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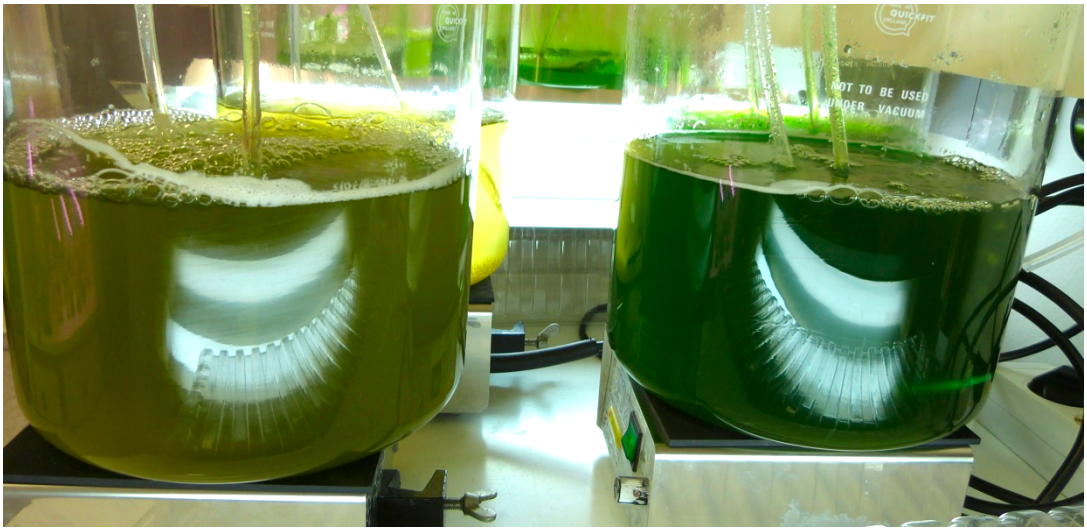

Cultivation of freshwater microalgae in wastewater from a Swedish pulp and paper mill

Master of Science Thesis in the Master Degree Program Innovative and Sustainable Chemical Engineering

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Freshwater microalgae were cultivated in wastewater from a wastewater treatment plant at a pulp and paper mill in Sweden. The growth was monitored and the cell composition of the microalgae was examined. The purpose of the project was to examine the possibility of combining flue gas treatment and wastewater treatment at the pulp and paper mill and investigate the possibility to produce valuable biomass products.

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Cover: Cultivations of microalgae in cultivation flasks used in the study.

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Abstract

Microalgae have potential to clean wastewater from inorganic nutrients while converting inorganic carbon dioxide in flue gas into valuable products. Wastewater from Nordic Paper Bäckhammar, a Swedish pulp and paper mill, was investigated for its ability to support growth of 15 freshwater microalgae species. Two promising species, *Scenedesmus obliquus* and *Dictyosphaerium pulchellum,* were further investigated in larger batch cultivations. Air with 1% carbon dioxide was bubbled through the cultures, and municipal wastewater was added in different amounts as additional nutrient source. Cell growth, nutrient reduction and cell content were evaluated. Specific growth rates of 0.013-0.082h^{-1} and 0.023-0.064h^{-1}, with generation times of 8.5-52.9h and 10.8-30.5h were obtained for *S. obliquus* and *D. pulchellum* respectively. Final dry weights reached 0.57-1.29g/l and 0.62-1.55g/l respectively. The results indicated that nutrient availability was a limiting factor for cell growth, and in particular nitrogen availability. The addition of municipal wastewater led to higher cell densities. Cell composition varied over the course of cultivation where total protein and carbohydrate content decreased and total lipid content increased for all cultures. The final lipid content of *S. obliquus* was 19-23% of dry weight, which is consistent with previously reported data. Literature data for *D. pulchellum* was not found, and the lipid content amounted to 33-35% of dry weight in the present study, slightly higher than *S. obliquus*. Nutrient reduction was comparable to other reported values; after eight days, close to 100% NH_4^+ was eliminated for both species. *D. pulchellum* seemed to reduce PO_4^{3-} faster than *S. obliquus* with 63-67% after eight days compared to 25-46%. After three weeks 79-98% PO_4^{3-} was eliminated for both species. Both *S. obliquus* and *D. pulchellum* showed potential for production of biomass while cleaning wastewater and are interesting candidates for further research with addition of flue gas. Of the two, *S. obliquus* has previously proved good growth in flue gas experiments, whereas not much research has been done on *D. pulchellum*.

Table of Contents

1. Introduction

Microalgae are photosynthesizing microscopic organisms, which fixate carbon from the atmosphere using sunlight, providing the oceans with life-sustaining energy (Andersen, 2005). Their abundance in species and habitats, and their fast growth make them interesting for a number of human uses, some of which have been explored for several decades (Sheehan, Dunahay, Benemann, & Roessler, 1998). This thesis is included in a larger project, aiming at combining bio-oil production with carbon dioxide fixation while utilizing wastewater, with the thesis' primary focus on microalgae cultivation in wastewater from Nordic Paper Bäckhammar pulp and paper mill.

1.1. Background

The pulp and paper industry is one of the most important industrial sectors in Sweden, representing 12% of Sweden's total turnover from industry (Skogsindustrierna, 2012). It also constitutes the sector with the largest carbon dioxide emissions (Grönkvist, 2010). To make use of the carbon dioxide in the flue gas and to add value to pulp and paper mills one can utilize microalgae, which consume inorganic carbon dioxide as their carbon source, converting it to biomass. The biomass can be used for a number of different purposes, bio-oil being one of them. Bio-oil can be used to substitute fossil oil used in a wide variety of products, or used as fuel by conversion to biofuels. Thus, inorganic carbon dioxide could be recirculated into biomass as energy-rich, highly desired, compounds. The concept of using flue gas for the purpose of microalgae production was studied by Brown (Brown, 1996) and regarded as viable and economic by Demirbas (Demirbas, 2011). Bark (Bark, 2012) and Engström (Engström, 2012) recently investigated microalgae growth with simulated flue gas from Swedish pulp and paper mills. They received promising results and suggested further research on the wastewaters from the mills as a potential source for microalgal nutrients.

The concept of using microalgae for wastewater treatment combined with algae biomass production traces back to as early as the 50's (Sheehan, Dunahay, Benemann, & Roessler, 1998). However, relatively few large-scale cultivation plants have been built, one reason being poor economical performance. There are numerous recent studies made on domestic and municipal wastewater as medium (Mostafa, Shalaby, & Mahmoud, 2012) (Kong, Li, Martinez, Chen, & Ruan, 2010) (Zhou, et al., 2012) (Min, et al., 2011) (Wang, et al., 2010) (Hammouda, Gaber, & Abdel-Raouf, 1995) (Sydney, et al., 2010) and some industrial wastewaters such as water from carpet industry (Bhatnagar, Chinnasamy, Singh, & Das, 2010), piggery wastewater (An, Sim, Lee, & Kim, 2003), and wastewater from an industrial park (Wu, Chen, Huang, & Lee, 2012). Some promising results from these studies have been obtained, motivating further investigation in using wastewater as growth medium. Since each wastewater is of different quality and each location exhibits different environments, there is no one solution for microalgae cultivation in wastewater, and each situation will require its unique solution.

Wastewaters from pulp and paper mills may offer an inexpensive source of nitrogen and phosphorous, the main nutrients required by algae. By reducing the levels of nitrogen and phosphorous in the wastewater, paper mills are aided to meet

environmental standards. Efficient combination of a carbon dioxide source and appropriate wastewater was described by Christenson and Sims (Christenson & Sims, 2011) as one of the challenges to make algae production cost effective. In this sense, pulp and paper mills serve as a suitable site for microalgal cultivation, since the carbon dioxide source and the wastewater can easily be combined. In addition, the waste heat from the process water can be used, reducing heating costs, which can be substantial in cold climates such as Sweden's.

In this master thesis, the possibility of using the wastewater from Nordic Paper Bäckhammar pulp and paper mill as growth medium for microalgae is investigated. The master's thesis is part of a Vinnova sponsored project, which is a collaboration between Nordic Paper Bäckhammar and Chalmers University of Technology among others, led by SP Technical Research Institute of Sweden. Within this project, a pilot scale cultivation pond will be built at the pulp and paper mill Nordic Paper Bäckhammar.

1.2. Problem formulation and aims

The purpose of this study is to investigate whether wastewater from Nordic paper Bäckhammar pulp and paper mill can support freshwater microalgal growth. The study is a screening of two different waters from different stages of the wastewater cleaning process at Nordic paper Bäckhammar, as well as the municipal wastewater in Bäckhammar, in combination with 14 different species of fresh water microalgae. Two selected species are studied further, investigating cell composition and nutrient reduction on a slightly larger scale. The aim is to find a suitable microalgae species for the purpose of biomass production, utilizing the waste streams from the pulp and paper mill.

1.3. Limitations

The scale-up study was restricted to two freshwater species that were selected from nine different species in a pre-screening trial. The experiments were performed with air inlet containing 1% carbon dioxide to improve growth; further investigations with flue gas were not made. Influence on temperature, light intensities and light cycles were not extensively investigated. To facilitate analyses, the wastewater was treated before cultivation, which does not correspond to practical management of a large-scale cultivation. Furthermore, only duplicates were made during one cultivation period.

2. Theory

2.1. Background to microalgae

Algae are phototrophic organisms of a wide diversity; over 30 thousand species are identified, and it is assumed that the actual number is substantially larger (Leite & Hallenbeck, 2012). Algae are found as eukaryotes in the kingdoms of Protozoa, Fungi, Chromista and Plantae (Leite & Hallenbeck, 2012). Microalgae are unicellular or filamentous phototrophic organisms originating from a eukaryotic heterotroph engulfing a cyanobacterium. The organism thus gained the ability of photosynthesis and these cells later evolved into plants (Madigan, Martinko, Dunlap, & Clark, 2009). Microalgae contain different pigments which make them green, red, brown or golden coloured, and are commonly divided into groups according to these colors (Masojídek, Moblízek, & Torzillo, 2004). Green algae exist in freshwater, saline water or in soil or lichens (Madigan, Martinko, Dunlap, & Clark, 2009). The microalgae included in this study are all green algae, also called chlorophytes, which are eukaryotic organisms from the plantae kingdom (Leite & Hallenbeck, 2012).

2.2. Oxygenic photosynthesis

Microalgae produce oxygen as they use the energy in the light to reduce CO_2 and incorporate the carbon into organic molecules, in the photoautotrophic process (Masojídek, Moblízek, & Torzillo, 2004). Photosynthesis occurs at the thylakoids, a system of membranes existing within the chloroplast (Tomaselli, 2004). They often have one single large chloroplast, while plants have 20 to 50 smaller ones in each cell (Mathews, van Holde, & Ahern, 2000).

The photosynthetic pigments extract energy from the light, which is converted into chemical energy by production of the high-energy molecule ATP and the reductant $NADPH_2$. Water molecules are converted into oxygen in the process, providing the hydrogen to form $NADPH_2$. These reactions, called the light reactions, constitute the first part of the photosynthesis and occur on the photosynthetic membranes (Masojídek, Moblízek, & Torzillo, 2004). The second part of the photosynthesis involves the dark reactions, which in eukaryotic algae take place in the stroma, the liquid inside the chloroplast. Here, CO_2 is reduced by $NADPH_2$ with the energy needed provided by ATP, and carbohydrates are formed (Masojídek, Moblízek, & Torzillo, 2004).

2.2.1. Light reactions

The main pigments are chlorophylls, carotenoids and phycobilins (Masojídek, Moblízek, & Torzillo, 2004). Each has different absorption ranges, and occurs in different groups of organisms. Chlorophyll *a* exists in the reaction centre of all oxygen producing photoautotrophs and it can also be found in light-harvesting antennae together with Chlorophyll *b* or *c*. Chlorophyll *b*, *c*, and *d* are accessory pigments providing a wider range of light for absorption. Carotenoids function as accessory pigment, part of the reaction centre or part of the protective system against for example high irradiance (Masojídek, Moblízek, & Torzillo, 2004).

There are two photosystems, PSI and PSII, each containing an electron transport chain of proteins. As the antennae pigments are subjected to photons of the appropriate energy, electrons are excited and the energy is transferred by resonance energy transfer between antennae molecules to the photosynthetic reaction center

(Mathews, van Holde, & Ahern, 2000). In PSII, as the energy reaches the photosynthetic reaction center an electron is emitted, which travels through an electron transport chain creating a proton motive force across the membrane. This proton motive force is used to produce ATP from ADP by an enzyme in the electron transport chain. The electron is transferred to the photosynthetic reaction center of PSI, where again light energy is absorbed, emitting an electron to go through the electron transport chain of PSI. Here the electron is used to reduce $NADP^+$ to $NADPH_2$. As the photosynthetic reaction center in PSII is lacking an electron, water is split to replace it, thereby forming oxygen. The final products of the photosystems are ATP, $NADPH_2$ and oxygen (Mathews, van Holde, & Ahern, 2000).

2.2.2. Dark reactions

The dark reactions constitute the second part of the photosynthesis, and occur without the presence of light and even more rapidly in the presence of light (Mathews, van Holde, & Ahern, 2000). The ATP and $NADPH_2$ formed in the light reaction are used in the dark reactions to fixate CO_2. In the carboxylation phase, CO_2 reacts with ribulose biphosphate forming two molecules of phosphoglycerate. Secondly, in the reduction phase, phosphoglycerate is reduced by $NADPH_2$ to form phosphoglyceraldehyde, which also requires ATP (Masojídek, Moblízek, & Torzillo, 2004). In the regeneration phase ribulose phosphate is formed again from different sugar phosphates to bind more CO_2. The products of the dark reactions are formed in the production phase, and include carbohydrates, fatty acids, amino acids, and organic acids. (Masojídek, Moblízek, & Torzillo, 2004)

2.2.3. Photorespiration

An alternative process to carboxylation is photorespiration, where oxygen reacts with ribulose biphosphate yielding serine, ammonia and CO_2 as products (Masojídek, Moblízek, & Torzillo, 2004). In this process organic carbon is instead lost. The rate of photorespiration depends on the ratio O_2/CO_2 and is minimized by removing oxygen or adding CO_2 (Masojídek, Moblízek, & Torzillo, 2004).

2.3. Heterotrophic and mixotrophic growth

Some microalgae species have the ability to use organic compounds as their carbon and energy source, thus eliminating the need of CO_2 and light. Some microalgae that are included in the project and are able of heterotrophic growth are *Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, *Chlorella vulgaris* (Lee, 2004) and *Chlorella protothecoides* (Heredia-Arroyo, Wei, & Hu, 2010). The maximum specific growth rate is generally lower for heterotrophic cultivations but cell densities and productivity can be higher than for photosynthetic growth (Lee, 2004). Some species, such as *Chlorella sorokiniana*, *Chlorella vulgaris*, *Scenedesmus obliquus* (Lee & Shen, 2004), *Scenedesmus quadricauda* (Zhao, Yu, Jiang, Zhang, & Tan, 2012) and *Chlorella protothecoides* (Heredia-Arroyo, Wei, & Hu, 2010) can grow in a mixotrophic mode; inorganic carbon and organic carbon are used simultaneously, thus combining the advantages of photosynthetic growth and heterotrophic growth (Lee & Shen, 2004).

2.4. Growth factors for microalgal cultivation

Microalgal growth is dependent on different factors, of which light, nutrients, pH and temperature are central.

2.4.1. Light

As the foundation for microalgae growth is photosynthesis, one of the most important factors for growth is light, which in a cultivation setting can be supplied continuously or in cycles with alternating light and dark periods. Sufficient light is needed for optimal algal growth but excessive light may lead to photoinhibition (Behrens, 2005). The rate of photosynthesis depends on the irradiance in a linear fashion if the algae are not light saturated (Masojídek, Moblízek, & Torzillo, 2004). At the saturation point the photosynthetic rate is at its maximum, and increased irradiance may cause photoinhibition, as described below, reducing the photosynthetic rate (Masojídek, Moblízek, & Torzillo, 2004). In a dense culture the light gradient will be steep, causing the cells closest to the light source to be exposed to excessive light and the inner cells to an insufficient amount of light. Therefore, the cultivation should be mixed continuously to prevent an uneven exposure to light (Behrens, 2005). In regards to cultivation in wastewater, coloration and particles in the wastewater can further limit light penetration in algal ponds used for wastewater treatment (Borowitzka, 1998).

Light adaption and photoinhibition

The natural variations in irradiance have provided photosynthetic plants with the ability to adapt to new conditions, to keep the balance of light and dark reactions. One mechanism works to increase the number of photosynthetic units at lower irradiance, to increase the efficiency of harvesting the light energy. At higher irradiance the photosynthetic units are instead reduced in number (Masojídek, Moblízek, & Torzillo, 2004). This change in photosynthetic capacity takes days, and so more rapid adaption methods are needed for the shorter time scale (Masojídek, Moblízek, & Torzillo, 2004). The dissipation of thermal energy as the pH gradient builds up is such a quick mechanism that exists is green algae (Briantais, Vernotte, Picaud, & Krause, 1979) and as the pH gradient builds up, the carotenoid alcohol, zeaxanthin, is formed (Masojídek, Moblízek, & Torzillo, 2004). This reduces the photosynthetic efficiency and is a photoinhibitory mechanism (Vonshak & Torzillo, 2004). Further reduction of photosynthetic efficiency occurs as, at high irradiance, the PSII reaction centres are altered and inactivated if not repaired continuously; a mechanism that is described as an emergency process by Masojidek *et al.* (Masojídek, Moblízek, & Torzillo, 2004). However, Behrenfeld *et al.* found that despite inactivated PSII centres the total photosynthetic rate was not reduced due to a simultaneous increase of electron turnover in the remaining functional PSII centres (Behrenfeld, Prasil, Kolber, Babin, & Falkowski, 1998).

2.4.2. Nutrients

Microalgae are made up of different substances, and these are thus needed for growth. The ones that they need in largest quantities are called macronutrients, and they are C, N, P, S, K, Na, Fe, Mg and Ca (Grobbelaar, 2004). In addition to the macronutrients algae also need various trace elements such as B, Cu, Mn, Zn, Mo, Co, V and Se (Grobbelaar, 2004).

CO_2 and O_2

The source of carbon for photoautotrophic growth is CO_2, and optimal microalgal growth requires higher CO_2 concentration than supplied by the atmosphere (Grobbelaar, 2004), thus motivating addition of CO_2. As the ratio CO_2/O_2 is important for minimizing photorespiration, removal of O_2 is another important factor. Excess O_2 may also lead to photooxidative damage (Behrens, 2005). As mentioned above,

mixing is important to make the light exposure to each cell more uniform. Other advantages that arise with good mixing are that the mass transfer becomes more efficient facilitating CO_2 uptake and release of O_2. In addition, temperature and pH is easier controlled (Behrens, 2005).

Nitrogen and phosphorous

The ratio of nitrogen and phosphorous available to the algae are important for cell growth. The Redfield ratio of 106C:16N:1P (stoichiometric ratio) is commonly used to indicate the required ratio of carbon, nitrogen and phosphorous (Grobbelaar, 2004). The nitrogen source can be nitrate, ammonium or urea, and it is not generally established which source leads to better growth (Grobbelaar, 2004). One study showed that the optimal photosynthetic capacity for a *Chlorella* species was obtained with ammonium as nitrogen source, and with nitrate as nitrogen source the algae showed a loss of chlorophyll (Ahmad & Hellebust, 1990). However another study showed that nitrate led to better growth for *Scenedesmus* sp. than did ammonium (Arumugam, Agarwal, Arya, & Ahmed, 2013). Phosphorous is the third of the most important macronutrients, and is taken up by the cells as phosphate (Grobbelaar, 2004). As phosphate easily bind to other ions and precipitate it often becomes the limiting nutrient. Cells may also take up additional phosphorous and store it for future use if phosphorous becomes limiting (Grobbelaar, 2004).

2.4.3. pH

pH is an important factor for microalgal growth and is also affected by the growth. As CO_2 is taken up, pH rises due to following reactions (Grobbelaar, 2004):

$$2HCO_3^- \rightleftharpoons CO_3^{2-} + H_2O + CO_2$$
$$HCO_3^- \rightleftharpoons CO_2 + OH^-$$
$$CO_3^{2-} + H_2O \rightleftharpoons CO_2 + 2OH^-$$

As evident from these reactions, called the bicarbonate-carbonate buffer system, OH^- ions are released as CO_2 is taken up, leading to the increase of pH. On the other hand, CO_2 will form acids in water; a fact that according to Grobbelaar (Grobbelaar, 2004) can be used to control pH by introducing CO_2 to the culture medium, thereby decreasing pH and simultaneously providing carbon source for growth. This principle was used by Bark (Bark, 2012) and Engström (Engström, 2012), who showed that by increasing the CO_2 concentration step-wise, pH can be balanced by the acids formed from CO_2 and the alkaline substances formed by algal growth.

In addition to the bicarbonate-carbonate buffer system, also the nitrogen source affects pH. As explained above, nitrate and ammonia are the main nitrogen sources, and when ammonia is used the pH is reduced due to the release of H^+ ions. In contrast, when nitrate is used the pH increases (Grobbelaar, 2004).

2.4.4. Temperature

Temperature is another important factor, and should be around 20-30°C according to Chisti (Chisti, 2007). Generally the cell growth increases with temperature until the temperature optimum is reached. Further increase of temperature reduces growth radically (Dauta, Devaux, & Piquemal, 1990). The optimal temperature for growth will vary between species and reported optimum temperatures of some of the species included in the project is summarized in Table 1.

Table 1: Reported temperature optima for some species.

Species	Temperature optimum
Chlorella vulgaris	30°C (Chinnasamy, Ramakrishnan, Bhatnagar, & Das, 2009) (Cassidy, 2011)
Ankistrodesmus falcatus	35°C (Talbot, Thébault, Dauta, & de la Noüe, 1991)
Botryococcus braunii	30°C (Yoshimura, Okada, & Honda, 2013)
Chlamydomonas reinhardtii	32°C (James, Hocart, Hillier, Price, & Djordjevic, 2013)
Chlorella sorokiniana	38°C (Patterson, 1970)
Scenedesmus obliquus	20-30°C (Martinez, Jimenez, & Yousfi, 1999)
***Scenedesmus* sp.**	30°C (Cassidy, 2011)

Temperature affects the sensitivity to high light intensity, in that the tolerance to high light intensities increases with increased temperature (Bouterfas, Belkoura, & Dauta, 2002), and sub-optimal temperatures induce photoinhibition even at lower light intensities (Vonshal, Torzillo, Masojidek, & Boussiba, 2001). The cultivation temperature will also affect cell composition (Hu, 2004). The affect of temperature on lipids has been shown in a study on *Chlorella sorokiniana,* where low and high temperatures caused a higher total lipid content and total unsaturated fatty acids increased with decreased temperature too keep the membrane fluidity (Patterson, 1970). However, Hu suggests that while the temperature affects the composition of different classes of lipids, the total lipid content is not as clearly affected (Hu, 2004).

2.4.5. Interaction with indigenous microorganisms

Cultivation of microalgae in wastewater adds another factor of interactions with indigenous microorganisms, since there exists protozoa and bacteria native to the wastewater. One study made on cultivation consortia of microalgae and bacteria (de-Bashan, Hernandez, Morey, & Bashan, 2004), resulted in increased growth and nutrient reduction; 100% ammonium, 15% nitrate and 36% phosphorous, compared to 75% ammonium, 6% nitrate and 19% phosphorous reduction obtained by the microalgae alone. Another study showed similar trends of enhanced nutrient reduction by interaction of microalgae and other microorganisms, where bacteria consumed organic nitrogen, supplying the microalgae with ammonium and thus preventing nitrogen limitation in the beginning of the cultivation (Lau, Tam, & Wong, 1995). According to Subashchandrabose *et al.* (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2011) it is urease bacteria, commonly found in the intestinal tract, that are able to produce ammonium as a product of urea metabolism, which can be utilized by the microalgae. They further reason that the cause for the improved growth is beneficial interactions between the microorganisms: Organic molecules released from microalgae may serve as substrate to bacteria. Furthermore, the oxygen released by microalgae can be used by bacteria, which also serves to reduce oxygen levels. High oxygen levels may otherwise inhibit photosynthesis as mentioned previously. Correspondingly, carbon dioxide generated by bacteria is used by the microalgae.

2.5. Cell composition and nutrient starvation

Microalgal cells are made up of three main constituents; proteins, carbohydrates and lipids. The relative composition of each is dependent on species and the cultivation environment (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). As nutrients become limiting in a microalgal culture, the cells react by accumulating high-energy compounds such as carbohydrates and lipids as energy storage. Nitrogen starvation is a common method to induce increased lipid or carbohydrate content (Hu, 2004). One study on *Scenedesmus obliquus* showed that after nitrate depletion, the percentage of sugars increased when cultivated in closed bioreactors or outdoor cultivations. Another study showed that the lipid content of *Scenedesmus obliquus* increased substantially under nitrogen depleted conditions to a total fatty acid content of about 45% while the protein content decreased from 56% to 8% (Breuer, Lamers, Martens, Draaisma, & Wijffels, 2012). Due to the cell composition's dependence of cultivation environment there are sources of varying reported cell compositions. In Table 2 reported lipid content are presented for some of the species that are used in the study.

Table 2: Recorded lipid content of some of the species included in the project.

Species	Lipid content (%)
Chlorella vulgaris	14-22 (Becker, 1994), 18-40 (Illman, Scragg, & Shales, 2000)
Ankistrodesmus falcatus	17 (Nascimento, et al., 2013), 28-40
Botryococcus braunii	25-75 (Chisti, 2007), 45 (Nascimento, et al., 2013)
Chlamydomonas reinhardtii	21 (Becker, 1994)
Chlorella sorokiniana	20-22 (Illman, Scragg, & Shales, 2000)
Chlorella protothecoides	11-23 (Illman, Scragg, & Shales, 2000)
Scenedesmus obliquus	12-14 (Becker, 1994), 17 (Nascimento, et al., 2013)
Scenedesmus dimorphus	16-40 (Becker, 1994)
Scenedesmus obtusiusculus	20-56 (Toledo-Cervantes, Morales, Novelo, & Revah, 2013)
Scenedesmus quadricauda	19.9 (Becker, 1994), 14-28(autotrophic), 31(mixotrophic) (Zhao, Yu, Jiang, Zhang, & Tan, 2012)
Chlorella saccharophila	41-64 (Herrera-Valencia, Contreras-Pool, Lopez-Adrian, Peraza-Echeverria, & Barahona-Perez, 2011)

2.6. Cultivation systems

Microalgae cultivation may be in laboratory scale for research reasons, or large-scale for commercial production of biomass. The type of reactor system best suitable will be dependent on the purpose of production and environmental conditions.

2.6.1. Modes of growth

Microalgal cultivations can be grown either in continuous mode or in batch mode. A continuous culture is fed with medium in the same rate as it is taken out, which allows the cell concentration to reach a steady state (Wood, Everroad, & Wingard, 2005).

In a batch culture the culture medium and algae are added in the beginning and no additional material is added during growth, however CO_2 and air can be added (Lee & Shen, 2004). Unlike steady state in a continuous culture the algae growth rate changes throughout the duration of the culture. A lag phase may occur at the beginning of the culture, where the cells are growing slowly due to the need for adaption to new conditions such as different light intensity or nutrient concentration (Lee & Shen, 2004). A larger inoculum or one that is adapted to similar conditions gives rise to a shorter lag phase (MacIntyre & Cullen, 2005). As the culture adapts to the new environment their growth enters the exponential phase as the cells grow and divide into new cells (Lee & Shen, 2004). Though the lag phase and the exponential phase are common for all microorganisms, photosynthetic microorganisms such as microalgae may display an additional, linear, phase resulting from the characteristics of the light penetration in the culture (Lee & Shen, 2004). As the cell density increases the light will eventually not suffice to saturate the light requirement for all cells, resulting in a limitation for biomass production, and giving rise to linear growth (Lee & Shen, 2004). As the cells consume nutrients and CO_2, at some point one substance will become depleted and the medium will be unable to support continued exponential cell growth. Thus the growth will slow down to a stationary phase (MacIntyre & Cullen, 2005).

2.6.2. Growth rate

Vegetative cell division is the common way for reproduction for microalgae, although sexual reproduction occurs occasionally (Tomaselli, 2004). The cell increases in size and divides into two cells, which in turn grow and divide into two cells each. The cell growth is therefore exponential if there are no limitations to growth. A common way to measure microbial growth is by specific growth rate, μ, in h^{-1}, or d^{-1}, describing the biomass growth related to the existing biomass (Lee & Shen, 2004). The specific growth rate, μ, can be determined by following relationship in batch cultivation assuming zero mortality (Wood, Everroad, & Wingard, 2005):

$$\mu = \frac{\ln\left(\frac{N_t}{N_0}\right)}{\Delta t}$$

N_0 and N_t are the population sizes at the beginning and the end of the time interval, Δt, respectively. Thus μ is the slope of a growth curve where the population size is expressed on a logarithmic scale. Doubling time is usually expressed per day and can be calculated as follows (Wood, Everroad, & Wingard, 2005):

$$k = \frac{ln2}{\mu}$$

To obtain three measurements in the exponential growth phase required for a reliable result, the inoculum should be small enough to be able to produce at least five generations within the exponential growth phase (Wood, Everroad, & Wingard, 2005).

2.6.3. Large-scale reactor systems

There are two main routes for large-scale microalgae cultivation systems; indoor photobioreactors or outdoor ponds. The main advantage with the closed photobioreactor is that it can achieve higher yields of biomass and the risk of

contamination can be controlled (Tredici, 2004). However the cost of this kind of system is high (Borowitzka, 2005) (Tredici, 2004). Outdoor ponds are almost exclusively the kinds currently in use for commercial algae production (Borowitzka, 2005). These open ponds can be economically more efficient than photobioreactors due to their ease of operation and cheaper construction. However, open ponds are limited to a few species of microalgae, whereas conditions in a closed system can be controlled to allow cultivation of a wider variety of species (Tredici, 2004). Among the species investigated in the project, *Chlorella* species and *Scenedesmus obliquus* have been successfully cultivated in outdoor ponds (Borowitzka, 2005).

Although there are a variety of types of outdoor ponds the most efficient kind in general is the raceway pond, which is rectangular, 20 to 30cm deep and provided with mixing by paddle wheels (Borowitzka, 2005). In addition to raceway ponds, circular ponds with rotating arms and natural ponds are the only types that are used commercially and ponds where circulation is provided by inclination and pumping systems have also been demonstrated on a larger scale (Tredici, 2004).

Outdoor ponds are generally more difficult to control in terms of for example temperature and pH, and the additional issue of contamination is present (Tredici, 2004). Contamination is inevitable in open systems, as bacteria, fungi and other microalgae will start growing in the system. One strategy of handling the contamination is to grow the cultures as batch cultures and start new monocultures regularly; another way of keeping contamination low is to operate the culture at optimal conditions for the desired species, favouring their growth (Borowitzka, 2005).

2.6.4. Harvesting

Harvesting of the algal biomass is likely to constitute a large portion of the total cost in a large-scale cultivation process due to the dilute cultivations and the small cell sizes (Grima, Belarbi, Fernández, Medina, & Chisti, 2003). To separate the biomass from the liquid phase centrifugation, filtration or sedimentation can be used, and flocculation of the cells may be needed before separation. Depending on the species and product one method may be favourable over others (Grima, Fernández, & Medina, 2004). Grima *et al.* propose sedimentation for wastewater cleaning purposes due to the large volumes whereas centrifugation may be suitable for high-value products.

2.7. Products from algal biomass

There is a range of products that can be produced from microalgae. Commercial production of microalgae includes the areas of human health food and functional food, feed additives, aquaculture, coloring substances, antioxidants and polyunsaturated fatty acids (Pulz & Gross, 2004). In respect to algal biomass cultivated in wastewater the uses of the biomass is somewhat narrowed, since it would be unsuitable for any food or feed uses. There has been a large interest in using microalgae for biofuel production, utilizing the high-energy compounds carbohydrates and lipids in the cells; an area more suited for wastewater cultivations. However, also for this purpose it is important to take into account the effect of accumulation of toxic compounds existing in the wastewater, such as heavy metals, on the final product.

For biodiesel production, high lipid content is desired. However, not only the total lipid content is of interest, but also the quality of the fatty acids is important for biodiesel production. The quality has to meet certain standards to comply with biodiesel regulations (Chisti, 2007). Microalagal oil is generally relatively high in polyunsaturated fatty acids, which increases the risk of oxidation during storage and may make the biofuel fail to meet the standards unless it is modified (Chisti, 2007).

In addition to biodiesel, other fuels that can be produced from microalgal biomass are ethanol by fermenting the carbohydrates, methane production by anaerobic digestion or thermal gasification, or direct combustion to produce heat and electricity (Sheehan, Dunahay, Benemann, & Roessler, 1998).

2.8. Nordic Paper Bäckhammar pulp and paper mill

Nordic Paper Bäckhammar is a pulp and paper mill in Värmland, Sweden, producing 200 000 tonnes of pulp by the Kraft pulping process (Nordic Paper, 2013).

2.8.1. The Kraft process

There are four chemical pulping processes used; the sulphate (Kraft) process, the sulphite process, the neutral sulphite semichemical (NSSC), and soda, where the Kraft process is the most common one (U.S. Environmental Protection Agency, 1990). In the Kraft process, the wood is fragmented into chips, and cooked in white liquor, containing the pulping chemicals sodium sulphide and sodium hydroxide. The cooking results in the pulp, now with some of the lignin removed, and the black liquor, which contains the pulping chemicals. The pulp is washed and the water from the washing is mixed with the spent cooking liquor, forming the weak black liquor. This black liquor is thickened in an evaporation plant and sent to the recovery boiler where it is combusted. The cooking chemicals are recovered as smelt and regenerated into white liquor by addition of calcium oxide (U.S. Environmental Protection Agency, 1990). Thus, the chemicals in the Kraft process are recovered, requiring only addition of make up chemicals.

2.8.2. Wastewater treatment at Bäckhammar pulp and paper mill

The wastewater treatment plant at the pulp and paper mill Nordic Paper Bäckhammar treats water from the pulp and paper process, the evaporation plant and the feed water generation process (Berggren, 1996). All waters flow in a common pipe to the treatment plant and are filtered through a coarse grid. The pH is then adjusted by pH control and automatic addition of either soda lye or sulphuric acid to keep pH at around 7.5. The water passes through a smaller filter of 1 mm and thereafter nutrients are added as diammonium sulphate and phosphoric acid to facilitate the subsequent microbiological treatment. The biological treatment is performed in two steps with similar design; a biofilm is attached to a mobile carrier material that is designed to improve oxygen transfer to the microorganisms. Aeration and agitation are provided by two blowing engines and an aeration system covering the bottom of the reactor. Spray water injections prevent foam formation. (Berggren, 1996)

After biological treatment the water is led to a chemical precipitation facility where the bacteria are separated from the water to form sludge at the bottom of the tank. Precipitation chemicals are added to improve sedimentation of the sludge and to precipitate remaining high molecular substances and PO_4-P. To further improve the separation flocculating polymers are added as needed. The sludge is thereafter

dewatered in the dewatering facility and finally deposited or incinerated (Berggren, 1996). The treated water is released to the river Visman (Nordic Paper, 2011). The release of total nitrogen was in 2010 158 kg/day, which was well below the limit of 250 kg/day. The corresponding release of total phosphorous was in 2010 3.4 kg/day and the limit is 3.5 kg/day (Nordic Paper, 2011).

2.9. The Vinnova sponsored project at Nordic Paper Bäckhammar

This thesis is part of a larger Vinnova-sponsored project, which is a collaboration between Nordic Paper Bäckhammar and Chalmers University of Technology among others, led by SP Technical Research Institute of Sweden. The aim of the three-year project is to test the production of bio-oil from microalgae by constructing a pilot scale sized open pond at the Nordic Paper Bäckhammar site, taking advantage of the waste streams at hand for beneficial purposes. The waste heat from the pulping process is to be used as a heat source, combined with the wastewater as growth medium. The flue gas from the pulp mill will supply the carbon dioxide needed for good growth. A picture of Nordic Paper Bäckhammar seen from the side of the wastewater treatment plant is shown in Figure 1.

Figure 1: Nordic Paper pulp and paper mill in Bäckhammar; the site of the construction of the pilot-scale algal cultivation. The wastewater treatment plant is shown in the foreground, with the two bioreactors to the left. By permission of Nordic Paper Bäckhammar, 13-06-07.

3. Methods and material

In a pre-screening, nine species of freshwater microalgae were investigated in combination with two wastewaters from Nordic Paper Bäckhammar. One wastewater from the municipal wastewater treatment plant in Bäckhammar was included as a source of extra nutrition. From the pre-screening, two of the species that were estimated to grow the best were selected to the scale-up study using cultivating flasks containing 2.5 L.

3.1. Medium and wastewaters

The medium used for pre-culturing and storage of the species was Bold Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V) (Culture Collection of Algae and Protozoa) (for full recipe see Appendix A). The two different wastewaters from Nordic Paper Bäckhammar were obtained from the effluent from the first and second biological cleaning step respectively (Hereafter referred to as bioreactor-1 and bioreactor-2 effluent respectively). The wastewater from the municipal wastewater treatment plant was obtained from the influent to the plant, before chemical treatment. Nutrient concentrations were measured at each facility before delivery and presented in Table 3. pH was measured before use. Each of the waters was filtered through 1.6 µm Whatman glass fibre filters to remove particles to simplify analyses. After pre-screening, new waters were obtained for the scale-up cultivations and treated in the same way.

Table 3: Nutrient concentration in wastewaters used in the pre-screening. Some values are missing due to limited analysis capacity at respective facility.

	Bioreactor-1	Bioreactor-2	Municipal wastewater
NO_3^--N (mg/l)	-	-	1.6
NH_4^+-N (mg/l)	3.61	2.66	9.6
Tot N (mg/l)	-	-	18.4
PO_4^{3-}-P (mg/l)	1.08	0.712	-
Tot P (mg/l)	-	-	2.2
pH	7.1	6.6	6.5

The filtered waters used for the scale-up study were each sent for analysis at AK Lab in Borås before cultivation and from each culture three weeks into the cultivation. The analysed species were NH_4^+-N, NO_3^--N, PO_4^{3-}-P, total nitrogen and total phosphorous. The nutrient levels of respective water blend in the beginning of the cultivation were calculated from the analysed nutrient levels in each of the waters. To avoid nutrients contained by the cells to interfere with the analysis, the cell suspensions in the middle and at the end of cultivation were centrifuged and filtered through Whatman 1.6 µm glass fibre filter to remove all algal cells.

3.2. Microalgae species

The species used in the project are summarized in Table 4. For the species that were ordered from CCAP, also the strain number is given.

Table 4: Species used in the project: name and strain number.

Species and strain
***Chlorella vulgaris* CCAP 211/11B**
***Ankistrodesmus falcatus* CCAP 202/15A**
***Botryococcus braunii* CCAP 807/1 or UTEX 572**
***Chlamydomonas reinhardtii* CCAP 11/32C**
***Chlorella sorokiniana* CCAP 211/8K**
***Chlorella protothecoides* CCAP 211/54**
***Scenedesmus obliquus* CCAP 276/50**
***Scenedesmus dimorphus* UTEX 417**
***Dictyosphaerium pulchellum* CCAP 222/2A**
***Scenedesmus obtusiusculus* CCAP 276/25**
***Scenedesmus quadricauda* CCAP 276/21**
***Chlorella saccharophila* CCAP 211/60**
***Selenastrum capricornutum* CCAP 278/4**
***Scenedesmus simris* 002**

Six of the species that were used in the pre-screening were available at SP Technical Research Institute of Sweden: *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Ankistrodesmus falcatus*, *Botryococcus braunii*, *Chlorella sorokiniana* and *Chlorella protothecoides*. These were included in the first pre-screening set. *C. vulgaris* and *C. sorokiniana* have shown the ability to reduce nitrogen levels in wastewater for the purpose of lipid production (Feng, Li, & Zhang, 2011) (Kim, Lingaraju, Rheaume, Lee, & Siddiqui, 2010) (Liu, Li, Qiao, Lin, & Wang, 2012) (de-Bashan, Trejo, Huss, Hernandez, & Bashan, 2008). Also *C. protothecoides* has demonstrated efficient reduction of nutrient levels in wastewater (Li, Zhou, Hu, Min, Chen, & Ruan, 2012), although the species has mainly been investigated for mixotrophic and heterotrophic growth as these modes of growth has proved more efficient for the species (Xu, Miao, & Wu, 2006) (Heredia-Arroyo, Wei, & Hu, 2010). *C. reinhardtii* and *A. falcatus* showed good results when subjected to simulated flue gas in the study by Bark (Bark, 2012). *B. braunii* is known for its high lipid content and has also showed potential for wastewater treatment purposes (Órpez, Martínez, Hodaifa, El Yousfi, Jbari, & Sánchez, 2009).

Three additional species were obtained from Eva Albers at Chalmers University of Technology: *Scenedesmus obliquus*, *Scenedesmus dimorphus* and *Dictyosphaerium pulchellum*. One strain of *S. obliquus* has been studied for the purpose of wastewater treatment (Martinez, Jimenez, & Yousfi, 1999), and showed good results in the study by Bark (Bark, 2012) when subjected to simulated flue gas. *S. dimorphus* has been demonstrated to efficiently remove nitrogen and phosphorous from heavily contaminated waste streams (González, Canizares, & Baena, 1997) and a strain of *D.*

pulchellum was according to Gentili[1] found to thrive seasonally in an outdoor cultivation in a facility in Umeå run by the Swedish University of Agricultural Sciences (SLU). *D. pulchellum* was also found to dominate a lake in England and found to tolerate a wide range of pH and environments with high turbidity (Irfanullah & Moss, 2006). These three species were used in the second set of pre-screening.

Five other species were investigated in a small-scale visual study, however were not possible to choose for scale-up due to time constraints. *S. simris* was received from Simris Alg. Four were ordered from CCAP (Culture Collection of Algae and Protozoa): *Scenedesmus obtusiusculus*, *Scenedesmus quadricauda*, *Chlorella saccharophila* and *Selenastrum capricornutum*. The ordered species were selected according to results from previous studies, particularly their growth rate in wastewaters and their lipid content. *S. capricornutum*, a microalga commonly used for toxicity tests, was chosen to give an indication of toxicity in the wastewaters. A summary of the qualities of the respective species that were ordered from CCAP is presented in Table 5.

Table 5: Summary of the qualities of the species that were ordered from CCAP. Focus for selection was whether the species had previous wastewater been cultivated in wastewater or in conditions with elevated CO_2 concentration, and lipid content.

Species	Note	Literature
Scenedesmus obtusiusculus	Showed high tolerance to CO2 and high lipid content under stress.	(Toledo-Cervantes, Morales, Novelo, & Revah, 2013)
Scenedesmus quadricauda	Has ~14-33% lipid content and capability to grow on olive oil mill wastewater.	(Zhao, Yu, Jiang, Zhang, & Tan, 2012), (Pinto, Pollio, Previtera, & Temussi, 2002)
Chlorella saccharophila	Has shown 41% lipid content and has been grown on carpet mill effluent with ~17-18% lipid content.	(Herrera-Valencia, Contreras-Pool, Lopez-Adrian, Peraza-Echeverria, & Barahona-Perez, 2011), (Chinnasamy, Bhatnagar, Hunt, & Das, 2010)
Selenastrum capricornutum (Raphidocelis subcapitata)	Used for toxicity test on wastewater from a pulp and paper mill. Sensitive to toxic compounds.	(Ahtiainen, Nakari, Ruoppa, Verta, & Talka, 2000) (Connecticut College)

3.3. Small scale visual screening of species

As a first screening of the nine algae species, a visual test was made to select the best-growing species for the scale-up experiments. The three different wastewaters were used as growth medium as well as a 1:1 mixture of the municipal wastewater with each of the pulp and paper mill wastewaters. The species pre-screening was divided into two sets with cultures starting at two different occasions. In the first set, the wastewaters without inoculated algae were also tried for observation of growth of indigenous species.

[1] Francesco Gentili (Assistant researcher, Institutionen för vilt, fisk och miljö, SLU Swedish University of Agricultural Sciences) email correspondence 2nd of May 2013.

To obtain approximately the same amount of biomass in each culture, OD_{750} was used as a rough estimation of the indirect cell concentration in the pre-cultures. From a visual inspection, it was decided to inoculate 250 µl of *B. braunii* as reference, and the volume of the other species was calculated according to OD_{750} (see Appendix B). For each of the pre-screening cultivation, 1 ml of pre-culture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 1 ml of corresponding wastewater. The calculated amount of each species was inoculated into the tissue culture flask containing 30 ml of respective water, and placed under 18 W fluorescent lights. Pictures were taken daily except weekends, and the sets were compared according to cultivation day. The organization of the species pre-screening is shown in Table 6.

Table 6: Organization of species pre-screening. Set 1 includes species 1 to 6 as well as flasks with only wastewater and without algae. Set 2 includes the three other species that were started at a later time. The sets were compared according to starting day of cultivation.

Set 1						
0a	1a	2a	3a	4a	5a	6a
0b	1b	2b	3b	4b	5b	6b
0c	1c	2c	3c	4c	5c	6c
0d	1d	2d	3d	4d	5d	6d
0e	1e	2e	3e	4e	5e	6e
0f	1f	2f	3f	4f	5f	6f

Set 2		
7a	8a	9a
7b	8b	9b
7c	8c	9c
7d	8d	9d
7e	8e	9e
7f	8f	9f

a = Bioreactor-1 effluent
b = Bioreactor-2 effluent
c = Municipal wastewater
d = Bioreactor-1 effluent + Municipal wastewater, ratio 1:1
e = Bioreactor-2 effluent + Municipal wastewater, ratio 1:1
f = Artificial 3N-BBM+V medium

0 = no species
1 = *Chlorella vulgaris*
2 = *Ankistrodesmus falcatus*
3 = *Botryococcus braunii*
4 = *Chlamydomonas reinhardtii*
5 = *Chlorella sorokiniana*
6 = *Chlorella protothecoides*
7 = *Scenedesmus obliquus*
8 = *Scenedesmus dimorphus*
9 = *Dictyosphaerium pulchellum*

3.4. Additional small-scale visual study

The five species that were not tested in the first two species screening tests were studied in a screening using the same kind of 50 ml tissue culture flasks. The same procedure for inoculation and cultivation was used as for the species screening but the wastewaters were limited to corresponding blends to the scale-up study, which was bioreactor-2 effluent with 10% and 75% municipal wastewater respectively. Cultures with artificial medium were used as control. For the species *S. capricornutum*, which is commonly used in toxicity tests due to its high sensitivity to toxic compounds (Connecticut College), also pure bioreactor-2 effluent and municipal wastewater were tested to see any potential indications of toxicity. The experimental setup for the small-scale visual screening is shown in Table 7.

Table 7: Organization of the small-scale visual study of the five additional species. Bioreactor-2 effluent with added 10% and 75% municipal wastewater were tried for each species, as well as artificial growth medium (3N-BBM+V). For *S. capricornutum* also unblended bioreactor-2 effluent and municipal wastewater was tested to investigate the effect of potential toxic compounds in each of the waters.

10a	11a	12a	13a	14a
10b	11b	12b	13b	14b
10c	11c	12c	13c	14c
			13d	
			13e	

a = 90% Bioreactor-2 effluent + 10% municipal wastewater
b = 75% municipal wastewater + 25% Bioreactor-2 effluent.
c = Artificial 3N-BBM+V medium
d = 100% Bioreactor-2 effluent
e = 100% Municipal wastewater

10 = *Scenedesmus quadricauda*
11 = *Scenedesmus obtusiusculus*
12 = *Chlorella saccharophila*
13 = *Selenastrum capricornutum*
14 = *Scenedesmus simris*

3.5. Small scale screening of mixed waters

By adding the municipal wastewater to the bioreactor-2 effluent, the nutrient concentration is increased. To investigate the effect of increased nutrients on algal growth, a series of different blends of the municipal wastewater and bioreactor-2 effluent were tested on three of the species in pre-screening set number one. The species were *A. falcatus*, *C. reinhardtii* and *C. sorokiniana*, and the blends were 0%, 10%, 25%, 50%, 75%, 90% and 100% of municipal wastewater in bioreactor-2 effluent. There was also a control with 3N-BBM+V medium for each species. The same type of tissue culture flasks was used in this screening as in the pre-screening and the inoculation of each species into respective blend of wastewater was performed in the same way as in the pre-screening.

3.6. Small scale screening with sterile-filtered waters

As the wastewaters contain native species of bacteria and other microorganisms, one smaller set of screening was performed using waters filtered through 0.2μm filters. This pore-size removes microorganisms (Kawachi & Noel, 2005), ensuring the absence of microorganisms and was compared to the other screening to see the effect of native microorganisms on the growth. This set was performed on the nine species from the first two screening sets, with bioreactor-2 effluent and the municipal wastewater separately. The inoculation was performed in the same way as the other parts of the pre-screening.

3.7. Scale-up cultivation

The results from the pre-screening were the basis of the layout choices of the larger scale cultivations. The equipment consisted of eight 6L glass flasks with lights from both sides as shown in the schematic image in Figure 2 and in the picture in Figure 3. The light followed a cycle of four light hours and 20 dark hours per day. The cultivation flasks are numbered 1 to 8 and will be referred to respective number throughout the report. Each of the two species was grown in bioreactor-2 effluent with 10% added municipal wastewater and in 25% bioreactor-2 effluent with 75% municipal wastewater. The total starting volume of each of the cultivations was 2.5L and each combination of species and water blend was cultivated in duplicates. Air

with 1% CO_2 with an approximate total flow rate of 1.4L/min divided into the eight flasks was introduced to the cultivation by tubes going to the bottom of the flasks. Tubes for gas outlet were attached to the top of the cultivation flasks. A descriptive image of a cultivation flask is shown in Figure 4. The gas flow created some agitation in the cultivations, and additional agitation was provided from day three by magnetic stirrers. The average temperature from day 3 and on was 30.4±2°C with heat added to the cultures by the stirrers and lamps. The average temperature of each of the cultivations is presented in Table 8. The cultivation flasks were autoclaved before addition of wastewater culture medium and provided with a sterile air filter each.

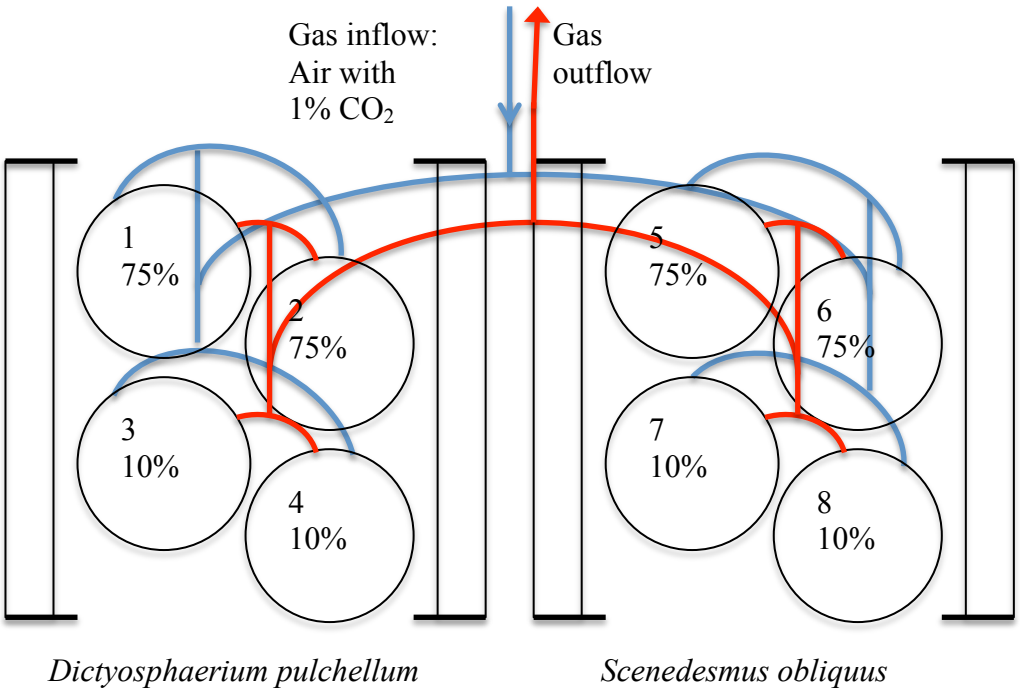


Figure 2: Schematic image of experimental setup with the gas connections included. The blue lines indicate gas inflow and the red lines indicate gas outflow. The gas flow was distributed into the cultivation flasks from one common tube by division into eight and the gas outflow was collected in a similar manner. The glass flasks are arranged in a zigzag pattern to provide light from both directions. Light is provided from the left and right of a group of four cultivation flasks.

Figure 3: Algae cultivation setup. To the left are the four cultures of *D. pulchellum* and to the right *S. obliquus*.

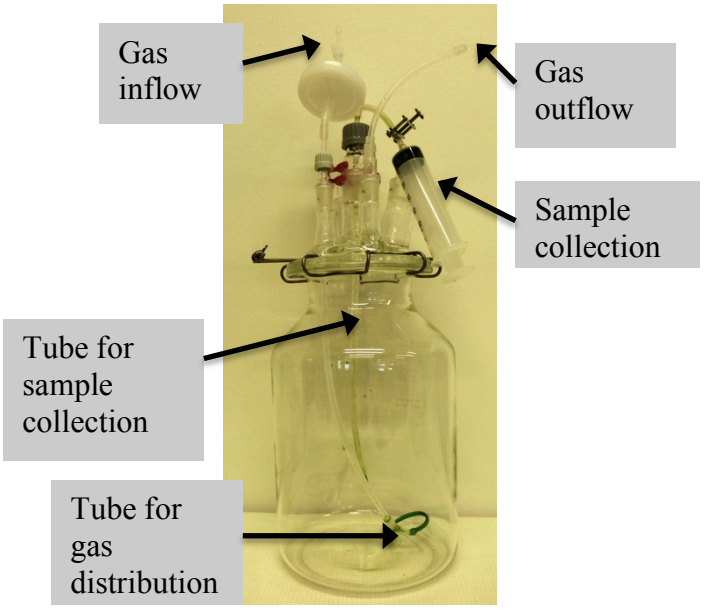


Figure 4: Setup of the cultivation flasks. The tube for the gas inflow is provided with a sterile air filter and a green loop at the bottom of the flask with small holes, which increases the pressure of the gas introduced to the flasks. The syringe is used for collecting samples and a clamp keeps the liquid in the sampling tube from re-enter the flask.

Table 8: Average temperature during the course of cultivation.

Culture	Average temperature, °C
1. *D. pulchellum*, 75%	30.3
2. *D. pulchellum*, 75%	32.7
3. *D. pulchellum*, 10%	31.3
4. *D. pulchellum*, 10%	31.3
5. *S. obliquus*, 75%	29.3
6. *S. obliquus*, 75%	30.7
7. *S. obliquus*, 10%	28.7
8. *S. obliquus*, 10%	28.8

The pre-cultures of the two selected species were grown with the addition of air and 1% carbon dioxide for a few days before inoculation. OD_{750} was measured and the inoculum size was determined with the intent to obtain OD_{750} of 0.02 as start value in the cultures. The inoculation was done in the same way as in the pre-screening by centrifuging the pre-culture at 5000 rpm for 10 min, discarding the supernatant and adding the same amount of the respective wastewater.

3.7.1. Analyses for cell growth

OD_{750}, dry weight and pH were measured daily Monday to Friday, sometimes twice a day. OD_{750} and dry weight were each plotted to produce growth curves of the cultures. In addition, the cultures were examined in microscope occasionally to visually determine whether the cultures were becoming contaminated or changed in morphology during growth. Dry weight was used to calculate specific growth rate in the beginning (approximately the first 90 h) of cultivation and the growth curves were studied to determine the effect of the different waters on the growth and which species obtained the highest biomass. Cell composition analyses were performed to determine the composition of carbohydrates, proteins and lipids at three points in the study; in the exponential growth phase, in the middle of the cultivation period and at the end of the cultivation.

OD

As discussed by Bark (Bark, 2012), OD is an indirect measurement of cell mass, which is more inaccurate if there is high absorbance of the light by the cells. Bark therefore examined different wavelength to find the wavelength with minimum absorbance. He found OD_{750} to meet this requirement, and this wavelength is therefore used throughout this study. There was assumed to be a linear relationship between OD_{750} and cell mass up to an OD_{750} value of around 0.7. If a sample showed a higher value than OD_{750} 0.7 the samples were diluted. OD_{750} was measured using a Cary 50 UV-Vis Spectrophotometer, where the absorbance was measured three times, after which the sample was mixed in the cuvette and measured three more times. This was repeated so that a total of nine measurements per sample were obtained. The mixing was done to prevent the cells from settling, which would give a lower OD value. The average of the measurements was calculated. A blank, constituting a sample of respective wastewater taken before inoculation that was kept throughout the cultivation, was used to zero before measurements of the samples.

Dry weight

Dry weight is a direct measurement of cell mass and provides a value of total cell mass. It does not however say anything about the cell number. A filter pore size of 1.6 µm was used since the species used are larger than that and will thus stay in the filter. The size of *S. obliquus* is in the range of 2-10 x 6-15 µm (John, 2011) and *D. pulchellum* has been reported to be in the range of 4.3-7.5 µm (Irfanullah & Moss, 2006). The filters were marked and placed in a constant variable room with a temperature of 23°C and air humidity of 50% RH overnight. The filters where then weighed. 20 or 10 ml of the cultivation broth, depending on cell density, was filtered and washed twice with 10 ml ammonium bicarbonate. Washing with a solution such as ammonium bicarbonate was recommended by Zhu *et al.* (Zhu & Lee, 1997) for marine microalgae and implemented in this study as a precautionary measure. After washing, the filters were dried in oven at 100°C overnight (approximately 24 h) and kept in the constant variable room (23°C, 50% RH) overnight (approximately 20 h). The filters were then weighed again and the difference was calculated. A blank, constituting a sample of wastewater taken before inoculation, was measured in the beginning of the cultivation period as reference and its net weight was subtracted from the cell culture samples to avoid any particles left in the wastewater to add mass to the samples. Double samples were made of all dry weight samples. For calculation example, see Appendix C.

3.7.2. Cell composition analysis

Cell composition was analysed at three points of the growth. The samples were frozen at the time of collecting the samples and defrosted at the time of analysis. The carbohydrate and the protein analyses were made at Chalmers University of Technology and the lipid analysis was made at SP Technological Research Institute of Sweden. The final concentration of each was compared to the dry weight measured at the time of sampling, providing the biomass weight percentage. For calculation example of respective constituent, see Appendix D-F.

Total carbohydrates

To determine total carbohydrates a modified sulphuric acid phenol method by Herbert *et al.* (Herbert, Phipps, & Strange, 1976) was used. The cell suspension was treated with phenol and sulphuric acid. The color that was developed, an indirect measure of total carbohydrates in the sample, was measured using a microtiter plate reader.

Glucose standards and the frozen samples were prepared. The standard curve was made from 0.5 g/l glucose solution and diluted in a series of five down to 0.025 g/l. For samples with low cell density, the cells were concentrated by centrifugation. Similarly, samples with high cell density were diluted to give an absorbance within the range of the standard curve. 30 µl of each standard dilution were pipetted into a microtiter plate with double samples. Similarly, 30 µl of the cell samples were pipetted in the microtiter plate in triplicates. 30 µl of 5% phenol solution was added to each well and the wells were stirred using a multipipette. 150 µl of concentrated sulphuric acid was added to each well and mixed carefully using the pipette. The microtiter plate was left for 30 min after which absorbance was measured in triplicates at 488 nm. The average of the triplicate measurements and triplicate samples was calculated, however highly deviating values were excluded. The double samples of the standard were not averaged but plotted in the standard curve as separate points. The blank was included in the standard curve instead of subtracted from all samples.

To avoid including extracellular carbohydrates from the growth medium in the total cellular carbohydrates, samples of culture medium were also prepared by centrifuging each sample and transferring the supernatant to new sample tubes. These were analysed in the same way as the cell samples in triplicates and the absorbance was subtracted from the absorbance of the cell samples. When using diluted samples, the supernatant was diluted correspondingly.

Total protein

The analysis of total protein content was performed using a Bio-Rad DC Protein assay kit. The cell suspension was treated with SDS, and the supernatant was used for analysing the total amount of protein.

90µl of each sample was mixed with 10 µl of 10% SDS making up a solution of 1% SDS. The samples were mixed by vortex and heated at 95°C for 5 minutes. They were then mixed by vortex again and centrifuged at 14680 rpm for 5 minutes. The supernatant was transferred to new tubes. A standard was prepared by using seven dilutions of the BSA protein standard and one blank containing milliQ water. To each standard solution 50 µl 10% SDS was added to make up a total of 500 µl. The final dilution was calculated. 20 µl of each sample (supernatant), including standard and blank, was transferred to a new eppendorf tube. 100 µl of reagent A was added as well as 800 µl of reagent B. The tubes were mixed by vortexing and 200 µl of each sample was transferred to a microtiter plate in duplicates. After 15 minutes the absorbance was measured at 750 nm. To calculate the concentration of protein in the samples, the standard curve equation was applied to all samples, and correction for the sample dilution (100 µl/90 µl) was made.

Lipids

The lipid analysis was performed by extracting the lipids from the cells and performing a methanolysis on the triacylglycerols forming fatty acid methyl esters (FAME). 1 ml of the samples, in duplicates, was centrifuged for 5 minutes at 7100 rpm. The supernatant was discarded and to selected samples, 10 µl 2.5 mg/ml of triglyceride C19 was added. 200 µl of chloroform/methanol (1:3) solution was added to each sample including a blank sample containing no cells. The samples were mixed by vortexing and treated with ultrasound for 30 minutes. 100 µl milliQ water was added to each sample, mixed by vortexing and centrifuged for 5 minutes at 7100 rpm. The bottom phase containing the oil was transferred to a new tube. The extraction was repeated once by adding 200 µl chloroform/methanol solution, treated with ultrasound for 15 and centrifuged for 2 minutes. The collected oil phase was washed by adding 100 µl 0.73% NaCl solution, vortexing and centrifuging for 2 minutes at 7100 rpm. The final oil phase was transferred to new tubes and let dry over night with lids open.

200 µl hexane was added to the dried samples, mixed and treated with ultrasound for 15 minutes. 100 µl of hexane with 10 mg/ml C17 was added together with 400 µl solution of 1.2 g Na in 100 ml MeOH. The samples were mixed and left for 30 minutes. 200 µl milliQ water was added and samples were mixed and centrifuged for 2 minutes at 7100 rpm. 200 µl of the upper phase, the hexane phase, was transferred to GC vials with airtight lids. A standard was prepared using FAME Mix C8-C24 100 mg/ml in hexane. Five dilutions were prepared and treated in the same way as the samples by first adding 100 µl hexane with C17 to 200 µl standard sample and then following the previous procedure. The standard solutions were prepared in duplicates.

The amount of each triglyceride was calculated by comparing the obtained peak areas to that of FAME C17 and applying the standard curves of each FAME to the samples. The yield of methanolysis was estimated using the triglyceride C19 that had been added to selected samples. For total lipid content, the amounts of the individual triglycerides were added.

4. Results

4.1. Small-scale visual pre-screening

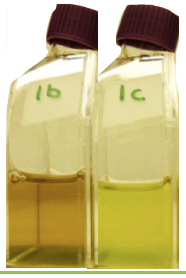
The results from the pre-screening are supported by some of the pictures that were taken. However, the growth was sometimes poorly visualised by images and thus the results are based on impressions and estimations that were noted during the course of the experiments.

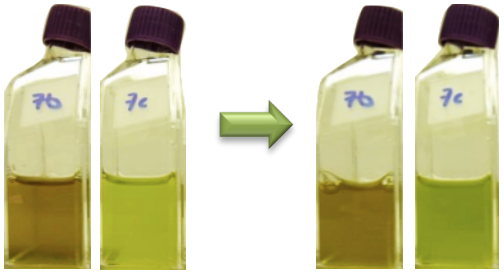
4.1.1. Species

The color and turbidity of the cultivations were the main indicators of growth in the pre-screening. In general, a green color indicates good growth, although the tone of green can vary between species and depending on cultivation conditions. Some samples were also examined under microscope to determine whether the cultures were contaminated, the shape and the size of the cells and also to get a rough estimate of the cell number. As some species attached to the bottom of the tissue culture flasks, they were de-attached using a pipette on day 12 for more representative pictures. A summary of the results is shown in Table 9.

Selected species from the pre-screening were *S. obliquus* and *D. pulchellum*. The choice was based on the growth that could be seen visually, the appearance of the cells under microscope and other factors such as how the species had grown in previous experiments, where they are found in nature, and what is known about them from literature. The specific motivations are further explained in the discussion section.

Table 9: Summary of species pre-screening. Pictures from day 3 and day 14 with b=bioreactor-2 effluent and c=municipal wastewater. The early pictures give an indication on how fast the algae grow in the medium and the later pictures how high cell density the culture can reach. After day 14, further growth in the wastewaters could not be detected.

Species	Growth in the beginning (day 3)	Final density (day12)	Comments
Chlorella vulgaris	Good 	Medium 	Form clusters. Attach to bottom of flask.
Ankistrodesmus falcatus	Poor 	Good 	In bioreactor-1 and -2 effluent, the cells' morphology is changed; they are bent and not uniform. Contaminated and attached to bottom of flask.
Botryococcus braunii	Good 	Medium 	Contaminated. Other species took over culture. Attach to bottom of flask.
Chlamydomonas reinhardtii	Good 	Good 	*C. reinhardtii* are out conquered in number by other species. Attach to bottom of flask.
Chlorella sorokiniana	Good 	Medium 	Form clusters. Contaminated. Attach to bottom of flask.

Species	Speed of growth (day 3)	Final density (day12)	Comments
Chlorella protothecoides	Poor 	Good	Form some clusters. Has a more yellow color than most others.
Scenedesmus obliquus	Good 	Good	Not completely uniform in shape. In all waters they are rounder than in the control.
Scenedesmus dimorphus	Medium 	Good	Very round cells compared to original culture, many are solitary.
Dictyosphaerium pulchellum	Poor 	Good	Often in groups of two or four.

Growth in the artificial medium was significantly slower than in the wastewaters, as s demonstrated with an example in Figure 5. On day 4, the municipal wastewater showed more growth than the artificial medium, however, the final density (day 22) in the artificial medium was substantially higher.

Figure 5: Left: C. protothecoides on day 4 in municipal wastewater (6c, left) and artificial growth medium (6f, right). Right: Same cultures on day 22. Growth in the artificial growth medium (6f, right) was slower than in the wastewaters (6c, left).

4.1.2. Additional small-scale visual study

The growth of the five additional species grown in wastewater is summarized below in Table 10 and corresponding pictures are shown in Figure 6. All species except for *C. saccharophila* grew tolerable in both waters. *S. capricornutum* showed less growth than the *Scenedesmus* species, however grew reasonably well in both waters.

Table 10: Cell growth of the five species in the small-scale visual study. Only *C. saccharophila* did not grow well in either water. The *Scenedesmus* species showed best performance.

Species	Growth
10. *Scenedesmus quadricauda*	Good
11. *Scenedesmus obtusiusculus*	Good
12. *Chlorella saccharophila*	Poor
13. *Selenastrum capricornutum*	Medium
14. *Scenedesmus simris*	Good

Figure 6: The five species cultivated in the two different blends of wastewaters used in the larger scale cultivations and artificial medium. 10=*Scenedesmus quadricauda*, 11=*Scenedesmus obtusiusculus*, 12=*Chlorella saccharophila*, 13=*Selenastrum capricornutum*, 14=*Scenedesmus simris*, a=90% bioreactor 2 effluent, 10% municipal wastewater, b= 25% bioreactor 2 effluent, 75% municipal wastewater.

As *S. Capricornutum* is known to be sensitive to toxicity, it was also grown in bioreactor-2 effluent and municipal wastewater separately. It grew tolerable in both waters as can be seen in Figure 7.

Figure 7: S. Capricornutum grown in bioreactor-2 effluent to the left and municipal wastewater to the right. Both waters show significant growth of the species.

4.1.3 Wastewaters

The pre-screening indicated that the municipal wastewater provided for better growth than did the both waters from the pulp and paper mill. There was no significant visual difference between the bioreactor-1 and -2 effluent, and therefore the study continued using the bioreactor-2 effluent since it was mainly considered for use in the pilot scale plant planned in the Vinnova financed project.

The wastewater blend series indicated that a higher percentage of municipal wastewater resulted in higher cell densities, as seen from the turbidity of the cultures in Figure 8. The result is represented by *A. falcatus*; the other tested species, *C. reinhardtii* and *C. sorokiniana*, showed the same trend.

Figure 8: Blend series with *Ankistrodesmus falcatus*, from 100% municipal wastewater to the far left to 100% bioreactor 2 effluent to the far right. Higher percentage of municipal wastewater results in higher cell density.

4.1.4. Screening with sterile-filtered waters

Pictures from the screening with sterile-filtered effluent from bioreactor 2 and the municipal wastewater are shown below in Figure 9. Species 1-5 grown in effluent from bioreactor-2 have attached to the tissue culture flasks, as they did in the other screening sets. There is no apparent difference in these cultures from the cultures with non-sterile waters.

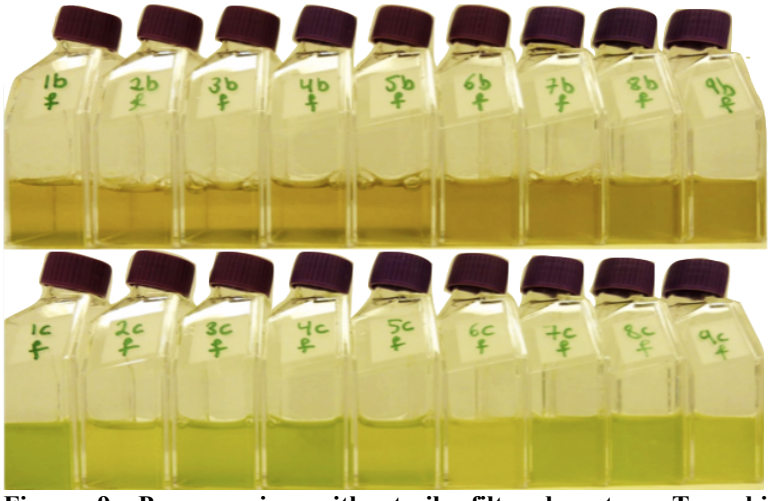


Figure 9: Pre-screening with sterile filtered waters. Top: bioreactor-2 effluent. Bottom: municipal wastewater. The species are arranged 1-9 in the same order as in the other pre-screening trials.

4.3. Scale-up cultivations

4.3.1. Growth curves and species performance

The dry weight based growth curves for all cultures are presented in Figure 10. The cultivations with 75% municipal wastewater all have higher dry weight throughout the cultivation period. The growth curves of the cultures with 10% municipal wastewater follow each other reasonably well during the latter part of the cultivation. The numbers and arrows mark phases in the cultivation period that require additional attention and are further explained below in this section. Growth curves by OD_{750} are presented in Appendix G.

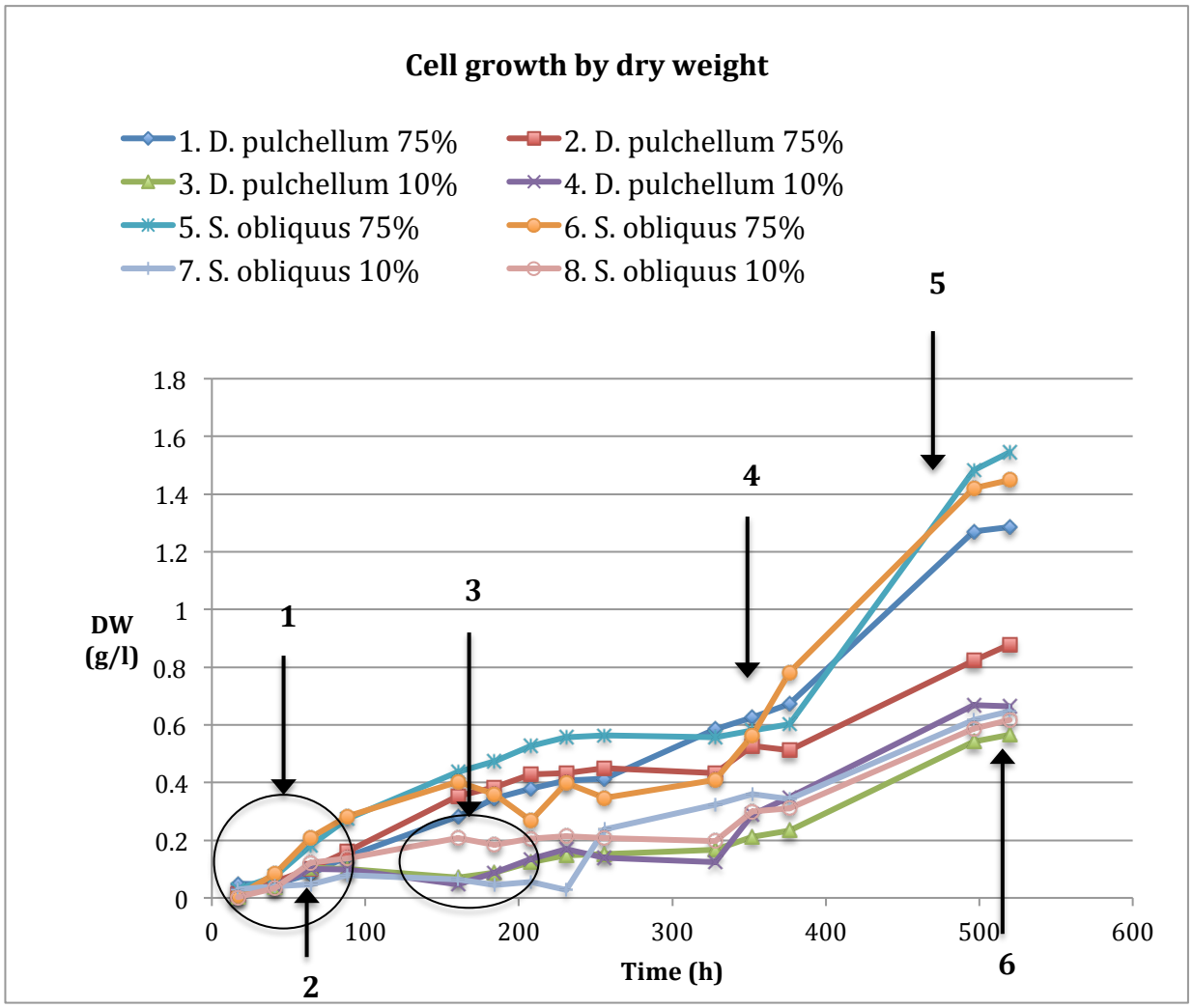


Figure 10: Growth curves based on dry weight for the eight cultures. The cultivations are referred to as culture 1 - culture 8 as is indicated in the legend. The four top growth curves are the cultivations with 75% municipal wastewater, and the lower ones are the cultivations with 10% municipal wastewater; there is a clear trend separating these two groups. The numbers and arrows point out periods in the cultivation that will be explained further.

1. The exponential phase of the growth curves are shown in a close-up in Figure 11. The specific growth rates during the exponential phase were calculated by fitting a linear regression to the semi-logarithmic graph using at least three measure points during the first 90 hours. The slope of the linear regression is equal to the specific growth rate; graphs are presented in Appendix G. The specific growth rate and the corresponding doubling time based on dry weight and OD_{750} are summarized in Table 11. Dry weight and OD_{750} give different results, showing contradictive trends. The cultures with 75% municipal wastewater have a lower specific growth rate than the cultures with 10% municipal wastewater based on dry weight, however this trend is not apparent according to OD_{750}. Furthermore, *S. obliquus* has a slightly higher growth rate than *D. pulchellum* in general based on dry weight whereas the opposite is true for the OD_{750} measurements. However, both methods show that culture 7, one of the replicates of *S. obliquus* in 10% municipal wastewater, has a significantly lower growth rate than the others.

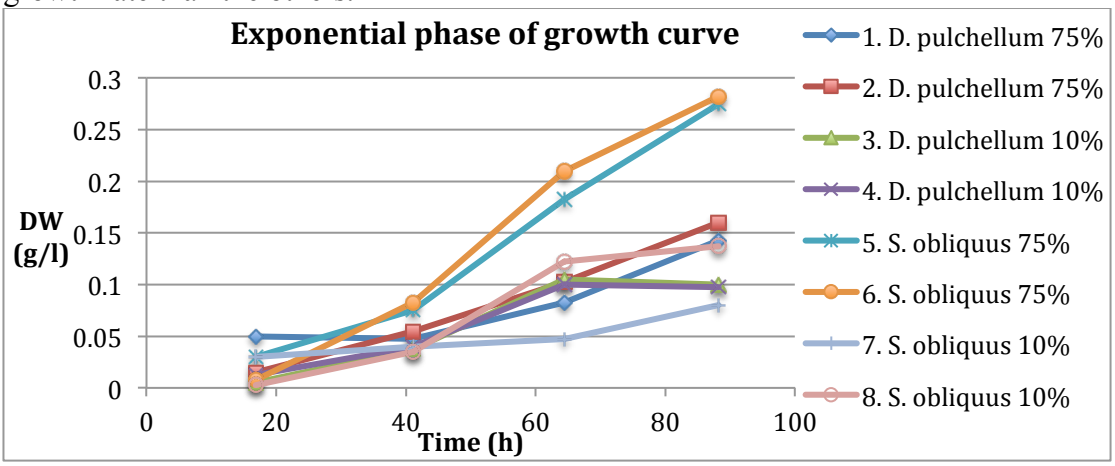


Figure 11: The exponential phase seen in more detail.

Table 11: Specific growth rates and corresponding doubling time calculated by dry weight and OD_{750}. The two measurements methods give different results and show different trends. The dry weight measurements show that *S. obliquus* have a higher specific growth rate while the opposite is true for OD_{750} measurements.

Culture	Specific growth rate by DW (h^{-1})	Doubling time by DW (h)	Specific growth rate by OD_{750} (h^{-1})	Doubling time by OD_{750} (h)
1. *D. pulchellum*, 75%	0.023	29.7	0.085	8.2
2. *D. pulchellum*, 75%	0.023	30.5	0.17	4.1
3. *D. pulchellum*, 10%	0.064	10.8	0.12	5.8
4. *D. pulchellum*, 10%	0.044	15.9	0.11	6.3
5. *S. obliquus*, 75%	0.032	21.9	0.059	11.7
6. *S. obliquus*, 75%	0.026	26.6	0.059	11.7
7. *S. obliquus*, 10%	0.013	52.9	0.042	16.5
8. *S. obliquus*, 10%	0.082	8.5	0.066	10.5

2. One of the replicates of *S. obliquus* in 10% municipal water (culture nr 7) obtained, after the first few days, a brown/yellow color compared to the replicate culture (culture nr 8), as seen in Figure 12. However, later on both cultures obtained similar colors. Microscopic checks made occasionally during the cultivation showed that bacteria, existing in the wastewater from start, continued to exist in the cultivations. On day 3, culture 7 contained more bacteria than culture 8, later in the cultivation period, there was no apparent difference between the cultures.

Figure 12: Top: *S. obliquus* in 10% municipal wastewater on day 3. The left culture (7) has a brown/yellow color compared to the right culture (8). Microscopic examination showed a larger amount of bacteria in this culture than in culture 8. Bottom: The same cultures on day 12. The colors of the both cultures are the same and there is no apparent difference between the cultures when examined under microscope.

3. On day 8, 10% additional municipal water was added to the cultures with initially 10% municipal wastewater (cultures 3,4, 7 and 8). Below, in Figure 13, the effect on cell growth is presented. The replicates of *D. pulchellum* have similar growth curves during this period, showing an increase in growth after the addition. One culture of *S. obliquus* (culture nr 8) shows similar growth rate after the addition as before. The other replicate of *S. obliquus*, (culture nr 7) shows no significant increase of growth at all during this period.

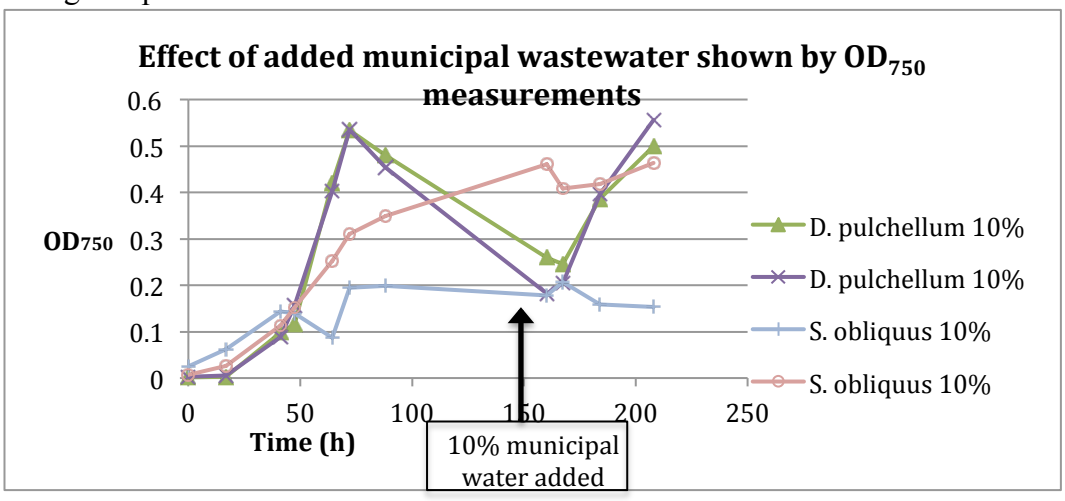


Figure 13: Effect of 10% added municipal water to cultures 3, 4, 7 and 8. Both *D. pulchellum* cultures show a clear increase after addition whereas *S. obliquus* does not show such a clear trend.

4. During the course of the cultivations, the algae attached to the cultivation flasks as can be seen in Figure 14. *D. pulchellum* seemed to be more inclined to attach to the glass flask than *S. obliquus*. Magnetic stirrers were used, however did not completely prevent attachment, especially to the sides of the cultivation flasks.

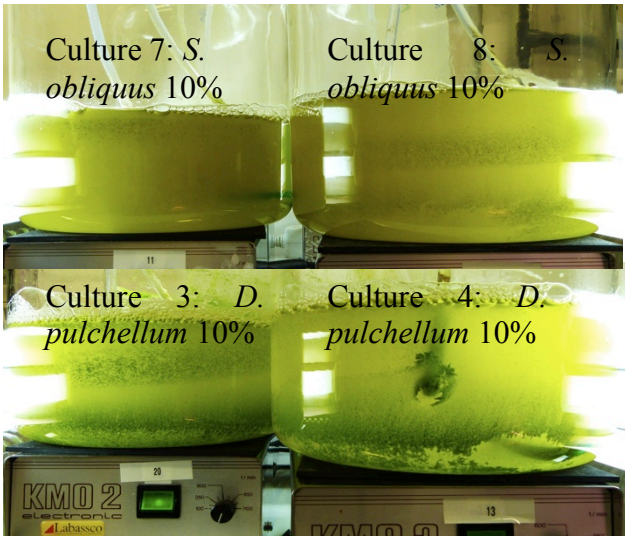


Figure 14: Day 15. : Top: *D. pulchellum* in 10% municipal wastewater. Bottom: *S. obliquus* in 10% municipal wastewater. The algae attach to the glass bottle even though agitation is provided by magnetic stirrers; *D. pulchellum* more so than *S. obliquus*.

5. Towards the end of the cultivation, one of the replicates with *D. pulchellum* in 75% municipal wastewater (culture nr 2) obtained flake-shaped larger particles throughout the cultivation flask. Figure 15 shows a picture of a sample taken from culture 2 on day 21.

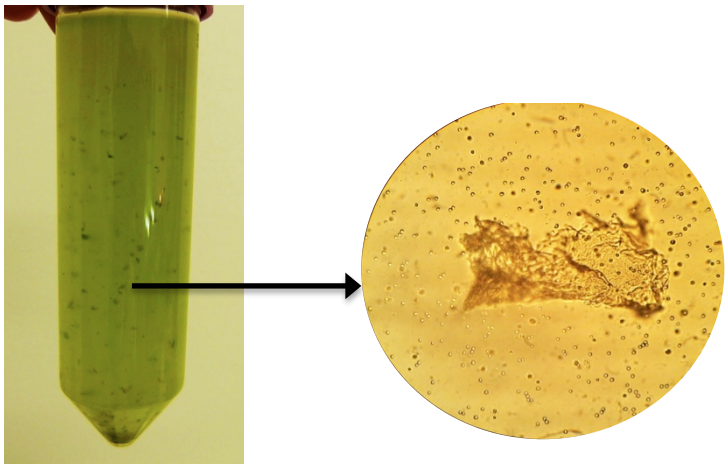


Figure 15: *D. pulchellum*, 75% (culture 2) at day 21. Sample from the culture to the left and image taken through the microscope to the right. There are large flake-like particles throughout the

6. The final dry weights as measured at the end of the cultivations on day 22 are presented in Table 12. Corresponding pictures taken through the microscope are shown for one of each species. For the cultures with 75% municipal wastewater, *S. obliquus* have a higher final dry weight. For the cultures with 10% municipal wastewater, final dry weights are substantially lower, and all within the same range.

Culture	Final dry weight (g/l)
1. *D. pulchellum*, 75%	1.29
2. *D. pulchellum*, 75%	0.88
3. *D. pulchellum*, 10%	0.57
4. *D. pulchellum*, 10%	0.67
5. *S. obliquus*, 75%	1.55
6. *S. obliquus*, 75%	1.45
7. *S. obliquus*, 10%	0.65
8. *S. obliquus*, 10%	0.62

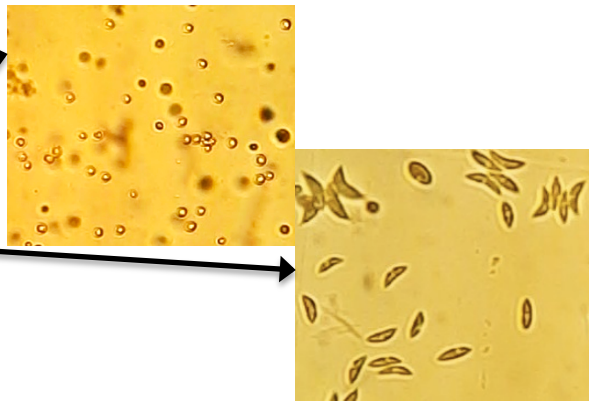


Table 12: Final dry weight as measured at 520h (day22). The cultures with 10% municipal wastewater have significantly lower dry weight. Between the species there is no clear trend, however *S. obliquus* 75% have a higher final dry weight than *D. pulchellum* 75%. Corresponding pictures taken through the microscope are shown to the right. The samples in the pictures are diluted.

4.3.2. Comparison of cell density measured by OD_{750} and dry weight

All cultures were followed by OD_{750} and dry weight throughout the cultivation period. OD_{750} measurements were made daily, sometimes twice a day, and dry weight measurements were also made daily except for some exceptions. Both methods were used for measuring cell mass, dry weight as a direct measurement, and OD_{750} as an indirect measurement. When comparing the results of both methods, the growth curves correspond well to each other as can be seen in Figure 16 and Figure 17. The OD_{750} growth curves have one additional measurement point at 426 hours, which shows the reduced cell mass increase at the end of the cultivations. When plotting dry weight with OD_{750} there is a strong linear relationship for all cultures with R-values above 0.926 and an average of 0.976 (see Appendix H).

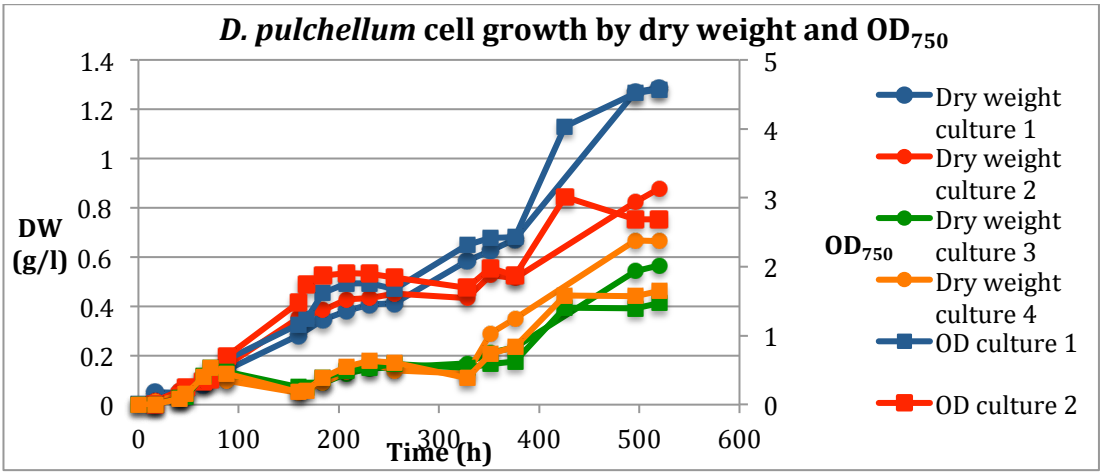


Figure 16: Comparison of dry weight and OD_{750} for *D. pulchellum*. Dry weight is on the left y-axis and OD_{750} on the right y-axis. The two measurements correspond well within duplicates.

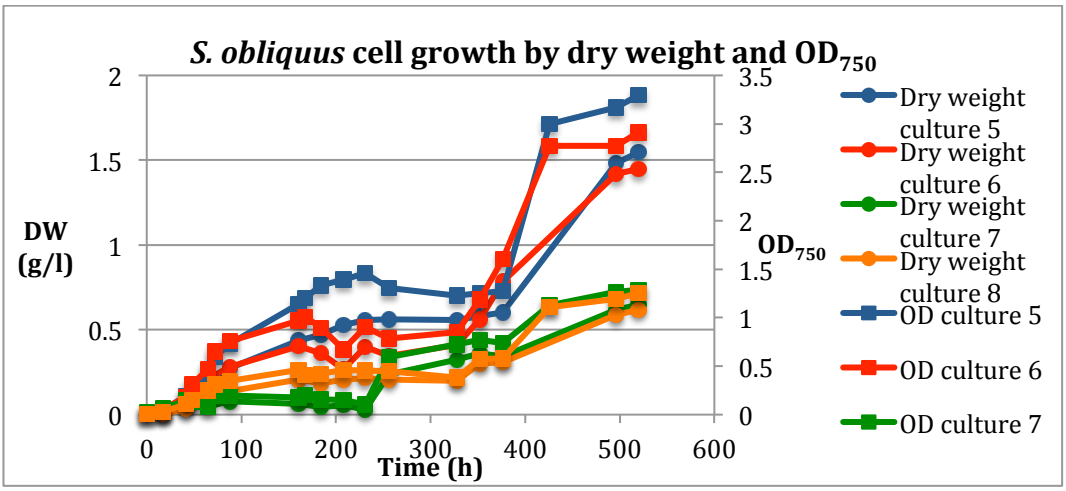


Figure 17: Comparison of dry weight and OD_{750} for *S. obliquus*. Dry weight is on the left y-axis and OD_{750} on the right y-axis. Also here, the methods correspond well within duplicates.

4.3.3. pH

pH was relatively stable throughout the course of cultivation, as seen in Figure 18. There is an increase in pH in the beginning of the cultivation, after which the pH is levelled out and kept between 7.5 and 8.5. The four cultures with 75% municipal wastewater have a higher pH than the cultures with 10% municipal wastewater throughout the cultivation.

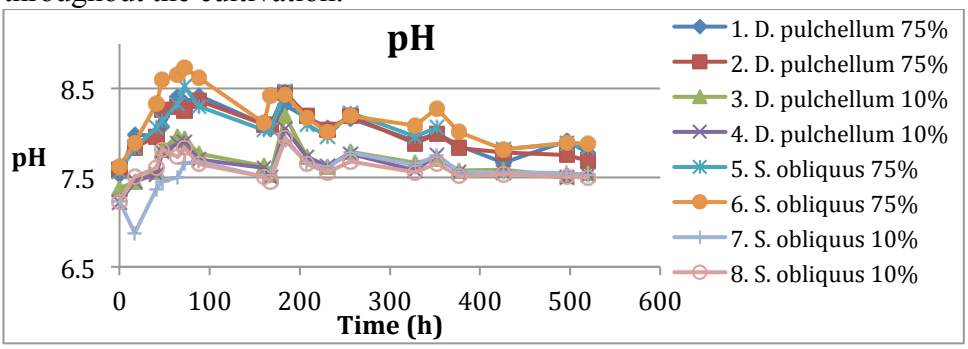


Figure 18: pH variations during the cultivation period. Except a significant increase in the beginning of the cultivations, pH is fluctuating slightly but relatively stable within a narrow range.

4.3.4. Wastewater content and nutrient consumption

Each wastewater was analysed prior to cultivation, and the nutrient levels in the beginning of each of the cultivation were calculated according to respective blend. Table 13 presents these calculated values as well as the analysis results made by the municipal wastewater treatment plant (MWTP) before delivery of the municipal wastewater and results of the bioreactor-2 water from the pulp and paper mill (Nordic Paper) in Bäckhammar. It is evident that the analysis results vary significantly between analyses, however the results from AK Lab are used for reduction calculations. The nutrient levels of each of the cultivations on day 17 were also analysed. (Complete analysis results are listed in Appendix I). The reduction of total nitrogen, ammonium, total phosphorous and phosphate over the course of the cultivation are presented in Table 14 and Table 15. The numbers for cultures 3,4,7 and 8 over three weeks are slightly underestimated since additional municipal wastewater was added on day 8. The nitrate levels were too low to quantify and are thus not included. The available molar N:P ratio (including NH_4^+ and PO_4^{3-}) is also included in the beginning and on day 17.

Table 13: Nutrient levels of each of the waters before delivery and at start of cultivation. (M.w. = municipal wastewater) Both bioreactor-effluent and the municipal wastewater have changed composition from delivery to cultivation start according to the analysis results.

Wastewater	Time of analysis	Tot N (mg/l)	NH_4^+-N (mg/l)	NO_3^--N (mg/l)	Tot P (mg/l)	PO_4^{3-}-P (mg/l)
75% m.w. (AK Lab)	Cultivation start	4.6	3.25	<0.03	0.45	0.32
10% m. w. (AK Lab)	Cultivation start	5.9	4.16	<0.03	0.424	0.294
m.w. (AK Lab)	Cultivation start	4.1	2.9	<0.03	0.46	0.33
Bior.-2 effluent (AK Lab)	Cultivation start	6.1	4.3	<0.03	0.42	0.29
m.w. (MWTP)	Before delivery	21.1	18.3	0.29	2.4	-
Bior.-2 effluent (Nordic Paper)	Before delivery	-	1.9	-	1.66	0.416

Table 14: Nutrient reduction in each culture calculated from start to day 17. Both NH_4^+ and PO_4^{3-} are reduced to a large extent over the cultivation period.

Reduction, entire cultivation period (%)					Available N:P ratio	
Culture	Tot N	NH_4^+-N	Tot P	PO_4^{3-}-P	Start	Day 17
1. *D. pulchellum*, 75%	67.4	99.8	92.0	97.2	10.2	0.7
2. *D. pulchellum*, 75%	58.7	99.8	86.9	96.3	10.2	0.6
3. *D. pulchellum*, 10%	>76.3	>97.4	>85.8	>91.5	14.1	0.3
4. *D. pulchellum*, 10%	>76.3	>99.8	>83.5	>89.8	14.1	0.3
5. *S. obliquus*, 75%	69.6	99.8	93.8	98.1	10.2	1.0
6. *S. obliquus*, 75%	69.6	99.7	93.3	98.1	10.2	1.5
7. *S. obliquus*, 10%	>69.5	>99.7	>74.1	>87.1	14.1	0.3
8. *S. obliquus*, 10%	>78.0	>99.7	>76.7	>78.6	14.1	0.2

Table 15: Nutrient reduction. Culture 3, 4, 7 and 8 are calculated for two periods: From start to day 8 when water was added and from day 8 to day 17. Reduction of NH_4^+ is large for all periods and cultures, PO_4^{3-} reductions are substantially lower.

	Reduction (%)			
	1st period (start - day 8)			
Culture	Tot N	NH_4^+-N	Tot P	PO_4^{3-}-P
3. *D. pulchellum* 10%	74.6	99.4	62.3	62.6
4. *D. pulchellum* 10%	74.6	99.6	57.5	67.0
7. *S. obliquus* 10%	67.8	99.6	36.3	45.6
8. *S. obliquus* 10%	72.9	99.5	0.9	25.2
	2nd period (day 8 – day 17)			
3. *D. pulchellum* 10%	20.5	97.4	68.4	81.1
4. *D. pulchellum* 10%	20.5	97.4	66.3	75.1
7. *S. obliquus* 10%	15.1	96.0	61.9	78.5
8. *S. obliquus* 10%	29.7	96.1	76.7	72.7

4.3.5. Cell composition

Total lipids, protein and carbohydrates as measured at three points during the course of cultivation are shown in Figure 19 to Figure 21. The cell constituents together did in some cases add up to more than 100% and in some cases below 50%. The apparent trends are that lipid content is increasing in all cultures, whereas both protein and carbohydrate content is decreasing. The lipid analysis indicates a higher final lipid content in *D. pulchellum* than in *S. obliquus,* whereas the other analyses do not show any similar consistent trends between species.

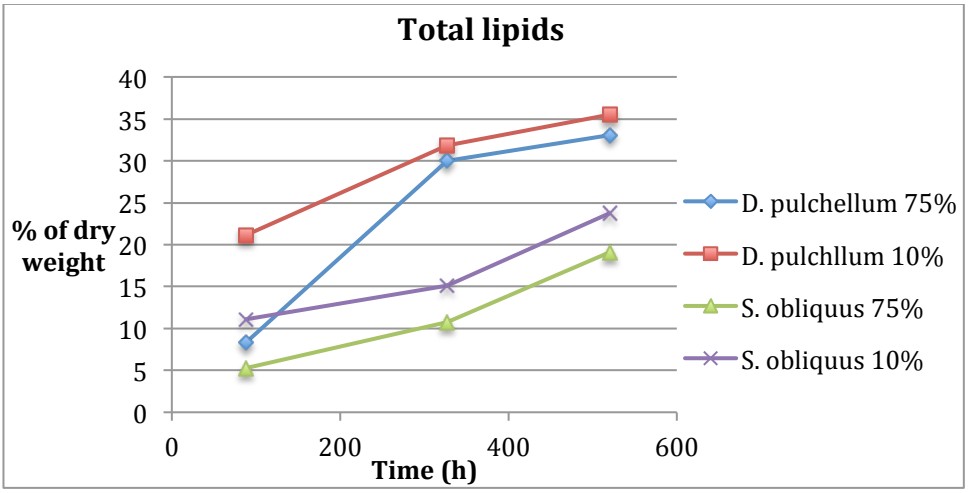


Figure 19: Total lipids at three points during the course of cultivation. The average of the duplicate cultures are shown as percentage of the corresponding dry weight. There is a clear trend of increasing lipid content during the course of cultivation.

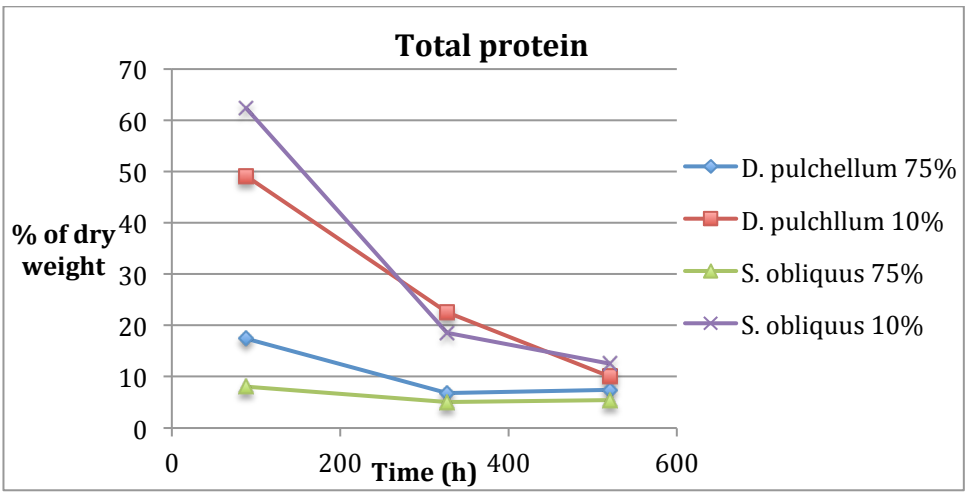


Figure 20: Total protein at three points during the course of cultivation. The average of the duplicate cultures are shown as percentage of the corresponding dry weight. The trend shows that protein content is decreasing over the course of cultivation.

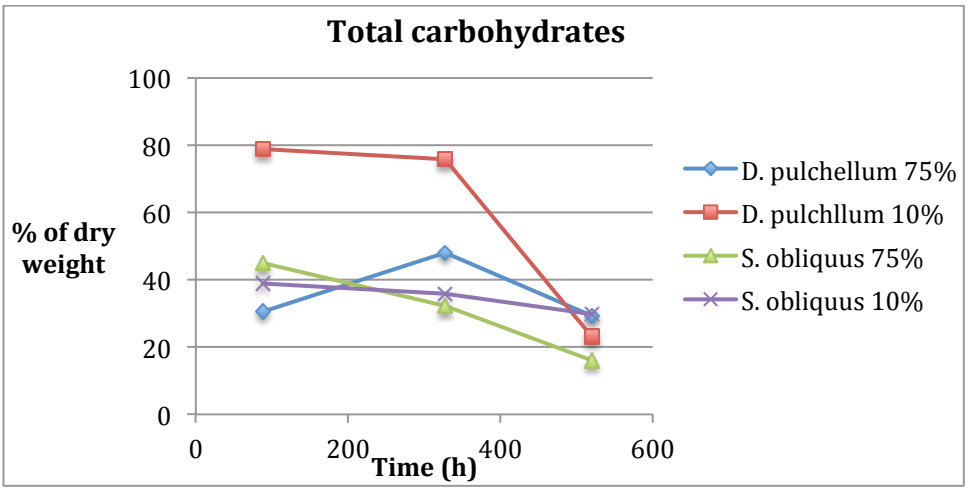


Figure 21: Total carbohydrates at three points during the course of cultivation. The average of the duplicate cultures are shown as percentage of the corresponding dry weight. There is no clear trend for change of carbohydrate content, however a majority of the cultivations shows reduced carbohydrate content over time.

The averaged final cell content is summarized in Table 16. The results indicate that *D. pulchellum* reaches higher total lipid content under these cultivation conditions than does *S. obliquus,* however the standard deviations for *D. pulchellum* is rather high. The more nutrient-poor cultivations show a higher lipid content on average for both species. There is also a visible trend in the averaged protein, where the more nutrient-poor cultivations have a slightly higher share of protein than do the cultures containing more nutrients.

Table 16: Average total lipids, protein and carbohydrates at the end of the cultivation period (day 23). *D. pulchellum* has higher lipid content than *S. obliquus.* There are no clear trends for total protein and total carbohydrates.

Species	Total lipids (% of dry weight)	Total protein (% of dry weight)	Total carbohydrates (% of dry weight)
***D. pulchellum* 75%**	33 ±5.2	7 ±1.6	30±6.2
***D. pulchellum* 10%**	35 ±11.2	10 ±2.7	23±1.9
***S. obliquus* 75%**	19 ±0.7	5 ±0.1	16±4.7
***S. obliquus* 10%**	23 ±0.3	12±0.6	30±0.7

5. Discussion

5.1. Selection of species for scale-up

The species that showed best growth from the first set of pre-screening of species were *A. falcatus*, *C. reinhardtii*, *C. sorokiniana* and *C. protothecoides*. *A. falcatus*, however, showed a change in morphology in the waters from bioreactor 1 and 2 (see Table 9). The shape was irregular and bended, suggesting that some substance in the water affected the shape. Because of this change in morphology and the fact that the culture was contaminated from start, *A. falcatus* was not used for the larger screening. *C. protothecoides* showed good visual growth after 12 days, better than both *C. reinhardtii* and *C. sorokiniana*. *C. protothecoides* is known to grow heterotrophic (Xu, Miao, & Wu, 2006) (Heredia-Arroyo, Wei, & Hu, 2010), which could result in higher cell densities than solely autotrophic growth. It was thus an interesting candidate for scale-up. Neither of the species in the second set, nor *C. protothecoides,* was attached to the tissue culture flasks, which is a good quality when growing them in larger flasks.

All species in the second set showed good growth, particularly the *Scenedesmus* species (see Table 9). However it was preferred to only choose one *Scenedesmus* species to keep a variety of species, and *S. obliquus* had proved to grow well in simulated flue gas (Bark, 2012). *D. pulchellum* had been found in the open pond in Umeå, Sweden, and was therefore interesting as a species that was competitive and thrived naturally in these kinds of environments. *D. pulchellum* was for these reasons chosen over *C. protothecoides*. *S. obliquus* and *D. pulchellum* were thus chosen for the scale-up screening.

5.2. Wastewaters

The screening of the water blends showed that there was increased growth in higher percentage of the municipal wastewater (see Figure 8), which was expected since this water is more nutrient rich according to the analyses (see Table 3). Although there would therefore be advantageous to grow the microalgae in pure municipal wastewater, a low concentration is preferred due to the main purpose of the study to utilize the wastewater from the pulp and paper mill. 10% municipal wastewater showed increased growth compared to pure bioreactor-2 effluent and was therefore chosen for the scale-up study.

The cultures in the sterile water screening showed no visual significant difference from the non-sterile screening (see Figure 9), indicating that the microorganisms native to the waters are either out-conquered by the algae when grown in these conditions or have no significant effect on the growth of the algae. The microscopic examinations during the run of the scale-up cultures showed microorganisms other than algae in all cultures. In the beginning of the experiment, one culture of *S. obliquus* (culture 7) showed larger amounts of other microorganisms than the other cultures, which is likely to have related to the brown/yellow color of that culture compared to the greener color of the duplicate culture (see Figure 12). However, later on the cultures obtained similar colors, indicating that the algae to bacteria ratio had become similar in the duplicate cultures. This effect indicates that there is a balance between the microalgae and other microorganisms in the culture. The yellow color that was obtained by several cultures, including culture 8, later on in the study, is likely to be a result of chlorophyll degradation due to nutrient deficiency or cell death.

However it could also be a result of increased bacterial growth as the algal growth decreased. Considering the high nutrient reductions obtained in this study and the rather high levels of bacteria in the wastewater, a beneficial relationship between microalgae and bacteria, such as is described by Lau *et al.* (Lau, Tam, & Wong, 1995) and Subashchandrabose *et al.* (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2011) and discussed previously in the theory section, is likely to have existed.

5.3. Comparison of species in the scale-up study

The specific growth rate in the exponential growth phase is marginally higher for *S. obliquus*, if neglecting culture nr 7 (see Table 11). Also the final density is somewhat higher for most cultures of *S. obliquus* (see Table 12), although the high variation between the duplicates indicates that these results may depend very much on small variations in cultivation conditions such as temperature and level of agitation. In a study on *S. obliquus* cultivated in secondarily treated urban wastewater at 30°C, the maximum specific growth rate was $0.0438h^{-1}$ (Martinez, Jimenez, & Yousfi, 1999) and another study received a maximum specific growth rate of $0.052\ h^{-1}$ in secondarily treated urban wastewater (Hodaifa, Martinez, Órpez, & Sánchez, 2012). Both studies thus reported values that are in the range of the obtained values in this study ($0.013\text{-}0.082h^{-1}$).

Temperature was relatively stable over the period of cultivation, but differed somewhat between cultures (see Table 8). A comparison of the replicate cultures of *S. obliquus* 75% indicates that temperature is an important growth factor. One of the cultures, culture 6, had on average 1.3°C higher temperature than did culture nr 5, which may be a reason for the higher final dry weight. The temperature optimum of *S. obliquus* has been reported to be 20-30°C (see Table 1), but is dependent on other factors, such as light intensity (Bouterfas, Belkoura, & Dauta, 2002). It is however likely that culture nr 6 is closer to the temperature optimum of *S. obliquus* in these conditions, which would thus be above 30°C. Culture nr 2 (*D. pulchellum* 75%) had the highest average temperature (32.7°C), which is above the temperature optimum for many algal species (see Table 1). The growth of culture nr 2 compared to its replicate culture nr 1 (with average temperature 30.3°C) is not as high, and formed flake-like aggregates after three weeks of cultivation (see Figure 15). It is thus possible that 32.7°C is above the temperature optimum for *D. pulchellum*, and becomes limiting for growth.

Limitation of growth due to insufficient light is a common occurrence for microalgae cultivation. Light limitation can often be detected by a linear phase in the growth curve as described previously. It is difficult to tell if this is a factor in this study since there is no representable growth curve between the exponential growth phase and the latter part of the cultivation. It is however likely that the color of the wastewater affected light penetration and consequently photosynthesis. The issue of coloured wastewaters have been reported in a study on olive oil mill wastewater as one of the main limiting factors (Hodaifa, Martinez, Órpez, & Sánchez, 2012), and is likely to increase with scale-up of cultivation system.

The nutrient analysis of the municipal wastewater before start of cultivations showed significantly lower values than the preliminary analysis made at the wastewater

treatment plant (see Table 13). One plausible explanation may be that nutrients were removed during filtration by preparing the wastewater for cultivation. There is however also likely that nutrients levels were reduced between analyses; NH_4^+ may have been reduced by NH_3 formation due to the slight alkaline conditions as described by Martinéz *et al.* (Martínez, Sánchez, Jiménez, Yousfi, & Munoz, 2000), or indigenous microorganisms may have altered the conditions. However, the significantly higher final biomass concentration of the cultures with 75% municipal wastewater (see Table 12) indicates that these cultures did contain more nutrients from the beginning than the second analysis shows.

The molar ratio of available N:P is lower than the Redfield ratio at the beginning of cultivation, and is further reduced at day 17 (see Table 14). Furthermore, Rhee reported a molar ratio N:P for *Scenedesmus* species to be 30:1 (Rhee, 1978), indicating that nitrogen becomes limiting before the end of cultivation. In general, the nutrient reductions obtained are very high over the course of the cultivation period (see Table 14), however most reported nutrient reductions are for a substantially shorter cultivation period. One study reported 95-100% NH_4^+ removal and 55-85% PO_4^{3-} removal after two days using immobilized algae (Ruiz-Marin, Mendoza-Espinosa, & Stephenson, 2010). It was suggested however that these values were high due to improved efficiency using immobilized cells. Another study with primary settled wastewater showed 90% NH_4^+ reduction after eight days and 80% phosphate reduction after ten days (Lau, Tam, & Wong, 1995). Another study with secondarily treated wastewater reported 100% NH_4^+ reduction after eight days and 98% P reduction after four days (Martínez, Sánchez, Jiménez, Yousfi, & Munoz, 2000). The reductions in the present study measured over the first 8 days show similar NH_4^+ reductions as previously reported, however the PO_4^{3-} reduction is lower (see Table 15). This lower reduction of PO_4^{3-} may be due to a more nitrogen limiting culture medium, or uncertainties in the analysis

The trends of cell composition change during the cultivation period are expected and consistent with literature findings. Total lipids are increasing with reduced nitrogen levels, which is the base for the common method of nitrogen starvation for the purpose of increased lipid content. Protein levels are decreasing over time, and are somewhat consistent with previously reported values of reductions. One study showed protein reduction from 56% to 8% protein (Breuer, Lamers, Martens, Draaisma, & Wijffels, 2012) for *S. obliquus,* and another study on wastewater with nitrogen limiting conditions reported 11.8% protein (Martínez, Sánchez, Jiménez, Yousfi, & Munoz, 2000). Although the trend is not very clear, carbohydrate levels are decreasing in general over the course of the cultivation. Final lipid content in *S. obliquus* is somewhat higher than reported findings (see Table 2), which likely is due to the nitrogen limiting conditions in the present study. Neither species seem to accumulate large amounts of carbohydrates; instead energy storage seems to be directed towards lipid accumulation.

5.4. Experimental setup issues

The equipment setup of the scale-up study was tried here for the first time. Some unexpected factors influenced the results of the study. After two days of cultivation it was noted that the algae had a tendency to attach to the glass flasks as well as the tubes inside the cultivation flask. To obtain more even mixing, magnetic stirrers were introduced. However, these stirrers caused unintentional heating of the cultivations,

causing somewhat uneven temperatures between cultures. In addition, the stirrers did not provide sufficient mixing and thus the samples did not, during the majority of the cultivation period, represent the overall cell density of the cultures. Only at the end of the cultivation period, when the volume was reduced to about half, could sufficient mixing be provided by shaking the cultivation flasks. Meaningful conclusions about cell growth can thus only be drawn from the very first and last parts of the cell growth curves. To avoid this issue, it is recommended to ensure sufficient mixing, for example by keeping the volume of cultivation medium sufficient low, when using this kind of setup.

5.5. Analyses reliability

The specific growth rates correspond reasonably well between duplicates with the exception of culture nr 7, which was discussed previously. Specific growth rate for the duplicates culture 1 and 2 varies significantly using OD_{750} measurements, however they correspond using dry weight measurements. The specific growth rate as measured by OD_{750} is different than that measured by dry weight. As the initial OD is quite low (below the lower limit of reliable measurements; OD_{750} 0.2), the measurements in the beginning of the cultivation may be unreliable, and thus the calculated specific growth rate may be incorrect. The growth rate calculated from the dry weight may also be unreliable due to the low cell concentration, however probably not to the same extent. Likewise, when measuring cell concentration, dry weight is to be considered the more reliable method, since OD is affected by cell size and how they group together; characteristics which may change over the cultivation period. OD_{750} for the both species are not comparable, as seen when comparing with dry weight. *S. obliquus* form distinct groups of four up to sixteen as seen in microscope. In addition, *S, obliquus* cells are larger than *D. pulchellum,* which may give a lower OD_{750} value compared to dry weight.

Cell composition analysis was quite uncertain, as is apparent when adding the different constituents together, which results in mass percentages well above 100% of dry weight in some instances, and below 50% in some cases. The least reliable analysis of the cell content analysis methods appeared to be the total carbohydrate analysis. Re-runs of samples differed significantly in results, and the small volumes involved imply large uncertainties of the results. The protein analysis involved an extraction of protein from the biomass, which may not have been performed to 100%. Also the lipid analysis involved uncertainties, due to incomplete extraction or removal of oil phase, however here an approximate extraction yield was accounted for. Other factors affecting the lipid analysis are contamination during the GC-analysis and integration of peak areas. In addition the methanolysis of triglycerides implies that all lipids are not fully accounted for using this analysis method. For all cell composition analyses, dry weight is used to calculate the weight percentage of each of the constituents. Uncertainties in dry weight measurements thus affect all results to a significant extent.

6. Conclusion

The main goal of the study was to determine whether microalgae are able to grow in wastewater from a pulp and paper mill. The results indicate that they can, however to a limited extent. The main limitation seems to be nutrient deficiency, which can be helped by adding a wastewater with higher nutrient levels. Neither wastewater that was tested seem to have severe toxic effects on algae. Since the effluents from bioreactor-1 and 2 displayed similar performance it was concluded that bioreactor-2 effluent is the more desirable growth medium as it is the latter step in the wastewater treatment. A diversity of algae was tested in the wastewaters and only one species (*Chlorella saccharophila*) did not grow to any significant extent in either water. All of the *Scenedesmus* species grew well and did not seem to differ significantly between each other. *D. pulchellum* had marginally slower growth but had a higher final lipid content than *S. obliquus*. Both species showed efficient nutrient reduction in the wastewaters, comparable to precious studies. As not much research has been made on *D. pulchellum,* further investigation of this species may be needed, for instance to examine its tolerance to flue gas and temperature. Any *Scenedesmus* species may be a good choice for further research and for scale-up.

7. Further research

There were some topics appearing in this study that is interesting for further research, one of them being characteristics of the wastewater. Degradation products of pharmaceuticals and heavy metals are commonly found in municipal wastewater and may be further analysed of its effect on the final biomass product and possible waste streams. If municipal wastewater should be used as an additional nutrient source it is also interesting to investigate the effect of storage on its composition, since the present study indicated significant changes of nutrient concentration during a rather short storage period. One issue with using wastewater is the turbidity and color, which causes light limiting conditions. An alternative to using wastewater as culture medium, and avoid issues of turbidity is to take advantage of the sludge that is produced in excess in any wastewater treatment plant. This nutrient rich sludge is available in abundance at the pulp and paper mill, and could be used to leach out the nutrients in a microalgae cultivation. Reduction of nutrients from the sludge would be beneficial for the pulp and paper mill as some of it is used as construction material in landfills, where leaching of nutrients is not desirable. Another area for research is the effect of flue gas combined with cultivation in wastewater, which could have potentially large influences on both cell growth and cell composition. For an outdoor large-scale cultivation, the conditions will be substantially different; sufficient mixing and light are important parameters, as is the effect of contamination, which is likely to occur in an outdoor setting.

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Appendix A

3N-BBM+V (Bold's Basal Medium with 3fold Nitrogen and Vitamins; modified)

Stock solutions in g / 1000 ml water	for 1 liter final medium
• 25.0 g $NaNO_3$	30.0ml
• 2.5 g $CaCl_2 \cdot 2\ H_2O$	10.0ml
• 7.5 g $MgSO_4 \cdot 7\ H_2O$	10.0ml
• 7.5 g $K_2HPO_4 \cdot 3\ H_2O$	10.0ml
• 17.5 g KH_2PO_4	10.0ml
• 2.5 g NaCl	10.0ml
• Trace element solution (see below)	6.0ml
• Vitamin B1 (see below)	1.0ml
• Vitamin B12 (see below)	1.0ml

Autoclave each stock solution (not the vitamins) separately before mixing the medium. Make up to 1 liter with distilled water and store cold.

Trace metal solution

Add to 1000 ml of distilled water 0.75 g Na2EDTA and the minerals in exactly the following sequence:

• $FeCl_3 \cdot 6\ H_2O$	97.0mg
• $MnCl_2 \cdot 4\ H_2O$	41.0mg
• $ZnCl_2 \cdot 6\ H_2O$	5.0mg
• $CoCl_2 \cdot 6\ H_2O$	2.0mg
• $Na_2MoO_4 \cdot 2\ H_2O$	4.0mg

Vitamin B1

0.12 g Thiaminhydrochloride in 100 ml distilled water. Filter sterile.

Vitamin B12

0.1 g Cyanocobalamin in 100 ml distilled water, take 1 ml of this solution and add 99 ml distilled water. Filter sterile.

Appendix B

Calculation of inoculum for pre-screening of species

Since the pre-culture biomass concentration varied substantially between species, the size of the inoculum of the pre-screening was determined according to OD_{750} of the pre-cultures. The reference volume was set to 250µl of the *B. braunii* pre-culture and the volumes for the other species were calculated based on OD_{750}. The measured OD_{750} values and the calculated volumes for pre-screening set 1 are summarized in Table 17.

Table 17: OD_{750} values of the pre-cultures and calculated volumes for inoculation to pre-screening set 1. The volumes are based on 250 µl of the *B. braunii* pre-culture.

Species	OD_{750} pre-culture	Volume (µl)
Chlorella vulgaris	1.0455	268
Ankistrodesmus falcatus	0.8077	346
Botryococcus braunii	1.1191	250
Chlamydomonas reinhardtii	0.99195	282
Chlorella sorokiniana	1.1712	239
Chlorella protothecoides	0.77545	361

$$V_{inoculum,SpeciesX} = \frac{250\mu l * OD_{750,B.braunii}}{OD_{750,SpeciesX}}$$

Example for *C. vulgaris*:

$$V_{inoculum,C.vulgaris} = \frac{250\mu l * 1.1191}{1.0455} = 268\mu l$$

The inoculation of the second species pre-screening run was calculated in the same way with values listed in Table 18.

Table 18: OD_{750} values of the pre-cultures and calculated volumes for inoculation to pre-screening set 2. The volumes are based on 250 µl of the *B. braunii* pre-culture.

Species	OD_{750} pre-culture	Volume (µl)
Botryococcus braunii	1.0306	250
Scenedesmus obliquus	0.20105	1282
Scenedesmus dimorphus	0.1945	1325
Dictyosphaerium pulchellum	0.17635	1461

Appendix C

Calculation of dry weight.

Calculation of total protein is demonstrated by an example calculation of the sample from culture 1, day 15. The filter weight before and after filtering is listed below. The dry weight was measured in duplicates using 20ml per duplicate.

Filters before	1a	0.0888g
	1b	0.0876g
Filters after	1a	0.1001g
	1b	0.0991g

- The difference in weight of each filter was calculated:
-

$$Weight\ sample\ 1a = 0.1001g - 0.0888g = 0.0113g$$
$$Weight\ sample\ 1b = 0.0991g - 0.0876g = 0.0115g$$

- The blank sample was subtracted from the cell sample. In this case the blank sample showed a negative value.

$$Net\ weight\ sample\ 1a = 0.0113g + 0.0003g = 0.0116g$$
$$Net\ weight\ sample\ 1b = 0.0115g + 0.0003g = 0.0118g$$

- The average of the samples was calculated:

$$Average\ sample\ 1 = \frac{0.0116g + 0.0118g}{2} = 0.0117g$$

- The weight per 1L was calculated:

$$Sample\ 1\ \frac{g}{l} = \frac{0.0117g}{20ml} * 20 * \frac{1000ml}{l} = 0.57g/l$$

Appendix D

Carbohydrate analysis – standard curves and calculation of cell content percentage.

The carbohydrate standard curves are shown below in Figure 22.

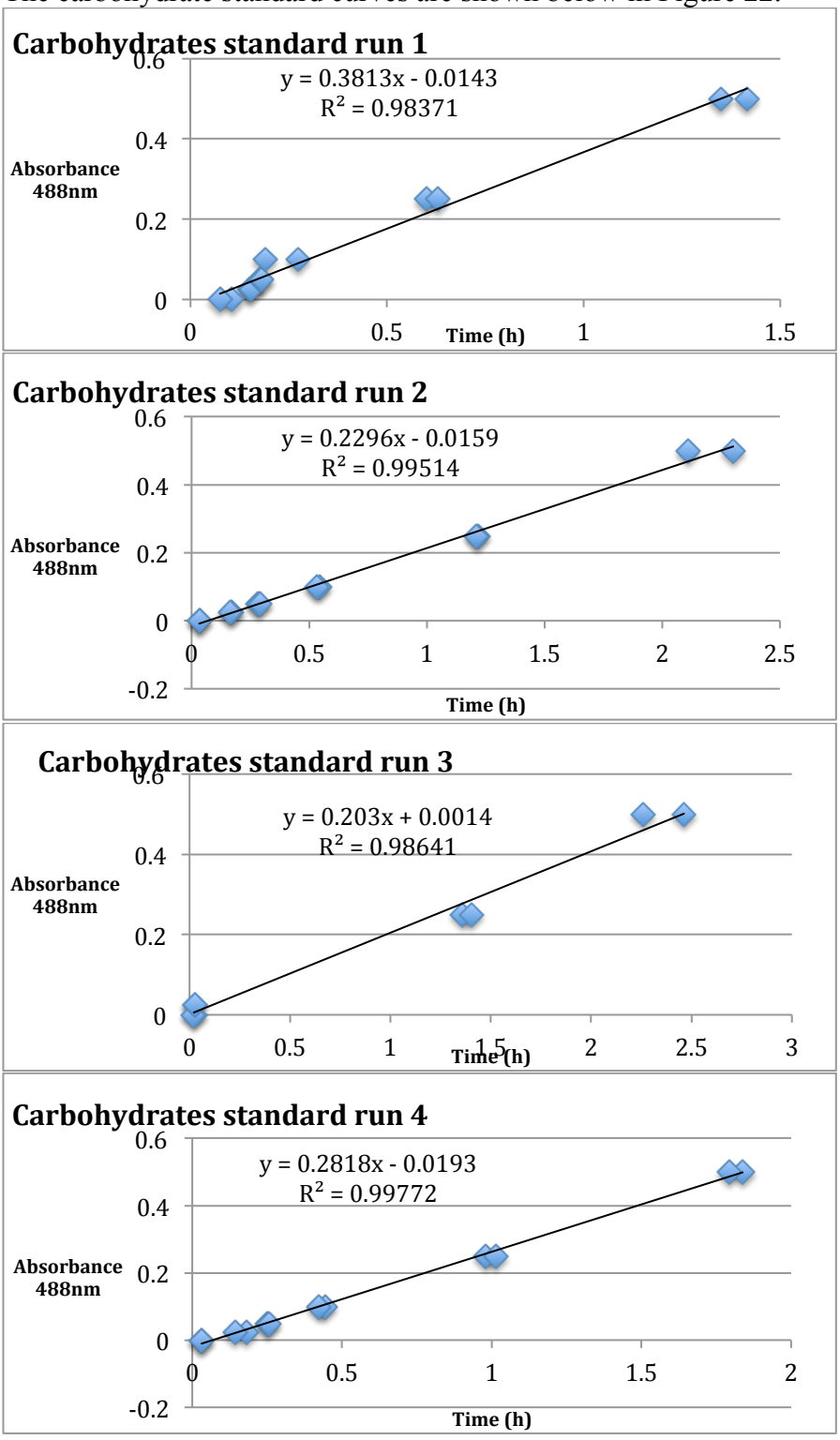

Figure 22: Carbohydrate standard curves.

The calculation of total carbohydrate content is explained by an example (culture 1 from day 15). The raw data obtained from the microtiter plate reader for the sample and corresponding supernatant is shown in Table 19 below.

Table 19: Raw data for sample from culture 1 day 15. Well E2-G2 contain triplicates of the cell sample, well H2-B3 contain the corresponding supernatant.

Well Row	Well Col	Content	Raw Data (488) 1 - 0 s	Raw Data (488) 2 - 86 s	Raw Data (488) 3 - 172 s
			0	86	172
E	2	Sample X50	0.4371	0.4377	0.4382
F	2	Sample X62	0.4493	0.447	0.4484
G	2	Sample X74	0.4251	0.424	0.4254
H	2	Sample X86	0.0974	0.0956	0.0985
A	3	Sample X3	0.1092	0.104	0.1064
B	3	Sample X15	0.1205	0.1218	0.1196

- The three measurement points of each well were averaged.

$$Well\ E2\ average = \frac{0.4371 + 0.4377 + 0.4382}{3} = 0.4377$$

- The same calculations were made for the other wells resulting in the averaged values below:

E2	0.4377
F2	0.4482
G2	0.4248
H2	0.0972
A3	0.1065
B3	0.1206

- The triplicates of the sample and corresponding supernatant were averaged:

$$Cell\ sample\ average = \frac{0.4377 + 0.4482 + 0.4248}{3} = 0.4369$$

$$Supernatant\ average = \frac{0.0972 + 0.1065 + 0.1206}{3} = 0.1081$$

- The averaged value was calculated into g/l carbohydrates by applying the corresponding standard curve equation to the value, in this case the standard curve from run 2.

$$Cell\ sample\ carbohydrate\ conc. = 0.4369 * 0.2246 - 0.0159$$
$$= 0.08223 g/l$$

$$Supernatant\ sample\ carb.conc. = 0.1081 * 0.2246 - 0.0159$$
$$= 0.00838 g/l$$

- The carbohydrate concentration of the supernatant was subtracted from the value of the corresponding cell sample.

$$Cell\ carbohydrate\ conc. = 0.08223 - 0.00838 = 0.07385 g/l$$

- If the sample was diluted, the value was corrected correspondingly. (Sample diluted 3x)

$$Corrected\ cell\ carbohydrate\ conc. = 0.07385 * 3 = 0.22155 g/l$$

- By dividing with the corresponding dry weight measured at the point the sample was taken, the biomass percentage of carbohydrates was obtained (dry weight culture 1 day 15: 0.57g/l):

$$Part\ carbohydrates = \frac{0.22155}{0.57} = 0.39 = 39\%$$

Appendix E

Protein analysis – standard curves and calculation of cell content percentage.

The protein standard curves are shown in Figure 23.

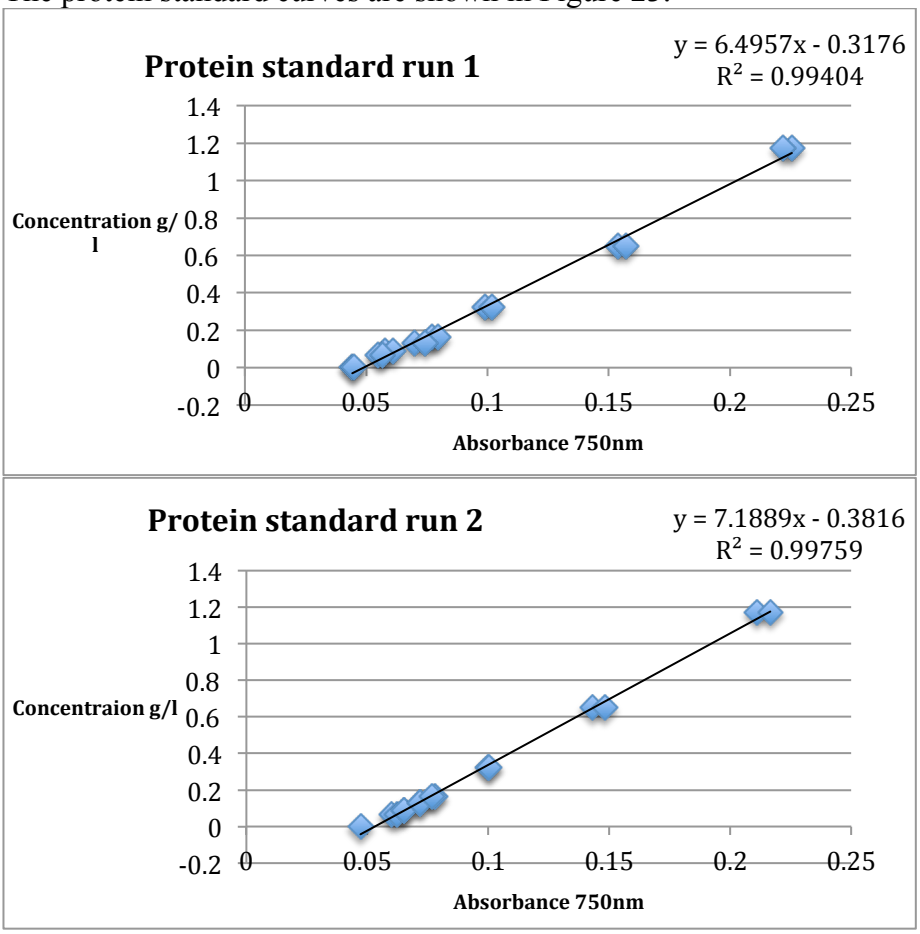

Figure 23: Protein standard curves.

Calculation of total protein is demonstrated with an example calculation of the sample from culture 1, day 15. The raw data is presented in Table 20.

Table 20: Raw data for sample from culture 1 day 15. Well A5 and B5 contain duplicates of the cell sample.

Well Row	Well Col	Content	Raw Data (750) 1 - 0 s	Raw Data (750) 2 - 86 s	Raw Data (750) 3 - 172 s
			0	86	172
A	5	Sample X5	0.0493	0.0502	0.05
B	5	Sample X17	0.0541	0.0533	0.0553

- The three measurement points of each well were averaged.

$$Well\ A5\ average = \frac{0.0493 + 0.0502 + 0.05}{3} = 0.04983$$

$$Well\ B5\ average = \frac{0.0541 + 0.0533 + 0.0553}{3} = 0.05423$$

- The duplicates of the sample were averaged:

$$Cell\ sample\ average = \frac{0.04983 + 0.05423}{2} = 0.05445$$

- The averaged value was calculated into g/l carbohydrates by applying the corresponding standard curve equation to the value, in this case the standard curve from run 1.

$$Cell\ sample\ protein\ conc. = 0.05445 * 6.4957 - 0.3176 = 0.03609 g/l$$

- The dilution of the cell sample (addition of 10µl SDS to 90µl sample) was corrected for:

$$Corrected\ cell\ sample\ protein\ conc. = 0.03609 * \frac{100}{90} = 0.0401$$

- By dividing with the corresponding dry weight measured at the point the sample was taken, the biomass percentage of carbohydrates was obtained (dry weight culture 1 day 15: 0.57g/l):

$$Part\ carbohydrates = \frac{0.0401}{0.57} = 0.070 = 7.0\%$$

Appendix F

Lipid analysis – standard curves and calculation of cell content percentage.

The standard curves that were used are shown below in Figure 24 with concentration on the y-axis and response on the x-axis. C19:0 was quantified by using C18:0, since the saturated FAMEs have similar standard curves within the same range of carbon chain length.

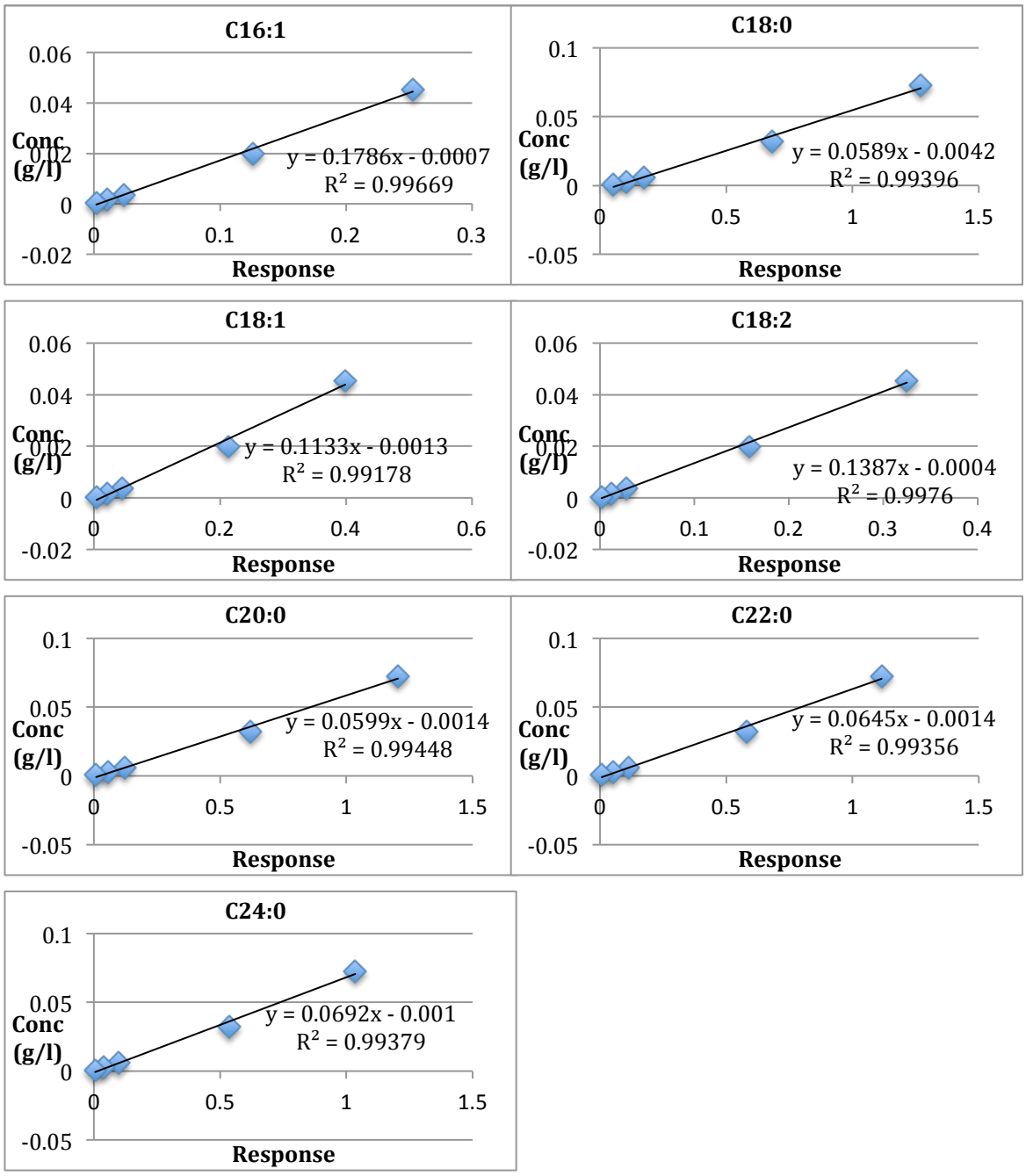

Figure 24: Standard curves of FAMEs. Response (Peak area divided with corresponding C17 peak area) on the x-axis and concentration on the y-axis.

The calculations of total lipids are illustrated by an example of one of the measured FAMEs. Corresponding calculations were made for all measured FAMEs and the values added to obtain the total lipid amount.

Raw data: Sample A, C16:0 = 12174612
Sample A, C17:0 = 6079240
Sample B, C16:0 = 12167891
Sample B, C17:0 = 6405125

$$Response\ sample\ A = \frac{12174612}{6079240} = 2.002654$$

$$Response\ sample\ B = \frac{12167891}{6405125} = 1.89971117$$

$$GC\ sample\ A\ concentration = 2.002654 * 0.062 - 0.0058 = 0.1183645 g/l$$
$$GC\ sample\ B\ concentration = 1.89971117 * 0.062 - 0.0058 = 0.111982 g/l$$

- Correcting for dilution (original cell suspension=1ml, final oil phase=0.3ml).
  $Sample\ A\ correction\ for\ dilution = 0.118365 * 0.3 = 0.03551$
  $Sample\ B\ correction\ for\ dilution = 0.111982 * 0.3 = 0.03359$
- The yield of the methanolysis was calculated by dividing the concentration of C19 with the theoretical value, which gives a yield of 0.448. Thus the yield was low enough to consider:

$$Sample\ A\ correcting\ for\ yield = \frac{0.03551}{0.448} = 0.07926\ g/l$$
$$Sample\ B\ correcting\ for\ yield = \frac{0.03359}{0.448} = 0.07498\ g/l$$

- The samples were averaged:

$$Averaged\ sample = \frac{0.07926 + 0.07498}{2} = 0.07712\ g/l$$

The calculated concentration is the mass of FAME. However since the weight of three FAMEs is very close to the weight of one triglyceride, no correction for this was made.

Appendix G

Cell growth by OD750 and calculation of specific growth rates.

The cell growth by OD is shown in Figure 25.

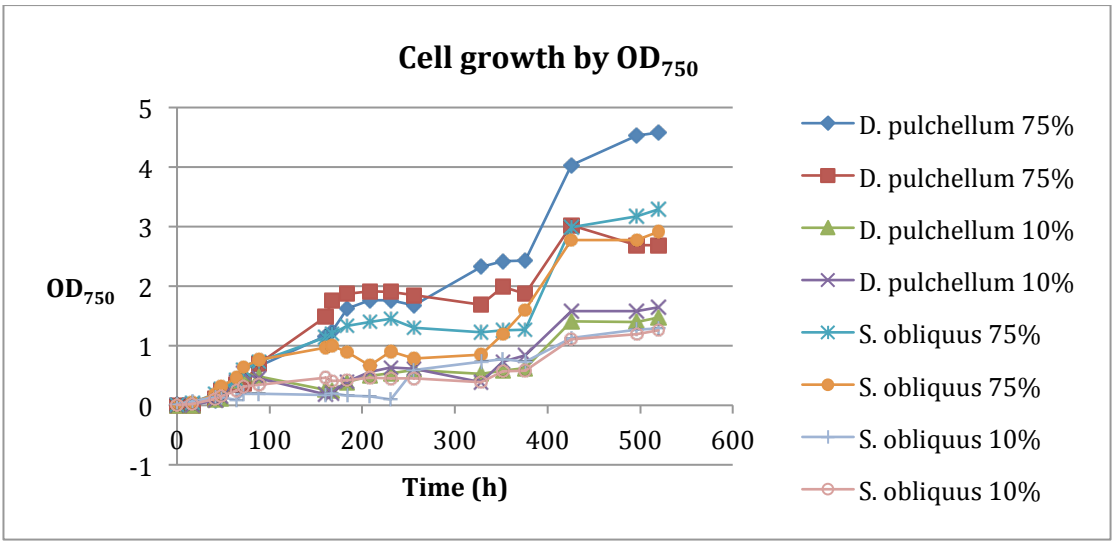

Figure 25: Cell growth by OD_{750}.

The specific growth rates were calculated based on dry weight and OD_{750}. The first measure points were plotted in a logarithmic scale and the estimated linear phase was used to create a linear regression. The specific growth rate is the slope of the linear regression. The graphs are seen in Figure 26 and Figure 27.

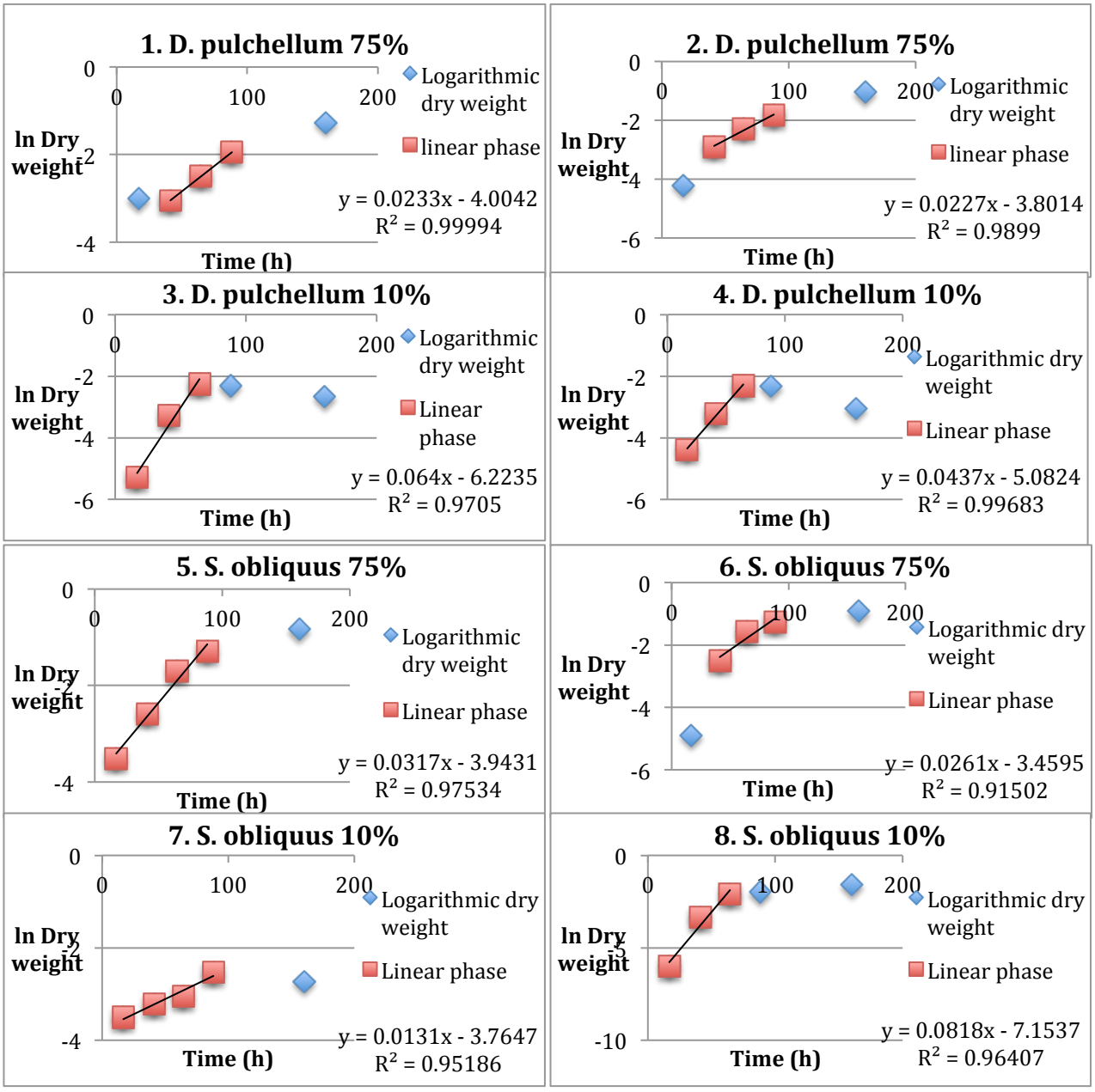

Figure 26: Linear regressions fit to the exponential phase of the logarithmic growth curve measured in dry weight.

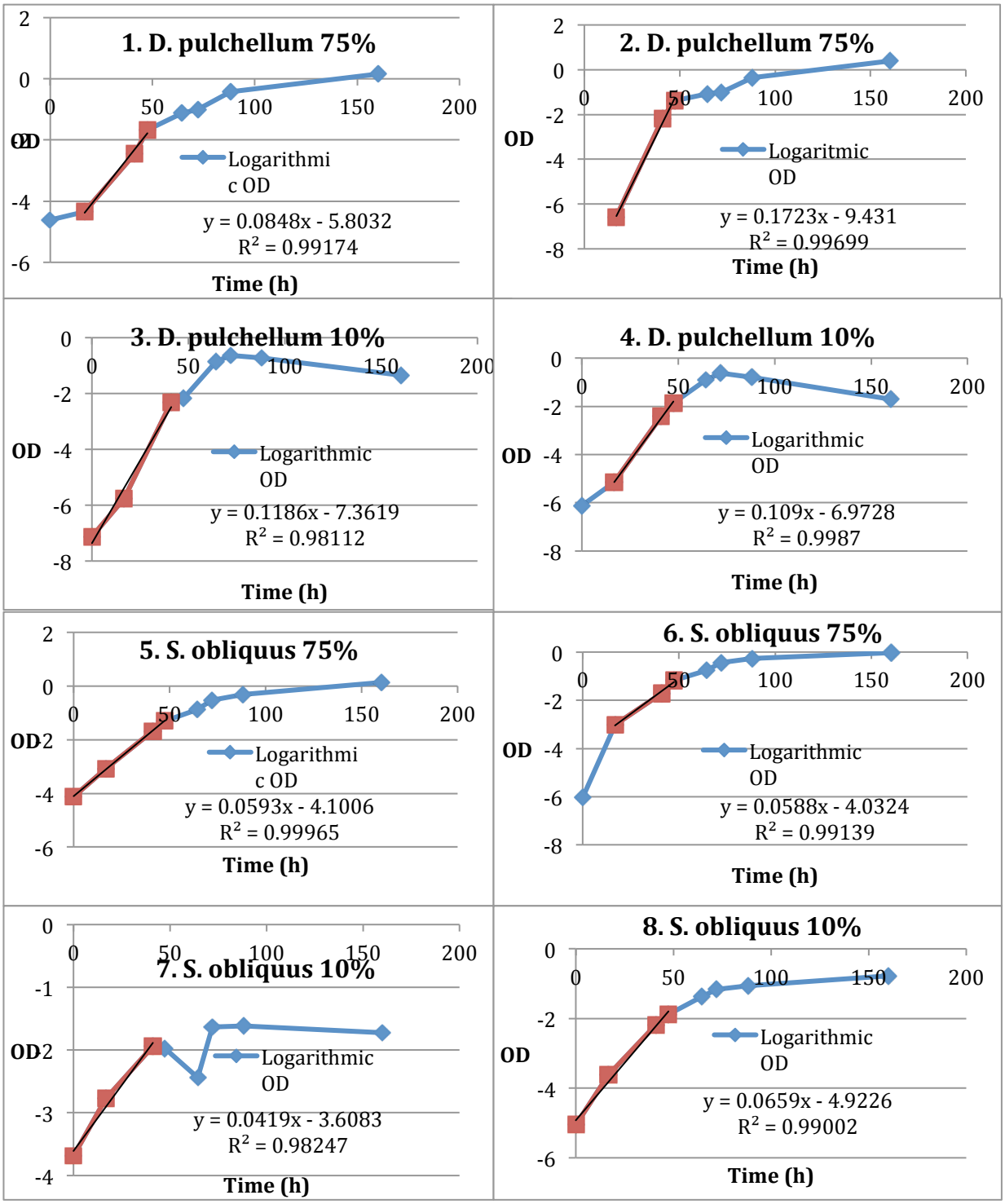

Figure 27: Linear regressions fit to the exponential phase of the logarithmic growth curve measured in OD_{750}.

The doubling time was calculated using the equation mentioned in the theory, and was for culture 1 (*D. pulchellum* 75%) calculated as:

$$k = \frac{ln2}{\mu} = \frac{0.6931}{0.0233h^{-1}} = 29.7h$$

Appendix H

Comparison of dry weight and OD_{750} as measure of growth.

By plotting dry weight to OD_{750} a linear relationship is obtained as shown in

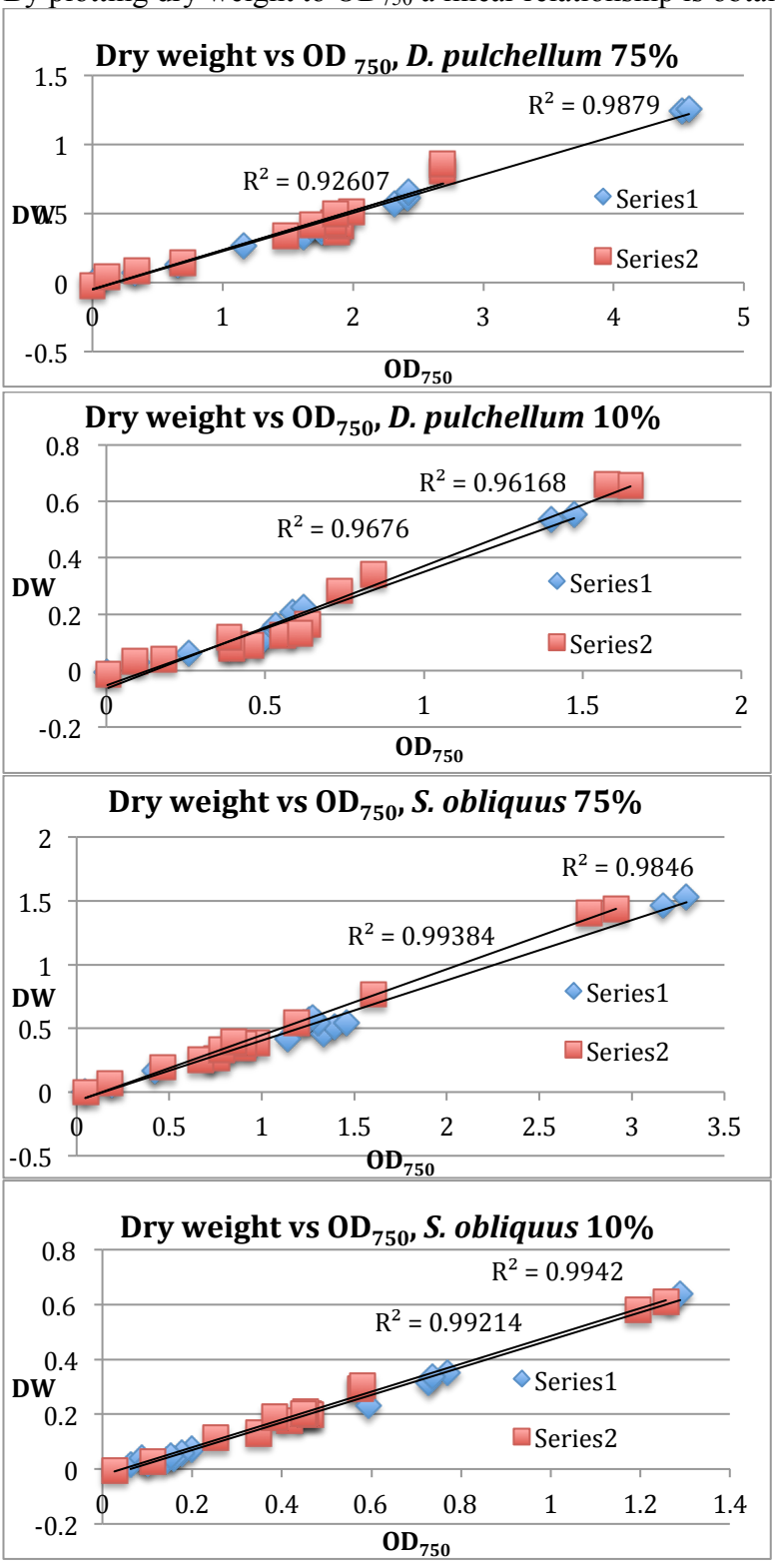

Figure 28: Dry weight plotted against dry weight to show how the two methods compare.

Appendix I

Water analysis results.

The results of the water analyses are shown in Table 21.

Table 21: Results of water analysis.

Culture	Time (hours)	Tot N mg/l	NH_4^+ (mg/l)	NO_3^- (mg/l)	Tot P (mg/l)	PO_4^{3-} (mg/l)
1. *D. pulchellum* 75%	0	4.6	3.25	<0.03	0.45	0.32
	376	1.5	0.006	0.678	0.036	0.009
2. *D. pulchellum* 75%	0	4.6	3.25	<0.03	0.45	0.32
	376	1.9	0.007	<0.03	0.059	0.012
3. *D. pulchellum* 10%	0	5.9	4.16	<0.03	0.424	0.294
	168	1.5	0.023	<0.03	0.16	0.11
	168	1.76	0.3107	<0.03	0.19	0.132
	376	1.4	0.008	<0.03	0.06	0.025
4. *D. pulchellum* 10%	0	5.9	4.16	<0.03	0.424	0.294
	168	1.5	0.017	<0.03	0.18	0.097
	168	1.76	0.3053	<0.03	0.208	0.1203
	376	1.4	0.008	<0.03	0.07	0.03
5. *S. obliquus* 75%	0	4.6	3.25	<0.03	0.45	0.32
	376	1.4	0.006	<0.03	0.028	0.006
6. *S. obliquus* 75%	0	4.6	3.25	<0.03	0.45	0.32
	376	1.4	0.009	<0.03	0.03	0.006
7. *S. obliquus* 10%	0	5.9	4.16	<0.03	0.424	0.294
	168	1.9	0.015	<0.03	0.27	0.16
	168	2.12	0.3035	<0.03	0.289	0.177
	376	1.8	0.012	<0.03	0.11	0.038
8. S. obliquus 10%	0	5.9	4.16	<0.03	0.424	0.294
	168	1.6	0.019	<0.03	0.42	0.22
	168	1.85	0.3071	<0.03	0.424	0.231
	376	1.3	0.012	<0.03	0.099	0.063