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Abstract

The scope of this project is to Study the long-term performance and wastewater nutrients removal efficiency by microalgae in a PBR under no favourable conditions. COD, TN, TP, NH_4^+-N, NO_3^--N, PO_4^{3-}-P, TSS and pH values will be measured on daily basis before and after microalgae treatment to evaluate the removal efficiency and if there exist cause-effect relationships between parameters. The parameters to be evaluated if they have any effect over the nutrient removal efficiency are temperature and HRT.

Results show a low removal efficiency when compared with other studies. In this case, due to the conditions under which the microalgae have been cultivated. The lack of CO_2 bubbling and the presence of inhibitory substances like the high concentration of ammonium in the wastewater could be the main reason.

Flow cytometry has shown to be a remarkable tool to identify changes in the communities and understand the niches of each species and the type of interaction between species in the media. Can be concluded that *C. sorokiniana* and *T. obliquus* are species able to coexist within the same environment without becoming competitors.

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Lars Rune not only helped to collect and bringing raw wastewater to the laboratory. Also, to remember that taking a break and getting out of the laboratory is also necessary. He has shown me my new passion: Surfing!

To all my classmates, especially to the "Master room crew" who always was able to make me laugh, even in hard times. Thank you, guys!

Last, but not least, I would like to thank my family for helping me to make this dream come true. I wish one day I will be able to return the effort you have made.

Abbreviations

COD: Chemical Oxygen Demand

TN: Total Nitrogen

TP: Total phosphorous

NH_4^+-N: Ammonium as nitrogen

NO_3^--N: Nitrate as nitrogen

PO_4^{3-}-P: Phosphate as Phosphorous

HRT: Hydraulic Retention time

PBR: Photobioreactor

TSS: Total Suspended Solids

WWTP: Wastewater Treatment Plant

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1. Introduction

Depletion of Earth's resources is an undeniable problem nowadays. The "use and throw" culture (also known as a linear economy) is facing its extinction and circular economy is the new goal to achieve. Water and phosphorous are critical resources in our economy and wastewater image have changed from waste to valuable resource. Not only water and phosphorus can be recovered from wastewater. Nitrogen and organic matter are compounds that can contribute to strengthening the circular economy. (Abu-Ghunmi, Abu-Ghunmi, Kayal, & Bino, 2016).

All those retrieved compounds can be used not only to close the loop and keep the cycle going, but they can also improve the economic efficiency of WWTP too. Retrieved nitrogen and phosphorous can become fertilizers for our fields and at the same time, the eutrophication effect of rivers and lakes is reduced or avoided. The organic matter can be digested and transformed into biogas as another product obtained from waste. Therefore, by promoting the resources recovery from wastewater we are obtaining economic and environmental benefits (Abu-Ghunmi, Abu-Ghunmi, Kayal, & Bino, 2016).

Microorganism-based technologies are used to reduce the levels of organic matter and nutrients in wastewater secondary treatment. WWT is currently based on the action of heterotrophic and nitrifying bacteria which provide acceptable levels of COD removal but at high operational costs and high CO_2 footprint (Posadas, et al., 2017). However, most of the nutrients remain in the effluent. The digestion process eases the release of nutrients from the biodegradable fraction of the waste. Organic Nitrogen is converted into ammonium and organic Phosphorus is hydrolyzed to soluble Phosphorus. Released nutrients are soluble and tend to form inorganic compounds. Typically, the soluble Phosphorus content in municipally digested wastes ranges from 50 to 500 $mg \cdot L^{-1}$ and Nitrogen concentrations are normally five times higher than soluble Phosphorus (Mehta, Khunjar, Nguyen, Tait, & Batstone, 2015).

These compounds are a valuable product for agriculture, but not for water bodies where they can trigger the eutrophication process. Those nutrients must be removed from the

effluent to avoid it., The European Commission Directive (1998) 98/15/EEC established upper limits for TN of 10 $mg \cdot L^{-1}$ and TP of 1 $mg \cdot L^{-1}$ of effluent nutrient concentrations when discharged into water bodies. However, even when nutrient concentrations are far below the above upper limits, there are still chances of eutrophication, since some species of microalgae were observed to display rapid growth even under TN of 0.28 $mg \cdot L^{-1}$ and TP of 0.01 $mg \cdot L^{-1}$ (Wang, et al., 2017).

Here, algal-bacteria symbiosis can provide a solution. The CO_2 released by bacteria is absorbed by algae, producing O_2 which is used by bacteria to degrade organic matter. Algae also contribute to reducing the concentration of nutrients in wastewater (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2011). However, microalgae can do much more than removing nutrients or consuming CO_2.

Microalgae potential uses have been studied for decades but mainly as a source of biodiesel, but it turned out that their exclusive use for biofuel production is not sustainable and expensive. Recently, the focus has changed to value-added bioproducts, and bioremediation (Asraful Alam & Wang, 2019). Other studies suggest the idea of extra biogas production through fermentation of algae biomass after nutrients have been removed as a more sustainable and economic solution (Collet, et al., 2011).

Wastewater can be an inhospitable environment. Choosing the right species is a key factor to achieve efficient levels of nutrients removal. *Chlorella* species rank among the top 10 best-performing microalgae in highly polluted environments. (De Godos, et al., 2010). For this reason, the species *Chlorella Sorokiniana* was chosen to perform this test.

There are several parameters that can affect algal growth and nutrient removal efficiency. Those parameters include light intensity, pH, temperature, nutrients concentration, CO_2 availability, O_2 concentration and competition by other species. All these values must be monitored to evaluate their effect on the purpose of this study.

The conditions chosen in most of the studies found were as close as possible to ideal conditions or using artificial wastewater. Nevertheless, each wastewater composition is unique and to achieve reliable results for future designs of algal treatment processes,

each case should be studied individually. Only a few studies were found to reproduce real-life conditions. Most of them focus on maximizing algae biomass production. Nearly all the wastewater treatment plants do not operate under those conditions.

1.1. Scope

This dissertation is a step further in the development of a project led by the University of Stavanger and IVAR. The planned process is the following:

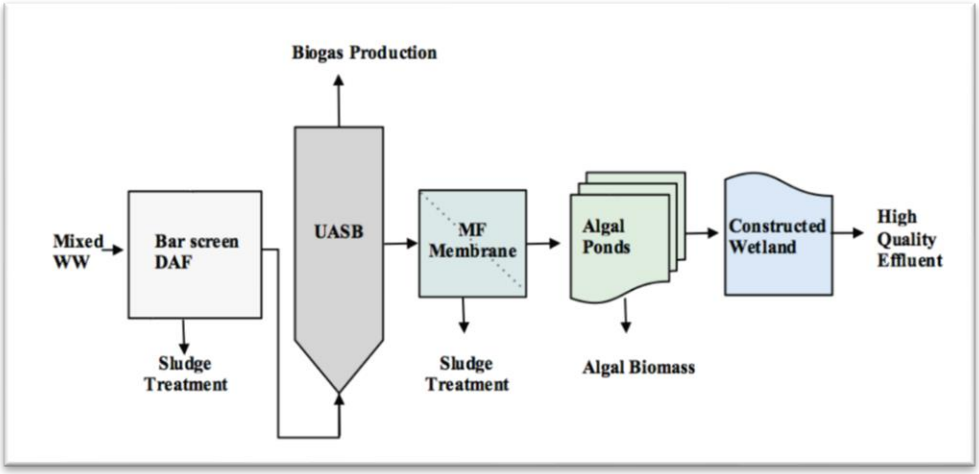

Figure 1-1: Process scheme of the project (Carlsen, 2018)

Wastewater samples were collected from the Wastewater Treatment Plant (WWTP) at Grødaland. Bar screening and dissolved air flotation (DAF) are the first treatment the wastewater undergoes in the WWTP. After preliminary treatment, Up-flow Anaerobic Sludge Blanket Reactor (UASB) and membrane microfiltration (MF) follow as a primary and secondary treatment in the laboratory at the University of Stavanger (Carlsen, 2018). The filtrated UASB effluent, after filtration, will be used as an inlet in the photobioreactor (PBR) for tertiary treatment emulating an algal pond.

1.2. Objectives

Study the long-term performance and wastewater nutrients removal efficiency by microalgae in a PBR under real-life conditions. COD, TN, TP, NH_4^+, NO_3, PO_4^{3-}, TSS and pH values will be measured on daily basis before and after microalgae treatment to evaluate the removal efficiency and if there exist cause-effect relationships between parameters. Alkalinity, VFA and light intensity will be measured also but not on a daily basis.

2. Theoretical Background

2.1. Wastewater

Human activities always produce waste. Some will end as wastewater and the quality and quantity depend on the source (Henze, van Loosdrecht, Ekama, & Brdjanovic, 2008). There are different sources of wastewater:

- Domestic wastewater: Discharged from non-industrial activities (Houses, Schools, shops, etc).
- Industrial wastewater: Wastewater produced in industrial activities.
- Infiltration or inflow: water that eventually enters the sewer from foundation drains, leaking pipes, submerged manholes, and groundwater infiltration.
- Stormwater: runoff from meteorological events (Riffat, 2013).

Depending on the source, wastewater has different characteristics and its main constituents and its consequences on the environment are shown in the following table:

Table 2-1: Wastewater constituents and their effects (Henze, van Loosdrecht, Ekama, & Brdjanovic, 2008)

Constituent	Example	Effects
Microorganisms	Pathogenic bacteria, virus and worms' eggs	Risk when bathing and eating shellfish
Biodegradable organic materials	Oxygen depletion in rivers, lakes and fjords	Fish death, odours
Other organic materials	Detergents, pesticides, fat, oil and grease, colouring, solvents, phenols, cyanide	The toxic effect, aesthetic inconveniences, bioaccumulation in the food chain
Nutrients	Nitrogen, phosphorus, ammonium	Eutrophication, oxygen depletion, toxic effect
Metals	Hg, Pb, Cd, Cr, Cu, Ni	Eutrophication, oxygen depletion, toxic effect
Other inorganic materials	Acids, i.e., hydrogen sulphide, bases	Corrosion, toxic effect
Thermal effects	Hot water	Changing living conditions for flora and fauna
Radioactivity	Hydrogen sulphide	Toxic effect, accumulation

Observing the effects that untreated wastewater may have on the environment, wastewater must undergo the proper treatment before its discharge.

2.2. Wastewater treatment

Each wastewater is different as well as the required treatment. A combination of physical, chemical and biological processes is used depending on the nature of the pollutant:

- Physical treatment: Mainly suspended solids are removed through sedimentation, screening and filtration. (Riffat, 2013)
- Chemical treatment: Enhanced removal of suspended solids by addition of chemicals which produce their coagulation and flocculation. (Tchobanoglous, Burton, & Stensel, 2003).
- Biological treatment: Original microorganisms from wastewater are used to consume and reduce the concentration of biodegradable organic matter and nutrients like nitrogen and phosphorous. (Riffat, 2013).

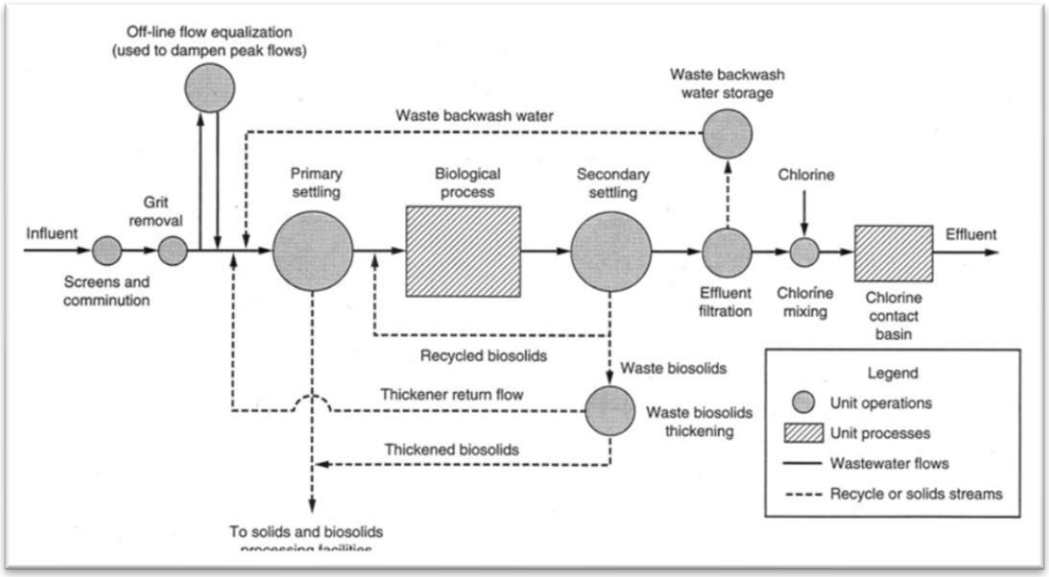

Figure 2-1: Conventional wastewater treatment flow scheme (Tchobanoglous, Burton, & Stensel, 2003).

Different levels of treatment can be achieved through a combination of previous operations and processes:

- Preliminary treatment: Gross solids that may damage equipment are removed.
- Primary treatment: Removal of suspended solids and organic matter through sedimentation.

- Advanced primary treatment: Addition of chemicals to enhance the removal of suspended solids and organic matter.
- Secondary treatment: Most of the organic matter and nutrients are removed via biological and chemical processes.
- Tertiary treatment: Removal of residual suspended solids. Disinfection and nutrient removal are also part of this treatment.
- Advanced: Removal of suspended solids after biological treatment (Tchobanoglous, Burton, & Stensel, 2003).

Flow diagram of standard wastewater treatment is shown in figure 2-1.

2.2.1. Biological treatment in secondary and advanced treatment

It is crucial removing organic matter from wastewater. If wastewater would be discharged into water bodies without treatment, the organic matter will be used as a carbon source by microorganisms. Aerobic microorganisms consume oxygen to oxidize organic matter. This would lead to an increased demand for oxygen, producing the eutrophication of the water body (Riffat, 2013). Wastewater contains different types of microorganisms. Some are pathogenic and need to be removed but other ease the treatment by consuming the organic matter and nutrients present in wastewater transforming it into simple end products and biomass as represented in the following equation (Tchobanoglous, Burton, & Stensel, 2003):

$$Organic\ material + O_2 + NH_3 + PO_4^{-3} \xrightarrow{Microorganisms} Biomass + CO_2 + H_2O$$

2.2.2. Anaerobic wastewater treatment

In terms of COD removal, wastewater anaerobic treatment is a highly effective process. The first step of the anaerobic degradation is the hydrolysis of complex organic material to its basic monomers. These monomers are then fermented to organic acids and hydrogen by the fermenting bacteria (acidogens). The volatile organic acids are transformed into acetate and hydrogen by the acetogenic bacteria. Lately, methanogens use hydrogen and acetic acid produced to produce methane and carbon dioxide (Ersahin, Ozgun, Kaan Dereli, & Ozturk, 2011). The reasons why the anaerobic process is a better choice to reduce COD than aerobic systems are the following:

- 90% less of sludge production
- Capable to work with strong COD load rates
- Energy production (Biogas)
- No chemicals required
- Plain technology with high efficiency
- No aeration required. (Henze, van Loosdrecht, Ekama, & Brdjanovic, 2008).

2.2.3. Advanced treatment

Secondary treatment effluent may meet mandatory requirements for discharge, making necessary additional treatment to reduce the level of contaminants. This additional treatment is defined as a process needed to remove suspended, colloidal, dissolved advanced nutrients such as nitrogen and phosphorus, specific heavy metals or inorganics and removal of emerging contaminants remaining on the secondary treatment effluent. (Tchobanoglous, Burton, & Stensel, 2003) (Riffat, 2013).

2.2.4. Microalgae based advanced treatment

Microalgae-bacteria consortia are capable to remove nutrients while producing biomass. During photosynthesis, microalgae release oxygen, which is used by the aerobic bacteria to degrade organic matter into CO_2, soluble phosphorus and different inorganic nitrogen sources. Then, microalgae uptake CO_2 and solubilized macro- and micronutrients to grow, resulting in a clean effluent and valuable biomass. Thus, in comparison to conventional wastewater treatment plants, the use of microalgae-bacteria consortia for wastewater treatment presents the advantage of the nutrient recovery. The wastewater composition affects nutrient uptake, existing an optimal C:N:P ratio, different for each species. Nutrient uptake also depends on environmental factors such as pH, temperature, light intensity, turbidity and watercolour, among others. The main genera present in photobioreactors used for wastewater bioremediation are *Chlorella* and *Scenedesmus*. In many occasions, a consortium of different microalgae is used to treat wastewater but depending on the characteristics of each wastewater, some genera grow and become the dominating species (Asraful Alam & Wang, 2019).

2.2.4.1. Nitrogen removal

Nitrogen compounds in wastewater come from mineralization of larger molecules like

proteins and urea (Riffat, 2013). The removal of nitrogen species is carried out by nitrifying bacteria (Riffat, 2013) or by assimilation carried out by microalgae. (Barsanti & Gualtieri, 2014). When organic matter is degraded, organic compounds are broken down into inorganic compounds like NH_3 or NH_4^+. Both species remain in equilibrium according to the following equation:

$$NH_4^+ \leftrightarrow NH_3 + H^+$$

pH and temperature affect this equilibrium. Higher pH values and temperature tend to lead the equilibrium to the right side of the equation (higher NH_3 concentration) and part of total nitrogen can be lost as NH_3 volatilization. NH_4^+ may also be removed as N_2 through the nitrification-denitrification process (Wang, et al., 2017). The different mechanisms for nitrogen removal are shown in figure 2-2.

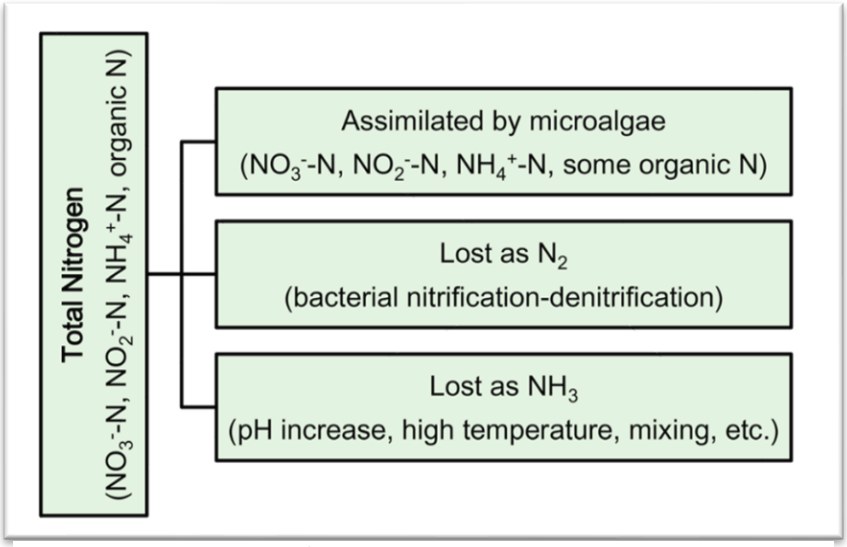

Figure 2-2: Mechanisms of nitrogen removal in microalgae-based advanced municipal wastewater treatment (Wang, et al., 2017).

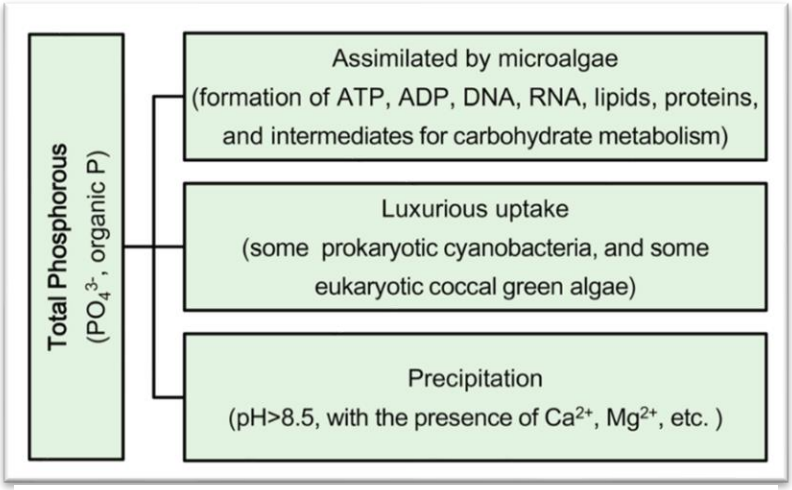

Figure 2-3: Mechanisms of phosphorus removal in microalgae-based advanced wastewater treatment (Wang, et al., 2017).

2.2.4.2. Phosphorus removal

Traditionally, phosphorus is removed through precipitation by its incorporation into biological suspended solids (microorganisms) or chemicals (metallic ions). Chemical precipitation of phosphate is necessary when previous treatment stages cannot achieve the required discharge criteria (Sedlak, 1991). Phosphorus as orthophosphate PO_4^{-3}-P is the most common form in wastewater and often is the only assimilable by microalgae. (Wang, et al., 2017). Phosphorus is used by microalgae for energy production and is a major component in proteins, lipids and DNA/RNA. (Barsanti & Gualtieri, 2014). Microalgae can remove more phosphorus by luxurious uptake: Phosphorus is stored in the cell as polyphosphate granules (Larsdotter, 2006). The different mechanisms for phosphorus removal are shown in figure 2-3.

2.2.5. Factors affecting nutrient removal in microalgae-based wastewater treatment

Factors affecting microalgae-based treatment can be divided into three categories:

- **Intrinsic factors:** The chosen microalgae strain should have specific properties to be a suitable candidate. These properties could be classified into three levels: necessary, basic and additional. For necessary properties, the strain must have (1) fast growth rate, (2) high nutrients requirements and (3) being able to survive under low concentrations of nutrients. In terms of basic properties, the strain should be (1) easy to harvest, (2) tolerant to environmental fluctuations and (3) resistant to bacterial or fungal contamination. Regarding additional properties, ideal strains should show (1) soluble algal product secretion and (2) accumulation of valuable products to offset the cost the treatment (Wang, et al., 2017). Interaction with other organisms is another intrinsic factor to consider. Predation by zooplankton can be devastating for microalgae systems. Bacteria may have different effects; some might compete with microalgae for nutrients. Alternatively, some bacteria can coexist with microalgae reaching a synergetic symbiosis (Wang, et al., 2017).
- **Environmental factors:** (1) N:P ratio: the according to stoichiometric equations, the ideal N:P ratio should be 5:1. Nevertheless, such a ratio is not often found in wastewater, where phosphorus is often limited (Wang, et al., 2017). (2) pH: due to photosynthesis, CO_2 is absorbed, what rise the pH of the culture. pH can reach 11 or more if CO_2 is limiting and bicarbonate is used as a carbon source. (Larsdotter, 2006). Depending on the nitrogen source, the pH may vary as well. NO_3^- as

nitrogen source tend to raise the pH meanwhile ammonia assimilation will reduce drop the pH (Larsdotter, 2006) according to the following equations (Ebeling, Timmons, & Bisogni, 2006):

- Ammonia as a nitrogen source (↓ pH):

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2$$

- Nitrate as a nitrogen source (↑ pH)

$$16NO_3^- + 124CO_2 + 140H_2O + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 138O_2 + 18HCO_3^-$$

(3) CO_2 enrichment: providing CO_2 as a carbon source can improve the algal growth and nutrient removal, but also should be considered that CO_2 will drop the pH and subsequently nitrogen removal will be reduced due to NH_3 volatilization. (Wang, et al., 2017). (4) Light: Light intensity plays an important role. Too high intensity may result in photoinhibition and too low will limit growth. At higher depths and concentrations, light intensity must be high in order to penetrate through the culture. (Barsanti & Gualtieri, 2014). Light cycles also affect algae growth and nutrient removal efficiency. It has been reported that the specific growth of microalgae under continuous light was higher than under light/dark cycles (Lee & Lee, 2001). Nevertheless, seems like light has a low influence on COD removal (Jämsä, et al., 2017) (4) Temperature: Algae optimal temperature range varies depending on the species. According to their temperature tolerance, they can be classified as polar organisms (<10°C), temperate (10 – 25°C), tropical (>20°C). Most commonly cultured species of microalgae tolerate temperatures between 16°C and 27°C. Temperature plays a major role in algal metabolism. Temperatures below 16ºC will reduce the growth rate and above 35ºC are lethal for most of the species. (Barsanti & Gualtieri, 2014).

- **Operational factors:** (1) Initial microalgal biomass concentration: Higher initial concentrations show a shorter lag phase and higher nutrients removal efficiency (Wang, et al., 2017). (2) Pre-treatment of secondary wastewater: A proper pre-treatment can improve microalgae cultivation by eliminating competitors or predators. (Wang, et al., 2017). (3) Hydraulic retention time (HRT): Too high HRT may lead to nutrient depletion while a too short HRT may lead to too high

nutrients level in the effluent. Additionally, biomass washout may occur leaving not enough microalgae biomass in the system for nutrient removal. (Wang, et al., 2017). (4) Mixing: Proper mixing prevents algae settling, eliminates thermal stratification (Wang, et al., 2017) and reduce the effect of internal shading in dense cultures and photoinhibition, as mixing exposes all cells to light for short periods of time (Larsdotter, 2006).

2.2.6. Carbon removal

In photosynthesis, inorganic carbon is absorbed together with light to produce sugars:

$$6H_2O + 6\,CO_2 + light \rightarrow C_6H_{12}O_6 + 6O_2$$

Some algal species can change their metabolism and use organic carbon as well. Species like Chlorella or Scenedesmus can swift their metabolism depending on the available carbon source and the light conditions (Larsdotter, 2006). But other factors, like pH, may affect the way carbon is assimilated. For pH values between 5 and 7, CO_2 uptake occurs by diffusion meanwhile for pH higher than 7, the most common form of inorganic carbon is HCO_3^- and is absorbed by active transport (Gonçalves, Pires, & Simões, 2016).

2.3. Microalgae

Microalgae are microorganisms from the kingdom Protista which can be found in a variety of aquatic environments. Aquatic algae are found from freshwater to salt lakes, with a broad range of pH, temperature, turbidity, O_2, and CO_2 tolerance. Phytoplankton produces around 50% of the oxygen we need, but when their population becomes too large due to pollution with nutrients like nitrogen and phosphorous, these blooms may cause oxygen depletion and reducing the transparency of water. Some species can be toxic as they produce toxins and poisons. (Barsanti & Gualtieri, 2014). For this reason, nutrients removal from wastewater is necessary. Although their similarities to plants, they do not contain plant-structures like roots, stems or leaves. Like all Eukaryotes, algae cells contain membrane-bound organelles. (Singh & Chandra Saxena, 2015). Algae show different forms such as single cells, filmy conglomerations, matted or branched colonies (Barsanti & Gualtieri, 2014). Algae can show different ways of nourishment, but there are two that are the most representative: autotrophy and heterotrophy. Autotrophic organisms obtain their energy through photosynthesis, absorbing light and CO_2 producing sugars and releasing O_2. Heterotrophs obtain their

energy from organic compounds. Mixotrophy is a type of metabolism in which the organisms can use inorganic and organic carbon sources. It has been reported that *Chlorella sorokiniana* can grow at night on glucose, while during the day they were mixotrophic using both glucose and CO_2. Mixotrophic growth outperforms photoautotrophic growth and that would be a definite option to achieve high growth rates (Richmond & Hu, 2013).

2.3.1. Microalgae cultivation factors in wastewater

Each wastewater is different. Knowing its characteristics is fundamental to determine the efficiency of algal-bacterial systems for wastewater treatment. Among those characteristics, there are some that are especially important:

- **Initial C:N:P ratio:** This ratio represents the biodegradability in the absence of inhibitory or unbiodegradable compounds. The optimum biodegradability ratio is 100:18:2 (Posadas, et al., 2017). This ratio is very similar to the Redfield ratio (106:16:1), a ratio which describes the elemental composition of plankton biomass in the following idealized chemical reaction for the formation of phytoplankton through photosynthesis and nutrient uptake (Tyrrell, 2019):

$$106CO_2 + 16HNO_3 + H_3PO_4 + 78H_2O \leftrightarrow (C_{106}H_{175}O_{42}N_{16}P) + 150O_2$$

  To overcome the carbon limitation in wastewater, CO_2 can be added. But not only, but CO_2 addition also contributes to pH control of the algal-bacterial broth (Posadas, et al., 2017).
- **NH_4-N:** Concentrations over 100mg/L and pH > 8, photosynthesis result inhibited due to NH_3 toxicity. Effluents with NH_4-N concentrations need to be diluted or supplied at low loading rates (Posadas, et al., 2017). But depending on the sources consulted, this value can be reduced down to 20 mg/L (Larsdotter, 2006).
- **Heavy metals:** photosynthesis and growth inhibitors and may produce morphological changes in the cell wall at low concentrations (Posadas, et al., 2017).
- **Toxic organic pollutants:** Reduce the activity of microalgae and bacteria. (Posadas, et al., 2017).
- **pH:** wastewaters with pH values outside of the optimal range (7 - 9) are hardly biodegraded without any pH adjustment. (Posadas, et al., 2017).

But not only wastewater characteristics affect to microalgae. There few parameters to keep in mind while cultivating microalgae:

- **Light:** Can be considered the main factor in determining the performance of microalgae. Microalgae can absorb light at wavelengths from 400 to 700 nm. This range is defined as photosynthetically active radiation (PAR) which is saturated at irradiances ranging from 100 to 200 μmols·m^{-2}·s^{-1} (Acién Fernández, Fernández Sevilla, & Molina Grima, 2017). However, water absorbs part of PAR. Moreover, in dense cultures occurs an internal shading effect. To avoid this effect, mixing is necessary to expose all cells to light for a short period of time, avoiding also the photoinhibition, increasing productivity (Larsdotter, 2006). The effect of light cycles is different depending on the length of exposure and the type of metabolism the strain has. (Lee & Lee, 2001) affirm that under continuous light, the nutrient removal of a photoautotrophic organism was higher than under continuous light, but this experiment was run in a short period (3 days). Nevertheless, (Posadas, et al., 2017) state that long light exposure and high irradiance may result in growth photoinhibition and culture photo-damage.
- **Temperature:** Most of the strains have an optimal temperature range of 20 – 30ºC. (Acién Fernández, Fernández Sevilla, & Molina Grima, 2017). Above this range, growth declines due to oxidative stress (Posadas, et al., 2017). On the other hand, at low temperatures, microalgae get photoinhibited by high intensities (Larsdotter, 2006). Photoinhibition in microalgae varies depending on the species and temperature. Optimal PAR increased with temperature until an optimal temperature was reached (Schmidt, Gagnon, & Jamieson, 2016).

2.3.2. Microalgae utilization

Microalgae have been used over hundreds of years as food, fodder, remedies and fertilizers (Barsanti & Gualtieri, 2014). Today microalgae potential has increased, and they can be used for fuel production, cosmetics and aquaculture. On the next table are summarized the areas in which microalgae are used.

Table 2-2: Uses for microalgae

Use	Example	References
Nourishment	Animal feed, Human food,	(Barsanti & Gualtieri, 2014)
Pharmaceutical	Bioactive products such as: Fatty acids, vitamins, carotenoids, toxins	(Barsanti & Gualtieri, 2014) (Posten & Walter, 2012)
Cosmetic	Anti-aging creams, anti-irritants, thalassotherapy	(Barsanti & Gualtieri, 2014)
Energy	Biofuels	(Posten & Walter, 2012)
Environmental	Wastewater treatment, fertilizer	(Richmond & Hu, 2013)

2.4. Quantification

Counting cells has two main purposes. First, estimate the size of the culture and second, assess the rate of cell division (Andersen, 2005). In this project, several methods were used: Neubauer haemocytometer, Flow cytometer, Microplate reading and a Coulter counter.

2.4.1. Neubauer Haemocytometer:

Although its simplicity, visual counting with counting chamber is one of the most used methods. It's a thick crystal slide with a size of 30 x 70 x 4 mm (width x length x height). The grid is divided into 9 squares of 1mm^2. The central square is divided into 25 medium squares of 0.2 mm^2 and each of these squares is subdivided into 16 small squares. The scope of Haemocytometers is counting cultures with concentrations within $25{\cdot}10^4$ - $25{\cdot}10^6$ cells/mL, but $1{\cdot}10^6$ would be the optimal concentration. Below the lower limit, the number of counted cells is too low to provide a reliable value of the concentration. Above the limit, the probability of counting errors increases and too much time consuming (Bastidas, 2013). The most common slide of this type is 0.1 mm deep. The volume in nine large squares is 0.0009 mL, with the 2 chambers having 18 squares with a total volume of 0.0018 mL (Karlson, Cusack, & Bresnan, 2010). In order to calculate cell concentration by counting on the squares, the following equation can be used (Bastidas, 2013):

$$Concentration = \frac{Number\ of\ cells \cdot 10{,}000}{Number\ of\ squares} \quad (1)$$

In case we need to dilute our sample, the dilution factor needs to be added to the previous equation (Bastidas, 2013):

$$Concentration = \frac{Number\ of\ cells \cdot 10{,}000}{Number\ of\ squares \cdot dilution} \quad (2)$$

The following image shows the grid in a Haemocytomer:

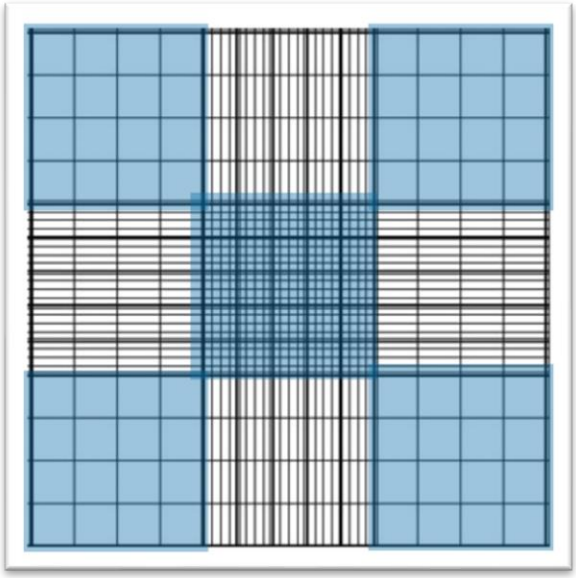

Figure 2-4:Counting chamber grid (Bastidas, 2013).

2.4.2. Flow cytometer

Flow cytometry has become a useful tool regarding microalgae culturing as flow cytometers, are able to make measurements of cells in solution as they pass by the instrument's laser at rates of 10,000 cells per second, way faster than optical counting (ThermoFisher Scientific, n.d.). In flow cytometry, cells are aligned by sheath fluid into a narrow stream onto which several light sources are focused. Each cell scatters light, angular intensity depends on the refractive index (Andersen, 2005). As the cell passes through the laser beam, light is scattered in all directions, and the light scattered in the forward direction at a low angle is proportional to the size of the cell or particle. Light may enter the cell and be reflected and refracted by the nucleus and other contents of the cell; thus, the 90° light scatter, may be considered proportional to the granularity of the cell.

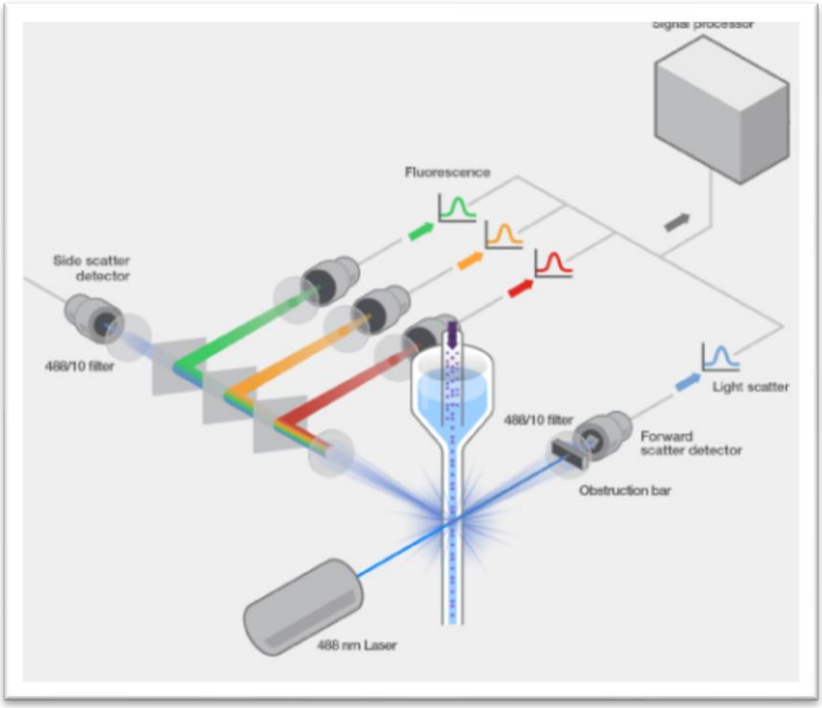

Figure 2-5: Flow cytometer compounds (ThermoFisher Scientific, n.d.)

There are 3 main components of a flow cytometer (fluidics, optics and electronics):

- **Fluidics:** Flow cytometers are equipped with a tank supplying the sheath liquid that carries the cells through the instrument. Cell suspensions are pushed through a capillary into a sheath fluid stream. Under laminar flow conditions, the sheath liquid aligns the cells into a narrow-centred stream (Andersen, 2005).
- **Optics:** When a particle passes through the light beam, light can be reflected or refracted. Light scatter detectors are located at 180° (forward scatter or FSC) and at 90° (side scatter or SSC) with respect to the light source. Both parameters are related to cell size, but the side scatter is more influenced by the cell surface and internal cellular structure (Andersen, 2005).
- **Electronics:** To be usable, analogic data from the photomultipliers must be converted to digital. Background noise, small particles and a large number of cells may overload the system. To avoid saturation of the conversion circuitry, only events of interest must be converted. Therefore, the operator needs to select one or several signals (called discriminators or triggers) As an example, to record chlorophyll fluorescing microalgae, it is best to choose red fluorescence as the discriminator and to select a threshold that is high enough so optical and electronic noise are left out but that is low enough so no cells are missed (Andersen, 2005).

2.4.3. Coulter counter

A Coulter Counter is an instrument able to count and size the cells in an electrolyte based on the Coulter principle. The Coulter principle states that particles pulled through an orifice, concurrent with an electric current, produce a change in impedance proportional to the volume of the particle. The sample is placed in a cuvette with electrolyte and two electrodes are introduced in the cuvette. A pump generates suction and the sample passes through the aperture, generating changes in the electric field between the electrodes which are registered and amplified by the electronics (Beckman Coulter Life Sciences, 2019).

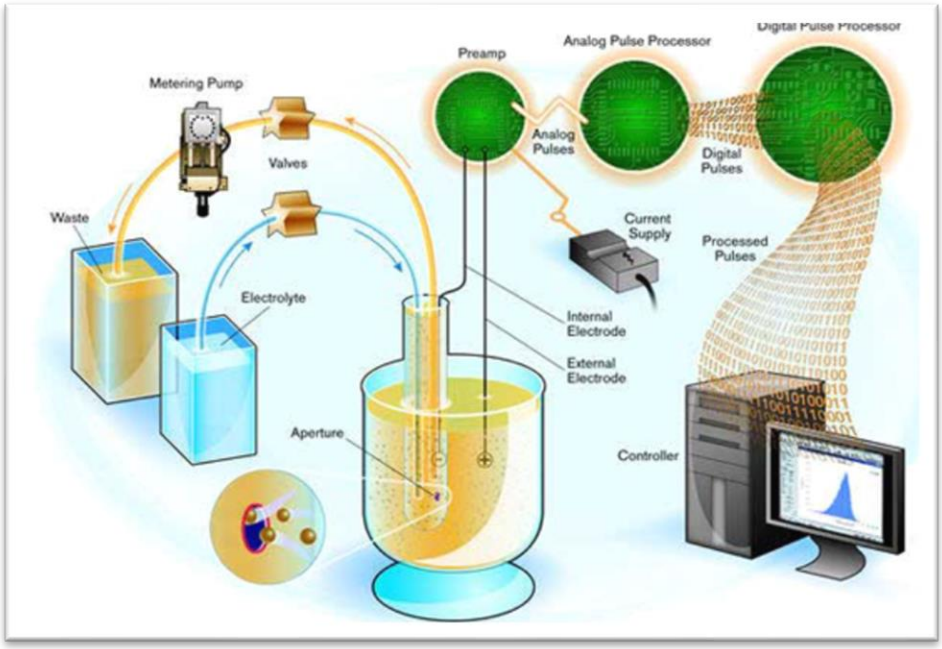

Figure 2-6: Coulter counter compounds (Beckman Coulter Life Sciences, 2019).

2.4.4. TECAN Microplate Reader

Microalgae growth can be analysed also by fluorescence exciting their pigments and interpretation of the emitted light, which is proportional to the concentration of pigments (Tecan, 2016). Plate-based technologies involve the growth of cells in wells of a plate that are analysed for changes between control and test samples by one or more spectrophotometric approaches such as absorbance, fluorescence, or luminescence. Flask-based approaches require larger spaces, more reagents, and greater media volumes. As a result, microplates facilitate the rapid analysis of significant sample sizes at reduced costs and with less waste (Haire, et al., 2018). Temperature, pH-value,

dissolved oxygen among other parameters may significantly affect the fluorescence quantum yield and therefore the results (Tecan, 2016)

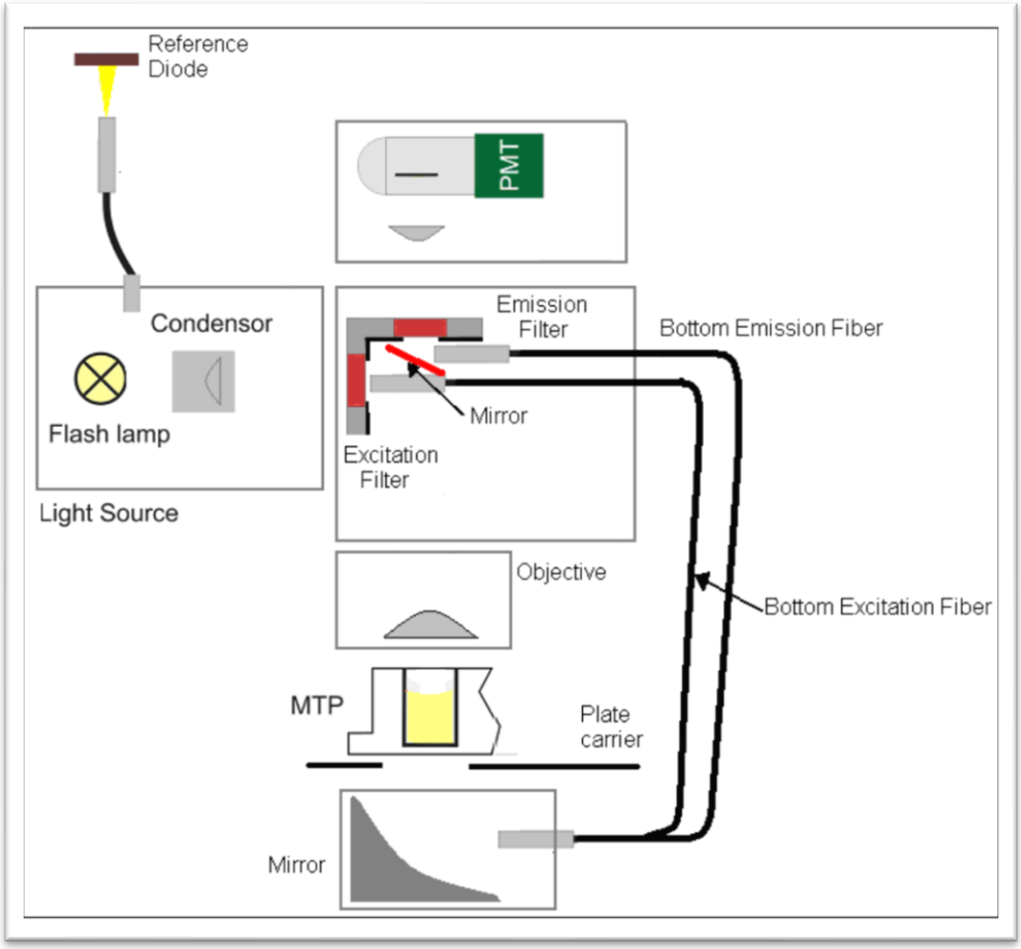

Figure 2-7:Fluorescence intensity bottom system. (Tecan, 2016)

3. Materials and methods

This thesis involves microalgae cultivation in different media, microalgae quantification comparison between different devices and nutrients removal efficiency of microalgae cultivated in a photobioreactor under non-ideal conditions.

3.1. Microalgae cultivation

Chlorella sorokiniana and *Tetradesmus obliquus* were cultivated in MWC media for counting methods comparison. Only *Chlorella sorokiniana* was cultivated in MWC media phosphorous limited and MWC media Nitrogen limited with NH_4Cl as nitrogen source and wastewater, different nitrogen source compared to the original MWC recipe as the wastewater used contain mainly NH_4 as a nitrogen source.

3.1.1. Strains

Chlorella Sorokiniana and *Tetradesmus obliquus* in suspension, kept at 6ºC in Erlenmeyer flasks with MWC+Se media dated 28/03/18 were used for this test.

3.1.2. Medias

3.1.2.1. MWC media

MWC media was prepared according to the recipe described in the Scandinavian Culture Collection of Algae and Protozoa (SCCAP):

- Chemicals:
  - Thiamine hydrochloride (C12H18Cl2N4OS), 99%. Producer: VWR chemicals.
  - Mangan (II)-chlorid-4-hydrate (MnCl2 • 4 H2O). Producer: Riedel-De Haenag SeelzeHannover
  - Copper (II)-sulfate-5-hydrate (CuSO4 • 5 H2O) Producer: Merck
  - Natriummolybdat-2-hydrate (Na2MoO4 • 2 H2O). Producer: Riedel-De Haenag SeelzeHannover
  - Zinksulfat-7-hydrate (ZnSO4•7H2O). Producer: Riedel-De Haenag Seelze-Hannover
  - Cobalt(III) chloride hexahydrate (COCl2•6H2O), >98%. Producer: Alfa Aesar.
  - Nickel (II) sulphate hexahydrate (NiSO4 •6H2O), >98%. Producer: Alfa Aesar.
  - Sodium orthovanadate (Na3VO4), 99.9%. Producer: Alfa Aesar
  - Potassium chromate (K2CrO4), >99.5%. Producer: Merck
  - Iron (III) chloride hexahydrate (FeCl3 · 6H2O), >99%. Producer: Merck
  - Di-potassium hydrogen phosphate (K2HPO4), >99%. Producer: Merck
  - Ethylenediaminetetraacetic acid disodium salt dihydrate (C10H14N2Na2O8 • 2 H2O), >99%. Producer: VWR
  - Calcium chloride dihydrate (CaCl2 · 2 H_2O), >99%. Producer: VWR chemicals.
  - Boric acid (H3BO3), >99%. Producer: Sigma- Life Science
  - Sodium hydrogen carbonate (NaHCO3), >99%. Producer: Merck.
  - Sodium nitrate (NaNO3). Produced by Merck.

- Sodium metasilicate nonahydrate (Na2O3Si · 9 H2O), >98%. Producer: Sigma Aldrich.
- D - (+)- Biotin (C10H16N2O3S), >98% . Producer: Alfa Aesar.
- Cyanocobalamin B12 (C63H88CoN14O14P), >98%. Producer: Alfa Aesar.
- Sodium nitrate (NaNO3), >99%. Producer: Emsure
- Sodium dihydrogen phosphate monohydrate (NaH2PO4 · H2O), >99%. Producer: Merck
- Magnesium sulphate heptahydrate (MgSO4 · 7 H2O), 99.7%. Producer: VWR Chemicals

- Equipment:
  - 1000 - 100 µL, 100 - 10µL Pipettes
  - 250 mL Erlenmeyer flasks
  - Digital microscope, VisiScope® BL254 T1
  - Autoclave Panasonic MLS-3781L
  - Orbital shaker Edmund Bühler SM-30
  - Laminar flow hood: Nuair. Model: NU-437-400E

3.1.2.2. Nutrients limited medias

To study the algae growth in phosphorous and nitrogen-limited media, three extra media were prepared:

- **N-media:** Following the MWC recipe but changing the Nitrogen source and changing initial concentration. NH_4Cl was used instead of $NaNO_3$ as a Nitrogen source. The initial concentration was 100 mg NH_4 /L. No phosphorous source was added.
- **P-media:** Following the MWC recipe but changing the initial PO_4^{-3} concentration to 25 mg/L. No nitrogen source was added.
- **N+P media:** Following the MWC recipe but changing the Nitrogen source and changing initial concentrations of N (100mg NH_4/L) and P (25 mg PO_4/L)

  In all these media, instead of using Tris buffer, (contains nitrogen), 4 g/L of $NaHCO_3$ (Chosen concentration to fulfil the Redfield ratio).

3.1.3. Procedure for media preparation

3.1.3.1. MWC media

MWC media was prepared according to a recipe from SCCAP site (SCCAP, 2019). All

the equipment was sterilized before use. Stock and trace element solutions were prepared following the recipe described on table 3-1:

Table 3-1:MWC media recipe from SCCAP (SCCAP, 2019).

Stock Solutions

Chemical	Concentration
CaCl2 • 2 H2O	36.80 g/L
MgSO4 • 7 H2O	37.00 g/L
NaHCO3	12.60 g/L
K2HPO4 • 3 H2O	11.40 g/L

Trace Elements Solution

Chemical	Quantity
C10H14N2Na2O8 • 2 H2O	4.36 g
FeCl3•6H2O	3.15 g
MnCl2 • 4 H2O	0.18 g
H3BO3	1.00 g
1% CuSO4 • 4 H2O	1 mL
2.2 % ZnSO4 • 7H2O	1 mL
1% COCl2 • 6H2O	1 mL
0.6% Na2MoO4 • 2 H2O	1 mL
dH2O	To 1000 mL
Vitamin Stock Solutions	
Compound	**Quantity**
Biotin	0.0005 g
Thiamine HCl (B1)	0.1 g
Cyanocobalamin (B12)	0.0005 g
Na2O3Si • 9 H2O	28.40 g/L

After adding all the compounds, pH was adjusted to 7.5 using NaOH 1M or HCl 1M. Later, the solutions were autoclaved.

3.1.3.2. Nutrient limited medias

The purpose of the limited media is studying the growth in a media which N is the limited compound. Also, in a media which phosphorous is the limited compound and finally, a media in which both nutrients are limited. By combining the N-media and P-media, a dilution series was prepared (1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) for each nutrient with an initial concentration of 100mg NH_4/L and 25 mg PO_4 /L. The same dilution series was prepared for the N+P media.

3.1.3.3. Wastewater

The wastewater used was collected from IVAR WWTP in Grødaland. This wastewater is a mix of household and industrial wastewater. The wastewater was kept at 6ºC to reduce the activity of microorganism before experiments. Prior to being used in this study, the wastewater was treated in an Upflow Anaerobic Sludge Blanket Reactor (UASB) and a tubular crossflow membrane filtration (Carlsen, 2018). The effluent from the membrane was fed into a photobioreactor to study the nutrient removal efficiency of microalgae.

3.1.4. Procedure for Algal cultivation:

Before starting cultivation, as microalgae were kept at 6ºC, to "wake up" microalgae from lethargy, a small volume from the initial suspension was inoculated into MWC media until microalgae achieved the logarithmic growth phase. Once at this point of growth, 2 mL of the suspended algae were transferred to autoclaved 250 mL Erlenmeyer flasks containing 100 mL MWC media for sub-culturing. The algal cultures were incubated at room temperature (24ºC), 80-90 rpm using as the light source a 58W fluorescent tube providing 54 µmol m^{-2} s^{-1} of light intensity with a light/dark regime of 18/6 hours. When algal cultures were in the logarithmic phase, they were sub-cultured. The cultures were shaken once a day to avoid self-shading and to ensure gas-transfer.

In addition, *Chlorella sorokiniana* was cultivated in microplates to study its growth in nutrient-limited media and see which nutrient affect the most to the growth and under what concentrations/ratios perform better. The materials used for this test were the following:

- Tecan Infinite F200 PRO. (Tecan)
- 96 wells TC-Treated Multiple Well Plates. (Costar)

• Breath easy: Sterile gas permeable membranes. (Diversified Biotech)

When cultivated in limited media, an original microalgae culture was diluted before inoculation to a concentration value around 10^5 cells/mL and each column of the plate contained a dilution factor of the nutrients, as described on the picture below:

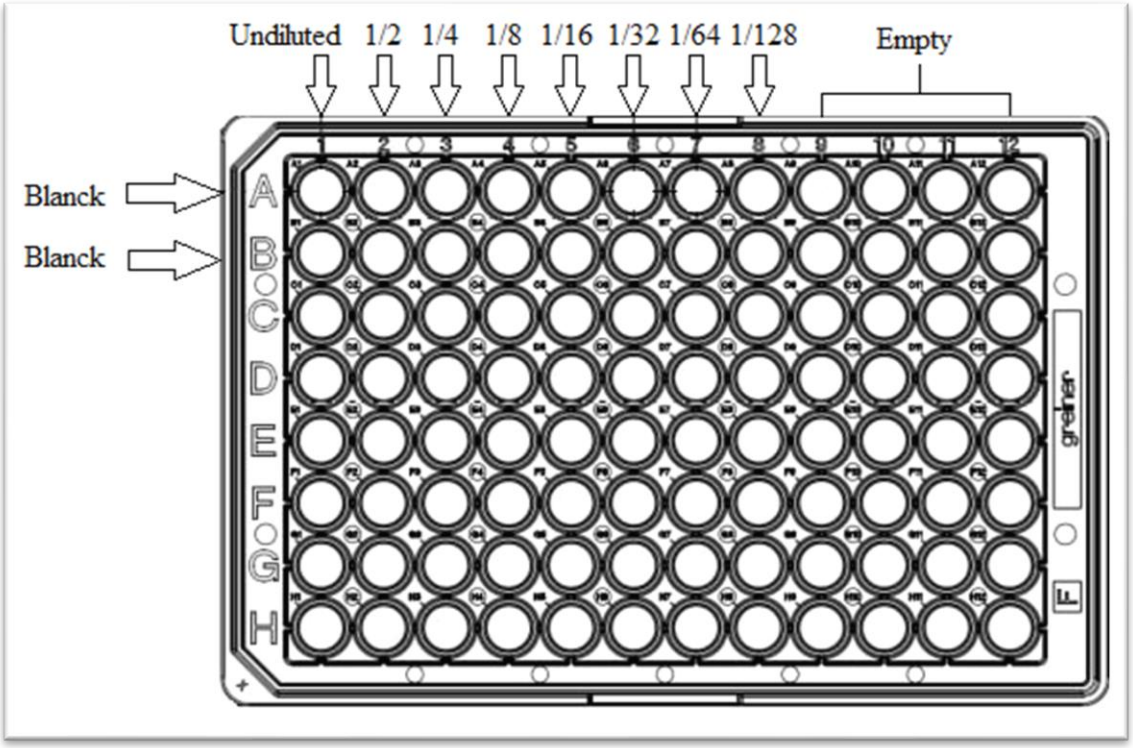

Figure 3-1: Plate distribution of dilution series and blanks. Image modified. Original image: (Green Bioresearch, 2019)

After inoculation, fluorescence was measured daily and, afterwards, the plate was covered with a sterile permeable gas membrane and placed back onto the orbital shaker.

3.2. Quantification

Counting chamber, flow cytometry and Coulter Counter were compared during quantification. To compare the methods, two species of microalgae were compared, *Chlorella sorokiniana* and *Tetradesmus obliquus.*

3.2.1. Equipment:

- Multisizer 4e Coulter Cell Analyzer
- Flow cytometer BD Accuri C6 Plus
- Neubauer counting chamber: Neubauer improved
- 1000 - 100 µL, 100 - 10µL Pipettes

- 250 mL Erlenmeyer flasks
- Digital microscope, VisiScope® BL254 T1
- Autoclave Panasonic MLS-3781L
- Orbital shaker Edmund Bühler SM-30.

3.2.2. Counting chamber analysis

The cultures were shaken to homogenize the suspension and separate microalgae, as they tend to create flocks. Counting started from day 0 (inoculation day) until day 6, as normally both species achieve the logarithmic phase on day 5 (observed from the previous counting). After placing the glass coverslip over the counting chamber, 10 μL of the culture were pipetted in one of the channels of the glass. When the cultures were too dense, original cultures were diluted with distilled water to achieve the right concentration for counting with this method, around $1 \cdot 10^6$ cells/mL (Bastidas, 2013). 5 minutes after pipetting, counting started using the microscope with 40x objective and the grid shown in figure 2-4. Equations 1 and 2 were used to calculate the concentration of the culture.

3.2.3. Flow cytometer analysis.

As described before, cultures were shaken to homogenize the suspension and separate microalgae, as they tend to create flocks. Counting started from day 0 (inoculation day) until day 6, as normally both species achieve the logarithmic phase on day 5 (observed from the previous counting). 1 mL of each culture, each day, was pipetted into glass cuvettes and placed in the flow cytometer. The flow speed in the flow cytometer was set in slow (14μL/min) and the volume read was 50μL. Flow cytometer Accuri C6 has two excitation wavelengths: 488 nm and 640nm. Signal receptors detect wavelengths of 533/30 nm (FL1), 585/40 nm (FL2), >670 nm (FL3), and 675/25 nm (FL4).

3.2.4. Coulter Counter analysis.

Each day after inoculation, 5mL of each culture were pipetted into plastic cuvettes and vortexed thoroughly for Coulter counter analysis. Two different tube apertures were used, 20 μm and 50 μm.

3.3. Role of each species

As it will be explained later in the results section, two species of microalgae (*Chlorella sorokiniana* and *Tetradesmus obliquus*) and bacteria (nitrifying bacteria) were present in the reactor. This situation brought up the idea that could be studied the role of each

species and how they contribute to remove the nutrients and if bacteria could affect the efficiency. Both species were cultivated separated, both together and both plus bacteria (only in wastewater) in MWC media and in wastewater. The species were cultivated in autoclaved Erlenmeyer flasks. 150 mL of MWC media and wastewater were added to the Erlenmeyer flasks according to the following scheme:

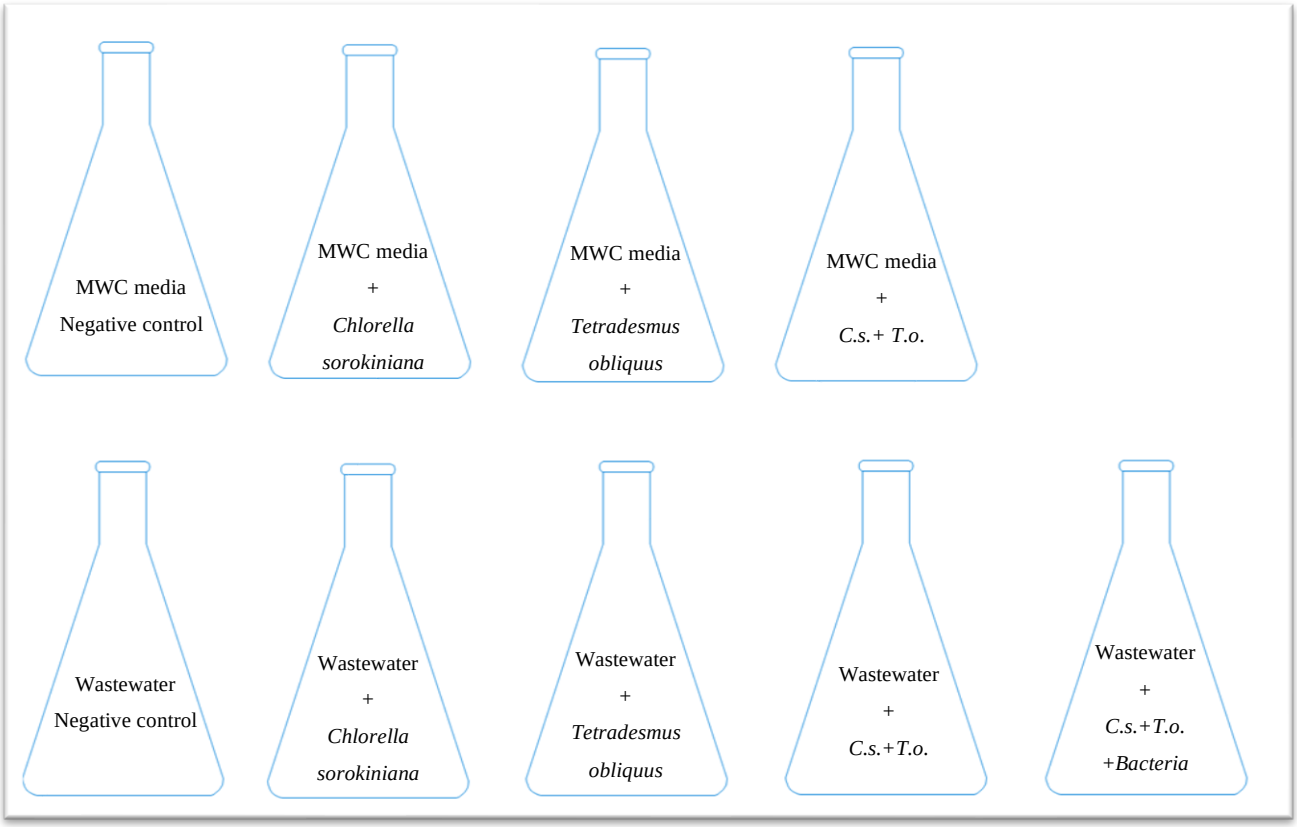

Figure 3-2: Mixotrophic test scheme

A negative control was used to have a reference to compare the results. To each Erlenmeyer containing MWC media, 2 mL of pre-cultivated cultures of each species were inoculated. The same for those containing wastewater except on in which 2 mL from photobioreactor culture were inoculated. From day 0 (Inoculation day), COD, NH_4-N, PO_4^{3-}-P and NO_3^--N were measured for 4 days. Every day, a sample of 5 mL was filtrated using glass fibre filters in a filtration unit to remove microalgae and proceed to measure the concentration of the previous components.

3.3.1. Equipment

- 250 mL Erlenmeyer flasks
- Autoclave Panasonic MLS-3781L

- Orbital shaker Edmund Bühler SM-30
- Thermoreactor: Spectroquant TR 620. Producer: Merk millipore
- Photometer: Spectroquant pharo 300. Producer: Merk
- Filtration unit
- Whatman Glass microfiber filters. Diameter 47 mm. 1.5 µm particle retention. Grade GF/C. Producer: VWR.

3.3.2. Chemicals:

- Spectroquant COD cell test (measuring range: 100-1500 mg/L COD). Product number: 109773. Producer: Merck
- Spectroquant Nitrate cell test (measuring range: 0.5-18 mg/L NO3-N). Product number: 114542. Producer: Merck
- Spectroquant Phosphate Cell Test (measuring range: 0.5-25.0 mg/L PO4-P. Product number: 114729. Producer: Merck
- Spectroquant Total Phosphorous Cell Test (measuring range: 0.5-25.0 mg/L PO4-P. Product number: 114763. Producer: Merck
- Spectroquant Ammonium Cell Test (measuring range: 4-80.0 mg/L NH4-N). Product number: 114559. Producer: Merck

3.3.3. Procedure for COD measurements

COD was measured in the inlet and outlet of the photobioreactor to evaluate its efficiency. Following the instructions, the sample is oxidized with a hot sulphuric solution of potassium dichromate using silver sulphate as a catalyst. The concentration of green Cr^{3+} ions is measured photometrically.

2 mL of sample were added to the reaction cell and mixed well. The cell was heated to 148ºC in the preheated thermoreactor for 2 hours. The cell was cooled to room temperature and measured in the photometer (Merk, Spectroquant COD Cell Test, 2019).

3.3.4. Procedure for Ammonium measurement

Ammonium nitrogen (NH_4-N) exists occurs partly in the form of ammonium ions and partly as ammonia. A pH-dependent equilibrium exists between the two forms. In strongly alkaline solution ammonia dominates as the form in which ammonium nitrogen exists. Ammonia reacts with hypochlorite ions to produce monochloramine, which

reacts with a substituted phenol to form blue indophenol derivative. This compound can be measured photometrically.

The volume of sample used was 0.1 mL. This volume was added to the respective reaction cell and mixed. 1 dose of NH_4-1K was added and the cell was shaken vigorously until reagent was completely dissolved. The sample was left for 15 minutes to react before measured in photometer (Merk, Ammonium Cell Test, 2019).

3.3.5. Procedure for Phosphates measurement

In sulfuric solution, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically. In this case digestion is not required. 1 mL of pretreated sample was added to the cell. 5 drops of Reagent P-2K were added and the cell mixed. After, 1 dose of Reagent P-3K was added and the cell shook vigorously until the reagent is completely dissolved. The cell was left to react for 5 minutes and then measured in the photometer (Merk, Phosphate Cell Test, 2019).

3.3.6. Procedure for Nitrates measurement

In sulfuric and phosphoric solution nitrate ions react with 2,6-dimethylphenol (DMP) to form 4 nitro-2,6-dimethylphenol that is determined photometrically. Wastewater nitrate values were low, but in the photobioreactor effluent, the values were high due to the presence of nitrifying bacteria. Effluent samples had to be diluted up to 10 times or even 15 in some situations to be able to read them. The procedure to measure nitrates starts adding 1 level blue micro-spoon of NO_3-1K into a reaction cell and mixing it for 1 minute. After, 1.5 mL of the sample are pipetted into the cell and allowing it to react for 10 minutes (Merk, Nitrate Cell Test, 2019).

3.4. Wastewater treatment using microalgae

3.4.1. Equipment:

- Peristaltic pump Ismatec Reglo ICC Digital
- Photobioreactor
- Air pump Quiet air pump 400. Producer: Ehein
- pH meter pHenomenal pH 1100L. Producer: VWR
- Thermoreactor: Spectroquant TR 620. Producer: Merk millipore
- Photometer: Spectroquant pharo 300. Producer: Merk

- Alkalinity measurement: Titroline 5000 auto-titration. Producer: instrument-teknikk AS.
- Filtration unit
- Whatman Glass microfiber filters. Diameter 47 mm. 1.5 µm particle retention. Grade GF/C. Producer: VWR.
- Photometer LI-250A. Manufacturer: LI-COR

3.4.2. Chemicals

- Spectroquant COD cell test (measuring range: 100-1500 mg/L COD). Product number: 109773. Producer: Merck
- Spectroquant Nitrate cell test (measuring range: 0.5-18 mg/L NO3-N). Product number: 114542. Producer: Merck
- Spectroquant Phosphate Cell Test (measuring range: 0.5-25.0 mg/L PO4-P. Product number: 114729. Producer: Merck
- Spectroquant Total Phosphorous Cell Test (measuring range: 0.5-25.0 mg/L PO4-P. Product number: 114763. Producer: Merck
- Spectroquant Ammonium Cell Test (measuring range: 0.5-16.0 mg/L NH4-N). Product number: 114544. Producer: Merck
- Spectroquant Ammonium Cell Test (measuring range: 4-80.0 mg/L NH4-N). Product number: 114559. Producer: Merck
- Spectroquant Total Nitrogen Cell Test (measuring range: 10-150 mg/L N). Product number: 114763. Producer: Merck
- Hydrogen Chloride (HCl), 0.1 M. Producer: Merck
- Sodium Hydroxide (NaOH), 0.1 M. Producer: Merck

3.4.3. Feeding the photobioreactor

Wastewater from Grødaland after preliminary treatment was kept in a cool room at 6ºC to feed an Up-flow Anaerobic Sludge Blanket Reactor (UASB). The effluent from this UASB reactor followed membrane microfiltration. Once filtrated, the effluent was poured into autoclaved bottles and kept at 6ºC in a fridge from which was pumped into the reactor at 0,155 mL/min in order to fulfil and HRT of 2,25 days.

3.4.4. Photobioreactor

Most of the studies choose ideal conditions to evaluate microalgae efficiency removing nutrients from wastewater. Conditions that in real life are hard to maintain as they would make the process too expensive. For this reason, microalgae have been cultivated in wastewater to study their efficiency to remove nutrients from wastewater under non-ideal conditions. In this study, using a Phenometrics 101 photobioreactor which has a 700 mL flask (150 - 600mL of working volume), temperature was set at 15ºC, bubbling only air to enhance mixing, no pH control, stirring at 150 rpm, 16/8 hours day/night light periods (light intensity changed sinusoidally with steps of 25 μmols·m^{-2}·s^{-1}). Light source peak intensity was 3000 μmols·m^{-2}·s^{-1}. Nevertheless, light intensity was measured at the bottom of a borosilicate flask containing 1 cm deep sample of distilled water, with a peak intensity of 1400 μmols·m^{-2}·s^{-1}. Same test was performed with 1 cm deep sample of from the photobioreactor in the same flask. In this case, light intensity at the bottom depends on microalgae concentration. The values ranged from 1000 to 700 μmols·m^{-2}·s^{-1}. The light intensity was also measured on the bottom of the reactor, showing results between 4 and 2 μmols·m^{-2}·s^{-1}. Although, the light intensity is still high at 1 cm deep, air bubbling and stirring ensure that all the cells are not exposed to that intensity for a long time. No matter what the maximum intensity of light source used is or what the geometry of a photobioreactor is, supplying enough photons to each cell in a 1 cm thick culture of $2 \cdot 10^9$ cell/mL is virtually impossible even with a light intensity of 1000 W/cm (Lee C.-G. , 1999).

The reactor was filled up to 500 mL with pre-treated wastewater. Once the water achieved the working temperature (15ºC), 50 mL of an existing culture of *Chlorella Sorokiniana* was inoculated into the reactor.

Chlorella Sorokiniana growth was evaluated in MWC media to find out its maximum growth rate.

According to figure 3-2, the growth rate for Chlorella Sorokiniana during the logarithmic phase was 1,7808 $\frac{1}{day}$, a value that was verified with the curve equation between days 2 – 3 and using the following equation (Borowitzka & Moheimani, 2015):

$$Growth\ rate = \frac{\ln(N_{t2}/N_{t1})}{t_2 - t_1}$$

In continuous cultivation mode, like in our photobioreactor, wastewater is retained in the reactor for a specific period to reach adequate nutrients removal efficiency. This term is defined as hydraulic retention time (HRT), which is calculated by the following equation:

$$HRT = \frac{V}{Q}$$

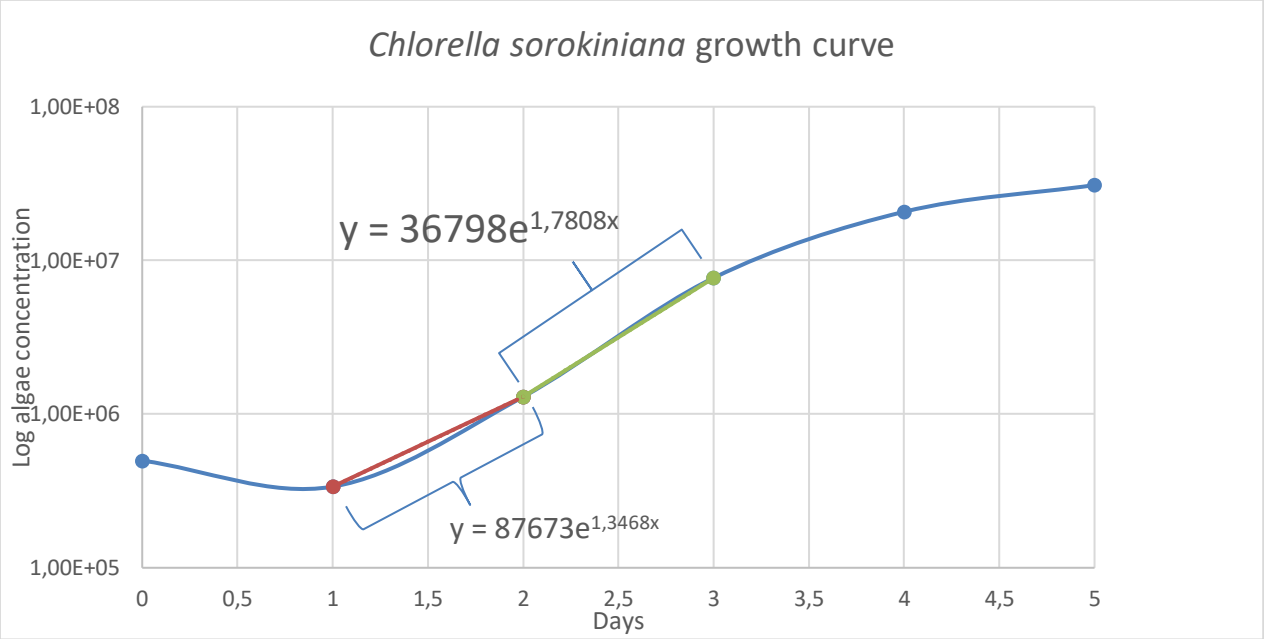

Figure 3-3: Logarithmic growth curve for Chlorella sorokiniana

where HRT is the hydraulic retention time (day), V is the working volume (m^3), and Q is the inlet flowrate (m^3/day) (Asraful Alam & Wang, 2019). According to Alam & Wang, microalgal-based systems, HRT usually ranges between 2 and 10 days in order to achieve acceptable nutrient removal efficiencies (Asraful Alam & Wang, 2019). Another used operational factor is the dilution rate, which assuming a steady state can be described as:

$$D = \frac{1}{HRT} = \mu_{max} - b = \frac{Q}{V}$$

Following the previous recommendation of an HRT between 2 - 10 days and taking as working volume 500mL. Initially, a quarter of the dilution rate was applied, which

provided an HRT of 2,25 days. Every day was kept record of pH, Algae concentration, Total Suspended Solids, COD_{in-out}, Total $Nitrogen_{in-out}$, $NH_{4in-out}$, Total $Phosphorous_{in-out}$, $PO_4^{-3}{}_{in-out}$ and $NO_{3in-out}$.

3.4.5. Procedure for Algae concentration

Using a syringe, 2ml of the sample were taken and placed in a glass cuvette and mixed vigorously to measure algae concentration in a flow cytometer setting the flow rate in "slow".

3.4.6. Procedure for Total Suspended Solids

To measure the Total Suspended Solids the method followed was Standard method 2540 D. Using a syringe, 50 mL of sample were filtrated using a previously weighted glass fibre filter. After, the filter was dried for 1 hour at 105ºC. After dried, was placed in a desiccator for 15 minutes to cool down at room temperature and weighed again.

3.4.7. Procedure for COD measurements

COD was measured in the inlet and outlet of the photobioreactor to evaluate its efficiency. Following the instructions, the sample is oxidized with a hot sulphuric solution of potassium dichromate using silver sulphate as a catalyst. The concentration of green Cr^{3+} ions is measured photometrically.

2 mL of sample were added to the reaction cell and mixed well. The cell was heated to 148ºC in the preheated thermoreactor for 2 hours. The cell was cooled to room temperature and measured in the photometer (Merk, Spectroquant COD Cell Test, 2019).

3.4.8. Procedure for Total Nitrogen measurement

Organic and inorganic nitrogen compounds are transformed into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor. In a solution acidified with sulfuric and phosphoric acid, this nitrate reacts with 2,6-dimethylphenol (DMP) to form 4-nitro 2,6-dimethylphenol that is determined photometrically.

1 mL of sample was pipetted into an empty cell together with 9 mL of distilled water and homogenised. 1 level blue micro spoon of reagent N-1K was added to the cell and mixed. 6 drops of reagent N-2K was added and mixed with the other contents of the cell. The cell was heated at 120ºC in the thermoreactor for one hour. When the cell was cooled to room temperature, 1 mL of the digested sample was added into the reaction cell together with 1 mL of reagent N-3K. The samples were mixed and left to react for 10 minutes, before being read (Merk, Nitrogen (total) Cell Test, 2019).

3.4.9. Procedure for Ammonium measurement

Ammonium nitrogen (NH_4-N) exists occurs partly in the form of ammonium ions and partly as ammonia. A pH-dependent equilibrium exists between the two forms. In strongly alkaline solution ammonia dominates as the form in which ammonium nitrogen exists. Ammonia reacts with hypochlorite ions to produce monochloramine, which reacts with a substituted phenol to form blue indophenol derivative. This compound can be measured photometrically.

When measuring ammonium in the sample, depending on the initial NH_4 concentration, different kits were used. Wastewater has a high concentration of ammonium and had to be diluted. The dilution factor used was 1/5. The volume of sample used depended on the kit used. 0.1 mL in the if the range was between 4.0 - 80.0 mg/l NH_4-N or 5 ml of sample when the range was between 0.010 - 2.000 mg/l NH_4-N. These volumes were added to the respective reaction cell and mixed. 1 dose of NH_4-1K was added and the cell was shaken vigorously until reagent was completely dissolved. The sample was left for 15 minutes to react before measured in photometer (Merk, Ammonium Cell Test, 2019).

3.4.10. Procedure for Total phosphorus measurement

In sulfuric solution, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically. To analyse total phosphorus, the sample needs to be digested previously. 1 mL of pre-treated sample was pipetted into a reaction cell together with 1 dose of reagent P-1K. The cell has heated at 120ºC for 30 minutes. After digestion, 5 drops of Reagent P-2K were added and the cell mixed. After, 1 dose

of Reagent P-3K was added and the cell shook vigorously until the reagent is completely dissolved. The cell was left to react for 5 minutes and then measured in the photometer (Merk, Phosphate Cell Test, 2019).

3.4.11. Procedure for Phosphates measurement

In sulfuric solution, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically. In this case digestion is not required. 1 mL of pretreated sample was added to the cell. 5 drops of Reagent P-2K were added and the cell mixed. After, 1 dose of Reagent P-3K was added and the cell shook vigorously until the reagent is completely dissolved. The cell was left to react for 5 minutes and then measured in the photometer (Merk, Phosphate Cell Test, 2019).

3.4.12. Procedure for Nitrates measurement

In sulfuric and phosphoric solution nitrate ions react with 2,6-dimethylphenol (DMP) to form 4 nitro-2,6-dimethylphenol that is determined photometrically. Wastewater nitrate values were low, but in the photobioreactor effluent, the values were high due to the presence of nitrifying bacteria. Effluent samples had to be diluted up to 10 times or even 15 in some situations to be able to read them. The procedure to measure nitrates starts adding 1 level blue micro-spoon of NO_3-1K into a reaction cell and mixing it for 1 minute. After, 1.5 mL of the sample are pipetted into the cell and allowing it to react for 10 minutes (Merk, Nitrate Cell Test, 2019).

3.4.13. Procedure for Alkalinity test

To measure the alkalinity of the inlet and outlet of the reactor was used the instrument Titroline 5000. The samples were diluted and placed on a low speed magnetic stirring device before being titrated with HCl 0.1 M to four pH values (6.7, 5.9, 5.2, and 4.3). If the pH value was below than 6.7, NaOH was added to the sample to correlate pH value to above 6.7. Amount of acid needed for each titration point was recorded into computer software TITRA 5, which calculated the alkalinity of the sample (Carlsen, 2018).

4. Results

Several tests have been run and the results are shown in this chapter: (1) Comparison of different methods for quantifying algae, (2) Microalgae growth in limited media, (3) Contribution of each species to nutrient removal efficiency and (4) Microalgae nutrient removal efficiency under non-ideal conditions. Due to the large amount of data collected from this last test and the different events that came about, the data will be presented as timelines in which the events will be marked with numbers and a description of what occurred in that event.

4.1. Counting methods comparison

Biomass is a key parameter in any study related to microalgae. Although there exist several methods to determine this parameter, all of them have pros and cons. The ideal method should be fast, precise with a low level of detection and requires a small volume of sample.

4.1.1. Comparison between a Haemocytometer and a flow cytometer:

The following tables compare the growth curves measured for 6 days with a counting chamber and a flow cytometer for *Chlorella sorokiniana* and *Tetradesmus obliquus*:

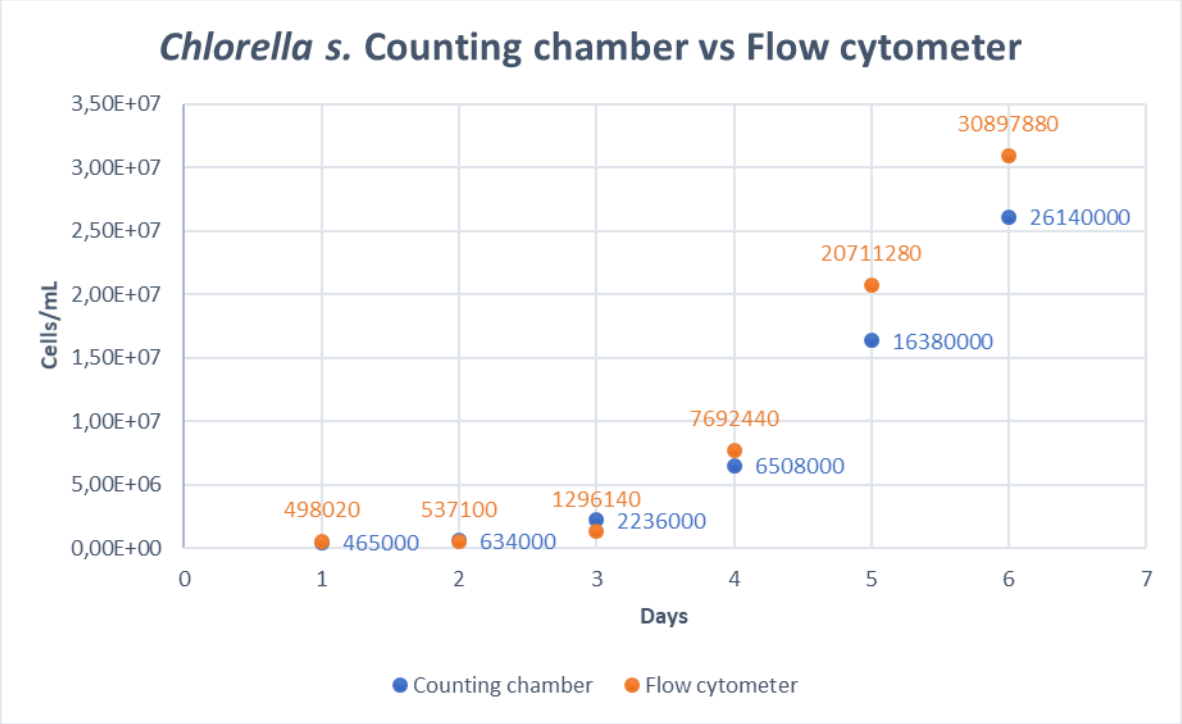

Figure 4-1: Counting values for Chlorella s. using a haemocytometer and a flow cytometer

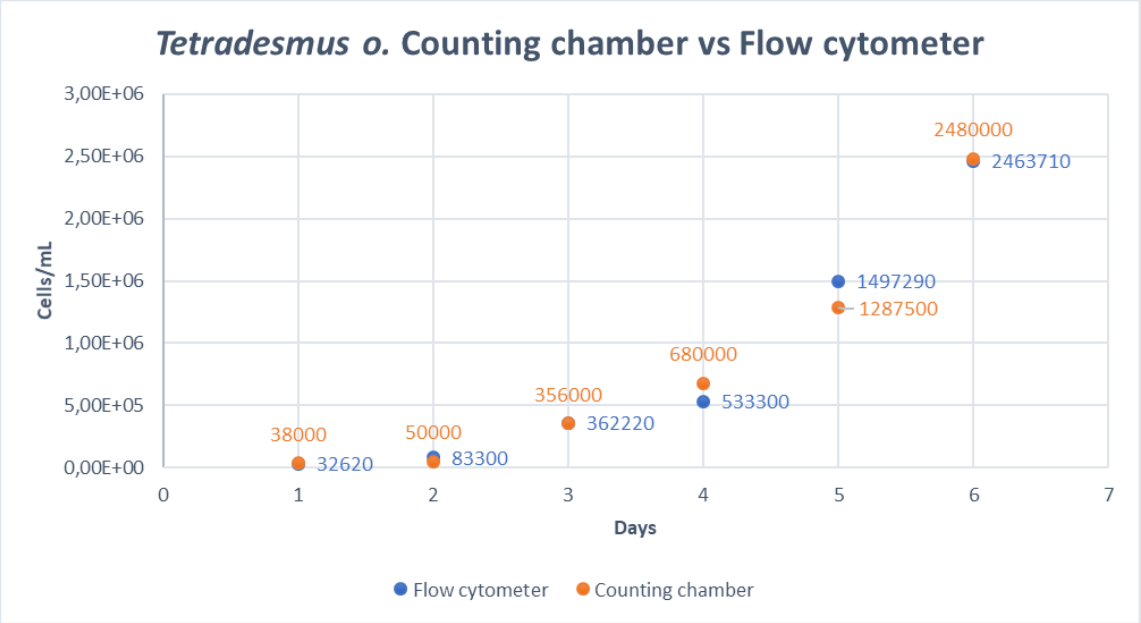

Figure 4-2:Counting values for Tetradesmus o. using a haemocytometer and a flow cytometer

Figures 4-1 and 4-2 show a clear correlation between counting values using both methods. For higher concentrations of Chlorella, it can be observed an increasing difference between the readings of the counting chamber and the flow cytometer.

4.1.2. Coulter counter readings

Coulter Counter reading can be observed in Appendix 1. These results are far from being similar to those obtained with the other two previous methods. For this reason, they were compared with beads solution in order to verify the and the outcomes were the same.

4.2. Microalgae growth in limited medias

Chlorella sorokiniana was cultivated for 5 days in limited media to evaluate how the limitation of nutrients affects its growth and which are the best N:P ratios. *Chlorella s.* was cultivated in three different media: N-limited media, P-limited media and normal media containing both nutrients. The figures 4-3, 4-4 and 4-5 show the growth in N-limited media, P-limited media and normal media respectively.

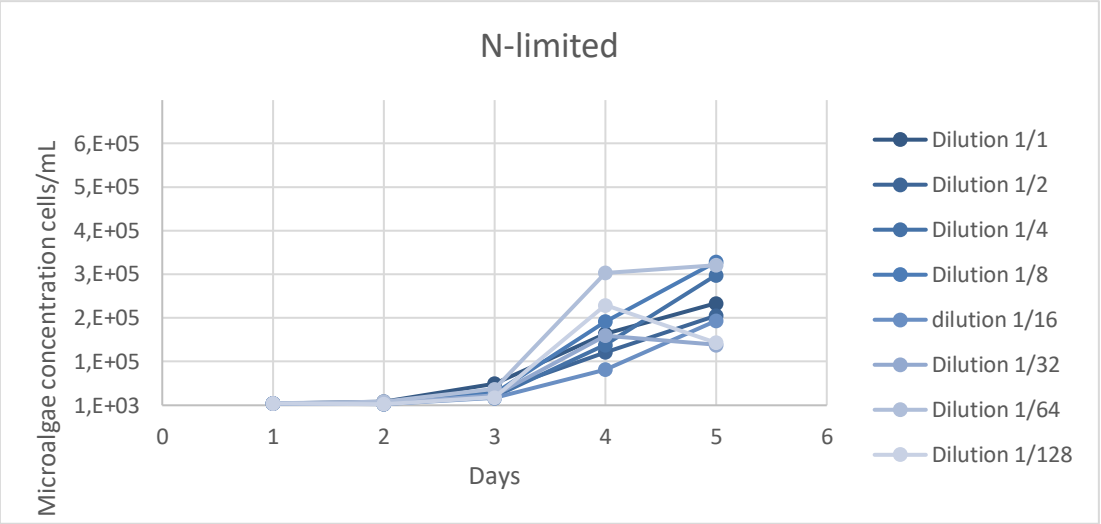

Figure 4-3: Chlorella s. growth in N-limited media

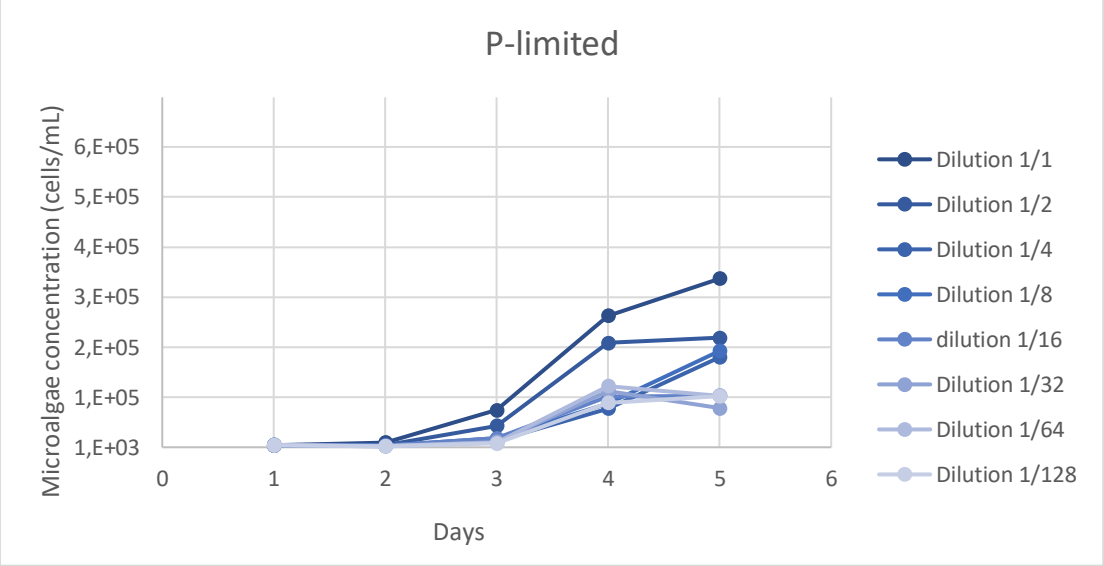

Figure 4-4: Chlorella s. growth in P-limited media.

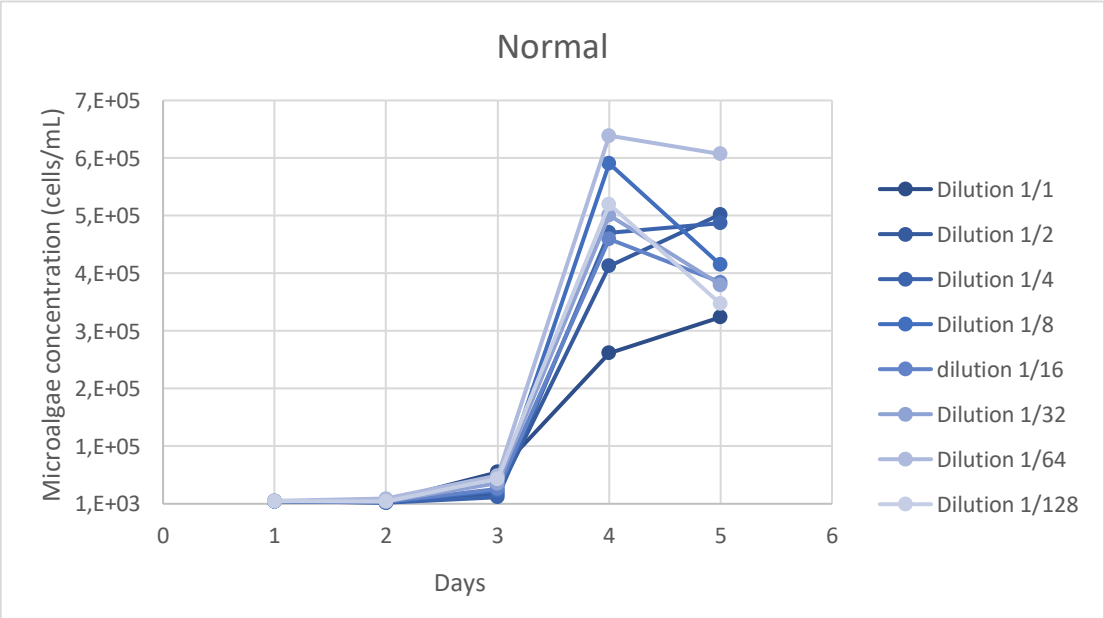

Figure 4-5:Chlorella s. growth in normal media

- N-Limited media: It could be observed that chlorella growth rate was faster in the 1/64 and 1/128 dilutions between days 3 and 4, but after it can be observed already how the population decay compared with the other dilutions in which growth continue.
- P-Limited media: The most concentrated media (1/1 and 1/2) show a shorter lag phase and the growth is substantially higher compared with the other dilutions.
- Normal media: All the dilutions show a similar lag phase, but the growth rate is appreciably higher when the media is more diluted but also decay phase starts earlier in the most diluted media.

The following table shows the growth rates for days 3 and 4 for each media and each dilution:

Table 4-1: Growth rates for Chlorella sorokiniana cultivated in microplates in the logarithmic phase

	Growth rates for days 3 and 4 for each dilution (day^{-1})							
	Dilution 1/1	Dilution 1/2	Dilution 1/4	Dilution 1/8	Dilution 1/16	Dilution 1/32	Dilution 1/64	Dilution 1/128
Normal	1,55	3,08	3,60	3,22	2,83	2,62	2,55	2,46
N-limited	1,17	1,33	1,89	1,94	1,53	1,92	2,06	2,47
P-limited	1,26	1,56	1,74	1,54	1,71	2,27	2,54	2,33

4.3. Contribution of each species to the nutrient removal efficiency

In the photobioreactor was found that two species were present, instead of one as it was planned. Also was observed nitrifying activity as nitrates concentration increased over time. This situation led to the idea of study how each species contributes to the nutrient removal efficiency and if the presence of bacteria affects the removal efficiency. Both species were cultivated separated, both together and both plus bacteria (only present in wastewater) in MWC media and in wastewater. The species were cultivated in autoclaved Erlenmeyer flasks. 150 mL of MWC media and wastewater were added to the Erlenmeyer flasks. The result can be seen in the appendix 2. Each plot represents how a nutrient changed over time in each media. It is interesting the fact that microalgae consumed NO3-N (figure 5-31) instead of NH4-N as nitrogen source (figure 5-29). It could be due to the lower levels of NH4-N in the media and NO3-N concentration was 6 times higher.

4.4. Microalgae nutrient removal efficiency under non-ideal conditions

50mL of a *Chlorella Sorokiniana* strain was inoculated into the photobioreactor with 500mL of pre-treated wastewater. The temperature was set at 15ºC, bubbling only air to enhance mixing, no pH control initially, stirring at 150 rpm, 16/8 hours day/night light periods and an initial HRT of 2,25 $days^{-1}$. On 07/08/2019, the HRT was changed to 5.05 $days^{-1}$ to observe if the nutrient removal efficiency improves. COD, TN, TP, NH_4^+, NO_3, PO_4^{3-}, TSS and pH values were measured on a daily basis before and after microalgae treatment. Every time wastewater feeding changed, it is marked with a green striped line. When a significant event occurred and an effect on the parameters was observed, it is marked with a number in each timeline. List and description of events:

1. Nitrifying Bacteria metabolism overcomes microalgae and acidifies the environment. Manual dosing of NaOH 1M once per day until pH = 8.
2. As pH was changing every day, automatic dosing (62.5 µL/6h) was set in order to try keeping the pH stable.
3. Automatic dosing stopped. pH value was too high (8.72). NH_4-N values started to rise.
4. Wastewater dosed to recover the nitrifying community. pH started to rise as the nitrifying community is struggling and microalgae metabolism rises the pH due to the side products. Started to dose HCl 0.1 M (50µL/10 min) to keep the pH stable at (7.6-7-9).
5. Acid dosing stopped. pH stable at 7.8.
6. pH started to drop again. Back to dosing NaOH.
7. Biofilm detached
8. Biofilm detached

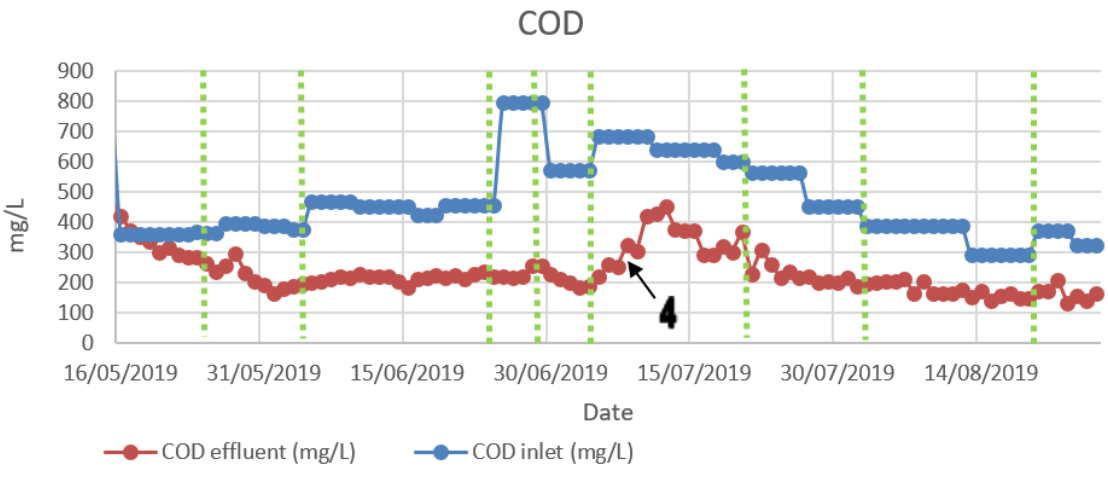

Figure 4-6: Timeline for COD concentration in the inlet and outlet

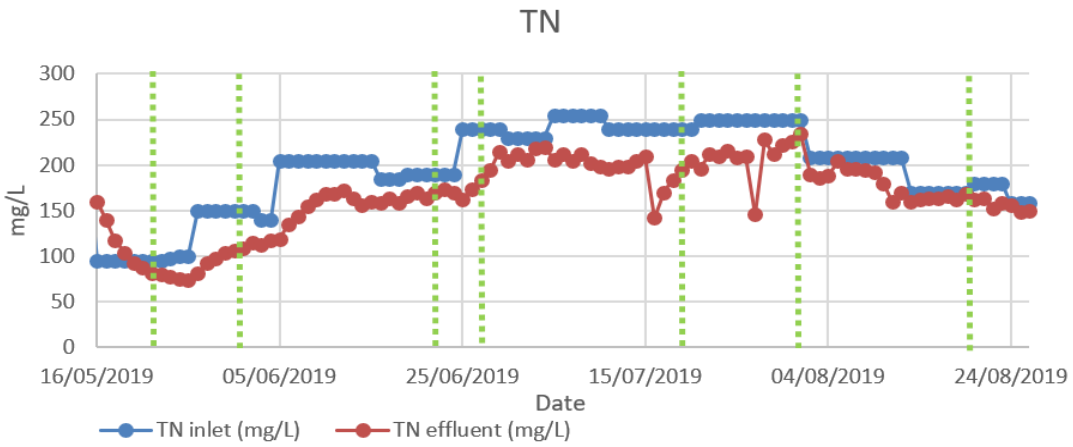

Figure 4-7: Timeline for Total Nitrogen in the inlet and outlet

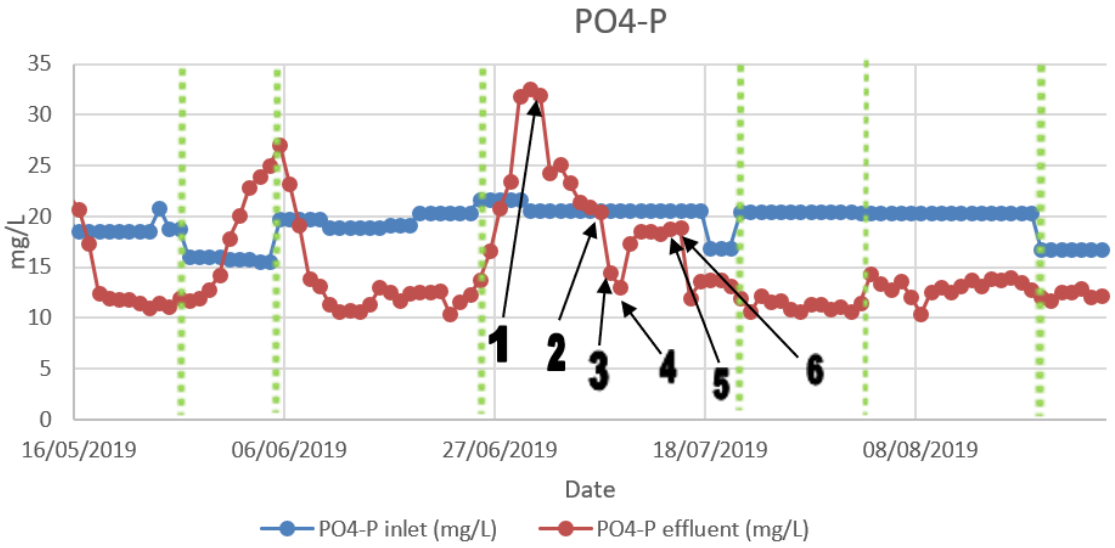

Figure 4-8:Timeline for Phosphates (PO_4-P) in the inlet and outlet

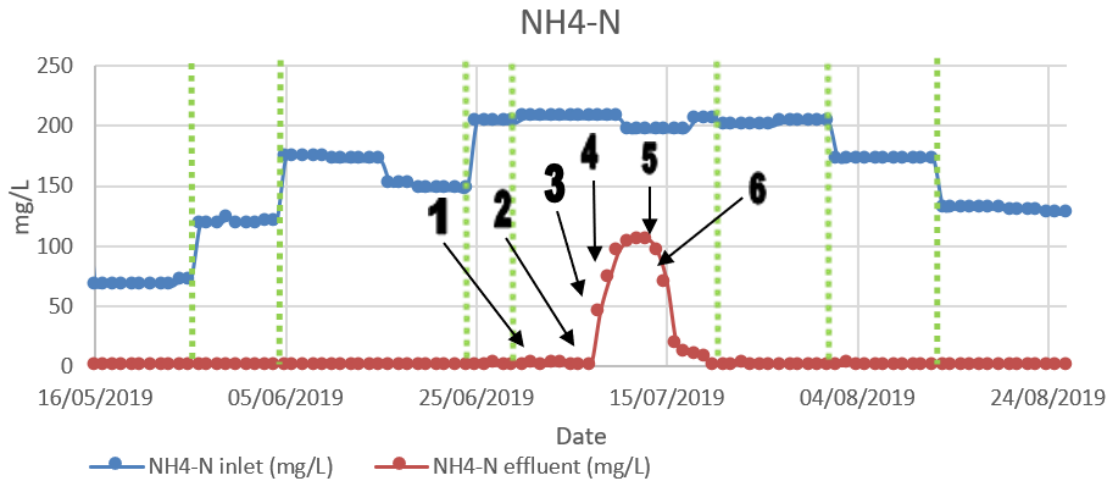

Figure 4-9:Timeline for NH_4-N in the inlet and outlet

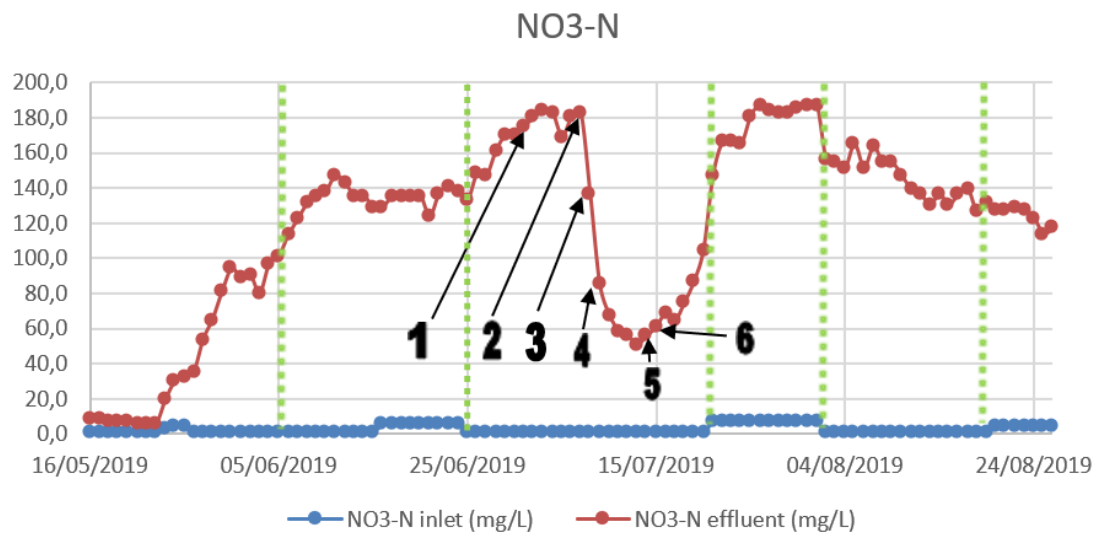

Figure 4-10:Timeline for Nitrates (NO_3^--N) in the inlet and outlet

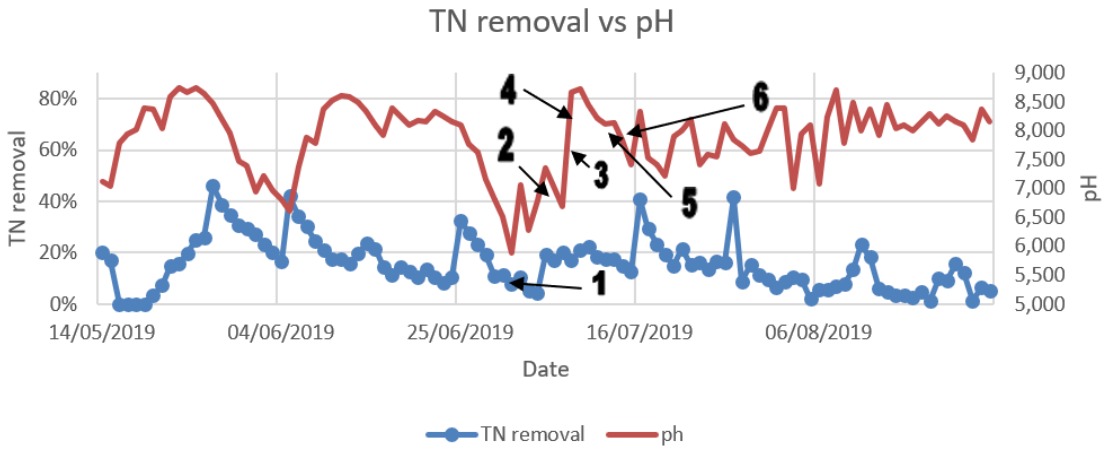

Figure 4-11: Effects of pH variation on TN removal

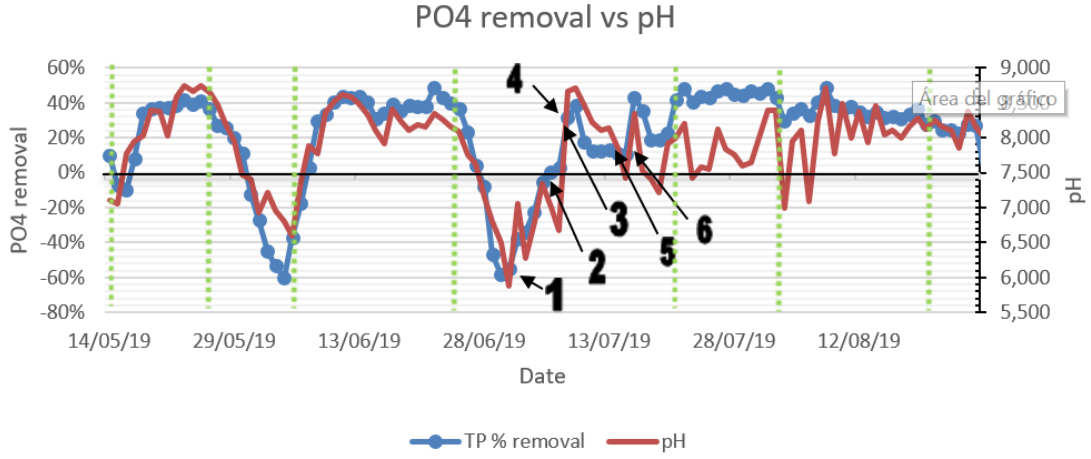

Figure 4-12: Effects of pH variation on PO_4^{3-}-P removal

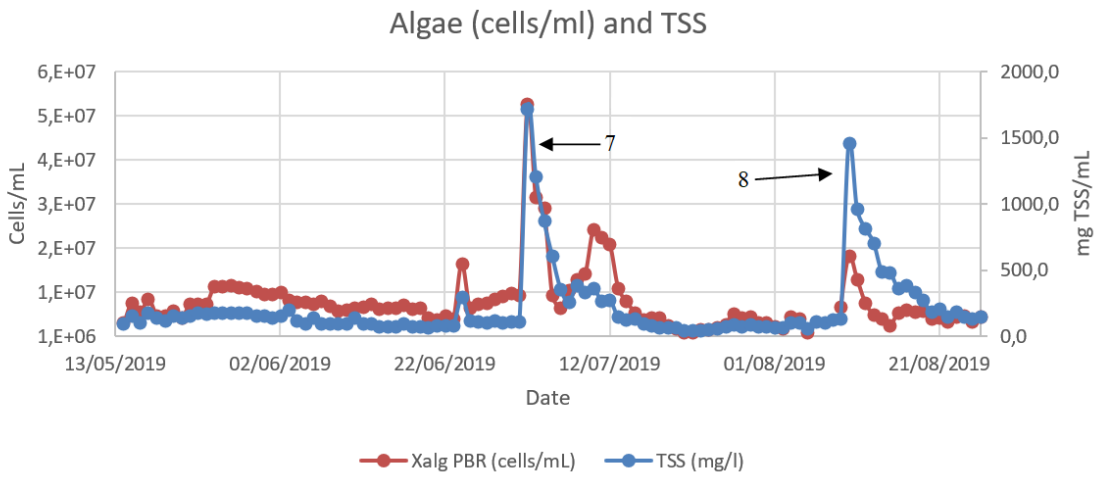

Figure 4-13: Algae concentration and Total Suspended Solids

- Figure 4-6: COD concentration did not experience significant variations due to pH changes. The only noteworthy change was from the date 09/07/2019 in which 10mL of pre-treated wastewater were added to try recovering the nitrifying community and COD concentration increased.

- Figure 4-7: Two substantial drops in Total Nitrogen were observed the dates 16/07/2019 and 27/07/2019. No significant pH changes occurred in those dates. These drops could be due to errors in the readings.

- Figure 4-8: Before event 1, when pH control started, can be observed strong variations in the PO_4^{3-}-P concentrations. After, PO_4^{3-}P start to stabilise and removal too, as observed in figure 4-12.

- Figure 4-9: Next day after NaOH dosing started, NH4-N values started to rise and continued like it. The first thought was that the nitrifying community was struggling. The type of connection used to dose NaOH (T type, more details in the discussion section) seems to be the source of this situation. To solve it, wastewater was dosed to try recovering the nitrifying community and the connection was changed.

- Figure 4-10: Initially there was no NO3 in the inlet and in the effluent values were low, around 10 days later NO3 values started to rise, proving the presence of nitrifying bacteria. When the event 3 occurred, values started to drop drastically.

- Figure 4-11: In this figure cannot be appreciated a strong relationship between pH values and Total Nitrogen removal.

- Figure 4-12: A strong relationship between pH values and PO_4^{3-}-P removal can be observed. Both lines are similar before and after pH control.

- Figure 4-13: In both 7 and 8 events, the biofilm formed inside the reactor was detached to observe if any change in the efficiency would occur, but no changes were observed. Only the TSS values changed proportionally to the microalgae concentration.

VFA and alkalinity values:

Table 4-2: Alkalinity and VFA values

	20/06/2019	25/06/2019	28/06/2019	07/07/2019	21/07/2019
Inlet PBR VFA (as mg/L Ac. Acid)	0	0	1,1	2	0
Inlet PBR Alkalinity (as mg/L $CaCO_3$)	376,6	434,4	459,5	455,7	453
Effluent PBR VFA (as mg/L Ac. Acid)	0	0	0	0	0
Effluent PBR Alkalinity (as mg/L $CaCO_3$)	13,5	18,1	11,9	10,5	14,6

It is seen that almost all the alkalinity is consumed. A result which was expected as microalgae metabolism consumes alkalinity according to the following equations formulated by Ebeling, Timmons and Bisogni assuming NH_4^+ as the main nitrogen source for algae (Ebeling, Timmons, & Bisogni, 2006):

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2$$

Adding the fact that chemolithotrophs like nitrifying bacteria use CO_2 as the inorganic-carbon source. When carbon dioxide dissolves in wastewater, carbonic acid is formed In wastewater some of the carbonic acid disassociates and forms the bicarbonate ion and the hydrogen ion. It is the bicarbonate ion that is used as the inorganic carbon source (Gerardi, 2002).

5. Discussion

5.1. Counting methods

The efficiency of wastewater treatment depends on several factors and each wastewater has a different composition. To discover the best species to be used for this purpose first needs to be identified which are the most resistant and with the best removal rates as Posadas et al. suggested (Posadas, et al., 2017) and expose them to the environment in which they are going to be consuming nutrients. Ideal situations can be used as a first approach to the proposed hypothesis, but those conditions are, in most cases, too expensive and unachievable in real conditions.

Once algae have been exposed to the new environment, keeping track of its growth is essential to verify if they are adapting well. 3 different ways have been studied and compared. The results obtained using Haemocytometer and flow cytometer showed similar values and trend. Differences between values could be produced by miscounting due to human error or the presence of algae flocks (although vortexing thoroughly) which made difficult to count all the cells present in those groups, especially in higher densities. Although some differences, the results from both methods follow the normal algae growth trend and can be considered as reliable methods for algae counting. Nevertheless, Coulter counter results are far from being like those obtained with the previous methods and do not show any trend. After reading the values obtained in day 0 and day 1, samples were read 3 times each to evaluate the variation among reads. Although each sample was vortexed thoroughly before each read, there were strong variations in most of the samples. (See annexe 1).

By reason of these results, a dilution series of one strain (*Chlorella sorokiniana*) was prepared to verify these results. Before, the device was cleaned twice thoroughly following the user's manual instructions, modifying the current intensity and gain values and removing the noise from the new readings. 20µm aperture tube was used at first, but noise values and variations in readings were unacceptable. It could be due to its high sensitivity. User's manual recommends to not place any other device that could produce vibrations or noise close to the Coulter counter. However, a computer is placed a few centimetres away from the device on the same table.

Hence, 50 μm aperture tube was set to reduce interferences. As a result of this change, the noise was reduced, and readings were more similar to each other but counting remained still high. 1:10 dilution was measured with the flow cytometer as well, in other to validate these results, showing a concentration of 4,238,180 cells/mL. If we compare this value with the 3μm column in figure 5-12 (*Chlorella sorokiniana* average size is 3μm) we can observe a substantial difference between both numbers.

Coulter counter counts by reading variations in an electric field between electrodes. An explanation for the previous counting differences between devices could be that membrane of some dead cells remains stable, occupying a volume and being counted as a living cell. Another explanation could be that as samples need to be vortexed in order to keep algae in suspension and to separate them (algae tend to create flocks), it creates bubbles within the sample, some of them minuscule and could be counted as cells, what could lead to overcounting errors as well.

5.2. Growth in limited medias

Chlorella Sorokiniana has been cultivated on microplates to study how the concentration of nutrients affects its growth. Tecan microplate reader was set as follows:

Mode	Fluorescence Top Reading
Excitation Wavelength	430nm
Emission Wavelength	600nm
Excitation Bandwidth	20nm
Emission Bandwidth	30nm

The results on the nitrogen-limited media show higher biomass production between days 3 and 4 in the highest dilutions (1/32, 1/64 and 1/128) what surprisingly contradicts the ideal ratio for Nitrogen and phosphorus stated by Wang et al. as optimal for algae growth. (Wang, et al., 2017). Nevertheless, can be observed in figures 4-4 and 4-3 a higher biomass production in the dilutions 1/1 and 1/2 in the P-limited media than in the N-Limited media, it fit better ratios between 5 and 30, as stated by Jeon Choi and Mok lee (Jeong Choi & Mok Lee, 2015). Biomass production is highly dependent on the N/P ratios as we can see. Also, this result means that a higher P-content in the wastewater favours biomass productivity more than a higher N-content in the wastewater. (Jeong Choi & Mok Lee, 2015).

5.3. Role of each species:

Initially, only *Chlorella sorokiniana* was supposed to be present in the photobioreactor. But then nitrates values started to rise. Analysing a sample from the photobioreactor to check if bacteria could be observed, two species of microalgae could be identified under the microscope and in the flow cytometer together with bacteria. The following image is a picture of a pure culture of *Chlorella sorokiniana:*

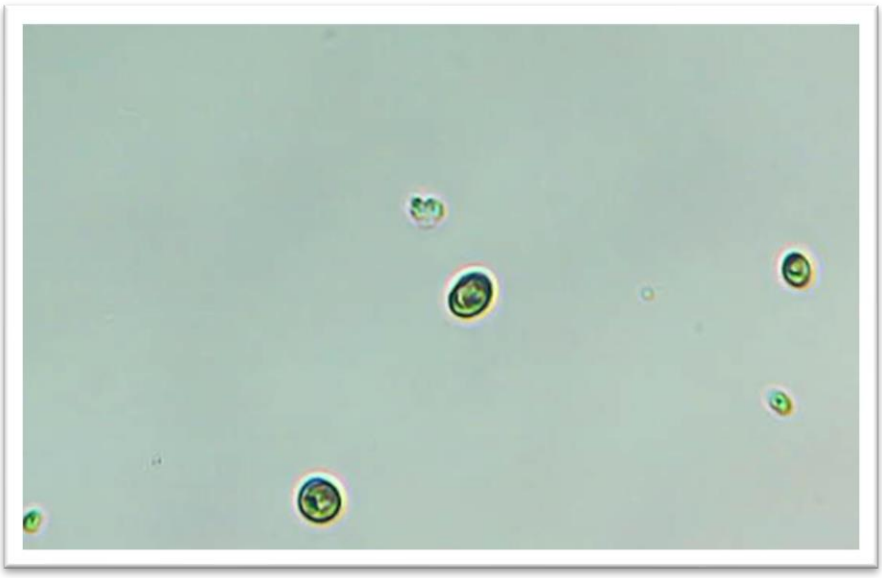

Figure 5-1:Chlorella sorokiniana under the microscope (optics x40)

The next image represents the signal that a pure culture of *Chlorella sorokiniana* generates in the channel FL1 (530 +/- 15nm) of the flow cytometer:

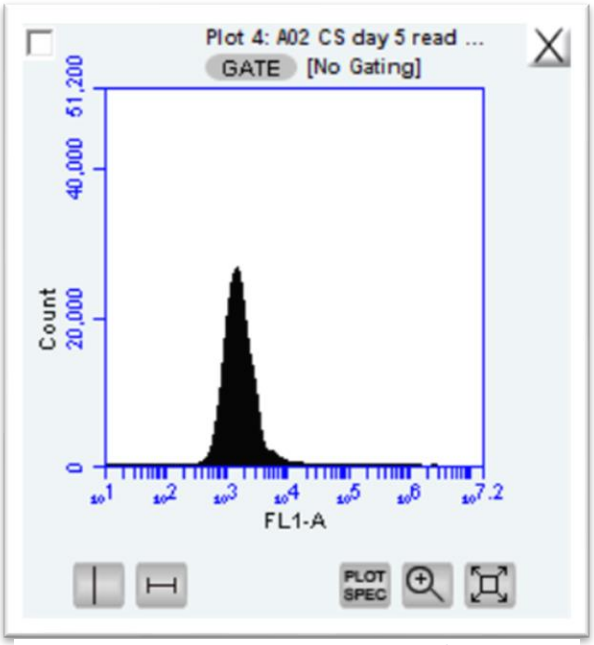

Figure 5-2: Signal in channel FL1 for Chlorella sorokiniana

The following image is a sample from the photobioreactor. The black arrow points microalgae different from *Chlorella sorokiniana*:

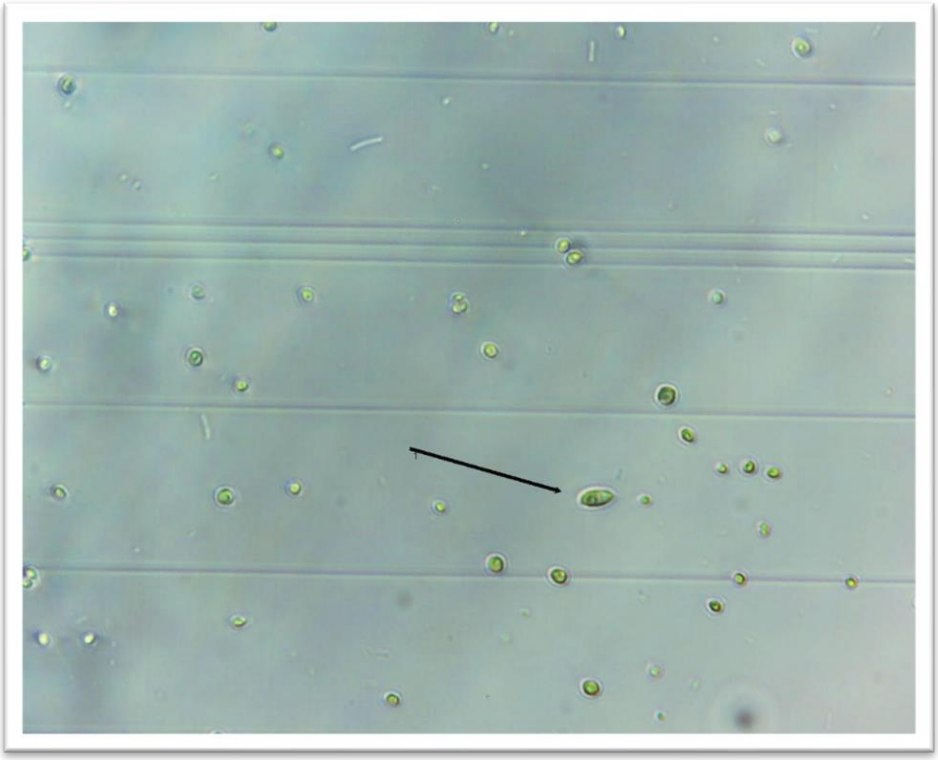

Figure 5-3: A different species identified in the photobioreactor

It was suspected that the photobioreactor was contaminated with *Tetradesmus obliquus (*Also known as *Scenedesmus obliquus)*. Species that have been involved in this project or even could be present in the wastewater used, as well as bacteria were not expected but they were present. Probably the ceramic membrane filtration unit system was contaminated. All the material used for this project (bottles, Erlenmeyer flasks, etc) were autoclaved before use. The species identified in the figure 5-3 was compared with a pure culture of *Tetradesmus obliquus:*

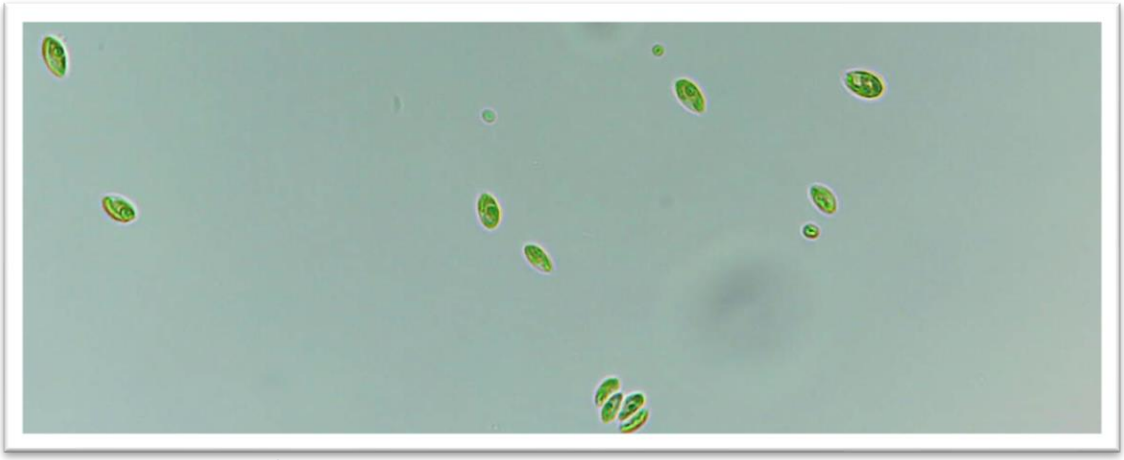

Figure 5-4: Pure culture of Tetradesmus obliquus

The signal that *T. obliquus* creates in the channel FL1 in the flow cytometer is the following:

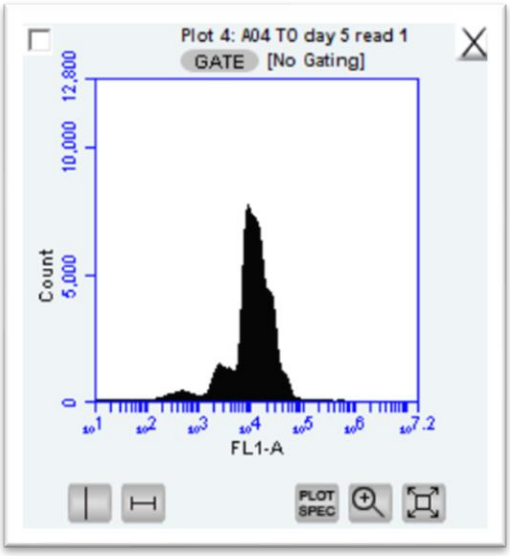

Figure 5-5: Signal in FL1 of T. obliquus

Comparing figures 5-2 and 5-5, the signal from *T. obliquus* is stronger than the one from *C. sorokiniana*. What is relatable to the difference in the size of both species. The signal read after a sample from the photobioreactor shows two peaks, one representing each species:

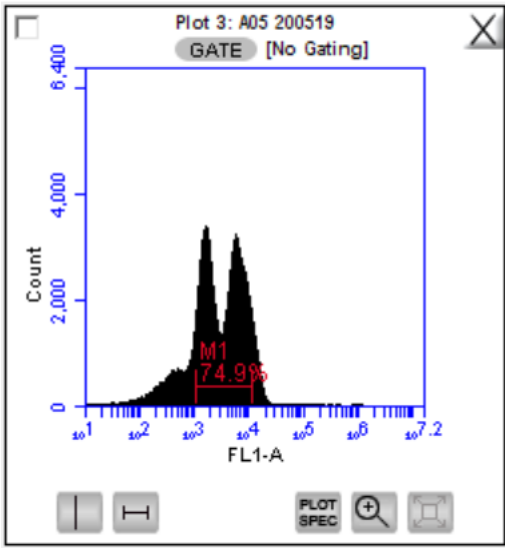

Figure 5-6: Signal in FL1 filter from both species present in the PBR

Initially, T. obliquus concentration was similar to C. *sorokiniana's* but over time, *T. obliquus* overtook *Chlorella's* concentration as can be observed in the figure:

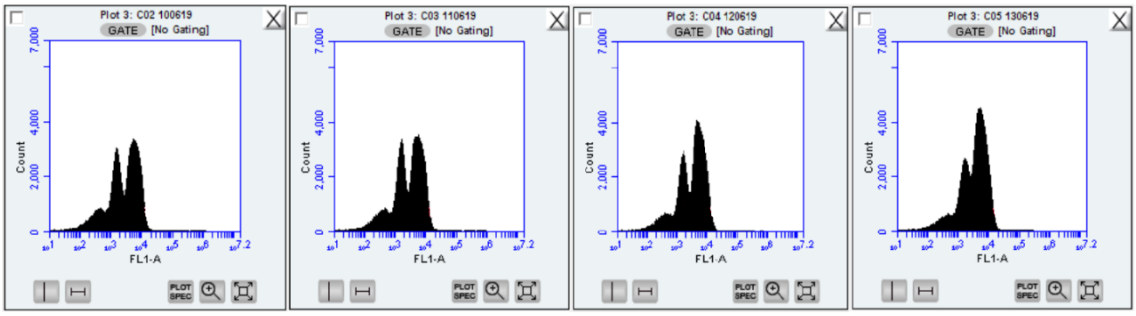

Figure 5-7: Changes in the concentrations for each species between June 10th and June 13th, 2019

It could be related to the niche that each occupies inside the PBR. It was observed that, when the biofilm was detached from PBR's walls (01/07/19), suddenly *C. sorokiniana* concentration raised dramatically, making almost inappreciable the signal from *T. obliquus*. It continued like it until 19/07/19, day in which the signal from *T. obliquus* started to appear again and increasing as the concentration also increased overcoming again *C. sorokiniana*:

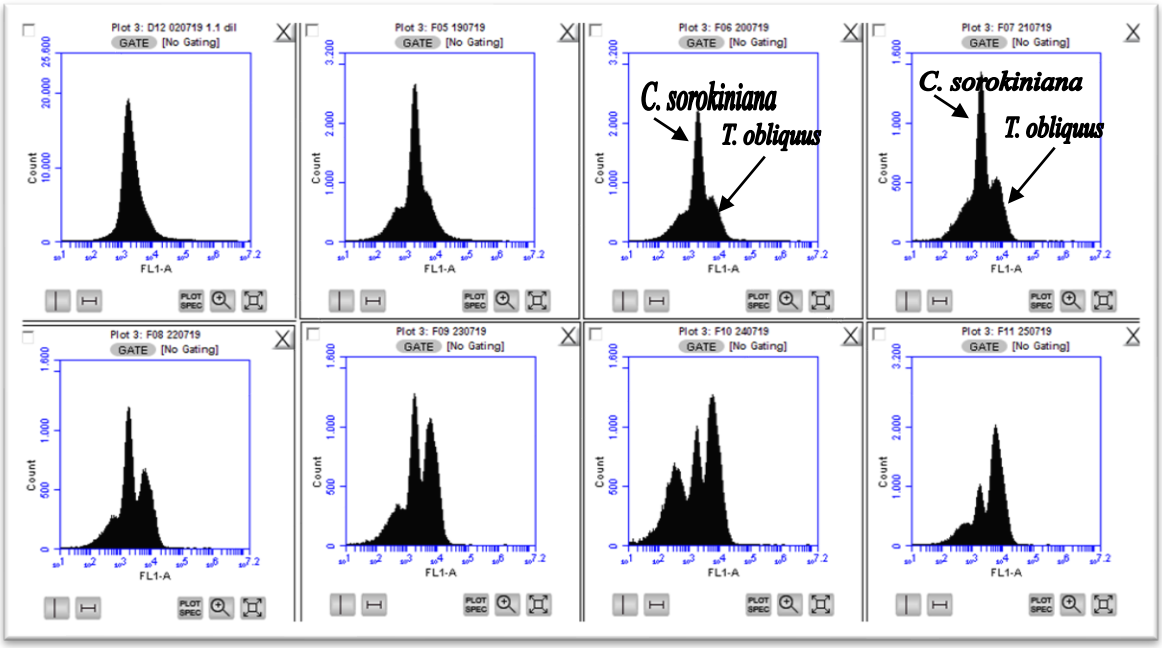

Figure 5-8: Changes in the signal of C. sorokiniana and T. obliquus overtime after detachment of biofilm

Another condition observed worth to be mention, is the morphology presented by *T. obliquus*. This species depending on the stress at which is exposed might change its morphology to protect itself from grazers or competitors. The environmental conditions not only determine which phenotypes will be produced, but also provide the arena where different morphologies experience different growth and survival and thus which

are subjected to selection. Competition and predation are considered the major selective forces responsible for organizing and structuring communities. In the presence of grazers or competitors, *T. obliquus* tends to create colony groups (Lurling, 2003). Observing the samples taken from the PBR, *T. obliquus* remained in the single-cell form (blue circles):

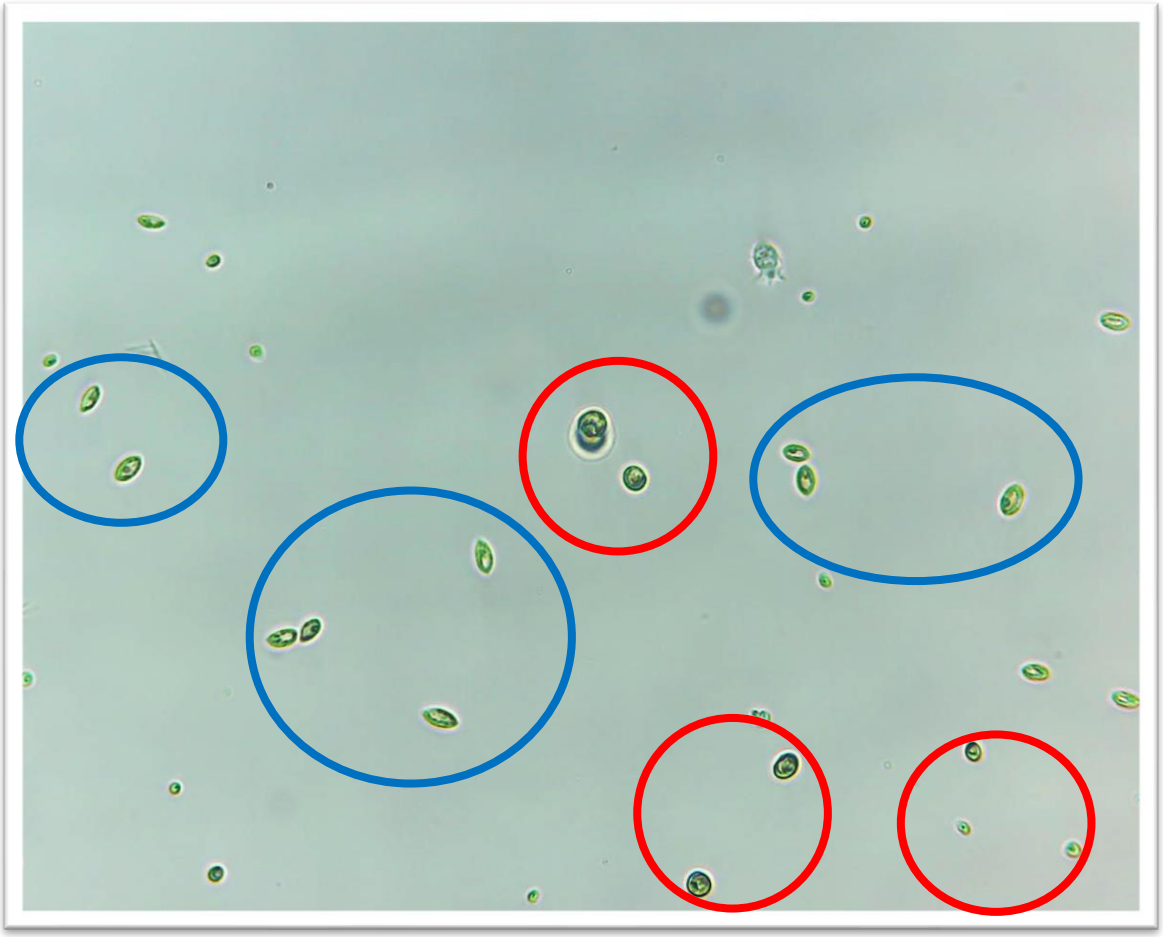

Figure 5-9: T.obliquus within the blue circules and C. sorokiniana within red circles.

As *C. Sorokiniana* numbers increased when the biofilm was removed, and *T. obliquus* remained in the single-celled form, together with the observation done by Lurling, merge the hypothesis that both species might live in the same environment occupying different niches (suspension and biofilm) and not representing a competitor to each other. One of the principles of community ecology is the enhancement on productivity when different organisms grow together. When functionally diverse algae are grown together, the resulting communities are more productive than monocultures of individual species. An explanation for increased productivity in mixed cultures is through resource-use complementarity. When species that have different growth requirements and they are grown together, competition between members of the

community is reduced compared with that experienced by individuals in dense monocultures. (Kazamia, Riseley, Howe, & Smith, 2014).

5.4. Microalgae nutrient removal efficiency under non-ideal conditions

Observing figure 4-6, an average of 50% of COD removal was achieved during this study. The value is lower than what found in other studies (Hammouda, Gaber, & Abdel-Raouf, 1995). Initially could be thought that the temperature chosen for this study could affect the COD removal, but Jämsä et al. found that temperature did not have a great influence on COD removal (Jämsä, et al., 2017). Another observation was although the HRT changed from 2,25 $days^{-1}$ to 5,05 $days^{-1}$, no significant differences were noted on the COD removal.

Total Nitrogen removal shows the most surprising values. Although there are peaks on Total nitrogen removal up to 46%, on average the removal reaches only 16%. Comparing the ammonium ion concentrations and total nitrogen in the inlet, ammonium ions represent around an 80% of the TN. The remaining nitrogen include nitrates and most likely other organic species of nitrogen, like urea (not measured in this study). The ammonium concentration in the inlet is higher than the toxicity limit, which is over than 25μM (Barsanti & Gualtieri, 2014). It could be a reason for the lower efficiency of microalgae in this study. Also, out of that 16% of removal efficiency, should be counted that part of ammonium ion in secondary municipal wastewater may also be removed through N_2 loss (due to bacterial nitrification-denitrification) and NH_3 volatilization (Wang, et al., 2017).

Phosphate values had a great correlation with pH values. At the beginning of this study, there was no pH control. It can be observed in figure 4-12, that before pH control started (29/06/2019) Phosphate removal rate fluctuated with the same trend as pH did. After pH control started, the phosphate removal started to become more stable achieving an average of 40% of removal.

Nitrates were almost inexistent at the beginning of this study in the PBR, but eventually, its production increased exponentially. When pH control began, the type of connection used to dose NaOH was the following:

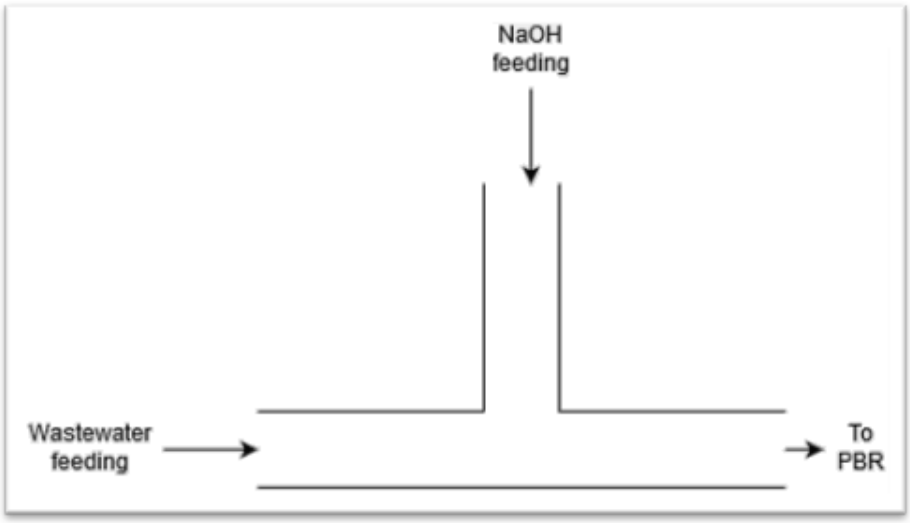

Figure 5-10: Initial NaOH dosing setting

With this type of connection, although the pump stopped dosing NaOH, wastewater remains in contact with the NaOH solution and by diffusion, OH^- ions could flow to the wastewater feeding, raising its pH. After observing the Ammonium levels increasing in the effluent, this connection was changed. The dosing tube was placed on the top of the PBR and from that moment NaOH solution was dripping into the PBR:

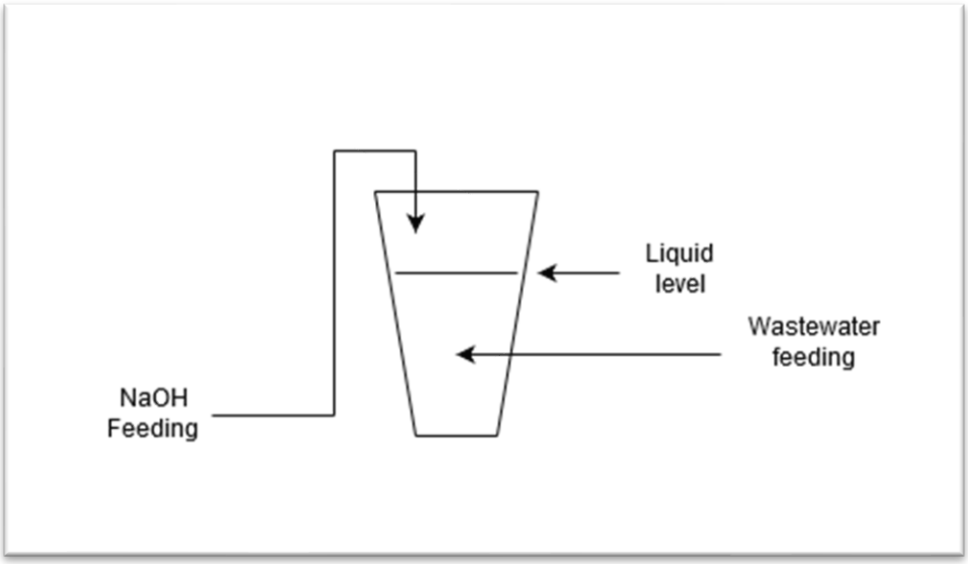

Figure 5-11:NaOH dosing setting after observing changes in NH4 levels in the effluent from PBR

After this modification, the target was trying to recover the nitrifying community. During this period, pH was kept at the ideal values for this type of bacteria, between 5.8 and 8.5 (Gerardi, 2002). It could be assumed that the nitrifying bacteria present were *Nitrosomonas* and *Nitrobacter*, as the last one required longer time to recover,

according to the NO_3^- levels in the effluent. *Nitrobacter* has a longer generation time (12 – 60 hours) than *Nitrosomonas* (8 – 36 hours) (Gerardi, 2002). After increasing the HRT, it could be observed a diminution in the NO_3^- levels in the effluent. Most of the Ammonium ion is converted into NO_3^- , making it the main nitrogen source available for algae.

6. Conclusion

In general, results show low removal efficiency when compared with other studies. In this case, due to the conditions under which the microalgae have been cultivated. The lack of CO_2 bubbling and the presence of inhibitory substances like a high concentration of ammonium in the wastewater could be the main reason. But, most of the studies are performed under ideal conditions and in batch mode or with much higher HRT's (Hammouda, Gaber, & Abdel-Raouf, 1995).

Using microalgae for wastewater treatment is a process that depends on many factors. Keeping them under control is crucial to maintain a stable environment for microalgae. Factors like temperature or mixing can be changed within wider ranges compared to others like pH or light intensity without affecting the nutrient removal efficiency. pH especially affects the removal efficiency on one of the key nutrients: phosphorous. To keep an acceptable removal pH must be kept between 8 and 8,3, a pH acceptable for all the species present on this study in the PBR. The situation created by setting up for NaOH for pH helped to show the sensitivity of the nitrifying bacteria to pH changes.

Regarding nitrogen species, the total nitrogen removal was low. Mainly due to the high levels of nitrates on the effluent. The rise on the HRT had a positive effect on its removal. Increasing it in further research could lead to better removal efficiencies. Such a change was not observed on COD removal which remained along with the study around 50%.

From all the counting methods used along with this study, flow cytometry has shown to be a remarkable tool to identify changes in the communities and understand the niches of each species and the type of interaction between species in the media. Can be concluded that *C. sorokiniana* and *T. obliquus* are species able to coexist within the same environment without becoming competitors.

Further research could be continuing the line of this study but providing CO_2 supply and connecting on series both PBRs that are available. This set up would increase the HRT and microalgae present in the second PBR will be fed with a media low in ammonium, which acts as an inhibitory substance.

7. References

Abu-Ghunmi, D., Abu-Ghunmi, L., Kayal, B., & Bino, A. (2016). Circular Economy and the Opportunity Cost of Not 'Closing the Loop' of Water. *Journal of Cleaner Production*.

Acién Fernández, F. G., Fernández Sevilla, J. M., & Molina Grima, E. (2017). Microalgae: The Basis of Mankind Sustainability. In *Case Study of Innovative Projects - Successful Real Cases.* doi:DOI: 10.5772/67930

Andersen, R. A. (2005). *Algal Culturing Techniques.* Elsevier Academic Press.

Asraful Alam, M., & Wang, Z. (2019). *Microalgae Biotechnology for Development of Biofuel and Wastewater Treatment.* Springer. doi:https://doi.org/10.1007/978-981-13-2264-8

Barsanti, L., & Gualtieri, P. (2014). *Algae: Anatomy, Biochemistry and Biotechnology.* CRC Press.

Bastidas, O. (2013). Retrieved from https://www.academia.edu/13150839/Technical_Note-

Beckman Coulter Life Sciences. (2019). Retrieved from https://www.beckman.com/support/faq/products/how-do-coulter-counters-work

Borowitzka, M. A., & Moheimani, N. R. (2015). *Algae for Biofuels and Energy.* Springer.

Carlsen, R. (2018). Optimization, quantification and treatment efficiency of algae grown on secondary wastewater effluent. M. Sc. Thesis. *M. Sc. thesis*.

Collet, P., Hélias, A., Lardon, L., Ras, M., Goy, R.-A., & Steyer, J.-P. (2011). Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresource Technology, 102*, 50-56.

De Godos, I., Vargas, V., Blanco, S., García González, M., Soto, R., García-Encina, P., . . . Múñoz, R. (2010). A comparative evaluation of microalgae for the degradation of piggery wastewater under photosynthetic oxygenation. *Bioresource Technology, 101*, 5150–5158.

Ebeling, J. M., Timmons, M. B., & Bisogni, J. J. (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems. *Aquaculture*(257), 346 - 358.

Ersahin, M., Ozgun, H., Kaan Dereli, R., & Ozturk, I. (2011). *Anaerobic Treatment of Industrial effluents: An Overview of Applications.* (F. S. García Einschlag, Ed.)

InTech.

Gerardi, M. (2002). *Nitrification and Denitrification in the Activated Sludge Process.* Wiley-Interscience.

Gonçalves, A., Pires, J., & Simões, M. (2016). A review on the use of microalgal consortia for wastewater treatment. *Algal research*.

Green Bioresearch. (2019). Retrieved from Green Bioresearch: https://elabsaver.com/product/96-well-cell-culture-plates-2/

Haire, T. C., Bell, C., Cutshaw, K., Swiger, B., Winkelmann, K., & Palmer, A. G. (2018). Robust Microplate-Based Methods for Culturing and in Vivo Phenotypic Screening of Chlamydomonas reinhardtii. *Frontiers in Plant Science, 9,* 235.

Hammouda, O., Gaber, A., & Abdel-Raouf, N. (1995). Microalgae and wastewater treatment. *Ecotoxicology and environmental safety, 31,* 205-210.

Henze, M., van Loosdrecht, M., Ekama, G., & Brdjanovic, D. (2008). *Biological wastewater treatment: Principles, modelling and design.* IWA publishing.

Jämsä, M., Lynch, F., Santana Sánchez, A., Laaksonen, P., Zaitsev, G., Solovchenko, A., & Allahverdiyeva, Y. (2017). Nutrient removal and biodiesel feedstock potential of green alga UHCC00027 grown in municipal wastewater under Nordic conditions. *Algal research, 26,* 65 - 73.

Jämsä, M., Lynch, F., Santana-Sánchez, A., Laaksonen, P., Petteri, G., Solovchenko, A., & Allahverdiyeva, Y. (2017). Nutrient removal and biodiesel feedstock potential of green alga UHCC00027 grown in municipal wastewater under Nordic conditions. *Algal Research*(26), 65–73.

Jeong Choi, H., & Mok Lee, S. (2015). Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. *Bioprocess and Biosystems Engineering, 38*(4), 761–766.

Karlson, B., Cusack, C., & Bresnan, E. (2010). *Microscopic and molecular methods for quantitative phytoplankton analysis.* Intergovernmental Oceanographic Commission of UNESCO.

Kazamia, E., Riseley, A. S., Howe, C. J., & Smith, A. G. (2014). An Engineered Community Approach for Industrial Cultivation of Microalgae. *Industrial Biotechnology, 10*(3).

Larsdotter, K. (2006). Wastewater treatment with microalgae - A literature review. *Vatten, 62,* 31 - 38.

Lee, C.-G. (1999). Calculation of Light Penetration Depth in Photobioreactors.

Biotechnology and Bioprocess Engineering .

Lee, K., & Lee, C. G. (2001). Effect of light/dark cycles on wastewater treatments by microalgae. *Biotechnology and Bioprocess Engineering*, *6*(3), 194 - 199. doi:https://doi.org/10.1007/BF02932550

Lurling, M. (2003). Phenotypic plasticity in the green algae Desmodesmus and Scenedesmus with special reference to the induction of defensive morphology. *Annales de Limnologie - International Journal of Limnology, 39*(2), 85 - 101.

Mehta, C., Khunjar, W., Nguyen, V., Tait, S., & Batstone, D. (2015). Technologies to Recover Nutrients from Waste Streams: A Critical review. *Critical Reviews in Environmental Science and Technology*(45:4), 385-427. doi:10.1080/10643389.2013.866621

Merk. (2019). *Ammonium Cell Test.* Retrieved from https://www.merckmillipore.com/GB/en/product/Ammonium-Cell-Test,MDA_CHEM-114559#anchor_PI

Merk. (2019). *Nitrate Cell Test.* Retrieved from https://www.merckmillipore.com/GB/en/product/Nitrate-Cell-Test,MDA_CHEM-114542#anchor_PI

Merk. (2019). *Nitrogen (total) Cell Test.* Retrieved from https://www.merckmillipore.com/GB/en/product/Nitrogen-total-Cell-Test,MDA_CHEM-114763#anchor_PI

Merk. (2019). *Phosphate Cell Test.* Retrieved from https://www.merckmillipore.com/GB/en/product/Phosphate-Cell-Test,MDA_CHEM-114729#anchor_PI

Merk. (2019). *Spectroquant COD Cell Test.* Retrieved from https://www.merckmillipore.com/GB/en/product/COD-Cell-Test-Hg-free,MDA_CHEM-109773#anchor_PI

Posadas, E., Alcántara, C., García Encina, P., Gouveia, L., Guieysse, B., Norvill, Z., . . . Muñoz, R. (2017). Microalgae cultivation in wastewater. In *Microalgae-Based Biofuels and Bioproducts* (pp. 67 - 91). Woodhead Publishing. doi:https://doi.org/10.1016/B978-0-08-101023-5.00003-0

Posadas, E., Alcántara, C., García-Encina, P., Gouveia, L., Guieysse, B., Norvill, Z., . . . Muñoz, R. (2017). Microalgae cultivation in wastewater.

Posten, C., & Walter, C. (2012). *Microalgal Biotechnology: Potential and Production.* De Gruyter.

Richmond, A., & Hu, Q. (2013). *Handbook of Microalgal Culture: Applied Phycology and Biotechnology.*

Riffat, R. (2013). *Fundamentals of wastewater treatment and engineering.* IWA publishing.

SCCAP. (2019). *Scandinavian Culture Collection of Algae and Protozoa*. Retrieved from http://www.sccap.dk/media/freshwater/3.asp

Schmidt, J. J., Gagnon, G. A., & Jamieson, R. C. (2016). Microalgae growth and phosphorus uptake in wastewater under simulated cold region conditions. *Ecological engineering*(95), 588 - 593.

Sedlak, R. (1991). *Phosphorus and nitrogen removal from municipal wastewater: Principles and practice. Second Edition.* CRC Press.

Singh, J., & Chandra Saxena, R. (2015). *Handbook of Marine Microalgae.* Academic Press. doi:https://doi.org/10.1016/B978-0-12-800776-1.00002-9

Subashchandrabose, S., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2011). Consortia of cyanobacteria/microalgae and bacteria: Biotechnological potential. *Biotechnology Advances, 29,* 896-907. doi:https://doi.org/10.1016/j.biotechadv.2011.07.009.

Tchobanoglous, G., Burton, F., & Stensel, H. D. (2003). *Wastewater engineering: Treatment and reuse.* Mc Graw Hill .

Tecan. (2016). *Instructions for Use for Infinite 200 PRO.* Retrieved from http://biomimetic-lab.vscht.cz/wp-content/uploads/2017/04/Infinite-200-PRO.pdf

ThermoFisher Scientific. (n.d.). (ThermoFisher Scientific) Retrieved from How a Flow Cytometer Works: https://www.thermofisher.com/es/es/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/flow-cytometry-basics/flow-cytometry-fundamentals/how-flow-cytometer-works.html#overview

Tyrrell, T. (2019). Redfield Ratio. In *Encyclopedia of Ocean Sciences* (3 ed., pp. 461-472). Academic Press. doi:https://doi.org/10.1016/B978-0-12-409548-9.11281-3.

Wang, J., Zang, T., Dao, G., Xu, X., Wang, X., & Hu, H. (2017). Microalgae-based advanced municipal wastewater treatment for reuse in water bodies. *Applied Microbiology and Biotechnology, 101,* 2659–2675. doi:https://doi.org/10.1007/s00253-017-8184-x

8. Appendixes

Appendix 1

Coulter counter results

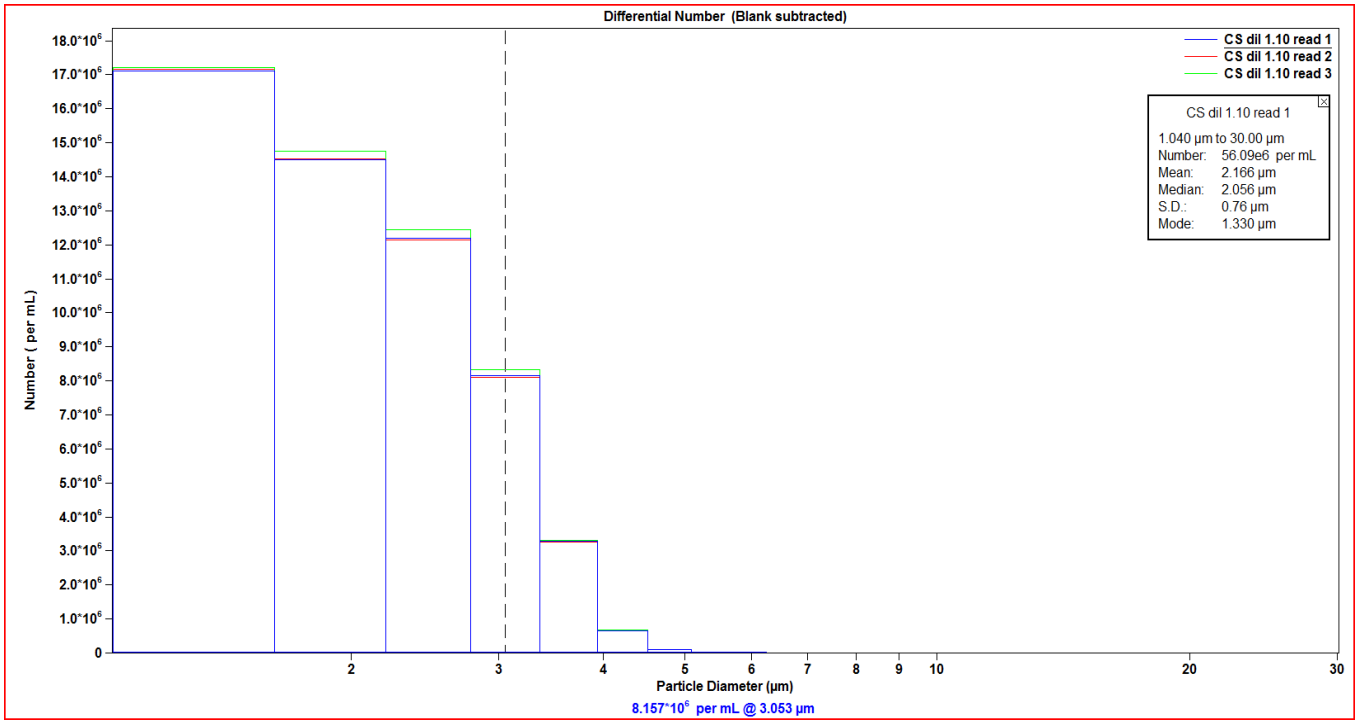

Figure 7-1: Coulter counter readings for dilution 1:10 of C. sorokiniana culture

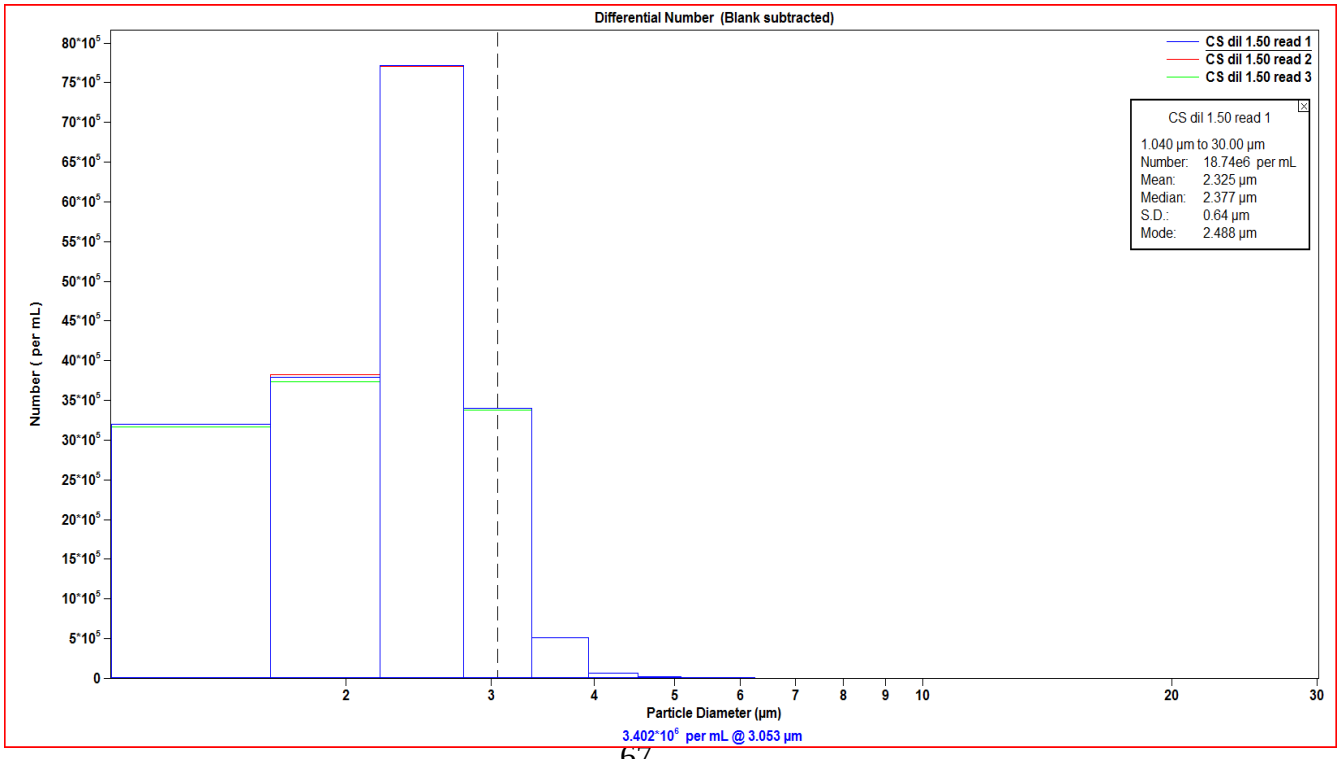

Figure 7-2: Coulter counter readings for dilution 1:50 of C. sorokiniana culture

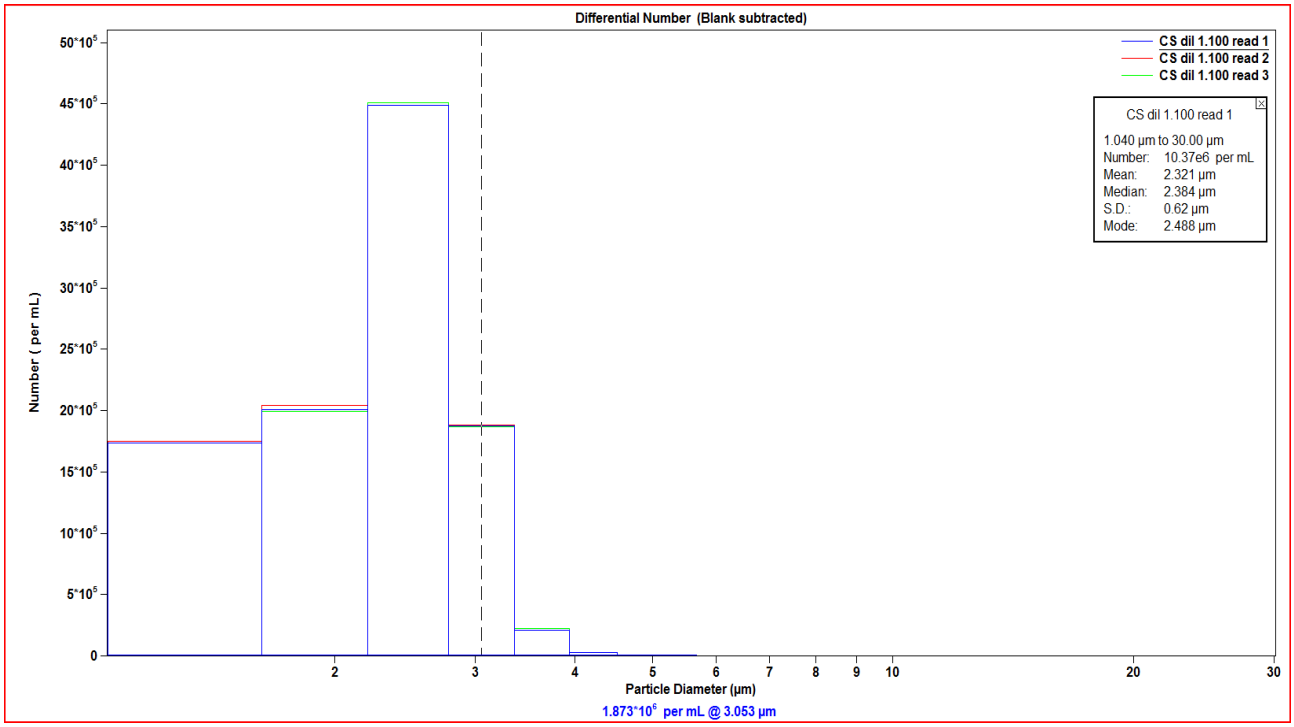

Figure 7-4: Coulter counter readings for dilution 1:100 of C. sorokiniana culture

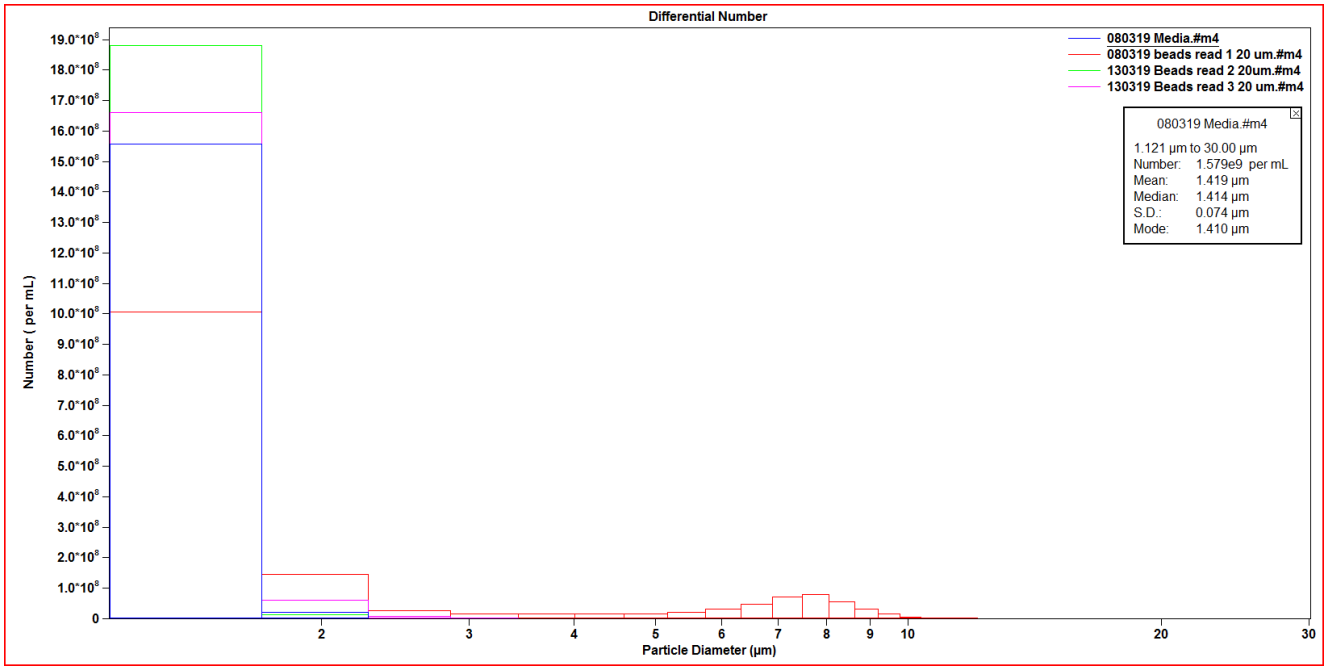

Figure 7-5: 2µ microbeads readings. Solution concentration: ~10^6 beads/mL

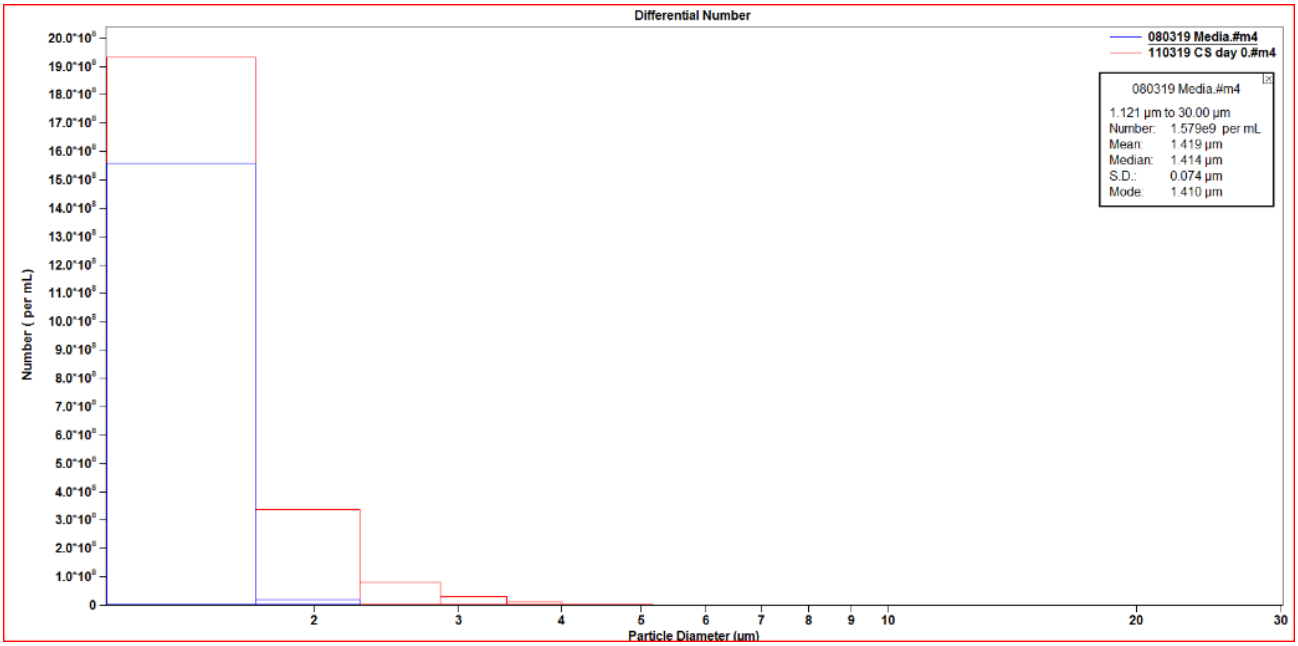

Figure 7-6: Coulter counter readings for C. sorokiniana culture on inoculation day.

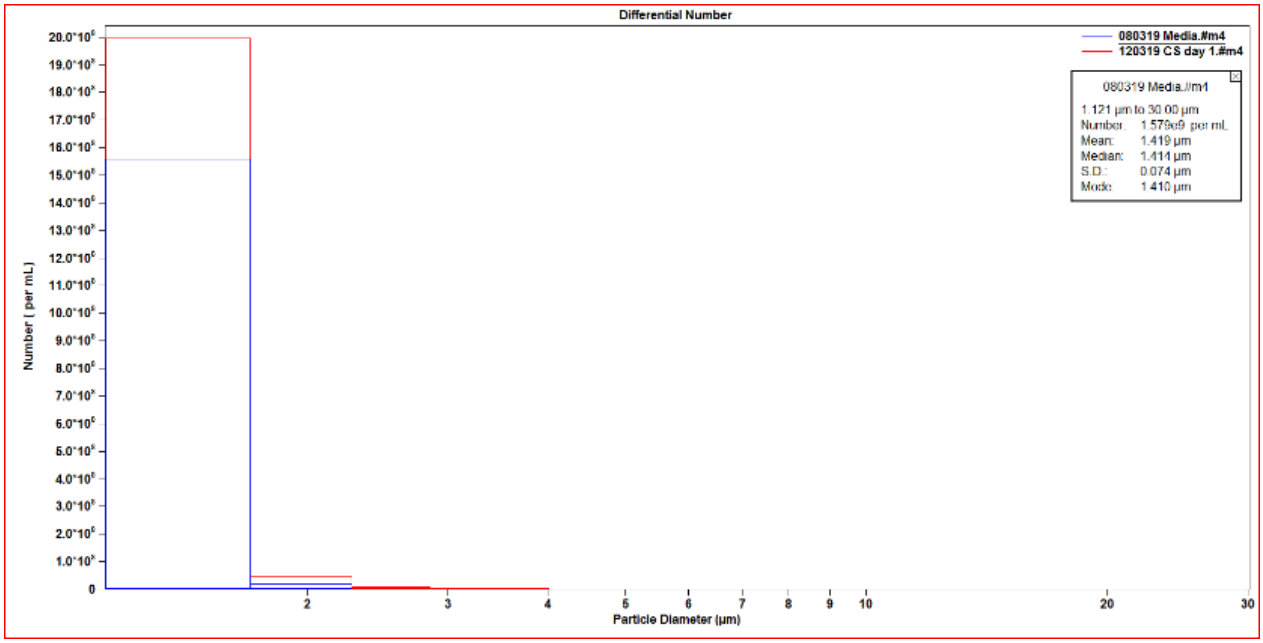

Figure 7-7: Coulter counter readings for C. sorokiniana culture. 1 day after inoculation day.

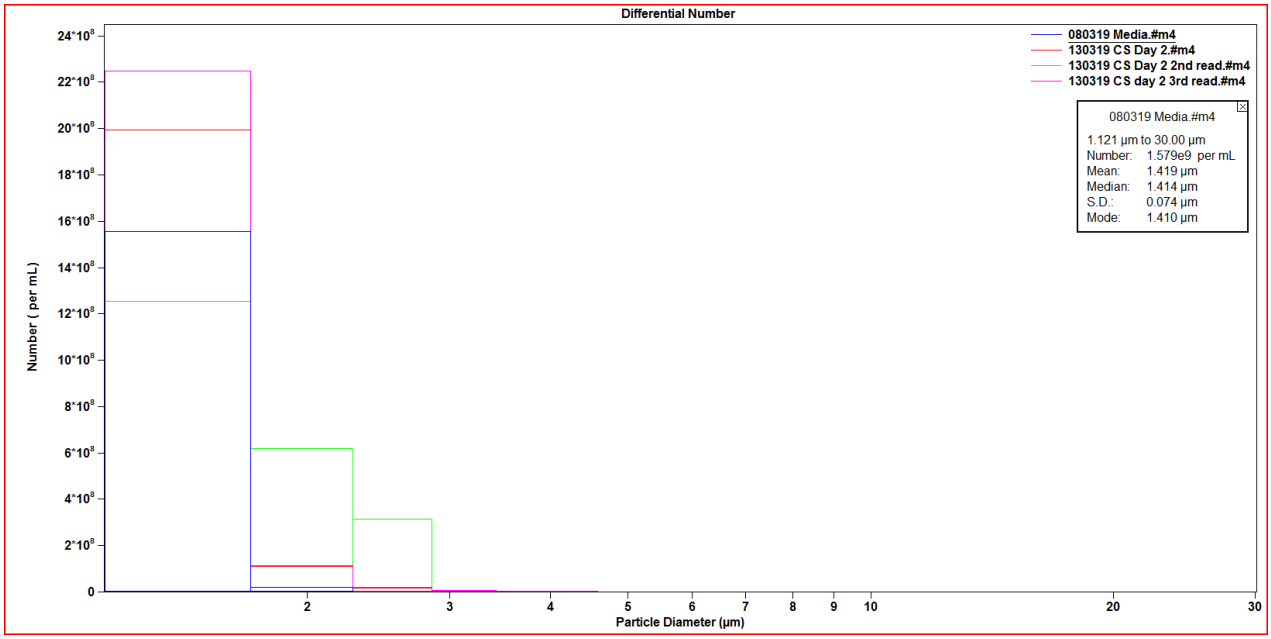

Figure 7-8: Coulter counter readings for C. sorokiniana culture. 2 days after inoculation day.

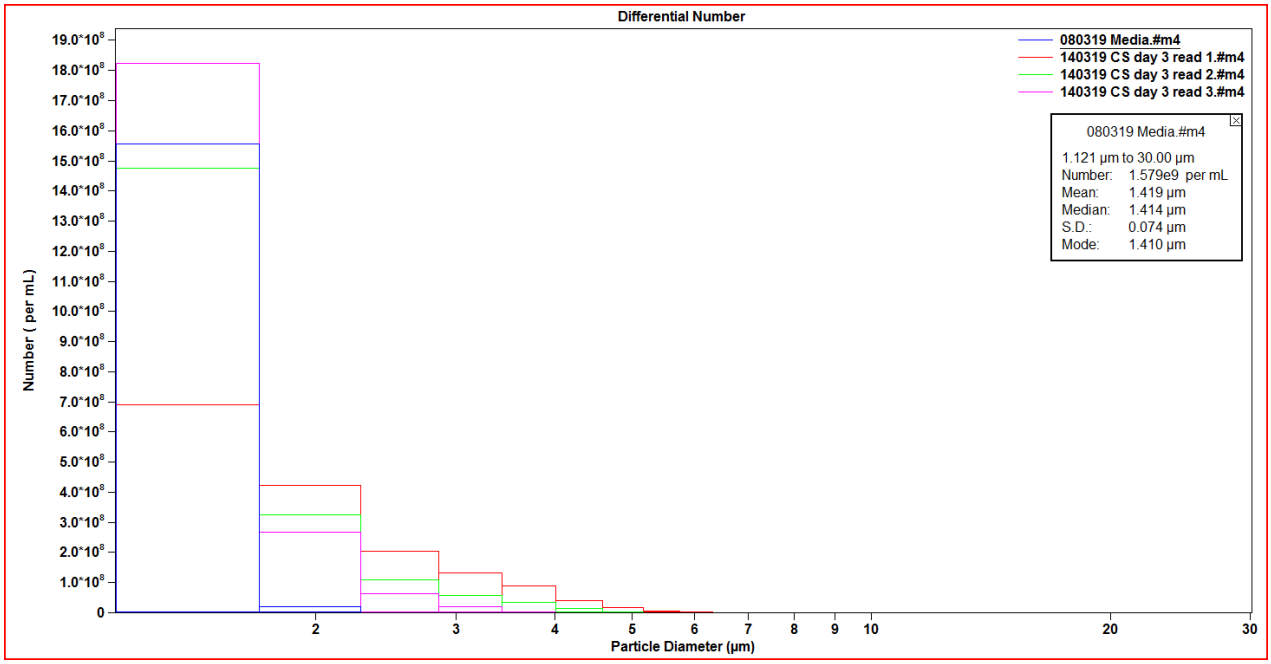

Figure 7-9: Coulter counter readings for C. sorokiniana culture. 3 days after inoculation day.

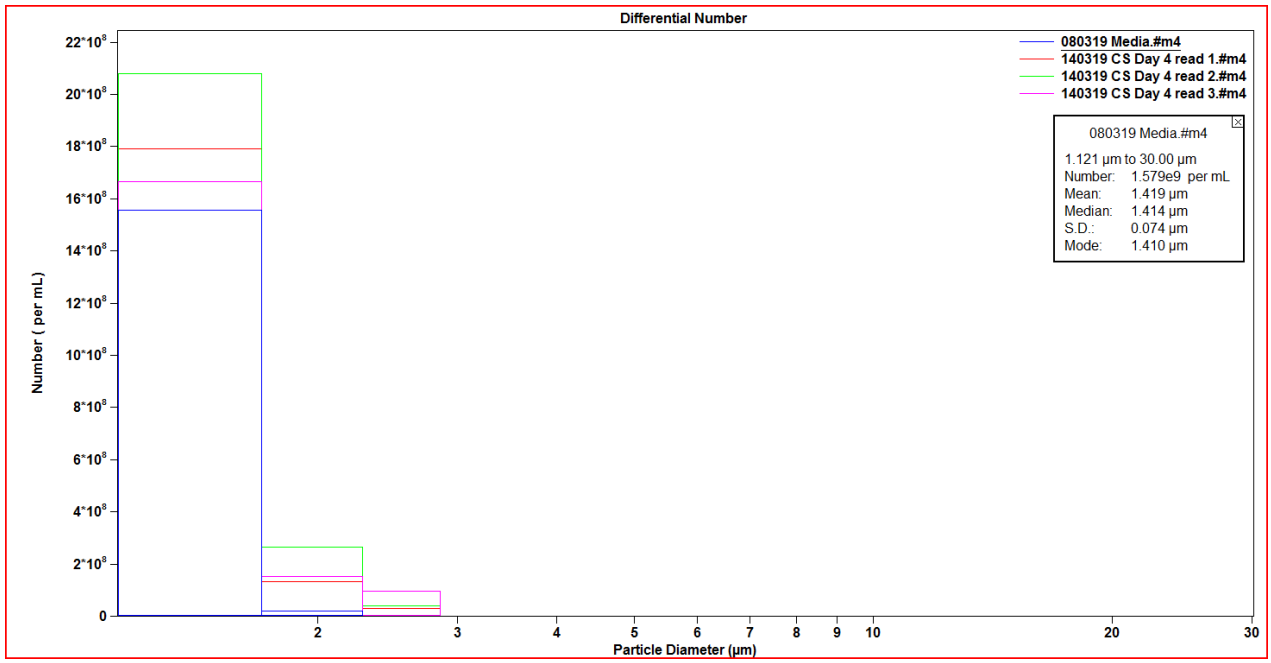

Figure 7-10: Coulter counter readings for C. sorokiniana culture. 4 days after inoculation day.

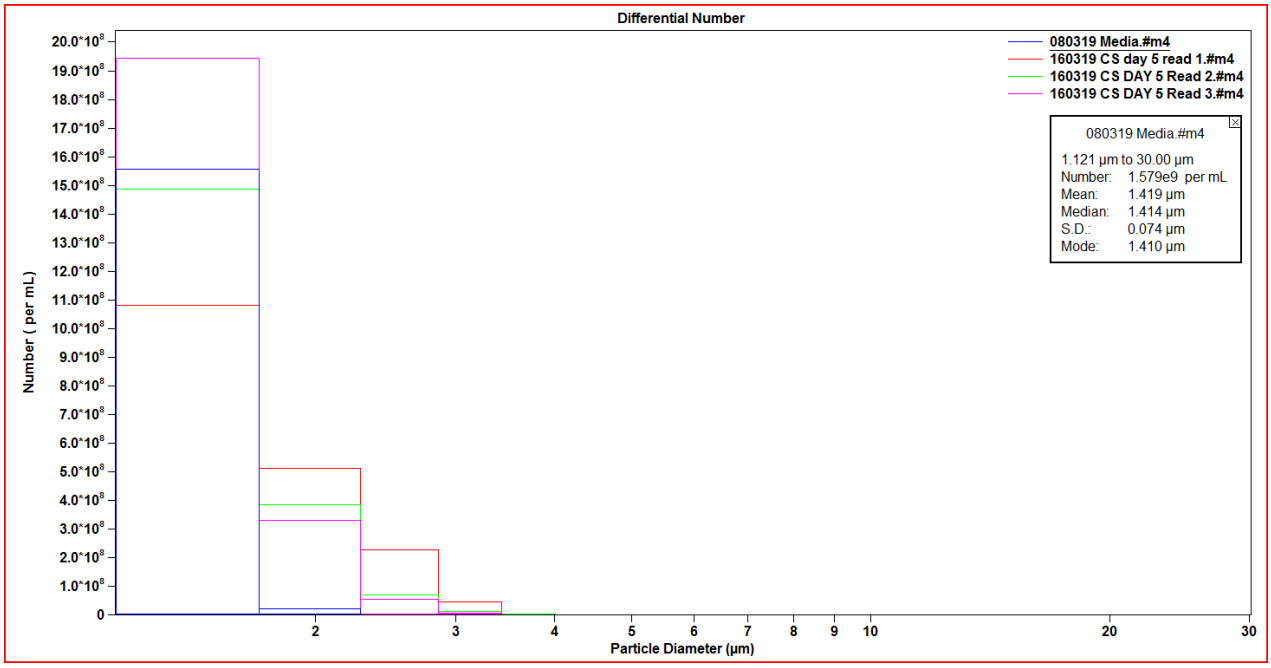

Figure 7-11: Coulter counter readings for C. sorokiniana culture. 5 days after inoculation day.

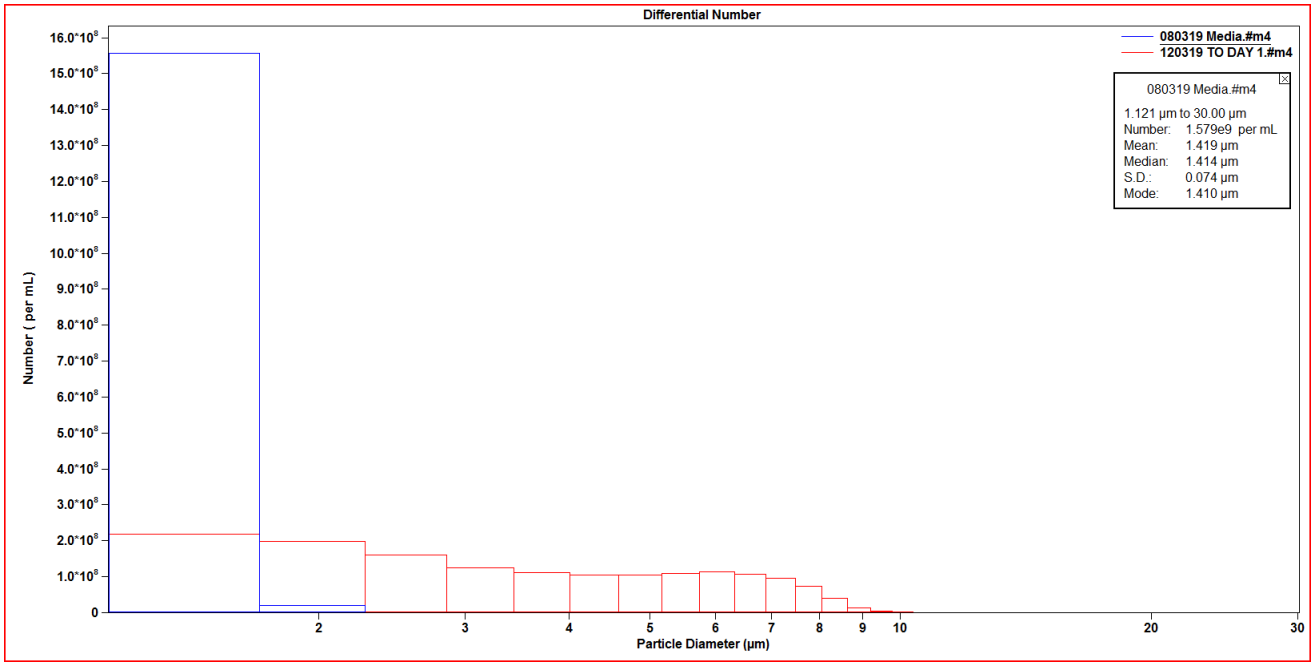

Figure 5-23: Coulter counter readings for T. obliquus culture. Inoculation day.

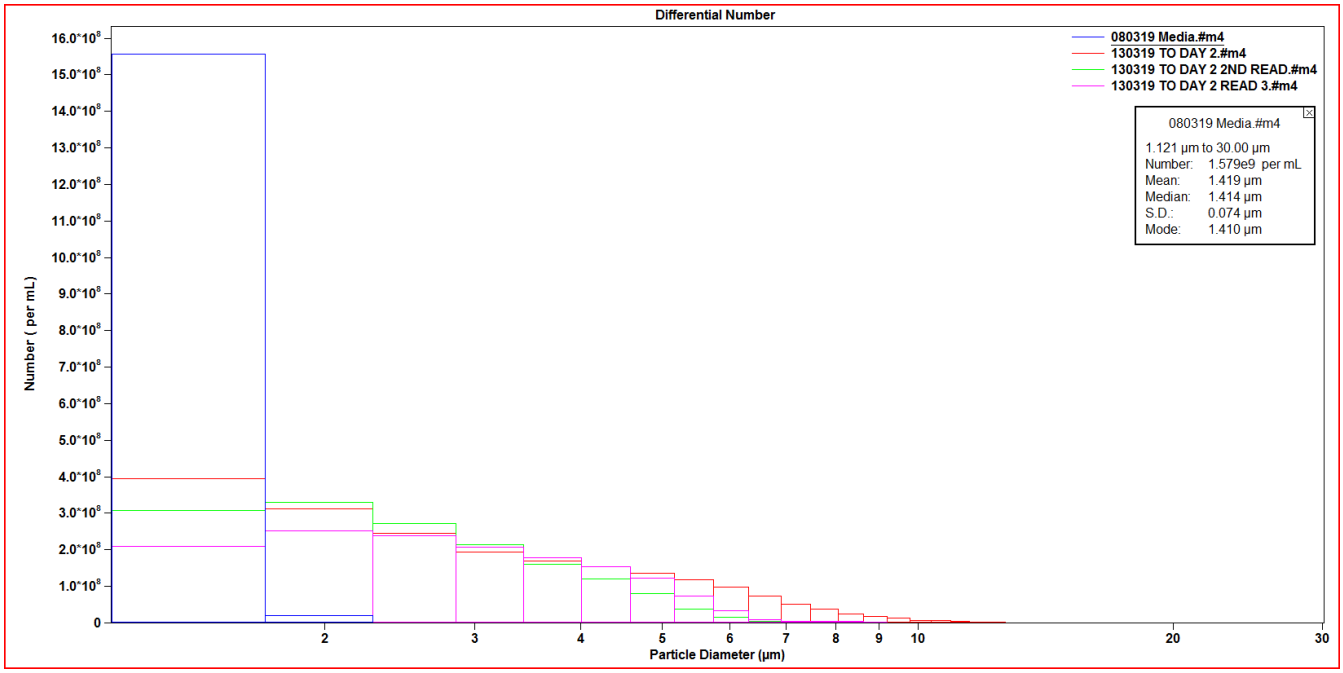

Figure 5-24: Coulter counter readings for T. obliquus culture. 1 day after inoculation day.

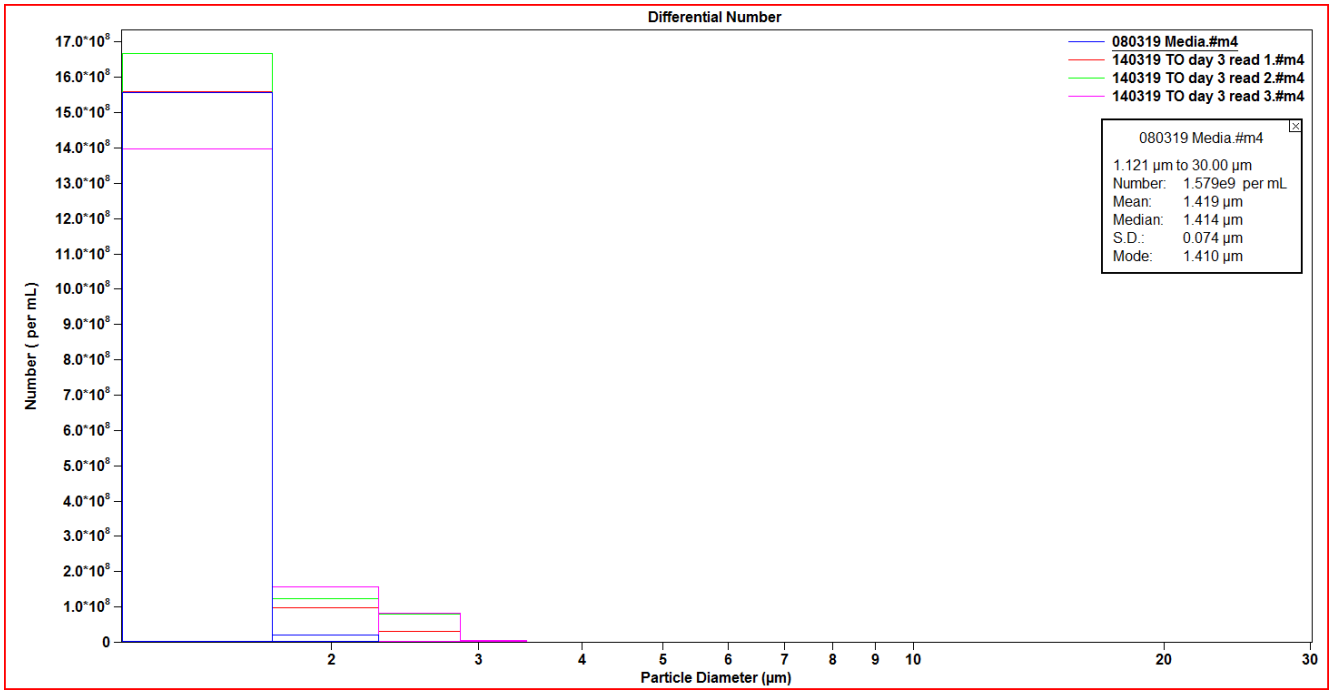

Figure 5-25: Coulter counter readings for T. obliquus culture. 2 days after inoculation day.

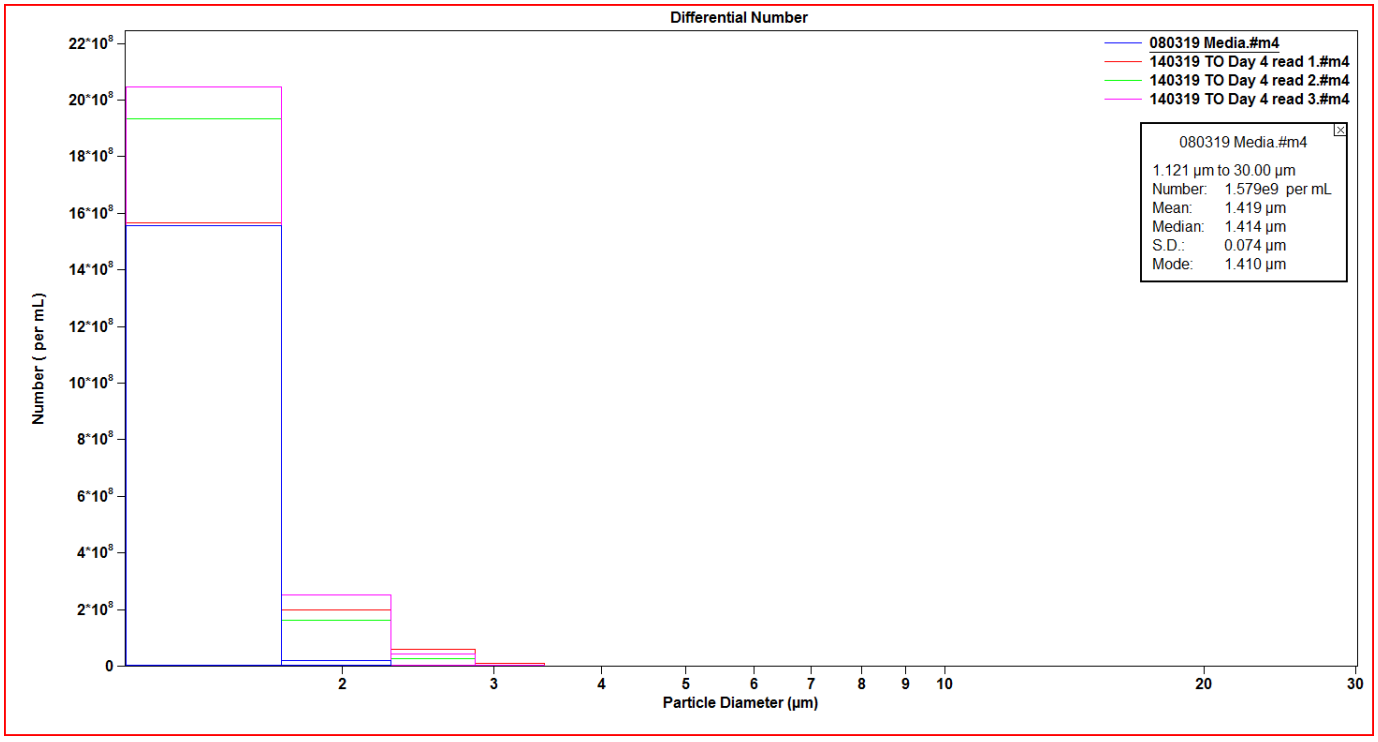

Figure 5-26: Coulter counter readings for T. obliquus culture. 3 days after inoculation day.

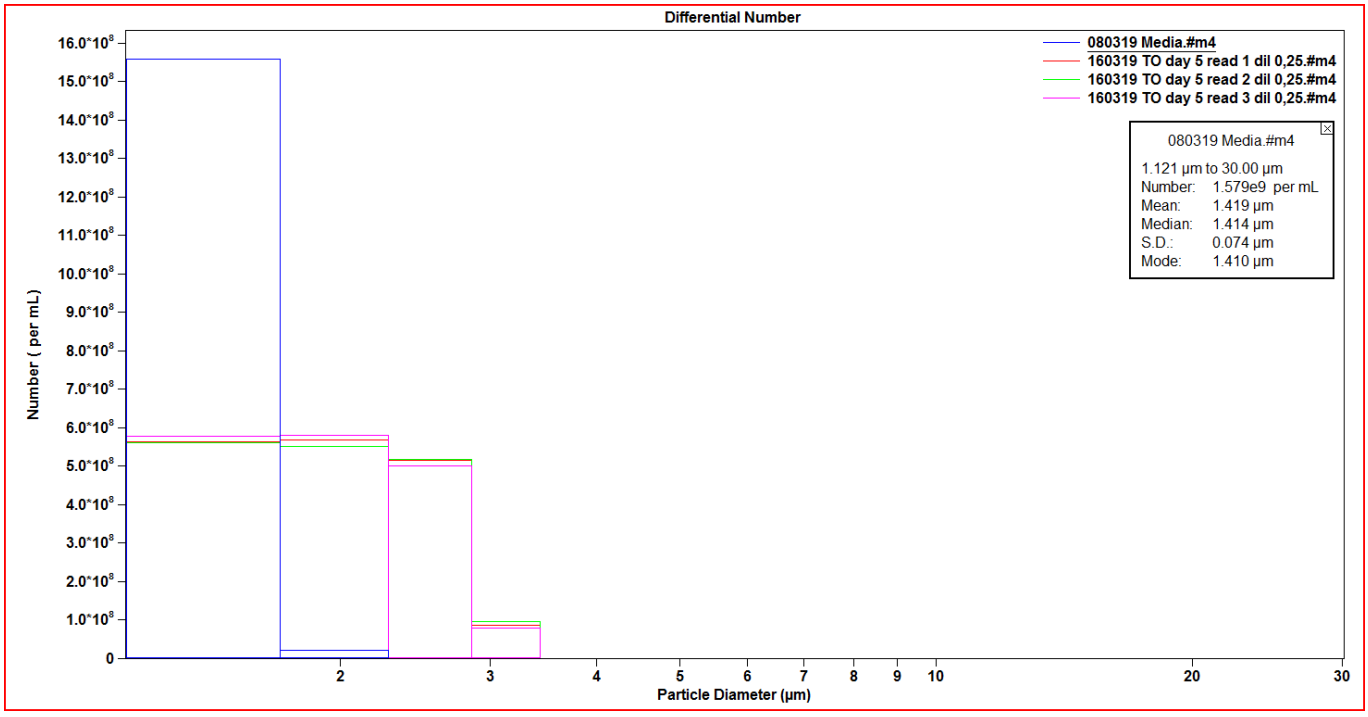

Figure 5-27: Coulter counter readings for T. obliquus culture. 4 days after inoculation day.

Appendix 2

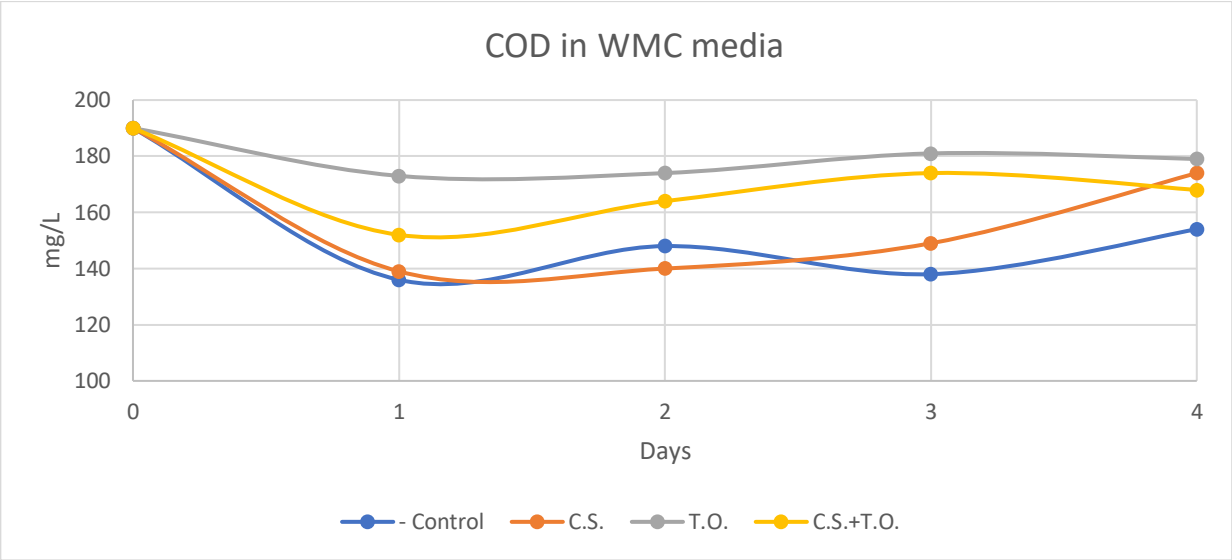

Figure 5-28: COD readings each day for each species of microalgae in MWC media.

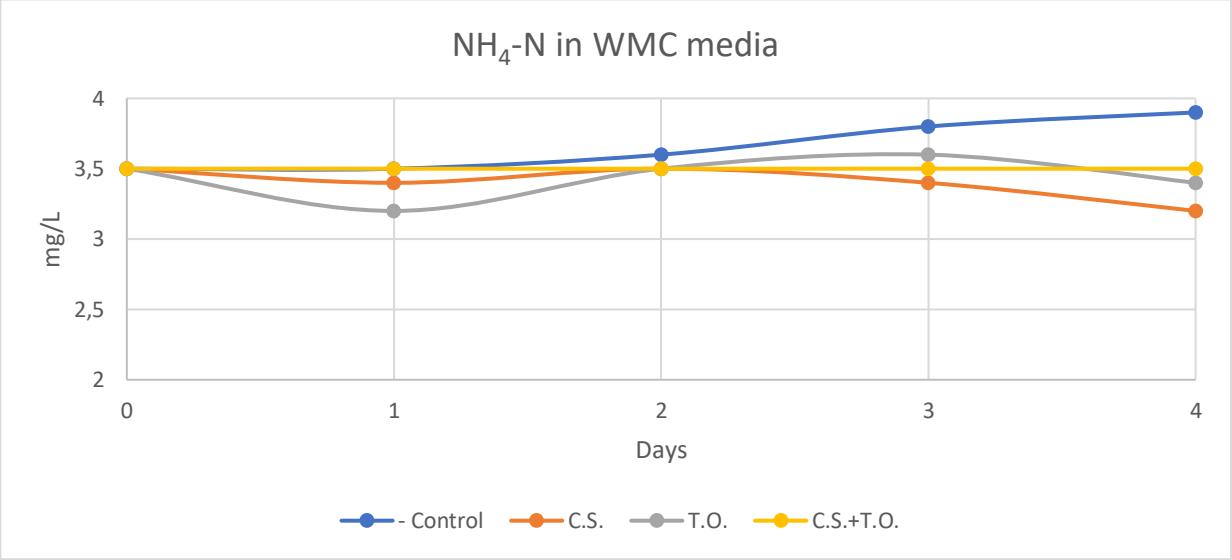

Figure 5-29: NH4-N readings each day for each species of microalgae in MWC media.

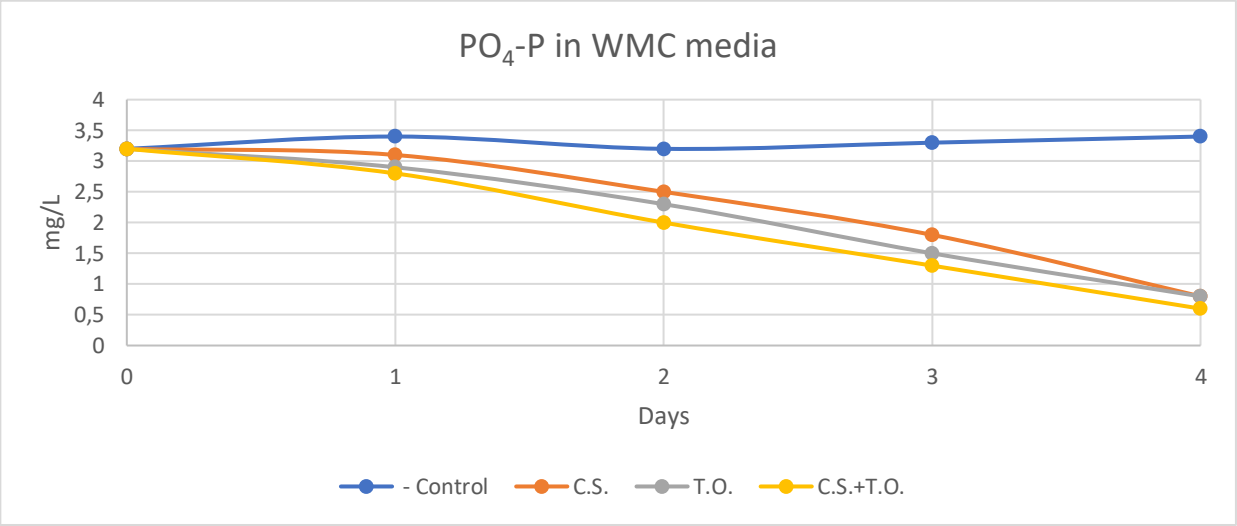

Figure 5-30: PO4-P readings each day for each species of microalgae in MWC media.

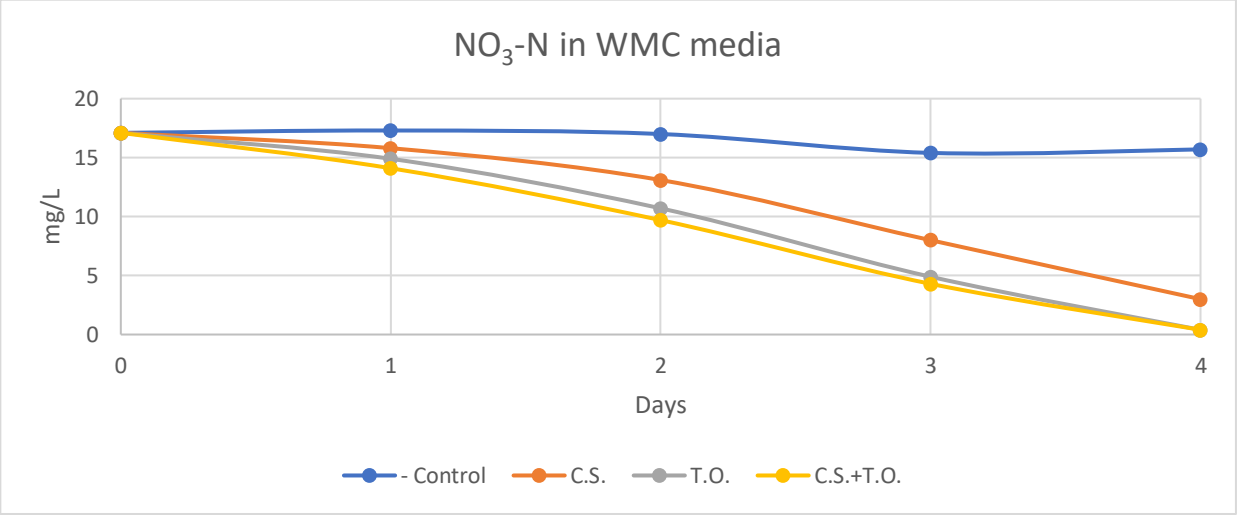

Figure 5-31: NO3-N readings each day for each species of microalgae in MWC media.

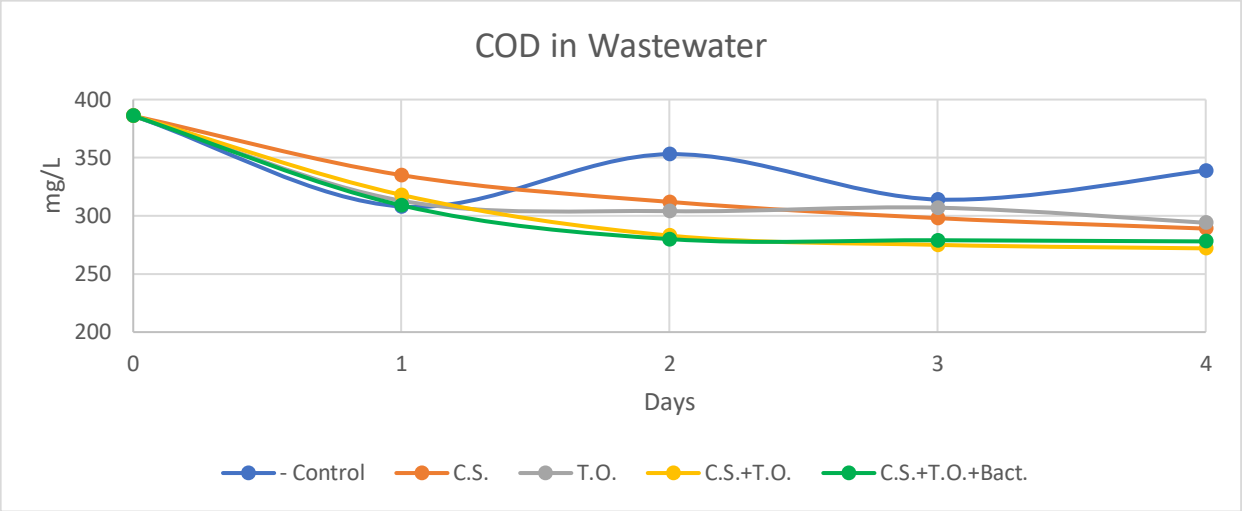

Figure 5-32: COD readings each day for each species of microalgae with and without bacteria in wastewater.

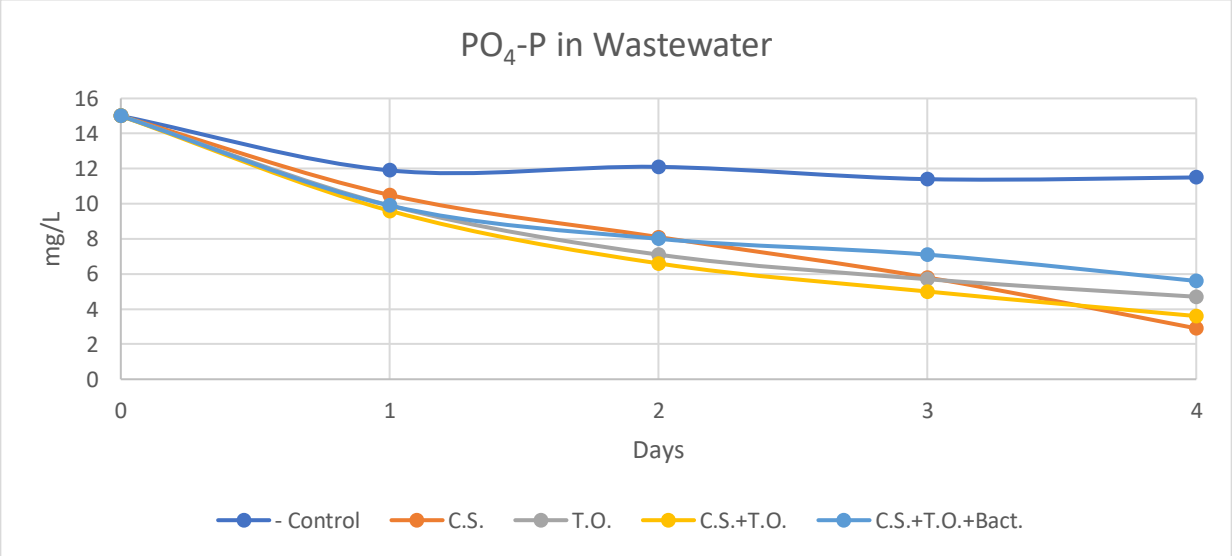

Figure 5-33: PO4-P readings each day for each species of microalgae with and without bacteria in wastewater.

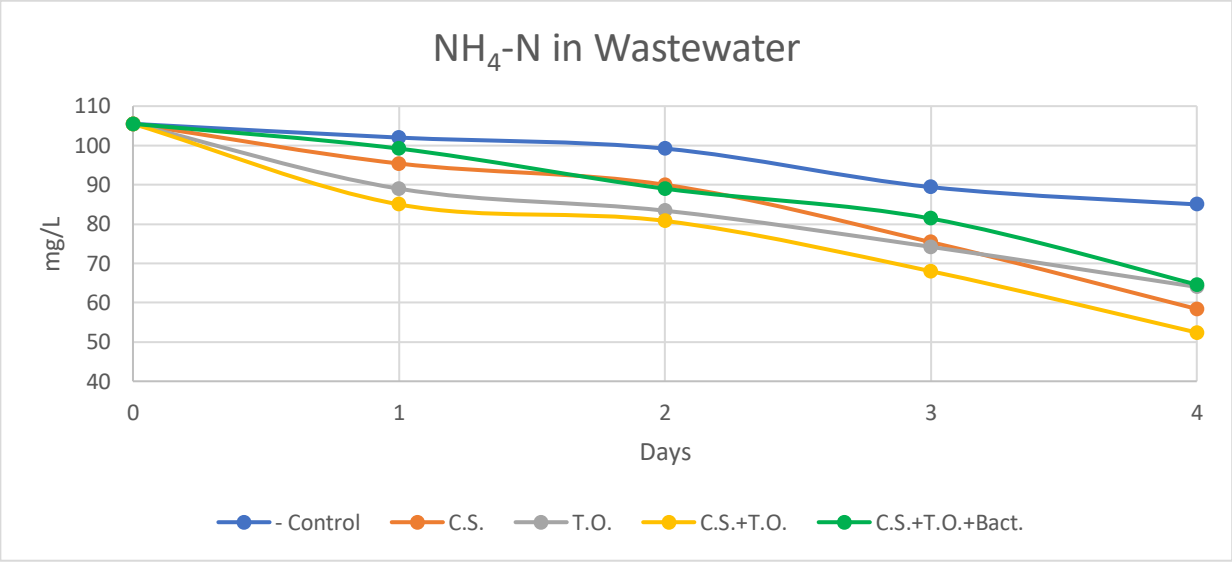

Figure 5-34: NH4-N readings each day for each species of microalgae with and without bacteria in wastewater.

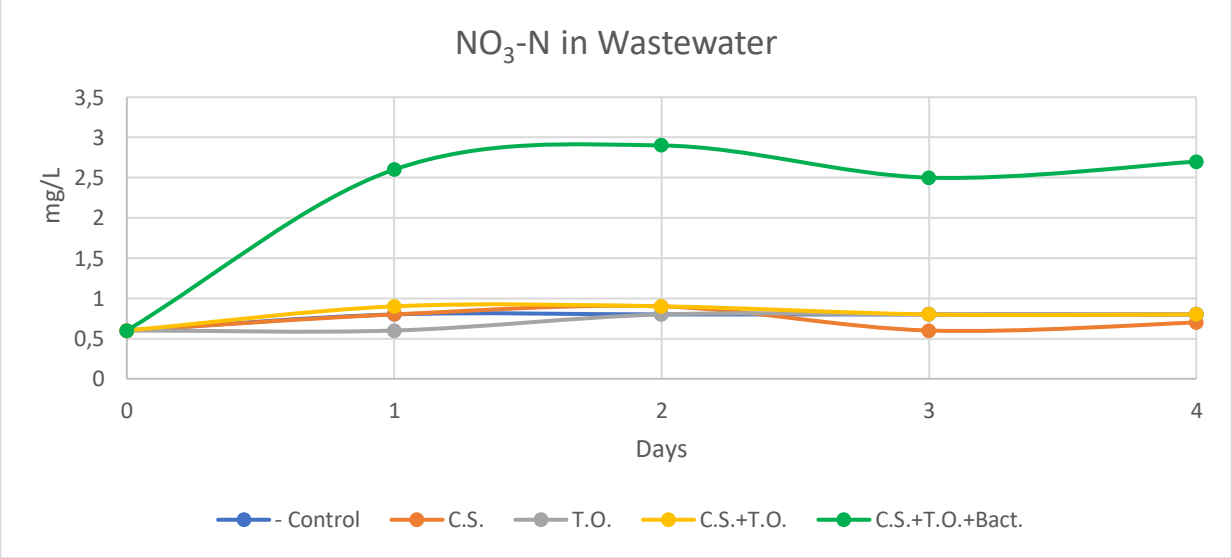

Figure 5-35: NO3-N readings each day for each species of microalgae with and without bacteria in wastewater.