UNIVERSITY OF VAASA

FACULTY OF TECHNOLOGY

ENERGY TECHNOLOGY

Katarina Martonen

CULTIVATION OF MICROALGAE IN WASTEWATER

Water treatment and biomass production

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Supervisor	Erkki Hiltunen
Instructor	Liandong Zhu

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Katarina Martonen

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SYMBOLS AND ABBREVIATIONS

COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
DW_0	Dry weight at time t_0
DW_1	Dry weight at time t_1
HRAP	High Rate Algal Pond
N_1	Dry biomass at time t_1
N_2	Dry biomass at time t_2
NH_4+	Ammonium
NH_4-N	Ammonium-nitrogen
OD	Optical density
P	Biomass productivity
PBR	Photobioreactor
r	Equal to µ
t	Time
T_2	Doubling time
TN	Total nitrogen
TP	Total phosphorous
µ	Specific growth rate
µm	Micrometer

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Author: Katarina Martonen
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Instructor: Liandong Zhu
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ABSTRACT:

The purpose of this thesis is to study whether cultivation of the microalgae *Scenedesmus dimorphus* is feasible in wastewater from the local municipal wastewater treatment plant Pått in Vaasa. Microalgae were cultivated in wastewater from three different points in the wastewater treatment process. The first point is after the pre-sedimentation pond, the second is after the sedimentation pond and the third is effluent that is about to be dispatched to the sea. The study was conducted in a laboratory, in two different temperatures and light intensities. Microalgal biomass accumulation was determined by optical density measurement and weighing, and growth parameters were calculated. Removal of total nitrogen, total phosphorous, ammonium and chemical oxygen demand from the wastewater was measured with photometric test kits.

The removal of nutrients was most efficient from the wastewater collected after the pre-sedimentation pond in both studied temperatures, however more efficient in 24°C than in 16°C. During the first 24 hours total phosphorous decreased by 60% to 77%. Removal of total nitrogen was most efficient in the wastewater collected after the pre-sedimentation pond, where the nitrogen appears in the form of ammonium. Under the conditions of the study all ammonium was removed during nine days of cultivation. No decrease in chemical oxygen demand was noticed, on the contrary, chemical oxygen demand increased by 123% to 175% in the wastewater collected after the sedimentation pond. The accumulation of biomass was quite similar in all three tested waters and most efficient in higher temperature and higher light intensity. The mean specific growth rate was (0.11-0.15) day^{-1} and the mean doubling time (4.7-6.5) days. The mean biomass increase was (0.56-1.18) g L^{-1} during the first nine days of cultivation, and the mean biomass productivity was (0.04-0.18) g L^{-1} day^{-1}.

KEYWORDS: Microalgae, wastewater treatment, nutrient removal, phycoremediation, *Scenedesmus*

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TIIVISTELMÄ:

Tämän tutkielman tarkoitus on selvittää onko mikrolevän *Scenedesmus dimorphus* kasvatus mahdollista Vaasan Påttin jätevesipuhdistamon jätevedessä. Mikrolevää kasvatettiin kolmessa jätevesipuhdistamon prosessin eri pisteestä kerätyssä vedessä. Ensimmäinen piste on esiselkeytyksen jälkeen, toinen jälkiselkeytyksen jälkeen ja kolmas on mereen päästettävää vettä. Tutkimus tehtiin laboratoriossa, kahdessa eri lämpötilassa ja kahdella valon eri intensiteetillä. Biomassan kertymistä tutkittiin punnitsemalla ja mittaamalla optista tiheyttä. Kokonaistypen, kokonaisfosforin, ammoniumin ja kemiallisen hapenkulutuksen vähenemistä jätevedessä tutkittiin fotometrisillä testikiteillä.

Ravinteiden poistaminen oli tehokkainta esiselkeytysaltaan jälkeen kerätyssä vedessä, kummassakin tutkitussa lämpötilassa. Tehokkaampaa se oli kuitenkin 24°C lämpötilassa kuin 16°C lämpötilassa. Ensimmäisen 24 tunnin aikana kokonaisfosfori väheni 60%-77%. Kokonaistypen poistaminen oli tehokkainta esiselkeytysaltaan jälkeen kerätyssä vedessä, missä typpi esiintyy ammoniumin muodossa. Tässä tutkimuksessa käytetyissä olosuhteissa kaikki ammonium poistui vedestä yhdeksän päivän viljelyn aikana. Kemiallisessa hapenkulutuksessa ei huomattu vähenemistä, päinvastoin, kemiallinen hapenkulutus lisääntyi 123%-175% selkeytysaltaan jälkeen kerätyssä vedessä. Biomassan kertyminen oli melko samanlaista kaikissa testipisteissä. Tehokkainta kertyminen oli korkeammassa lämpötilassa ja suuremmalla valon intensiteetillä. Spesifinen kasvunopeus oli (0,11-0,15) pvä$^{-1}$ ja keskimääräinen kaksinkertaistumisaika oli (4,7-6,5) päivää. Biomassan keskimääräinen kertyminen viljelyn ensimmäisten yhdeksän päivän aikana oli (0,56-1,18) g pvä$^{-1}$, ja keskimääräinen biomassan tuottavuus oli (0,04-0,18) g l^{-1} pvä$^{-1}$.

AVAINSANAT: Mikrolevä, jäteveden puhdistus, *Scenedesmus*

1. INTRODUCTION

The growing concern for the future of our planet is accelerating the quest for renewable sources of energy. The increasing amount of carbon dioxide in our atmosphere, released by combustion of fossil fuels, is contributing to global warming. The search for renewable and sustainable sources of energy, to compensate for fossil fuels, has drawn attention to the possibility of utilizing microalgae as a feedstock for energy production. Microalgae are a promising option for the production of biofuel because of their suitable lipid content. Algae biomass can also be used as a substrate for biogas production. High value products such as food, nutritional supplements, omega-3 fatty acids, proteins, pigments, pharmaceuticals, biodegradable plastics and animal feed are produced from microalgae. The residual biomass from production can be used as fertilizer or soil improvement (Gouveia 2011; Christenson & Sims 2011).

The most sustainable way of growing microalgae for biofuel would be to utilize wastewaters derived from municipal, agricultural and industrial services. Microalgae effectively utilize carbon dioxide through photosynthesis, and take up nutrients like nitrogen and phosphorous from the water. Microalgae cultivation in wastewater simultaneously produces valuable biomass at the same time as it reduces eutrophication of natural water bodies. Remediation of wastewaters with microalgae is an environmentally safe and economical way of wastewater treatment (Pittman, Dean & Osundeko 2011; Christenson & Sims 2011).

Production of first generation biofuels uses food crops as feedstock, while production of second generation biofuels uses nonedible remains of food production, or sole biofuel crops. Third generation biofuels are derived from microorganisms, such as microalgae. Biofuels from microalgae have less or no impact on food availability and agriculture, and can be locally produced. Optimistic calculations show that biofuel production from microalgae would use less land and water than the traditionally grown oilseed crops, while producing more biodiesel. The greatest challenge for microalgae production is to find cost effective and energy efficient ways of mass production and harvesting (Gouveia 2011; Gerbens-Leenes, Xu, De Vries & Hoekstra 2014).

This thesis will focus on microalgae cultivation and remediation of wastewater with microalgae. The study will evaluate the feasibility of microalgae cultivation in wastewater from the local municipal wastewater treatment plant Pått in Vaasa, Finland. The work is restricted to laboratory experiments.

The thesis is conducted within the project TransAlgae. TransAlgae is a cross-border project in the Botnia Atlantica region, with partners in Finland, Sweden and Norway. One focus of the TransAlgae project is utilizing waste streams for growing algae in a Nordic climate, and finding new solutions for renewable energy.

This work starts with an introduction of the basic biology and growth requirements of microalgae, and a presentation of different cultivation systems. Hereafter follows a short presentation of general wastewater treatment, and how microalgae is used in wastewater treatment. The wastewater treatment process at Pått is presented to explain the process and the composition of the water. Hereafter the research questions and the experiments are presented in detail. Finally, the results are presented and discussed, and conclusions are made and summarized.

2. CULTIVATION OF MICROALGAE

In order to achieve maximal biomass yield from algae cultivation, it is important to know the ideal growth conditions for the chosen species. There are countless numbers of microalgae species with variable requirements on their environment. Optimal light, temperature, pH, salinity and mixing should be provided, as well as the right nutrients in the right proportions. There are many cultivation systems, that all have their own advantages and restrictions. There are many techniques of harvesting algae, which so far is the most energy demanding part of the algae cultivation process.

2.1. The biology of microalgae

Algae consist of a wide mix of photosynthesizing organisms. Depending on morphology and size, algae are divided into micro- and macroalgae. Macro-algae are multiple cell organisms that resemble plants, while microalgae are a diverse range of single celled primary producers. Microalgae are found practically everywhere where there is light and humidity at least at some time of the year. Microalgae are found in marine and fresh waters, deserts, hot springs and on snow and ice. The number of species of microalgae is greatest in the seas and lakes (Lindholm, 1998: 15; Rajkumar & Zahira, 2013: 1-7). Microalgae are categorized in divisions based on their characteristic form and structure, and specific structural, chemical and functional features. The most important groups of microalgae in terms of abundance are green algae (Clorophyceae), diatoms (Bacillariophyceae), blue-green algae (Cyanophyceae) and golden algae (Chrysophyceae). The total estimated number of algae species are 200 000 to 800 000, of which 35 000 are described in literature (Rajkumar & Zahira, 2013: 7).

Algae are considered to account for more than half of the primary production and use of carbon dioxide on our planet, and they have a very important role on the climate. The oxygen in our atmosphere origins from photosynthesizing blue-green algae

(cyanobacteria). In addition, algae play an important role as a base for different nutrient chains in the environment. Algae utilize a lot of different nutrients and play a fundamental role in the circulation of macronutrients like nitrogen and phosphorous (Lindholm, 1998:12-18).

Microalgae can utilize many types of trophy, and are also capable of shifting metabolism and source of nourishment as a reaction to changes in the conditions of their environment. The most common and important trophy amongst microalgae is autotrophy. Autotrophic organisms obtain energy by absorbing sunlight and reducing CO_2 by oxidizing the substrate (commonly water) and releasing O_2. Heterotrophic organisms on the other hand utilize organic compounds produced by other organisms as their energy source. Photoautotrophic organisms utilize sunlight and carbon dioxide from the atmosphere to create chemical energy through photosynthesis. Photoautotrophic organisms only require inorganic mineral ions for growth. Most microalgae belong to the photoautotroph, but they still need minimal quantities of organic compounds, such as vitamins, for their growth. The energy source for mixotrophic organisms comes through performed photosynthesis, both through organic compounds and CO_2. A subtype of mixotrophy is amphitrophy, in which the organism can live either autotrophically or heterotrophically, depending on the availability of carbon source and light. Photoheterotrophic (photo-organotroph) organisms require the energy from light for utilization of organic compounds. A small group of algae belongs to the chemoautotrophs / chemoheterotrophs, and these are able to oxidize inorganic compounds for energy. Phagocytotic algae absorb particles of nutrient into food vesicles for digestion. The distinction between all these different strategies of trophy is not always clear, and change between the various possibilities are likely under most growth conditions. (Mata, Martins, & Caetano, 2010: 222-223; Grobbelaar 2013: 123-124)

2.2. Environmental requirements of microalgae

Microalgae have a high photosynthetic efficiency and a high growth rate, and are able to double their mass in as short time as 3.5 hours (Zhu, Li, Guo, Huang, Nugroho & Xia 2017: 296). Under suitable conditions, they commonly double their biomass within 24 hours (Mata et al. 2010: 223). Most algal species have a high content of lipids, normally between 20% to 50% of dry weight (Zhu et al. 2017: 296) Some algae strains have a lipid content of up to 70% (Gerbens-Leenes et al, 2014: 8551). For optimal growth, microalgae need appropriate nutrient supply, the possibility of gas exchange and the delivery of photosynthetically active radiation.

Although microalgae are able to survive in the harshest of environments, when cultivating algae, the optimal conditions are of great interest (Rajkumar & Zahira 2013: 7; Griffiths 2013). There are many designs for algae cultivating systems, and the common goal of all is to optimize the growth environment and the productivity of the algae (Griffiths 2013: 51).

2.2.1. Light

The first limiting factor of algae growth is light. The algae need photosynthetically active radiation to capture carbon dioxide and to produce oxygen and organic material. If the intensity of the light is too low, the photosynthetic rate of the algae cells is non-existent or minute. When the light intensity increases, the photosynthetic efficiency increases up to a point where the cells are light saturated. From this point, increasing light intensity no longer increases photosynthesis. When the light intensity gets too high, the photosynthetic apparatus is damaged by the excess radiation and the cells are photo-inhibited. As a result, the photosynthetic rate decreases with increasing light intensity. For most algae, the saturation point is reached at about 1700 to 2000 µmol m^{-2} s^{-1} (Griffiths 2013: 52-53). In the dark, during nighttime, the algae cells use oxygen for their own respiration, releasing carbon dioxide (Riffar 2013: 45).

The challenge of the cultivation system is to make sure that all algae cells receive enough irradiation. The light intensity declines with culture depth, as the light is absorbed by the cells and shading the cells below. Light does not penetrate more than a few centimeters in a thick algal culture. Photosynthesis is most effective in relatively dilute densities of algae (Griffiths 2013: 54).

Direct sunlight can often be too intense and cause photo-inhibition at the surface. At the same time, algae cells deeper down may suffer from photo-deprivation, as the radiation has been absorbed or reflected by cells closer to the surface. To deal with this challenge cultivations must be designed with a large surface to volume ratio and adequate mixing of the algae mass to make sure all cells are illuminated for an appropriate amount of time (Christenson & Sims 2011: 689).

Artificial light can be used for cultivating algae, mainly as a supplement for light supply during nighttime or cloudy days. From an energy efficiency point of view, natural sunlight needs to be the main source of light. Artificial lighting elevates operational costs and thereby put higher restraints on biomass yields. Light accessibility can be managed by reactor design. To dilute strong sunlight, the reactors (tubular or plate-) can be placed to overlap and shade each other. Internal illumination can be used to make the illuminated surface-area bigger in relation to the volume (Griffiths 2013: 55).

The design of cultivation systems is a trade-off between many factors regarding light. A more dilute cultivation facilitates deeper illumination, but at the same time harvesting is more expensive. A thin culture layer and shallow depth increases reactor material expenses and/or requirements on land area and makes the mixing inefficient (Griffiths 2013: 54).

2.2.2. Temperature

Temperatures naturally fluctuate diurnally and seasonally. After light, temperature is the second most important limiting factor for algae growth. The optimal temperature for most algae species ranges between 20°C and 30°C. The temperature requirements, as well as

other basic requirements, are species specific. Temperatures below optimum reduce the growth rate of the algae. However, most algae species tolerate temperatures up to around 15°C lower than the optimal temperature range. Algae are more vulnerable to temperatures higher than their optimum, only a few degrees can lead to cell death. Elevated temperatures decrease the net efficiency of photosynthesis as the rate of respiration increases. At higher temperatures, CO_2 becomes less soluble faster than O_2, which accelerates this effect (Mata et al. 2010: 223; Griffiths, 2013: 55). During nighttime low temperatures can even be advantageous, as it reduces respiration rate. According to Chisti (2007), as much as 25% of the biomass that is produced during daylight hours can be lost during night due to respiration.

The relationship between temperature and light can cause problems, especially in outdoor cultures. Early morning hours with a combination of intensive light and a temperature below optimum can cause photo-inhibition, since the cells are too cold to process incoming photons. Closed photo-bioreactors often suffer from too high temperatures, and mostly require some kind of heat exchange system (Griffiths 2013: 55-56).

2.2.3. Salinity

Microalgae species tolerate salinity differently and have different salinity optima. The range for the optimum can vary if salinity increases due to evaporation during hot weather. Salinity affects the growth and cell composition of the algae through osmotic stress, and through changes in the intercellular ionic ratios due to permeability of selective membranes. In cultures, salinity is easy to control through adding fresh water or salt (Mata et al. 2010: 223).

2.2.4. pH

Most algae species can tolerate quite large fluctuations in pH. Most freshwater eukaryotic algae prefer acidic environments (pH 5-7), and cyanobacteria prefer alkaline environments (pH 7-9) (Lorenz, Friedl & Day 2005: 155). During photosynthetic carbon fixation, OH^- ions accumulate in the liquid. pH gradually rises, and pH measurements as high as 11 are not unusual in dense algal cultures where no CO_2 is added (Grobbelaar 2013: 125). However, pH levels of over 10 and 11 can be inhibitory for photosynthesis (Sawyer, McCarty, & Parkin 2003: 560). Elevated pH in the range 10.2-12.0 can cause auto-flocculation of algae (Molina Grima, Fernández & Medina 2013: 271).

2.2.5. Mixing

Microalgae cells live in suspension in the water, mostly unable to move by themselves. The algae are dependent on movement and currents in the water. In a cultivation system with no mixing the algae mass will settle to the bottom of the cultivation vessel. To maximize production, stirring of the water-pillar is very important. Mixing keeps the algae cells evenly distributed in the culture media and the nutrients available for the algae cells. Mixing makes sure that all algae cells get evenly distributed light, which is of great importance in denser cultures where the algae cells circulate from dark to light zones. It decreases the negative effect of shading deeper into the reactor, and reduces photo-inhibition at the surface. Mixing also promotes gas exchange with the surrounding environment and evens out differences in temperature. Too harsh mixing and too much turbulence can still stress and damage the algae (Mata et al. 2010: 223).

Mixing can be provided mechanically or by aeration. In open ponds, mixing is usually provided by a paddlewheel or a rotating arm. Mechanical mixing can also be provided by stirring or pumping. An effective way of providing mixing is aeration in different forms of gas transfer systems. Gas bubbling can also simultaneously fulfill other purposes, including supply of CO_2 and nutrients, pH control and O_2-stripping. Bubbling with CO_2

is popular, but unfortunately, most of it is lost to the atmosphere. Mixing is energy demanding, since it must be more or less constant to prevent the algae mass from settling. The rate and efficiency of mixing is therefore a trade-off between energy requirements, cell damage and growth rate (Griffiths 2013: 57-58).

2.3. Nutrient requirements of microalgae

The three most important nutrients are carbon, nitrogen and phosphorous. In addition, the algae needs small amounts of micronutrients. Addition of nutrients to the water can be expensive, and because of this, production of algae in wastewater makes the production more cost efficient. In addition to the production of biomass, algae can effectively take up and remove nutrients from the wastewater. Combining algae production and wastewater treatment would be a great opportunity for wastewater plants to reduce costs (Christenson & Sims 2011).

Nutrients, except for light and carbon, are available for the algae cells in the growth medium, such as the wastewater. Lack of or shortage of any nutrient might cause disturbance in metabolism and decrease productivity and growth (Griffiths 2013: 56).

2.3.1. Carbon

The dry weight of the algae cell constitute by 40% to 50% of carbon (Chisti, 2007; Mata et al. 2010). Algae utilize carbon dioxide from the atmosphere for photosynthesis, and release oxygen into the atmosphere. When trying to maximize production, the provision of carbon dioxide can be a challenge. Carbon dioxide addition increases biomass growth and the lipid contents of the algae cells. When growing algae in open ponds, mass transfer can be a problem, and to closed reactors carbon dioxide need to be added (Christenson & Sims 2011).

In open ponds, diffusion of CO_2 from the atmosphere can at most sustain algae biomass production of 10 to 12 g of dry weight m^{-2} day^{-1} (Grobbelaar 2013: 127). Atmospheric CO_2 is not enough to satisfy the carbon demand of autotrophic production in high yielding cultivation systems. Therefore, supply of CO_2 and/or HCO_3^- are of great importance. The most important buffer present in freshwater is the bicarbonate-carbonate-system (CO_2 - H_2CO_3 - HCO_3^- - CO_3^{2-} -system), that controls and maintains specific pH-levels. CO_2 can be provided for the photosynthesis through the following reactions (Grobbelaar 2013: 125):

$$2HCO_3^- \leftrightarrow CO_3^{2-} + H_2O + CO_2 \quad (1)$$

$$HCO_3^- \leftrightarrow CO_2 + OH^- \quad (2)$$

$$CO^2{}_3^- + H_2O \leftrightarrow CO_2 + 2OH^- \quad (3)$$

Algae utilize CO_2 for photosynthesis. As the CO_2 concentration is reduced below the equilibrium concentration with air, this causes an increase in pH. The alkalinity of the water changes as the pH rises, which result in that CO_2 can be extracted for algae growth from bicarbonates and carbonates according to the equations 1 and 3 (Sawyer, McCarty & Parkin 2003: 557-560).

Carbon concentrations, mixing and pH are closely linked. Reactions of CO_2 with H^+, OH^-, H_2O and NH_3 are promoted by the mixing. In this way, the mixing affects CO_2 uptake rates, which in turn affects the pH. Addition of CO_2 should be controlled by a pH meter to prevent pH rise over acceptable levels. In long tubular reactors, CO_2 needs to be added at certain points, as the pH rises along the ways when CO_2 is consumed (Griffiths 2013: 56).

Heterotrophic and mixotrophic algae species can fill up some or all of its carbon requirements by using organic carbon sources such as glucose or acetate (Griffiths 2013: 56). It is a challenge for engineering to find ways to optimize carbon dioxide delivery and allowing adequate release of oxygen at the same time. Flue gases can be utilized, but often the distance between production sites makes the use less cost efficient. The removal of

excess oxygen is a big challenge for production in closed reactors, because too high oxygen levels can inhibit the photosynthesis process (Christenson & Sims 2011:689).

2.3.2. Nitrogen

For microalgae growth, nitrogen is the second most important nutrient after carbon. The nitrogen content of the algal biomass varies between one to more than ten percent, depending on its availability. The demand for nitrogen varies between groups and species. Nitrogen limitation is typically expressed by discoloration of the algae culture, and accumulation of organic compounds. The discoloration is caused by a decrease in chlorophylls and increase in carotenoids. The accumulated carbon compounds are for instance polysaccharides and polyunsaturated fatty acids (Grobbelaar 2013: 126-127).

Algae are able to utilize a variety of nitrogen compounds. Similar growth rates have been recorded with supply of nitrate (NO_3^-), ammonium (NH_4^+) and urea. Some cyanobacteria are capable of reducing N_2 to NH_4^+, catalyzed by the enzyme nitrogenase (Grobbelaar 2013: 126-127). Many microalgae prefer ammonium as nitrogen source, as it does not need to be reduced before amino acid synthesis. When ammonium is available, no other forms of nitrogen sources are utilized. However, high ammonium concentrations can be toxic (Sirin & Sillanpää 2015: 82). Microorganisms usually prefer ammonium nitrogen as a source of nitrogen. For cultivation it is important to keep in mind that ammonia can be lost from the growth media by volatilization. The pH of the growth media is affected by the nitrogen source. When ammonium is utilized, pH could decrease during growth phase due to the release of H^+ ions, and when nitrate is utilized pH increases. In cultivations, nutrients are usually supplied in excess, but they can also be intentionally limited, for instance for production of polyunsaturated fatty acids or β-carotene (Grobbelaar 2013: 126-127).

2.3.3. Phosphorous

Algal biomass contains less than one percent phosphorous, but still it is a very important nutrient for growth and cellular processes. Phosphorous is preferably supplied as orthophosphate (PO_4^{2-}). Phosphorous is an important growth limiting factor, because it is easily bound to other ions, like carbonate (CO_3^{2-}) and iron. This causes precipitation and makes the phosphorous unavailable for uptake by the algae. Algae can also store excess phosphorous in polyphosphate bodies (luxury storage), to be used when supply is limited, which makes phosphorous supply possible both internally and externally. The supply of phosphorous influences the lipid contents and carbohydrates of the produced biomass. The ratio between nitrogen and phosphorous is also important. One way of keeping the dominance of a preferred algae, is to keep the N/P ratio of the culture right for that specific species (Grobbelaar 2013: 127).

Algae are known to excrete alkaline phosphatases when phosphorous is limiting. This makes organic phosphorous available to the algae for reabsorption. It is possible that the excreted organic substances serve as an energy source for the algae. Mixotrophic algae use this energy at night. The production of extracellular organic substances alter diurnally, with a 6 hours delay behind the growth curve and decrease during the dark period (Grobbelaar 2013: 124).

2.3.4. Micronutrients

In addition to carbon, nitrogen and phosphorous microalgae need small amounts of micronutrients. Important micronutrients include sulfur (S), potassium (K), sodium (Na), iron (Fe), magnesium (Mg), calcium (Ca) and trace elements such as boron (B), copper (Cu), manganese (Mn), zinc (Zn), molybdenum (Mo), cobalt (Co), vanadium (V) and selenium (Se). The trace elements are important in enzyme reactions and biosynthesis of many compounds (Grobbelaar 2013: 127).

2.4. Cultivation systems

There is a great variety of different cultivation systems for microalgae. The systems include open and closed systems with suspended and immobilized cultures. The main goal for all systems is to achieve optimal productivity by trying to fulfill the optimal growth conditions mentioned in the previous chapter. Other aims are low costs of production and maintenance, and maximum use of land area.

2.4.1. Open systems

Algae are most commonly cultivated in open systems, both in commercial production and industrial processes, as well as in wastewater treatment. An open system can be a natural water body, a pond or a cascade system. The building and operating costs of open systems are relatively low, but they are vulnerable to influences from the surrounding environment. It is impossible to maintain a monoculture of a single species, and the risk of contamination is high. The only successful largescale commercial cultivation of monoculture in open systems is accomplished with species that live in for example high pH or salinity that other species cannot endure. The growth conditions of open systems are exposed to weather and climate, which makes the maintenance of constant irradiance and temperature difficult. The cost of harvesting is usually high as the concentration of the algae mass is relatively diluted (Griffiths 2013: 58-59).

One of the first systems used for algae cultivation were circular ponds, in which the water is mixed with a rotating arm placed in the middle of the pond. The most commonly used system in commercial production is the raceway pond. Raceway ponds have been in use since the 1950's, and are oval shaped shallow ponds where the water is circulated by a paddle wheel. The depth is usually 15 to 20 cm. Raceway ponds can be built and operated at reasonable costs, but the productivity often suffers from contamination, inadequate mixing and use of photosynthetically active radiation and inefficient use of carbon dioxide. Biomass concentration is normally around 0.5 g L^{-1}. Raceway ponds should in

theory be able to produce 60 g m^{-2} day^{-1}, but in practice, they have only been able to produce 10 to 25 g m^{-2} day^{-1}. Cascade systems are able to produce higher algal densities, up to 10 g L^{-1}. The productivity is still around the same as for a raceway pond. In a cascade system, the water slowly runs down a slight slope under a sheet of glass, which gives a very thin culture depth of less than 1 cm (Christenson & Sims 2011: 690-692; Griffiths 2013: 59-60).

2.4.2. Closed systems

Many different designs of closed reactors have been developed. A closed reactor, usually called photobioreactor (PBR), can usually provide higher cell densities and biomass yields, and accordingly reduced cost of harvesting. Contamination and environmental parameters are easier to control and more sensitive strains of algae can be cultivated. The capital and operating costs are higher than for an open system (Griffiths 2013: 61). Production in closed reactors has been reported to be 20 to 40 g m^{-2} day^{-1} (Christenson & Sims 2011: 690-692).

The goal of most PBR:s is to maximize the provision of light by optimizing the surface to volume ratio, while still maintaining reasonable cultivation volume, operating cost, mixing and cleaning. There is a tradeoff between optimal light penetration depth and temperature control. PBR:s demand a quite turbulent mixing to prevent sedimentation. In addition, cultivation of sensitive species demands sterile environment. Sterilization needs to be done by chemicals, which is expensive. Scale up is most likely done by multiplying units rather than maximizing reactor size (Griffiths 2013:58-68).

The most commonly used closed reactors are tubular or flat-plate reactors. Most closed reactor systems need some kind of circulation between a unit for illumination, and a unit for gas exchange provision and harvest. Tubular reactors can be vertical, horizontal or helical shaped. Vertical tubular reactors can be so called airlift or bubble column reactors, where the mixing and gas transfer is provided through bubbling with air, or air enriched with CO_2, from the bottom of the reactor. The advantage of vertical tubular reactors is the

optimization of light capture, but the length of the tubes is restricted by the accumulation of O_2. With the helical tubular reactor land area required can be reduced, but even light provision is restricted (Griffiths 2013: 62-65). The greatest problem to overcome with tubular reactors, is the toxic accumulation of oxygen. Moreover, challenges are dealing with overheating, negative pH and CO_2 gradients, fouling to surfaces and first and foremost high material and maintenance costs (Christenson & Sims 2011: 690-692). Flat plate panel reactors provide the algae suspension a uniform light distribution with a large surface area. The flat plates can be vertical or placed at an angle towards the sun. The top of the reactor can be open to improve gas transfer, and the need for pumping is reduced if the culture is mixed with air (Griffiths 2013: 65-66).

Algae production in large scale for high value products like, food, feed, pharmaceuticals or chemicals can be profitable in closed reactors. In production of algae biofuel, however, the energy balance must be positive. The energy recovered must exceed the energy input. Because of this, production of algae for biofuel is done in open raceway systems, despite the lower yield, as they are easier to scale up and less energy demanding (Griffiths 2013: 72).

2.4.3. Immobilized cultures

Because the recovery of biomass from suspended algae cultures is challenging, methods of cultivating immobilized or attached algae cultures have been developed. The economics of immobilized algae cultures for large-scale production is yet prohibitive, and has only been confined to the laboratory. The future prospects due to increased algal densities and low water and area requirements make immobilized algae cultivation worth developing.

In laboratory studies, matrix- immobilized algae have shown to be beneficial, both in nutrient removal from wastewater, as well as in enhanced hydrocarbon production and lipid content. Algal biofilms provide great hope for the future cultivation and recovery of microalgae. Biofilms are already used in the wastewater treatment industry. The

attachment of algae to the biofilms also seem to benefit from the bacteria in the wastewater. The development of large-scale biofilm production of algae, in combination with wastewater treatment, could contribute to a better and more cost effective production, dewatering process and harvesting procedure (Christenson & Sims 2011: 690-692).

2.5. Harvesting techniques

The greatest challenge for algae production is the harvesting, in other words the removal of the algae from the water. The recovery has been estimated to contribute to 20% to 30% of total production costs. The microscopic size of the microalgae makes the separation challenging. The size of unicellular eukaryotic algae is typically 3 µm to 30 µm and cyanobacteria as small as 0.2 µm to 2.0 µm. In addition to the small size of the algae, the concentrations of the algae are normally relatively dilute which also requires the use of large quantities of water. Cultures with a concentration of 200 to 600 mg of algae per liter are common. The challenge is to lower the costs of harvesting and to create methods of harvesting that allow the use of the algal biomass for the production of bio-products. Methods of harvesting can be divided into mechanical, chemical, electrical and biological ones (Christenson & Sims 2011: 692-694).

Before further processing into biofuel, the algae mass is usually dried, and to reach this the algae mass goes through one or many stages of dewatering. Usually the microalgae slurry in open ponds contain 0.05% of dry weight. Sedimentation or flocculation may raise the concentration of dry weight to 2%. After mechanical dehydration and centrifugation, the concentration of dry weight reaches 30%. The wet slurry can either be converted directly to biofuel, or further dried to 85% dry weight. The last step of thermal drying is very energy demanding (Gerbens-Leenes et al. 2014).

2.5.1. Chemical harvesting techniques

The algae suspension can be treated with chemicals to increase the particle size. This chemical flocculation is usually performed prior to any other method of harvesting. Microalgae cells are negatively charged. Electrolytes are added to neutralize the charge of the algae cells and synthetic polymers are added to flocculate the cells. The challenge is to find flocculants that do not obstruct the use of the algae and/or sludge in the downstream process. The charge of the cells is usually neutralized with aluminum sulfate ($Al_2(SO_4)_3$) and ferric chloride ($FeCl_3$). The use of aluminum and sulfate has shown to be inhibiting for bacteria in wastewater sludge and aluminum treated sludge is also problematic in disposal and land application. The use of natural polymers as flocculants is less studied, but would be beneficial as they do not pollute the biomass the same way. Successful results have been reached at laboratory scale using the polysaccharide chitosan and cationic starch. (Christenson & Sims 2011: 692-694).

2.5.2. Mechanical harvesting techniques

Mechanical techniques for recovering suspended algae include centrifugation, filtration, sedimentation and dissolved air flotation. Attached algae can also be mechanically harvested when using biofilm by scraping the algae off the surface. Separating algae from the water by centrifugation is a fast and reliable way of harvesting. This type of method is suitable for all kind of algae, but the challenge is the high operating and investment costs. Because of the high costs, centrifugation is not considered suitable for large-scale use. Different methods of filtration can be used to harvest filamentous algae strains and larger species of algae at a relatively low cost. However, for suspended microalgae cost and energy demand of filtration are high due to fouling and replacement costs of membranes. Tangential flow filtration is considered the most effective (Chen, Yeh, Aisyyah, Chang, & Lee 2011: 77-79; Christenson & Sims 2011: 692-694). Sedimentation of algae can be done at low cost, but is very slow and can give concentrations of solids of

1.5% (Christenson & Sims 2011: 692-694). To speed up the process of sedimentation, this method can be used in combination with chemical methods (Chatsungnoen & Chisti 2015). Dissolved air flotation is used in removal of sludge in wastewater treatment. In algae recovery this method is considered more efficient than sedimentation. This method is also used in combination with chemical flocculation treatment (Christenson & Sims 2011: 692-694).

2.5.3. Electrical and magnetic harvesting techniques

Attempts have been made to separate algae from the water by electrophoresis. Charged algae are driven out of the solution by an electric field. Microalgae are carried to the surface by the hydrogen generated by the water electrolysis. The advantage of this method is that no chemicals need to be added (Chen et al. 2011: 77-79). The challenges of the application of this method in large scale are high power requirement and high cost (Christenson & Sims 2011: 692-694). In an external magnetic field, algae cells can be captured by functional magnetic particles. Fe_3O_4 nanoparticles have been used under slightly acidic conditions. This new method is relatively fast and simple and has high recovery efficiency. The method is limited by the difficulty in producing functional magnetic particles (Molina Grima, Fernández, & Medina 2013: 277).

2.5.4. Biological harvesting techniques

Spontaneous flocculation of algae can be divided into autoflocculation and bioflocculation. Autoflocculation occurs at high pH levels. The negatively charged algae cells are neutralized by positively charged calcium phosphate precipitate and flocculation occurs. The pH increase is caused by dissolved carbon dioxide, which further leads to supersaturation of calcium and phosphate ions. Bioflocculation is used to describe flocculation caused by secreted polymers. Adding flocculating microbes has also given

great results in recovery. Both methods have achieved algae recovery of up to over 90 % (Christenson & Sims 2011: 693-694).

3. WASTEWATER TREATMENT

There is no universal process for cleaning wastewater, as every location and situation is unique. The composition of the wastewater is complex, and the sources of pollution different. The source can be domestic or municipal -sewage from urban and rural areas, and it can periodically contain varying amounts of rainwater and melting snow. Wastewater can origin from manufacturing or industrial plants or be run off from agricultural land or waste disposal plants (Abdel-Raouf, Al-Homaidan, & Ibraheem 2012: 259; Riffar 2013: 76). Wastewater is usually a combination of water from many origins, depending on in which way the water is collected (Rawat, Kumar, & Bux 2013: 181).

To effectively treat wastewater, it is important to characterize and identify the chemical compounds. The concentrations of the compounds are used as a measure of the quality of the wastewater (Rawat et al. 2013: 184). The composition of the wastewater varies depending on the origin. It consists of a variety of organic and inorganic materials and man-made compounds (Abdel-Raouf et al. 2012: 259-260). The majority of the chemical components in municipal wastewater are carbohydrates, proteins, lipids and urea. Urea comes from urine, and forms large quantities of nitrogenous matter in the wastewater system (Rawat et al. 2013: 184). Other biodegradable products found in wastewater are ammonia, fats, lignin, soaps, oils and other synthetic chemicals that consists of carbon, hydrogen, oxygen, sulphur, phosphorous and iron. Parameters that describe wastewater are total dissolved solids, pH, temperature, colour and odor (Rawat et al. 2013: 183-184). Industrial wastewater can contain heavy metals and toxic compounds, and runoff from rain and melting snow can contain petroleum compounds, silt and pesticides (Riffar 2013: 76). Many microorganisms flourish in wastewater, especially bacteria, viruses and protozoa. The microorganisms are mostly harmless, but they also contain pathogenic microorganisms (Abdel-Raouf et al. 2012: 259-260).

The main goal of wastewater treatment is to avoid eutrophication and pollution of natural water bodies. The wastewater treatment process needs to fulfill regulations and limits to protect the public health and the environment. The main objective of the wastewater

treatment is to reduce and remove suspended solids, biodegradable organic matter, pathogens and toxic compounds (Riffar 2013: 75).

Wastewater can be treated physically, chemically and/or biologically. Physical treatment methods remove suspended solids by e.g. sedimentation or filtration. Chemical treatment methods aim to destruct or convert contaminants through chemical reactions, e.g. flocculation sedimentation, disinfection or precipitation. Biological treatment methods aim to convert or destruct contaminants, and to reduce biodegradable organic matter and nutrients with the help of microorganisms (Riffar 2013: 78).

The treatment technology of sewage can be divided into preliminary, primary, secondary, tertiary and quaternary treatment levels. The preliminary treatment removes the coarse materials. Larger objects are removed when the sewage passes through bars (space 20-60 mm), and grit and silt are settled by reducing the velocity of the flow while letting organic matter continue to the next phase. In the primary treatment stage of the process the main part (up to 70%) of the remaining solids settle by gravity in sedimentation tanks. Sometimes chemical coagulants are used. The secondary treatment processes organic matter and solids. A mixed population of heterotrophic bacteria utilizes the remaining organic matter for growth and energy. There are multiple ways to achieve the aerobic oxidation of biological oxygen demand (BOD). The microbial population can be fixed on a surface of biofilm, or suspended in reactors, called activated sludge. Biological oxidation systems are effective in removing pathogenic bacteria. Sometimes a combination of biological and chemical treatments are used (Abdel-Raouf et al. 2012: 260; Riffar, 2013: 79-80).

The advanced treatment steps include the tertiary and quaternary processes. In the tertiary treatment process, organic ions are removed either biologically or chemically. Compared to the chemical method, the biological method is not as expensive and does not cause secondary pollution. The quaternary process removes heavy metals, remaining organic compounds and soluble minerals. Methods used in advanced treatment processes are complex, and designed to target certain nutrients like phosphorous or nitrogen. The more steps there are in the cleaning process, the more expensive the process is. Compared to the primary treatment stage the tertiary process is approximately four times more

expensive, and the quaternary eight to sixteen times more expensive. (Abdel-Raouf et al. 2012: 260-261)

3.1. Microalgae in the wastewater treatment process

The research of algae based wastewater treatment started in the 1950's, when the combination of wastewater treatment and protein production was of interest. Microalgae have been widely used in pond wastewater treatment since the early 1950's (Rawat et al. 2013: 186). Already in the late 1950's the use of algae in wastewater treatment for removal of nitrogen and phosphorous as well as provision of oxygen for bacterial respiration was suggested (Abdel-Raouf et al. 2012: 263).

Since 2000, the treatment of wastewater with algae has been termed *phycoremediation* (Rawat et al. 2013: 185). Phycoremediation of wastewater utilizes microalgae in large scale for the removal of pollutants and produces non-hazardous end products. Micro-algal cultures assimilate huge amounts of nutrients as well as reduce biological and chemical oxygen demand (BOD and COD). The increase in pH caused by photosynthesis can further accelerate the removal of nutrients by ammonia stripping or phosphorous precipitation (Rawat et al. 2013: 185). As a part of the biological process in the secondary treatment stage, microalgae produce oxygen that enhances growth of bacteria. Microalgae absorb nutrients and produce oxygen through photosynthesis, while bacteria degrade organic matter and nutrients utilizing the oxygen produced by the microalgae (Brenner & Abeliovich 2013).

Microalgae have been proven to effectively remove nitrogen, phosphorous and chemical oxygen demand and even pathogens from wastewater. The use of microalgae is more cost efficient than activated sludge processes and other secondary treatment processes. The microalgae also reduce emissions of greenhouse gases and require low energy. The formation of sludge is reduced as well (Sirin & Sillanpää 2015).

Microalgae are usually applied in the tertiary treatment of domestic wastewater, in maturation ponds. Microalgae can also be utilized in small-to medium scale municipal wastewater treatment systems. There are different pond technologies available, all relatively simple to operate. (Rawat et al. 2013: 180-181). High rate algal ponds (HRAP) are the most cost-effective reactors for wastewater treatment. HRAP:s used in wastewater treatment usually consist of different departments for different means, which are called Advanced Pond Systems. In addition to the HRAP, the Advanced Pond Systems have anaerobic digestion pits, ponds for algal maturation and ponds for algal settling. One of the most commonly used wastewater treatment systems are activated sludge systems. In comparison, the HRAP:s require about 50 times more land area, without consideration of the land area needed for the disposal of the waste activated sludge. The operational cost of the Advanced Pond System is only 20% of the Activated Sludge system, and the construction cost is less than half (Park, Craggs, & Shilton 2011).

Biological wastewater treatment systems utilizing algae remediation are also called oxidation ponds or waste stabilization ponds. In areas with warm climate, waste stabilization ponds are common. They are recommended by the WHO as the process to choose when resources and skills are limited. This is due to the simplicity and reliability of the systems, and the efficiency of pathogen destruction from water. In developed countries, however, this kind of process has lost ground to the Activated Sludge process. The wastewater treatment plants do not have the capacity for the space demanding oxidation ponds to keep up with the increasing amounts of wastewater. In developed countries, the incomplete purification efficiency and the high consumption of land resources render oxidation ponds noneconomic (Brenner & Abeliovich 2013: 595-601).

However, the prospect of producing renewable biofuel from algae produced in wastewater, has led to new interest in the area also in developed countries. There is, however, a basic contradiction between intensive production of algal biomass and wastewater treatment. Wastewater treatment with algae need long retention time and exhaustion of nutrients, while effective biomass production require high nutrient concentration. The economy suffers from the further treatments steps of the water required due to the incomplete wastewater treatment (Brenner & Abeliovich 2013: 595-

601). Another major challenge to be overcome is the economics of the harvesting of the algal biomass (Rawat et al. 2013: 185).

3.2. Algae species used for phycoremediation

Microalgae are efficient in fixing carbon dioxide by photosynthesis and removing nutrients from wastewater. Some of the most common microalgae species studied and used in wastewater treatment are *Chlorella, Oscillatoria, Scenedesmus, Ankistrodesmus, Botryococcus, Synechocystis, Lyngbya, Gloeocapsa, Spirulina, Chroococcus* and *Anabaena*. These are all species that flourish in eutrophic waters rich in nitrogen and phosphorous, converting the nutrients into biomass. *Chlorella (vulgaris*) species have been used for wastewater treatment all around the world. Algal species of special interest are species with for instance extreme temperature tolerance, capacity of heavy metal accumulation and mixotrophic growth as well as production of high value by-products. Nutrient removal from wastewater in cold climates have for instance been studied with a strain of *Phormidium* that was isolated from a polar environment with temperatures below 10°C. The selection of suitable strains of microalgae for wastewater treatment and biomass production is very important. The most studied algal species are *Chlorella, Scenedesmus* and *Ankistrodesmus,* which have been grown in different kind of wastewaters originating from various industrial effluents (Rawat et al. 2013: 186-187).

Several studies have proven the potential for nutrient removal from wastewater by microalgal biomass production. Removal of total nitrogen (TN) from the wastewater reach the range of 74-92%, total phosphorous (TP) 74-100% and ammonium (NH_4^+) 96-99% in studies conducted by Sacrístan de Alva et al. (2013), Zhu et al. (2014) and Gentili (2014). Zhu et al. (2014) observed a 77% decline in chemical oxygen demand (COD).

Recent studies also focus on the lipid content of and lipid extraction from the algae biomass produced in wastewater (Sacristán de Alva, Luna-Pabello, Cadena & Ortíz 2013).

4. THE WASTEWATER TREATMENT PROCESS AT PÅTT

The Pått municipal wastewater treatment plant is located by the sea shore at Palosaari in Vaasa, Finland. Annually the wastewater treatment plant treats 6 to 7 million cubic meters of wastewater from the city of Vaasa and the neighboring municipalities Mustasaari and Maalahti. Variations in the amounts of wastewater mainly depend on amounts of rain and melting snow (Vaasan Vesi 2017a).

When reaching the wastewater treatment plant, the sewage first passes through the preliminary treatment where the coarse material is removed by two stairbars. Grit and sand, that comes to the treatment plant with the surface water, are separated in basins. The coarse waste is collected and transported to combustion at Westenergy, the local waste-to-energy plant, and the sand is transported to the local waste management company Stormossen (Vaasan Vesi 2017b). The water then continues to the pre-sedimentation pond. This unit for removing mud and silt was ready for use in 2011. Solids settle to the bottom of the pond by gravity, further led to the sludge preparation unit, and dried. A precipitation chemical can be added to further diminish the load in the downstream process (Vaasan Vesi 2017c).

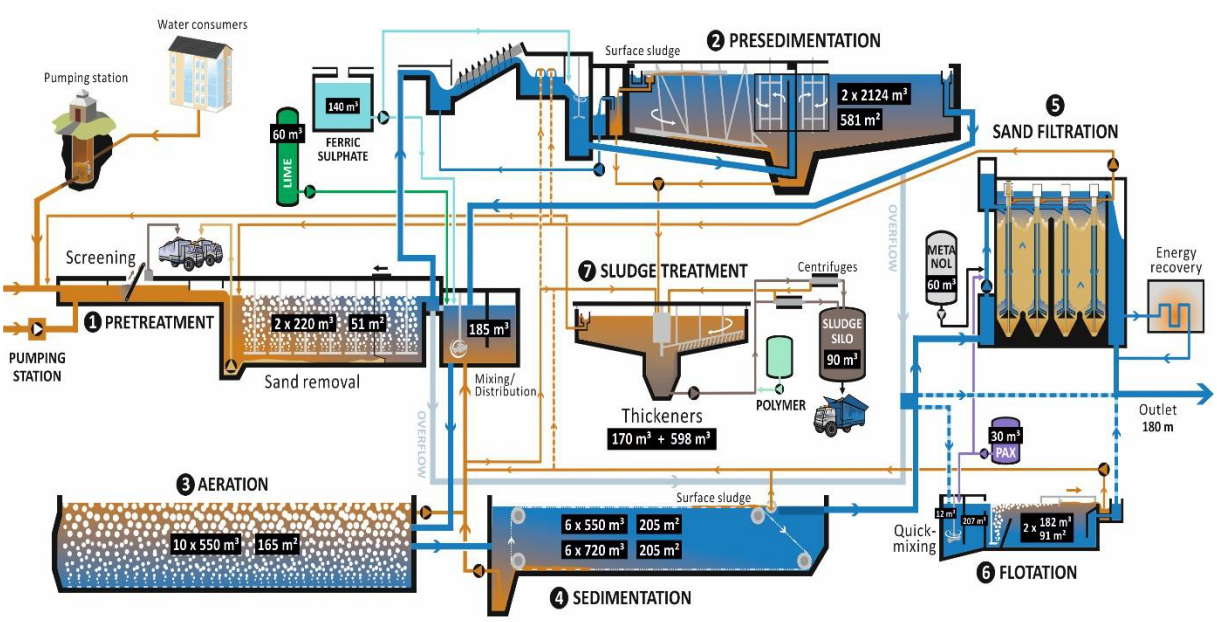
Active sludge that is formed during the process is added into the wastewater in the ten aeration ponds that come next in the process. The aeration provides bacteria and protozoa in the sludge with the oxygen needed to decompose organic compounds (Vaasan Vesi 2017d). Sludge consisting of the remaining substances, dead bacteria and precipitated phosphates is separated from the water in the sedimentation ponds, of which there are twelve. The sludge from the bottom of the ponds is pumped to the mixing pond and mixed with the incoming water and then redistributed to the aeration ponds. Some of the sludge is removed through the sludge-treatment, and the sludge from the surface is led back to the pre- sedimentation pond (Vaasan Vesi 2017e).

The purified water from the surface of the post-sedimentation ponds is led to the after-treatment, the sand-filtration unit. This unit was built in 2012 to meet the new standards for denitrification (Vaasan Vesi 2017f). The water is filtered through a bed of sand that is four meter thick, where nitrogen, phosphorous and remaining suspended solids are

removed. A precipitation chemical is added if necessary, and methanol works as a source of carbon for the denitrification process (Vesala 2014: 13).

After the water has passed the sand-filtration unit, the water is led out to the sea through a pipe with the length of 180 meter (Vaasan Vesi 2017h). Sludge from the different parts of the process is dewatered by passing through ponds for thickening to centrifugation. The dewatered sludge is transported to the local waste management company Stormossen, where 800 000 m³ (7,8 GW) of biogas is produced annually (Vaasan Vesi 2017i). The water treatment process is illustrated in Picture 1.

The wastewater treatment plant also contains a flotation unit, which can be taken into use in difficult situations when rainwater and melting snow put a too great load on the biological processes. The rainwater and the water from melting snow can be led pass the biological process to the flotation unit where precipitation chemicals and dispersion water is added. The sludge floats up to the surface and the water beneath is dispatched to the sea (Vesala 2014: 13). The flotation unit also guarantees the water to meet the standards of the environmental regulations even in situations of disruption and poisonous discharge, as all water can be led directly to and through the flotation unit (Vaasan Vesi 2017g).



Picture 1. A schematic picture of the water treatment process (Karlsson, 2017).

The sewage treatment is regulated and supervised by the Regional State Administrative Agencies of Western and Inner Finland. The latest requirements (of 10.7.2017) state that at least 95 % of phosphorous and BOD must be removed before dispatch to the sea. COD must be reduced by at least 85 %, and nitrogen at least 70 % annually. The removal of nitrogen is calculated as an annual average, and measured when the temperature of the incoming sewage is over 12°C. The amount of nutrients allowed in water dispatched to the sea are 10 mg L^{-1} BOD, 75 mg L^{-1} COD and phosphorous 0.3 mg L^{-1} (Vaasan Vesi 2017a).

The water treatment process at Pått is a biological process and is thus vulnerable to changes. The process is constantly supervised by an automatically working surveillance system that analyzes the water in different phases of the process. The wastewater treatment plant has a laboratory of its own (Vaasan Vesi 2017a). In the laboratory the pH, conductivity, solids, NH_4-N, NO_3, TP, solute P and alkalinity of the incoming and outgoing water are examined daily. COD and TN are tested a few times a week, as well as waters from different stages of the process. In addition, the Environmental laboratory of the City of Vaasa tests the water for faecal bacteria (*enterococcus*, *E.coli*) and BOD (Koivisto 2017).

In 2016 the results for purification at Pått was 98% for phosphorous, 66% for nitrogen, 95% for BOD and 85% for COD (Vaasan Vesi 2017j). The removal of nitrogen is facing challenges during the cold time of the year. The nitrification process in the aeration ponds works all year round, but the denitrification process in the after-treatment unit has limitations due to low temperatures. In practice, the denitrification process works properly from May/June to December/January, during which time the reduction of nitrogen is between 80-100%. During the cold time of the year, the reduction goes down to between 35-50% (Koivisto 2017).

The amounts of nitrogen and phosphorous in the incoming water are (40-70) mg L^{-1} total nitrogen (TN) and (5-9) mg L^{-1} total phosphorous (TP). The concentrations after the pre-sedimentation pond are (30-60) mg L^{-1} TN and (2-5) mg L^{-1} TP, and after the sedimentation pond (25-50) mg L^{-1} TN and (0.7-2.0) mg L^{-1} TP. The water dispatched to

the sea contains (4-40) mg L^{-1} TN and (0.1-0.4) mg L^{-1} TP. The pH of the water is between 7 and 8 at these measuring points (Koivisto 2017).

The nitrogen arrives to the wastewater treatment plant almost entirely in the form of ammonium (NH_4^+) (Koivisto 2017). At Pått nitrogen is removed from the wastewater in a biological process consisting of a combination of active sludge and filtration (Vesala 2014: 16). Nitrification is an aerobic biological process where bacteria (*Nitrosomas, Nitrobacter*) oxidize ammonium nitrogen (NH_4^+) and ammonia (NH_3) in a two-step process through the form of nitrite (NO_2^-) to nitrate (NO_3^-). The bacteria require enough oxygen to complete the nitrification process, and that is the reason for the aeration (Vesala 2014: 16-17).

The second step of the nitrogen removal happens in the after treatment unit and is called denitrification. In the denitrification process, heterotrophic bacteria reduce nitrate (NO_3^-) through the form of nitrite (NO_2^-) to nitrogen gas (N_2) which is released into the atmosphere. Denitrifying bacteria (*Achromobacter, Aerobacter, Alcaligenes, Basillus, Brevibacterium, Flavobacterium, Lactobasillus, Micrococcus, Proteus, Pseudomonas* and *Spirillium*) can utilize both dissolved oxygen and nitrate as a source of oxygen for their metabolism. If oxygen is present, it is used first. Therefore, the denitrification process occurs under anaerobic conditions. The process also needs a source of carbon to work. To some extent, carbon from the organic compounds of the wastewater can be utilized, but to maximize the denitrification process carbon is added in the form of methanol (Vesala 2014: 18-19).

The amount of water treated at the Pått wastewater treatment plant was 7.42 million cubic meters in 2016 (Vaasan Vesi 2017l). The average amount of water flowing through the plant is 17 000 m^3 day^{-1} (Koivisto 2017). The flow of sewage water is very dependent on the weather-conditions, as rain and melting snow raises the amounts of incoming water (Vesala 2014: 11). There are also significant diurnal variations. The flow during nighttime is approximately 300 m^3 h^{-1} and daytime 2000 m^3 h^{-1} (Koivisto & Vesala 2017). The water flows through the whole treatment process in approximately 24 hours, with average flow velocity (17 000 m^3 day^{-1}) in 26.4 hours. The phases of the process where the water linger for the longest amount of time are the pre-sedimentation ponds, the aeration and the

sedimentation ponds. With average flow velocity, the water lingers in the pre-sedimentation pond for approximately 6 hours, and the sedimentation pond a little less than 11 hours (Koivisto 2017). The temperature of the incoming wastewater to Pått is 14°C during summertime. The temperature of the water do not rise above 12°C until June. During winter, the temperature of the water is less than 10°C, mostly around 8°C to 9°C. Under periods when the snow is melting, the temperature of the incoming water is 5°C to 6°C (Koivisto & Vesala 2017).

5. DESCRIPTION OF THESIS

The aim of this thesis is to cultivate the microalgae *Scenedesmus dimorphus* in wastewater from the local municipal wastewater treatment plant Pått. The research questions of this thesis are:

Is it feasible to grow microalgae in wastewater from the municipal wastewater treatment plant Pått in the city of Vaasa?

What point of the wastewater treatment process is most suitable for cultivation of microalgae, in terms of production of algae biomass?

At what point of the treatment process do the microalgae remove most nutrients from the wastewater?

Is there a difference in growth of algal biomass and nutrient removal at different temperatures?

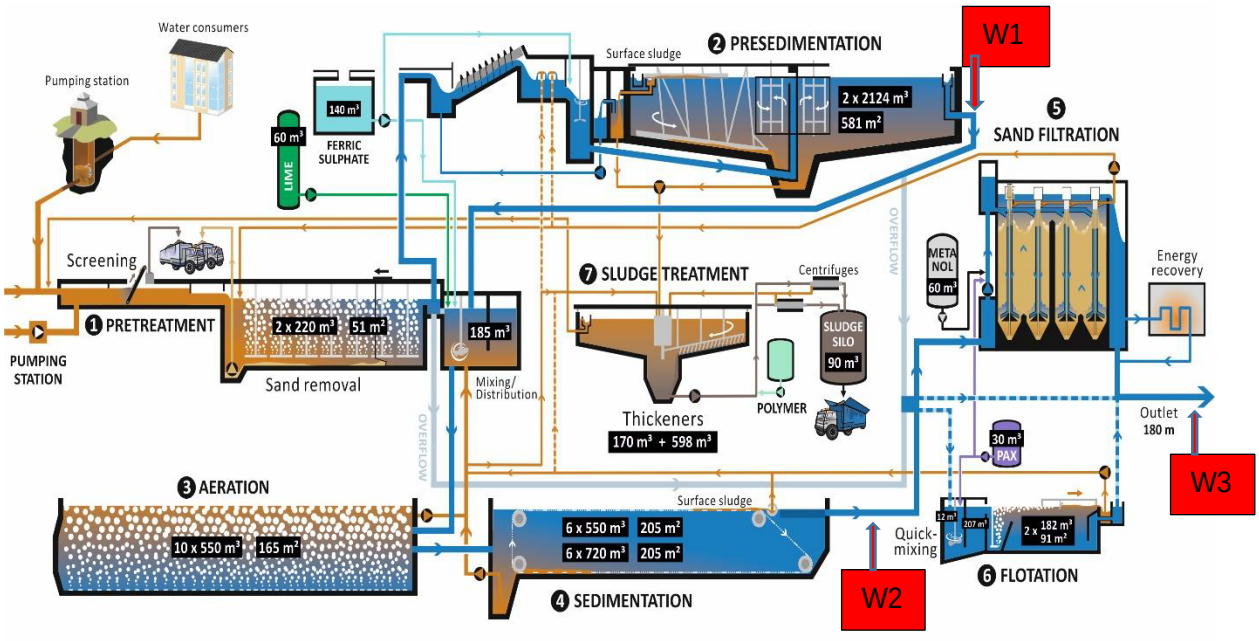
The hypothesis is that *Scenedesmus dimorphus* will effectively remove COD, TP, TN and NH_4^+ from the wastewater. *Scenedesmus dimorphus* will grow most efficiently in the water with most nutrients and better in 24°C than in 16°C.

6. MATERIALS AND METHODS

The algae used in the experiment is a species of green algae (Clorophyta), *Scenedesmus dimorphus*. In the Baltic area *Scenedesmus* flourish during summertime in eutrophic lakes and in puddles on seashore rocks enriched by birdmanure (Lindholm, 1998: 50-51).

6.1. Experiment I: Cultivation in 24°C

In agreement with the personnel at the local wastewater treatment plant Pått, water samples were collected from three different points in the water treatment process. The first point (W1) is after the pre-sedimentation pond, the second (W2) point is from after the sedimentation pond, and the third (W3) is water from the beginning of the pipe that leads the cleaned water out to the sea. The points are shown in Picture 2. Hereafter the water-samples will be referred to as W1, W2 and W3. One important criterion for the chosen points W1 and W2 was that these are the places in the process where the water lingers for the longest period. The water moves quickly through the system, and has normally passed through the process in approximately 24 hours.



Picture 2. The points from which the water samples were collected are shown as red boxes in the schematic picture of the water treatment process (Karlsson 2017).

6.1.1. The pilot study 1

Before the real experiment was started, a pilot study was conducted (30.5-8.6.2017). In the pilot study the algae were cultivated for ten days under the same conditions as later in the actual experiment. The algae cultivated in the pilot study were collected and used in the experiment. In the pilot study algae were cultivated in duplicates in water from the three mentioned points of the water treatment process and in Bristol Medium. The accumulation of biomass was monitored by measuring optical density every day, and water analyses were conducted at the beginning and at the end of the pilot study after 10 days. The largest increase in biomass occurred in W1 and W2, water collected from after the pre-sedimentation pond and the sedimentation pond. The growth was 600% and 598%, respectively. The least growth, 251% occurred in the Bristol medium (W4). The pH was quite high during the whole pilot study (10 to 11 in W1 and W2, 8 to 9 in W3 and Bristol). During the pilot study all ammonium was removed and 90% of the total nitrogen

was removed from the wastewaters. There was an increase of COD in all cylinders. The colour of the algae cultivation turned from bright green to yellow during the pilot study.

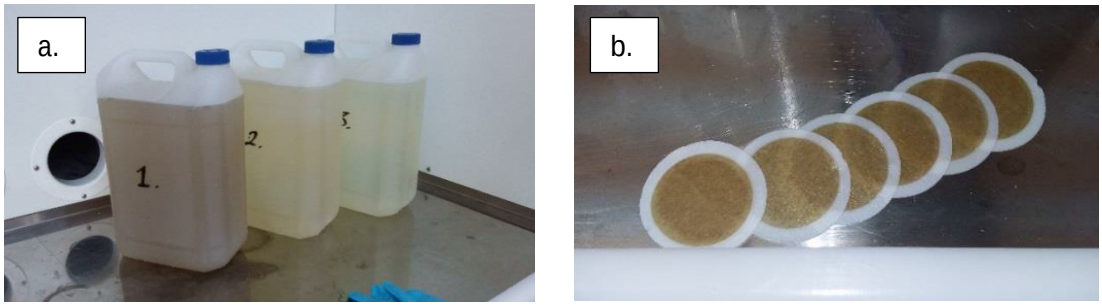
6.1.2. Description of experiment I

The water samples for the experiment were collected on 12.6.2017 (Picture 3), and the experiment was carried out during 18 days from 13.6 to 30.6.2017. Water analyses were performed on day one (13.6), day two (14.6, after 24 hours), day 9 (21.6) and day 18 (30.6). Water analyses were performed in duplicates with Merck Spectroquant Cell tests for Chemical oxygen demand (COD), Total Nitrogen (TN), Total Phosphorous (TP) and ammonium (NH_4^+). Optical density, pH and temperature were measured every second day. The colour of the algae cultivation was also followed up, described and photographed.

Picture 3. Water sample collected after the pre-sedimentation pond at Pått wastewater treatment plant.

Water from the chosen points at Pått wastewater treatment plant was collected in the afternoon. The Bristol medium that was used in the pilot study was not included in this experiment, as the water properties differ a lot from the wastewater. The water was collected and transported in 5-liter canisters. The water was then brought to the laboratory, where approximately 1.8 liters of each sample was filtered to remove any excess particles from the water (Picture 4). The water collected after the presedimentation pond was rich in particles. The samples were filtered through 1.6 µm micro-glassfiber Munktell filterpapers (Picture 5 a-b). After the filtration the samples were autoclaved for 30 minutes. In the evening, the samples were moved to the refrigerator to cool down over night.

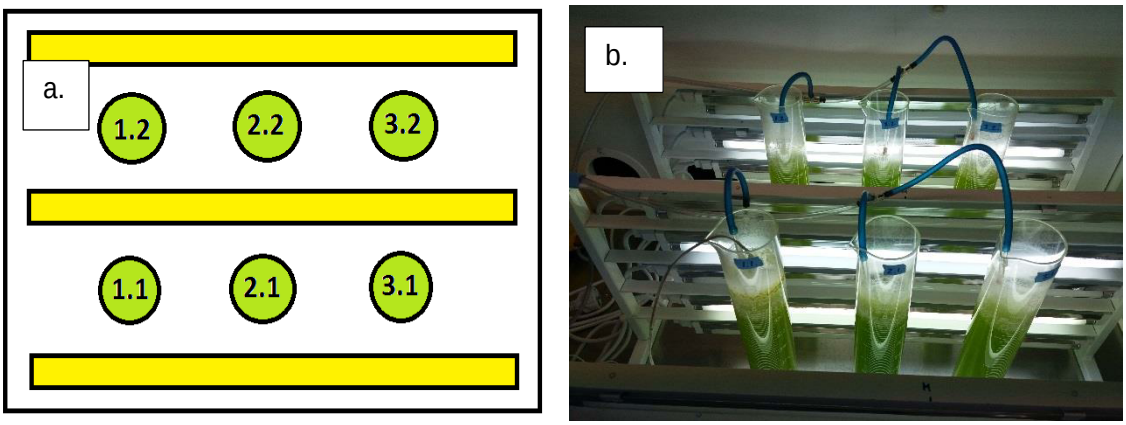
Picture 4. The water is filtrated to remove particles



Picture 5. a. The collected water samples from (1) after the pre-sedimentation, (2) the sedimentation pond and (3) water about to be dispatched to the sea. b. The filterpapers in the picture are from the filtration of W1.

The experiment was started the following morning. Six 500 ml measuring cylinders were placed in two rows in a frame of three rows of fluorescent lamps in a fume hood. Each row of lamps contains five lamps (cool white fluorescent light). Only the second and fourth lamp from the bottom of each row was used. The light was on 24 hours a day during the whole experiment. The light intensity in the light rack was 147 µmol m^{-2} s^{-1} ± 28 µmol m^{-2} s^{-1}.

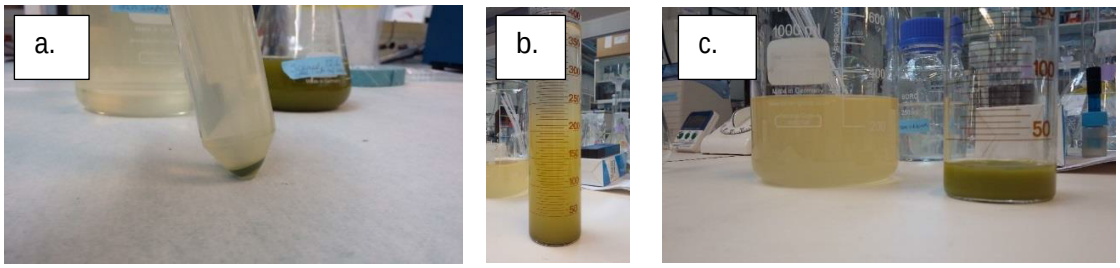
The measuring cylinders in the first row were numbered 1.1, 2.1 and 3.1, and in the second row 1.2, 2.2 and 3.2 (Picture 6. a-b). The first pair of cylinders (1.1 and 1.2) were filled with 450 ml of water 1 each, the second pair (2.1 and 2.2) with 450 ml of water 2 each and the third pair (3.1 and 3.2) with 450 ml of water 3 each. All of the measuring cylinders were aerated at medium speed with an aquarium pump (Sera Precision Air 275 R Plus) through plastic hoses and thin glass pipettes.



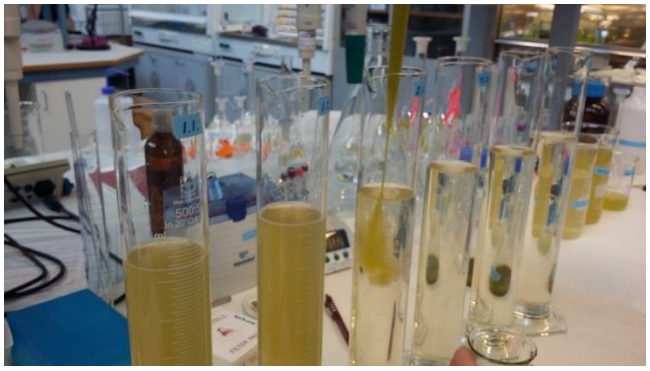
Picture 6. a. The measuring cylinders' placing in the light rack from above. b. The measuring cylinders were placed between three rows of fluorescent lamps. From the left the cylinders front and back contain water from the pre-sedimentation pond (W1), the post-sedimentation pond (W2) and water to be dispatched to the sea (W3).

The algae mass from the pilot study had been collected and was used in the actual experiment. Thus, the algae were acclimatized to growing in the wastewater. The

collection of the algae mass from cylinders 1.1, 2.1 and 3.1 of the pilot study was done through centrifugation. The centrifuged algae mass was studied in a microscope, to make sure the centrifugation had not damaged the algae cells. The algae from the rest of the cylinders (1.2, 2.2 and 3.2) were left standing over night for separation by gravity. Each of the measuring cylinders in the actual experiment was incubated with 20 ml of *Scenedesmus dimorphus* suspension separated by gravity (Picture 7 b and c), and 10 ml of suspension separated by centrifugation (Picture 7 a).



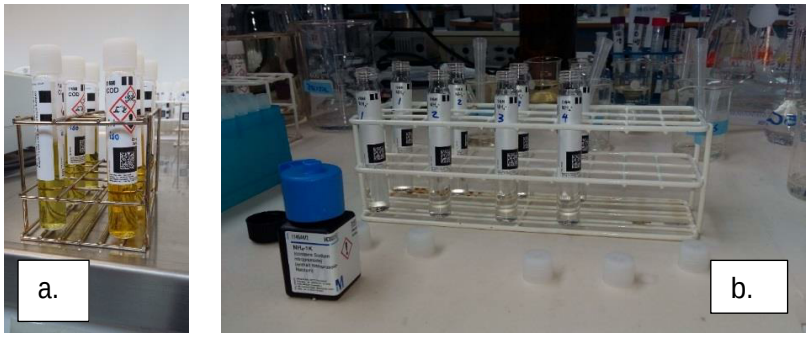
Picture 7. Algae separated through centrifugation (a) and gravitation (b and c).



Picture 8. Inoculation with algae suspension from the pilot study.

Water analyses were performed in duplicates with Merck Spectroquant measuring kits (Picture 9 a-b) for COD, TN, TP and NH_4^+ on water samples taken from the inoculated measuring cylinders 40 minutes after inoculation. The water samples were centrifuged in 4500 rpm for 10 minutes to separate the algae from the water. Prior to the water analyses,

the supernatant was filtered through a 0.45 µm syringe filter (PALL Acrodisc 32 mm Syringe Filter with 0.45 µm Supor Membrane).



Picture 9. Water analyses were performed with Merck Spectroquant Cell tests (a and b).

Optical density, temperature and pH were measured every second day at 10 am. Optical density (OD) measurement is a method of determining biomass concentration to determine growth conditions and growth stages. This method, which is also known as absorbance or turbidity, is used in studies of microalgae cultivation, algae physiology and biotechnology to measure biomass of microalgae and other unicellular organisms. Optical density measurement is a much faster and more convenient way to determine cell concentration than by the alternative way of counting cells in the microscope. The amount of light absorbed at wavelength 600 nm to 750 nm is related directly to cell biomass. The absorbance of the sample is read by a spectrophotometer. The sample of the algal culture is diluted to a concentration that presents an OD value less than 1 at 680 nm or 750 nm. A correlation curve between OD values and samples of algae cultures sampled for dry weight is determined. When using optical density as a measurement of biomass, it should be kept in mind that there is a risk of inaccuracy due to changes in optical properties caused by changes in cell morphology and composition (Lee et al. 2013: 40). During this experiment optical density at 680 nm (OD_{680}) was measured and recorded. The algae suspension was diluted 1:3 with distilled water (1 ml algae suspension and 2 ml distilled

water) prior to measurement. From day nine onwards the algae suspension was so dense, it had to be diluted 1:6 to keep the absorbance value under 1.

The cylinders were properly stirred all the way to the bottom before the start of the optical density measurements. Despite the aeration some of the algae tend to settle to the bottom of the measuring cylinder. The further the experiment proceeded the more algae settled to the bottom. The algae were stirred to make the algae mass evenly distributed in the measuring cylinder for the optical density measurement. The water level in the cylinders was kept constant at 480 ml by adding distilled water up to that point after the measurements. The aeration cause evaporation of (11.2 ± 4.6) ml daily, varying from cylinder to cylinder and from day to day. The air flowing to every cylinder should have been the same amount, but there may have been a slight difference in the amount of air reaching each cylinder, due to the order of the cylinders, and the random way the tip of the pipette touched the bottom of the cylinder. The airflow was not restricted in any way, and the pipettes were all the same.

The correlation between optical density and dried biomass of the different waters was pre-determined during the pilot test. This was done on 1.6 µm micro glass-fiber filter papers (Munktell) pre-dried in the oven for 2 hours at 110°C. The filter papers were marked and weighed, and kept in a desiccator. The correlation between the optical density and the dried biomass was determined separately for each water-sample. The water from the algae cultivation was diluted 1, 2, 5, 10 and 20 times, each at a volume of 50 ml (Picture 10). Optical density was measured and the dilution of volume 50 ml was filtered through the weighed filters. The filter papers with the attached dried algal biomass were then dried in the oven at 80°C for 18 hours. After that the filter papers were allowed to cool down in the desiccator after which they were weighed and the algae mass could be calculated and the correlation between optical density and dry weight could be determined. The linear relationship between optical density and dry weight was calculated with Microsoft Excel (Appendix 1).

Picture 10. Algae biomass diluted 1, 2, 5, 10 and 20 times for determination of correlation between optical density and dry biomass.

In addition to calculating the linear relationship between optical density and biomass, other growth parameters were calculated. The specific growth rate (μ) during the first nine days of the cultivation was calculated, according to the following expression (Zhu, Hiltunen, Shu, Zhou, Li & Zhongming 2014: 105):

$$\mu\ (day^{-1}) = \ln (N_2/N_1) / (t_2-t_1), \qquad (4)$$

where N_1 and N_2 are the dry biomass (g/l) at time t_1 and t_2. The biomass productivity (P) was also determined, according to the expression

$$P = (DW_i - DW_0) / (t_i - t_0), \qquad (5)$$

where DW_i and DW_0 represent the dry biomass (g/l) at time t_1 and $t_{0,}$ where t_0 is the initial time. The doubling time was calculated, according to the following expression

$$T_2 = 0.6931 / r, \qquad (6)$$

where r is equal to μ when mortality is zero, and 0.6931 comes from the natural logarithm of 2 (Wood, Everroad, & Wingard 2005: 272).

6.2. Experiment II: Cultivation in 16°C

The aim of the second experiment was to cultivate *Scenedesmus dimorphus* in a cooler wastewater temperature and lower light intensity than in the previous experiment, and to examine nutrient removal and biomass accumulation under these conditions. The personnel at Pått was interested in nutrient removal by microalgae in the summertime at a water temperature of 16°C (Koivisto & Vesala 2017).

6.2.1. The pilot study 2

Before the real experiment was started a pilot study was conducted (13.-15.9.2017). *Scenedesmus dimorphus* was grown in wastewater for three days. The waters were 24 hours composite samples from after the pre-sedimentation pond (W1) and from after the sedimentation pond (W2). The cultivation was situated in a climate chamber with the intention to keep the temperature at 16 degrees and under day and night conditions with 12 hours dark and 12 hours light. The algae was cultivated in beakers placed on magnetic stirrers in the same light rack used in the previous experiment. There was no place for more than four magnetic stirrers in the light rack, which led to the decision to only study nutrient removal from W1 and W2. Two lamps per row (totally 6 lamps) were on between 7 am and 7 pm, and during nighttime the lights were switched off. Problems were encountered in keeping the temperature stabile, due to the warming effect of the lights. Despite efforts to adjust the temperature of the climate chamber the temperature fluctuated between 7.2°C and 19.7°C. The nutrient removal was modest. Ammonium was reduced by 25% in 24 hours and 32% in 48 hours in W1. TN was reduced by 21% after 24 hours and 44% after 48 hours in W1 while an increase in TN surprisingly occurred in

W2. The results for COD and TP were contradictory and showed both increase and decrease. The pH was lower than in the previous experiment, 8.77 ± 0.35, which could be a consequence of lower photosynthesis. During nighttime pH slightly declined which could indicate respiration of the algae. The combination of sudden bright light in the morning in combination with the cold night temperatures could also have caused photo-inhibition. At the end of the pilot study, the algae were collected and grown in room temperature to be used in the following experiment.

6.2.2. Description of experiment II

The experiment was carried out during nine days from 26.9 to 4.10.2017. The water samples used in the experiment were 24 hours composite samples collected by the personnel at Pått for their own water analyses. The water samples were from the same two points of the wastewater treatment process at Pått as in the first experiment, from after the pre-sedimentation pond (W1) and from the sedimentation pond (W2). The samples were picked up the 26.9 at 8 am and brought to the laboratory. The samples were poured into glass bottles and 1.4 liters of each water was autoclaved for 30 minutes, and the bottles were then left to cool down in a water bath.

The algae used in the pilot study, mixed with a little new algae from the batch cultivation, had been left to separate by gravity since the previous day. The algae had separated well and the supernatant was poured out. The thick algae suspension was poured into centrifuge tubes and centrifuged in 4500 rpm for 6 minutes. The supernatant was poured away and the algae were washed with tap water and centrifuged again. The supernatant was poured out, and the algae mass was mixed again with tap water, to make 45 ml of dark green thick inoculum for the experiment.

The autoclaved waters were poured into four 800 ml beakers, two beakers with W1 (1.1 and 1.2) and two beakers with W2 (2.1 and 2.2). At first 300 ml of respective water was poured into the beakers. The beakers were inoculated with 10 ml each of the prepared

dense algae inoculum. The beakers were then filled up to 500 ml with the respective wastewater.

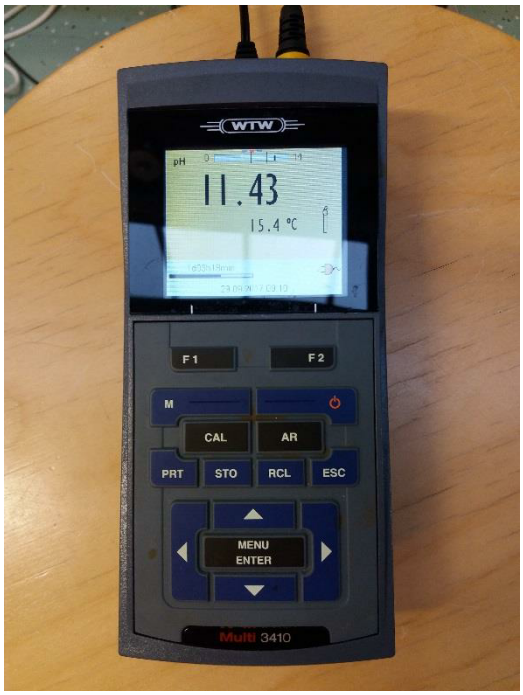
The beakers were placed on magnetic stirrers in the light rack situated in the climate chamber (Picture 11). Only two lamps were used during this experiment, lamp 3 in row 1 and 3 of the light rack. The light was on continuously 24 hours a day during the experiment. The mean light intensity in the light rack was 29,7 µmol m^{-2} s^{-1} within the range 5 to 110 µmol m^{-2} s^{-1}. The beakers were positioned in the light rack in the following order; 1.1 front left, 1.2 front right, 2.1 hind left and 2.2 hind right (Picture 12).

Picture 11. The setup of the experiment in the climate chamber.

For water analyses and biomass determination, 40 ml from each inoculated beaker was collected and centrifuged in 4500 rpm for 10 minutes. The unfiltered supernatant was used for water analyses. The algae mass was diluted with distilled water and filtered through pre-dried and pre-weighed filter-papers with pore size 1.2 µm. The filter-papers with the attached algae mass was dried in the oven in 90 °C for 18 hours, after which they were put in a desiccator to cool down before weighing. Water analyses were conducted on day 1, 2, 3 and 9. Biomass accumulation was measured by collection and weighing every second day, on day 1, 3, 5, 7 and 9. Before the collection of the algae suspension for measurements, the beakers were properly stirred. The amount of water removed for

the tests was not replaced as it would have diluted the algae suspension and the nutrients. The pH and the temperature were recorded continuously with a WTW Multi 3410 logging device (Picture 12). The sensor of the logging device was placed in beaker 1.1, and was programmed to take measurements every 30 minutes. The growth parameters were calculated according to the same equations as used in experiment I.

Picture 12. The placing of the beakers in the climate chamber. The sensor of the pH logging device was placed in beaker 1.1.



Picture 13. The WTW Multi 3410 meter used in Experiment II for logging of pH and temperature.

6.3. Margins of error

The accuracy of the measurements has a margin of error that needs to be noted at every step of process. It is more a rule than an exception that measurement results are associated with errors. This applies both to standardized methods of analysis and to routine analysis. Both systematic and random errors need to be prevented and avoided. Systematic errors affect the accuracy of the method of analyses, and may be a result of for example wrong sample volume, wrong pH, wrong reaction time or mixture. In systematic errors all the analysis results deviate from the real value in the same manner. Random errors affect the precision of the analysis results, and are minimized by good operating techniques and calculation of the mean value of multiple measurements (Hiltunen, Linko, Hemminki, Hägg, Järvenpää, Saarinen, Simonen & Kärhä 2011: 14-19; Docslide SQ NOVA 60 manual 2017: 23).

6.3.1. Duplicates and standard deviations

The cultivation experiments were conducted in duplicates with the waters collected from different points. The mean value of the measurements were calculated, and the standard deviations noted in the graphs and tables.

6.3.2. Accuracy of pH measurements

The pH-meter used in the first experiment was an Extech Instruments Exstik PH100, calibrated between pH 7 and 10. The pH-meter indicates when it needs to be recalibrated, and should give accurate results in between. According to product specifications the accuracy of the Exstik 100 is ± 0.01 pH in the range 0.00 to 14.00. In the second experiment pH was measured with a logging WTW Multi 3410 meter. According to product specifications the accuracy of the WTW Multi 3410 meter is ± 0.004 pH within the range -2 to 20 pH.

6.3.3. Accuracy of pipettes

The pipettes used for the measurements are adjustable Eppendorf Research Plus pipettes of dimensions 1 ml, 5 ml and 1000 µl. The pipettes are to be calibrated regularly. Despite the calibration of the pipettes, the human factor plays a large role when using a pipette. Typical for the accuracy of using a pipette is that it varies greatly with routine and style.

6.3.4. Accuracy of Spectroquant photometer

The Spectroquant® NOVA 60 photometer is an instrument for routine water analysis. More than 170 methods for Spectroquant® cell and reagent tests can be programmed. The accuracy of the Spectroquant is given separately for each different cell test (Sigma-Aldrich 2017).

6.3.5. Accuracy of Merck Spectroquant Cell Tests

Measurements of nutrients were conducted with Merck Spectroquant Cell Tests. The production control for all of the Merck tests is determined in accordance with ISO 8466-1 and DIN 38402 A51. According to product specifications the following accuracies are determined for the used tests (Table 1):

Test	Test Range (mg L^{-1})	Accuracy
COD	5.0-80.0 mg L^{-1}	± 1.8 (mg L^{-1} COD)
	50.0-500.0 mg L^{-1}	± 13.0 (mg L^{-1} COD)
TN	0.5-15.0 mg L^{-1}	± 0.6 (mg L^{-1} N)
	10.0-150.0 mg L^{-1}	± 5.0 (mg L^{-1} N)
TP	0.05-5.00 mg L^{-1}	± 0.06 (mg L^{-1} PO4-P)
	0.5-25.00 mg L^{-1}	± 0.4 (mg L^{-1} PO4-P)
NH4	0.6-20.6 mg L^{-1}	± 0.4 (mg L^{-1} NH4)
	5.2-103.0 mg L^{-1}	± 1.9 (mg L^{-1} NH4)
NH4-N	0.5-16.0 mg L^{-1}	± 0.4 mg L^{-1} (NH4-N)
	5.2-103.0 mg L^{-1}	± 1.9 (mg L^{-1} NH4-N)

Table 1. Given accuracy of the Merck Cell Tests used in the experiments. (Merckmillipore 2017a; Merckmillipore, 2017b; Merckmillipore, 2017c; Merckmillipore, 2017d; Merckmillipore, 2017e; Merckmillipore, 2017f; Merckmillipore, 2017g; Merckmillipore, 2017h).

6.3.6. Accuracy of the Spectrophotometer

The photometric absorbance accuracy of the Shimadzu UV1601 Spectrophotometer is ± 0.004 Abs (at 1.0 Abs) and ± 0.002 Abs (at 0.5 Abs). At the beginning of each measurement the spectrophotometer is calibrated (Auto Zero) with two cuvettes filled with distilled water, of which one is left in the spectrophotometer as reference zero when the absorbance of the cuvette with algae suspension is measured. The same cuvette was used for measurement of all different waters. In between measurements the cuvette was thoroughly rinsed with distilled water.

6.3.7. Accuracy of the scale

The scale used to weigh the filter-papers and the attached biomass is a Sartorius TE214S Lab balance scale. According to product specifications the accuracy of the scale is ± 0.1 mg (Scalenet 2017).

6.3.8. Accuracy of biomass measurements

The accumulation of biomass was determined by measuring optical density at 680 nm. Each measuring cylinder was inoculated with the same volume of algae suspension at the beginning of the experiment. The mean starting point of the optical density was 0.542 ± 0.011 g L^{-1} in experiment I and 0.272 ± 0.018 g L^{-1} in experiment II. Still it is not possible to inoculate the exact same amount of algae cells of the same lifecycle stage, which already at the starting point gives some uncertainty to the continuation of the experiment. The algae biomass tends to sediment to the bottom of the cylinder despite the aeration or magnetic stirring. Before the optical density measurement the algae mass was stirred properly to make the suspension as homogenous as possible. Still some algae attached to the bottom of the cylinders, to the glass pipes providing the aeration, and to the inside of the measuring cylinders above the water line due to the bubbling caused by the aeration. For the optical density measurement 1 ml of algae suspension was collected with a pipette and diluted with distilled water to proportions 1:3 or 1:6. The representativeness of the collected 1 ml sample can be random despite the efforts of mixing and providing a homogenous suspension. Two different people conducted the measurements using the exact same techniques, but the human factor still needs to be taken into account. As the biomass accumulated clumping and flocculation of algae mass increased, which also could affect the results of the measurements. The determination of biomass accumulation by optical density must be regarded as an estimate of the biomass.

6.3.9. Accuracy of correlation curve between optical density and biomass

The linear relationship between optical density and the biomass was determined by measuring optical density and weighing the biomass at different dilutions of the algae suspension. The regression line was calculated in Microsoft Excel. The R squared (R^2) is a number between 0 and 1, which describes how well the line statistically correlates with the data. The accuracy of the calculated correlation lines are ($x=OD_{680}$): W1: $R^2=0.9954$,

W2: R^2=0.9938 and W3: R^2=0.9857. The linear relationship between optical density and dry biomass in the different waters was determined at the end of the pilot study. The obtained correlation curves differ from each other in steepness. This provides quite different pictures whether one chooses to pick one correlation curve for all waters, or to use each correlation curve for each water. The reliability of the optical density as a measurement of biomass can be questioned. The colour of the algae cultivation had changed remarkably, which indicates changes in cell composition and optical properties

7. RESULTS

In the following chapter the results of the two experiments are explained separately, first the results of experiment I followed by the results of experiment II. After this a comparison between the results of the two experiments after 24 hours and after 9 days are illustrated.

7.1. Results from experiment I

The air temperature in the fume hood, where the algae cultivation was situated, was 24.0°C ± 0.8 °C. The correlation between OD_{680} and the dry weight of the algae biomass for the different waters was W1: $y=0.658x$ ($R^2=0.995$), W2: $y=0.565x$ ($R^2=0.994$), W3: $y=0.418x$ ($R^2=0.986$), where x is the optical density measurement. To illustrate the estimation of biomass accumulation the correlation for W2 was chosen, and applied to the three different data sets. The correlation was chosen because it represents a mean value of the correlations, and has a good coefficient of determination (R^2).

The concentration of algae biomass in the beginning of the experiment was (0.92 ± 0.02) g L^{-1}. The algae started growing immediately and the growth continued steadily for all waters during the first 9 days, after which the algae mass in W2 did not grow in the same pace as W1 and W3 for a few days (Figure 1.). The growth, nevertheless, continued for W2 throughout the experiment, whereas the growth in W3 planed out towards the end of the experiment. In W1 there was even a small decline in biomass during the last four days.

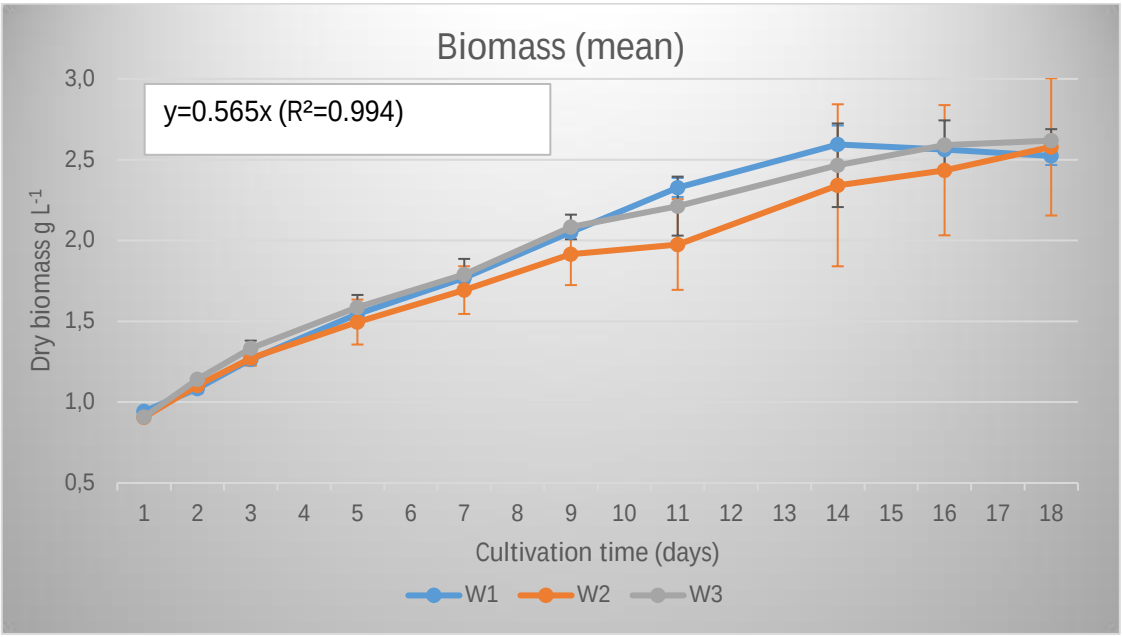


Figure 1. Accumulation of biomass during experiment I.

The growth parameters of the different waters were calculated (Table 2.). Specific growth rate, doubling time and biomass productivity were calculated on data from the first nine days of cultivation when the growth was steady, whereas the increase in biomass represents the whole duration of the experiment.

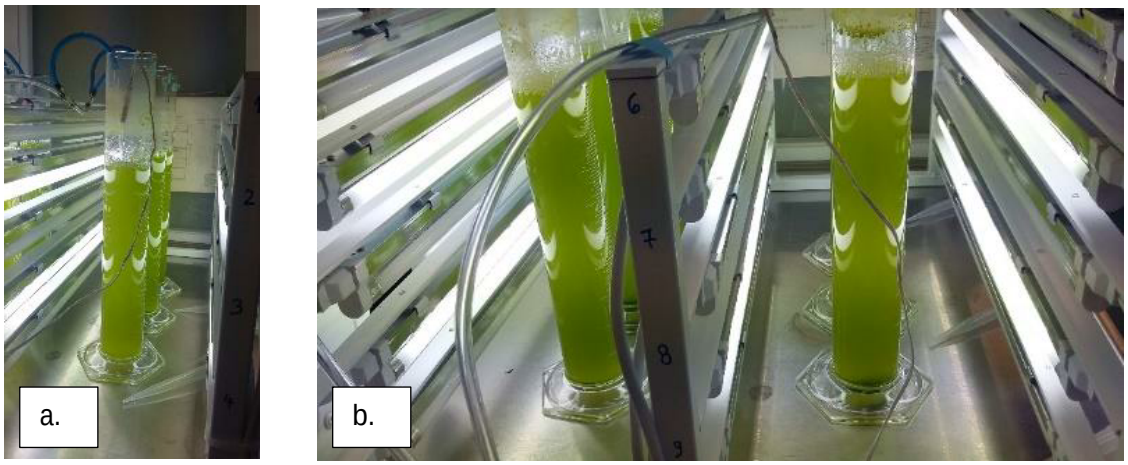
Table 2. Growth parameters of *Scenedesmus dimorphus* grown in wastewater (mean ± std. dev.).

	Specific growth rate (μ day^{-1})	Doubling time (days)	Biomass increase (g L^{-1})	Biomass productivity (g L^{-1} day^{-1})
W1	0.107 ± 0.040	6.47 ± 0.13	1.58 ± 0.05	0.146 ± 0.013
W2	0.112 ± 0.058	6.42 ± 0.78	1.67 ± 0.42	0.157 ± 0.036
W3	0.109 ± 0.068	5.74 ± 0.53	1.71 ± 0.10	0.182 ± 0.041

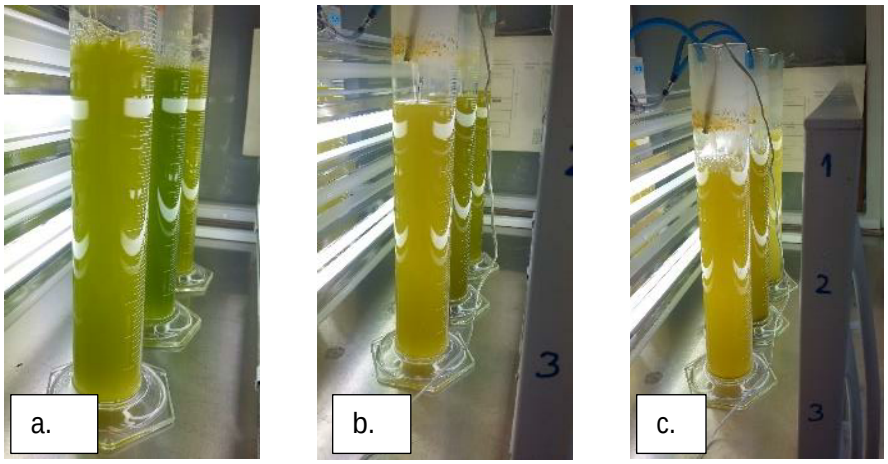
The specific growth rate was the biggest in W2, however, only slightly bigger than in the other two wastewaters. The shortest doubling time and the biggest biomass productivity was found in W3, which also had the largest increase in biomass at the end of the experiment.

The colour of the algae cultivation changed during the experiment. The algae mass from the pilot study was yellow in colour when inoculated into the cylinders with fresh wastewater (Picture 14). Overnight, from day one to day two, the colour changed from dark yellow to bright green, as the algae got new nutrients (Picture 15 a). The color then slowly turned more and more yellow towards the end of the experiment. The change in colour is illustrated in pictures 15 a-b, 16 a-c and 17 a-c.

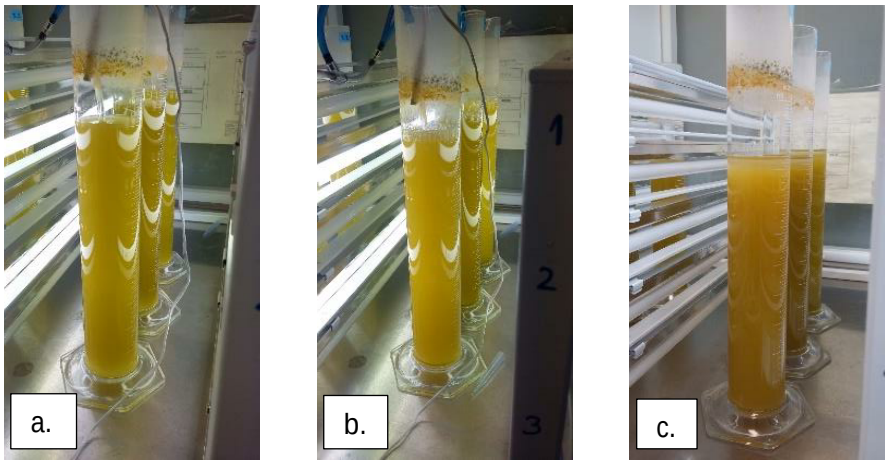
Picture 14. Algae cultivation experiment I, day 1 directly after inoculation.



Picture 15. Algae cultivation, day 2 (a) and day 4 (b).



Picture 16. Algae cultivation, day 7 (a), 11 (b), and 14 (c).



Picture 17. Algae cultivation day 16 (a) and 18 (b and c).

During the first 24 hours total nitrogen (TN) was removed from the water samples W1 by 86 %, W2 by 57 % and W3 by 87 % respectively. The nitrogen concentration was declining also in the tests made on day 9 and 18, as illustrated in Figure 2. By the end of the experiment, 83 % of TN was removed from W1, 90 % from W2 and 89 % from W3, respectively.

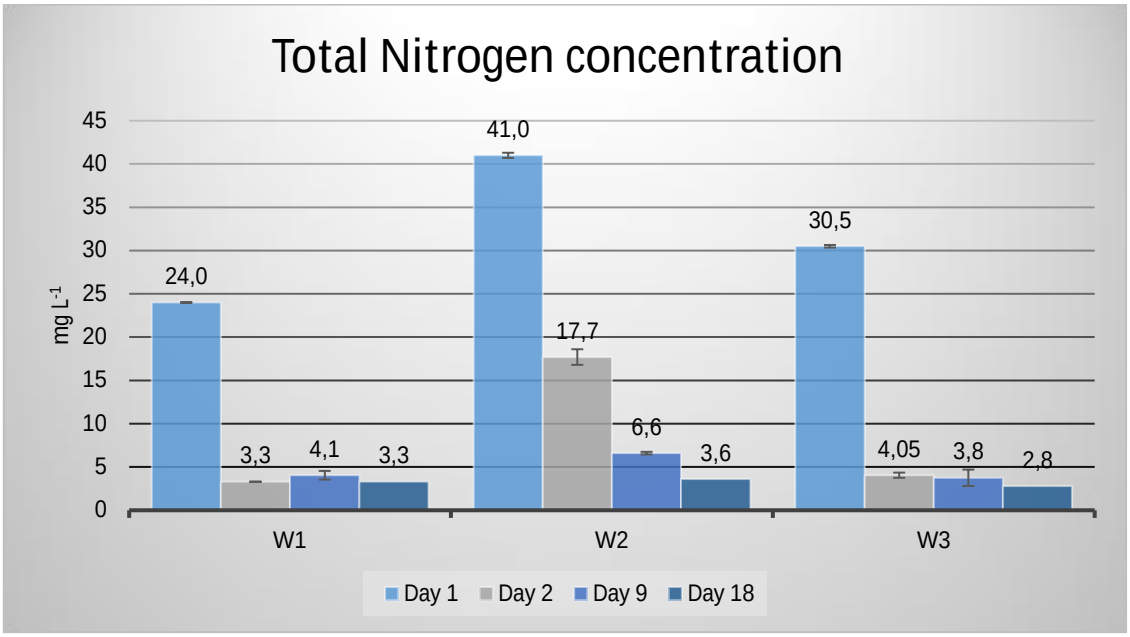


Figure 2. Removal of nitrogen during experiment I. The figure shows the three water samples on day 1, 2, 9 and 18.

Ammonium was only found in W1, the water collected after the pre-sedimentation. No ammonium was found in the other waters, because of the nitrification process at Pått that turns ammonium into nitrate. The amount of ammonium in W1 was 23.2 mg L^{-1}. All ammonium was removed during the first 24 h. The ammonium and nitrate could have been removed by the algae, or by so called ammonium stripping caused by the aeration in combination with the high pH elevated by photosynthesis.

The pH rose during the first 24 hours from 9.2 (W1), 8.7 (W2) and 8.9 (W3) to between 11.0 and 11.2. The pH stayed above 10.0 during the first 9 days, after which it gradually started to decline in most cylinders except for W2 (2.1 and 2.2 in Figure 3) where it started rising again. This new rise in pH synchronizes with the new rise in growth for W2 as shown in the biomass curve (Figure 1).

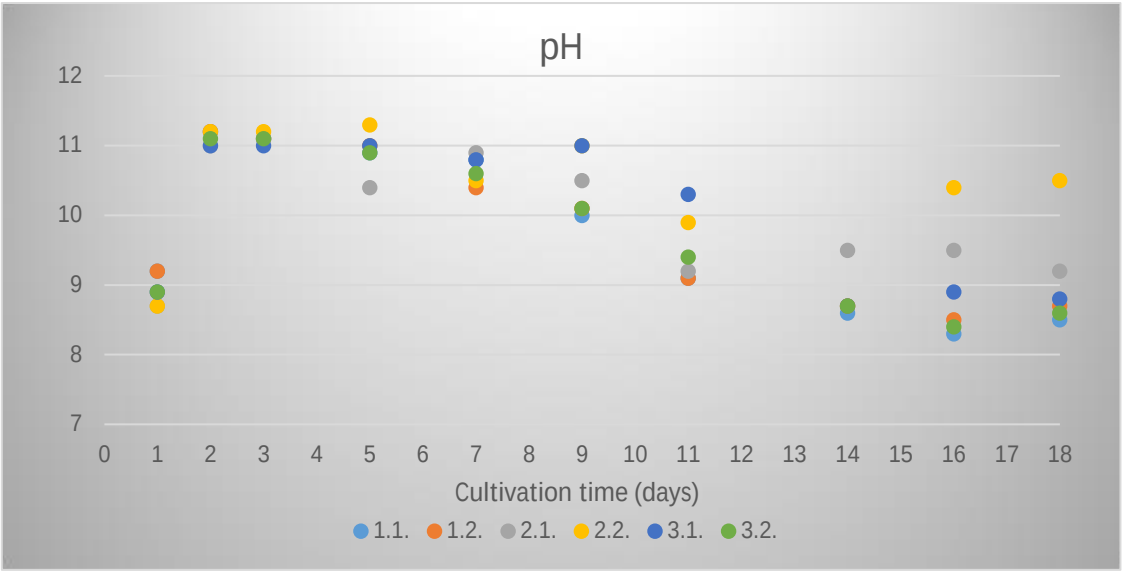


Figure 3. The pH of the different cylinders during experiment I.

The removal of total phosphorous (TP) was tested on day 1, day 2 and day 18. Unfortunately, the tests of suitable range ran out, and the new ones did not arrive on time for testing on day 9. The concentration of TP in the water samples was quite low. During the first 24 hours 77% of the TP had disappeared from W1, and 61% from W2. The decline in W3 was not as large, only 5%, but the amounts of phosphorous were also very small, as shown in the following graph. By the end of the experiment, 86% of TP had been removed from W1, 72% from W2 and 40% from W3, respectively (Figure 4).

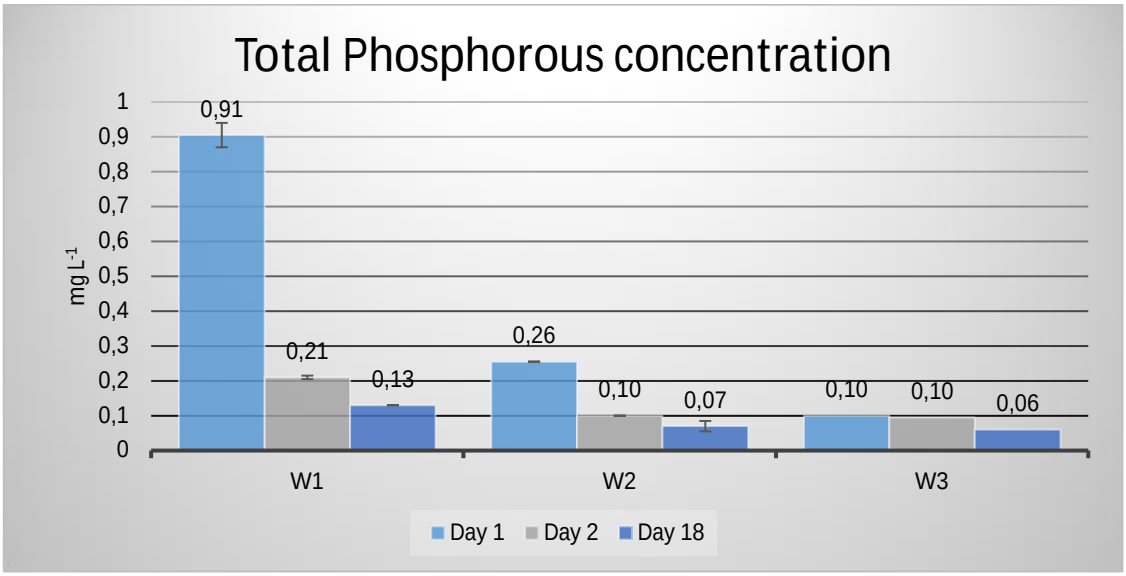


Figure 4. Removal of phosphorous in waters W1, W2 and W3 on day 1, 2 and 18.

The algae cultivation was not successful in removing chemical oxygen demand (COD) over the course of the entire experiment. The first test after 24 hours showed a decline in COD for W1. W1 showed an increase in COD on day 9, yet by the end of the experiment it was slightly declined. For waters W2 and W3, the increase from day 1 to day 18 was clear (Figure 5).

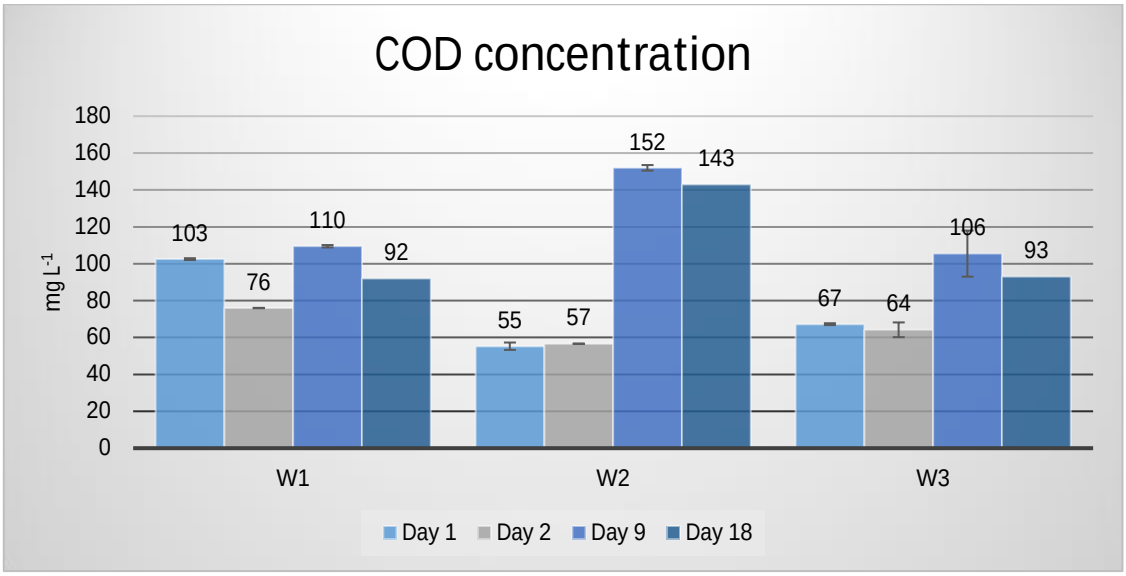


Figure 5. Development of COD during experiment I. The figure shows the development of COD in W1, W2 and W3 on day 1, 2, 9 and 18.

7.2. Results from experiment II

The color of the algae suspension was green at the beginning of the experiment and stayed green during the whole experiment. The mean temperature in beaker 1.1 was 15.5°C ± 0.5°C. The pH at the beginning of the experiment was a little bit above 9. The pH stayed around 9.5 for the first 36 hours, after which the pH started to rise. The pH increased during the following 30 hours up to 11.4, after which it stabilized between 11.2 and 11.3, as seen in Figure 6. Both the temperature and pH are illustrated in the graphs below. In both a peak can be seen on day 4 (Figures 6 and 7). The logging device ran out of batteries and had to be recharged for a few hours. The break in the measurements caused the peaks. The smaller peaks in the graph of the temperature is a consequence of the cooling system of the climate chamber. The lights of the light rack have a warming effect on the climate chamber, while the cooling fan of the climate chamber cools down the air on a regular basis.

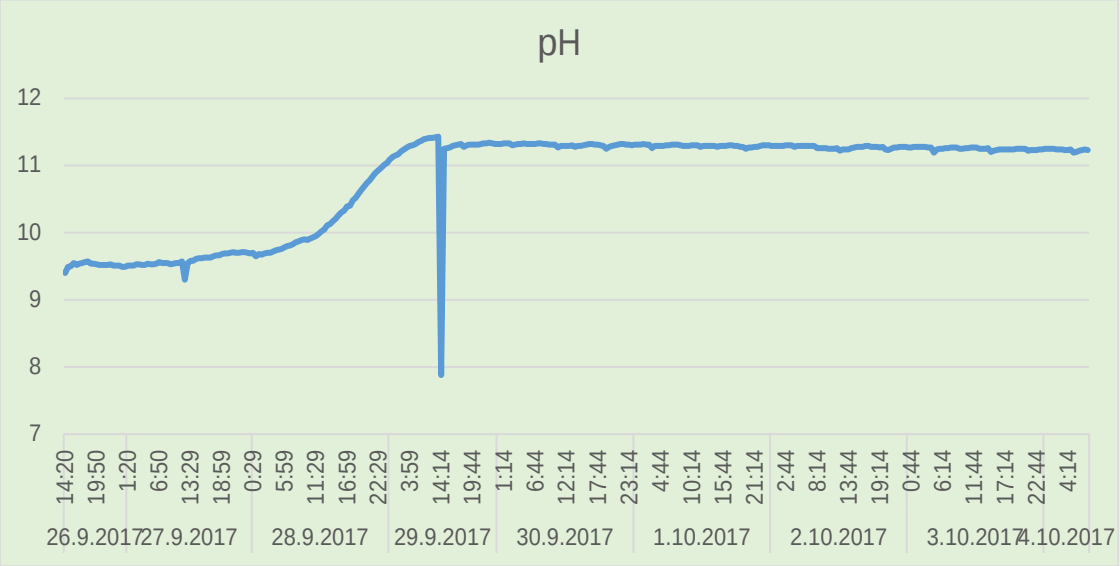


Figure 6. The graph shows the pH level of the experiment.

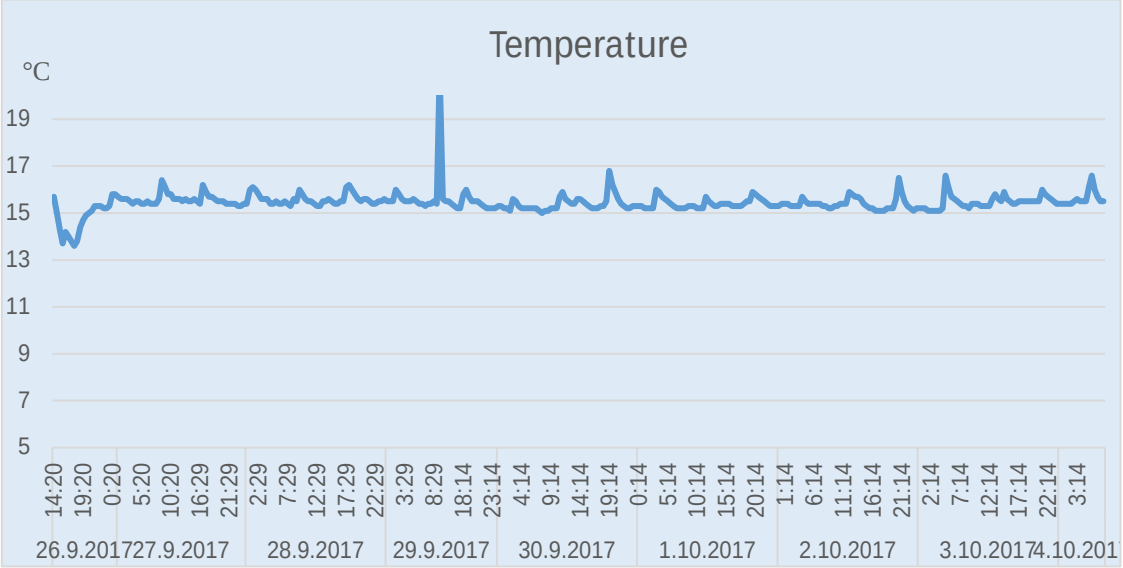


Figure 7. The temperature during the experiment.

The algae grew nicely during the cultivation in the climate chamber (Figure 8). The growth slightly slowed down during the last two days of the experiment. There is no difference in the accumulated biomass in the two waters, as seen in Figure 8. W2 showed a slightly higher specific growth rate and a shorter doubling time. The increase in biomass was also bigger in W2, but there was also a larger variance between the beakers. The growth parameters are shown in Table 3.

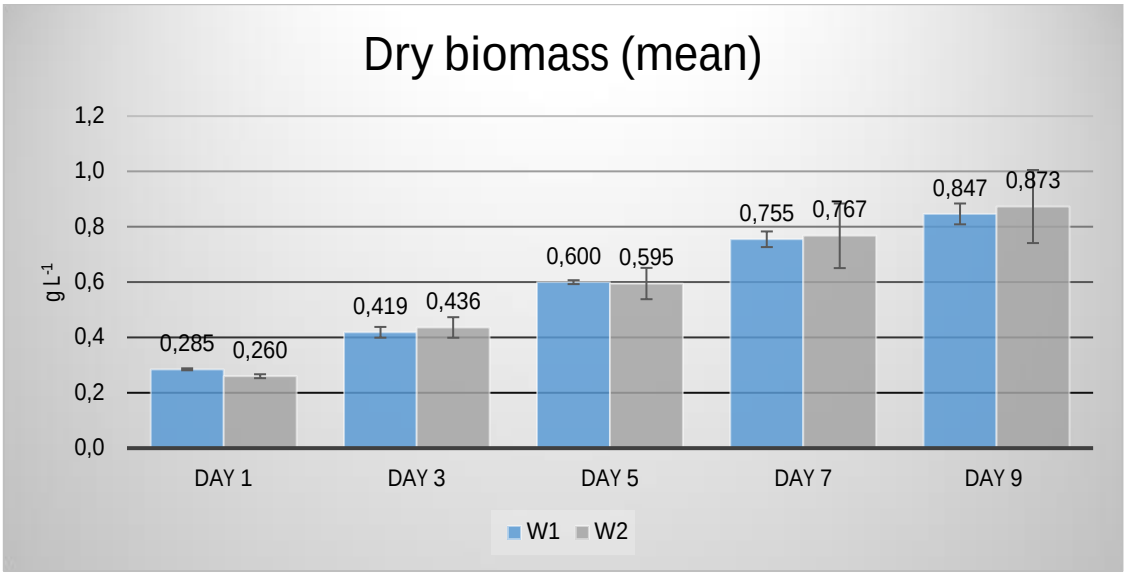


Figure 8. The accumulation of biomass during nine days of cultivation in 16°C.

Table 3. Growth parameters of *Scenedesmus dimorphus* grown in wastewater in 16°C (mean ± std. dev.).

	Specific growth rate (μ day^{-1})	Doubling time (days)	Biomass increase (g L^{-1})	Biomass productivity (g L^{-1} day^{-1})
W1	0.136 ± 0.059	5.10 ± 0.15	0.56 ± 0.03	0.037 ± 0.023
W2	0.151 ± 0.079	4.65 ± 0.69	0.61 ± 0.14	0.042 ± 0.031

The removal of total nitrogen is illustrated in Figure 9. Due to the nitrification process at Pått most of the total nitrogen is in the form of ammonium in W1 and in the form of nitrate in W2. The ammonium concentrations are illustrated in Figure 10. Ammonium nitrogen (NH_4-N) presents the weight of the nitrogen excluding the weight of hydrogen in the ammonium molecule. In W1 82 % of the nitrogen is in the form of ammonium (TN 28 mg L^{-1} and NH_4-N 22.9 mg L^{-1}). In W1, 47.9% ± 0.2% of the ammonium in W1 was removed during the first 24 hours, and 91.9% ± 0.7% during 48 hours. By the end of the cultivation there was no ammonium left in W1. The amount of ammonium left in W2 at the beginning of the experiment was very small (1.58 mg L^{-1}), and was almost completely removed during 24 hours. The effective removal of ammonium is reflected in the halving of TN during the first 24 hours in W1. The removal of nitrate was not as fast as the removal of ammonium. During the first 24 hours, 5.7% of TN was removed from W2. However, there was a notifiable difference between the beakers, with a removal of 2.8% in beaker 2.1 and a removal of 8.6% in beaker 2.2. After 48 hours 19.7% ± 3.6% of TN was removed from W2, and 43.7% ± 1.1% after nine days.

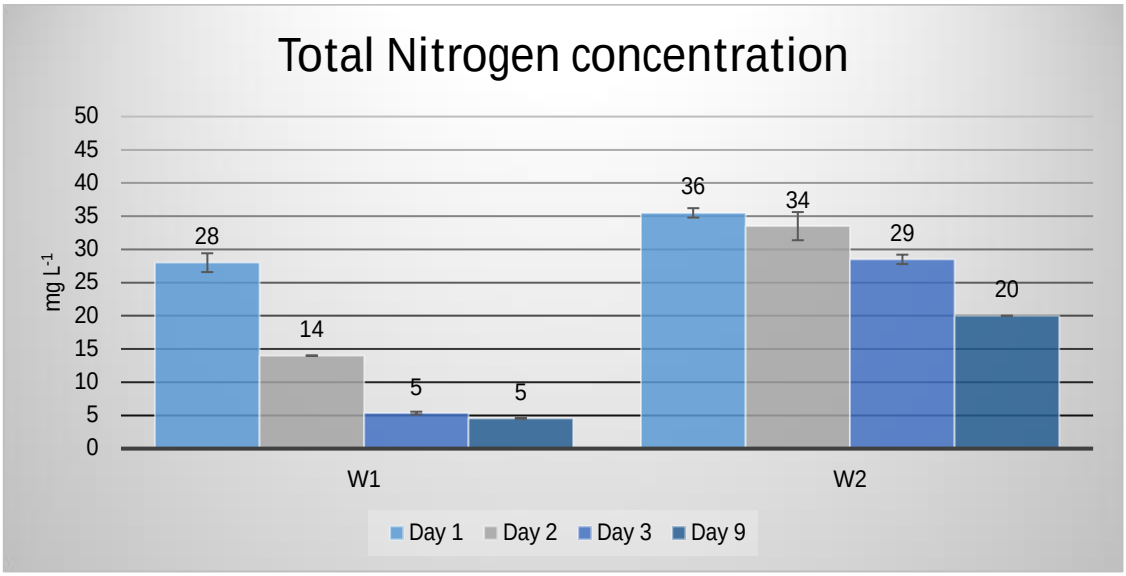


Figure 9. The removal of total nitrogen from the wastewater during 9 days.

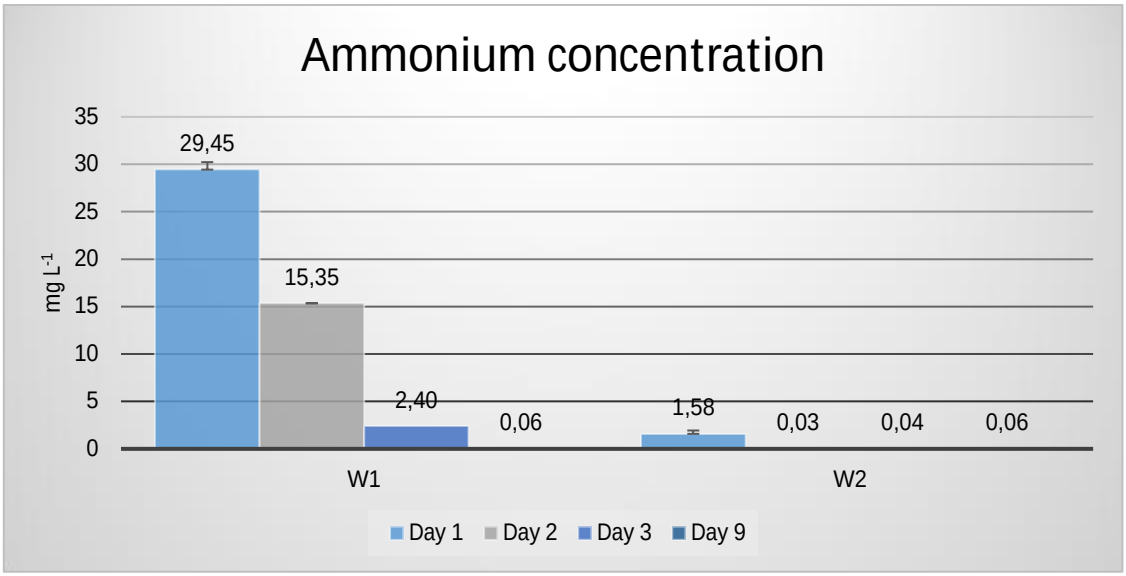


Figure 10. The ammonium concentration of W1 and W2 on day 1, 2, 3 and 9.

The concentration of phosphorous was not high in the waters at the start of the experiment, 1,5 mg L^{-1} in W1 and 0,4 mg L^{-1} in W2. After 24 hours 63 % of TP was removed from W1, after 48 h 72% and 77% by the end of the experiment. In W2, 60% of TP was removed during 24 hours, after which there were only some small fluctuations in

the concentration. The concentration of total phosphorous is illustrated in Figure 11 below.

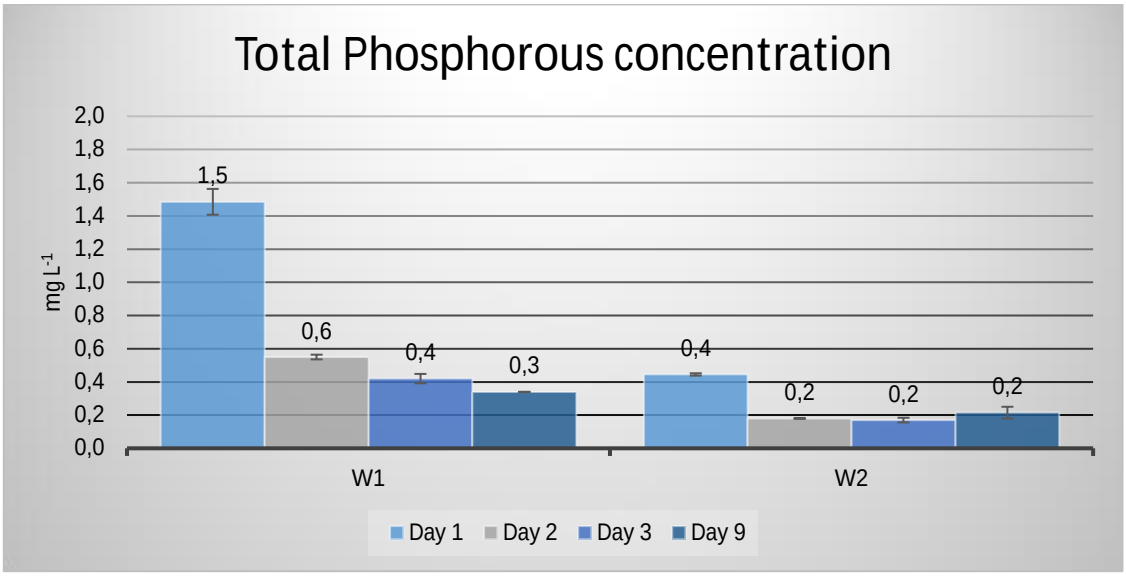


Figure 11. The concentration of Total Phosphorous in W1 and W2 on day 1, 2, 3 and 9.

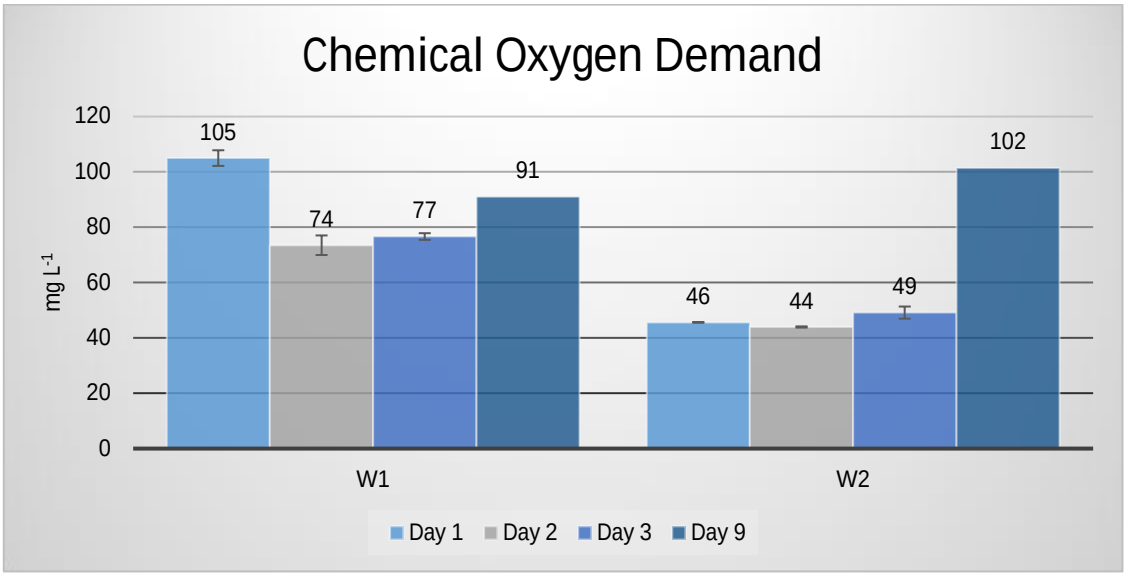


Figure 12. Chemical oxygen demand in W1 and W2 on day 1, 2, 3 and 9.

The chemical oxygen demand (COD) decreased in both waters during the first 24 hours. The reduction was bigger in W1, as shown in Figure 12. During 24 hours, COD was

reduced by 30% in W1 and 4% in W2. After the first reduction, COD started increasing in both waters. By the end of the experiment, the total reduction of COD in W1 was 13% compared with the starting point. However, the COD increased by 123% in W2.

7.3. Comparison between the results from experiment I and experiment II

The duration of experiment I was longer (18 days) than experiment II (9 days). However, the results are comparable during the first nine days of cultivation. The biomass at the starting point of the experiment was denser in experiment I, as shown in Figure 13. The mean biomass in the beginning was 0.924 g L^{-1} ± 0.022 g L^{-1} in experiment I, and 0.285 g L^{-1} ± 0.018 g L^{-1} in experiment II. In both experiments, the biomass density at the starting point was a little less in W2. In experiment I the biomass in W1 was 0.943 g L^{-1} versus 0.905 g L^{-1} in W2, in experiment II the biomass in W1 was 0.285 g L^{-1} versus 0.260 L^{-1} in W2. The specific growth rate was higher, and the doubling time was faster in both waters in experiment II. The biomass increase and the biomass productivity was bigger in experiment I (Table 4).

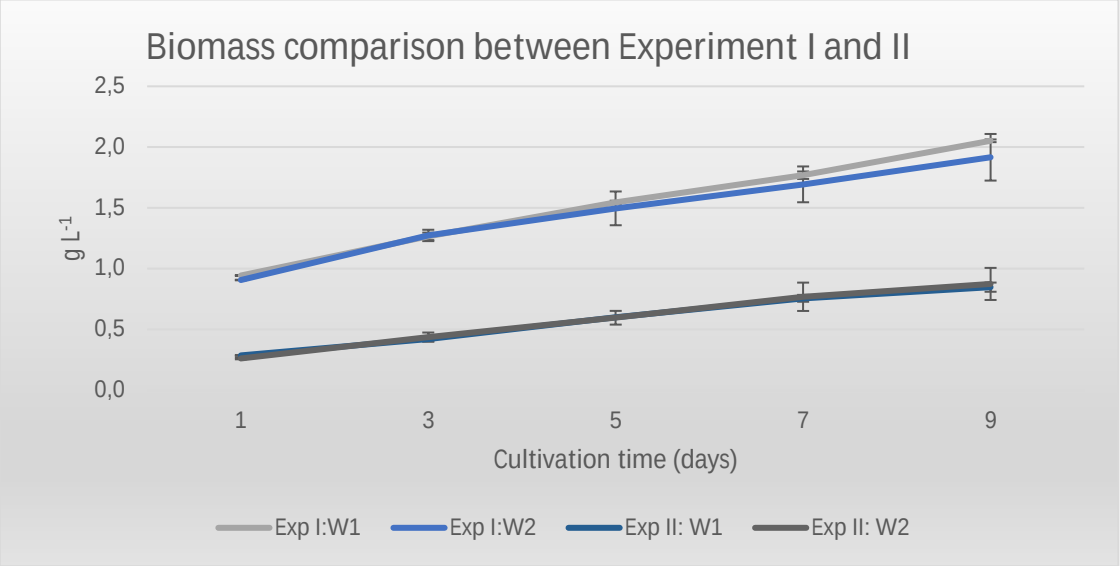


Figure 13. The biomass accumulation curve of W1 and W2 in experiment I and II during nine days.

Table 4. Comparison of growth parameters during 9 days of cultivation of *Scenedesmus dimorphus* in wastewater in 24°C (Exp. I) and 16°C (Exp. II) (means ± std. dev.).

	Specific growth rate (μ day^{-1})	Doubling time (days)	Biomass increase (g L^{-1})	Biomass productivity (g L^{-1} day^{-1})
Exp I: W1	0.107 ± 0.040	6.47 ± 0,13	1.11 ± 0.01	0.146 ± 0.013
Exp II:W1	0.136 ± 0.059	5.10 ± 0,15	0.56 ± 0.03	0.037 ± 0.023
Exp I: W2	0.112 ± 0.058	6.42 ± 0,78	1.01 ± 0.19	0.157 ± 0.036
Exp II:W2	0.151 ± 0.079	4.65 ± 0,69	0.61 ± 0.14	0.042 ± 0.031
Exp I: W3	0.109 ± 0.068	5.74 ± 0,53	1.18 ± 0.11	0.182 ± 0.041

The removal of nutrients in the two experiments have both similarities and differences. The concentration of nutrients in the beginning of the experiments are in the same range and comparable. The concentrations can be seen in Table 5.

Table 5. Starting point (mg L^{-1}) of nutrients in the experiments.

		COD	TN	TP	NH4
W1	I	103	24	0,9	23
	II	105	28	1.5	29
W2	I	55	41	0,3	0
	II	46	36	0,4	1.6

A comparison of nutrient removal during Experiment I and II can be seen in Figure 14 a-d. Instead of removal, an increase in the chemical oxygen demand was seen in both experiments. In W1, a decrease happened during 24 hours, and after 9 days, a small increase was seen in experiment I. In W2, a small decrease in COD occurred during experiment II after 24 hours, but in both experiments, COD had more than doubled after nine days.

Total phosphorous removal was efficient in both waters already after 24 hours, and almost no difference between the two experiments can be seen. Unfortunately, there are no records of TP removal on day 9 for experiment I. In W1 the TP removal is a little lower in Experiment II than in experiment I, but the starting concentration was also higher.

The removal of ammonium was more or less complete in both waters and both experiments after nine days. In experiment I ammonium was completely removed already

after 24 hours, while the process was a little bit slower in experiment II. The concentration of NH_4 in W2 was under the test range in the first experiment. For the second experiment, tests of a smaller range were acquired.

The removal of total nitrogen in W1 reflects the effective removal of ammonium. The removal of the nitrogen that is in the form of nitrate is slower than the removal of ammonium. The removal of TN from W2 in experiment II was not as effective as in experiment I.

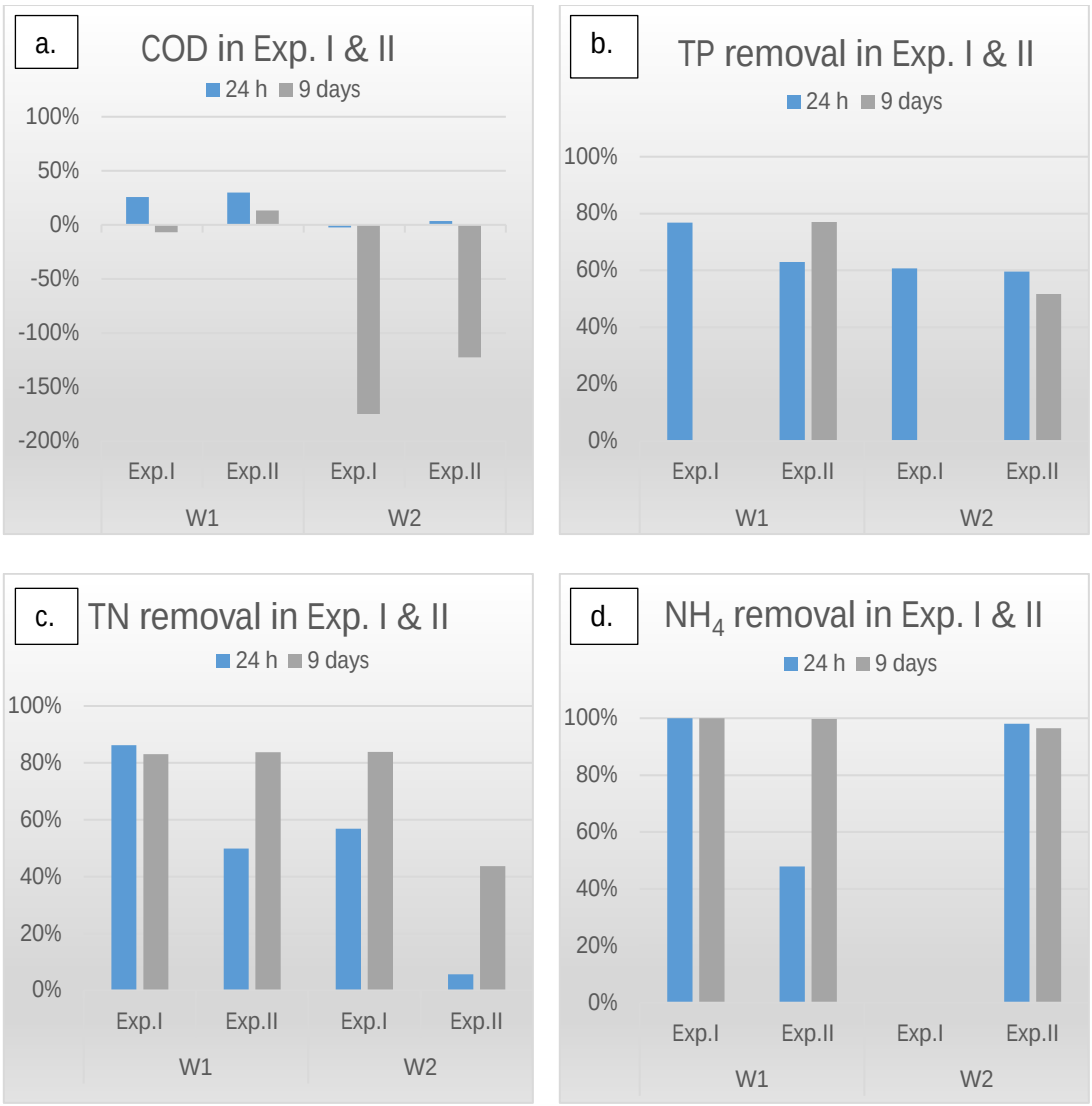


Figure 14 a-d. Comparison of nutrient removal in experiments I and II. The blue bars show the removal in percentage after 24 hours, the grey bar after 9 days. In each figure the removal in W1 is presented to the left and removal in W2 to the right.

When analyzing the results it is important to keep in mind that the pretreatment of the waters for the laboratory experiments have some impact on the nutrient values. Table 6 shows a comparison between the results of the water analyses made at Pått and the water analyses made in the present study.

Table 6. Results of the water analyses (mg L^{-1}) made at Pått and at University of Vaasa in June and in September 2017. The water was filtered prior to autoclaving in June.

12.6.2017						
	W1		W2		W3	
	Pått	UVA	Pått	UVA	Pått	UVA
COD	163.0	102.5	61.2	55.3	68.8	67.3
TN	32.5	24.0	40.5	41.0	27.9	30.5
NH4+/-N	38.9	23.2			0.0	
TP	2.8	0.9	1.1	0.3	0.2	0.1
26.9.2017						
	W1		W2			
	Pått	UVA	Pått	UVA		
COD	195.0	105.0	48.4	45.6		
TN	28.2	28.0	28.9	35.5		
NH4-N	32.1	22.9	-	1.2		
TP	2.6	1.5	0.5	0.4		

Differences can be noticed especially in COD and TP values, where the differences are particularly large in W1. Filtration has a huge impact on the values of COD and TP. The water samples at Pått are not filtrated nor autoclaved prior to water analyses. The water analyses are made for ammonium (NH_4^+) at University of Vaasa and for ammonium nitrogen (NH_4-N) at Pått.

On day 18 of Experiment I, the water samples for the duplicate test for water analyses were filtered through a filter with bigger pore-size. 1.2 µm filters were used for the supernatant instead of 0.45 µm as in sample 1, because of a lack of equipment in the laboratory. The bigger pore-size resulted in two and three-fold amounts of nutrients in the water analyses, as shown in Table 7. The sample 2 (the duplicate) could not be compared

with the previous tests. The water samples in experiment II were not filtrated but they had to be centrifuged to remove the algae cells.

Table 7. The table shows the results of different pore-size for sample-filtration on the test results for TP and COD on day 18 of experiment I. Sample 1 is filtrated with pore-size 0.45µm, and sample 2 is filtrated with 1.2 µm.

TP			COD		
	Sample 1(mg L^{-1})	Sample 2(mg L^{-1})		Sample 1 (mg L^{-1})	Sample 2 (mg L^{-1})
W1	0.13	0.32	W1	92	306
W2	0.07	0.18	W2	143	314
W3	0.06	0.17	W3	93	302

The differences in nutrients due to the pretreatment are reflected in the proportions of the nutrients, particularly in the ratio between nitrogen and phosphorous. Table 8 shows the molar N:P ratios in the waters. The N:P ratio is higher in the pretreated water than in the original wastewater.

Table 8. The molar ratios of nitrogen and phosphorous in W1, W2 and W3.

	W1	W2	W3
Pått 12.6.2017	25	84	308
Pått 26.9.2017	24	118	
Exp. I	59	356	674
Exp. II	41	199	

8. DISCUSSION

In this study the accumulation of biomass and removal of nutrients by the microalgae *Scenedesmus dimorphus*, cultivated in wastewater in two different temperatures, were measured. There were no differences in the biomass accumulation between the different test points. In the first experiment, the algae grew very well in the water dispatched to the sea, W3, which was surprising as one could expect the algae to grow more in water with more nutrients. The most important growth parameters are light, temperature and carbon. Since methanol is added as a carbon source for the denitrification process in the after treatment unit at Pått, the good growth rate and biomass productivity in W3 may be influenced by the availability of an organic carbon source. At the start of the first experiment, the COD value of W3 is higher than that of W2. However, the difference falls into the margin of error for the test kit.

In both experiments, W2 showed a large standard deviation in growth of biomass. The mean value does not differ from W1. However, in both experiments the difference in biomass growth between 2.1 and 2.2 is large, representing both the best biomass growth and the least. It is uncertain what this might depend on.

In comparison to other studies, the specific growth rate [mean (0.107-0.136) μ day^{-1}] and the doubling time [mean (4.65-6.47) days] of this study are quite low, while the biomass increase [mean (0.56-1.18) g L^{-1}] and the biomass productivity [mean (0.037-0.182) g L^{-1} day^{-1}] are quite good. Zhu et al. (2014) cultivated *Chlorella zofingiensis* in artificial wastewater. The specific growth rate of *C. zofingiensis* in that cultivation experiment was (0.208-0.260) μ day^{-1}, and the doubling time (2.67-3.34) days. The biomass increase was 0.277 g L^{-1} and 1.004 g L^{-1} and the biomass productivity (0.018-0.067) g L^{-1} day^{-1}. The initial biomass concentration of the cultivation that was carried out for 15 days was less than 0.1 g L^{-1}. Eustance et al (2013) studied nitrogen removal from wastewater with two microalgae strains, *Scenedesmus* sp. and *Monoraphidium* sp. The algae was supplied urea, nitrate or ammonium and sparged with 5 % CO_2 or air, with or without pH regulation. Regardless of the substrate, both algae strains showed better specific growth rates sparged with CO_2. The mean specific growth rate (during exponential phase) of

Scenedesmus sp. was (0.90-1.18) μ day^{-1} with added CO_2, and (0.22-0.55) μ day^{-1} sparged with air. The final biomass concentrations were (0.34-2.41) g L^{-1} (CO_2) and (0.26-1.23) g L^{-1} (air), respectively. The mean specific growth rate of *Monoraphidium* sp. during the exponential growth phase was (0.43-0.49) μ day^{-1} sparged with air and (1.09-1.58) μ day^{-1} sparged with CO_2. The final biomass concentration was (0.37-1.13) g L^{-1} (air) and (1.26-1.89) g L^{-1} (CO_2) respectively. The cultivation experiment went on for 26 days.

Scenedesmus dimorphus showed higher specific growth rate and shorter doubling time in W2 compared to W1 in both 24°C as well as in 16°C. For both W1 and W2 the specific growth rate and doubling time was better in 16°C (Exp. II). It is important to keep in mind that the concentration of the algae mass at the starting point was much lower in Experiment II, which have impact on the growth parameters. This fact makes the growth parameters of the two experiments less comparable. A more diluted algae cultivation facilitates more effective growth, while a doubling of a denser culture renders more biomass than the doubling of a more dilute cultivation. The biomass increase was higher in both waters at 24°C (Exp. I). However, the starting density of the cultivation was also higher in Experiment I. In Experiment I, over the course of 9 days, the biomass increase was the highest in W3, and the least in W2. In Experiment II the biomass increase was slightly higher in W2 than in W1. Regarding the biomass increase, however, the large standard deviation of W2 needs to be noticed.

The discoloration of the algae cultivation in the first experiment could depend on nitrogen deprivation. The algae cultivation turned from bright green to a more and more yellow shade during the experiment. Discoloration is a typical response to nitrogen limitation. The discoloration is caused by a decrease in chlorophylls and an increase in carotenoids. There is also an accumulation of organic carbon compounds and oils (Grobbelaar 2013: 126-127). Nitrogen appears mostly in the form of ammonium in W1, and of nitrate in W2 and W3. Very little ammonium was found in W2, a consequence of the nitrification process in the aeration ponds at Pått. Before the water reaches the sedimentation ponds (W2) ammonium turns into nitrate. Due to the pre-treatment, the results for total nitrogen and ammonia show smaller amounts in the analyses made at University of Vaasa than at Pått. The TN value of the water analyses made at University of Vaasa is also lower in W1

than in W2. The water samples used in the experiments are autoclaved, which probably causes some loss of ammonia or ammonium due to the heating.

The removal of nutrients from wastewater by microalgae have been observed in numerous studies. Sacrístan de Alva et al. (2013) cultivated *Scenedesmus acutus* in municipal pre-treated (primary settling) and post-treated (activated sludge) wastewater. They obtained the highest nutrient removal and biomass and lipid accumulation in municipal pretreated wastewater. The removal of phosphates was 65% and the removal of organic nitrogen and ammonia 92%. A high removal of COD (77%) was observed. The highest content of lipids (28.3%) was also retrieved in microalgae that was cultivated in the municipal pretreated wastewater. Zhu et al. (2014) cultivated *Chlorella zofingiensis* in artificial wastewater. pH regulation of the algae cultivation increased the TN removal from 45% to 74%, and the TP removal from 92% to 100%. The pH regulated group also had the highest lipid productivity (37.5 mg L^{-1} day^{-1}) with a biodiesel yield of 19.4% of dry weight. Sirin & Sillanpää (2015) also observed high removal of TN and TP between 74% to 90% when cultivating *Nannochloropsis oculata* in municipal wastewater. Gentili (2014) studied microalgal growth and lipid production as well as nutrient removal on mixed municipal and industrial wastewater (pulp-, paper- and dairy-), while adding CO_2 by flue gas bubbling. Three strains of microalgae species were tested, *Scenedesmus dimorphus*, *Selenastrum minutum*, and a locally isolated strain of *Scenedesmus* sp. All three strains reduced the total nitrogen to the same level found on the time of the final harvest already in two days. Ammonium was reduced between 96% to 99%, and phosphate between 91% to 99%. The highest biomass and lipid yield (37% of dry matter) was found in *Selenastrum minutum* (Gentili 2014).

The results of this study are in accordance with the observations above. During experiment I all ammonium was removed during the first 24 h. The ammonium could have been removed by the algae or by so-called ammonium stripping caused by the aeration in combination with the high pH elevated by photosynthesis. When the pH of the water rises above 11, ammonium starts converting to dissolved ammonia. The ammonia gas can be driven away from the water by aeration of the water column (Lindquist 2003: 85). This makes it difficult to draw conclusions about the ammonium and total nitrogen removal in experiment I. However, in experiment II the aeration was excluded to

eliminate that interference. Still almost half of the ammonium was removed during the first 24 hours, and 92 % of the ammonium was removed after 48 hours. The pH rose above 11 approximately 60 hours after the start of the second experiment, and no air bubbles were provided to lift gas to the surface.

In experiment I, the biomass accumulation was the most effective in W1 during the first 14 days of cultivation. However, the biomass in W1 stopped growing and started to decline by cultivation day 14. Perhaps, the effective uptake of ammonium leads to the algae faster reaching a state of nutrient limitation that slows down the growth of biomass.

Quite contrary to the expectations, after initial decrease in W1 and no change in W2, COD increased instead of decreased during the experiments in the long run. The increase in COD was particularly great in W2 in both experiments. An increase in COD has been reported in other studies as well. Gentili (2014) found that COD increased in the supernatant of three different wastewater mixes when cultivating *Selenastrum minutum*. Microalgae are known to excrete organic compounds in the substrate. It is a normal function of algal cells to release extracellular organic substances. These can be carbohydrates, amino acids, peptides, phenolic compounds, enzymes and vitamins. They are recycled in the aquatic food chain. It is also suggested that the exudation products also serve other functions, as to reduce heavy metal toxicity, reduce sinking rate of cells, to prevent grazing and to help in utilizing nutrients in scarce situations. The production and composition of these excreted products vary with the growth status and growth phase of the algae, the photosynthetic rate, with nutrient conditions and of course with species (Burkiewicz & Synak 1996). The increase in COD in W2 can depend on the chemical composition of the water. The most evident difference between W1 and W2 is the nitrogen composition as mentioned in the previous texts.

One reason why the algae showed signs of nitrogen deprivation although the water was not totally depleted of nitrogen may be the proportions of nitrogen, phosphorous and carbon. The literature often refers to the Redfield ratio for phytoplankton to apply for microalgae. This ratio comes from the average chemical composition of phytoplankton that is 106C: 16N: 1P. This ratio is often considered when examining possible nutrient deprivation. According to this molar ratio, most growth media have too much nitrogen in

relation to carbon, and may become carbon limited. When the pH of the algae cultivation rises quickly to 9 and higher, this can be an indication that carbon is limited. A N:P ratio higher than 16 makes the algae phosphorous limited (Harrison & Berges 2005: 26). The preferred N:P ratios are species specific, and vary between 8-45. Addition of nitrogen or phosphorous may be required to reach the proper ratio even when using wastewater as a growth medium for microalgae (Christenson & Sims 2011: 688). The optimal N:P ratio is the ratio where neither N nor P is limiting growth (Thrane, Hessen & Andersen 2017).

In the experiments the N:P ratio in W1 was 59 in experiment I, and 41 in experiment II, while in W2 it was 356 in experiment I, and 199 in experiment II. In W3 the ratio was even higher, 674. However, in the untreated wastewater the N:P ratio is lower in W1, in the range 24-25. The pre-treatment of the water for the laboratory tests, especially the filtration of the wastewater in experiment I removes some of the phosphorous and makes the N:P ratio even bigger.

Arbib, Ruiz, Álvarez-Díaz, Garrido-Pérez, Barragan & Perales (2013) studied *Scenedesmus obliquus* growth in wastewaters with N:P ratios 1, 3, 5, 9, 13, 22 and 35. They examined the effect of the different N:P ratios on growth rate and nutrient removal (in temperature 20°C ± 1°C). They found that the final concentration of biomass was divided into two groups. Those with N:P ratio below 9 had lower final biomass concentration than those with ratio 13 or above. They concluded that for overall maximum productivity N:P ratio in wastewater should be in the range 9-13. They also found that the removal of nitrogen and phosphorous was most efficient in the same particular range for *Scenedesmus obliquus*. A cultivation with N:P ratio below this range would probably be nitrogen limited, and a cultivation with a ratio higher than this would probably be phosphorous limited (Arbib et al. 2013). Thrane et al. (2017) examined how the optimal N:P ratio of the microalgae *Chlamydomonas reinhardtii* responded to changes in the temperature of the environment. The temperatures in the experiment was between 10.3°C and 18.1°C, and the N:P ratio between 7.8 and 96.4. They found that with increasing temperature, the optimal N:P ratio also increased. They suggest that at high temperatures the algae cells require more nitrogen relative to phosphorous, and vice versa. The optimal N:P ratio for *Chlamydomonas reinhardtii* increased from 26.5 to 36.5 over the examined temperature gradient (Thrane et al. 2017).

Based on these findings, it is more likely that the cultivations during the experiments were phosphorous-limited, especially in W2 and W3. The first experiment showed signs of nutrient deprivation by the change in colour from green to yellow over the course of 18 days. The second experiment showed no signs of nutrient deprivation within 9 days. The N:P ratio of W1 is closest to the optimum range found by Arbib et al. (2013), and could indicate that W1 is more suitable for algae cultivation. The real, untreated wastewater has a N:P ratio of around 22-24, which is even closer to the optimum ratio for *Scenedesmus obliquus* found by Arbib et al.

Based on the results of this study, the removal of nutrients was most efficient in W1. The removal of total nitrogen was more efficient in W1 because of the efficient removal of ammonium. The removal of total nitrogen was more efficient at higher temperature and light intensity than at lower. The removal of total phosphorous was slightly more efficient in W1, but there was no big difference between the different temperatures. There was no high removal of chemical oxygen demand from W1 in either experiment, but the COD did not increase as it did in W2 over the course of 9 days. Considering the flow rate of water through the municipal wastewater plant Pått, the nutrient removal during 24 hours is of most interest.

9. CONCLUSIONS AND FUTURE RESEARCH

The results of this study show that *Scenedesmus dimorphus* can be grown in wastewater from the municipal wastewater treatment plant Pått. This study indicates that *Scenedesmus dimorphus* is most effective in nutrient removal from the water collected after the pre-sedimentation pond (W1).

There were no large differences in biomass accumulation between the different test points. Based on the results of this study, the water to choose varies depending on the aim and duration of the cultivation. If the goal is to maximize algal biomass production, the water to be dispatched to the sea (W3) showed the best result both at the time span of 9 and 18 days. Biomass accumulation was effective in W1 during the first 14 days of cultivation. The results of this study indicates that the water collected after the pre-sedimentation pond (W1), might be the best choice for cultivation of *Scenedesmus dimorphus*, when restricting the cultivation time to 14 days or less. If the aim is to cultivate *Scenedesmus dimorphus* for a longer period, the water dispatched to the sea (W3) may be to prefer.

Based on the results of this study, cultivation of *Scenedesmus dimorphus* in 24°C is more efficient in both nutrient removal and production of biomass than cultivation in 16°C is. However, during the experiments the light intensity was also lower during cultivation in 16°C, which also affected the growth of the algae.

There has been discussion about building a sludge pipe from the wastewater treatment plant Pått to the local waste disposal plant Stormossen. Today the sludge is transported from Pått to Stormossen by trucks. The proposed advantage with growing microalgae in the wastewater is that it would make the sludge more slippery and the sludge would run more easily in the pipe. Simultaneously the algae biomass would contribute to the biogas production at Stormossen. The land area at the municipal wastewater treatment plant Pått is restricted. The plant is situated by the shore in the urban area of Palosaari in the city of Vaasa, and there is no room for expansion of the area. Cultivation of microalgae at the wastewater treatment plant would need land area that is not currently available. However,

there has been discussion about building a walk along the shore, which would leave a quite large amount of wetland available for maturation ponds.

At the moment, the most likely possibility for using microalgae at Pått would be to use it in the existing water treatment process to remove nutrients. However, there is a basic contradiction between the time it takes to remove nutrients and to accumulate biomass, and the time it takes for the water to run through the process at the wastewater treatment plant. Some adjustments to the process would have to be made, and the most likely way would be to lead some of the water from the treatment process to the algae cultivation. The laboratory scale experiments indicate that the most suitable point for microalgae would be in or in connection to the pre-sedimentation pond (W1). The challenge would be to remove the microalgae before further steps of the process and from the water before reaching the sea. Before microalgae can be used in wastewater treatment and/or for production of biofuel or biogas, a cost-effective and energy efficient harvest method must be developed. This is a universal problem for algae cultivation, and the area of algae research in which most effort and input is required.

More experiments need to be conducted at lower temperatures and at different light intensities. An even shorter time between water analyses, for example 6 h, 12 h, 18 and 24 h is of interest, because of the flow rate of the water running through Pått. In the laboratory, controlling the pH of the cultivation would be of interest to avoid interference with ammonium removal. Addition of carbon dioxide to the cultivation is also necessary to maximize biomass accumulation.

Experiments on untreated wastewater have to be done. The pretreatment made to the water prior to the experiments of this study changes some of the properties of the wastewater. The pH of the untreated wastewater is lower, and the ratio of nutrients is slightly different. The untreated wastewater also contains microorganisms. The microorganisms of the wastewater can have a both positive and negative influence on algae growth. The next step would be to try microalgae cultivation at pilot scale in wastewater from Pått. Biogas production is a growing trend in Ostrobothnia. Further studies on macro algae (seaweed) in wastewater treatment would also be interesting.

SUMMARY

The aim of this thesis was to study the feasibility of cultivating the microalgae *Scenedesmus dimorphus* in waters from the local municipal wastewater treatment plant Pått. The cultivation of algae in wastewater from three different points in the wastewater treatment process was evaluated. The cultivation took place in the laboratory under two different temperatures and light intensities. Growth of biomass and removal of chemical oxygen demand, total phosphorous, total nitrogen and ammonium were measured.

The removal of nutrients was most efficient in the first test point, water collected after the pre-sedimentation pond. At this point of the wastewater treatment process the nitrogen is mostly in the form of ammonium, which is easily available for the microalgae. The nutrient removal was more effective in the higher temperature than the lower. There was no large difference in biomass accumulation between the test points, but the biomass accumulation was also greater in the higher temperature and stronger light intensity.

The laboratory study indicates that cultivation of microalgae in wastewater at the local wastewater treatment plant Pått could be feasible. However, further studies need to be done at pilot scale with wastewater that is not pre-treated. Before application of microalgae in the wastewater treatment process, the efficient removal of the microalgae from the wastewater must be solved. Currently, the most probable adjustment would be to lead some of the water from the pre-sedimentation pond to a separate maturation pond for algae cultivation. The accumulated algae biomass could be used as a substrate for biogas production or as a raw material for production of biofuels.

REFERENCES

Abdel-Raouf, N., Al-Homaidan, A., & Ibraheem, I. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences, 19*, 257-275.

Arbib, Z., Ruiz, J., Álvarez-Díaz, P., Garrido-Pérez, C., Barragan, J., & Perales, J. (2013). Photobiotreatment: Influence of Nitrogen and Phosphorous Ratio in Wastewater on Growth Kineticts of Scenedesmus obliquus. *International Journal of Phytoremediation, 15*(8), 774-788.

Brenner, A., & Abeliovich, A. (2013). Water Purification: Algae in Wastewater Oxidation Ponds. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 595-601. Ed. A. Richmond & H. Qiang, Chichester: John Wiley & Sons.

Burkiewicz, K., & Synak, R. (1996). Biological Activity of the Media After Algal Cultures can Result from Extracellular Carbohydrates. *Journal of Plant Physiology, 148*, 662-666.

Chatsungnoen, T., & Chisti, Y. (2015). Harvesting micro-alage by flocculation-sedimentation. *Algal Research*, 271-283.

Chen, C.-Y., Yeh, K.-L., Aisyyah, R., Chang, J.-S., & Lee, D.-J. (2011). Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology* 102, 71-81.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances, 25*, 294-306.

Christenson, L., & Sims, R. (2011). Production and harvesting of microalgae for wastewater treatment, biofuels and bioproducts. *Biotechnology Advances* 29, 686-702.

Docslide SQ NOVA 60 manual-en-2014-06. (2017). Retrieved from SQ NOVA 60 manual-en-2014-06. [cited 27.9.2017] Available from Internet: https://docslide.net/documents/sq-nova-60-manual-en-2014-06.html

Gentili, F. G. (2014). Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases. *Bioresource Technology* 169, 27-32.

Gerbens-Leenes, P., Xu, L., De Vries, G., & Hoekstra, A. (2014). The blue water footprint and land use of biofuels from algae. *Water Resourses Research*, 8549-8563.

Gouveia, L. (2011). *Microalgea as a Feedstock for Biofuels.* Heidelberg: Springer.

Griffiths, M. (2013). Microalgal Cultivation Reactor Systems. In *Biotechnological Applications of Microalgae: Biodiesel and value-added products*, 51-75. Ed. F. Bux. Boca Raton: CRC Press.

Grobbelaar, J.U. (2013). Inorganic Algal Nutrition. In *Handbook of Microalgal Culture; Applied Phycology and Biotechnology* 2 719). Ed. A. Richmond & H. Qiang. Chichester: John Wiley & Sons.

Harrison, P. J., & Berges, J. A. (2005). Marine Culture Media. In *Algal Culturing Techniques*, 21-33. Ed. R. A. Andersen. London: Elseviewer Academic Press.

Hiltunen, E., Linko, L., Hemminki, S., Hägg, M., Järvenpää, E., Saarinen, P., Simonen S., Kärhä, P. (Eds.). (2011). *Laadukkaan mittaamisen perusteet.* Espoo: Mittatekniikan keskus.

Karlsson, L. (2017). Bilder. Message to Katarina Martonen, Vaasa 22.8.2017. Personal communication.

Koivisto, M. (2017). Tapaaminen 16.8. Message to Katarina Martonen, Vaasa 18.8.2017. Personal communication.

Koivisto, M., & Vesala, M. (2017, 8 16). (K. Martonen, & C. Nuortila, Interviewers) Vaasa.

Lee, Y.-K., Chen, W., Shen, H., Han, D., Li, Y., Jones, H., Timlin, J.A., Hu, Q. (2013). Basic Culturing and Analytical Measurement Techniques. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 37-67. Ed. A. Richmond & Q. Hu. Chichester: John Wiley & Sons.

Lindholm, T. (1998). *Algfenomen och algproblem.* Åbo: Åbo Akademi.

Lindquist, A. (Ed.). (2003). *About Water Treatment.* Helsingborg: Kemira Kemwater.

Lorenz, M., Friedl, T., & Day, J. (2005). Perpetual Maintenance of Actively Metabolizing Microalgal Cultures. In *Algal Culturing Techniques*, 145-156. Ed. R. Andersen. Elseviewer/Academic Press.

Mata, T., Martins, A., & Caetano, N. (2010). Microalgae for biodiesel and other applications: A review. *Renewable and Sustainable Energy Reviews, 14*, 217-232.

Merckmillipore (2017a). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/COD-Cell-Test,MDA_CHEM-101796#anchor_PI

Merckmillipore (2017b). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/COD-Cell-Test,MDA_CHEM-114690#anchor_PI

Merckmillipore (2017c). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Nitrogen-%28total%29-Cell-Test,MDA_CHEM-114537#anchor_PI

Merckmillipore (2017d). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Nitrogen-%28total%29-Cell-Test,MDA_CHEM-114763#anchor_PI

Merckmillipore (2017e). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Phosphate-Cell-Test,MDA_CHEM-114543#anchor_PI

Merckmillipore (2017f). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Phosphate-Cell-Test,MDA_CHEM-114729#anchor_PI

Merckmillipore (2017g). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Ammonium-Cell-Test,MDA_CHEM-114544#anchor_PI

Merckmillipore (2017h). [27.9.2017] Available online: http://www.merckmillipore.com/FI/en/product/Ammonium-Cell-Test,MDA_CHEM-114559#anchor_PI

Molina Grima, E., Fernández, F., & Medina, A. (2013). Downstream Processing of Cell Mass and Products. In *Handbook of Microalgal Culture - applied phycology and biotechnology*, 267-309. Ed. A. Richmond & H. Qiang. Chichester: John Wiley and Sons.

Park, J., Craggs, R., & Shilton, A. (2011). Wastewater treatment high rate algal ponds for biofuel production. *Bioresource Technology*, *102*, 35-42.

Pittman, J., Dean, A., & Osundeko, O. (2011). The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology, 102*(1), 17-25.

Rajkumar, R., & Zahira, Y. (2013). The Biology of Microalgae. In *Biotechnological Applications of Microalgae: biodiesel and value added products*, 7-15. Ed. F. Bux. Boca Raton: CRC Press.

Rawat, I., Kumar, R., & Bux, F. (2013). Phycoremediation by High-Rate Algal Ponds (HRAPs). In *Biotechnological Applications of Microalgae: Biodiesel and value-added products*, 179-199. Ed. F. Bux. Boca Raton: CRC Press.

Riffar, R. (2013). *Fundamentals of Wastewater Treatment and Engineering.* Boca Raton: CRC Press.

Sacristán de Alva, M., Luna-Pabello, V. M., Cadena, E., & Ortíz, E. (2013). Green microalga Scenedesmus acutus grown on municipal wastewater to couple nutrient removal with lipid accumulation for biodiesel production. *Bioresource Technology, 146*, 744-748.

Sawyer, C. N., McCarty, P. L., & Parkin, G. F. (2003). *Chemistry for Environmental Engineering and Science* (5th ed.). New York: McGraw-Hill Higher Education.

Scalenet (2017). [27.9.2017]. Retrieved from Sartorius Operating Instructions. Available online: http://www.scalenet.com/pdf/Sartorius_GD_GE.pdf

Sigma-Aldrich (2017). [27.9.2017] Retrieved from www.sigmaaldrich.com. Available online: http://www.sigmaaldrich.com/catalog/product/mm/109751?lang=fi&region=FI

Sirin, S., & Sillanpää, M. (2015). Cultivating and harvesting of marine alga Nannochloropsis oculata in local municipal wastewater for biodiesel. *Bioresource Technology, 191*, 79-87.

Thrane, J.-E., Hessen, D. O., & Andersen, T. (2017). Plasticity in algal stochiometry: Experimental evidence of a temperature-induced shift in optimal supply N:P ratio. *Limnology and Oceanography, 62*, 1346-1354.

Vaasan Vesi (2017a). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/pattin-puhdistamo

Vaasan Vesi (2017b). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/esikasittely

Vaasan Vesi (2017c). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/esiselkeytys

Vaasan Vesi (2017d). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/ilmastus

Vaasan Vesi (2017e). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/selkeytys

Vaasan Vesi (2017f). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/hiekkasuodatus

Vaasan Vesi (2017g). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/flotaatio

Vaasan Vesi (2017h). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/purkuputki

Vaasan Vesi (2017i). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/lietteenkuivaus

Vaasan Vesi (2017j). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/lietteenkuivaus

Vaasan Vesi (2017l). [14.8.2017] Available online: http://www.vaasanvesi.fi/web/sv/avainluvut

Vesala, M. (2014). *Jälkisuodatusyksikön kemikaalien ja olosuhteiden optimointi.* Tekniikka ja liikenne, Ympäristöteknologia. Vaasa: Vaasa University of Applied Sciences.

Wood, M., Everroad, R., & Wingard, L. (2005). Measuring growth rates in microalgal cultures. In *Algal Culturing Techniques,* 269-285. Ed. R. Andersen. London: Elseviewer Academic Press.

Zhu, L., Hiltunen, E., Shu, Q., Zhou, W., Li, Z., & Zhongming, W. (2014). Biodiesel production from algae cultivated in winter with artificial wastewater through pH regulation by acetic acid. *Applied Energy, 128,* 103-110.

Zhu, L.-D., Li, Z.-H., Guo, D.-B., Huang, F., Nugroho, Y., & Xia, K. (2017). *Cultivation of Chlorella sp. with livestock waste compost for lipid*. Bioresource Technology 223, 296-300.

Appendix 1

Trendline calculation. Correlation between OD680 and dried Biomass