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Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge

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Abstract: Two microalgae, *Chlorella vulgaris* and *Scenedesmus acuminatus*, were batch cultivated separately in two types of diluted liquid digestates. The first digestate (ADPP) was obtained from a mesophilic laboratory digester treating biosludge from a pulp and paper industry wastewater treatment plant. The second digestate (ADMW) was collected from a full-scale mesophilic anaerobic digester treating mixed municipal wastewater treatment sludge. The highest biomass production (as volatile suspended solids, VSS), 8.2–9.4 g L^{-1}, was obtained with *S. acuminatus* in ADPP. *C. vulgaris* in ADMW had the lowest biomass production, reaching 2.0 g L^{-1}. Both microalgae removed ammonium efficiently from ADPP (99.9% removal rate) while the final ammonium removal efficiencies from ADMW with *S. acuminatus* and *C. vulgaris* were only 44.0% and 23.8%, respectively. The phosphate removal efficiencies from both ADPP and ADMW were higher than 96.9% with both microalgae. The highest carbohydrate content (60.5%) was obtained with *S. acuminatus* cultivated in ADPP. *S. acuminatus* in ADPP showed one of the highest biomass production yields that has been reported for microalgae in real wastewater-derived nutrient sources. Consequently, this combination is promising for developing biorefinery and biofuel applications in the pulp and paper industry.

Keywords: microalgae; digestate; high biomass yield; nutrient removal; biorefinery

1 Introduction

The pulp and paper industry typically consumes large amounts of wood and water and is among the largest producers of industrial wastewater in the world (Ashrafi et al. 2015). Thus, wastewater treatment is an indispensable part of this industry. However, traditional aerobic wastewater treatment produces vast amounts of biosludge, which is mechanically dewatered as such or mixed with primary sludge and then typically incinerated or landfilled (Stoica et al. 2009). While anaerobic digestion (AD) of the generated biosludge was studied in the 1980s (Puhakka et al. 1988), the recent developments towards biorefineries and circular economy thinking have led to a renewed interest in applying AD for biosludge treatment, as its energy balance is more positive and it enables simpler nutrient recovery as compared to incineration (Kinnunen et al. 2015). A microalgae-utilising biorefinery concept has been proposed to produce microalgae biomass and to recover nutrients using the liquid effluent of pulp and paper mill-digested residue as a nutrient source for the microalgae (Kinnunen and Rintala 2016; Kouhia et al. 2015). However, pulp and paper mill wastewaters can contain compounds such as lignins, humic acids, furans and dioxins (Ali and Sreekrishnan 2001), which can inhibit microbial growth and, thus, hinder utilisation of the microalgal biomass for products such as biodiesel, biomethane and bioethanol, which require large amount of biomass and cost-efficient cultivation. Microalgal cultivation in pulp and paper mill digestates has been studied previously (Polishchuk et al. 2015; Kinnunen and Rintala 2016) but resulted in low biomass production (0.2 g volatile suspended solids (VSS) per L) (Kinnunen and Rintala 2016).

The cultivation of various microalgal species has been studied using various other waste streams as well (Jia et al. 2016; Molinuevo-Salces et al. 2016; Nam et al. 2016; Posadas et al. 2016). Municipal wastewater is one of the most often used wastewaters due to its large volumes and

accessible collection (Tan et al. 2015), and it has been shown to be promising for simultaneous microalgal biomass production and nutrient recovery (Cai et al. 2013a; Tan et al. 2015). In addition to studies on municipal wastewater, microalgae cultivation has also been studied using the liquid fraction of the digestate from AD of municipal wastewater sludge. Tan et al. (2015) succeeded in cultivating *Chlorella pyrenoidosa* outdoors using a diluted liquid fraction of anaerobically digested biosludge, obtaining a maximum biomass concentration of 1.86±0.09 g-VSS L^{-1} during summer, with the photobioreactor temperature ranging from 27.5 to 42.6 °C. This indicates the feasibility of large-scale outdoor microalgal cultures using effluents from AD of sludges as a nutrient source. However, the growth yields and nutrient recovery efficiency of different microalgal species can be different, even under similar conditions (Abdel-Raouf et al. 2012), which makes it important to find an optimal microalgal species for each application.

The aim of the present study was to assess the feasibility of cultivating microalgal biomass in pulp and paper mill biosludge digestate. Utilising this concept, microalgae cultivation could be integrated in pulp and paper industry biorefinery to produce microalgal biomass (e.g. to biofuel applications while recovering nutrients from the liquid digestate). The digestate from a municipal wastewater treatment plant was used as a reference cultivation medium. The cultivation of two microalgal species, *Chlorella vulgaris* and *Scenedesmus acuminatus*, which were chosen due to their high growth rates and yields as well as their broad use in wastewater treatment studies (Bohutskyi et al. 2015; Wang et al. 2015; Zuliani et al. 2016), was compared in these two digestates.

2 Materials and methods

2.1 Microalgal strains and growth medium for seed cultures

Chlorella vulgaris (SAG 211-11b) and *Scenedesmus acuminatus* (SAG 38.81) were obtained from the SAG Culture Collection of Algae at the University of Göttingen, Germany as culture suspensions. *C. vulgaris* had been grown in Jaworski's medium (Lakaniemi et al. 2011) and stored frozen at -85 °C for 4 years. After thawing, *C. vulgaris* was inoculated to 100 mL N-8 medium and cultivated in 250 mL Erlenmeyer flasks on an orbital shaker (150 rpm) under fluorescent lamps (Osram L 18W/965 bio lux, Germany) at a light intensity of 40 µmol photons m^{-2} s^{-1} as a seed culture. *S. acuminatus* was inoculated to N-8 medium immediately after obtaining it from the culture collection and cultivated under the same conditions as *C. vulgaris*. The N-8 medium consisted of (g L^{-1}): KNO_3, 0.5055; KH_2PO_4, 0.7400; Na_2HPO_4, 0.2598; $MgSO_4 \cdot 7H_2O$, 0.0500; $CaCl_2 \cdot 2H_2O$, 0.0175; $FeNaEDTA \cdot 3H_2O$, 0.0115 and micronutrient ($ZnSO_4 \cdot 7H_2O$, 0.0032; $MnCl_2 \cdot 4H_2O$, 0.013; $CuSO_4 \cdot 5H_2O$, 0.0183; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.0070). The pH of the N-8 medium is naturally 6.5. *C. vulgaris* grew well with that initial pH, whereas there was no growth of *S. acuminatus* in the N-8 medium with an initial pH of 6.5. Based on a previous study by Xu et al. (2015), NaOH was added to adjust the pH to 8.0 for *S. acuminatus* cultivation.

2.2 Digestates

Digestates from two different sources were studied for microalgal growth. The first digestate (ADPP) was collected from a mesophilic laboratory-scale (6 L) completely stirred tank reactor (hydraulic retention time 14 d and organic loading rate 2.1 kgVS m^{-3} d^{-1}) treating biosludge from a pulp and paper industry wastewater treatment plant. The reactor set-ups were as described in Kinnunen et al. (2015), but the biosludge used in this study originated from different pulp and paper mills compared with the data reported in Kinnunen et al. (2015), and thus the digestate characteristics are not directly comparable. The second digestate (ADMW) was collected from a

mesophilic anaerobic digester (typically operated at a hydraulic retention time of 20–25 d and organic loading rate of 2.0 kgVS m^{-3} d^{-1}) treating mixed sludge in a municipal wastewater treatment plant (Rahola, Tampere, Finland). The digestates were stored at 4 °C until prepared for the cultivation experiments.

To remove particulate solids, both digestates were centrifuged at 5200 rpm for 4 min, and the separated supernatant was filtered through a glass fibre filter (Whatman GF/A, UK) under non-aseptic conditions (not meant to sterilise the wastewater). After filtration, the filtered digestates were stored at 4 °C before use. This study includes two separate cultivation experiments with both digestates. As the filtered digestates were prepared at different times and from different batches of digestates for the two cultivation experiments (Experiments I and II), there were some differences in the digestate compositions (Table 1). Considering that the PO_4^{3-}-P level may be not sufficient in ADMW, an additional experiment was performed with 0.548 g L^{-1} K_2HPO_4 added to the ADMW to enhance microalgal cultivation and nitrogen removal efficiency. Thus, the N/P ratio was adjusted to 7.5, and this ratio was selected as it has been used for high nutrient removal during microalgae cultivation in municipal wastewaters (Cai et al. 2013b; Tan et al. 2015).

2.3 Microalgal cultivation in digestates

Experiment I was done to select the optimal dilution factor of the liquid digestates for microalgal growth. The digestates were diluted with distilled water, using dilution factors of 5x, 3x and 1.5x and 10x, 7x, 3.5x, 2x and 1x for the ADPP and ADMW, respectively. Using the selected dilution factors, Experiment II was conducted to further study the biomass production, carbon and nutrient removal efficiency and chemical composition of the produced biomass (carbohydrate, lipid and protein). All cultivations were performed in duplicates.

Experiment I was conducted in 1-L photobioreactors, which consisted of a 1-L glass bottle (PYREX) closed with a plastic cap and having two tubes as the gas inlet and outlet. The cultures were bubbled from the bottom with 5% CO_2 in the air (v/v) at a flow rate of 0.105 L min^{-1} using a glass distribution tube (porosity 0, ⌀ 22mm, Duran Group, Germany). The photobioreactors were continuously illuminated using white fluorescent lamps (Osram L 18W/965 De Luxe cool daylight, Germany) from two sides of the reactors. It is commonly believed that each microalgal strain has a particular light intensity that is the most optimal for biomass growth (Ho et al. 2012; Xu et al. 2015). Based on preliminary tests (data not shown) in which the microalgae were cultivated separately in N-8 medium at different light intensities, 150 µmol photons m^{-2} s^{-1} and 240 µmol photons m^{-2} s^{-1} were chosen as the light intensities for the cultivation of *C. vulgaris* and *S. acuminatus*, respectively. The inoculum culture was centrifuged to separate cells from the N-8 medium before being mixed with the desired digestate. To identify the accurate microalgal growth medium compositions in Experiment II, samples were taken for analysis of initial dissolved nutrients after inoculation. Each microalgal genus was inoculated to its respective photobioreactor to provide an initial optical density (OD) of 0.20. The initial total culture volume in the reactors was 350 mL for ADPP (the availability was limited) and 700 mL for ADMW. The temperature of the reactors was maintained at 22±2 °C. Distilled water was added to adjust for the water lost through evaporation each time before taking samples for analyses. All cultivations (each combination of different microalgal species with different dilution of digestate) were carried out for 11–12 d.

Experiment II using the selected dilution factors was conducted using similar conditions as Experiment I. The difference was that the initial culture volume in all the cultivations was 700 mL to provide enough volume for the more extensive sampling and more reliable comparison of the

150 growth of the two microalgae in the two digestates. The cultivation duration in Experiment II was
151 14 d.

152 **2.4 Analyses and calculations**

153 The culture pH was measured using a WTW 3110 pH meter (WTW, Germany) with a SenTix® 41
154 electrode (WTW, Germany) in Experiment I and a WTW 330 pH meter (WTW, Germany) with a
155 Slimtrode electrode (Hamilton, Germany) in Experiment II. The light intensity was measured from
156 the outer surface of the photobioreactors by a MQ-200 Quantum Meter (Apogee, USA). The
157 optical density (OD) of the culture samples was measured at a wavelength of 680 nm using a
158 Shimadzu UV-1700 Pharmaspec spectrophotometer after proper dilution with deionised water to
159 give absorbance values between 0.2–0.7. Light microscopy was carried out using a Zeiss Axioskop
160 2 equipped with an AxioCam MRc camera. The microalgae cells were first sonicated for 10 min
161 and then observed under the light microscope. Volatile suspended solids (VSS) were measured by
162 filtering 5–15 mL culture solution through a glass fibre filter (Whatman GF/A). Each filter
163 containing the suspended solids was dried at 105 ºC overnight, weighed and then burned in a 550
164 ºC muffle furnace for 2 h and weighed again. VSS was determined gravimetrically as a difference
165 of the filters after treatment at these two temperatures. The filtrate from VSS filtration was used in
166 the analysis of soluble chemical oxygen demand (COD_s), dissolved organic carbon (DOC) and
167 nutrient (N, P) concentration.
168 COD_s was determined using the dichromate method according to the Finnish Standard SFS 5504.
169 DOC was measured with a total organic carbon analyser (Shimadzu Model TOC-5000) with an
170 ASI-5000 autosampler. Total nitrogen was measured as total Kjeldahl nitrogen (TKN) with the
171 Tecator Kjeltec Systems (FOSS Tecator Digestor 8 and KT 200 Kjeltec, Sweden), and total

phosphorus (TP) was measured with a Hach kit LCK349 (0.05–1.5 mg L^{-1} PO_4-P) or LCK350 (2.0–20.0 mg L^{-1} PO_4-P), according to the manufacturer's instructions. NH_4^+-N was measured with an ion selective electrode (Thermo Scientific Orion ISE meter). The ammonium removal rate was calculated as ARR=(C_0-C_t) t^{-1}, where C_0 is the ammonium concentration on day 0, and C_t is the ammonium concentration when the ammonium concentration had fallen below 0.5 mg L^{-1}, which indicated >99% NH_4^+-N removal. The possible significance of ammonium stripping was estimated by calculating the fraction of unionised ammonium with the following equation (Emerson et al. 1975) as rate of ammonia stripping has been shown to correlate well with free ammonia concentration (Zimmo et al. 2003):

$$unionised\ NH_3(\%) = \frac{100}{1 + 10^{(pK_a - pH)}}, \quad (1)$$

where $pK_a = 0.09018 + \frac{2729.92}{T}$ and $\mathrm{T} = \mathrm{temperature}(^{o}\mathrm{K})$.

NO_3^-, NO_2^- and PO_4^{3-} were measured using an ICS-1600 ion chromatograph (Dionex, USA) with an AS-DV autosampler, Ion- Pac AS4A-SC anion exchange column and ASRS-300 suppressor (2 mm). The eluent contained 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$, and the eluent flow rate was 1 mL min^{-1}.

The composition of the produced microalgal biomass (proteins, carbohydrates, and lipids) was measured from the freeze-dried biomass. Before freeze-drying, the algal culture was centrifuged at 5200 rpm for 2 min, and the supernatant was discarded. The harvested microalgae samples were dried in a vacuum freeze dryer (Christ ALPHA 1-4 LD plus) for 24 h. The protein content of the produced biomass was measured with a protein assay kit, based on the method of Bradford (Bio-Rad Protein Assay Dye Reagent Concentration; Protein Standard II). The total carbohydrate concentration of the algal biomass was measured with the anthrone method after hot alkaline extraction (Chen and Vaidyanathan 2013). In short, 10 mg dried microalgal pellets were

resuspended in 0.2 mL distilled water and then heated in 0.4 mL 40% (w/v) KOH at 90 °C for 1 h. After cooling down, the sample was mixed with 1.2 mL cold absolute ethanol and stored in a fridge at −20 °C overnight. The pellet was resuspended in 1.5 mL distilled water after discarding the supernatant. An aliquot (0.2 mL) of the sample was mixed and vortexed with 0.4 mL of pre-chilled 75% H_2SO_4 solution (stored at 4 °C) in a test tube. To this, 0.8 mL of the anthrone reagent (2 g L^{-1} in 75% H_2SO_4, freshly prepared) was added, and then the mixture was subsequently boiled at 100 °C for 15 min. After cooling, the absorbance was read at 578 nm using a Shimadzu UV-1700 Pharmaspec spectrophotometer. The blank absorbance of the sample was read by reacting 0.2 mL of the sample with 1.2 mL 75% H_2SO_4 without the anthrone reagent. The amount of carbohydrate was estimated using a standard curve created using d-glucose. The total lipid content of the biomass was measured by extracting the lipids with chloroform/methanol and determining the lipids gravimetrically. An aliquot (50 mg) of freeze-dried microalgal biomass was mixed with 10 mL of chloroform/methanol (2/1, v/v) and then sonicated for 5 min. After sonication, the mixture was reacted for 4 h on a magnetic stirrer at 1000 rpm. Then, 5 mL of distilled water were added to the mixture and centrifuged together at 3000 rpm for 2–3 min. Lipids remained in the chloroform after centrifugation, and then the chloroform (8 mL) was placed in a pre-weighted tube. The nitrogen was sparged to remove chloroform for 2 h and lipid content was left in the tube; the tube was then weighed again.

3 Results

3.1 Selection of the dilution factor for the digestates

The growth of *Chlorella vulgaris* and *Scenedesmus acuminatus* was tested with different dilutions with the pulp and paper mill digestate (ADPP; 5x, 3x and 1.5x) and municipal sludge digestate

(ADMW; 10x, 7x, 5x, 3.5x, 2x and 1x) to study the growth of the two microalgae in the two digestates at similar initial ammonium concentrations. As shown in Table 2, both *C. vulgaris* and *S. acuminatus* had the highest biomass production in 2x diluted ADMW and 1.5x diluted ADPP. Compared with the growth of both microalgae in ADMW, the biomass production in ADPP was much higher (maximum VSS=9.4±0.8 g L^{-1} of *S. acuminatus* and VSS=5.1±0.6 g L^{-1} of *C. vulgaris*). In fact, the obtained biomass production was among the highest reported for microalgal cultivations that have been conducted in real wastewater (Table 3). The biomass production of both microalgae was lower in undiluted ADMW, and it is likely that microalgal growth was limited by the higher ammonium concentration (840 mg L^{-1}) and brownish colour of the undiluted digestate. The initial ammonium concentrations in 2x diluted ADMW and 1.5x diluted ADPP were 420 mg L^{-1} and 230 mg L^{-1}, respectively, whereas the corresponding phosphate concentrations were 1.0 mg L^{-1} and 16.0 mg L^{-1}, respectively. As the biomass production was the highest at these conditions, 2x diluted ADMW and 1.5x diluted ADPP were selected for the more detailed study of biomass production, nutrient removal and algal biomass composition in Experiment II.

3.2 Algal growth and nutrient removal efficiency

In Experiment II, the microalgal growth was studied in more detail using the selected dilutions with both ADPP and ADMW. Of the two different digestates, both microalgae grew better in ADPP when compared to ADMW and reached their highest biomass concentrations (*C. vulgaris*: 2.9 g L^{-1}; *S. acuminatus*: 8.2 g L^{-1}) on day 14 (Fig. 1a). In ADMW, *S. acuminatus* reached a maximum biomass concentration of 2.9 g L^{-1} and *C. vulgaris* of 2.0 g L^{-1}. The biomass concentration of *S. acuminatus* in ADPP was higher than that detected for the other cultivations from day 2 onwards. On day 7, the biomass concentration of *S. acuminatus* in ADPP was already

4.9 g L^{-1}, while in the other cultivations biomass concentrations remained below 3.0 g L^{-1} on day 14.

Both microalgae were able to remove ammonium efficiently from ADPP, in which the ammonium concentration decreased from 240 mg L^{-1} to 0.1 mg L^{-1} during cultivation of both microalgal species, resulting in a 99.9% removal efficiency (Fig. 2b). Interestingly, the same amount of ammonium and phosphorus was removed by both algae in ADPP, even though the biomass production for *S. acuminatus* (8.2 g L^{-1}) was more than two times higher than that for *C. vulgaris* (2.9 g L^{-1}). The ammonium was, however, removed faster by *S. acuminatus* (26.5 mg L^{-1} d^{-1}) than by *C. vulgaris* (17.1 mg L^{-1} d^{-1}). From ADMW, which had an initial ammonium concentration of 410 mg L^{-1}, the ammonium removal efficiencies were much lower, being only 44.0% and 23.8% with *S. acuminatus* and *C. vulgaris*, respectively (Fig. 1b).

The initial phosphate concentration in ADPP was 8.0 mg L^{-1} while in ADMW it was much lower (1.3 mg L^{-1}, Fig. 1c), apparently due to phosphorus removal using chemical precipitation in the municipal wastewater treatment plant. The phosphate levels in ADPP and ADMW decreased rapidly to below the detection limit of 0.1 mg L^{-1} by both microalgae, in 4 days with ADPP and 2 days with ADMW. Thus, the phosphate removal efficiencies were higher than 96.9% in all four cultivations (Fig. 1c). An additional experiment performed to assess the effects of phosphate addition to the ADMW (initial phosphate concentration was 73.8±1.8 mg L^{-1}, added as K_2HPO_4) resulted in 99% removal of phosphate within 9 days with *S. acuminatus* and 14 days with *C. vulgaris* but similar biomass production and ammonium removal efficiency as cultivations without extra phosphate (Fig. 1).

3.3 COD and DOC during microalgal cultivation in the digestates

It is essential to measure COD in wastewater treatment, as it is a typical indicator of the water quality. The initial COD_s value in the 2x diluted ADMW was 1259±5 mg L^{-1}, which was approximately two times the initial value present in the 1.5x diluted ADPP having COD_s of 600±34 mg L^{-1}. In ADPP, the COD_s removal efficiencies of *C. vulgaris* and *S. acuminatus* were 27.6% and 36.1%, respectively (Fig. 2a). In ADMW, the highest COD_s removal efficiency was obtained with *C. vulgaris* (55.4%), while *S. acuminatus* was able to remove 48.7% of the initial COD_s. DOC is a typical parameter measured from microalgal cultivations, as DOC is usually released during microalgal photosynthesis and can support bacterial growth (Watanabe et al. 2005; Hulatt and Thomas 2010). The DOC concentration in ADPP was stable and remained close to the initial value during the whole cultivation period (Fig. 2b). A similar amount of DOC was removed from ADMW by the two microalgae (26.0% by *C. vulgaris*, 24.8% by *S. acuminatus*) (Fig. 2b).

3.4 Chemical composition and morphological changes of the microalgae

Among all studied cultures, *S. acuminatus* in ADPP had the highest carbohydrate content (60.5%) per dry weight, whereas a carbohydrate content of only 6.8% was measured from the dried cells of *C. vulgaris* cultivated in ADPP (Table 3). Similarly, the carbohydrate content of *S. acuminatus* and *C. vulgaris* grown in ADMW were 44.3% and 6.3%, respectively.

C. vulgaris is spherical in shape while *S. acuminatus* is spindle-shaped (Fig. 3). No morphological differences in the *C. vulgaris* cells in the two digestates were observed between day 4 and day 14 (these cultivation days represent nitrogen-sufficient and nitrogen-limited conditions in ADPP). The cell size (diameter) of *C. vulgaris* was about 5–10 μm in both studied digestates during the whole cultivation period. However, clear morphological changes of *S. acuminatus* were detected in both digestates between day 4 and day 14. In ADPP, the cell length of *S. acuminatus* increased

from 20 to 22.5 μm on overage while the width increased from 6.25 to 7.5 μm on average. In ADMW, the cell length of the *S. acuminatus* decreased from an average of 30 to 25 μm while the width increased from an average of 8.75 to 11.25 μm. Slightly different types of changes in cell morphology were observed in a previous study, in which the cell length size of *Scenedesmus* sp. was found to increase from 4.5 to 5.3 μm while the cell width size decreased from 3.36 to 2.44 μm when cultivated under a nitrate-limited condition (Pancha et al. 2014). Thus, there was no clear correlation between nitrogen availability and the cell size.

4 Discussion

This study was carried out in batch to select microalgal species that enable high biomass production and efficient nutrient removal from pulp and paper mill biosludge digestate and to assess the potential of pulp and paper mill biosludge digestate as a cultivation medium compared to the more commonly used municipal wastewater treatment digestate. The biomass production of *S. acuminatus* cultivated in ADPP (8.2–9.4 g L^{-1}) in this study was among the highest obtained when microalgae have been cultivated in real wastewater, while several studies have reported high microalgal biomass production (7.22–12.4 g L^{-1}) in artificial growth medium (Table 3).

The selection of medium dilution plays an important role in microalgal cultivation since the dilution will change the medium turbidity (thus light penetration) and nutrient concentrations (Posadas et al. 2016; Wang et al. 2010; Xia and Murphy 2016). High ammonia concentrations have been shown to inhibit microalgal growth, whereas too low nutrient concentrations can limit growth (Britto and Kronzucker 2002; Tan et al. 2015). In contrast to our study, Franchino et al. (2013) chose higher dilution ratios (1:10, 1:15, 1:20 and 1:25) as optimum to ensure the microalgal growth due to the high digestate medium turbidity. However, higher dilutions reduced the

concentrations of nutrients, which could result in lower microalgal biomass production (Franchino et al. 2013; Wang et al. 2010). Instead of clean water, Bohutskyi et al. (2016) mixed 1–20% anaerobic digestion centrate (ADC) with primary and secondary wastewater effluents separately to cultivate several types of microalgal strains, and they found that 5–10% ADC succeeded in improving microalgal growth and productivity in both effluents due to the additional nutrients and optimum nitrogen-to-phosphorus ratio.

The present study shows a high microalgal biomass yield is possible in the liquid digestates from pulp and paper wastewater treatment plant biosludge. The growth of *S. acuminatus* appeared to be similar level in both ADPP (8.2–9.4 g-VSS L^{-1}) and ADMW (2.2–2.9 g-VSS L^{-1}) in Experiment I and II, while *C. vulgaris* growth differed more between the two experiments, with both digestates being higher in ADPP in Experiment I (5.1 vs. 2.9 g-VSS L^{-1}) and in ADMW in Experiment II (2.0 vs. 1.2 g-VSS L^{-1}). Even though a strict comparison between the two cultivations is not justified due to different sampling dates and slightly different cultivation conditions, this shows the repeatability of the high biomass production of *S. acuminatus* in ADPP. On the other hand, the growth of *C. vulgaris* appeared to be more sensitive to cultivation conditions even when including the differences in the compositions of the digestates in Experiments I and II (Table 1). Similarly, in the previous study, the growth of *C. vulgaris* has been found to vary (0.31–0.19 g-VSS L^{-1}) when using even synthetic growth medium (Kinnunen and Rintala 2016).

Several possible reasons (e.g. algal species, medium characteristics and microbial community) could explain the different growth yields in the cultivations of this study. Kinnunen and Rintala (2016) obtained a concentration of 0.17 g L^{-1} (VSS) when *Scenedesmus sp*. was cultivated in a liquid digestate from a different pulp and paper mill. The growth of this different *Scenedesmus* species was much lower than the biomass production obtained with *S. acuminatus* in ADPP in this

study. Lignin, which ends up in pulp and paper mill wastewaters, is an amorphous polymer that is difficult for microorganisms to degrade (Higuchi 1990). In addition, some of the polyphenolic compounds in softwood knots, such as pinosylvins, have antimicrobial activity (Välimaa et al. 2007), while lignin and its derivatives are quite toxic to certain microorganisms, such as microalgae and cyanobacteria (Ball et al. 2001). It has been reported that *S. subspicatus* was much more resistant than *C. vulgaris* and *Microcystis aeruginosa* to the chemicals released from barley straw (e.g. 2 phenyl-phenol, p-cresol and benzaldehyde) (Murray et al. 2010). This indicates that *C. vulgaris* was more susceptible to the chemical compounds likely present in ADPP, which may have caused the much lower biomass production obtained with *C. vulgaris* than with *S. acuminatus* in ADPP. When microalgae are cultivated in wastewaters or digestates, microbes are always present and might affect the growth of microalgae. In the present study, the indigenous microbial communities of the two digestates (ADPP and ADMW) were likely different since they originated from different types of sources and had very different chemical compositions. Studies have shown that certain bacteria can enhance bacterial growth, whereas certain bacteria can inhibit it (Croft et al. 2005; Santos and Reis 2014). For example, De-Bashan et al. (2004) reported that *Azospirillum brasilense* strain Cd stimulated the growth of *C. vulgaris* and *C. sorokiniana* when they were co-immobilised in small alginate beads. Interestingly, a similar genus, *Azospirillum lipoferum*, was found in an aerated plug-flow lagoon that was used to treat pulp and paper mill effluent (Yu and Mohn 2001). However, De Bashan et al. (2004) did not study the effect of *Azospirillum brasilense* on *S. acuminatus*, and therefore it is not possible to compare the effect of *Azospirillum* to the growth of *C. vulgaris* and *S. acuminatus*. Lee et al. (2016) assumed that the reason for the slow growth of *S. quadricauda* in municipal wastewater might be related to *Alcaligenes*, which was an abundant bacterium in the wastewater. Some species of *Alcaligenes* genus have been shown to

cause cell lysis and the death of certain cyanobacteria (Manage et al., 2000), and others have been shown to have nitrification and denitrification abilities that may affect ammonium removal and nitrogen availability to the microalgae (Joo et al. 2005). The interactions between bacteria and microalgae have been shown to be very species specific, even in the same medium (Schäfer et al. 2002). In our study, certain a bacterium present in the studied ADPP may have enhanced the growth of *S. acuminatus* but not the growth of *C. vulgaris.* Alternatively, a certain bacterium could have inhibited *C. vulgaris* but not *S. acuminatus*.

The present results demonstrate efficient nutrient (ammonium and phosphorus) removal by both microalgae from ADPP, while different nutrient removal efficiencies were obtained in ADMW with the two different microalgal strains. Beuckels et al. (2015) reported that *C. vulgaris* was able to accumulate more nitrogen into biomass than *S. obliquus*. This likely happened in this study with ADMW, as the decrease in NH_4^+-N concentration was higher with *C. vulgaris* than with *S. acuminatus* (Fig. 1b), although the biomass growth of *C. vulgaris* was somewhat lower (Fig. 1a). Several possible ammonium transformations (algal uptake, ammonia stripping, bacterial growth and nitrification) can happen in algae–bacteria consortium systems, such as microalgal cultures in unsterilised wastewater (Bohutskyi et al. 2015; González-Fernández et al. 2011; He et al. 2013; Zimmo et al. 2003). In this study, the nitrate and nitrite levels in both liquid digestates were low (<1.0 mg L^{-1}) during the whole cultivation. This means the possibility of ammonium removal by nitrification was small. As the pH varied in all cultures between 7.5 and 8.0 and the average temperature was 22°C, the theoretical fraction of unionised ammonia in all cultivations was 1.4%–4.4%. This suggests that some stripping of the unionised ammonia may have occurred but that the main portion of the removed ammonium from the digestates was used for microbial growth. The removed phosphorus could be taken up into the microalgal cells as polyphosphates and/or cell

components or precipitate from the medium due to high pH (Cai et al. 2013a, b). Thus, it seems that the higher initial phosphate concentration of ADPP was not the reason for the higher biomass production observed in ADPP than in ADMW.

While there was no big difference in DOC removal with *C. vulgaris* and *S. acuminatus* when the same digestate was used, the difference in DOC trends between ADPP and ADMW emphasise their differences as a cultivation medium. One reason for stable DOC in ADPP could be that the released DOC from photosynthetic microalgal cells equalled to the consumed DOC for growth of hetertrophic organisms (such as bacteria). Decrease in COD_s suggests, however, that higher level of organic compounds was degraded during the cultivation than was released as DOC by the microalage. COD_s was not fully removed during the cultivations, indicating that treatments other than biological methods could be required for further COD_s removal after microalgal harvesting.

The nutrient and carbon removal levels from ADPP were similar with both *C. vulgaris* and *S. acuminatus*, but the biomass production of *S. acuminatus* was much higher than that of *C. vulgaris*. Based on the typical biochemical composition of microalgae, it is estimated that about 50% of the microalgal biomass is carbon (Chisti 2008). Thus, 1.0–4.1 g L^{-1} carbon was required to produce the microalgal biomass, as the obtained VSS values ranged between 2.0 and 8.2 g L^{-1} for the two microalgae (Fig. 1a). However, the total removed dissolved carbon from the digestates was below 150 mg L^{-1}. Hence, CO_2 supply contributed to the microalgal growth as the main carbon source, indicating that most of the microalgal biomass was produced via photoautotrophic growth.

Based on the chemical formulas of the main components of microalgae (carbohydrate: $C_6H_{10}O_5$, lipid: $C_{57}H_{104}O_6$ and protein: $C_{1.9}H_{3.8}ON_{0.5}P_{0.031}$) (Kouhia et al. 2015), nitrogen only appears in proteins. It is assumed that microalgae using the same amount of nitrogen should produce the same amount of protein. However, in this study, despite the similar ammonium removal, the protein

content of *C. vulgaris* was 6–13.8 percentage units higher than that of *S. acuminatus* in the same digestate, whereas *S. acuminatus* contained significantly more carbohydrates and produced more biomass than *C. vulgaris*. Nitrogen deficiency can cause a reduction in protein content (Diniz et al. 2016) along with an enhancement of energy-rich products, such as carbohydrates and lipids (de Farias Silva and Bertucco 2015; Siaut et al. 2011). In this study, the produced microalgal biomass likely contained mainly proteins at the beginning due to the sufficient nitrogen in the cultures, and the microalgal carbon was allocated to energy-rich compounds after the ammonium was consumed completely. Similarly, when microalga *Chlamydomonas reinhardtii* was exposed to environmental stress such as nitrogen starvation, starch accumulation was first observed and reached high levels by day 2 (approximately 60 μg per million cell), and after extended nitrogen limitation (5 days), oil accumulation reached a maximal level (40 μg per million cell) (Siaut et al. 2011). The carbohydrate and lipid contents of *C. vulgaris* in ADMW and ADPP were in a similar range, while the protein content of *C. vulgaris* in ADMW was higher than that in ADPP (Table 3), likely due to the higher initial nitrogen concentration of ADMW compared to that of ADPP. However, as the sum of the analysed biochemical components (58.8%–71.1%) from *C. vulgaris* was much lower than 100%, it is not certain whether carbohydrate or lipid accumulation occurred in *C. vulgaris*. The sum of proteins, lipids and carbohydrates in *C. vulgaris* has also been reported to be lower than 70% in previous studies (Lakaniemi et al. 2011; Sydney et al. 2010). Burczyk et al. (2014) suggested that low levels of polyamines (PAs) in the cell walls of microalgae might enhance the action of lytic enzymes, and they found that the PA content in *C. vulgaris* strain 140 was 4 to 5 times higher than that in *S. obliquus* strain 633. Thus, it is possible that in this study and also in the previous studies reporting sums of proteins, carbohydrates (sugars) and lipids to be clearly below 100%, the high PA content in *C. vulgaris* may have hindered the cell lysis during the

analysis of the biochemical components. In addition, carbohydrates might have been lost due to the alkali dissolution during the measurement (Kane and Roth 1974).

5 Conclusion

Chlorella vulgaris and *Scenedesmus acuminatus* were shown to be able to grow and remove nutrients in liquid digestates from both a pulp and paper industry wastewater treatment plant (ADPP) and a municipal wastewater treatment plant (ADMW). *S. acuminatus* in 1.5-times diluted ADPP enabled the highest biomass production of 8.2–9.4 g L^{-1}, which is among the highest yields reported for microalgae cultivated in wastewaters. The maximum biomass yield was also much higher than the growth of *C. vulgaris* in 1.5-times diluted ADPP (2.9 g L^{-1}) as well as the growth of *S. acuminatus* (2.9 g L^{-1}) and *C. vulgaris* (2.0 g L^{-1}) in 2-times diluted ADMW. Phosphate and ammonium removal efficiencies were high with both microalgae from ADPP (over 97%). Both algae were able to remove phosphate from ADMW, although the ammonium removal efficiencies remained low (24–44%). According to the results obtained in this study, cultivation of *S. acuminatus* in pulp and paper mill biosludge digestates is a promising approach for producing a carbohydrate-rich biomass with a high yield and cheap nutrient supply (e.g. for biogas and bioethanol production). Future studies on semi-continuous or continuous cultivation systems and biomass harvesting could further promote the practical applications.

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Figure captions:

Fig. 1 Microalgal biomass concentration (as g VSS L^{-1}) (a), the soluble ammonium-N (b) and phosphate-P concentrations (c) during the cultivation of *Chlorella vulgaris* and *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted), the municipal wastewater treatment plant (ADMW; 2x diluted) and the municipal wastewater treatment plant supplied with phosphorus (ADMW+phos.; 2x diluted). The results of VSS and phosphate-P are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation. The results of ammonium-N are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error.

Fig. 2 COD removal efficiency (a) and DOC concentration (b) during the cultivation of *Chlorella vulgaris* and *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted). The results are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation.

Fig. 3 Microscope photos of the microalgal cells: *Chlorella vulgaris* in ADPP (a)(c); *Chlorella vulgaris* in ADMW (b), (d); *Scenedesmus acuminatus* in ADPP (e), (g) and *Scenedesmus acuminatus* in ADMW (f), (h) on day 4 and day 14, respectively. Digestates were from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted)

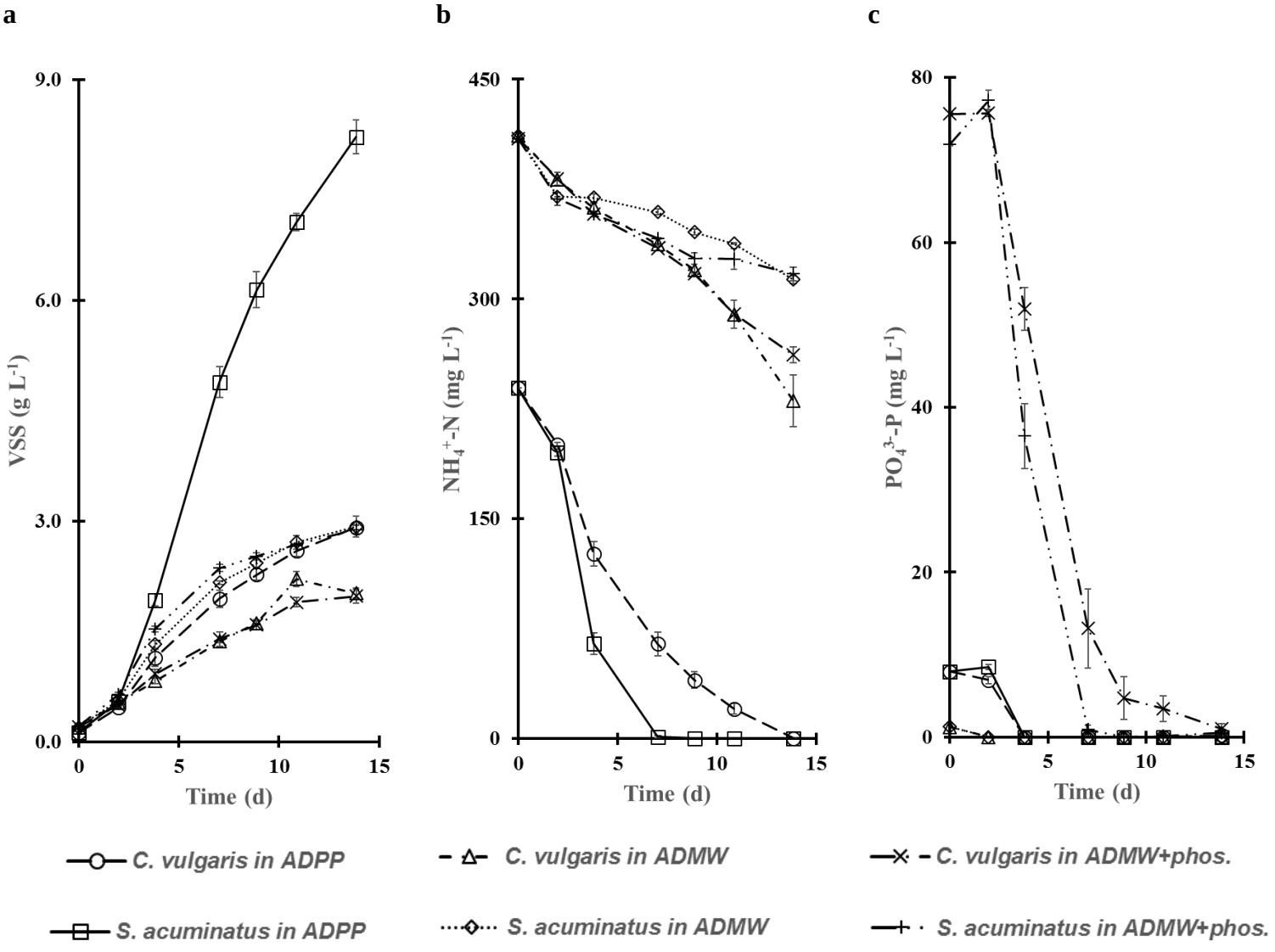
Table captions:

Table 1 Characteristics of the filtered digestates originating from the pulp and paper wastewater treatment plant (ADPP) and the municipal wastewater treatment plant (ADMW). Two batches (Experiment I and II) of both filtered digestates were used. The results are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error

Table 2 Ammonium-N and phosphate-P concentrations and biomass production of *Chlorella vulgaris* and *Scenedesmus acuminatus* cultivated in diluted digestates from a pulp and paper mill wastewater treatment plant (ADPP) and a municipal wastewater treatment plant (ADMW). The results of biomass production as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation. The results of ammonium-N and phosphate-P are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error

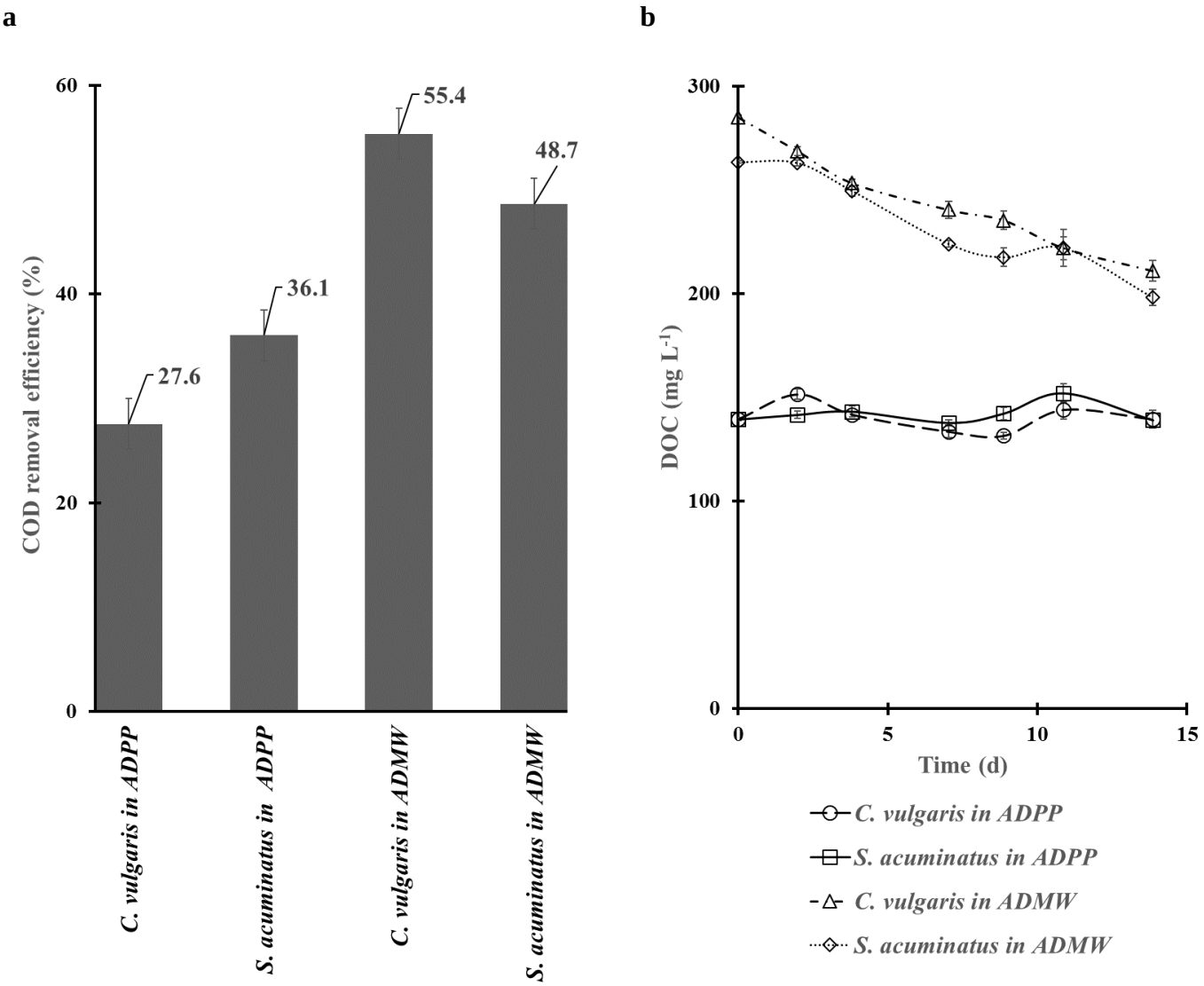
Table 3 Maximum biomass concentrations and chemical compositions of the produced biomass from selected studies in which microalgae have been cultivated in real wastewaters and synthetic media. The digestates were from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted)

628 **Figure 1**



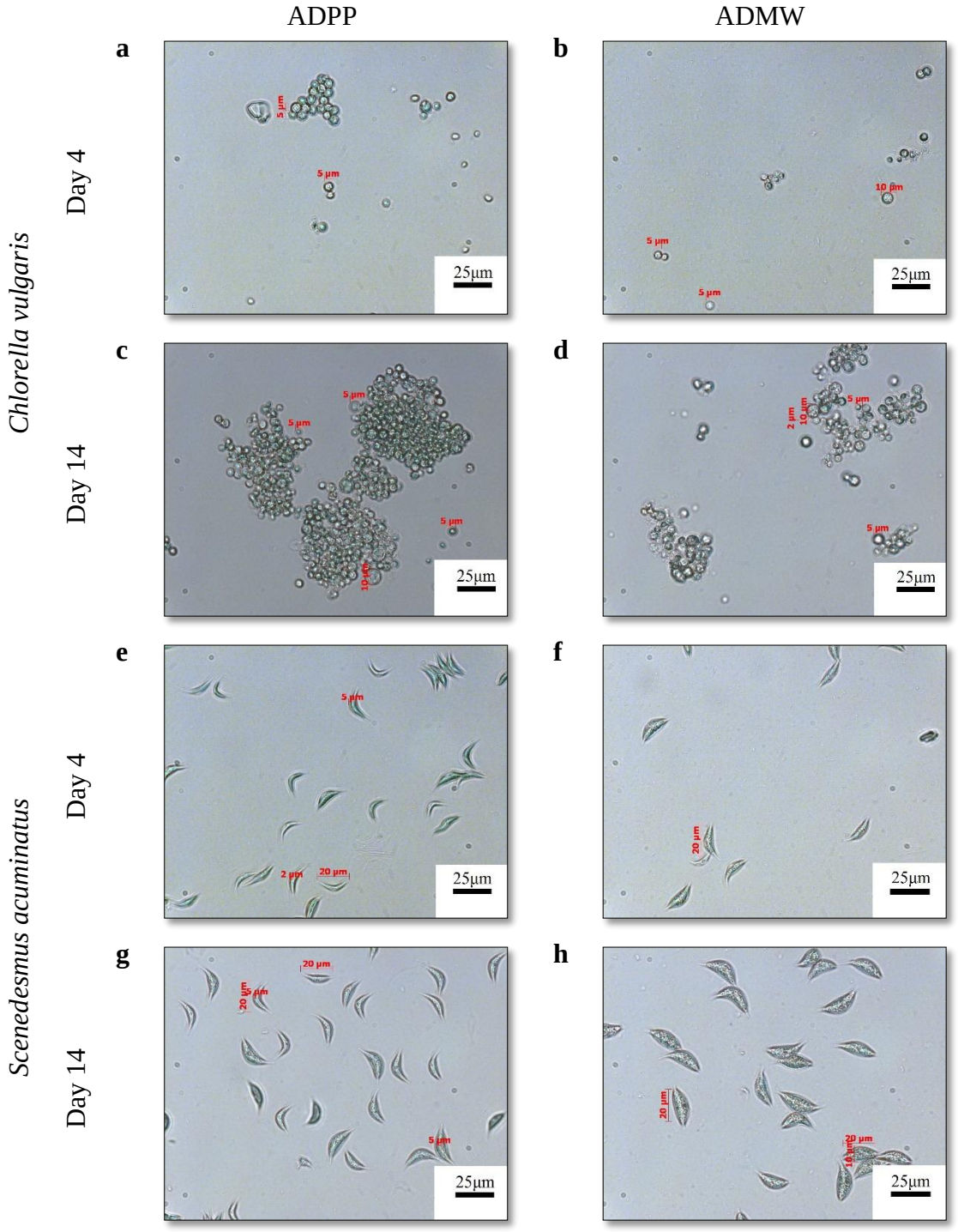
629

630 **Figure 2**



631

632 **Figure 3**



633

Table 1

	ADPP		ADMW	
	Experiment I	Experiment II	Experiment I	Experiment II
pH	8.5	8.5	8.3	8.6
DOC (mg L^{-1})	370±40	210±2	530±20	560±20
COD_s (mg L^{-1})	910±30	900±70	1850±40	2500±15
TKN (mg L^{-1})	350±10	360±20	840±40	1000±150
NH_4^+-N (mg L^{-1})	350±50	360±1	840±130	820±10
NO_3^- (mg L^{-1})	<0.5	<0.5	<0.5	<0.5
NO_2^- (mg L^{-1})	<0.5	<0.5	<0.5	<0.5
TP (mg L^{-1})	28±1	20±1	10±1	14±2
PO_4^{3-}-P (mg L^{-1})	24±1	12±0.1	2.0±0.2	2.5±0.1

DOC= dissolved organic carbon
COD_s = soluble chemical oxygen demand
TKN= total Kjeldahl nitrogen
TP= total phosphorus.

Table 2

	ADPP			ADMW			
Dilution factor	5x	3x	1.5x	10x	7x	3.5x	2x	1x
Ammonium-N	70±10	115±15	230±35	84±10	120±20	240±40	420±65	840±130
Phosphate-P	4.8±0.2	8±0.3	16±0.7	0.20±0.0	0.29±0.0	0.57±0.1	1.0±0.1	2.0±0.2
C. vulgaris VSS ($g\ L^{-1}$)	1.9±0.2	3.0±0.1	5.1±0.9	0.6±0.1	0.6±0.2	1.1±0.1	1.2±0.1	0.9±0.1
S. acuminatus VSS ($g\ L^{-1}$)	6.1±3.1	6.2±2.3	9.4±1.1	0.8±0.1	0.9±0.1	1.7±0.1	2.2±0.1	2.1±0.2

641 **Table 3**

Medium	Microalgae	Maximum biomass concentration (g L^{-1})	Carbohydrates (%)	Lipids (%)	Proteins (%)	Reference
ADPP	*Chlorella vulgaris*	2.91[a)]	6.8	21.7	30.3	This study
ADPP	*Scenedesmus acuminatus*	8.22 [a)]	60.5	19.9	24.3	This study
ADMW	*Chlorella vulgaris*	2.02 [a)]	6.3	23.0	41.8	This study
ADMW	*Scenedesmus acuminatus*	2.92 [a)]	44.3	35.9	28.0	This study
Anaerobic digested poultry litter	*Scenedesmus bijuga*	0.38	22.9	9.5	39.0	Singh et al. (2011)
Human urine	*Chlorella sorokiniana*	9.3	n.a.[b)]	n.a.	n.a.	Tuantet et al. (2014)
Anaerobic digested municipal wastewater	*Chlorella pyrenoidosa*	1.97	13.9	10.9	60.7	Tan et al. (2015)
Anaerobic treated Piggery wastewater	*Chlorella vulgaris*	3.24	n.a.	32	n.a.	Marjakangas et al. (2015)
Swine manure	*Chlorella vulgaris*, *Scenedesmus obliquus* and *Chlamydomonas reindhardtii*	1.25	50	20	25	Molinuevo-Salces et al. (2016)
Anaerobic digested sewage	*Scenedesmus sp.* and/or *Chlorella sp.*	0.42	n.a.	n.a.	n.a.	Viruela et al. (2016)
Tris–acetate–phosphate medium	*Chlamydomonas reinhardtii* UTEX 90	12.4	59.7	n.a.	9.2	Choi et al. (2010)
Modified Basal Medium	*Chlorella vulgaris* FSP-E	7.22	50.4	n.a.	n.a.	Ho et al. (2013)

642 Note: This table gives an indication of the range of microalgal biomass production and cell compositions obtained in
643 various studies but the given values cannot be explicitly compared as the studies have been conducted using different
644 growth conditions (photobioreactor design, light intensity, CO_2 addition, nutrient concentration etc.).
645 [a)] Only biomass values from Experiment II are reported, as biomass composition was not measured in Experiment I
646 [b)] n.a.=data not available