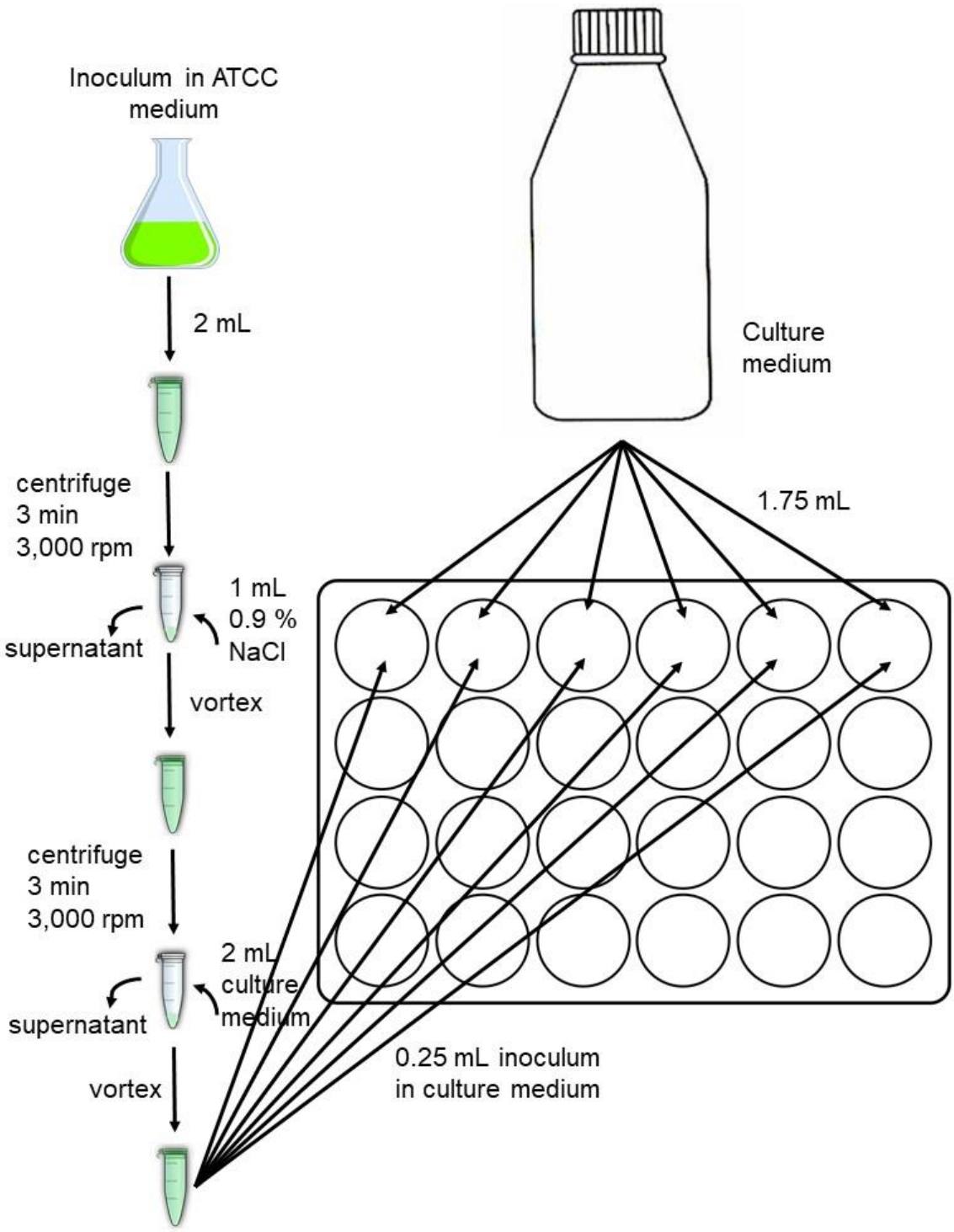

Figure 9: Scheme of inoculation procedure of 24-well plates.

Following these steps, 24-well plates were filled. Moreover, additional plates were used for blanks. For this purpose, 2 mL of each medium were transferred into two wells. Illustrations of plate 1 (inoculated) and plate 6 (medium blanks) are given in Figure 10 as examples.

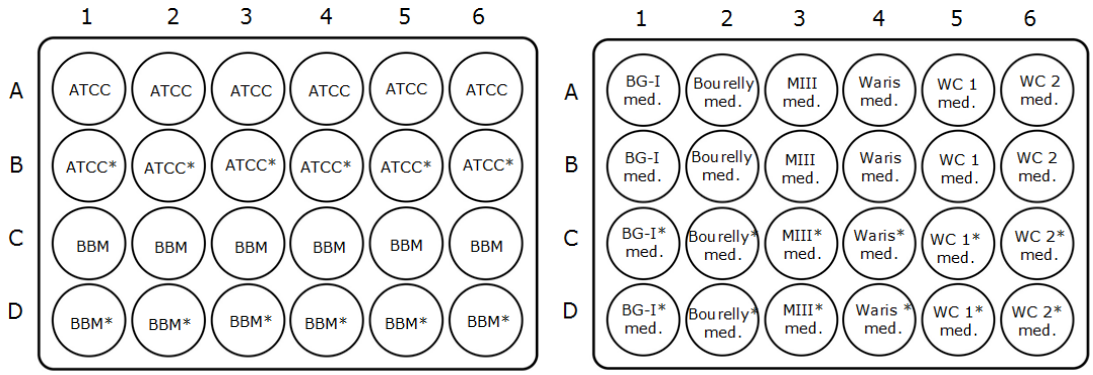

Figure 10: Illustration of cultivation plate and plate containing blank medium. (*vitamins added, med. = medium)

The plates were cultivated under an illumination of approximately 2,500 Lux (fluorescent lamp 18 W), approximately 23 °C (room temperature not adjusted) and a day/night cycle of 16:8 hours for 7 days. They were incubated on a shaker at 100 rpm. The plates containing the blanks were also put on the same shaker to assure the same conditions for cultivation plates and plates with blanks. Possible changes in the absorbance of the medium were eliminated by subtracting the blank. After inoculation and then approximately every 24 hours, the OD_{436} of each sample and the blanks was measured in a plate reader. For the measurements, the plates were taken from the shaker and samples were homogenised using a pipette. The OD of the blank media was subtracted from the OD of the culture suspension. As in the first experiment no significant changes of the OD of pure media were observed, this was not done in the experiment with *N. limnetica* (CCALA). Here, the blanks were only measured once and the values were used as blanks for all measurements.

Using the obtained optical density of the cultures, growth curves were created with the OD as a function of time. Moreover, the growth rate (μ) between one point and its subsequent measuring point was calculated for each measuring point using Equation 6.

$$\mu = \frac{\ln(c_2) - \ln(c_1)}{t_2 - t_1}$$

Equation 6

μ ..... *Growth rate [day^{-1}]*

c_1 ..... *Biomass concentration at timepoint 1*

c_2 ..... *Biomass concentration at timepoint 2*

t_1 ..... *Timepoint 1*

t_2 ..... *Timepoint 2*

Cultivation in 100-mL shaking flasks

The aim of this experiment was to optically evaluate which of the media (ATCC, BBM, BG-I, Bourelly, MIII, Waris and WC) provided the best conditions for reproducible experiments. The focus was on the aggregation of cells, which makes sampling difficult. This experiment was carried out with the strain *N. limnetica* (ecoduna). The inoculum was incubated in ATCC medium at 3,000 Lux (fluorescent lamp 18 W), approximately 23 °C (room temperature not adjusted) and a day/night cycle of 16:8 hours for 20 days.

To 35 mL of each medium, 5 mL of inoculum were added. One flask of each medium containing vitamins and one flask of each medium without vitamins were inoculated. The flasks were incubated at 3,000 Lux (fluorescent lamp 18 W), approximately 23 °C (room temperature not adjusted) and a day/night cycle of 16:8 hours for 6 days.

They were optically controlled once a day. On day 6, the pH was measured in all flasks. Moreover, the TS and the OD_{436} were determined as described above on day 6.

Cultivation in 2-L flasks

The aim of this experiment was to find a medium that supports fast and good growth of *N. limnetica*. Three media in which *N. limnetica* showed most promising growth rates and behaviour in the previous experiments were further investigated. Therefore, *N. limnetica* (ecoduna) and *N. limnetica* (CCALA) were cultivated in ATCC, BG-I, and MIII media in 2-L flasks. The inoculum was incubated in ATCC medium at 3,000 Lux (fluorescent lamp 18 W), a day/night cycle of 16:8 hours and approximately 23 °C (room temperature not adjusted) for 14 days. For both organisms the same setup was used. Thus, the setup is described once and differences are indicated.

ATCC, BG-I, and MIII media were prepared according to the recipes that can be found in section 3.3.1. 2-L flasks (1 L working volume) were inoculated with 250 mL algae suspension (750 mL medium). The flasks were incubated at approximately 23 °C (room temperature not adjusted) and 2,500 Lux (fluorescent lamp 36 W) and a day/night cycle of 16:8 hours for 14 days. They were aerated with ambient air through sterile filters as already described in section 3.4.4 (Figure 6). The flasks were placed next to each other, spread over two shelves. To avoid errors caused by potentially different conditions in different positions, they were randomised after each sampling (three times a week). The experiments were carried out in 3-fold determinations. Before taking samples, the flasks were weighed and the amount of water that had evaporated was estimated and refilled with RO-H_2O. After that, 25 mL of sample were taken and the flasks were weighed again. This weight was noted as new target value for the next sampling. From each flask, pH and biomass concentration were analysed and growth rates for each medium were calculated according to Equation 6 (see above).

The growth rates, total biomass concentration, and growth curves of all media were compared to evaluate which was the most suitable cultivation medium for *N. limnetica*.

3.5.3 Scale-up and recirculation in a flat plate reactor

The aim of this experiment was to cultivate *N. limnetica* (CCALA) at a larger scale and to evaluate the effects of recirculating process water after harvesting. Moreover, the suitability of cross-flow filtration and centrifugation as harvesting methods was assessed.
This experiment was carried out with the strain *N. limnetica* (CCALA). The inoculum was incubated in ATCC medium at 5,000 Lux in a day/night cycle of 16:8 hours and 20 °C.

Reactor design

A flat plate reactor built of glass plates with a maximum working volume of 20 L and a surface area to volume ratio of 0.25 cm^{-1} was used for these recirculation experiments. Figure 11 shows an illustration of the photobioreactor and its dimensions.

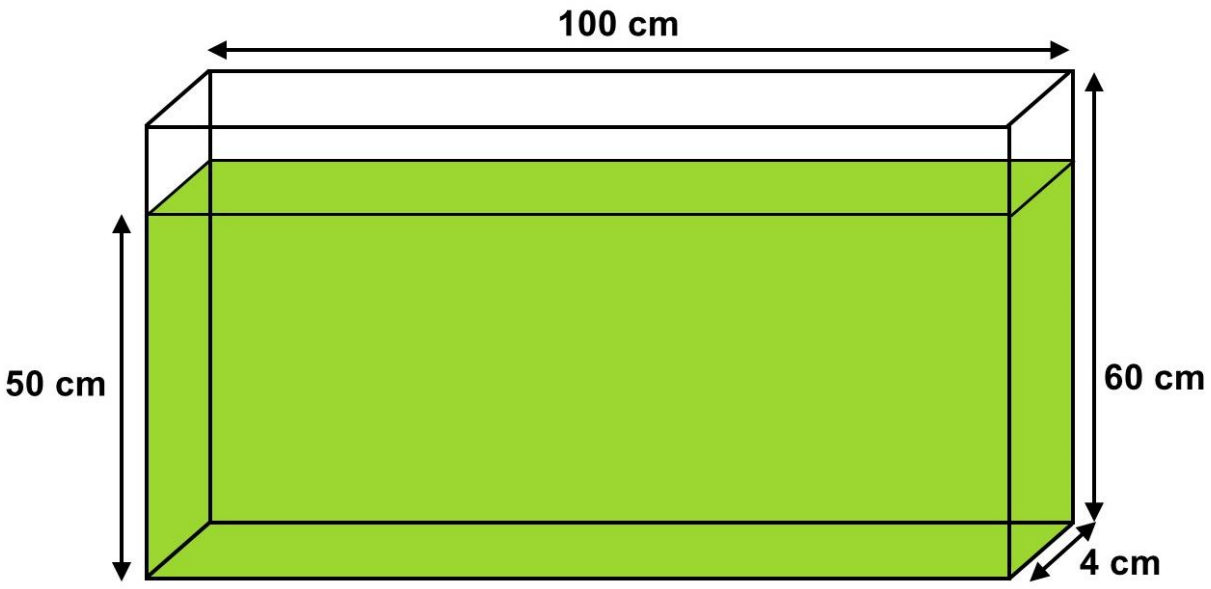

Figure 11: Illustration of flat plate reactor.

On the back of the reactor, a wooden plate with 12 LED light bands were fixed. Four LED-bands were coupled to one switch and were simulating daylight when mixed. Thus, the light intensity could be adjusted to the culture density and filling volume. Aeration and mixing of the reactor were achieved by an aquarium airstone connected to a pump. CO_2-supply could be installed on demand; a CO_2-bottle with an electric valve was turned on at a certain cell density. Lights and CO_2 valve were connected to electricity via a timer switch to enable a day/night cycle. The reactor was fixed onto a wooden stillage with wheels so that it could be moved around.
Figure 12 shows the set up flat plate reactor with 20 L of *N. limnetica* (CCALA) culture in ATCC medium. All lights are turned on and the CO_2 supply is installed and turned on as well in this figure.

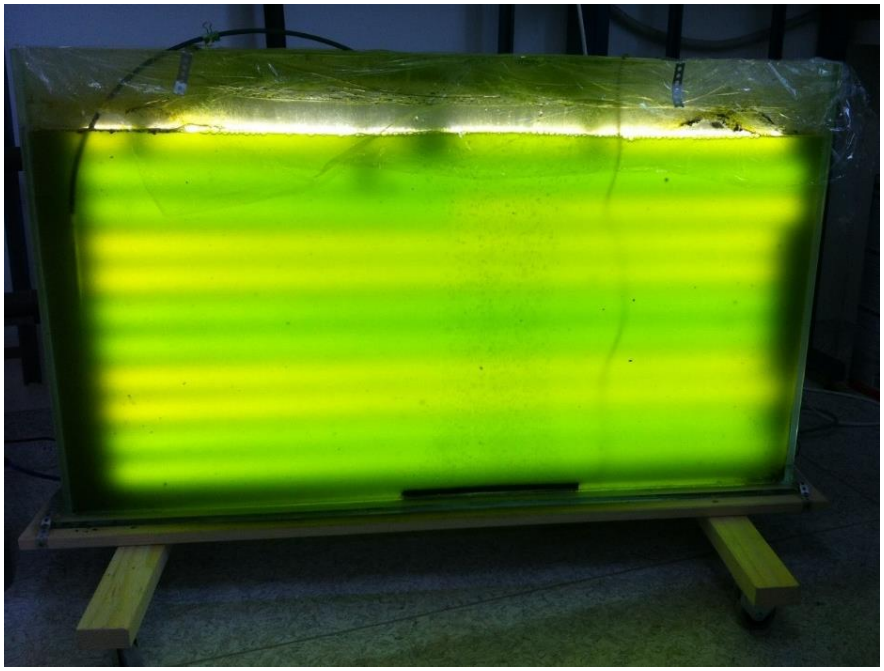

Figure 12: Flat plate reactor filled up to maximum working volume of 20 L, all lights on and CO_2 supply installed.

Cultivation and growth monitoring

Cultivation was started with half the maximum working volume: 9 L ATCC medium were filled into the flat plate reactor and 1 L of inoculum was added. During the whole experiment, the day/night cycle was set to 18:6 hours and the room temperature was 20 °C. The temperature in the reactor was not controlled. Only one light switch (four light bands) was turned on at the beginning. When the OD_{436} of the culture was above 1 (on day 6), 5 L of ATCC medium were added to the reactor. On the following day (day 7), the second light switch was turned on. On the 12th day of cultivation, the reactor was filled up with ATCC medium to the maximum working volume (20 L). This was set as the starting point of the first cycle.

Every weekday, RO-H_2O was filled into the reactor to reach the 20 L mark and refill evaporated liquid. Approximately 30 minutes later, a sample was taken for analysis. The OD_{436} was measured in a spectrophotometer every weekday for monitoring growth of *N. limnetica* (CCALA). The growth rate was calculated as described above (Equation 6 in section 3.5.2). Moreover, the pH of all samples was measured using pH stripes. When the OD_{436} exceeded 1.5, the CO_2 supply was installed to maintain the pH below 10. Moreover, the third set of light bands was turned on at that point. When the growth rate declined and did not recover, a harvest and recirculation process was conducted to start a new cycle.

Harvesting and recirculation

The harvesting experiments aimed at a quick harvest to ensure that the reactor could be filled up as fast as possible. For harvesting biomass, 15 L of the culture suspension were removed and the residual 5 L were left in the reactor as inoculum for the consecutive cycle.

The first harvest was primarily performed by cross-flow filtration, whose setup is shown in Figure 13. The flow varied but it was very low. After 12 hours, 9.4 L of the suspension were filtered and the filtration was stopped. The remaining cell suspension (5 L) was stored at 4 °C over the weekend, then filtration continued for 6 hours. After that, another 3.5 L of process water were separated by filtration from the biomass. As the filtration was getting even slower, it was stopped. The rest of the suspension (1.5 L) was centrifuged for 10 minutes at 10,000 rpm (19,690 g Sorvall centrifuge, Fiberlite rotor).

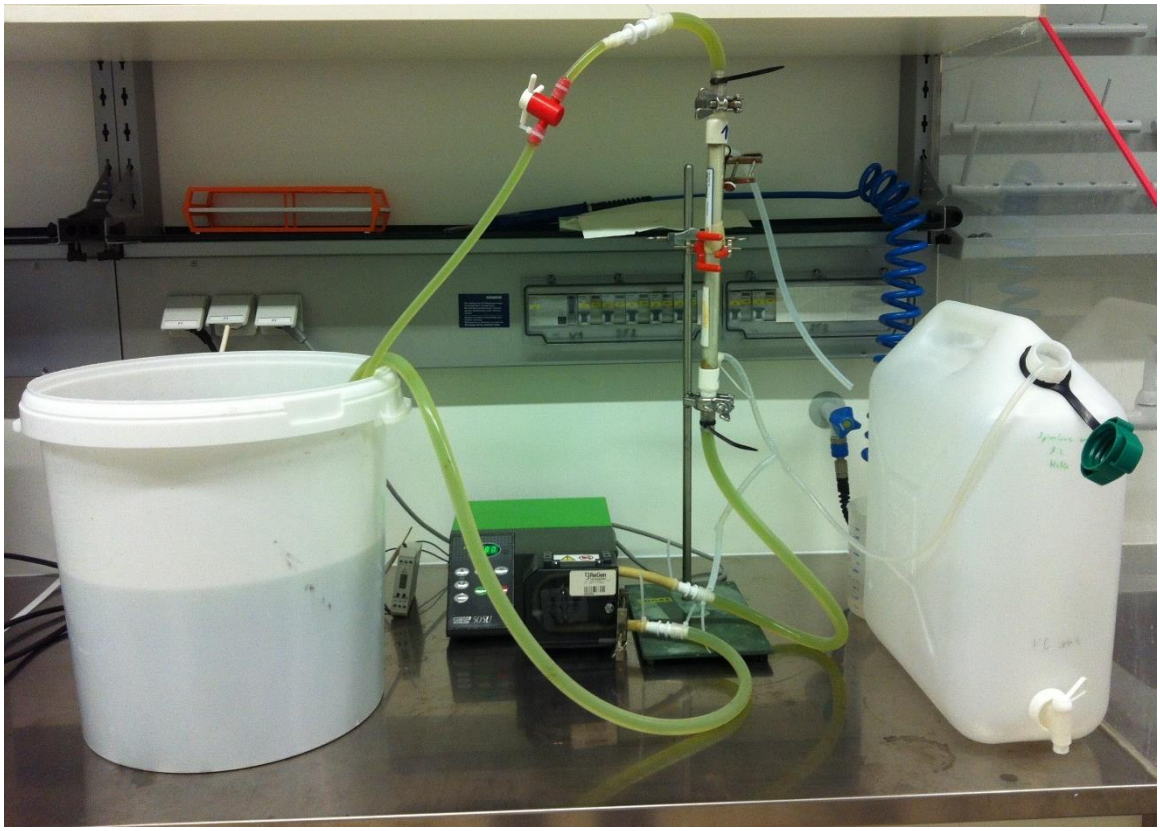

Figure 13: Setup of cross-flow filtration unit for biomass harvest.

After cleaning the filtration unit, it was reused for the second harvest. 8.5 L were filtered in 6 hours. Thus, the remaining 6.5 L were centrifuged again under the same conditions as before.

For consecutive harvests, the whole culture suspension was centrifuged; cross-flow filtration was no longer used.

For recirculation experiments, the following procedure stayed the same. After separating the biomass from the process water, samples of both were frozen to be analysed later. The process

water was enriched with all nutrients needed for 15 L of ATCC medium and reintroduced to the reactor. Then, the reactor was filled up to 20 L working volume with RO-H_2O. This was set as the starting point of a new cycle. After the fourth harvest, the experiment was ended.

Analyses

Table 2 shows the analysis schedule for the cultivation in the flat plate reactor. On holidays, no samples were taken.

Table 2: Analysis schedule for flat plate reactor.

	Culture suspension		Biomass				Process water		
	OD_{436}	pH	TS	Lipid content	Protein content	Pigment content	IC	NPOC	ICP
Monday	x	x							
Tuesday	x	x							
Wednesday	x	x							
Thursday	x	x							
Friday	x	x							
before harvest	x	x	x	x	x	x	x	x	x
after recirculation	x	x							

4 Results and discussion

In order to achieve the aim of optimising nutrient compositions for cultivating *Nannochloropsis limnetica* and to evaluate possible effects of process water recirculation, three steps were performed. Firstly, a theoretical comparison of cultivation media was conducted (see section 4.2). Secondly, growth in selected cultivation media was compared (see section 4.3) and finally, recirculation experiments were performed (see section 4.4). To enable cultivation experiments (step two and three), several pre-experiments were necessary (see section 4.1 and 4.3).

4.1 Comparison of different methods for growth evaluation

Since pigments account for absorption maxima of whole cells but their content can vary over cultivation (due to different conditions and culture age) growth curves based on OD values may not necessarily reflect the growth of an algal culture. On the other hand, OD measurements at wavelengths that are influenced by pigments exclude errors caused by non-photosynthetic organisms such as bacteria. These errors are not eliminated when using the TS as growth parameter. (Griffiths et al., 2011)

For this reason, an absorbance spectrum of an *N. limnetica* culture was recorded to find a wavelength suitable for usage as growth parameter throughout a cultivation. Furthermore, OD measurements and TS analysis were compared.

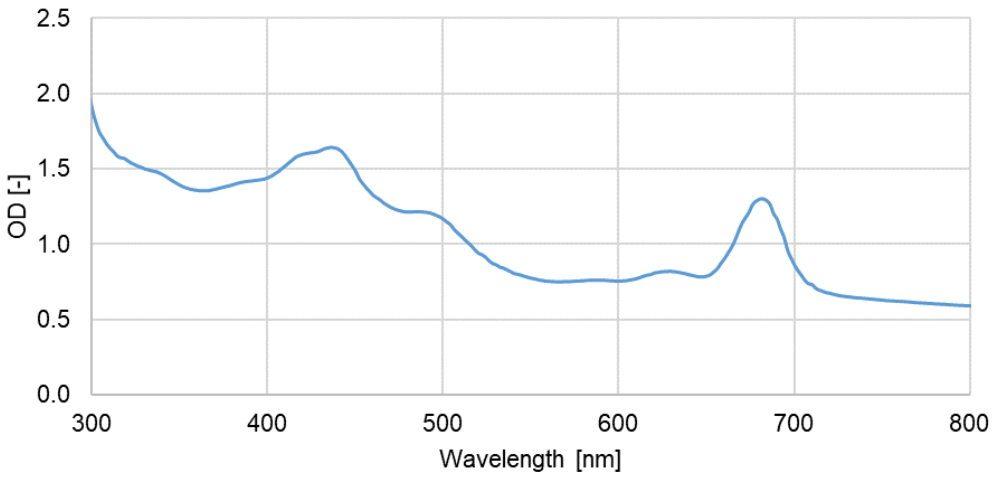

Figure 14: Absorbance spectrum of *N. limnetica* (ecoduna).

The recorded spectrum of the undiluted inoculum (Figure 14) shows absorption maxima at $\lambda_1 = 436$ nm and $\lambda_2 = 682$ nm. A plateau was detected at $\lambda_3 = 485$ nm. No maximum was detected at $\lambda = 750$ nm. Thus, OD_{436}, OD_{485} and OD_{682} were measured in subsequent experiments.

N. limnetica cultures form small aggregates, especially cultures of *N. limnetica* (ecoduna). For this reason, it is not only difficult to take homogenous samples, this also complicates OD measurements, which was also experienced by Fietz et al. (2005). To evaluate the influence of factors such as flocculation of cells and measurement errors, and to find a suitable method for evaluating algal growth, OD measurements and biomass concentration (TS) of a dilution series of *N. limnetica* (CCALA) were compared (Figure 15). This comparison shows that the difference between the OD at different wavelengths increases with cell concentration. The highest divergence was observed at 100 % cell suspension. There, the difference between OD_{436} and OD_{682} is 0.16 absorbance units. This difference is considered acceptable for measurements used for an estimation of algal growth. Therefore, measurements at those three wavelengths are considered equivalent and only one was used in further experiments. As it returns the highest signal, it was decided to use OD_{436} for further OD measurements.

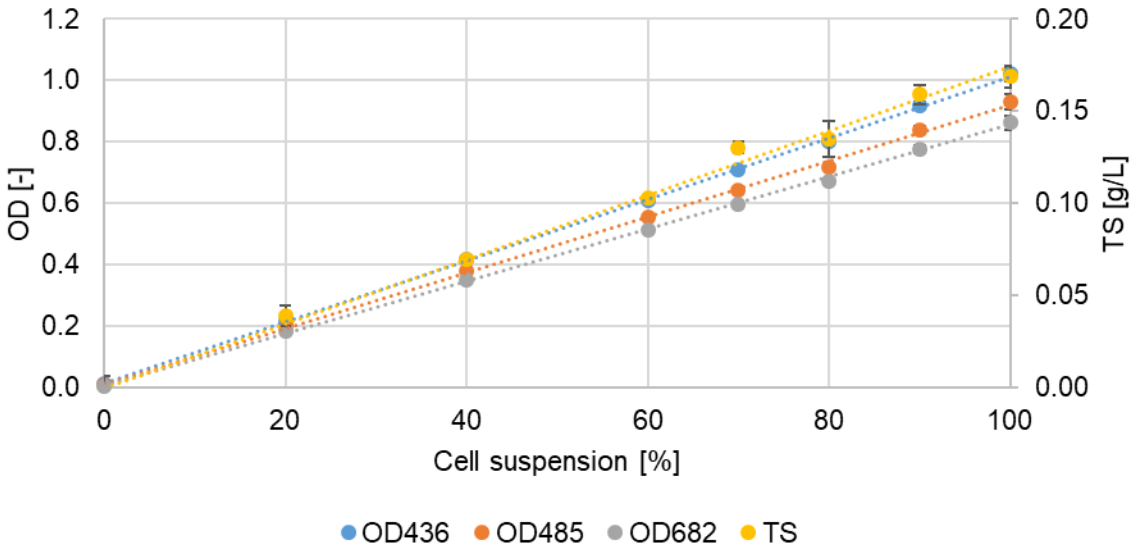

Figure 15: OD_{436}, OD_{485}, OD_{682} and TS of a dilution series of *N. limnetica* (CCALA) culture.

The biomass concentration determined by TS analysis shows more irregularities than OD measurements. OD graphs are more linear than the one obtained from TS values. Overall, all four trend lines have a coefficient of determination of above 0.99 (R^2_{OD436} = 0.9996, R^2_{OD485} = 0.9989, R^2_{OD682} = 0.9996, R^2_{TS} = 0.9934) and thus all methods are considered suitable.

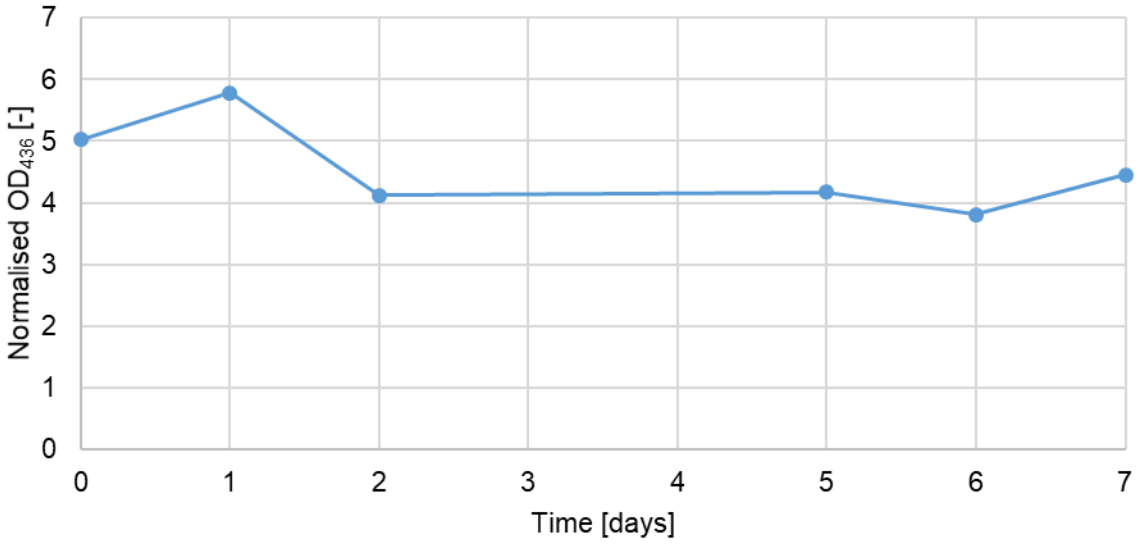

Figure 16: OD_{436} normalised to TS over 7 days cultivation of *N. limnetica* (ecoduna).

The normalised OD_{436} (ratio OD_{436} to TS) of a culture of *N. limnetica* (ecoduna) over the time course of 7 days (Figure 16) shows that the ratio of OD_{436} to TS is not constant over this time period, which is consistent with the findings by Griffiths et al. (2011). Nevertheless, the observed variation is low. In the beginning of cultivation, OD measurements would underestimate the dry weight according to this graph. This is also reflected in the analysis of the dilution series where the difference of the results between TS and OD measurement is higher at low culture density. As the differences between the two methods were considered to be at an acceptable level, it was decided that both can be used as growth parameter.

4.2 Theoretical media comparison

Recipes for cultivating *N. limnetica* were sought and their elemental composition compared. Following this, media for cultivation experiments were chosen. Table 3 provides a list of all media that were found for cultivating *N. limnetica* as well as their recipes, origin and the information whether the medium was chosen for further cultivation experiments.

Table 3: Cultivation media that were found and considered for cultivating *N. limnetica*.

Medium	Source	Chosen	Reason
ES Ag (Basal medium)	Sammlung von Algenkulturen Göttingen (SAG)	-	Contains soil extract → not reproducible
Bold's Basal Medium (BBM)	Culture Collection of Autotrophic Organisms (CCALA), Andersen et al. (2005)	✓	
Bourelly	Noda et al. (2017), Krienitz and Wirth (2006)	✓	
Waris	Krienitz et al. (2000), UTEX (2018)	✓	
MIII	Modified version used in Fietz et al. (2005), Körner and Nicklisch (2002)	✓	
M99F	Fietz et al. (2005)	✓	
Woods Hole Medium	Fawley and Fawley (2007)	-	No recipe was found
ATCC	IFA-Tulln	✓	
BG-I	IFA-Tulln	✓	
WC	Krienitz et al. (2000), Andersen et al. (2005)	✓	

All chosen media were further investigated and compared regarding their composition. In the following paragraph, only the macro elements are discussed. Generally, the nutrient composition differs in the various media without an obvious trend. The concentrations of all macro elements present in the media are shown in Figure 17.

The two media MIII and M99F are very similar, since M99F is a variation of MIII. Both of them contain a high variety of elements and they contain a few macro elements in an amount above average (C, Ca, Cl, Mg, S and Si). They have much lower K, P and N contents than the other media. The fact that the other media contain high amounts of N was seen as indicator that a high amount of N is important for growth. As M99F contains less N than MIII, it was omitted.

Waris is a rather "thin" medium, with low concentrations of most macro elements. This is especially true for N (1 mmol/L), compared to other media. Moreover, besides the main nutrients N, P and K it only contains Ca, Mg and S; for this reason, it is interesting to test. Contrary to Waris, BG-I is a very nutrient-rich medium. It specifically contains a lot of N and Na (14 mmol/L). Furthermore, the amount of Fe in BG-I is highest out of all analysed media. WC is the only medium containing Tris buffer, which makes it expensive. It has many different nutrients, but in low to moderate amounts and it does not contain Fe. Bourelly contains high amounts of K and C. The other elements are present at average quantities. Bourelly does not contain Cl or Si. ATCC has only little C, Ca, and Cl and no Fe. It contains low to moderate amounts of the other elements. BBM does not contain any C, which might be an advantage as heterotrophic bacteria will not find a C-source. It contains a high amount of EDTA, K and P.

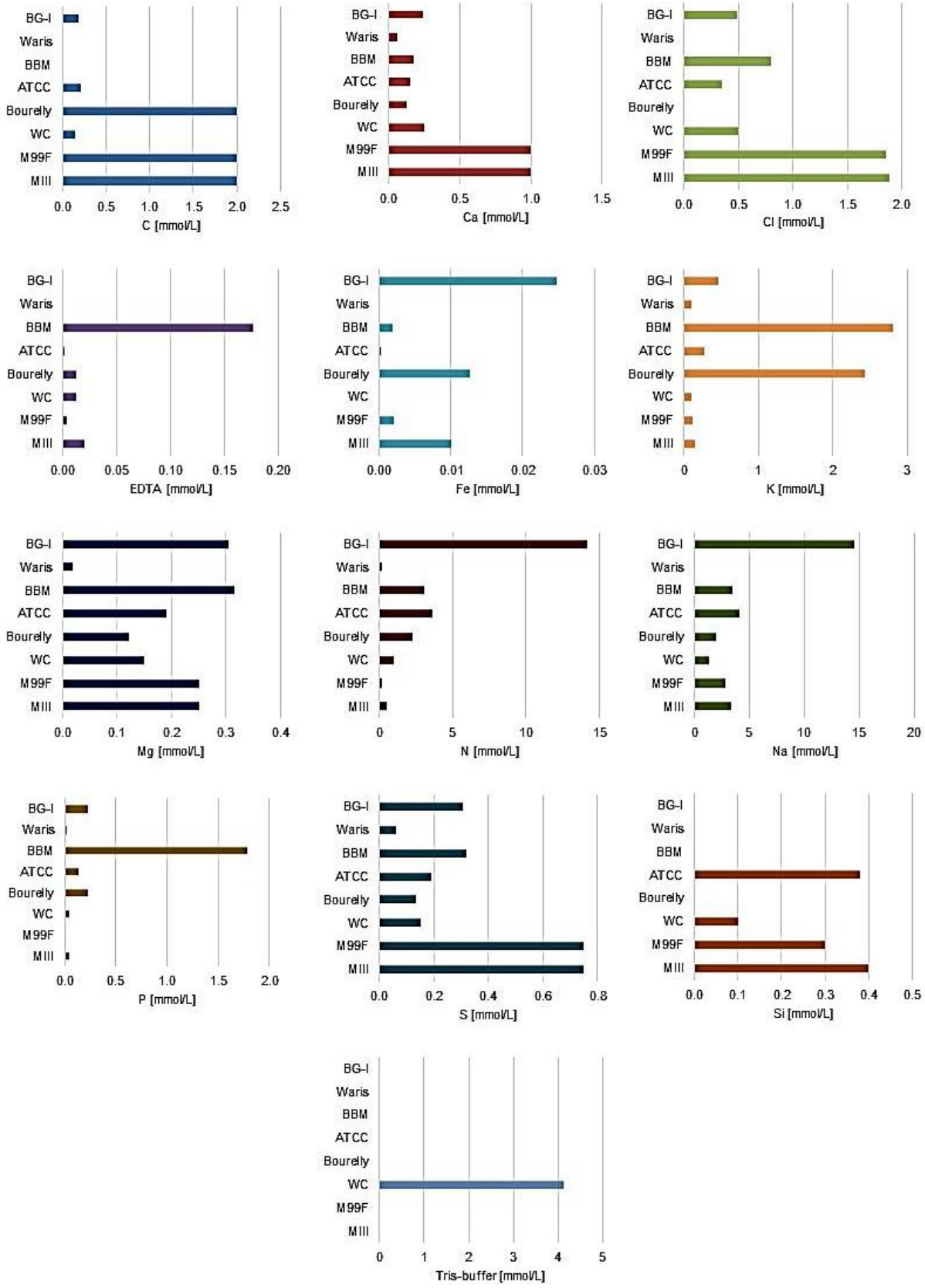

Figure 17: Macro element composition of cultivation media.

As it was not found that some of the media showed a significantly higher or lower potential than others and also no similarities (except for M99F and MIII) were found, it was decided to test the following media in the herein conducted experiments: ATCC, BBM, Bourelly, BG-I, MIII, Waris, WC.

Only three of the media recipes, namely those of Bourelly, M99F and WC, suggested the addition of vitamins. Vitamin B12, Thiamine and Biotin are present in all of them, but the amounts differ tremendously (Figure 18). Based on these recipes, it was decided to prepare a vitamin solution with a concentration of 0.015 µmol/L Vitamin B12, 0.296 µmol/L Thiamine, and 0.020 µmol/L Biotin.

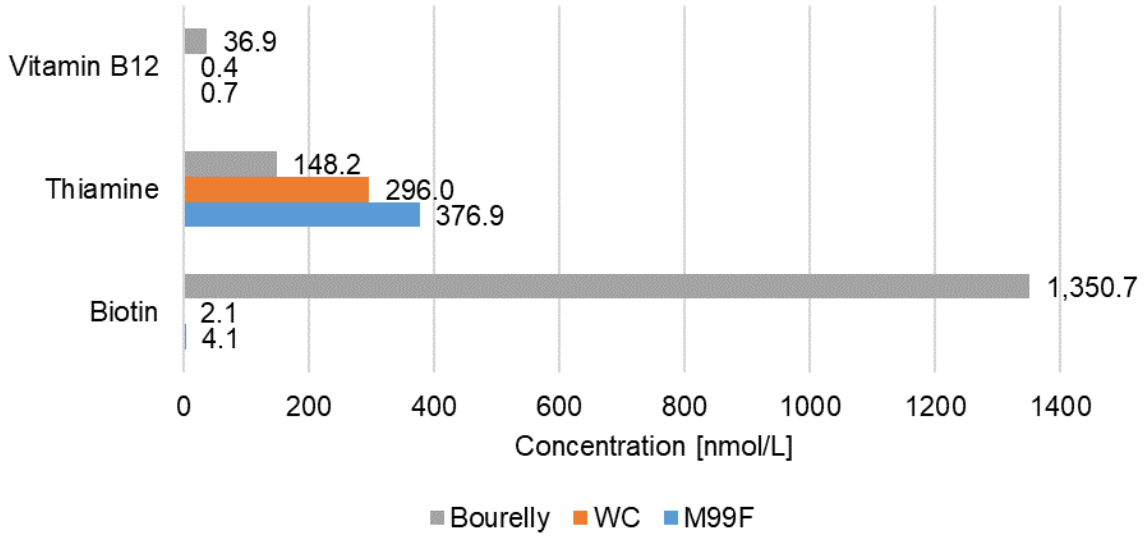

Figure 18: Concentration of vitamins in cultivation media.

The analysis of trace elements resulted in the following observations. The concentration of trace elements is below average in MIII and M99F. The trace element solutions for ATCC, Waris and BBM contain only few different elements but BBM contains a very high amount of B. The trace element composition of Bourelly is similar to that of the other media with a tendency to higher concentrations. Also, WC medium did not show significant differences to other media. BG-I contains various trace elements in amounts above average and is the only medium containing Cr. Therefore, it was decided to use the composition of the trace element solution for BG-I medium for all media in the described experiments.

4.3 Growth evaluation and selection of most promising media

4.3.1 Pre-experiments in 24-well plates and flasks

Cultivation experiments in 24-well plates were conducted to test the growth of *N. limnetica* in various media in parallel. A series of dilutions was performed before to determine the required inoculation density. To reach the desired start-OD of 0.1 - 0.2, 0.25 mL of inoculum were transferred to 1.75 mL of medium (cultivation of *N. limnetica* (ecoduna)). Since the inoculum for the cultivation of *N. limnetica* (CCALA) was denser, a higher dilution (10-fold – 0.2 mL inoculum to 1.8 mL medium) was necessary to obtain a comparable start-OD (0.1 - 0.2).

The cultivation of *N. limnetica* in 24-well plates was successful in the media ATCC, BG-I and MIII (example for successful cultivation shown in Figure 19). In the media BBM, Bourelly, Waris and WC *N. limnetica* did not grow (exemplary shown in Figure 20). Results obtained with the strains of ecoduna and CCALA are similar in the conducted experiments.

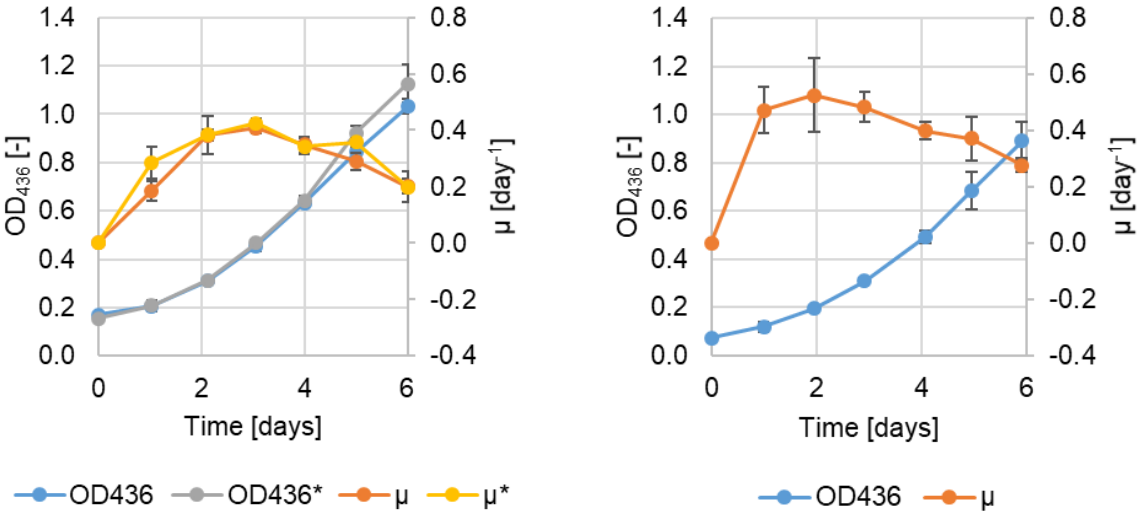

Figure 19: OD_{436} and growth rate of *N. limnetica* in ATCC over 6 days cultivation. Left: *N. limnetica* (ecoduna), right: *N. limnetica* (CCALA) (*vitamins added)

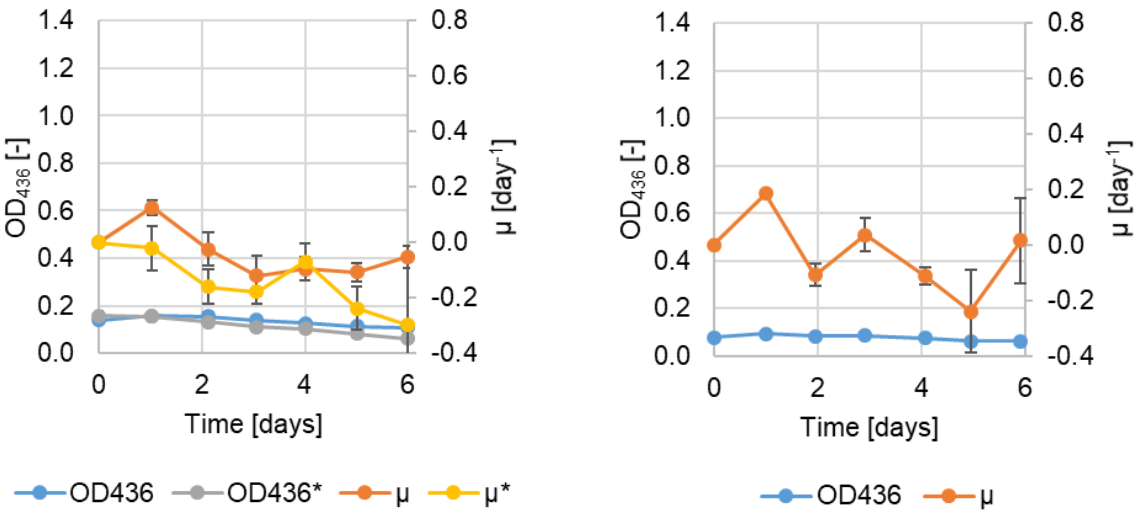

Figure 20: OD_{436} and growth rate of *N. limnetica* in Bourelly over 6 days cultivation. Left: *N. limnetica* (ecoduna), right: *N. limnetica* (CCALA). (* vitamins added)

Figure 21 shows the maximum OD as well as the highest growth rate of *N. limnetica* (ecoduna) in all tested media. As stated above, it is clearly visible that BBM, Bourelly and Waris are not suitable for cultivation of *N. limnetica*. OD_{436} is highest in ATCC and BG-I while maximum growth rates are comparable in ATCC, BG-I, MIII and WC. The adjustment of the pH-value in WC (pH 8.7 and pH 8) does not significantly influence growth of *N. limnetica* (ecoduna). These results are confirmed by those obtained in 100-mL flasks (compare Figure 21 and Figure 24) and comparable to those of *N. limnetica* (CCALA) (Figure 22).

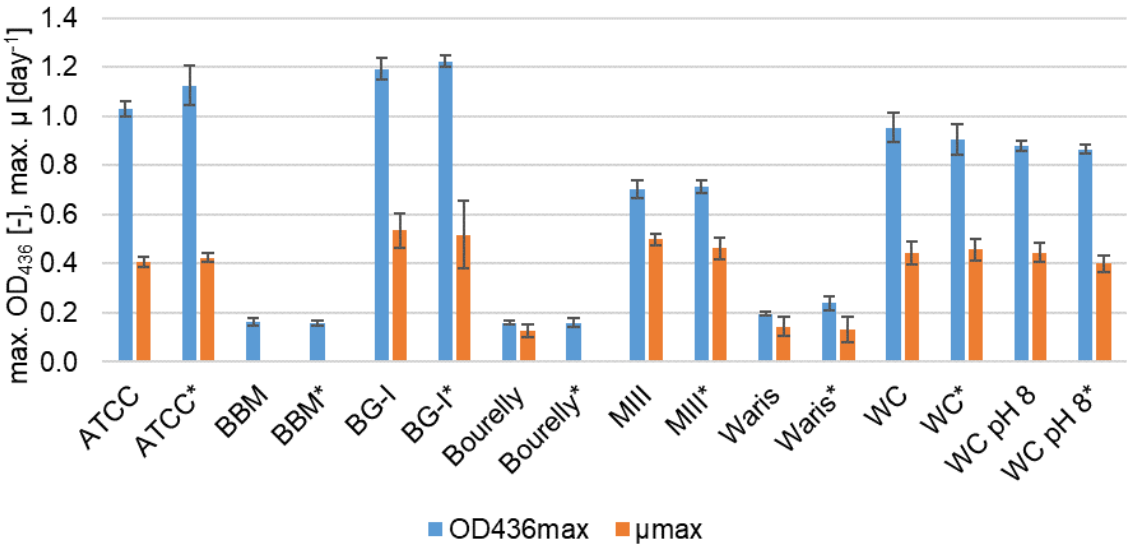

Figure 21: Maximum OD_{436} and growth rates in cultivation of *N. limnetica* (ecoduna). (*vitamins added)

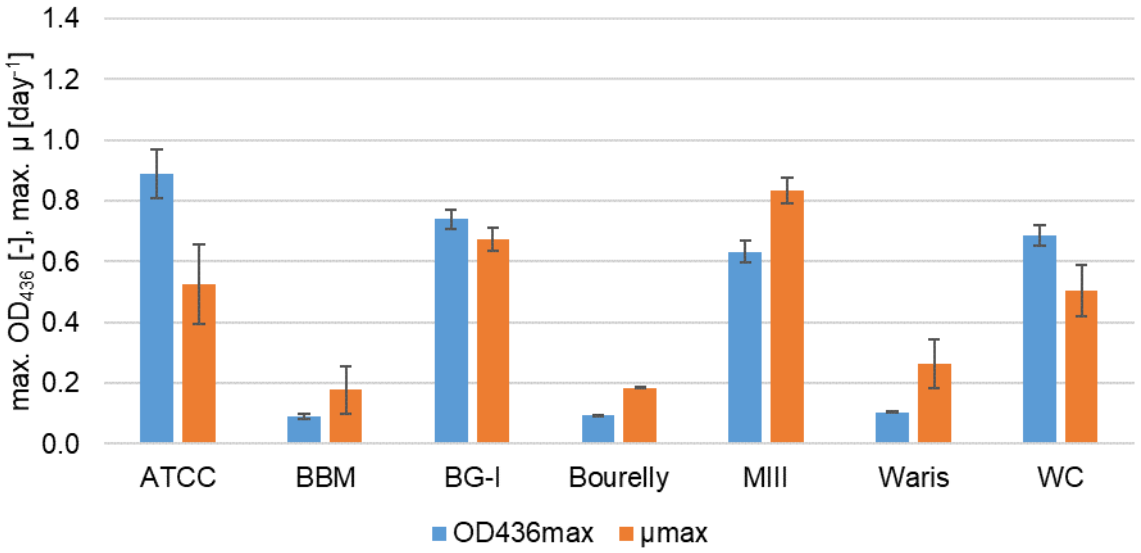

Figure 22: Maximum OD_{436} and growth rates in cultivation of *N. limnetica* (CCALA).

The behaviour of *N. limnetica* (ecoduna) could be studied during the cultivation in 100-mL flasks. In all of the tested media, biomass flocculates but the flocs have different sizes. Usually, they sink to the bottom of the flask. The flocs are middle-sized and dark green in ATCC. In BBM, they are very light green and middle-sized. They are also middle-sized in MIII, Waris, and WC but in MIII and WC, they become smaller over time. The flocs are bigger in BG-I and very small in Bourelly. Nevertheless, there is no correlation between floc formation and growth. The appearances of *N. limnetica* (ecoduna) in different media after 1 week cultivation are shown in Figure 23. The colour of the culture suspensions after 1 week reflects the OD_{436}, which is significantly lower in yellowish and light green cultures (Figure 23 and Figure 24).

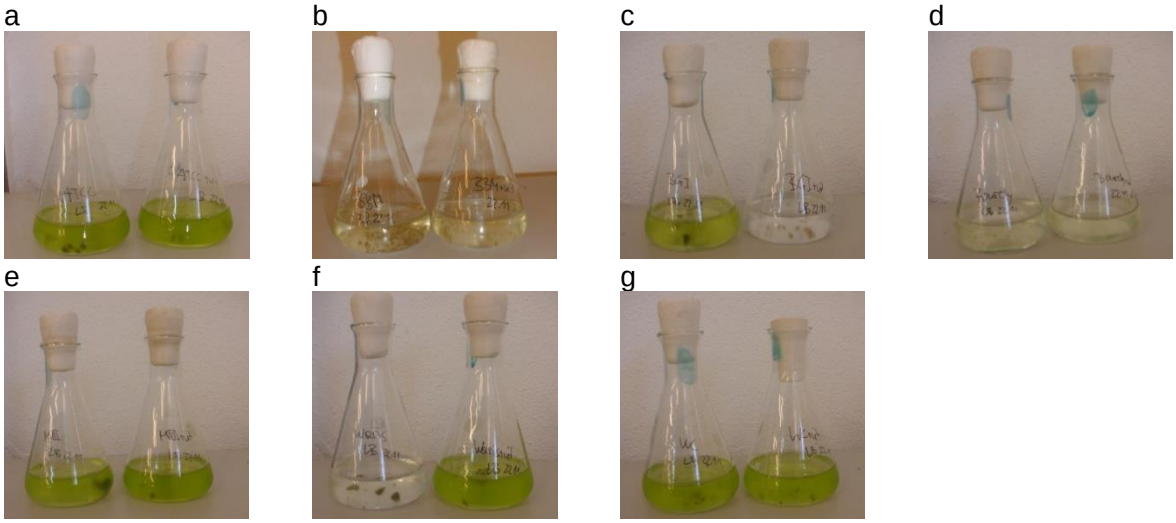

Figure 23: Appearance of *N. limnetica* (ecoduna) in different media in 100-mL flasks after 1 week cultivation. ATCC, ATCC* (a), BBM, BBM* (b), BG-I, BG-I* (c), Bourelly, Bourelly* (d), MIII, MIII* (e), Waris, Waris* (f), WC, WC* (g); (* vitamins added).

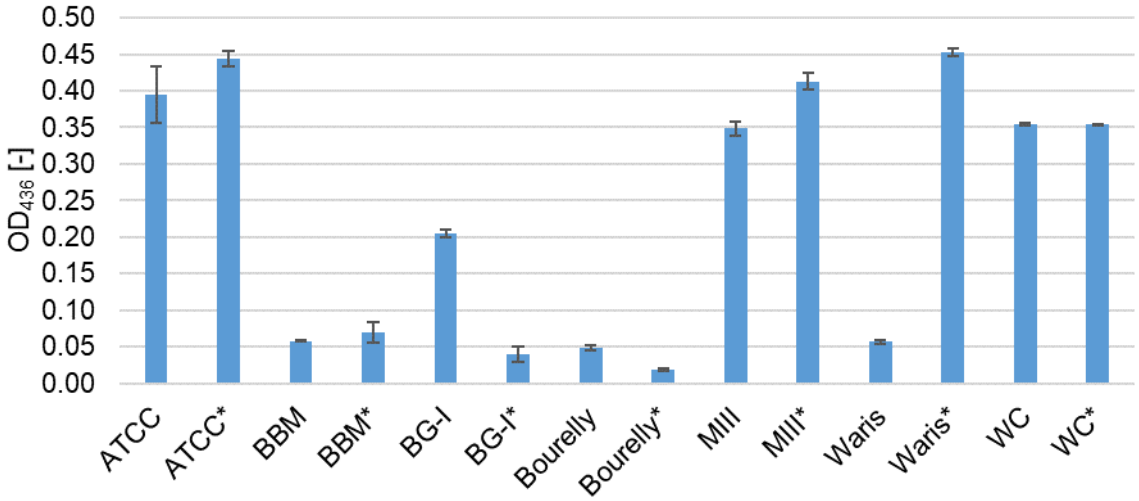

Figure 24: OD_{436} of *N. limnetica* (ecoduna) after 1 week cultivation in 100-mL flasks. (* vitamins added)

Vitamin addition does not show a systematic advantage or disadvantage. This result is in accordance with the findings of Fietz et al. (2005). Again, it is assumed that this is also the case for *N. limnetica* (CCALA).

In summary, cultivation problems occurred in BBM, Bourelly, and Waris. A possible explanation for those problems might be the altered trace element solution. Possibly the different overall composition is not suitable for these media. ATCC, BG-I, MIII and WC provide good growth conditions for both *N. limnetica* strains. Nevertheless, WC was excluded from further investigation because it contains Tris-buffer, which makes its production expensive. Since growth in this medium was not outstanding, it is considered not to be economic. Thus, ATCC, BG-I and

MIII were chosen for further experiments. A summary of the results of the small-scale experiments can be found in Table 4.

Table 4: Summary of small-scale experiments.

Medium	Description of growth	Further investigation
ATCC	Good growth in 24-well plates and flasks	✓
BBM	No growth in 24-well plates and flasks	-
BG-I	Better in plates, moderate in flask without vitamins	✓
Bourelly	No growth in 24-well plates and flasks	-
MIII	Good growth in both (higher OD in flasks)	✓
Waris	Little growth in plates, high growth only in flask with vitamin solution	-
WC	Good growth in 24-well plates and flasks, but due to Tris-buffer not economic	-

4.3.2 Cultivation in 2-L flasks

After cultivation experiments in 24-well plates and 100-mL flasks, the herein selected media were used for scaling up the cultivation and having a closer look at differences in growth using those three media.

In both experiments, there are some pH fluctuations over time but the pH does not exceed the range of 7 to 9. The increasing pH value over time can be explained by the bicarbonate equilibrium $H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$. HCO_3^- is converted to CO_2 which is then used by algae. Therefore, H^+-concentration decreases, which means that the pH increases. (Chi et al., 2011) Changes in pH can be avoided by adding buffer to the medium or by sparging with CO_2. Nevertheless, this is neither economical nor sustainable as unused CO_2 will be released into the atmosphere. (Scherholz and Curtis, 2013)

The growth curves of both *N. limnetica* strains in three different media over 14 days of cultivation are shown in Figure 25 (biomass concentration – TS) and Figure 26 (growth rate). For *N. limnetica* (ecoduna), TS was used to estimate the biomass concentration and to calculate the growth rate. Problems occurred during sampling (flocculation of the culture) that led to high standard deviations, especially after calculating the growth rate (Figure 26). Therefore, it was decided to use OD_{436} instead of TS in the consecutive experiment to monitor the growth of *N. limnetica* (CCALA). In order to facilitate comparison of both strains (section 4.3.3), the final TS of both was measured.

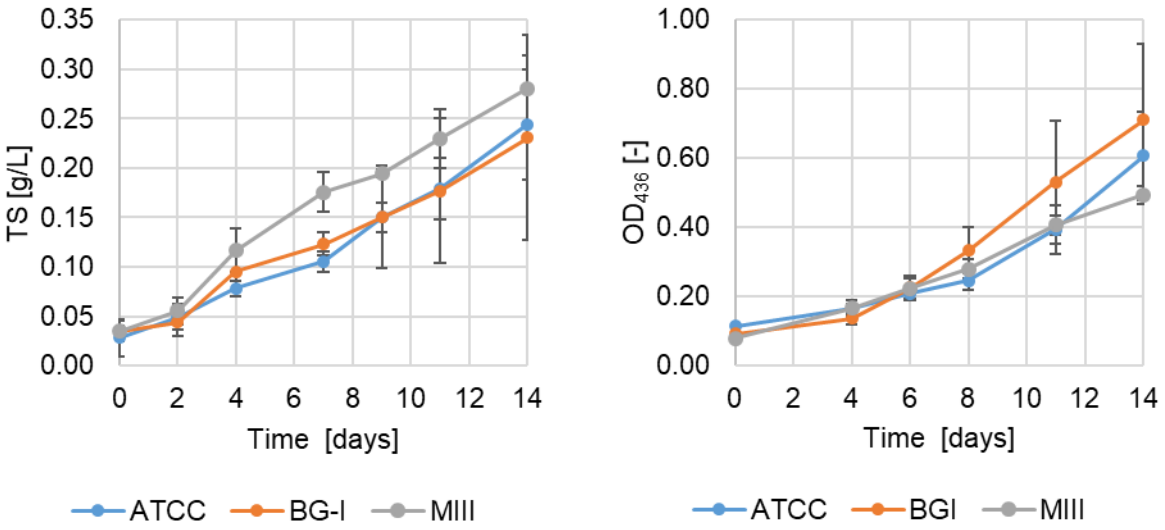

Figure 25: Biomass concentration (TS or OD_{436}) of *N. limnetica* cultivation in 2-L flasks over 14 days. Left: *N. limnetica* (ecoduna), right: *N. limnetica* (CCALA)

In comparison with the small-scale experiment (24-well plates), the growth rate is smaller at this larger scale. This is a typical upscaling-effect. Especially light is a limiting factor after upscaling as it is absorbed by algal cells and cannot reach the core part of the cultivation vessel or flask (Sipaúba-Tavares et al., 2013).

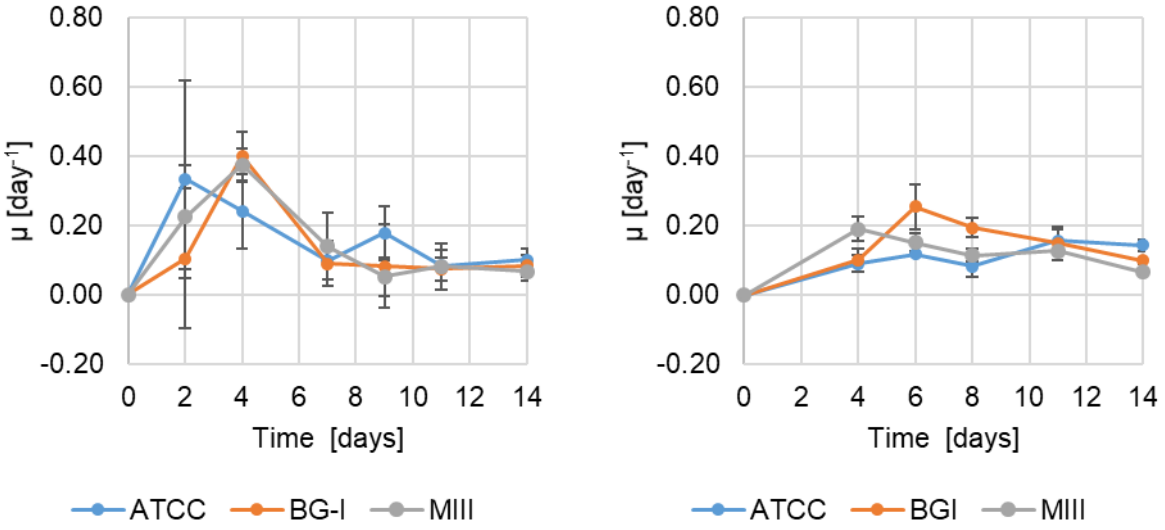

Figure 26: Growth rate of *N. limnetica* cultivation in 2-L flasks over 14 days. Left: *N. limnetica* (ecoduna), right: *N. limnetica* (CCALA)

As can be seen in Figure 25, the strain obtained from ecoduna achieved the highest biomass concentration in MIII medium (0.28 g/L ± 0.03 g/L). In this medium, the TS values are slightly higher than in the other media over the whole time span. Nevertheless, the values of all three

media are very similar. Consequently, the course of the growth rates of all three media is also similar, which is illustrated in Figure 26. It rises in the beginning until day 2 to 4 and after that, the growth rates decrease to approximately 0.1 day^{-1}. The strain obtained from CCALA attained the highest OD_{436} values in ATCC medium (0.90 ± 0.21). Again, the course of the growth curve is similar in all three media. This is also reflected in Figure 26, as the growth rate develops similarly in all media. The growth rate of the strain obtained from ecoduna is high in the beginning and levels out at approximately 0.1 day^{-1} after day 7 while the growth rate of *N. limnetica* (CCALA) is relatively stable around 0.15 day^{-1} over the whole experiment. Nevertheless, especially at high values of the growth rate, there are high standard deviations in the experiment with *N. limnetica* (ecoduna), as already mentioned. Therefore, the differences between both strains could also have been caused by measurement errors when using TS as growth parameter. The differences between both strains are discussed in more detail in section 4.3.3.

In conclusion, the cultivation in all three media seems to be similarly promising. None of the media shows to be unsuitable and none of the media sticks out as much more promising than the others. Therefore, the decision of which medium to use in recirculation experiments was based on practicability. It was decided to use ATCC because this medium is easy to prepare and to handle. As experience shows, a concentrate can be made and then diluted, which saves time and space. This is also relevant for industrial applications.

4.3.3 Comparison of CCALA and ecoduna strain

In the course of conducting the experiments, it turned out that *N. limnetica* (ecoduna) differs from the strain obtained by CCALA in terms of optical appearance in flask cultivation, even though they appear equal when examined under the microscope (see Figure 5, section 3.2). The most obvious difference observed is flocculation. *N. limnetica* (ecoduna) tends to form small aggregates. This phenomenon of autoflocculation is also observed when cultivating the strain ordered from CCALA but at a much lesser extent. In general, flocs complicate sampling of *N. limnetica* as the cultures cannot be homogenised properly. Therefore, high standard deviations are observed when analysing samples of the culture suspension which is in contrast to the conducted pre-experiment (section 4.1). This is not appropriate for laboratory experiments as results can only be interpreted as an approximation as experienced in the executed experiments described above.

In addition, differences were observed in the maximum growth parameters achieved by the two different organisms. Figure 27 gives an overview of the highest growth rates that are achieved in the small-scale experiment in 24-well plates and the larger-scale experiment in 2-L flasks of both strains.

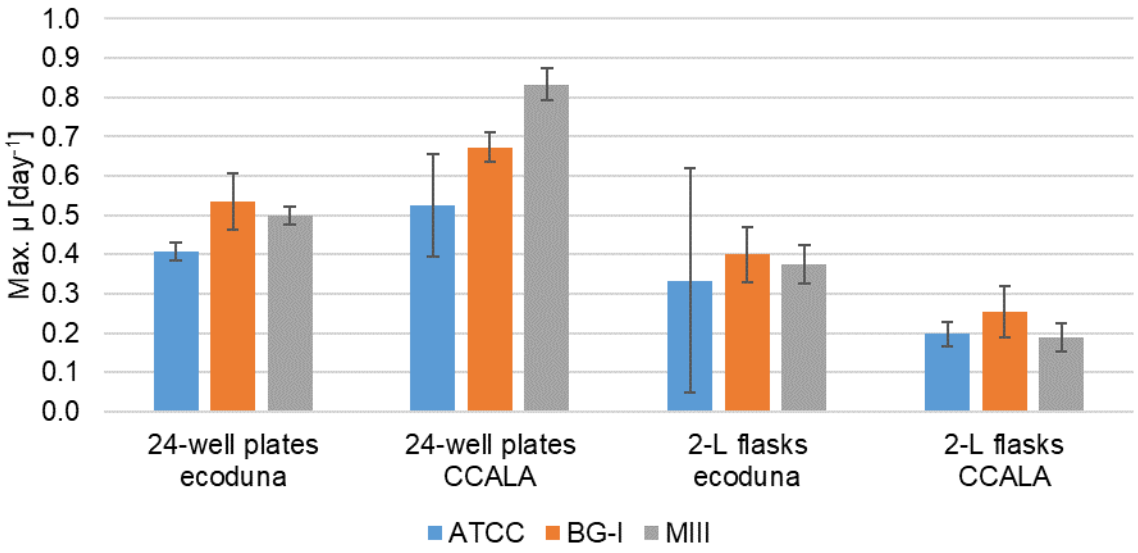

Figure 27: Comparison of maximum growth rates of *N. limnetica* (ecoduna) and *N. limnetica* (CCALA) in experiments in 2-L flasks and 24-well plates.

As can be seen in this figure, growth rates of the strain obtained from CCALA are higher than those of that obtained from ecoduna in the small-scale experiment. 24-well plates have a considerably low volume and layer thickness. Therefore, the suspension is subject to being heated up by the light energy and more water can evaporate than at a larger scale. Both of these are stress factors for algae. Possibly, the strain obtained from CCALA is more robust to these parameters than that obtained from ecoduna and therefore retains higher growth rates. In the larger-scale experiment, this is reversed, which leads to the conclusion that when stress factors such as heat and evaporation are present at a lower extent, the strain obtained from ecoduna can achieve higher growth rates.

Figure 28 gives a comparison of the maximum OD of the two strains that was measured in the small-scale experiment in 24-well plates and a comparison of the highest biomass concentrations in the larger-scale experiment (2-L flasks).

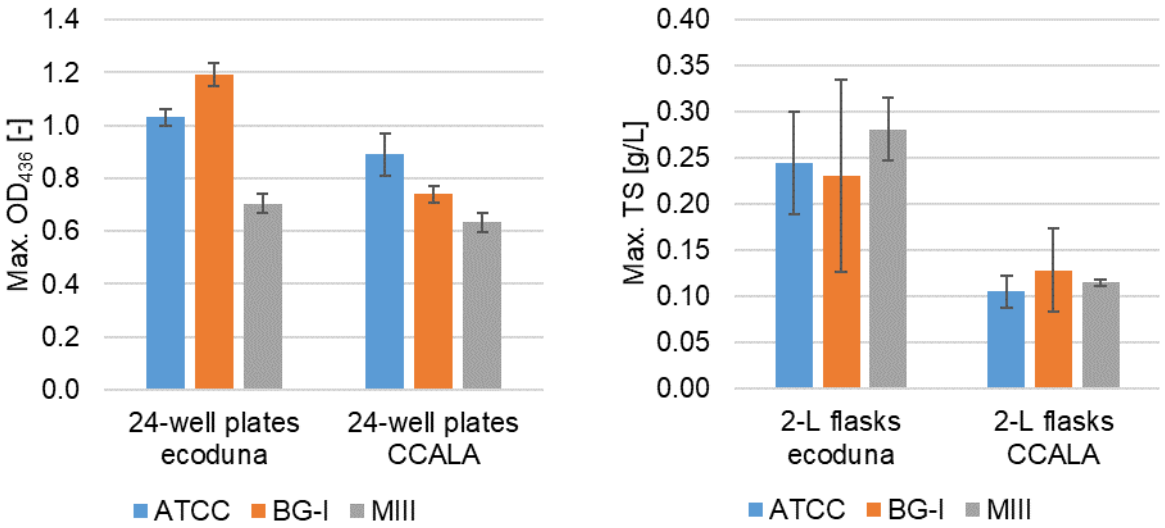

Figure 28: Comparison of maximum OD_{436} (left) and TS (right) of *N. limnetica* (ecoduna) and *N. limnetica* (CCALA) in experiments in 2-L flasks and 24-well plates.

In both experiments, the biomass concentration or OD_{436} of the strain obtained from ecoduna is higher than that of *N. limnetica* (CCALA). This indicates that with this strain, a higher biomass yield can be achieved. Nevertheless, large measurement errors are visible in TS measurements, especially in TS measurements of *N. limnetica* (ecoduna). This illustrates the problem of sampling caused by flocculation described above.

As the experiments were not performed simultaneously, the observed differences could possibly be due to environmental conditions. Nevertheless, as indicated in section 1, different *N. limnetica* strains exist. Probably, *N. limnetica* (ecoduna) is a different ecotype than *N. limnetica* (CCALA).

To conclude, it cannot be said with confidence that one of the two strains has a higher growth rate, but higher final biomass concentration was achieved by *N. limnetica* (ecoduna). Still, sampling problems occurring with this strain complicate its cultivation for research purposes. Therefore, *N. limnetica* (CCALA) was found to be more suitable for recirculation experiments.

4.4 Scale-up and recirculation in a flat plate reactor

After selecting ATCC as most convenient medium and *N. limnetica* (CCALA) as more suitable strain, a recirculation experiment was performed in a flat plate photobioreactor with a working volume of 20 L. The aim was to study the effects of recirculating process water on the performance of *N. limnetica* and to compare filtration and centrifugation as harvesting methods.

4.4.1 Cultivation

Growth behaviour

N. limnetica was cultivated in a flat plate reactor for 74 days. The medium was recycled three times during this time period, resulting in four cycles of cultivation.

Generally, it was observed that mixing using an aquarium airstone is not efficient enough. The glass plates of the reactor had to be cleaned manually every day using a rubber on a rod. Biofilms formed quickly, especially in the corners. These biofilms and also flocculation of the cells occurred more frequently after each recirculation.

Figure 29 shows the course of OD_{436} and growth rate of *N. limnetica* over the cultivation. Recirculation events are characterised by the sudden drop of the OD_{436} and marked by a vertical line (day 22, day 40, and day 56).

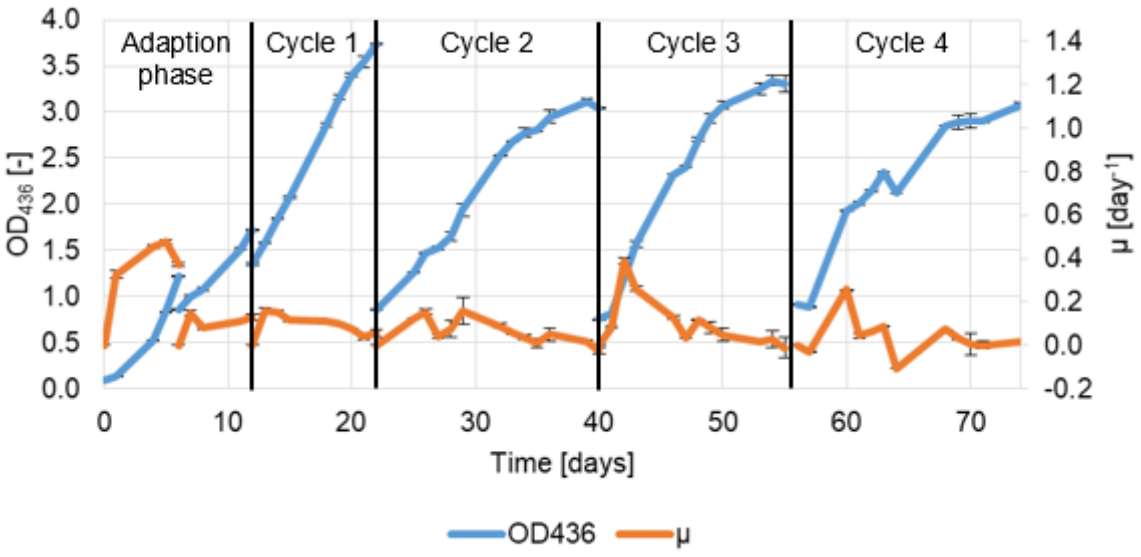

Figure 29: OD_{436} and growth rate of *N. limnetica* (CCALA) over four recirculation cycles in flat plate reactor.

Until day 12, the inoculum was allowed to adapt to the conditions in the plate reactor and to develop a higher start-OD. After starting with 10 L working volume, additional 5 L were added on day 7 and another 5 L were added on day 12. These media addition events are visible in the

graph as gaps (dilution of the culture suspension) on the respective days. After filling the reactor up to 20 L on day 12, the culture had reached its start-OD of cycle 1.

The cultivation was stable; start-OD_{436} (0.75-1.0) and final OD_{436} (3.0-3.75) of all cycles are in a narrow range and comparable. The growth rate is also consistently between -0.1 day^{-1} and 0.2 day^{-1} with two outliers (0.39 day^{-1} on day 42 and 0.26 day^{-1} on day 43). The recirculation of process water does not have an effect on the growth rate in any other way. During the last cycle (fifth cycle; not shown), *N. limnetica* was overgrown by another alga, supposedly *Chlorella* sp. Therefore, the cultivation was stopped and the fifth cycle could not be evaluated. It is not clear how many cycles would have been possible without a decrease in biomass formation if the culture had not been overgrown. At least four recirculation cycles without negative effects on algal growth are possible. To the authors knowledge, no results of recirculation experiments with *N. limnetica* have been published yet, therefore it was not possible to compare the results found in this experiment. To find out which substances accumulate during the cultivation, the process water was analysed. Moreover, the biomass was analysed to investigate the status of the cells. The results are described below.

Harvesting procedure

Major problems were experienced when a cross-flow filtration unit was used to separate the biomass from the process water. The flow was very low (1 L/h on average) during the first harvest and just a little higher (1.4 L/h) during the second harvest. The reason for the increased flow was that the filtration unit was cleaned with RO-H_2O before the second harvest was conducted. But even with a clean membrane, the flow rate is still low. Due to the low flow and the resulting long time the harvesting procedure takes, the culture suspension, which is used as inoculum for the next run, remains in the reactor for several hours. During this time, there is no appropriate mixing as the reactor design and the air pump are not suitable for such a low level of suspension. This, additionally to the fact that the process is time-consuming, led to the conclusion that the cross-flow filtration unit used is not suitable for harvesting *N. limnetica*. As already mentioned, membrane clogging is a frequently observed problem (Milledge and Heaven, 2013) that leads to the experienced slow-down of the process. Due to the observed problems, filtration was only used for the first two harvests but not for the third and fourth.

In both cases (harvest 1 and 2), the remaining culture suspension was centrifuged (as described in section 3.5.3) after cross-flow filtration. Surprisingly, after the first harvest the supernatant was coloured red (Figure 30) in contrast to the transparent permeate of the filtration process. After the second harvest, just a light difference in colour of permeate and supernatant was observed (Figure 31); the permeate was again transparent whereas the supernatant was yellow. The difference in colour of the process water indicates that more particles and extracellular components are separated when using cross-flow filtration than when using centrifugation. During both harvests, a red biofilm was observed on top of the green algae biomass (compare

Figure 32). This red colour might be caused by a bacterial contamination. It is not clear if this contamination influenced the course of the experiment and in which extent. Nevertheless, it is assumed that the red colour comes from a bacterium that lives in symbiosis with *N. limnetica* and does not harm the cultivation.

Because of the problems mentioned above, it was decided that centrifugation is more suitable for harvesting in this particular setting. At industrial scale, factors such as robustness as well as maintenance and overall costs etc. need to be considered. Nevertheless, literature confirms the conclusion that filtration is often less suitable for harvesting microalgae, especially for small cells (< 10 µm) (Milledge and Heaven, 2013).

Figure 30: Process water after centrifugation. Pictures are from first harvest.

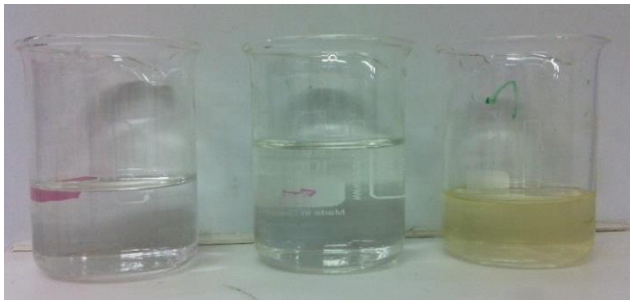

Figure 31: Colour comparison. From left to right: RO-H_2O, process water after cross-flow filtration, process water after centrifugation. Pictures are from second harvest.

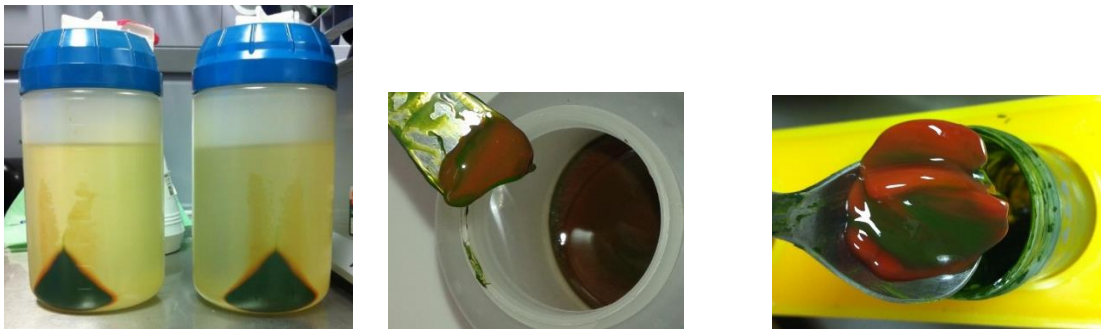

Figure 32: Red biofilm on top of green algae biomass. Left: Culture suspension after centrifugation, second harvest. Middle: Biomass after centrifugation, first harvest. Right: Biomass after centrifugation, second harvest.

4.4.2 Biomass analyses

Lipids

The lipid concentration in the biomass does not significantly alter; it is constantly between 27 % and 35 % (Figure 33). This means that the recirculation of process water does not influence the lipid production of *N. limnetica*. The product that is especially aimed for in *N. limnetica* production is therefore not influenced by process water recirculation. As opposed to this, Zhang et al. (2016) found that the lipid content of *Nannochloropsis oceanica* biomass decreases when cultivated in recirculated process water. In prospective experiments, the pattern of polyunsaturated fatty acids should be analysed to find out whether process water recirculation influences it.

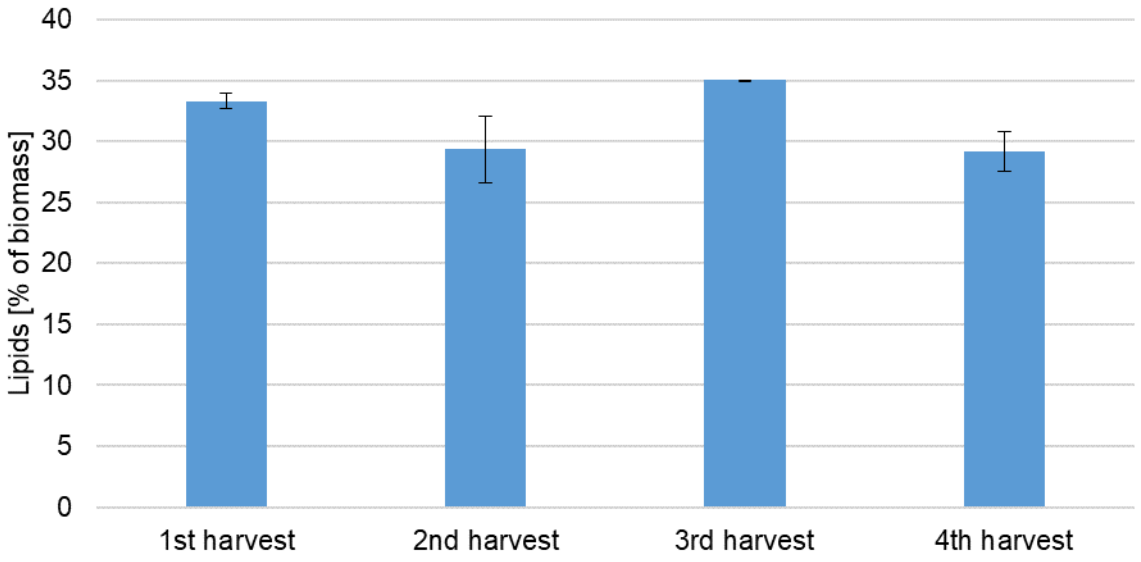

Figure 33: Lipid content of biomass after each harvest.

Pigments

The pigment composition (content of chlorophyll$_a$ and total carotenoids) decreases with each harvest (Figure 34). This is consistent with the results of the study conducted by Rodolfi et al. (2003). Pigments are important for harvesting light energy for photosynthesis (Masojídek et al., 2013). Therefore, lower pigment concentrations in the biomass result most probably in reduced growth, especially when the organism is grown phototrophically. Nevertheless, it was observed in this experiment that algal growth did not decline with a decrease in pigment content. However, some algae can grow mixotrophically. This means that additionally to utilising light energy to convert inorganic CO_2 to biomass, the organism assimilates organic carbon sources (Sforza et al., 2012). Consequently, the same growth rates could also be achieved with a lower pigment content in the biomass because the organism would receive part of the needed energy from the organic carbon source. As in this cultivation, the organic matter increased (Figure 35) when at

the same time the pigment content decreased, *N. limnetica* might have used the accumulated organic matter as C-source and might have grown mixotrophically. It has not yet been investigated if *N. limnetica* is able to grow mixotrophically but this phenomenon was observed for *Nannochloropsis salina* by Sforza et al. (2012) and for *Nannochloropsis* sp. by Cheirsilp and Torpee (2012). Whether mixotrophic growth is indeed possible for *N. limnetica*, should be investigated in future experiments.

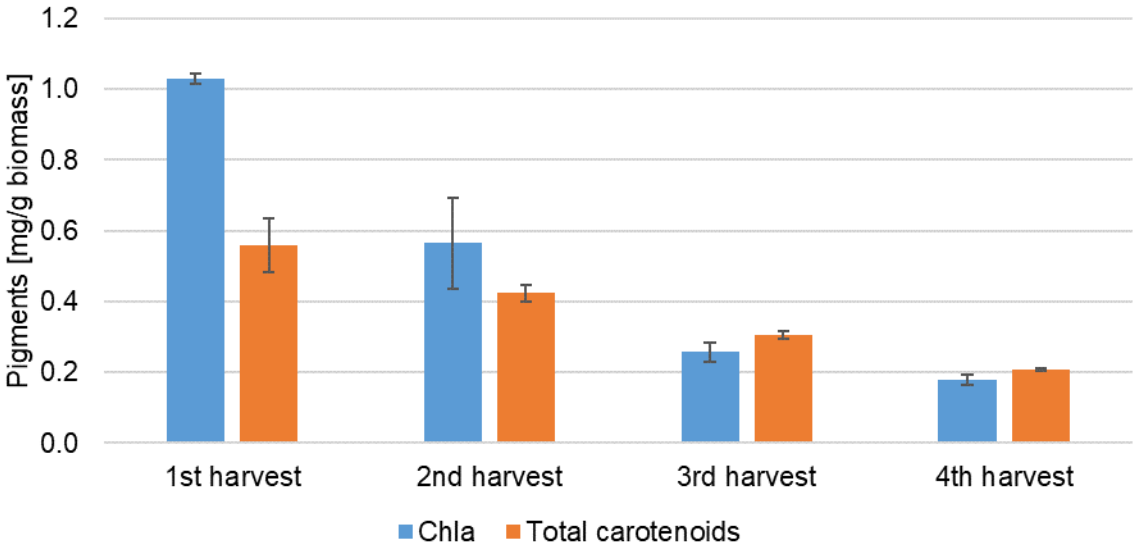

Figure 34: Pigment composition of biomass after each harvest.

Interestingly, the chlorophyll$_a$:carotenoids ratio changes after the third harvest. Whereas in the samples of the first two harvests the chlorophyll$_a$ content is higher than the carotenoids content, in the samples of the third and fourth harvest the carotenoid content is higher than the chlorophyll$_a$ content. It was observed that the colour of the culture suspension turned from green to yellowish towards the end of the cultivation. This effect can be explained by the described change in pigment composition. Carotenoids harvest light energy and transfer it to chlorophyll$_a$ but, more interestingly, they protect the cells from e.g. high irradiance (Masojídek et al., 2013). Some carotenoids, especially xantophylls, are produced in elevated concentrations when the organism is grown under unfavourable conditions such as high irradiance, salinity, and low nutrient availability (Borowitzka, 2016; Masojídek et al., 2013). In this cultivation, light intensity and spectra were constant and cannot be responsible for the shift in pigment composition. Moreover, the content of organic matter in the process water rises significantly after the third harvest, which correlates with the observed change in pigment composition. Therefore, it is possible that the high content of organic matter leads to unfavourable environmental conditions that cause the increased production of carotenoids.

4.4.3 Process water analyses

Organic matter

The concentration of non-purgeable organic carbon (NPOC) in the process water, which was measured after each harvest, rises with each recirculation (Figure 35). This indicates the accumulation of organic matter in the process water. Organic matter can consist of proteins, lipids, organic acids, phytohormones, and allelopathic chemicals released by the cells (Liu et al., 2016). The observed rise in organic matter is consistent with the results of Zhang et al. (2016). They found an increase of total organic carbon after each cycle when cultivating *N. oceanica* in recirculated medium. Moreover, Depraetere et al. (2015) found that process water contains high amounts of large polysaccharides (> 3,000 Da) after the cultivation of *Arthrospira platensis* (cyanobacterium).

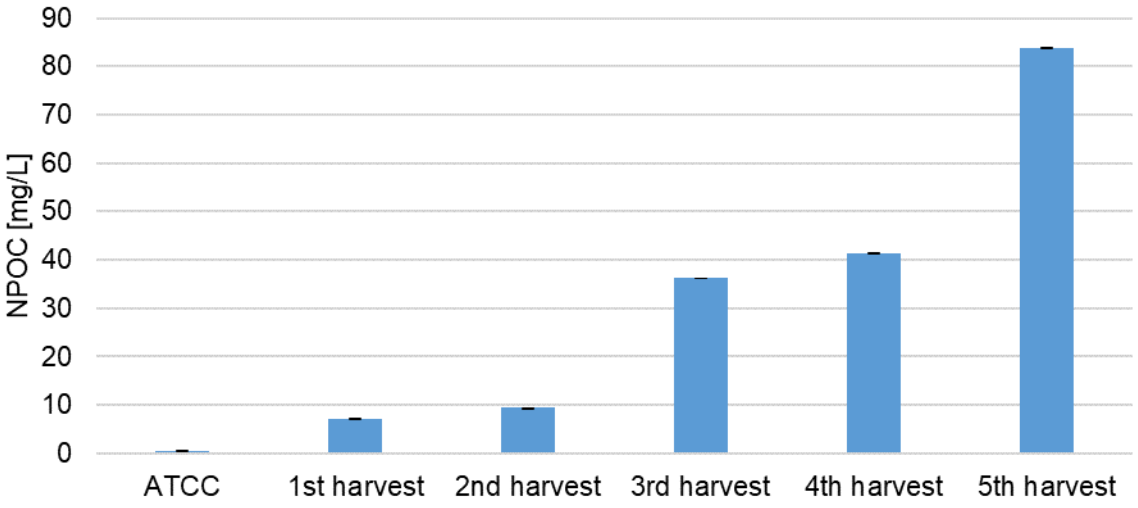

Figure 35: NPOC content of process water after each harvest.

Extracellular polymeric substances protect cells from unfavourable stress such as fluctuations of pH-values, temperature, and nutrient concentrations (Schnurr and Allen, 2015). Thus, the increasing concentration of organic matter might be an indicator for the cells dealing with environmental stress.

Especially exopolysaccharides are responsible for cell to cell interactions, adhesion and biofilm formation (Liu et al., 2016). They are known to have antibacterial properties and might potentially be used in pharmaceutical applications (Liu et al., 2016). As they are excreted to the medium through active secretion or through cell lysis (Schnurr and Allen, 2015), they can easily be recovered (Liu et al., 2016). Some of these extracellular substances (proteins, lipids, organic acids, phytohormones, and allelopathic chemicals) might inhibit the growth of microalgae after reaching a certain threshold; therefore, it could be necessary to remove them before recirculating the process water (Morocho-Jácome et al., 2016). Zhang et al. (2016) removed the total organic

carbon < 50 kDa and showed that growth inhibition was significantly reduced when using this treated process water. It is not clear, though, which of the organic substances might be responsible for growth inhibiting effects. When cultivating *Arthrospira platensis* on medium with added excreted polysaccharides, no significant decrease in growth compared to fresh medium without polysaccharides was observed (Depraetere et al., 2015). This suggests that polysaccharides do not inhibit growth, at least of *Arthrospira platensis*. Nonetheless, another potential problem is that exopolysaccharides increase the viscosity of the culture suspension and increase difficulties at harvesting (Depraetere et al., 2015). Furthermore, previous studies showed that fatty acids can inhibit growth of cyanobacteria and green algae (Nakai et al., 2005; Song et al., 2017).

Finally, it can be stated that the increasing NPOC concentration in this experiment could have influenced growth performance and biomass composition of *N. limnetica*. No reduced growth rate was detected, but possibly, growth could even be enhanced when organic matter is removed before recirculation. To confirm the hypothesis that NPOC inhibits growth and to evaluate harvesting efficiencies, NPOC removal experiments should be carried out. Additionally, the NPOC should be characterised to find out which organic substances are present in the process water.

Nutrient analysis

Samples of fresh ATCC medium and samples of process water after each harvest were analysed using IC and ICP analysis. Nonetheless, during IC analysis, complications in the form of measuring errors with unknown origin occurred, even after repeated analysis of different batches of the samples. Therefore, only the first (ATCC medium) and last (process water after fourth harvest) sample were evaluated. The results of the IC analysis are shown in Figure 36. ICP analysis of all samples was evaluated.

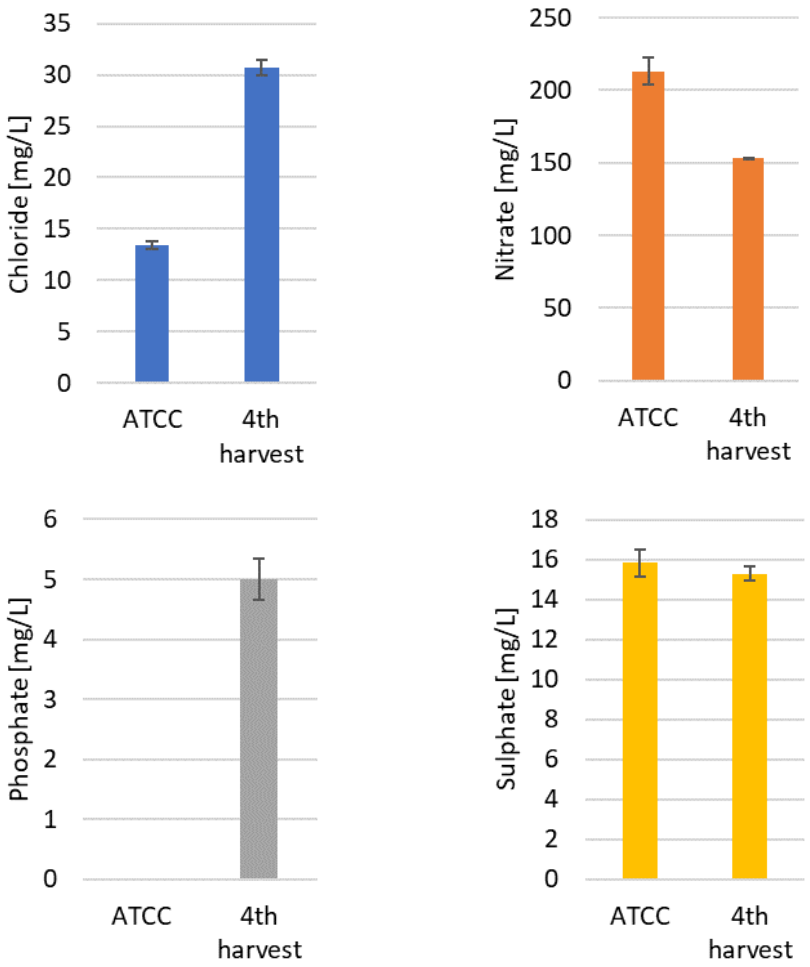

Figure 36: Concentration of chloride, nitrate, phosphate and sulphate in ATCC medium and in process water after the fourth harvest. Results obtained by IC analysis.

The chloride concentration doubles during the cultivation as it increases from 13 mg/L in fresh medium up to 30 mg/L after the fourth harvest. This means that chloride is present in excess, which may lead to an inhibition of growth as *N. limnetica* is a freshwater strain. Chloride is added to the culture medium in the form of $FeCl_3$ and $CaCl_2$. As ICP analysis shows, calcium accumulates during the first three cycles but its amount decreases during the fourth cycle. Thus, it can be assumed that the addition of calcium can be reduced and a lower amount of $CaCl_2$ can be added after recirculation in order to regulate the amount of both elements in the culture suspension. On the contrary, the amount of iron decreases after each cycle, from 0.8 mg/L in the initial medium to below the detection limit of 0.1 mg/L after the last harvest. As already mentioned, iron is an important media compound, especially for photosynthetic organisms (Borowitzka, 2016). Therefore, the amount of $FeCl_3$ added to the process water after recirculation should be increased.

The amount of nitrate decreases from 213 mg/L to ca. 153 mg/L after the fourth harvest. Thus, nitrate is consumed by the algae strain. Concerning the macro elements, nitrate is provided in the form of $NaNO_3$. The amount of sodium significantly increases over the cultivation. In the initial

medium 88.0 mg/L of sodium are present whereas after the third and fourth harvest, more than 200 mg/L accumulate. Especially in combination with the above-mentioned rise of chloride, this can inhibit the growth of *N. limnetica* as it is a freshwater strain. As nitrate concentration decreases while sodium concentration increases, it can be assumed that $NaNO_3$ dissociates and the nitrogen available from NO_3^- is consumed by the algae while Na^+ remains in solution. The nitrogen concentration in the natural environment of *N. limnetica* (4.6 mg/L) is more than 10 times lower than in ATCC medium (51 mg/L). Therefore, it may be possible to reduce the amount of $NaNO_3$. Nevertheless, this could possibly lead to a lower growth rate as the organism metabolised most of the available nitrogen. Sodium is additionally provided as Na_2CO_3 and Na_2SiO_3. It should be investigated if the addition of these compounds can be reduced in order to regulate the accumulation of sodium.

In most of the samples, the amounts of phosphorus (ICP analysis) and phosphate (IC analysis) are below the detection limits of 0.2 mg/L and 3.9 mg/L, respectively. For this reason, it is not possible to reliably evaluate the development of phosphorus or phosphate concentration. Nevertheless, it can be assumed that it does not significantly influence the performance of the cultivation because the amounts present are low. This reflects natural conditions, as in natural environments phosphorus is often a limiting nutrient (Markou et al., 2014). Presumably, all phosphorus available is consumed; it is provided as K_2HPO_4. The amount of potassium changes inconsistently throughout the cultivation (10 mg/L in ATCC medium, ca. 5 mg/L after the first and second harvest, 15.5 mg/L after the third and 11.9 mg/L after the fourth harvest). The consumption of potassium might depend on environmental conditions. Thus, the potassium and phosphate content of the process water should be determined after the harvest and K_2HPO_4 should be added accordingly.

The amount of sulphate present in ATCC and process water after the fourth harvest is similar (15 – 16 mg/L). It is added to the medium in various forms in combination with trace elements, but predominantly as $MgSO_4$. ICP analysis shows that the trend of the amount of magnesium and sulphur present in the process water is similar: The amount of both elements increases up to the third harvest and after the fourth harvest, the amount is decreased again, which fits the result of the IC analysis mentioned above. This suggests that magnesium and sulphur are not consumed in the amount they are provided. Magnesium is an important part of chlorophyll$_a$ (Masojídek et al., 2013) and therefore necessary for photosynthesis. Thus, a shortage of magnesium would be detrimental. Likewise, sulphur limitation would influence growth negatively (e.g. reduce photosynthesis) (Giordano and Prioretti, 2016). As both elements accumulate over three cycles, it can be assumed that the amount of $MgSO_4$ added after recirculation can be reduced. Nevertheless, care should be taken and the accumulation monitored to prevent shortage of either of the two compounds.

It can be said that, with the exception of aluminium, the amount of trace elements is stable over the cultivation and their addition of 1 mL/L after recirculation is suitable. The amount of

aluminium decreases significantly with each cycle. Therefore, it can be concluded that this element is not provided in the right quantity. Nevertheless, an increase in the addition of trace element solution might lead to an imbalance of other trace elements. A more suitable measure would be the independent addition of another aluminium source.

One should always bear in mind that not only the amount of single nutrients affects algal growth but also the ratio between them; especially the C:N:P ratio is crucial (Markou et al., 2014). This should be considered when adapting the addition of nutrients.

5 Conclusions and outlook

The aim of this thesis was to tackle the problem of high water and nutrient amounts needed for algae cultivation by firstly optimising nutrient composition for the cultivation of *Nannochloropsis limnetica* and, secondly, by investigating the effects of recirculating the spent growth medium that contains, among other substances, the residual nutrients. In the course of the recirculation experiment, two harvesting methods, namely cross-flow ultrafiltration and centrifugation, were compared. The herein conducted experiments showed that there are two different ecotypes of *N. limnetica*, whereby the strain provided by CCALA is easier to handle since less autoflocculation occurs. Among the tested media, ATCC was selected since it showed the best results with regard to growth and since it is simple to prepare, and both are important issues in algae cultivation. The recirculation of process water, which was conducted in a flat plate photobioreactor, hardly affects the growth and lipid content of *N. limnetica*, but the ratio of chlorophyll$_a$ to carotenoids. Additionally, chloride, sodium, calcium, and potassium as well as organic matter accumulated in the process water. Concerning the harvesting method, it was shown that cross-flow ultrafiltration is not suitable whereas centrifugation is effective. At the current state of knowledge, it can be concluded that process water could be recycled up to four times without affecting growth and lipid content and therewith the application possibilities of *N. limnetica* most likely not even at larger scales.

Nevertheless, the accumulation of nutrients and of organic matter could have, possibly negative, effects on growth, biomass composition as well as harvest and finally the efficiency of the whole cultivation process. In case of nutrients (chloride, sodium, calcium, potassium), corrosion due to elevated salt concentrations and membrane fouling as well as reduced separation efficiency during harvest may occur. The harvesting process can also be influenced by the increasing organic matter, which increases the viscosity of the process water. Especially at industrial scale, problems in cultivation and harvest are closely connected with expenses in time and cost and need to be minimized for example by a precise nutrient-monitoring of the process water and addition of scarce nutrients only.

Based on the conducted experiments (four recirculation cycles), it should be possible to reduce the consumption of fresh water and the production of waste water by approximately 65 % and 70 %, respectively. In case of a large-scale cultivation facility with 1000 L cultivation medium per year, savings of 650 L fresh water could be obtained. However, a more precise statement requires additional information concerning the characteristics and composition of the organic matter and the effect of the compounds on growth and biomass composition of *N. limnetica*, and their effect on contaminations. A further interesting question in this context would be if *N. limnetica* could use the accumulating organic matter as an alternative energy source for mixotrophic growth. Before implementing process water treatment in an algae production process, it would be necessary to estimate costs and benefits of process water recycling and

treatment, but for this purpose and to gain reliable data, experiments at larger scales would be necessary.

Finally, it can be said that several indicators for cellular stress were identified in spite of a stable growth rate. Further experiments should be conducted to optimise the cultivation. Possibly, medium recycling can even enhance the yield of the cultivation if the identified stress factors are removed. Moreover, nutrient addition should be optimised and the effect of organic matter removal on growth rate, harvesting efficiency, and pigment production should be analysed. Nevertheless, it was shown that the recirculation of process water for at least four cycles does neither inhibit growth nor reduce the lipid content of the biomass. The water demand can be reduced significantly by implementing water recirculation into an algae cultivation process without entailing loss in quality.

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