INVESTIGATION OF THE GROWTH AND OPERATIONAL CONDITIONS FOR THE ENRICHMENT OF *CHLORELLA VULGARIS* AND ITS USE IN ANAEROBIC DIGESTATE TREATMENT

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INVESTIGATION OF THE GROWTH AND OPERATIONAL CONDITIONS FOR THE ENRICHMENT OF *CHLORELLA VULGARIS* AND ITS USE IN ANAEROBIC DIGESTATE TREATMENT

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ABSTRACT

INVESTIGATION OF THE GROWTH AND OPERATIONAL CONDITIONS FOR THE ENRICHMENT OF *CHLORELLA VULGARIS* AND ITS USE IN ANAEROBIC DIGESTATE TREATMENT

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Master of Science, Environmental Engineering
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Recently, microalgae have proved its prosperity in wastewater treatment technologies with high nutrient removal efficiency and robustness. Thus, the aim of this master thesis focused to investigate the optimum conditions for enrichment of *Chlorella vulgaris* under autotrophic and mixotrophic conditions and to research the treatment of anaerobic digestate with *C. Vulgaris*.

In this thesis study, *C. Vulgaris* culture was first enriched under autotrophic conditions ammonium-N. The optimum illumination period of hourly frequencies (24h:0, 8h:8h:8h, 6h:6h:6h:6h, 12h:12h) were investigated in batch mode. This thesis is the first to investigate the effect of intermittent illumination in the literature with hourly illumination frequencies. The highest total ammonium nitrogen (TAN) (80%) and PO_4^{-3}-P (40%) removal efficiency and caratenoid production (800µg/mL) were obtained at 24h:0 (light:dark) illumination. Thereafter, the optimum N:P ratio (6,8,10) was investigated in semi-continuous photobioreactors (PBRs) with 8-day HRT. The highest TAN (almost 100%) and PO_4^{-3}-P (60%) removal efficiencies were obtained with N:P ratio of 8.

C. Vulgaris culture was then enriched under mixotrophic conditions in batch PBRs. Then, the effect of 2-, 4- and 8- day HRT was investigated in semi-continuous PBRs. Accordingly, almost 100% TAN, 100% PO_4^{-3}- P and 50% sCOD removal efficiencies were obtained at 4-day HRT. Afterwards, the optimum Nitrogen Loading Rate (NLR) and Phosphorus Loading Rates (PLR) were investigated at constant HRT of 4 days and the almost 100% TAN, PO_4^{-3}-P and 50% sCOD removal efficiencies were achieved, at NLR of 8 mg N/L.d, PLR of 1 mg P/L.d. and OLR of 100 mg/L.d sCOD Finally, mixotrophic *C. Vulgaris* culture enriched under optimum conditions defined above were used for the treatment of an anaerobic digestate containing chicken manure, poppy seeds, unpretreated and pretreated corncob. The microalgae-bacteria consortium exhibited almost 100% TAN, 80% PO_4^{-3}-P and 50% TOC removal efficiencies and an overall improvement in microalgal growth performance was observed. Results revealed that microalgal systems can be operated as complementary systems to anaerobic digesters to achieve further nutrient removal and create an opportunity of algal biomass for value-added products.

Keywords: *Chlorella vulgaris*, Microalgal Enrichment, Microalgal Wastewater Treatment, Anaerobic Digestate

ÖZ

CHLORELLA VULGARIS'IN ZENGİNLEŞTİRİLMESİNDE BÜYÜME VE ÇALIŞMA KOŞULLARININ ARAŞTIRILMASI VE ANAEROBİK ÇÜRÜTÜCÜ ÇIKIŞ SUYUNUN ARITIMINDA KULLANIMI

Subaşı, Irmak
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Son zamanlarda, mikroalgler, yüksek besin giderme verimliliği ve sağlamlığı ile atıksu arıtma teknolojilerindeki başarısını kanıtlamıştır. Bu nedenle, bu yüksek lisans tezinin amacı, ototrofik ve miksotrofik koşullar altında *C. Vulgaris*'in zenginleştirilmesi için optimum koşulların araştırılması ve anaerobik çürütücü çıkış suyunun arıtılmasıdır.

Bu tez çalışmasında, *C. Vulgaris* kültürü ilk olarak ototrofik koşullarda amonyum-azotu ile zenginleştirilmiştir. Daha sonra, kesikli modda, optimum aydınlatma periyodu saatlik frekanslar ile (24h:0, 8h:8h:8h, 6h:6h:6h:6h, 12h:12h) araştırılmıştır. Bu tez, literatürde aralıklı aydınlatmanın etkisini saatlik aydınlatma frekansları ile araştıran ilk tezdir. En yüksek TAN (%80) ve PO_4^{-3}-P (%40) giderme verimliliği ve karetenoid üretimi (800µg/mL) 24h:0 (aydınlık:karanlık) aydınlatmada elde edilmiştir. Daha sonra, 8 günlük HBS ile işletilen yarı-sürekli fotobiyoreaktörlerde (FBR'ler) optimum N:P oranı (6,8,10) araştırılmıştır. En yüksek TAN (takriben %100) ve PO_4^{-3}-P (%60) giderim verimleri N:P 8 oranı ile elde edilmiştir.

Daha sonra, *C. Vulgaris* kültürü kesikli FBR'lerde, miksotrofik koşullar altında zenginleştirilmiştir. Devamında, yarı-sürekli FBR'lerde 2, 4 ve 8 günlük HBS'nin

etkisi araştırılmıştır. Buna göre, 4 günlük HBS'de takriben%100 TAN, %100 PO_4^{-3}-P ve %50 sCOD giderim verimleri elde edilmiştir. Daha sonra, optimum Azot Yükleme Hızı (AYH) ve Fosfor Yükleme Hızları (FYH) araştırılmış ve takriben %100 TAN, PO_4^{-3}-P ve %50 çKOİ giderim verimleri AYH 8 mg N/L.g, FYH 1 mg P/L.g ve OYH 100 mg/L.g çKOİ koşullarında elde edilmiştir. Son olarak, tavuk gübresi, haşhaş tohumu, ön işleme tabi tutulmamış ve ön işleme tabi tutulmuş mısır koçanı içeren anaerobik çürütücü çıkış suyunun arıtılması için, yukarıda tanımlanan optimum koşullar altında zenginleştirilmiş miksotrofik *C. Vulgaris* kültürü kullanılmıştır. Mikroalg-bakteri konsorsiyumu takriben %100 TAN, %80 PO_4^{-3}-P ve %50 TOK giderim verimleri elde edilmiş ve mikroalg büyüme performansında genel bir gelişme gözlemlenmiştir. Sonuçlar, mikroalgal sistemlerin, daha fazla besin giderimi sağlamak ve katma değerli biyokütle üretim fırsatı yaratmak adına anaerobik çürütücülere tamamlayıcı sistemler olarak çalıştırılabileceğini ortaya koymuştur.

Anahtar Kelimeler: *Chlorella vulgaris*, Mikroalgal Zenginleştirme, Mikroalgal Atıksu Arıtımı, Anaerobik Çürütücü Çıkış Suyu

To The Worldwide Pandemic

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ABBREVIATIONS

μ	Specific growth rate (d^{-1})
AD	Anaerobic Digestate
ATP	Adenosine Triphosphate
BBM	Bold's Basal Medium
BOD	Biochemical Oxygen Demand
CO_2	Carbon Dioxide
COD	Chemical oxygen demand
C. Vulgaris	*Chlorella vulgaris*
DO	Dissolved Oxygen
HRAP	High-Rate Algal Ponds
HRT	Hydraulic retention time
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO_3^--N	Nitrate nitrogen
NO_2^-	Nitrite
NO_2^--N	Nitrite nitrogen
NO_x	Nitrogen Oxide
OD	Optical Density
PO_4-P	Orthophosphate phosphorus

PAR	Photosynthetically active radiation
PBR	Photobioreactor
sCOD	Soluble Chemical Oxygen Demand
TOC	Total Organic Carbon
tCOD	Total Chemical Oxygen Demand
TAN	Total ammonium nitrogen (NH_4^+-N + NH_3-N)
TN	Total nitrogen
TS	Total solids
TSS	Total suspended solids
WW	Wastewater
VS	Volatile solids
VSS	Volatile suspended solids
vvm	Volume gas per volume of broth per minute

CHAPTER 1

INTRODUCTION

Climate change is a major issue endangering humanity's future, which grasps scientists' and engineers' great attention. The rapid change in atmospheric conditions is jeopardizing agriculture and water security that awaits majority of the human population (FAO, 2008). Caused by the trapping of greenhouse gases inside the earth's atmosphere, climate change causes increase in atmospheric and sea temperatures, which has never been seen before in the earth's history. In numbers, total greenhouse gas emissions in 2018 declared to be 55.3 $GtCO_2e$. 37.5 $GtCO_2$ of that amount are found to be due to fossil CO_2 emissions from energy production and industrial activities, says emissions gap report prepared by the United Nations Environment Program (UNEP) in 2019 (Fawzy et al., 2020). Hence, fossil-based, and land-use-related CO_2 emissions are 74% of the total global greenhouse gas emissions (Fawzy et al., 2020). Consequently, driving mankind towards further exploitation of the natural ecosystems; the impacts of the global warming will be catastrophic.

Thereof, many researchers are on exploration of the newest means to decrease the anthropogenic contribution to the climate change and mitigate the impacts. Those are involving the employment of technologies for atmospheric CO_2 sequestration (Fawzy et al., 2020), sustainable food and energy production leading to reduction in CO_2 emissions (FAO, 2008). Microalgae, interestingly, can achieve all the above in one cell. Microalgae can be utilised either as a biofuel or as a food source due to its rich lipid, protein, and carbohydrate content. Moreover, it has a great potential for CO_2 sequestration (Singh and Ahluwalia, 2013). In fact, microalgae have 10-50

times higher CO_2 fixation efficiency than terrestrial plants (Costa et al., 2019). Therefore, microalgal-mediated treatment facilities have the potential to improve the existing global warming mitigation strategies (Wang et al., 2008; Lam and Lee, 2012). Moreover, microalgae species have a high nutrient removal capacity and high durability against ambient changes (Singh and Ahluwalia, 2013; Gonçalves et al., 2017). Thus, implementing the algal units to Wastewater Treatment Plants (WWTPs) will offer opportunities for both increasing negative emission and manufacturing of value-added products for a circular economy.

Despite many advantages, the researchers have always been challenged by the optimization of hydraulic retention time (HRT), nitrogen, phosphorus, and organic loading rates (NLR, PLR and OLR), nutrient, and organic carbon sources, and physical conditions (i.e., light, temperature, reactor configuration) for improved growth and maximum nutrient removal efficiencies.

The aim of this master thesis is therefore, to enrich *C. Vulgaris* under different environmental conditions and to investigate its use in treatment of anaerobic digestate (AD). The objectives of this thesis are as follows:

- To enrich *C. Vulgaris* under autotrophic conditions and investigate the effect of some environmental and operational parameters on culture.
- To determine the optimum nitrogen source, illumination period and N:P ratio for autotrophic conditions, leading to the highest nutrient removal efficiency and growth performance.
    - ❖ This thesis is the first to investigate intermittent illumination with hourly frequency
- To enrich *C. Vulgaris* under mixotrophic conditions and investigate the effect of HRT and nutrient loading rate on culture's enrichment and nutrient removal performance.

- To determine the optimum HRT and nitrogen-phosphorus loading rates for mixotrophic conditions.
- To investigate the treatment of original wastewater that is AD, on C. Vulgaris culture.
- To investigate the effect of pretreated and unpretreated corncob-fed digestates on nutrient removal and growth performance of C. Vulgaris culture.
- To investigate the effect of bacteria consortium on nutrient removal and growth performance of C. Vulgaris culture.

    - ❖ This thesis is to first to investigate the use of *C. Vulgaris* in treatment of an AD that consists of digested chicken manure, poppy seeds, and corncob which might affect the microalgal-bacterial consortium.

This master thesis is composed of six chapters. The first chapter provides a summary of the background information about the research topic of this thesis, as well as a summary of the inspiration and the scope of this master thesis. Chapter 2 reviews the literature and previous research performed on microalgal metabolisms, growth modes (autotrophic, mixotrophy and heterotrophy), operational and environmental conditions for its enrichment, and its utilization in real wastewaters. Chapter 3, Chapter 4 and Chapter 5 reveal separate experimental studies; thus, each of these chapters involve its focused introduction, related materials and methods, results discussion, and conclusion sections. Chapter 3 elaborates the enrichment of *C. Vulgaris* culture under autotrophic conditions. This chapter includes the identification the best nitrogen source, illumination period and N:P ratio for the enrichment of this culture. Chapter 4 touches down the details of mixotrophic enrichment and synthetic wastewater treatment with *C. Vulgaris* culture. Chapter 5 involves a sequenced batch test with 2 nutrient loading (feeding) stages where the axenic *C. Vulgaris* culture is compared to xenic *C.Vulgaris*-bacteria mix culture in nutrient removal and enrichment. The effect of pretreated and unpretreated corncob

fed digestates is discussed in this chapter. Chapter 6 summarizes all results briefly and gives the major conclusions of the thesis together with the future recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 *Chlorella vulgaris*

Among the many microalgae species, *Chlorella vulgaris* earned scientific attention in early 1900s soon after its discovery by Martinus Willem Beijerinck (Beyerinick, 1890). Since 1900s, in Germany, it attracted scientists as an unconventional food source that can be utilized as a solution for poverty, nowadays, in Japan it is cultured immensely for many medical purposes to prevent various diseases and optimizing immune system etc. On the other hand, in 1950s, engineers started to study it for CO_2 sequestration and develop technologies for phytoremediation (Justo et al., 2001; Morris et al., 2009; Oswald, 1988) Moreover, due to the rising demand for alternative energy sources, engineers suggested algae as a third-generation biofuel since it requires much less land space than terrestrial plants and it has a higher potential of oil production in a shorter period. *C. Vulgaris*, here, attracted scientists and engineers who are searching for the best algae alternative thanks to its ability to accumulate high amounts of lipids under mixotrophic conditions and value-added residue remaining after lipid extraction (Demirbas, 2011; Wang et al., 2013)

2.1.1 Morphology

From 3.4 billion years, found in fossil rocks in Western Australia, till now microalgae have maintained its cell structure (Safi et al., 2019). The evidential estimations show that those microorganisms are ancestors of plants, having a primitive cell structure and a large surface to volume body ratio which enables them

to uptake larger amounts of nutrients (Gupta et al., 2016). Yet, their photosynthetic mechanism is like higher plants representing a complicated and expertly organized forms of life (Bhola et al., 2011).

Algae are photosynthetic eukaryotic microorganisms that are found in various aquatic habitats like rivers, oceans, ponds, and wastewater (Lavajoo and Dehghani, 2016) Earliest algal division has shown in the "three domains of life (Archaea, Bacteria, Eukarya)" which is based on small subunit rRNA phylogeny (Figure 2.1), under eukaryotic domain (Bhattacharya and Medlin, 1998).

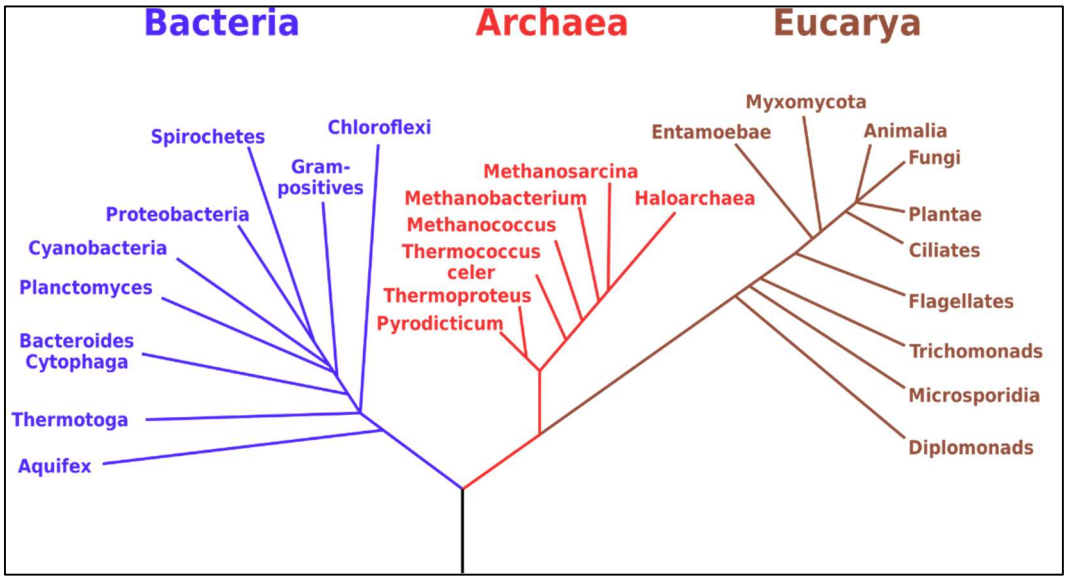

Figure 2.1. Three domain of life (Bhattacharya and Medlin, 1998)

Lately eukaryotic algae (both microalgae and macroalgae) have been classified under six divisions named Rhodophyta (red algae), Dinoflagellata (Pyrrophyta), Chlorophyta (green algae), Chromophyta, Cryptophyta and Euglanophyta based on structure of flagellate cells, the nuclear division process (mitosis), the cytoplasmic

division process (cytokinesis), and the cell covering (Enamala et al., 2018) (Figure 2.2).

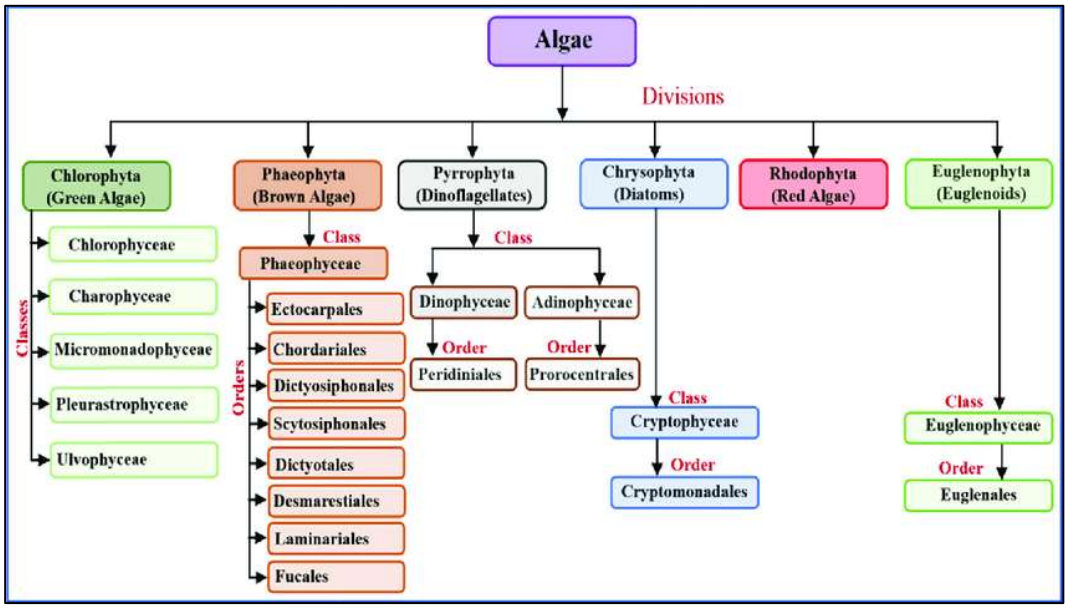

Figure 2.2. Algae divisions and classes (Enamala et al., 2018)

C. Vulgaris is classified under Chloropyhta division, Trebouxiophyceae class and the order of Chlorellales which is a unicellular spherical microscopic cell, having 2–10 µm diameter (Yamamoto et al., 2004; Yamamoto et al., 2005).

C. Vulgaris has a changing cell wall structure throughout its lifetime. The daughter cells have a 2 nm thin electron-dense unilaminar layer gradually getting thicker to the size of 17–21 nm after its maturation. A microfibrillar layer is formed as a chitosan-like layer composed of glucosamine that creates a rigid cell wall (Yamamoto et al., 2004; Yamamoto et al., 2005). The cytoplasm of the cell is a gel-like substance composed of water, proteins, and minerals (Safi et al., 2014). The internal organelles are mitochondria, a small nucleus, vacuoles, a single chloroplast, and the Golgi body (Safi et al., 2014). The double layer chloroplast is composed of

an outer membrane which is permeable to ions, and an inner membrane which has a specific protein transport function. The chloroplast, on the other hand is where photosynthesis occurs by converting CO_2 into carbohydrates (Kim and Lee, 2009).

2.1.2 Reproduction and Growth Kinetics

C. Vulgaris reproduces asexually by auto sporulation with an average doubling time of 24h. Under ideal conditions, the alga multiplies itself to four daughter cells, each with its own cell wall. Then the mother cell is consumed by the daughter cells following their liberation from the mother cell during the maturation period. (Figure 2.3) (Yamamoto et al., 2004; Yamamoto et al., 2005).

C. Vulgaris has five stages of growth that can be classified as lag phase, exponential phase, phase of declining relative growth, stationary phase, and death phase. In lag phase (Figure 2.3) culture is adapting itself to upscaling while it has a very little increase in cell density. In the second phase which is exponential phase, cell density is increasing as a function of time which is represented by a relation between specific growth rate and the change in cell density in time, as it is presented in Equation 2.1. In this phase the specific growth rate (μ) is mainly dependent on the light intensity and temperature. Phase of declining relative growth is the period where the cell division gets slow, dictated by the nutrient concentration, pH, carbon dioxide concentration and the light intensity. After this phase, with the balanced growth rate and limiting factors, the algal culture enters a stationary phase where the cell density is stable. In the last phase, with the decreasing nutrient concentration, variation in pH and temperature or possible contaminations cell density decreases sharply (Schuler and Kargi, 2002).

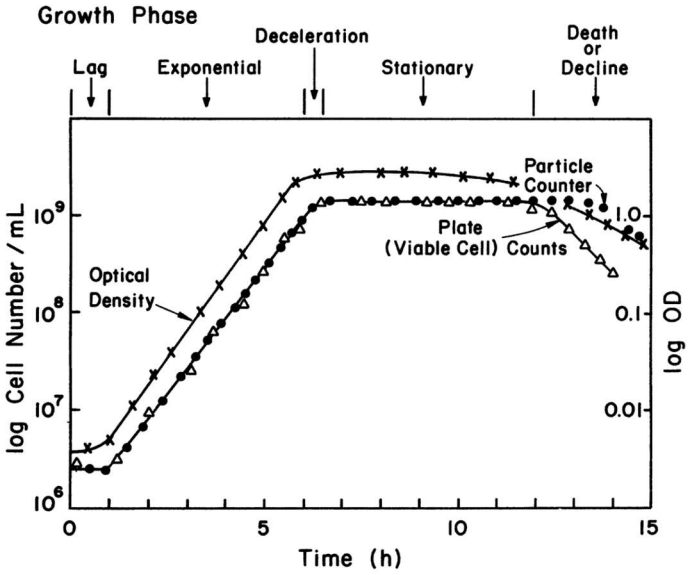

Figure 2.3. Typical growth phase of microorganisms (Schuler and Kargi, 2002)

$\frac{dX}{dt} = \mu . X$ ..... Equation 2.1

2.1.3 Metabolic Pathways

2.1.3.1 Photosynthesis and Respiration

Oxygenic photosynthesis is a unique process in which light is used as a source of energy for photoautotrophs to produce organic matter. Carbohydrate production is based on a simple equation of oxygenic photosynthesis, which shows all the necessary requirements of photosynthesis (Bauer et al., 2021).

This process is divided into two stages, known as light reactions and dark reactions. Light energy is converted to chemical energy in photosynthetic membrane light reactions, producing a biochemical reductant $NADPH_2$ and a high-energy compound

adenosine triphosphate (ATP). $NADPH_2$ and ATP are used in the biochemical during the dark phase, which occurs in the stroma.

2.1.3.2 Nitrogen Metabolism

Nitrogen uptake mechanisms in microalgae can be reviewed in two different pathways as nitrate uptake mechanism and ammonium uptake mechanism. Nitrate uptake mechanism has been broadly studied and explained over *Chlamydomonas reinhardtii* (Figure 2.4), which is sharing same division (chlorophyta) and similar cellular characteristics with *C. Vulgaris* (Chioccioli et al., 2014) being a good model for understanding the details of nitrate uptake mechanism in eukaryotic microalgae species (Sanz-luque et al., 2015).

The pathway for nitrate assimilation to amino acid in eukaryotic microalgae involves four steps: First step is the transport mechanism of nitrate into the cell (Sanz-luque et al., 2015). which requires ATP-dependent permease systems that NRT2 protein is responsible for the transport of nitrate and/or nitrite species to cross the cell cytoplasmic membrane as well as NRT1 proteins (Sanz-luque et al., 2015; Vega, 2018). NRT2 protein works along with the NAR2 protein to fully function this transport system (Sanz-luque et al., 2015). Later, a cytosolic Nitrate Reductase (NiR) reduces nitrate to nitrite that is also transported into the chloroplast. Inside the chloroplast, Nitrate Reductase (NiR) catalyses this species to ammonium (Fernandez and Galvan, 2008; Guerrero et al., 1981). In here, the transport of the nitrite to the chloroplast is performed by the NAR1 proteins (Peakman et al., 1990; Suppmann and Sawers, 1994; Rexach et al., 2000). As the final step, ammonium is incorporated to carbon skeleton of amino acids by the help of glutamine synthetase/glutamine oxoglutarate amino transferase or glutamate synthase (GS/GOGAT) cycle (Lea and Miflin, 1975) (Figure 2.4).

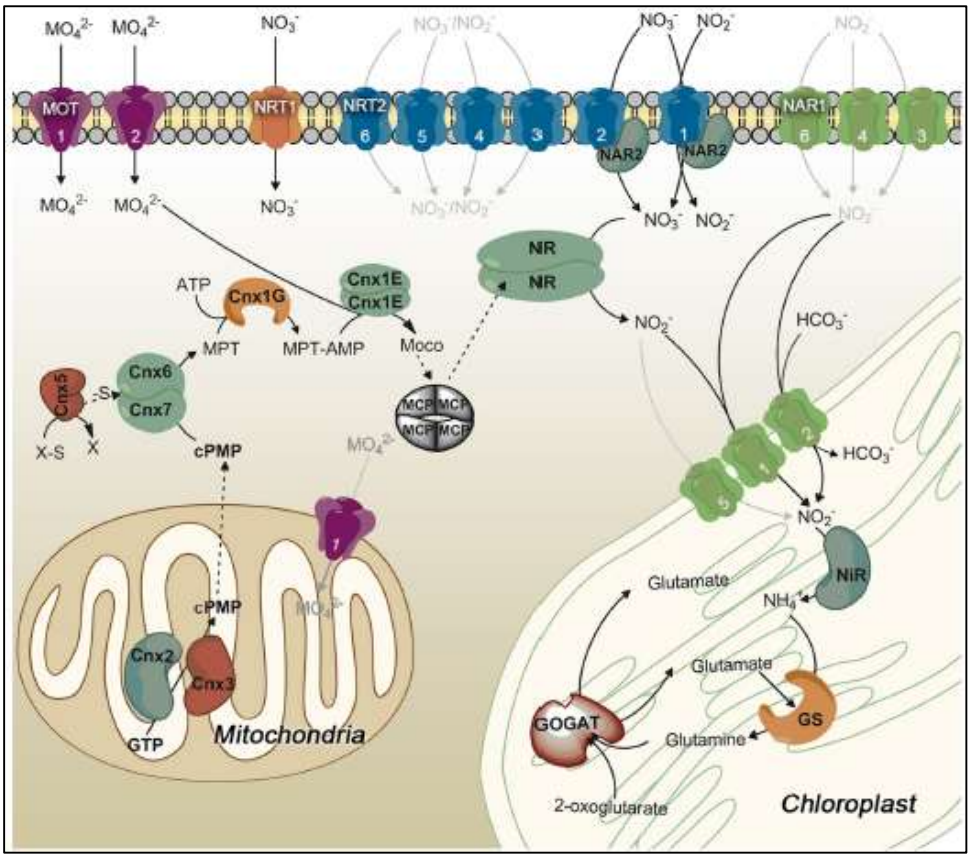

Figure 2.4. Nitrate uptake mechanism in *Chlamydomonas* (Sanz-luque et al., 2015)

Ammonium, on the other hand, is the most preferred nitrogen source for algae, since it entails less energy for its assimilation compared to nitrate (Liu et al., 2016). Transportation of ammonia across the cell membrane is accomplished by the proteins of Ammonium Transporter Family (AMT). Herein, ammonium is present in all compartments of the cell. The assimilation primarily begins with GS pathway that glutamate joins with the ammonium, which later produces a glutamine molecule via glutamine synthetase (GS) (Figure 2.5). The cycle is completed with GOGAT pathway which a-ketoglutarate is involved into reaction to produce two molecules of glutamate with the help of glutamate synthase (GOGAT) (Figure 2.5). Alternatively,

the assimilation may occur through a single reaction called amination of a-ketoglutarate, a metabolic intermediate of the Krebs cycle, into glutamate by the glutamate dehydrogenase (GDS). Glutamate is later incorporated into biomass by transamination and macromolecule formation (Liu et al., 2016; Wu et al., 2015).

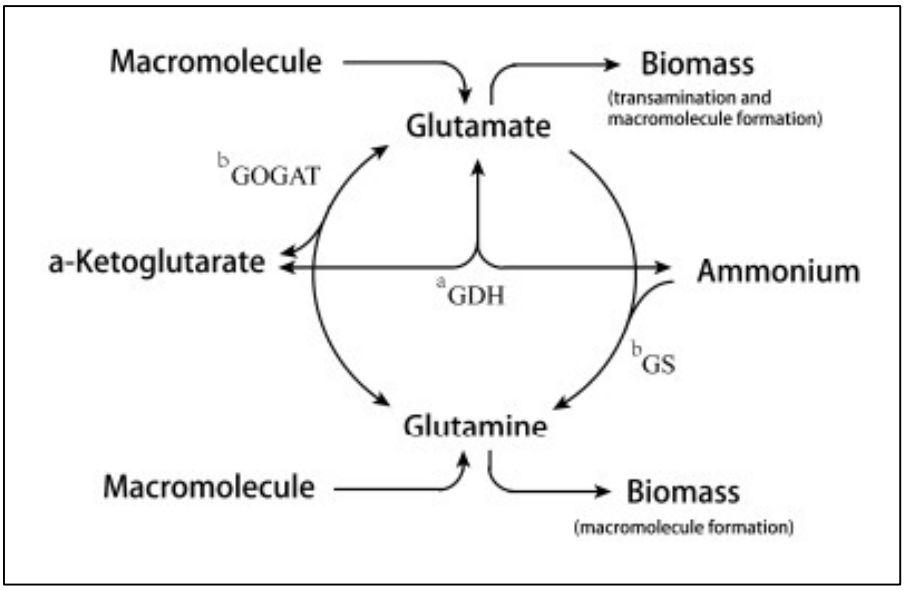

Figure 2.5. GOGAT cycle (Liu et al., 2016)

Since α-ketoglutarate (2-oxoglutarate), a metabolic intermediate of the Krebs cycle, is an essential substrate in both the GS-GOGAT pathway and GDH pathway for nitrogen assimilation, carbon metabolism and nitrogen metabolism are closely connected (Lu et al., 2018). In microalgae cultivation, parameters of the C/N ratio, light intensity and quality, and carbon forms could be adjusted to enhance carbon assimilation, further promoting the nitrogen assimilation (Han et al., 2019).

2.1.3.3 Phosphorus Metabolism

Phosphorus removal in microalgal systems, occurs simultaneously with nitrogen assimilation through photosynthesis and respiration (Molinuevo-salces et al., 2019). Although the algal growth mainly depends on soluble inorganic phosphate and orthophosphate species, organic phosphorus species are also included in its phosphorus metabolism (Singh et al., 2018). Algal cells can exhort phosphatase to utilize organic phosphorus to synthesize inorganic phosphorus transporters that are used for inorganic phosphorus absorption (Donald et al., 1997). However, being mostly available for the biological metabolism of microalgae, inorganic phosphorus uptake refers to the main mechanism within the algal cells (Singh et al., 2018). The affinity of various inorganic phosphate species is defined by their charge, the pH of the cell membrane. According to that, mostly absorbed species are HPO^{-}, HPO^{2-}, and PO_4^{-3} (Solovchenko et al., 2020). The kinetics of the uptake mechanism is also determined by many factors such as pH, salinity, growth, temperature, and starvation of the algal cells (Powell, et al., 2009).

The starvation of the algal cells defines two specific phosphorus uptake mechanism. The first one, namely, overshoot or overcompensation mechanism is known as the excess accumulation of the phosphorus in P-starved algal cells when they are exposed to a P-rich environment (Chopin et al., 1997). The other one, luxury uptake, also refers to an excess phosphorus phenomenon, however, in a P-sufficient environment this time (Eixler et al., 2006). With these mechanisms, the excess phosphorus is often stored as inorganic polyphosphate (Poly-P) (Singh et al., 2018) which is a linear unbranched polymer of several inorganic phosphorus molecules (Singh et al., 2018). Poly-P is not used as often as inorganic phosphorus. However, when the inorganic phosphorus is lacking, Poly-P can be utilized in metabolic activities such as synthesis of phosphorylated compounds including ATP, and in adjusting intracellular pH homeostasis and osmotic pressure (D. Singh et al., 2018;

Su, 2021; Whitton et al., 2015). This phenomenon can be beneficial for a wastewater treatment system to remove excess phosphorus. However, the excess accumulation of the phosphorus within the cell also may cause hindering the growth performance (Q. Li et al., 2018). In the beginning, the nutrients' uptake was based on the Monod's Growth fashion where the microbial growth rate depends on the external concentration of a limiting nutrient. However, this was demonstrated to be deflected from this fashion for some limiting nutrients such as N, P, Si, or Vitamin B12, in algal studies. According to the findings, the rate of nutrient uptake can surpass the limit which is necessary for growth based on nutrient availability and cell condition and growth can continue even after the depletion of external growth-limiting nutrients by deployment from storage reservoirs (Droop, 1973; Rhee, 1973). Hence, researchers have developed different models to have a better understanding for phosphate utilization.

The first one is "Quota Model" which suggests that uptake is shaped by external substrate concentration; while growth is affected by internal substrate concentration (a P pool); and in a steady-state system, the specific rate of uptake is always the product of the specific growth rate and internal substrate concentration that is identified as "P quota" (Droop, 1973). Although the model takes P pool into consideration, the limitations of the sole P pool perspective has found to be unrealistic by other researchers, that has led them to develop other models (Droop, 1973). The second model is "Phosphate Interaction Model" (Figure 2.6) that has multiple internal P pools of soluble inorganic phosphate (SIP), soluble organic phosphate (SOP), and polyphosphate (Poly-P). The external inorganic phosphorus (P_{ex}) basically provides the internal pools and can be converted to poly-P of organic phosphate. The external utilization of inorganic phosphate has Michaelis–Menten kinetics pattern, while the relation between internal phosphate pools is also following Michaelis–Menten kinetics (John and Flynn, 2000).

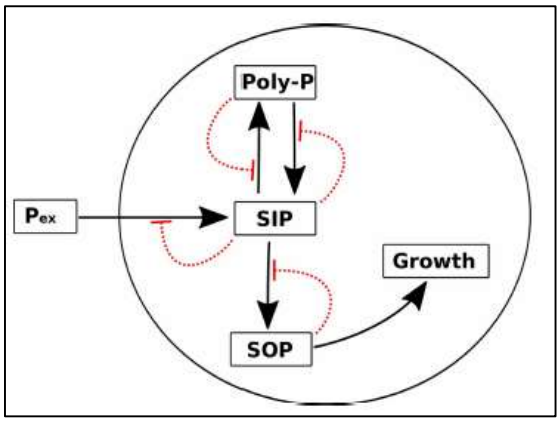

Figure 2.6. Phosphate Interaction Model (PIM) where SIP, SOP and Poly-P pools are in relation (Singh et al., 2018)

Extended Phosphate Interaction Model (ExPIM), on the other hand, is the revised version of the previous that covers the influence of light on growth and P uptake mechanism additionally (Figure 2.7). ExPIM also considers the degradation of cell components such as proteins and RNA during starvation and restoration of those components to support growth (Dauta, et al., 1990)

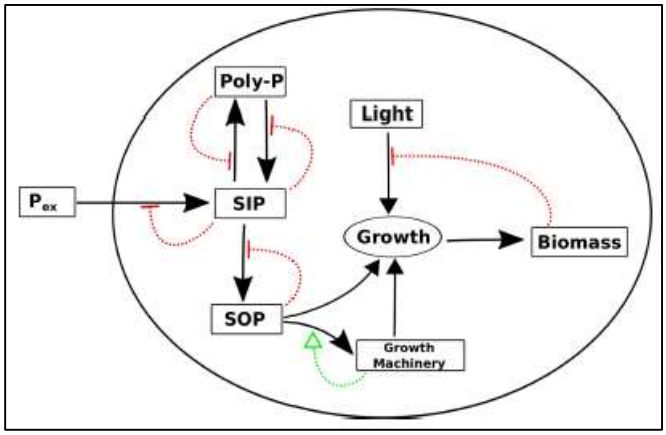

Figure 2.7. Extended Phosphate Interaction Model (ExPIM) (Singh et al., 2018)

Carbon Metabolism

2.1.3.4 Carbon Metabolism

Green algae have different modes of mechanisms that carbon is fixed differently due to the availability of the organic/inorganic carbon and the light (Hammed et al., 2016). Those can be listed autotrophy, heterotrophy and mixotrophy as presented in Table 2.1. While these mechanisms can proceed separately depending on the condition and availability, there is a possibility to observe a shift between mechanisms or simultaneous operation within the cell (Bell, 2013; Liang et al., 2009; Perez-Garcia et al., 2011) All carbon regimes are schematically demonstrated in Figure 2.8 below.

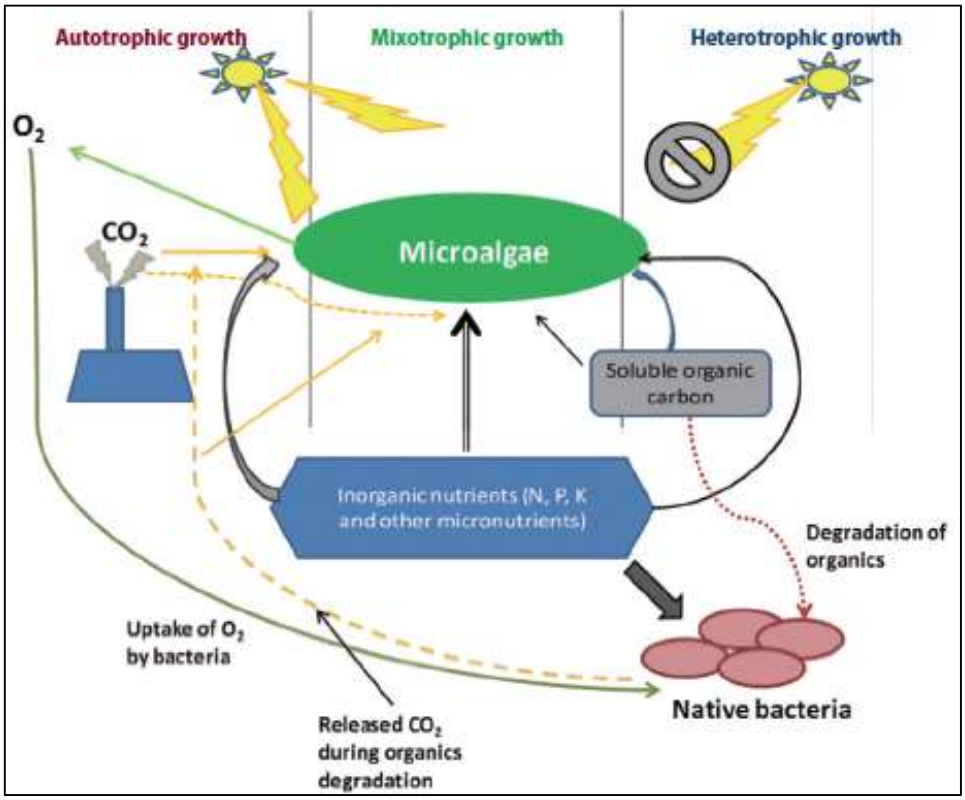

Figure 2.8. A demonstration for carbon regimes (Hammed et al., 2016)

Table 2.1. Growth modes of microalgae (Perez-Garcia et al., 2011)

Growth Mode	**Energy Source**	**Carbon Source**	**Light Availability**	**Metabolism Variability**
Photo-autotrophic	Light	Inorganic	Obligatory	No switches between sources
Heterotrophic	Organic Carbon	Organic	No requirement	Switches between sources
Mixotrophic	Light and Organic	Inorganic and Organic	Not obligatory	Simultaneous between utilization

Heterotrophic Metabolism

Heterotrophic growth is a no photosynthetic, dark metabolism in which organic substrate assimilation produces energy via oxidative phosphorylation, along with oxygen consumption as the final electron acceptor (Morales-Sánchez et al., 2015; Perez-Garcia et al., 2011). In heterotrophic mechanism glucose is assimilated through Embden–Meyerhof Pathway (EM) and Pentose Phosphate Pathway (PPP) after a direct uptake with a cytoplasmic membrane bond protein as represented schematically in Figure 2.9 (Morales-Sánchez et al., 2013; Perez-Garcia et al., 2011). Glucose, however, is firstly phosphorylated to glycerol phosphate and later is oxidized to triose phosphate (Mendes et al., 2007; Taborda et al., 2021). Later, the glycerol is converted to glyceraldehyde-3-phosphate and glycerate, which are intermediates involved in the EMP pathway of glycolysis, to produce pyruvate that also joins to the Tricarboxylic Acid Cycle (TCA cycle) (Yazdani and Gonzalez, 2007) Acetate or acetic acid, on the other hand, is metabolized by acetylation of coenzyme A by acetyl CoA synthetase and it produces acetyl coenzyme A (acetyl-CoA) which later goes under glyoxylate cycle to form malate in cytoplasm and, TCA to form citrate in mitochondria. The metabolites of these cycles are joined later to

carbon skeleton and used for energy source as ATP, and energy for reduction (NADH) (De Swaaf et al., 2003; Mendes et al., 2007).

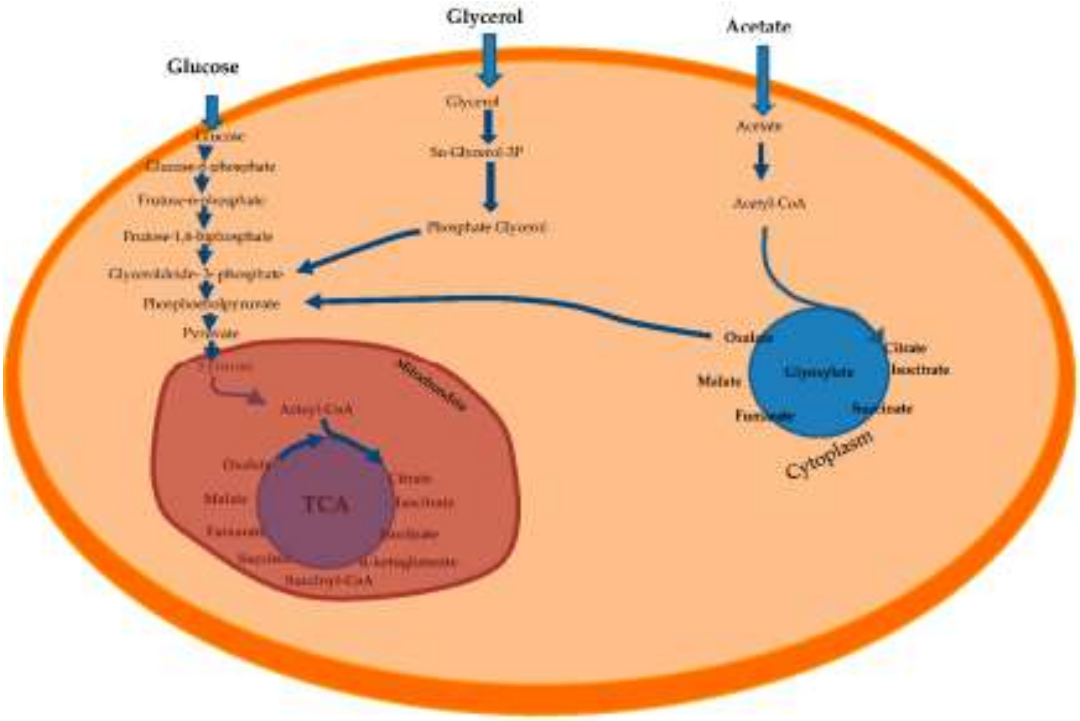

Figure 2.9. Carbon metabolism inside a microalgal cell (da Silva et al., 2021)

Autotrophic Metabolism

Autotrophic metabolism has three sub metabolisms: A photosynthetic reduction in a light cycle, CO_2 concentration and dark cycle (Perez-garcia and Bashan, 2015). The photosynthesis occurs thru a photon absorption by algal pigments, such as chlorophylls, carotenoids and phycobilin to form electrons. During the light reactions, donated electrons are transferred to two types of photosynthetic units, called PSI and PS II. They transform water and photons into ATP and the electron carrier nicotinamide adenine dinucleotide phosphate (NADPH) in addition to the

cytochrome b6f complex. The products of light reactions are later used to fix CO_2 in the Calvin cycle in fallowing dark reactions (Carvalho et al., 2011; Hildebrand et al., 2013; Pilon et al., 2011). CO_2 diffusion in water and bicarbonate consumption in stroma are both slow processes. CO_2 concentration delivers sufficient CO_2 to the stroma of chloroplasts while diminishing O_2 impediment to ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO) (Nelson and Cox, 1982). The CO_2 concentration of the molecule is performed by using carbonic anhydrase, which are zinc-metalloenzymes that catalyse the rapid interconversion of inorganic carbon species ($CO_2 + H_2O \rightarrow HCO^{3-} + H^+$) (Moroney et al., 2011). Dissolved inorganic carbon transporters stimulate inorganic carbon and CO_2 uptake (Pilon et al., 2011). HLA3 at the plasma membrane, LCIA at the chloroplast membrane, and CCP1 and 2 at the chloroplast are examples of inorganic carbon transporters (Ohnishi et al., 2010; Pollock et al., 2004). Rhesus proteins also function as CO_2 channels in microalgae (Soupene et al., 2002), and Rhesus-1 protein is a bidirectional CO_2 channel in *Chlamydomonas reinhardtii* (Soupene et al., 2004).

Many species can switch between the carbon sources, named as photoheterotrophy where growth cells use light to generate energy, to fix nitrogen and organic matter as a carbon source without CO_2 (Chen et al., 2011). Since organic carbon and light are mandatory for photoheterotrophic cultivation, it is seldom used as a method to produce microalgae biomass (W. Wang et al., 2014), and thus, the photoheterotrophic culture is not discussed further in this thesis.

Mixotrophic Metabolism

Mixotrophic metabolism is the simultaneous occurrence of both utilization of inorganic carbon and organic carbon in the presence of light (Kang et al., 2004), in other words, autotrophy and heterotrophy appear concurrently (J. Wang et al, 2014). Photosynthesis fixes CO_2, that is induced by light, while aerobic respiration organic

compounds are absorbed via aerobic respiration, that is affected by the availability of organic carbon (Perez-Garcia et al., 2011).

Respiration and photosynthesis are interrelated activities within a microalgae cell (Yang et al., 2015). For example, consumption of polysaccharides, that are formed in chloroplasts, happens through photosynthesis by mitochondria. The attainment of ATP production in mitochondria by chloroplasts increases the efficiency of photosynthesis as a result (Yang et al., 2015). Mixotrophic microalgae can utilize organic or inorganic sources and light in various combinations. Mixotrophy makes microalgae more adaptable because they can collect carbon and energy requirements from organic or inorganic sources and light simultaneously (Chen et al., 2011).

2.2 Important Parameters in Microalgal Treatment

The growth kinetics of eukaryotic microalgae is influenced by many key parameters such as light, nutrient source, carbon source, temperature, and pH (Eriksen, 2008; Posten, 2009). The productivity of the culture and the maximum treatment efficiency are retained by the optimization of those parameters and maintaining those parameters. This section examines the details of the key growth parameters.

2.2.1 pH

The optimization of pH determines the characteristics of metabolism and the biosynthesis of the secondary metabolites due to proton concentration released in the water (Khalil et al., 2010). Although the known facts show that microalgae species prefer pH around 7.0 to grow, there are many studies indicating the enrichment of different microalgae species in different pH ranges between pH 2.5 to 11.5 (Sakarika and Kornaros, 2016). For *C. Vulgaris*, optimum pH values are differentiated regarding the purpose of the study. For example, the optimum growth of the culture

was observed under pH 6.5-7.0, the accumulation of lipid occurred at pH range of 7.0- 8.5 (Sakarika and Kornaros, 2016), while biomass productivity was observed to be promoted in alkaline environment of pH 9-10 (Daliry et al., 2017). Despite of the knowledge on optimum pH ranges, there are many factors that change pH in the culture media, such as CO_2 concentration, the presence of ammonium and nitrate as nitrogen source, making the optimization of this parameter quite challenging.

2.2.2 Temperature

Optimum temperature for microalgal growth and treatment performance may vary among different species. Yet, in general microalgae species can handle wide range of temperatures of 15 to 30°C, achieving photosynthesis and cell division properly (Daliry et al., 2017). Additionally, below 15°C, each increase in temperature affects photosynthetic activity positively, until it reaches to over 30°C. This trend is frankly related to enzyme activity and the Calvin cycle (Falkowski and Owens, 1980) Moreover, in some studies it has been demonstrated that at temperatures above 25°C, the protein synthesis within the cell may reduce (Konopka and Brock, 1978). Likewise, Deniz, (2020) suggests an optimum growth of *C. Vulgaris* at 25°C.

2.2.3 Nutrient Source

In algal metabolism, as previously mentioned in this thesis, the source of macronutrients (nitrogen and phosphorus) determines the pathway of the assimilation and the secondary metabolites which resultantly affect the growth kinetics and treatment performance of the culture. Especially, the source of nitrogen is a key parameter for the overall performance of a culture. Thus, it should be well optimized specific to the conditions. For example, ammonium is the most preferred nitrogen source for most microalgae species including *C. Vulgaris* (Liu et al., 2016)

since the pathway of the assimilation does not include the reduction of nitrate and nitrite species to ammonium, it requires less energy to complete assimilation (Fernandez and Galvan, 2008; Guerrero et al., 1981). Hence, the growth performance was observed to be optimum with ammonium in the studies comparing other nitrogen sources of urea and nitrate, while lipid content was found to be maximum with urea (Altın et al., 2018). However, since ammonium consumption causes a decrease in pH, an inhibitory effect of ammonium can be observed and hence, a biological or a chemical buffer might be needed during the cultivation to avoid reverse effect of decreased pH, while with the assimilation of urea or nitrate such inhibition is not a matter of concern (Eustance et al., 2013).

Phosphorus, on the other hand, plays an important role for algae in growth, lipid production, fatty acid production and other metabolic activities. For orthophosphate uptake, microalgae often adapt to phosphorus in its inorganic form of $H_2PO_4^-$ or, HPO_4^2 (Becker, 1994). Yet, orthophosphates can also be induced upon organic phosphates by phosphatases at the cell surface (Larsdotter, 2006).

2.2.4 Nitrogen to Phosphorus (N:P) Ratio

Decreased availability of key nutrients for microalgae often results in the limited growth and treatment performance, and altered composition of the organism (Beardall et al., 2001). The optimum nitrogen to phosphorus (N:P) ratio, hence, is a key parameter to be optimized for a healthy culture. This ratio has been investigated in many studies for a given species (Rasdi and Qin, 2015), and for *C. Vulgaris*, they found out that the best N:P ratio of the media is in the range of 5-15 (g:g). However, this optimum ratio also varies according to the desired performance of nutrient removal, biomass productivity and/or lipid and protein content (Choi and Lee, 2015; Anbalagan et al., 2016; Aslan and Kapdan, 2006).

2.2.5 Carbon Source

The most common inorganic carbon sources of microalgae can be listed as, ambient CO_2, CO_2 from flue gases, and soluble chemically fixed CO_2 ($NaHCO_3$ and Na_2CO_3) (Znad et al., 2012). HCO^{3-} and CO_3^{-2}, are the most preferable forms of inorganic carbon species (Carvalho et al., 2006). Providing inorganic carbon to an algal culture mostly occurs through aeration. However, the atmospheric CO_2 concentration of 0.039% (Putman et al., 2016) is not often adequate for an optimum algal growth. At this point, enriched CO_2 can be an option to reach to the growth limits of species (Larsdotter, 2006). Yet, at high CO_2 concentrations, pH regulation becomes difficult, and chemical precipitation of salts including CO_3^{2-}, OH^-, and PO_4^{3-} occurs, causing cell damage (Carvalho et al., 2006).

Organic carbon sources, then again, are glucose, starch, sucrose, acetate, glycerol, which may have different effects on algal growth. For example, the introduction of the complex organic carbons, such as sucrose or glucose may lead the biosynthesis of various lipids and carbohydrates in algal cell. Moreover, such complex sugars may result in a possible reduction in anabolism of photosynthetic proteins and pigments (W. B. Kong et al., 2013). Moreover, it was revealed in a study conducted by Bashir et al. (2019), that sucrose and glucose supplementation considerably increased *P. lutheri* growth and cell density in mixotrophic cultures. Furthermore, Chinnasamy et al., (2009) demonstrated 3–10 times higher biomass yields of *Chlamydomonas globose*, *Chlorella minutissima*, and *Scenedesmus bijuga* with glucose, sucrose, or acetic acid supplementation. Glucose and sucrose, on the other hand, result in an increased growth of *Chlorella kessleri* under mixotrophic and heterotrophic conditions.

2.2.6 Aeration

Aeration is a parameter that helps microalgal system with mixing and preventing precipitation as well as the supplementation of CO_2. Moreover, it can homogenize the culture mass and improves the light penetration. The type of culture of open system or photobioreactor (PBR) and culture system scale determine the aeration rate (Daliry et al., 2017). For example, for *C. Vulgaris*, 6% CO_2 concentration and 0.4 vvm are found to be optimal for an enhanced growth (Anjos et al., 2013). Yet, other studies used different aeration rates of 0.07 vvm (Cheng et al., 2006), 0.5 vvm (Feng et al., 2011), 0.22 vvm (Fan et al., 2007), 0.67 vvm (Woertz et al., 2009) and 0.1 vvm (Li et al., 2013) in continuous mode, with *C. Vulgaris* as optimum aeration rates for growth.

2.2.7 Light Intensity and Illumination Period

Light is a key factor that aids the algal cells to grow as well as a photon source for photosynthesis to convert CO_2 into organic compounds, such as carbohydrates and proteins. The intensity of light determines the cellular activity of algae. If a culture is enriched under light limitation, cellular mechanisms can tend to convert carbon to amino acids. Yet, under the saturated light intensities, carbon to sugar, and starch production are enhanced, and the maximum growth rate is stabilized (Daliry et al., 2017). Different strains of algae require different light intensities to grow in optimum levels. For example, optimum light intensities for *Chlorella kessleri* and *Chlorella protothecoide* were stated as 120 µmol. $m^{-2}.s^{-1}$ and 30 µmol. $m^{-2}.s^{-1}$, respectively (Y. Li et al., 2012) while, for *Scedenesmus Obliquus* this values was 150 µmol. $m^{-2}.s^{-1}$ (Sforza et al., 2014). For *C. Vulgaris*, however, the optimum light intensity was found as 50-200 µmol. $m^{-2}.s^{-1}$ depending on the culture age and other enrichment conditions (Daliry et al., 2017; Khalili et al., 2015).

The period of illumination has various effects on each species. Some studies show that non-continuous illumination stabilizes improved growth rates due to cell division that occurs under dark conditions in a photosynthetic cell. However, in some cases, cells need both dark and light conditions for its growth. Not only cell division but also, chlorophyll production, lipid, carbohydrate content and nutrient removal efficiencies are affected by illumination periods. The optimum illumination periods were suggested differently. Daliry et al. (2017) suggested 16:8 (light:dark) period while Deniz (2020) states that 24:0 (light:dark) period results in optimum growth rate on *C. Vulgaris*.

2.2.8 Solids Retention Time (SRT) and Hydraulic Retention Time (HRT)

SRT, similarly, is the parameter that determines the rate of microbial growth and the amount of sludge to be disposed of in biological systems. Hence it can be concluded that the longer the SRT the more opportunity for the improvement of microbial species (Clara et al., 2005). However, being directly related with the light penetration, when more algal cells stay inside system with longer SRT, N and P removal efficiencies can be lowered (Xu et al., 2014). Therefore, it is crucial to adjust HRT and SRT values within the system to control the light while enabling the most active biomass to live (M. Xu et al., 2015).

HRT determines the influent nutrient loading rate and the effluent quality of the water. Moreover, it controls the biomass activity through the biomass concentration, solid-liquid separation efficiency and the dilution that causes increased /decreased light penetration as a result (Cromar and Fallowfield, 1997; García et al., 2000). Hence, it is well known that HRT optimization is a key step for algal research.

In many researches it was studied that the HRT of 2 days to 8 days is preferred for the microalgal based processes in municipal wastewater treatment (Larsdotter, 2006;

Muñoz and Guieysse, 2006; Posadas et al., 2014). Determining an optimal HRT in algal systems is important since it also serves to define the N, P, and light requirements for maintaining the microalgae culture in the PBR. While long HRT allows for the optimization of operating conditions, it may also result in nutrient starvation (Larsdotter, 2006).

All in all, especially in chemostat systems with equal HRT and SRT (Molina Grima et al., 1996), it is vital that HRT does not exceed the time required to maintain the growth rates of fast-growing microalgae in PBRs (Larsdotter, 2006). Hence, the optimum HRT for a chemostat system can be set in the range of 2-8 days. However, it should be optimized within the system.

2.2.9 Nitrogen and Phosphorus Loading Rates

Nitrogen loading rate (NLR) and phosphorus loading rate (PLR) applied in a PBR are quite important in determining the quality of culture as well as the nutrient and organic removal performance. Various studies show different optimum nutrient loading rates for each species. For example, a study exhibited that (Ruiz et al., 2011), NLR of 4 mg/L.d and PLR of 1 mg/L.d result in the best biomass growth rate in a *C. Vulgaris* culture. In another study performed by Zarrinmehr et al. (2020) the NLR of 11 mg/L.d. resulted in the best growth performance in an *Isochrysis galbana* culture. Whereas, in the same study, the maximum chlorophyll content was obtained in the culture fed with an NLR of 5.5 mg/L.d. Moreover, another study demonstrated that a PLR of 4.5 mg/L.d resulted in a higher cell number to *C. muelleri* culture, while the highest dry weight concentration and chlorophyll-a content were found to be at 2.25 mg/L.d PLR (Lovio-Fragoso et al., 2019). On the other hand, the highest total lipid was exhibited in the medium with the lowest PLR of 0.2 mg/L.d (Lovio-Fragoso et al., 2019). Şentürk and Yıldız (2020) revealed in a study performed with *C. Vulgaris* that, although the higher PLR promotes chlorophyll, carotenoid, and

lipid content, the P removal efficiency is reduced gradually by increasing PLR from 5 mg/L.d to 20 mg/L.d.

2.2.10 Organic Loading Rate

Organic loading rate and initial COD concentration are also important parameters that determines the fate of algal enrichment. In a study, conducted by Bashir et al. (2019), sucrose loading rates of 0.015, 0.3, 0.8, 3 and 6 g/L.d were provided to *P. Lutheri*. It was demonstrated that the culture with 0.3 g/L.d sucrose (0.32 g/L.d COD loading) exhibited the highest OD and dry weight results, indicating that an optimal growth was sustained in this condition. In another study that various glucose loading rates were applied to a *C. Vulgaris* culture for 4 days of cultivation, the cultures with 1.75 and 3.0 g/L.d glucose supplementation (1.9 g/L.d and 3.2 g/L.d COD loading) showed the maximum growth while demonstrating optimal chlorophyll and caratenoid production (W. B. Kong et al., 2013). Moreover, Daliry et al. (2017) also states 3.3 g/L.d glucose loading rate to lead to the optimum growth in *C. Vulgaris* culture. Another investigation performed with *C. pyrenoidosa* culture found that 0.2 g/L.d COD loading rate resulted in 80-100 % COD removal, whereas 0.5 and 0.8 g/L.d COD loading supplied cultures had maximum organic removal of 60 and 40 %, respectively, where the organic carbon source is acetate (Gupta et al., 2017). It may be concluded that high concentration of COD and organic loading rates can cause an inhibitory effect on culture depending on the source of organic carbon.

2.2.11 Optimum Conditions for *C. Vulgaris*

The parameters for an optimum condition of the enrichment and nutrient removal performance for *C. Vulgaris* have been studied by many researchers. *C. Vulgaris* is a quite robust species that has abundance in its natural environment and the

operational and environmental parameters can be applied in a wide range (Daliry et al., 2017). However, it should be noted that the optimum values of those parameters may vary according to the environmental conditions where the cultivation of *C. Vulgaris* is realised. Thus, in each case, the optimum parameters should be tested around the literature values and the best one should be investigated. The range for the optimum environmental and operational parameters are listed in Table 2.2.

Table 2.2. Optimum conditions for *C. Vulgaris* enrichment

Process Parameters	**Optimum Condition**	**Reference**
pH	6.5-10	Deniz, 2020
Temperature	25-30 °C	Konopka and Brock, 1978
Growth Metabolism	Mixotrophic	Daliry et al., 2017
Nitrogen Source	NH_4^+	Liu et al., 2016
Phosphorus Source	$H_2PO_4^-$	Larsdotter, 2006
N:P Ratio	5-15 g/g	Aslan and Kapdan, 2006
Inorganic Carbon Source	HCO_3^- and CO_3^{-2}	Carvalho et al., 2006
Organic Carbon Source	Glucose	Daliry et al., 2017
Aeration	0.4 vvm	Anjos et al., 2013
Light Intensity	50-220 µmol m^{-2}/s^{-1}	Khalili et al., 2015; Daliry et al., 2017
Illumination Period	24:0 (light: dark)	Deniz, 2020
HRT	2-8 days	Larsdotter, 2006
NLR	4-11 mg/L.d	Ruiz et al., 2011; Zarrinmehr et al., 2020
PLR	1-5 mg/L.d	Şentürk and Yıldız 2020; Ruiz et al., 2011
OLR	0.2 g/L.d	Gupta et al., 2017

2.3 Microalgae Cultivation Systems

Microalgal cultures can be enriched in various systems for its commercial use. In literature systems are usually grouped into two categories: Open Pond Systems and PBRs.

2.3.1 Open Ponds

Open ponds are mostly concrete or compacted earth systems which comes in different shapes. The fundamental criteria that are provided by a pond are enough sunlight, an appropriate hydrodynamic force, and a closed loop channel mixing to uniform the cells when compared to closed systems (Zuccaro et al., 2019).

There are many important design parameters for these systems to be operated correctly like temperature of the wastewater, dimensions of the pond and light input (Zuccaro et al., 2019). Unfortunately, these systems are vulnerable to seasonal changes that causes uncontrolled change in ionic composition. Moreover, the more the algal culture becomes weak due to seasonal changes, higher the possibilities would be for microorganisms such as fungus, bacteria, virus, rotifers, Cladocerans (e.g., Daphnia), Amebae, Cyclopoid copepods, Ciliates, and Chironomid midges to predominate the algal culture (Zuccaro et al., 2019).

Contrariwise, microbial community interactions in open ponds provide a mutual benefit to populations. The most famous one is the bacteria and microalgal symbiosis, which benefits extracellular products and hence, improves removal efficiencies and algal growth performance (Kumar et al., 2015; Ryu et al., 2014). More than 80% of algal biomass is produced in open ponds around the world, owing to the low capital cost required for these systems. The use of closed PBRs, on the other hand, is expected to increase in demand and sales by 2024 due to profits associated with these production systems (Zuccaro et al., 2019).

2.3.2 Closed Photobioreactors (PBRs)

PBRs are designed to have control over microalgal generation in higher biomass efficiencies (Wang et al., 2012). Although the cultivation mode incurs further

expenditures in terms of light illumination, CO_2 and cultivation feedings compared to open ponds (Zuccaro et al., 2019), many advantages make these systems appealing. First, PBRs need less maintenance in the systems that are influenced by variables such as pH, temperature, light, the provided CO_2 concentration. Moreover, loss of water by evaporation and stripped CO_2 can be prevented by capturing (Singh and Sharma, 2012).

However, PBRs have a few limitations, including biofilm formation, which leads to oxygen accumulation in the culture. Biofilm formation can cause a detrimental effect on photosynthetic growth and may prevent light penetration. The function of light, the role of circulation, the role of mass transfer, the materials used in the structure, and the temperature are still the essential factors to consider in building a PBR (Zuccaro et al., 2019).

2.4 Applications of Microalgal Cultures

2.4.1 Commercial Productions

Due to its composition (Table 2.3.), algae have been always a sustainable source for many commercial applications. Axenic algal cultures have been employed for edible purposes for centuries. However, since 1950s the rise in worldwide population led the scientists to develop algal cultures for unconventional protein sources and antibiotic sources (Borowitzka, 1995). Marching on to 1970s, algal cultures were prompted by the scientists for renewable energy sources and the first aquaculture emerged in Mexico. No later than 1980, there were 46 factories over Asia that mass-produces microalgae, being mostly *C. Vulgaris* (Pulz and Scheibenbogen, 2007).

Table 2.3. General composition of different algae (% of dry matter) (Tandon and Jin, 2017)

Microalgae	Protein Content	Carbohydrate Content	Lipid Content
Anabaena cylindrica	43–56	25–30	4–7
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Porphyridium cruentum	28–39	40–57	9–14
Scenedesmus obliquus	50–56	10–17	12–14
Spirulina maxima	60–71	13–16	6–7
Synechococcus sp.	63	15	11

2.4.1.1 Biofuel Production

In microalgal cells, triacylglycerol is the main type of energy storage, accounting for 60–70% of the dry cell weight (Hu et al., 2008; Scott et al., 2010). Three fatty acid (FA) chains are attached to a glycerol backbone in each TAG molecule. Depending on the degree of unsaturation, each FA molecule is classified as saturated FA (SFA), monosaturated FA (MUFA), or polyunsaturated FA (PUFA). The abundance of those molecules determines how the extracted oil is tailored for various uses such as nutrient supplements, emulsifiers, biofuels, and so on (Sharma et al., 2018). The biofuels can be listed as, biodiesel, bioethanol, biohydrogen, biomethane, and bioelectricity (Sharma et al., 2018).

Microalgae have been considered a promising future biofuel feedstock due to their diverse characteristics regarding their growth characteristics and grow on non-productive land and use poor-quality water, the ability to remove pollutants from wastewater and sequester CO_2 from flue gases (Bennion et al., 2015; Chisti, 2007; Frank et al., 2013; López Barreiro et al., 2013).

Biodiesel synthesis by transesterification of lipids, bioethanol production through fermentation of algal biomass, biogas production through anaerobic digestion, and thermochemical conversion are some of the different processes for turning microalgae into biofuel. However, for the environmental scientists the best approach would be the anaerobic digestion in the application of microalgae for biofuel production. By recovering nutrients from the removed leftover biomass and producing electricity from the methane biogas, anaerobic digestion can help to solve some of the issues coming from the capital-intensive dewatering process, high number of residues left from the lipid extraction and constant requirement for fertilizers (Molina Grima et al., 2003; Pragya et al., 2013).

The potential of a flue gas from a sugar factory to promote microalgae growth for biofuel and biofertilizer production is assessed in a study conducted by Zewdie and (Zewdie and Ali, 2020). They have reported that according to the integrated process model biodiesel, upgraded biogas, and bio-fertilizer, were produced, with production capabilities of 188 tons/year, $1.97x10^6 m^3$/year, and 42 tons/year, respectively. The electricity and thermal energy demands were also found to be 1822.13 and 3244.99 MWh/year, respectively, for the manufacturing of these items.

2.4.1.2 Nutrient Production for Human Consumption

Microalgae have gained attention past decades over its important Polyunsaturated fatty acids (PUFA) content that has a pharmaceutical value for human and animal health (Khozin-Goldberg et al., 2011; Martins et al., 2013; Ruxton et al., 2004). PUFA is a raw matter of fish oil, i.e. nowadays famous supplement. However, when the PUFA has been obtained from fish, there are possibilities of contamination with heavy metals, odour, environmental impacts (degradation in marine environment) and of course possible depletion of resources (De Swaaf et al., 2003; Greene et al., 2013; Martins et al., 2013). Microalgae, however, is a great candidate for a healthier

option to obtain PUFA from for the human consumption. Additionally, it is environmentally friendly and a “vegetarian and vegan “alternative (Khozin-Goldberg et al., 2011). Furthermore, Spirulina, has also the essential amino acids (EAAs) as well as many minerals like iron, making it a prominent source of plant-based protein. The protein content of Spirulina Maxima has seven times more protein than soybeans, on the same area of land (Kosaric et al., 1974). Moreover, Matondo et al., (2016) states an important essence that malnourished kids with ages between 6 months to 5 years old in the Democratic Republic of Congo have exhibited considerable improvement in their health conditions when they were fed with Spirulina.

To utilize microalgae species, they need to be harvested. Many researchers reported harvesting results changing from 40 to 150 ton/ha.year (Chisti, 2013) (dry matter) (Pedroni, et al., 2003). However, to harvest 100-ton microalgal biomass, up to 200 of CO_2, 10 ton of N, and 1 ton of P should be provided to that biomass. In a conventional algal production process, those nutrients are provided by chemical fertilizers to the system (Acién Fernández et al., 2018). Conversely, nowadays, an innovative approach to diminish this consumption, highlights the importance of the utilization of flue gases and wastewater as nutrients source (Acién Fernández et al., 2012).

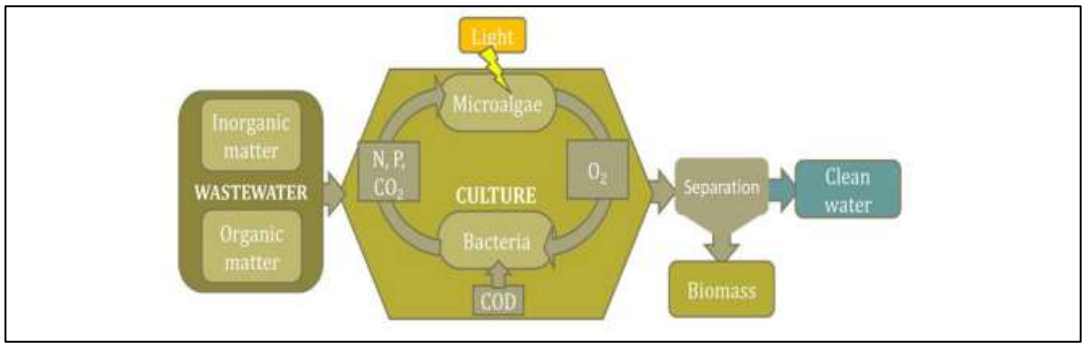

Figure 2.10. Use of wastewater as nutrient source for algal enrichment (Acién Fernández et al., 2018)

Acién Fernández et al. (2018) suggests that the N and P present in the effluents of wastewater treatment plants can be recovered as effective biomass by growing microalgae on them (Figure 2.10). Approximately 1 kg of dry microalgae biomass can be produced over per m^3 of sewage, while more than 10 kg of dry microalgae biomass can be produced over per m^3 of manure(Gabriel Acién et al., 2016).

A great example was demonstrated in a study performed by Kosaric et al. (1974). In this study, researchers enriched *S. Maxima*, which is the well-known high protein alga, in effluents from the London Municipal Waste Treatment Plant. The composition of product algae and the nitrogen and phosphorus removal performance were investigated, as well as the development of optimal growth conditions were performed. The benefits of this procedure in tertiary wastewater treatment and the single-cell protein quality were studied (Kosaric et al., 1974). It can be concluded that utilization of wastewaters for algal enrichment and valorisation of the biomass used in wastewater treatment can be a cost effective and environmentally friendly applications.

2.4.1.3 Pigment Production

Natural pigments are one of the most important groups to be studied among the diversity of chemicals produced by microalgae. Natural pigments derived from microalgae have health benefits (antioxidant, anticancer, and anti-inflammatory) and can be used to substitute commercial colorants with advantages (Rodrigues et al., 2015). Phycobiliproteins (up to 8% of dry weight), carotenoids (typically 0.1–0.2% of dry weight, but up to 14 percent in some species), and chlorophylls (0.5–1.0% of dry weight) are the three pigment groups present in microalgae (D'Alessandro and Filho, 2016). *C. Vulgaris*, *Spirulina platensis, Haematococcus pluvialis*, and *Dunaliella salina* were revealed to be the most important microalgal sources of chlorophylls, phycocyanin, astaxanthin, and β-carotene, respectively (Silva et al., 2020).

It is a fact that the production of pigments may be challenging due to the demand for strict control of operational conditions such as nutrients, pH, temperature, aeration rate, CO_2 concentration, and light regime, inoculum stage and size (Singh and Sharma, 2012). Stress management techniques have been suggested by researchers to increase biomass and high-value chemical yields. This technique basically depends on the control of significant deviation from the optimal conditions of the culture, resulting in modifications at all levels of the functionality of organism. These stress factors can be assessed in two categories: nutritional and physical stress. Physical factors are described as manipulations in operation conditions and external elements that affect microalgae growth, while nutritional factors are described as manipulations of culture. These manipulations can be listed as high light intensities, temperature, pH, salinity, and electromagnetic fields (Ördög et al., 2012; Rao et al., 2007; Wang et al., 2008; Yeesang and Cheirsilp, 2011).

A review study prepared by Benavente-Valdés et al. (2016) suggests that, limiting the N and P and increasing the salinity are viable methods of improving biomass, pigments, and lipids production in Chlorophyceae species. Controlling irradiance and photoperiod in cultures has been highlighted for being critical for the development of metabolic processes in microalgae for the synthesis of high-value chemicals (Benavente-Valdés et al., 2016).

2.4.2 Wastewater Treatment

Culturing microalgae for the commercial purposes additionally may serve as a wastewater treatment system. Conventional treatment systems are usually energy intensive due to their aeration system. Furthermore, due to a lack of local technical expertise, management of these sewage treatment plants in rural areas is difficult for the primary and secondary settling tanks. The WWTP frequently faces challenges such as nitrification reduction (due to a decrease in nitrifier activity), bulking and foaming (due to the excessive growth of filamentous bacteria), and so on (De-Bashan et al., 2004; Khin and Annachhatre, 2004). The high operational and maintenance requirements of WWTPs including solid waste material handling make it economically unfeasible. Furthermore, tertiary treatment for total removal of organic ions via chemical treatment is prohibitively expensive and has the potential to generate additional pollution (Abdel-Raouf et al., 2012).

Though, due to the low operational costs and less requirement for technical skill in system operation, algae-mediated wastewater treatment has started to be considered a potential solution for wastewater treatment in peri-urban and rural settings. Algal wastewater is powerful withinside the elimination of nutrients (C, N and P), coliform bacteria, heavy metals and the discount of chemical and biochemical oxygen demand, elimination and/or degradation of xenobiotic compounds and different contaminants (Abdel-Raouf et al., 2012; Cai et al., 2013; Rawat et al., 2011). Hence

algal-mediated compartments can be integrated to conventional WWTP as tertiary or quandary treatment. Figure 2.11 shows 3 examples to install the microalgae process (Tao, 2019).

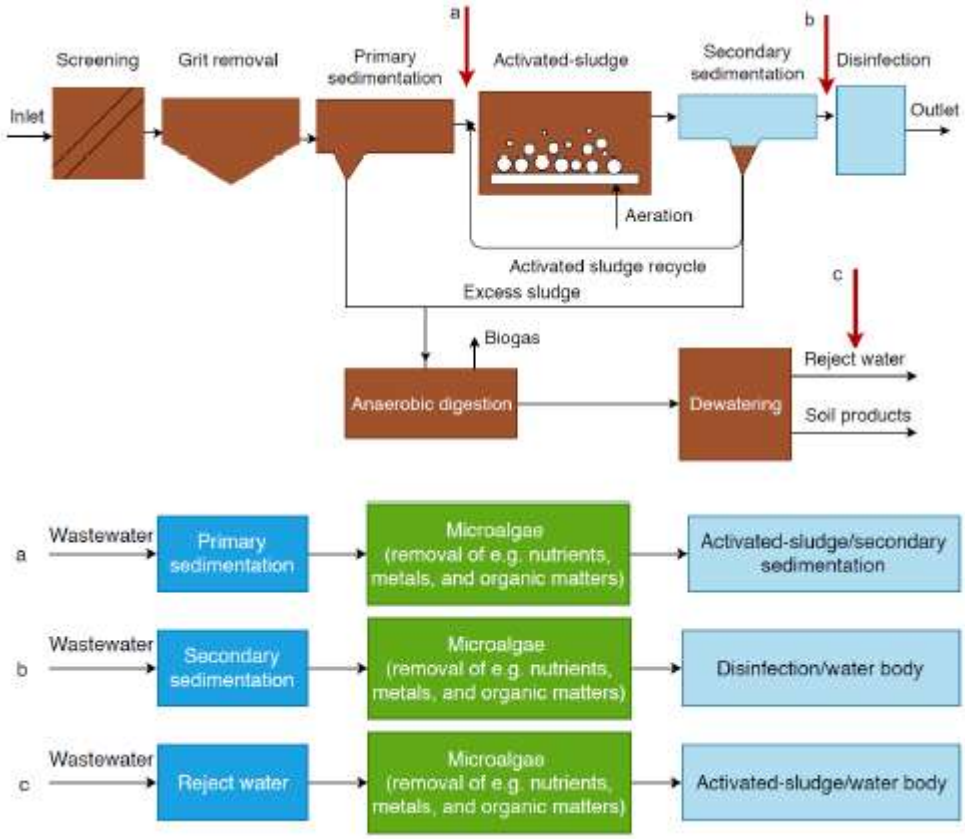

Figure 2.11. A conventional WWTP and possible points to install microalgae processes (Tao, 2019)

Moreover, algal treatment has many advantages over the conventional wastewater treatment systems. For example, less sludge formation, less energy requirement, reduction in greenhouse gas emissions, lower capital and maintenance costs, and the

opportunity of value-added products formation from the algal biomass comes with the algal remedy (Cai et al., 2013).

Algal systems have many areas of use such as human sewage, cattle wastes, agro-commercial wastes, commercial wastes, swine effluent, meat processing waste and different agricultural waste substrates (Abdel-Raouf et al., 2012; Al-Jabri et al., 2021; Cai et al., 2013). Table 2.4 summarizes different wastewaters and microalgae species that are used in the treatment of those.

As it can be seen in Table 2.4, the attractive features of *C. Vulgaris* have been reasons for its preference in treatment of various wastewaters (P. J. He et al., 2013). In addition to the ones mentioned earlier in this chapter, *C. Vulgaris*, has rapid growth rate and a short generation time that reduces the necessary start-up time of treatment systems (Canovas et al., 1996). Additionally, it has exhibited an enhanced growth performances as well as the potential in wastewater treatment and biodiesel production. Moreover, *Chlorella* sp. has high efficiency of removal of nitrogen and phosphorus (more than 80%) of nutrients in primary and secondary treatment effluents (L. Wang, Min, et al., 2010(L. Wang, Min, et al., 2010); Lau et al., 1995; Pittman et al., 2011). Since *C. Vulgaris* is resistant to invaders and can withstand the harsh environmental conditions of wastewaters, its use is ideal for large-scale production (Safi et al., 2014), and in the treatment of agricultural, and domestic wastewater, and high strength wastewaters (Borowitzka, 1999).

Table 2.4. Microalgae species in various wastewater treatment

Microalgae	Wastewater	References
Swine manure	*Chlamydomonas* sp. *Microspora* sp. *Chlorella* sp.	Godos et al., 2009
Digested Swine Manure	*Oocystis* sp. *Chlorella* sp. *Protoderma* sp. *Chlamydomonas* sp.	Molinuevo-Salces et al., 2010
Fish Processing WW	*Chodatella* sp. *Microspora* sp. *Scenedesmus* sp.	Riaño et al., 2011
Slaughterhouse WW	*Chlamydomonas subcaudata,* *Teilingia* sp. *Anabaena* sp.,	Hernández et al., 2016
Municipal sewage water	*Chlorella* sp. *Spirulina* sp.	Li et al., 2011
Agro-industry WW	*Scenedesmus obliquus* *C.vulgaris*	Godos et al., 2010
Pharmaceutical WW	*C. sorokiniana*	Escapa et al., 2015
Landfill leachate	*C.vulgaris* *Acutodesmus obliqus*	Khanzada, 2020 Sforza et al., 2014
WW from biomass to energy process	*Tetraselmis sp.* *Picochlorum sp.* *C. Vulgaris*	Das et al., 2020 Du et al., 2012
Wastewater from mines	*Micratinium reisseri*	Ji et al., 2014
Chicken Manure	*C. Vulgaris* *Scenedesmus obliquus*	Han et al., 2017
Olive Mill WW	Microalgae Consortia *C. Vulgaris*	Caprio et al., 2018

Members of the Proteobacteria, Bacteroidetes, Firmicutes, Synergistetes and Actinobacteria phyla found in wastewaters may predominate the algal consortia and create a biotic stress that may deteriorate culture health (Higgins et al., 2018; Jiang et al., 2021; Su et al., 2011; Yang et al., 2018). Nevertheless, a mutualist and/or commensalist interaction of bacteria with alga is always possible (Jiang et al., 2021).

Ecological studies performed on lichens and artificial environments have demonstrated that natural interactions include nutrient substrates, signal chemicals, and gene expression that is driven by evolution (Ramanan, et al., 2016). Figure 2.12. shows the interaction nature of microalgae and bacteria (Jiang et al., 2021).

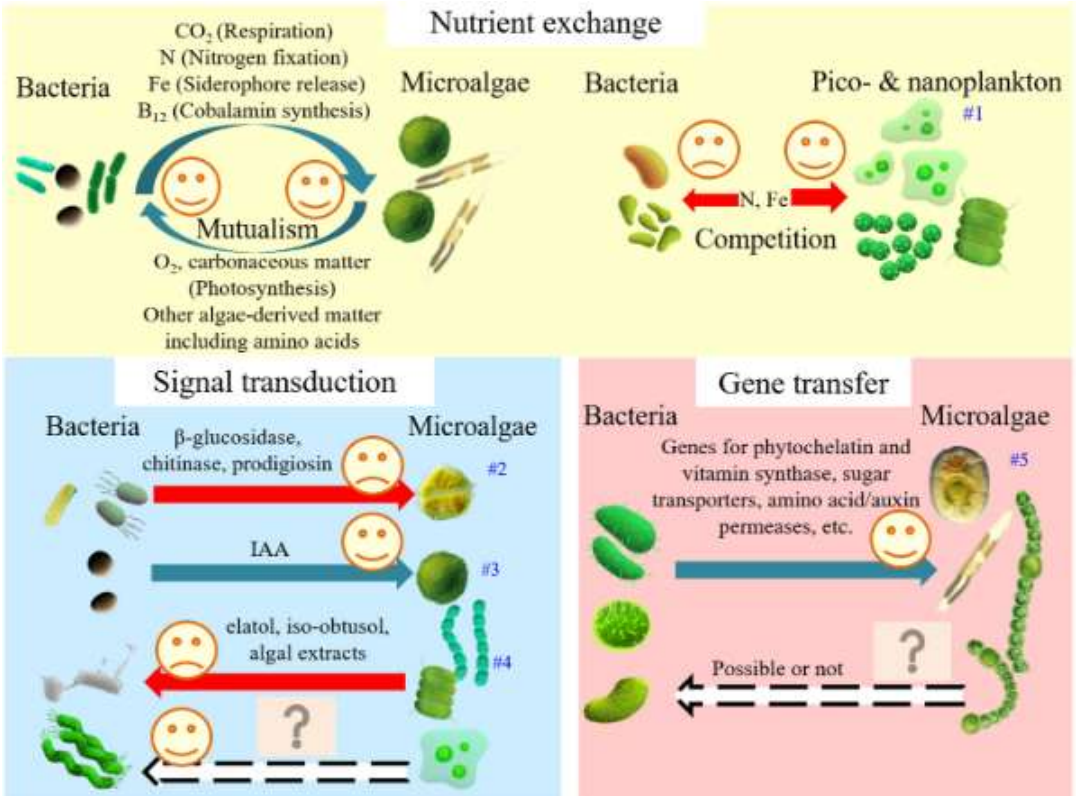

Figure 2.12. The interactions between microalgae and bacteria in natural lichen or simply designed artificial co-culture systems (Jiang et al., 2021)

As can be seen in the figure above, algal-bacterial interaction has so many effects on algal species that improves the treatment and growth performance. Nutrient interactions basically depend on a mutualistic exchange of necessary nutrients for both party (Jiang et al., 2021). Those nutrients can be listed as O_2 and CO_2, organic

carbon sources, amino acids, and vitamins. In signal transduction, however, chemicals produced by bacteria and algae serve as an activation/inhibition starter rather than a consumable nutrient (X. Wang et al., 2010). For example, indole-3-acetic acids and tryptophan, that are released from species like *Pseudomonas Putida* and *Sulfitobacter* sp., enhances the growth of microalgae and improves the overall algal quality and nutrient removal efficiencies (S. A. Amin et al., 2015).

In this interaction algal-bacterial, *C.Vulgaris* may have advantages over other algal species due to its robustness and the ability to accumulate high amount of lipids as storage materials, under such stresses. Hence, it can be concluded that, *C. Vulgaris* is appropriate to cultivate in wastewater, thus use it for wastewater treatment and biofuel generation as value-added product (Wirth et al., 2020). In Table 2.5, many researchers have studied the utilization of algal-bacterial interaction for wastewater treatment purposes.

Table 2.5. Studies using algal-bacterial co-culture and removal performances (Mujtaba and Lee, 2016)

Co-culture (microalgae / bacteria)	**Wastewater**	**Characterization**	**N removal (%)**	**P removal (%)**	**References**
C. Vulgaris / A. brasilense	Synthetic	$NH4^{+}$: 3 mg/L $PO4^{-3}$: 12 mg/L	91	75	De-Bashan et al., 2002
C. Vulgaris / B. licheniformis	Synthetic	$NH4^{+}$: 20 mg/L TP: 4 mg/L	86	93	Liang et al., 2013
C. Vulgaris / Pseudomonas putida	Synthetic	TN: 50 mg/L TP:10 mg/L	80	60	Mujtaba et al., 2015
C. microporum / WW bacteria	Municipal	TN: 39.5 mg/L TP: 5.3 mg/L	88	89	C. S. Lee et al., 2015
C. Vulgaris / WW bacteria	Municipal	TN:29-246 TP:1.4-19.6	97	98	He et al., 2014

Co-culture (microalgae / bacteria)	Wastewater	Characterization	N removal (%)	P removal (%)	References
C. pyrenoidosa / WW bacteria	Leachate	TN: 1786 mg/L TP: 4 mg/L	95	95	X. Zhao et al., 2014
S. obliquus / activated sludge	Piggery	TN: 44 mg/L TP: 19 mg/L	36	65	Godos et al., 2010
C. sorokiniana / activated sludge	Piggery	TN: 44 mg/L TP: 19 mg/L	21-25	23-54	Godos et al., 2010
C. sorokiniana / activated sludge	Swine	TN: 180 mg/L TP:15 mg/L	99	86	González et al., 2008
C. sorokiniana / anaerobic sludge	Agro-industrial	TN: 12 mg/L TP: 50 mg/L	83-95	58-81	Hernández et al., 2013
C. Vulgaris / WW bacteria[a]	Chicken Manure Supernatant	$NH4^+$: 20-162 TP: 34.5-94.8 mg/L	50-80	3-88	Wirth et al., 2020
C. Vulgaris / WW bacteria[b]	Anaerobic Fermentation Effluent	$NH4^+$: 21-300 mg/L TP: 2-34.7 mg/L	16-76	50-83	Wirth et al., 2020
C. Vulgaris / WW bacteria[c]	Municipal	$NH4^+$: 8-50 mg/L TP: 2-34.5 mg/L	80-100	80-100	Wirth et al., 2020

[a] *Actinobacteria* (55%), *Bacilli* (27%), *Gammaproteobacteria* (7%),
[b] *Clostridia* (33%), *Bacteroidia* (27%), *Bacilli* (8%)
[c] *Beta-* and *Gammaproteobacteria* (23–23%), *Actinobacteria* (13%)

All things considered; it can be stated that microalgae have many characteristics that attract the interest of scientists. As a summary of algae-mediated wastewater applications, the following remarks should be made:

- Microalgae is a fast-growing microorganism and adapts the environment quickly, that may shorten the start-up time of the wastewater treatment processes.

- Microalgae can be used as value-added products such as biofuel, vitamins, proteins, drugs.
- Microalgal treatment can be preferred as a wastewater treatment option due to the reduction in greenhouse gas emissions, lower capital, and maintenance costs as well as high nutrient removal performance.
- Microalgae can be used in many wastewaters as a treatment facility depending on parameters such as HRT, NLR, PLR, OLR, type of wastewater, bacterial composition etc.
- Microalgae-Bacteria interaction can be utilized for the treatment of various wastewaters and can remove nutrients up to 100%.
- Microalgae-Bacteria interaction may create an environment that algal growth and treatment performances are improved.
- *C. Vulgaris* may be selected over other algal species due to its resistant nature against challenging environmental factors, ability to accumulate high amount of lipids, and potential for economical applications in many areas of use.

CHAPTER 3

DETERMINATION OF OPTIMUM ENVIRONMENTAL AND OPERATIONAL PARAMETERS TO ENRICH *CHLORELLA VULGARIS* UNDER AUTOTROPHIC CONDITIONS

3.1 INTRODUCTION

In the world with a rising population, while uncontrolled wastewater discharge jeopardizes social-economical balances, environmental safety, water security and human health (Asgharnejad et al., 2021; Molinuevo-salces et al., 2019), CO_2 levels in the atmosphere is alarming governments nowadays. Therefore, various wastewater treatment technologies as well as carbon capture methods have gained popularity and become focus for research and development studies (Hong et al., 2019; Song et al., 2019; Yu et al., 2021).

Microalgae-mediated wastewater treatment systems can be solution for many problems mentioned above. First, photosynthesis by microalgal species accounts for 50% of global oxygen production (Cadoret et al., 2012), that is critical for the mitigation of global warming effects. Additionally, with algal-mediated wastewater treatment facilities, a reduction of pollution in water bodies is possible. Most importantly, autotrophic microalgal systems are great sources for reduction of CO_2 emissions (Molinuevo-salces et al., 2019). Due to those many advantages, algal-mediated wastewater treatment systems are in top topics of the scientist' agenda.

Especially for the systems that aim both wastewater treatment and carbon sequestration, autotrophic algal systems are crucial. In autotrophic systems, contamination is less likely, and the production costs are lower. Moreover, cellular lipid contents were found to be higher in autotrophic cultivation (Saxena et al., 2020). Moreover, Borowitzka,

(1999) suggests that for the large-scale production of algae, the outdoor autotrophic cultivation method is the optimal to achieve food grade algal biomass production.

However, there are limitations and challenges for algal systems. These challenges can be listed as, the requirement for land, the effect of wastewater characteristics, the influence of environmental and operational conditions, and biomass harvesting and valorisation (Molinuevo-salces et al., 2019). Hence the optimization of algal systems for many parameters became an art of science.

Among parameters optimized, N source is a critical parameter. The matter of choosing the best nitrogen sources for algal enrichment has been a very challenging issue for the researchers. Although the algal metabolism supports NH_4-N as mentioned before, some studies reported that NH_4-N may not be as effective as other nitrogen sources (Gour et al., 2018; Khalili et al., 2015; Zhan et al., 2016). Zhan et al. (2016) revealed that nitrite-nitrogen and nitrate-nitrogen were beneficial for *Chlorella* species, while ammonium-nitrogen has an inhibitory effect on algal growth. On the contrary, Altın et al (2018) obtained the best results in specific growth rate, biomass production rate and doubling time with NH_4^+-N while Xu et al., (2001) demonstrated that NO_3-N results in higher performances in growth, however, NH_4-N provides a more stable growth for *Ellipsoidion* sp. Hence, the optimum nitrogen source should be well optimized specific to the culture and physical conditions where the study is performed.

Illumination period, on the other hand, is a parameter that may be manipulated to get better performances from microalgae. It was exhibited in many studies that, photosynthetic microalgae have response mechanisms to induce the pigment production and photosynthetic activity with cyclic light: dark periods (Chiarini and Quadrio, 2021; Sforza et al., 2014; Stella, 2016). These mechanisms increase the capacity of the photosynthetic organisms to withstand high light stresses (Stella, 2016). Studies show that light:dark period applied on microalgae may result in higher

growth rates as well as pigment formation and lipid production (Wong et al., 2017). In a study conducted by Fakhri et al. (2017), five different illumination periods (12:12, 16:8, 24:0, 8:16, 0:24) demonstrated different results for each parameter like biomass production rate, lipid production rate and saturated fatty acid content. Hence, it can be concluded that for each species and desired parameters to be improved, illumination period should be optimized.

Another important parameter that should be optimized is N:P ratio. Several studies have found that the N:P ratio is important from both an ecological and a biotechnological standpoint. According to Figler et al. (2021), high nutrient concentrations combined with unfavourable N:P ratios may result in insufficient growth or nutrient removal. However, since every wastewater has different N:P ratio, the effect of this parameter on the very species of interest and its nutrient removal performance should be investigated.

This chapter of the thesis focuses on determination of the optimum nitrogen source, illumination period and N:P ratio for *C. Vulgaris* leading to the maximum growth and nutrient removal performance under autotrophic conditions. To this purpose, it was initially aimed at cultivating *C. Vulgaris* under autotrophic conditions. To investigate N source and illumination period 2 sets of batch PBRs were operated. Moreover, to determine the optimum N:P ratio, semi-continuous PBRs were set following the investigation of N source and illumination period. As a side note, this study is the first to report the investigation results of optimum intermittent illumination periods with hourly frequency.

3.2 MATERIAL AND METHODS

This section includes key details on the operational conditions, experimental set ups and analytical methods that are applied in the enrichment of the autotrophic *C.*

Vulgaris culture, investigation of effect of nitrogen sources, N:P ratio and illumination period.

3.2.1 Inoculum

An axenic *C. Vulgaris* culture (Figure 3.1) was obtained from "İstanbul Microalgae Biotechnologies Research and Development Centre" in an agar plate to be cultivated in liquid 3-Fold Bold's Basal Medium with vitamins (3N BBM +V) in accordance with the recommendation of "The UTEX Culture Collection of Algae" under sterile conditions.

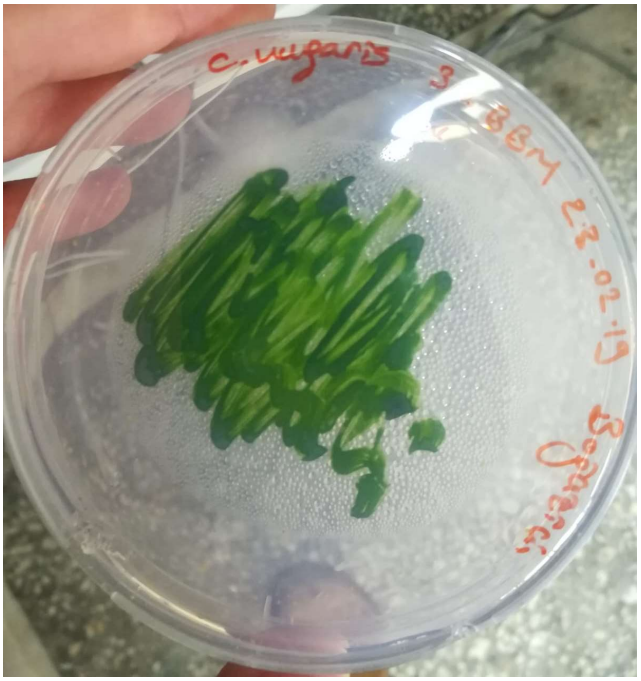

Figure 3.1. *C. Vulgaris* agar culture

C. Vulgaris culture was stored under the conditions recommended by UTEX, at 20 ±3 °C temperature, under 3 klux red led light lighting with 24-hour continuous illumination (UTEX Culture Collection of Algae).

3.2.2 Synthetic Wastewater

To enrich the *C.Vulgaris* culture, as UTEX recommended, "3-Fold Bold's Basal Medium with vitamins" was used. This synthetic media recipe was originally developed by Aghajanian (1979) from Bold's Basal Medium (Bischoff and Bold, 1963) after the discovery of three-fold nitrogen addition to BBM is working for cyanobacteria (Thomas and Montes, 1978). Later, vitamins addition to that medium was developed by UTEX researchers observing that the maximum efficiency from culture can be obtained with the contribution of vitamins. However, unlike the UTEX recipe of 3N-BBM stated, soil water mixture, which is a carbon source, was not added to the synthetic medium in any of the cultivation stages, since the cultivation mode was preferred to be conducted as photoautotrophic instead of mixotrophic.

The details related to the components of the synthetic wastewater and their concentrations are given in Table 3.1.

Table 3.1.3N-BBM + Vitamins medium (UTEX Culture Collection of Algae)

Component	**Final Concentration**
$NaNO_3$	8.82 mM
$CaCl2 \cdot 2H_2O$	0.17 mM
$MgSO_4 \cdot 7H_2O$	0.3 mM
K_2HPO_4	0.43 mM
KH_2PO_4	1.29 mM
NaCl	0.43 mM
P-IV Metal Solution	**Final Concentration**
$Na_2EDTA.2H_2O$	2 mM
$FeCl_3.6H_2O$	0.36 mM
$MnCl_2.4H_2O$	0.21 mM
$ZnCl_2$	0.037 mM
$CoCl_2.6H_2O$	0.0084 mM
$Na_2MoO_4.2H_2O$	0.017 mM
Vitamin Solution	**1 mL/L (for each)**
Cyanocobalamin	0.027 g/200 mL dH_2O
Thiamine HCl	0.067 g/200 mL dH_2O
Biotin	0.005 g/200 mL dH_2O

3.2.3 Photobioreactors (PBRs)

Two different types of PBRs were chosen for the experiments. 250 mL Erlenmeyer Flasks were used in the first step batch cultivation of *C. Vulgaris* to adopt the culture in liquid medium (Figure 3.2.). For initially scaling up of the culture and in all treatment studies, bubble column PBRs, that have 1 L volume, 8 cm diameter and 24 cm height, were used (Figure 3.3.).

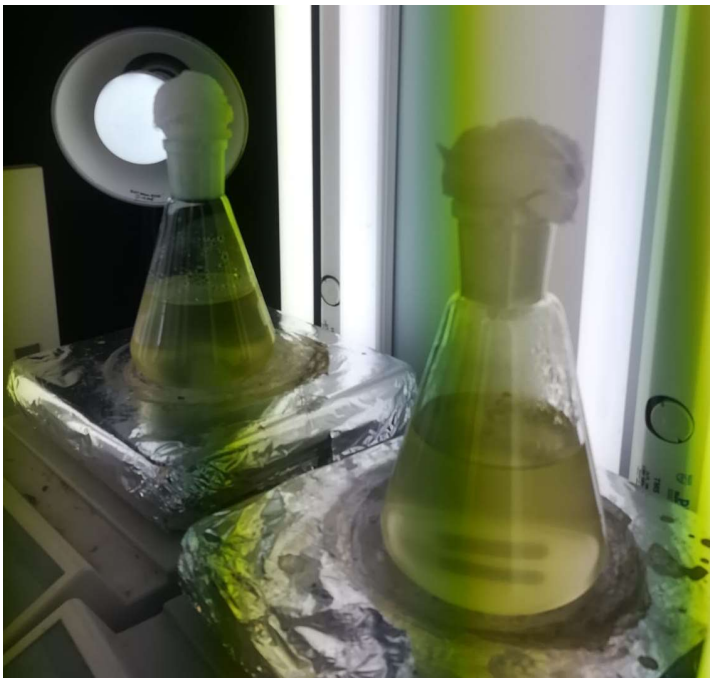

Figure 3.2. Small scale Erlenmeyer Flask PBRs (250 mL)

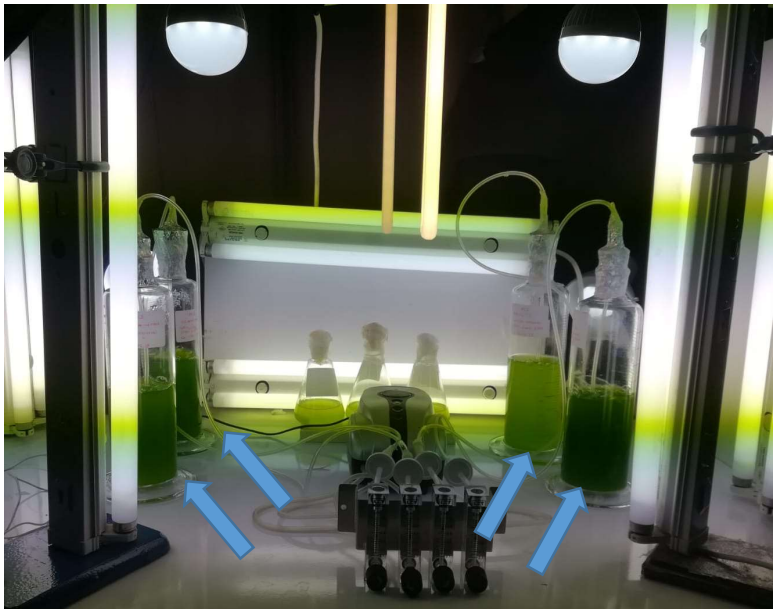

Figure 3.3. Bubble column PBRs (1 L)

3.2.4 Analytical Methods

During the experimental studies, density, pH, temperature, photosynthetically active radiation (PAR), dry weight, total ammonia nitrogen (TAN), ortho-phosphate (PO_4^{-3}-P), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), chlorophyll-a were measured.

pH: pH meter (Eutech, CyberScan, pH510) and pH probe (Sensorex, p350) were used to measure pH value.

Temperature: Temperature values of the PBRs were measured with 9263 A Plus digital thermometer. It should be noted that, the ambient temperature in the sets was measured in a container that has water inside, that would represent the temperature of the PBRs.

Optical Density: HACH spectrophotometer DR 2800 with 1-cm light path was used to measure optical density values at optimum wavelength determined for enriched *C.Vulgaris* culture. To determine the optimum wavelength, optical density values were read at different wavelengths and the highest absorbance value was obtained at 680 nm. Detection limit is between 0.1 and 1, so for samples with optical densities higher than 1, dilution is necessary.

Photosynthetically Active Radiation (PAR): PAR was measured through a hand device called PAR meter (Light SCOUT).

Dry Weight (DW): The dry weight measurement of the microalgal culture was carried out according to the Standard Method (APHA, AWWA, WEF, 2005). According to this method, samples taken from the PBR are primarily filtered through Sartorius brand filters (0.7 m), then dried overnight in oven at 105 °C in 30 mL of crucibles. They are held in the desiccators prior to weighing to remove the moisture.

Total Ammonia Nitrogen (TAN): TAN (NH_4^+-N + NH_3-N) analysis is based on the Titrimetric Method specified in the Standard Method, and Gerhardt Vapodest 40.0 is used for distillation. Samples withdrawn from the PBRs were filtered through the Sartorius brand filters (0.7 µm) for this analysis and diluted according to the required ranges. Related calibration curves are given in APPENDIX A.

Orthophosphate (PO_4^{-3}-P): For PO_4^{-3}-P analysis, the Ion Chromatography (IC) (Dionex) device has been utilized. For this, samples withdrawn from the PBRs were first filtered out of the syringe filters (0.45 µm). Then, by measuring the conductivity of the samples, the samples were diluted with distilled water, to a value of below 300 µS. IC device was set to a lowest limit of 100 psi, highest limit of 300 psi pressure, 5 Hz of data collection rate, temperature of 35°C, suppressor current of 50 mA and flow rate of 1 mL/min (APPENDIX B). The calibrations curve for PO_4^{-3}-P analysis is given in APPENDIX B.

Nitrate-Nitrogen (NO_3^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_3^--N analysis is given in APPENDIX B.

Nitrite-Nitrogen (NO_2^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_2^--N analysis is given in APPENDIX B.

Chlorophyll-a, Pheophytin-a and ß-Carotene: Pigments measurements were done according to the Standard Methods 10200H (APHA, AWWA, WEF, 2005). Optical density ratio of 664b/665a (OD (664b/665a)) gives insight about health of microalgal culture. Ratio of 1.7 represents the healthiest situation while 1.0 represents death of culture. When chlorophyll-a content of the culture is higher, the ratio would be closer to 1.7; however, when pheophytin-a concentration is high, the ratio would be closer to 1. Pheophytin-a is the chlorophyll-a molecule that lost its Mg^{+2} ion and cannot

function in photosynthesis reactions anymore. Moreover, for chlorophyll-b, and-c and total carotenoid, OD analyses at 647 and 630, 470 nm were performed (Dere et al., 1998). Equations 3.1-3.5 were used to determine chlorophyll-a, pheophytin-a, chlorophyll-b, chlorophyll-c and total caratenoid, respectively.

Chlorophyll a, $mg/m^3 = 26.7(OD_{664}-OD_{665})\ V_1/(V_2L)$ ……..…..... (Equation 3.1)

Pheophytin a, $mg/m^3 = 26.7(1.7xOD_{665}-OD_{664})\ V_1/(V_2L)$ …..... (Equation 3.2)

Chlorophyll b, $mg/L = 21.03\ (OD_{647}) – 5.43\ (OD_{664}) – 2.66\ (OD_{630})$ (Equation 3.3)

Chlorophyll c, $mg/L = 24.52(OD_{630}) – 7.60(OD_{647}) – 1.67(OD_{664})$… (Equation 3.4)

Total Carotenoid $\mu g/mL = (10^3(OD_{470}) – 1.90C_a – 63.14xC_b)/214$ …(Equation 3.5)

Where,

C_a: chlorophyll a

C_b: chlorophyll b

All analyses, except OD, were performed as duplicate, and averaged values were used in the figures and/or tables. OD analysis consists of three lines of work; standard deviation values are presented in the figures. In the calculation of the specific growth rate (μ) of the microalgal culture, the following Equation 3.6 was used (Krzemińska et al., 2014; F. Liang et al., 2013). Equation 3.7 (Liu et al., 2011) was used to determine double the number of cells (t_d) and Equation 3.8 (F. Liang et al., 2013) was used to calculate the biomass production rate (BPR). These values were calculated considering the steady-state conditions of the PBRs. The steady-state conditions in the studies were defined as “the point where the parameter value does not change more than 10% in three consecutive days” (Kılıç, 2017).

$\mu = \ln(N_2/N_1)/(t_2-t_1)$ …… (Equation 3.6)

$t_d = \ln(2)/\mu$ ……...… (Equation 3.7)

$dX/dt = (X_2-X_1)/(t_2-t_1)$ ……..…… (Equation 3.8)

N_1: OD value at time t_1

N_2: OD value at time t_2

μ: Specific growth rate (day^{-1})

t_d: Doubling time (days)

dX/dt: Biomass production rate (mg/L.d)

X_1: Dry weight of the microalgae at time t_1 (mg/L)

X_2: Dry weight of the microalgae at time t_2 (mg/L)

3.2.5 Experimental Setup

3.2.5.1 Cultivation of *C. Vulgaris*

Liquid Medium Cultivation

In liquid medium cultivation, inoculation was performed from the solid agar culture by mixing a loop of cells into a 1 mL of 3-N BBM+V (Thiamine and Cyanocobalamin) and then adding this 1 mL into 250 mL flasks that contains 150 mL of 3N BBM +V (Figure 3.4).

Firstly, to enrich the culture in sterile conditions, mediums and necessary materials were heat-treated at 121°C for 20 min in autoclave and the culture was inoculated under sterile conditions in laminar flow bio-safety cabin. The medium was prepared according to the recipe given in Table 3.1, and pH was set to 6.2 as UTEX Algae Culture Collection Centre recommended, with 1N NaOH and 1N HCl solutions.

The flasks were operated for 20 days. 150 µmol.m^{-2}.s^{-1}(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for 12:12 light: dark (L:D) illumination period for all PBRs. The PBRs were operated at an average temperature of 25 ± 3°C. During the first enrichment of cultures, no additional air supply was provided to the PBRs to prevent contamination. Instead, PBRs were mixed via magnetic stirrers at 100 rpm in order to increase the diffusion of air to the culture.

Figure 3.4. *C. Vulgaris* culture enriched in liquid medium

No analysis was performed since, with this set, it was intended to transfer *C. Vulgaris* culture from solid medium to liquid medium. In order to not to interfere with the

sterile conditions of the culture, pH change was not observed, and the goal was set to achieve a fresh stock culture that was not contaminated.

Solid Medium Cultivation

The obtained *C. Vulgaris* culture in solid media, was transferred to other replicant petri dishes to maintain a source culture. Recommended by UTEX, the petri dishes included 3N-BBM+V synthetic media (Table 3.1) and agar (Sigma Aldrich) mixture to create a solid medium. Petri dishes were incubated at 20±3°C under continuous illumination with 3 klux red led light (Kendirlioglu and Çetin, 2017). Given the consumable compounds (nitrate and phosphate) are declining in 3N-BBM+V synthetic media during the incubation period, the culture was transferred to replicant petri dishes every 10 days.

3.2.5.2 Cultivation of *C. Vulgaris* in Batch PBRs

Set 1: Cultivation of C. Vulgaris with Nitrate Source

The culture enriched with the previous set in the liquid medium was transferred to 1 L volume PBRs. The increase in the number of microalgal cells during operation is a limiting factor for culture to grow to a certain level. Hence, it is important to increase the scale of the PBRs during cultivation period, and thus reduce the space stress on culture to observe an improved growth (Fanesi et al., 2021).

Experiments were carried out by operating four glass PBRs, each with a total volume of 1L and an effective volume of 800 mL, in batch mode (Figure 3.5). B Three of them (M1, M2 and M3) were replicates and inoculated with microalgal culture, which was enriched in liquid medium (Section 3.2.5.1). The fourth PBR (ORI) was directly inoculated from agar culture (solid medium) to see the effect of scale on newly enriched culture and to ensure the continuity of the original culture. A 15% (v/v) inoculation ratio was used for M1, M2 and M3 PBRs. To do this, 20 mL culture

was acquired from each PBR installed in the previous set (Section 3.2.5.1) and mixed, creating an inoculation for 1 L PBRs. To avoid contamination of the culture, all materials, including the glass PBRs, were heat-sterilised in autoclave at 121°C for 20 min and PBRs were inoculated in the laminar flow bio-safety cabins under sterile conditions. The medium was prepared according to the recipe given in Table 3.1, and the pH of the medium was set to 6.3-6.4 with 1N NaOH and 1N HCl solutions.

PBRs were operated for 16 days. 150 μmol.m^{-2}.s^{-1}(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for 12:12 L:D (light:dark) illumination period. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs with a flowrate of 0.4-0.6 L/min (0.5-0.75 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination.

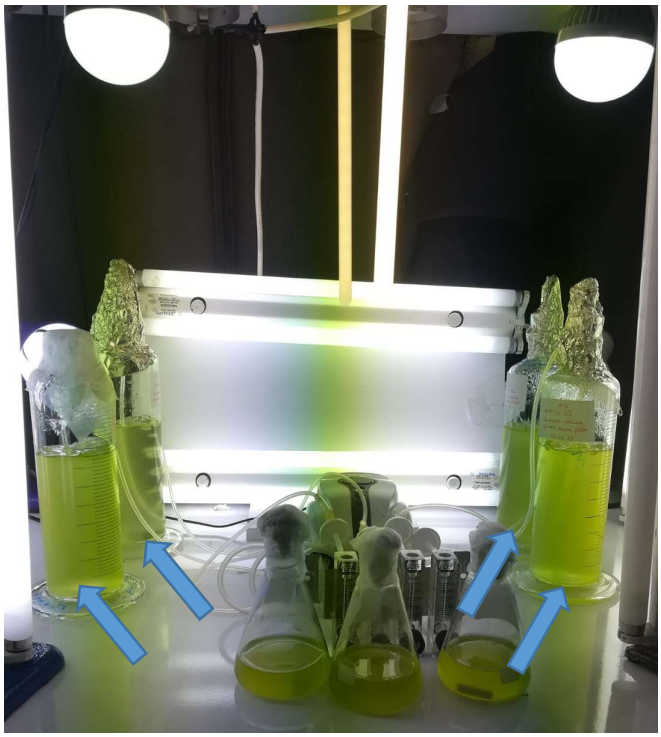

Figure 3.5. Scaled up enrichment of *C. Vulgaris* culture in nitrate medium

To detect doubling time, specific growth rate and biomass production rate, optical density and dry weight analyses were performed every other day, as well as pH monitoring.

Set 2: Cultivation of C. Vulgaris with Ammonium Source

The enriched *C. Vulgaris* culture in previous PBRs of Set 1 (Section 3.2.5.2) used nitrate as nitrogen source. However, the importance of working with a culture adapted to the content of real waste should also be considered. Since most of the domestic and industrial wastes have ammonium as the predominant nitrogen species instead of nitrate or nitrite, it was decided that they should be enriched with an ammonium (NH_4)-containing synthetic waste. Since this is the case, a modified

synthetic wastewater, in which NO_3^--N was replaced with equimolar NH_4^+-N, was prepared.

The ammonium nitrogen is the most preferred nitrogen species for an algal metabolism. However, consumption of ammonium lowers the pH of the nutrient medium with a released hydrogen ion from the cell wall as seen in Equation 3.9, to maintain the neutral state of the cell (Fuggi et al., 1981; Xin et al., 2010) leading to negative effects on algal growth. Hence, as previous studies show that, HEPEs and PIPES buffers are used to keep the pH of the medium at the optimum value specific to the species. Yet, HEPES / PIPES buffers may not be suitable to be used in biological systems due to their metal interaction (Ferreira et al., 2015). Hence, to ensure the further use of the culture, the pH of the medium was manually adjusted with 1 N NaOH to pH 7.8 (Eustance et al., 2013) during its operation.

$$100CO_2 + 12NH_4^+ + 70H_2O + H_2PO_4 \rightarrow CH_{178}O_{36}N_{12}P + 118O_2 + 11H^+ \quad \text{(Equation 3.9)}$$

Apart from the ammonium adaptation, the effect of two different illumination periods was also investigated. 12:12 (L:D) illumination, which is recommended by UTEX, and the continuous illumination, which is claimed to have an improved effect on the culture compared to other modes (UTEX Culture Collection of Algae) were put on comparison. For this purpose, as it was summarized in Table 3.2, two parallel batch PBRs with continuous illumination, named as C1 and C2, and the other two test batch PBRs with 12:12 (L:D) illumination, named as T1 and T2 were installed. PBRs were inoculated from previous PBRs, M1 and M3 from Section 3.2.5.2, Set 1, having the highest specific growth rates. Experiments were carried out by operating four glass PBRs each with a total volume of 1L and an effective volume of 800 mL, in batch mode. Yet, this time inoculation ratio was decreased from 15% to 15 ‰ by

using only 6 mL seed culture from each PBR (M1 and M3) to avoid further self-shading effect (Sforza et al., 2014).

Table 3.2. Set 2 PBRs and details related to the set-up conditions

PBR type	**Name**	**Operation mode**	**Illumination**
Control	C1	Batch	Continuous
Control	C2	Batch	Continuous
Test	T1	Batch	12:12 (L:D)
Test	T2	Batch	12:12 (L:D)

To avoid contamination of the culture, all materials including the glass PBRs were heat-sterilised in autoclave at 121°C for 20 min and PBRs were inoculated in the laminar flow bio-safety cabins under sterile conditions. The pH of the mediums was set to 6.3-6.4 with 1N NaOH and 1N HCl solutions.

PBRs were operated for 20 days. 150 $\mu mol.m^{-2}.s^{-1}$(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) with continuous and 12:12 (L:D) were provided for illumination. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs with a flowrate of 0.4-0.6 L/min (0.5-0.75 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination.

To detect doubling time (t_d), specific growth rate (μ) and biomass production rate, optical density and dry weight analyses were performed every other day, as well as pH monitoring. Additionally, NH_4^+-N and PO_4^{-3}-P analyses were conducted for the first and the last day of operation. To control the purity of the culture, samples from the PBRs were observed under microscope.

3.2.5.3 Set 3: Effect of Illumination Period on *C. Vulgaris* for Microalgal Treatment and Pigment Production

Illumination period was put on focus of this study to reveal the possible stress factors for *C. Vulgaris* and to obtain optimum period leading to the highest results in growth performances and nutrient removal efficiencies, as well as the pigment production. One of the stresses was exhibited as light stress that can be manipulated as intermittent illumination (Gong and Bassi, 2016). To investigate the effect of this stress factor, four different illumination periods were selected, those were 12h:12h (L:D), 8:8:8 (L:D: L), 6:6:6:6 (L:D:L:D) and continuous illuminations. For this purpose, four batch PBRs with different illuminations, named as I12, I6, I8 and I24, were set (Table 3.3). Experiments were carried out by operating four glass PBRs each with a total volume of 1L and an effective volume of 800 mL, in batch mode. A 20‰ (v/v) inoculation rate was used for those PBRs to prevent any possible self-shading where the possibilities were high with intermittent illumination (Sforza et al., 2014).

Table 3.3. Set 3 PBRs and details related to the set-up conditions

PBR type	Name	Operation mode	Illumination
Test	I24	Batch	Continuous
Test	I12	Batch	12:12 (L:D)
Test	I8	Batch	8:8:8 (L:D:L)
Test	I6	Batch	6:6:6:6 (L:D:L:D)

The inoculum which was acclimated to NH_4-containing synthetic wastewater, were obtained from the previous PBRs, C1 and C2, from Section 3.2.5.2, set 2. To avoid contamination of the culture, all materials included glass PBRs were heat-sterilised in autoclave at 121°C for 20 min and PBRs were inoculated in the laminar flow bio-safety cabins under sterile conditions. The medium was prepared according to the

NH_4-containing version of the one given in Table 3.1, and the pH of the medium was set to 6.3-6.4 with 1N NaOH and 1N HCl solutions.

PBRs were operated for 20 days. 150 μmol.m^{-2}.s^{-1}(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for each illumination period. The PBRs were operated at an average temperature of 28 ± 3°C. Aeration was supplied to all PBRs with a flowrate of 0.4-0.6 L/min (0.5-0.75 L/L/min, vvm) flowrate with air pumps (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination. pH was manually adjusted every 2 days to 7.8 with NaOH solution (Eustance et al., 2013).

To detect doubling time (t_d), specific growth rate (μ) and biomass production rate, optical density and dry weight analyses were performed every other day, as well as pH monitoring. Additionally, NH_4^+-N and PO_4^{-3}-P analyses were conducted for the first and the last day of the operation. Moreover, pigment analysis for the detection chlorophyll-a, -b, -c and β-Carotene were performed every other day.

3.2.5.4 Set 4: Determination of Optimum Nitrogen: Phosphorus (N:P) Ratio for *C. Vulgaris* for Enrichment and Wastewater Treatment

For enrichment of *C. Vulgaris*, a synthetic wastewater with a certain N:P ratio (approximately 2.3 (N:121 mg/L and P:53 mg/L)) was used, as UTEX recommended. However, according to many studies, optimum treatment and enrichment performance were obtained for N:P ratios of 5–15 (Anbalagan et al., 2016; Aslan and Kapdan, 2006; Choi and Lee, 2015b). Therefore, three different N: P ratios of 6, 8 and 10 were chosen to be investigated in this study. To accomplish this goal, TAN input values were set at 120 mg/L, close to the TAN value of the enrichment sets (121 mg/L), and PO_4^{-3}-P values were determined accordingly as 20,

15 and 12 mg/L for N:P ratios of 6, 8 and 10, respectively. Except for TAN and PO_4^{-3}-P concentrations, all other contents in the prepared medium were set as the same as in the 3N BBM+ V medium (Table 3.1).

Experiments were carried out by operating five glass PBRs each with a total volume of 1L and an effective volume of 800 mL, in semi-continuous mode (Figure 3.6). As summarized in Table 3.4, three PBRs, two control PBRs were conducted as control PBRs, contained either nitrate or ammonium. The standard 3N-BBM + V medium was added to all PBRs (Table 3.1). The HRT of the system was set at 8 days (Kılıç, 2017) by performing a 100 mL volume exchange every day.

Table 3.4. Set 4 PBRs and details related to the nutrient concentrations

PBRs		**N-Source**	**Influent Concentrations (mg/L)**	
Type	**Name**		**N**	**PO_4^{-3}-P**
Control	CNO3	NO3	120	52
Control	CNH4	TAN	120	52
Test	T6	TAN	120	20
Test	T8	TAN	120	15
Test	T10	TAN	120	12

To inoculate the PBRs, cultures obtained from the previous PBRs in Section 3.2.5.3., set 3 were mixed, and 5% inoculation was used. Because PBRs in the semi-continuous mode were going to be subjected to a 100 mL volume exchange every day, there was a possibility for loss of biomass. Hence, the inoculation percentage was increased to 5% from the previously used range of 15-20‰. The reason why a percentage less than the values given in the literature (10% and 20% (Papurello et al., 2019)) was preferred is that the system was tried to be kept away from the disadvantage of a self-shading for a longer time.

To avoid contamination of the culture, all materials included glass PBRs were heat-sterilised in autoclave at 121°C for 20 min and PBRs were inoculated in the laminar flow bio-safety cabins under sterile conditions. The pH value of the influent synthetic wastewater was adjusted at 7.5 ± 3 (Choi and Lee, 2013; Eustance et al., 2013). In order to prevent pH decrease caused by ammonium, 50 mM $NaHCO_3$ solution was fed to the system together with the feeding daily applied (Lohman et al., 2015).

PBRs were operated for 87 days. 150 $\mu mol.m^{-2}.s^{-1}$(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) with continuous illumination was provided. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs with a flowrate of 0.4-0.6 L/min (0.5-0.75 L/L/min, vvm) flowrate with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination.

As it will be discussed later in Section 3.3.4., a P accumulation was observed in the content of the PBRs. As a solution for this phenomenon starvation periods were applied in the PBRs. P-starved algal cells accumulate excess amount of P when they are exposed to a P-rich environment, which is known as overshoot or overcompensation mechanism (Chopin et al., 1997). Starvation periods were designed by imitating natural environmental conditions. Hence, luxury uptake, also refers to a phenomenon, that the excess P is often stored as inorganic polyphosphate (Poly-P) (Singh et al., 2018) may be stimulated with P starvation (Solovchenko et al., 2019). To this purpose, the PBRs were fed with their regular feed at irregular intervals (at intervals of 3, 4, 5 and 2 days) for approximately 40 days of operation, after they reached a steady-state condition in terms of growth and TAN removal performances. During this period (of irregular-interval feeding period) the TAN and PO4-P removal efficiencies were assessed as well as chlorophyll-a concentrations to observe the effect of nutrient starvation stress on *C. Vugaris*. Moreover, pH was

observed during this period to have a control over the balance in the system. Yet, dry weight and OD values were not examined. After the irregular-interval feeding period, PBRs were operated at 8-day HRT again.

Figure 3.6. Control and test PBRs conducted in Set 4

To detect doubling time, specific growth rate and biomass production rate, optical density and dry weight analysis were performed, as well as pH monitoring. Additionally, NH_4^+-N and PO_4^{-3}-P analyses were conducted every other day.

3.3 RESULTS AND DISCUSSION

3.3.1 Results of *C. Vulgaris* Cultivation Studies in Liquid and Solid Medium

Experiments conducted within this set were to enhance a healthy stock culture without causing contamination. For this reason, the system was not tampered with any analysis. During the operation of the PBRs (250 mL), an increasing green colour was observed in the PBRs at the end of 20 days. For cultures in the solid composition, the same was intended to maintain environmental conditions during the 20-day operation, and growth was observed in solid agars. Related figures are shared in APPENDIX C.

3.3.2 Results of *C. Vulgaris* Cultivation Studies in Batch PBRs

3.3.2.1 Results of Set 1: Cultivation of *C. Vulgaris* with Nitrate Source

The purpose of this experiment was to enrich autotrophic microalgal culture in a larger scale PBR with nitrate in batch mode. Therefore, four PBRs namely, M1, M2 and M3 (replicates) (each from liquid culture) and ORI (directly from an original agar plate culture) were operated. In these PBRs, nutrient removal efficiency was not aimed to be detected. However, the biomass production rate, doubling time and specific growth rate were determined with analysis. The results of Set 1 are shown in Figure 3.7.

As shown in Figure 3.7.a, pH increased in all PBRs till Day 10, while after Day 14, pH decreased in the M1 and M3 PBRs due to the indigenous decay (Salgueiro et al., 2016). Because the operation of the PBRs M2 and ORI was stopped on Day 14, the similar pH decrease was not observed in these PBRs. The pH decrease has also been

observed in studies that enrich the *C. Vulgaris* culture with nitrate. According to Zhao et al. (2016), the reason for pH raise is that free carbon dioxide is used by algal cells for photosynthesis and a shift in the chemical equilibrium system is occurring because there is a limited amount of CO_2 in the environment during the growth of microalgae. As a result, the concentration of bicarbonate decreases. While the concentration of carbon increases, hydroxyl ions and pH increase as well (Equation 3.10 and 3.11).

$2HCO_3 \rightleftharpoons CO_3^{2-} + H_2O + CO_2$ ……..…….... (Equation 3.10)

$CO_3^{2-} + H_2O \rightleftharpoons HCO_3^- + OH^-$ ……..…… (Equation 3.11)

In this set, M1 and M3 PBRs were in lead compared to other two PBRs (M2 and ORI) in terms of growth performances. Thus, as mentioned previously, the operation of M2 and ORI PBRs, was stopped at the end of Day 14 (Figure 3.7). As shown in Figure 3.7, M1 and M3 PBRs have followed a similar pattern for growth performances for almost the entire operation time. OD values of M1 and M3 PBRs were recorded as 3.129 ±0.005 and 3.127±0.02 at the end of the operation time (Figure 3.7.b.), being comparable with literature values (Sharma, 2012), given for a 16-day operation. On the other hand, the OD values of M2 and ORI PBRs were recorded as 1.62±0.001 and 2.35±0.002, respectively. Such delays in growth may have been due to the problems encountered with the air flow meter during the operation of M2 PBR. Furthermore, the relatively low OD values of ORI PBR was attributed to the fact that the initial OD value of the ORI PBR was slightly less than that of others.

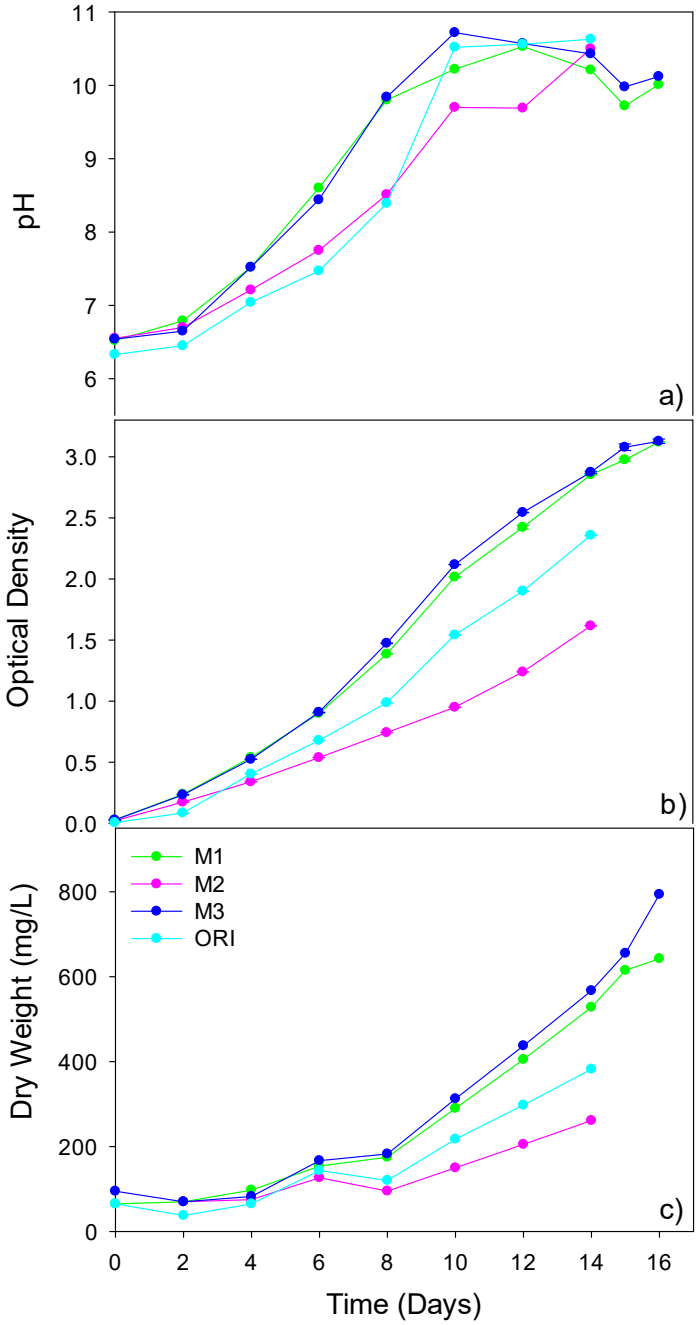

Figure 3.7. Results of M1, M2, M3 and ORI PBRs a) pH b) Optical Density c) Dry Weight

According to the results, specific growth rates were calculated as 0.30 $days^{-1}$ for M1, M2 and M3; which were comparable with the literature values (0.13-0.27 $days^{-1}$) (Lam and Lee, 2012; Mohsenpour et al., 2021; Wong et al., 2017). The ORI PBR was observed to have a greater specific growth rate than the other PBRs with 0.42 $days^{-1}$, although the OD value was smaller than that of M1 and M3 PBR. This might be due to the possible aging phenomena, that is suggested by Winokur (1949). He claims that aging cells of *C. Vulgaris* might cause a drop in growth performances. The algal cells in ORI PBR were younger than the pre-enriched algal cells in other PBRs. Since this is the case, the algal cells in ORI PBR demonstrates a higher specific growth rate. Doubling time of the cells, were calculated for M1, M2 and M3 PBRs as 2.35, 2.25 and 2.34 days, respectively, while this value was 1.64 days for the ORI PBR.

According to the results of the dry weight analysis (Figure 3.7.c), the biomass production rate was found to be 36 mg/L.d for the M1 PBR and 43.7 mg/L.d for the M3 PBR. However, the biomass production rates of the M2 and ORI PBRs were calculated as 13.67 and 22.67 mg/L.d, respectively. These values were comparable with the ones in a study researching the effect of N and P concentrations performed by Wong (2016) that works on *C. Vulgaris* culture. M3 exhibits a higher biomass production rate than the one in Wong (2016)'s study (average 38.75 ± 0.96 mg/L.d). The reason behind this fact might be the existence of vitamins in M3 PBR and higher illumination intensity applied (150 PAR for this study, 70 PAR for Wong (2016)).

3.3.2.2 Results of Set 2: Cultivation of *C. Vulgaris* in Batch PBRs with Ammonium Source

The aim of set 2 was to cultivate autotrophic *C. Vulgaris* culture using ammonium as the N source. The effect of different illumination periods of continuous illumination (namely, C1 and C2 replicates) and 12:12 (L:D) illumination (namely

T1 and T2 replicates) were also investigated with four PBRs in batch mode. To realise the investigation of these parameters, N as (TAN) and P removal performances were observed. The results of Set 2 are presented in Figure 3.8 and Figure 3.10.

As it is expected, the pH of all PBRs using NH_4-containing medium displayed an abrupt decrease for each analysis day as it can be seen in Figure 3.8.a. To prevent the inhibition of the cultures due to pH drop, pH was increased to a range of 7.8 and 8.2 with an intervention whenever pH decreased below 7.0. As mentioned before, the drop in pH can be explained by microalgae cells releasing the proton from the ammonium to maintain the cell's neutral state and reducing the pH of its medium (Fuggi et al., 1981; Xin et al., 2010). The ambient temperature of the setup where C1, C2 placed and T1, T2 placed are given in Figure 3.8.b.

According to the OD change demonstrated in Figure 3.8.c, the C1 and C2 PBRs followed a similar pattern of growth for almost the entire duration of operation. Yet, the OD values of PBRs T1 and T2 followed a slightly a lower pattern than the cultures enriched by continuous illumination, that were C1, C2 PBRs. C1, C2, T1 and T2 PBRs reached to OD values of 3.19±0.02, 3.13±0.005, 3.07±0.02 and 2.7±0.005 on Day 20, respectively. This is consistent with other studies in the literature. For example, in a study conducted by Sharma (2012), to enrich *C. Vulgaris* culture, with same illumination periods, it was revealed that PBRs operated under 12:12 illumination had much less OD values than those operated under continuous illumination. However, the T2 PBR did not have as much OD as C1, C2 and T1 PBRs. This result is solely associated with the failure of air flow meter for the T2 PBR.

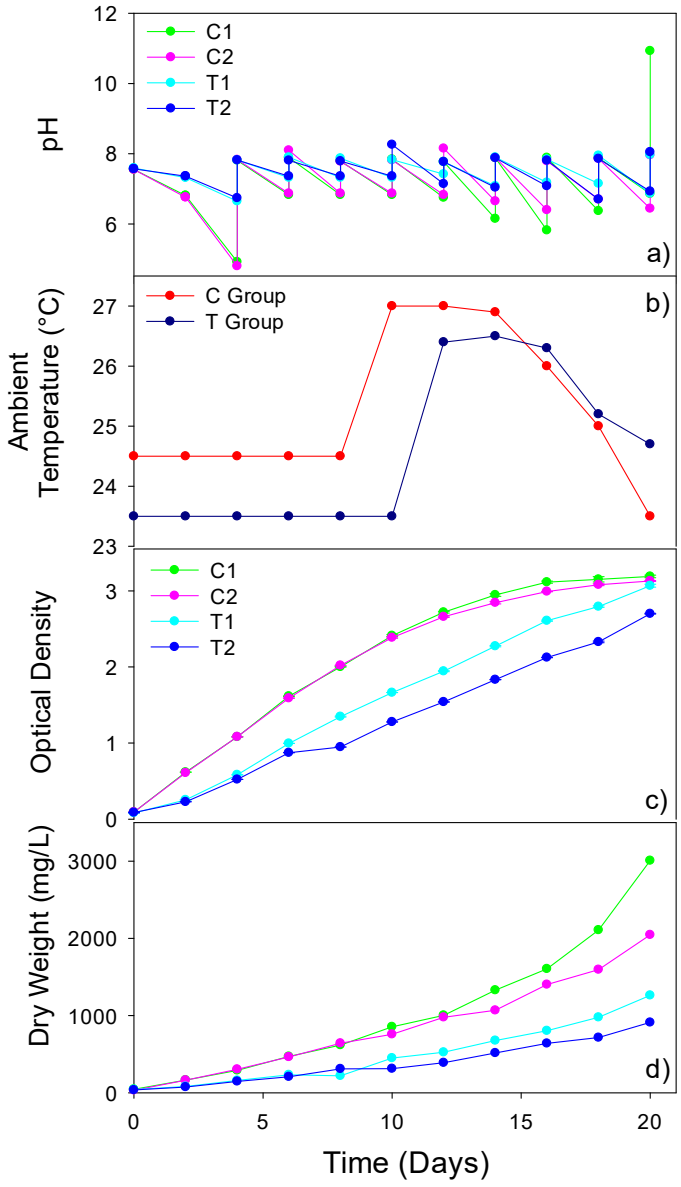

Figure 3.8. Results for growth performances of the PBRs in Set 2 a) pH b) Ambient Temperature °C c) Optical Density d) Dry Weight

According to the calculations based on OD, the specific growth rates of the C1, C2, T1 and T2 PBRs were 0.22, 0.22, 0.18 and 0.17 $days^{-1}$, respectively. These results were lower than the results obtained from previous PBRs in Set 1, M1 and M3, which

had nitrate as nitrogen source. A similar observation is in a study performed by Soni et al. (2017), which enriches *Chlorella pyrenoidosa* culture using three different sources of nitrogen. In Soni et al. (2017)'s study, it was observed that the OD values obtained in NH_4-containing medium (average 0.40) were much less than the OD values obtained in nitrate containing ones (average 1.65). Similarly, a study conducted by Tam and Wong (1996) observed that the specific growth rate of *C. Vulgaris* culture enriched in Bristol medium containing nitrate is higher than the culture enriched in NH_4-containing medium with the same concentration. It is known that most microalgae species, including *C. Vulgaris*, prefer ammonium as a nitrogen source because the pathway of assimilation requires less energy to complete (Liu et al., 2016; Fernandez and Galvan, 2008; Guerrero et al., 1981). Yet, as ammonium consumption causes a decrease in pH, an inhibitory effect of ammonium can be observed (Eustance et al., 2013). This might be the simplest reason to observe a limited growth in NH_4-containing mediums of C1, C2, T1 and T2 PBRs. As mentioned before, the specific growth rates results obtained from previous PBRs in Section 3.3.2.1, Set 1, M1 and M3 PBRs were 0.30 day^{-1}, while C1, C2, T1 and T2 PBRs demonstrated specific growth rates in the range of 0.22-0.17 day^{-1}.

The doubling time of the cells were also calculated and values for C1, C2, T1 and T2 were found to be 3.15, 3.16, 3.86 and 3.97 days, respectively. The increase in the doubling time of the culture since the previous experiment in Set 1 might be explained with the slowdown of culture, because of NH_4-containing medium (Soni et al., 2017). Apart from the effects of ammonium on growth, these results should also pose an aspect on the cell's maturity as suggested by Winokur (1949) on the aging of *C. Vulgaris*.

Time-dependent variations in dry weight concentration (Figure 3.8.d) followed a pattern like those in OD values. Maximum dry weight reached were 3000, 2046, 1262 and 912 mg/L, and biomass production rates were 148, 100, 61, 44 mg/L.d for

C1, C2, T1 and T2 PBRs, respectively. This reveals that the application of continuous illumination is the optimum illumination period since C1 and C2 showed higher specific growth rates and biomass production rates compared to that of T1 and T2 PBRs as seen in Figure 3.8.

Additionally, as seen in Figure 3.7 (Set 1) and Figure 3.8 (Set 2), the results of biomass production rates results obtained in Set 2 were higher than those given in the previous Set 1, (Section 3.3.2.1). For example, biomass production rates of M1 and M3 PBRs were 36 to 43.7 mg/L.d, where the nitrogen source is nitrate, and the illumination time is the same as T PBRs that were exposed to 12:12 (L:D) illumination, while T1 and T2 biomass production rates were calculated as 61 and 44 mg/L.d. This demonstrates that the NH_4-containing medium exhibits better growth performance in terms of biomass production rate compared to the NO_3-containing medium as the results shared in Set 1, Section 3.3.2.1. Hence, the optimum N source was chosen as ammonium. Since it is more compatible with the further studies that will be realised with real anaerobic digestate, which has high ammonium concentration, as N source ammonium was decided to be used in the following experiments.

Moreover, the biomass production rates were comparable with a literature study performed by Wong et al. (2017) with the *C. Vulgaris* culture in different nutrients (maximum 114 mg/L. d). The expected NH_4-containing medium inhibition was not observed for the biomass production rates. As Winkour (1949) suggests, although the algal cells age and slowdown their specific growth rate, the matured cells would have higher dry weight values and resultantly biomass production rates.

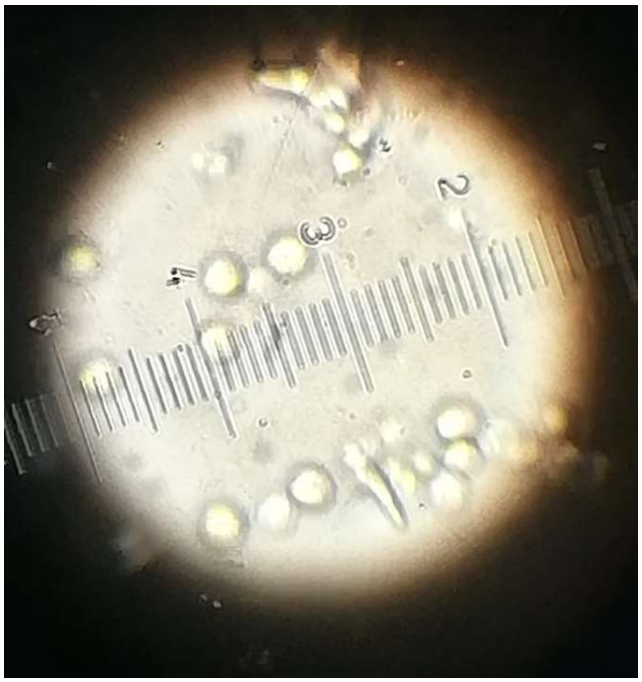

Figure 3.9. Microscopic Image of *C. Vulgaris* Cells (100 X Magnification)

Microscope observation reveals that the observed microorganisms were *C. Vulgaris*, and the size of the cell is about 2 μ (Figure 3.9).

As presented in Figure 3.10, the TAN removal efficiencies of the C1, C2, T1 and T2 PBRs were 91%, 80%, 40% and 12%, respectively (Figure 3.10.c). This reveals that not only for growth performances but also for TAN removal performances continuous illumination shows better results. Moreover, the treatment efficiencies of PBRs operated at continuous illumination were comparable to the literature values. According to a study conducted by Mayhead et al. (2018), a *C. Vulgaris* culture, demonstrated 95% TAN removal efficiency when initial TAN concentration was 104 mg/L which is close to the TAN value of Set 2 (Figure 3.10.a). Furthermore, in a study performed by Choi and Lee (2013), it was revealed that *C. Vulgaris* offers a treatment performance of 50% when initial NH_4^{+3}-N concentration is around 85

mg/L. Hence it can be concluded that for the PBRs operated under continuous illumination, an optimum and comparable TAN removal efficiency can be obtained.

For PO_4^{-3}-P removal, an unexpected increase in P concentration was observed in T1 and T2 PBRs (Figure 3.10.b and d). For the PBRs C1 and C2, removal efficiencies were found to be 37% and 28%, respectively. For this unexpected P concentration increase in T PBRs, it might be speculated that an indigenous decay of the culture might have occurred. In a study conducted by Mayhead et al. (2018), the PO_4^{-3}-P removal was recorded as 98% where the initial PO_4^{-3}-P concentration of the F2P medium is 2.5 mg/L while N:P ratio is 2.4, which is close to the value of Set 2. Despite the close N:P ratios, the relatively low PO_4^{-3}-P removal efficiencies in C1 and C2 PBRs, can be explained with high concentration of initial PO_4^{-3}-P (52 mg/L). In Mayhead's study, it was stated that the higher the N:P ratio applied, the higher PO_4^{-3}-P removal efficiencies were observed (Mayhead et al., 2018). Moreover, Whitton et al. (2016) suggests that greater N:P ratio exhibits a better microalgal growth that results in higher nutrient removal efficiencies. Hence, it can be concluded that both initial PO_4^{-3}-P concentration and N:P ratio studied in Set 2 did not support the P uptake phenomena for *C. Vulgaris.*

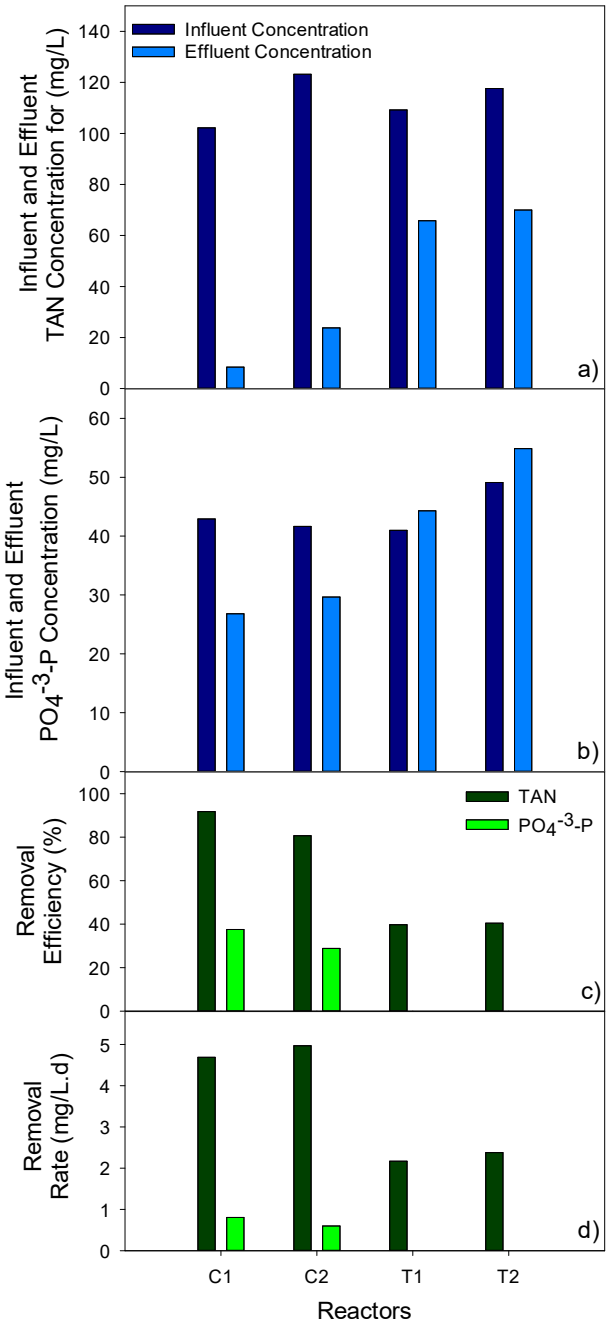

Figure 3.10. Results for nutrient removal performances in Set 2 a) Influent and effluent TAN concentrations b) Influent and effluent PO_4^{-3}-P concentrations, c) Removal efficiencies for TAN and PO_4^{-3}-P, d) Removal rates for TAN and PO_4^{-3}-P

3.3.3 Results of Set 3: Effect of Illumination Period on *C. Vulgaris* for Microalgal Treatment and Pigment Production

The aim of Set 3 was to investigate the effect of illumination periods of 12:12 (L:D), 8:8:8 (L:D:L), 6:6:6:6 (L:D:L:D), and 24:0 (L:D) on the growth and nutrient removal performances of *C. Vulgaris* culture. For that purpose, four different PBRs were set in batch mode. To realise the investigation of these parameters, N as (TAN) and P removal performances were observed along with the biomass production rate, doubling time and specific growth rate and Chlorophyll-a,b,c and carotenoids concentration of the culture. The results of Set 3 are displayed in Figure 3.11-Figure 3.14.

As seen in Figure 3.11.a. pH of the cultures enriched in NH_4-containing medium decreased after the first feeding from the initial 7.8 - 8.2 pH range to pH 7. To prevent the inhibition of the culture due to pH drop, pH was increased to a range of 7.8 - 8.2 with an intervention at the end of the sampling period as it can be seen in Figure 3.11.a. As seen in the Figure 3.11.b the ambient temperature of the set-ups increased from 22 to 28 °C during the operational period which is in optimum ranges for *C. Vulgaris* growth (Daliry et al., 2017)

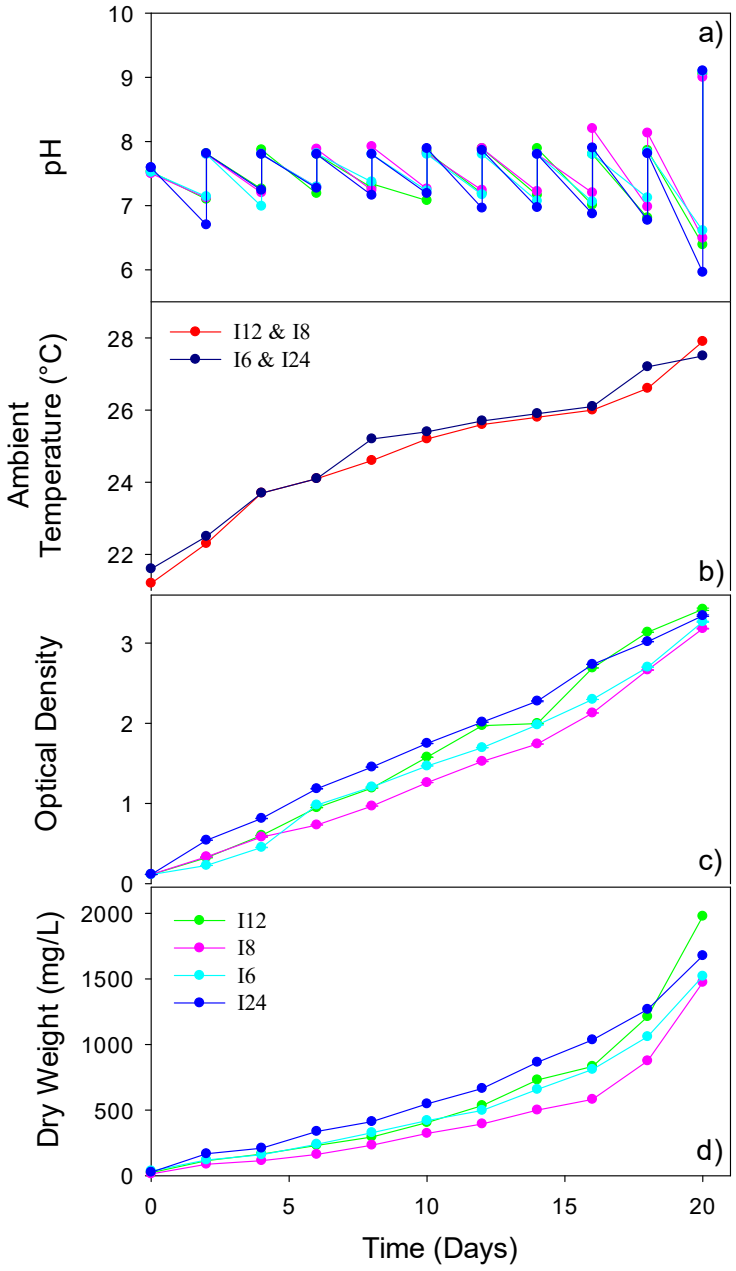

Figure 3.11. Results for growth performances of the PBRs in Set 3 a) pH b) Ambient Temperature c) Optical Density d) Dry Weight

Figure 3.11.c. demonstrates the change in OD values of the PBRs. The OD values of I12, I8, I6 and I24 PBRs were recorded as 3.4±0.01, 3.2±0.004, 3.3±0.007 and 3.30±009, respectively by the end of the operational period. While specific growth rates of the all PBRs were calculated to be 0.17 day^{-1}. Hence, it can be concluded that, in terms of growth performance, illumination periods did not make a dramatic difference which were no more than 10% between the PBRs. As Figure 3.11.d demonstrates, I12 PBR had the maximum dry weight value of 1977 mg/L while I8, I6 and I24 PBRs had dry weights of 1473, 1520, and 1676 mg/L, respectively. The biomass production rates of the PBRs were I12, I8, I6 and I24 were 97, 73, 74, 83 mg/L.d, respectively. Moreover, doubling time of all the PBRs was found to be 4 days. Once again, the results show clearly that the differences between growth performances showed by the PBRs were no more than 10%. Hence, it can be concluded that, in terms of growth, illumination period did not exhibit any differentiating performance.

As can be seen in Figure 3.12.a., TAN concentration gradually decreased in the PBRs and the removal efficiencies were recorded as 53%, 33%, 51% and 78% at Day 20 for I12, I8, I6 and I24 PBRs, respectively (Figure 3.12.a). Along with this, removal rates were calculated as 3.2, 2.0, 3.0 and 4.6 mg/L.d for I12, I8, I6 and I24, respectively (Figure 3.12.b). These results reveal that continuous illumination led to the highest TAN removal efficiency, followed by illumination of 12 hr in total (I12 and I6). Interestingly, illumination with hourly frequency of 8 hours (I8, 8:8:8, L:D:L) let to the lowest TAN removal efficiency, i.e only 42% of the efficiency obtained at the continuous illumination. Despite the total illumination period of 16 h, I8 PBR's TAN removal efficiency was also lower than I12 PBR with 12 hr illumination and even that of I6 PBR illuminated with hourly frequency of 6 hrs. A balanced L:D period or continuous illumination might be more advantageous compared to an unbalanced but higher illumination period. I12 and I6 PBRs had similar TAN removal efficiencies. Apparently, the effect of 12 hr total illumination

period is independent of the illumination frequency. This might be again attributed to a balanced L:D period, which remains to be researched.

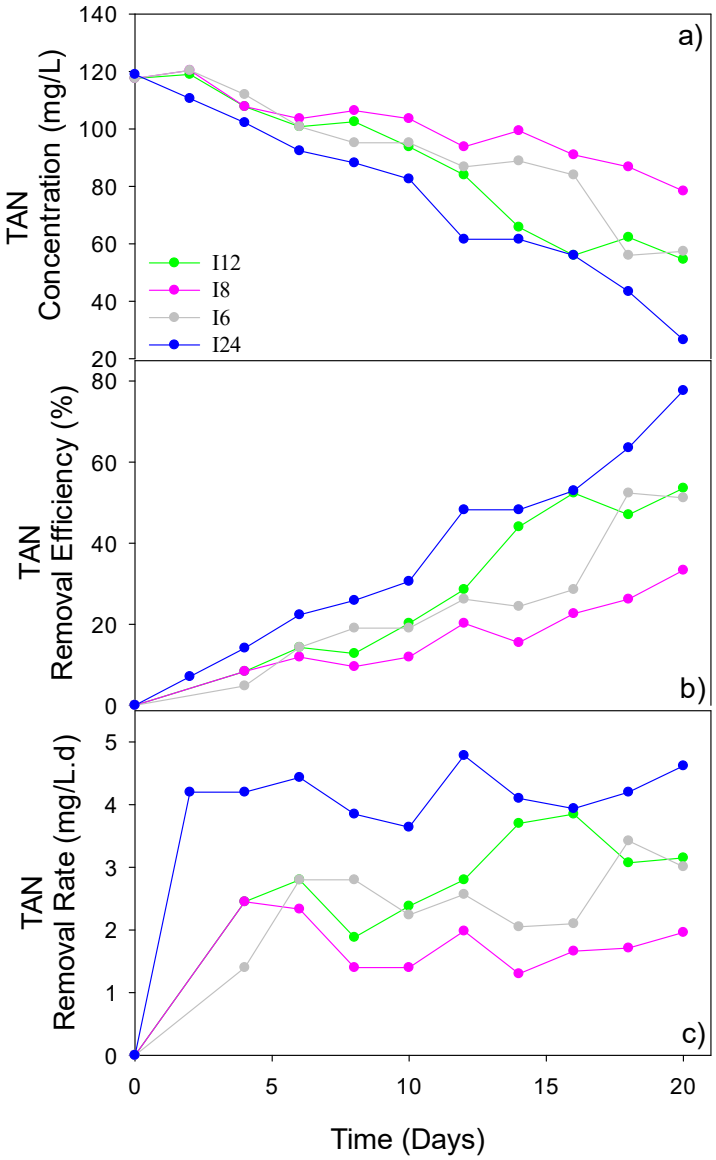

Figure 3.12. Results for nutrient removal performances of Set 3 a) TAN concentrations b) TAN removal efficiencies and c) TAN removal rates

PO_4^{-3}-P removal performance, on the other hand, was not following a similar pattern as TAN follows (Figure 3.13.a, Figure 3.13.b and Figure 3.13.c). As Figure 3.13.a, b, and c demonstrate, PO_4^{-3}-P concentration increases in the PBRs. The final PO_4^{-3}-P removal was recorded in I24 PBR as 41% and the removal rate reached to 0.9 mg/L.d at Day 20. The maximum observed P removal efficiency was recorded as 60% and 2.3 mg/L.d for I24 PBR at Day 12. I12, on the other hand, exhibited a maximum P removal rate of 2.5 mg/L.d at Day 2. The low P removal efficiencies might be attributed to the high N:P ratio of 2.3, that does not support the ultimate P storage metabolisms inside the algal cells (Mayhead et al., 2018) is also observed in T PBRs of Set 2 (Figure 3.10, Section 3.2.5.2). Another potential explanation might be attributed to the total exposed light time. As Krzemińska et al. (2014) states, the quantity of illumination determines the photosynthetic activity. Hence, the decreasing pattern in P removal efficiencies in I12 (with total light time of 12 h), I8 (with total light time of 16 h) and I6 (with total light time of 12 h) might be due to the lower total light time exposed on algae when compared to the that of I24 (continuous illumination).

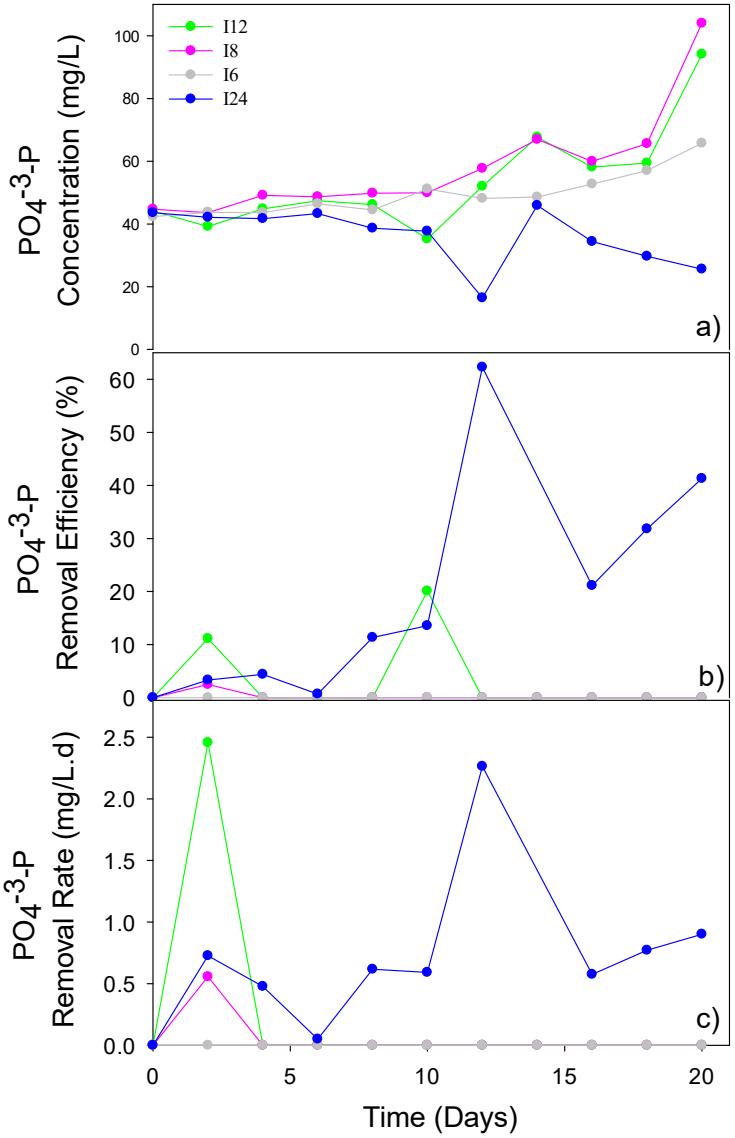

Figure 3.13. Results for nutrient removal performances a) PO_4^{-3}-P concentrations b) PO_4^{-3}-P removal efficiencies and c) PO_4^{-3}-P removal rates

The major issue observed in Figure 3.13, was the P accumulation. This issue might have been occurring due to the indigenous decay of the algal cells that releases P they stored back into the medium. This might be valid for nitrogen as well. Yet, when

the nitrogen molecules released back into the system, young cells perform photosynthesis. As it was provided in Section 3.2.5.2., Set 2, Equation 3.9, with photosynthesis where CO_2 is consumed, the consumption of 1 mole of P with 12 moles of $NH4^+$. This might be a potential explanation for the observed TAN removal (Figure 3.12), removal performance that reached to 80% and accumulation of P in the system. Hence, the released P may not be as efficiently removed as nitrogen in the system. Moreover, it should be noted that TAN removal is solely depends on photosynthetic mechanism. Ammonia stripping was not a matter of concern, considering the pH levels of 8.2 to 7.0. Additionally, it should be noted that, the experiments were performed elaborately and the analysis for P removal performance were repeated in another machinery and the same results were obtained. This should eliminate any experimental error possibility where results were reflected falsified.

The reasons for release and ultimate accumulation of P remains uncertain. However, another potential explanation might be extracellular polymeric substances (EPS) released by the I12, I8 and I6 PBRs that were exposed to intermittent illumination which is a stress factor. It might be speculated that the intermittent light might have induced EPS production and EPS might have been release by algal cells in the PBRs (Xiao and Zheng, 2016). Resultantly the EPS might have increased P concentration in the PBRs due to its phospholipid structure. The difference in the increasing trend of OD and dry weight presented in Figure 3.11.b and c, might support this speculation. EPS might have interfered the OD experiments, yet the dry weight values were more precise in terms of algal growth detection. The difference between two trends might be pointing out the EPS released in the medium from the algal cells.

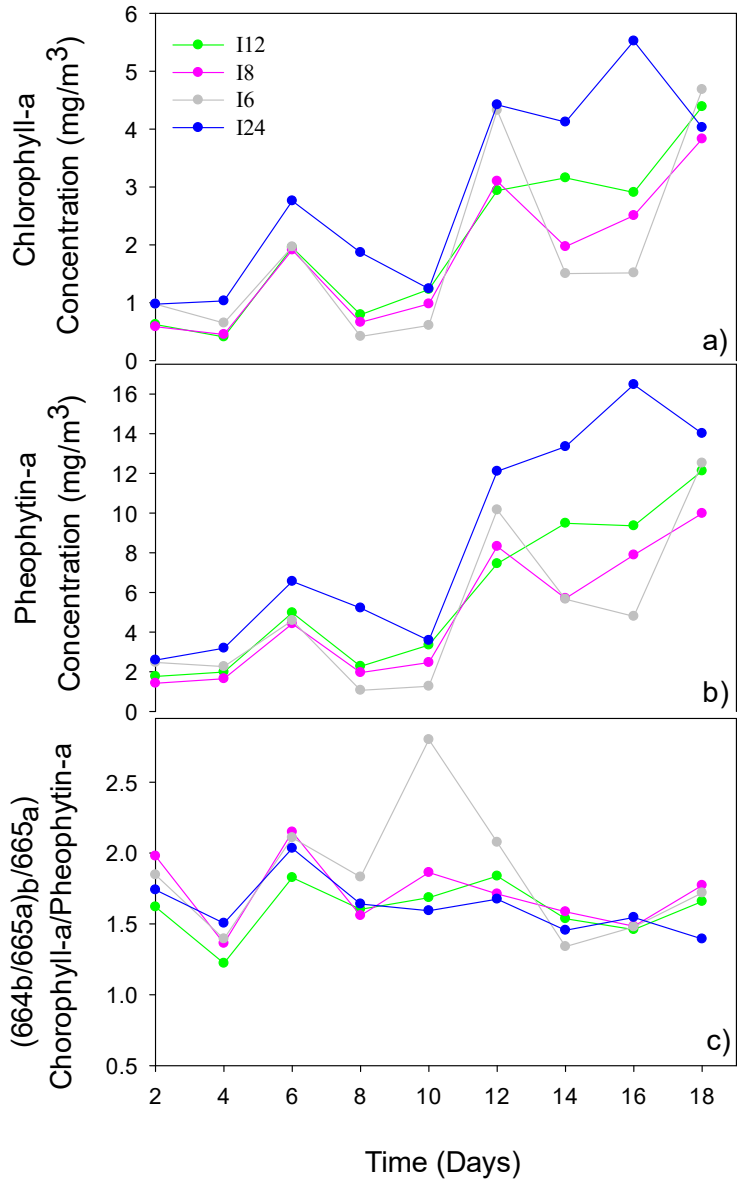

Figure 3.14. Results for Pigment Concentrations of PBRs in Set 3 a) Chlorophyll-a b) Pheophytin-a c) Chlorophyll-a/ Pheophytin-a ($664_b/665_a$)

The total chlorophyll-a concentrations obtained during the operational period is presented in Figure 3.14.a. for different illumination periods. It was noted that

chlorophyll-a concentration fluctuated in each PBR and reached up to 4.4, 3.8, 4.7 and 5.25 mg/m^3 for I12, I8, I6 and I24, respectively. A seen in Figure 3.14.b, Pheophytin-a concentrations of the cultures in I12, I8, I6 and I24 PBRs were found to be the maximum of 12.1, 10, 12.5 and 16.5 mg/m^3, respectively. However, Pheophytin-a content is a molecule formed after a degraded chlorophyll-a molecule lost its Mg^{+2} (Steinman et al., 2007). Hence, it might be said that pheophytin-a concentration simply represents a culture which is in its decay phase. For the determination of this situation, a ratio of chlorophyll-a to pheophytin-a absorbance is used. In Figure 3.14.c,the changes in this ratio can be observed. When the ratio is close to 1.0 it shows a decaying culture while when it is 1.7, the culture is perfectly healthy. According to that, all cultures exhibit a healthy pattern for their viability through whole operation time.

Like chlorophyll-a, chlorophyll-b and -c are important pigments (Figure 3.15) for commercial purposes as well (Ferreira et al., 2016). Hence, investigation of the potential effects of illumination is quite important. A study conducted by Ferreira et al. (2016) states that nitrogen starvation and low light intensity decreases total chlorophyll content (Chlorophyll-a and Chlorophyll-b). In this study, Chlorophyll a, b, c contents were found to increase in all PBRs. Therefore, it can be interpreted that, the intermittent illumination did not affect the microalgal culture negatively, in terms of chlorophyll-a, -b, and -c production. According to the results presented in Figure 3.15.a., I24 reached the highest chlorophyll-b concentration with 1.8 mg/L. Likewise, for chlorophyll-c concentration, I6 PBR showed the best performance with 3.4 mg/L (Figure 3.15.b).

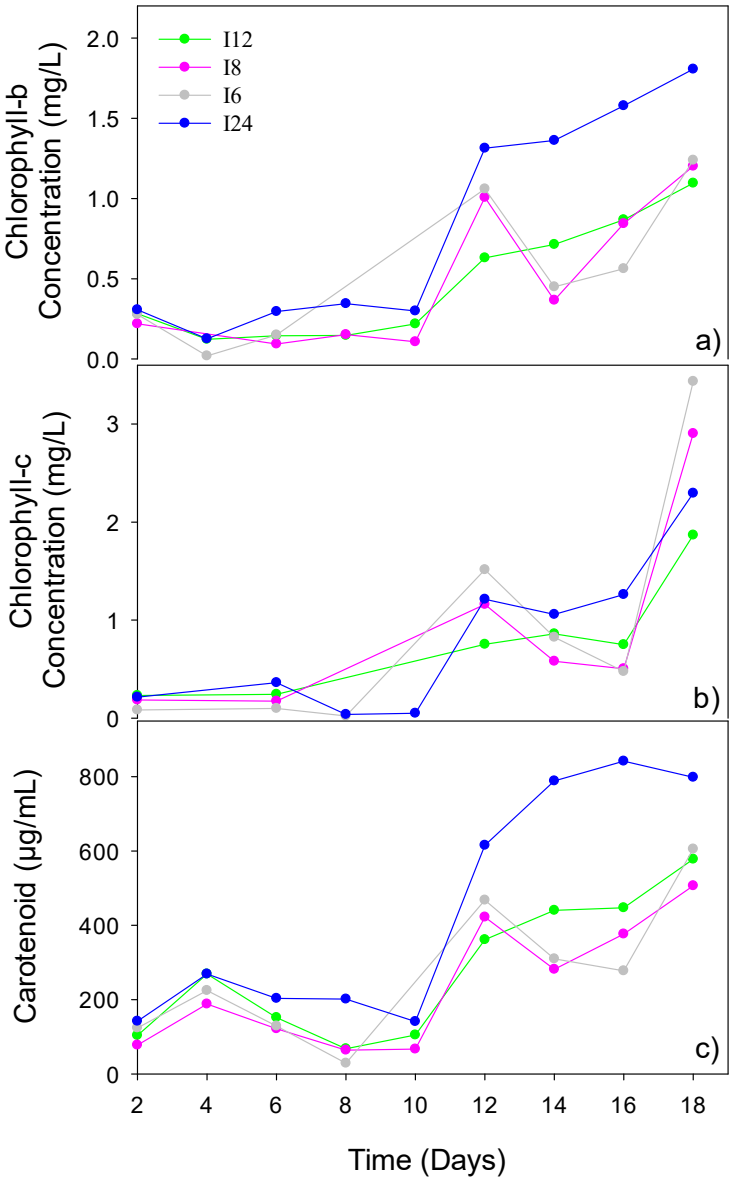

Figure 3.15. a) Chlorophyll-b (mg/L) b) Chlorophyll-c (mg/L) c) Carotenoid (µg/mL)

Carotenoid is an important molecule for microalgae due to its advantages improving effect on light harvesting (Sun et al., 2018) and being an indicator for environmental

stress and photo-oxidative damage (Wong et al., 2017). For example, primary carotenoid like lutein can transfer absorbed energy to chlorophylls, thus broadening the light-absorbing spectrum of microalgae (Saini et al., 2018). As well as their beneficial effects on algae, these molecules have a great market potential. For instance, carotene, astaxanthin, and lutein have gained popularity nowadays due to their antioxidant effects (Sun et al., 2018). The production of carotenoids can be increased under stress conditions related to light intensity, illumination period, light spectrum, salt, temperature etc (Gong and Bassi, 2016). As it can be seen in Figure 3.15.c, the photo-oxidative stress can be observed in all PBRs with the final carotenoid concentration of 578, 506, 605, and 798 µg/mL in I12, I8, I6 and I24 PBRs, respectively. It was observed that in I6 and I8 PBRs, carotenoid production exhibited a peak of on Day 12. Yet, the highest production was obtained from I24 PBR. Thus, regarding the chlorophyll production, it can be said that intermittent illumination (I6, I8 and I12) has a positive effect on algal growth for chlorophyll-a and-c production. However, the chlorophyll-a, -b and -c concentrations obtained from the four PBRs (I6, I8, I12 and I24) did not differentiate with more than 15%. Moreover, for carotenoid production, the intermittent illumination period did not exhibit as high carotenoid concentration as continuous illumination showed. Hence, considering the ease of operation, the optimum illumination for both algal growth and nutrient removal performances, continuous illumination was chosen. The following experiments were conducted with continuous illumination based on the results obtained in here, Set 3.

3.3.4 Results of Set 4: Determination of Optimum N:P Ratio for Enrichment of *C. Vulgaris* and Nutrient Removal

The aim of Set 4 was to determine the optimum N:P ratio for enrichment of *C. Vulgaris* growth and nutrient removal. For that purpose, five different PBRs were

operated in semi-continuous mode with an 8-day HRT. Those were three test PBRs (T6, T8, T10) all fed with $NH_4^+$and two control PBRs (CNH4 and CNO3). The details are given in Section 3.2.5.4., Set 4. To realise the determination of optimum N:P, nutrient removal performances were observed along with the growth activity. The results of the analyses as well as the microscopic images and view of PBRs and contents are presented in Figure 3.16-Figure 3.24.

From day one, the content of the T6, T8, T10 and CNH4 PBRs were settling down at the bottom of the PBRs as seen in Figure 3.16. However, in CNO3 PBR this problem was not observed.

Figure 3.16. *C. Vulgaris* is settling at the bottom of the PBRs in Set 4

It can be speculated that the reason for such an abrupt settling of the culture is related to algal aging. According to the many studies, it was observed that the mature cells may cause a settling problem due to their increasing dry weight as seen in Figure 3.22.d, which might be beneficial for the harvesting purposes (Nautiyal et al., 2014; Wen et al., 2016). Moreover, as suggested by Nautiyal et al., (2014), algal cells have the pectin material in their cell walls that makes them negatively charged. This characteristic can be used for the coagulation purposes with the help of positively charged content of the medium depending on the ionic strength. Hence, the reason only NH_4-containing PBRs experiencing the settling problem may be supporting this fact. Although Nautiyal et al., (2014) stated that the positive charge might come from bacteria or fungus, since the sterile conditions were well maintained in this experiment, this would not be considered as a reason.

A microscope analysis was performed throughout the whole operation periodically to ensure the contamination is not a matter of concern and cells were healthy. As can be seen in Figure 3.17, PBRs were free from contamination and seems healthy at Day 4. However, due to the settling problem the colour of the cells seems pale compared to the ones in later days.

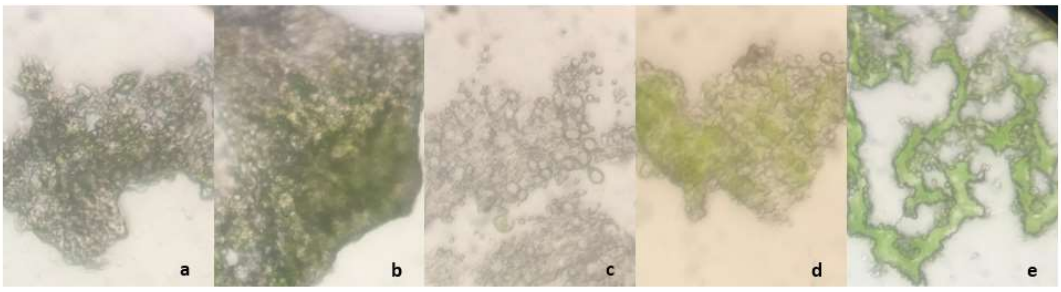

Figure 3.17. Light Microscope Images from the PBRs at Day 4 (a) T6, (b) T8, (c) T10, (d) CNH4 (e) CNO3

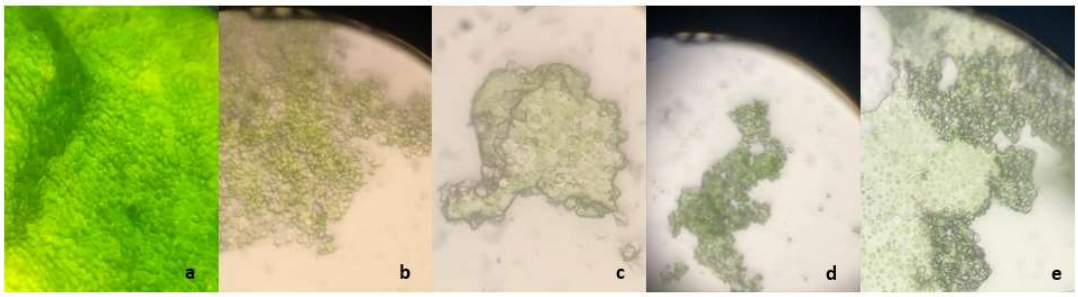

Figure 3.18. Light Microscope Images from the PBRs at Day 20 (a) T6, (b) T8, (c) T10, (d) CNH4 (e) CNO3

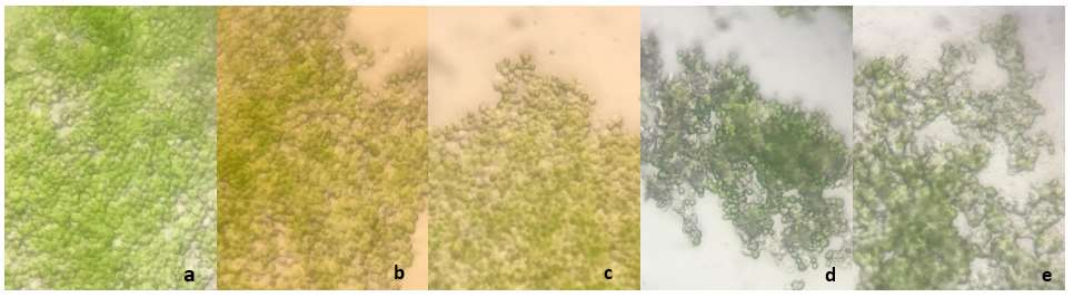

Figure 3.19. Light microscope images from the PBRs at Day 36 (a) T6, (b) T8, (c) T10, (d) CNH4 (e) CNO3

The settling problem observed in the T6, T8, T10, and CNH4 PBRs causes an HRT-SRT inequity that would alter the contact time of the culture with the nutrients. Moreover, since the contact surface of the culture would decrease, the steady-state conditions would not have been reached early. To overcome this problem, the PBRs were re-inoculated with the young cells of the CNO3 PBR. As given in Figure 3.18, at Day 20 cells started to get greener and more in number, which was associated with the re-inoculation performed at Day 6. Prior to that, under sterile conditions, settled algal cells were removed from the PBRs to prevent them from dragging the young cells down with their surface charge. Later, PBRs were operated as usual and allowed

to recover. After this treatment, the OD values of the T6 PBR started to increase by Day 8. Furthermore, CNH4 PBR (from Day 10), T8 (from Day 14), and T10 (from Day 18) also showed a similar improvement as CNO3 PBR and did not settle anymore. However, by Day 24, CNH4 PBR encountered the same settling problem (Figure 3.20). The number of the cells in the samples withdrawn on from Day 36 demonstrates that the cell density in test PBRs has risen, while in control PBRs it has not (Figure 3.19). Although no significant decrease in the OD value was detected until the last day of operation, microalgae in control PBRs settled through the whole operation. A similar situation was never observed again in other test PBRs.

Figure 3.20. Settling in CNH4 on Day 24

In order to comment on the settling observed in CH4 after Day 24, pH values of the reactors were considered (Figure 3.21.b). CNH4 PBR had pH of 9.2 to 9.5 between Days 24-40, which is slightly lower than that of other PBRs. Yet, pH values were similar in all PBRs after Day 40. It should be noted that pH was stabilized with 50 mM $NaHCO_3$ solution provided to the all NH_4-containing PBRs with each feeding, resulting in a pH range of 9.5-10.3 (Figure 3.21.b), which was appropriate for algal growth (Deniz, 2020) However, in an experiment conducted by Leite and Daniel (2020), it was stated that the harvesting efficiency of microalgae increases from approximately 10% to 40% in this pH range of 9 to 10. Yet, the same pH range was obtained for other PBRs (T6, T8, T10 and CNO3) as well, thus the settling of the CNH4 PBR cannot be attributed to the harvesting of microalgae.

It should be noted that, as it was discussed previously in Section, 3.2.5.4, to overcome the P accumulation problem in the system, between Day 40-76, a starvation period was applied. In this period daily feed was given to the system in different frequencies like in every 3, 4, 5 days instead of daily. Hence, the nutrient loading rates were decreased, and system was left to be deficient from P. The results of starvation period can be followed in Figure 3.21-Figure 3.24.

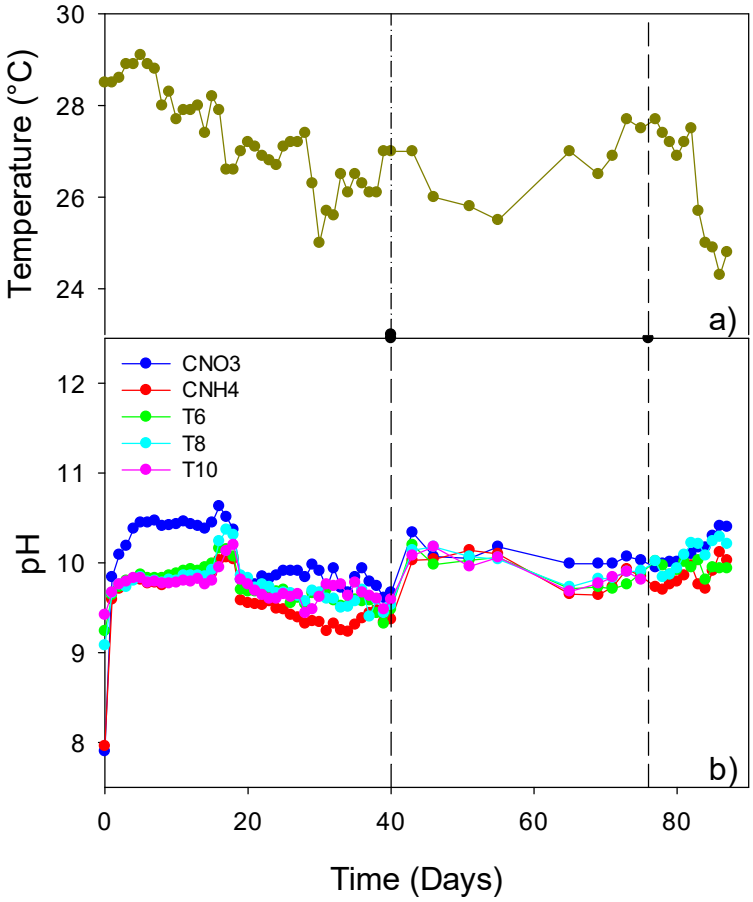

Figure 3.21. Results for Set 4 a) Ambient Temperature °C b) pH (Dashed lines represent the days where starvation period starts and ends)

Figure 3.21.a. and b demonstrate the results of temperature and pH. The ambient temperature decreased from 28ºC to 24 ºC. This is simply due to seasonal changes occurring through three months of operational period. Moreover, the range of 28-24 ºC is still in the optimum ranges for *C. Vulgaris* growth (Daliry et al., 2017)., hence, there is no expected issue related to the temperature change. pH usually follows a trend around 9.5-10.3. Yet, in CNO3 PBR, since there is no NH_4^+, NO_3^- culture had a relatively higher pH pattern following through the operational period.

As presented in Figure 3.22.a., OD demonstrated a fluctuating pattern for all PBRs other than that of CNO3 for the first 20 days. This is due to the settling problem happened in T6, T8, T10 and CNH4 PBRs. OD values of CNO3 PBR were rising faster than the other PBRs for the first 20 days. However, after Day 20, the trend changed in OD values for all PBRs. CNH4 started to show a slightly decreasing pattern. Although it starts to increase after Day 30, the OD values in CNH4 never reached the levels that others reach on Day 40. On Day 40, the OD value of T6 PBR was found to be 1.87±0.006 that appears to be ahead of the other PBRs. OD values of CNO3, CNH4, T8 and T10 PBRs at Day 40 were 1.38±0.005, 1.02±0.003, 1.42±0.008, and 1.07±0.004, respectively. Resultantly, T6 and T8 PBR reached to the highest OD values by Day40. This might be attributed to the relatively supporting N:P ratio of 6 and 8.

Time-dependent changes in dry weight can be followed in Figure 3.22.b. Dry weight values of the PBRs followed a similar pattern to the OD values. Dry weight values of CNO3, CNH4, T6, T8 and T10, PBRs reached to 695, 460, 890, 640, and 625 mg/L, respectively. Again, CNH4 exhibited the lowest growth performance throughout the first 40 days. On the other hand, among test PBRs T6 displayed the highest dry weight values.

The OD and dry weight analyses were not performed between Day 40-Day 76 that is during the starvation period due to a possible fluctuation that might be observed in this period. Hence, only chlorophyll-a analysis was performed to observe any possible stress effect on microalgal cultures.

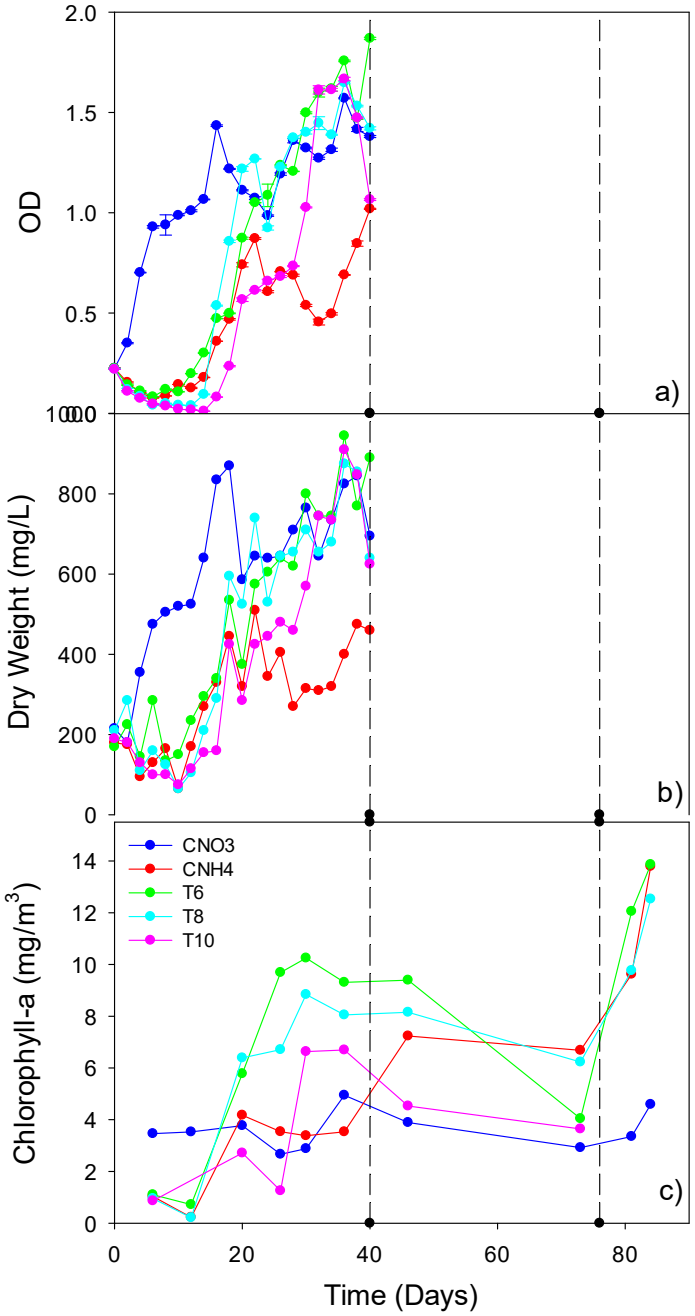

Figure 3.22. a) Optical Density b) Dry Weight c) Chlorophyll-a Concentration (Dashed lines represent the days where starvation period starts and ends)

As can be seen in the time-dependent change of chlorophyll-a concentration in Figure 3.22.c, T6, T8 and T10 PBRs were ahead of the control PBRs and chlorophyll-a concentration increases in all PBRs towards Day 40. In starvation period (between Days 40-76), chlorophyll-a concentration decreased, most probably due to the (predetermined) nutrient deficiency (Jalal et al., 2013). However, after the 8-day HRT operation was applied again (between Days 76-87) where starvation period was ended, chlorophyll-a concentration increased again and reached to approximately 12-14 mg/m^3 for T6, T8, T10 and CNH4 PBRs, which is comparable to the values found in the literature (Amin et al., 2018). It can be concluded that nutrient starvation reduces chlorophyll-a concentrations, since nutrients are needed during chlorophyll synthesis (Jalal et al., 2013). In the CNO3 control PBR, where the nitrogen source is nitrate, an average of 4 mg/m^3 chlorophyll-a concentration was detected after Day 4 to Day 87 which can be explained by the fact that the ammonium nitrogen is assimilated more quickly by microalgae than nitrate nitrogen (Perez-Garcia et al., 2011).

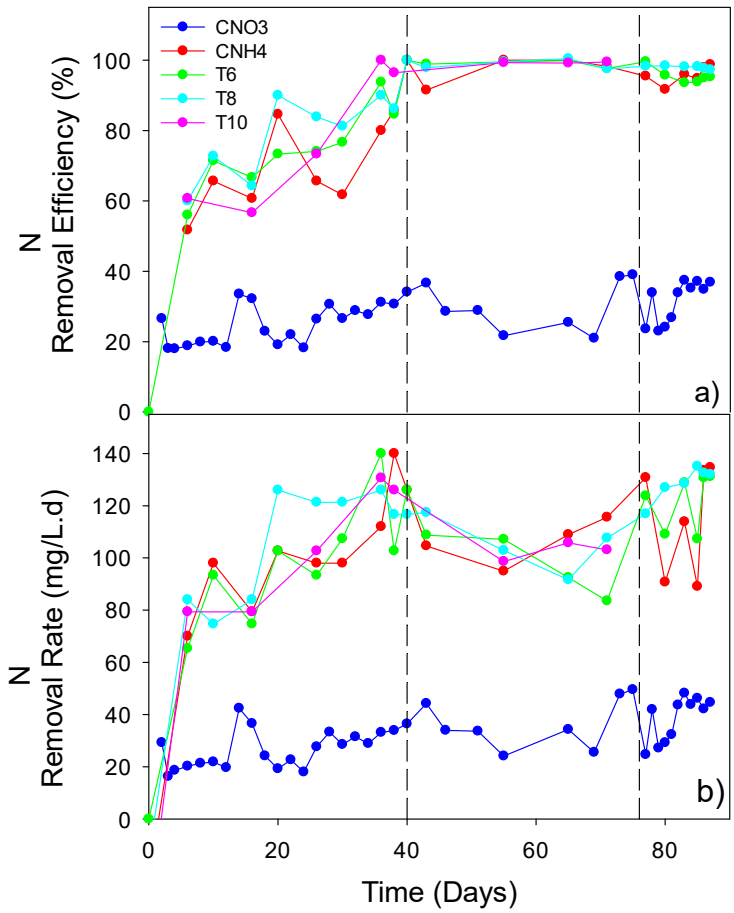

Figure 3.23. a) N Removal Efficiencies (TAN and NO_3^-N) for b) N Removal Rates (TAN or NO_3^--N) (Dashed lines represent the days where the starvation period starts and ends.)

Figure 3.23. shows that all NH_4-containing PBRs (CNH4, T6, T8, T10) demonstrated an increasing trend for both TAN removal efficiency between 60% - 100% and TAN removal rate between 60-120 mg/L.d. CNH4 initially was fed with same initial TAN concentration (120 mg/L), but lower N:P ratio of 2.3, reached to its steady-state with 80% TAN removal efficiency on Day 36 and reached 100% TAN removal efficiency and 140 mg/L.d removal rate on Day 40. For T6, T8 and

T10 PBRs steady-state condition was reached on Day 36 with 90% TAN removal efficiency, and on Day 40, PBRs reached to complete TAN removal efficiency and average removal rate of 140 mg/L.d. CNO3 PBR, on the other hand, followed a low removal pattern with an average of 20% NO3-N removal efficiency which reached to 30% on Day30 keeping the steady-state condition till Day 40 with average 40 mg/L.d N removal rate.

From Day 40 to 87, the test PBRs (T6, T8 and T10) and the NH_4-containing control PBR (CNH4) kept their steady-state condition which was already reached on Day 36. On Day 76, where 8-day HRT operation was started again, T6, T8 and CNH4 PBRs were still at their steady-states at around 96%, 97% and 99% TAN removal efficiencies, respectively. On the other hand, T10 PBR achieved 100% TAN removal efficiency at 71st day. However, the N removal efficiencies remained low for CNO3 PBR. On Day 87, the N removal efficiency was recorded as 37% (Figure 3.23.a).

For the differences in N removal efficiencies between ammonium containing and nitrate containing PBRs, the possibility of ammonia stripping was considered. According to (Anthonisen et al., 1976) at certain temperature, pH and TAN concentration, ammonium is converted into ammonia and stripped out from the system. Hence the possibility of this process was calculated through the Equation 3.12 and 3.13.

$$Kb/Kw = e^{(6344/(273+T(°C))} \quad \text{(Equation 3.12)}$$

$$NH_3\text{-N (mg/L)} = (\text{TAN (mg/L)} \times 10^{pH}) / (Kb/Kw+10^{pH}) \quad \text{(Equation 3.13)}$$

Where,

Kb: The ionization constant of the ammonia equilibrium equation

Kw: The ionization constant of water

To determine the highest possible ammonia concentration that can be removed by stripping, the maximum conditions (influent TAN concentration, highest pH achieved during operation and maximum temperature) were considered Accordingly, at influent TAN concentration of 120 mg/L, the highest pH was recorded as 10.31 and the highest temperature is 25°C. Under these conditions, 91% of the TAN concentration is converted to NH_3 (ammonia) form. Whether the ammonia is stripped or not is determined by Henry's constant (H: 0.0161 atm.L/mol, 25°C, Nazaroff and Alvarez-Cohen, 2001) As a result, it was determined that only 0.07% of ammonia dissolved in water could transfer to the gas phase in a day. Hence, since the stripping level is quite negligible, this possibility does not need to be considered. Additionally, as it is stated in the literature, the NH_3 creates a highly toxic effect on microalgae and may cause chlorophyll biodegradation, leading to whitening or yellowing of the culture (Collos and Harrison, 2014). Since this was not observed in any PBR, it can be said that the calculated theoretical ammonia level for the worst-case scenario may not be real. Hence, the high N removal performance in T6, T8, T10 and CNH4 PBRs compared to that of CNO3 PBR can be associated with the culture already acclimated to the NH_4-containing medium (Set 2). In fact, it was also observed that, for *C. Vulgaris* cultures acclimated to ammonium nitrogen source, the rate of assimilation of ammonium nitrogen is higher than the one of nitrate nitrogen (Perez-Garcia et al., 2011).

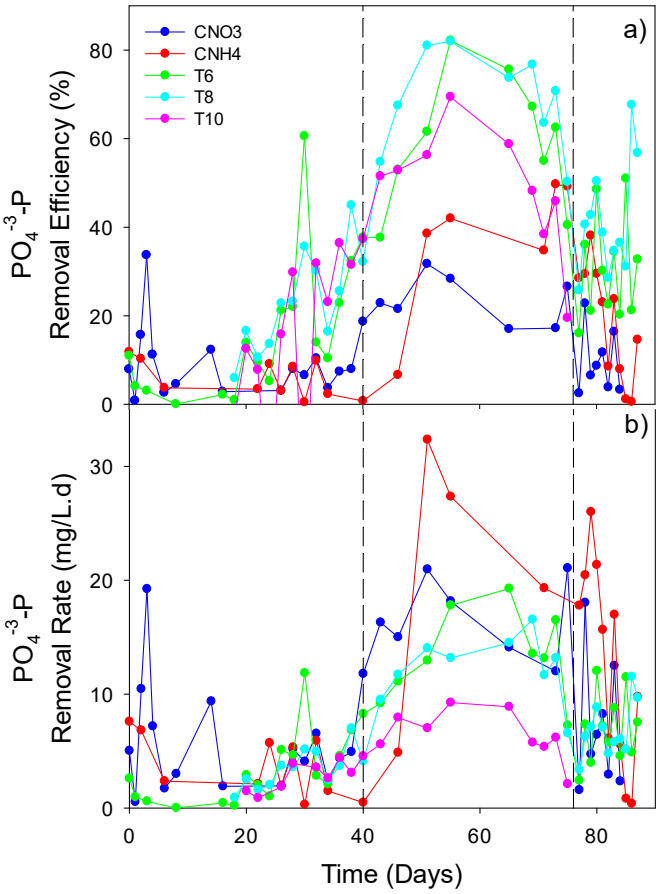

Figure 3.24. a) PO_4^{-3}-P Removal Efficiencies b) PO_4^{-3}-P Removal Rates (Dashed lines represent the days where the starvation period starts and ends.)

As Figure 3.24 demonstrates, the PO_4^{-3}-P removal efficiencies and rates fluctuate between Day 0-Day 40 for all PBRs. As it was observed for the whole operation, in terms of P removal steady-state conditions could not be obtained in any of the PBRs during the first 40 days as well. The trend in those days follows an average P removal efficiency of 5%-10% for CNO3 and CNH4 PBR, respectively. For T6, T8 andT10 PBRs, the average P removal efficiencies, between Day 0-Day 40, were 15%, 25% and 30%, respectively. The highest P removal efficiencies observed in the first 40

days were 19%, 10%, 61%, 45% and %38 for CNO3, CNH4, T6, TT8 and T10, respectively. The corresponding highest removal rates were recorded as 12, 7, 12, 7 and 5 mg/L.d for CNO3, CNH4, T6, T8 and T10. It should be reminded that the P given to those PBRs were 52 mg/L for CNO3 and CNH4, 20, 15 and 10 mg/L for T6, T8 and T10 respectively. The P removal efficiencies and rates were low compared to the literature studies. In studies that investigate the effect of N:P ratio, the P removal efficiencies were demonstrated as 90%-100% approximately (Lee et al., 2013; Mayhead et al., 2018; Sayadi et al., 2016). In researches conducted by Mayhead et al. (2018), N:P ratios were set between 6 to 8 and approximately 95-100% P removal efficiencies were obtained. Figler et al. (2021) claims that inadequate phosphate removals were caused from Bold's Basal Medium's inappropriate N:P ratio (N:P is 2.3). Hence, the low P removal efficiencies in CNO3 and CNH4 can be attributed to 3N BBM+V. However, there should be other reasons why the desired P removal efficiencies could not be reached in T6, T8 and T10 PBRs, where the N:P ratios were 6, 8, and 10, respectively. One might be the insufficiency of the inorganic carbon source provided to the PBRs.

In order to determine if the total inorganic carbon supplied to the PBRs is sufficient or not, a calculation was performed over the stoichiometric equation of microalgae in which N and P are consumed photo-autotrophically with given total inorganic carbon concentration. In APPENDIX D, the calculations of partitioning of applied CO_2 gas, and $NaHCO_3$ buffer in PBRs are given. Hence, the total dissolved inorganic carbon in the medium ($CO_{2\,(aq)}$ and HCO_3^-) supplied in a day was calculated assuming that all forms of inorganic carbon sources remained, and the total dissolved inorganic carbon was completely available for microalgae to be consumed. As result, the available inorganic carbon was found to be 0.05 mol/day which is not limited for P removal. The theoretical maximum PO_4^{-3}-P concentration that can be consumed with this inorganic carbon level was found to be 0.0005 mol/day. However, when the removal rate in the PBRs is considered, the maximum PO_4^{-3}-P removal rate was

0.0002 mol/day. Hence, it can be concluded that, higher PO_4^{-3}-P removal rate is possible with the inorganic carbon concentration supplied daily. Therefore, this low P removal efficiency may not have been caused from the limited amount of inorganic carbon sources in the system. Yet, since the calculations were performed on the assumption of all dissolved inorganic carbon forms were available for algae's consumption, an alkalinity experiment should be performed to make sure of this assumption.

The reason behind the P accumulation and the inefficient removal performances may be linked to "luxury uptake". As discussed in Section 2.1.3.3, a microalgae culture, which is exposed to high concentration of P for a period, not able to uptake the ambient P unless there is starvation of this nutrient (Aitchison and Butt, 1973; Cembella et al., 1984; Chopin et al., 1997). This might be another explanation for the unconsumed P. Algal cells may not have stored the excess P due to the P-rich environment considering that the cultures of this Set 4 were obtained from Set 3 operated with 3N BBM+V in Set 3, having 52 mg/L P.

During the starvation period (Day 40-Day 76), the PO_4^{-3}-P removal efficiency started to show an increasing trend for all PBRs (Figure 3.24.a and Figure 3.24.b). However, since HRT was not steady between days 40-76, no steady-state condition was obtained in P removal efficiency as well, thus, a fluctuating pattern was displayed. This increasing trend might be a clear indication of the effect of "luxury uptake" phenomenon on microalgal systems. The operation of the T10 PBR was stopped when the PO_4^{-3}-P removal efficiency fell below 20%, respectively, on Day 75. For the CNO3 PBR, after a starvation period, maximum P removal efficiency of 32% was observed on Day 51. In CNH4 PBR, this value was recorded as 50% on Day 73. On the other hand, T6 and T8 PBRs reached 82% and %81 maximum P removal efficiencies, respectively, on Day 55. These results may prove the positive effect of starvation period on the algal culture.

As it was discussed previously in Section 3.2.5.4, to stimulate luxury uptake phenomena, starvation periods were applied with disrupting HRT during Days 40-76 with irregular feeding. Hence, with every feeding operation HRT was changed. However, since this feeding approach (phenomenon) is not applicable in terms of engineering systems and since this phenomenon is still in the research phase, the system was again adjusted to 8-days HRT operation, during Days 76-87. In this period a decreasing trend was observed in the PO_4^{-3}-P removal efficiencies, but the T8 PBR showed approximately 60% PO_4^{-3}-P removal efficiency while, this value remained around 30% for T6 in the last three consecutive analysis days.

Although in the first operational period first 40 days, prior to starvation period, it seemed that the T6 PBR exhibited highest performances in P removal compared to the other PBRs, later it was revealed that the T8 PBR overcame the P accumulation problem after the starvation periods, and it is more responsive to the stress factors. According to the specific growth rate and biomass production rate results, T6 and T8 PBRs were also ahead of the other PBRs (T10, CNH4 and CNO3). However, in terms of TAN removal efficiencies, the T6, T8 and T10 PBRs did not demonstrate a major difference (no more than 10%) to put any N:P ratio forward. Yet, the comparable difference between the test PBRs appears in the starvation period. It was determined that the T8 PBR has overcome the P accumulation problem after starvation periods and is ahead of T6 PBR in terms of PO_4^{-3}-P removal efficiency in this period. Therefore, it was decided to continue with the T8 culture and N:P of 8 (g/g) in the following studies.

3.4 CONCLUSION

This study aimed to enrich a healthy autotrophic *C. Vulgaris* culture and determine the optimum nitrogen source, illumination period and optimum N:P ratio leading to the highest growth and nutrient removal performance under autotrophic conditions.

For this purpose, 4 sets of experiment were conducted, and the parameters were investigated. The results of these studies can be listed as,

- A culture was enriched in a 3 N BBM+V medium as recommended by UTEX.
- *C. Vulgaris* was able to be enriched in batch mode in both NO3-(Set 1) and NH4-containing (Set 2) mediums. However, when both Set 1 and Set 2 results are compared in terms of growth performance, the optimum N source was chosen as ammonium.
- For illumination period and the frequency effect, 24:0, 12:12, 8:8:8 and 6:6:6:6 (L:D) illumination periods were compared. It was revealed that for the highest growth and nutrient removal performances, continuous illumination should be conducted. In the case of intermittent illumination, a balanced L:D period might lead to higher TAN removal compared to a longer and unbalanced illumination period. This should be further investigated.
- The optimum N:P ratio was determined as 8 (g/g). However, as it was revealed that, P accumulation may be overcome with starvation period to stimulate "Luxury uptake" phenomenon that can assimilate excess P. This phenomenon should be researched further, and the starvation approach should be developed for further applications.

CHAPTER 4

DETERMINATION OF OPTIMUM ENVIRONMENTAL AND OPERATIONAL PARAMETERS TO ENRICH *CHLORELLA VULGARIS* UNDER MIXOTROPHIC CONDITIONS

4.1 INTRODUCTION

The utilization of technology in every aspect has a substantial contribution to global warming, either negatively or positively. For example, wastewater treatment plants (WWTPs) and waste management systems have a 3% contribution to global CO_2 emissions (Jaromin-Glen et al., 2020). Hence, achieving both more CO_2 sequestration and less CO_2 emissions within a treatment plant has become prominent while propagating new alternatives for biodiesel and crop production. For this aspect, microalga has proved itself to be the perfect candidate as it can sequester 513 tonnes of CO_2 while producing 100 tonnes of dry biomass per year (Bilanovic et al., 2009). This fact promotes an idea of net zero balance in algal systems where emitted biofuel combustion can be assimilated by microalgae (Kumar et al., 2010). To develop such systems, the selection of the cultivation mode for microalgae should be performed accordingly.

The photoautotrophic cultivation mode ensures contamination-free culture as well as constant CO_2 sequestration. On the other hand, heterotrophy can secure the high cell density without any requirement of illumination which makes the system cheaper and easier (Perez-Garcia et al., 2011). However, mixotrophic microalgal cultivation has been the most advantageous process compared to other modes (that is autotrophy and heterotrophy) (Zhan et al., 2017). In mixotrophy, after the organic carbon content is consumed totally by heterotrophic mechanism, algal cells begin to assimilate CO_2

autotrophically (Zhan et al., 2017). Thus, microalgae can improve its biomass production and lipid content and consumes inorganic carbon to produce oxygen (Abe et al., 2007). Furthermore, oxygen production can reduce ventilation costs in mixotrophic metabolism. Moreover, it was demonstrated in a study performed by Kang et al. (2004)that net photosynthetic rate of mixotrophy was much higher and rapid than that of autotrophy. Yet, Cecchin et al. (2018) reported that, a ratio of variable to maximum fluorescent (Fv/Fm) values, which indicates the wellness of photosynthesis, showed similarity for both conditions.

For biomass production rate, *C. Vulgaris* showed a significantly better performance in mixotrophic conditions compared with the autotrophic and heterotrophic conditions according to the study realized by(Heredia-Arroyo et al., 2011). A study on *Neochloris oleoabundans* revealed that when cultivated with carbon-rich manure, the algae's cell density was 150 percent higher than in autotrophic culture conditions, implying that mixotrophy was a useful cultivation method for increasing microalgae biomass (Giovanardi et al., 2013). In a study conducted by Cecchin et al. (2018), *Chlorella sorokiniana* exhibited a faster and higher growth under mixotrophic conditions compared to autotrophic conditions Likewise, a study conducted with *C. Vulgaris* culture revealed that lipid production was the highest under the mixotrophic conditions (Yeh et al., 2012). Additionally, (Day and Tsavalos, 1996) reported that the lipid content of *Tetraselmis* operated under mixotrophic/ photoheterotrophic conditions was 5.8 times higher compared to the ones operated under heterotrophic conditions.

Recent studies exhibited facts that in Chlorella species, cells have lower reducing power, lower chlorophyll content, increased carotenoid pigments, and lower iron-associated reducing antioxidant power, which can be associated with higher cellular survival rates in mixotrophic growth compared to cells in photoautotrophic growth (Ani Azaman et al., 2017). Additionally, phenolic compounds, which are more

abundant in the cells enriched in mixotrophic conditions, may protect the cells against radical and reactive species (Shetty and Sibi, 2014). The survival of the cells may be beneficial for any alterations implemented on algal culture such as HRT increase, nitrogen loading rate (NLR) and phosphorus loading rate (PLR) increase.

Although there are many advantages, mixotrophic enrichment has its own drawbacks. For example, mixotrophic operation of microalgal treatment is usually costly compared to photoautotrophic operation due to addition of organics. Moreover, these systems are more prone to contamination and there might be a competition between algae and other microorganisms due to the presence of organic carbon (Perez-garcia and Bashan, 2015; Zhan et al., 2017).

Because of the simultaneously occurring organic carbon reduction and nitrogen and phosphorus removal, mixotrophic growth conditions are the best for enriching an algal culture to adjust the system for wastewater treatment. However, mixotrophic metabolism is not simply addition of autotrophic and heterotrophic reactions as it is understood from the definition. In fact, there is a shift between those two metabolisms depending on the ambient conditions and this shift affect the removal performance of nutrients and organic carbon (Park et al., 2021). Hence, the contribution of heterotrophic and autotrophic mechanisms should be investigated and understood for a correct utilization and manipulation of mixotrophic cultures.

According to the Equation 4.1. (Autotrophic metabolism) and Equation 4.2. (Heterotrophic metabolism), inorganic and organic carbon can be tracked down and the stoichiometric consumption of each can be calculated. However, in mixotrophic metabolism (Equation 4.3.), the inorganic carbon produced by heterotrophic respiration also contributes to the inorganic carbon source for autotrophy that makes the examination and determination of the true contribution of both mechanisms to the mixotrophic one difficult (Park et al., 2021).

$H_2O + HCO_3^- \rightarrow C_{Biomass} + 1/2O_2 + 3OH^-$ ……..... (Equation 4.1)

$(1+a)\ CH_2O + O_2 \rightarrow C_{Biomass} + aCO_2 + (1+a)\ H_2O$ ……..… (Equation 4.2)

$bHCO_3^- + cCH_2O \rightarrow (b+(c-a))\ C_{Biomass} + 3OH^- + aCO_2$….. (Equation 4.3)

Overall, mixotrophic cultivation of microalgae exhibits many advantages over autotrophic and heterotrophic enrichment, especially for the systems that alterations on operational and environmental parameter will be performed.

According to the many studies performed on microalgal wastewater treatment, the cultivation mode has a profound impact on nutrient removal and growth performance of the algae. For instance, a study conducted by Osorio et al. (2020) showed that *C. Vulgaris* has 9% P removal under autotrophic conditions while it can achieve 32% removal efficiency under mixotrophic conditions. In a similar study, (Babaei et al., 2018) claimed that *C. Vulgaris* has a higher nutrient removal efficiency under mixotrophic conditions compared to the one operated under autotrophic conditions. Moreover, it was suggested that culture enriched under mixotrophic conditions, can adopt better to ammonium-N assimilation when it is shifting from nitrate nitrogen, compared to ones enriched under autotrophic conditions (Babaei et al., 2018)

This chapter of the thesis focuses on the investigation of the effect of mixotrophic conditions on microalgal growth and nutrient removal performance. Moreover, under mixotrophic conditions, the optimum HRT, NLR, PLR, and OLR was aimed to be researched and determined. For this reason, a *C. Vulgaris* culture was initially enriched under mixotrophic conditions with different C sources, in batch mode. Later, the optimum C source was determined and the enriched *C. Vulgaris* culture was inoculated into bubble-column PBRs to be operated in semi-continuous mode. The effect of 2-, 4- and 8-day HRT and the determination of optimum one was

performed. Additionally, the effect of NLR and PLR was investigated along with the OLR with gradual increase applied in semi-continuous PBRs.

4.2 MATERIALS AND METHOD

This section includes key details on the operational conditions, experimental set ups and analytical methods that are applied in the enrichment of the *C. Vulgaris* culture under mixotrophic conditions.

4.2.1 Preliminary Study

A preliminary study was conducted in content of this Chapter 4, to overcome the possible P removal problems that were encountered in Chapter 3, Sections 3.3.3 and 3.3.4. The low level of P removal efficiencies and P accumulation was associated with the finding that there is a limit for N and P consumption under autotrophic conditions (Aslan and Kapdan, 2006; Ruiz et al., 2011). According to a study conducted with *C. Vulgaris*, the desired treatment efficiencies can be observed with an initial concentration of less than 22 mg/L NH_4^+-N and 7.7 mg/L PO_4^{-3}-P (Aslan and Kapdan, 2006). In a similar study, the initial N and P concentration range, which provides the best nutrient removal efficiency, was found to be approximately 6-20 mg/L NH_4^+-N and 1-6 mg/L PO_4^{-3}-P (Ruiz et al., 2011).

For the preliminary study, an autotrophic semi-continuous PBR, T8, from Section 3.3.4 was operated at initial TAN and P concentrations which were half of the concentrations applied as previously in Section 3.2.5.4 (120 mg/L N and 15 mg/L P for an N:P ratio of 8). In other words, preliminary PBR was fed with initial concentrations of 64 mg/L TAN and 8 mg/L P, and the N: P ratio was maintained at 8, as it was determined in Section 3.3.4.

The results obtained by the preliminary study proved that the halved concentrations satisfied almost 100% of the removal efficiency for both TAN and PO_4^{-3}-P. The results are presented in APPENDIX E.

4.2.2 Inoculum

A *C. Vulgaris* culture was obtained from the preliminary study, explained in Section 4.2.1. (APPENDIX E), was used for the inoculation of the PBRs conducted in chapter 4.

4.2.3 Synthetic Wastewater

For the mixotrophic enrichment (Set 5), a synthetic wastewater given in Table 4.1, was used. The synthetic wastewater was derived from the one recommended by UTEX Culture Collection of Algae (Table 3.1). However, for creating mixotrophic conditions, a sCOD source was added. Moreover, influent TAN and P concentrations were halved as it was discussed in Section 4.2.1 and set to 64 mg/L N and 8 mg/L P. Yet, salt, metal, and vitamin concentrations were kept as same as the recipe provided by The UTEX Culture Collection of Algae (Table 4.1). The N and P concentrations in synthetic wastewater were changed through the experimental studies according to the results obtained in each experimental set-up. Thus, the changes were specifically mentioned in the Experimental Procedure Section (Section 4.2.6), in each experiment's procedure.

Table 4.1. Synthetic wastewater designed for mixotrophic enrichment of *C. Vulgaris* culture

Component	**Final Concentration**
NH_4Cl	4.41 mM
$CaCl2{\cdot}2H_2O$	0.17 mM
$MgSO_4{\cdot}7H_2O$	0.3 mM
K_2HPO_4	0.21 mM
KH_2PO_4	0.65 mM
NaCl	0.43 mM
P-IV Metal Solution	**Final Concentration**
$Na_2EDTA.2H_2O$	2 mM
$FeCl_3.6H_2O$	0.36 mM
$MnCl_2.4H_2O$	0.21 mM
$ZnCl_2$	0.037 mM
$CoCl_2.6H_2O$	0.0084 mM
$Na_2MoO_4.2H_2O$	0.017 mM
Vitamin Solution	**1 mL/L (for each)**
Cyanocobalamin	0.027 g/200 mL dH_2O
Thiamine HCl	0.067 g/200 mL dH_2O
Biotin	0.005 g/200 mL dH_2O

4.2.4 Photobioreactors (PBR)

For mixotrophic experiments in Chapter 4, a first step batch cultivation was performed prior to semi-continuous operation, to ensure the optimum form of acetate as well as acclimation of the culture to mixotrophic conditions. For this purpose, 500 mL Erlenmeyer Flasks were used.

To scale-up the culture and all following synthetic wastewater treatment experiments, glass bubble column PBRs, with 1 L volume, 8 cm diameter and 24 cm height, were used.

4.2.5 Analytical Methods

During the experimental studies, density, pH, temperature, photosynthetically active radiation (PAR), dry weight, total ammonia nitrogen (TAN), ortho-phosphate (PO_4^{-3}-P), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), chlorophyll-a were measured.

pH: pH meter (Eutech, CyberScan, pH510) and pH probe (Sensorex, p350) were used to measure pH value.

Temperature: Temperature values of the PBRs were measured with 9263 A Plus digital thermometer. It should be noted that, the ambient temperature in the sets was measured in a container that has water inside, that would represent the temperature of the PBRs.

Optical Density: HACH spectrophotometer DR 2800 with 1-cm light path was used to measure optical density values at optimum wavelength determined for erniched *C.Vulgaris* culture. To determine the optimum wavelength, optical density values were read at different wavelengths and the highest absorbance value was obtained at 680 nm. Detection limit is between 0.1 and 1, so for samples with optical densities higher than 1, dilution is necessary.

Photosynthetically Active Radiation (PAR): PAR was measured through a hand device called PAR meter (Light SCOUT).

Dry Weight (DW): The dry weight measurement of the microalgal culture was carried out according to the Standard Method (APHA, AWWA, WEF, 2005). According to this method, samples taken from the PBR are primarily filtered through Sartorius brand filters (0.7 m), then dried overnight in oven at 105 °C in 30 mL of crucibles. They are held in the desiccators prior to weighing to remove the moisture.

Total Ammonia Nitrogen (TAN): TAN analysis is based on the Nessler method (Crosby, 1968). Samples from the PBR were filtered through the cellulose acetate filters (0.45 μm) for this analysis and diluted according to the required ranges. Related calibration curves were given in APPENDIX F.

Orthophosphate (PO_4^{-3}-P): PO_4^{-3}-P analysis, was performed by Ion Chromatography (IC- Shimadzu Prominence HIC-SP). The working conditions of the IC were set to have the highest-pressure limit of 150 bar, oven temperature of 45°C, and the flow rate of 0.8 mL/min. Prior to the analysis, samples were filtered through 0.45 μm cellulose acetate filter. The calibrations curve for PO4-3-P analysis is given in APPENDIX G.

The limit of detection (LOD) and the limit of quantification (LOQ) values for low and high concentration with the calibration curves are given in APPENDIX G.

Moreover, for PO4 detection Lovibond LR-Tablet Kits were also used. The details related to calibration of the kits were given in APPENDIX H. The calibrations curve for PO_4^{-3}-P analysis is given in APPENDIX H.

Nitrate-Nitrogen (NO_3^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_3^--N analysis is given in APPENDIX G.

Nitrite-Nitrogen (NO_2^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_2^--N analysis is given in APPENDIX G.

Soluble Chemically Oxygen Demand (sCOD): sCOD was determined by EPA approved digestion method (for COD range of 0-1500 mg/L), using heat PBRs (Aqualytic AL 38) for 2 hours. Results were obtained spectrophotometrically with spectrophotometer (SN 05827, PC Multidirect).

Total Organic Carbon (TOC): Total organic carbon concentration of the samples was determined using Shimadzu 5000A model TOC analyser that employs 680 °C combustion catalytic oxidation method. The calibration curve for TOC analysis is given in APPENDIX I.

It should be noted that, the TOC analysis was performed for the soluble portion of the sample, representing sCOD. Moreover, sCOD results of Set 7 were converted from the TOC data. For this conversion Equation 4.4 was used (Dubber and Gray, 2010).

$sCOD=49.2+3.0(TOC)$ …….…….. (Equation 4.4)

Chlorophyll-a and Pheophytin-a: Pigments measurements were done according to the Standard Methods 10200H (APHA, AWWA, WEF, 2005). Optical density ratio of 664b/665a (OD (664b/665a)) gives insight about health of microalgal culture. Ratio of 1.7 represents the healthiest situation while 1.0 represents death of culture. When chlorophyll-a content of the culture is higher, the ratio would be closer to 1.7; however, when pheophytin-a concentration is high, the ratio would be closer to 1. Pheophytin-a is the chlorophyll-a molecule that lost its Mg^{+2} ion and cannot function in photosynthesis reactions anymore. Equation 4.5 and 4.6 were used to determine chlorophyll-a and pheophytin-a (Dere et al., 1998).

Chlorophyll a, $mg/m^3 = 26.7(OD_{664}-OD_{665})\ V_1/(V_2L)$ ……..…..... (Equation 4.5)

Pheophytin a, $mg/m^3 = 26.7(1.7xOD_{665}-OD_{664})\ V_1/(V_2L)$ ……..... (Equation 4.6)

Where,

C_a: chlorophyll a

C_b: chlorophyll b

All analyses were performed as at least duplicate, and averaged values were used in the figures and/or tables. For the analyses performed in triplicates, standard deviation values are presented in figures. In the calculation of the specific growth rate (μ) of the microalgal culture, the following Equation 4.7 was used (Krzemińska et al., 2014; F. Liang et al., 2013). Equation 4.8 (Liu et al., 2011) was used to determine double the number of cells (t_d) and Equation 4.9 (F. Liang et al., 2013). was used to calculate the biomass production rate (BPR). These values were calculated considering the steady-state conditions of the PBRs. The steady-state conditions in the studies were defined as "the point where the parameter does not change more than 10% in three consecutive days" (Kılıç, 2017).

$\mu = \ln(N_2/N_1)/(t_2-t_1)$ …… (Equation 4.7)

$t_d = \ln(2)/\mu$ ……...… (Equation 4.8)

$dX/dt = (X_2-X_1)/(t_2-t_1)$ ……..…… (Equation 4.9)

N_1: OD value at time t_1
N_2: OD value at time t_2
μ: Specific growth rate (day^{-1}) (SGR)
t_d: Doubling time (days)
dX/dt: Biomass production rate (mg/L.d) (BPR)
X_1: Dry weight of the microalgae at time t_1
X_2: Dry weight of the microalgae at time t_2

4.2.6 Experimental Setup

In this part of the thesis, the experimental procedures for mixotrophic enrichment in batch mode (Set 5), investigation of optimum HRT in semi-continuous mode (Set 6) and investigation of optimum NLR, PLR and OLR in semi-continuous mode (Set 7)

was given. It should be noted that, after 87 days of operation a contamination was observed in Set 6. Thus, prior to Set 7, a new *C. Vulgaris* culture was enriched in batch mode under mixotrophic conditions to be utilized in Set 7. The related details to this batch enrichment of *C. Vulgaris* were given in APPENDIX J

4.2.6.1 Set 5: Cultivation of *C. Vulgaris* in Batch PBRs under Mixotrophic Conditions

The aim of this batch PBR experiment was to investigate the effects of mixotrophic cultivation mode on nutrient removal and growth performance of *C. Vulgaris*. For this purpose, firstly, seven 500 mL batch PBRs with a 300 mL effective volume were set to enrich the *C. Vulgaris* culture obtained from the preliminary study mentioned in Section 4.2.1 (Table 4.2 and Figure 4.1). Six of them were designed to be mixotrophic, as test PBRs containing sCOD, and one was designed as an autotrophic as, a control PBR. The sCOD concentrations of each PBRs were 1000 mg/L. The TAN and P concentrations were set as 64 mg/L N and 8 mg/L P in all reactors. Six PBRs were set as duplicates of three different test groups that each one with different form of acetate (Table 4.2.). Equal amounts of (molar) acetic acid, sodium acetate or acetic acid-sodium acetate buffer were added to the test PBRs as organic carbon source. Although it is anticipated here that acetic acid and sodium acetate may have similar effects, test PBRs containing these carbon sources were nevertheless set up to determine the effect of buffer solution on nutrient removal and growth performance as well as pH.

Table 4.2. Properties of the PBRs and initial concentrations of nutrients and organic carbon

PBR		sCOD source	Cultivation Mode	Initial Concentrations (mg/L)		
Type	**Name**			**TAN**	**PO_4^{3-}-P**	**sCOD**
Control	Control	-	Autotrophic	64	8	-
Test	HAc 1	Acetic Acid	Mixotrophic	64	8	1000
Test	HAc 2	Acetic Acid	Mixotrophic	64	8	1000
Test	NaOAc 1	Sodium Acetate	Mixotrophic	64	8	1000
Test	NaOAc 2	Sodium Acetate	Mixotrophic	64	8	1000
Test	Buffer 1	HAc-NaOAc	Mixotrophic	64	8	1000
Test	Buffer 2	HAc-NaOAc	Mixotrophic	64	8	1000

The reason of using different sCOD sources was to investigate if it was possible to simultaneously eliminate the problems encountered in balancing the pH of the system. As it is known, during the consumption of ammonium nitrogen, microalgae reduce the pH of the environment by throwing an extra proton into the liquid media while assimilating the ammonia into the cell to maintain the neutral state of their cells (Fuggi et al., 1981; Xin et al., 2010). As a solution to this situation, the bicarbonate buffer was given to the system. However, it may cause a risk of ammonia stripping induced by the increased pH. Thus, it was decided to search for a sCOD source that will replace the bicarbonate buffer while minimizing the pH increase.

In biological systems, there is a balance between weak bases and conjugated salts or conjugated weak bases (Mohan, 2003). As seen in Equation 4.10 below, the equilibrium shifts to the acetic acid side as the pH decreases, while it shifts to the acetate side as the pH increases. It was observed in microalgal systems using acetic acid that the pH increases after consumption of acetic acid depending on the system pH (Huang et al., 2017). On the other hand, in microalgal systems, the pH is also balanced by the introduction of acetic acid into the system when the system pH increases (Aslan and Kapdan, 2006). Results observed in the literature show that the

regulation of pH in microalgal systems is a research topic all by itself, specific to the conditions of each system. Therefore, the effects of a buffer of acetic acid-acetate were aimed to be investigated.

$CH_3COOH_{(aq)} \leftrightarrow H^+ + CH_3COO^-_{(aq)}$…….…. (Equation 4.10)

$pH = pK_a + \log([CH_3COOH] / [CH_3COO^-])$ ….……. (Equation 4.11)

Buffer solutions generally work most effectively in a range where pH equals pKa, that is around “isoelectric"”point, where no net charge is observed (Mohan, 2003). To obtain such balance, as can be understood from Equation 4.10, the weak acid and conjugated salt solution in the buffer in equal molarity should exist (Mohan, 2003). According to this fact, as it can be seen in Equation 4.11, pH should be set at 4.75 considering the pKa value of acetic acid (pK_{HAc}=4.75). However, it is known that using such a low pH buffer solution in microalgal systems is risky for the health of the culture. Moreover, in fact, the pH range of 6.5-6.8 is given as BBM pH by UTEX. Thus, since this buffer can be effective up to around pH 6 (Mohan, 2003), the pH values of all PBRs, were set as 6.0-6.8 to satisfy both working conditions of the buffer and culture’s health.

The PBRs in this Set 5 were inoculated with a ratio of 33% from the previous PBR where halved influent TAN and P concentrations were experimented on (Section 4.2.1, preliminary study). The PBRs were operated for 5 days after the first feeding. When the N and P removals were reached to 80% (4th day), PBRs were fed on the 5th day to obtain the same initial nutrient and organic carbon concentrations and observed for another 4 days.

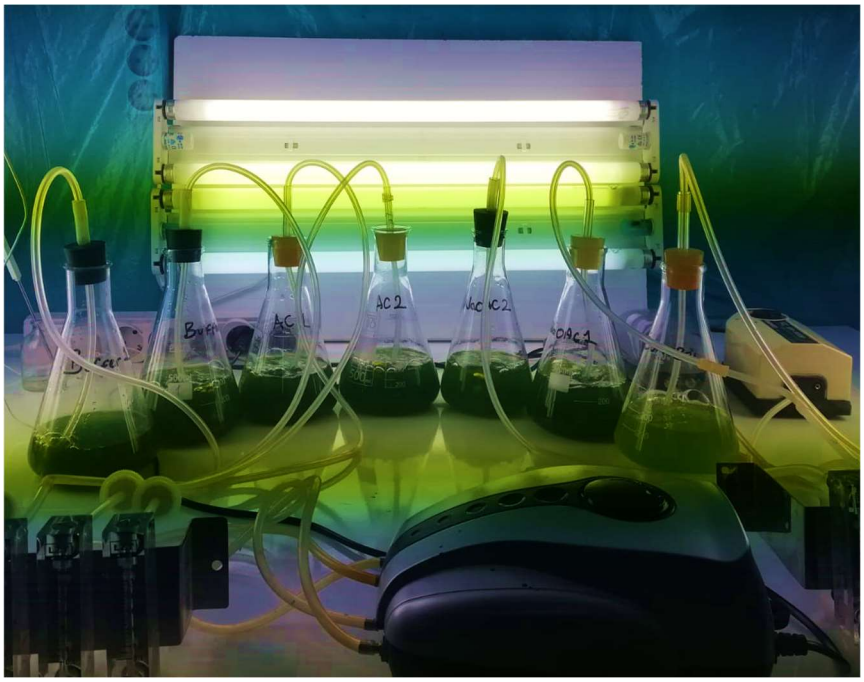

Figure 4.1. Seven batch PBRs set for the mixotrophic enrichment

The PBRs were operated at an average temperature of 25 ± 3°C. PBRs were operated for 9 days in total. 100 µmol.m^{-2}.s^{-1} (100 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for continuous illumination period. Aeration was supplied to all PBRs with a flowrate of 0.4-0.6 L/min flowrate (1.3-2.0 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 µm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination. pH of the PBRs was set to 6.5±0.3 and manually arranged to its initial pH value (of 6.5) everyday with 2 N NaOH and HCl.

TAN, PO4-P and sCOD analyses were performed to monitor the nutrient and organic removal efficiencies of the PBRs. In addition, dry weight and optical density experiments were carried out every other day as well. For the test PBRs, the average of the results of duplicate PBRs were calculated and each PBR was analysed for at

least two times for experiments. The results were presented with related standard deviations in the results section. After 2nd feeding, due to intentional pH alteration for Buffer 2 PBR, the results were evaluated separately from its replicate Buffer 1 PBR. Hence, the results do not include standard deviation for those PBRs at this feeding period.

4.2.6.2 Set 6: Investigation of the Effect of HRT on Mixotrophic Semi-Continuous PBRs at Constant Loading Rate

The aim of Set 6 is to investigate the effect of HRT on mixotrophic *C. Vulgaris* culture at constant loading rate. For this purpose, three semi-continuous replicate PBRs, namely MX1, MX2 and MX3 (Figure 4.2), with a total of 1 L and an effective volume of 800 mL were operated under mixotrophic conditions. PBRs were inoculated with a ratio of 25% from the cultures enriched in HAc PBRs (Set 5, Section 4.2.6.1), that has the highest growth performance and N, P and sCOD removal efficiencies. These PBRs were fed with a synthetic wastewater with inlet concentrations of 64 mg/L N and 8 mg/L P as it was tested in preliminary study mentioned in Section 4.2.1. The source of sCOD was acetic acid and was administered at an inlet value of about 500 mg/L sCOD (Table 4.3). All PBRs were initially operated with a constant 8-day HRT. During the whole operation of Set 6, PBRs were operated at a NLR of 8 mg/L.d N, PLR of 1 mg/L.d P and OLR of 62.5 mg/L.d COD.

Figure 4.2. Semi-Continuous PBRs in Set 6

Table 4.3. Properties of semi-continuous PBRs of Set 6

PBR		N Source	sCOD Source	Inlet Concentration (mg/L)		
Type	Name			N	PO_4-P	sCOD
Test	MX1	TAN	Acetic Acid	64	8	500
Test	MX2	TAN	Acetic Acid	64	8	500
Test	MX3	TAN	Acetic Acid	64	8	500

Due to the measures taken during the COVID-19 pandemic and quarantine processes, on March 24, 2020, a program was made to spend the least amount of time in the laboratory. It was only aimed at the time to maintain the culture during the intensive COVID-19 measures. Thus, after 24-day of operation, an intermediate feeding was put in operation starting with the COVID-19 measures which still led to an 8-day HRT of. Yet, no analysis was performed during this period. After the

measures were left behind, the PBRs were first cleaned and operated with daily feeding at an 8-day HRT. The PBRs were operated at a NLR of 8 mg/L.d N, PLR of 1 mg/L.d P and OLR of 62.5 mg/L.d COD. After MX1, MX2 and MX3 PBRs reached to steady-state N and P removal at HRT of 8 days operation, on Day 20, the HRT of MX1 and MX2 was reduced to 4 days, and then to 2 days. The HRT of MX3 PBR was kept as 8 days. The influent N, P and sCOD concentrations of MX1 and MX2 decreased in parallel to the decrease in their HRT (Table 4.4). In this way, the effect of HRT on the nutrient removal and growth performance of the PBRs were investigated at constant NLR of 8 mg/L.d N, PLR of 1 mg/L.d P and OLR of 62.5 mg/L.d COD. To evaluate the removal rates more effectively, kinetic studies were also carried out for all PBRs when they reached steady-state at each HRT level studied.

Table 4.4. Details related to the nutrient and organic carbon content of PBRs in Set 6

PBR		**N Source**	**sCOD Source**	**Inlet Concentration (mg/L)**			**HRT**
Type	**Name**			**TAN**	**PO_4^{-3}-P**	**sCOD**	
Test	MX1	TAN	HAc	64	8	500	
Test	MX2	TAN	HAc	64	8	500	8 Days
Test	MX3	TAN	HAc	64	8	500	
Test	MX1	TAN	HAc	32	4	250	
Test	MX2	TAN	HAc	32	4	250	4 Days
Control	MX3	TAN	HAc	32	4	250	
Test	MX1	TAN	HAc	16	2	125	
Test	MX2	TAN	HAc	16	2	125	2 Days
Control	MX3	TAN	HAc	16	2	125	

100 $\mu mol.m^{-2}.s^{-1}$(100 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for continuous illumination. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs

with a flowrate of 0.4-0.6 L/min flowrate (0.5-0.75 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 µm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination. pH of the influent synthetic wastewater was set to 6.0±0.3. A figure that shows the PBRs set after COVID-19 intensive measure period is provided below (Figure 4.3).

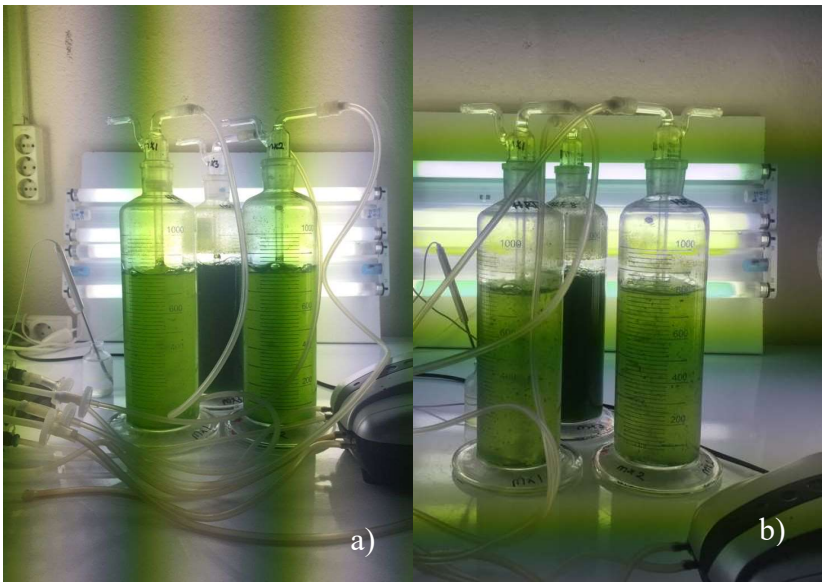

Figure 4.3. MX1-MX2-MX3 PBRs set after COVID-19 intensive measure period a) MX1 and MX2 4-day HRT operation b) MX1 and MX2 2-day HRT operation

TAN, PO4-P and sCOD analyses were performed every other day to monitor the nutrient and organic removal efficiencies of the PBRs. In addition, dry weight, optical density, and chlorophyll-a experiments were carried out every other day.

During the operation of the PBRs with 2-day HRT of, on Day 86, a sudden colour change was observed in all PBRs (Figure 4.4). Microscope observations revealed the existence of a species called *Lecane inermis* (Figure 4.5), which is a type of rotifer

in all cultures (Fyda et al., 2015). To understand when the contamination first started to appear, the previous samples were also examined under the microscope. It was found that the adult rotifers and their eggs were encountered for the first time on Day 83. Since there is a chance that this grazer species can harm the culture's health (Gong et al., 2015), the nutrient removal and growth data prior to the Day 83 were only considered and discussed in this Chapter 4. Fortunately, all PBRs had reached to their steady-state condition before Day 83 and it was possible to observe the HRT effect for all three HRTs studied (8, 4 and 2 days). It should be noted that all data obtained after Day 83 were under the influence of contamination, thus not involved in related results and discussion part (Section 4.3.2.)

Figure 4.4. Colour change observed in MX1, MX2 and MX3 PBRs

To overcome this contamination, many methods were suggested by researchers (Day et al., 2017; Montemezzani et al., 2015; Touloupakis et al., 2016). Those can be listed

as, introduction of pesticide, allelopathy, chemical usage, pH adjustment, CO_2 purging, filter etc. Due to the risk of disruption of operational conditions, chemical addition was avoided in fighting against this infection. However, filtering and CO_2 purging methods were applied on all cultures as suggested by Ma et al. (2017) to create a low pH that can suffocate the grazers while microalgae do not get affected. Although a purging set of 5 days were applied, no complete removal of the infection was observed since the grazers were shifted to dormant phases and protected themselves under CO_2 stress (Pajdak-Stós et al., 2014) (Figure 4.5). As this was the case, it was decided to terminate the PBRs.

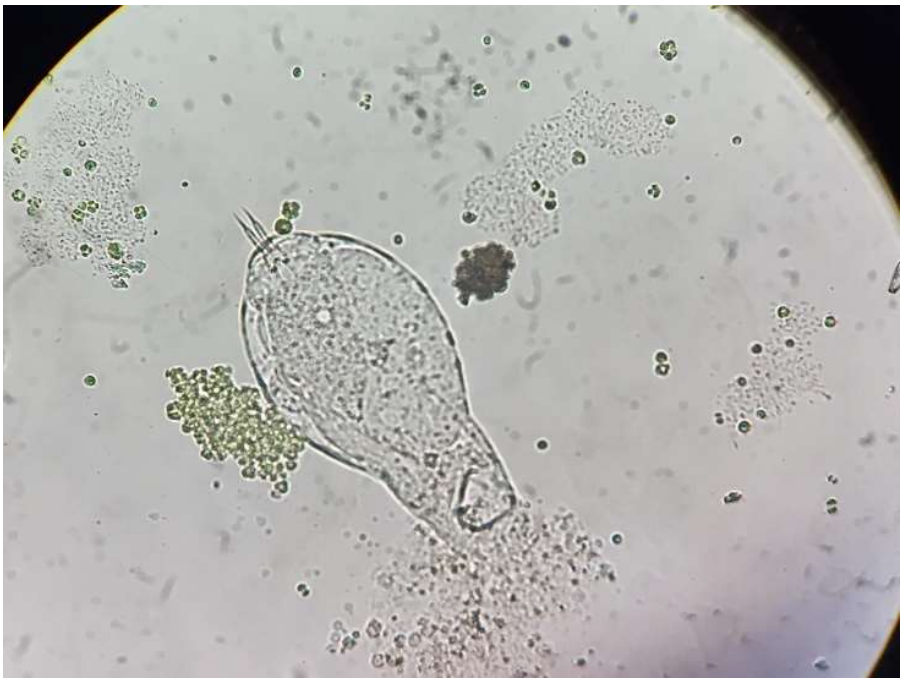

Figure 4.5. *Lecane inermis* bursting out from dormant phase captured under microscope (40X magnification)

4.2.6.3 Set 7: Investigation of Optimum Loading Rates in Mixotrophic Semi-Continuous PBRs at Constant HRT

The aim of Set 7 was to investigate the optimum NLR, PLR and OLR at constant HRT application, leading to the highest nutrient removal and growth performance.

As mentioned in Section 4.2.6., due to the contamination of *C. Vulgaris* with Rotifer *Lecane Inermis*, a new culture was obtained from Istanbul Microalgae Biotechnologies Research and Development Centre both in agar plates and liquid media. The new culture was cultivated in 250 mL-PBRs with three different mediums to determine the best medium for the following Set 7. The details of this enrichment study and the related results are given in APPENDIX J. As given in APPENDIX J, the new uncontaminated *C. Vulgaris* culture was enriched. The optimum medium content with best growth was found to be LB medium. LB medium contained 40 mg/L TAN, 5 mg/L P and 315 mg/L sCOD initial concentrations. These values were determined considering that the daily concentrations would be 8 mg/L.d, TAN 1 mg/L.d P and 62.5 mg/L.d. sCOD for the five days of experimental operation.

In Set 7 two semi-continuous replicate PBRs, namely LR1 and LR2, with a total of 1 L and an effective volume of 800 mL were operated under mixotrophic conditions (Figure 4.6). Each PBR was inoculated with a ratio of 25% from re-enriched *C. Vulgaris* culture mentioned in APPENDIX J. The PBRs were operated with loading rates of NLR of 8 mg/L.d N, PLR of 1 mg/L.d P and OLR of 62.5 mg/L.d sCOD. The HRT of the PBRs was set to 4 days regarding to the results of Set 6, Section 2.4.6.2, where the effect of HRT was investigated.

Figure 4.6. LR-1 and LR-2 PBRs

After 15 days of operation at constant NLR, PLR, and OLR (as sCOD), PBRs reached to steady-state conditions in terms of nutrient removal and growth performances. At this point, NLR and PLR were tripled to be adjusted as 24 mg/L.d N and 3 mg/L.d P. OLR was kept constant at 62.5 mg/L.d sCOD. At this point a gradual decrease in pH was observed which was followed by a decrease in P removal efficiency. Although OLR was increased to 200 mg/L.d sCOD on Day 36 to prevent this, the P removal efficiency kept deteriorating. Hence, on Day 95, NLR, and PLR was decreased to 8 mg N/L.d and 1 mg P/L.d, respectively and OLR was decreased to 100 mg/L.d, sCOD, to make sure that enough organic carbon was supplied to the system for P removal, and steady-state conditions were observed after this shift.

150 $\mu mol.m^{-2}.s^{-1}$(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for a continuous illumination. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs

with a flowrate of 0.4-0.6 L/min flowrate (0.5-0.75 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination. The pH of the feeding medium was set to 6.5 ± 3 (Ma et al., 2017).

TAN, PO4-P and sCOD analyses were performed every other day to monitor the nutrient and organic removal efficiencies of the PBRs. In addition, dry weight, optical density, and chlorophyll-a experiments were carried out every other day.

4.3 RESULTS AND DISCUSSION

4.3.1 Results of Set 5: Cultivation of *C. Vulgaris* in Batch PBRs under Mixotrophic Conditions

The aim of the study was to enrich a mixotrophic *C. Vulgaris* compare the mixotrophic and autotrophic growth conditions and to identify the optimum sCOD source with the highest growth and treatment performance. For this purpose, seven batch PBRs, namely Autotrophic Control, HAc1, HAc, 2, NaOAc 1, NaOAc 2, Buffer 1 and Buffer 2, were set.

As stated in Section 4.2.6.1, the PBRs were fed twice. After the consumption of N and P over 80% in the end of the 1st feeding period (which corresponds to Day 4), the 2nd feeding was performed on Day 5 and the PBRs were operated for another four days. For the first feeding period, the replicates of HAc, NaOAc and Buffer PBRs were operated similarly, while for the second feeding period the replicates Buffer 1 and Buffer 2 PBRs were treated differently in terms of pH adjustment hence, they were evaluated differently. The results of analysis are given in

Figure 4.7 and Figure 4.8. Except for the Buffer replicates in 2nd feeding period, all analyses data are the average values of replicate reactors.

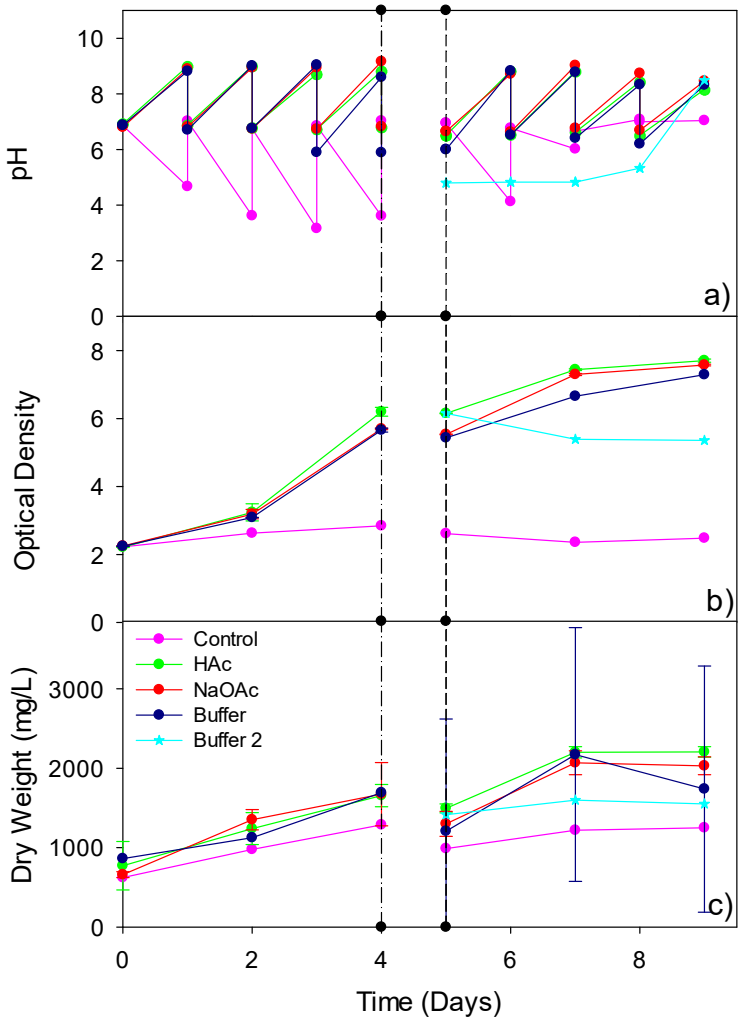

Figure 4.7. The Growth Performances of The PBRs for Set 5 a) pH b) Optical Density c) Dry Weight

As stated in Section 4.2.5., 2N NaOH and/or 2N HCl solution was used daily to adjust the pH at 6.5±0.1 (Figure 4.7.a). Although the pH range of HAc-NaOAc buffer

is 4.5±1, since this pH level is not applicable for microalgae, the PBRs were started at pH of 6.5±0.5. However, this situation prevented the buffer solutions from working effectively. As seen in

Figure 4.7.a, after the 1st nutrient feeding, the pH levels increased up to 9.0 in the same way for all mixotrophic cultures, and the type of sCOD source did not have any effect on pH. However, as NH_3 and protons (H^+) were formed due to NH_4^+ assimilation in ammonium-containing mediums, pH decreased in the autotrophic control PBR. Hereby, the applicability of the buffer system was questioned as it was not working in the necessary pH range of 6.5-10.0 (Deniz, 2020) for algal systems.

After the 2nd feeding, the pH change was observed between the Day 5 and Day 9. Here, to determine the effect of pH on the buffer solution, unlike the 1st feeding period, the pH of the Buffer 2 PBR was adjusted to the pH of HAc-NaOAc buffer which is pH 4.8. Then, it was observed that the pH remained constant at pH 4.8 between Day 5 and Day 7, and afterwards increased gradually. This indicates that the equimolar HAc-NaOAc buffer solution shows the expected effect at the correct pH range, but it was concluded that the buffer capacity was enough for 3 days. Yet, as will be mentioned later in this section, it is understood that the growth and treatment performance of the Buffer 2 PBR was also affected by this pH range (4.75 - 5.3). The decrease in the pH value of Control PBR (Figure 4.7.a) gradually slowed down between Days 6 and 9, which can be associated with the slower activity of the culture after reaching its growth limits.

As expected, OD values of the mixotrophic test PBRs reached to 6.2±0.01, 5.7±0.02, and 5.7±0.03 for Hac, NaOAc and Buffer PBRs in the 1st feeding period while, in the 2nd feeding period these numbers levelled up to 7.7±0.05, 7.6±0.03, and 7.3 for HAC, NaOAC (Figure 4.7.b) and Buffer 1 PBR, while it remained at 5.36 for Buffer 2 PBR (Figure 4.7.b). This fact simply proves that the low pH (<6.5) has an adverse effect on algal growth performance. However, it can also be concluded that the

results are worthy of the risk as the OD values of the Buffer 2 PBR were much higher compared to the literature values, and Hac-NaOAC buffer can be applied if the growth performance can be kept in a certain level. In a similar study conducted by Kim et al. (2013), with 4000 mg/L sCOD source, the highest OD obtained was recorded as 1.5. Moreover, when the OD results of the all-test PBRs were compared with the autotrophic control PBR with OD level of 2.85 and 2.5 in the 1st and 2nd feeding period respectively, the positive effect of organic carbon on growth performances can be seen clearly (Li et al., 2014).

Specific growth rates were calculated based on OD values for all PBRs for the 1st and 2nd feeding periods. The specific growth rate of the autotrophic control culture was calculated as 0.06 day^{-1} in the 1st feeding period (Day 0-4). In the 2nd feeding period it gave a declining trend (Day 5-9), hence no specific growth rate was shared. The specific growth rates of mixotrophic cultures were calculated as 0.26, 0.23 and 0.23 day^{-1} for HAc, NaOAc, and Buffer PBRs respectively in the 1st feeding period (Day 0-Day 4). The Buffer 2 PBR also shows a declining trend in this period as its pH decreased over time. The specific growth rate for HAc, NaOAc and Buffer 1 PBRs, exhibited a specific growth rate of 0.1 day^{-1} in the 2nd feeding period (Day 5-9). The doubling time of the autotrophic control PBR was found to be approximately 11 days and 3 days for HAc, NaOAc and Buffer cultures for the 1st feeding period.

The results of the dry weight analysis are shown in (Figure 4.7.c) and dry weight levels have a similar pattern as that of the optical density results. Accordingly, in the 1st feeding period, the control PBR reached a dry weight of about 1300 mg/L and it remained the same during 2nd feeding period. In mixotrophic cultures, the average dry weight values were 1654 ±140, 1670±400 and 1690 ±700 mg/L for Hac, NaOAc and Buffer PBRs, respectively in the 1st feeding period on Day 4. While those values reached to 2200±65, 2000±110, 1700 and 1550 mg/L for HAc, NaOAc, Buffer 1 and Buffer 2 PBRs in the 2nd feeding period on Day 8, respectively. Biomass production

rates of the cultures were calculated based on dry weight results, as 166, 220, 253, 207 mg/L.d for Control, HAc, NaOAc, and Buffer PBRs, respectively, in the 1st feeding period. In the 2nd feeding period these values were 65, 178, 183, 133, and 34 mg/L.d for Control, HAc, NaOAc, Buffer 1 and Buffer 2 PBRs, respectively. The lower increase in obtained OD and dry weight values in the 2nd feeding (Day 5-Day 9) compared to the 1st one (Day 0-Day 4) is due to the cultures had reached a certain growth limit. This situation generally occurs in two ways: first, the cells reach very high densities, and secondly, very small cells become suspended in the medium (Flynn, 2020). The first case was valid for this experiment, as the cell density increased noticeably, the inside of the PBR was shaded, and the light transmission was negatively affected (self-shading). This resulted in the inhibition of growth because of the system's self-limiting in the next process.

In (Figure 4.8.a and b)., TAN removal efficiency and removal rates of mixotrophic and autotrophic cultures are shown. According to the results, the TAN removal efficiency reached to 99.8 ± 0.2%, 100±0.0% and 100±0.03% for HAc, NaOAc and Buffer PBRs in the 1st feeding period (Day 4). The highest TAN removal rates on Day 2 were recorded as 30±3 mg/L.d for HAc, NaOAc and Buffer PBRs. In this period, the autotrophic control PBR was able to achieve only 57% TAN removal efficiency and 8 mg/L.d average TAN removal rate on Day 4. For 2nd feeding period TAN removal efficiencies of HAc, NaOAc and Buffer 1 and Buffer 2 PBRs were 99.1±1.1%, 100±0.0, 100% and 84%, respectively. The highest TAN removal rates, in this period, were recorded as 33±13, 36±2, and 42 mg/L.d for HAc, NaOAc and Buffer 1 PBRs, respectively on Day 6. The highest TAN removal rate exhibited by Buffer 2 was recorded as 10.7 mg/L.d on Day 9. As seen in the results, Buffer 2 PBR was adversely affected (Gensemer et al., 1993) due to the low pH during the 2nd feeding period (Figure 4.7.a). As seen in (Figure 4.8.a and b), no nitrogen removal was observed in the autotrophic control PBR in the 2nd feeding period.

In Figure 4.8.c and d, PO_4^{-3}-P removal efficiency and removal rate in the PBRs are shown. Accordingly, in the 1st feeding period, the P removal efficiency of the PBRs reached to 85±4%, 87.9±2%, 79±8% for HAc, NaOAc and Buffer PBRs, respectively by Day 4. The highest P removal rates were found as 3.7, 5.2 and 2.8 mg/L.d for HAc, NaOAc and Buffer PBRs, respectively on Day 2. In the autotrophic control PBR, these values were 78% and 1.9 mg/L.d, respectively. In the 2nd feeding period, P removal efficiency was 83±8%, 84±4%, 81% and, 76% for HAc, NaOAc, Buffer 1 and Buffer 2 PBRs, respectively, while the highest removal rates were 2.8±1, 3.8±1 and 6.7 mg/L.d, for HAc, NaOAc, Buffer 1, respectively, on Day 6. The highest P removal rate achieved by Buffer 2 PBR was 1.6 mg/L.d. In autotrophic PBR no P removal was observed during the 2nd feeding period. P removal rates were exhibited a declining trend. This decline in the removal performances in the 2nd feeding period may also be associated with microalgal cells reaching the growth limit and algal aging (Flynn, 2020).

The results of the analysis on sCOD removal efficiency and removal rate are shown in Figure 4.8.e and f. According to the results, HAc, NaOAc and Buffer PBRs demonstrated 87±6%, 80±9%, and 95±2% sCOD removal efficiencies, respectively. The highest removal rates were recorded as 475±10, 470±40, and 560±40 mg/L.d for HAc, NaOAc and Buffer PBRs, respectively, on Day 2. In the 2nd feeding period, these values were decreased to 84±4%, 72±2%, 76%and 56% for HAc, NaOAc, Buffer 1 and Buffer 2 PBRs, respectively. The highest sCOD removal rates in this period were found as 818±120, 560±110, 880±70 and 260±74 for HAc, NaOAc, Buffer 1 and Buffer 2 PBRs, respectively.

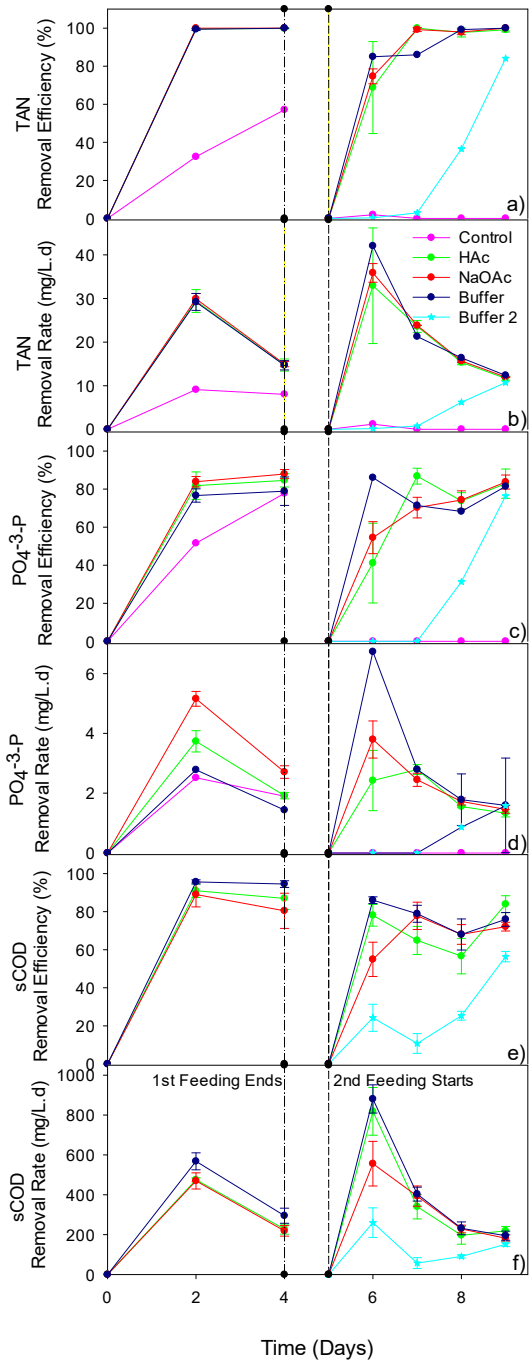

Figure 4.8. The Nutrient-Organic Removal Performances of The PBRs for Set 5 a) TAN Removal Efficiency b) TAN Removal Rate c) PO_4^{+3}-P Removal Efficiency d) PO_4^{+3}-P Removal Rate e) sCOD Removal Efficiency f) sCOD Removal Rate

To conclude, it has been found firstly in Set 5 that mixotrophic conditions significantly improve microalgal treatment and growth performance compared to the autotrophic counterpart. As mentioned earlier, one of the purposes of using a buffer solution containing equimolar HAc-NaOAc was to stabilize the system at a certain pH while providing an organic carbon source. However, low pH range may diminish microalgal growth and treatment performance compared to the ones operated with suitable pH range. In addition, the effect of this solution, which corresponds to 1000 mg/L sCOD, on pH stabilization lasts for 3 days. Therefore, it is understood that the use of this buffer solution in algal systems may not be as sustainable as manual pH stabilization. The other carbon sources, namely HAc and NaOAc were chosen to see the effect of each source in mixotrophic enrichment. It was found that the nutrient removal performances were similar. As expected, no more than about 10% difference in specific growth rate, cell doubling time and biomass production rate was observed between HAc and NaOAc PBRs. Likewise, in terms of nitrogen, phosphorus and sCOD removal efficiencies and removal rates (Figure 4.8) these PBRs had a similar trend with less than 10% difference. Therefore, this study revealed that, acetic acid (HAc) or sodium acetate (NaOAc) can be used as carbon source instead of buffer solution (HAc+NaOAc) to provide mixotrophic conditions. To make a choice HAc, was chosen since HAc PBRs were slightly ahead in terms of growth performance in PBRs.

4.3.2 Results of Set 6: Investigation of The Effect of HRT at Constant Nutrient Loading Rate on Mixotrophic Semi-Continuous PBRs

The aim of Set 6 was to reveal the effect of HRT on growth and nutrient removal performance on mixotrophic *C. Vulgaris* culture at constant loading rate. As aforementioned in Section 4.2.6.2., three semi-continuous PBRs were set, and they were initially operated in 8-day HRT for 24 days until COVID-19 measures started. Figure 4.9 and Figure 4.10 demonstrates the results of operation of these three replicates operated at 8-day HRT for 24 days.

According to the Figure 4.9.a, influent pH was kept around 6.0±0.02 and the pH of the effluent of all PBRs was stabile around 10.0±0.8 without any intervention with buffer. Figure 4.9.b, the ambient temperature was represented indicating that the operational conditions were in the optimum range for *C. Vulgaris* (22 to 28 °C). As seen in Figure 4.9.c, OD values of all PBRs displayed an increasing trend till Day 18 where they reached to steady-state. PBRs exhibited the highest 2.6±0.04, 3.1±0.7 and 2.4±0.5 OD values for MX1, MX2and MX3 PBRs, respectively, at Day 22. Specific growth rates of the PBRs were calculated as 0.51, 0.46 and 0.17 day^{-1} for MX1, MX2 and MX3 PBRs. On Day 24, a decrease in OD values was detected in all PBRs. This situation was associated with the increase in the cell mass of the cultures to a certain level (Flynn, 2020). After a certain stage, the activity of the culture decreases as the light is blocked and self-shading increases due to the intense growth. The doubling time of the PBRs were calculated as 2, 1 and 4 days for the PBRs MX1, MX2 and MX3, respectively.

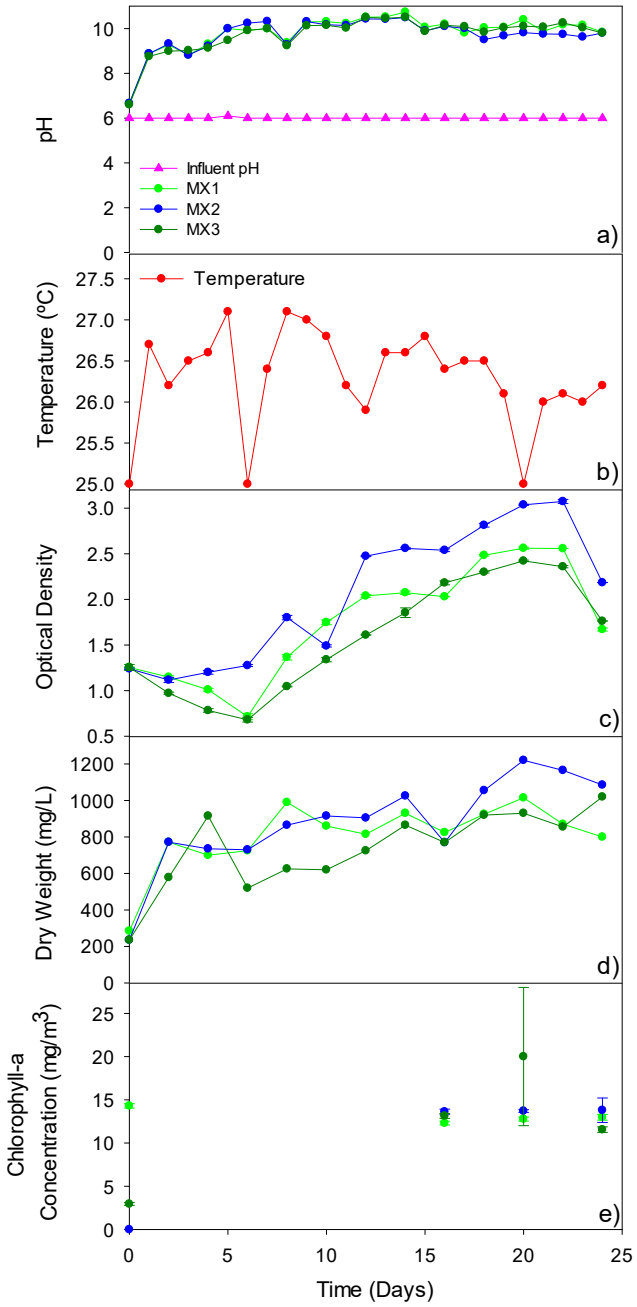

Figure 4.9. The Growth Performances of PBRs before COVID-19 Measures a) pH b) Ambient Temperature °C c) Optical Density d) Dry Weight e) Chlorophyll-a Concentration

The OD results of MX1, MX2 and MX3 were satisfactory when compared to the values observed in the literature. In a similar study performed for *C. Vulgaris* grown in mixotrophic conditions with an acetate source (approximately 1000 mg/L COD), the OD value (at 660 nm) was observed to be the highest 1.0 (Kong et al., 2011). In another study, the specific growth rates of 3 different microalgal cultures (*Neochloris oleabundans, Dunaliella sp., Botryococcus brauni*) enriched for 20 days in a medium containing 2000 mg/L glycerol (approximately 2000 mg/L sCOD) were approximately 0.19, 0.22, and 0.23 day^{-1} respectively (Choi and Lee, 2015). Accordingly, a culture of *C. Vulgaris* enriched in a medium with a concentration of 500 mg/L sCOD exhibited a comparable specific growth rate (0.17 day^{-1}).

In Figure 4.9.d, the results of the dry weight analysis performed are observed. The PBRs showed an increasing trend for dry weight values and reached to steady-state on Day 20. Accordingly, it was observed that the MX1, MX2 and MX3 PBRs reached to maximum dry weight values of 1015, 1220 and 1020 mg/L, respectively, on Day 20. These values are comparable with the literature studies. For instance, in a similar study conducted with *Chlorella pyrenoidosa* containing 1000 mg/L sCOD, the maximum dry weight reached was 200 mg/L (Gupta et al., 2017). Moreover, according to the calculations performed on the Day 20, the biomass production rates were determined as 50.7, 61.0 and 46.4 mg/L.d for MX1, MX2 and MX3. The chlorophyll results, as seen in Figure 4.9.e, showed that 13±0.3, 13.8±1.4 and 11.6±0.6 mg/m^3 chlorophyll-a were produced in MX1, MX2 and MX3.

TAN removal efficiency of the PBRs is shared in Figure 4.10.a. Accordingly, MX1, MX2 and MX3 PBRs demonstrated a constantly increasing trend for TAN removal performance and they reached to steady-state on Day 8. Almost 100% TAN removal efficiency was obtained by MX1, MX2 and MX3, respectively, on Day 8.

PO_4^{-3}-P removal efficiencies of the PBRs are demonstrated in Figure 4.10.b. According to the results shown, the MX1, MX2 and MX3 PBRs reached the steady-

state with almost 100% removal efficiency on Day 10 for MX1 and MX2, and by Day 16 for MX3.

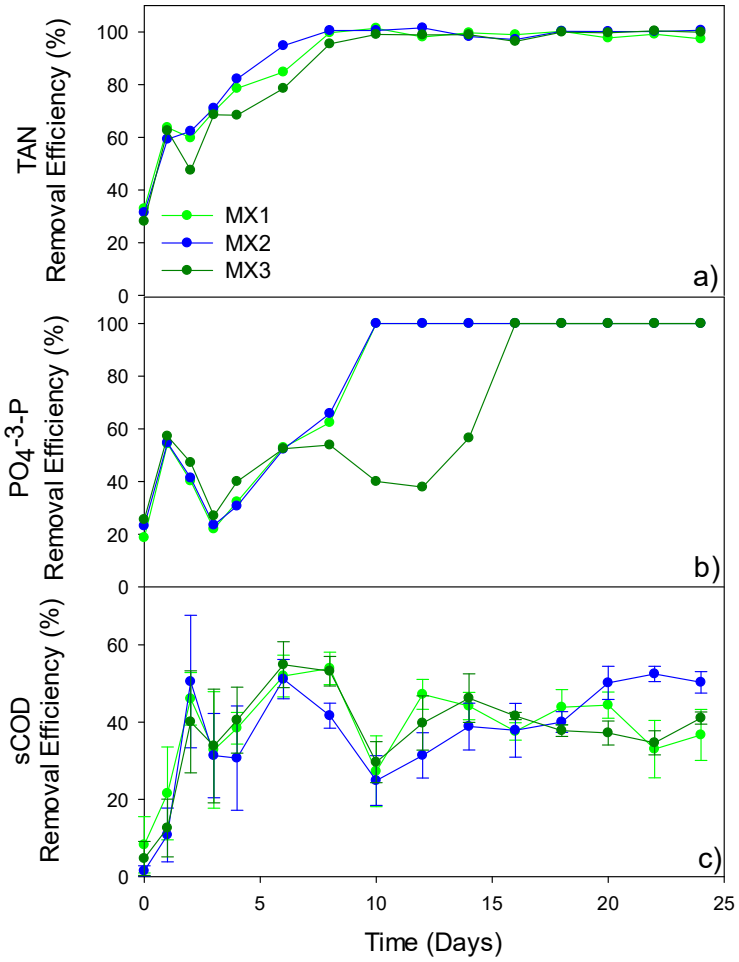

Figure 4.10. The Nutrient and Organic Removal Performances of PBRs before COVID-19 Measures a) TAN Removal Efficiencies b) PO_4^{-3}-P Removal Efficiencies c) sCOD Removal Efficiencies

In Figure 4.10.c, the sCOD removal efficiency of the PBRs are given. sCOD removal efficiencies of PBRs revealed a fluctuating trend. Yet, all reactors reached to steady-

state on Day 20. The maximum sCOD removal efficiency of the MX1, MX2 and MX3 PBRs were 44±3%, 53±2% and 46±6%, respectively, on Day 20. Contrary to TAN and P removal performance, the PBRs could only remove sCOD in 50% level. Similarly, H. Wang et al. (2012) on the microalgal treatment of dilute piggery wastewater, it was observed that the maximum sCOD removal achieved in a medium containing 500 mg/L sCOD reached approximately 36%. In this case, H. Wang et al. (2012) speculated that the sCOD removal efficiency is dependent on the initial concentration of sCOD. This speculation can also be supported by the fact that the algal culture enriched with an initial 1000 mg/L sCOD reaches 80% removal efficiency level in the previous batch experiments carried out in Set 5, Section 4.3.2. However, further research is needed to confirm these speculations. The use of the continuous illumination mode is seen as another reason for the lower sCOD removal efficiency observed in this experiment. In a study by (K. Lee and Lee, 2001) conducted on *C. Kessleri* strain enriched in mixotrophic conditions in continuous lighting and 12 h:12 h (light: dark) modes, culture enriched in 12 h:12 h (light: dark) mode showed higher sCOD removal performance. This may be since organic carbon assimilation takes place in the dark by microalgal metabolism thus higher the dark period, higher the sCOD removal. This assimilation mechanism only depends on self-shading darkness in the culture provided with continuous illumination (Kılıç, 2017). Such a situation seems to be valid for MX1, MX2 and MX3 PBRs with OD values between 2.0 and 3.0, and MX2, whose OD values are higher than the others, exhibits the highest sCOD removal efficiency.

This study, carried out up to Day 24, revealed that a mixotrophic microalgal culture with similar growth and removal performance, operated at an 8-day HRT, was enriched with a high removal performance of TAN (almost 100%) and P (almost 100%) in all three PBRs. Due to COVID-19, these PBRs were operated in such a way as to ensure the viability of microalgal cultures only.

Following the COVID-19 measures, first, the PBRs were operated with a daily feeding pattern at 8-days HRT, and then their performance was tested. After observing steady-state conditions for all PBRs, the HRTs of two of the PBRs (MX1 and MX2) were decreased to 4-day followed by 2 days. MX3, which was designed as control PBR, was operated at 8-day HRT till the end of the experiment. The second part of this study, namely operation period after COVID-19 measures, lasted 83 days (Day 1 to Day 83), till the contamination of the reactors with a rotifer as mentioned in Section 4.2.6.3. The analysis results of the reactors operated for 83 days under different HRT conditions are shown in Figure 4.11, Figure 4.12, Figure 4.13.

In Figure 4.11.b, the changes in pH of the PBRs are presented. Accordingly, pH was observed around 9.0 ±0.6 in all 8-4-2-day HRT periods without the need for any buffer solution. This is due to the acetic acid-water chemistry described in the Equation 4.10, Section 4.2.6.1. In Figure 4.11.c, ambient temperature results are shared. As can be seen, the ambient temperature fluctuates between 28 and 29 °C, which is in optimum ranges for *C. Vulgaris* growth (Daliry et al., 2017).

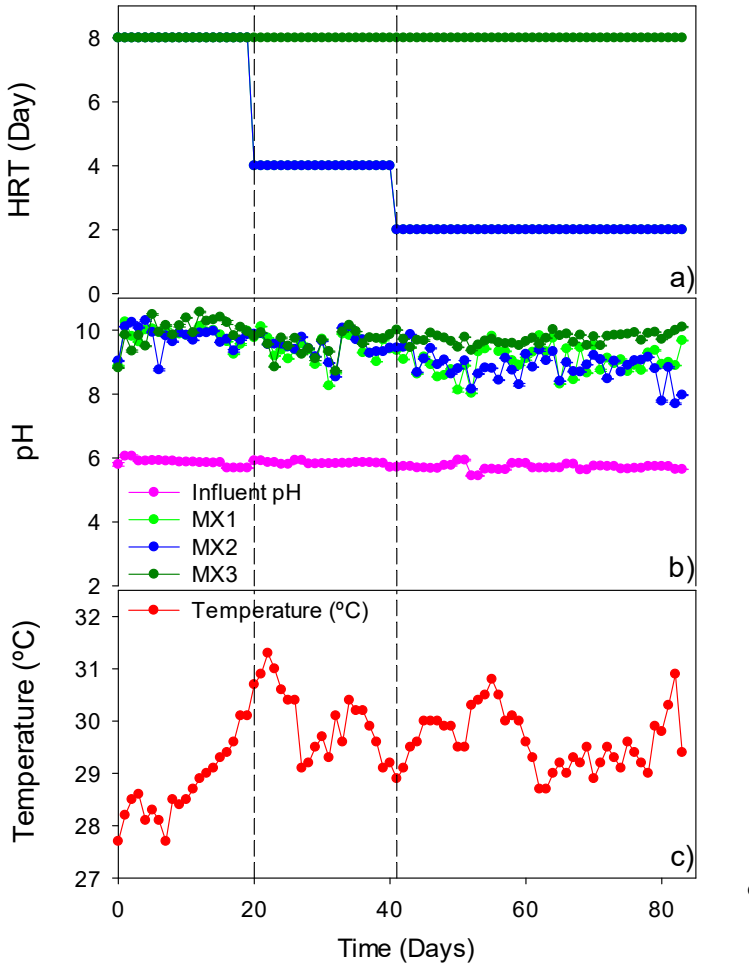

Figure 4.11. Results of Set 6 studied at different HRTs a) HRT b) pH c) Ambient Temperature °C

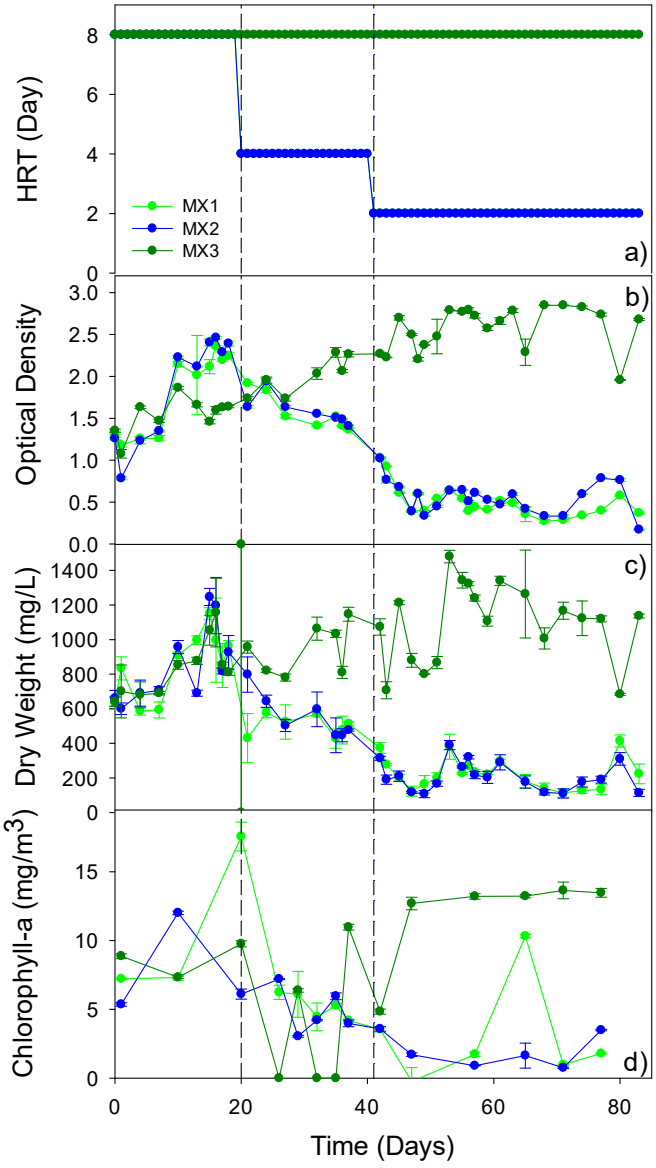

Figure 4.12. Results of Set 6 studied at different HRTs a) HRT b) Optical Density c) Dry Weight d) Chlorophyll-a Concentration

Time dependent OD changes of the PBRs are shown in Figure 4.12.b OD demonstrates a gradual increase for all PBRs for 8-day HRT operation. Maximum OD values for 8 days of HRT were observed as 2.4 ± 0.04, 2.5 ± 0.02 for MX1 and

MX2 on Day 16, respectively, while it was 1.9 ± 0.02 for MX3 on Day 10. All PBRs reached steady-state conditions on Day 16 at 8-day HRT operation period. Yet, despite the same operational conditions, the differences observed in OD of all PBRs on Day 16-20. This can be attributed to the difference in the distance of the PBRs from the light source. The specific growth rates calculated for MX1, MX2 and MX3 were 0.28, 0.21 and 0.14 day^{-1}, respectively during the 8-day HRT operation. As can be seen in the Figure 4.12.b there was a decrease in OD values of MX1 and MX2 on Day 21, after 4-day HRT operation started to be applied. It might be due to the shortening of the time the culture remained in the PBR. Accordingly, that OD value levelled down till is 1.0 ± 0.01 for MX1and MX2. For the MX3 PBR, which continued with 8-day HRT, the OD value increased and reached 2.3±0.1. During the 2-days HRT operation period, the OD values of MX1 and MX2 PBRs also fluctuated, and an average OD of 0.5 ± 0.1 was observed for both PBRs. In this situation, the culture developed a wall adhesion, most probably due to the "slow recovery" (Anbalagan et al., 2016) stress caused by the 2-days HRT operation. In daily operation, the walls of the PBRs were scraped from the adhered culture with the help of a sterile loop and OD values of those were measure. Yet, the culture was settling down, and detection of OD in the PBR became difficult.

A similar trend to OD was also observed in the dry weight results. According to Figure 4.12.c, dry weight values of all PBRs were increasing for 8-day HRT operation. The maximum dry weight values were found to be 1150±5 and1246±49 mg/L for MX1 and MX2, respectively, Day 15. For MX3, the maximum dry weight values reached was 1480±36 mg/L on Day 53. The biomass production rates for the MX1, MX2 and MX3 PBRs in HRT 8-dasy operation were calculated as 51.2, 46.2 and 15.6 mg/L.d, respectively. MX1 and MX3 reactors reached to steady-state. However, the dry weight values for MX1 and MX2 PBRs gradually decreased during 4-day and then 2-day HRT operation, but increased for the MX3 PBR, which continued to operate for HRT 8 days. As seen in Figure 4.12.d, chlorophyll-a values

were fluctuating during 8-day HRT and then 4-day HRT operation period. The maximum chlorophyll-a values were recorded as 18±1.1, 6.0±0.4 and 13.5±0.3 mg/m^3 for MX1, MX2 on Day 20 and for MX3 on Day 77, respectively. The values gradually decreased in MX1 and MX2 as the HRT was decreased to 4-and 2-day HRT. Results demonstrated that at an 8-day HRT, the cellular growth of microalgae increases with the increase of the hydraulic holding time when it comes to chlorophyll-a production (Xu et al., 2015).

TAN removal efficiencies of the PBRs are shown in Figure 4.13.b The maximum TAN removal efficiencies for all PBRs are 100±0% at 8-day HRT operation period. During 4-day HRT operation, 100±0% TAN removal performance remained stable for MX1 and MX2 PBRs until the Day 20, in which the HRT was reduced from 8 days to 4 days. On Day 20, TAN removal efficiencies of MX1 and MX2 went down till 90 ±1% level and reached to 100±0% back again on Day 26. No major fluctuation was observed in MX1, MX2 and MX3 when HRT was decreased to 2 days.

Figure 4.13.c demonstrates PO_4^{-3}-P removal efficiencies of PBRs. The maximum P removal efficiencies for all PBRs were also 100±0%, in the 8-day HRT operation period. During the 4-day and 2-day HRT operation all PBRs remained its almost 100% P removal performance.

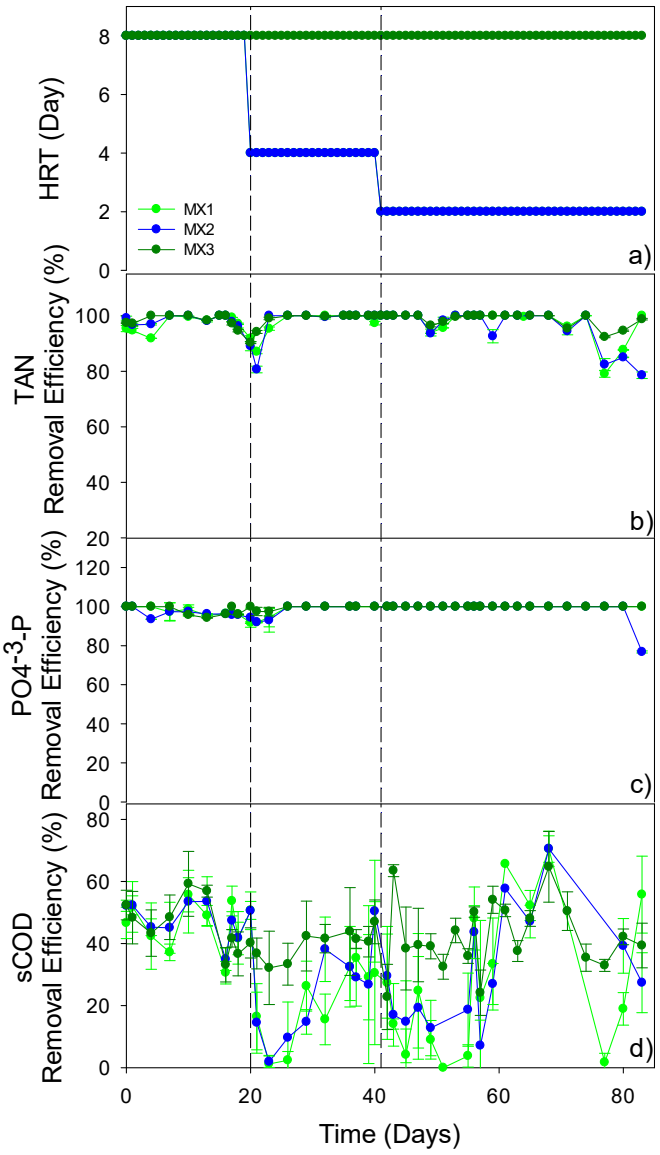

Figure 4.13. Results of Set 6 studied at different HRTs a) HRT b) TAN Removal Efficiencies c) PO4-3-P Removal Efficiencies d) sCOD Removal Efficiencies

Figure 4.13.d sCOD removal efficiencies are shown. Throughout the whole operation period, the PBRs exhibited a fluctuating pattern in sCOD removal efficiencies. The average sCOD efficiency for MX3 was recorded as 41±9 % during

8-day HRT operation (Day 1-Day 83). MX1 and MX2 exhibited average sCOD removal efficiencies of 46±8 and 48±6%, respectively, at 8-day HRT operation. At 4-day HRT operation, MX1 and MX2 showed 26±8% and 28±13% average sCOD removal efficiencies, respectively. For 2-day HRT operation, these values were 41±19% and 32±20% for MX1 and MX2, respectively. The maximum sCOD removal efficiencies were 56±6%, 54±8% and 65±11% for the MX1, MX2 and MX3 PBRs, respectively, at 8-day HRT operation period. At 4-day HRT operation, MX1 reached maximum 35±7%, and MX2 reached to 50±16% sCOD removal efficiency. At 2-day HRT operation those values were 70±9% and 47±5% for MX1 and MX2, respectively.

Kinetic Studies Performed for Set 6 (The Effect of HRT)

TAN, P and sCOD removal rates of the PBRs were also determined by the kinetic experiment that lasted for 24 hours and the effect of HRT was investigated. The data were presented in the Figure 4.14 and Figure 4.15. As can be seen in Figure 4.14, where the effect of 4- and 8-day HRT were compared, TAN and P concentrations were consumed totally in the first 3 hours in all PBRs. According to the results of this experiment, TAN removal rate of the MX1 and MX2 PBRs was 57 mg/L.d, and 56 mg/L.d, respectively. The P removal rate was 6 mg/L.d for MX1 and MX2. TAN and P removal rates of the MX3 PBR were 53 and 6 mg/L.d, respectively. sCOD removal rates were recorded as 520 mg/L.d. where sCOD removal efficiency was 40% for the MX1. MX2 exhibited sCOD removal efficiency of 20%, with a removal rate of 342 mg/L.d. MX3 demonstrated removal efficiency of 30%, while the removal rate was 432 mg/L.d.

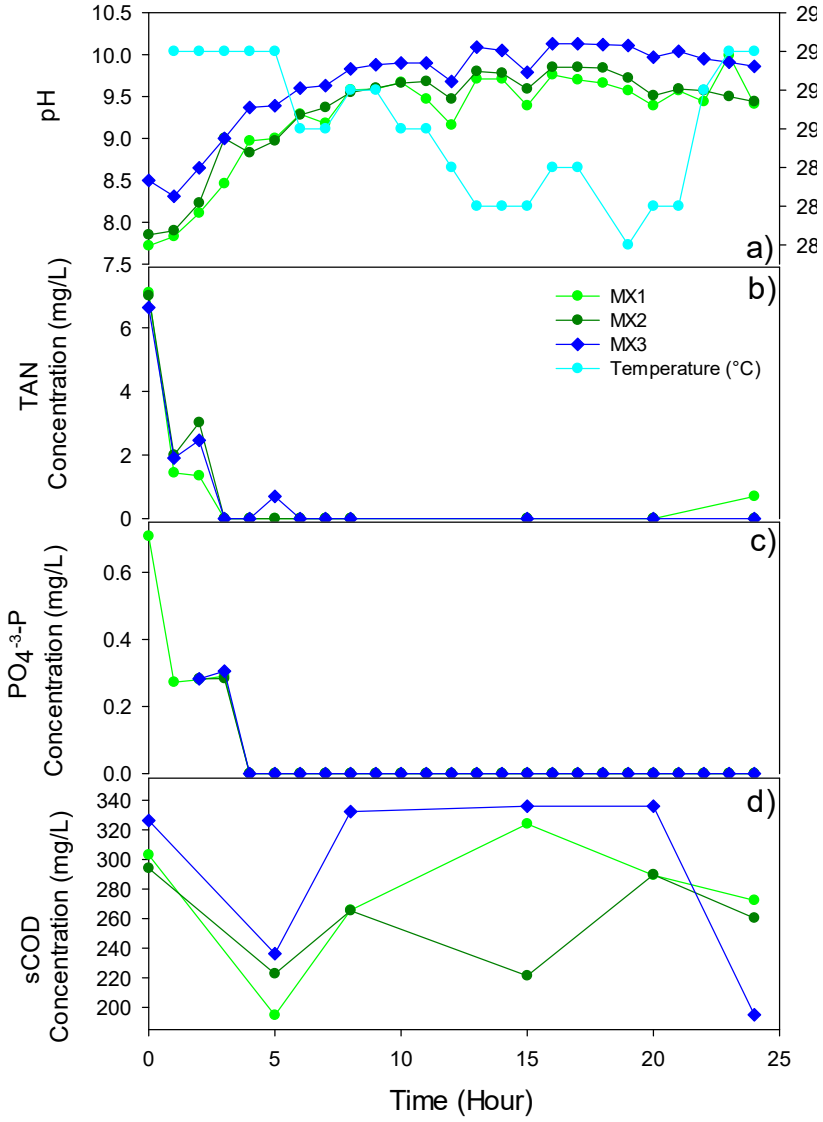

Figure 4.14. Results for kinetic studies of MX1, MX2 (4-day HRT) and MX3 (8-day HRT) a) pH-Temperature, b) TAN concentration c) PO_4^{-3}-P concentration d) sCOD concentration

The result of the kinetic study performed during 2-day HRT operation are given in Figure 4.15 in the comparison of 2-day and 8-day HRT. Accordingly, while the TAN

removal rates for the MX1 and MX2 PBRs were 21 mg/L.d, this value was found as 75 mg/L.d for the MX3 PBR. Similarly, P removal rates for MX1 and MX2 PBRs were 3.2 mg/L.d. For the MX3 PBR, this value is 6 mg/L.d. These values show the negative effect of 2-day HRT operation on the PBRs when the removal rates are compared, although the removal efficiencies for all PBRs were almost 100%. The highest sCOD removal efficiency in MX1 and MX2 PBRs were observed as 40% and 50%, respectively. sCOD removal rates were recorded as 116 and 255 mg/L.d for MX1 and MX2 respectively. The highest 30% removal efficiency was found in the MX3 PBR, the removal rate was 200 mg/L.d.

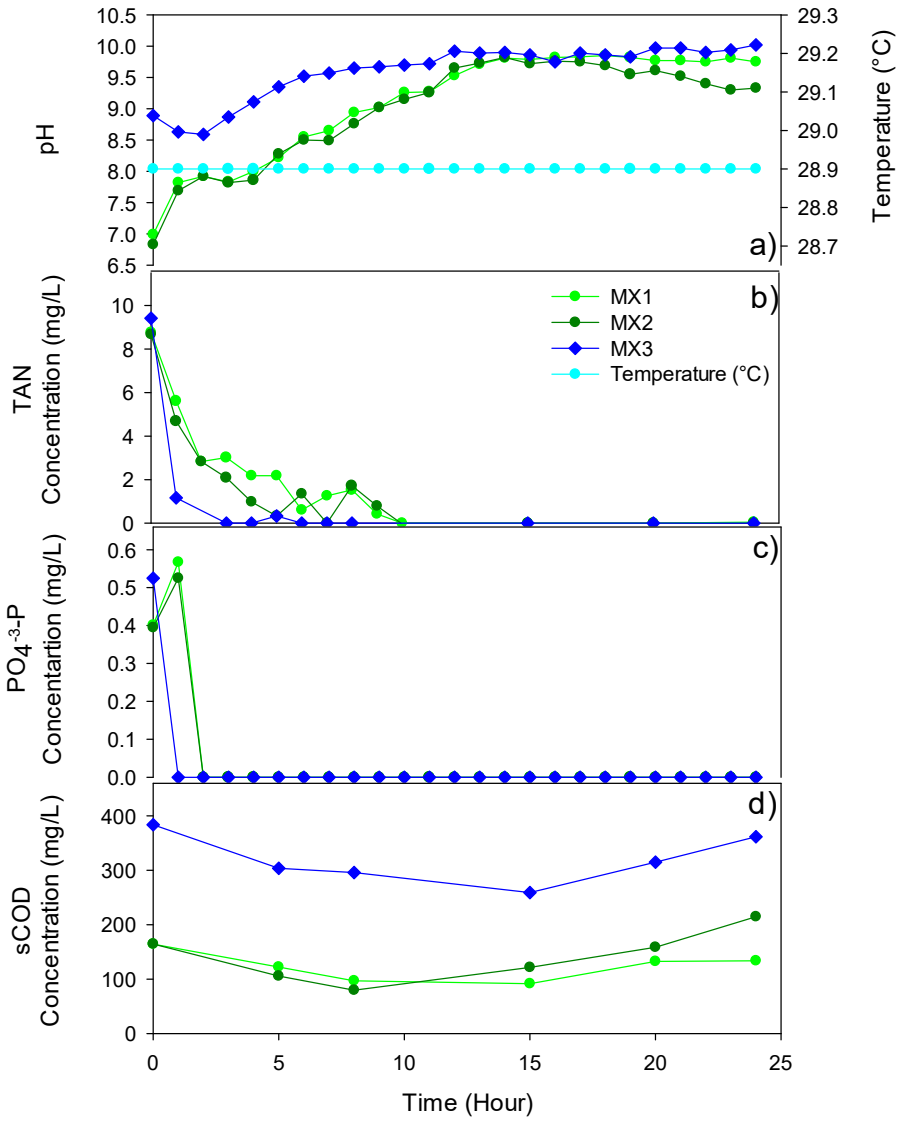

Figure 4.15. Results for kinetic studies of MX1, MX2 (2-day HRT) and MX3 (8-day HRT) a) pH-Temperature, b) TAN concentration, c) PO4-P concentration d) sCOD concentration

According to the results obtained from the both kinetic studies, at 4-day HRT operation, TAN removal rate appeared to be slightly higher when compared to those

at 8-day HRT. P removal rate were the same for both 4-day and 8-day HRT operation. Moreover, the highest sCOD removal rate was recorded in 4-day HRT operation in MX1 reactor. At 2-day HRT TAN removal rates obtained from MX1 and MX2 PBRs were three times lower than those obtained in MX3, at 8-day HRT. Moreover, the P removal rates were two times lower than those obtained in MX3, at 8-day HRT.

The negative effects of the decrease in HRT are evident in this study as well as in the literature. For example, in a similar study conducted by (Toledo-Cervantes et al., 2019), when HRT was reduced from 4 days to 2 days in a mixed algal culture, a 30% decrease in nitrogen removal efficiency and a 50% decrease in P removal efficiency were experienced. In addition, Cromar and Fallowfield, (1997) also mention the positive contribution of HRT increase to nitrogen and phosphorus treatment performance.

It should be noted that reducing the HRT to optimum levels increases the applicability of the systems in terms of time and capital cost since the lower HRT values allows the reactors to be operated with lower total volumes. Moreover, the higher HRT may lead an increase in the possibility of contamination due to the longer turnover time (i.e time required for the contamination to grow). However, to ensure the performance of algal PBRs at the optimum level, it is especially important to reduce the HRT gradually and to evaluate the effect of each HRT. The data in kinetic study reveal that 4-day HRT is optimal in terms of algal performance. Moreover, the operation of 4-day HRT ensures the flexibility of the microalgal system for further utilization as complementary treatment systems for the wastewater treatment plants where lower HRTs are usually applied (i.e. activated sludge). It should be also noted that with lower HRT microalgae acts more responsive to the alterations applied on parameters due to the less turnover time required. Furthermore, while in 8-day HRT operation the dilution ratio (12.5%) of the reactor makes the system more prone to

self-shading, the dilution ratio of the reactor at 4-day HRT operation (25%) prevents this possibility.

4.3.3 Results of Set 7: Investigation of Optimum Loading Rates in Mixotrophic Semi-Continuous PBRs at Constant HRT

The aim of Set 7 was to investigate the effect of nitrogen loading rates (NLR) and phosphorus loading rates (PLR) on mixotrophic microalgal cultures operated as constant HRT. 4-day HRT was applied as optimum HRT during the experimental period, regarding the previous mixotrophic semi-continuous PBR study (Set 6, Section 4.3.2.). After the successful enrichment of the obtained *C. Vulgaris* culture (APPENDIX J), LR1 and LR2 replicate PBRs were set and operated for 111 days. The results of OD, dry weight, pH, chlorophyll-a and TAN, PO_4^{-3}-P, sCOD removal efficiency of LR1 and LR2 PBR are shared in in Figure 4.16, Figure 4.17 and Figure 4.18.

In the scope of Set 7, NLR and PLR was increased periodically at constant 4-day HRT operation. As it was previously applied in Set 6, Section 4.3.2, initially the NLR was set for 8 mg N /L.d and PLR was set for 1 mg P /L.d. On Day 16, NLR and PLR values were tripled to 24 mg/L.d N and 3 mg/L.d P, respectively (dashed lines). As can be seen in Figure 4.16, when NLR and PLR increased 3-fold, it directly affected pH in a diminishing trend. 50 mM $NaHCO_3$ buffer was used to prevent pH decrease from Day 24. However, due to the quarantine protocols because of COVID-19 measures period on the weekends, daily feeding could not be provided uninterruptedly, and still a decrease in pH was observed in buffer deficiencies. To prevent this situation, buffer concentration doubled at those times and pH was stabilized. However, even if the pH balance was provided with the help of buffer solution, the decrease in TAN and P removal efficiency was seen as a sign that sufficient organic carbon concentration was not provided in the PBR. A study

conducted by Fica and Sims (2016) supports this speculation, where a significant decrease was observed in the TAN and P removal rates of PBRs with reduced organic load. For this reason, it was thought that the increase in nitrogen and phosphorus concentration should coincide with the total organic carbon concentration. Therefore, the amount of sCOD was nearly tripled and the OLR was increased from 62.5 mg/L.d to 200 mg/L.d on Day 36 (dashed lines) (Figure 4.18). After this intervention, an improvement was observed in the TAN removal efficiencies for both PBRs. Yet, the deterioration in P of LR1 and LR2 reactors could not been prevented with the increase in sCOD loading rate. Thus, on Day 96, NLR and PLR were reduced to 8 mg N/L.d and 1 mg P/L.d back again. OLR, on the other hand, was only halved to 100 mg/L.d to ensure organic carbon adequacy. At this point, the buffer was removed from the medium of the PBRs since it was not necessary. Although the OD and dry weight values continued to decrease, nitrogen and P removal efficiencies were almost 100% again (Figure 4.16, Figure 4.17and Figure 4.18).

In Figure 4.16.b, average pH values for both PBRs were observed as 9.2 ± 0.01 during the 8 mg/L.d NLR, 1 mg/L.d PLR and 62.5 mg/L.d OLR (as sCOD) operation. After Day 16, NLR, PLR and OLR (as sCOD) were changed to 24 mg N/L.d, 3 mg P/L.d PLR and 62.5 mg/L.d (as sCOD), respectively, pH decreased to 6.0 ± 0.01. To overcome the pH decrease, 50 mM of $NaHCO_3$ was added to the PBRs on Day 24, with daily feeding operation.

Between Day 24-96, the buffer addition maintained the pH at an average of 9.3 ± 1. After Day 96, with the change in loading rates to 24 mg N/L.d, 3 mg P/L.d PLR and 62.5 mg/L.d as sCOD, the buffer addition was removed from the operation and pH was kept at 9.8±0.5. As seen in Figure 4.16.c the ambient temperature fluctuates between 26 and 28°C during the whole operation period.

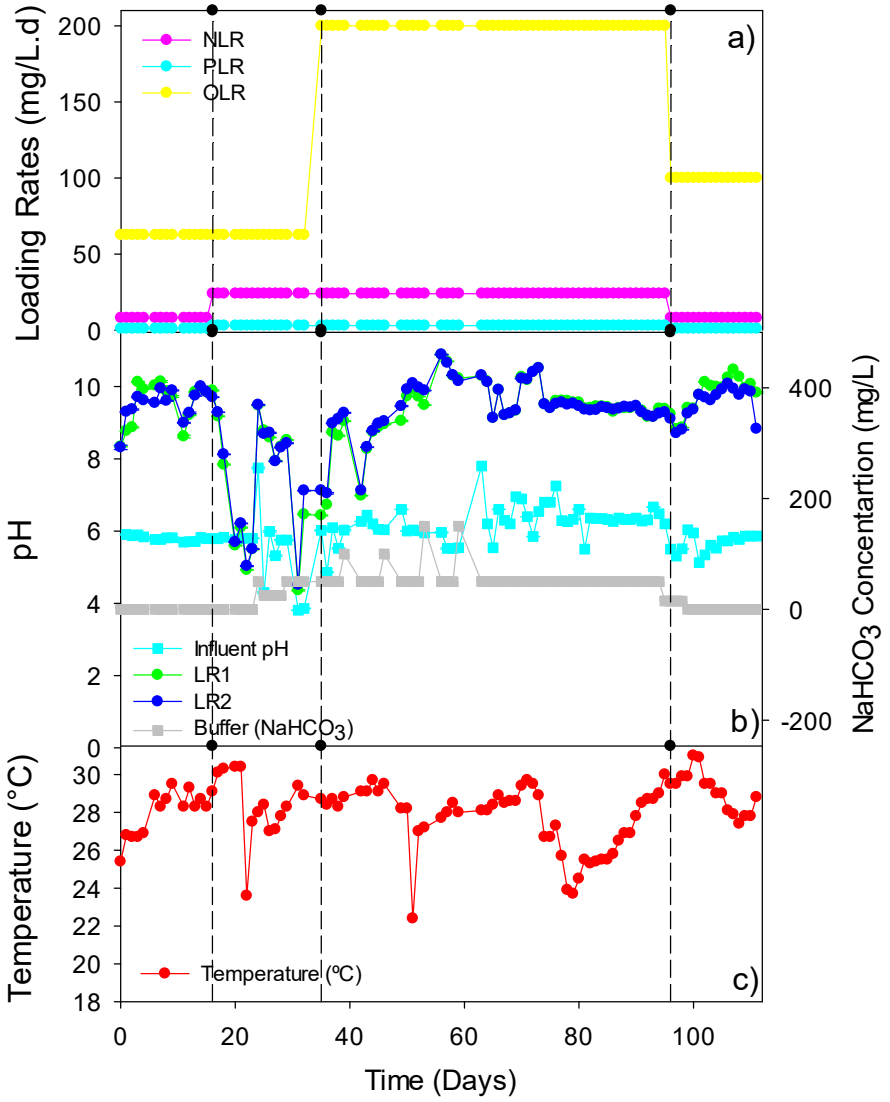

Figure 4.16. Results of Set 7 studied at different Loading Rates s a) Loading Rates b) pH c) Ambient Temperature

As it can be seen in Figure 4.17.b, Figure 4.17.c and Figure 4.17.d, an increase was observed in OD, dry weight, and chlorophyll-a values for both PBRs, in the period when NLR, PLR and OLR was set as 8 mg N/L.d, 1 mg P/L.d and 62.5 mg/L.d sCOD

(Day 1-15) and NLR, PLR and OLR was set as 24 mg N/L.d, 3 mg P/L.d and 62.5 mg/L.d sCOD as sCOD (Day 16-35). The maximum OD values reached by the PBRs were recorded as 1.4±0.0 and 1.5±0.0 for LR1 and LR2 respectively on Day15. These values reached to 2.01±0.0 for LR-1 and 2.1±0.0 for LR-2, while specific growth rates of the PBRs were calculated as 0.30 and 0.28 day^{-1} and the doubling times were calculated as 2.3 and 2.4 days for LR1 and LR2 PBRs on Day 35. These values are comparable to the ones presented in the literature. For instance, Whitton et al. (2016) determined in a similar study that the specific growth rate obtained from enrichment of *C. Vulgaris* strain was 0.17 day^{-1}. Dry weight values were recorded as 573±46 and 566±31 for LR1 and LR2, respectively, on Day 15. After OLR increased to 200 mg/L.d sCOD, biomass production rates were calculated as 21.3 and 19.1 mg/L.d for the LR1 and LR2 PBRs, respectively, with the maximum 1300±102.6 mg/L 1180±222.7 mg/L dry weight values observed when NLR, PLR and OLR was set as 24 mg N/L.d, 3 mg P/L.d and 200 mg/L.d sCOD (Day 50). After Day 53, the OD and dry weight values demonstrated a decreasing trend.

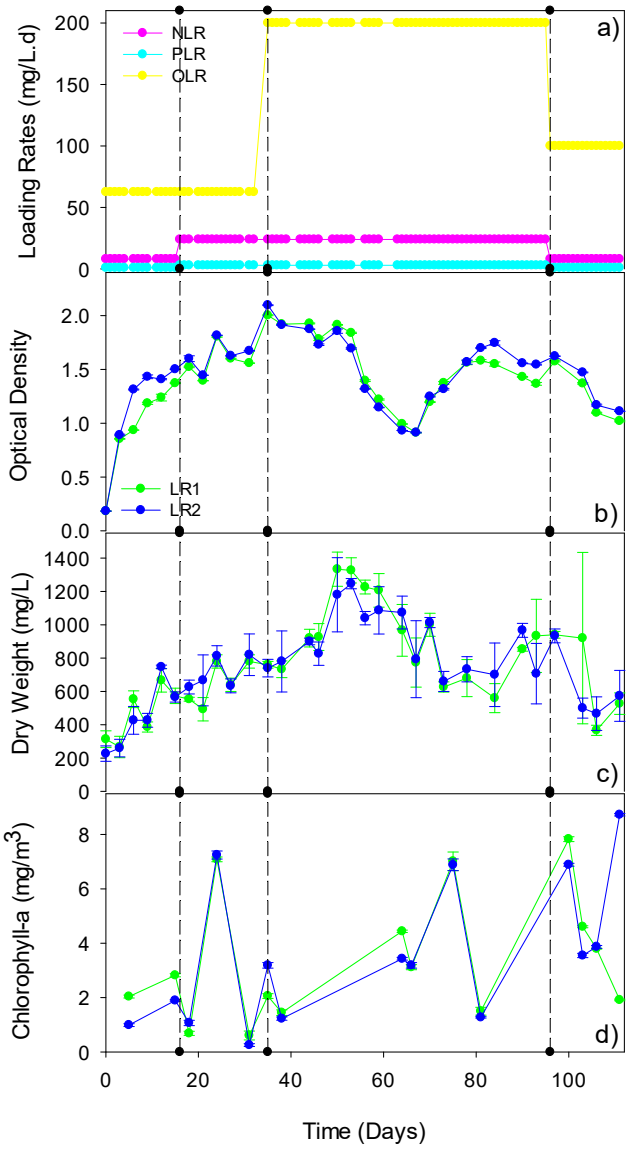

Figure 4.17. Results of Set 7 studied at different Loading Rates a) Loading Rates b) Optical Density c) Dry Weight d) Chlorophyll-a Concentration

The highest chlorophyll-a concentrations exhibited by the PBRs are 2.8±0.06 and 1.9±0.03 mg/ m^3 LR1 and LR2 PBRs, respectively on Day 15. After the NLR and PLR was tripled to 24 mg/L.d N and 3 mg/L.d P, these values reached to 7.8±0.1 and

7.2±0.2 mg/m^3 for the LR1 and LR2 PBRs, respectively (Figure 4.17.d.). These values are below the literature values (Basak et al., 2021). This situation can be attributed to the young age of the culture and the negative effects of the increase in nitrogen, phosphorus, and organic load of this culture. In addition, in a study investigating the effect of nitrogen and phosphorus load on chlorophyll-a concentration (Smith, 1982), it was revealed that similar chlorophyll-a concentrations were obtained at low phosphorus levels and that the chlorophyll content increased with the increase in phosphorus concentration. After OLR increased to 200 mg/L.d sCOD, Chlorophyll-a/pheophytin-a ratios were found as approximately 1.0 which represents the death of the culture (Dere et al., 1998), between Day 36 and Day 96. However, after Day 100 (where 8 mg/L.d NLR, 1 mg/L.d PLR and 100 mg/L.d OLR (as sCOD)), both PBRs reached to exactly chlorophyll-a/pheophytin-a ratio of 1.7 that demonstrates the healthiness of the culture. This situation might be associated with the fluctuations occurring in the PBR performance due to instability of the pH because of increased NLR and PLR. After NLR and PLR decreased to their optimized level and pH is stabilized, cell viability might have been increased.

As seen in Figure 4.18.b, in the period where 8 mg N/L.d NLR, 1 mg P/L.d PLR and 62.5 mg/L.d OLR (as sCOD) (Day 1-15) the TAN removal efficiencies of the PBRs are 99.9±0.0%. However, in the period where 24 mg N/L.d NLR, 3 mg P/L.d PLR and 62.5 mg/L.d OLR (as sCOD) (Day 16-35), TAN removal efficiencies decreased to the levels of average 68±7% and 67±% for both PBRs. On Day 36, when OLR was increased from 62.5 to 200 mg/L.d sCOD, TAN removal efficiencies reached to 81±7% for both PBRs. On Day 103, where 8 mg/L.d NLR, 1 mg/L.d PLR and 100 mg/L. OLR (as COD) were applied, almost 100% efficiency was obtained for both PBRs.

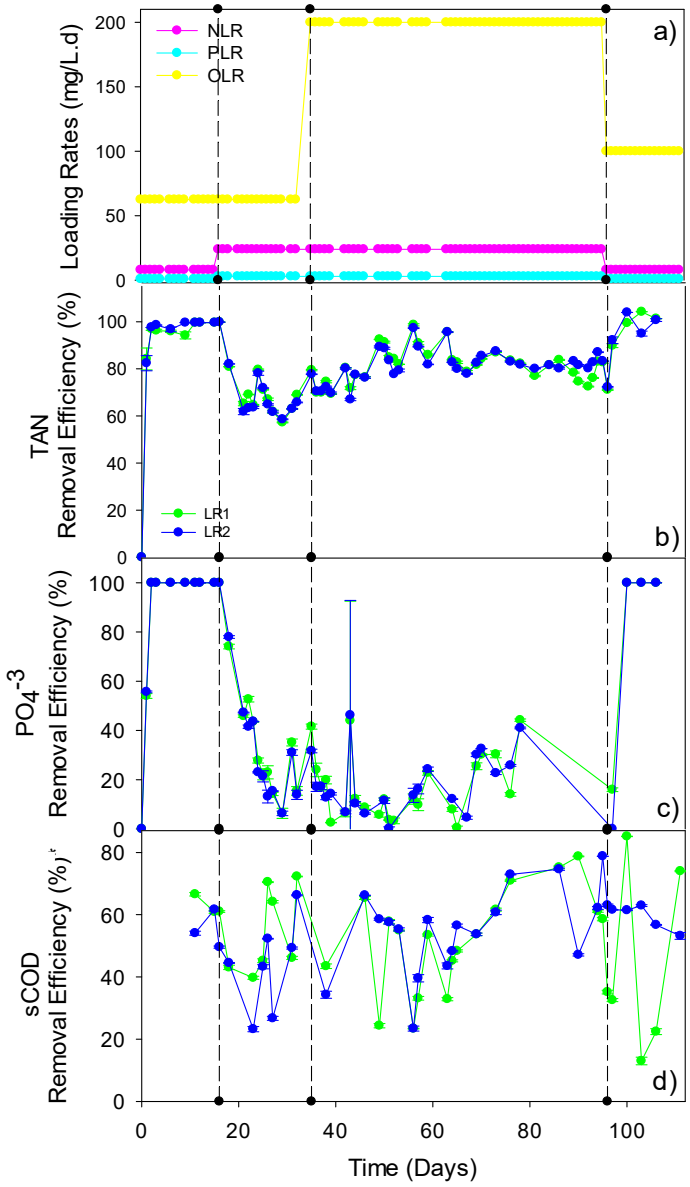

Figure 4.18. Results of Set 7 studied at different Loading Rates a) Loading Rates b) TAN Removal Efficiencies c) PO_4^{-3}-P Removal Efficiencies d) sCOD Removal Efficiencies (Calculated over TOC-COD conversion) (Section 4.2.5, Equation 4.4)

LR1 and LR2 PBRs exhibited almost 100±0% P removal efficiencies during Day 1-15 where 8 mg N/L.d NLR, 1 mg P/L.d PLR and 62.5 mg/L.d OLR (as sCOD)

(Figure 4.18.c.). When those values were set as where 24 mg N/L.d NLR, 3 mg P/L.d PLR and 62.5 mg/L.d OLR (as sCOD) (Day 16-35), P removal efficiencies started to fluctuate along with the pH values as observed in Figure 4.16.b. After that alteration P removal efficiency decreased to 6.4±2.1% for both PBRs. sCOD was increased to 200 mg/L.d OLR on Day 36 in order to fix this adverse effect. After this intervention the removal efficiencies reached up to maximum 44.2±0.5% and 41.0±0.4% for LR1 and LR2, respectively on Day 78. However, this level of P removal efficiencies was not sustained and after Day 15, average P removal efficiencies were 20±19% and 18±19% for LR1 and LR2, respectively. Moreover, P removal efficiencies went down till 0% on Day 99. After Day 100, when 8 mg N/L.d NLR, 1 mg P/L.d PLR and 100 mg/L.d OLR (as sCOD) were applied, P removal efficiencies in LR1 and LR2 reached to 100±0%.

As seen in the Figure 4.18.d., the maximum recorded sCOD removal efficiency was 66.6±0.6% and 61.6±0.5% for LR1 and LR2 PBRs respectively on Day 15. Yet, when NLR, PLR and OLR were changed to 24 mg N/l.d, 3 mg P/L.d and 62.5 mg/L.d sCOD, the fluctuation on sCOD removal efficiency did not get affected and continued through the whole operation. Moreover, when OLR was increased to 200 mg/L.d sCOD on Day 36, the average removal efficiencies were not affected. Hence no steady-state condition was observed for sCOD removal efficiency and the average sCOD removal efficiencies for LR1 and LR2 PBRs were recorded as 52.9±15.4% and 51.9±15%r, respectively for both all.

Kinetic Studies Performed for Set 7 (The Effect of LRs)

In this experiment, a kinetic study was applied to the PBRs to find the removal rates in the PBRs (Figure 4.19). TAN, P, and sCOD analyses were performed on the samples taken hourly from the PBRs. In this way, the removal rates by the PBRs in the conditions where24 mg/L.d NLR, 3 mg/L.d PLR and 200 mg/L.d OLR as sCOD

were applied, were determined. Accordingly, the highest TAN removal efficiency obtained in the LR1 and LR2 PBRs was 85% and 87%, respectively, and the TAN removal rates were 91 and 86 mg/L.d, respectively (Figure 4.19.a). As can be seen in (Figure 4.19.c) P concentration firstly increases and then decreased to the levels of maximum P removal rates of 2 and 1 mg/L.d for LR1 and LR2, respectively, which correspond to the highest P removal efficiencies of 20% and 14% for LR1 and LR2, respectively. Then P concentration increased in the 24^{th} hour again in the PBRs. Hence, overall P removal efficiency corresponds to 0% in both PBRs. In the results of kinetic study performed in Set 6, Section 4.3.2, when 8 mg/L NLR 1 mg/L PLR and 62.5 mg/L OLR were applied, the P removal efficiency were recorded as almost 100% with 6 mg/L.d. Hence it might be an indication of inhibitory effect of increased loading rates, where 24 mg/L.d NLR, 3 mg/L.d PLR and 200 mg/L.d OLR (as sCOD) were applied. For both PBRs, highest sCOD removal efficiencies were recorded as 30%, and removal rate was found as 245 mg/L.d.

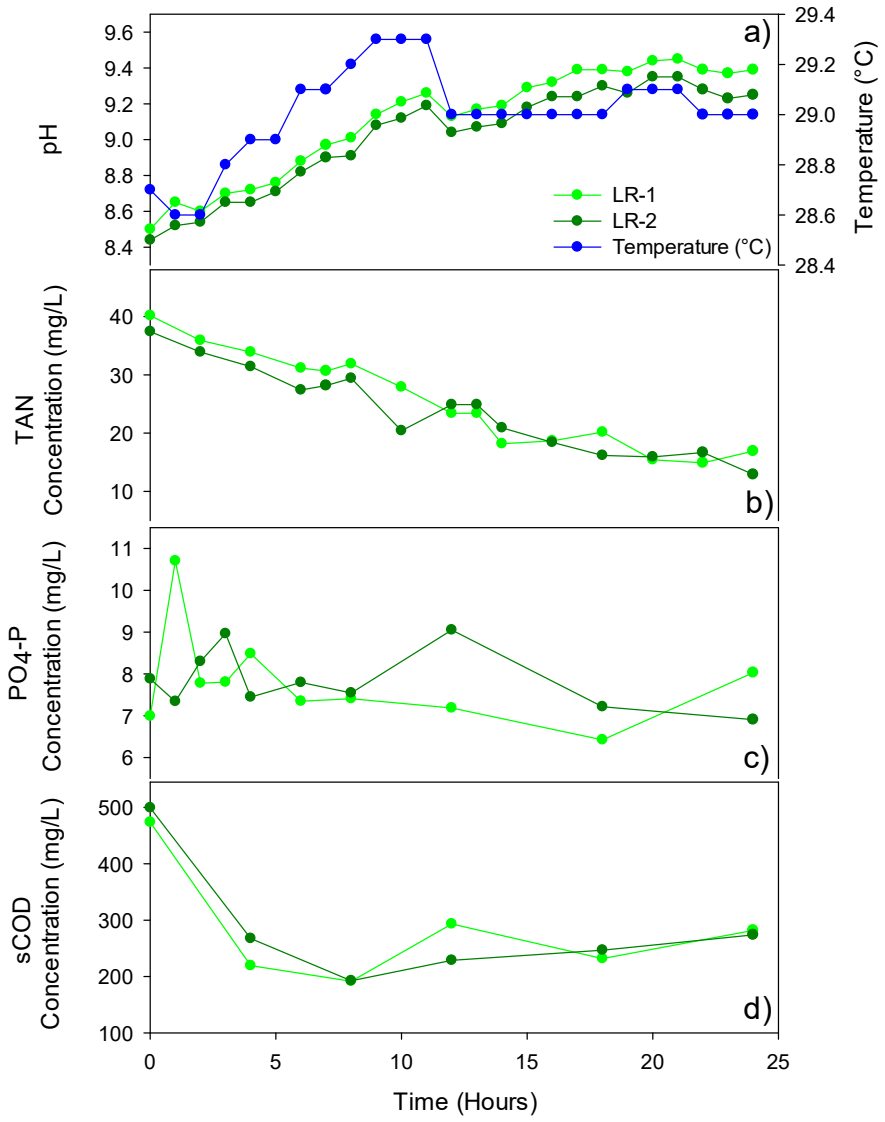

Figure 4.19. Results for kinetic studies of LR-1 and LR-2 a) pH-Temperature, b) TAN concentration c) PO_4^{-3}-P concentration d) sCOD concentration

As mentioned in Section 4.2.5.3., to understand the inorganic and organic carbon adequacy for increasing NLR and PLR, a mass balance study was performed over Equation 4.12 and Equation 4.13, on Day 70, where 24 mg/L.d NLR, 3 mg/L.d PLR

and 200 mg/L.d OLR (as sCOD) were applied, on Day 70, where 24 mg/L.d NLR, 3 mg/L.d PLR and 200 mg/L.d OLR (as sCOD) were applied. To achieve this, the TAN, P, sCOD and alkalinity removal efficiencies and rates were detected, and an elementary analysis was also performed to understand the algal culture's composition since it might not be matching with the literature. The details of the mass balance calculation are given in APPENDIX K.

$106HCO_3 + 16NH_4 + HPO_4 + 92H_2O \Longrightarrow C_{106}H_{263}O_{110}N_{16}P + 92OH + 106O_2$…….
……..……..……...(Equation 4.12)

$106CH_3COO^- + 16NH_4 + HPO_4 + 92H + 106O_2 \Longrightarrow C_{106}H_{263}O_{110}N_{16}P + 106H_2O + 106CO_2$……..…(Equation 4.13)

According to the calculations performed for the total inorganic (0.016 M) and organic carbon (0.009 M) concentrations supplied to the system on Day 70 (APPENDIX K), it was concluded that the carbon was not limited in the system for the consumption of nutrients. The NH4 concentration, which can be consumed with these nonlimiting supplied organic and inorganic carbon sources, were calculated as 3 mM. However, experimental analyses revealed that the concentration of NH4 consumed on Day 70 were 1.74 mM, which is less than the theoretically expected consumption, in the case of consumption of all supplied carbon. Hence, the amounts of alkalinity and organic carbon consumed in the PBRs were also taken into consideration. It was experimentally revealed that, considering the consumed alkalinity (HCO3) (0.0016M) together with the CO_2 gas supplied to the system (0.01 M) and consumed organic carbon (TOC) (0.004 M), the total NH4 consumed was 1.9 mM. The NH4 consumption mechanism could be revealed via autotrophic and heterotrophic mechanism as it was exhibited in APPENDIX K.

Yet, the mechanism behind the HPO4 consumption cannot be detected as per no results were acquired from elemental analysis regarding to the P percentage in the microalgae composition.

From the experimental results and mass balances performed over Equation 4.12 and Equation 4.13, it can be interpreted that autotrophic mechanism was the dominant mechanism as it consumes more nitrogen compared to the heterotrophic one (APPENDIX K). This might be reasonable due to the operational conditions which were suitable for autotrophic conditions as the illumination period was chosen as continuous. As Park et al. (2021) stated, light conditions applied in PBRs play an important role in determining the contribution of autotrophy and heterotrophy during a mixotrophic culture. Constant illumination might have created an adequate time to photoautotrophic mechanism process to dominate the system. On the other hand, the heterotrophic mechanism, which is the dark respiration in microalgal systems, is limited to the self-shading in the PBR where the HRT was 4 days.

Overall, increase in NLR and PLR caused a possible inhibitory effect on nutrient removal performance as well as growth performance. The TAN and P removal efficiencies were up to 100% where 8 mg/L.d NLR, 1 mg/L.d PLR and 62.5 mg/L.d OLR were applied (Day 1-Day 15). However, when loading rates were increased to 24 mg/L.d NLR, 3 mg/L.d PLR and 62.5 mg/L.d OLR, on Day 16, a deterioration was observed in both TAN and P removal efficiencies. On the other hand, OD and dry weight values were followed an increasing trend. To prevent this decreasing trend in TAN and P removal efficiencies, on Day 36 OLR was increased to 200 mg/L.d. With this intervention, TAN removal efficiencies demonstrated a slight improvement. However, P removal efficiencies could not be recovered and went down till 0%. Moreover, OD and dry weight trends started to decrease after Day 53. In a study conducted by (Fernandes et al., 2017), it was stated that a decrease in the

removal performance was observed with the increase of N and P load. Thus, the loading rates were decreased to 8 mg/L.d NLR, 1 mg/L.d PLR and 100 mg/L.d OLR.

4.4 CONCLUSION

The purpose of this study was initially to enrich a mixotrophic *C. Vulgaris* culture. The optimal HRT, NLR, PLR, and OLR under mixotrophic conditions were also aimed to be researched and determined. The result of this Chapter 4 can be summarized as follows,

- The N and P inlet concentrations and loading rates are determining the removal performance of microalgae. When the 120 mg/L N and 15 mg/L P inlet concentrations were halved to 64 mg/L N and 8 mg/L P, N and P removal efficiencies reached almost 100%.
- Mixotrophic growth conditions proved to be better for algal culture in both growth performance and treatment efficiencies, compared to the autotrophic growth conditions.
- Optimization of HRT reveals its importance being one of the most important parameters that determines the nutrient removal efficiencies and growth performance of *C.Vulgaris* with an optimum stress and less wash-out possibilities.
- The optimum HRT was determined as 4 days not only because it showed complete removal efficiency, but also being more applicable for the systems where microalgae can be further used as complementary (i.e. lower reactor volumes, less time for response to alterations). Moreover, it decreases the possibility of any problems that may emerge from self-shading.

- NLR, PLR, and OLR was increased to the limits where *C. Vulgaris* shall achieve a certain level of nutrient removal performances, and the limits were defined. According to the results, 8 mg/L NLR, 1 mg/L PLR and 62.5 mg/L OLR (as sCOD) were determined to be the optimum conditions for *C. Vulgaris.* This corresponds to influent TAN, P and sCOD concentrations of 32 mg/L, 4 mg/L and 250 mg/L, respectively.
- Increasing NLR and PLR in mixotrophic conditions proved the difference in contribution of autotrophic and mixotrophic mechanisms. In this set, it is shown that autotrophic mechanism is dominant.

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CHAPTER 5

INVESTIGATION OF THE USE OF *CHLORELLA VULGARIS* IN TREATMENT OF ANAEROBIC DIGESTATE

5.1 INTRODUCTION

Microalgae is a promising and environmentally friendly alternative for conventional biological wastewater treatment systems, being a renewable source for biomass and a feasible method for CO_2 fixation (Oswald, 1988; Singh and Saxena, 2015; Almomani et al., 2019). Usage of mixotrophic microalgae in wastewater treatment system allows both organic and inorganic carbon removal along with nitrogen and phosphorus reduction which allows an environmentally safe water discharge. Moreover, photosynthetic activity of the microalgae procures the necessary oxygen for heterotrophic bacteria to biodegrade carbonaceous content of the wastewater. Additionally, microalgae have demonstrated to be a potential source of energy thanks to its value-added biomass that can be utilised for biofuel production (Arun et al., 2020).

Another advantage of microalgal systems is that the algal cells can utilise both organic nitrogen (such as urea) and inorganic nitrogen (in the form of ammonia/ammonium), as well as nitrite and nitrate (Ross et al., 2018). Furthermore, the assimilation of both nitrogen and phosphorus molecules can occur simultaneously via photosynthesis. This phenomenon reduces the complexity of the treatment process (Gouveia et al., 2016; Sturm and Lamer, 2011; Masojídek et al., 2013).

Microalgal treatment processes create a potential to decrease greenhouse gas emissions due to CO_2 sequestration property of the algal systems. A study conducted by Kohlheb et al. (2020) exhibited that high rated algal ponds can contribute to CO_2 sequestration and eutrophication potentials with 458.27×10^{-3} kg CO_2 equiv./m^3 and 158.01×10^{-6} kg PO_4 equiv./m^3 respectively. Moreover, several studies have found that microalgae and associated microorganisms in wastewater treatment produce negligible N_2O emissions (Fagerstone et al., 2011; Guieysse et al., 2013). According to a study conducted by Alcántara et al. (2015), microalgae wastewater treatment system has an emission factor of 0.0047 percent g N_2O-N g^{-1}N-input. Since this is the case, throughout the years, many researchers conducted wastewater treatment studies with various algal species in real wastewaters. As seen in Table 5.1, most of the studies reached up to over 80-85% nutrient and organic removal efficiencies that is a clear demonstration of a possibility for safe water discharge activity and sanitary systems.

Table 5.1. Microalgae cultivation in various wastewaters and resultant nutrient removal performances

Microalgal Species	Wastewater Type	Parameter	Removal Efficiency (%)	References
C. Vulgaris	Agro-industrial Wastewater with by-products	$NO_2^- + NO_3^-$ Total-P sCOD	100 100 73.6	(Melo et al., 2018)
C. Vulgaris	Primary Settled Municipal Wastewater	NH3 sCOD PO_4^{3-}(from 3.2 mg/L to 0.1 mg/L)	91 67 98	(Evans et al., 2017)
Scenedesmus sp. *Chlorella sp.* *Chlamydomonas sp.* *S. Dimorphous*	Palm Oil Mill Wastewater	COD BOD Ammonia-N Phosphorus	86 86.5 99.5 98.8	(Mohd Udaiyappan et al., 2017)
C. Vulgaris	Rubber Wastewater	COD TKN	93.4 79.3	
Spirulina	Starch Processing Wastewater	COD Ammonium-N PO4-P	94 93 99	
Chlorella sp.	Industrial WW	Mn+2	84.9	
C. Vulgaris	Domestic Wastewater	Total-N Total-P	99.6 91.2	(Calicioglu and Demirer, 2019)
C. Vulgaris	Municipal Wastewater	Total-N Total-P	80 100	(Znad et al., 2012)

In addition to the mainstream wastewater, microalgae have also demonstrated prospering results in treating digester effluents as in many examples presented in Table 5.2 such as food waste, agricultural waste, industrial waste, and many others (Bauer et al., 2021). It was revealed that, due to the effluent characteristics of digestates, microalgal systems seem to have better performance in terms of its biomass production rate, nutrient utilization rate, lipid production, carbohydrate, and protein content (Koutra et al., 2018; Veronesi et al., 2015; Zuliani et al., 2016) compared to synthetic growth mediums. Additionally, in the literature, it is also revealed that the high nutrient removals, like 90-100 % for NH_4-N and 80-100 % PO_4-P, were obtained in piggery manure (Li et al., 2014) cattle manure (Franchino et al., 2013) and laying hen (Ülgüdür et al., 2019) digestates.

There are ample reasons why digester effluents are beneficial for microalgal biomass production and nutrient removal. Firstly, digester effluents are full of nutrients (N and P) and organics which are required to supplement microalgal enrichment (Uggetti et al., 2014). Secondly, the benefits of the utilization of microalgal species in such wastewaters comes from the harmony of the microalgal-bacterial coexistence. Many studies showed that microalgae and bacteria have a synergetic effect on each other's physiology and metabolism. Although the interaction of microalgae with bacteria is often regarded as a contamination during commercialization, recent researches demonstrated that bacteria promote microalgal growth in many ways (Ramanan et al., 2016). For instance, microbial consortium produces CO_2 inducing microalgae’s photosynthetic activity. Moreover, the bacterial consortium secretes promoting agents, such as thiamine metabolites, cobalamin, biotin, and a well-known plant growth hormone Indole-3 Acetic Acid that enhances microalgal growth (Higgins et al., 2018, 2016; Qi et al., 2017). Plant growth promoting bacteria (PGPB), commonly found in digestates and manure, were found to be affiliated with plant growth facilitating activities (Qi et al., 2017; Kumsiri et al., 2018). This order is also claimed to have the antifungal property that prevents

fungal contamination on microalgae, as well as secretion of phosphate solubilizing agents and siderophores which are iron-chelating substances that can prevent microalgae to be inhibited by those (Passari et al., 2015).

Many species belong to "plant growth promoting bacteria (PGPB)" group, such as *Pseudomonas, Bacillus, Enterobacter*, and *Erwinia* which were focused by the researchers (Kumsiri et al., 2018; Qi et al., 2017). Especially, in recent years, a species called *Pseudomonas Putida* was studied together with various microalgae species specifically on its growth promoting properties and co-culture removal performance (González-González and De-Bashan, 2021; Mujtaba et al., 2015; Qi et al., 2017; Wirth et al., 2020). As a bacterial partner of microalgae, *Pseudomonas Putida* has its own fame in promoting the overall performance of microalgal systems. However, not only bacterial partners but also the lignocellulosic substances in the digester effluents play a role in promoting microalgal activity. The lignocellulosic compounds are composed of hemi-cellulose, cellulose, and lignin. Further hydrolysis of these compounds into acetates, sugars and phenolics, respectively, enhances their bioavailability for microalgal growth (Miazek et al., 2014). In a study conducted by Choi and Lee (2019) suggests that when corncob extract was used as an external carbon source in a *C. Vulgaris* culture, the biomass could be harvested more than four times compared to the *C. Vulgaris* culture enriched in a regular growth medium.

Table 5.2. Microalgae cultivation on anaerobic digestates and resultant growth and nutrient removal performances

Microalgal Species	**Digestate Origin**	**Pre-treatment**	**Operation**	**Biomass Cultivation**	**Removal Performance**	**References**
C. Vulgaris	Dairy Manure	Dilution	Semi continuous (30 d) with 2% CO_2	1.3 g/L	TAN 100% TP 89.2% COD 55.4%	L. Wang et al., 2010
C. Vulgaris - nitrifying - denitrifying activated sludge	Vinasse	Dilution	Continuous (175 d) with synthetic biogas sparging	0.6 g/L	TN 37% TP 71% COD 51%	Serejo et al., 2015
C. Vulgaris, *N. oleoabundans,* *S. obliquus*	Cattle slurry and raw cheese whey	Dilution	Batch (21 d) in CO_2 incubator	0.26 g/L.d	TAN 99.9% PO4-P 97.3%	Franchino et al., 2013
Nannochloropsis salina	Municipal wastewater	Dilution	Batch (10 d) with air sparging	0.092 g/L.d 0.92 g/L	TN 100% TP 100%	Cai et al., 2013
Scenedesmus dimorphus	Diluted food waste and animal manure	Dilution	Batch	0.042 g/L.d	TN *72% TP* 100%	Abu Hajar et al., 2017
Chlorella sp. *Scenedesmus bijuga*	Poultry litter	Centrifugation, Dilution	Batch (12 d)	0.076 g/L.d 0.612 g/L	TN 60% TP 80%	Singh et al., 2011
C. Vulgaris, *A. platensis*	Agricultural Biogas Plant	Centrifugation, Dilution	Batch (14 d) with air sparging	2.49 g/L 32.99 g/L	TN* 81% TP* 94%	Kisielewska et al., 2021
Chlorella sp.	Food waste	Centrifugation, Dilution	Batch	-	PO4-P 72% TAN 100%	Yu et al., 2019

*The highest results among others

Overall, digester effluents have many advantages to be chosen as a source for a microalgal-bacterial co-culture, to achieve better nutrient removal and growth performances for microalgae. Moreover, as Xia and Murphy (2016) suggest, complementary systems with digesters and microalgal systems should be encouraged to be engineered further due to the advantages of greenhouse gas emission reduction and further nutrient removal.

Therefore, the focus of this study is to offer an alternative side stream treatment following digesters, that improves nutrient removal, provides a sustainable value-added product, and allows for CO_2 sequestration within the plant. For this purpose, two different anaerobic digestates obtained from Çelik-Çağlar's study (2021) were subjected to microalgal treatment creating two different microalgae-bacteria mixed cultures. Those two anaerobic digestates differ in their corncob type they contained one being pretreated and the other not being pretreated (Çelik-Çağlar, 2021). Hence the aim of this study can be listed as follows:

- To investigate the nutrient removal performance in microalgal treatment of anaerobic digestate.
- To observe microalgal-bacterial consortium interaction and overall performance improvement in microalgal growth and nutrient removal performance due to the existence of bacterial consortium.
- Pretreatment of corncob results in increase in soluble COD content. Therefore, the digestate containing pretreated corncob might have been enriched better in terms of plant promoting bacteria (i.e. P. Putida) which might improve the microalgal-bacterial consortium. Thus, in this study it was also aimed to observe the effect of pretreated and unpretreated corncob fed ADs would affect microalgal-bacterial consortium performance in terms of growth and nutrient removal.

5.2 MATERIALS AND METHOD

The nutrient removal performance in microalgal treatment of anaerobic digestates and the interaction of microalgal-bacterial consortium was aimed to be investigated. As mentioned earlier in Section 5.1, the overall nutrient removal and growth performance were expected to be enhanced with microalgae-bacteria co-existence. Moreover, the effect of pretreated and unpretreated corncob fed ADs on microalgal-bacterial consortium was also subjected to the investigation. Hence, 30 PBRs were set in batch mode with two sequential stages where two different nutrient and organic carbon loadings were applied. The PBRs were designed to compare microalgae-bacterial consortium with the solely bacterial consortium coming from those ADs and the solely microalgal culture. Moreover, to distinguish algal activity, PBRs were designed to be compared under light and dark conditions simultaneously. The inoculum, substrates, and operational conditions utilized in these studies are explained in detail below.

5.2.1 Inoculum

An axenic *C. Vulgaris* culture had been obtained from previous experiment mentioned in Chapter 4, Section 4.3.3. of this thesis. The culture had been operated in a semi-continuous PBR with 4-day HRT, N:P of 8 g/g and NLR of 8 mg/L.d and PLR of 1 mg/L.d. The TAN and PO^{-3}_4-P removal efficiencies of the culture reached up to 100% while organic carbon (TOC) removal efficiencies were approximately 50% (on average). The optical density of the microalgal culture was measured 1.68±0.0 at the time of use for the experiments conducted in Chapter 5. A microscopic observation was realised to confirm that the culture was axenic and free from contamination (Figure 5.1). The initial concentrations of the environmental parameters in *C. Vulgaris* solution used for the experiments are given inTable 5.3.

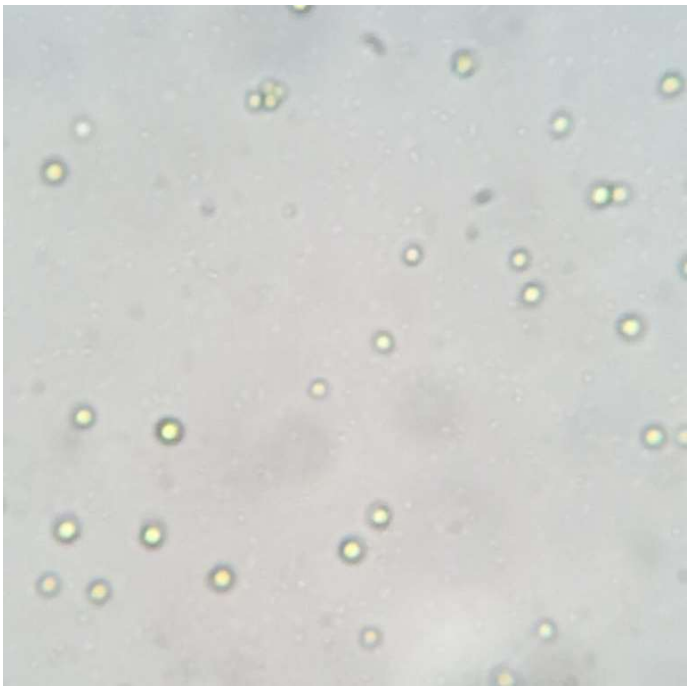

Figure 5.1. *C. Vulgaris* Cells (40 X Magnification)

Table 5.3. The initial concentrations of the environmental parameters in *C. Vulgaris* solution.

Parameter	**Result**
pH	8.90±0.01
TS (g/L)	2.7±0.4
VS (g/L)	0.6±0.04
TOC (mg/L)	111.2±0.11
tCOD (mg/L)	3483±7
TAN (mg/L)	1.8±0.02
NO2-N	1.2±0.3
NO3-N	1.6±0.01
TN (mg/L)	300±1.4
PO4-P (mg/L)	0.00
TP (mg/L)	9.0±0.04
OD (680 nm)	1.7±0.0

5.2.2 Original Wastewater

As original wastewater in this study, two anaerobic digestates (AD) were obtained from a study conducted by Çelik-Çağlar, (2021). Both lab-scale digester PBRs had anaerobic digestate taken from Afyon Energy Plant. The digesters of the Afyon Energy Plant were fed with chicken manure (83%) and poppy seeds (17%) (Çelik-Çağlar, 2021). Afyon Energy Plant has two digesters. The first digester intakes chicken manure and poppy seeds that later feeds the second digester (Çelik-Çağlar, 2021), The first digester effluent had been utilized as co-substrates in Çelik-Çağlar's study. As explained schematically in Figure 5.2, The two lab-scale digesters were additionally fed with unpretreated and pretreated (Hydrothermal pretreatment at 150 °C) corncob. The effluent of these two digesters, operated in Çelik-Çağlar's study, were subjected to be treated in the study, Chapter 5. Hence, in following sections the anaerobic digestates coming from Çelik-Çağlar's study will be named as UPCD (unpretreated corncob anaerobic digestate) and PCD (pretreated corncob anaerobic digestate) (Table 5.4).

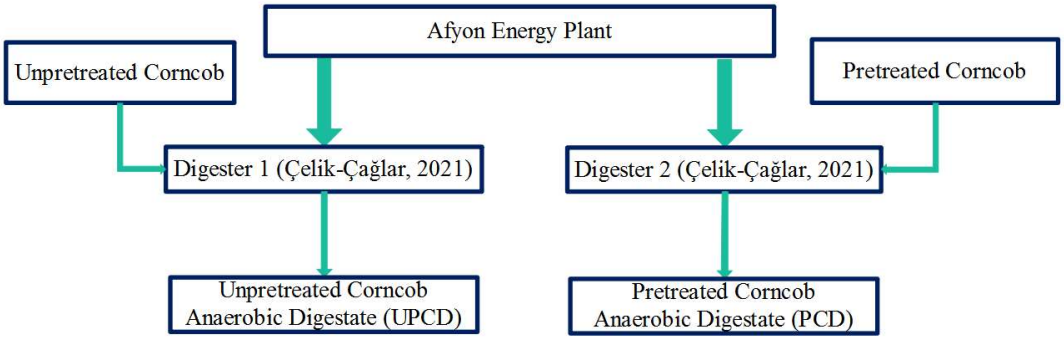

Figure 5.2. The schematic diagram of Çelik-Çağlar's study and Unpretreated Corncob Digestate (UCPCD)-Pretreated Corncob Digestate (PCD)

Table 5.4. The two digestate produced in Çelik-Çağlar's study (2021) and the operational conditions of the two related reactors

Anaerobic Digestates produced	**Inoculum**	**Co-Substrate**	**Substrate**	**Operational Conditions**
Un-pretreated corncob fed-AD (UPCD)	Ankara Central Municipal Wastewater Treatment Plant Digesters	First Digester-Afyon Energy Plant (Chicken manure (83%) and Poppy seeds (17%))	Raw Corncob	HRT 15 -10 day OLR (2, 3 and 4.5 g VS/L.d)
Pretreated corncob fed-AD (PCD)	Ankara Central Municipal Wastewater Treatment Plant Digesters	First Digester-Afyon Energy Plant (Chicken manure (83%) and Poppy seeds (17%))	HP_150 Pretreated CC	HRT 15 -10 day OLR (2, 3 and 4.5 g VS/L.d)

The anaerobic digestate is a great source of many "plant growth promoting bacteria" (Kumsiri et al., 2018) that can assure better conditions for a microalgal-bacterial co-culture. Moreover, according to the results obtained in Çelik-Çağlar's study (2021) pretreated and unpretreated corncob had different composition of cellulose, hemicellulose, and lignin. For instance, in pretreated corncob soluble portion of lignin was higher, while in unpretreated corncob cellulose and hemicellulose content was found to be higher. This fact may lead a variation between two habitats in the digestates and hence, in the microalgal-bacterial consortium. Moreover, the hydrolysation compounds might be different in those two PBRs (sugars, acetic acid, phenolics etc.) (Miazek et al., 2014). Thus, the microalgal-bacterial co-culture will be affected differently in each PBR.

Henceforth, in this thesis, both anaerobic digestates (UPCD and PCD) were studied with axenic microalgal culture. Herewith, both anaerobic digestates were put into investigation to reveal the effect of pretreated and unpretreated corncob on growth and nutrient removal performance on microalgal-bacterial co-culture. Many studies related to chicken manure digestate treatment via microalgae and corn-cob treatment

were presented in the literature (Altunoz et al., 2017; Choi and Lee, 2019; Duangjan et al., 2016; X. Han et al., 2017; Kumsiri et al., 2018; Malolan et al., 2020). However, this study is the first to combine all three substrates (chicken manure, poppy seeds, and corncob digestate) for microalgal-bacterial co-culture enrichment while assessing the nutrient removal and microalgal growth performance.

The two digestates were obtained periodically from Çelik-Çağlar's study and stored at -20°C. The digestates were collected at different operational periods (Table 5.4) and were mixed to achieve a well homogenous content to be used further in this study. The characterization of the two well homogenized digestates, namely unpretreated corncob digestate (UPCD) and pretreated corncob digestate (PCD), are given in Table 5.5. The digestates were used as both substrate and inoculum as the bacterial consortium coming from the digestates were expected to emerge a co-culture with microalgae.

Table 5.5. Characterization results of the two digestates

Parameter	UPCD	PCD
pH	7.7±0.01	7.7±0.01
TS (g/L)	29±4.2	19±2.5
VS (g/L)	17±3.2	10±2.2
TOC (mg/L)	889±0.4	1196±1.0
tCOD (mg/L)	18162±42	16313±74
TAN (mg/L)	507±0	498±0
NO2-N (mg/L)	6.4	6.0
NO3-N (mg/L)	3.4	3.5
TN (mg/L)	1068.3±0.2	1061.2±1.0
PO4-P (mg/L)	36.4	37.3
TP (mg/L)	520.0±0	607.5±0.1

5.2.3 Photobioreactors (PBRs)

In this study, the anaerobic digestates coming from Çelik-Çağlar's study, namely UPCD (unpretreated corncob anaerobic digestate) and PCD (pretreated corncob anaerobic digestate) (Hydrothermal pre-treatment at 150ºC) were the inoculum/substrate sources for co-cultures, while microalgal culture was set for inoculum as well.

In total, 30 batch PBRs were used, which can be categorized as 10 different groups of triplicates (Table 5.6). These groups cover two test and three control groups. Each group also covers a triplicate of light and dark version, that was either subjected to light or dark conditions to investigate the effect of illumination. Hence, the test groups for co-cultures contain UPCD or PCD and *C. Vulgaris* culture (abbreviated in this study as "MA") with a ratio of 1:1 (v/v). These PBRs were named as "UPCD+ MA Light", "UPCD+ MA Dark" and "PCD+ MA Light", "PCD+ MA Dark". To investigate the bacterial and algal activity separately and distinguish their nutrient removal mechanism (Wang et al., 2016), control PBRs were also designed for UPCD, PCD and *C. Vulgaris*. These PBRs were named as "UPCD Light", "UPCD Dark"; "PCD Light", "PCD Dark", "MA Light" and "MA Dark".

Table 5.6. Properties of PBRs conducted in Chapter 5

PBR Types	Number of PBRs	
	Light	Dark
Control (MA*)	3	3
Control (UPCD: Digestate with Unpretreated CC*)	3	3
Control (PCD: Digestate with Pretreated CC*)	3	3
Test (MA+UPCD)	3	3
Test (MA+PCD)	3	3

*MA: Microalgae, *CC: Corncob

Abovementioned each PBR had 250 mL total volume and 200 mL working volume. The PBRs were autoclaved initially, and UV sterilised before use. A photograph demonstrating the configuration of the PBRs is presented in Figure 5.3.

Figure 5.3. PBR configuration of Chapter 5

5.2.4 Analytical Methods

During the experimental studies, optical density, pH, temperature, photosynthetically active radiation (PAR), dry weight, total ammonia nitrogen (TAN), ortho-phosphate (PO_4^{-3}-P), TOC, tCOD, TN, TP, TS, VS, chlorophyll-a were measured.

pH: pH meter (Eutech, CyberScan, pH510) and pH probe (Sensorex, p350) were used to measure pH value.

Temperature: Temperature values of the PBRs were measured with 9263 A Plus digital thermometer. It should be noted that, the ambient temperature in the sets was measured in a container that has water inside, that will represent the temperature of the PBRs.

Optical Density: HACH spectrophotometer DR 2800 with 1-cm light path was used to measure optical density values at optimum wavelength determined for erniched *C.Vulgaris* culture. To determine the optimum wavelength, optical density values were read at different wavelengths and the highest absorbance value was obtained at 685 nm. Detection limit is between 0.1 and 1, so for samples with optical densities higher than 1, dilution is necessary.

Photosynthetically Active Radiation (PAR): PAR was measured through a hand device called PAR meter (Light SCOUT).

Total Solids (TS) and Volatile Solids (VS): TS and VS were carried out according to the Standard Method 2540-B and 2540-E, respectively. Samples were filtered through glass fibre filters (1.2 µm pore size) and dried at 105 °C for at least 1 hour to get the TS results. Later samples were put into 550 °C oven to detect VS (APHA, AWWA, WEF, 2005).

Total Ammonia Nitrogen (TAN): TAN analysis is based on the Nessler method (Crosby, 1968). Samples from the PBR were filtered through the cellulose acetate filters (0.45 µm) for this analysis and diluted according to the required ranges. Related calibration curves were given in APPENDIX F.

Orthophosphate (PO_4^{-3}-P): For PO_4^{-3}-P analysis, were performed in Ion Chromatography (IC- Shimadzu Prominence HIC-SP). The working conditions of the IC were set to have the highest-pressure limit of 150 bar, oven temperature of 45°C, and the flow rate of 0.8 mL/min. Prior to the analysis, samples were filtered through 0.45 µm cellulose acetate filter.

The limit of detection (LOD) and the limit of quantification (LOQ) values for low and high concentration with the calibration curves are given in APPENDIX G.

Soluble Ortho Phosphorus (SOP): Determination of SOP were performed according to the Ascorbic Acid Method given in Standard Methods 4500-P (APHA, AWWA, WEF, 2005). Detection of the result were carried out spectrophotometrically, at 880 nm (HACH, DR 2800).

Soluble Chemically Oxygen Demand (sCOD): sCOD was determined by EPA approved digestion method (for COD range of 0-1500 mg/L), using heat PBRs (Aqualytic AL 38) for 2 hours. Results were obtained spectrophotometrically with spectrophotometer (SN 05827, PC Multidirect).

Total Organic Carbon (TOC): Total organic carbon concentration of the samples was determined using Shimadzu 5000A model TOC analyser that employs 680 ºC combustion catalytic oxidation method. The calibration curve for TOC analysis is given in APPENDIX I.

It should be noted that, the TOC analysis was performed for the soluble portion of the sample, representing sCOD and related TOC conversion for Chapter 5 was given in APPENDIX I.

Total Chemical Oxygen Demand: Determination of Total Chemical Oxygen Demand (tCOD) was performed by EPA approved digestion method (for COD range of 0-1500 mg/L) and detection were performed spectrophotometrically (SN 05827, PC Multidirect).

Nitrate-Nitrogen (NO_3^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_3^--N analysis is given in APPENDIX G.

Nitrite-Nitrogen (NO_2^--N): IC devise has been used with the same conditions mentioned above. The calibrations curve for NO_3^--N analysis is given in APPENDIX G.

Total Nitrogen: Low-range test kit vials (Catalog No: 535560, Lovibond GmbH, Aqualytic, Germany) were used for the measurement of TN.

Total Phosphorus: Low-range test kit vials (Catalog No: 535560, Lovibond GmbH, Aqualytic, Germany) were used for the measurement of TP.

Chlorophyll-a and Pheophytin-a: Pigments measurements were done according to the Standard Methods 10200H (APHA, AWWA, WEF, 2005). Optical density ratio of 664b/665a (OD (664b/665a)) gives insight about health of microalgal culture.

Ratio of 1.7 represents the healthiest situation while 1.0 represents death of culture. When chlorophyll-a content of the culture is higher, the ratio would be closer to 1.7; however, when pheophytin-a concentration is high, the ratio would be closer to 1. Pheophytin-a is the chlorophyll-a molecule that lost its Mg^{+2} ion and cannot function in photosynthesis reactions anymore. Equation 5.1 and 5.2 were used to determine chlorophyll-a and pheophytin-a (Dere et al., 1998).

Chlorophyll a, $mg/m^3 = 26.7(OD_{664}-OD_{665})\ V_1/(V_2L)$ …….…..... (Equation 5.1)

Pheophytin a, $mg/m^3 = 26.7(1.7xOD_{665}-OD_{664})\ V_1/(V_2L)$ ……..... (Equation 5.2)

Where,

C_a: chlorophyll a

C_b: chlorophyll b

All analyses were performed as at least duplicate, and averaged values were used in the figures and/or tables. For the analyses performed in triplicates, standard deviation values are presented in figures. In the calculation of the specific growth rate (μ) of the microalgal culture, the following Equation 5.3 was used (Krzemińska et al., 2014; F. Liang et al., 2013). Equation 5.4 (Liu et al., 2011) was used to determine double the number of cells (t_d) and Equation 5.5 (F. Liang et al., 2013) was used to calculate the biomass production rate (BPR). These values were calculated considering the steady-state conditions of the PBRs. The steady-state conditions in the studies were defined as “the point where the parameter does not change more than 10% in three consecutive days” (Kılıç, 2017).

$\mu = \ln(N_2/N_1)/(t_2-t_1)$ …… (Equation 5.3)

$t_d = \ln(2)/\mu$ ……...… (Equation 5.4)

$dX/dt = (X_2-X_1)/(t_2-t_1)$ ……..…… (Equation 5.5)

N_1: OD value at time t_1

N_2: OD value at time t_2

μ: Specific growth rate (day^{-1}) (SGR)

t_d: Doubling time (days)

dX/dt: Biomass production rate (mg/L. days) (BPR)

X_1: Dry weight of the microalgae at time t_1

X_2: Dry weight of the microalgae at time t_2

5.2.5 Experimental Setup

PBRs were operated in two stages in batch mode. The first stage lasted for 168 hours, while the second stage lasted 156 hours. As mentioned in Section 5.2.3, 15 PBRs were subjected to continuous light illumination and 15 of them were kept under dark to distinguish photosynthetic activity. For this purpose, 100 $\mu mol.m^{-2}.s^{-1}$ (100 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) were provided. The PBRs were operated at an average temperature of 25 ± 3°C. Aeration was supplied to all PBRs with a flowrate of 0.6 L/min flowrate (3 L/L/min, vvm) with air pump (RESUN Air Pump AC-9602) (Anjos et al., 2013; Ruiz et al., 2013). The ends of air inlet and outlet pipes were sealed with 0.45 μm filters (Hidrofobic Minisart Syringe Filter) to prevent contamination. Initial pH of the PBRs was set to 7.0±0.3.

The 1st stage of the operation aimed at adaptation of microalgal-bacterial consortium to the new environmental conditions. The 2nd stage was conducted to observe the activity of the co-culture and to compare its activity to sole bacterial consortium and sole microalgal culture.

The soluble portion of the PBRs, that constitutes TAN, PO_4^{-3}-P and TOC, was set to be equal in all PBRs. The digestates were diluted to reach to the certain TAN, PO_4^{-3}-P and TOC loading rates that algal culture can handle as experimented in Chapter 4. Accordingly, microalgae can deliver an almost 100% removal efficiency for 8

mg/L.d N and 1 mg/L.d P, while over 50% removal for 20-40 mg/L.d TOC. Hence, the concentrations of the PBRs were aimed to be set for a system to be operated for 144 hours. All PBRs were diluted with synthetic wastewater and distilled water to reach to the targeted concentrations of N, P and TOC as shown in Table 5.7. N, P and TOC were added in the form of NH_4Cl, K_2HPO_4 - KH_2PO_4 and HAc, respectively.

Table 5.7. The initial targeted concentrations of the parameters in 1st Stage

		Targeted Initial Concentrations (mg/L)		
PBRs	**Content (1:1-100 mL:100mL)**	**NH_4-N**	**PO_4-P**	**TOC**
Control (MA)	Microalgae (1): Synthetic WW (1)	60	9	250
Control (UPCD)	Distilled Water (1): UPCD[a] (1)	60	9	250
Control (PCD)	Distilled Water (1): PCD[a] (1)	60	9	250
Test (MA+UPCD)	Microalgae (1): UPCD[a] (1)	60	9	250
Test (MA+PCD)	Microalgae (1): PCD[a] (1)	60	9	250

[a]Diluted UPCD and PCD

Unfortunately, initial conditions could not meet the targeted concentrations for 1st Stage. As seen in Table 5.8, due to a possible solids' content interruption, initial concentrations were falsified during the preparation of set-up and caused deviated PO_4^{-3}-P, TAN and TOC initial concentrations. Hence, with the second loading in the 2nd Stage, the analysis was also carried out carefully and the initial concentrations were fixed to be the approximately same in all PBRs. All PBRs were diluted with synthetic wastewater this time to reach to the targeted concentrations of N, P and TOC as shown in Table 5.8. N, P and TOC were added in the form of NH_4Cl, K_2HPO_4 - KH_2PO_4 and HAc, respectively. HAc added to the PCD and UPCD was 6 mM in distilled water, while to MA this value was 11.2 mM.

Table 5.8. The initial targeted concentrations of the parameters in 1st Stage and 2nd Stage

	1st Stage Initial Concentration (mg/L)			2nd Stage Initial Concentration (mg/L)		
PBRs	**NH4-N**	**PO4-P**	**TOC**	**NH4-N**	**PO4-P**	**TOC**
Control (MA)	57	7	245	100	10	230
Control (UPCD)	75	24	230	100	14	250
Control (PCD)	65	16	250	100	13	260
Test (MA+UPCD)	70	25	200	100	14	250
Test (MA+PCD)	57	15	200	100	11	260

In this experiment, TAN, PO_4-P and TOC analyses were performed daily to monitor the nutrient and organic removal efficiencies of the PBRs. In addition, total and volatile solids, optical density, and chlorophyll-a experiments were performed for initial and final conditions as well.

5.3 RESULTS AND DISCUSSION

5.3.1 1st Stage: First Nutrient Loading

In the 1st Stage, as discussed in Section 5.2.5, due to the high solid content of the ADs the initial concentrations deviated from the desired plan. Therefore, an extra nutrient loading seemed appropriate to conduct after the 1st Stage. However, since the initial conditions could not be set as targeted, the adaptation period of both microalgal-bacterial consortium in terms of co-existence, was worth examining.

Microalgae culture used in this study was an axenic culture. Although bacterial consortium is considered a potential source of contamination for microalgal cultures, "plant growth promoting bacteria (PGPB)" can improve microalgal activity via the release of plant growth hormones, hence facilitating the formation of a co-culture (Fuentes et al., 2016; Ramanan et al., 2016). The bacterial consortium (i.e. digestate) used in this study was initially anaerobic. Adaptation of the bacterial consortium to

the new aerobic conditions would be provided in this study via aeration started to take place. After this adaptation period, PGPB activity might begin to release the plant growth hormones and allow the two consortia (microalgae and bacteria) to co-exist. This might be due to the fact that the bacterial consortium may release the plant hormones (i.e. Indole-3 acetic acid). In the 2nd Stage, the synergetic effect resultant of microalgal-bacterial consortium was indeed observed with improved the nutrient removal efficiencies and growth performances.

For this reason, until the nutrient concentrations were completely removed, the PBRs were operated for 168 hours and then for the following 48 hours they were in an idle state prior to the 2nd Stage. The results of pH, temperature, TAN, PO_4-P and TOC removal as well as OD, TS-VS and chlorophyll data are shared for the 1st stage in Figure 5.4-Figure 5.9.

It should be noted that the removal efficiencies of the reactors obtained in the 1st stage improved in the second nutrient loading. This indeed revealed the adaptation of the microalgae and the microorganisms in the digestates to the environmental conditions as described above. In this respect, the detailed comparison and discussion of the reactor groups were done for the 2nd stage. Yet this section, namely Section 5.3.1, was still presented to reveal the adaptation of the cultures and to simply put the difference between the control and test reactors as well as the light and dark reactor groups.

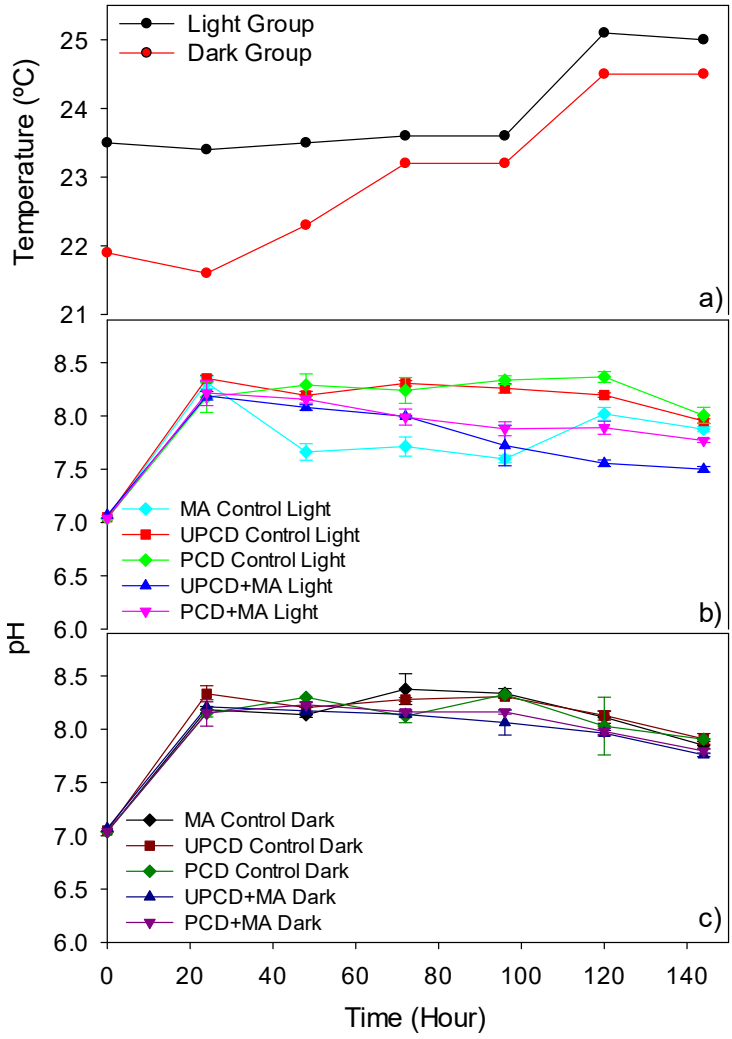

Figure 5.4. a) Temperature alterations for PBRs for the 1st Stage b) pH alterations in light group PBRs for the 1st Stage and c) pH alterations in dark group PBRs for the 1st Stage

As seen in Figure 5.4, where temperature and pH results are shown, temperature was recorded as 24.0±0.75 ºC (Figure 5.4.a) for the light group PBRs and 23±1.2ºC for the dark group PBRs. The pH of all the PBRs, as seen in Figure 5.4.b and c, reached over 8.5 ±0.1 in the first 24 hours. Later, the pH decreased slightly in the PBRs due to TAN removal, where the proton was released in the media in turn decreasing the pH (Junaidi et al. , 2020).

It should be noted that, at the hour 144 (T_{144}), the sampling volumes were not enough to perform all the analyses. Hence, at the hour 168 (T_{168}) another sampling was performed and the growth analysis for OD, chlorophyll-a and TS-VS was performed in T_{168}. Herein, the operational and environmental conditions were the same for both T_{144} and T_{168}.

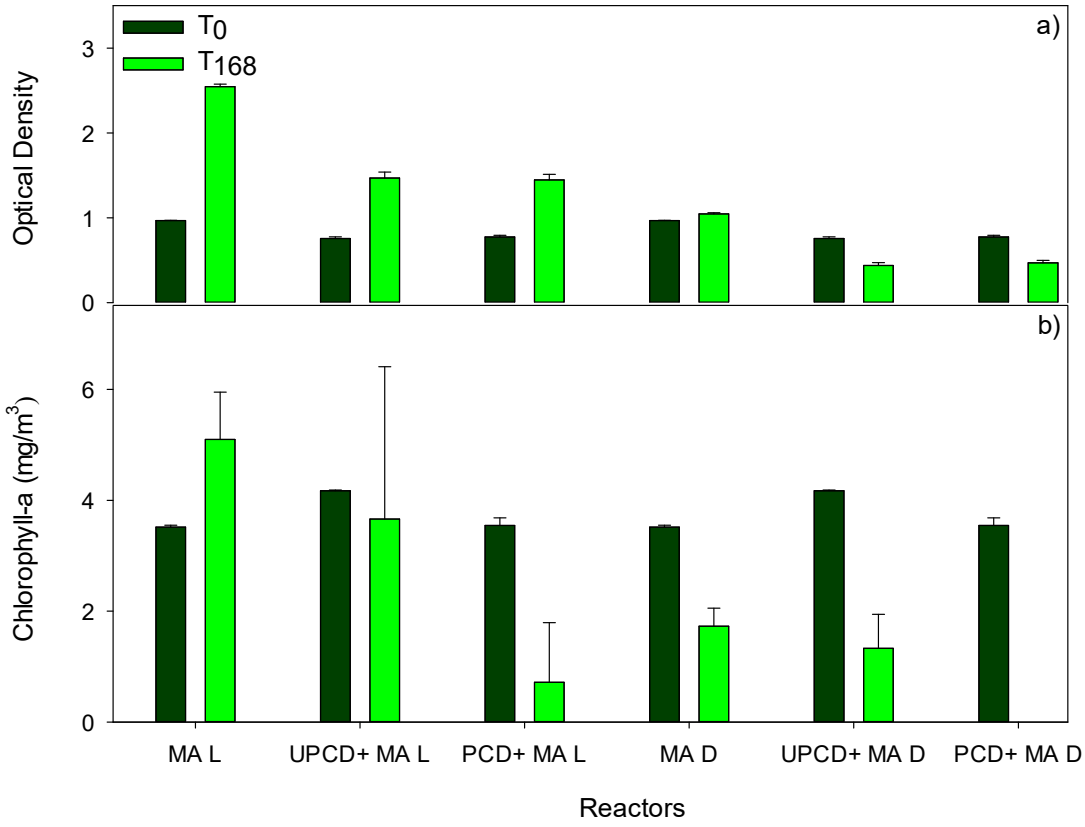

Figure 5.5. a) OD and b) Chlorophyll-a results for light and dark group PBRs of the 1st Stage at T_0 and T_{168} (D: Dark and L: Light)

Optical density and chlorophyll-a results are presented in Figure 5.5. Accordingly, OD values reached 2.55±0.00 for MA Light PBR while this value was 1.5 ±0.02 for both UPCD+MA Light and PCD+MA Light PBRs (Figure 5.5.a). On the other hand, 0.97±0.00 was reached by MA Dark PBRs and the OD values dropped to 0.76±0.02,

0.78±0.02 for UPCD+MA Dark and PCD+MA Dark PBRs (Figure 5.5.a). These results simply indicate that illuminated algal activity exhibited a better growth performance, compared to the ones that were not illuminated. Moreover, in UPCD+MA Dark and PCD+MA Dark PBRs, the OD values showed a diminishing trend. This might be due to the bacterial consortium of UPCD and PCD outcompeting the microalgal culture because of the inadequate light intensity that microalgae require to grow (Gurung et al., 1999; Ramanan et al., 2016). For the chlorophyll-a results, however, only MA Light PBR exhibited an increasing pattern (Figure 5.5.b), while other PBRs had a reduced concentration of chlorophyll-a. This might be simply related to the adaptation period as abovementioned, in which the initial stage of microalgae and bacterial consortium was still taking place. Since the adaptation of both consortia was not completed, the chlorophyll-a production in UPCD+MA and PCD+MA PBRs did not occur. Moreover, a study conducted by Ayre et al. (2017) suggests that increased availability of CO_2 and related pH change can contribute to the acclimatization of microalgae to turbidity and strength of the wastewater and resultantly allows microalgae to produce more chlorophyll-a. Hence, the supplied CO_2 via air in this study may not be enough to allow the cultures to stage out the adaptation period and chlorophyll-a production could not be observed.

It should be noted that, the chlorophyll-a analyses were troublesome to be performed due to the high solids' content in UPCD+MA and PCD+MA PBRs. Thus, some of the results was not able to be recorded as in PCD+MA Dark PBR (Figure 5.5). However, as time passed, in the 2nd stage, the texture of the slurry changed and became more agglomerated with a lighter colour. Hence, in the 2nd stage chlorophyll-a analyses were performed more easily and results were more precise.

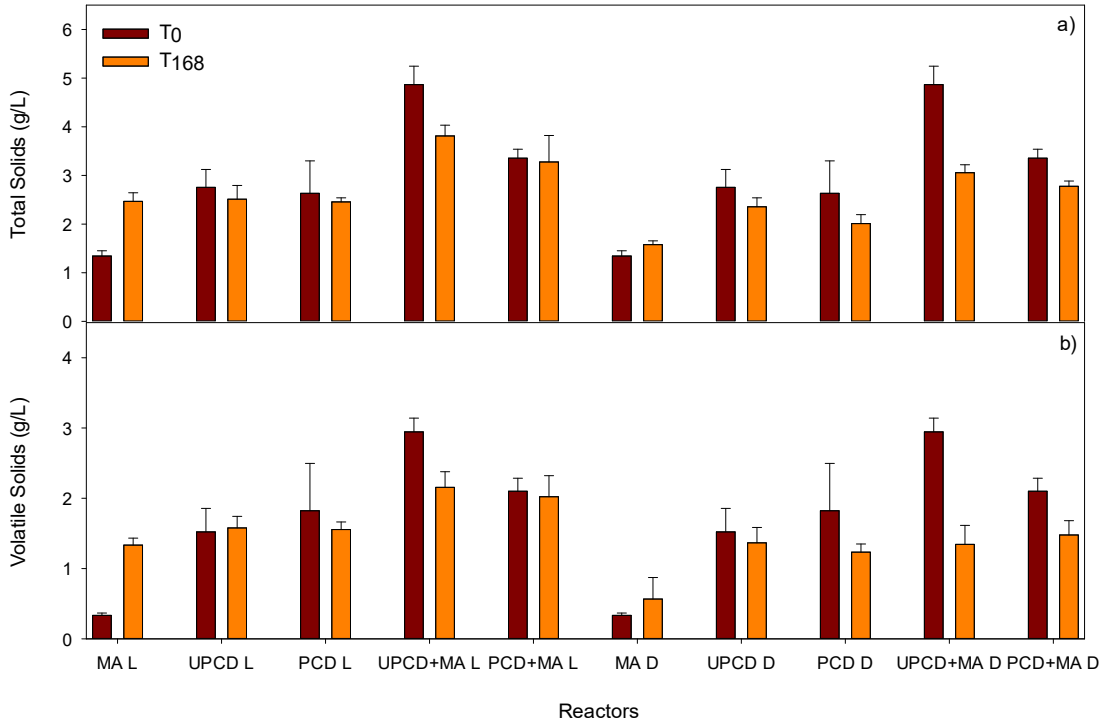

Figure 5.6. a) Total Solids and b) Volatile Solids Content of the Light and Dark Group PBRs of the 1st Stage at T_0 and T_{168} (D: Dark and L: Light)

As seen in Figure 5.6, TS and VS values are exhibited. The diminishing pattern of TS and VS concentrations in all PBRs except MA Light, might demonstrate the effects of the adaptation process of microalgae with UPCD and PCD bacterial cultures. The TS concentrations at T_{168} were 2.5±0.2, 3.8±0.2, and 3.3±0.5 g/L in MA Light, UPCD+MA Light and PCD+MA Light, respectively (Figure 5.6.a). Then again, UPCD Light and PCD Light PBRs exhibited 2.5±0.3 g/L TS concentrations at T_{168}. Furthermore, for the MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs TS concentrations were recorded as 1.6±0.1, 2.4±0.2, 2±0.2,3±0.2 and 3±0.1 g/L at T_{168}. The VS values obtained by those PBRs were 1.3±0.1, 2.2±0.2 and 2.0±0.3 g/L for MA Light, UPCD+MA Light and PCD Light, respectively (Figure 5.6.b), and 1.6±0.2 g/L for both UPCD and PCD Light PBRs. The VS concentration of dark group PBRs were recorded as 0.6±0.3, 1.4±0.2,

1.2±0.1, 1.3±0.3 and 1.5±0.2 g/L for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs. The VS/TS ratios were recorded for all PBRs as given in Table 5.9. VS/TS ratio corresponds to the biomass content with respect to total solids. Hence the higher the ratio, the higher the amount of biomass available in the system. According to the results, MA Light and Dark reactors showed an increase in their biomass ratio, however, MA Light reactors, as expectedly, showed a higher increase in this value. In UPCD Light and Dark PBRs VS/TS ratio increased while, in all other PBRs this ratio was decreasing in both light and their dark counterparts.

Table 5.9. VS/TS Ratios for All PBRs at T_0 and T_{168}

	VS/TS Ratio (%)	
	T_0	**T_{168}**
MA Light	24.8	54.1
UPCD Light	55.2	62.8
PCD Light	69.2	63.3
UPCD + MA Light	60.5	56.6
PCD + MA Light	62.6	61.7
MA Dark	24.8	35.9
UPCD Dark	55.2	58.0
PCD Dark	69.2	61.3
UPCD + MA Dark	60.5	44.0
PCD + MA Dark	62.6	53.2

TAN removal efficiencies are given in Figure 5.7. Accordingly, it can be observed that, all PBRs reached to a maximum of 95-100% TAN removal efficiencies (Figure 5.7.a). A similar pattern in the dark group PBRs was also observed for TAN removal efficiencies. According to the results, for all dark group PBRs, 82-100% TAN (Figure 5.7.b) removal efficiencies were recorded. As can be seen in the TAN removal efficiency results, light group reactors and dark group reactors exhibit almost the same TAN removal performance. Similarly, between the test and control reactors, there is no major difference (≤10%) in terms of TAN removal efficiencies. Hence, it can be interpreted that, in addition to the microalgal TAN removal as

observed in MA control reactors, nitrification might be also the other possible mechanism in the UPCD, UPCD+MA, PCD and PCD+MA reactors (both in light and dark reactors). In the dead zones of the reactors where the sparging of the air might have not been enough to reach, anaerobic niches might have survived that would make the denitrification activities possible. Although the results of NO_2-N and NO_3-N concentrations obtained from the PBRs tested for T_0 and T_{144} show no observable increase (APPENDIX L), there might be a potential nitrification and denitrification process performed in the possible anaerobic niches and nitrate emerging from these processes might be consumed by microalgae. Another potential explanation to non-observable NO_2-N and NO_3-N changes might be aerobic denitrification process which was proposed by Robertson and Kuenen (1984), a process reducing the NO_3^- into gaseous nitrogen forms in the presence of oxygen gas, which allows the nitrification and denitrification processes to be performed simultaneously.

The TAN removal rates reached by all the PBRs are presented in Table 5.10. Accordingly, MA Light, UPCD Light and PCD Light PBRs reached 1.8±0.1, 1.7±0.04 and 1.7±0.2 mg/L.hr TAN removal rates, respectively. However, UPCD+MA Light and PCD+MA Light PBRs exhibited 1.3±0.4 and 1.1±0.1 mg/L.hr TAN removal rates (Table 5.10.) in the first 24 hours. The TAN removal rates were recorded as 1.2±0.01, 1.4±0.2 and 1.5±0.1 mg/L.hr for MA Dark, UPCD Dark and PCD Dark PBRs, respectively. On the other hand, both UPCD+MA Dark and PCD+MA Dark reached 0.8±0.2 mg/L.hr for TAN removal rates which were less than their controls. Apparently, microalgal-bacterial consortium reactors have slightly negatively affected the TAN removal. This might be attributed to the adaptation period in which microalgae and the bacterial consortium was surpassed their initial stages. Hence, in this period, there might be slowing down of the consortia of microalgae-bacterial co-culture, where their individual activities slowed down due to the environmental conditions' alterations.

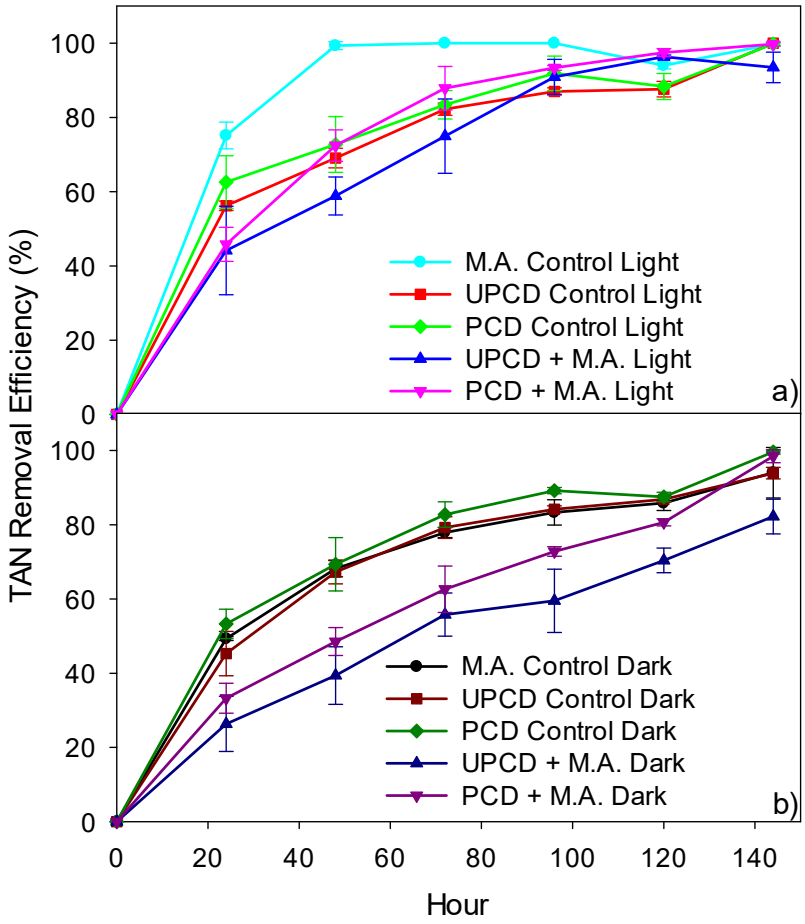

Figure 5.7. Results of 1st Stage a) TAN removal efficiency of light group b) TAN removal efficiency of dark group

As seen in Figure 5.8, the highest PO^{-3}_4-P removal efficiency was recorded in the MA Light PBRs (almost 100%), in comparison to the other PBRs. This value was followed by the MA Dark PBRs where P removal efficiency reached up to 40%. This indicates an expected difference in the P removal performance of microalgal autotrophic and heterotrophic metabolism due to the light/dark conditions. Other PBRs, UPCD, UPCD+MA, PCD and PCD+MA (both light and dark replicates) removed PO^{-3}_4-P with a removal efficiency of 10-30%. This can be attributed to the Phosphate Accumulating Organisms (PAOs) which might be a part of the bacterial consortium. As Tchobanoglous et al. (2014) states, PAOs release P from their

polyphosphate storage while producing polyhydroxy butyrate storage products (PHB). Once aeration is introduced to the system, polyphosphate bonds are formed and PAOs uptake P from the media.

It should be noted that chemical P removal from the system was also possible via Fe, Ca, and Al. Although the considerable amount of precipitation occurs in the high pH range (pH>11), Takács et al. (2006) showed that in a broad range of pH, P can be precipitated with Fe and removed from the system in extremely low concentrations (<0.2 mg P/L). Thus, in this part of the thesis, the chemical P removal cannot be considered as the main removal mechanism.

The P removal rates reached by the PBRs are presented in Table 5.10. According to the results, 0.14±0, 0.11±0.02, 0.06±0.03, 0.21±0.1 and 0.01±0 mg/L.hr P removal rates were recorded for MA Light, UPCD Light, PCD Light, UPCD+MA Light and PCD+MA Light PBRs, respectively. For MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs those values were 0.08±0, 0.1±0.05, 0.08±0.05, 0.13±0.03 and 0.04±0.01, respectively. For MA, UPCD+MA and PCD+MA PBRs, the light and dark replicates differ from each other as illumination influenced photosynthesis realised by microalgae. Hence, it can be said that, in addition to PAOs activity in the bacterial consortium, MA activity has a distinctive effect on P removal as well. In UPCD and PCD PBRs, light and dark replicates show almost the same P removal rates that indicates the PAOs activity which does not depend on illumination.

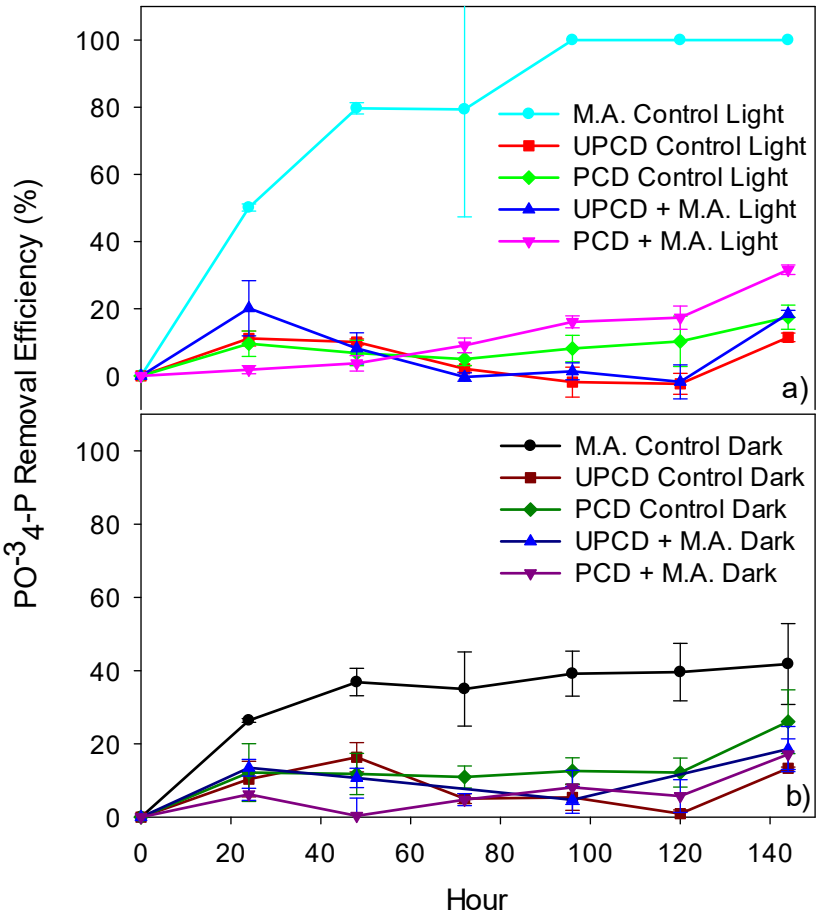

Figure 5.8. Results of the 1st Stage a) PO^{-3}_4-P removal efficiency of light group b) PO^{-3}_4-P removal efficiency of dark group

Figure 5.9 presents the results of the TOC removal efficiencies of the PBRs throughout the 1st Stage. Accordingly, MA Light, UPCD Light and PCD Light PBRs reached to 44±0.8% TOC removal efficiency at T_{144}. UPCD+MA Light and PCD+MA Light reached to 23±2% TOC removal efficiency in T_{144}. MA Dark PBR reached to 55±0.2, UPCD and PCD Dark PBRs reached to 45±2% and UPCD+MA Dark and PCD+MA Dark PBRs reached to 30±2% TOC removal efficiencies. The TOC removal rates are provided in Table 5.10. As can be seen in the results for the achieved TOC removal rates, the TOC removal performances were similar in light and dark replicates of each PBRs. This might be attributed to the fact that the

mechanisms that consume TOC does not depend on the light in UPCD, PCD Control and UPCD+MA and PCD+MA PBRs. The possible aerobic denitrification and PAOs activity, as abovementioned, removes soluble organic carbon along with those activities. Moreover, in MA Light and MA Dark reactors, similar TOC removal rate results were obtained. This can also be due to the heterotrophic mechanism of microalgae, in which illumination is not a driving factor.

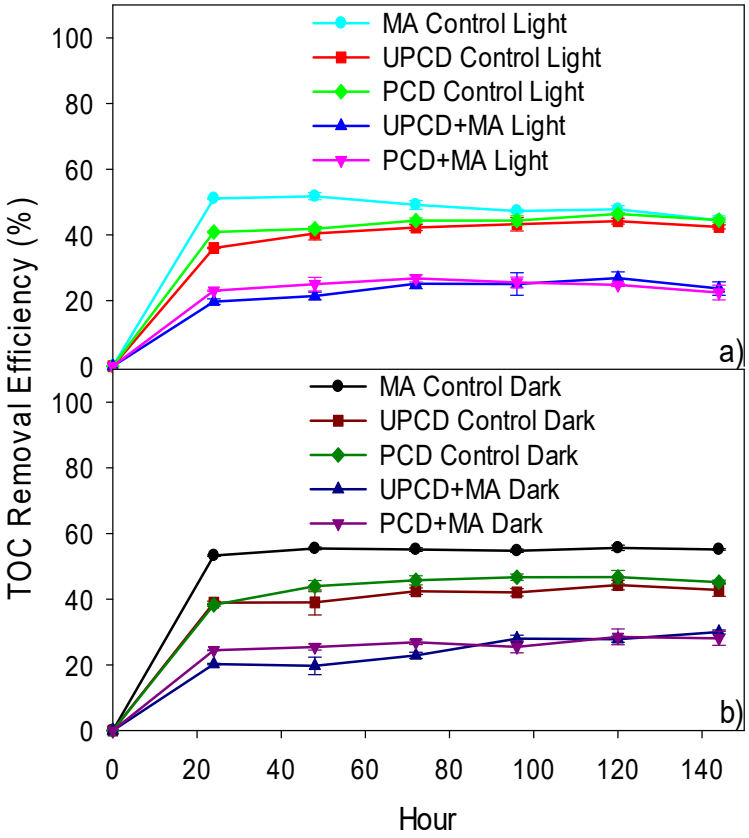

Figure 5.9. Results of the 1st Stage a) TOC removal efficiency of light group b) TOC removal efficiency of dark group

Table 5.10. TAN, PO_4^{-3}-P and TOC Removal Rates of PBRs for the 1st Stage

	TAN (mg/L.hr)	**PO_4^{-3}-P (mg/L.hr)**	**TOC (mg/L.hr)**
MA Light	1.8±0.1	0.14±0	5.2±0.04
UPCD Light	1.7±0.04	0.11±0.2	3.5 ±0.03
PCD Light	1.7±0.2	0.06±0.03	4.4±0.02
UPCD+MA Light	1.3±0.4	0.2±0.1	1.5±0.08
PCD+MA Light	1.1±0.1	0.01±0.01	2±0.02
MA Dark	1.2±0.01	0.08±0.01	5.4±0.03
UPCD Dark	1.4±0.2	0.1±0.05	3.8 ±0.05
PCD Dark	1.5±0.1	0.08±0.05	4.1±0.04
UPCD+MA Dark	0.8±0.2	0.13±0.03	1.6±0.03
PCD+MA Dark	0.8±0.2	0.04±0.01	2.1±0.02

5.3.2 2nd Stage: Second Nutrient Loading

After a certain period of the 1st Stage, the 2nd Stage was initiated with a different nutrient loading. PBRs were operated for 156 hours. The results of pH, temperature, TAN, PO_4-P and TOC removal efficiencies and rates as well as OD, TS-VS and chlorophyll data are shared in Figure 5.10-Figure 5.16.

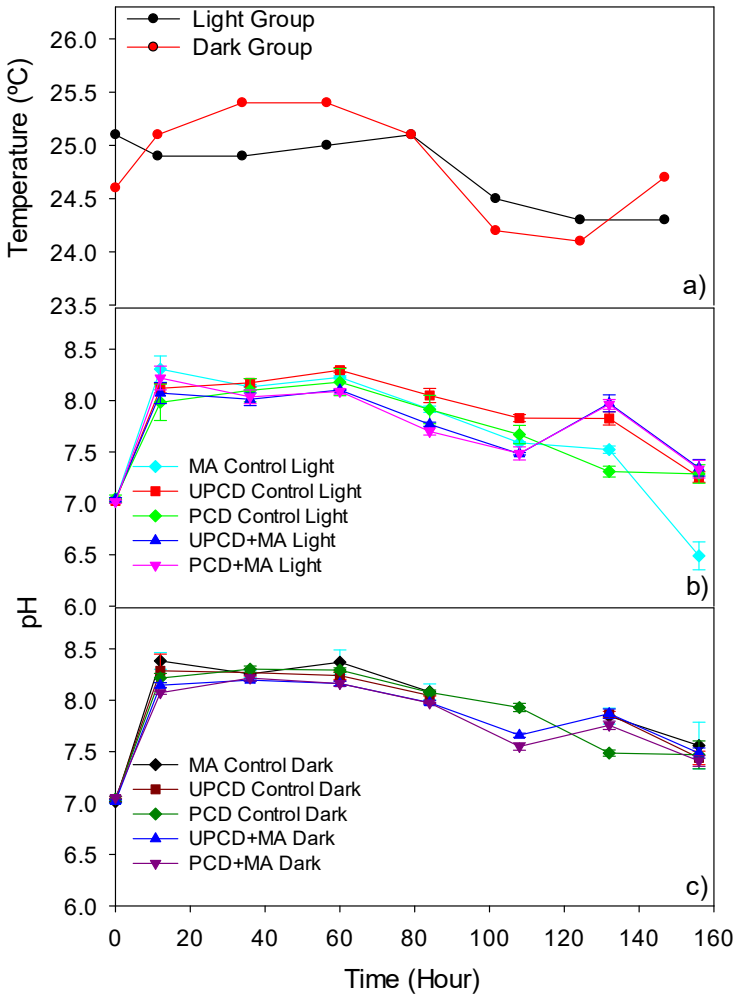

Figure 5.10. a) Temperature results for PBRs of the 2nd Stage b) pH in light group PBRs and c) pH in dark group PBRs

As seen in Figure 5.10, where temperature and pH results are shared, temperature shows (Figure 5.10.a) an average of 24.8±0.35 ºC for both light PBRs and dark PBRs. pH of MA Light PBRs, as seen in Figure 5.10.b, reached over 8.3 ±0.1 in first the 12 hours, while pH of UPCD+MA Light and PCD+MA Light PBRs reached 8.1±0.1 and 8.2±0.1 in the same period, respectively. UPCD and PCD Light PBRs exhibited a similar pH alteration during the operation with 8.1±01 and 8±0.2, respectively. For the dark PBRs, those values are 8.4±0.1, 8.3±0.1, 8.2±0.1, 8.1±0.2

and 8.1±0.2 for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs, respectively. The change in pH depends on TAN and TOC consumption depending on the source as this was also observed in Chapter 4, Section 4.3.1. where acetate and its forms were used as the organic carbon source. As it was discussed in Chapter 4, Section 4.2.5, when acetate/acetic acid was introduced into the system, the pH increased (Huang et al., 2017), while with the consumption of TAN, pH decreased (Chapter 3, Section 3.2.5.3). Hence, the balance between the two mechanisms of TAN and TOC removal defines the pH level in the system. To conclude, the increase in pH is generally observed in mixotrophic systems with acetic acid. Here, the MA control PBRs were fed with 11.2 mM acetic acid, and UPCD and PCD control PBRs were fed with 6mM acetic acid to achieve the targeted TOC concentration of 250 mg/L (Section 5.2.5). Hence, the acetic acid added may explain the pH increase observed in all control reactors. The same trend in the pH was also observed in UPCD+MA and PCD+MA PBRs. Hence, although the organic carbon source coming from UPCD and PCD was not determined, this might be indicating that the organic carbon source was acetic acid. Moreover, it should be noted that the pH levels observed in all PBRs are ideal for growing freshwater microalgae while avoiding ammonia toxicity and phosphate precipitation (Heubeck et al., 2007).

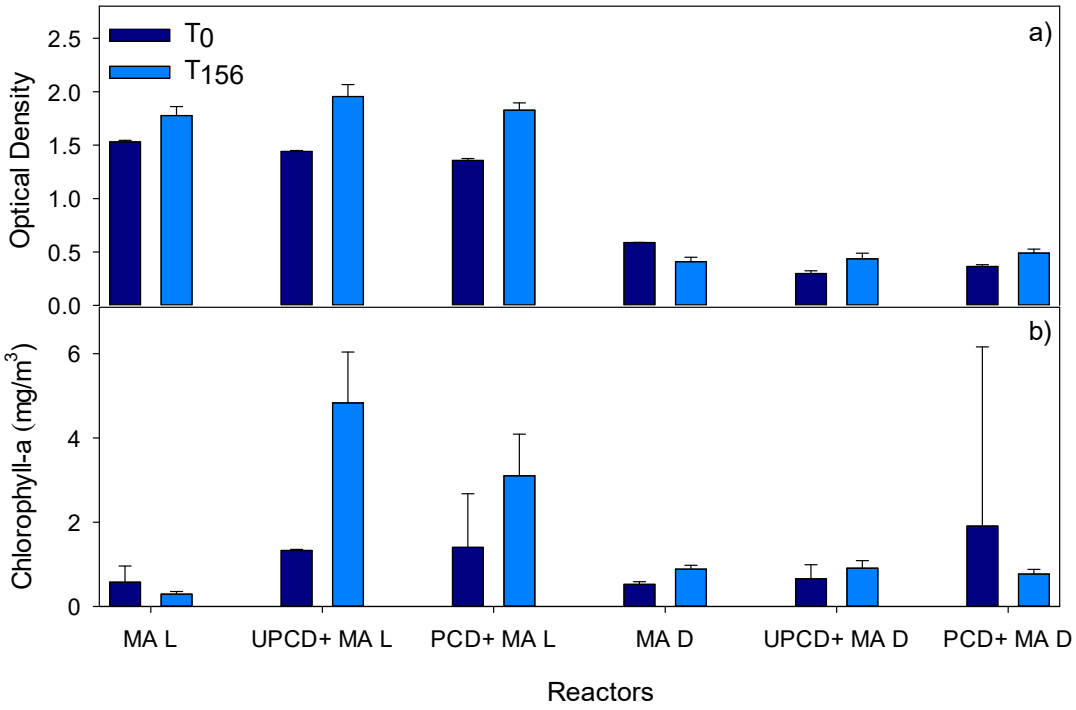

Figure 5.11. a) OD and b) Chlorophyll-a results for light and dark group PBRs of the 2nd Stage at T_0 and T_{156} (D: Dark and L: Light)

Optical density and chlorophyll-a concentration results are displayed in Figure 5.11. Accordingly, a clear demonstration of the effect of illumination on OD results can be seen. OD values of MA Light, UPCD+MA Light and PCD+MA Light PBRs reached to 1.8±0.1, 2.0±0.1 and 1.8±0.1, respectively, at T_{156} (Figure 5.11.a). The effect of microalgal-bacterial consortium on the growth performance on an algal culture can be observed in the OD results. The specific growth rate of the UPCD+MA Light and PCD+MA Light PBRs was 0.05±0.01 day^{-1} while a lower specific growth rate was exhibited by MA Light PBR with 0.02±0.01 day^{-1}. In a similar study conducted with *C. Vulgaris* enriched at 10% dilution of sterile chicken manure digestate (Rajagopal et al., 2021), the OD values reached a maximum of 0.5. In the characteristics of 10% digestate in Rajagopal et al. (2021), the TAN concentration was recorded as approximately 500 mg/L and the TOC was

approximately 1000 mg/L while in this section of this thesis, the 2nd stage initial TAN and initial TOC concentrations were approximately 100 mg/L and 250 mg/L, respectively. Despite the lower TAN and TOC values in this study compared to Rajagopal et al. (2021), the reason behind the higher growth performance observed, might be the composition of microbial consortium. Moreover, a similar study conducted with enriched *C. Vulgaris* culture with unsterilized filtered anaerobic digestates, exhibited similar OD results (OD 2.0) (Zuliani et al., 2016). These studies (Rajagopal et al., 2021; Zuliani et al., 2016) along with the 2nd Stage results presented herein, support the hypothesis that the bacterial consortium improves the overall growth performance of microalgae.

The similar pattern also accounts for chlorophyll-a production for UPCD+MA Light and PCD+MA Light PBRs. UPCD+MA Light PBRs reached 4.83±1.2 mg/m^3 chlorophyll-a concentration at T_{156} while this value was 3.1±1 mg/m^3 for PCD+MA Light PBRs. The increase in chlorophyll-a concentrations of those PBRs were approximately 4-fold and 2-fold for UPCD+MA Light and PCD+MA Light PBRs, respectively from T_0 to T_{156}. In MA Light PBR the chlorophyll-a value was recorded as 0.6±0.4 mg/ m^3 at T_0 while, 0.3±0.06 mg/ m^3 $T_{156.}$ As it can be interpreted from these results, it can be said that UPCD has a higher improving effect on pigment production compared to PCD. This might be pointing out the effect of corncob treatment. Unlike the possible expected result for PCD having more active bacterial consortium due to the hydrolysed sugar and the other by-product content that are available for bacterial consortium, the UPCD came forward in enhancing the chlorophyll-a content. This might be due to the already hydrolysed sugar and the other by-product content that might be inhibitory for microalgae in PCD containing PBRs. For example, as concluded in a study conducted by Morales-Sánchez et al. (2013), although heterotrophic cultivation of *Neochloris oleoabundans* revealed that this strain could grow on 10 g/L glucose or 10 g/L cellobiose, when xylose or arabinose were used as carbon sources, no *Neochloris* growth was observed. It is not only limited to sugar type, but also other by-products may also exhibit inhibition on microalgal activity. Hence, the sugar break down is important to be well analysed in

order to claim that the hydrolysation of the lignocellulosic content, as PCD have, could be beneficial for microalgal growth.

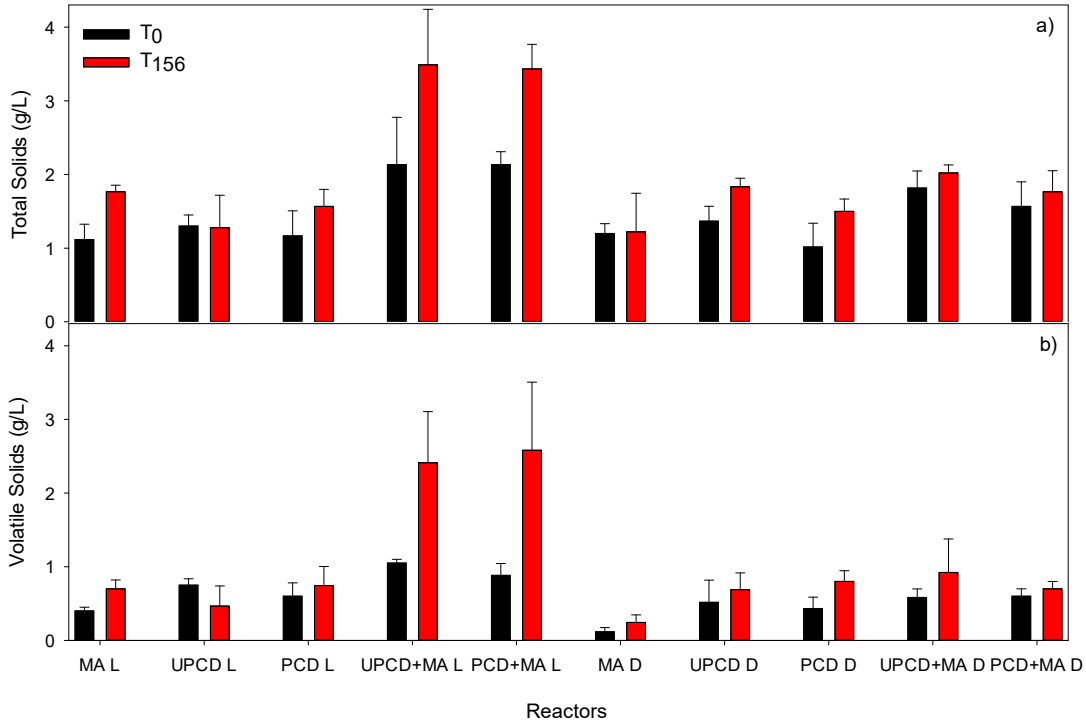

Figure 5.12. a) Total Solids and b) Volatile Solids content of the light and dark group PBRs of the 2nd Stage at T_0 and T_{156} (D: Dark and L: Light)

As seen in Figure 5.12, TS and VS concentrations of the PBRs for T_0 and T_{156} were presented. Accordingly, TS values increased to 3.5±0.8 g/L for both UPCD+MA Light and PCD+MA Light PBRs (Figure 5.12.a). These values are comparable for UPCD+MA Light and PCD+MA Light PBRs with the TSS results presented by Marazzi et al. (2020) that investigates the effect of bacterial consortium on microalgal culture. According to Marazzi et al. (2020), the maximum TSS values for microalgal cultures reached to approximately 2g/L. Furthermore, the biomass production rate of UPCD+MA Light and PCD+MA Light PBRs, calculated over TS values, were found to be 0.2±0.01 g/L.d while MA, UPCD and PCD Light PBRs had

at most 0.1±0.0 g/L.d of biomass production rate. These results can also be considered as an indication for a phenomenon that microalgae-bacterial co-existence has a positive effect on microalgal growth performance. For the dark PBRs, the biomass production rates were recorded as, 0.003, 0.07, 0.07, 0.3 and 0.3 g/L.d (calculated over TS) for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD Ma Dark, respectively. The biomass production rates calculated over VS values for UPCD+MA Light and PCD+MA Light was 0.21±0.1 and 0.26±0.1 g/L.d, respectively. The maximum VS values for UPCD+MA Light and PCD+MA Light PBRs among all others were recorded as 2.4±0.7 and 2.6±0.9 g/L, respectively (Figure 5.12.b). On the other hand, the biomass production rates for UPCD+MA Dark and PCD+MA Dark PBRs were recorded as 0.05±0 and 0.02±0 g/L.d, where the maximum VS values were found to be 0.9±0.5 and 0.7±0.1 g/L for UPCD+MA Dark and PCD+MA Dark PBRs. The MA Light and Dark reactors showed the biomass production of 0.05±0.01 and 0.02±0.01 g/L.d, respectively. Hence, the effect of the microbial consortium on microalgal growth (possibly due to plant growth hormones) was demonstrated.

In Table 5.12 VS/TS ratio of the PBRs were provided. Accordingly, the highest increase in this ratio was observed in both UPCD+MA Light and PCD+MA Light reactors. The observed results can simply be attributed to the effect of microalgal-bacterial consortium in which the growth performances improved with the help of illumination. Hence, it can be said that the microalgal culture was positively affected from this co-existence. Moreover, VS/TS results can be supported with the production of chlorophyll-a, as above-mentioned. As observed in the biomass production rate, the increase in VS/TS ratio of MA Light PBRs was much less (10%) than the increase in UPCD+MA Light and PCD+MA Light PBRs (40% and 80%, respectively). This is, similarly, an indication for the contribution of the bacterial consortium on the microalgal growth performance. The other observable difference in improvement of biomass content was recorded for UPCD+MA Light and PCD+MA Light. Herein, PCD+MA Light demonstrated a higher increase in its biomass content (80%) than that of UPCD+MA Light (40%). This might be due to

the difference of the bacterial consortium between the two, which might have been caused by the pretreatment, that alters the hemi-cellulose, cellulose and soluble lignin content.

Table 5.11. VS/TS Ratios for All PBRs at T_0 and T_{156}

PBRs	VS/TS Ratio (%)	
	T_0	T_{156}
MA Light	35.8	39.6
UPCD Light	57.7	36.5
PCD Light	51.4	47.5
UPCD + MA Light	49.2	69.1
PCD + MA Light	41.4	75.1
MA Dark	9.7	20.0
UPCD Dark	37.8	37.6
PCD Dark	42.6	53.3
UPCD + MA Dark	32.1	45.6
PCD + MA Dark	38.3	39.6

To sum up, UPCD improved the chlorophyll-a production and in return, microalgal growth in UPCD+MA Light reactors. On the other hand, PCD increased the VS concentration in PCD+MA Light reactors. This might be attributed to the improvement of a specific bacterial consortium in UPCD, that might be benefitted from microalgae as well as improving microalga growth (mutualism). Moreover, as can be seen in Figure 5.13, the UPCD+MA Light and PCD+MA Light (Figure 5.13. b and c) PBRs demonstrated an immense visual difference compared to their dark versions as well as MA Light PBRs (Figure 5.13.a). These photographs, in particular that of test PBRs which had green- brown colour at the end of the 2nd stage, well revealed the effective microalgal growth in UPCD+MA and PCD+MA Light PBRs.

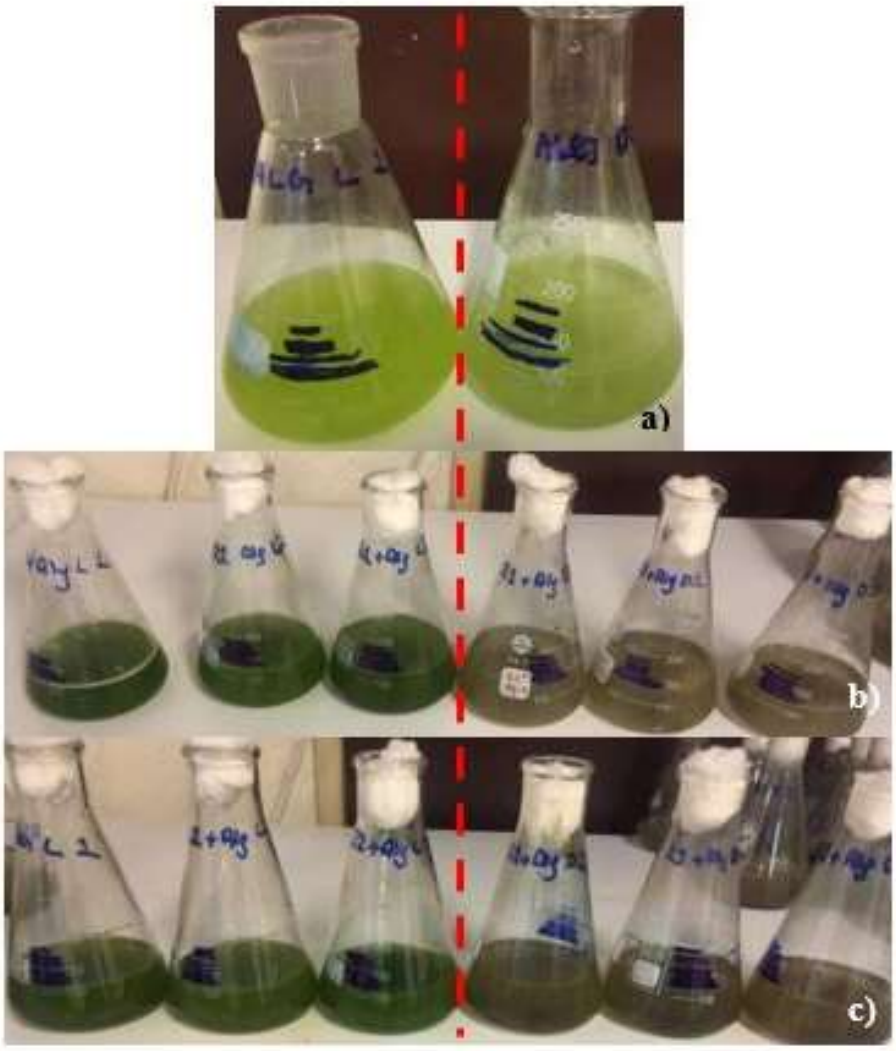

Figure 5.13. The a) MA Light and Dark, b) UPCD+MA Light and Dark and c) PCD+MA Light and Dark PBRs at the end of the 2nd Stage (PBRs on the left of the dashed line are the "light" groups and PBRs on the right of the dashed line are the "dark" group)

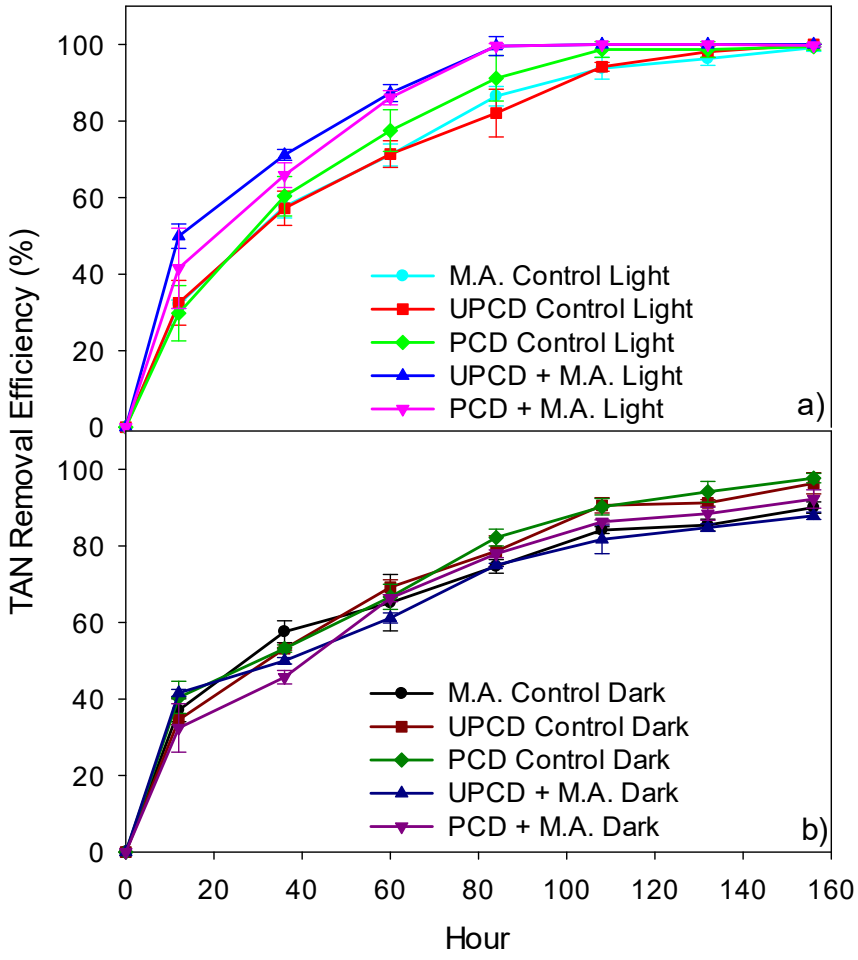

Figure 5.14. Results of 2nd stage a) TAN removal efficiency light group b) TAN removal efficiency dark group

In Figure 5.14, the TAN results of the 2nd Stage are demonstrated. Accordingly, all the PBRs reached almost 100% TAN removal efficiency (Figure 5.14.a and b). Yet, the TAN removal rates show difference between the PBRs for the first 12 hours. As seen in Table 5.12., among light group, UPCD+MA and PCD+MA Light PBRs had the highest TAN removal rates of 4.1±0.3 and 3.6±0.6 mg/L.hr. While MA Light, UPCD Light and PCD Light exhibits 2.7±0.1, 2.8±0.6, and 2.5±0.6, mg/L.hr TAN removal rates, respectively. The higher TAN removal for microalgal-bacterial consortium PBRs (UPCD+MA and PCD+MA Light) underlines the effect of a

possible co-culture at the time. The TAN Removal rate results belonging to the dark group PBRs, on the other hand, were 3.2±0.4, 3.1±0.1 3.4±0.4, 3.6±0.1 and 2.7±0.6 mg/L.hr for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark, respectively. The reason that MA Dark, UPCD Dark and PCD Dark PBRs exhibited better removal rates in the first 12 hours than their light counterparts, might be due to the ammonia stripping phenomena. As seen in Figure 5.10.b, pH vs time graph, in the first 12 hours a peak observed from the pH 7.0 to 8.2-8.4. This might have caused ammonia stripping in all the PBRs. However, for UPCD+MA Light and PCD+MA Light PBRs, the stripping and microalgal TAN removal mechanism possibly worked together and generated the difference between the test and control PBRs. In order to assess the occurrence of a stripping mechanism in the PBRs a theoretical calculation was performed (APPENDIX M).

$Kb/Kw = e^{(6344/(273+T(°C))}$ ……...…… (Equation 5.6)

$NH_3\text{-}N\ (mg/L) = (TAN\ (mg/L) \times 10^{pH}) / (Kb/Kw+10^{pH})$ ….…….. (Equation 5.7)

Where,

Kb: The ionization constant of the ammonia equilibrium equation

Kw: The ionization constant of water

Accordingly, at the operated pH (8.2-8.4) and temperature (25.5°C), with an initial TAN concentration By Henry's constant (H: 0.0161 atm.L/mol, 25°C, Nazaroff and Alvarez-Cohen, 2001), it was determined that only 0.07% of ammonia dissolved in water could transfer to the gas phase.

Furthermore, in a study conducted by Mujtaba et al. (2017) investigating the effect of co-culture of pure immobilized *C. Vulgaris* and pure suspended *P. Putida* on nutrient removal, the maximum TAN removal reached by the co-culture was recorded at 85% (for an initial TAN concentration of 50 mg/L). Although the initial TAN concentration was higher in the 2nd stage of the study, the reason that the higher

TAN removal efficiency was observed, may be due to the pure *P.Putida* culture used in Mujtaba et al. (2017). An example for the opposite situation can be observed in a study conducted with an activated sludge (AS) and *C. Vulgaris* culture (Wang et al., 2016), where the complete TAN removal (of 50 mg/L) was reached by the co-culture in 20th hour. The tests performed on the microbial consortium showed that the dominant bacteria in AS was *P. Putida*. Hence, it can be said that, instead of a pure culture, a mixed bacterial consortium might be a better option for developing wastewater treatment systems coupled with microalgae. In the 2nd Stage of this thesis, the reason behind achieving complete TAN removal by UPCD+MA Light and PCD+MA Light PBRs after 94 hours might be the adaptation period of microalgae-bacteria consortium to reach status of co-existence. Moreover, the microalgae concentration, the illumination intensity and VS-TS concentration might be determining factors in this case. Thus, a better removal can be speculated to be observed in a third nutrient loading. As abovementioned in the 1st Stage, nitrification can be interpreted as a possible mechanism in the UPCD, UPCD+MA, PCD, and PCD+MA reactors (both in light and dark reactors). Although there is no observable increase in the NO_2-N and NO_3-N concentrations in the PBRs at T_0 and $T_{156.}$ (APPENDIX L), there may be a potential of nitrification and denitrification processes performed by aerobic and anaerobic niches, respectively, and nitrate emerging from these processes may be consumed by microalgae or by aerobic denitrification proposed by Robertson and Kuenen (1984), which converts NO_3 into gaseous nitrogen forms in the presence of oxygen gas.

As it was demonstrated in Figure 5.15, the PO_4-P removal efficiencies of MA Light, UPCD+MA Light and PCD+MA Light PBRs were much higher compared to UPCD and PCD Light PBRs. UPCD+MA Light and PCD+MA Light PBRs achieved 99±0.9% and 100±0.3% P removal efficiencies, respectively (Figure 5.15. a), while MA Light PBRs reached 65±10% P removal efficiency in the first 36 hours. On the other hand, UPCD Light and PCD Light PBRs only achieved 10±3% removal efficiency for the same period (Figure 5.15.a). Moreover, the maximum observed P removal efficiency at the 36th hour was 14.5±5.7% for MA Dark PBRs. UPCD Dark

PBRs only achieved 11.5±1.3%, and PCD Dark PBRs achieved 4±5%. Furthermore, UPCD+MA Dark and PCD+MA Dark PBRs reached to 8±1.7% and 10±2.3 P removal efficiencies (Figure 5.15.b).

UPCD+MA Light PBRs and PCD+MA Light PBRs showed the best performance in terms of P removal efficiency among other PBRs due to the co-existence of a microalgal-bacterial consortium. The difference between the dark and light group test PBRs, where the microalgal-bacterial co-existence took place, demonstrated the positive effect of illumination in algal P removal mechanism which was an additional P removal process besides photosynthesis. In a similar study conducted by Wang et al. (2016), also almost 100% P removal efficiency was achieved (from 12mg/L TP) in 6 hours. On the other hand, the P removal completed in 36 hours in UPCD+MA Light and PCD+MA Light PBRs (from 12 mg/L P as well). This might be associated with the adaptation period where microalgal-bacterial consortium did not completely adapt to each other that delayed the P removal for few days. The maximum P removal rates reached by MA Light, UPCD Light, PCD Light, UPCD+MA Light and PCD+MA Light PBRs were 0.3±0.1, 0.2±0.1, 0.1±0, 0.5±0, and 0.7±0.1 mg/L.hr, respectively (Table 5.12). On the other hand, these values were 0.2±0.03, 0.2±0.04, 0.05±0.2, 0.2±0.1 and 0.1±0.05 for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs, respectively (Table 5.12). As can be seen in the results of P removal rates, the light group reactors exhibited higher performance than that of their counterparts in dark conditions. Moreover, UPCD+MA Light and PCD+MA Light showed higher P removal rates than that of the MA Light PBRs and much higher rates than that of PCD and UPCD controls. That indicates the positive effect of the bacterial consortium on P removal activity of microalgae.

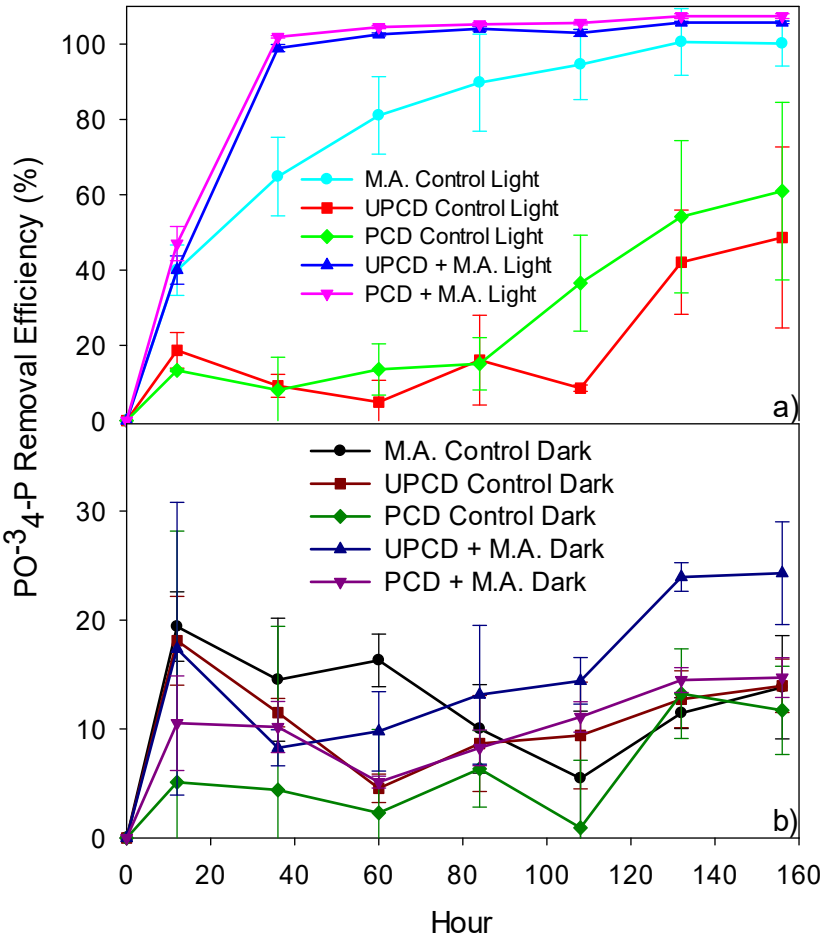

Figure 5.15. Results of 2nd stage a) PO^{-3}_4-P removal efficiency light group b) PO^{-3}_4-P removal efficiency dark group c) PO^{-3}_4-P removal rates light group d) PO^{-3}_4-P removal rate dark group

The TOC alteration throughout the operation time is given in Figure 5.16. As seen in the results, maximum TOC removal observed for PBRs was approximately 53±1 %, 52±2%, 55±2%, 54±1%. and 55±0% for MA Light, UPCD Light, PCD Light, UPCD+MA Light and PCD+MA Light PBRs (Figure 5.16.a) with a negligible difference among each other. Dark PBRs' results were also similar to each other as 55±0.8%, 57±1%, 56±1, 57±0.6 and 55±2 for MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs (Figure 5.16.b). In a similar study conducted with *C. Vulgaris* and AS under light conditions (Serejo et al., 2015), COD

removal was recorded as approximately 50% for the mixed culture as well. According to a mass balance calculation over carbon experiments, Serejo et al. (2015) claims that the main C removal mechanism was assimilation into microalgae biomass. Hence, while microalgae utilize carbon to grow heterotrophically or mixotrophically, they alter their metabolic pathways in response to the availability of organic substrates such as organic acids or glucose and that might be associated with the level of consumption of TOC in all PBRs.

The maximum TOC removal rates of the PBRs were found as 3.6±0.2, 3.4±0.4, 4.1±0.2, 3.9±0.04, 4±0.1 mg/L.hr for MA Light, UPCD Light, PCD Light, UPCD+MA Light and PCD+MA Light, respectively (Table 5.12). For dark groups PBRs these values were 3.5±0.1, 4±0.2, 4±0.1, 3.7±0.04, 3.9±0.3 mg/L.hr (MA Dark, UPCD Dark, PCD Dark, UPCD+MA Dark and PCD+MA Dark PBRs) (Table 5.12). The TOC removal rates for Light group and Dark group exhibit similar performances. This might be attributed to the organic carbon removal mechanisms in the PBRs which are independent of illumination conditions. In the microalgal case, the consumption of TOC arose from the heterotrophic mechanism that occurs under dark conditions. The TOC removal in the UPCD and PCD PBRs possibly depends on the aerobic denitrification which does not require any illumination either.

Table 5.12. TAN, PO_4^{-3}-P and TOC Removal Rates of PBRs for the 2nd Stage

	TAN (mg/L.hr)	**PO_4^{-3}-P (mg/L.hr)**	**TOC (mg/L.hr)**
MA Light	2.7±0.1	0.3±0.1	3.6±0.2
UPCD Light	2.8±0.6	0.2±0.1	3.4 ±0.4
PCD Light	2.5±0.6	0.1±0	4.1±0.2
UPCD+MA Light	4.1±0.3	0.5±0	3.9±0.04
PCD+MA Light	3.6±0.6	0.7±0.1	4.0±0.1
MA Dark	3.2±0.4	0.2±0.03	3.5±0.1
UPCD Dark	3.1±0.1	0.2±0.04	4.0±0.2
PCD Dark	3.4±0.4	0.05±0.2	4.0 ±0.1
UPCD+MA Dark	3.6±0.1	0.2±0.1	3.7±0.04
PCD+MA Dark	2.7±0.6	0.1±0.05	3.9±0.3

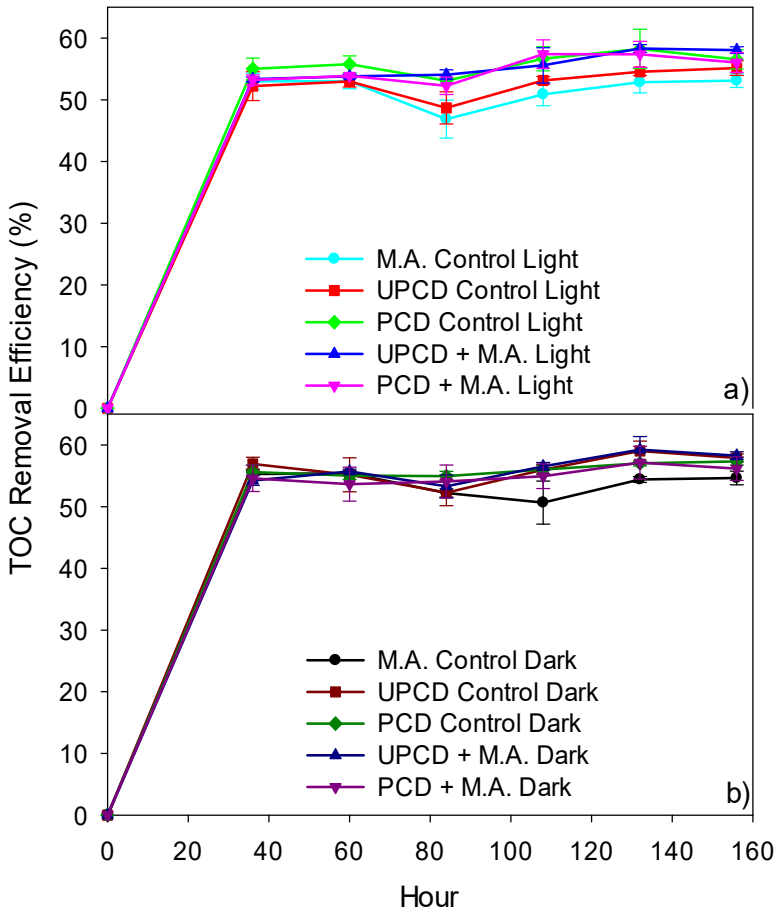

Figure 5.16. Results of 2nd Stage a) TOC removal efficiency light group b) TOC removal efficiency dark group

All in all, considering the removal rates observed in 2nd stage, It can be interpreted that, compared to UPCD, PCD can achieve slightly higher performance in terms of PO_4-P and TOC removal, while UPCD is slightly better in TAN removal in their light group test PBRs. Yet, the results are not enough to support the hypothesis that microalgal-bacterial co-existence improves the microalgal treatment performance in this stage. This might be attributed to the abovementioned phenomena: where an adaptation period that can facilitate the microalgal-bacterial co-existence is required. Hence, to observe the distinguishable nutrient removal performances, this study should be extended, and the co-culture reactors should be operated for a longer

period. The growth performances showed the expected results as PCD+MA Light PBRs exhibited 80% increase in its VS/TS ratio. UPCD+MA Light PBRs, on the other hand, showed higher increase in OD and chlorophyll-a concentration compared to MA Light and PCD+MA Light PBRs. Hence, it can be said that the synergetic effect of PCD and UPCD might have different effects on the bacterial consortia that ultimately affects microalgal growth in different ways (i.e chlorophyll-a production, dry cell mass, OD). Thus, for a stronger conclusion of the comparison of both digestates, further research should be conducted.

Elemental Analysis

As seen in Table 5.13, an elemental analysis was performed on the PBRs at T_{156} to have a better understanding of the real effects of the microalgal-bacterial consortium on the quality of microalgae as a value-added product. Because of the differences in cellular composition between microalgae, UPCD and PCD, microalgae have a higher carbon and hydrogen content. UPCD had more potential to experience a self-shading effect due to its higher OD results (2.0) compared to MA and PCD+MA (1.8). This might be attributed to the lower C% in the cell observed in the elemental analysis, although it exhibited better N and P removal performance (Table 5.13). Wang et al., (2016). suggests that microalgae store nutrient in their mass while ADs convert nutrient. Considering the N and C % results, the final product of the microalgal-bacterial consortium can be suggested for further utilization as a feedstock or for fertilizer production (Wang et al., 2016).

Table 5.13. Elemental (C/H/N/S) analysis of harvested biomass from MA Light, UPCD+MA Light and PCD+MA Light PBRs of 2nd Stage

	C%	**H%**	**N%**	**S%**
MA Light	56±0.7	8±0.3	4±0.1	0.2±0.1
UPCD+MA Light	45±0.3	6±0.1	4±0	0.2±0.1
PCD+MA Light	51±0	7±0.1	5±0.1	0.2±0.1

5.4 CONCLUSION

The aim of this study was to investigate the nutrient removal performance of microalgal treatment of anaerobic digestate, to observe the interaction of the microalgal-bacterial consortia and to assess the overall performance improvement in microalgal growth and nutrient removal due to the presence of the bacterial consortia. Moreover, the effect of pretreated and unpretreated corncob feed on the overall microalgal-bacterial consortium activity was aimed to be observed via differences in growth and nutrient removal performance in microalgae-bacteria mixed cultures.

- It was revealed that a two-stage nutrient loading improves the nutrient removal efficiencies as well as growth performances of all sole cultures (control) and co-cultures. Thus, this indicates an adaptation period that the cultures went through under the studied conditions.
- It was observed that for N and P removal performance, growth performance and chlorophyll-a production, microalgal-bacterial consortium created a positive synergetic effect on the algal culture.
- It was demonstrated that, within the systems where microalgae can be implemented as a complementary (tertiary) treatment, complete N and P removal can be possible to achieve.
- The effect of pretreated and unpretreated corncob revealed itself on OD and chlorophyll-a results. Unpretreated corncob anaerobic digestate was slightly advanced according to the observed OD and chlorophyll-a increase. Moreover, for P removal and TOC removal efficiencies, UPCD came forward.
- According to the elemental analysis results, the microalgae harvested from mixed PBRs (with AD) can be used as feed or fertilizer.
- Overall, the nutrient removal and microalgal growth performance was demonstrated to be improved via a microalgal-bacterial consortium after adaptation of the cultures to the operational and environmental conditions.

In Figure 5.17, a microalgal system was proposed to be implemented in Afyon Energy Power Plant, as a complementary system, where the digestates were obtained for Çelik-Çağlar 's study (2021) and in turn for this thesis. As seen in Figure 5.17 , a microalgal system receiving the effluent of the digesters would be beneficial in the treatment of nutrients as it delivers complete TAN and P removal. Such a system would display higher removal rates compared to the sole microalgal systems as well as an energy plant without a microalgal system. In addition to that, microalgae can be utilized later as a value-added product for biofuel production as it meets the criteria to be processed for biofuel production purposes.

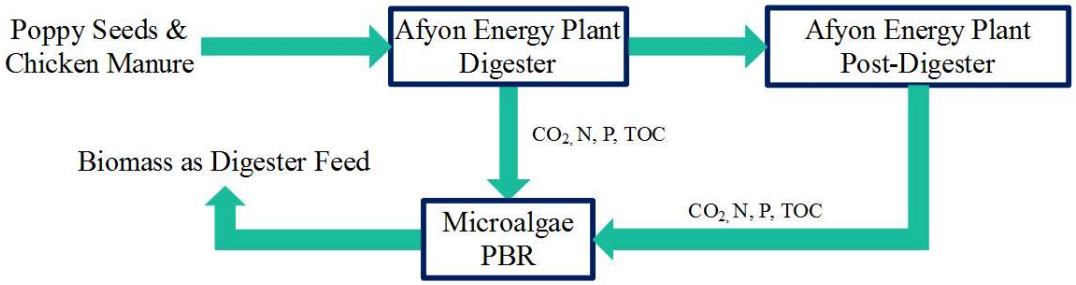

Figure 5.17. A schematic diagram representing the complementary system with Afyon Energy Plant and Microalgae

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

This study aims to determine the optimum operational and environmental conditions for *C. Vulgaris* under autotrophic and mixotrophic conditions. In addition, the purpose of this study was to see how a bacterial consortium would affect the nutrient removal and growth performance of a *C. Vulgaris* culture supplied with an original anaerobic digestate. The substrates of the anaerobic digestate were chicken manure, poppy seeds, and corncob.

- *C. Vulgaris* was enriched in an NH_4-containing medium under autotrophic conditions to allow for better growth performance and better acclimation to original wastewater treatment compared to the culture enriched in NO_3-containing medium.

- The effect of illumination periods of 24:0, 12:12, 8:8:8, and 6:6:6:6:6 (L:D) was investigated. This study was the first conducted in the literature.
    - The results revealed that continuous illumination was necessary for an optimal nutrient removal performance as well as the highest β-carotene production.
    - A phosphorus accumulation problem was encountered and speculated to be emerging from the EPS release due to the stress caused by intermittent illumination.

- The ideal N:P ratio was investigated for *C. Vulgaris* culture under autotrophic conditions.
    - The optimum N:P ratio was determined to be 8 (g/g) with complete TAN removal and maximum P removal efficiency (80%).
    - It was proved that although UTEX recommends the 3-N BBM+ Vitamins medium with N:P of 2.3 (g/g), for an optimal nutrient removal

performance and a responsive and flexible system, N:P 2.3 should not applied.

- P accumulation was observed, and a starvation period was attempted to overcome this event and to stimulate the "Luxury uptake" phenomenon, which can assimilate excess P.
- In the starvation period, the reactor operated with N:P of 8 was more responsive compared to others.

- A mixotrophic *C. Vulgaris* culture was aimed to be enriched. It was also intended to research and determine the optimum HRT, NLR, PLR, and OLR under mixotrophic conditions.
    - With inlet concentrations of 120 mg N/L and 15 mg P/L, the average removal efficiencies of TAN and P were almost 100% and 30%, respectively.
    - With inlet concentrations of 64 mg N/L and 8 mg P/L, the TAN and P removal efficiencies reached almost 100%.

- Mixotrophic growth conditions proved to be better in comparison to autotrophic conditions in terms of both growth performance and treatment efficiencies.
    - The buffer of HAc-NaOAC with equimolar concentrations, was tested in *C. Vulgaris* culture for the first time in the literature.
    - It was observed that the buffer works for an initial pH of 4.5-5.5 in keeping the pH steady. However, in this initial pH range microalgae were negatively affected. Hence, utilization of this buffer may not be proper for biological systems.

- HRT optimization demonstrated its significance as one of the most important parameters determining nutrient removal efficiencies and growth performance of *C.Vulgaris* with minimal stress and wash-out possibilities.
    - The 4-day HRT was determined to be the optimum HRT demonstrating complete nutrient removal efficiency at constant loading rate of 8 mg N/L.d

NLR, 1 mg P/L.d PLR and 62.5 mg sCOD/L.d OLR. The nutrient removal performance was close to that of 8-day HRT, yet much higher than that of 2-day HRT.

- It exhibited lower growth performance compared to 8-day HRT and higher growth performance compared to 2-day HRT. Yet, the level of growth was comparable to the literature studies. Hence it became preferable since the system is faster and self-shading problems can be avoided.

- The NLR, PLR, and OLR were increased to the point where *C. Vulgaris* was aimed to achieve a certain level of nutrient removal performance, and the limits were established. The optimal loading rates were determined to be 8 mg/L.d, 1 mg/L.d, and 62.5 mg/L.d, NLR, PLR, and OLR (as sCOD), respectively at constant 4-day HRT. Increasing NLR and PLR negatively affected the nutrient removal efficiency.
  - Increasing NLR and PLR in mixotrophic conditions demonstrated the distinction between autotrophic and mixotrophic mechanisms.
  - The *C. Vulgaris* culture in this study revealed that the dominant mechanism is autotrophic growth mechanism.

- The synergetic effect of microalgae-bacteria consortium was investigated.
  - It was found that microalgae-bacterial co-existence have a positive synergetic effect on the algal culture in terms of N and P removal performance, growth performance, and chlorophyll-a production.
  - The effect of pretreated and unpretreated corncob on OD and chlorophyll-a results was revealed. According to the observed OD and chlorophyll-a increase, unpretreated corncob anaerobic digestate was slightly better.
  - Overall, the use of a microalgae-bacteria consortium improved nutrient removal and microalgal growth performance. As a result, discharge standards within the system can be met where microalgal systems can be implemented in energy plants to provide complete removal of TAN and P.

RECOMMENDATIONS

The investigation of intermittent illumination should be researched further to understand the true effect of stress and the mechanisms behind it. Although as a first step this study exhibited many useful results, the EPS analysis can be beneficial to understand the P release in the medium as well as the effect of stress. Moreover, for the long-term effect of the illumination period, a semi-continuous study can be performed with the same conditions and thus, the change in the chlorophyll-a, -b,-c and β-Carotene concentration would be observed in detail.

The microalgal-bacterial consortium development was investigated. A phylogenetic analysis can be performed, in order to observe a complete co-culture formation and thus the change in the microalgae-bacteria consortium could be well defined. Furthermore, understanding the content of the bacterial consortium would not be adequate, but also plant growth mechanism should be well understood for further manipulations in the application. For this purpose, indole 3- acetic acid can be analysed. Additionally, for a long-term observation of the synergetic effect of microalgal-bacterial consortium, a semi-continuous system could be operated.

Moreover, an extensive study should be performed to identify complete hydrolysed form of lignin, cellulose, and hemi-cellulose content in UPCD and PCD, those were fed with pretreated and unpretreated corncob. Hence, the true effect of pre-treatment of corncob, which affected the bacterial consortium, and the heterotrophic activity of microalgae can be detected more accurately.

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APPENDICES

A. Gerhardt-TAN Calibration Curve

The low and high range calibration curves obtained from the calibration using the NH_4Cl stock solution in the TAN analyses, performed with the distillation unit (Gerhardt Vapodest 40), are shown in Figure A.1. and Figure A.2., respectively.

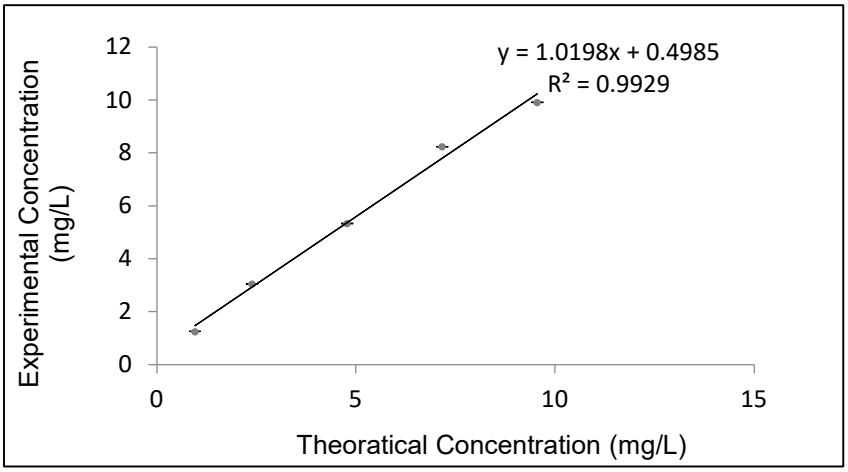


Figure. A 1. Low Range TAN Calibration

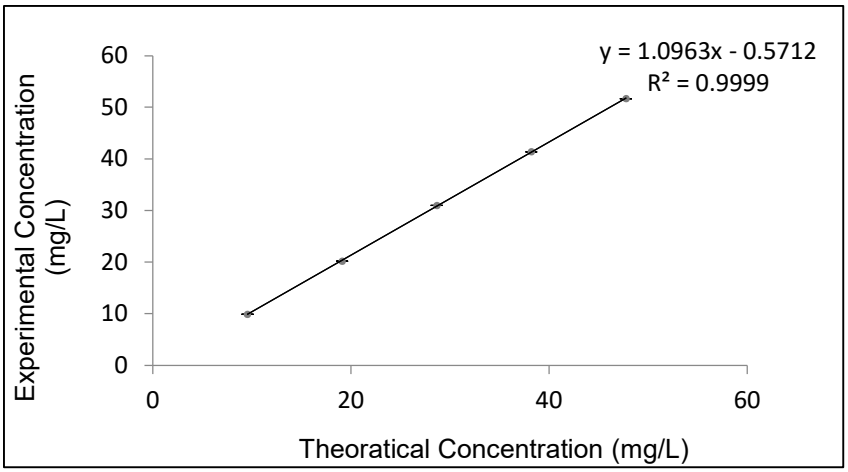


Figure. A 2. High range TAN calibration

B. IC (Thermo Scientific)-PO_4 Calibration Curve

The high range and the low range calibration curves of IC (Thermo Scientific) are shown in Figure B.1 and B.2. According to the results, LOD (limit of detection) of the machine gives LOD 0.0382 mg/L while LOQ gives 0.1159 mg/L PO_4 concentration.

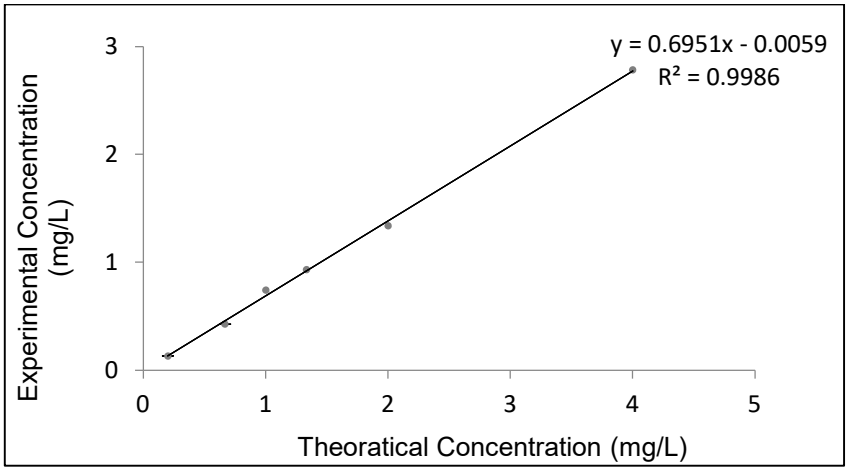


Figure B. 1. Low range PO4 calibration in IC

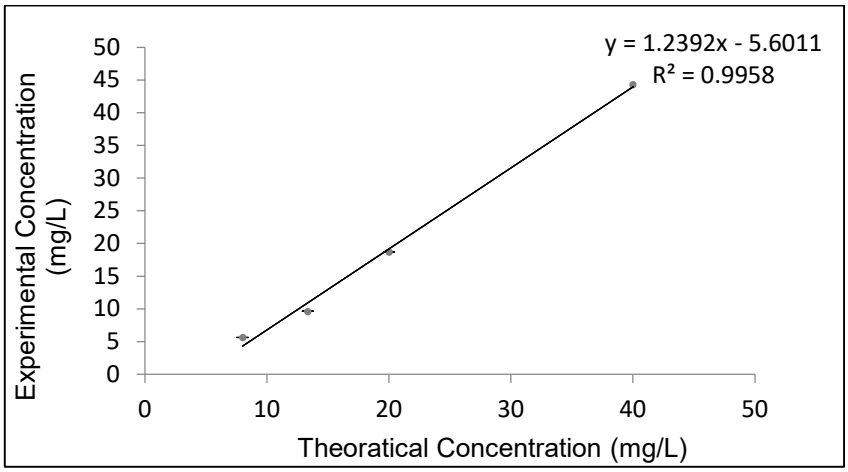


Figure B. 2. High range PO_4 calibration in IC

C. Liquid and Solid Medium Cultivation of *Chlorella vulgaris*

Figure C. 1. Enrichment of *C. Vulgaris* in solid medium

Figure C. 2. Enrichment of *C. Vulgaris* in liquid medium

D. Phosphorus Removal Calculation for Autotrophic Metabolism of *C. Vulgaris*

The CO2 dissolved in water can be calculated with Henry's Constant. Under normal conditions, Henry's Law applies that the amount of dissolved gas in a liquid is proportional to its partial pressure in the headspace above the liquid (Herman, 2017).

The headspace of the PBRs is simply the atmospheric pressure since the PBRs were operated as open systems. Hence,

$P_{CO} = X_{CO2} H_{CO}$

$P_{CO2} = Partial\ pressure\ of\ CO2\ in\ air (0.0004\ atm)$

$H_{CO} = 0.0334\ moles/L.atm$ (at 25 ºC)

$X_{CO} = 0.00001$

The CO_2 given to one PBR in a day can be calculated by multiplying the molar concentration of CO_2 (calculated above), flow of air given to the PBR daily (L/min) and duration of aeration is given (min).

$Q_{air} = 0.4\ L/min$

$Q_{air} = Time\ duration\ air\ is\ supplied\ (daily = 1440\ min)$

$X_{CO2,daily} = X_{CO}\ Q_{air} T_{CO}$

$X_{CO2,daily} = 0.0077\ moles/day$

After calculating the CO2 mole concentration given daily (0.0077 moles/day), the addition of inorganic carbon as buffer ($NaHCO_3$) can be calculated.

$$M_{NaHC} = 0.005\ moles/L.day$$

$$M_{NaHCO3} = 0.05\ moles/L.day\ V_{reactor} = 0.05\ (0.8\ L)$$

$$M_{NaHCO3} = 0.04\ moles/day$$

As photoautotrophic metabolism of *C. Vulgaris* states for both 1 mole of HCO_3 or CO2 sources, 0.01 moles of P can be consumed.

$106HCO_3^- + 16NH_4^+ + HPO_4^{-2} + 92H_2O \Longrightarrow C_{106}H_{263}O_{110}N_{16}P + 92OH^- + 106O_2$

$100CO_2 + 12NH_4^+ + 70H_2O + H_2PO_4 \rightarrow CH_{178}O_{36}N_{12}P + 118O_2 + 11H^+$

Thus, 0.0077 moles CO2 and 0.04 moles HCO_3 may lead to removal of 0.000477 moles of P consumes at the total. Hence, the maximum theoretical consumption of P can 0.00048 moles/day, considering that all inorganic carbon supplied is totally consumed.

E. Preliminary Study: Enrichment of Semi-Continuous *Chlorella vulgaris* with Halved NLR and PLR

In this preliminary study, an autotrophic *C. Vulgaris* culture was operated in semi-continuous mode. One PBR was set with a total volume of 1L and effective volume of 800 mL and it was operated with the same environmental conditions as Set 4 operated (Section 3.3.4). PBR was fed with initial concentrations of 64 mg/L TAN and 8 mg/L P, and the N: P ratio was maintained at 8, as it was determined in Section 3.3.4. As can be seen in Figure E.1., *C. Vulgaris* PBR reached to almost 100% of the removal efficiency for both TAN and PO_4^{-3}-P.

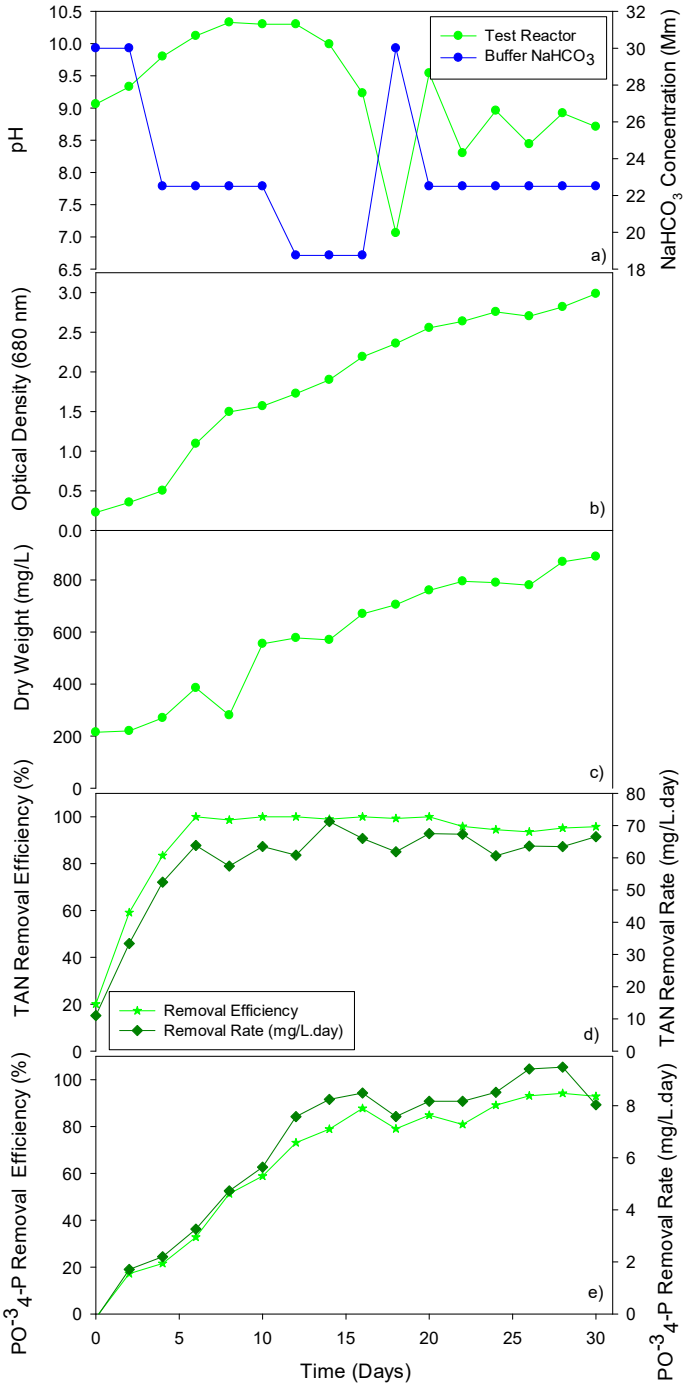


Figure E. 1. Results of preliminary study for growth and nutrient removal performances

F. Nessler-Calibration Curve

The calibration curve obtained using NH_4Cl stock solution in NH_3-N analysis performed with the Nessler Method (HACH) is shown in Figure F.1. LOD and LOQ for ammonia measurements using the Nessler method are $3.12*10^{-17}$ and $9.48*10^{-17}$ mg/L TAN.

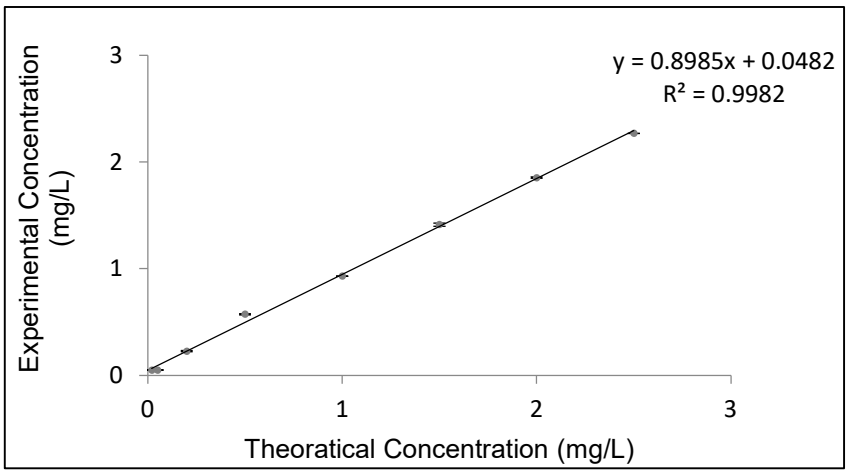


Figure F. 1. Nessler-TAN Calibration Curve

G. IC (Shimadzu)- PO_4, NO_2 and NO_3 Calibrations

The PO4 Calibration performed in IC (Shimadzu) is shown in Figure F.1. The LOD was calculated as 0.024 while LOQ was found to be 0.072 mg/L PO_4.

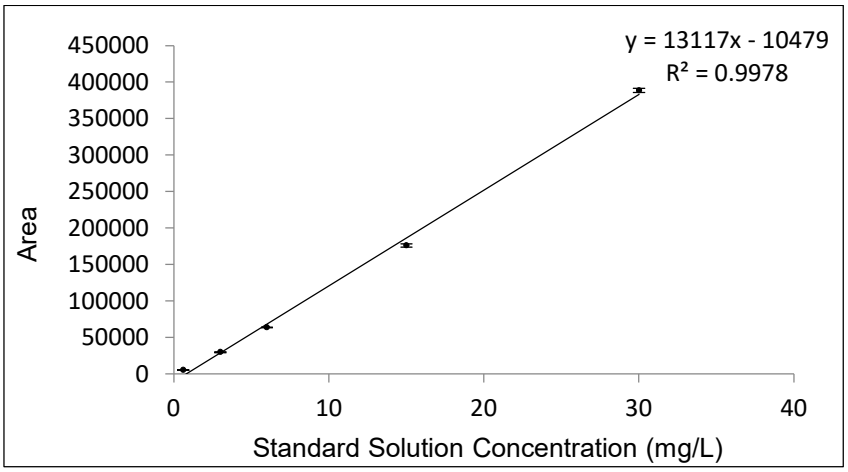


Figure G. 1. PO_4 Calibration in IC (Shimadzu)

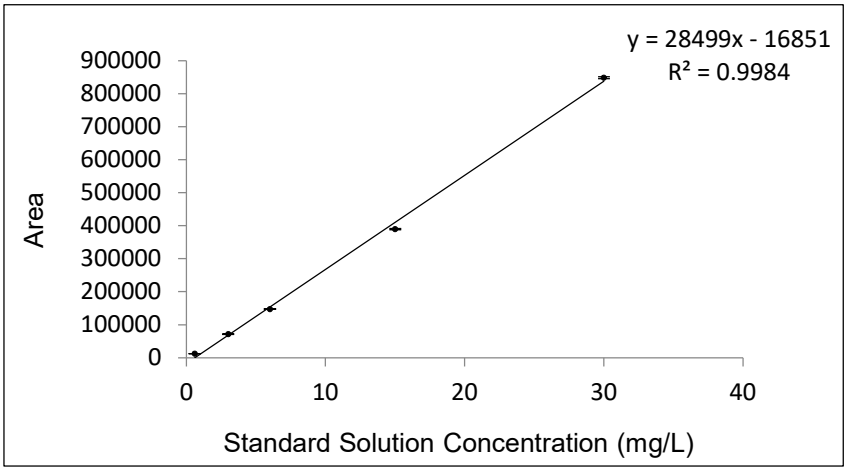


Figure G. 2. NO_3 Calibration in IC (Shimadzu)

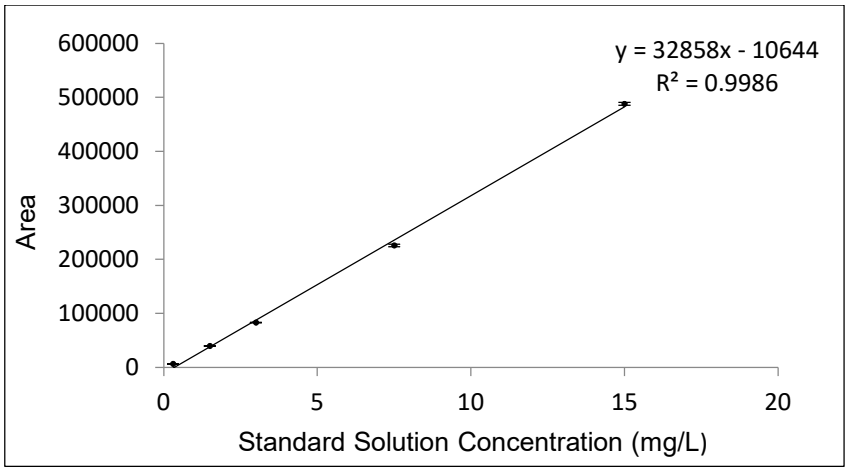


Figure G.3. NO_2 Calibration in IC (Shimadzu)

H. Lovibond-PO4 Tablet Kit Calibration

The PO4 tablet kit calibration was performed, and the results are presented in Figure H.1.

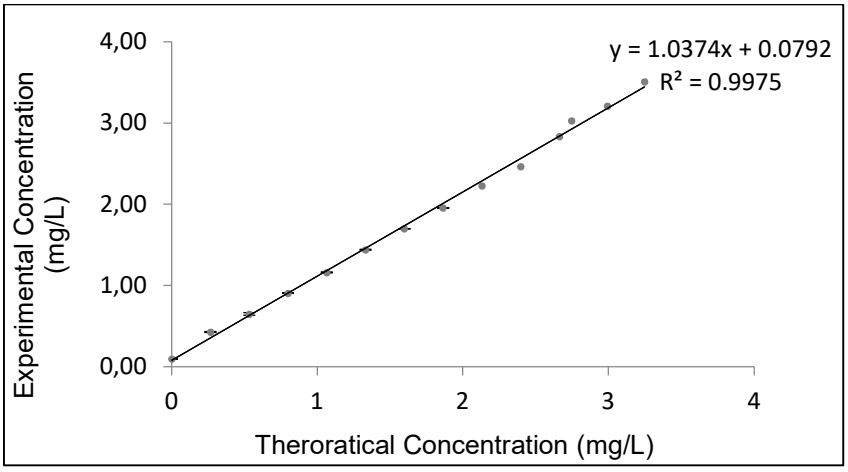


Figure H. 1. PO4 Calibration in Lovibond Tablet Kit (Shimadzu)

I. TOC (Shimadzu)- Total Organic Carbon Calibration

The TOC calibration results are shown in Figure H.1. According to the calculations, LOD is 0.02 mg/L and LOQ is 0.07 mg/L TOC.

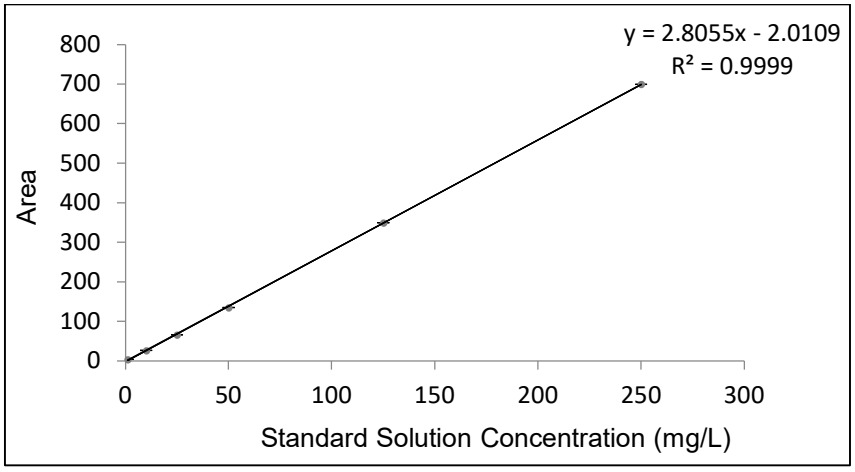
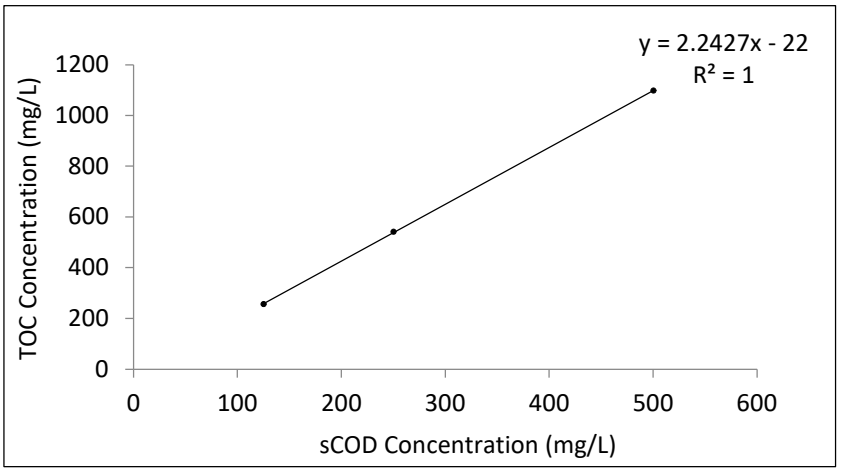


Figure I. 1. TOC Calibration in TOC Machine (Shimadzu)



A correlation between TOC and sCOD was performed as shown in Figure I.2. The following equation was formulated as shown below.

sCOD=2.24(TOC) -22

J. Enrichment of New Culture After the Contamination of *Lecane Inermis* (Prior to Set 7)

Due to the contamination of the cultures with Rotifer *Lecane inermis* contamination encountered in Section 4.3.3., Set 6, a new *C. Vulgaris* culture was obtained from, İstanbul Microalgae Biotechnologies Research and Development Centre both in agar plates and liquid media.

To enrich the new culture in a healthy way and to increase contamination measures, the set stand was taken to an isolated room and the entrances to the room were restricted. In the first stage, to ensure the continuity of the culture, *C. Vulgaris* cells from solid media are transferred to agar plates that are prepared with vitamin-enriched 3-layer Bold's Basal Medium (Aghajanian, 1979) recommended by "The UTEX Algae Culture Collection Center of Algae". Accordingly, the solid medium was operated for 20 days. 3 klux lightning with continuous red LED lighs was provided for all agars continuously. The temperature was kept at 20±3°C (Kendirlioğlu and Çetin, 2017) (Figure J.1).

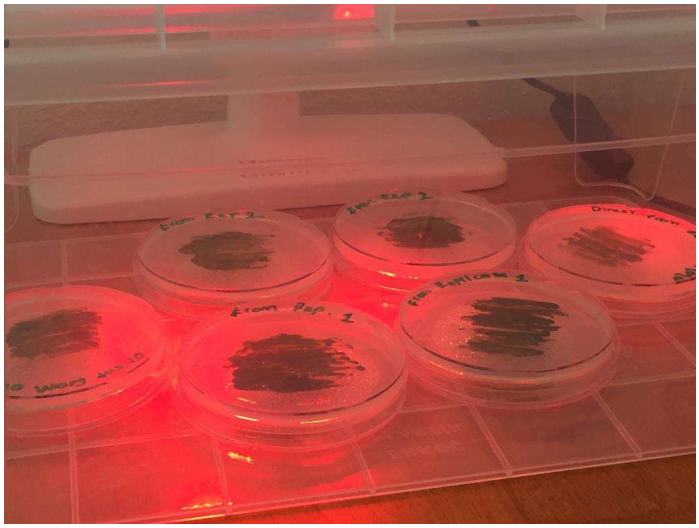


Figure J.1. Re-enriched *C. Vulgaris* culture on agar plates

Later, six PBRs named as LB-1, LB-2, MB-1, MB-2, PB-1, PB-2 were set in batch mode in 250 mL PBRs with 150 mL working volume. Three different mediums were used in the enrichment of the new culture. The media are named as LB (Loading BBM), MB (Mixotrophic BBM), and PB (Photoautotrophic BBM) and the related details are presented in Table J.1. LB stands for the medium that has five-day loading of nitrogen, phosphorus and organic concentrations, and the daily loading rates were set as NLR: 8 mg/L.d, PLR:1 mg/L.d and OLR:62.5 mg/L.d. PB stands for 3N-BBM+ Vitamins medium (As it was described in Table 3.1, Section 3.2.3.). MB medium, on the other hand, was designed as a mixotrophic version of the 3N-BBM + Vitamins medium containing 1000 mg/L sCOD. Again, in these media, the NaNO3 was replaced with equimolar NH4Cl to adapt the culture to the working conditions of the next experimental set up. PBRs were operated in batch mode for 5 days.

Table J.1. Properties of three different mediums

	Concentration (mg/L)		
Medium	**NH_4-N**	**PO_4-P**	**sCOD**
LB	40	5	312,5
PB	120	52	-
MB	120	52	1000

150 µmol.m^{-2}.s^{-1}(150 PAR) lighting with 18 W cool-white florescent lamps (OSRAM, L 18W/685) was provided for 12:12 light: dark (L:D) illumination period for all PBRs (Kılıç, 2017; Wang and Huang, 2005) as UTEX recommended for newly enriched cultures. The pH value of the inlet medium (synthetic wastewater) was set as 6.8 ±3 for MB and PB PBRs and 5.8 ±3 for LB PBRs (Ma et al., 2017). The ambient temperature of the system was set to the optimum 25±3°C. To keep the PBRs free of contamination at the beginning, no air was supplied. Instead, the diffusion of air was provided with magnetic stirrers at 100 rpm mixing.

Figure J.2. Enrichment of the new culture in 250 mL Erlenmeyer Flasks

From the LB, PB and MB PBRs, which were enriched for 7 days with all contamination precautions taken, in the first stage, the PB PBRs were bleached. MB PBRs, on the other hand, could not show full development, but the OD value of LB PBRs increased to about 0.3. Hence, LB PBRs were selected to be used in Section 4.3.3, Set 7. The LB PBRs were observed under the microscope for contamination and no foreign species were found. At this point, three LB PBRs were re-inoculated into a similar medium and the PBRs were multiplied.

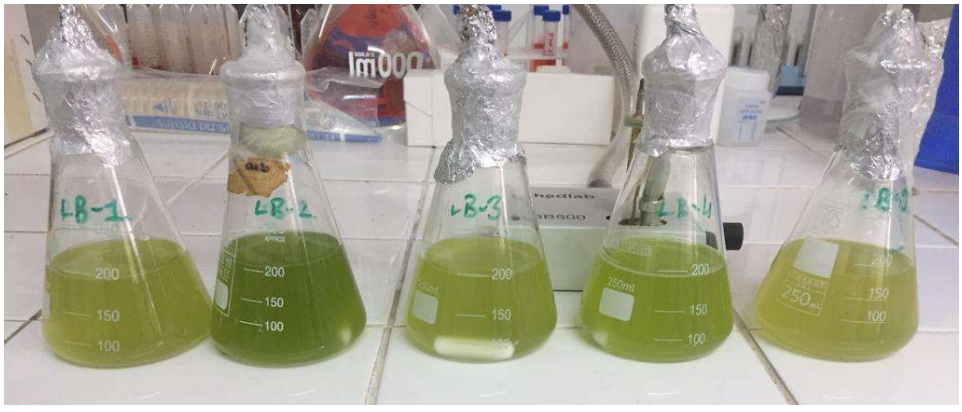


Figure J.3. Multiplied LB PBRs

K. Organic and Inorganic Carbon Adequacy Calculations

To track down inorganic and organic carbon through autotrophic and heterotrophic processes for the determination of dominant metabolism, firstly an elemental analysis for the algal culture was performed in Central Laboratory, METU as it is seen in the Table K.1.

Table K.1. Elemental Composition of Microalgal Culture from LR-1 and LR-2

	C (%)	**N (%)**	**H (%)**	**S (%)**
LR-1	48.34	6.61	9.46	0.33
	47.64	6.58	8.87	0.27
	47.85	6.56	9.24	0.76
LR-2	45.43	6.43	9.39	0.49
	46.48	6.60	9.02	0.76
	47.75	6.67	9.65	0.29

According to the results obtained from the elemental analysis, C/N ratio of the mass formula of *C. Vulgaris* As the formula given below

$$\frac{\%C}{MW_{Carbon}} = X_{Carbon}$$

$$\frac{\%47.94}{12\ g/mole} = 3.99$$

$$\frac{\%N}{MW_{Carbon}} = X_{Nitrogen}$$

$$\frac{\%6.58}{14\ g/mole} = 0.47$$

X: Mole fraction of the element

MW: Molecular weight of the element (g/mole)

Thus;

$$\frac{C}{N} = \frac{3.99}{0.47} = 8.49$$

According to the result of the C/N (a/b, in Equations K.1 and K.2) ratio, from the consumed organic and inorganic carbon, the consumed nitrogen can be found for both autotrophic and heterotrophic metabolism. However, the entire empirical formulas cannot be calculated using Equation K.1 and K.2, as no results for P and O were sustained in the analysis.

Autotrophic Metabolism:

$8.5HCO_3 + 1NH_4 + dHPO_4 + cH_2O \rightarrow C_aH_dO_eN_bP + cOH + fO_2$ ……

……...Equation K.1.

Heterotrophic Metabolism:

$8.5CH3COO^- + 1NH_4 + dHPO_4 + cH + aO_2 \rightarrow C_aH_dO_eN_bP + aH_2O + fCO_2$

……...……...… Equation K.2.

Table K.2. gives the resultant NH_4, inorganic carbon (alkalinity) and organic carbon consumption by molarity for the PBRs, namely, LR1 and LR2.

Table K.2. Experimental Results of the Total Consumed Molarities (M) of Each Species

	LR1	LR2
NH_4^+	0.00174	0.00169
HPO_4	0.00006	0.00006
HCO_3	0.0016	0.0018
$CH3COO^-$	0.0040	0.0040

The available $NH4^+$ molarity can be calculated over the HCO_3 and $CH3COO^-$ amounts supplied to the reactors, as can be seen below;

$X_{CO2,daily} = 0.010\ M$

$X_{HCO3,daily} = 0.006\ M$

Hence, total inorganic C given to the system is 0.016 M in a day

$X_{orgC,daily} = 0.009\ M$

Hence, total organic C given to the system 0.009 M in a day

Autotrophic mechanism,

$$\frac{Molarity\ of\ Given\ Inorganic\ Carbon}{Molarity\ of\ Available\ NH4} = 8.5$$

$$\frac{0.016\ M}{Molarity\ of\ Available\ NH4} = 8.5$$

$$Molarity\ of\ Available\ NH4\ with\ inorganic\ C = 0.0019\ M$$

Heterotrophic mechanism,

$$\frac{Molarity\ of\ Given\ Organic\ Carbon}{Molarity\ of\ Available\ \ NH4} = 8.5$$

$$\frac{0.009\ M}{Molarity\ of\ Available\ NH4} = 8.5$$

$$Molarity\ of\ Available\ NH4\ with\ organic\ C = 0.0011\ M$$

So total Molarity of NH4 in both mechanisms is:

$$Molarity\ of\ Total\ Available\ NH4, theoratically = 0.0030\ M$$

$$Molarity\ of\ Total\ Consumed\ NH4, experimentally = 0.0017\ M$$

Hence, the total C given to the system is not limited. However, less NH4 (0.0017 M) than calculated (0.0030 M) was consumed in the system.

Thus, if the calculation was performed over consumed alkalinity (HCO3) (0.0016M) together with supplied CO2 (0.01 M) and consumed TOC (0.004 M), the amounts of NH4 to be consumed are calculated as follows.

Autotrophic mechanism,

$$\frac{Molarity\ of\ total\ Inorganic\ Carbon}{Molarity\ of\ Consumed\ NH4} = 8.5$$

$$\frac{0.0116\ M}{Molarity\ of\ Consumed\ NH4} = 8.5$$

$$Molarity\ of\ Consumed\ NH4\ with\ inorganic\ C = 0.0014\ M$$

Heterotrophic mechanism,

$$\frac{Molarity\ of\ Consumed\ Organic\ Carbon}{Molarity\ of\ Consumed\ NH4} = 8.5$$

$$\frac{0.004\ M}{Molarity\ of\ Consumed\ NH4} = 8.5$$

$$Molarity\ of\ Consumed\ NH4\ with\ organic\ C = 0.0005\ M$$

$$Molarity\ of\ Total\ Consumed\ NH4 = 0.0019\ M = 1.9\ mM$$

Total amount of ammonium consumed in the PBRs is almost equal to the expected amount to be consumed when the carbon sources considered are the consumed alkalinity (HCO3), consumed TOC (organic carbon) and all daily supplied CO2.

L. Results For NO_2-N And NO_3-N

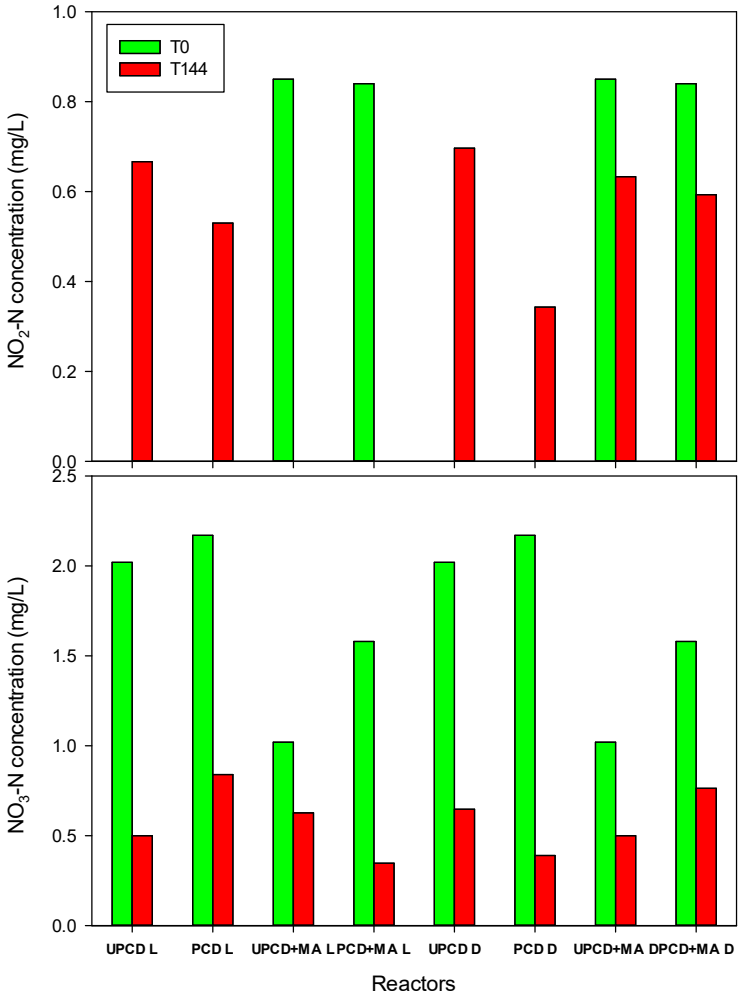


Figure J. 2. The NO_2-N and NO_3-N change in concentrations at 1st Stage

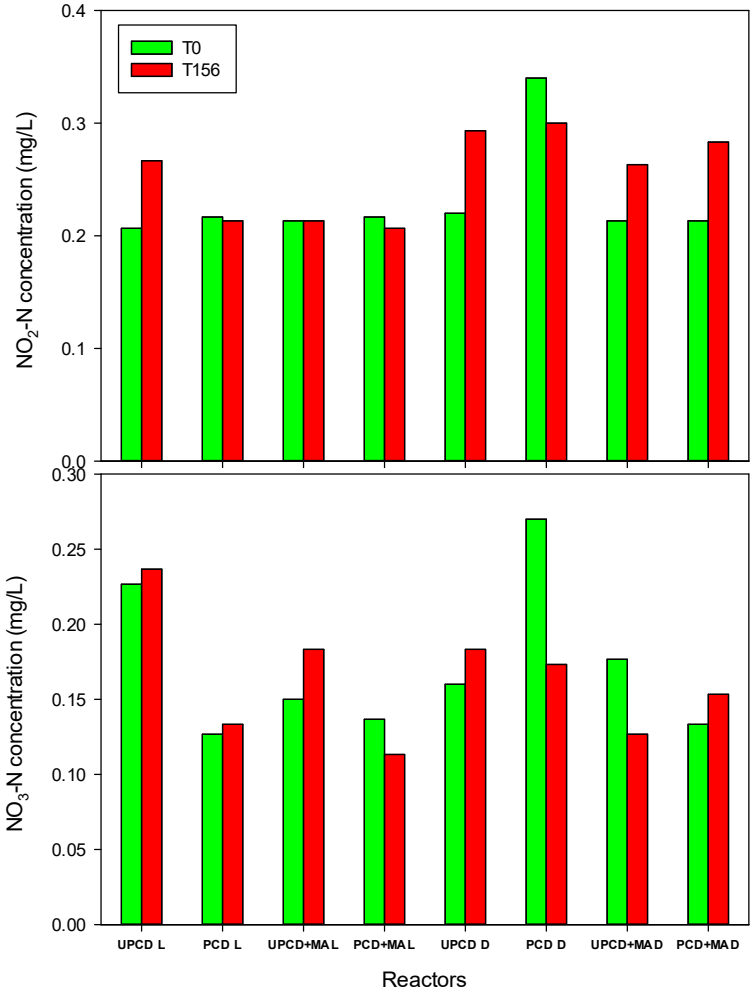


Figure J. 3. The NO2-N and NO3-N change in concentrations at 2nd Stage

M. Ammonia Stripping Calculation

To determine the highest possible ammonia concentration in soluble form and the maximum TAN concentration that can be removed by stripping in Chapter 3 and Chapter 4, Equation M.1 and M.2 was performed according to the calculation described in Anthonisen et al. (1976), the highest inlet TAN concentration, the highest pH value and temperature values obtained during operation were considered. was taken, and accordingly, it was calculated that 15% of the TAN concentration was converted from the $NH4^+$ (ammonium) form to the NH_3 (ammonia) form when the initial TAN concentration was 100 mg/L, the pH was 8.4, and the temperature was 25°C.

$K_b/K_w = e^{(6344/(273+T(°C))}$ …….…… (Equation M.1)

NH_3-N (mg/L) = (TAN (mg/L) x 10^{pH}) / (Kb/Kw+10^{pH}) …… (Equation M.2)

Subsequently, the concentration of the ammonia compound in the air at equilibrium was calculated using Henry's constant (H: 0.0161 atm.L/mol, 25°C, Nazaroff and Alvarez-Cohen, 2001). As a result, it was determined that only 0.07% of ammonia dissolved in water could be in the gas phase to be stripped out.

For Chapter 3:

Kb/Kw= $e^{(6344/(273+T(°C))}$ = 1760019286.24

NH3-N (mg/L) = (120 (mg/L) x $10^{10.3}$) / (1760019286.24+$10^{10.3}$)

NH_3-N (mg/L) =91 mg/L

Stripping to the air:

$P_{NH3} = X_{NH3} H_{NH}$

$P_{NH3} = Partial\ pressure\ of\ CO2\ in\ air(0.0004\ atm)$

$H_{NH3} = 0.0161\ moles/L.atm$ (at 25 ºC)

$\boldsymbol{X_{NH3} = 5.3}$

Hence,

$P_{NH3} = 0.13\ atm$

And from P.V=n.R.T

n (X_{NH3}, in air)=0.004 mole/L in air which corresponds to 0.07% (X_{NH3} (in liquid)/X_{NH3} (in air))

For Chapter 4:

Kb/Kw= $e^{(6344/(273+25(°C))}$ = 1760019286.24

NH3-N (mg/L) = (100 (mg/L) x $10^{8.4}$) / (1760019286.24+$10^{8.4}$)

NH_3-N (mg/L) =15.2 mg/L

Stripping to the air:

$P_{NH3} = X_{NH3}H_{NH3}$

$P_{NH3} = Partial\ pressure\ of\ CO2\ in\ air(0.0004\ atm)$

$H_{NH3} = 0.0161\ moles/L.atm$ (at 25 ºC)

$\boldsymbol{X_{NH3} = 0.9}$

Hence,

$P_{NH} = 0.014\ atm$

And from P.V=n.R.T,

n (X_{NH3}, in air)=0.0006 mole/L in air which corresponds to 0.07% (X_{NH3} (in liquid)/X_{NH3} (in air)).