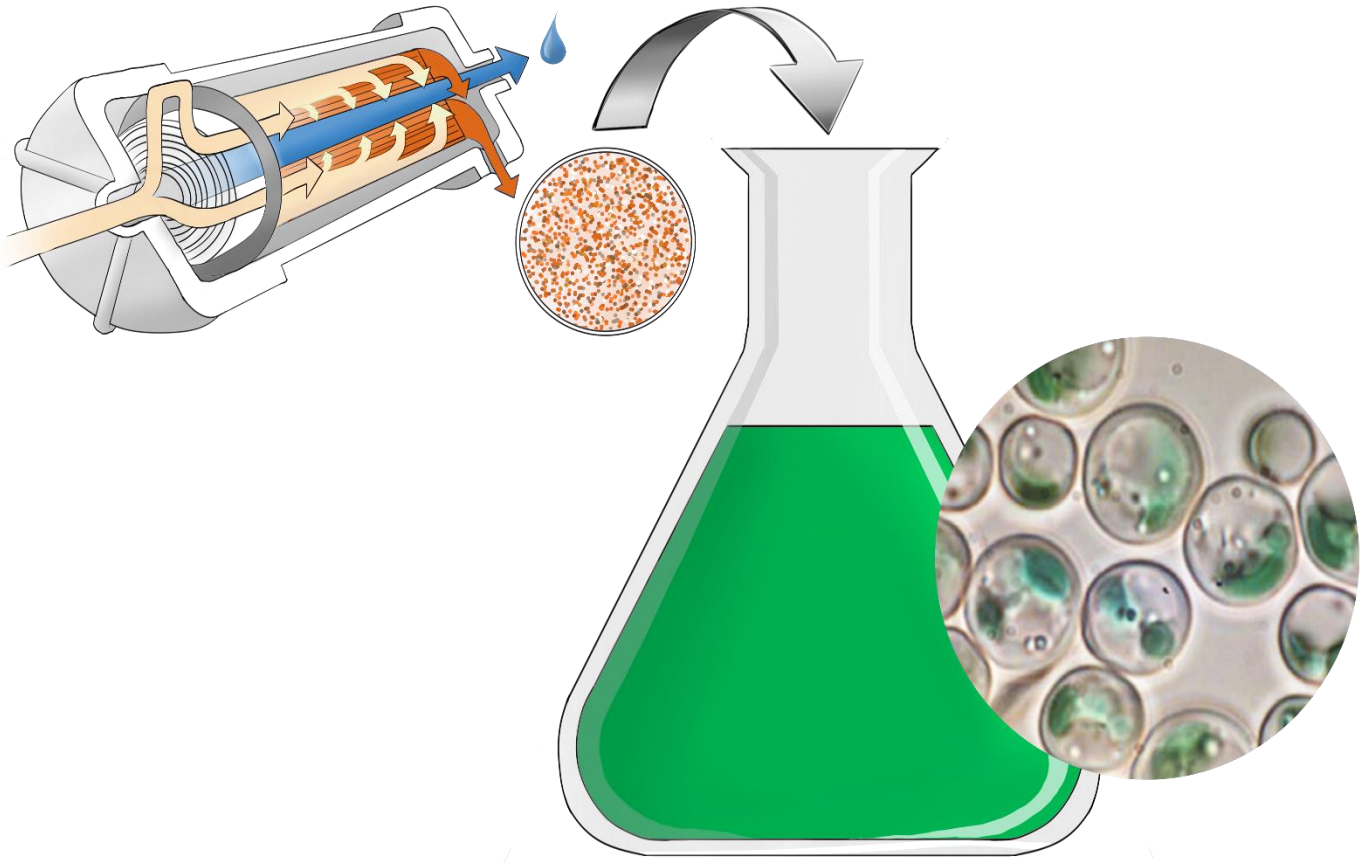
MICROALGAL CULTIVATION ON RECOVERED NUTRIENTS

Cultivation of the extremophilic microalgae Galdieria sulphuraria on reverse osmosis concentrate from water and resource recovery pilot plant of New Energy and REsources from Urban Sanitation (NEREUS)



Microalgal cultivation on recovered nutrients

Cultivation of the extremophilic microalgae Galdieria sulphuraria on reverse osmosis concentrate from water and resource recovery pilot plant of New Energy and REsources from Urban Sanitation (NEREUS)

By

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This thesis was performed jointly at:

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This thesis cannot be made public until new notice by the TU Delft (David Weissbrodt) and company (Paula van den Brink) main supervisors.

Preface

This thesis distills the knowledge gained through a period of 8 months in the microalgal cultivation on recovered nutrients. This thesis is the final part of my MSc. degree for the faculty of Civil Engineering & Geosciences under the department of Water Management at Delft University of Technology. This thesis is of great interest for people who are involved in the field of microalgae and biotechnology. The research described in this thesis was carried out jointly at: 1) Weissbrodt Lab for Environmental Life Science Engineering, Environmental Biotechnology Section, Department of Biotechnology, Faculty of Applied Sciences 2) NEREUS, Evides Industriewater, Delft Blue Innovations research facility at Delfluent – Harnaschpolder wastewater treatment plant.

Introductory information is given in Chapter 1, with special focus on the microalgae *G. sulphuraria*. Chapter 2 provides the background information. The materials, experimental set-up and procedures for the different experiments are given in Chapter 3. Chapter 4 describes the results of the microalgal cultivation for all the different experimental factors investigated. Chapter 5 contains the conclusions and recommendations.

This thesis has been successful due to all the people who spent their time, effort and willingness to guide and help me in understanding the topic of the project. Firstly, I would like to thank my main TU Delft supervisor Dr. Ir. David Weissbrodt and main company supervisor Paula van den Brink from Evides Industriewater for their expertise and support throughout the thesis. In addition, they also helped me with the supply of important data, designing of the lab experiments, gave feedbacks for improvement of the outcomes of the experiments and gave advice on the structuring of this report. Secondly, I would like to thank my other university supervisors Dr. Ir. Ralph Lindeboom and Prof. Dr. Ir. Merle de Kreuk for their general guidance, help with discussion of results, help with judgement of the thesis and willingness to take their time to answer my questions. Moreover, I would like to express my appreciation for the supervision of my other company supervisor Otto Schepers from Evides Industriewater for his help and support throughout the thesis. Furthermore, I would like to extend my thanks to the people in the Environmental Biotechnology Section who made it a great time during my thesis work and helped me throughout the thesis. Finally, I would like to thank my family and friends for their unwavering support. They were always there by my side to encourage and help me as much as they could during the thesis. The insightful talks with my family members and and friends have added in lifting my spirit and motivation many times.

This thesis gave me a very broad insight in the field of microalgae and biotechnology and I would definitely like to continue to work in this field in the coming future. Lastly, I am convinced that this report can be of great use for readers and for future research about the same or other related topics.

Abstract

Galdieria sulphuraria (*G. sulphuraria*) is a eukaryotic, extremophilic, spherical and unicellular species of red algae. *G. sulphuraria* can grow at very low pH-values (pH 0.05 – 5.0) and high temperatures (35 – 56 °C). The growth conditions of *G. sulphuraria* make it suitable for axenic cultivation because the low pH and high temperature minimalize the risk of microbial contamination. Next to its ability to remove nutrients in wastewater treatment, *G. sulphuraria* is a prospective producer of a valuable product, Phycocyanin (PC), a thermostable blue pigment-protein complex, which is used as, among others food additive and food colorant.

These characteristics of *G. sulphuraria* lead its selection by Evides Industriewater for the uptake of the ammonium present in the reverse osmosis (RO) concentrate of New Energy and REsources from Urban Sanitation (NEREUS). NEREUS focuses on the re-use of nutrients present in wastewater, among others ammonium. One of the goals of NEREUS is to re-use the ammonium present in the RO concentrate with the use of algae. In order to recover ammonium from the RO concentrate of NEREUS, it is necessary to test whether *G. sulphuraria* is capable of growing on such medium. The possibility of cultivating of *G. sulphuraria* on the RO concentrate from water and resource recovery pilot plant of NEREUS was investigated in this thesis.

The objectives of this thesis were to find the optimal growing conditions and assess the biomass growth and nutrients consumption. Screening experiments with synthetic Allen medium, which is usually used for the cultivation of the *G. sulphuraria,* were conducted to obtain the best growing conditions of *G. sulphuraria*. In order to understand the best growing conditions for the cultivation of *G. sulphuraria*, the effects of several factors were investigated, which are: 1) different metabolism, 2) different nitrogen sources and concentrations (ammonium: 100 – 1000 $mgNH_4^+$-N/L and nitrate: 247 $mgNO_3^-$-N/L), 3) different carbon sources (glucose, bicarbonate and CO_2) and different glucose concentrations (C:N = 5:1 and 10:1), 4) different phosphate concentrations (N:P = 37:1 and 7.2:1), 5) culture densities. Ammonium with mixotrophic metabolism turned out to be the best nitrogen source. Biomass concentration on ammonium was four times higher than on nitrate. Increasing the ammonium concentration from 200 $mgNH_4^+$-N/L to 1000 $mgNH_4^+$-N/L resulted in around 25% more biomass and no firm conclusions could be drawn from the experiment performed with different phosphate concentrations. No significant increase in the growth of *G. sulphuraria* was observed between Carbon:Nitrogen (C:N) ratio = 5:1 and 10:1. Furthermore, culture densities higher than 0.7 g/L of biomass resulted to a slower growth of *G. sulphuraria*.

Experiment with synthetic RO concentrate shows that there was light limitation involved during the cultivation. Highest and fastest growth (μ_{max} = 0.78 day^{-1}) was observed in the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium culture. Growth inhibition was observed in cultures containing RO concentrate of NEREUS. Still, *G. sulphuraria* did grow on RO concentrate of NEREUS. This work is contributing to the scientific and engineering community in the field of microalgae.

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Chapter 1 Introduction

1.1 NEREUS project

The New Energy and REsources from Urban Sanitation (i.e. NEREUS) project is part of Evides Industriewater (i.e. EIW). EIW is one of the leading water partner companies for instance of in the industry sector in the Netherlands. EIW provides a wide variety of products and services, such as: process water, wastewater treatment, water re-use, water services and water supply. Moreover, EIW also conducts research and looks at sustainable and innovative technological concepts in order to contribute to a better environment (Evides, 2016). The NEREUS project is one of the sustainable and innovative technological projects of EIW.

The NEREUS project aims to enhance the development of the circular, sustainable economy and the alteration of wastewater into valuable resources such as water**,** nutrients (e.g. cellulose, phosphate, ammonium) and energy that could be re-used in the Interreg 2Seas area (NEREUS Project, 2018a). The Interreg 2 Seas area covers the coastal regions along the Southern North Sea and the Channel area as shown in Figure 1 (Interreg 2 Seas area, n.d.).

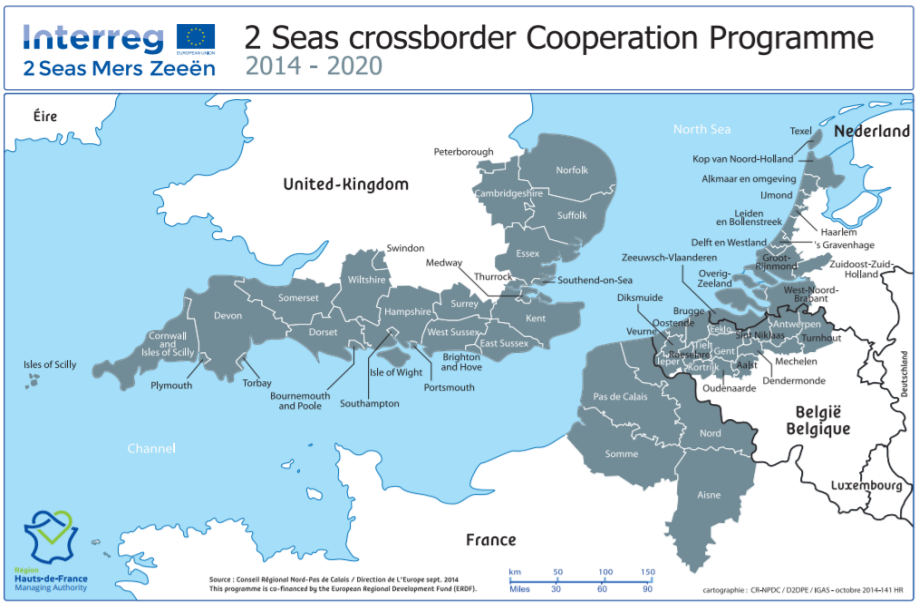

Figure 1: Interreg 2 Seas area (Interreg 2 Seas area, n.d.)

The pilot plant setup of the NEREUS project is located in the Delft Blue Innovations research facility at Delfluent – Harnaschpolder (HNP) wastewater treatment plant (WWTP). An overview of the different processes within the pilot plant of the NEREUS project is shown in Figure 2.

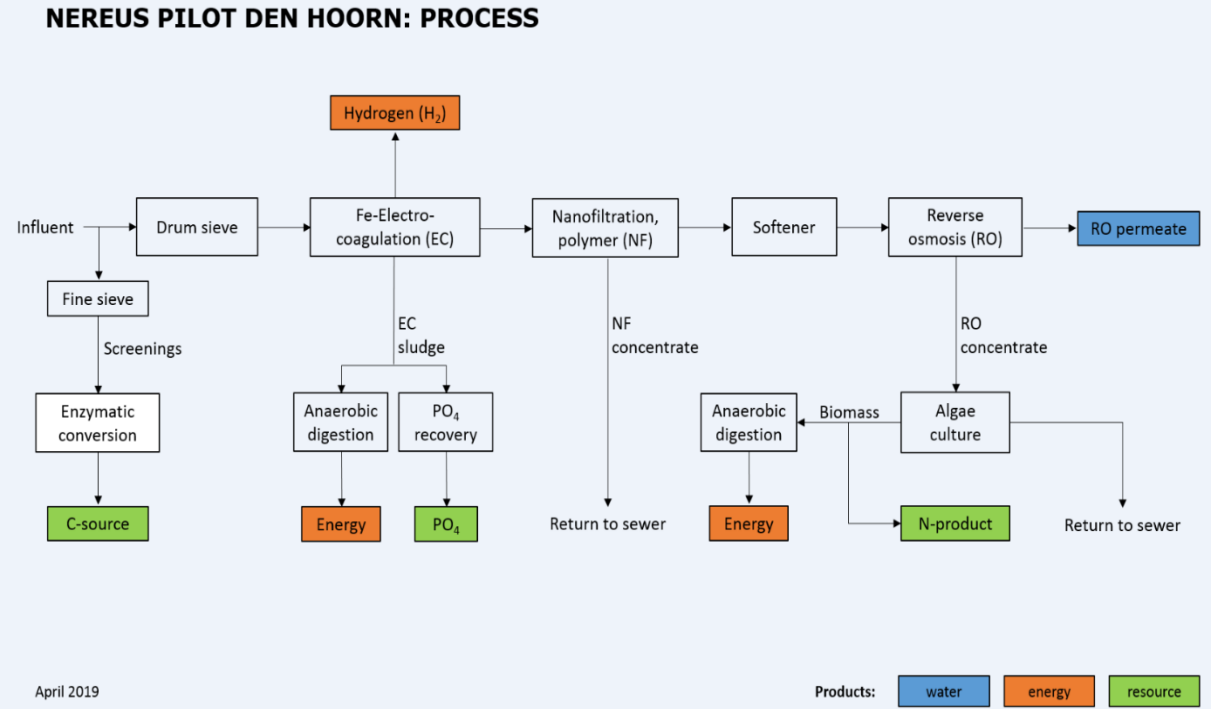

Figure 2: Process scheme of the different process units within the NEREUS project (NEREUS Project, 2018c)

NEREUS aims at the re-use of wastewater because of the increase in water scarcity all around the world**.** There are four main processing water treatment units which are: drum sieve, electrocoagulation, nanofiltration and reverse osmosis (Figure 2). Cellulose is the first nutrient to be recovered from the screenings. Most of the phosphates will be recovered by the electrocoagulation and will go to the electrocoagulation sludge. Phosphates are finite nutrients, crucial for agricultural purposes and are currently not recovered from wastewater. The NEREUS project is working on the recovery of these phosphates so that these phosphates can be re-used in a better manner and thus preventing the need for more phosphate to be mined.

Moreover, the other nutrient that the NEREUS project is focusing on is ammonium. Another goal of NEREUS is to re-use the ammonium present in the RO concentrate with the use of algae. The ammonium will be used as a source of nitrogen for the growth of the algae culture (NEREUS Project, 2018a). Wastewater also contains energy that could be used as a sustainable source of energy in order to reduce CO_2 emissions, which is also an aim of the NEREUS project (NEREUS Project, 2018a).

1.2 General Overview of *Galdieria sulphuraria* and its application

Galdieria sulphuraria (*G. sulphuraria*) belongs to the microalgae group. Microalgae are a diverse group of photosynthetic unicellular organisms, living in freshwater or saline environments, which make use of sunlight, carbon dioxide and water to make their own food. Microalgae have high potential as a source of pigments, antioxidants and biofuels for industrial operations (Milledge, 2012). *G. sulphuraria* is a eukaryotic, extremophilic, spherical and

unicellular species of red algae (Algenuity, 2016) . It has biomass formula: $C_{106}H_{263}O_{110}N_{16}P$ (Redfield et al., 1963) and belongs to the Cyanidiales group, a group of single-cell organisms of identical-looking characteristics (Sloth et al., 2006). Figure 3 shows a sketch of the ultrastructural characteristics of *G. sulphuraria* (Merola et al., 1981). The *G. sulphuraria* cells are about 25 µm in diameter and are green since *G. sulphuraria* contains low levels of phycoerythrin, a red protein-pigment complex which is present in red algae. Phycoerythrin is a light-harvesting phycobiliprotein, supplementary to the main chlorophyll pigments responsible for photosynthesis (Madigan, 2012).

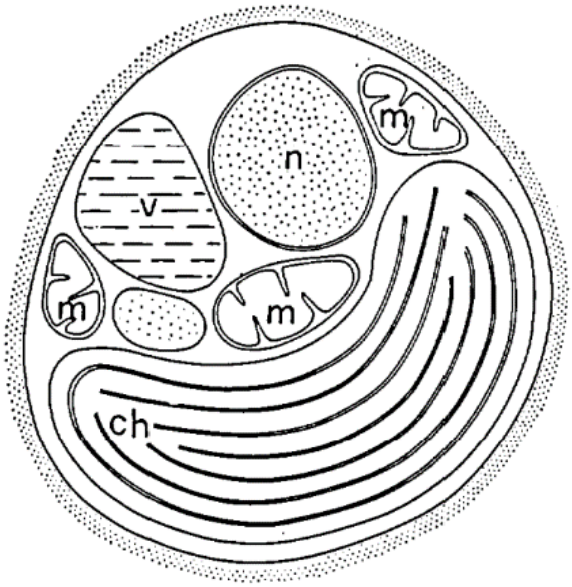

Figure 3: Sketch of the ultrastructural characteristics of *G. sulphuraria* (Merola et al., 1981), where: ch = chloroplast, m = mitochondria, n = nucleus and v = vacuole.

G. sulphuraria can grow at very low pH-value (pH 0.05 – 5.0) and high temperatures (35 – 56 °C). It is mostly found in sulfuric acid hot springs (Reed & Bhattacharya, 2009). It can also be found in toxic metal-rich environments containing toxic metals such as arsenic, aluminum, cadmium, mercury (Schonknecht et al., 2013). Besides, *G. sulphuraria* is also highly tolerant of high salt concentrations (Schonknecht et al., 2013). The growth conditions of *G. sulphuraria* make it suitable for axenic cultivation because the low pH and high temperature minimalize the risk of microbial contamination (Hirooka & Miyagishima, 2016).

Moreover, *G. sulphuraria*, is known for its exceptional metabolic flexibility, growing autotrophically, heterotrophically and as well mixotrophically (Barbier et al., 2005; Gross & Schnarrenberger, 1995). *G. sulphuraria* can grow with inorganic carbon sources (e.g. carbon dioxide) as well as organic carbon sources (e.g. glucose and glycerol). Autotrophic growth regime means growing with carbon dioxide as source of carbon and light as energy source while heterotrophic growth regime means growing with organic carbon and no light. Mixotrophic growth regime is when organic carbon, carbon dioxide and light are simultaneously supplied to

the micro-organisms. Light intensities 80 – 100 µmol photons $m^{-2}s^{-1}$ for cultures using light as source of energy are required for optimum biomass growth and pigment accumulation (Sloth et al., 2006). *G. sulphuraria* can grow on more than 50 different carbon sources for heterotrophic and mixotrophic growing cultures (Barbier et al., 2005). However, the growth of the latter can be inhibited when glucose concentration is above 200 g/L (B1umbak et al., 2011). Figure 4 shows light microscopic images of *G. sulphuraria* cells grown under illumination in the absence of glucose (autotrophically) and in darkness in the presence of glucose (heterotrophically) (Schonknecht et al., 2013).

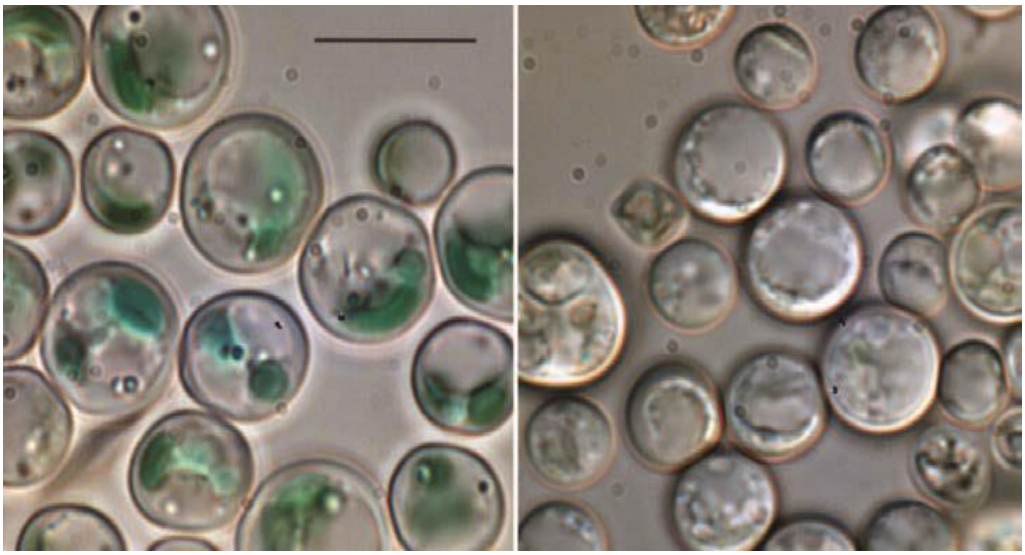

Figure 4: Light microscopic images of *G. sulphuraria* cells grown autotrophically (left) and heterotrophically (right) (Schonknecht et al., 2013).

Nitrogen (N), phosphorus (P) and carbon (C) are the key nutrients for the growth of *G. sulphuraria* and all living organisms in general (Tuantet, 2015). RO concentrate from NEREUS water and resource recovery pilot plant contains ammonium (NH_4^+) as source of nitrogen. Moreover, nitrogen in wastewater is mostly present in the form of nitrate (NO_3^-) and ammonium while phosphorus in wastewater is usually present in the form of phosphate (PO_4^{3-}) (BAHR et al., 2008). Having the ability to consume nutrients such as N, P and C during its metabolism, *G. sulphuraria* can be implemented in water treatment processes. For instance, single-step removal of nutrients by mixotrophic metabolism of *G. Sulphuraria* has earlier been researched (Henkanatte-Gedera et al., 2015). *G. sulphuraria* were cultivated on urban wastewater with ammonium concentration of 40 mg/L NH_4^+–N and phosphate concentration of 10 mg/L PO_4^{3-} (Selvaratnam et al., 2014). In addition, In addition, not all strains of *G. sulphuraria* can grow on both NO_3^- and NH_4^+ (Gross & Oesterhelt, 1999). Some do not grow on medium containing NH_4^+ as source of nitrogen (Hirooka & Miyagishima, 2016) and many strains of *G. sulphuraria* grow on nitrate as source of nitrogen source (Gross, 1999). Moreover, microalgae also require very small amounts of iron (Fe) and micronutrients such as copper (Cu), manganese (Mn), and zinc (Zn) for rapid growth. These micronutrients and Fe together are referred to as trace elements. (Tuantet et al., 2014)

Next to its ability to remove nutrients in wastewater treatment, *G. sulphuraria* is a prospective producer of valuable products (Sloth et al., 2017). It produces a high-value compound, Phycocyanin (PC), a thermostable blue pigment-protein complex (Graziani et al., 2013). Phycocyanin produced by *G. sulphuraria* is of the type C-phycocyanin (C-PC) (Eriksen, 2008). C-PC is a light-harvesting phycobiliprotein and a vital photosynthetic pigment in *G. sulphuraria* (Albertano et al., 2000; Diner, 1979). The C-PC content in *G. sulphuraria* is affected by the temperature of cultivating medium. Cultivating *G. sulphuraria* at temperatures around 34 °C yield to a higher C-PC cell content than higher temperatures (~42°C) although cell growth is higher at higher temperatures (Sloth et al., 2017).

C-PC is soluble in water and is known for its fluorescent properties (Eriksen, 2008). C-PC is used as a fluorescent dye and probe (Eriksen, 2008). C-PC has also been found to have more various purposes. C-PC is used as food additives and food colorants (Eriksen, 2008; Prasanna et al., 2007). It can also be used as nutraceuticals in health foods and has pharmaceutical potentials due to its anti-oxidative and radical scavenging properties (Benedetti et al., 2004; Bermejo et al., 2008; Bhat & Madyastha, 2000; Eriksen, 2008; Romay et al., 1998; Soni et al., 2008). Moreover, C-PC is used in cosmetics and biotechnology (Eriksen, 2008). Furthermore, many studies suggests that C-PC also has various different anti-inflammatory and anti-carcinogenic potential properties in medicine and diagnostics (González et al., 1999; Liu et al., 2000; Romay et al., 1998; Roy et al., 2007; Subhashini et al., 2004).

G. sulphuraria cultivation is considered in bioremediation projects because of its ability to grow under a large variety of conditions. The bioremediation projects where *G. sulphuraria* was considered were for the recovery of precious metals (gold and palladium) and rare-earth elements (neodymium, dysprosium, copper, lanthanium) (Ju et al., 2016; Minoda et al., 2015). Wastewater treatment project where *G. sulphuraria* was used was for the removal of nitrogen and phosphorus from various wastewater streams (Selvaratnam et al., 2014).

1.3 Knowledge gaps

Studies on *G. sulphuraria* have been recently done mainly because of its ability as a prospective producer of Phycocyanin. However, the cultivation of the extremophilic *G. sulphuraria* from most studies were investigated using Allen medium or wastewater as medium. The RO concentrate from NEREUS is interesting because it contains high ammonium concentration (≈ 200 mg/L NH_4^+ – N) which can be used as nitrogen source for the *G. sulphuraria*. The growth of *G. sulphuraria* on the RO concentrate from the water and resource recovery pilot plant of NEREUS has not been investigated before. However it is necessary to test whether *G. sulphuraria* is capable of growing on such medium.

1.4 Research question

"Can the microalgae G. sulphuraria be cultivated on reverse osmosis concentrate from the water and resource recovery pilot plant of NEREUS?"

1.5 Objectives

The aim of this master thesis was to study the growth of the microalgae *G. sulphuraria* using reverse osmosis (RO) concentrate as medium to cultivate them. The scope of this thesis is to provide EIW with an overview of the best conditions needed to cultivate the microalgae *G. sulphuraria* and how the nutrients present in their wastewater can be optimally taken up and used by *G. sulphuraria* for their biomass growth. The objectives of this thesis are:

1. Determination of the optimal growing conditions and possible limitations in the growth of *G. sulphuraria*.
2. Determination of the effect of ammonium concentration on biomass growth.
3. Assessment of the pigment formation.
4. Assessment of the biomass growth, nutrients consumption and ammonium removal efficiencies with synthetic and real RO concentrate.

1.6 Hypotheses

G. sulphuraria can growth under all the three metabolisms (autotrophic, heterotrophic and mixotrophic). However, each metabolism will result to different growth and growth rates. It can be hypothesized that mixotrophic metabolism will result in higher growth and growth rates because glucose is available in the medium for biomass production and light is available as source of energy. Autotrophic cultures obtain light energy but the glucose is produced by photosynthesis. Heterotrophic cultures do not obtain light energy but glucose is available in the medium for biomass production. Nutrients such as carbon, nitrogen and phosphorus are known to be among the most important nutrients that microalgae need to grow. Nitrogen and phosphorus deficiency result in lower growth and in accumulation of lipids in the cell (van der Hulst, 2012). Moreover, carbon limitation also results in lower growth of *G. sulphuraria*. It can hypothesized that nutrient limitations have an influence on the physiology and growth of *G. sulphuraria*. In addition, light is a very important parameter for growth of microalgae. Higher culture density allows less light to pass through the medium, thus lowering the growth of *G. sulphuraria*. RO concentrate from NEREUS may contain unwanted nutrients or metals because its source is from municipal and domestic wastewater. Therefore, there might be inhibition in the growth of *G. sulphuraria* when grown on RO concentrate from NEREUS.

Chapter 2 Scientific Background information

2.1 Reverse osmosis (RO) concentrate

Reverse osmosis (RO) is a well-known water treatment technology for the production of freshwater (Subramani & Jacangelo, 2014). Global and domestic use of the RO technology to produce pure water has risen extremely in recent years (Greenlee et al., 2009; Peñate & García-Rodríguez, 2012). One of the major drawbacks with RO technology is the concentrate. produced during the process, which is not very environmentally friendly because it contains high salt concentrations and often a lot of nutrients which can cause eutrophication if disposed into surface water discharge (Chislock et al., 2013; Subramani & Jacangelo, 2014). In addition, high salt concentrations discharge into surface water can result into death of aquatic lives. The common disposal possibilities of RO concentrate are as follows: surface water discharge (lakes, rivers, oceans), deep well injection, evaporation ponds and land application (Bergman, 2007; Malmrose et al., 2004). Besides, the cost of disposal is the determining factor for the choice of the disposal option (Ahmed et al., 2001; Arnal et al., 2005; Malaeb & Ayoub, 2011).

Cultivation of microalgae *G. sulphuraria*, is an alternative method for the removal of the nutrients in the RO concentrate, provided the macro-elements such as nitrogen and phosphorus are present for the growth of the *G. sulphuraria.* EIW is experienced with the RO technology and has been applying this technology for quite a long time. RO concentrate of NEREUS is very different from usual RO concentrate. Usual RO concentrate is quite saline and is widely applied on sea and surface water. The source water of the RO concentrate is municipal and household wastewater and the pre-treatment train involves a drum sieving followed by electrocoagulation and nanofiltration and finally is the RO unit. The composition of the RO concentrate of the NEREUS water treatment pilot is shown in Table 1 (EIW, 2019).

Note: The possibility of growing *G. sulphuraria* with urine can also be investigated and some background information about urine as a medium can be found in Appendix A.1

Table 1: composition of the RO concentrate of the NEREUS water treatment pilot

pH	8.25 - 8.5
Turbidity	11.4 - 12.2 FNU
Conductivity	6150 - 6500 µS/cm
Ammonium	150 - 200 mg NH4 -N
Phosphate	0 - 0.1 PO4 -P
Iron	1.24 - 3.88 mg/L

2.2 Allen Medium

Allen medium is the medium which is usually used for the culture of the microalgae *G. sulphuraria* (M. B. Allen, 1959). Allen medium consist of all the necessary nutrients that *G. sulphuraria* requires to grow. Allen medium was first prepared with ammonium as source of nitrogen (M. B. Allen, 1959). However, Allen medium which is available commercially on the market is prepared using nitrate as source of nitrogen (Cyanosite, n.d.; UTEX, 2019). Allen medium consist of 2.3 g/L HEPES buffer, 1.5 g/L $NaNO_3$, 0.0375 g/L K_2HPO_4, 0.0375 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L Na_2CO_3, 0.025 g/L $CaCl_2 \cdot 2H_2O$, 0.058 g/L $Na_2SiO_3.9H_2O$, 0.006 g/L Citric Acid.H_2O, 0.75 mg/L $Na_2EDTA.2H_2O$, 0.097 mg/L $FeCl_3.6H_2O$, 0.041 mg/L $MnCl_2.4H_2O$, 0.005 mg/L $ZnCl_2$, 0.002 mg/L $CoCl_2.6H_2O$ and 0.004 mg/L $Na_2MoO_4 \cdot 2H_2O$ (Cyanosite, n.d.; UTEX, 2019).

2.3 Growing metabolism

There are three types of metabolism, namely: autotrophic (Phototrophic), Mixotrophic and heterotrophic metabolism. Figure 5 below shows a schematic overview about the three metabolisms (Shoener et al., 2019).

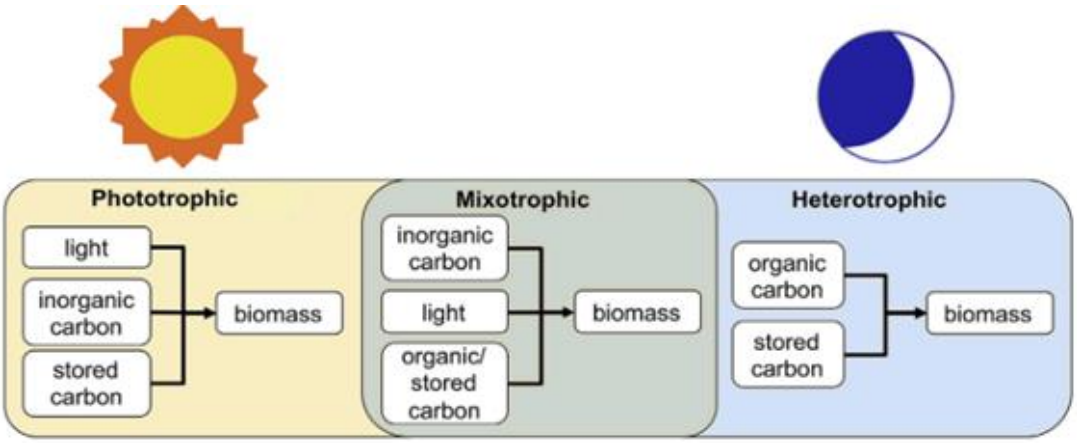

Figure 5: Schematic overview about the energy and carbon sources that are used by microalgae in each of the three metabolisms (Shoener et al., 2019).

Microalgae must have a source of carbon and a source of energy for the synthesis of cell components.

Autotrophic metabolism

Autotrophic growth is the most common way for the cultivation of microalgae. Autotrophs perform photosynthesis to obtain their substrates for biomass production. Photosynthesis consists of two steps: the light dependent step and the light independent step. The light dependent step is where light energy is used to produce the energy-rich compound ATP and O_2 while the light independent step, the Calvin cycle, uses the previously formed ATP from the

light dependent step to convert CO_2 and water into organic compounds (glucose) as shown below (Thangaraj, 2010):

Overall photosynthesis reaction: light energy + $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$

The overall growth reaction of the microalgae under autotrophic metabolism is as shown below (Eze et al., 2018):

Overall growth reaction: $16NH_4^+ + 106H_2O + 106CO_2 + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 14H^+ + 104O_2$

Heterotrophic metabolism

Heterotrophic growth occur in the absence of light. Heterotrophic organisms obtain their source of energy and substrate from organic carbon compounds such as glucose. Oxidation of organic compounds such as glucose to carbon dioxide and water occurs via cellular respiration as is shown below (Alberts, 2009):

Cellular respiration reaction: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ + energy

Oxygen is very essential for heterotrophic organisms because they obtain their source of energy by oxidation of the organic carbon source. Therefore, limitation in oxygen supply will lead to limitations in the growth of heterotrophic organisms.

The overall growth reaction of the microalgae under heterotrophic metabolism is as shown below:

Overall growth reaction: $16NH_4^+ + 17.67C_6H_{12}O_6 + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 14H^+$

Mixotrophic metabolism

Mixotrophs requires both an inorganic and organic source of carbon as well as light to grow. Microalgae grown under mixotrophic regime grow faster than those under heterotrophic or autotrophic regimes because the specific growth rates of microalgae grown under mixotrophic cultures is approximately the sum of the autotrophic growth rate and heterotrophic growth rate (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011).

Since mixotrophs require both an inorganic and organic source of carbon for their growth, the resulting overall growth reaction is more complex.

2.4 Carbon and nitrogen source

Glucose is the most commonly used carbon source for growing of microalgae. Microalgae cultures grown with glucose produces higher growth and respiration rates than with other substrate because glucose possesses more energy content per mol (≈2.8 kJ/mol of energy) compared with other substrates (Boyle & Morgan, 2009; Perez-Garcia et al., 2011). Glucose assimilated are used for cell synthesis, respiration and part of it is also storage (Perez-Garcia et al., 2011).

Ammonium is the most preferred nitrogen source for algae (Perez-Garcia et al., 2011). Ammonium is energetically a more efficient nitrogen source, requiring less energy for its uptake (Goldman, 1977; Grobbelaar, 2007; Shi, Zhang, & Chen, 2000; Syrett & Morris, 1963; Wilhelm et al., 2006). Nitrate can also be used as source of nitrogen for growth of microalgae. When microalgae are grown in medium containing nitrate, nitrate is first transported across the membrane and then reduced to ammonia by enzymes before it can be assimilated (Buchanan, Gruissem, & Jones, 2000). Nitrate is first reduced to nitrite and the latter later being reduced to ammonium. The process of converting the nitrate into ammonium consumes a lot of energy, carbon and protons (Buchanan et al., 2000)

2.5 Limitations in growth of microalgae

Algae requires optimum conditions for them to be able to grow optimally. The most important variables which can cause limitations in the growth of microalgae are:

- pH
- Temperature
- Light intensity
- Carbon dioxide, oxygen and necessary nutrients in the medium
- Culture density

2.6 Phycocyanin (PC)

Phycocyanin (PC) is a member of the group of light-harvesting proteins, namely: phycobiliproteins. Phycobiliproteins are among the most abundant proteins in many cyanobacteria, algae and microalgae. Phycobiliproteins are not vital for the functioning of the cells, instead they are also selectively used up when cells are limited to nitrogen and therefore are phycobiliproteins also intracellular nitrogen storage compounds (Allen & Smith, 1969; Boussiba & Richmond, 1980; Lewitus & Caron, 1990; Sloth et al., 2006; Yamanaka & Glazer, 1980).

The three common phycobiliproteins are phycoerythrin (PE), allophycocyanin (APC) and PC. PE contains phycoerythrobilin chromophores while PC and APC contain phycocyanobilin chromophores. Pre-fixes were used to rename phycobiliproteins in order to characterize them according to their phycobilin content and phycocyanins with only phycocyanobilin chromophores were named C-phycocyanin (C-PC) (MacColl, 1998). C-PC constitute of two polypeptide subunits, the α- and β-subunit polypeptides respectively (Stec et al., 1999). Figure 6 shows the ribbon representation of the α- and β-subunit polypeptides of C-PC (Padyana et al.,

2001). Moreover, the two subunits form αβ monomers which is the functional unit of C-PC (Eriksen, 2008).

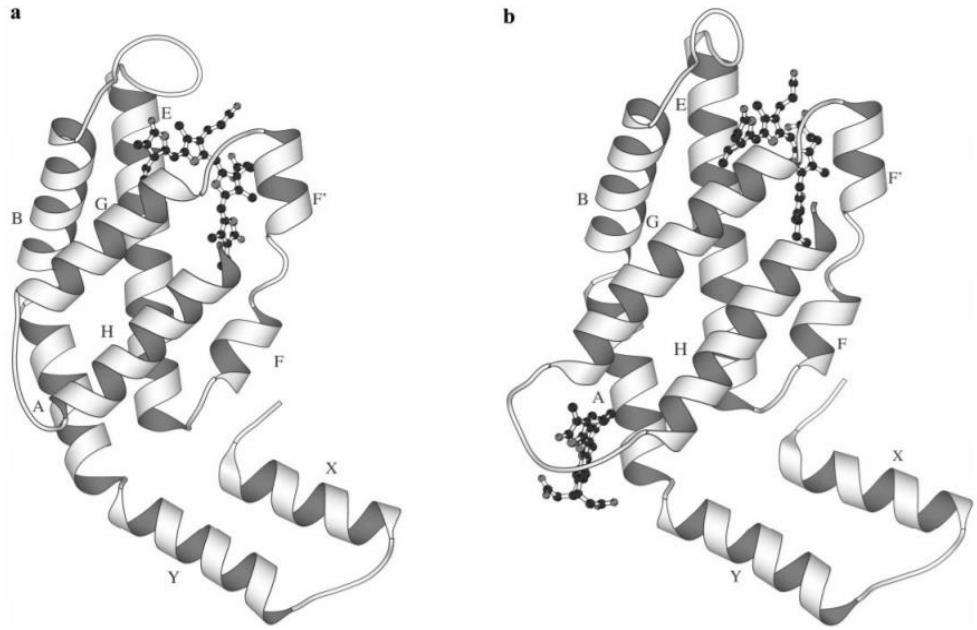

Figure 6: Ribbon representation of (a) α-subunit and (b) β-subunit of C-PC, (Padyana et al., 2001).

The concentration of dissolved C-PC can be determined by the spectrophotometry method as shown in the formula below (Moon et al., 2014):

$$\text{C-}\mathbf{Phycocyanin\ (mg/mL)} = \frac{\mathbf{OD_{620} - (0.474 * OD_{652})}}{\mathbf{5.34}}$$

The purity of C-PC is very important because the purity needed for a certain product varies from application to application. Purity of isolated C-PC is expressed by a purity number which is defined as the optical density (OD) ratio of the 620 nm and 280 nm wavelengths (OD_{620}/OD_{280}).

$$\mathbf{Purity\ no.} = \frac{\mathbf{OD_{620}}}{\mathbf{OD_{280}}}$$

Optical density measurement at 280 nm wavelength represents the abundance of all proteins present in solution. (Mishra & Shrivastav, 2008; Parmar et al.,2011) Optical density measurement at 620 nm wavelength represents the wavelength where C-PC has a maximum absorbance. However, 618 nm and even 615 nm wavelength are also very commonly used in literature as the wavelength where C-PC has a maximum absorbance.

Chapter 3 Experimental Methods

3.1 Materials

Microalgae strain culture

The red microalgae *G. sulphuraria* 107.79 was initially obtained from the University of Göttingen.

Chemicals

Ethanol (96 %) was purchased from Boomlab. Sulfuric acid (H_2SO_4), ammonium sulfate ($(NH_4)_2SO_4$) ($\geq$ 99.0%), dipotassium phosphate (K_2HPO_4), D-(+)-Glucose ($C_6H_{12}O_6$) ($\geq$ 99.5%), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), zinc chloride ($ZnCl_2$) and copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) were used in the experiments and were purchased from Sigma-Adrich.

Media

Three different media were used for the experiments and these were:

- Allen medium was purchased from UTEX Culture Collection of Algae of University of Texas and was only used for the starter cultures of *G. sulphuraria* 107.79. The concentrations of the nutrients in Allen medium can be seen in section 2.2.
- Synthetic RO medium was prepared by adding macro-nutrients: $(NH_4)_2SO_4$, K_2HPO_4 and $C_6H_{12}O_6$ and micro-nutrients with concentrations as follows: 0.041 mg/L $MnCl_2.4H_2O$, 0.005 mg/L $ZnCl_2$, 0.05 mg/L $CuSO_4$, 0.097 mg/L $FeCl_3.6H_2O$. The concentrations of the macro-nutrients are given in the experimental procedures, section 3.2.
- RO concentrate was obtained from the water and resource recovery pilot plant of NEREUS. The concentrations of the nutrients are given in the experimental procedures, section 3.2.

Incubator

All experiments were performed in an incubator (Model: AlgaeTron AG 130-ECO) (Figure 7) available at NEREUS pilot plant or in a lab incubator (Figure 8) available at the Weissbrodt Group for Environmental Life Science Engineering, Environmental Biotechnology Section, Department of Biotechnology, TU Delft..

Figure 7: Incubator (Model: AlgaeTron AG 130-ECO)

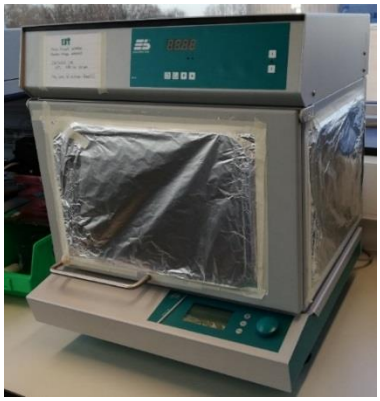

Figure 8: Incubator at EBT

3.2 Experimental set-up and procedures

Standard growing conditions

All experiments were performed under some standard conditions which are as follows:

- pH = 2
- Temperature = 35 ℃
- 14h/10h light/dark cycle
- Shaking speed = 145 rpm.
- Light intensity = 80 µmol photons $m^{-2}s^{-1}$

3.2.1 Screening experiments

Starter culture of microalgae *G. sulphuraria*

Starter culture of microalgae *G. sulphuraria* was cultivated in 500 mL Erlenmeyer flasks under the standard conditions described in section 3.2 in the AlgaeTron incubator. The starter cultures were grown autotrophically in Allen medium. pH-adjusted Allen medium was prepared before the cultivation. Concentrated sulphuric acid was added dropwise into the Allen medium until a pH value of 2 was obtained.

40 mL of previous starter cultures of *G. sulphuraria* 107.79 was pipetted equally into four 15 mL centrifuge tubes (4 x 10 mL) and centrifuged for 10 min at 1500 rpm with the use of a lab centrifuge (Model: Hermle Z 207 A). After centrifugation, the supernatant above the algae pellets was discarded and the algae pellets were re-dissolved with 1 mL of the pH-adjusted Allen medium. The dissolved pellets were then pipetted into the sterilized Erlenmeyer flasks and the pH-adjusted Allen medium was added and mixed properly until a homogeneous algae .

Finally, cellulose steristoppers was used for sealing. The cellulose steristoppers are air permeable and therefore allow air to pass through and diffuse in the microalgae culture. Sampling was done every 3 - 4 days to analyse the growing behaviour of the *G. sulphuraria*. The biomass growth of the microalgae was measured in terms of absorbance and cell counting.

Experiment 1: Dry weight measurements

The dry weight of a mixotrophic culture was measured in order to establish a good correlation with the absorbance value. Several dilutions of the mixotrophic culture were performed to determine the dry weight of the cultures at different cell densities. The dry weight analysis consists in filtering a known volume of sample and drying it 24 hours at 103°C in an oven. Whatman filters were used for the filtering and the filters were dried before the experiment and preserved in a desiccator at room temperature. The filtration was performed with a laboratory vacuum pump on 19 mL of sample. Each sample was weighted precisely on a weighing balance

measuring up to 3 decimal places. The difference between the empty filter (m_1) and the filter with dried biomass (m_2) gives the concentration of biomass as follows:

$$Dry\ weight\ \left(\frac{g}{L}\right) = \frac{m_2 - m_1}{Volume\ of\ sample} * 1000$$

Experiment 2: Cultivation of *G. sulphuraria* with different ammonium concentrations, different glucose concentrations and under different metabolisms

The goal of Experiment 2 was to assess the growth capability of *G. sulphuraria* in synthetic Allen medium with different ammonium concentrations and under different metabolisms. Experiment 2 was carried out using 48 well-plates (Figure 9) and *G. sulphuraria* was cultivated autotrophically, heterotrophically and mixotrophically under the standard conditions described in section 3.2 in the lab incubator available at Faculty of Applied Sciences, Department of Environmental Biotechnlogy (EBT), Delft University. The heterotrophic experiments were performed using glucose as organic carbon source and three different C:N ratios were tested, 5:1, 10:1 and 15:1 respectively. Two different C:N ratios, 5:1 and 10:1 were investigated for the mixotrophic experiments. Autotrophic growth does not require glucose as carbon source. The 48 well-plates have a growth area of 0.95 cm^2 per well and a well volume of 1.6 mL. All experiments were performed in duplicate.

Varying ammonium concentrations experiments were performed with seven different ammonium concentrations: 100, 200, 400, 600, 800 and 1000 mgNH_4^+-N/L. A wide range of ammonium concentrations were tested in order to investigate whether the ammonium concentration present in the RO concentrate is sufficient for the cultivation of *G. sulphuraria*. All the experiments were performed with a Nitrogen:Phosphorus (N:P) ratio = 37 because the N:P ratio in Allen medium is also 37.

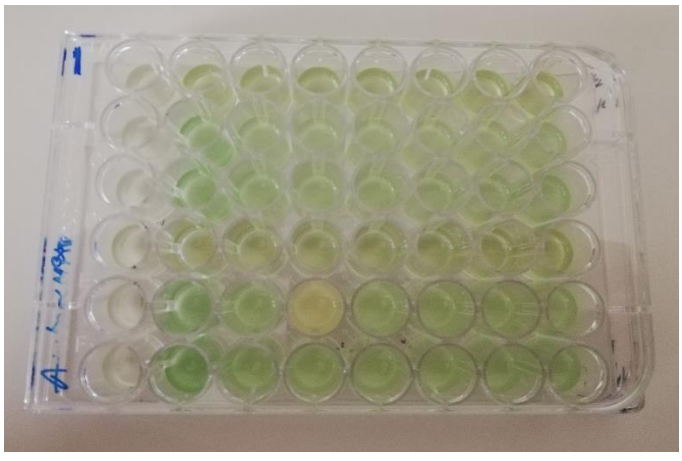

Figure 9: 48 well-plates with microalgae

A definite volume of the autotrophically algae starter culture was pipetted into the 48 well-plates and a certain volume of the stock solution was pipetted into the well-plates such that the total volume of the culture in the well-plates is equal to 1.0 mL and the initial optical density at 800 nm of the culture is equal or very close to 0.1 at the start of the experiments. Finally, the

well-plates were sealed with a breathe-easy membrane. The breathe-easy membrane is permeable to oxygen, carbon dioxide and water vapor. Daily sampling was done to analyse the growth of the *G. sulphuraria*. The biomass growth of the microalgae and pigment formation were measured in terms of absorbance.

Experiment 3: Cultivation of *G. sulphuraria* with different phosphate concentration, with bicarbonate supplementation and with carbon dioxide supplementation

The goal of Experiment 3 was to assess whether there are phosphate and carbon dioxide limitations on the growth of *G. sulphuraria*. Experiment 3 was carried out in 100 mL serum bottles (Figure 10) under the standard conditions described in section 3.2 in the lab incubator available at Faculty of Applied Sciences, Department of Environmental Biotechnlogy (EBT), Delft University. The culture medium was inoculated with autotrophically grown algae starter culture cells and the initial optical density at 800 nm of the culture was equal or very close to 0.1 at the start of the experiments. All experiments were performed in duplicate and with 50 mL of microalgae culture. *G. sulphuraria* was cultivated mixotrophically with ammonium concentration = 1000 mgNH_4^+-N/L and C:N ratio = 10:1.

The effect of phosphate were performed with two different phosphate concentrations, N:P ratio = 37:1 and 7.2:1 respectively. The N:P ratio = 37:1 and 7.2:1 were selected because an N:P ratio = 37:1 is present in Allen medium and according to the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, the theoretical physiologically optimal N:P ratio needed for growth of *G. sulphuraria* is 7.2:1. After inoculation, the anaerobic serum bottles were sealed with the breathe-easy membrane.

Moreover, we investigated the effect of the supplementation of bicarbonate (2.0 g/L) and CO_2 gas to the microalgae culture. The experiments were performed with N:P ratio = 37:1. The bicarbonate was weighed in the anaerobic serum bottles itself and as soon as the culture medium was added the anaerobic serum bottles were tightly sealed with a stopper. For the experiment with addition of CO_2 gas, the culture medium was first poured into the anaerobic serum bottles and tightly sealed with a stopper before CO_2 gas bubbling was performed. The medium was flushed with CO_2 gas at a flowrate of 0.7 L/min for a duration of 2 minutes. Therefore, the 50 mL of medium was flushed with 1.4 L of CO_2 gas and at room temperature and pressure that is equal to 1.4/24 = 0.06 moles of CO_2 gas per 50 mL = 1.2 moles/L of CO_2 gas.

For all experiments, daily sampling was done to analyse the growth of the *G. sulphuraria*. The biomass growth of the microalgae and pigment formation were measured in terms of absorbance.

Figure 10: Cultivation of *G. sulphuraria* with different phosphate concentration and with bicarbonate and carbon dioxide supplementation in 100 mL serum bottles

Experiment 4: Cultivation of *G. sulphuraria* with different culture densities

The goal of Experiment 4 was to assess the growth of *G. sulphuraria* in synthetic medium with different culture densities. Firstly, 200 mL of algae culture was cultivated mixotrophically in a 500 mL Erlenmeyer flask under the standard conditions described in section 3.2 in the AlgaeTron incubator with ammonium concentration = 600 mgNH_4^+-N/L, N:P ratio = 37:1 and C:N ratio = 5:1. The culture medium was inoculated with autotrophically grown algae starter culture cells and the initial optical density at 800 nm of the culture was equal or very close to 0.1 at the start of the experiments. When an absorbance of about 0.9 was achieved, half of the algae culture solution was used for the preparation of two dilutions, namely: absorbance = 0.3 and 0.6 respectively. Demi-water was used for the dilutions and a total volume of 100 mL of culture was prepared. The algae cultures with absorbance = 0.3, 0.6 and 0.9 respectively were cultivated in 250 mL Erlenmeyer flasks for 9 days under the standard conditions described in section 3.2 in the AlgaeTron incubator. After inoculation and dilution, the Erlenmeyer flasks were sealed with cellulose steristoppers. Daily sampling was done to analyse the growth of the *G. sulphuraria*. The biomass growth of the microalgae was measured in terms of absorbance. Monitoring of the ammonium, phosphate and glucose uptake by *G. sulphuraria* was done at the start, midway and end of the experiments respectively in order to determine whether there was any limitation during the experiments. Samples of the culture were filtered using 0.45 µm filters and the filtrate was used to measure the ammonium, phosphate and glucose concentration in it.

3.2.2 Experiments with synthetic and real RO concentrate

Experiment 5: Growth and nutrients consumption of *G. sulphuraria* in synthetic RO concentrate

The goal of Experiment 5 was to assess the biomass growth of *G. sulphuraria* and monitor the ammonium, phosphate and glucose uptake by *G. sulphuraria* in synthetic RO concentrate medium. *G. sulphuraria* was cultivated mixotrophically in a 500 mL Erlenmeyer flask (Figure 11) under the standard conditions described in section 3.2 in the AlgaeTron incubator. Synthetic RO concentrate with ammonium concentration representative to the ammonium concentration in real RO concentrate was prepared and used as medium. The synthetic RO concentrate medium also contains phosphate as source of P, glucose as source of C and small amounts of trace elements. 200 mL of microalgae culture was cultivated with ammonium concentration = 200 mgNH_4^+-N/L, N:P ratio = 37:1 and C:N ratio = 5:1. The culture medium was inoculated with autotrophically grown algae starter culture cells and the initial optical density at 800 nm of the culture was equal or very close to 0.1 at the start of the experiment. After inoculation, the Erlenmeyer flasks were sealed with cellulose steristoppers. Experiment with synthetic RO concentrate was set up to mostly monitor the ammonium, phosphate and glucose uptake by *G. sulphuraria*. Samples of the culture were filtered using 0.45 µm filters and the filtrate was used to measure the ammonium, phosphate and glucose concentration in it on a daily basis for 17 days of culture period. In addition, the biomass growth of *G. sulphuraria* was also daily monitored and measured in terms of absorbance for a duration of 17 days.

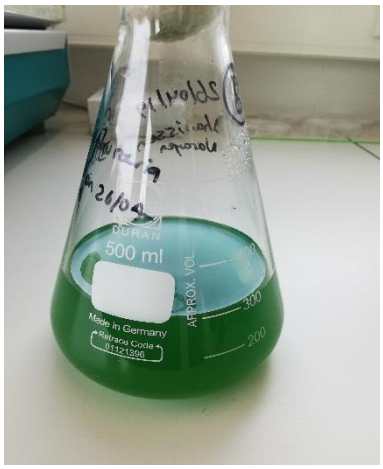

Figure 11: Cultivation of *G. sulphuraria* with synthetic RO concentrate in a 500 mL Erlenmeyer flask

Experiment 6: Growth and nutrients consumption of *G. sulphuraria* in real RO concentrate

The goal of Experiment 6 was to demonstrate the ability of *G. sulphuraria* to grow in real RO concentrate and assess its nutrients consumption. Also, it was to compare with *G. sulphuraria* grown in synthetic RO concentrate medium. *G. sulphuraria* was cultivated mixotrophically in 500 mL Erlenmeyer flasks under the standard conditions described in section 3.2 in the AlgaeTron incubator. Real RO concentrate from NEREUS contains very low phosphate concentration (0 - 0.1 mg/L) and therefore phosphate was added. Glucose and small amounts of trace elements was also added to the real RO concentrate. Three experiments were

performed, a mix of real RO concentrate and synthetic RO concentrate (20% real RO concentrate + 80% synthetic RO concentrate and 40% real RO concentrate + 60% synthetic RO concentrate) and 100% real RO concentrate. All the experiments were performed with 200 mL of microalgae culture and were cultivated with starting ammonium concentration = 200 mgNH_4^+-N/L, N:P ratio = 37:1 and C:N ratio = 5:1 respectively. The culture medium was inoculated with autotrophically grown algae starter culture cells and the initial optical density at 800 nm of the culture was equal or very close to 0.1 at the start of the experiment. After inoculation, the Erlenmeyer flasks were sealed with cellulose steristoppers. More nutrients were added to the cultures when the nutrients concentration in the culture were very low. Experiment with real RO concentrate was set up to monitor the biomass growth of *G. sulphuraria*. They were daily measured in terms of absorbance for a cultivation period of 14 days. Moreover, the ammonium, phosphate and glucose uptake by *G. sulphuraria* in real RO concentrate was also daily determined during the 14 days of cultivation. Samples of the culture were filtered using 0.45 µm filters and the filtrate was used to measure the ammonium, phosphate and glucose concentration in it.

3.3 Analytical methods

Absorbance measurements

- The biomass growth of the microalgae and the pigment formation for the experiments in the well-plates were monitored by means of measuring the absorbance (optical density) value of the culture at 800 nm and 618 nm respectively with a plate reader (Tecan infinite M200 Pro).

- The biomass growth of the microalgae and the pigment formation for the experiments in Erlenmeyer flasks and anaerobic serum bottles were monitored by means of measuring the absorbance (optical density) value of the culture at 800 nm and 618 nm respectively with a UV/visible spectrophotometer (Hach DR 3900, Germany).

Ammonium and phosphate measurements

- Ammonium and phosphate concentrations in the culture medium were measured by colorimetry using Hach test kits. LCK 302, 303 and 304 cuvette test kit were used to measure ammonium concentrations. Phosphate concentrations in the medium was measured with the use of LCK 349 and 350 cuvette test kit.

Glucose measurements

- The glucose concentration in the culture medium was measured using a glucose meter (Medisana MediTouch2).

pH measurements

- pH measurement was done using a portable multi-meter (Model: Hach HQ30D).

3.4 Modelling the cultures cultivated on synthetic and real RO concentrate using Aquasim simulation software

The cultures cultivated on synthetic and real RO concentrate were also modelled using the mathematical simulation software Aquasim. Stoichiometric and kinetic parameters of growth were assessed from the biomass production and substrate consumption profiles using Aquasim. The growth and substrate uptake for the batch cultures was modeled by the Monod model (Monod, 1949):

$$\frac{dC_x}{dt} = \mu C_x = \frac{\mu_{max} C_s}{C_s + K_s} C_x \quad \text{and} \quad \frac{dC_s}{dt} = q_s C_s = \left(\frac{\mu_{max} C_s}{C_s + K_s} - m_s\right) * 1/Y_{x/s,max} * C_x$$

where: C_x = concentration of biomass

C_s = concentration of substrates

μ_{max} = the maximum specific growth rate

K_s = substrate affinity

m_s = maintenance rate on substrate

$Y_{x/s,max}$ = yield of biomass per amount of substrate

The factor of $\frac{I}{K_I + I}$ (Monod, 1949) was added to the Aquasim model to account for light limitation, where I = light intensity and K_I = half saturation intensity. I was calculated from the Lambert-Beer equation (Mäntele & Deniz, 2017):

$$A = \log_{10}\left(\frac{I_0}{I}\right) = \epsilon l C_x \quad (2)$$

where: A = absorbance

I_0 = *initial light intensity*

ϵ = molar absorption coefficient

l = pathlengh of the light through the sample

C_x = concentration of biomass

All the above parameters were used for the Aquasim model. μ_{max}, $Y_{x/s,max}$, K_s, m_s, ϵ and K_I were derived by parameter fitting.

3.5 Alternative method for determination of the maximum specific growth rate

The maximum specific growth rate (μ_{max}) for the cultures where the substrate concentrations were not measured were calculated as follows:

$$\mu_{max} = \frac{(\text{Ln}\,(DW_2) - \text{Ln}\,(DW_1))}{t_2 - t_1}$$

where: DW_1 = dry weight of biomass at the start of the exponential phase (g/L)

DW_2 = dry weight of biomass at the end of the exponential phase (g/L)

t_1 = time at the start of the exponential phase (day)

t_2 = time at the end of the exponential phase (day)

Chapter 4 Results and Discussion

4.1 Results and discussion from screening experiments

4.1.1 Correlation between biomass concentration and absorbance

Beer-Lambert's law shows a direct correlation between the absorbance and concentration of biomass according to equation 2 in section 3.1. There is a linear relationship up to a certain part (Mehta, 2012). The Beer-Lambert relationship breaks down and follow a non-linear relationship at high analyte concentrations (Mehta, 2012). High concentrations can result in an alteration in the absorption wavelength of the biomass and alter the refractive index of the solution, thus affecting the resulting absorbance (Mehta, 2012). Figure 12 shows the correlation between the biomass concentration and the absorbance at 800 nm of a mixotrophic culture of *G. sulphuraria*. From Figure 12, a linear relationship until absorbance of about 0.6 was observed. From Figure 12, it can also be observed that from absorbance 0 till 0.6 there is a smaller change in the biomass concentration compared to absorbance 0.6 till 1.8.

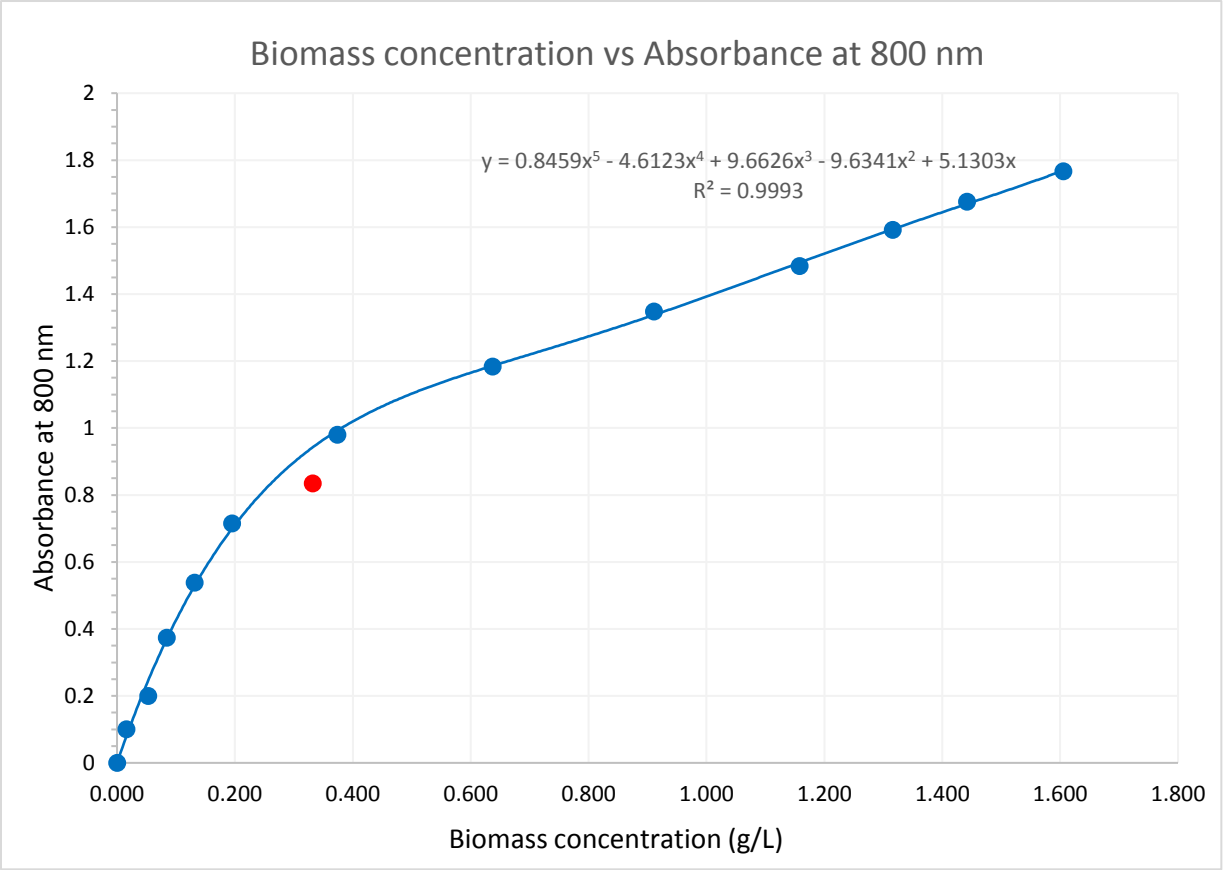

Figure 12: Correlation dry weight and absorbance at 800 nm of mixotrophic culture of G. sulphuraria (pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Note: The data point marked red was considered as an outlier and was not taken into account for the regression line. This is because a worse fitted regression line as well as a lower correlation coefficient, R^2 (R-squared) was obtained when the red data point was taken into account.

4.1.2 Mixotrophic metabolism with ammonium as source of nitrogen resulted as the best metabolism

4.1.2.1 Metabolisms when ammonium was used as source of nitrogen

Ammonium was firstly used as source of nitrogen to cultivate *G. sulphuraria* and the growth of *G. sulphuraria* under the three metabolisms, auto-, mixo- and heterotrophic metabolism was monitored. Figure 13 shows the biomass growth under auto-, mixo- and heterotrophic metabolism respectively with ammonium as source of nitrogen. The experiment was performed with ammonium concentration = 200 mgNH_4^+-N/L, C:N ratio = 5:1 and N:P ratio = 37:1.

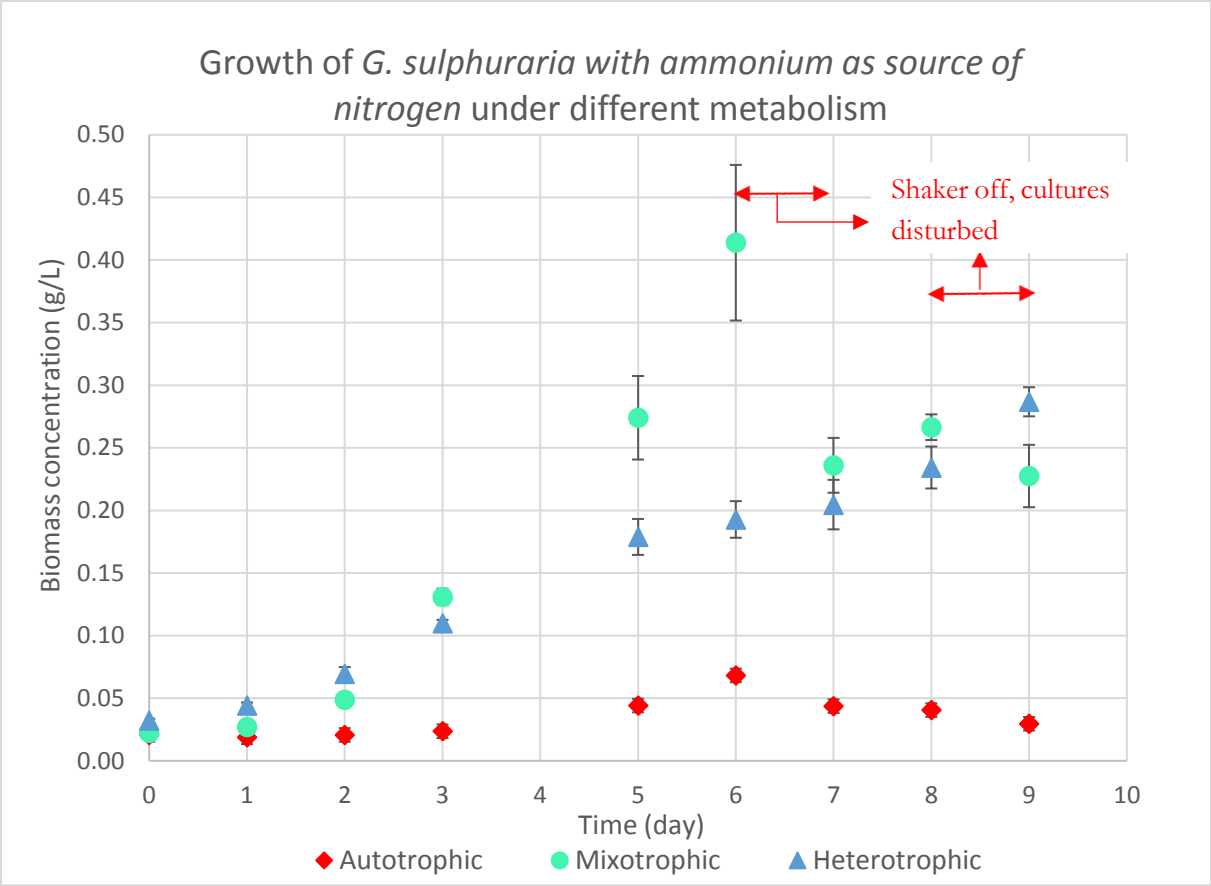

Figure 13: Biomass growth under auto-, mixo- and heterotrophic metabolism respectively with ammonium as source of nitrogen (Conc. of NH_4^+ on day 0 = 200 mg NH_4^+- N/L, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Note: Between the measurement of day 6 and the one of day 7 and the measurement of day 8 and the one of day 9, the shaker inside the incubator was incidentally turned off and cultures were disturbed.

From Figure 13, it can be observed that the mixotrophic metabolism gave more favorable results than the heterotrophic and autotrophic metabolism. Considering the first 6 days of cultivation, it can be observed that the mixotrophic culture resulted in an average biomass concentration of 0.41 g/L while the heterotrophic and autotrophic cultures produced an average biomass concentration of 0.19 g/L and 0.07 g/L respectively. The mixotrophic culture produced about twice more biomass than the heterotrophic culture and about six times more biomass than the autotrophic culture for the first 6 days of cultivation. The results of this experiment is in agreement with literature, where it stated that cultures using an organic carbon source (mixotrophic and heterotrophic cultures) results to higher cell density and biomass productivity than cultures without an organic carbon source (autotrophic cultures) (Chen, 1996). Results of this experiment is also in agreement with literature, where it stated that mixotrophic cultures results in much higher biomass concentration than heterotrophic cultures (Sloth et al. 2006).

In addition, the growth rates under autotrophic and heterotrophic metabolism were lower compared to that from mixotrophic metabolism. The maximum biomass specific growth rates (μ_{max}) for the three metabolisms when ammonium was used as nitrogen source were calculated from data obtained up to day 6 and they are shown in Table 2. The lag phase of the autotrophic culture was longer (2 days) compared to those of the mixo- and heterotrophic cultures (≈ 1 day).

Table 2: μ_{max} values of *G. sulphuraria* for different culture metabolisms using ammonium as source of nitrogen (Conc. of NH_4^+ on day 0 = 200 mg NH_4^+- N/L, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Culture	μ_{max} (day^{-1})
Autotrophic	0.30 ± 0.02
Heterotrophic	0.35 ± 0.03
Mixotrophic	0.54 ± 0.01

Usually, the specific growth rate of mixotrophic cultures is approximately the sum of the cell specific growth rates of the autotrophic and heterotrophic cultures (Perez-Garcia et al., 2011). This might explain the findings that *G. sulphuraria* grew faster under mixotrophic conditions and the results from Table 2 are in line with the findings of Perez-Garcia et al., 2011. The mixotrophic and heterotrophic cultures used glucose as organic carbon source for growth. Glucose possesses more energy content per mol (≈ 2.8 kJ/mol of energy) compared with other substrates (Boyle & Morgan, 2009; Perez-Garcia et al., 2011). Carbon dioxide is used as carbon source for autotrophic cultures. Under autotrophic metabolism microalgae first perform photosynthesis to convert the carbon dioxide to glucose which will afterwards be used for biomass growth. This is why microalgae cultured with glucose as carbon source (mixotrophic and heterotrophic cultures) have higher growth rates and shorter lag phase than cultures without glucose (autotrophic cultures). Under heterotrophic growth conditions, organic carbon source such as glucose is used both as carbon and energy sources for biomass formation. Under mixotrophic growth conditions, light partially provides the energy and the carbon source can mostly be used for biomass formation, thus resulting in higher growth and growth rates of the mixotrophic cultures compared to heterotrophic cultures.

Note: Day 10 and 11 were during the weekend and no sampling was done. On day 12, the experiment was stopped and no sampling was done because a lot of evaporation occurred during the period from day 9 till day 12. Evaporation was insignificant from day 0 till day 9.

4.1.2.2 Metabolisms when nitrate was used as source of nitrogen

After that the growth of *G. sulphuraria* under auto-, mixo- and heterotrophic metabolism using ammonium as source of nitrogen was investigated, the growth of *G. sulphuraria* under auto-, mixo- and heterotrophic metabolism using nitrate as source of nitrogen is investigated in this section. Figure 14 shows the biomass growth under auto-, mixo- and heterotrophic metabolism respectively with nitrate as source of nitrogen. The experiment was performed with nitrate

concentration = 247 mg NO_3^-- N/L, C:N ratio = 5:1 and N:P ratio = 37:1.

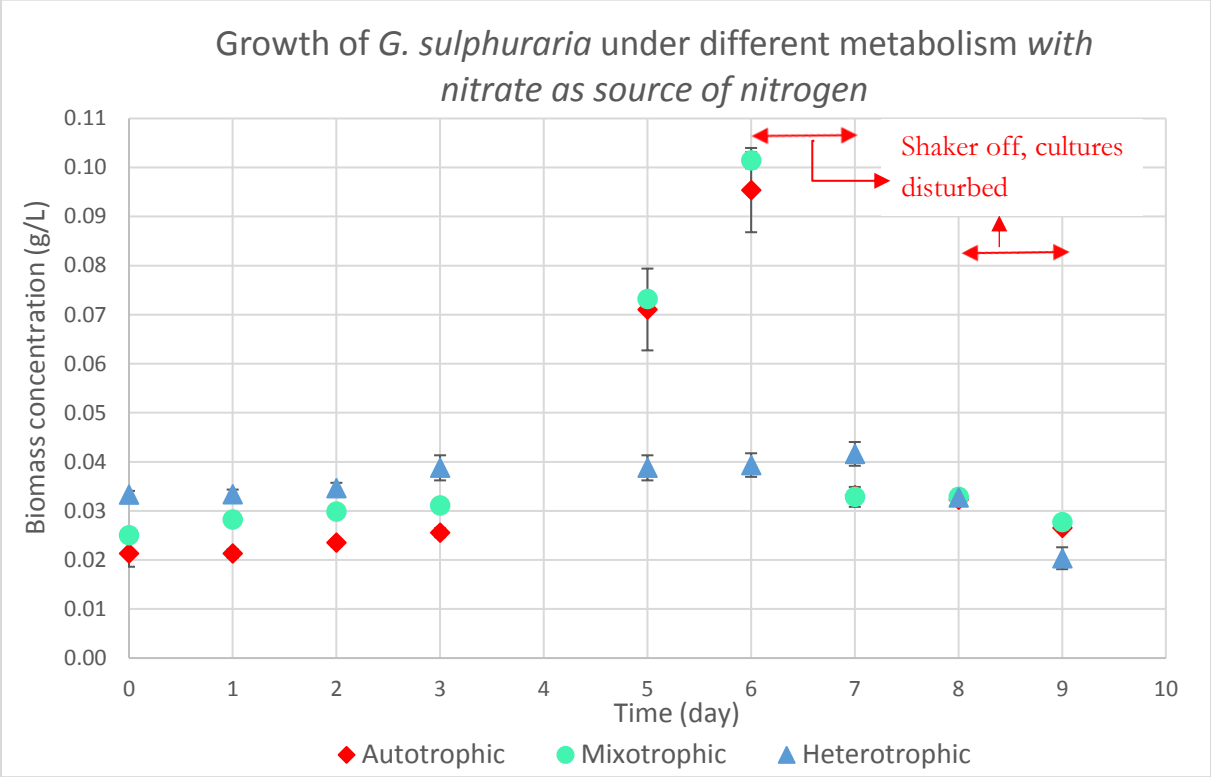

Figure 14: Biomass growth under auto-, mixo- and heterotrophic metabolism respectively with nitrate as source of nitrogen (Conc. of NO_3^- on day 0 = 247 mg NO_3^-- N/L, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Note: Between the measurement of day 6 and the one of day 7 and the measurement of day 8 and the one of day 9, the shaker inside the incubator was incidentally turned off and cultures were disturbed.

From Figure 14, it can be observed that auto- and mixotrophic metabolism gave better results than heterotrophic metabolism. Considering the first 6 days of cultivation, it can be observed that the mixotrophic culture resulted in an average biomass concentration of 0.10 g/L while the autotrophic and heterotrophic cultures produced an average biomass concentration of 0.095 g/L and 0.039 g/L respectively. It has to be noted that the heterotrophic culture started with higher biomass concentration (0.033 g/L) than the mixotrophic (0.025 g/L) and autotrophic (0.021 g/L) culture. The heterotrophic culture built up the least biomass from day 0 till day 6, 0.039 g/L and resulted in the lowest maximum specific growth rate, 0.074 day^{-1}, compared to those from autotrophic (0.30 day^{-1}) and mixotrophic (0.26 day^{-1}) metabolism. The heterotrophic results of this experiment is in agreement with literature, where it stated that heterotrophic cultures assimilate nitrate less rapidly in the dark than in the light and that darkness may have a negative consequence on the uptake of nitrates (Perez-Garcia et al., 2011). Light reduces cofactors such as pyridine nucleotides and ferredoxins which are used as electron donors for nitrate reduction to nitrite and ammonium respectively (Kraepelin, 1977). Table 3 shows the μ_{max} values which

were calculated from data obtained up to day 6 for the three metabolisms when nitrate was used as nitrogen source.

Table 3: μ_{max} values of *G. sulphuraria* for different culture metabolisms using nitrate as source of nitrogen (Conc. of NO_3^- on day 0 = 247 mg NO_3^-- N/L, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Culture	μ_{max} (day^{-1})
Autotrophic	0.30 ± 0.03
Heterotrophic	0.074 ± 0.02
Mixotrophic	0.26 ± 0.002

The auto- and mixotrophic cultures produced almost same amount of biomass, 0.095 and 0.10 g/L respectively. In addition, they grew almost at the same speed, with maximum specific growth rates of 0.30 and 0.26 day^{-1} for the autotrophic and mixotrophic cultures respectively. The possible reasoning for having almost the same biomass concentration after 6 days of cultivation and almost the same maximum specific growth rate for the autotrophic and mixotrophic cultures is such that up to 6 days of cultivation the exponential part of the growth curve was not over yet and therefore for a better comparison of the mixotrophic culture with the autotrophic culture a longer cultivation period is required when nitrate is used as source of nitrogen.

4.1.2.3 Ammonium is a better source of nitrogen than nitrate

From section 4.1.2.1 and 4.1.2.2, the growth of *G. sulphuraria* under auto-, mixo- and heterotrophic metabolism using ammonium and nitrate respectively have been investigated. Comparing Figure 13 and Figure 14 it can be deduced that *G. sulphuraria* grew faster with ammonium than nitrate under mixo- and heterotrophic metabolism. Considering the first 6 days of cultivation, the mixotrophic culture with ammonium produced about four times more biomass than the mixotrophic culture with nitrate while the heterotrophic culture with ammonium produced about five times more biomass than the heterotrophic culture with nitrate. Ammonium is energetically a more efficient nitrogen source, requiring less energy for its uptake (Goldman, 1977; Grobbelaar, 2007; Shi et al., 2000; Syrett & Morris, 1963; Wilhelm et al., 2006). When microalgae are grown in medium containing nitrate, nitrate is first transported across the membrane and then reduced to ammonia by enzymes before being used for growth of the microalgae (Buchanan et al., 2000). Nitrate is first reduced to nitrite and the latter later being reduced to ammonium. The process of converting the nitrate into ammonium consumes a lot of energy, carbon and protons (Buchanan et al., 2000). This is why *G. sulphuraria* grows faster with medium containing ammonium as nitrogen source than medium containing nitrate as nitrogen source.

4.1.3 Higher ammonium concentrations resulted in higher growth of *G. sulphuraria*

Nitrogen is quantitatively one of the most important element contributing to microalgal biomass, about 1 to 10% dry weight. From previous results, it has been observed that mixotrophic metabolism with ammonium as source of nitrogen resulted to highest biomass production and therefore this section investigated the effect of ammonium concentration on the mixotrophic growth of *G. sulphuraria*. Figure 15 shows how the growth of *G. sulphuraria* under mixotrophic metabolism varies with increase in ammonium concentration. The experiment was performed with C:N ratio = 5:1 and N:P ratio = 37:1. Figure 15 shows the ammonium varying experiments with six ammonium concentrations: 100, 200, 400, 600, 800 and 1000 mgNH_4^+-N/L respectively.

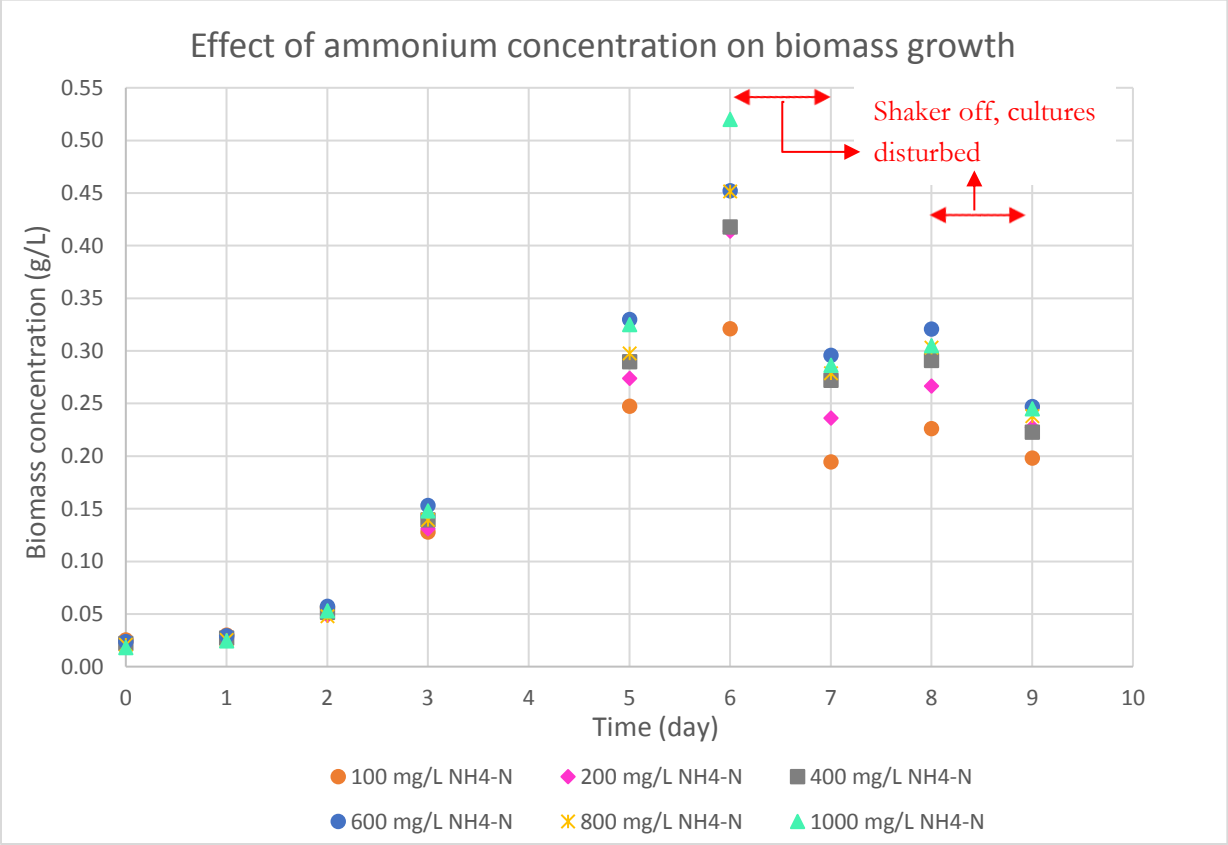

Figure 15: Effect of ammonium concentration on biomass growth (Mixotrophic metabolism, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Note: Between the measurement of day 6 and the one of day 7 and the measurement of day 8 and the one of day 9, the shaker inside the incubator was incidentally turned off and cultures were disturbed.

Considering the first 6 days of cultivation, it can be observed from Figure 15 that an increase in ammonium concentration results in an increase in biomass growth of *G. sulphuraria* if the growth with ammonium concentration = 600 mgNH_4^+-N/L is excluded. After 6 days of

cultivation, ammonium concentration = 100, 200, 400, 600, 800 and 1000 mgNH_4^+-N/L resulted in biomass concentration = 0.321, 0.414, 0.418, 0.452, 0.452, 0.520 g/L respectively. The results with ammonium concentration = 600 mgNH_4^+-N/L is an outlier and possible reasoning for the higher expected growth can be due to human and experimental errors during preparation of medium, for instance, errors while weighing the amount of chemicals for medium or errors while measuring volumes. The μ_{max} values of *G. sulphuraria* under the different ammonium concentrations are shown in Table 4.

Table 4: μ_{max} values of *G. sulphuraria* under different ammonium concentrations (Mixotrophic metabolism, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Ammonium concentration (mgNH_4^+-N/L)	μ_{max} (day^{-1})
100	0.47 ± 0.01
200	0.54 ± 0.01
400	0.55 ± 0.004
600	0.55 ± 0.003
800	0.57 ± 0.03
1000	0.60 ± 0.08

It can be seen from Table 4 that the growth rate of *G. sulphuraria* with ammonium concentration = 100 mgNH_4^+-N/L was lower (0.47 day^{-1}) compared to the growth rates of higher ammonium concentrations (0.54, 0.55, 0.55, 0.57 and 0.60 day^{-1} for ammonium concentrations = 200, 400, 600,800 and 1000 mgNH_4^+-N/L respectively). The lower growth rate *G. sulphuraria* with ammonium concentration = 100 mgNH_4^+-N/L was due to nutrient limitation (C and P) as shown in the theoretical calculations below:

Maximum biomass concentration obtained with the culture having ammonium concentration = 100 mgNH_4^+-N/L on day 6 was 0.321 g/L. Using the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, it can be deduced that for 0.321 g/L of biomass, 0.115 g/L of carbon = 0.115*(180/12) = 1.73 g/L of glucose, 2.80 mg/L of PO_4^{3-}-P and 20.3 mg/L of NH_4^+-N was required. Concentrations of macro-nutrients supplied for the culture with ammonium concentration = 100 mgNH_4^+-N/L are as follows: 1.25 g/L of glucose, 2.70 mg/L and 100 mg/L of NH_4^+-N. Therefore, it can be seen that theoretically there was limitations in glucose and phosphate concentrations in the culture with ammonium concentration = 100 mgNH_4^+-N/L on day 6 during the experiment. Cultures with lower ammonium concentration had lower phosphate and glucose concentrations compared to the others cultures with higher ammonium concentrations because the C:N and N:P ratio was kept constant in this experiment at 5:1 and 37:1 respectively.

Considering the first 6 days of cultivation, biomass produced and growth rate of *G. sulphuraria* with ammonium concentration = 200 mgNH_4^+-N/L was very close to those with higher ammonium concentrations. From theoretical calculations, no nutrient limitation was obtained for cultures with ammonium concentration = 200, 400, 600, 800 and 1000 mgNH_4^+-N/L. The

theoretical glucose, phosphorus and nitrogen concentrations required for the maximum biomass concentration reached on day 6 for the culture with ammonium concentration = 200 mgNH_4^+-N/L is described below:

Maximum biomass concentration obtained with the culture having ammonium concentration = 200 mgNH_4^+-N/L on day 6 was 0.414 g/L. Using the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, it can be deduced that for 0.414 g/L of biomass, 0.148 g/L of carbon = 0.148*(180/12) = 2.23 g/L of glucose, 3.62 mg/L of PO_4^{3-}-P and 26.1 mg/L of NH_4^+-N was required. Concentrations of macro-nutrients supplied for the culture with ammonium concentration = 200 mgNH_4^+-N/L are as follows: 2.50 g/L of glucose, 5.40 mg/L and 200 mg/L of NH_4^+-N. Therefore, it can be seen that theoretically there was no limitations in glucose, phosphate and ammonium concentrations in the culture with ammonium concentration = 200 mgNH_4^+-N/L on day 6 during the experiment. The same procedure was followed for the cultures with higher ammonium concentrations (400, 600, 800 and 1000 mgNH_4^+-N/L) and it was found that they were no nutrient limitation. Since the experiment was disturbed after day 6, a clear complete growth curve was not be obtained. If a complete growth curve was obtained, it would be observed that the growth will be higher at higher ammonium concentrations because the cultures with lower ammonium concentration would be earlier be limited in phosphate and glucose concentration because the C:N and N:P ratio was kept constant in this experiment at 5:1 and 37:1.

From theoretical calculations it was observed that the phosphate and glucose were the nutrients which were more vulnerable to limitations and higher ammonium concentrations of ammonium ensured higher concentrations of phosphate and glucose in the medium thus resulted to higher growth. It must be noted that the error bars were not included in Figure 15 because the data points were very to each other and thus no clear distinction were observed between the error bars. The standard deviations of the data points can be found in the Appendix A.2.

4.1.4 Higher glucose concentration resulted in no significant change on the growth of *G. sulphuraria* under mixo- and heterotrophic metabolism

Glucose is an important source of carbon as nutrient. From section 4.1.3, it has been observed that glucose limitation can have impact on the growth of *G. sulphuraria*. Therefore, this section investigated the effect of glucose concentration on the growth of *G. sulphuraria* for both mixotrophic and heterotrophic metabolism. Figure 16 shows the biomass concentrations reached after 6 days of cultivation for the glucose varying experiments with two C:N ratios = 5:1 (glucose concentration = 2.5 g/L) and 10:1 (glucose concentration = 5.0 g/L). According to the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, the theoretical physiologically optimal C:N ratio needed for growth of *G. sulphuraria* is 5.7:1. The experiment was performed under mixo- and heterotrophic metabolism, ammonium concentration = 200 mgNH_4^+-N/L and N:P ratio = 37:1. The aim of this experiment was to investigate whether a higher glucose concentration will lead to higher growth of *G. sulphuraria*.

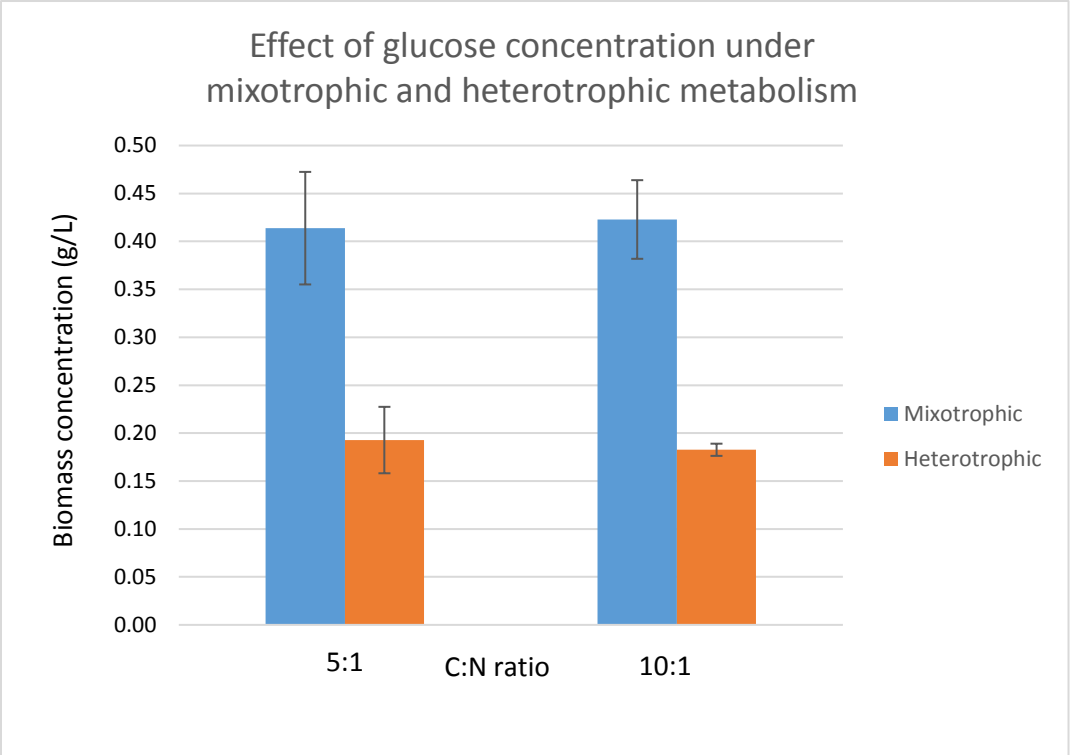

Figure 16: Effect of glucose concentration under mixotrophic and heterotrophic metabolism (Conc. of NH_4^+ on day 0 = 200 mg NH_4^+- N/L, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

It can be observed from Figure 16 that the mixotrophic growth of *G. sulphuraria* after 6 days of cultivation resulted in biomass concentrations of 0.414 g/L and 0.423 g/L for C:N ratio = 5:1 and 10:1 respectively. The heterotrophic growth after 6 days of cultivation resulted in biomass concentrations of 0.193 g/L and 0.183 g/L for C:N ratio = 5:1 and 10:1 respectively. From Figure 16 it can concluded that doubling the glucose concentration does not significantly increase the growth of *G. sulphuraria*. A C:N ratio = 5:1 produced almost the same amount biomass as a C:N ratio = 10:1 and this is in agreement with the μ_{max} values obtained. Both C:N ratio = 5:1 and 10:1 resulted in the same or almost the same μ_{max} values as shown in Table 5.

Table 5: μ_{max} values of *G. sulphuraria* for mixotrophic and heterotrophic cultures with different C:N ratios (Conc. of NH_4^+ on day 0 = 200 mg NH_4^+- N/L, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Culture	C:N ratio	μ_{max} (day^{-1})
Autotrophic	5:1	0.54 ± 0.01
	10:1	0.56 ± 0.06
Heterotrophic	5:1	0.35 ± 0.03
	10:1	0.35 ± 0.01

Maximum biomass concentrations was obtained with the mixotrophic cultures, 0.414 g/L and 0.423 g/L for C:N ratio = 5:1 (glucose concentration = 2.5 g/L) and 10:1 (glucose concentration = 5.0 g/L) respectively. Using the biomass formula of *G. sulphuraria* : $C_{106}H_{263}O_{110}N_{16}P$, it can

be deduced that for 0.414 g/L biomass, 0.148 g/L of carbon = 0.148*(180/12) = 2.23 g/L of glucose was required. For 0.423 g/L biomass, 0.151 g/L of carbon = 0.151*(180/12) = 2.27 g/L of glucose was required. Therefore, theoretically there was no limitations of glucose concentration in any of the cultures on day 6 during the experiment.

4.1.5 Supplementation of bicarbonate and CO_2 gas did not promote to better growth of *G. sulphuraria*

Carbon is the most important nutrient in the biomass composition of microalgae. Considering the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, it can be seen that carbon content is higher than that of nitrogen and phosphorus. Carbon consists of about 36% of weight percentage of the biomass. From previous section, section 4.1.4, the growth of *G. sulphuraria* using glucose as source of carbon was investigated and in this section the effect of bicarbonate and carbon dioxide addition on biomass growth was investigated in order to monitor whether these additions will result to higher biomass concentrations because of the low amount of carbon dioxide in the air. Figure 17 shows how the growth of *G. sulphuraria* varies when carbon dioxide and bicarbonate was added to the culture. The experiment was performed with ammonium concentration = 1000 mgNH_4^+-N/L, N:P ratio = 37:1 and C:N ratio = 10:1.

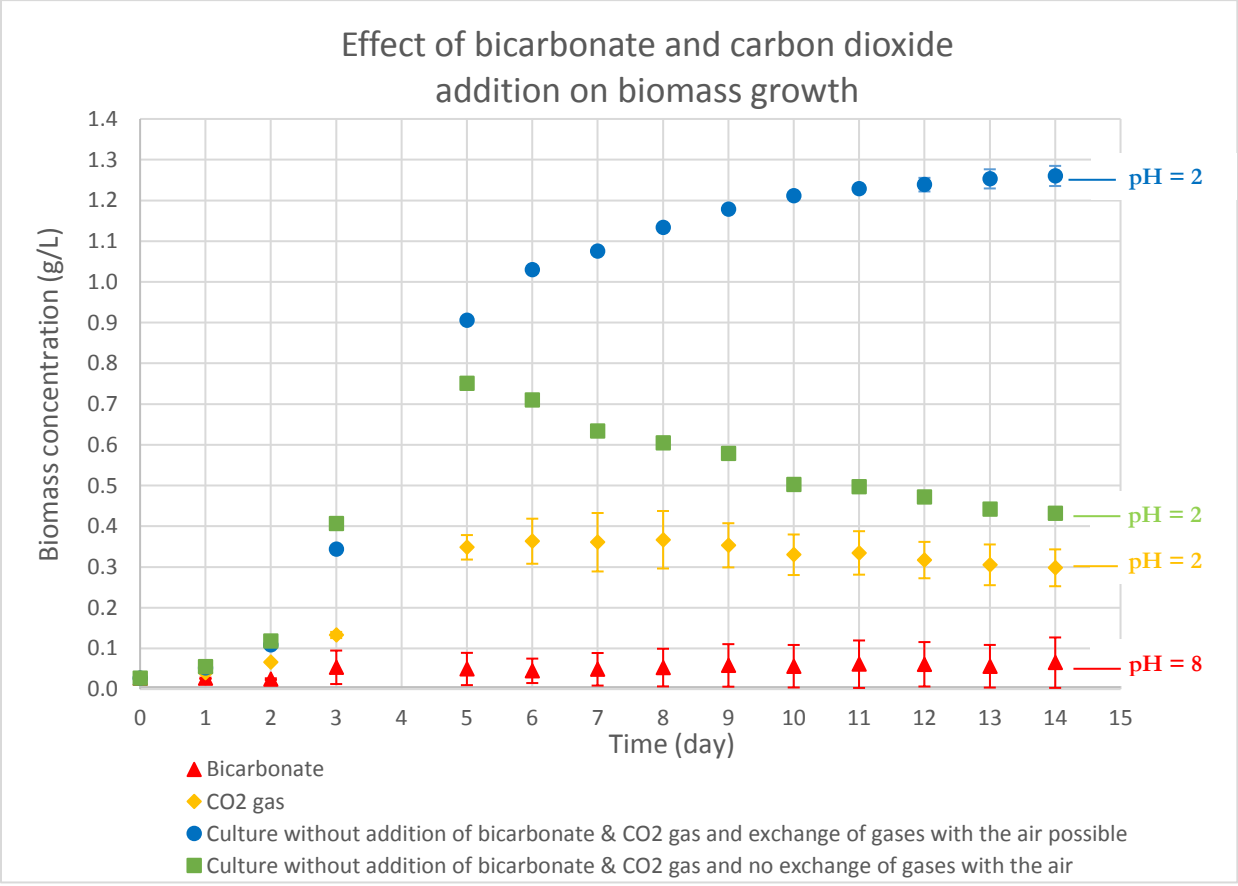

Figure 17: Effect of bicarbonate and carbon dioxide gas supplementation on biomass growth (Mixotrophic metabolism, conc. of NH_4^+-N/L on day 0 = 1000 mg NH_4^+-N , C:N ratio on day 0 = 10:1, N:P ratio on day 0

= 37:1, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

From Figure 17 it can be observed that *G. sulphuraria* did not grow well in the culture with bicarbonate addition and built up very little biomass, a maximum average biomass concentration of about 0.05 g/L. This is because the pH of the culture was 8 and the growing pH range for *G. sulphuraria* is pH 0.05 – 5.0. Also, this results shows that *G. sulphuraria* requires an acidic environment to be able to grow efficiently and optimally. Moreover, from Figure 17 it can be observed that the growth of *G. sulphuraria* in the culture with CO_2 gas addition was better than the culture with bicarbonate addition since it resulted to a maximum average biomass concentration of 0.36 g/L after 6 days of cultivation and this is mostly because the culture with CO_2 gas addition was at pH = 2. Lower biomass concentration of *G. sulphuraria* was observed in the culture with CO_2 gas addition than in the cultures without addition of bicarbonate and CO_2 gas. The culture without addition of bicarbonate & CO_2 gas and exchange of gases with the air possible reached an average biomass concentration of 1.02 g/L after 6 days of cultivation while the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air reached an average biomass concentration of 0.705 g/L after 6 days of cultivation.

Theoretically, the volume of carbon dioxide gas added to the culture at the start of the experiment was not limited during the experiment. From Figure 17, it can be assumed that the maximum average biomass concentration of *G. sulphuraria* which resulted from the culture with CO_2 gas addition was 0.36 g/L. Using the biomass formula of *G. sulphuraria:* $C_{106}H_{263}O_{110}N_{16}P$, it can be deduced that for 0.36 g/L biomass, 0.13 g/L of carbon = 0.13*(44/12) = 0.47 g/L of CO_2 = 0.011 moles/L CO_2 was required theoretically. Since 1.17 moles/L CO_2 was supplied to the culture by CO_2 gas bubbling, there was an excess of CO_2 in the medium and thus no limitation of CO_2 during the experiment. The calculation done above took into consideration that all the carbon source were provided by CO_2 but this experiment was performed mixotrophically, that is, glucose was also present and also contributed for carbon. Therefore, the lower growth and lower maximum specific growth rate of *G. sulphuraria* for the culture with CO_2 gas addition (0.57 day^{-1}) than the cultures without addition of bicarbonate and CO_2 gas was not due to limitation of CO_2. Table 6 shows the μ_{max} values of *G. sulphuraria* for the culture with carbon dioxide addition and the cultures without bicarbonate and carbon dioxide addition. It has to be noted that the μ_{max} of the culture with bicarbonate addition was not calculated because there was very little and varying biomass growth, ≈ 0.05 g/L. The standard deviation for the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air could not be computed because of the absence of duplicates due to non-availability of space in the incubator at that time.

Table 6: μ_{max} values of *G. sulphuraria* for the culture with carbon dioxide addition and the cultures without bicarbonate and carbon dioxide addition (Mixotrophic metabolism, conc. of NH_4^+-N/L on day 0 = 1000 mg NH_4^+-N , C:N ratio on day 0 = 10:1, N:P ratio on day 0 = 37:1, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Culture	μ_{max} (day^{-1})
CO_2 gas addition	0.57 ± 0.003
Without addition of bicarbonate & CO_2 gas and exchange of gases with the air possible	0.74 ± 0.003
Without addition of bicarbonate & CO_2 gas and no exchange of gases	0.67

Comparing the results from Table 6, it can be observed the culture with CO_2 gas addition resulted in a lower μ_{max} (0.57 day^{-1}) compared to that of the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air (0.67 day^{-1}). The lower growth rate of the culture with CO_2 gas addition might be due to oxygen limitation in culture with CO_2 gas addition because the only difference between the two cultures is that the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air had more oxygen and less carbon dioxide at the start of the experiment than the culture with carbon dioxide addition. The culture with CO_2 gas addition had part of the oxygen present in the medium and headspace flushed out during the flushing of the medium with CO_2 gas. The theoretical oxygen concentrations required for the maximum biomass concentration reached on day 5 for the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air is described below:

Maximum biomass concentration obtained with the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air on day 5 was 0.746 g/L. Using the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, it can be deduced that for 0.746 g/L of biomass, 0.370 g/L of oxygen (O) = 0.370*(32/16) = 0.740 g/L of oxygen gas was required. Concentration of oxygen present in the headspace is as follows: air consist of 20.95% of oxygen and the volume of oxygen present in 1L of air = 0.2095 L. 1 mole of ideal gas occupies 24 L of volume at room temperature and pressure. Therefore, 0.2095 L of oxygen gas is equivalent to 0.00873 mol O_2/L of air, which is equal to 279.4 mg O_2/L. Therefore, it can be seen that theoretically there was limitations in oxygen in the culture without addition of bicarbonate & CO_2 gas and no exchange of gases with the air during the experiment.

With a concentration of 279.4 mg O_2/L, it is theoretically expected to be finished when a biomass concentration of 0.282 g/L is reached. Therefore, after having reached a biomass concentration of 0.282 g/L, the culture was either supplied with oxygen produced during photosynthesis, if photosynthetic O_2 was produced or it continued growing under oxygen limitation until a saturation point was reached on day 5 (0.746 g/L of biomass concentration). From day 6 till day 14, a gradual decrease in the growth can be observed from Figure 17. Similar behaviour was observed with the culture with CO_2 addition because after reaching a biomass concentration of 0.36 g/L on day 6 there was no build-up in biomass and was afterwards

followed by the gradual decrease in the growth. Moreover, considering the growth curve of the culture without addition of bicarbonate & CO_2 gas and exchange of gases with the air possible from Figure 17, it can be observed that it continued growing after day 5 and this might be mainly because the culture had continuous exchange of oxygen from the air to the medium because there was no glucose, ammonium and phosphate limitations at the end of the experiment. The conclusion of this section is that oxygen is a very important parameter for the growth of *G. sulphuraria.*

Note: From literature, it was found out that when G. sulphuraria 074G were grown mixotrophically, no photosynthetic O_2 evolution was detected and cellular respiration was in the same range as under heterotrophic growth (Oesterhelt et al., 2007). During cellular respiration oxygen is consumed and the energy for the process is obtained from the assimilated glucose. Photosynthesis of G. sulphuraria 074G was downregulated in the presence of glucose as carbon source (Oesterhelt et al., 2007).

4.1.6 Higher phosphate concentration resulted in slightly higher growth of *G. sulphuraria*

Phosphate is also a very important element contributing to microalgal biomass. From section 4.1.3, it has been observed that phosphate limitation can have impact on the growth of *G. sulphuraria*. Therefore, this section investigated the effect of phosphate concentration on the growth of *G. sulphuraria*. Figure 18 shows the phosphate varying experiments with two N:P ratios = 37:1 and 7.2:1. The N:P ratio = 37:1 and 7.2:1 were selected because an N:P ratio = 37:1 is present in Allen medium and according to the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, the theoretical physiologically optimal N:P ratio needed for growth of *G. sulphuraria* is 7.2:1. The experiment was performed with ammonium concentration = 1000 mgNH_4^+-N/L and C:N ratio = 10:1. The experiment was performed with high ammonium and glucose concentrations to ensure that they are not limited at any time during the experiment and that the effect of phosphate concentration on biomass growth can be independently of these limitations be investigated.

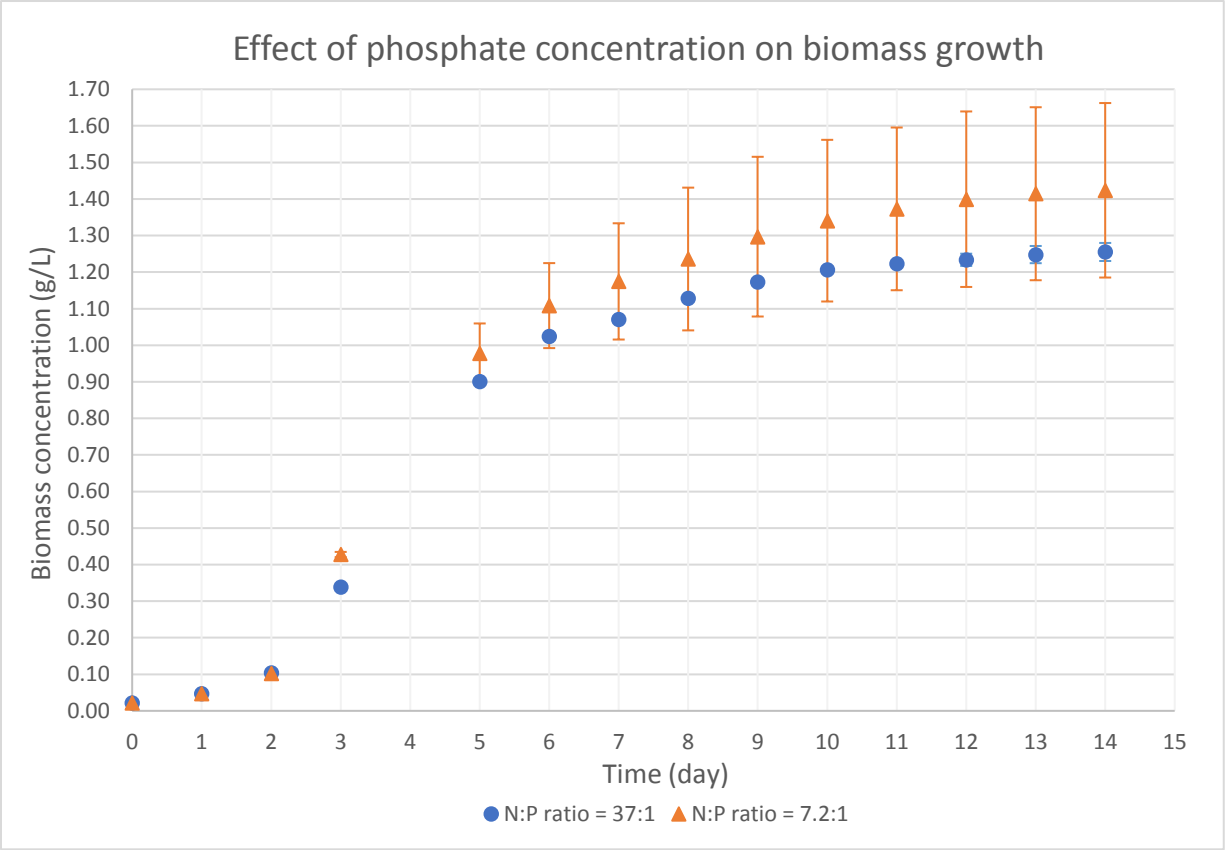

Figure 18a: Effect of phosphate concentration on biomass growth (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 1000 mg NH_4^+-N/L, C:N ratio on day 0 = 10:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

It can be observed from Figure 18a that the culture mixotrophic of *G. sulphuraria* with N:P ratio = 7.2:1 resulted to a higher average biomass concentration (1.42 g/L) than with N:P ratio = 37:1 which resulted to an average biomass concentration of 1.26 g/L. Based only on the average biomass concentrations of *G. sulphuraria* after 14 days of cultivation, one may conclude that the N:P ratio = 7.2:1 produced about 13% more biomass than *G. sulphuraria* with N:P ratio = 37:1. However, from Figure 18a, it can be seen that the culture with N:P ratio = 7.2:1 had a higher standard deviation than the culture with N:P ratio = 37:1 and this is because one of the two cultures grown with N:P ratio = 7.2:1 had grown relatively higher than the other (see Figure 18b). From Figure 18b, it can be observed that the culture with N:P ratio = 7.2:1 – culture 2 was within the same range as the cultures with N:P ratio = 37:1 while the culture with N:P ratio = 7.2:1 – culture 1 was not. No presence of external organisms and phosphate precipitation were observed when the cultures with the N:P ratio = 7.2:1 and 37:1 were analysed under microscope. It is therefore necessary to repeat the measurements before drawing any firm conclusions. The μ_{max} values of *G. sulphuraria* under the different phosphate concentrations are shown in Table 7.

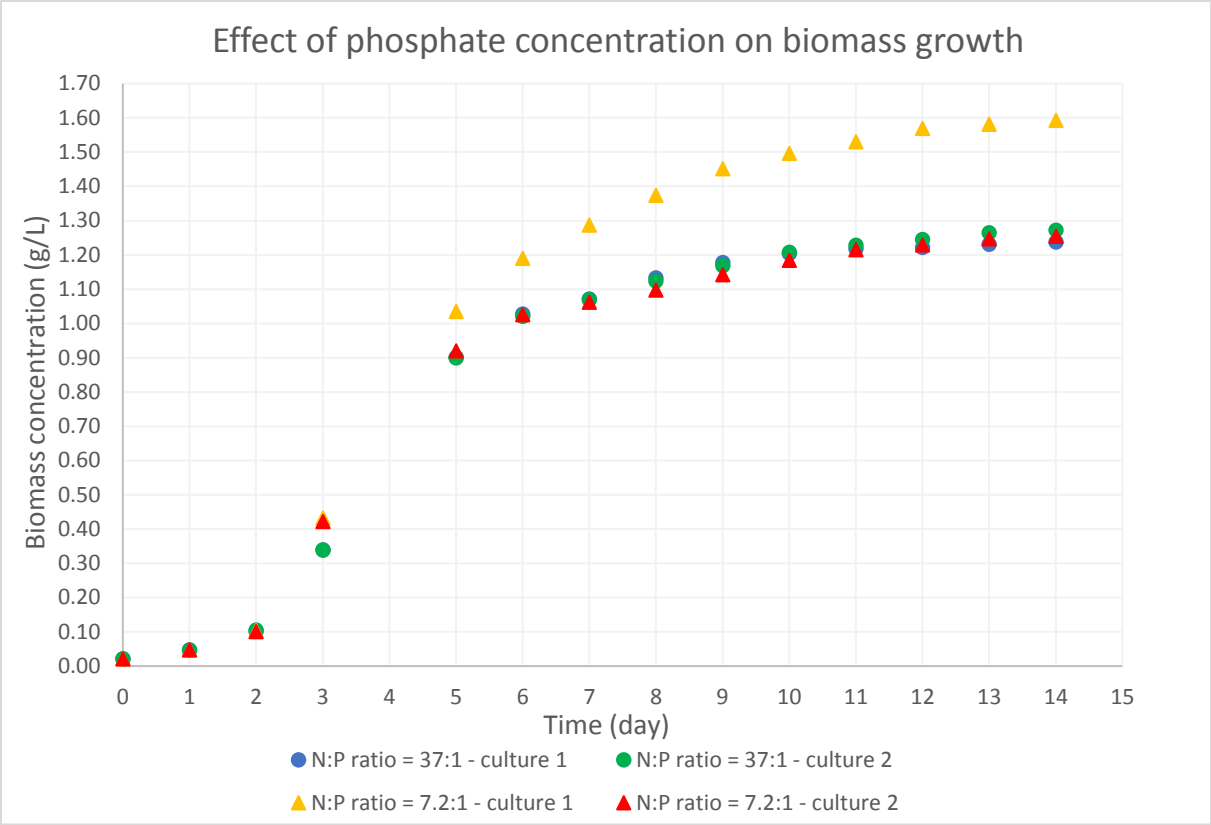

Figure 18b: Effect of phosphate concentration on biomass growth - single measurements (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 1000 mg NH_4^+-N/L, C:N ratio on day 0 = 10:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Table 7: μ_{max} values of *G. sulphuraria* under different phosphate concentrations (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 1000 mg NH_4^+-N/L, C:N ratio on day 0 = 10:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Phosphate concentration (mg PO_4^{3-}-P/L)	N:P ratio	μ_{max} (day^{-1})
27.0	37:1	0.74 ± 0.003
138.9	7.2:1	0.76 ± 0.02

From Table 7, it can be observed that there was a very small significant difference between the maximum specific growth rates of *G. sulphuraria* under N:P ratio = 37:1 and 7.2:1 respectively. Adding about five times more phosphate to a culture results to almost the same maximum growth rate of the *G. sulphuraria* (0.76 day^{-1}) as a culture having five times less phosphate (0.74 day^{-1}).

The theoretical phosphorus concentrations required for the maximum biomass concentration reached for the cultures with N:P ratio = 37:1 and 7.2:1 respectively are shown below:

Maximum average biomass concentration obtained with the culture having N:P ratio = 37:1 and 7.2:1 was 1.26 and 1.42 g/L respectively. Using the biomass formula of *G. sulphuraria*: $C_{106}H_{263}O_{110}N_{16}P$, it can be deduced that for 1.26 g/L of biomass, 11.0 mg/L of PO_4^{3-}-P was

required while for 1.42 g/L of biomass, 12.4 mg/L of PO_4^{3-}-P was required. Concentrations of phosphorus supplied to the cultures are shown in Table 7 and were as follows: 27.0 and 138.9 mg/L of PO_4^{3-}-P for the cultures with N:P ratio = 37:1 and 7.2:1 respectively. Therefore, it can concluded that theoretically there was no limitations in phosphate concentrations in any of the two cultures during the experiment. Dosing as less external nutrients (phosphate and glucose) as possible is also the goal of the NEREUS project and cultivation of *G. sulphuraria* will be economically more feasible if a phosphate concentration = 27.0 mg PO_4^{3-}-P/L (N:P ratio = 37:1) is used.

Note: From day 6 till day 14 of the cultivation period, there was a relative decreased in the amount of biomass produced compared to the amount of biomass produced during the first 5 days of cultivation. Also, the growth curve reached almost a plateau from day 12 till day 14 even when there were still a lot of nutrients present in the medium. Possible reasoning for the decreased in the growth of G. sulphuraria from day 6 onwards might be due to light limitation because of the very dense culture and the effect of culture density on growth of G. sulphuraria was investigated in section 4.1.7.

4.1.7 Culture densities higher than 0.7 g/L of biomass resulted to slower growth of *G. sulphuraria*

Culture density is closely correlated to microalgae growth. From previous section, section 4.1.5, it was deduced that the growth of *G. sulphuraria* was probably limited by light when the *G. sulphuraria* culture became too dense. Therefore, this section investigated the effect of culture density on the growth of *G. sulphuraria*. Figure 19 shows the effect of culture density on growth of *G. sulphuraria*.

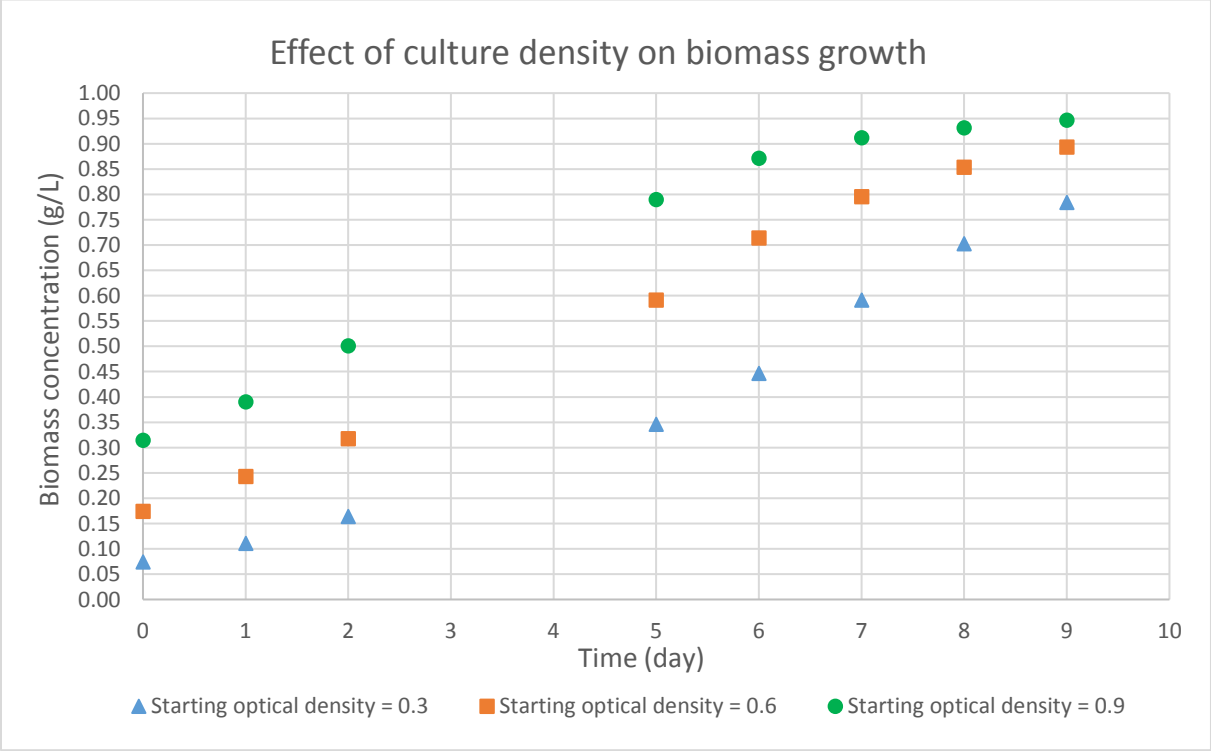

Figure 19: Effect of culture density on biomass growth (Mixotrophic culture, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaking speed = 145 rpm). Note: Standard deviation not included because the cultures were not grown in duplicate.

From Figure 19, it can be observed from the culture with starting optical density = 0.6 and 0.3 that there was a significant decrease in their rates of growth for culture densities higher than 0.7 g/L of biomass. Besides, taking day 5 as reference point, it can be observed that the culture with starting optical density = 0.9 grew at a slower rate compared to the culture with starting optical density = 0.6 and 0.3. This is because light was limiting at higher culture densities and not the nutrients. To verify whether light was the limiting factor at higher culture densities, the glucose concentration was measured at the start and at the end of the experiments in order to determine the change in glucose uptake for each culture and the results are shown in Table 8. The change in biomass concentration for each culture was also calculated from the measurements done at the start and at the end of the experiments and are shown in Table 8.

Table 8: Change in glucose uptake and biomass concentration for each culture (Mixotrophic culture, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaking speed = 145 rpm).

Starting optical density of culture	Change in biomass concentration, ΔDW (g/L)	Change in glucose concentration, Δglu (g/L)	Ratio (ΔDW/Δglu)
0.30	0.71	1.49	0.48
0.60	0.72	1.51	0.48
0.90	0.63	1.35	0.47

From Table 8, it can be observed that the change in biomass formed was proportional to the change in glucose concentration uptaken, resulting in similar ratio of change in biomass concentration to change in glucose concentration (0.47 – 0.48) for all the cultures. Light was indeed limiting at higher culture densities since the change in biomass formed was proportional to the change in glucose concentration uptaken for all cultures. A high biomass density results in a denser culture and in a slower growth of *G. sulphuraria* because denser culture allows less incoming light to pass through the medium if the light path is too long. The results of this experiment is in agreement with literature, where it stated that a higher biomass concentration prevent algae cells from the incoming radiation if the light path is too long (van der Hulst, 2012).

Note: All the three cultures had the same ratio of amount of substrates to the amount of biomass at the start of the experiments and all of them were cultivated under the same conditions. Table 9 shows the ratio of the glucose concentration to the biomass concentration ($\frac{DW_{start}}{Glu_{start}}$) for each culture at the start of the experiments. It can be observed from Table 9 that the $\frac{DW_{start}}{Glu_{start}}$ resulted in almost the same value for all the cultures (≈ 20 -21).

Table 9: $\frac{DW_{start}}{Glu_{start}}$ for each culture at the start of the experiment (Mixotrophic culture, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaking speed = 145 rpm). Note: Standard deviation not included because the cultures were not grown in duplicate

Starting optical density of culture	Biomass concentration at the start of experiment, DW_{start}, (g/L)	Glucose concentration at the start of experiment, Glu_{start}, (g/L)	$\frac{DW_{start}}{Glu_{start}}$
0.30	0.074	1.53	20.7
0.60	0.174	3.44	19.8
0.90	0.314	6.26	19.9

4.1.8 Higher ammonium concentration, phosphate concentration or glucose concentration resulted to no significant change in the ratio of absorbance at 618 nm to absorbance at 800 nm.

The ratio of absorbance at 618 nm to the absorbance at 800 nm ($\frac{A_{618\,nm}}{A_{800\,nm}}$) gives an idea of the amount of pigment produced with respect to the amount of biomass produced and this can be a determining factor for the cultivation of *G. sulphuraria* for phycocyanin production. The higher the $\frac{A_{618\,nm}}{A_{800\,nm}}$, the higher the amount of pigment produced with respect to the amount of biomass produced. Table 10, 11 and 12 shows the $\frac{A_{618\,nm}}{A_{800\,nm}}$ at different ammonium concentrations, different phosphate concentrations and different glucose concentrations respectively. Table 10 and 12 only shows data up to day 6 because after day 6 cultures were disturbed due to the shaker inside the incubator which was incidentally turned off.

From Table 10, it can be observed that there was no significant change in the $\frac{A_{618\,nm}}{A_{800\,nm}}$ when the ammonium concentration was increased from 100 to 1000 mgNH_4^+-N/L. Moreover, from Table 11, it can be observed that increasing the phosphate concentration by five times resulted in no significant change in the $\frac{A_{618\,nm}}{A_{800\,nm}}$. Increasing the glucose concentration from a C:N ratio = 5:1 to a C:N ratio = 10:1 also did not result to a significant change in the $\frac{A_{618\,nm}}{A_{800\,nm}}$ as seen from Table 12. Furthermore, it was observed from Table 10, 11 and 12 that during the growth of the *G. sulphuraria*, there no significant change in the $\frac{A_{618\,nm}}{A_{800\,nm}}$ at the start of the experiment compared to the $\frac{A_{618\,nm}}{A_{800\,nm}}$ at the end of the experiment. The $\frac{A_{618\,nm}}{A_{800\,nm}}$ was determined in order to know what was the optimal time for pigment extraction during the cultivation period. However, from the results obtained from absorbance measurements, the optimal time for pigment extraction during the cultivation of *G. sulphuraria* could not be determined.

Table 10: $\frac{A_{618\,nm}}{A_{800\,nm}}$ at different ammonium concentrations (Mixotrophic metabolism, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

	A 618nm / A 800nm					
Ammonium concentration (mgNH4+-N)	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 5**	**Day 6**
100	1.116	1.107	1.083	1.078	1.075	1.072
200	1.135	1.136	1.125	1.104	1.091	1.085
400	1.128	1.106	1.088	1.091	1.086	1.069
600	1.132	1.137	1.114	1.096	1.089	1.073
800	1.108	1.108	1.102	1.089	1.083	1.088
1000	1.122	1.117	1.093	1.086	1.077	1.083

Table 11: $\frac{A_{618\,nm}}{A_{800\,nm}}$ at different phosphate concentrations (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 1000 mg NH_4^+-N/L, C:N ratio on day 0 = 10:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

		A 618nm / A 800nm												
		Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
N:P ratio	**37:1**	1.115	1.106	1.081	1.088	1.076	1.073	1.070	1.065	1.067	1.066	1.067	1.064	1.066	1.063
	7.2:1	1.111	1.101	1.085	1.085	1.084	1.080	1.079	1.075	1.072	1.062	1.065	1.062	1.064	1.062

Table 12: $\frac{A_{618\,nm}}{A_{800\,nm}}$ at different glucose concentrations (Conc. of NH_4^+ on day 0 = 200 mg NH_4^+- N/L, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

	A 618nm / A 800nm					
C:N ratio	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 5**	**Day 6**
5:1	1.135	1.136	1.125	1.104	1.091	1.085
10:1	1.142	1.134	1.129	1.097	1.088	1.083

4.2 Results and discussion from synthetic and real RO concentrate experiments

4.2.1 Cultivation of *G. sulphuraria* with synthetic RO concentrate medium

4.2.1.1 Biomass and nutrient consumption models with no light limitation included in the model

Figure 20 shows the biomass and nutrient consumption models with no light limitation included in the model. The experiment was performed with ammonium concentration = 200 mgNH_4^+-N/L, N:P ratio = 37:1 and C:N ratio = 5:1. The biomass and nutrient consumption models were obtained from Aquasim.

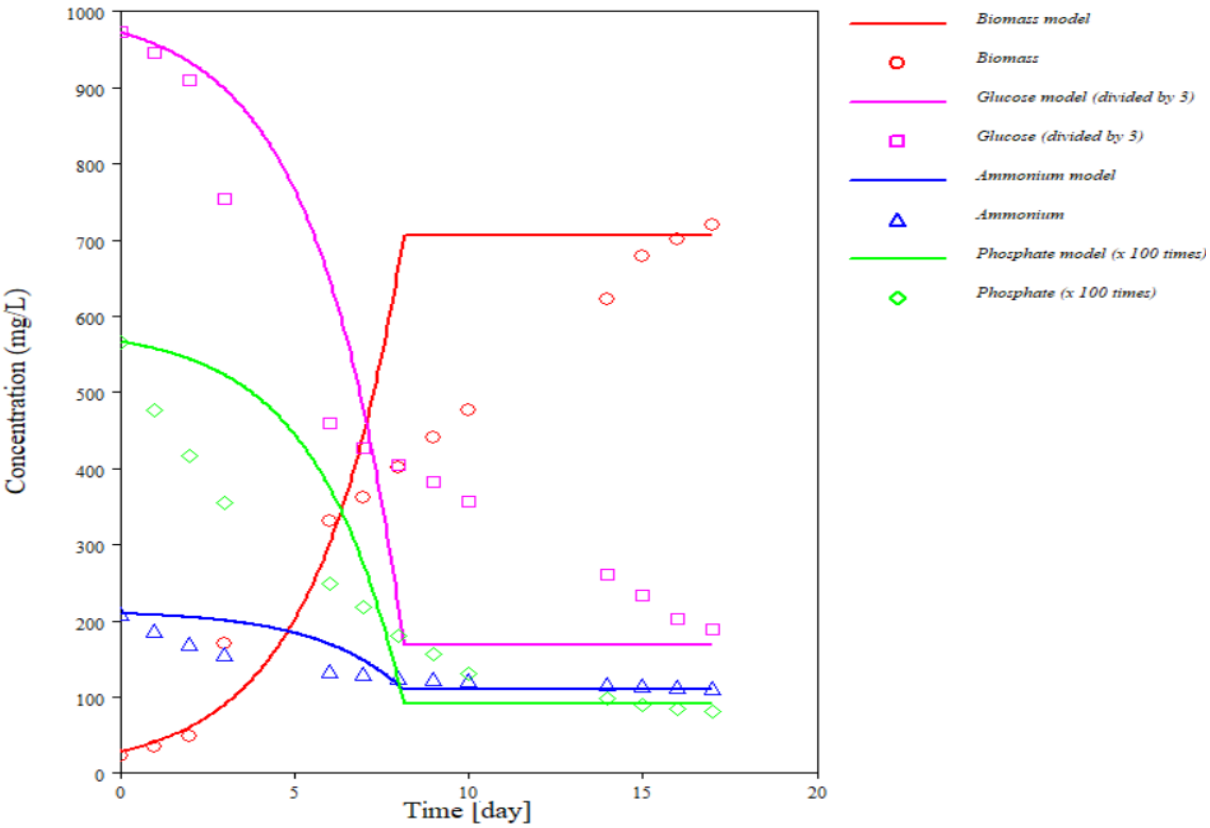

Figure 20: Biomass and nutrient consumption models with synthetic RO concentrate medium and with no light limitation included in the model (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L , C:N ration on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: For good visualization purpose, the glucose concentration and glucose model are shown three times less. The phosphate concentration and phosphate model shown are 100 times more. The rates of the processes, kinetics, μ_{max} and biomass yields were determined with the concentrations obtained during sampling.

From Figure 20, it can be observed that the biomass and nutrient consumption fitted Aquasim models did not fit very well with the data sets obtained from the daily sampling. The data sets in Figure 20 shows how the nutrients present in the medium were uptaken by the *G. sulphuraria* with time and it also shows the biomass growth pattern for the 17 days of culture period. The difference between the models and the data obtained from sampling can be due to limitations

in the growth during the culture period. From the biomass model it can clearly be observed that after day 6 there were limitations in the growth because the fitted models deviated from the data sets and the model did not account for external limitation factors such as light for example. The effect of light limitation was also investigated and implemented in the rates of the processes. The models with light limitation are shown below in Figure 21.

4.2.1.2 Biomass and nutrient consumption models with light limitation included in the model

Light is one of the most important variable in algae growth. Figure 21 shows the biomass and nutrient consumption models with synthetic RO concentrate medium with light limitation included in the model.

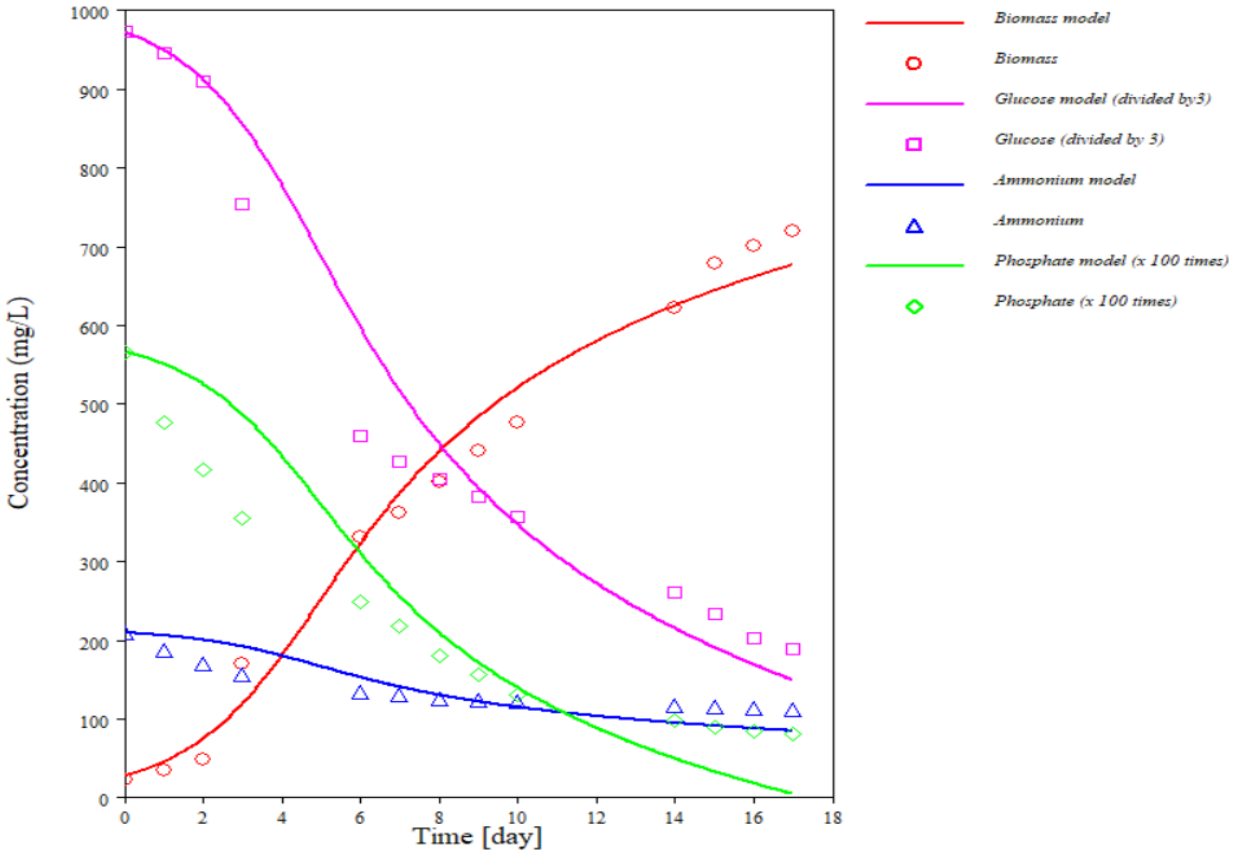

Figure 21: Biomass and nutrient consumption models with synthetic RO concentrate medium under light limitation included in the model (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L , C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: For good visualization purpose, the glucose concentration and glucose model are shown three times less. The phosphate concentration and phosphate model shown are 100 times more. The rates of the processes, kinetics, μ_{max} and biomass yields were determined with the concentrations obtained during sampling.

From Figure 21 it can be observed that better fitted models were obtained with the implementation of the light limitation and therefore confirms that light was limiting during the

culture period, especially after day 6. However, perfect fitted models were not obtained, and this might be because of other limiting factors which were not investigated. The μ_{max} and maximum biomass yields for the experiment with synthetic RO concentrate were obtained from Aquasim by parameter fitting and are shown in Table 13.

Table 13: μ_{max} and maximum biomass yields for the experiment with synthetic RO concentrate medium at temperature = 35 ℃ and pH = 2

μ_{max} (day^{-1})	$Y_{x/glc,max}$ (g of TSS g^{-1})	$Y_{x/NH4^+,max}$ (g of TSS g^{-1})	$Y_{x/PO4^{3-},max}$ (g of TSS g^{-1})
0.65	0.26	5.22	116

From Sloth et al., (2017), experiments were performed at almost the same temperature (34°C) and C:N ratio = 10:1 resulted in μ_{max} = 0.65 day^{-1}, yield of biomass on glucose, $Y_{x/glc}$ = 0.51 g of TSS g^{-1} and yield of biomass on ammonium, $Y_{x/NH4+}$ = 14.75 g of TSS g^{-1}. Moreover, from Sloth et al., (2017), experiments were also performed at temperature 40 °C and C:N ratio = 10:1 resulted in μ_{max} = 1.22 day^{-1}, yield of biomass on glucose, $Y_{x/glc}$ = 0.55 g of TSS g^{-1} and yield of biomass on ammonium, $Y_{x/NH4+}$ = 13.7 g of TSS g^{-1}. From this we can deduce that μ_{max} is the most sensitive to small changes in temperature. Other literatures obtained μ_{max} = 1.04 - 1.26 day^{-1} and yield of biomass on glucose, $Y_{x/glc}$ = 0.41 – 0.48 g of TSS g^{-1} at higher temperatures (40 - 42°C) (Graverholt & Eriksen, 2007; Schmidt, Wiebe, & Eriksen, 2005; Sloth et al., 2006). The yield of biomass on phosphate was not determined in those literatures. The μ_{max} obtained in this experiment was similar to that from Sloth et al., (2017) but in our case a higher μ_{max} value was expected at temperature 35 °C if there was no light limitation. The maximum yield of biomass on glucose and ammonium obtained from this experiment, 0.26 g of TSS g^{-1} and 5.22 g of TSS g^{-1} respectively, was lower compared to the ones obtained from literatures. Storage of substrates might have occurred during the experiment, leading to lower concentration of the substrates present in the medium.

4.2.2 Cultivation of *G. sulphuraria* with real RO concentrate medium

The experiment with real RO concentrate was conducted with a mix of real RO concentrate and synthetic RO concentrate (20% real RO concentrate + 80% synthetic RO concentrate and 40% real RO concentrate + 60% synthetic RO concentrate) and 100% real RO concentrate as shown in Figure 23. A mix of real RO concentrate and synthetic RO concentrate was considered because the first time *G. sulphuraria* was grown on 100% real RO concentrate, no growth was observed and the possible reason why that the *G. sulphuraria* did not grow on 100% RO concentrate was because the RO concentrate on that particular day of the experiment had an unusual high iron concentration of about 15.0 mg/L. From Wang et al., 2010, it was observed that the growth of Microcystis aeruginosa, cyanobacterium, produces phycocyanin as pigment, was inhibited at concentration of iron = 1.38 mg/L and therefore, it was highly probably that the *G. sulphuraria* did not grow on 100% RO concentrate because of the very high iron concentration. Figure 22 shows a comparison of the *G. sulphuraria* culture grown for the first time on 100% real RO concentrate having an iron concentration = 15.0 mg/L with the *G. sulphuraria* culture grown on synthetic RO concentrate.

Figure 22: No growth of *G. sulphuraria* for the first time growth on 100% real RO concentrate having an iron concentration = 15.0 mg/L (left) and *G. sulphuraria* culture grown on synthetic RO concentrate (right) (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L , C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Moreover, a mix of real RO concentrate and synthetic RO concentrate was considered in order to monitor whether there is any inhibition with increase in real RO concentrate because of the possible presence of various different types of metals, including heavy metals, in the RO concentrate as its source is municipal and household wastewater. It can be observed from Figure 23 that the first 5 days of the experiment was performed with C:N ratio = 5:1 and N:P ratio = 37:1, same conditions as the synthetic RO concentrate experiment. After 5 days of cultivation almost all the phosphate and glucose in the cultured were consumed and therefore more phosphate and glucose were added to the culture for continuation of the growth of the *G. sulphuraria*. The C:N and N:P ratio after 5 days of cultivation was higher than that of the first 5 days of cultivation in order to make sure that there is sufficient nutrient in the culture because the next sampling was scheduled for day 10 due to public holiday issues.

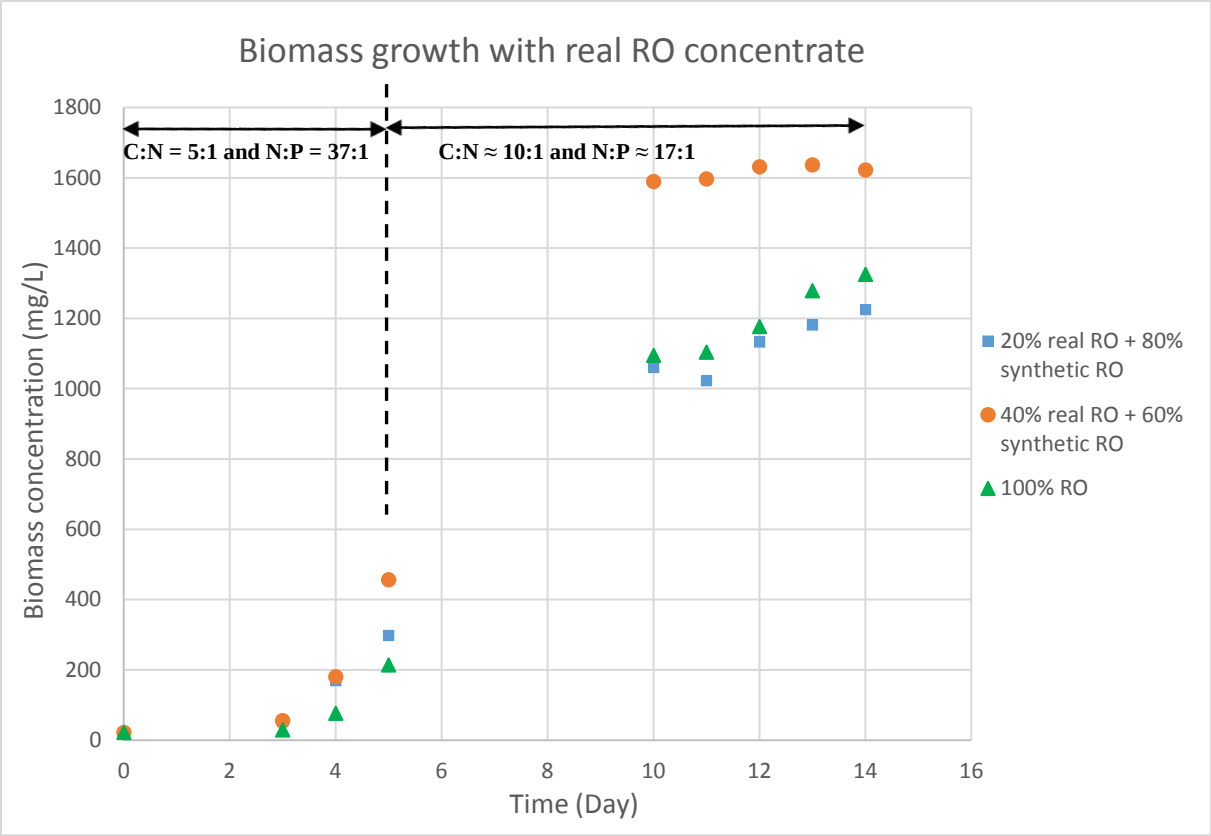

Figure 23: Biomass growth with real RO concentrate (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L , pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: Standard deviation not included because the cultures were not grown in duplicate.

From Figure 23 it can be observed that the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium produced the highest growth of *G. sulphuraria*, biomass concentration ≈ 1.6 g/L after 14 days of cultivation. The mix of 40% real RO concentrate and 60% synthetic RO concentrate medium produced the highest specific growth rate (0.77 day^{-1}) than the other two cultures (0.56 day^{-1} and 0.48 day^{-1}) as shown in Table 14. The 20% real RO concentrate and 80% synthetic RO concentrate medium and 100% real RO concentrate medium attained almost the same growth after 14 days of cultivation. The two mixed RO concentrate media grew almost at the same rate during the first 4 days and after that the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium grew at a faster rate. The 100% RO concentrate had a longer lag phase (≈ 3 days) compared to the mix RO concentrate media (< 3 days) because the *G. sulphuraria* needed to acclimate itself to the new environment.

Table 14 shows the μ_{max} and maximum biomass yields for the experiment involving real RO concentrate. The μ_{max} and maximum biomass yields were determined from the growth and nutrient consumption during the first 5 days because there was data from day 0 till day 5 and from day 10 till day 14 and the exponential growth took place between day 0 and day 5.

Table 14: μ_{max} and maximum biomass yields for the experiments with real RO concentrate at temperature = 35 ℃ and pH = 2.

Medium	μ_{max} (day^{-1})	$Y_{x/glc,max}$ (g of TSS g^{-1})	$Y_{x/NH4^+,max}$ (g of TSS g^{-1})	$Y_{x/PO4^{3-},max}$ (g of TSS g^{-1})
20% real RO conc + 80% synthetic RO conc	0.56	0.12	2.19	41.7
40% real RO conc + 60% synthetic RO conc	0.77	0.14	2.58	50.0
100% real RO conc	0.48	0.10	1.52	38.5

The biomass and nutrient consumption fitted Aquasim models with real RO concentrate medium for the first 5 days of cultivation under light limitation can be found in Appendix A.3. The 14 days of nutrient consumptions of *G. sulphuraria*, for each media can be found in Appendix A.4. From Table 14, it can be seen that the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium produced the best results compared to the other two media.

4.2.2.1 Comparison of results from synthetic RO concentrate and real RO concentrate

The μ_{max} and maximum biomass yields values from the experiments with the mixed RO concentrate, 100% real RO concentrate and synthetic RO concentrate are shown in Table 15.

Table 15: μ_{max} and maximum biomass yields values from the experiments with the mixed RO concentrate, 100% real RO concentrate and synthetic RO concentrate at temperature = 35 ℃ and pH = 2.

Medium	μ_{max} (day^{-1})	$Y_{x/glc,max}$ (g of TSS g^{-1})	$Y_{x/NH4^+,max}$ (g of TSS g^{-1})	$Y_{x/PO4^{3-},max}$ (g of TSS g^{-1})
100% synthetic RO conc	0.65	0.26	5.22	116
20% real RO conc + 80% synthetic RO conc	0.56	0.12	2.19	41.7
40% real RO conc + 60% synthetic RO conc	0.77	0.14	2.58	50.0
100% real RO conc	0.48	0.10	1.52	38.5

Comparing the μ_{max} values of Table 15, it can be seen that the μ_{max} values from the two mixed RO concentrate media (0.56 day^{-1} and 0.77 day^{-1}) were closer to the μ_{max} obtained from the synthetic RO concentrate medium (0.65 day^{-1}) than that from the 100% real RO (0.48 day^{-1}). The μ_{max} from the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium was higher than that from the other 2 media involving real RO concentrate and also higher than that obtained from the synthetic RO concentrate medium. This was not expected because the mix of 20% real RO concentrate and 80% synthetic RO concentrate medium is closer to the

synthetic RO concentrate medium in terms of compositional ingredients and therefore the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium was expected to have a lower μ_{max} than the mix of 20% real RO concentrate and 80% synthetic RO concentrate medium. The higher μ_{max} obtained from the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium than the μ_{max} of the other media might be due to the presence of other metals in the real RO concentrate, which are present in optimum concentrations for faster growth of *G. sulphuraria*. Real RO concentrate from NEREUS may contain many more nutrients or metals other than ammonium that algae can feed on because its source is from municipal and domestic wastewater. For instance, iron, which is essential for cell growth is present in the RO concentrate from NEREUS. For this experiment, the measured iron concentration in the RO concentrate from NEREUS (100% real RO concentrate) was 3.88 mg/L and the concentration of iron in the two mixed RO concentrate media as shown in Table 16.

Table 16: Iron concentration in the medium

Medium	Fe (mg/L)
20% real RO conc + 80% synthetic RO conc	0.82
40% real RO conc + 60% synthetic RO conc	1.70
100% real RO conc	3.88

From literature, a study done on Microcystis aeruginosa, cyanobacterium, which produces phycocyanin as pigment shown that the μ_{max} is limited to iron concentration below 0.69 mg/L and is inhibited when concentration of iron is 1.38 mg/L. The maximum μ_{max} is observed at iron concentration = 0.69 mg/L (Wang et al., 2010). Besides, comparing data from Table 14 and Table 16, it can be observed that there is a transitional point at iron concentration = 1.70 mg/L and that μ_{max} for *G. sulphuraria* was limited when iron concentration was 0.82 mg/L and was inhibited when concentration of iron was 3.88 mg/L.

However, we cannot conclude that the higher μ_{max} obtained with the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium was due to the iron concentration (1.70 mg/L) because there might be some other untested nutrients or metals, which were present in optimal concentrations in the RO concentrate from NEREUS and which contributed or were even the main reasons behind the higher μ_{max}. This is because the μ_{max} obtained from the synthetic RO concentrate medium was 0.65 day^{-1} with iron concentration of 0.02 mg/L, which is slightly a lower μ_{max} than that with the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium containing iron concentration of 1.70 mg/L and on the other hand the μ_{max} obtained from the synthetic RO concentrate medium is slightly higher than that with the mix of 20% real RO concentrate and 80% synthetic RO concentrate medium containing iron concentration of 0.82 mg/L.

In addition, comparing the maximum biomass yields from Table 15, it can be seen that the maximum biomass yields from the mix and 100% real RO concentrate media were not in the same range as the maximum biomass yields obtained from the synthetic RO concentrate medium. Figure 24 shows the change in biomass concentrations and the respestive amount of

each nutrient consumed for the synthetic and real RO concentrate experiments.

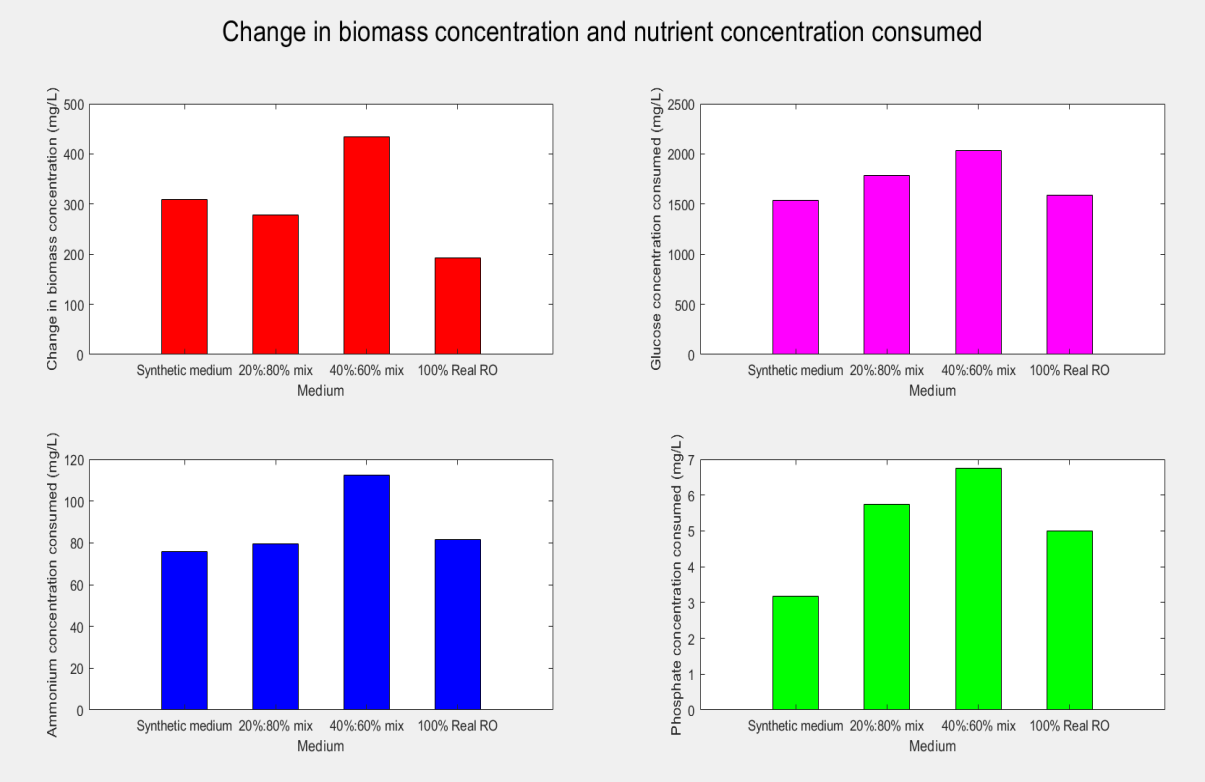

Figure 24: Showing the change in biomass concentration and the respective amount of each nutrient consumed for the synthetic and real RO concentrate experiments. Note: Data sets used to make Figure 23 were as follows: the mixed RO concentrate and 100 real RO concentrate results were data from day 0 till day 5 while the synthetic RO concentrate results were data from day 0 till day 6. The slight difference is due to the fact that the both experiments were not conducted at the same time and depending on when the experiments were started, the days of sampling were different due to no sampling during weekends. Standard deviation not included because the cultures were not grown in duplicate.

From Figure 24, it can be deduced that the culture grown on the mix of 20% real RO concentrate and 80% synthetic RO concentrate and the culture grown on 100% real RO concentrate produced less biomass in 5 days of cultivation than the culture grown on synthetic RO concentrate in 6 days of cultivation. In addition, the mixed RO concentrate media consumed more substrates in 5 days of cultivation than the culture grown on synthetic RO concentrate in 6 days of cultivation Therefore, less biomass was produced while more substrates were consumed by *G. sulphuraria* grown on the mix of 20% real RO concentrate and 80% synthetic RO concentrate medium and on the 100% real RO concentrate medium, resulting in lower biomass yields than those obtained from the culture grown on synthetic RO concentrate as shown in Table 15. From Figure 24, it can be observed that the culture cultivated on the mix of 40% real RO concentrate and 60% synthetic RO concentrate produced more biomass in 5 days of cultivation than the culture grown on synthetic RO concentrate in 6 days of cultivation. However, the resulting biomass yields for the culture cultivated on the mix of 40% real RO concentrate and 60% synthetic RO concentrate were still lower than those obtained from the culture grown on synthetic RO concentrate and this was also observed from Table 15.

The nutrients in the real RO media were consumed more rapidly but the amount of biomass produced was not in equilibrium with the amount of nutrients consumed. It might be that there were some other organisms feeding on the nutrients in the media because from wastewater there is high possibility of having organisms in the RO concentrate. Samples of the culture during the cultivation period were analysed under the microscope to check whether there was any other organisms in the culture but no other organisms were found. Moreover, the possibility of storing nutrients within the cell, that is not all nutrients consumed are directly used for growth but are stored for use during periods where they are limited might be a reason for the faster nutrients uptake from the culture. For instance, the fast uptake of glucose can be due to accumulation of lipids inside the cells of the *G. sulphuraria*.

4.2.2.2 Considerations for NEREUS pilot plant for a continuous chemostat system

A chemostat is a bioreactor to which medium is continuously added, while the culture is also continuously removed at the same rate to keep the culture volume constant. Figure 25 shows a design of the continous chemostat system.

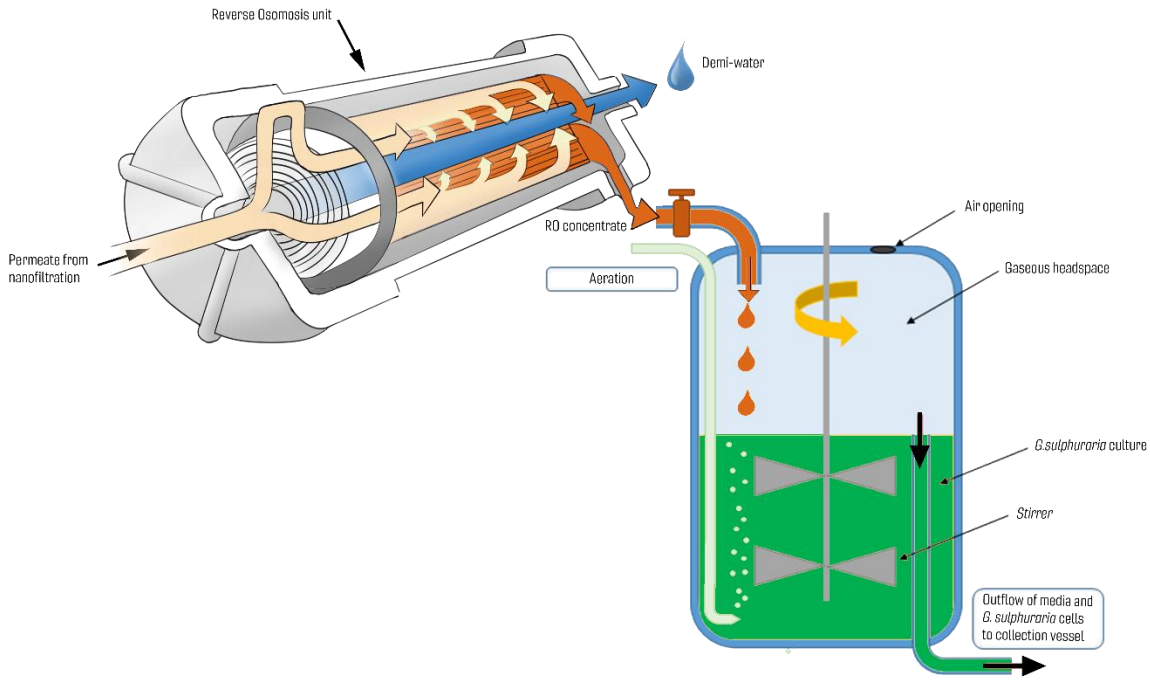

Figure 25: Design of the continous chemostat system

Moreover, using Table 14, Appendix A.4 and data from NEREUS: flowrate of RO concentrate = 50 L/h (1200 L/d) and average ammonium concentration in medium = 200 mgNH_4^+-N /L, the volume of a reactor needed to treat the ammonium present in the medium via the cultivation of *G. sulphuraria*, the amount of biomass that can be produced and value of the biomass produced are shown in Table 17. Table 17 shows the key parameters and calculations for a

continuous chemostat system, the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium was compared with the 100% real RO concentrate medium. The volume of the reactor was calculated from the flowrate, average ammonium concentration and the maximum ammonium consumption rate, as is shown below:

$$\textbf{Volume of reactor} = \frac{\textbf{Flowrate} * \textbf{average ammonium concentration}}{\textbf{Maximum ammonium consumption rate}}$$

The amount of biomass produced is calculated as follows:

$$\textbf{Amount of biomass produced} = \textbf{Flowrate} * \textbf{average ammonium concentration} * Y_{x/NH_4^+,max}$$

The value of dry biomass is 100 euros/kg (Wijffels et al., 2010). Therefore the value of the biomass produced was calculated as follows:

$$\textbf{Value of biomass produced} = \textbf{amount of biomass produced in kg} * \textbf{100 euros/kg}$$

The same amount of ammonium considered in both media was the same. It has to be noted that only 40% of the amount of ammonium in the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium originates from the RO concentrate of NEREUS and the rest of the 60% is external ammonium. The calculations accounts for 100% removal of the ammonium present in the medium.

Table 17: Key parameters and calculations for a continuous chemostat system

	Medium	
	40% real RO + 60% synthetic RO	**100% real RO**
Flowrate of medium (m^3/d)	1.2	1.2
Average NH4+ concentration in medium (mg NH4+-N/L)	200	200
Maximum NH4+ consumption rate (mgNH4+-N/L/d)	30.6	17.25
Volume of reactor needed (m^3)	7.8	13.9
$Y_{x/NH4+,max}$ (g of TSS g^{-1})	2.58	1.52
Amount of biomass produced (kg/d)	0.62	0.36
Value of biomass produced (euros)	62	36

The pigments (phycocyanin) values were not calculated because the price varies a lot, depending on the purity of the product. The price varies from 1000 euros/kg to 15 million euros/kg (Sigma-Aldrich, n.d.; Tabernero et al., 2012). From Table 17, it can be observed that a smaller volume of reactor (7.8 m^3) will be required to treat the ammonium with the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium than with the 100% real RO

concentrate (13.9 m^3). In addition, it can also be observed that the amount of biomass produced from the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium is about 1.7 times that from the 100% real RO. The only problem with the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium is that only 40% of the ammonium from the RO concentrate from NEREUS will be re-used for algae growth and the 60% of the external ammonium added will increase the operational and capital costs of ammonium solely. However, having a smaller volume means less energy for operation of the reactor and producing more biomass means more product can be extracted and therefore more income. Comparing the benefits and drawbacks of both media, it can be concluded that growing *G. sulphuraria* on the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium will be more profitable than on the 100% real RO concentrate.

4.2.3 Overall discussion

Ammonium with mixotrophic metabolism turned out to be the best nitrogen source. This is because ammonium is energetically a more efficient nitrogen source, requiring less energy for its uptake (Goldman, 1977; Grobbelaar, 2007; Shi et al., 2000; Syrett & Morris, 1963; Wilhelm et al., 2006). The mixotrophic culture produced about twice more biomass than the heterotrophic culture and about six times more biomass than the autotrophic culture. Biomass concentration on ammonium was four times higher than on nitrate. Mixotrophic metabolism resulted to the best metabolism because the specific growth rate of mixotrophic cultures is approximately the sum of the cell specific growth rates of the autotrophic and heterotrophic cultures (Perez-Garcia et al., 2011). Under mixotrophic growth conditions, light partially provides the energy and the organic carbon source can mostly be used for biomass formation, thus resulting in higher growth and growth rates of the mixotrophic cultures compared to heterotrophic and autotrophic cultures. Higher ammonium concentrations resulted to higher growth and growth rates of the *G. sulphuraria* because lower ammonium concentration was faster limited in phosphate and glucose concentration because the C:N and N:P ratio was kept constant in this experiment at 5:1 and 37:1. Therefore, it was observed that phosphate and glucose play an important role in the growth of *G. sulphuraria*. Higher glucose concentration resulted to no significant change in the growth of the *G. sulphuraria* because both C:N ratio of 5:1 and 10:1 was not limited after 6 days of cultivations according to the theoretical calculations. However, supplementation of bicarbonate and CO_2 gas to the culture did not result to higher biomass concentrations because with bicarbonate addition, the pH of the medium was 8 and at that pH the *G. sulphuraria* cannot grow. Adding carbon dioxide to the medium was also not efficient because during the flushing of the medium with carbon dioxide most of the oxygen in the headspace and liquid was removed and *G. sulphuraria* requires oxygen for their growth. Moreover, no firm conclusions could be drawn from the experiment performed with different phosphate concentrations because it was observed from Figure 18b that the culture with N:P ratio = 7.2:1 – culture 2 was within the same range as the cultures with N:P ratio = 37:1 while the culture with N:P ratio = 7.2:1 – culture 1 was not. Furthermore, a significant decrease in the rates of growth for culture densities higher than 0.7 g/L of biomass was observed because light was limiting at higher culture densities. Verification of whether light was the limiting factor at

higher culture densities was done by measuring the change in biomass formed for each culture as well as the change in glucose concentration uptaken. For all the cultures the change in biomass formed was proportional to the change in glucose concentration uptaken, showing that light was indeed the limiting factor. When the *G. sulphuraria* was grown on the RO concentrate of NEREUS, it was found out that the mix of 40% real RO concentrate and 60% synthetic RO concentrate medium resulted in higher biomass concentrations (≈ 1.6 g/L) compared to the other RO concentrate cultures (≈ 1.2 – 1.3 g/L) after 14 days of cultivation. The mix of 40% real RO concentrate and 60% synthetic RO concentrate medium produced the highest specific growth rate (0.77 day^{-1}) than the other two RO concentrate cultures (0.56 day^{-1} and 0.48 day^{-1}). However, lower biomass yields were obtained from the cultures cultivated on RO concentrate of NEREUS than those obtained from the culture grown on synthetic RO concentrate as can be observed from Table 15. The lower biomass yields can be due to growth inhibitory components in RO concentrate of NEREUS since its source is municipal and domestic wastewater. Besides, another reason for the lower biomass yields might be the possibility of stored nutrients within the cell, that is not all nutrients consumed are directly used for growth but are stored for use during periods where they are limited.

Chapter 5: Conclusions and recommendations

5.1 Conclusions

Combining the findings of the objectives lead to the conclusion of this study and the possibility to answer the research question as follows:

Can the microalgae G. sulphuraria be cultivated on reverse osmosis concentrate from water and resource recovery pilot plant of NEREUS?

G. sulphuraria can be cultivated on the reverse osmosis concentrate from the pilot plant of NEREUS as shown in Figure 23. The mix of 40% real RO concentrate and 60% synthetic RO concentrate medium gave the most promising results, highest growth and highest μ_{max} (0.77 day^{-1}), for the cultivation of *G. sulphuraria*. *G. sulphuraria* which grew under synthetic RO concentrate medium produced better-fitted biomass and nutrient consumption models when light limitation was implemented in the mathematical model. Growing *G. sulphuraria* on its wastewater will contribute to a better economy for NEREUS because less chemicals will be required to remove nutrients such as N and P present in wastewater. However, growth inhibitory components in RO concentrate of NEREUS may pose a challenge to process productivities because of the resulting low biomass yields. Still, it can be concluded that *G. sulphuraria* is a promising candidate for use in RO concentrate of NEREUS as consumer of the ammonium present in the RO concentrate.

The optimal growing conditions and possible limitations in the growth of G. sulphuraria were as follows: Ammonium with mixotrophic metabolism turned out to be the best nitrogen source. The mixotrophic culture produced about twice more biomass than the heterotrophic culture and about six times more biomass than the autotrophic culture. Biomass concentration on

ammonium was four times higher than on nitrate. Higher ammonium concentrations resulted to higher growth and growth rates of the *G. sulphuraria*. An ammonium concentration = 200 mgNH_4^+-N/L produced promising results in terms of biomass growth and therefore the ammonium concentration present in the reverse osmosis concentrate provides enough source of nitrogen for cultivation of *G. sulphuraria.* Moreover, no significant increase in the growth of *G. sulphuraria* was observed from the increase of the C:N ratio of 5:1 to 10:1 and therefore a C:N ratio of 5:1 is sufficient for the growth of *G. sulphuraria*. In addition, no firm conclusions could be drawn from the experiment performed with different phosphate concentrations. Adding bicarbonate and carbon dioxide gas to the culture did not promote to better mixotrophic growth of *G. sulphuraria*. Besides, it was found that *G. sulphuraria* did not grow well under oxygen limitation and therefore in closed reactors, aeration with air or oxygen gas is needed for optimum growth of the microalgae. Culture densities higher than 0.7 g/L of biomass resulted in a significant decrease in the rates of growth of *G. sulphuraria.*

5.2 Recommendations

This research thesis leads to some interesting aspects which require further research and the following recommendations are proposed:

- **Investigation of the effect of mixing**

  The effect of mixing on the growth rate and amount of biomass produced can be investigated to find out how the growth changes when mixing changes. When the shaker was off, a sudden drop in the growth was observed and therefore, more knowledge is needed to truly know the reasons behind it.

- **Investigate the effect of aeration (air/oxygen) of the culture**

  The effect of aeration of the culture on the biomass growth can be investigated to find out how the growth changes when oxygen in the culture changes. Oxygen is a very important parameter for growth of *G. sulphuraria*.

- **Repeat the experiment performed with different phosphate concentrations**

  Since no firm conclusions could be drawn from the experiment performed with different phosphate concentrations, the experiment should be repeated.

- **Repeat the experiment performed with synthetic and real RO concentrate media**

  Since the experiments performed with synthetic and real RO were performed only once, repeating the experiments are important.

- **Investigate on the viability of the well-plate set-up over the Erlenmeyer set-up.**

  The well-plate set-up is a fast way of varying variables at a time but it's feasibility is very important and needs further research especially on the aspects such as whether there is efficient mixing in the wells compared to the Erlenmeyer set-up, whether the algae receive enough light for growth and also whether the well-plate is suitable for experiments under high temperature because the problem of evaporation.

- **Measuring of the photosynthetic oxygen under autotrophic and mixotrophic metabolism.**

  Monitor whether photosynthetic oxygen is produced when *G. sulphuraria* is cultivated mixotrophically. Also, monitoring of the photosynthetic oxygen produced during autotrophic cultivation will give more information about the growing mechanism of the *G. sulphuraria.*

- **Monitoring the growth of *G. sulphuraria* under specific mix of RO concentrate from NEREUS and synthetic RO concentrate.**

  Though preliminary results were obtained on the growth of *G. sulphuraria* under mix of RO concentrate from NEREUS and synthetic RO concentrate, more knowledge is required to know if the growth of *G. sulphuraria* is optimum at a specific mix. This will give rise to a better characterization of the best medium needed to grow *G. sulphuraria* to optimize profitability.

- **Investigation of the effect of iron on biomass growth and growth rate of *G. sulphuraria* with synthetic medium.**

  The effect of iron on biomass growth and growth rate could not fully be investigated from previous experiments done on RO concentrate from NEREUS because of the presence of other nutrients or metals present in the RO concentrate. Further research is needed to find out exactly what happens with varying iron concentrations in the medium.

- **Analysing the composition of the metals present in the RO concentrate from NEREUS and investigate the effect of these metals.**

  Analysis can be done using Ion Chromatography (IC) and investigating how the growth changes in the presence of the metals present in the RO concentrate of NEREUS will provide more information about the inhibitors present in the RO concentrate of NEREUS.

- **Monitoring of pigment formation by performing extraction of pigments**

  Performing extraction of pigments will give more insights about the amount of pigments formed during the cultivation period and these results may provide information regarding the optimal time for pigment extraction with respect to the amount of biomass formed.

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Appendix

A.1 Urine

Urine is a sterile transparent-amber color liquid. Urine is generated by the kidneys and contains a diversity of water-soluble compounds which are eliminated from the human bloodstream.(Tuantet, 2015) Urine contains high concentrations of urea, inorganic salts, creatinine, organic acids as well as toxins and pigments (Bouatra et al., 2013). The composition of urine is highly dependent on a person's health, diet and exercise. N, P and Potassium (K) form the major elements present in urine (Tuantet, 2015). There are also low concentrations of trace metals, such as Fe, Cu, Mn and Zn, which causes rapid growth of microalgae, present in urine (Ronteltap et al., 2007; Tuantet et al., 2014). Besides, differences in gender and races have been found to show different urine composition (Rose et al., 2015). Concentrations per element present in urine vary widely (Table 18) (Zhang et al., 2014).

Table 18: Elemental composition of urine (Zhang et al., 2014).

Element	Fresh human urine (mg/L)
Nitrogen	4850 ± 1730
Phosphorus	155 ± 65
Potassium	877 ± 304
Magnesium	25.4 ± 17.0
Calcium	46.2 ± 39.7
Zinc	0.125 ± 0.069
Molybdenum	< 0.1
Manganese	< 0.1
Copper	< 0.1
Iron	< 0.1

Urine is the vital nutrient source in domestic wastewater because more than half of the nutrient load of domestic wastewater originates from urine (Tuantet, 2015; Tuantet et al., 2014). Urine contributes to only 1% of the total wastewater volume. This 1% of urine, however, contains 40% of the phosphorus load, 69% of the nitrogen load, and 60% of the potassium load of domestic wastewater (Zeeman et al., 2008; Zeeman & Kujawa-Roeleveld, 2011). Therefore, if source-separated urine is applied, a large proportion of the nutrients present in domestic wastewater may be directly recovered and re-used for other purposes such as fertilizer in agriculture (Höglund, 2001), depending on whether there are regulatory restrictions in the countries it will be applied (Lienert & Larsen, 2010). There are very few countries that actually implement the re-use of urine on a large scale and some of the reasons are misconceptions and prejudice towards the safety and hygiene, as well as the technical challenges (Andersson, 2015; Johansson et al., 2000; Rosemarin et al., 2008).The growing world's population increases the demand for crop fertilizers. However, readily available phosphorus reserves are expected to deplete in the coming century (Vaccari, 2009). From the above arguments, re-use of human urine can be a valuable step towards improved sustainability and lowering costs of e.g. fertilizer.

The main component and nitrogen form in fresh urine is urea ($CO(NH_2)_2$), a waste product from the amino acid metabolism (Tuantet, 2015). In addition, urine also contains a lot of phosphorus in the form of phosphate (93-100%) of the total amount of phosphorus in urine (Kuntke, 2013; Udert et al., 2006). Urea is readily hydrolyzed to ammonia (NH_3), ammonium (NH_4^+) and bicarbonate (HCO_3^-) by the catalyzing microbial enzyme 'urease' according to the Equation below (Udert et al., 2003):

$$CO(NH_2)_2 + 2H_2O \rightarrow NH_3 + NH_4^+ + HCO_3^-$$

The reaction above is called 'ureolysis' (Tuantet, 2015). Hydrolyzation can be accomplished within hours to days, dependent on the amount of urease and whether the urine is being mixed. Apart from urease, urea amidolyase is a second enzyme that is known to be able to hydrolyze urea (Udert et al., 2003). However, denaturation of microbial urease occurs at pH values below 5 (Mobley et al., 1995; Udert et al., 2003). During ureolysis pH of the medium increases and precipitation of inorganic salts like struvite ($MgNH_4PO_4 \cdot 6H_2O$), hydroxyapatite ($Ca_5(PO_4)_3(OH)$) and calcite ($CaCO_3$) occurs (Hao et al., 2008; Udert et al., 2003). Precipitation of inorganic salts lowers the availability of the inorganic nutrients such as phosphate and magnesium (Tuantet, 2015). Urine hydrolysis can be done by letting urine stored in containers or bottles stand on a shaker with continuous mixing at 30 °C (Tuantet et al., 2014). The composition of urine changes with time when stored. Degradation of urine can be prevented if the urine is stored at 4℃ to inhibit bacterial growth (Abdalla, 2003).

The median pH value of urine is 6.2 (Rose et al., 2015). Ammonia and ammonium resulting from the urea hydrolysis as shown in Equation above are present in an equilibrium governed by pH and temperature (Emerson et al., 1975). Figure 26 shows the ammonia and ammonium distribution as a function of pH (Kunz & Mukhtar, 2016). Ammonia is much more toxic to organisms than ammonium. Several studies have shown growth inhibition and die-off of microalgae when the equilibrium shifts from ammonium to ammonia and that may occur when temperature or pH increases (Azov & Goldman, 1982; Källqvist & Svenson, 2003; Konig et al., 1987)

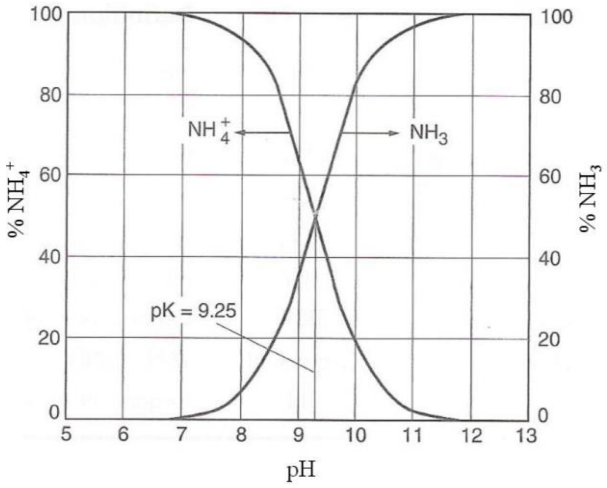

Figure 26: Ammonia and ammonium distribution as a function of pH (Kunz & Mukhtar, 2016).

A.2 Standard deviations for experiment on effect of ammonium concentration on biomass growth

Table 19: Standard deviations for experiment on effect of ammonium concentration on biomass growth (Mixotrophic metabolism, C:N ratio on day 0 = 5:1, N:P ratio on day 0 = 37:1, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

Ammonium-N concentration (mg NH4-N/L)	Day 0	Day 1	Day 2	Day 3	Day 5	Day 6	Day 7	Day 8	Day 9
100	0.001	0.001	0.001	0.003	0.009	0.008	0.012	0.029	0.013
200	0.003	0.002	0.005	0.006	0.033	0.062	0.022	0.010	0.025
400	0.002	0.002	0.003	0.012	0.027	0.041	0.047	0.041	0.018
600	0.001	0.001	0.005	0.003	0.006	0.024	0.024	0.036	0.009
800	0.002	0.001	0.003	0.011	0.013	0.092	0.015	0.052	0.021
1000	0.000	0.000	0.001	0.011	0.040	0.195	0.028	0.030	0.019

A.3 The biomass and nutrient consumption fitted Aquasim models with real RO concentrate medium for the first 5 days of cultivation under light limitation

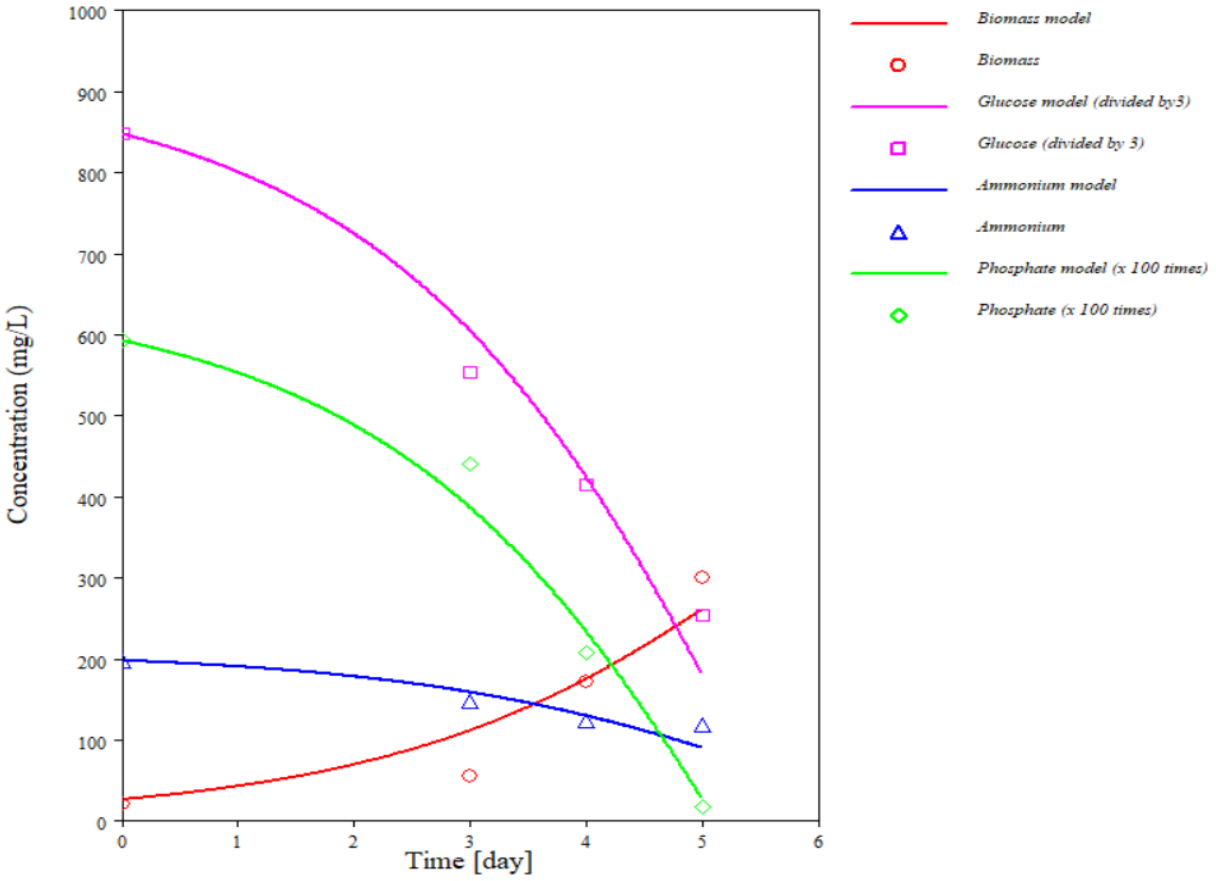

Figure 27: Biomass and nutrient consumption models with mix of 20% real RO concentrate and 80% synthetic RO concentrate medium under light limitation (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: For good visualization purpose, the glucose concentration and glucose model are shown three times less. The phosphate concentration and phosphate model shown are 100 times more. The rates of the processes, kinetics, μ_{max} and biomass yields were determined with the concentrations obtained during sampling.

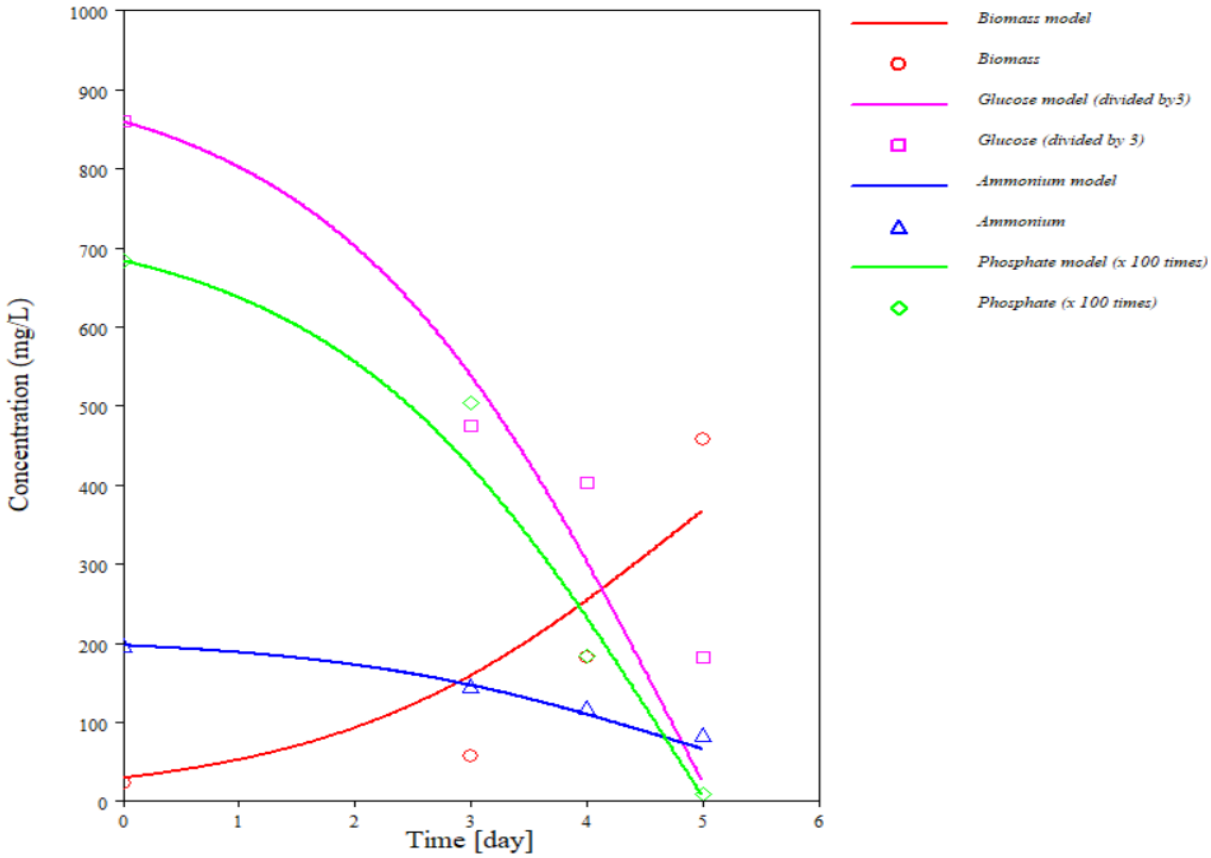

Figure 28: Biomass and nutrient consumption models with mix of 40% real RO concentrate and 60% synthetic RO concentrate medium under light limitation (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: For good visualization purpose, the glucose concentration and glucose model are shown three times less. The phosphate concentration and phosphate model shown are 100 times more. The rates of the processes, kinetics, μ_{max} and biomass yields were determined with the concentrations obtained during sampling.

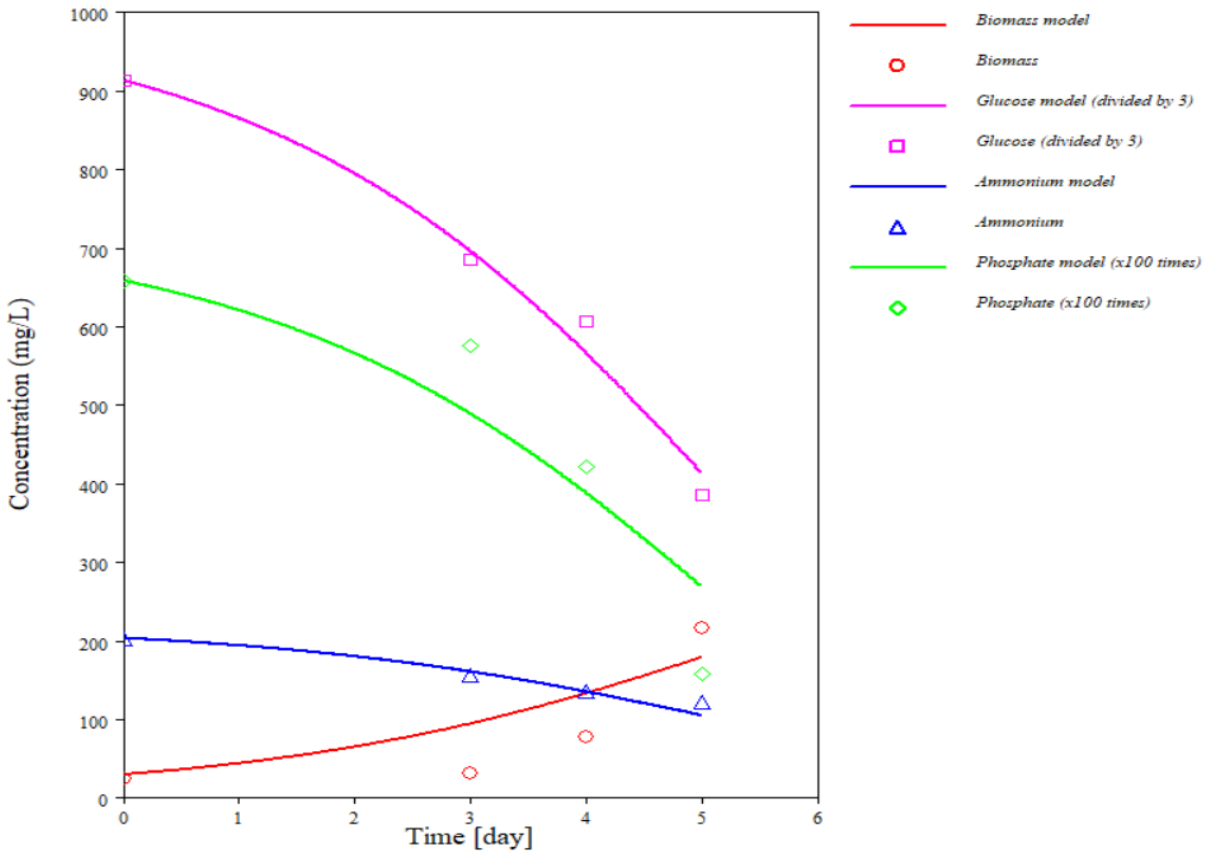

Figure 29: Biomass and nutrient consumption models with 100% real RO concentrate under light limitation (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm). Note: For good visualization purpose, the glucose concentration and glucose model are shown three times less. The phosphate concentration and phosphate model shown are 100 times more. The rates of the processes, kinetics, μ_{max} and biomass yields were determined with the concentrations obtained during sampling.

A.4 Nutrients consumption of *G. sulphuraria* for the 14 days of cultivation period

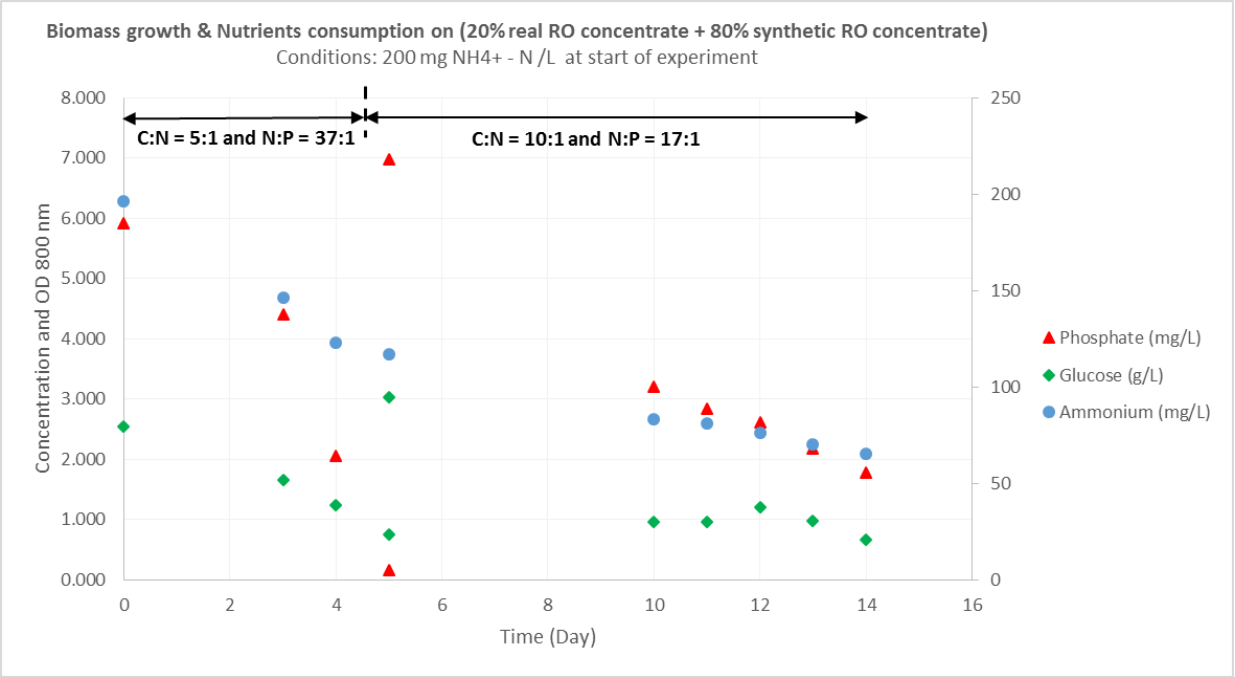

Figure 30: Nutrient consumption with mix of 20% real RO concentrate and 80% synthetic RO concentrate medium (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

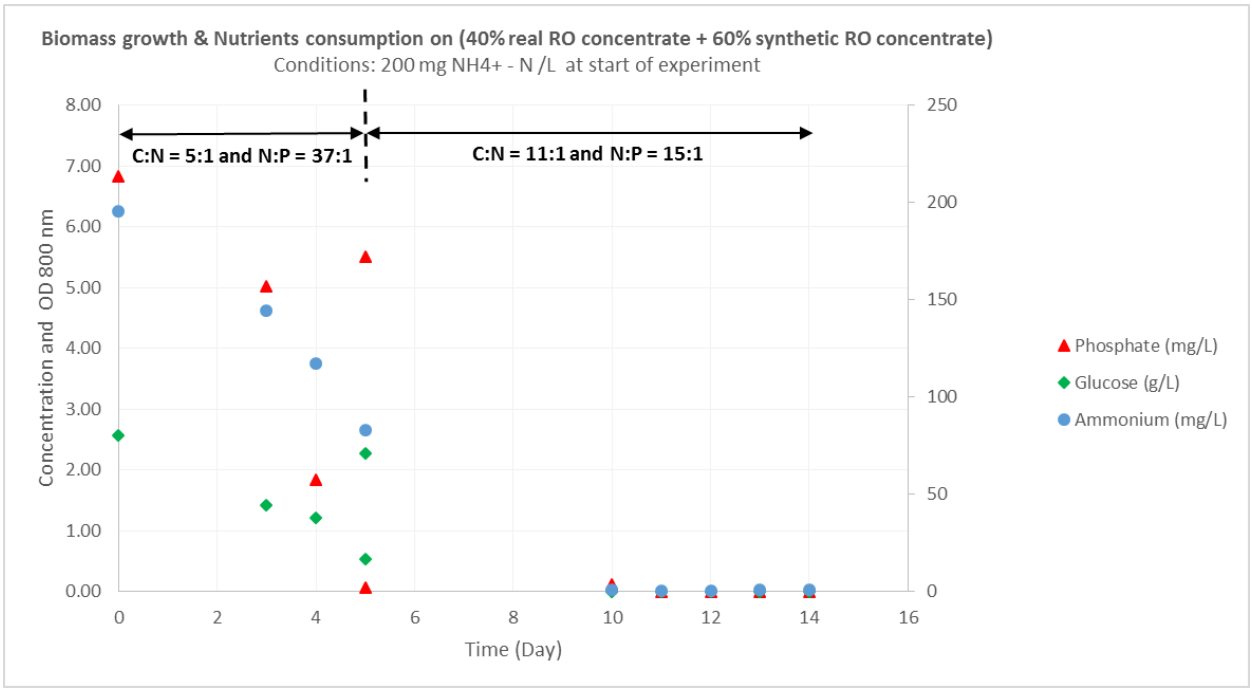

Figure 31: Nutrient consumption with mix of 40% real RO concentrate and 60% synthetic RO concentrate medium (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).

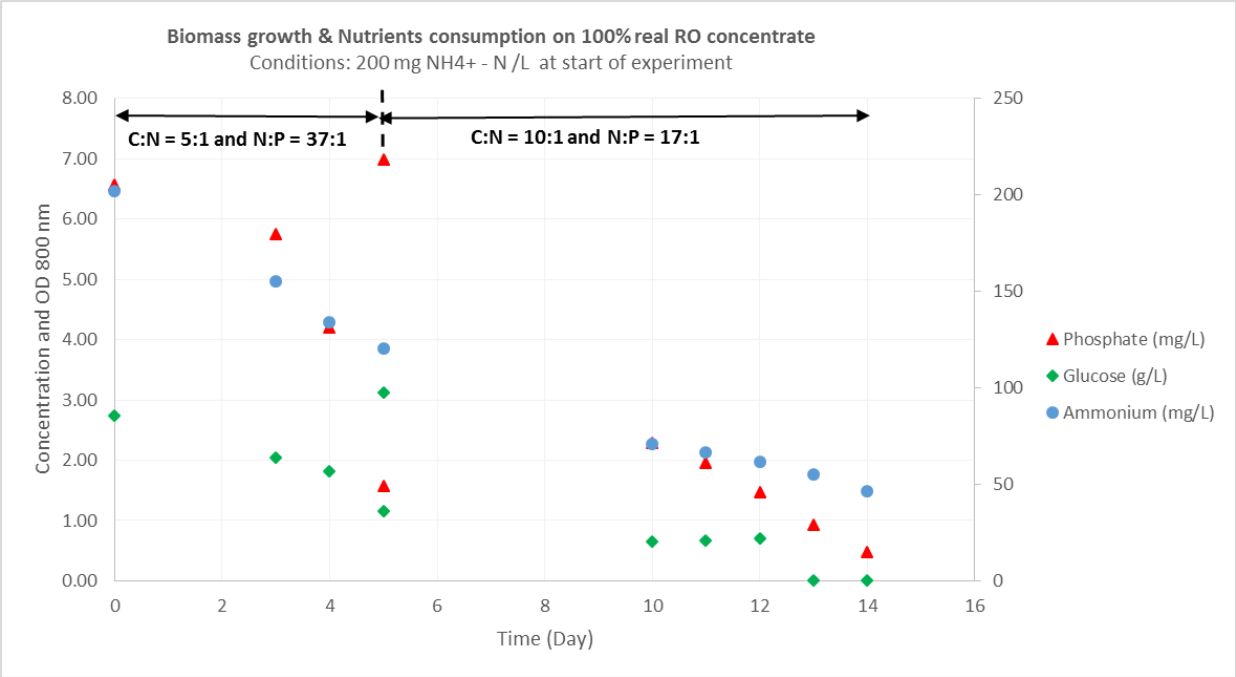

Figure 32: Nutrient consumption with 100% real RO concentrate medium (Mixotrophic metabolism, conc. of NH_4^+ on day 0 = 200 mg NH_4^+-N/L, pH = 2, temperature = 35 ℃, light intensity of 80 µmol photons $m^{-2}s^{-1}$, light cycle of 14:10, shaker at 145 rpm).