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Abstract

The main objective of this project is to identify algal strains, which efficiently grow in the specific secondary wastewater effluent from IVAR Wastewater Treatment Plant (WWTP) at Grødaland. Algal cultivation will be optimized using different strategies including changing environmental factors such as light intensity, temperature, and mixing. It is essential that the algae simultaneously remove large amounts of nutrients, while producing algal biomass. To identify the best algal strains for wastewater treatment, the amount of nutrients, chemical oxygen demand (COD), alkalinity, and total suspended solids (TSS) will be measured before and after algal treatment. To accurately and effectively study the algal growth, it is fundamental to determine the most reliable and rapid quantification method. Therefore, algal enumeration based on flow cytometry, direct counting using counting chambers, microplate readings, and optical density (OD) measurements will be studied and compared.

The results indicated a good linearity for OD_{540}, OD_{680}, and OD_{750} measurements and flow cytometry for axenic cultures of *C. vulgaris, C. sorokiniana, T. obliquus,* and *M. salina* cultivated in MWC+Se (freshwater species) and L1 (marine species) growth media. For algae cultivated in wastewater the flow cytometer provided an accurate and reliable cell count, in addition to being an easy and rapid quantification method. For the most reliable result, one should conduct flow cytometry and direct counting with counting chamber simultaneously. Algal strains *T. obliquus, C. vulgaris,* and especially *C. sorokiniana* grew well in pure secondary effluent, achieving maximum growth rates of 1.28 d^{-1}, 1.33 d^{-1}, and 1.99 d^{-1}, respectively. In wastewater diluted with unfiltered lake water, *C. sorokiniana* reached maximum growth rate of 2.06 d^{-1}, followed by *C. vulgaris* with growth rate 1,60 d^{-1} and *T. obliquus* with growth rate 1.39 d^{-1}. *M. salina* did not grow in pure wastewater, but grew well in wastewater diluted with filtered seawater with a growth rate of 1.55 d^{-1}. The results indicated that applying algae *T. obliquus* and *C. sorokiniana* for wastewater treatment would result in great nutrient removal. *T. obliquus* removed 80% TP, 71% TN, and 70% NH_4^+, while *C. sorokiniana* removed up to 72% TP, 70% TN, and 73% NH_4^+. These algae also present effective biomass production of up to 1.35 g/L and 1.05 g/L, respectively. Based on growth characteristics, nutrient removal, and biomass productivity, *C. sorokiniana* and *T. obliquus* seems to be the best algal species to treat the mixed wastewater effluent from IVARs WWTP at Grødaland.

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Abbreviations

BOD	Biochemical Oxygen demand
COD	Chemical Oxygen Demand
COD_s	Soluble Chemical Oxygen Demand
DAF	Dissolved Air Floatation
EDCs	Endocrine Disrupting Compounds
FCM	Flow Cytometry
FSC	Forward Scatter Parameter
MF	Microfiltration
OD	Optical Density
SSC	Side Scatter Parameter
TDS	Total Dissolved Solids
TN	Total Nitrogen
TP	Total Phosphorus
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket Reactor
WW	Wastewater
WWTP	Wastewater Treatment Plant

1. Introduction

The world is experiencing continuous population growth, increased urbanization, and industrialization. Hence, larger volumes of domestic, industrial, and agricultural wastewater are generated, posing a threat to aquatic ecosystems and water quality. Poorly treated wastewater contains excessive amounts of nutrients. If released to the aquatic environment, the result is potentially eutrophication of the recipient water. Eutrophication includes algal blooms, oxygen depletion and loss of species diversity. Conventional treatment methods for removing nutrients from wastewater include anaerobic digestion, nitrification, and denitrification, or chemical methods such as precipitation with salt ions. These methods have several drawbacks such as cost and complexity [1]. To avoid these drawbacks, using microalgae for wastewater treatment has been receiving increasing interest the last years. Using microalgae for treatment have shown to efficiently remove nitrogen and phosphorous without addition of chemicals. Algae-based wastewater treatment generates O_2, mitigate CO_2, and produce valuable algal biomass and biofuels [2].

Algal-based wastewater treatment is sustainable and environmentally friendly as it potentially mitigates CO_2 emissions. Algae can bind approximately 1.85 to 2.5 kg CO_2 per kg dry biomass. Increased CO_2 partial pressure compared to the atmosphere is essential to ensure maximum growth without carbon limitation. Flue gas from industry is a possible source of additional CO_2 for algal cultivation [3].

Laboratorial cultivation of microalgae can convert the energy of sunlight to more than 5% to chemical energy in the form of biomass. Many algae can reach doubling times of approximately 5 hours and growth rates of over 3 d^{-1}. They can contain lipid contents over 50%. As they have simple structure, lacking stems, leaves, and roots, the entire algal biomass can be harvested and utilized [3].

Wastewater of different sources is typically rich in nitrogen, phosphorous, and trace elements essential for algal cultivation [4]. Several microalgal strains have been studied for their ability to treat wastewater. Freshwater microalgae, such as *Chlorella* sp., have been shown to be efficient in removing both nutrients and chemical oxygen demand (COD) in various types of wastewater streams [5]. Some of these wastewater streams include municipal wastewater [6],

digested manure [7], and industrial wastewater [8], [9]. Freshwater algae *Chlorella vulgaris* have shown to efficiently reduce pH, total dissolved solids (TDS), biochemical oxygen demand (BOD), COD, nitrate, ammonia, phosphate, sulphate, calcium, magnesium, sodium, potassium, several heavy metals, and coliform bacteria from water [10]. *Chlorella sorokiniana* have successfully been cultivated in industrial wastewater, resulting in over 99% of phosphate and ammonia removal. The *C. sorokiniana* batch culture produced 1.33 g of biomass L^{-1} day^{-1} [11]. *H. pluvialis,* an algae commonly used for astaxanthin production, has successfully been cultivated in domestic secondary effluent. This species has been found to produce large amounts of biomass, while simultaneously removing 98% of total phosphorous (TP) and 94% total nitrogen (TN) [12]. Seawater microalgae *Nannochloropsis salina (*synonym: *Microchloropsis salina)* has been cultured in anaerobic digestion effluent, resulting in 100% nitrogen and phosphorous removal, while simultaneously producing lipids [13]. *Scenedesmus Obliquus* (synonym: *Tetradesmus Obliquus*) have great potential for advanced wastewater treatment and lipid production. After 6 days of cultivation in secondary wastewater effluent, *T. Obliquus* removed over 95 % of TP and TN [14].

Measuring growth is important in algal research for establishing growth curves and for determining biomass productivity. There are several types of cell quantification instruments and methods used today. Using microscopy for cell enumeration is a common and easy way of determining algal growth. However, researchers increasingly prefer to use automated cell counters for more rapid cell counts [15]. Microplate readings are a fast, easy, and low-cost method used for determination of growth kinetics of microalgae by measuring both optical density (OD) and fluorescence intensity. However, one should only use low-density cultures for analysis, due to light and gas-transfer limitations in dense cultures [16]. Flow cytometer has emerged as algal quantification analysis the last years. Light absorbing pigments in algae makes the flow cytometer an excellent instrument for algal analysis, as the method measure cell fluorescence and particle size [17].

Even though many studies on microalgae-based wastewater treatment show great results, all wastewater effluent streams are unique. It is therefore essential to establish algal growth characteristics of selected algae and the algal treatment efficiency of the specific wastewater from IVARs Wastewater Treatment Plant (WWTP) at Grødaland, before applying treatment method in full-scale systems. Several quantification methods were studied and compared to accurately and effectively determine changes in algal cell density.

1.1 Scope of Work

This study is part of a wastewater treatment project conducted by the University of Stavanger in cooperation with IVAR WWTP at Grødaland. The planned process is illustrated in Figure 1.1.

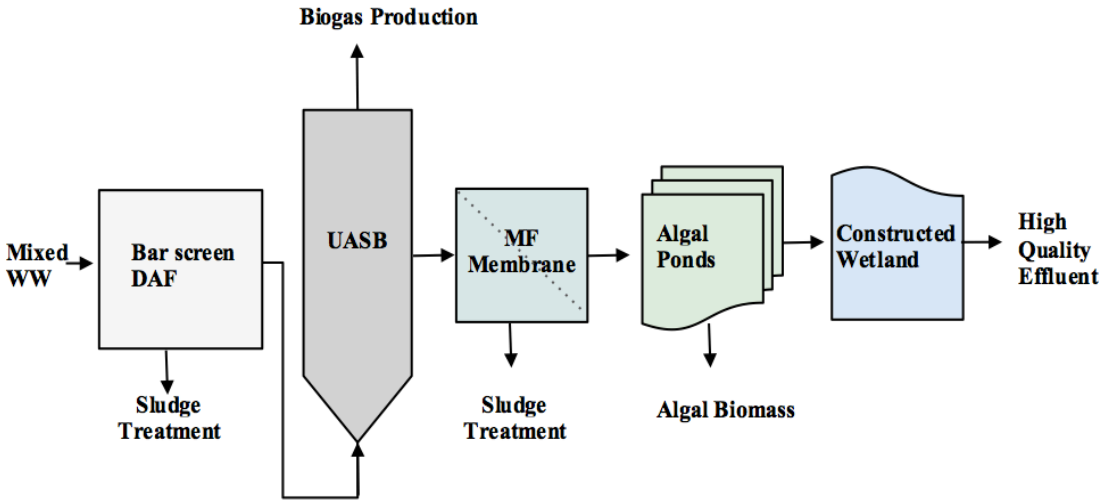


Figure 1.1: *Process illustration of the planned lab-scale wastewater treatment project by the University of Stavanger and IVAR Wastewater Treatment Plant at Grødaland.*

The raw wastewater (WW) is a mixture of municipal wastewater from Varhaug and industrial wastewater from Kviamarka næringsmiddelpark and Norsk Protein AS. The water is first to be treated by IVAR using bar screen, fat, sand, and grit removal along with dissolved air floatation (DAF). After preliminary treatment, Upflow Anaerobic Sludge Blanket Reactor (UASB), and membrane microfiltration (MF) are used as the primary and secondary treatment step, respectively, and is conducted by the University of Stavanger. Following, algal ponds will be used to treat the secondary effluent as the tertiary treatment step. Before algal pond treatment can be conducted it is necessary to identify the most efficient algal strains for wastewater treatment.

1.2 Objectives

The aim of this project is to identify algal strains able to efficiently grow in a specific mixed wastewater stream from IVAR WWTP at Grødaland, while simultaneously removing nutrients and soluble chemical oxygen demand (COD_s.) It is necessary to compare the efficiency and accuracy of different algal quantification technologies as drawbacks have been linked to many commonly used methods. To characterize growth of axenic algal strains in secondary effluent, several quantification methods will be used, including flow cytometer, optical density (OD) measurement, counting chambers, and microplate readings measuring fluorescence intensity and OD_{750}. All methods will be tested and compared, in order to find the most accurate and efficient algal quantification analysis. Concentration of nutrients (TN, nitrate, nitrite, ammonium, TP, and phosphate), alkalinity, and COD before and after algal treatment will be analyzed in order to determine algal treatment efficiency and characteristics. Total suspended solids (TSS) will be measured as an indication of total algal biomass production.

2. Theoretical Background

2.1 Wastewater Treatment

Wastewater is liquid waste released from private homes, industry, and commercial establishments together with surface runoff water, infiltration, and stormwater [18]. Wastewater treatment is essential to protect the environment and the public health. Untreated wastewater will become septic and the degradation of organic matter can lead to release of toxic gases to the environment. Wastewater contains nutrients, such as phosphorous (P) and nitrogen (N), that stimulates algae blooms in recipient waters. This may lead to eutrophication, oxygen depletion, and death of species [19]. Wastewater may contain other toxic compounds depending on the process used including endocrine disrupting compounds (EDCs), heavy metals, and possible carcinogens [18]. EDCs in industrial wastewater are components of emerging concern, as it can lead to the feminization of fish [20]. Industrial wastewater can also contain toxic compounds dependent on type of process being used. Stormwater can contain pesticides, petroleum, and particles from urban and agricultural runoff [18] . Therefore, treatment of wastewater, that is, reducing levels of solids, organic matter, pathogens, and toxic compounds, is essential before releasing the effluent to recipient waters [19].

2.1.1 Conventional Wastewater Treatment

Wastewater treatment includes physical, chemical, and biological methods. Physical treatment includes physical forces such as sedimentation, filtration, and screening for removing suspended solids from the wastewater. In chemical treatment, chemicals are added for coagulation-flocculation or precipitation mechanisms to remove contaminants. Biological treatment uses microorganisms for degrading biodegradable organic matter and removing nutrients, for example by using activated sludge process or membrane bioreactor [18]. Different levels of operations are necessary to achieve sufficient contaminant removal. Conventional treatment methods are shown in Figure 2.1.

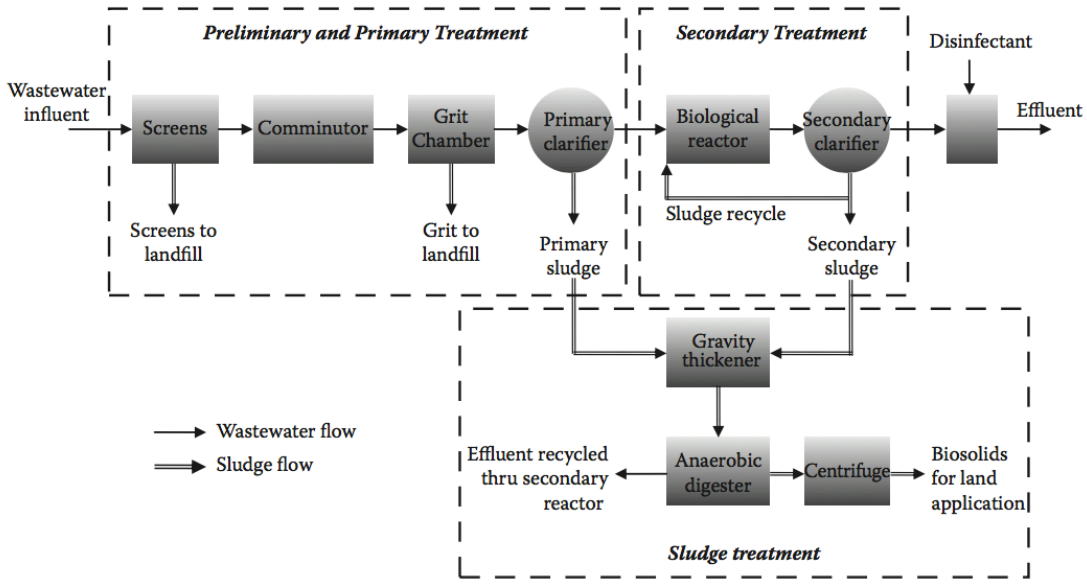


Figure 2.1: *Example of a conventional wastewater treatment process [18].*

Preliminary treatment is the first level of treatment. Here larger objects, solids, and grit are removed from the influent [19]. This is important to avoid operational problems in pumps and equipment downstream. Screens, grit chamber, and floatation are examples of preliminary treatment [18]. *Primary treatment* is a physical process that removes suspended solids and organic matter, typically by sedimentation [19]. Chemicals are added in *enhanced primary treatment* leading to coagulation-flocculation of suspended solids, resulting in enhanced solids removal in primary clarifiers. Biological processes are used in *secondary treatment* to reduce BOD by removing biodegradable organic matter and suspended solids. Secondary treatment typically consists of a biological reactor followed by a sedimentation tank. A combination of chemical and biological treatment is used when nitrogen and phosphorus removal is required [18]. In *tertiary treatment*, residual suspended solids are removed. This is usually achieved using filtration or micro-screens. Disinfection and nutrient removal is also included [19]. *Advanced treatment,* such as activated carbon or ion exchange, is used for the removal of residual suspended solids and other components due to toxicity. The residuals remaining after each treatment step is called sludge. The preliminary treatment step produces waste low in organic content that can be disposed in landfills. A waste rich in organic material is produced from primary and secondary clarifiers and will need an additional treatment step termed sludge treatment. *Sludge treatment* reduce amount of organic matter, liquid, and pathogens in the sludge. Liquid can be reduced using processes such as dissolved air floatation, centrifugation, and gravity thickening. For removing organic content and

pathogens, anaerobic or aerobic digestion, composting, heat- or air-drying are examples of processes commonly used. After treatment, the sludge can be utilized as soil conditioner and fertilizer in agriculture [18].

2.1.2 Microorganisms in Wastewater Treatment

Wastewater contains a variety of microorganisms including bacteria, viruses, and protozoa. The majority of them are harmless, however, some microorganisms are pathogens and must be removed before releasing the effluent to recipient waters. Microorganisms can be utilized for the oxidation of organic matter to acceptable end products while simultaneously removing nutrients. The natural biodegradation properties of microorganisms needs be thoroughly understood to enhance the removal rate in biological treatment systems [18, 19].

The aerobic biodegradation of organic material by microorganisms is represented by the following non-stoichiometric Equation 2.1 [19].

$$Organic\ material + O_2 + NH_3 + PO_4^{3-} \xrightarrow{microorganisms} new\ cells + CO_2 + H_2O \qquad (2.1)$$

As seen from Equation 2.1, the microorganisms utilize oxygen (O_2) and nutrients ammonia (NH_3) and phosphate (PO_4^{3-}) for the conversion of organic matter into simple end products (CO_2 and H_2O) and biomass (new cells). Microorganism's utilization of oxygen creates oxygen demand in the recipient water. This can lead to oxygen depletion in the water.

2.1.3 Measuring Organic Matter in Wastewater

Several methods can be utilized for measuring the amount of organic matter in wastewater, which include measuring the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) [18].

BOD is a parameter used for measuring the biodegradable part of the organic matter in wastewater. Biochemical oxygen demand is the amount of oxygen utilized by aerobic microorganisms to degrade organic matter. The BOD test takes about 5 days. COD measures

the biodegradable and non-biodegradable parts of the organic matter. This test only takes a few hours. The test utilizes potassium dichromate, which is a strong oxidant, to oxidize organic matter in acidic conditions. The amount of oxygen required can be calculated from amount of oxidant consumed [18].

2.1.4 Advanced Wastewater Treatment

If the concentrations of suspended solids, dissolved solids, and other constituents remaining after conventional secondary treatment do not meet the regulatory limits for discharge, tertiary wastewater treatment or advanced wastewater treatment should be used. Additional treatment steps are added to conventional wastewater treatment to sufficiently remove residual suspended solids and organic material, pathogens, and nutrients to limit the eutrophication of sensitive recipient waters, and inorganic constituents such as heavy metals [18].

When selecting treatment method one should consider the nature of the wastewater, use of wastewater effluent, economic and environmental feasibility. If the secondary wastewater effluent contains too high levels of organic and inorganic colloidal and suspended solids, filtration is commonly used as advanced wastewater treatment. The types of filtration commonly used include depth filtration, surface filtration, and membrane filtration. Removal of some dissolved organic constituents can be achieved by carbon adsorption, reverse osmosis, chemical precipitation or oxidation, electrodialysis, and distillation. Chemical processes or membrane filtration can remove dissolved inorganic matter [19].

2.1.5 Microalgae-based Advanced Wastewater Treatment

Algal cultivation system has been studied for a long time. Already during World War II, Germany applied algal cultivation in open ponds for food supplement. Several countries in Eastern Europe, Japan and Israel followed, and began cultivating algae in open ponds for the production of food in the 1970s. Open pond systems are commonly applied in industry due to its simplicity and low-cost. However, environmental conditions can be challenging to control, resulting in low biomass production and contamination of the algal culture. One should utilize highly selective microalgae to prevent contamination by other microorganisms and

microalgae. An alternative is the closed photobioreactor, which have shown to increase photosynthetic activity and biomass production. One drawback is the high cost and that only specific algae can be used for cultivation [21].

Many studies have shown that microalgae have great potential for removing nutrients from wastewater. Algae have been used to treat wastewater for a long time, as W.J Oswald invented the high-rate algal ponds in the 1950s [3]. This process takes advantage of the algae and bacterial symbiotic relationship. Bacteria use organic waste from wastewater along with oxygen, to produce bacterial biomass, CO_2 and nutrients. Algae utilize nutrients and CO_2 for the production of new algal biomass and oxygen. This will in turn provide the components for bacterial aerobic degradation of organic waste [22]. Microalgae for removal of contaminants, such as nutrients and toxic compounds, are commonly termed phycoremediation. Phycoremediation is utilized for nutrient removal from municipal wastewater, treatment of acidic and metal wastewaters, carbon capture, xenobiotic biotransformation, and as algae-based biosensors for the detection of harmful compounds [23].

Using algae-based wastewater treatment rather than conventional treatment methods have several advantages. These include cost effectiveness, low energy use, reduced sludge formation and decreased greenhouse gas emissions, and production of high-value algal biomass, for example fatty acids for biofuels [22]. Microalgae-based systems leave low residual nutrient concentrations without adding extra chemicals. However, drawbacks include a relatively long treatment time, complicated processes separating algae with treated wastewater and reduced performance under bacterial contamination and zooplankton predation [2].

2.1.6 Nitrogen Removal in Microalgae-based Wastewater Treatment

Nitrogen is present in wastewater in various forms, including ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and organic nitrogen [2]. Nitrogen is assimilated by microalgae producing substances like proteins, enzymes, peptides, chlorophylls, genetic material (DNA, RNA), and energy transfer molecules (ATP, ADP) [24]. Bacterial nitrification-denitrification leads to some nitrogen being lost as nitrogen gas (N_2). Nitrogen is also lost as NH_3 as a result

of volatilization, affected by pH, temperature, and mixing [2]. Nitrogen removal mechanisms in wastewater treatment using microalgae are summarized in Figure 2.2.

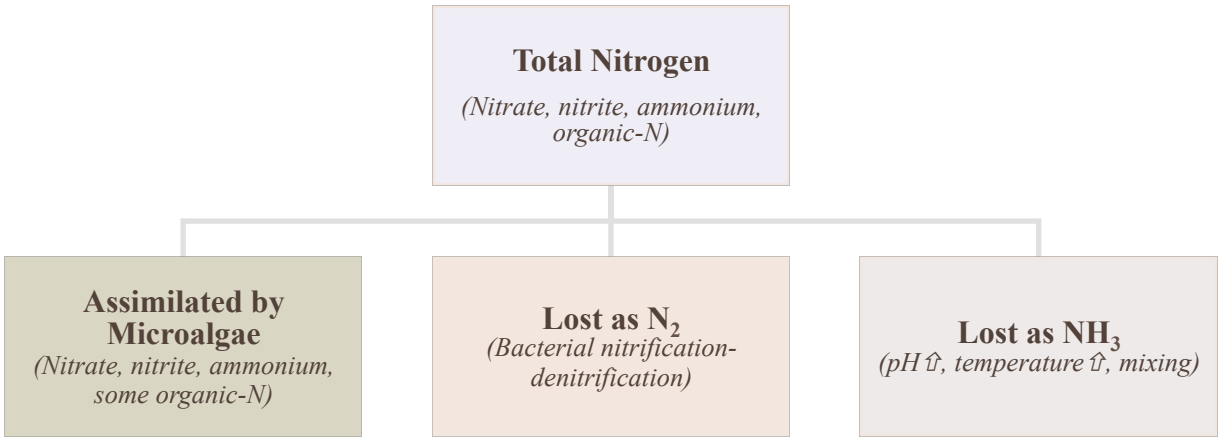


Figure 2.2: *Principal of nitrogen removal in microalgae-based wastewater treatment [2].*

2.1.7 Phosphorous Removal in Microalgae-based Wastewater Treatment

Autotrophs assimilate dissolved phosphorous into organic phosphorous. Phosphorous is utilized by microalgae to form energy transfer molecules, genetic material, phospholipids for cell membranes, proteins [24], and intermediates for carbohydrate metabolism. Some cyanobacteria and eukaryotic coccal green algae have the ability to accumulate phosphate as polyphosphate granules. Phosphorous is commonly removed from wastewater by precipitation with the presence of Ca^{2+}, Mg^{2+}, high pH, and dissolved oxygen [2]. Phosphorous removal mechanisms in wastewater treatment using microalgae are summarized in Figure 2.3.

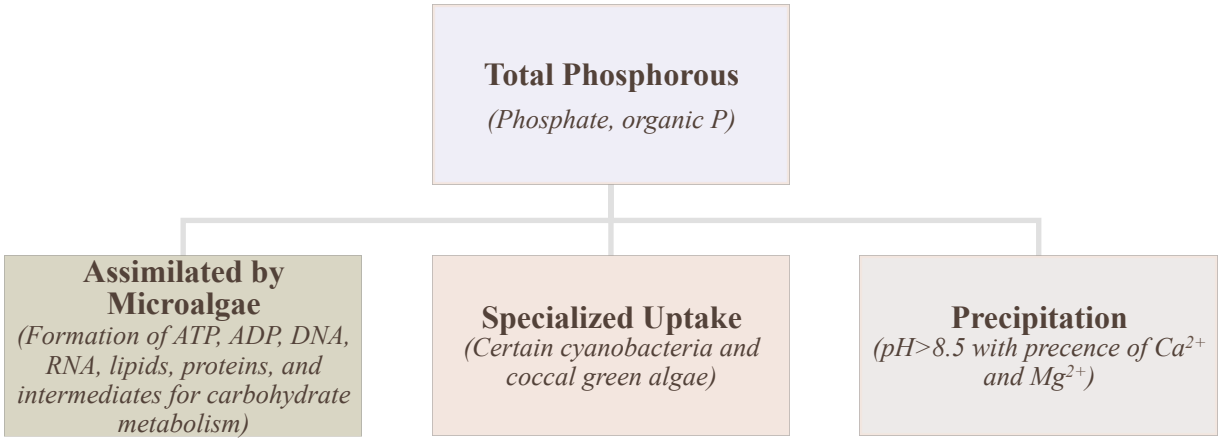


Figure 2.3: *Principal of phosphorous removal in microalgae-based wastewater treatment [2].*

2.1.8 Carbon Removal in Microalgae-based Wastewater Treatment

Carbon dioxide can be assimilated from the atmosphere and from industrial exhaust gas by the microalgal photosynthesis process [24]. Some microalgae are also capable of using organic carbon through heterotrophic processes, while others are mixotrophic using both inorganic and organic carbon sources [5]. The pH value in the culture has an effect on carbon uptake by algae. At pH values ranging from 5 to 7, CO_2 is taken up through diffusion. Bicarbonate is taken up by active transport at pH values above 7 [1].

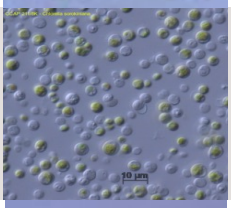
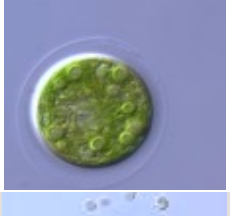
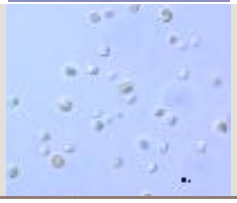
2.1.9 Species and Strain Selection for Wastewater Treatment

A desirable property microalgae used in wastewater treatment is rapid growth. Rapid growth is required for high productivity and reduces the risks of contamination. Small, single planktonic cells grown in suspension will grow faster compared to larger, colonial or filamentous cells or cells that grow attached to surfaces. However, larger filamentous and colonial cells are easier to harvest [25]. Ideal microalgal strains for wastewater treatment have

properties such as a high nutrient requirements, compared to average N and P content of biomass, as well as the ability to reduce low concentrations of nutrients to even lower levels [2].

The following algal species; *Chlorella vulgaris, Chlorella sorokiniana*, *Tetradesmus obliquus* (synonym: *Scenedesmus obliquus*) *Haematococcus pluvialis*, and *Microchloropsis salina* (synonym: *Nannochloropsis salina*) were selected for this study based on their ability to purify wastewater. As mentioned in Chapter 1, these species have all been identified as microalgae with the ability to assimilate large amount of nutrients from wastewater, while simultaneously producing biomass. Characteristics for selected algal strains are described in Table 2.1.

Table 2.1: *Characteristics of selected algal strains.*

Algal strain	Growth rate μ, d^{-1}	Composition, % P: Protein L: Lipid C: Carbohydrates	Morphology	Average diameter size, μm	References
Chlorella Vulgaris 211-11b	1.61	P: 24-58 L: 5-58 C:12-55		2-5	[26-28]
Tetradesmus obliquus 276/3A	1.13	P: 50-56 L: 12-14 C:10-17		5-10	[29-31]
Chlorella sorokiniana 211/8K	2.4-6.48	P: 37.7 L: 20.9 C: 27.5		3	[32-35]
Haematococcus pluvialis 34/7	0.72	P: 21.1 L: 22.2 C: 38.0		4-20	[36-39]
Microchloropsis Salina 849/3	1.30	P: 17.8 L: 16.9 C: 8.9		2.5	[40-43]

2.2 Microalgae

To achieve successful wastewater treatment using microalgae, it is important to understand algal biology and the factors affecting their growth.

Algae are classified phototrophs as they require light for their metabolic functions. Most algae are also autotrophs, as they use carbon dioxide as their sole source of carbon [44]. Some are heterotrophic (using organic carbon as their sole source for as carbon), while others are mixotrophic (using both inorganic and organic carbon) [1]. Algae have simple structures without any roots, stems or leaves. Aquatic algae can occur in almost every type of aquatic environments as they tolerate a broad range of pH, temperature, O_2, and CO_2 concentrations. Some grow attached to substrates such as plants, soil, trees, and animals, while others grow suspended in water. Algae can occur in any illuminated body of water, including under the polar ice. Benthonic algae grow in shallow waters attached to the bottom, within the sediments, or on plants or on animals [24].

Algal cells have highly variable cell structures. The blue-green algae, cyanobacteria, are prokaryotic cells similar to bacteria and are relatively simple cells. Eukaryotic algae have organelles such as a nucleus, chloroplasts, mitochondria, endoplasmic reticulum, and Golgi apparatus. Algae exist in a variety of morphologies varying with different cell life stages. Some are coccoid, filamentous, amoeboid, capsoid, flagellate, and sarcinoid [15].

Microalgae are single-celled structures of a few microns in size, with the ability of forming many-celled colonies up to 60 meters long, called kelp. Microalgae are responsible for producing about half of the oxygen on earth and are the most important primary biomass producers, forming the basis of the marine food chain. Plankton can grow to a biomass of 2-6 tons per hectare and year, and algal blooms can form up to 50 tons [3].

2.2.1 Cultivation of Microalgae

Microalgal cultivation is an increasing area of interest due to microalgae having the ability to convert sunlight and CO_2 into high-value products. The synthesis of sustainable products from

sun light, water and CO_2 can provide renewable sources of biofuels and chemical energy, while simultaneously help mitigate climate change [3].

An algal culture consists of three main components: the culture medium containing the nutrients and trace elements, the algal cells growing in the medium and the air where CO_2 is released from the medium. The most important parameters controlling algal growth are nutrients, pH, salinity, temperature, and light. These conditions are species-specific [24].

2.2.2 Factors Affecting Algal Growth

Microalgal growth can be affected by both biotic factors, such as the presence of pathogens and competition by other microalgal species, and abiotic factors such as temperature, light, pH, nutrients and vitamins, salinity, and mixing [1].

Optimal culture temperature will vary with type of media and algal strains used for culturing. The most common cultured species tolerates temperatures from 16 to 27^{o}C, where 18 to 20^{o}C is commonly utilized for culturing [24].

Light is essential for cultivation algae as it is their main source of energy. The intensity of illumination varies with depth and density of the algal culture. Great depth and high cell density needs increasing light intensity to penetrate the culture. However, if the light intensity is too high this can cause photo-inhibition or possibly overheating. Commonly used light intensity range from 100 to 200 $\mu E/sm^2$ (5-10% of full daylight). Diurnal cycles are often applied as many microalgal species do not grow well under constant illumination [24].

While some algal species grow in acidic or basic environments, the optimal pH for the cultivation of most species ranges from 8.2 to 8.7. Aerating and addition of CO_2 can be used to control pH in cultures [24]. A high supply of CO_2 can lead to acidification of the culture conditions, which will inhibit the growth of microalgae. Therefore, pH control is necessary to promote growth [45].

Nutrients such as carbon, nitrogen, phosphorous, and several microelements are essential for growth of microalgae. A sufficient supply of carbon is vital for algal growth due to algal

biomass consist of 50% carbon [46]. Carbon is present in various forms in the liquid phase, including CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}. Generally, microalgae prefers CO_2 as carbon source. Nitrogen is an important compound in algal nutrition. Microalgal dry mass contains about 7% nitrogen. Nitrogen is essential for cellular and protein productivity and chlorophyll synthesis [45]. Nitrate, ammonia, and urea are widely used as nitrogen source for algal cultivation. Changes in nitrogen supply can potentially influence metabolic pathways, leading to altered composition of the algae [46]. Dry algal biomass contains approximately 1% of phosphorous. Phosphorous is essential for several metabolic pathways and cellular regulations [45]. Algae prefer phosphorus in the form of inorganic phosphate, such as $H_2PO_4^-$ or HPO_4^{2-} [46].

Microelements (such as sulphur, iron, magnesium, potassium, sodium, copper, manganese, zinc, cobalt, molybdenum) are essential for microalgal growth. Sulfur is needed for protein synthesis and photosynthetic activity. Iron is a catalyst in the production of chlorophyll and is needed for cellular metabolism. Magnesium is essential in nitrogenase activity in cellular metabolism. Copper is important for optimal photosynthesis and molybdenum for nitrogen assimilation [45].

Different types of vitamin B are essential for algal growth. Approximately 50% of microalgal species need cobalamin (B12), 20% need thiamine (B1), and <5% need biotin (B7) for growth. B12 is important to transfer methyl groups and methylating toxic components. B7 is a cofactor for essential for carboxylase enzymes involved in fatty acid synthesis. B1 has an important role in the carbon metabolism [24].

Marine algae grow optimally in salinity ranges from 20 to 24 g/L, slightly lower than their natural habitat [24].

Agitation of microalgal cultures is essential to avoid sedimentation of algae. Proper mixing provides illumination and nutrients for all cells in culture and enhances gas transfer between culture medium and air. CO_2 addition may be necessary in dense cultures or for pH control [24].

To provide the best media for selected species one should consider the conditions of the species natural habitat. Some species grow best in eutrophic environments, while other prefers

oligotrophic conditions. If the species are r-selected, they are characterized by rapid growth rate, autotrophic metabolism, and flexible environmental tolerance. K-selected species grow at a slower rate, employ mixotrophic metabolism, and grow in stable environmental conditions [24].

2.2.3 Batch Culture Cultivation Method

Batch cultures are the most used algae culturing method for its low cost and simple culture system. The batch culture often consists of 250 mL Erlenmeyer culture flask with gauze bung. As the system is closed, with no input or output of resources, the algal cell density increase until exhaustion of limiting substances. The cells will die after exhaustion unless subculturing by applying a small volume of the sample to new media containing the essential nutrients [24]. Agitation is necessary to ensure nutrient and gas exchange between cells, media and air. The cultures can be illuminated by natural or artificial light [15].

The growth of the algal population in a batch culture typically shows a sigmoidal growth curve as shown in Figure 2.4.

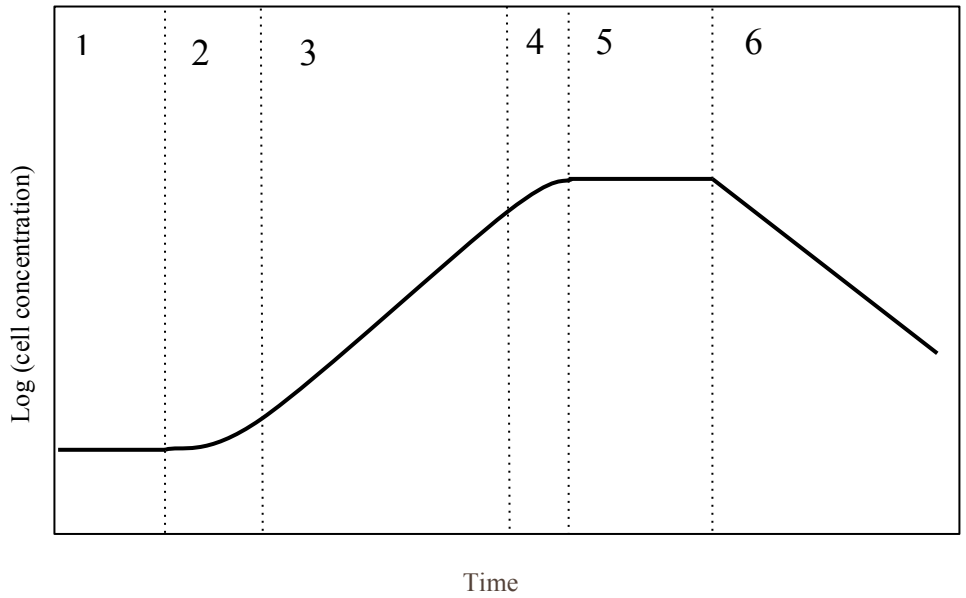


Figure 2.4: *Typical growth characteristics of an algal batch culture [24].*

Using the batch cultivation method, algal growth goes through several phases as seen in points 1 to 6 in Figure 2.4. These phases include the lag phase, acceleration phase, exponential phase, retardation phase, stationary phase, and the death phase, respectively [24]. The various phases represents algal population alternations due to changing environmental conditions [46].

The growth rate (μ), divisions per time, can be calculated using Equation 2.2 [25].

$$\mu = \frac{ln(N_{t_2}/N_{t_1})}{t_2 - t_1} \tag{2.2}$$

where

N_{t1} is the cell density at time 1 (t_1)

N_{t2} is the cell density at time 2 (t_2)

2.2.4 Algal Biomass Utilization

Algae can be utilized in human and animal food, pharmaceuticals, cosmetics, and as fertilizer [24]. Various applications of algae are summarized in Table 2.2.

Table 2.2: *Examples of algal biomass utilization.*

Application	Examples	Reference
Nutrition	Animal feed, human food, aquaculture	[3, 24, 45]
Pharmaceuticals	Bioactive molecules and toxins for development of new medicines, antioxidants, vitamins, anticoagulants, anti-cancer activity	[3, 15, 45]
Extracts	Agar (microbiological culture substrate), alginates (medical dressing, textile industry), carrageenan (gelling and thickening in food)	[24]
Cosmetics	Hair care, anti-wrinkle, sun creams, pigments in cosmetics	[3, 24, 45]
Energy	Bio-methane, biofuels, bio-hydrogen, bioethanol	[3, 15, 45]
Environmental field	Wastewater treatment, fertilizer, CO_2 sequestration	[3, 15, 24, 45]
High-value chemicals	Carotenoids (e.g. β-carotene), recombinant plasmids (e.g. enzymes, vaccines, antibodies, growth factors)	[15]

2.3 Quantification of Algae

Measuring the abundance of cells is essential to determine the growth rates of algal cultures [25]. Estimating growth rates require measurements of the change in biomass over time [47]. Some methods for algal quantification includes flow cytometer, microplate readings, OD measurement, and counting chambers.

2.3.1 Flow Cytometer

Flow cytometry has emerged over the last years as a method of enumerating algal cells. The method includes measurements of light scatter and fluorescence of the algal sample, while

passing light beams at rates up to 1000 cells per second. In addition to cell counts, it can also determine other parameters such as lipid content and lipid composition [25].

Flow cytometry analyses cells in suspension. The sheath fluid aligns the cells in a 10-20 μm narrow steam, which passes through light sources resulting in scattering light. Algal cells contain fluorescent compounds such as chlorophyll and carotenoids. Where the absorption spectrum corresponds with the excitation source, the fluorescent compound will emit fluorescence at a higher wavelength. Photodiodes detect these emitted lights and transform them into digital signals [47]. The flow cytometer measure the intensity of light scatter. The forward scatter parameter (FSC) is linked to cell size, while the side scatter parameter (SSC) is related to shape and composition of the cell [17].

The flow cytometer consists of fluidics, optics, and electronics as illustrated in Figure 2.5. The fluidics system transports the sample from the sample tube to the flow cell. In the flow cell, the sample passes the laser and is either sorted by cell sorters or transported to waste. The optical system is responsible for collecting and transporting lights in the instrument using lenses and filters. It also includes the detection system, which generates a current induced by the action of light. The electronics process and digitalize the photocurrent from the detector [48].

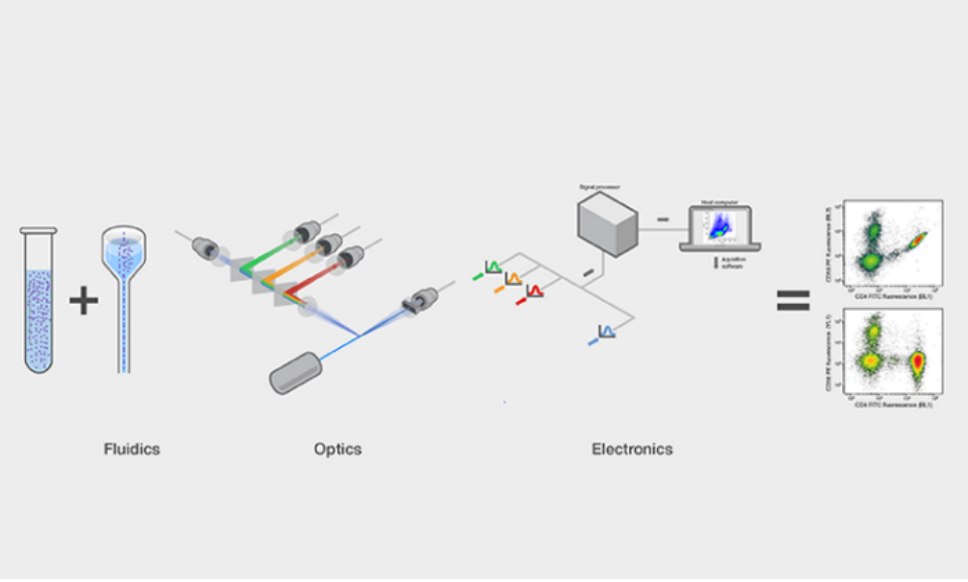


Figure 2.5: *The components of the flow cytometer [48].*

2.3.2 Optical Density (Absorbance)

Measuring optical density (OD) or absorbance of the algal culture is a rapid and commonly used method for estimating cell density. Measuring absorbance at 550 or 750 nm will avoid interference with absorbance of algal pigments such as chlorophyll [25]. Amount of light absorbed by an algal suspension is an indication of cell mass in the sample [49]. It is essential to establish a relationship between cell count and absorbance. This correlation will change with growth rate and cell size [25].

The amount of light absorbed by the algal cells is an indication of amount of algal cells in the sample. When determining OD, algal cultures need to be diluted to achieve OD_{680} and OD_{750} value less than 1 to achieve the linear range of measurement [15].

2.3.3 Microplate Readings

Newly available fluorescence plate readers, such as Tecan Infinite F200 PRO, can be used to measure algal growth by monitoring change in chlorophyll fluorescence over time [47]. Measurement results depend on environmental factors such as temperature, pH, and gas-transfer. The Tecan Infinite F200 can also measure absorbance (OD) of algal cell cultures. Fluorescence intensity (FI) can be measured for determination of amount of fluorescent compounds in multiwell-plates [50]. The fluorescence intensity system of the Infinite F200 PRO consists of a light source, fluorescence optics, and fluorescence detection system, as illustrated in Figure 2.6.

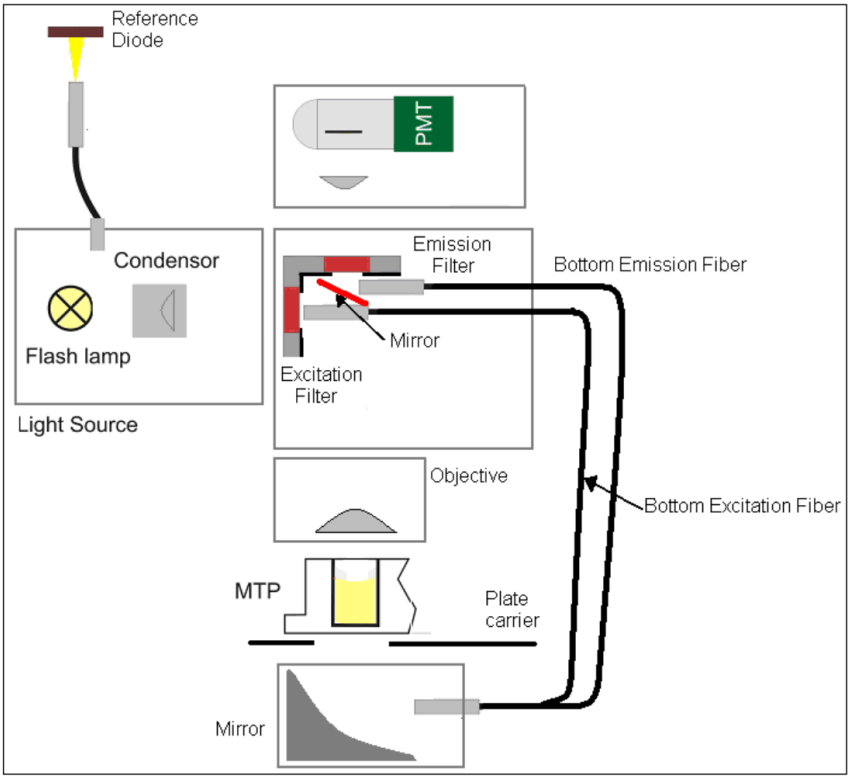


Figure 2.6: *The components of the light intensity bottom system in TECAN Microplate Reader [50].*

Flashlight is focused through the silt and the excitation filter before it enters the optical system. Light is guided by excitation bottom fiber to the bottom optics probe. Here, the light is focused by an elliptical mirror through the bottom of the microplate, and into the well containing the fluorescent compounds. These compounds emit light, which is focused on the excitation bottom fiber. The light passes a mirror through the emission filter, reaching the fluorescence detection system. Low light intensities are detected using a photomultiplier tube (PMT) [50].

2.3.4 Neubauer Haemocytometer Counting Chamber

Cell number and concentration can easily be counted using optical microscopy. The Neubauer chamber remains as one of the most common methods for enumerating cultured cells [51].

The Improved Neubauer haemocytometer is a thick, microscope slide, found to be excellent in quantifying single-celled algae. The chamber has a grid/framework etched onto it, which can be observed under the microscope using 10 or 40x objective [25].

The haemocytometer is suitable for cells less than 100 μm in diameter [25]. The concentration range for cell enumerating using a Neubauer chamber is 250 000 cells/mL to 2.5 million cells/mL. If the concentration of cells is below 250 000 cells per mL, the enumeration will not match the original concentration in the sample. If the number of cells exceeds 2.5 million cells/mL, the sample should be diluted to achieve reliable cell count. A recommended dilution concentration is 1 million cells/mL [51].

In the Neubauer haemocytometer, the chamber depth is 0.1 mm. The framework is made up by nine big squares of 1x1 mm, which gives 0.1 mm^3 (0.1 μL) volume per big square. Squares are also subdivided into smaller squares of known size. Since the volume of each square is known, one can easily calculate number of cells per unit volume [25].

Enumerating algal cells can be done by counting number of cells in the big squares in the Neubauer chamber, as seen in Figure 2.7. When determining cell concentration in the sample, Equation 2.3 can be used [51].

$$Concentration = \frac{Number\ of\ Cells\ x\ 10\ 000}{Number\ of\ Squares} \quad (2.3)$$

If a dilution is applied, the concentration can be calculated using Equation 2.4 [51].

$$Concentration = \frac{Number\ of\ Cells\ x\ 10\ 000}{Number\ of\ Squares\ x\ dilution} \quad (2.4)$$

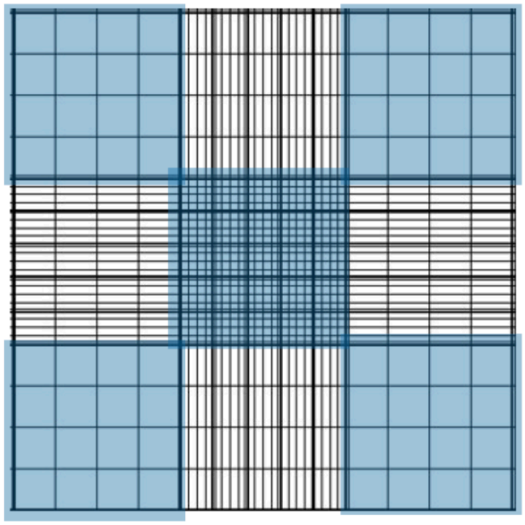


Figure 2.7: *The counting squares in the Improved Neubauer counting chamber [51].*

2.4 Specific Objectives

This study aims to identify algal strains that effectively grow in wastewater from IVAR WWTP in Grødaland, while sufficiently remove nutrients and COD. To do this, selected algal strains (Table 2.1) are to be grown in wastewater for a specific time period. The best algal strains will be used in algal pond as the tertiary treatment step. To study algal nutrient removal in wastewater, important wastewater characteristics such as nitrogen, nitrite, nitrate, phosphorous, phosphate, ammonium, COD, TSS, and alkalinity, will be measured before and after algal treatment. Algae are to be cultivated in different types of media, including growth media and wastewater, under different environmental conditions, to examine efficient cultivation methods. When examining algal growth, several different quantification methods are available, each with its own advantages and limitations. In interest of finding the most effective and accurate way to monitor algal growth, flow cytometer, microplate readings (measuring OD_{750} and fluorescence intensity), and counting chambers are to be examined and compared. By using the quantification data, the growth rates of each algal strain will be determined.

3. Materials and Methods

In this chapter, the materials and methods for algal quantification, algal cultivation, and measurements of algal wastewater treatment efficiency are described.

3.1 Algal Cultivation

Freshwater and marine algae were cultivated in specific growth media and both pure and diluted secondary wastewater effluent to study algal growth characteristics and growth kinetics.

3.1.1 Algal Strains

Freshwater algae in suspension *Chlorella vulgaris* (CCAP 211/11B), *Tetradesmus obliquus* (CCAP 276/3A), *Haematococcus pluvialis* (CCAP 34/7), *Chlorella sorokiniana* (CCAP 211/8K), and marine algae in agar *Microchloropsis salina* (CCAP 849/3) were received from the Culture Collection of Algae and Protozoa in Scotland the 22th of February 2018. The freshwater and marine species were cultivated the 23rd of February 2018, and maintained in MWC+Se and L1 growth media, respectively.

3.1.2 Wastewater

The wastewater used in this study was provided from IVAR WWTP in Grødaland. This wastewater is a mix from household and industrial wastewater (Norsk Protein AS and Kviamarka næringsmiddelpark). 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. The wastewater was frozen for storage prior experiments. The effluent from the membrane was used as algal cultivation media to determine algal treatment efficiency. Nutrients, TSS, Alkalinity, and COD content in the wastewater were measured before and after algal treatment.

3.1.3 Natural Water

Natural lake water and seawater were used in this study for algal cultivation. The lake water was sampled from Hålandsvatnet, Stavanger. Seawater was collected at IRIS using a pipeline from Byfjorden (North: 58^{o} 57' 48`` East: $5^{o}$ 43` 8``) at 80 m depth from Byfjorden (Randaberg, Norway).

3.1.4 Chemicals

- Thiamine hydrochloride ($C_{12}H_{18}Cl_2N_4OS$), 99%. Producer: VWR chemicals.
- Selenous acid (H_2SeO_3), 98%. Producer: Sigma Aldrich.
- Mangan (II)-chlorid-4-hydrat ($MnCl_2 \cdot 4\ H_2O$). Producer: Riedel-De Haenag Seelze-Hannover
- Copper(II)-sulfate-5-hydrat ($CuSO_4 \cdot 5\ H_2O$) Producer: Merck
- Natriummolybdat-2-hydrat ($Na_2MoO_4 \cdot 2\ H_2O$). Producer: Riedel-De Haenag Seelze-Hannover
- Zinksulfat-7-hydrat ($ZnSO_4 \cdot 7H_2O$). Producer: Riedel-De Haenag Seelze-Hannover
- Cobalt(III) chloride hexahydrate ($COCl_2 \cdot 6H_2O$), >98%. Producer: Alfa Aesar.
- Nickel(II) sulphate hexahydrate ($NiSO_4 \cdot 6H_2O$), >98%. Producer: Alfa Aesar.
- Sodium orthovanadate (Na_3VO_4), 99.9%. Producer: Alfa Aesar
- Potassium chromate (K_2CrO_4), >99.5%. Producer: Merck
- Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), >99%. Producer: Merck
- Di-potassium hydrogen phosphate (K_2HPO_4), >99%. Producer: Merck
- Ethylenediaminetetraacetic acid disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2\ H_2O$), >99%. Producer: VWR
- Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$), >99%. Producer: VWR chemicals.
- Boric acid (H_3BO_3), >99%. Producer: Sigma- Life Science
- Sodium hydrogen carbonate ($NaHCO_3$), >99%. Producer: Merck.
- Sodium selenate anhydrous (Na_2SeO_3), >99.8%. Producer: Alfa Aesar.
- Sodium nitrate ($NaNO_3$). Produced by Merck.
- Sodium metasilicate nonahydrate ($Na_2O_3Si \cdot 9\ H_2O$), >98%. Producer: Sigma Aldrich.

- D - (+)- Biotin ($C_{10}H_{16}N_2O_3S$), >98% . Producer: Alfa Aesar.
- Cyanocobalamin B_{12} ($C_{63}H_{88}CoN_{14}O_{14}P$), >98%. Producer: Alfa Aesar.
- Sodium nitrate ($NaNO_3$), >99%. Producer: Emsure
- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), >99%. Producer: Merck
- Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7\ H_2O$), 99.7%. Producer: VWR Chemicals

3.1.5 Equipment

- Laminar flow hood: Nuair. Model no. NU-437-400E
- Incubator: Innova S44i Eppendorf.
- Autoclave: Panasonic MLS-3781L.
- Microscope Olympus BX61
- 250 mL Erlenmeyer flasks
- Sterilized loop
- Bunsen burner
- Disposable serological pipettes. Producer: VWR

3.1.6 Procedure for Growth Media Preparation

Freshwater media, MWC+Se, and marine media, L1, were prepared for algal cultivation. The media was autoclaved and stored in refrigerator until use.

3.1.6.1 Freshwater Media MWC+Se

MWC+Se was prepared as described by the Scandinavian Culture Collection of Algae and Protozoa [52].

Stock solutions and trace element solutions were prepared according to recipe in Table 3.1. The pH value was adjusted to 4 - 4.5 to retain solubility of the metals. Vitamin primary stock solutions were prepared according to recipe in Table 3.1. For the vitamin B_{12} and Biotin stock solution approximately 11% water (0.89 mL of dH_2O added for each 1 mg B_{12}) and 4% water (9.6 mL of dH_2O added for each 1 mg Biotin) were added for crystallization, respectively. The vitamin stock solutions were stored in freezer until use.

Table 3.1: *Concentration of compounds used in preparation of stock solutions, trace element solutions and vitamin primary stock solution.*

Stock Solutions	
Compound	**Quantity**
$CaCl_2 \cdot 2\ H_2O$	36.80 g/L
$MgSO_4 \cdot 7\ H_2O$	37.00 g/L
$NaHCO_3$	12.60 g/L
$K_2HPO_4 \cdot 3\ H_2O$	11.40 g/L
$NaNO_3$	85.00 g/L
$Na_2O_3Si \cdot 9\ H_2O$	28.40 g/L
Trace Element Solution	
Compound	**Quantity**
$C_{10}H_{14}N_2Na_2O_8 \cdot 2\ H_2O$	4.36 g
$FeCl_3 \cdot 6H_2O$	3.15 g
$MnCl_2 \cdot 4\ H_2O$	0.18 g
H_3BO_3	1.00 g
1% $CuSO_4 \cdot 4\ H_2O$	1 mL
2.2 % $ZnSO_4 \cdot 7H_2O$	1 mL
1% $COCl_2 \cdot 6H_2O$	1 mL
0.6% $Na_2MoO_4 \cdot 2\ H_2O$	1 mL
dH_2O	to 1000 mL
Vitamin primary stock solution	
Compound	**Quantity**
Biotin	0.1 g/L
Cyanocobalamin (B_{12})	1 g/L

3.1.6.2 Seawater media L1

The procedure for L1 preparation is written as described by media recipe of the Scandinavian Culture Collection of Algae and Protozoa [53].

Stock solution for major elements, primary stock solutions, and vitamin mix were prepared according to recipe in Table 3.2.

Table 3.2: *Concentration of compounds used in preparation of stock solutions, trace element solutions, and vitamin stock solution.*

Stock solution for major elements	
Compound	**Quantity**
$NaNO_3$	75 g/L
$NaH_2PO_4 \bullet H_2O$	5 g/L
Primary trace elements stock solutions	
Compound	**Quantity**
$CuSO_4 \bullet 5\ H_2O$	2.45 g/L
$Na_2MoO_4 \bullet 2\ H_2O$	19.9 g/L
$ZnSO_4 \bullet 7H_2O$	22 g/L
$CoCl_2 \bullet 6H_2O$	10 g/L
$MnCl_2 \bullet 4\ H_2O$	180 g/L
H_2SeO_3	1.3 g/L
$NiSO_4 \bullet 6H_2O$	2.7 g/L
Na_3VO_4	1.84 g/L
K_2CrO_4	1.94 g/L
Vitamin stock solution	
Compound	**Quantity**
Biotin	0.0005 g
Thiamine HCl (B_1)	0.1 g
Cyanocobalamin (B_{12})	0.0005

For the trace metal working stock solution, 4.36 g $C_{10}H_{14}N_2Na_2O_8 \bullet 2\ H_2O$ and 3.15 g $FeCl_3 \bullet 6H_2O$ were added to a 1000 mL volumetric flask, along with 0.25 mL of $CuSO_4 \bullet 5\ H_2O$, 3 mL of $Na_2MoO_4 \bullet 2\ H_2O$, and 1 mL of $ZnSO_4 \bullet 7H_2O$, $CoCl_2 \bullet 6H_2O$, $MnCl_2 \bullet 4\ H_2O$, H_2SeO_3 , $NiSO_4 \bullet 6H_2O$, Na_3VO_4 and K_2CrO_4. Distilled water was filled to 1000 mL.

For the final preparation of L1 media 1 mL $NaNO_3$, 1 mL $NaH_2PO_4 \bullet 2\ H_2O$, 1 mL of trace elements working stock solution and 1 mL of vitamin mix stock solution, as described in Table 3.2, were added to a 1000 mL volumetric flask. Filtered seawater was filled up to 1000 mL and pH was adjusted to 8 before autoclaving the finished media.

3.1.7 Procedure for Algal Cultivation

The inoculation of algal cultures was done in the laminar flow hood using sterile technique. All equipment was autoclaved before use.

C. vulgaris, T. obliquus, H. pluvialis, C. sorokiniana, and *M. salina* were provided by CCAP. Characteristics of each algae are described in Table 2.1 To prepare algal cultures in growth media, 2 mL of each of the suspended freshwater algae were transferred to three 250 mL Erlenmeyer flasks containing 100 mL autoclaved MWC+Se media. An aliquot of *M. salina* were transferred to 100 mL marine media F/2 or L1, using a sterilized loop. The algal cultures were incubated at 18-20^{o}C, 80-90 rpm and photosynthetic LED light of 15-100 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours. When algal cultures were in late logarithmic phase, they were sub-cultured for maintenance. The algal cultures were regularly examined and checked for contamination and cell viability using microscope. The cultures were shaken once a day to avoid self-shading and to ensure gas-transfer.

When preparing cultures for experiments, algal suspension was transferred to wastewater to be treated. The wastewater used for culturing was either pure or diluted, depending on the experiment. 100-200 mL of pure secondary effluent, secondary effluent mixed with 50% natural water (lake water and seawater) or 50% growth media, were used as algal culture media. The wastewater was frozen for storage. For some experiments, the wastewater was filtered before use as it was found that TSS increased after freeze-thaw processes. Cultures were incubated as described above.

C. vulgaris, C. sorokiniana, T. obliquus, and *M. Salina* were cultivated in both diluted and pure wastewater over 9-14 days for purifying the wastewater. Algae consortia from Lake Hålandsvatnet in Stavanger were also tested for treatment. To determine algal treatment efficiency the amount of nutrients (TP, TN, phosphate, nitrite, nitrate, and ammonium) and COD were measured using Spectroquant cell tests from Merck. Production of TSS was measured as an indication of algal biomass productivity.

3.2 Algal Quantification

With the aim to identify the most accurate and effective method for algal quantification, data from flow cytometry, OD measurements, counting chamber counts, and microplate readings were compared.

3.2.1 Chemicals

- C6 Flow Cytometer starter kit. Producer: BD Biosciences
- 70% Ethanol
- Algal Cultures

3.2.2 Equipment

- UV-VIS Spectrophotometer UVmini-1240. Producer: Shimadzu
- Brand UV cuvettes, 1.5 mL semi-micro. Producer: Sigma Aldrich
- Flow Cytometer: BD Accuri C6. Producer: BD Bioscience
- Disposable serological pipettes. Producer: VWR
- Microplate reader: Tecan Infinite F200 PRO. Producer: Tecan
- 24 Well Cell Culture Cluster of Polystyrene. Producer: Costar
- Breathe Easy: Sterile Gas Permeable Membranes. Producer: Diversified Biotech
- Microscope VisiScope series 200. Producer: VWR
- Neubauer counting chamber: Neubauer Improved. 0.100 mm depth, 0.0025 mm^2. Producer: Assistant Germany
- Lens Tissue Paper. Producer: Karl Hecht Assistent

3.2.3 Flow Cytometer Analysis of Algae Cell Numbers

Flow Cytometry is an emerging method for quantifying algal cells. The principal behind the method is described in Chapter 2.3.1.

The BD Accuri C6 flow cytometer was used in this study. The system consists of a microprocessor controlled, peristaltic pump that continuously collects sample material and preforms a count per mL without having to add counting beads. The Accuri C6 contains two lasers of 488 and 640 nm and four signal detectors of 533 nm (FL1), 585 nm (FL2), 670 nm (FL3), and 675 nm (FL4).

Size beads were analyzed on the Accuri to determine where various algal cells of different sizes appear on the scatter plot. Once a day, 1 mL of algae suspension was added to sample vials and vortexed quickly to homogenize sample, before analysis on the C6. All samples were analyzed using a flow rate of 14 μL/min with a 10 μm core size. As the C6 monitors the volume of sample analyzed, the concentration was determined directly from the software (BD Accuri C6 Plus).

All five algal species showed a high florescence on trigger FL4 (red fluorescence) on the C6. By using the size of the algae, combined with florescence, algae were easily distinguished from background noise such as bacteria and debris. This was done by gating the algal population appearing on the forward scatter trigger (FSC) and the fluorescence trigger FL4 (red fluorescence 675 nm), as illustrated in Figure 3.1.

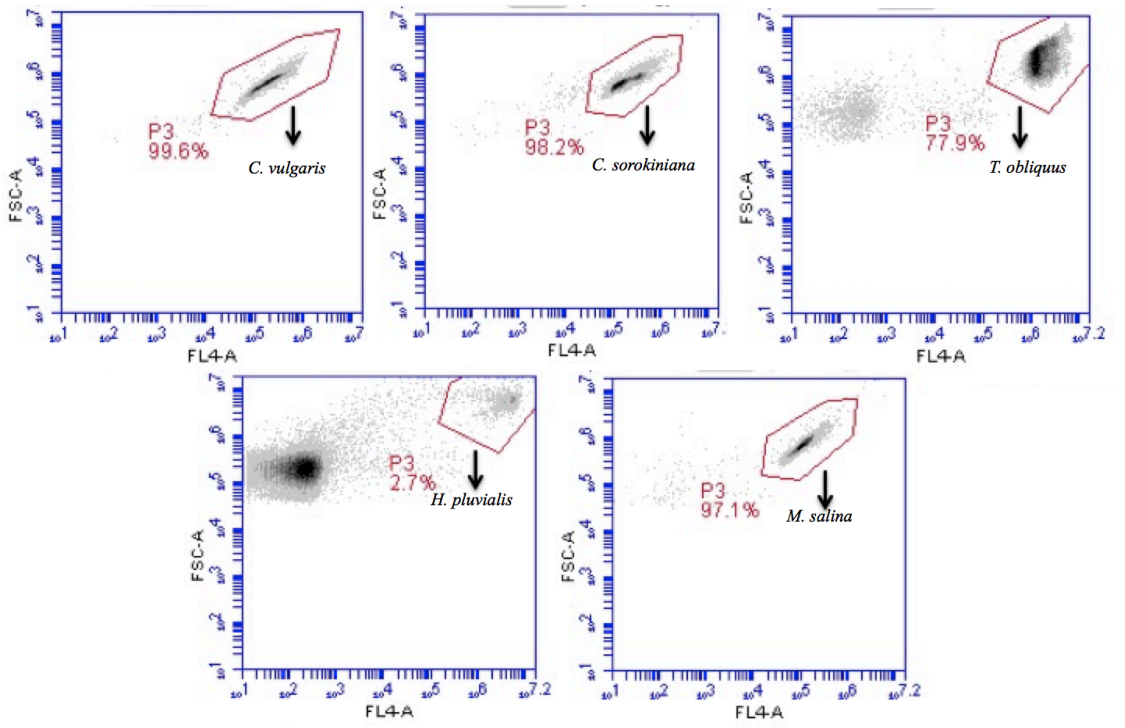


Figure 3.1: *Method developed for distinguishing selected algae from background noise and debris using the forward scatter trigger and the fluorescence trigger FL4 (675 nm) on Flow Cytometer BD Accuri C6.*

3.2.4 OD Measurement of Cell Growth

Measuring the optical density of algae cultures is a common method for estimating cell density. Theory behind the method is described in Chapter 2.3.2.

OD measurement of algal cells growing in MWC+Se and L1 media was done to establish a relationship between absorbance and amount of cells in the culture. 1 mL of homogenized algal cell suspension from the algal batch cultures were added to disposable UV cuvettes and analyzed once a day by measuring absorbance on the spectrophotometer. All samples were run on 540 nm, 680 nm, and 750 nm to find the most accurate wavelength for estimating algal cell density.

3.2.5 Microplate Readings for Quantification of Algal Cell Numbers

Microplate readings can be used for monitoring fluorescence intensity and OD in algae cultivated in microplates. Theory about the method can be found in Chapter 2.3.3.

The procedure used was based on a method by Van Wagenen et al [16]. 2 mL of homogenized algal cell suspension was added to clear-bottomed 24 well microplates. Sterile Breathe Easy gas permeable membranes added to cover the microplates to reduce evaporation. The microplates were incubated at 18-20^{o}C, 80-90 rpm, and light intensity of 15-100 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours. The microplates were analyzed for growth once a day using TECAN Infinite M200 Pro microplate reader. The growth curve was determined by measuring florescence intensity at 440nm excitation, 690nm emission, and optical density measurements at 750 nm (OD_{750}). A calibration curve with all examined algal strains at four concentrations determined by flow cytometry was used to estimate concentration of the cells.

3.2.6 Direct Cell Counting

Direct counting using counting chambers are traditional and widely utilized method of enumerating cells in culture. Information about the method can be found in Chapter 2.3.4.

The Improved Neubauer counting chamber was used for direct cell counting. The glass cover slip was added to the chambers central area, before homogenous algal suspension from the batch cultures was pipetted into the counting chamber. Dense cultures were diluted with distilled water prior analysis. The sample was enumerated manually using an optical microscope with 40x objective. A total of 16 squares (corresponding to one big blue square as seen in Figure 2.7) were counted for each sample. For calculating concentration in the sample, Equation 2.3 and 2.4 were used. After each sample, the counting chamber and cover slip was carefully cleaned with 70% Ethanol and wiped dry using lens paper.

3.3 Measurement of Algal Wastewater Treatment Efficiency

The ability of algae to purify wastewater was studied by measuring removal of COD, phosphate, phosphorous, nitrite, nitrate, nitrogen, ammonium, and alkalinity before and after cultivation. Production of TSS was measured as an indicator of algal biomass productivity.

3.3.1 Chemicals

- Spectroquant COD cell test (measuring range: 100-1500 mg/L COD). Product number: 109773. Producer: Merck
- Spectroquant Nitrate cell test (measuring range: 0.5-25.9 mg/L NO_3-N). Product number: 114563. Producer: Merck
- Spectroquant Phosphate Cell Test (measuring range: 0.5-25.0 mg/L PO_4-P. Product number: 114729. Producer: Merck
- Spectroquant Ammonium Cell Test (measuring range: 0.5-16.0 mg/L NH_4-N). Product number: 114544. Producer: Merck
- Spectroquant (total) Cell Test (measuring range: 10-150 mg/L N). Product number: 11473. Producer: Merck
- Spectroquant Nitrite Cell Test (measuring range: 0.010-0.700 mg/L NO_2-N). Product number: 114547. Producer: Merck
- Hydrogen Chloride (HCl), 0.1 M. Producer: Merck
- Sodium Hydroxide (NaOH), 0.1 M. Producer: Merck
- Filter for TSS measurement: Glass microfiber filter, 1.5 μm particle retention. Grade GF/C. Producer: VWR.

3.3.2 Equipment

- Thermoreactor: Spectroquant TR 620. Producer: Merck
- Photometer: Spectroquant Pharo 300. Producer: Merck
- Alkalinity measurement: TitroLine 5000 Auto-titration. Producer: Instrument-teknikk AS, Oslo
- Technical Buffer pH 4.01 (50 mL). Producer: VWR
- Technical Buffer pH 7.00 (50 mL). Producer: VWR

3.3.3 Procedure for COD Measurement

COD measurement was used to analyze the oxidizable organic matter in the wastewater before and after algal treatment. The sample is oxidized with hot sulphuric solutions of potassium dichromate using silver sulphate is the catalyst. Concentration of green Cr^{3+} - ions is then measured photometrically [54].

2 mL of sample were added to the reaction cell and mixed well. The cell was heated to 148°C in the preheated thermoreactor for 120 minutes. The cell was cooled to room temperature and measured in the photometer.

3.3.4 Procedure for Nitrate Cell Test

In sulphuric and phosphoric solutions, nitrate ions react with the compound 2,6-dimethylphenol (DMP). This reaction forms 4-nitro-2,6-dimethylphenol, which can be measured photometrically [55].

1 mL of filtered sample was pipetted into the reaction cell along with 1 mL reagent NO_3-1K before mixing the cell. The cell was left for 10 minutes to react and was thereafter measured in the photometer.

3.3.5 Procedure for Nitrite Cell Test

Nitrite ions reacts with sulfanilic acid to form diazonium salt in acidic solution. The salt reacts with N-(1-naphtyl)etylenediamine dihydrochloride to form red-violet azo dye, which can be measured photometrically [56].

5 mL of the sample was pipetted into the reaction cell and shaken vigorously until reagent was completely dissolved. After 10 minutes reaction time, the sample was measured photometrically.

3.3.6 Procedure for Total Nitrogen Cell Test

Treating the organic and inorganic nitrogen with an oxidizing agent in the thermoreactor, will lead to production of nitrate. Nitrate, in a acidic solution containing sulphuric and phosphoric acid, will react with DMP to form 4-nitro-2,6-dimethylphenol which can be measured photometrically [57].

First, the sample had to be digested. 1 mL of sample was pipetted into an empty cell along with 9 mL of distilled water, before mixing the sample. 1 level blue microspoon of reagent N-1K (in the cap of the N-1K bottle) 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 120 ^{o}C 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 along with 1 mL of reagent N-3K. The sample were mixed and left to react for 10 minutes, before analyzed photometrically.

3.3.7 Procedure for Phosphate and Total Phosphorous Cell Test

Spectroquant Phosphate Cell Test determines amount of both orthophosphate and total phosphorous in the sample. Orthophosphate will, in a sulphuric solution, react with molybdite ions and form molybdophosphoric acid. Asorbic acid will reduce molybdophosphoric acid to phosphomolybdenum blue (PMB), which can be measured photometrically [57].

Determining total phosphorous in the sample requires a digestion step. This was done by adding 1 mL of sample along with 1 dose of reagent P-1K to the cell. The cell was mixed and heated at 120^{o}C in the thermoreactor for 30 minutes. For the measurement of phosphate, no digestion step was needed.

1 mL of sample (digested sample for TP measurement, not digested sample for phosphate measurement) was added to the reaction cell and contents were mixed. 5 drops of reagent P-2K was added to the cell and mixed. 1 dose reagent P-3K was added and the cell was shaken

vigorously until reagent was completely dissolved. After 5 minutes reaction time, the sample was measured in the photometer.

3.3.8 Procedure for Ammonium Cell Test

Ammonium nitrogen (NH_4-N) exists in a pH dependent equilibrium in forms of ammonium ions and ammonia. In a strong alkaline solution ammonia dominates as the form in which ammonium nitrogen exists. Ammonia reacts with hypochlorite ions and produces monochloramine, which reacts with a substituted phenol to form blue indophenol derivative. This compound can be measured photometrically [58].

When estimating ammonium in the sample, 0.5 mL of sample was added to the 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.

3.3.9 Procedure for TSS Analysis

Standard method 2540 D was used for the TSS analysis. The sample was homogenized by shaking before filtering using a pre-weighed GF/C Glass microfiber filter of 1.5 μm. Afterwards, the filter was dried for one hour in an oven of 103-105 ^{o}C, cooled in a desiccator and weighed. The increase of weight of the filter represents the TSS of the sample.

3.3.10 Procedure for Alkalinity Analysis

The alkalinity of samples was measured using the instrument Titroline 5000. The sample were diluted and placed on a low speed magnetic stirring device before being titrated with HCl 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 were recorded into computer software TITRA 5, which calculated the alkalinity of the sample.

4. Results

This chapter presents results obtained from the conducted experiments. The result is sub-divided in three chapters: (1) Comparing Methods for Quantifying Algae, (2) Algal Growth Characteristics, and (3) Algal Wastewater Treatment Efficiency.

4.1 Comparing Methods for Quantifying Algae

Quantitative algal analysis is important in algal research to determine growth and biomass productivity of algal strains. Measuring algal growth will help identify algal strains with the ability grow efficiently in wastewater, while producing valuable algal biomass in the process. Several analytical methods are used for algal quantification today, including flow cytometry, counting chambers, optical density measurements, and microplate readings. There are observed both advantages and drawbacks with each of these methods. Ideally, quantification methods should be rapid, accurate, and precise, a low limit of detection and using a small volume of sample [59]. This experiment was done to determine the most effective and accurate way to quantify algae in different types of media.

4.1.1 Comparing OD Measurements and Flow Cytometry for Algal Cells Cultivated in Growth Media

For freshwater algae and marine algae cultivated in MWC+Se and L1, respectively, daily flow cytometry and OD measurements were conducted for 10 days to examine the linear relationship between the two different quantification methods. The result for *C. vulgaris, C. sorokiniana, T. obliquus, H. pluvialis, and M. salina* is shown in Figure 4.1-4.5, respectively.

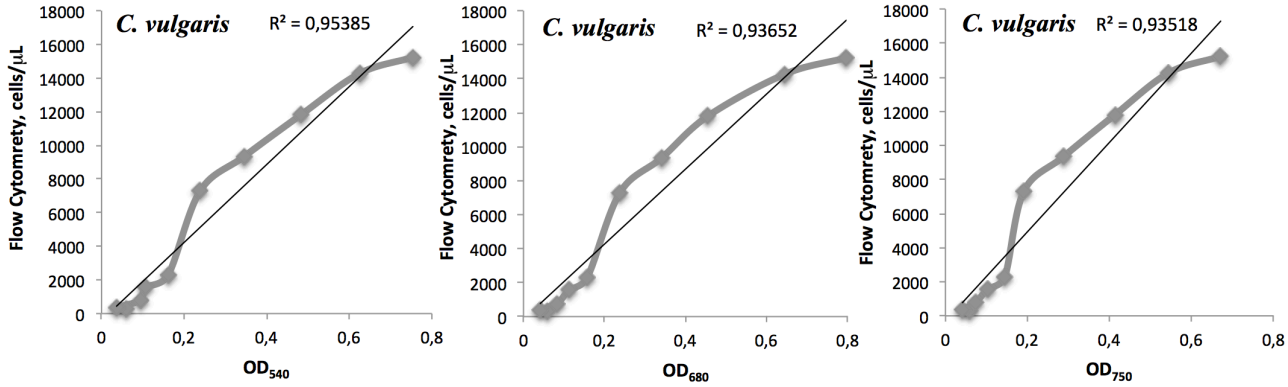


Figure 4.1: *The linear relationship between flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for C. vulgaris grown in growth media MWC+Se.*

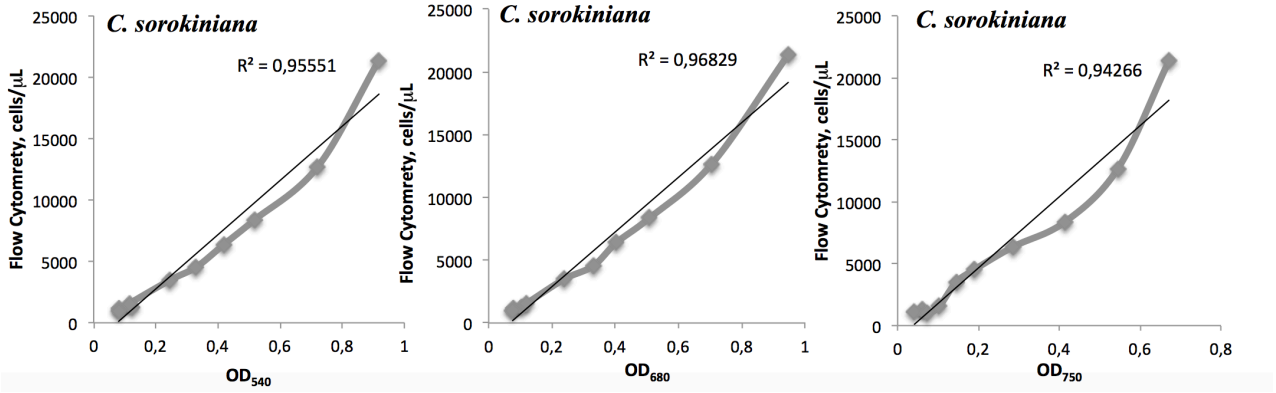


Figure 4.2: *The linear relationship between flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for C. sorokiniana grown in growth media MWC+Se.*

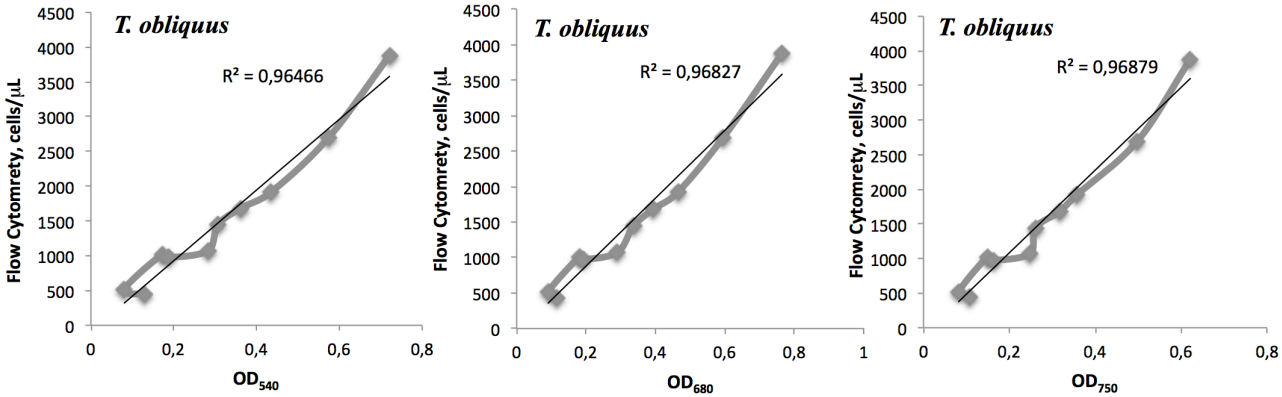


Figure 4.3: *The linear relationship between flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for T. obliquus grown in growth media MWC+Se*

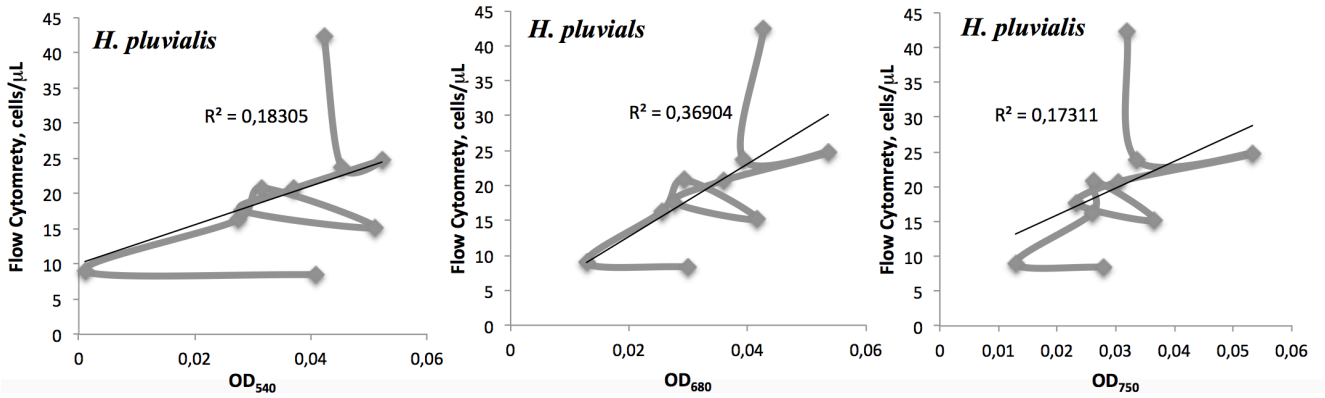


Figure 4.4: *The linear relationship between flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for H. pluvialis grown in growth media MWC+Se*

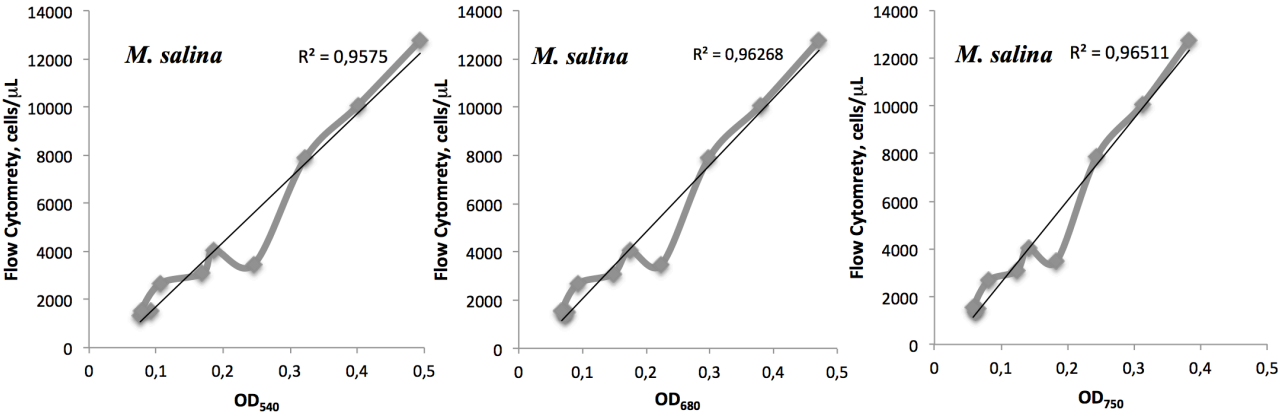


Figure 4.5: *The linear relationship between flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for M. salina grown in growth media L1.*

As seen from Figure 4.1, 4.2, 4.3, and 4.5, the linearity of flow cytometer data and OD_{540}, OD_{680}, and OD_{750} measurements for *C. vulgaris, C. sorokiniana, T. obliquus, and M. salina* corresponds well with $R^2 > 0.94$. The comparisons between the two methods for *H. pluvialis* in Figure 4.4 show a poor linearity of $R^2 < 0.37$.

4.1.2 Comparing Flow Cytometry, Counting Chamber, and Microplate Readings for Algal Cells Grown in Wastewater

Enumerations of algal cells cultivated in wastewater were conducted once a day using flow cytometer, counting chamber, and microplate readings (measuring fluorescence intensity and OD_{750}). The results from each quantification method were used to determine if the different quantification methods corresponded with each other. As *H. pluvialis* grew poorly throughout several experiments, these algae were excluded from the results. Freshwater algae *C. vulgaris, C. sorokiniana, T. obliquus,* and marine algae *M. salina* were cultivated in pure wastewater and wastewater diluted with 50% natural water, including lake water for freshwater algae and seawater for marine algae.

Comparison of the different quantification analysis for algae cultivated in pure and diluted wastewater is illustrated Figure 4.6 and Figure 4.7, respectively.

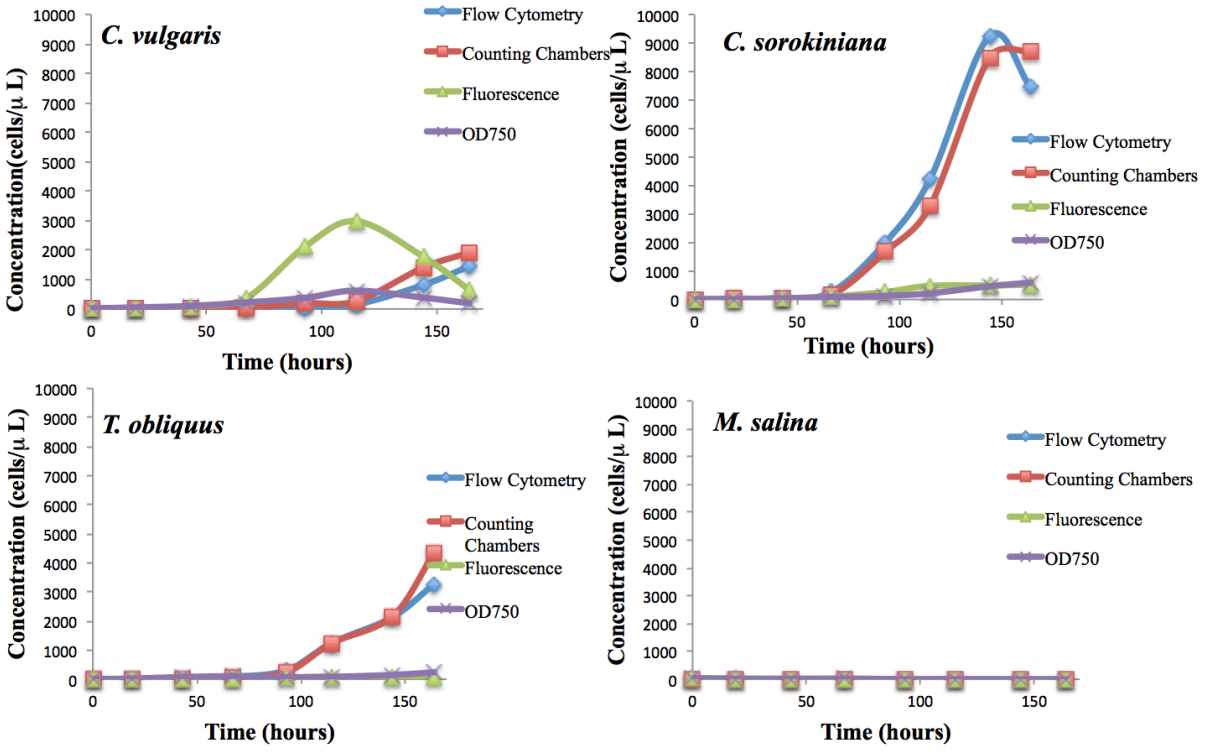


Figure 4.6: *Comparison of quantitative algal analysis methods for C. sorokiniana, C. vulgaris, T. obliquus, and M. salina grown in pure wastewater*

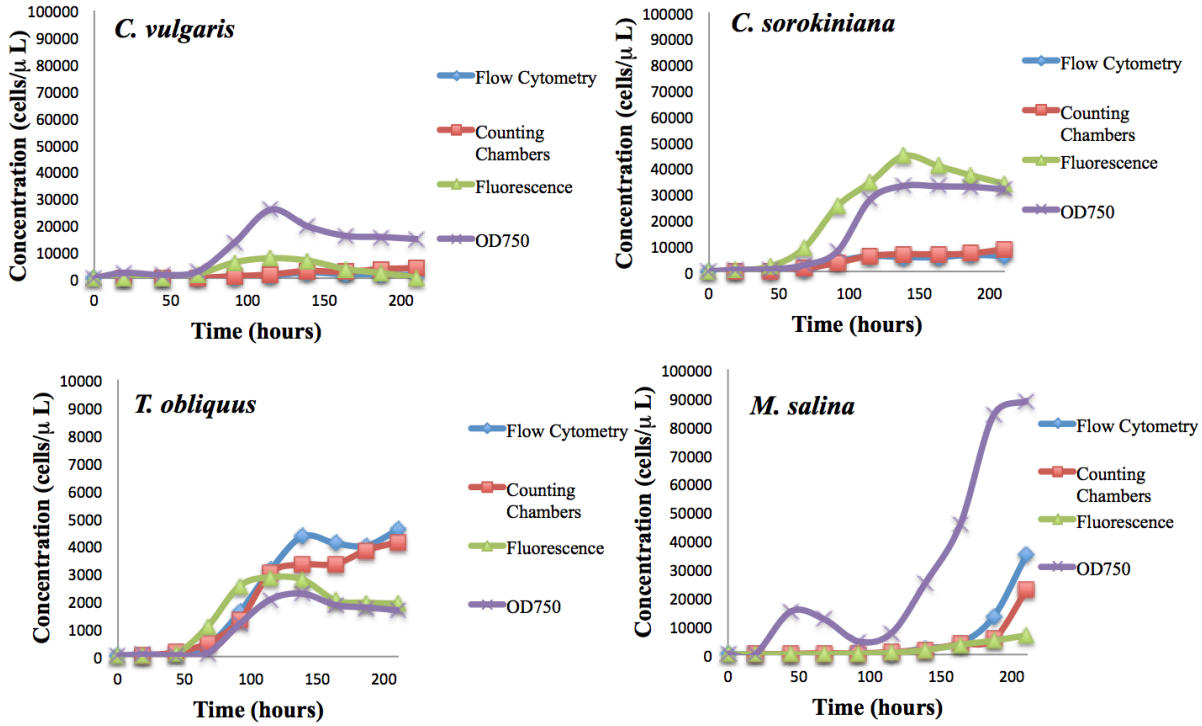


***Figure 4.7**: Comparison of quantitative algal analysis methods for C. sorokiniana, C. vulgaris, T. obliquus, and M. salina grown in wastewater diluted with natural water*

As seen from Figure 4.6 and 4.7, the results indicate variable fluorescence intensity and OD measurements for algae cultivated in pure and diluted secondary wastewater effluent. Estimation of algal concentration based on flow cytometry and counting chamber corresponds quite well. Similar findings were achieved in repeated experiments under identical conditions (Figure A.1.1 and Figure A.1.2 in Appendix 1).

4.2 Growth Characteristics and Optimization of Algal Cultivation

Algae were grown in different types of media using different environmental conditions for the characterization and optimization of the cultivation for the different algal strains. The algae were cultivated in growth media, pure wastewater, and wastewater diluted with media or natural water (lake water for freshwater species and seawater for marine species). Different strategies were used to achieve highest possible growth rates.

4.2.1 Algae Cultivated in Growth Media MWC+Se and L1

Growth media MWC+Se were used for freshwater algae and growth media L1 for marine algae. Growth curves based on daily flow cytometry analysis for algae *C. vulgaris, C. sorokiniana, T. obliquus, H. pluvialis,* and *M. salina* are shown in Figure 4.8.

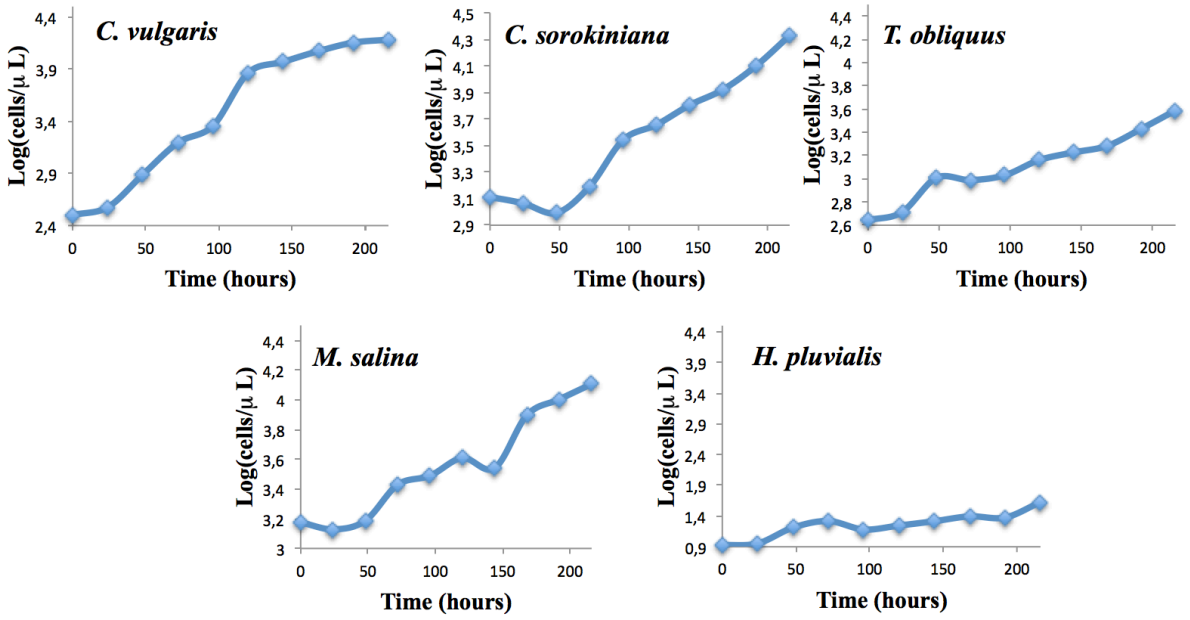


***Figure 4.8**: Established growth curves based on Flow Cytometry for algal species C. vulgaris, C. sorokiniana, T. obliquus, and H. pluvialis grown in MWC+Se media and M. Salina in L1 media.*

As seen from Figure 4.8, *C. vulgaris, C. sorokiniana, T. obliquus,* and *M. salina* achieve high cell density during cultivation. *C. vulgaris* exhibit short lag-phase, but reach stationary phase

faster compared to the other algae. The growth curve of *H. pluvialis* illustrates slow growth and low cell density.

Growth rates of the different algal strains cultivated in growth media were calculated using values from the identified logarithmic phases in growth curves presented in Figure 4.8, and Equation 2.2 (Chapter 2.2.3). The result is shown in Table 4.1.

Table 4.1: *Calculated growth rates based on data from identified logarithmic phases of algae grown in specific growth media along with Equation 2.2.*

Algal strains	Growth media	Growth rate μ, day^{-1}
C. vulgaris	MWC+Se	0.77
C. sorokiniana	MWC+Se	0.63
T. obliquus	MWC+Se	0.67
H. pluvialis	MWC+Se	0.37
M. salina	L1	0.56

The result in Table 4.1 indicates that *C. vulgaris* grows quite fast in MWC+Se, compared to the other strains. Algae H. *pluvialis* presented slow growth and low cell density throughout all experiments.

4.2.2 Algae Cultivated in Secondary Wastewater Effluent

Algae were cultivated in batch cultures containing pure secondary effluent (both filtered and unfiltered), secondary effluent diluted with 50% growth media, or with 50% unfiltered and filtered natural water (lake water for freshwater species and seawater for marine species). This was done to examine algal growth efficiency in different kinds of wastewater conditions. As the algae examined are to be used for wastewater treatment in algal ponds, it is of interest to study growth characteristics mixed with natural water sources. Additional growth curves from other experiments can be found in Appendix 3.

For algae grown in pure wastewater, different strategies were tested. When cultivating algae in unfiltered wastewater, environmental conditions included light intensity of 15 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 18°C, and 80 rpm. Algae cultivated in filtered wastewater were grown at light intensity of 100 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 20 °C and 90 rpm. The secondary wastewater effluent was frozen for storage prior algal treatment experiments. The unfiltered wastewater is secondary effluent was not filtrated after thawing, while filtered wastewater is secondary effluent filtered after thawing. Unidentified algae consortia from Hålandsvatnet (Figure A.2 in Appendix 2) were also included in the experiment of algal cultivation in filtered wastewater, while *H. pluvialis* were excluded due to poor growth. Growth curves based on daily flow cytometry analysis for algae cultivated in wastewater are shown in Figure 4.9.

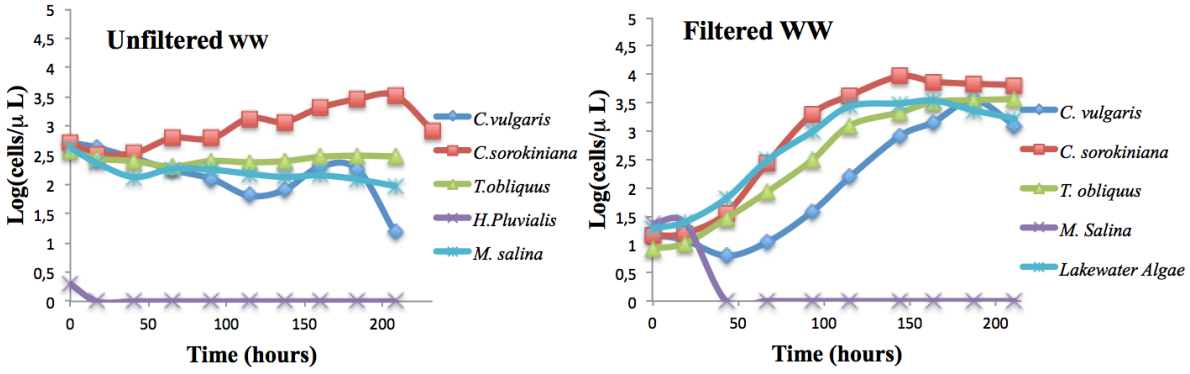


***Figure 4.9**: Growth curves of algae cultivated in unfiltered and filtered secondary wastewater effluent.*

The growth curves in Figure 4.9 indicate that algal strain *C. sorokiniana* grows effectively in both filtered and unfiltered wastewater. Especially in filtered wastewater, this algal strain is exhibiting short lag-phase and reaching high cell density before entering stationary phase compared to other algae. This confirmed in other experiments, illustrated in Figure A.3.1-A.3.2 in Appendix 3. This suggests a high adaptability and viability of *C. sorokiniana* in this specific type of wastewater. *C. vulgaris*, *T. obliquus*, algae consortia from Hålandsvatnet, and especially *C. sorokiniana* exhibit excellent growth in filtered wastewater, where *C. sorokiniana*, *T. obliquus* and algae consortia present short lag-phases. *C. vulgaris* use some more time before entering the logarithmic phase, but eventually reaches the same cell density as *T. obliquus* and algae consortia. In the unfiltered wastewater effluent, none of the other algal strains than *C. sorokiniana* grow well, as they do not reach any logarithmic phase. *M. salina* indicated poor growth in pure wastewater during all experiments, as seen in Figure 4.9 and Figure A.3.1 in Appendix 3.

As the algae tested in this study are to be used for wastewater treatment in algae ponds, wastewater was diluted with both filtered and unfiltered natural water to study the growth. Secondary wastewater effluent was filtered after thawing due to aggregates forming after freezing, before being mixed with 50% natural water. Algae were cultivated at light intensity of 100 µmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 20°C, and 90 rpm. Growth curves based on daily flow cytometry analysis for algae cultivated in diluted wastewater are shown in Figure 4.10.

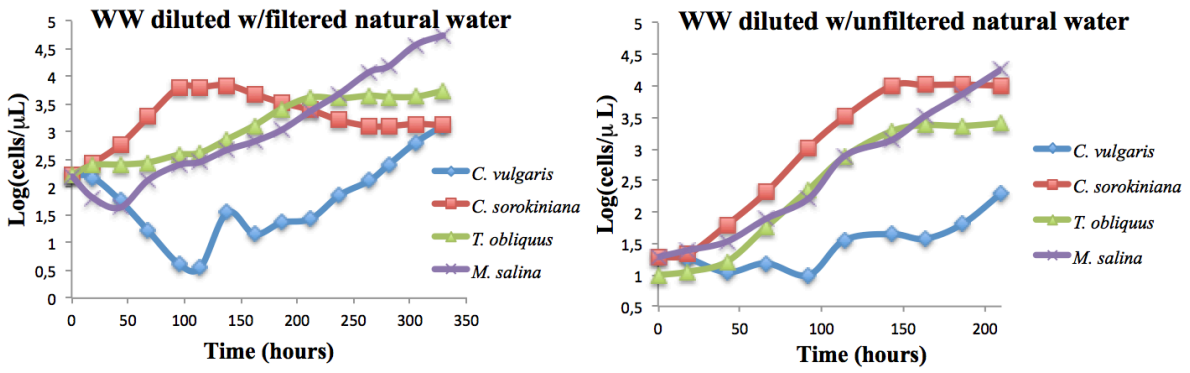


***Figure 4.10**: Growth curves of algae cultivated in wastewater diluted with filtered and unfiltered natural water.*

The growth curves illustrated in Figure 4.10, indicates efficient growth of *C. sorokiniana, T. obliquus,* and *M. salina* in diluted wastewater, especially when diluted with unfiltered natural water. *C. vulgaris* do not reach high cell density in diluted wastewater during experimental period, compared to the other algae.

Algae were cultivated in wastewater diluted with 50% of growth media. This was done with interest of studying the effect of adapting algae to the wastewater prior cultivation in pure wastewater. Algae were incubated at light intensity of 15 µmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 18°C, and 80 rpm in growth media. When adapted algae were transferred to filtered wastewater, cultivation conditions were changed to light intensity of 100 µmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 20°C, and 90 rpm. Growth curves based on daily flow cytometry analysis for algae cultivated in diluted wastewater are shown in Figure 4.11.

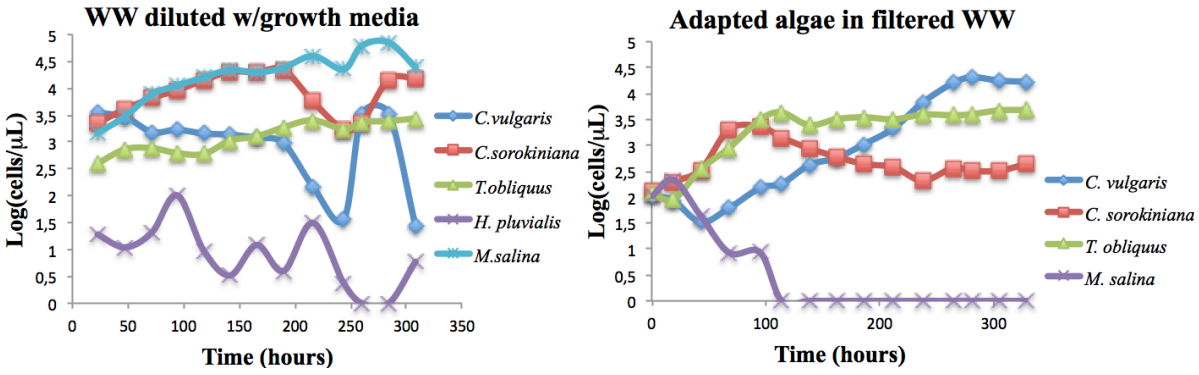


Figure 4.11: *Growth curves of algae cultivated in wastewater diluted with 50% growth media to adapt algae to wastewater and growth curves of adapted algae cultivated in pure wastewater.*

The results in Figure 4.11 imply the adapting the algae prior cultivating in pure wastewater, could have an effect on *C. vulgaris* growth, as the cells show a very long and steady logarithmic phase, reaching high cell density. The results for *C. sorokiniana, T. obliquus,* and *M. salina* did not indicate a pronounced effect of adapting algae, when compared to the results in Figure 4.9.

Growth rates of different algal strains were calculated using Equation 2.2 and values from identified logarithmic phase in growth curves shown in Figure 4.9-4.11 and A.3.1-A.3.2 in Appendix 3. Values left blank were due to lack of algal growth or highly variable growth, making it difficult to identify the logarithmic phase to be used in Equation 2.2. The calculated growth rates are summarized in Table 4.2.

Table 4.2: *Established growth rates (μ) for algae cultivated in pure and diluted wastewater.*

Algal strains	Wastewater diluted w/filtered natural water	Wastewater diluted w/unfiltered natural water	Wastewater diluted w/growth media	Unfiltered wastewater	Filtered wastewater	Adapted algae in filtered wastewater
C. vulgaris	0.76	0.75 – 1.60	-	-	1.23 – 1.33	0.89
C. sorokiniana	1.19	1.38 – 2.06	0.39	0.45	1.46 – 1.99	1.80
T. obliquus	0.63	1.31 – 1.39	0.34	-	1.26 – 1.28	1.26
M. salina	0.80	1.02 – 1.55	0.84	-	-	-
Lake water algae consortia	-	-	-	-	1.56	-

The established growth rates in Table 4.2 indicates that *C. vulgaris, C. sorokiniana, T. obliquus,* and lake water algae consortia grew very well in filtered wastewater. Especially suited for cultivating in this type of pure mixed secondary effluent for growth purposes, seems to be strain *C. sorokiniana,* which is able to grow in all of the tested culture conditions. *M. salina* did not grow in pure wastewater. However, the algae grew well when cultivated in diluted wastewater, indicating that this species could be used in an algal pond diluted with filtered seawater. *H. pluvialis* exhibited poor growth in all experiments, so growth rates were not possible to calculate.

4.3 Algal Treatment Efficiency

To determine algal wastewater treatment efficiency, selected wastewater parameters were examined before and after algal cultivation, and compared with a negative control. These parameters included COD, phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), total nitrogen (TN), total phosphorous (TP), and alkalinity (mg $CaCO_3$/L). COD were not measured for wastewater diluted with seawater due to high Cl^- content is making the test unreliable. Nutrient and COD measurements were done using Spectroquant Cell Kits. Production of TSS after wastewater treatment were analyzed and compared to a negative control as an indication of algal biomass production.

In the experiments presented in this chapter, algae were grown in both pure and diluted wastewater under identical environmental condition using light intensity of 100 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 20°C, and 90 rpm. Unidentified algae consortia from Hålandsvatnet (Figure A.2 in Appendix 2) were also included in the experiment of algal treatment of pure wastewater for 9 days. Algae *H. pluvialis* were excluded due to poor growth.

The chemical composition of the secondary wastewater effluent measured before algal treatment is presented as the mean value from two identically conducted experiments. Inorganic N/P ratios were calculated using obtained inorganic N values (NH_4^+, NO_2^- and NO_3^-) and, inorganic P values (PO_4^{3-}). The result is shown in Table 4.3.

Table 4.3: *Characteristics of secondary wastewater effluent*

Parameter	Wastewater	Wastewater diluted w/unfiltered lake water	Wastewater diluted w/unfiltered seawater
TSS, mg/L	50	100	-
COD_s, mg/L	250	243	-
PO_4^{-3}, mg/L	12.6	7.55	8
NH_4^+, mg/L	54.3	41.0	37.2
NO_2^-, mg/L	0.003	0.005	0.002
NO_3^-, mg/L	0.3	0.5	0.2
TN, mg/L	59.3	46.8	41.5
TP, mg/L	14.3	8.1	9.0
Inorganic N/P ratio	4.3	5.5	4.7
Alkalinity, mg$CaCO_3$/L	2071	1494	1377

Algal nutrient, COD_s, and alkalinity removal by algae cultivated in diluted wastewater after 9 and 11 days are presented in Figure 4.12 and 4.13, respectively.

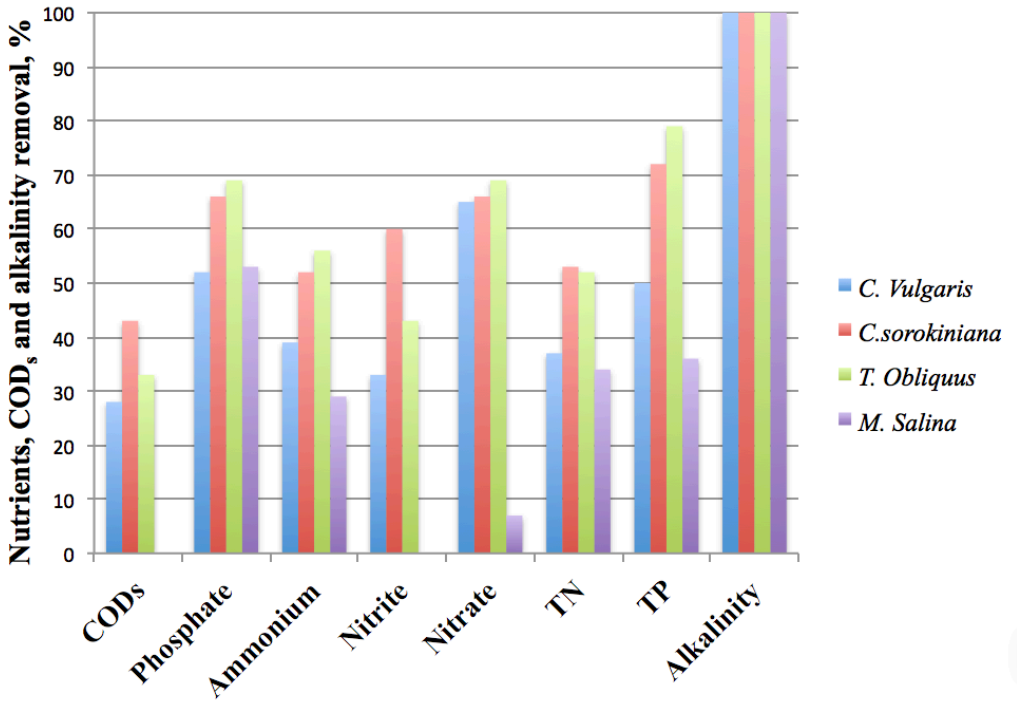


Figure 4.12: *Algal nutrient, COD, and alkalinity removal from wastewater diluted with unfiltered natural water after 9 days.*

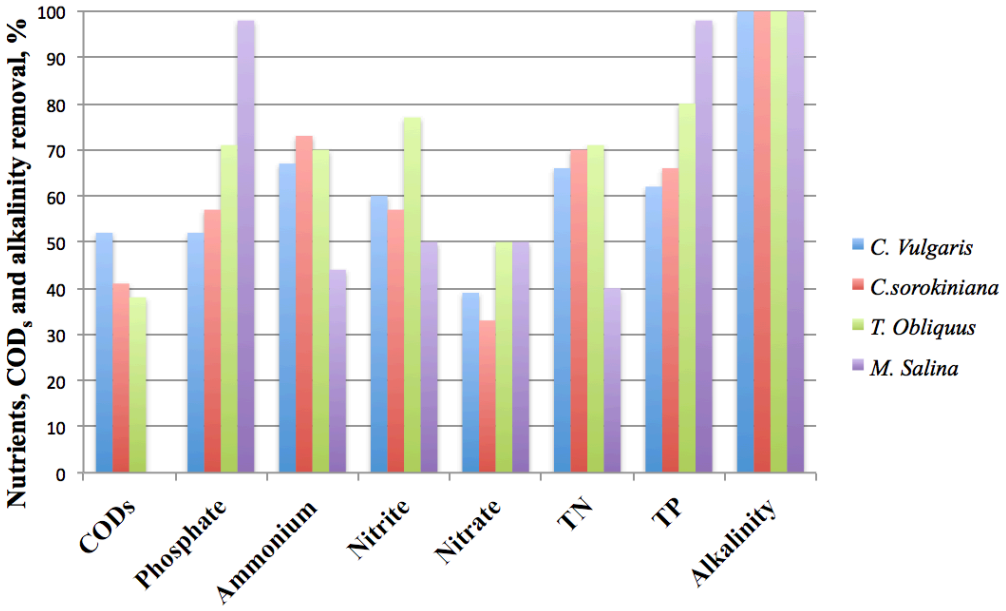


Figure 4.13: *Algal nutrient, COD, and alkalinity removal from wastewater diluted with unfiltered natural water after 11 days.*

Figure 4.12 and 4.13 indicates generally higher algal nutrient removal after 11 days compared to 9 days of algal treatment. The results imply that *C. sorokiniana* and *T. obliquus* is the most efficient algal strains for removing nutrients in wastewater diluted with lake water. *T. obliquus* is able to remove up to 80% TP, 71% TN, and 70% NH_4^+, while *C. sorokiniana* remove up to 72% TP, 70% TN, and 73% NH_4^+. *C. vulgaris* demonstrates COD_s removal efficiency of 52%, followed by *C. sorokiniana* and *T. obliquus*, with maximal removal of 43% and 38% respectively. *M. salina* shows high removal percentage of phosphate and TP of 98% after 11 days.

Algal nutrient, COD_s, and alkalinity removal efficiency of pure wastewater after 9 and 11 days are shown in Figure 4.14 and 4.15.

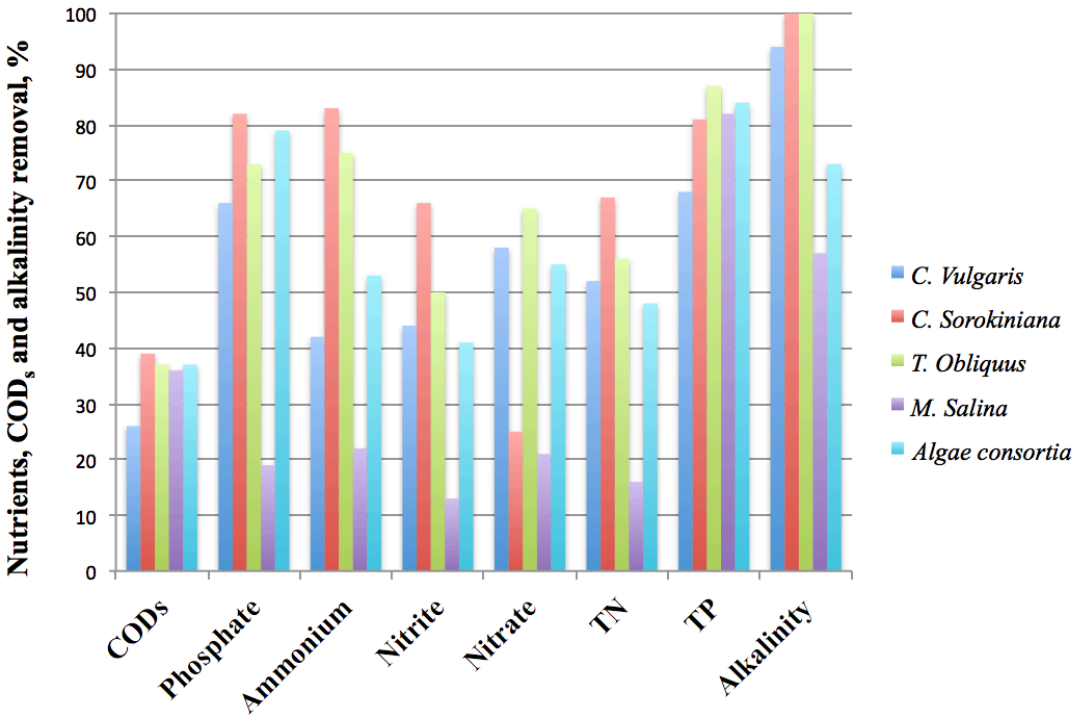


Figure 4.14: *Algal nutrient, COD, and alkalinity removal from pure wastewater after 9 days.*

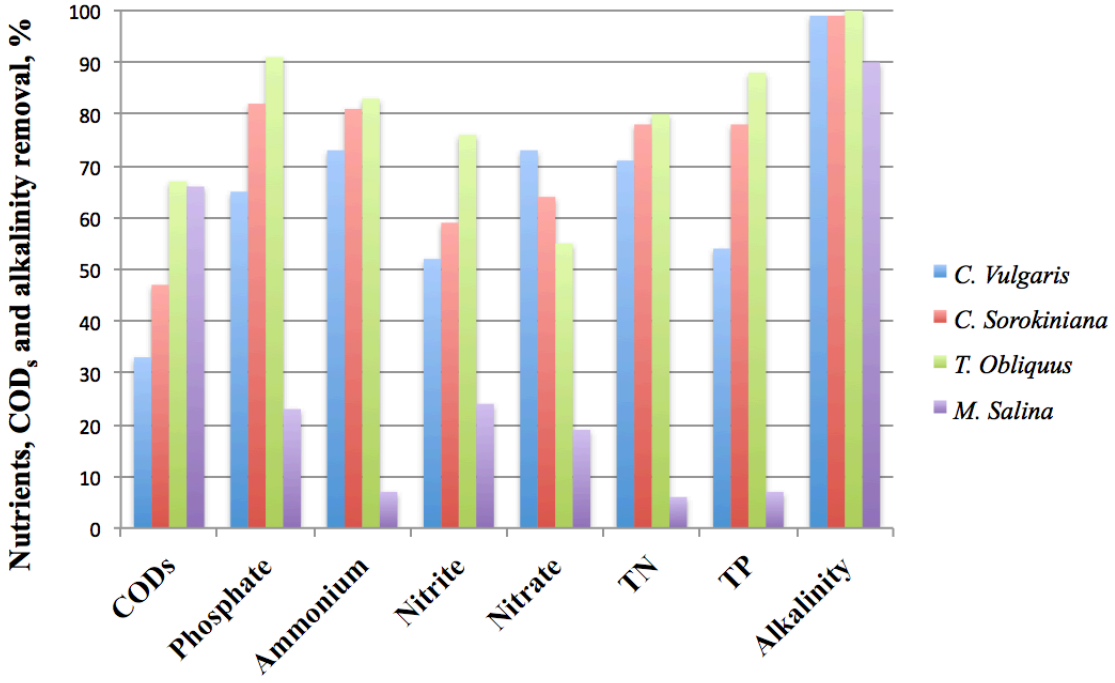


Figure 4.15: *Algal nutrient, COD, and alkalinity removal from pure wastewater after 11 days.*

As seen from Figure 4.14 and 4.15 *T. obliquus* and *M. salina* shows highest COD_s removal efficiency of 67% and 66%, respectively. The result indicate high nutrient removal efficiency by algae *T. obliquus*, with a TP removal of 88% and TN of 80%, followed by *C. sorokiniana,* which present TP and TN removal 81% and 78%, respectively. *T. obliquus and C. sorokiniana* removes up to 83% ammonium in wastewater. Marine algae *M. salina* presents generally lower nutrient removal, however, this species is able remove 82% of TP. Algae consortia from Hålandsvatnet show high TP removal of 84% after 9 days. The results imply that increasing algal treatment time of pure wastewater from 9 to 11 days, leads to generally higher treatment efficiency.

TSS production of algal strains cultivated in pure and diluted wastewater after 11 days was measured as an indication of dry algal biomass production. The result is presented in Table 4.4.

Table 4.4: *TSS production after 11 days of algae cultivated in pure and diluted wastewater.*

Wastewater condition	gTSS/L by *C. vulgaris*	gTSS/L by *C. sorokiniana*	gTSS/L by *T. obliquus*	gTSS/L by *M. salina*
Pure wastewater	0.75	1.05	1.35	0.25
Diluted wastewater	0.50	1.00	1.25	0.40

The result in Table 4.4 indicates effective biomass production by *T. obliquus*, producing up to 1.35 gTSS/L in pure wastewater. *M. salina* shows lowest TSS production of 0.25-0.40 gTSS/L.

5. Discussion

5.1 Algal Quantification Methods

Before applying algal-based wastewater treatment for a specific wastewater stream, it is essential to study algal growth characteristics and establish growth kinetics for different algal strains. To predict and track algal growth in different environments most accurately, it is necessary to compare several quantification methods. It is difficult to determine the most accurate algal cell quantification method, as it requires the true concentration to be known. To obtain reliable algal quantification data one should conduct several methods simultaneously.

The change in optical density for algae grown in specific growth media was studied and compared to algal enumeration based on flow cytometry. Freshwater species *C. vulgaris, C. sorokiniana, T. obliquus,* and *H. pluvialis* were cultivated in MWC+Se media, while marine species *M. salina* were cultivated in L1 media. The results in Figure 4.1-4.5 present a good linear relationship for OD measurements at 540nm, 680nm, and 750nm compared to flow cytometry for all algal species, except for *H. pluvialis*. *H. pluvialis* cultures were very difficult to maintain and found challenging to achieve high cell density. Göksan et al. [60] reported that problems related to *Haemotococcus pluvialis* cultivation include low growth rates, low cell concentration and lack of selective growth medium. Throughout the experiments, *H. pluvialis* were shown to be fragile and easily ruptured, making the quantification methods unreliable. Ruptured cells will result in low cell counts using flow cytometry, but still result in light scattering and/or absorbance using OD measurements. This will give a false indication of algal growth in the culture. Errors linked to OD measurements could be explained by changes in chlorophyll content over time. Griffiths et al. [49] reported that algal species *Chlorella, Scenedesmus,* and *Nannochloropsis* had a variable chlorophyll content depending on age and culture conditions, leading to significant error in OD measurements as algal quantification method. However, the results in this study suggest that OD measurements and Flow cytometry could be used to estimate growth of axenic cultures of *C. vulgaris, C. sorokiniana, T. obliquus,* and *M. salina* in specific growth media MWC+Se and L1.

For algal cells cultivated in pure and diluted secondary wastewater effluent, several quantification methods were studied, including flow cytometry, counting chambers, and

microplate readings (measuring fluorescence intensity and OD_{750}). As seen from Figure 4.6 and 4.7, estimated cell concentrations obtained from flow cytometry and counting chamber analysis correlate well, unlike the values obtained using fluorescence intensity and OD_{750} measurements. The result in Figure 4.6 and 4.7 suggests that cell concentration estimated by direct counting is not typically higher or lower compared to cell concentration estimated by the flow cytometry. Errors in Neubauer chamber counting occur in the range of 20-30%, possibly due to errors linked to pipetting and sample loading into the counting chamber [51]. Another possible error with counting cells manually is the variability in counted cells due to human errors such as visualization errors due to cell aggregation and debris [61]. This was experienced with the small *M. salina* with diameter of 2.5 μm [43]. *M. salina* cultures were difficult to distinguish from debris in the wastewater and/or natural water, possibly resulting in a higher or lower count. Therefore, another counting chamber should be considered when enumerating *M. salina.* Andersen [47] describes Petroff-Hausser counting device as appropriate counting chamber when enumerating cells with sizes less than 1-5 μm.

When applying direct cell counting, the method will simultaneously provide information about contamination and viability of the cells while counting. The method is also simple and low-cost. However, direct counting is time-consuming. In addition, when analyzing a sample only a tiny amount of volume is pipetted into the counting chamber, resulting in a tiny amount of cells to be analyzed. If the sample is not completely homogenous, the count will not represent the actual cell concentration. The algal cultures usually grew dense after some days and had to be diluted prior to counting. This can be a source of error, as an imprecise dilution will result in unreliable cell count [61].

Flow cytometry has been used for estimating algal biomass for a long time. It provides a rapid and accurate algal quantification analysis, and can also give information about DNA, protein, pigment, and lipid content [62]. Higher counts by flow cytometry compared to counting chamber, as seen for *T. obliquus* and *M. salina* in Figure 4.7 can possibly be due to other algae or organic matter present in the wastewater diluted with a natural water source. Identifying correct cell type and contamination in the sample can be more difficult using flow cytometry compared to using counting chamber. However, using the fluorescence trigger FL4 (red fluorescence) and forward scatter parameter (FSC) (size of algal cells) make this method quite accurate, as red fluorescence is linked to total chlorophyll content [17].

To obtain reliable data, it is important that the algae stay suspended as single cells. *H. pluvialis* cells were fragile and were shown to easily deform, resulting in inaccurate data. A drawback with the flow cytometer is that it is very expensive to purchase and use, compared to counting chambers. Lower counts obtained with the flow cytometer can be explained by cell aggregates leading to the flow cytometer counting several cells as one. *T. obliquus* was observed to commonly grow in cells of 2 or 4. This could explain the lower count by Flow cytometry, as observed in Figure 4.6. The error could possibly be avoided by using a counting chamber.

Optical density is one of the simplest and fastest methods of indicating algal growth [62]. However, OD measurements have been found to be unreliable for several algal species due to changes in pigment content during growth. When using this method, it is essential to establish a relationship between cell numbers and absorbance. This correlation will become inaccurate as the culture grows and cells change in size [25]. OD_{750} gives too high values when wastewater is diluted with lake water as seen in for *C. vulgaris, C. sorokiniana,* and *M. salina* in Figure 4.7. These high values can possibly be explained by the unfiltered natural water source containing a lot of particles and organic matter, which lead to an increase in light scattering.

Microplate based method can be used to observe microalgal growth rates in low-density microplate cultures. However, dense cultures lead to light and gas-transfer limitations which can effect microalgal growth [16]. These limitations can be seen in Figure 4.6 and Figure 4.7, as the algal cultures cultivated in microplates seem to reach stationary phase much faster, compared to batch cultures counted by flow cytometry and counting chambers. In addition, when using microplate readings, it is not possible to distinguish algal species from each other. When wastewater is diluted with unfiltered natural water, the result will be unreliable because of algae present in the sample. Using flow cytometer and counting chamber makes this process easier, as long as the algae are not from the same strain.

As seen from Figure A.2 in Appendix 2, the lake water used for diluting wastewater contained different types of algae. This could have contributed to counting errors in analysis methods as some of the algae were of the same size and morphology as the strains used in the experiment.

Although all the methods have some limitations, the results suggest that both flow cytometry and direct counting can be used to monitor algal growth in wastewater quite accurately as the cell counts correlate well with each other. Similar conclusions were drawn by Rutten et al. [63], who found a good correlation between enumerating algae using flow cytometry and microscope counts.

5.2 Algal Growth Characteristics

Studying algal growth characteristics and growth kinetics in specific types of media is essential to optimize algal cultivation for wastewater treatment. Cultivation of algae includes a lot of trial and error in order to find the most optimal cultivation conditions. A problem linked to presenting algal growth characteristics, is that algal growth depends on many factors such as the age of the algal culture (number of algal subcultures), at which growth phase the algae is at when cultivated and at which density the algae were cultivated in specific media. These factors will probably vary between compared strains used in the experiments, leading to bias in the results and conclusions. To decrease these biases one could study at the optimal density to cultivate algae, and use algal cultures of the same age in experiments.

Growth curves from algae cultivated in growth media MWC+Se and L1 (Figure 4.8) indicated that all species, except for *H. pluvialis*, reached high cell density during the experimental period. *H. pluvialis* was difficult to grow in culture and it never reached high cell densities. To optimize the growth of *H. pluvialis*, other growth media and other environmental conditions than the ones included in this study should perhaps be considered. Katsuda et al. [37] achieved a high growth rate of 0.72 d^{-1} by cultivating *H. pluvialis* in Kobayashi's basal medium using flashing blue LED lights. As mentioned in Chapter 2.1.9, larger cells will grow slower compared to small cells [25]. Therefore, this experiment should have been conducted for a longer time period to allow the *H. pluvialis* cultures to reach stationary phase so it could be determined how high cell densities this species can achieve.

The results in Table 4.1 shows that *C. vulgaris, C. sorokiniana, T. obliquus, H. pluvialis,* and *M. salina* cultivated in growth media reached growth rates of 0.77 d^{-1}, 0.73 d^{-1}, 0.67 d^{-1}, 0.37 d^{-1}, and 0.56 d^{-1}, respectively. These values are much lower compared growth rates achieved in other studies. As summarized in Table 2.1, other studies resulted in growth rates of 1.61 d^{-1}

for *C. vulgaris,* 2.4-6.48 d^{-1} for *C. sorokiniana*, 1.13 d^{-1} for *T. obliquus*, 0.72 d^{-1} for *H. pluvialis* and 1.30 d^{-1} for *M. salina*. However, these studies utilized other types of growth media and environmental conditions than used in this study. Commonly, the other studies conducted algal experiments at higher temperatures and increased light intensity. This is probably why they achieved such rapid cell growth, as illumination often is the limiting factor for algal growth. Martinez et al. [30] found that increasing the temperature to 30°C led to a maximum growth rate of *T. obliquus* of 1.13 d^{-1}. This is significantly higher than growth rate of 0.67 d^{-1} achieved in this study at 18°C. *C. sorokiniana* have been found to achieve very fast growth rates in earlier studies. Kumar et al. [34] achieved growth rate of 2.4 d^{-1} when increasing temperature to 30°C. Janssen et al. [33] cultivated *C. sorokiniana* under continuous illumination of 630 μmol m^{-2} s^{-1}, resulting in a maximum growth rate of 6.48 d^{-1}. These are very fast growth rates for *C. sorokiniana* when compared to the growth rate of 0.63 d^{-1} achieved in this study. The conditions used in this study included light intensity of 15 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 18 °C, and 80 rpm. These are ideal conditions when the goal is maintaining algal cultures in incubator over time, prior experiments. However, when the goal is rapid algal growth, it seems to be essential to increase light intensity and temperature.

When algae were grown in unfiltered wastewater, as illustrated in Figure 4.9, the only algae able to grow were *C. sorokiniana*. The poor growth of all other strains could be explained by unfavorable environmental conditions, such as too low illumination at only 15 μmol m^{-2} s^{-1} and 18°C. One other possible explanation could be formation of aggregates in wastewater formed after thawing. Wastewater was shown to increase in TSS after freezing for storage. This could lead to a more turbid wastewater, making it difficult for the light to penetrate the culture. Due to this, the wastewater was filtered using 1.5 μm filter before algal cultivation in following experiments. Also, the algae were inoculated at quite high cell-density in unfiltered wastewater, which may have a poor effect on cell growth. To avoid this effect, one should consider inoculating algal cells at lower densities at the beginning of experiments. Several experiments should be conducted to find the most optimal density to culture the algal cells.

In the growth experiments using filtered wastewater (Figure 4.9), enhanced growth was achieved for all species, except for *M. salina*. Low salinity or possibly other toxic substances present in the wastewater may have inhibited growth of *M. salina* in pure wastewater. Figure

A.3.1 in Appendix 3 presents results obtained from additional experiments, which confirm poor growth of *M. salina* in pure wastewater. The growth rates of algae cultivated in filtered wastewater in Table 4.2, ranging from 1.23 to 1.99 d^{-1}, were higher compared to most of the other experiments conducted in this study. One reason for this was probably due to illumination, temperature and rpm were increased to 100 μmol m^{-2} s^{-1}, 20°C, and 90 rpm, respectively. Another explanation could be that filtering out the formed aggregates after freezing enhanced algal growth. Lake water algae consortia from Hålandsvatnet (as seen in Figure A.2, Appendix 2) grew efficiently in the filtered wastewater. However, the consortia will most likely include algal species able to produce toxins. Therefore, this type of algal consortia might not be suitable for use in wastewater treatment. An idea might be to identify which algal strains the lake water consortia include to determine if there is any toxin producing species present.

When cultivating algae in diluted wastewater, the result in Figure 4.10 imply that using unfiltered instead of filtered natural water is the best choice to achieve efficient algal growth. This corresponds with calculated growth rates in Table 4.2, as growth rates from algae cultivated in wastewater diluted with filtered water were ranging from 0.63 d^{-1} to 1.19 d^{-1}, while using unfiltered water resulted in higher growth rates ranging from 0.75 d^{-1} to 2.06 d^{-1}. All species, except *C. vulgaris,* achieved high cell density during the experimental period. Using unfiltered natural water will provide other microorganisms, such as bacteria, which possibly accelerate algal growth due to algal and bacterial symbiotic relationship, as previously mentioned in Chapter 2.1.4. One possible error in the growth curve of algae cultivated in unfiltered lake water is the presence of other algal species in lake water, as illustrated in Figure A.2, Appendix 2. The flow cytometer could count these as the targeted algal strains, as some of the endogenous lake water species had the similar cell sizes and fluorescence as the pure cultures used in this study, resulting in higher cell numbers of the cultivated algal species in samples than actually present.

Figure 4.11 illustrates a little to no effect of adapting the algae to wastewater by mixing it with 50% growth media, before transferring adapted algae to pure wastewater. When comparing growth of unadapted algae in pure wastewater in Figure 4.9 and Figure A.3.1 in Appendix 3, the result indicated that there is no effect of adapting algae *C. sorokiniana*, *T. obliquus,* and *M. salina*. However, the algae were only adapted over one generation. Adapting algae over several generations could have a better effect on algal growth due to more time for

selection. The results imply that *C. vulgaris* might have a positive effect of being adapted, due to achieving very long and steady logarithmic phase and reaching high cell density.

5.3 Algal Treatment Efficiency

To identify algal strains able to purify the secondary wastewater effluent from IVAR WWTP at Grødaland, treatment efficiency of the selected algae were studied. This was done by measuring amount of nutrients and COD_s in wastewater before and after algal treatment. Algal production of TSS was measured as an indication of algal biomass productivity.

The algae were cultivated in filtered wastewater and filtered wastewater diluted with unfiltered natural water, and incubated at a light intensity of 100 μmol m^{-2} s^{-1} with a light/dark regime of 16/8 hours, 20°C, and 90 rpm. After a lot of trial and error, this strategy of cultivating algae for algal treatment were found to be most efficient and suitable for use in algal treatment ponds. Based on this, these experiments were selected be presented in this study. Algal treatment efficiency and algal biomass production from other experiments are presented in Table A.4.1 – A.4.5 in Appendix 4.

The optimal inorganic N/P ratio has been proposed to be in the range from 6.8 to 10 [64]. Measured inorganic N/P ratio before algal cultivation were a bit lower from optimal conditions as results in Table 4.3 showed N/P ratio of 4.3 in pure wastewater, 5.5 in wastewater diluted with lake water, and 4.7 in wastewater diluted with seawater. Algal growth does not seem to be severely limited by phosphorous nor nitrogen. However, addition of nitrogen could possibly enhance algal growth and treatment efficiency, as nitrogen limitation leads to a reduction of growth and photosynthesis [24].

As previously mentioned in Chapter 2.1.6, nitrogen can be assimilated by microalgae in the form of nitrate, nitrite, ammonium, and some organic-N [2]. The wastewater composition, shown in Table 4.3, indicates that most of the nitrogen in the wastewater is present as ammonium and is readily available for algal assimilation. The results in Figure 4.12-4.15 show high ammonium removal by *C. sorokiniana* and *T. obliquus* (>70% in diluted wastewater and 83% in pure wastewater). *C. vulgaris* removed up to 67% and 73% ammonium in diluted and pure wastewater, respectively. Higher ammonium content in pure

wastewater, compared to diluted wastewater might stimulate algae to remove more nitrogen. Similar results were obtained by Wang et al. [64], where Chlorella sp. removed up to 83% ammonium from pure municipal wastewater.

M. salina removed low amounts of ammonium, especially in pure wastewater, as seen in Figure 4.15. This can be confirmed by poor growth by *M. salina* in pure wastewater, as illustrated in Figure 4.9. The results commonly show lower nitrate removal compared to ammonium removal for all species. This may imply limited nitrification and that the algae used in this experiment prefer ammonium as nitrogen source. Algae consortia remove more nitrate than ammonium, indicating nitrification processes. It is possible that some ammonium could have been removed by ammonia volatilization. The low alkalinity at the end of experiments imply that pH have increased which may lead to NH_3 stripping from wastewater [2].

The characteristics of the secondary wastewater effluent, as shown in Table 4.3, imply that most of the phosphorus in wastewater is in the form of phosphate. As previously described in Chapter 2.1.7, microalgae assimilate phosphate and organic phosphorous for important cell functions. Phosphorous can also be removed by precipitation at an elevated pH [2]. As seen from the results of microalgal nutrient removal in diluted wastewater (Figure 4.12 and 4.13), *M. salina* removed up to 98% TP, followed by *T. obliquus* with a maximal removal of 80%. Phosphorous removal in pure wastewater (Figure 4.14 and 4.15) was most efficiently preformed by *T. obliquus* with a removal of 88%. *C. vulgaris* and *C. sorokiniana* removed >61% and >71% in pure and diluted wastewater, where *C. sorokiniana* demonstrated most efficient nutrient removal. Wang et al. [64], presented a phosphorous removal of 90% by *Chlorella* sp. in pure municipal wastewater. The reason for higher removal percentage is possibly due to a higher N:P ratio of 6, compared to a N:P ratio of 4.3 in the pure wastewater used in this experiment.

As previously mentioned in Chapter 2.1.8, microalgae is able to remove organic carbon through mixotrophic or heterotrophic metabolism [5]. As seen from Figure 4.12-4.15, maximal COD removal was preformed by *T. obliquus* of 67 % in pure wastewater and by *C. vulgaris* of 52% in diluted wastewater. Reasons why COD were not removed at a higher percentage might be that the organic carbon remaining in the secondary effluent is inert or

slowly degradable matter. Therefore, it is probable that algae are using CO_2 from the air as sole carbon source, not preforming mixotrophic or heterotrophic metabolism [24].

CO_2 can be the limiting nutrient in algal cultivation when using atmospheric CO_2 as inorganic carbon source. This can lead to growth inhibition of some algal species [21]. Arbib et al. [65] reported significantly improved algal biomass productivity and nutrient removal by adding flue gas to a *T. obliquus* culture. Shen et al. [14] also found that carbon supplement enhanced algal nutrient removal as *T. obliquus* cultivated in municipal wastewater with supplement of additional CO_2 achieved high TN and TP removal efficiencies of 98% and 96%, respectively. As seen in Figure 4.12-4.15, all algal species removed up to all of the alkalinity in the wastewater, implying shortage of inorganic carbon. Therefore, adding external CO_2 could possibly enhance algal growth and nutrient removal.

It might be effective to expand algal treatment time to 11 days or longer as the results indicated increasing algal treatment efficiency when increasing treatment time.

Amount of TSS was measured before and after algal treatment of wastewater. This can be used as an indication of total algal biomass production [66]. The result in Table 4.4 indicates effective biomass production by *T. obliquus*, producing up to 1.35 gTSS/L in pure wastewater, followed by a slightly lower biomass production by *C. sorokiniana* (up to 1.05 mg/L) and *C. vulgaris* (0.75 mg/L*)*. *M. salina* shows lowest TSS production of 0.25-0.40 gTSS/L. Comparing with these values with other results gained in other studies indicates that this is very effective biomass production. Ramaraj et al. [66] reported a total biomass production ranging from 0.07 g/L to 0.26 g/L for algae consortia cultivated in a natural water media. Biomass productivity of algae cultivated in effluent from a submerged membrane anaerobic bioreactor by Ruiz-Martinez et al. [67] resulted in a maximum biomass level of 0.6 g/L.

6. Conclusion

Overall conclusions of experiments presented in Chapter 4-6 are summarized to answer the thesis objectives as described in Chapter 1.2.

The result indicated that OD_{540}, OD_{680}, and OD_{750} measurements and flow cytometry can be utilized as a reliable quantification method for axenic algal cultures of *C. vulgaris, C. sorokiniana, T. obliquus,* and *M. salina* cultivated in growth media MWC+Se (for freshwater species) and L1 (for marine species). OD measurements presented good linearity ($R^2 > 0.94$) with data based on flow cytometry for all species, except *H. pluvialis* ($R^2<0.37$).

Estimated cell concentrations from algae cultivated in secondary wastewater effluent obtained from flow cytometry and counting chamber correlate well. Data obtained from fluorescence intensity and OD_{750} measurements show highly variable result, suggesting that these methods should not be conducted. Despite limitations, the results suggest that both flow cytometry and direct counting using the Improved Neubauer counting chamber can be used to monitor algal growth in wastewater quite accurately.

Algae strain *C. sorokiniana* presented ability to grow in wastewater in all of the tested culture conditions. This suggests a high adaptability and viability of *C. sorokiniana* in this specific type of wastewater.

Increasing light intensity, temperature and agitation from 15 μmol m^{-2} $s^{-}1$, 18°C, and 80 rpm to 100 μmol m^{-2} s^{-1}, 20°C, and 90 rpm, respectively implied more efficient algal growth in wastewater. *T. obliquus, C. vulgaris* and especially *C. sorokiniana* cultivated in pure wastewater presented maximum growth rates of 1.28 d^{-1}, 1.33 d^{-1}, and 1.99 d^{-1}, respectively. The same species achieve increasing maximal growth rates in wastewater diluted with unfiltered lake water. Experiment indicated that marine algae *M. salina* was not suited for cultivating in pure wastewater, but achieved maximal growth rate of 1.55 d^{-1} when cultivated in wastewater diluted with filtered seawater.

The results imply satisfactory nutrient removal achieved when applying algae *T. obliquus* and *C. sorokiniana* for wastewater treatment. *T. obliquus* removed up to 80% TP, 71% TN, and 70% NH_4^+ removal, while *C. sorokiniana* removed up to 72% TP, 70% TN, and 73% NH_4^+. These algae also present effective biomass production of up to 1.35 g/L and 1.05 g/L, respectively. This high biomass productivity suggests that microalgal cultivation in wastewater could offer a potential for biofuel production. Even higher nutrient removal and biomass productivity might be possible when increasing treatment time and/or applying external source of CO_2.

Treating wastewater with microalgae represent a promising alternative to already existing wastewater treatment technologies. Based on growth characteristics, nutrient removal, and biomass productivity, *C. sorokiniana* and *T. obliquus* seems to be the best algal species to treat the mixed wastewater effluent from IVARs WWTP at Grødaland.

7. Further Research

This study indicates great potential for microalgae for tertiary wastewater treatment step. A recommendation for further research is to apply mixed microalgae culture systems and mixed algal-bacteria consortia for improved treatment efficiency and higher biomass productivity. Other studies have shown great results for using these strategies to effectively purify wastewater streams [68-70].

The results gained from various experiments showed that algae often used up all of the alkalinity in the culture, indicating shortage of inorganic carbon. In future experiments, the effect of adding external source of CO_2 to control alkalinity and pH and to provide carbon for algal growth, should be studied. Posadas et al. [71] presented great effect of applying external source of CO_2 for pH and alkalinity control, as well as increasing removal of COD, TP, and TOC.

Increasing light intensity, temperature and agitation from 15 μmol m^{-2} s^{-1}, 18°C, and 80 rpm to 100 μmol m^{-2} s^{-1}, 20°C, and 90 rpm indicated more efficient algal growth in wastewater. To identify the most optimal conditions for growth of selected algae in this specific wastewater, this should be investigated further.

As experiments conducted for a longer time period indicated higher nutrient removal, one should conduct additional research to find the optimal algal treatment retention time.

The studied algal species, especially *C. sorokiniana* and *T. obliquus,* presented great potential for wastewater treatment. The next step should be up scaling by using these identified effective algal strains for wastewater treatment, for example using photobioreactors. One could also secure more accurate data by doing additional experiments for statistical analysis.

Another recommendation for further research to study the mixotrophic growth strategy to optimize algal productivity compared to autotrophic growth, as adding organic carbon to photobioreactors have shown to increase algal productivity and growth [72].

8. References

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Appendix

Appendix 1: Comparison of Different Algal Quantification Methods for Algae Grown in Wastewater

Appendix 2: Image of Microalgal Consortia from Lake Hålandsvatnet, Stavanger

Appendix 3: Additional Growth Curves for Algae Grown in Wastewater

Appendix 4: Algal Nutrient and COD Removal in Additional Experiments

Appendix 1: Comparison of Different Algal Quantification Methods

Measurements of algal concentrations cultivated in both diluted and pure wastewater were conducted once a day using flow cytometer, counting chambers, and microplate readings (measuring fluorescence intensity and OD_{750}). Comparison of the different quantification analysis is illustrated Figure A.1.1 and Figure A.1.2.

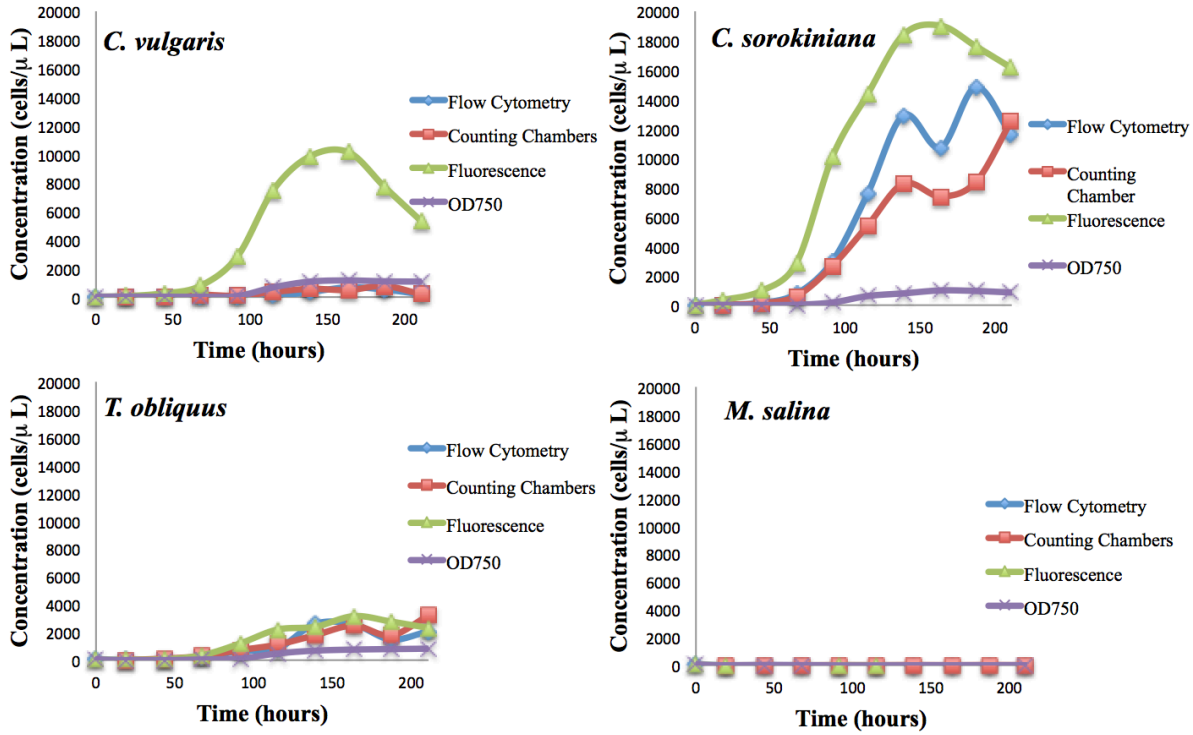

Figure A.1.1: *Comparison of quantitative algal analysis methods for C. sorokiniana, C. vulgaris, T. obliquus, and M. salina grown in pure wastewater.*

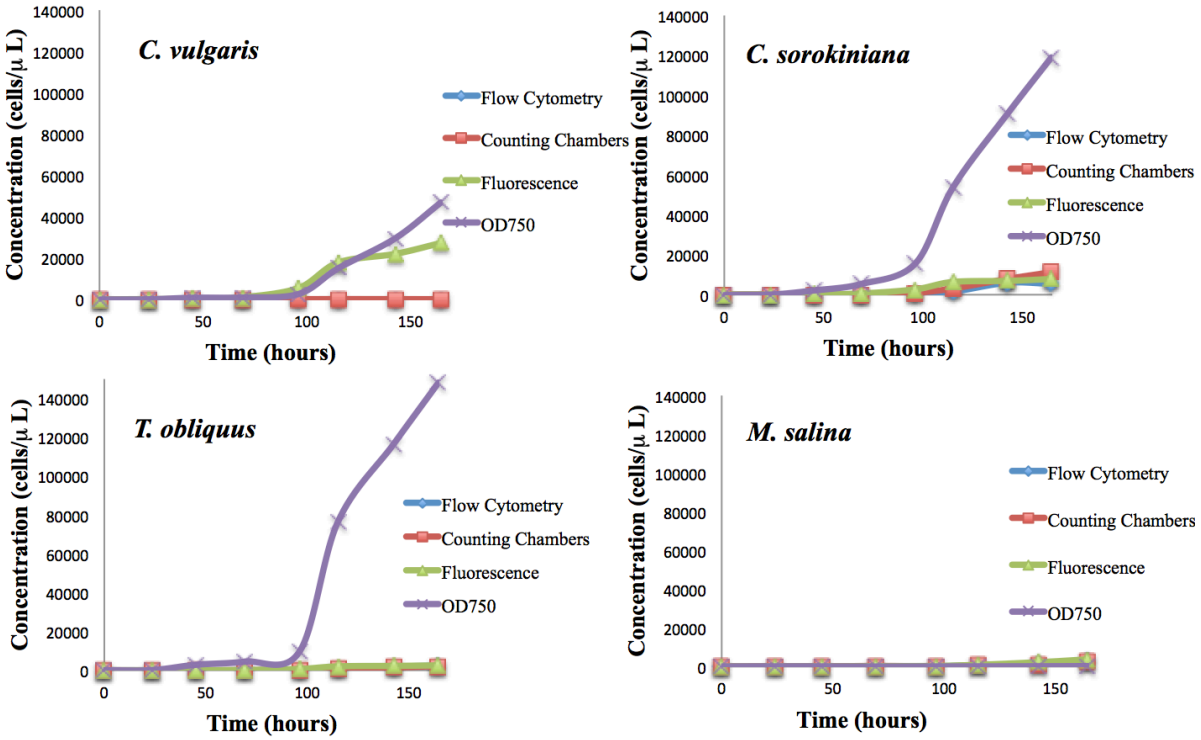

Figure A.1.2: *Comparison of quantitative algal analysis methods for C. sorokiniana, C. vulgaris, T. obliquus, and M. salina grown in wastewater mixed diluted 50% natural water (lake water for freshwater algae and seawater for marine algae).*

Appendix 2: Image of Microalgal Consortia from Lake Hålandsvatnet, Stavanger

Image taken microscopically (40X objective) for the illustration of microalgal consortia present in Lake Hålandsvatnet in Stavanger, is shown in Figure A.2.

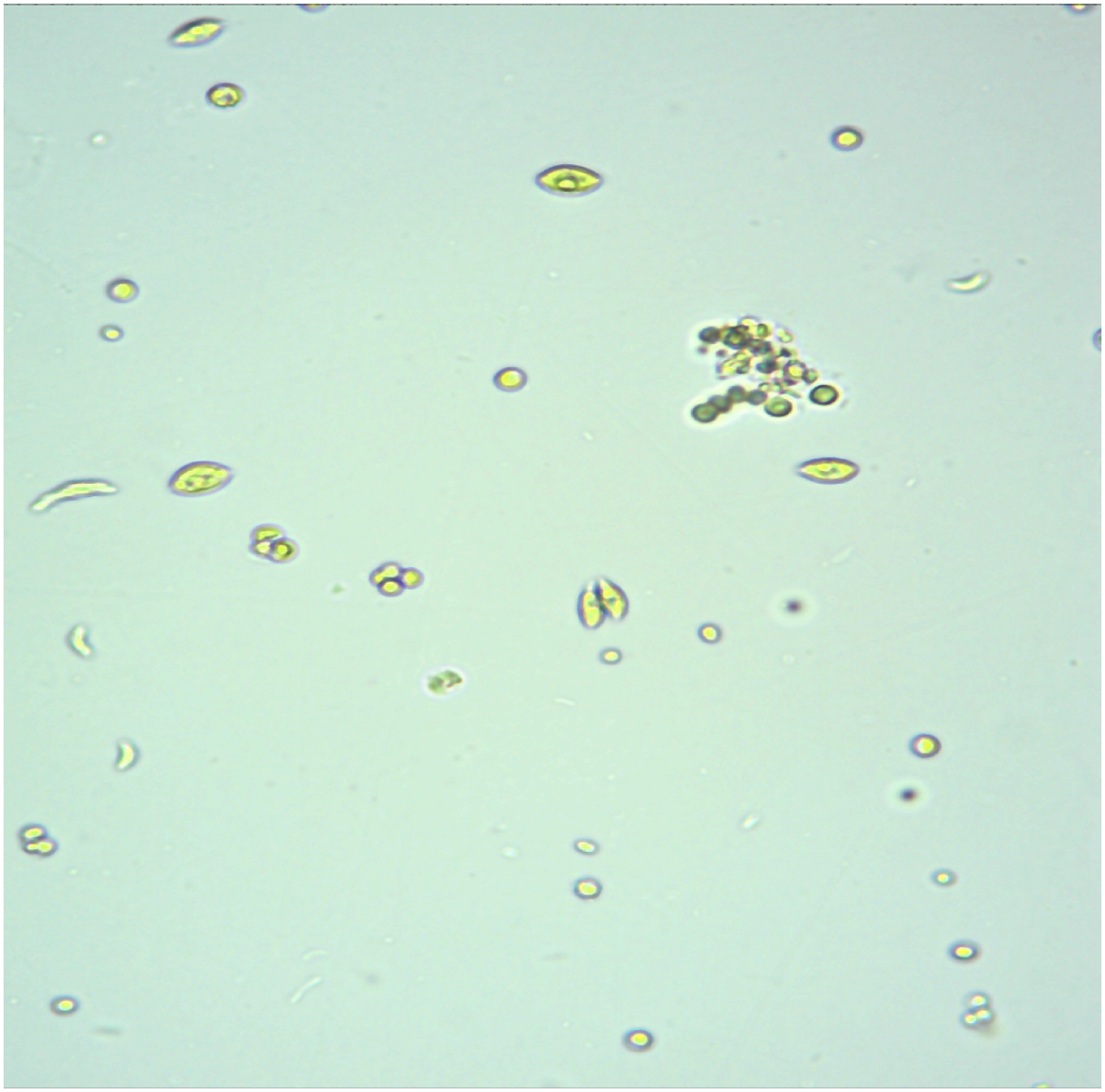

Figure A.2: *Microalgal consortia from Lake Hålandsvatnet, Stavanger.*

Appendix 3: Additional Growth Curves

Additional growth curves were established for *C. vulgaris, C. sorokiniana, T. obliquus,* and *M. salina* cultivated in pure and diluted filtered wastewater is shown in Figure A.3.1 and A.3.2. The growth curves are based on data obtained from daily flow cytometry analysis.

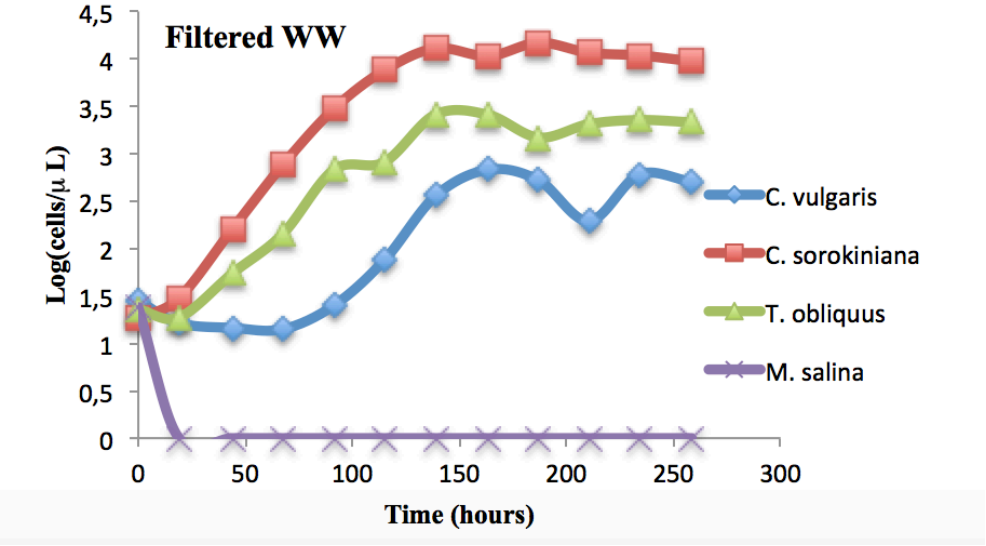

***Figure A.3.1**: Algae cultivated in pure wastewater.*

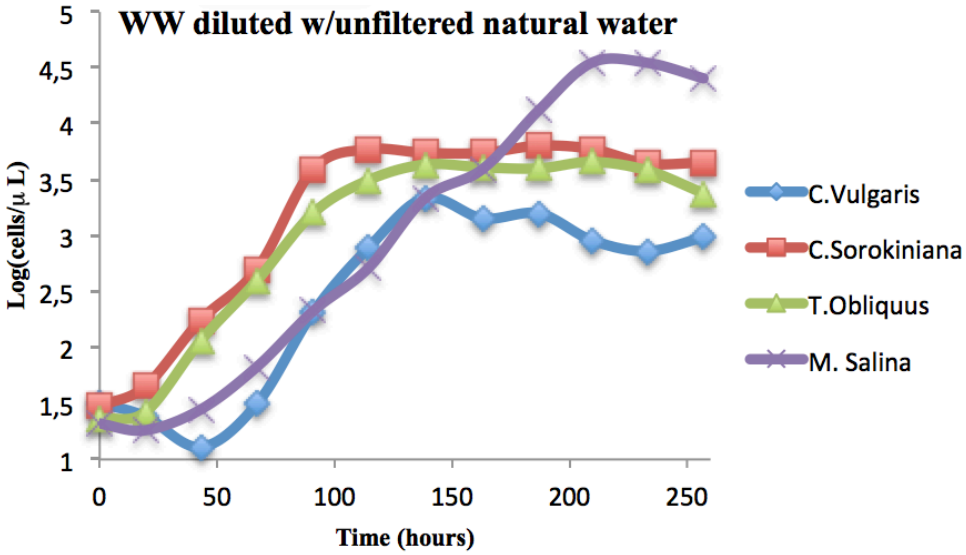

***Figure A.3.2:** Algae cultivated in wastewater diluted with unfiltered natural water.*

Appendix 4: Algal Treatment Efficiency in Additional Experiments

Algal nutrient and COD removal, and TSS production in additional experiments are shown in Table A.4.1 to Table A.4.5.

Table A.4.1: *Percentage nutrient removal of algae cultivated in unfiltered wastewater after 9 days of treatment.*

Parameter	*C. vulgaris*	*C. sorokiniana*	*T. obliquus*	*H. pluvialis*	*M. salina*
COD_s	52	69	38	33	34
PO_4^{3-}	42	59	57	74	69
NH_4^+	64	63	59	64	59
NO_2^-	0	0	0	0	0
NO_3^-	64	64	60	62	60
TN	41	55	55	73	64
TP	94	78	72	90	99
Alkalinity	53	100	74	100	90

Table A.4.2: *Percentage nutrient removal of algae cultivated in wastewater diluted with 50% growth media after 3 and 12 days of treatment.*

Parameter	*C. vulgaris*		*C. sorokiniana*		*T. obliquus*		*H. pluvialis*		*M. salina*	
	Day 3	Day 12	Day 3	Day 12	Day 3	Day 12	Day 3	Day 12	Day 3	Day 12
COD_s	16	3	14	29	42	33	29	40	6	10
PO_4^{3-}	44	91	48	96	34	97	51	32	85	99
NH_4^+	47	100	52	100	44	100	41	66	32	92
NO_2^-	0	0	0	0	0	0	0	0	0	0
NO_3^-	0	0	0	0	0	0	0	0	0	0
TN	-	81	-	50	-	84	-	50	-	87
TP	-	75	-	5	-	88	-	5	-	97
Alkalinity	100	100	51	91	99	95	38	91	96	97

Table A.4.3: *Percentage nutrient removal of algae cultivated in wastewater diluted with 50% filtered natural water after 14 days of treatment.*

Parameter	*C. vulgaris*	*C. sorokiniana*	*T. obliquus*	*M. salina*
COD_s, mg/L	0	0	0	-
PO_4^{-3}, mg/L	65	66	94	69
NH_4^+, mg/L	42	93	98	34
NO_2^-, mg/L	57	59	83	60
NO_3^-, mg/L	8	13	21	-25
TN, mg/L	50	62	85	56
TP, mg/L	49	50	88	65
Alkalinity, mg$CaCO_3$/L	98	95	95	86

Table A.4.4*: Percentage nutrient removal of adapted algae cultivated in wastewater after 14 days of treatment.*

Parameter	*C. vulgaris*	*C. sorokiniana*	*T. obliquus*	*M. salina*
COD_s, mg/L	41	49	49	71
PO_4^{-3}, mg/L	76	57	90	3
NH_4^+, mg/L	97	92	99	1
NO_2^-, mg/L	53	69	69	42
NO_3^-, mg/L	57	79	70	9
TN, mg/L	52	65	66	8
TP, mg/L	75	60	87	12
Alkalinity, mg$CaCO_3$/L	94	98	99	61

Table A.4.5: *TSS production after algae cultivated in pure and diluted wastewater.*

Wastewater	Treatment time, days	gTSS/L by *C.vulgaris*	gTSS/L by *C.sorokiniana*	gTSS/L by *H.pluvialis*	gTSS/L by *T.obliquus*	gTSS/L by *M.salina*
Unfiltered	9	0.233	0.250	0.400	0.333	0.400
Diluted w/media	12	7.28	7.95	1.95	8.62	14.62
Diluted w/filtered natural water	14	0.10	0.20	-	0.15	0.20
Filtered (adapted algae)	14	0.40	0.50	-	0.65	0.10