Cultivation of Scenedesmus acuminatus in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge

Cultivation of *Scenedesmus acuminatus* in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge

Ran Tao*, Aino-Maija Lakaniemi, Jukka A. Rintala

Laboratory of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI- 33101 Tampere, Finland

* Corresponding author at: Tampere University of Technology, P.O. Box 541, FI- 33101 Tampere, Finland

E-mail address: ran.tao@tut.fi

Abstract:

Different undiluted liquid digestates from mesophilic and thermophilic anaerobic digesters of pulp and paper industry biosludge with and without thermal pretreatment were characterized and utilized for cultivating *Scenedesmus acuminatus*. Higher *S. acuminatus* biomass yields were obtained in thermophilic digestates (without and with pretreatment prior to anaerobic digestion (AD): 10.2±2.2 and 10.8±1.2 g L^{-1}, respectively) than in pretreated mesophilic digestates (7.8±0.3 g L^{-1}), likely due to differences in concentration of sulfate, iron, and/or other minor nutrients. *S. acuminatus* removed over 97.4% of ammonium and 99.9% of phosphate and sulfate from the digestates. Color (74–80%) and soluble COD (29–39%) of the digestates were partially removed. Different AD processes resulted in different methane yields (18–126 L CH_4 kg^{-1} VS), digestate compositions, and microalgal yields. These findings emphasize the importance of optimizing each processing step in wood-based biorefineries and provide information for pulp and paper industry development for enhancing value generation.

Keywords: wastewater treatment; pulp and paper industry; digestate characteristics; microalgal growth; nutrient recovery

1 Introduction

Due to environmental pollution and climate change, the European Union has promoted a binding goal of reducing greenhouse gas emissions by at least 40% in each member country by 2030 compared to 1990, including a 27% share of renewable energy for the EU (European Council, 2014). With the rapid growth of and heavy dependence on fossil fuels in Asia (Lee et al., 2017) as well as in other regions (e.g., North America, Latin America, and Africa) (Tan et al., 2017), a series of policies and legislations to encourage a low-carbon economy and green growth should be implemented. Biomass, which refers to all organic material originating from plants (e.g., algae, trees, and crops), can be converted into biofuels and energy carriers and is therefore a major renewable energy feedstock (McKendry, 2002). Compared with terrestrial plants, microalgae have great potential as a sustainable bioenergy feedstock due to, e.g., higher growth rates, no requirements for arable land, and the potential of wastewater treatment to recover nutrients (Guldhe et al., 2017).

However, before microalgae can be commercially utilized in low-value products such as energy and fuels (Arenas et al., 2017), higher biomass yields need to be generated to make the process more economically feasible. Since wastewater can provide the water and nutrients for the microalgae, many studies have been carried out to cultivate microalgae in different kinds of wastewaters, including municipal, agricultural, and industrial wastewater (Lv et al., 2017; Guldhe et al., 2017; Kinnunen and Rintala, 2016). Microalgal cultivation

in anaerobic digestion (AD) effluents, as a specific waste stream, has shown significant potential for biorefinery applications due to efficient nutrient removal and accumulation of high-value products (e.g., astaxanthin, carotenoids, and omega-3 fatty acids) in microalgal biomass (Polishchuk et al., 2015; Xia and Murphy, 2016). The integration of AD effluents from pulp and paper industry biosludge and microalgal cultivation (hereafter referred to as integrated AD&MC system) has been studied to produce biomass and recover nutrients from wastewater (Kinnunen and Rintala, 2016; Polishchuk et al., 2015). The results of our previous study (Tao et al., 2017) indicated the possibility of high-yield microalgal biomass production and efficient nutrient removal when *Scenedesmus acuminatus* was cultivated in liquid digestates from the AD of pulp and paper industry biosludge.

The pulp and paper industry is a water- and energy-intensive biomass-refining industry that typically treats its wastewaters in aerobic systems, which generate a large amount of primary sludge and biosludge. The AD of the generated sludge has gained increasing attention within the pulp and paper industry due to, e.g., methane production as a renewable energy (Kinnunen et al., 2015; Veluchamy and Kalamdhad, 2017) and the possibility for nutrient recovery. Thermal pretreatment prior to AD is one of the main approaches used to enhance methane production from pulp and paper industry biosludge (Kamali et al., 2016; Kinnunen et al., 2015). To understand the effect of thermal pretreatment temperatures (80 °C, 105 °C, 121 °C, and 134 °C) on the potential for methane production from biosludge in the pulp and paper industry, Kinnunen et al. (2015) carried out methane potential batch assays at 35 °C. They reported that methane production was increased by 39–140% compared to untreated biosludge with increasing pretreatment temperatures, except for methane production from biosludge treated at the lowest temperature, 80 °C,

which was lower than that obtained from untreated biosludge. However, although increased pretreatment temperatures increased methane production, costs and energy consumption increased as well (Kinnunen et al., 2015). To our knowledge, the first full-scale AD plant integrated with a pulp mill for digesting pulp mill sludge is currently being planned in Finland (Liikanen, 2016).

Previous studies have shown that biosludge with different treatments (pretreatment and AD) can result in different methane production yields and digestate compositions (Asunis, 2015; Kinnunen et al., 2015). To optimize an integrated AD&MC system for maximum bioenergy (methane and microalgal biomass) production, it is important to study each component and thus provide an overview of the AD&MC system itself. The aim of this work was to study *S. acuminatus* cultivation in various types of liquid digestates from the AD of pulp and paper industry biosludge, which to our knowledge has not been studied before. The objective was to provide scientifically and practically relevant information to pulp and paper industry biorefineries that consider implementing AD of biosludge and microalgal cultivation in the resulting liquid digestate. The following research questions were addressed: (1) How do different AD conditions change the composition of the digestates and in turn affect the growth of *S. acuminatus*? (2) Can *S. acuminatus* grow in and simultaneously remove nutrients from undiluted digestates from pulp and paper mill biosludge? The microalga *S. acuminatus* was chosen due to its high growth rate and ability to grow in various types of waste streams (Adamsson, 2000; Tao et al., 2017).

2 Materials and Methods

2.1 Microalgal strain and liquid digestates

Scenedesmus acuminatus (SAG 38.81) was obtained as a culture suspension from the SAG Culture Collection of Algae at the University of Göttingen, Germany. The stock culture was maintained in 100 mL of modified N-8 medium (Praveenkumar et al., 2014) in a 250-mL Erlenmeyer flask on an orbital shaker (150 rpm) and continuously illuminated using fluorescent lamps (Osram L 18W/965 Biolux, Germany) at a light intensity of 40 µmol photos m^{-2} s^{-1}. Since there was no growth of *S. acuminatus* in the modified N-8 medium with an initial pH of 6.5, the pH was adjusted to 8.0 by adding 5 M NaOH. Based on a previous study by Xu et al. (2015), 8.0 is an optimal initial pH for the cultivation of *Scenedesmus* sp.

Four types of digestates characterized in this study were collected from anaerobic, semi-continuously fed, completely stirred tank reactors (5 L liquid volume) treating biosludge from a pulp and paper industry wastewater treatment plant (Asunis, 2015). Three different pulp and paper mill biosludge digestates used in the microalgal cultivation experiments of the present study were anaerobically digested at 55 °C (thermophilic digestate, T), anaerobically digested at 55 °C after thermal pretreatment at 121 °C for 10 min (pre-treated thermophilic digestate, Tp), and anaerobically digested at 35 °C after thermal pretreatment at 121 °C for 10 min (pre-treated mesophilic digestate, Mp). The fourth pulp and paper mill biosludge digestate referred to in this paper was anaerobically digested at 35 °C (mesophilic digestate, M) (Asunis, 2015) and utilized for the cultivation of *S. acuminatus* in our previous study (Tao et al., 2017). The digestates were centrifuged at 5200 rpm for 4 min, and the supernatant was filtered through a glass fiber filter (Whatman GF/A, UK).

After filtration, the liquid digestates (Fig. S1 in the Supplementary Material) were stored at 4 °C before being used.

The microalgal growth results with the mesophilic digestate (M) are not directly comparable to the three digestates used for microalgal cultivation in the present study because, in our previous study, *S. acuminatus* was grown in 1.5-times diluted mesophilic digestate M (Tao et al., 2017), whereas in this study *S. acuminatus* was cultivated in undiluted digestates. Therefore, growth yields of *S. acuminatus* in digestate M were not compared to the microalgal cultivation results obtained in this study. However, the composition of the digestate M was provided in order to show more clearly how the digestate characteristics change depending on the AD temperature and presence or absence of a pretreatment step.

2.2 Photobioreactors

S. acuminatus was grown separately in the three different digestates (digestate refers to liquid, filtered digestate) for 21 days in photobioreactors (four replicates with each digestate), which consisted of a 1-L glass bottle (Pyrex) sealed with a plastic cap, with two tubes penetrating the cap serving as the gas inlet and outlet. Air with 5% CO_2 (v/v) at a flow rate of 0.105 L min^{-1} was sparged from the bottom by a glass distribution tube (porosity 0, ⌀ 22 mm, Duran Group, Germany). The photobioreactors were continuously illuminated using white fluorescent lamps (Osram L 18W/965 De Luxe Cool Daylight, Germany) with a light intensity of 240 µmol photos m^{-2} s^{-1} (Xu et al., 2015) from two sides of the reactors. *S. acuminatus* was inoculated to the photobioreactors to provide an initial optical density (OD_{680}) of 0.2. The initial total culture volume in the reactors was 600 mL.

The temperature of the reactors was maintained at 22±2 °C. Water evaporated during the cultivation due to the constant sparging, and therefore distilled water was added to compensate for the evaporated water volume (marked with lines on the photobioreactors) each time before taking samples for analyses.

2.3 Analytical methods

The culture pH was measured using a WTW 330 pH meter (WTW, Germany) with a Slimtrode electrode (Hamilton, Germany). The light intensity was controlled by measuring the average value of six sites on two sides of the photobioreactors' outer surface by a MQ-200 quantum meter (Apogee, USA).

Volatile suspended solids (VSS) were measured by filtering 10–15 mL of culture solution through a glass fiber filter (Whatman GF/A) to assess microalgal biomass production. Each filter containing the suspended solids was dried at 105 ºC overnight, then weighed and burned in a 550 ºC muffle furnace for 2 h before being weighed again. VSS was determined gravimetrically as the difference between the filters after treatment at these two temperatures. The supernatant after VSS filtration was used in the analysis of digestate color (ODd_{680}) and turbidity, soluble chemical oxygen demand (soluble COD), soluble biochemical oxygen demand (BOD_7s), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and nutrient (N, P, S) concentrations. The OD was measured at a wavelength of 680 nm using a Shimadzu UV-1700 Pharmaspec spectrophotometer after proper dilution with distilled water to give absorbance values between 0.2–0.7. Turbidity was measured with a TN-100/T-100 turbidimeter. OD_{680} was also measured from non-filtrated samples to assess microalgal biomass production (ODm_{680}).

155 The growth rates were calculated using the following equation:

156 $$\mu = \frac{\ln(X_t/X_0)}{t - t_0} \quad (1)$$

157 where X_0 is the concentration of biomass measured as VSS (g L^{-1}) at initial time (t_0) and X_t
158 is the concentration of biomass at a specific time (t).

159 Soluble COD was determined using a dichromate method according to the Finnish Standard
160 SFS 5504. The determination of BOD_7s was achieved with a WTW OxiTop
161 Control/OxiTop measuring system. DOC and DIC were measured with a total organic
162 carbon analyzer (Shimadzu Model TOC-5000) with an ASI-5000 autosampler. NH_4^+-N was
163 measured with an ion-selective electrode (Thermo Scientific Orion ISE meter). The
164 nutrients' (ammonium, phosphate, and sulfate) removal rate was calculated as NRR = (C_0 −
165 C_t) t^{-1}, where C_0 is the nutrient concentration on day 0, and C_t is the nutrient concentration
166 after decreasing to below 0.1 mg L^{-1}, which represents > 99.9% nutrient removal. NO_3^-,
167 NO_2^-, PO_4^{3-}, and SO_4^{2-} were measured using an ICS-1600 ion chromatograph (Dionex,
168 USA) with an AS-DV autosampler, Ion-Pac AS4A-SC anion exchange column, and ASRS-
169 300 suppressor (2 mm). The system was operated in isocratic mode using an eluent
170 containing 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$, and an eluent flow rate of 1 mL min^{-1}.

171 # 3 Results and Discussion

172 ## 3.1 Characteristics of the liquid digestates

173 The four pulp and paper industry biosludge digestates originating from digesters operating
174 at different temperatures to treat biosludge with and without thermal pretreatment had

different characteristics (Table 1). The initial pH of all the digestates was above 8.0, and the buffering capacity was good because the pH remained relatively stable in all cultivations despite efficient ammonium utilization, the uptake of which usually decreases culture pH, as shown by, e.g., Goldman and Brewer (1980). The ODd_{680} of the thermophilic digestates were higher than those of the mesophilic digestates. In addition, the ODd_{680} of the digestates indicated that pretreatment leads to increased color, as their ODd_{680} were slightly higher than those without pretreatment. Digestate Tp showed the darkest color (ODd_{680}: 0.63±0.08; turbidity: 320 NTU) of all the digestates. However, the ODd_{680} of digestate T (0.59±0.06) was higher than that of Mp (0.35±0.01), while its turbidity (280 NTU) was lower than that of Mp (290 NTU). The correlation between ODd_{680} and turbidity is unclear, likely due to the different wavelengths used in the two measurements. Substances in the liquid digestates responsible for their color may include clay, silt, finely divided inorganic and organic matter, soluble-colored organic compounds, plankton, and other microscopic organisms (Wang et al., 2010). The turbidity of liquid digestates may vary, ranging from, e.g., 2960 to 51400 NTU in the liquid fraction of mainly manure digestates from 11 full-scale co-digestion plants (Akhiar et al., 2017). The turbidities of our samples were much lower than those in Akhiar et al. (2017), likely due to different sampling methods. In the study of Akhiar et al. (2017) the liquid fractions of the digestates were separated from the solids either by screw press, centrifugation or vibrating screen, whereas in this study digestates were centrifuged and then filtered through glass fiber filters with a nominal pore size of 1.6 µm. The dark color of the medium, which results in poor light penetration, is one of the issues that could reduce microalgal growth (Wang et al., 2010; Xia and Murphy, 2016). For example, in a study by Wang et al. (2010) where *Chlorella* sp. were cultivated in

198 a liquid fraction (filtered through glass microfiber filters with pore size of 1.5 µm) of
199 anaerobically digested dairy manure (turbidity: 1800–1900 NTU) with different dilutions
200 (10-, 15-, 20-, and 25-times) for 21 days, the inverse correlation between turbidity and
201 specific algal growth rates (R^2 = 0.982) indicated that high turbidity may limit algal growth.
202 However, dilution for the benefit of microalgal growth increases total wastewater treatment
203 volume and might actually reduce microalgal growth due to a reduction in nutrients and
204 trace element concentrations.

205 The thermophilic digestates (T and Tp) had on average 65 mg L^{-1} higher ammonium
206 concentrations compared with the mesophilic digestates (M and Mp). In addition, the
207 pretreatment also led to increased ammonium concentration in the digestate especially in
208 the case of thermophilic digestion. The digestate Tp had on average 100 mg L^{-1} higher
209 ammonium concentration than digestate T (Table 1). Ammonium was available in all the
210 digestates as a nitrogen source for microalgal growth, while nitrate and nitrite
211 concentrations were below 1.0 mg L^{-1}. The sulfate-S concentration in digestate Mp was
212 much lower than corresponding concentrations in the other three digestates (Table 1). The
213 total phosphorus content was similar (27–30 mg L^{-1}) in all the digestates, and
214 approximately 50% of the phosphorus existed in the form of phosphate — except in
215 digestate M, where the phosphate share was slightly higher (64.3%). Xin et al. (2010) have
216 reported an optimal N/P ratio (mass per mass) for *Scenedesmus* sp. LX1 growth to range
217 between 5 and 8, while *Scenedesmus* sp. in the study of Rhee (1978) required an N/P ratio
218 of approximately 13.5 to grow without limitations by either nutrient. The optimal ratio is
219 also species-specific. The N/P ratios of the digestates in this study ranged from 12 to 18
220 (Table 1) and were thus somewhat higher than the reported values. However, no extra

phosphate was added to the digestates since it did not help with microalgal biomass production or ammonium removal in the digestates of sewage sludge in our previous study (Tao et al., 2017).

A phenomenon similar to that with ammonium was observed with soluble COD values of the different digestates. The thermophilic digestates had higher soluble COD values than the mesophilic digestates; and when the digestates produced at the same digestion temperature were compared, those generated with pretreatment resulted in higher soluble COD values than those without pretreatment (Table 1). The BOD_7s/soluble COD ratios were lower than 1:20 in the measured digestates (T, Tp, and Mp), which means that most of the organic material left in the liquid digestates after anaerobic digestion was not easily biodegradable. The DIC concentration (520–690 mg L^{-1}) of each digestate was higher than the corresponding DOC concentration (150–540 mg L^{-1}).

3.2 Cultivation of *S. acuminatus* in the liquid digestates

3.2.1 Microalgal biomass production

Microalgal biomass production as indicated by VSS in the three studied digestates (T, Tp, and Mp) was as shown in Fig. 1. The ODm_{680} and VSS had a positive correlate in each digestate (T: $R^2 = 0.96$; Tp: $R^2 = 0.96$; Mp: $R^2 = 0.97$). The final microalgal biomass concentration after 21 days of batch cultivation was higher with both thermophilic digestates (T, Tp: 10.2±2.2–10.8±1.2 g L^{-1}) than the concentration obtained with the mesophilic digestate (Mp: 7.8±0.3 g L^{-1}). Despite the relatively high initial ammonium concentrations (380–480 mg L^{-1}) in all cultures, no clear lag phase was observed in microalgal growth. The biomass concentration started to stabilize on day 15–18. *S.*

acuminatus in digestate Tp initially grew more slowly than in digestates T and Mp, likely due to its higher initial ammonium concentration potentially inhibiting or slowing down photosynthesis (Abeliovich and Azov, 1976) as well as poorer light penetration (due to the darker color of the digestate). Before day 9, the *S. acuminatus* biomass concentration in digestate T (6.0 g-VSS L^{-1} at day 9) was the highest, followed by *S. acuminatus* in digestate Mp (4.9 g-VSS L^{-1} at day 9) and Tp (4.4 g-VSS L^{-1} at day 9). After day 9 and day 15, the VSS concentration in digestate Tp exceeded that in digestates Mp and T, respectively. The highest specific growth rates for all digestates were obtained during different periods (Table 2). These values are relatively high, as previous studies have reported growth rates ranging from 0.41 to 1.06 day^{-1} (Diniz et al., 2017; Wang et al., 2010).

The results of this study show that liquid digestates from pulp and paper wastewater treatment plant biosludge digestion can support high microalgal biomass yields and thus confirm the results of our previous study (Tao et al., 2017). In addition, in this study high microalgal biomass concentrations were obtained in the liquid digestates without dilution. To our knowledge, this has not been reported before. The light path in this study was not optimized, but it was shown that the color of the digestates was not a problem in the simple cultivation systems used. Thus, the microalgae should also grow well in more optimized short-path photobioreactors without dilution of the digestate. Bacteria were observed in the cultures, which was expected since the digestates were not sterilized in this study. Thus, the measured VSS values did include some bacteria associated with the microalgae. However, majority of the biomass was likely microalgae. For example, Hulatt and Thomas (2010) found an increased number of bacteria during 30-day microalgal cultivation, but reported that less than 1% of carbon of the total biomass comprised of bacteria.

The influence from pulp and paper mill digestates on microalgal growth is also species-specific. For example, Kinnunen and Rintala (2016) previously reported that the highest biomass concentration (less than 0.2 g-VSS L^{-1}) was obtained with *Scenedesmus* sp. originating from Lake Pyhäjärvi (Tampere, Finland) in 4-times diluted liquid digestate from pulp and paper industry biosludge AD after optimizing the dilution. Although the biosludge used in Kinnunen and Rintala (2016) and in this study were from the same pulp and paper mill, the different characteristics of the digestates (likely due to changes in, e.g., wood source, pulp mill operation parameters, and seasons) and microalgal strains clearly affected the obtainable biomass quantity.

3.2.2 Nutrient removal from liquid digestates

S. acuminatus removed nutrients efficiently from the digestates (Fig. 2). The ammonium concentration decreased from an initial 380–480 mg L^{-1} to less than 0.2–10 mg L^{-1}. The ammonium removal efficiency in the thermophilic digestates was over 99.9%, which was slightly higher than that obtained in the mesophilic digestate (97.4%). The pH fluctuated between 7.8 and 8.4 (Fig. S2 in Supplementary Material) and showed a decreasing trend likely due to ammonium uptake, which is known to reduce pH (Goldman and Brewer, 1980). The overall ammonium removal rates during the 21-day cultivation period were similar in all cultures (T: 18.3 mg L^{-1} day^{-1}; Tp: 23.3 mg L^{-1} day^{-1}; and Mp: 17.8 mg L^{-1} day^{-1}). However, a clear change in the ammonium removal rate was seen in all digestates after day 7, likely due to exhaustion of phosphate and sulfate (Fig. 2). Ammonium removal rates before and after day 7 were 43.1 and 5.9 mg L^{-1} day^{-1}, 34.5 and 17.7 mg L^{-1} day^{-1}, and 26.0 and 13.8 mg L^{-1} day^{-1} for digestate T, Tp, and Mp, respectively. This finding indicates that the exhaustion of phosphate and sulfate from the cultures could slow

289 ammonium uptake as previously shown also by Xin et al. (2010). Several ammonium
290 transformations (e.g., algal uptake, ammonia evaporation, bacterial growth, and
291 nitrification) can occur in algae–bacteria consortium systems (González-Fernández et al.,
292 2011). According to the average temperature (22 °C) and observed pH range (7.8–8.4), the
293 theoretical fraction of unionized ammonia in all cultivations was 2.8%–10.3% (the equation
294 used for calculation shown in Tao et al., 2017). In addition, only low levels of nitrate and
295 nitrite (< 3 mg L^{-1}) were found in all cultivations. These data suggest that ammonium
296 stripping and nitrification may have occurred, but that the main portion of the removed
297 ammonium from the digestates was used for microbial growth.

298 Sulfate concentration increased in all cultures from day 0 to day 2 (Fig. 2c). The resulting
299 sulfate likely originated from other sulfur compounds present in the digestates. During
300 anaerobic digestion, sulfate can be converted to sulfide by sulfate-reducing bacteria, and
301 result in the presence of H_2S and HS^- in the liquid phase (Cirne et al., 2008). H_2S and HS^-
302 could be converted into sulfate during cultivation via chemical and biological reactions in
303 the cultures supplied with air (Chen and Morris, 1972). Additionally, microalgae are
304 capable of releasing enzymes that can split inorganic sulfur from organic compounds and
305 make the sulfur available for algal growth (Giordano and Raven, 2014; Kertesz, 2000).
306 After the initial increase, however, sulfate was completely removed by day 7-9. Phosphate
307 removal, on the other hand, started immediately and phosphate was completely removed by
308 day 7 in all cultures. The overall phosphate and sulfate removal rates were 2.28 and 2.39
309 mg L^{-1} day^{-1}, 1.63 and 1.68 mg L^{-1} day^{-1}, and 2.13 and 0.45 mg L^{-1} day^{-1} for digestates T,
310 Tp, and Mp, respectively. The removal rates of both phosphate and sulfate in digestate T
311 were the highest among all digestates. Phosphorus was likely removed from the digestates

312 through adsorption on the microalgal surface, intracellular uptake, and precipitation (Cai et
313 al., 2013). In the present study, VSS continued to increase even though phosphate was no
314 longer detected from the liquid digestates after day 7, which indicates that initial
315 phosphorus level in the digestates was high enough to support microalgal growth.

316 Based on the results of this study, Initial sulfate concentrations in liquid digestates could
317 affect ammonium removal efficiency and microalgal biomass production. This hypothesis
318 is supported by the fact that the cultivations in digestates T and Tp had similar initial sulfate
319 concentrations (15–17 mg L^{-1}) that enabled over 99.9% ammonium removal and similar
320 microalgal biomass production, while the different initial sulfate concentrations in
321 digestates T and Mp (17 vs. 3 mg L^{-1}), which had similar initial ammonium concentrations,
322 resulted in different ammonium removal efficiencies and algal biomass yield. Biological
323 nitrogen (N) uptake is catalyzed during photosynthesis by nitrogenase, which contains
324 iron–sulfur clusters (Zheng and Dean, 1994). A shortage of either sulfur or iron can, thus,
325 decrease the microalgal growth rate (Kumaresan et al., 2017; Liu et al., 2008). Sulfate is a
326 primary sulfur source for microalgae in aquatic environments, but the effect of sulfate
327 concentration on microalgal growth has not been widely studied. Mera et al. (2016)
328 reported that the growth of microalga *Chlamydomonas moewusii* was quite similar at
329 sodium sulfate concentrations of 0.1–3 mM (SO_4^{2-}-S: 3.2–96 mg L^{-1}), but microalgal
330 biomass yields were lower at higher and lower sodium sulfate concentrations. In a study by
331 Lv et al. (2017), similar *Chlorococcum* sp. growth at SO_4^{2-}-S levels from 6–90.3 mg L^{-1}
332 was obtained, but was much lower at 0 mg L^{-1} sulfate. Due to the small number of related
333 studies, the effect of sulfate and combined effect of iron and sulfate on microalgal growth
334 should be further studied in the future. However, it should be also noted that other

335 micronutrients and trace elements that were not measured in this study could have caused
336 some differences in microalgal growth.

337

338 **3.2.3 Soluble COD, DOC, DIC, and color changes**

339 In this study, microalgal cultivation removed soluble COD and DIC to a certain extent; 29–
340 39% removal and 47–57% removal, respectively (Fig. 3a, d). DOC acted somewhat
341 contradictory to soluble COD, as the DOC level increased in the mesophilic digestate (Fig.
342 3c). Soluble COD removal efficiency from the thermophilic digestates (38% and 39%) was
343 higher than that from the mesophilic digestate (29%). The total removed dissolved carbon
344 (<1 g L^{-1}) from the digestates was lower than the total carbon present in the biomass (3.9–
345 5.4 g L^{-1}), when assuming that approximately 50% of the total produced biomass is carbon
346 (Chisti, 2008). Hence, the cultivation was mixotrophic as both organic and inorganic
347 carbon was utilized, but mainly photoautotrophic as CO_2 was the main carbon source used
348 for microalgal growth.

349 COD represents the concentration of chemical oxidizer needed to oxidize all the oxidizable
350 organic or inorganic materials in wastewater, and DOC is used to reflect the dissolved
351 organic carbon content of a sample. In most microalgal studies, either DOC or COD has
352 been measured during microalgal cultivation (Eloka-Eboka et al., 2017; Guldhe et al., 2017;
353 Wang et al., 2010), yet the correlation between COD and DOC in microalgal cultures
354 remains unclear. For example, Marjakangas et al. (2015) reported an increase in both
355 soluble COD and DOC concentrations, likely due to a stress caused by an initial pH
356 decrease after *C. vulgaris* CY5 was mixotrophically cultivated in anaerobically treated
357 piggery wastewater. Thus, it seems that changes in COD and DOC depend on growth

conditions. In our study, organic carbon release from photosynthetic microalgal cells might explain the observed increase in DOC during the cultivations in mesophilic digestate. The decrease in soluble COD suggests that organic materials from the digestates were consumed during cultivation and that the amount of consumed materials was higher than the organic carbon released by the microalgae during normal photosynthetic growth. Some studies have reported relatively high COD removal efficiencies (75–80%) from liquid digestates integrated with microalgal cultivation (Yan and Zheng, 2014; Yang et al., 2015). Soluble COD in this study was not easily biodegradable and was not therefore fully removed. Further removal of soluble COD would be possible with non-biological treatments, e.g., chemical oxidation, if deemed necessary. However, further soluble COD removal would probably not be needed as the COD load (both low flow and COD concentration) from algae treatment reject waters would be minimal compared to the effluent COD load from the activated sludge plant the sludges originates, which may be up to tens of tons COD per day (e.g., Regional State Administrative Agency of Eastern Finland, 2016). Furthermore, in practice the effluent from algae treatment could be circulated to the beginning of the activated sludge process, as is typically done with dewatering reject waters after AD in municipal wastewater treatment plants.

The OD_{680} of the digestates were measured after removing the microalgae to demonstrate their color change during cultivation (Fig. 3b). The OD_{680} values in all digestates decreased until day 9 but remained stable afterward. At the end of the batch cultivations, the color removal efficiencies in T, Tp, and Mp were 80%, 74%, and 79%, respectively. The mechanism of color removal is not clear based on the results of this study. However, Graham and Wilcox (2000) suggested that lignin (one cause of color) could be converted

into other non-colored materials by microalgal metabolism. Tarlan et al. (2002) also reported that the main mechanism of color removal from pulping effluents with a mixed culture of microalgae was metabolic conversion of colored molecules to non-colored molecules rather than adsorption. Thus, the possible reason for the lower removal efficiency of COD (29–39%) than color (74–80%) in this study was that the colored organic molecules were converted into non-colored organic molecules.

3.2.4 Integration of methane production and microalgal cultivation in the digestate

To evaluate the different integrated AD&MC systems, the performance of each processing step is shown in an overview (treatment methods of biosludge, microalgal cultivation conditions, and bioenergy production) (Table 2). During the 21-day cultivation, approximately 35% more microalgal biomass (as VSS) was obtained in the thermophilic digestates than in the mesophilic digestate. This is a promising discovery, as methane production in thermophilic digestion with pretreatment was higher than that obtained in the corresponding mesophilic process; likewise, methane production in thermophilic digestion without pretreatment was also higher than that obtained in mesophilic digestion without pretreatment (Table 2). This finding indicates that the highest methane production and microalgal biomass yields can be obtained in the same integrated AD&MC system.

The effect of sludge pretreatment before digestion on microalgal cultivation is not, however, fully clear based on the results of this study. Asunis (2015) reported that thermal pretreatment increased the methane yield by 100% in thermophilic AD, while the increase was 460% in mesophilic AD. The difference caused by pretreatment prior to thermophilic digestion on microalgal biomass production in the digestate was not significant. Although

maximum methane and microalgal biomass production were obtained with the same process (thermophilic AD with pretreatment), other factors should be considered, including the cost and energy burden of thermal pretreatment.

4 Conclusions

The cultivation of *Scenedesmus acuminatus* was successful in different undiluted digestates from pulp and paper industry biosludge treated at different AD conditions (mesophilic vs. thermophilic, with and without thermal pretreatment). *S. acuminatus* grew well (7.8–10.8 g L^{-1}) and removed nutrients efficiently (over 97%) from all the digestates. Color (74–80%) and soluble COD (29–39%) were partially removed. The digestates from the thermophilic process with pretreatment generated the highest microalgal biomass concentrations, which is a promising discovery for pulp and paper industry algae-based biorefinery applications as maximum methane production was also obtained at the same conditions.

Acknowledgments: This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 [grant number 643071]. We would like to thank Viljami Kinnunen and Ramasamy Praveenkumar for their suggestions about the experimental set-up. We would also like to thank Tarja Ylijoki-Kaiste for her help in the laboratory.

Appendix A. Supplementary data

Figure S1. The photos of liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp) before (day 0) and after cultivation (day 21).

Figure S2. pH evolution during the cultivation of *Scenedesmus acuminatus* in the liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

References

1. Abeliovich, A.H.A.R.O.N., Azov, Y., 1976. Toxicity of ammonia to algae in sewage oxidation ponds. Appl. Environ. Microbiol. 31, 801-806.

2. Adamsson, M., 2000. Potential use of human urine by greenhouse culturing of microalgae (*Scenedesmus acuminatus*), zooplankton (*Daphnia magna*) and tomatoes (*Lycopersicon*). Ecol. Eng. 16, 243-254.

3. Akhiar, A., Battimelli, A., Torrijos, M., Carrere, H., 2017. Comprehensive characterization of the liquid fraction of digestates from full-scale anaerobic co-digestion. Waste Manage. 59, 118-128.

4. Arenas, E.G., Palacio, R., Juantorena, A.U., Fernando, S.E.L., Sebastian, P.J., 2017. Microalgae as a potential source for biodiesel production: techniques, methods, and other challenges. Int. J. Energy Res. 41, 761-789.

5. Asunis, F., 2015. Thermal pretreatment to enhance anaerobic digestion of pulp and paper mill biosludge (Unpublished master's thesis). Universita degli Studi di Cagliari Facolta di Ingegneria e Architettura, Italy and Tampere University of Technology, Finland. Retrieved from http://people.unica.it/giorgiadegioannis/files/2017/08/Master-Thesis-AF_17-11_v07_FINAL_FULL.pdf

6. Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew. Sustainable Energy Rev. 19, 360-369.

7. Chen, K.Y., Morris, J.C., 1972. Kinetics of oxidation of aqueous sulfide by oxygen. Environ. Sci. Technol. 6, 529-537.

456 8. Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26,
457 126-131.

458 9. Cirne, D.G., Van Der Zee, F.P., Fernandez-Polanco, M., Fernandez-Polanco, F., 2008.
459 Control of sulphide during anaerobic treatment of S-containing wastewaters by adding
460 limited amounts of oxygen or nitrate. Rev. Environ. Sci. Bio. 7, 93-105.

461 10. Diniz, G.S., Silva, A.F., Araújo, O.Q., Chaloub, R.M., 2017. The potential of
462 microalgal biomass production for biotechnological purposes using wastewater resources.
463 J. Appl. Phycol. 29, 821-832.

464 11. Eloka-Eboka, A.C., Inambao, F.L., 2017. Effects of CO_2 sequestration on lipid and
465 biomass productivity in microalgal biomass production. Appl. Energy 195, 1100-1111.

466 12. European Council. Conclusions (23 and 24 October 2014). 2030 Climate and Energy
467 Policy Framework. EUCO 169/14; 2014. Retrieved from
468 http://www.consilium.europa.eu/uedocs/cms_data/docs/pressdata/en/ec/145397.pdf.

469 13. Giordano, M., Raven, J.A., 2014. Nitrogen and sulfur assimilation in plants and algae.
470 Aquat. Bot. 118, 45-61.

471 14. Goldman, J.C., Brewer, P.G., 1980. Effect of nitrogen source and growth rate on
472 phytoplankton‐mediated changes in alkalinity. Limnol. Oceanogr. 25, 352-357.

473 15. González-Fernández, C., Molinuevo-Salces, B., García-González, M.C., 2011. Nitrogen
474 transformations under different conditions in open ponds by means of microalgae–bacteria
475 consortium treating pig slurry. Bioresour. Technol. 102, 960-966.

476 16. Graham, L.F., Wilcox, L.W., 2000. Algae. Prentice-Hall, Englewood Cliffs, NJ.

17. Guldhe, A., Ansari, F.A., Singh, P., Bux, F., 2017. Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation. Ecol. Eng. 99, 47-53.

18. Hulatt, C.J., Thomas, D.N., 2010. Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion?. Bioresour. Technol. 101, 8690-8697.

19. McKendry, P., 2002. Energy production from biomass (part 1): overview of biomass. Bioresour. Technol. 83, 37-46.

20. Kamali, M., Gameiro, T., Costa, M.E.V., Capela, I., 2016. Anaerobic digestion of pulp and paper mill wastes–An overview of the developments and improvement opportunities. Chem. Eng. J. 298, 162-182.

21. Kertesz, M.A., 2000. Riding the sulfur cycle–metabolism of sulfonates and sulfate esters in Gram-negative bacteria. FEMS Microbiol. Rev. 24, 135-175.

22. Kinnunen, V., Rintala, J., 2016. The effect of low-temperature pretreatment on the solubilization and biomethane potential of microalgae biomass grown in synthetic and wastewater media. Bioresour. Technol. 221, 78-84.

23. Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Res. 87, 105-111.

496 24. Kumaresan, V., Nizam, F., Ravichandran, G., Viswanathan, K., Palanisamy, R., Bhatt,
497 P., Arasu, M.V., Al-Dhabi, N.A., Mala, K., Arockiaraj, J., 2017. Transcriptome changes of
498 blue-green algae, *Arthrospira* sp. in response to sulfate stress. Algal Res. 23, 96-103.

499 25. Lee, C.T., Hashim, H., Ho, C.S., Van Fan, Y., Klemeš, J.J., 2017. Sustaining the low-
500 carbon emission development in Asia and beyond: Sustainable energy, water, transportation
501 and low-carbon emission technology. J. Clean. Prod. 146, 1-13.

502 26. Liikanen, J. (2016, December 7). Biofuel from Äänekoski. Paper and Timber Journal.
503 Retrieved from http://www.paperijapuu.fi/biofuel-from-aanekoski/

504 27. Liu, Z.Y., Wang, G.C., Zhou, B.C., 2008. Effect of iron on growth and lipid
505 accumulation in *Chlorella vulgaris*. Bioresour. Technol. 99, 4717-4722.

506 28. Regional State Administrative Agency of Eastern Finland (2017, March 31)
507 Environmental permit for Savon Sellu. Retrieved from
508 https://tietopalvelu.ahtp.fi/Lupa/Lisatiedot.aspx?Asia_ID=1297010

509 29. Lv, J., Guo, J., Feng, J., Liu, Q., Xie, S., 2017. Effect of sulfate ions on growth and
510 pollutants removal of self-flocculating microalga *Chlorococcum* sp. GD in synthetic
511 municipal wastewater. Bioresour. Technol. 234, 289-296.

512 30. Marjakangas, J.M., Chen, C.Y., Lakaniemi, A.M., Puhakka, J.A., Whang, L.M., Chang,
513 J.S., 2015. Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on
514 sterilized and non-sterilized anaerobically pretreated piggery wastewater. Biochem. Eng. J.
515 103, 177-184.

516 31. Mera, R., Torres, E., Abalde, J., 2016. Effects of sodium sulfate on the freshwater
517 microalga *Chlamydomonas moewusii*: implications for the optimization of algal culture
518 media. J. Phycol. 52, 75-88.

519 32. Polishchuk, A., Valev, D., Tarvainen, M., Mishra, S., Kinnunen, V., Antal, T., Yang,
520 B., Rintala, J., Tyystjärvi, E., 2015. Cultivation of *Nannochloropsis* for eicosapentaenoic
521 acid production in wastewaters of pulp and paper industry. Bioresour. Technol. 193, 469-
522 476.

523 33. Praveenkumar, R., Kim, B., Choi, E., Lee, K., Cho, S., Hyun, J.S., Park, J.Y., Lee,
524 Y.C., Lee, H.U., Lee, J.S., Oh, Y.K., 2014. Mixotrophic cultivation of oleaginous *Chlorella*
525 sp. KR-1 mediated by actual coal-fired flue gas for biodiesel production. Bioprocess
526 Biosyst. Eng. 37, 2083-2094.

527 34. Rhee, G.Y., 1978. Effects of N: P atomic ratios and nitrate limitation on algal growth,
528 cell composition, and nitrate uptake. Limnol. Oceanogr. 23, 10-25.

529 35. Tan, S., Yang, J., Yan, J., Lee, C., Hashim, H., Chen, B., 2017. A holistic low carbon
530 city indicator framework for sustainable development. Appl. Energy 185, 1919-1930.

531 36. Tao, R., Kinnunen, V., Praveenkumar R., Lakaniemi, A.M., Rintala, A.J., 2017.
532 Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid
533 digestates from anaerobic digestion of pulp and paper industry and municipal wastewater
534 treatment sludge. J. Appl. Phycol. doi: 10.1007/s10811-017-1175-6

535 37. Tarlan, E., Dilek, F.B., Yetis, U., 2002. Effectiveness of algae in the treatment of a
536 wood-based pulp and paper industry wastewater. Bioresour. Technol. 84, 1-5.

537 38. Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and
538 phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a
539 freshwater microalga *Scenedesmus* sp. Bioresour. Technol. 101, 5494-5500.

540 39. Veluchamy, C., Kalamdhad, A.S., 2017. Biochemical methane potential test for pulp
541 and paper mill sludge with different food/microorganisms ratios and its kinetics. Int.
542 Biodeterior. Biodegradation 117, 197-204.

543 40. Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic
544 digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae
545 *Chlorella* sp. Bioresour. Technol. 101, 2623-2628.

546 41. Xia, A., Murphy, J.D., 2016. Microalgal cultivation in treating liquid digestate from
547 biogas systems. Trends Biotechnol. 34, 264-275.

548 42. Xu, X., Shen, Y., Chen, J., 2015. Cultivation of *Scenedesmus dimorphus* for C/N/P
549 removal and lipid production. Electron. J. Biotechnol. 18, 46-50.

550 43. Yan, C., Zheng, Z., 2014. Performance of mixed LED light wavelengths on biogas
551 upgrade and biogas fluid removal by microalga *Chlorella* sp. Appl. Energy 113, 1008-
552 1014.

553 44. Yang, L., Tan, X., Li, D., Chu, H., Zhou, X., Zhang, Y., Yu, H., 2015. Nutrients
554 removal and lipids production by *Chlorella pyrenoidosa* cultivation using anaerobic
555 digested starch wastewater and alcohol wastewater. Bioresour. Technol. 181, 54-61.

556 45. Zheng, L., Dean, D.R., 1994. Catalytic formation of a nitrogenase iron-sulfur cluster. J.
557 Biol. Chem. 269, 18723-18726.

Figure Captions

Fig. 1. Biomass concentration as volatile suspended solids (VSS) during the cultivation of *Scenedesmus acuminatus* in the liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

Fig. 2. The soluble ammonium-N (a), phosphate-P (b), and sulfate-S concentrations (c) during the cultivation of *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp). The nitrate and nitrite concentrations are not shown since they remained below 3 mg L^{-1} in all cultures.

Fig. 3. Soluble COD concentration and removal efficiency (a), ODd_{680} of the cultivation medium (b), DOC concentration (c), and DIC concentration (d) during the cultivation of *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

Tables

Table 1 Composition of the liquid digestates from the anaerobic digestion of the pulp and paper industry biosludge produced under thermophilic conditions without pretreatment (T) and with pretreatment (121 °C) for 10 min (Tp) and under mesophilic conditions without pretreatment (M) and with pretreatment (121 °C) for 10 min (Mp).

	T	Tp	M [a)]	Mp
pH	8.2	8.3	8.5	8.3
Alkalinity (mg L^{-1} $CaCO_3$)	2700	3100	n.a.[b)]	2600
ODd_{680}	0.59 ± 0.06	0.63 ± 0.08	0.34 ± 0.01	0.35 ± 0.01
Turbidity (NTU)	280	320	n.a.	290
NH_4^+-N (mg L^{-1})	380 ± 20	480 ± 20	350 ± 50	380 ± 15
NO_3^- (mg L^{-1})	<1.0	<1.0	<1.0	<1.0
NO_2^- (mg L^{-1})	<1.0	<1.0	<1.0	<1.0
TP [a)] (mg L^{-1})	33 ± 3	27 ± 1	28 ± 1	33 ± 2
PO_4^{3-}-P (mg L^{-1})	16 ± 3	15 ± 3	18 ± 1	15 ± 1
N:P[c)]	12.1 ± 2.3	17.6 ± 1.5	12.5 ± 2.0	11.6 ± 1.0
SO_4^{2-}-S[a)] (mg L^{-1})	17 ± 1.0	15 ± 0.1	17 ± 0.9	3 ± 0.1
Soluble COD (mg L^{-1})	1200 ± 130	2000 ± 130	910 ± 30	1170 ± 10
BOD_7s [a)] (mg L^{-1})	110 ± 5	60 ± 100	n.a.	60 ± 5
BOD_7s/soluble COD [a)]	0.09 ± 0.04	0.03 ± 0.77	n.a.	0.05 ± 0.50
DOC (mg L^{-1})	300 ± 4	540 ± 110	370 ± 40	150 ± 0
DIC (mg L^{-1})	570 ± 10	690 ± 46	520 ± 5	680 ± 47 [a)]

a) The values with ± sign include standard errors (n = 2)

b) n.a. = data not available

c) N:P (mass per mass): N refers to NH_4^+-N and P refers to TP

585 **Table 2** Integrated processes of anaerobic digestion of pulp and paper industry biosludge and

586 *Scenedesmus acuminatus* cultivation in the undiluted liquid digestates from the anaerobic digestion of the

587 biosludge

	Pretreatment	AD temperature (°C)	Cultivation duration (d)	Methane yield (L CH_4 kg^{-1} VS)	Highest obtained biomass concentration (g-VSS L^{-1})	Highest specific growth rate (d^{-1})
M	No	35	14	18[a)]	8.8 ± 0.8[b)]	0.99[b)] (day 4–7)
Mp	Yes	35	21	101[a)]	7.8 ± 0.3	0.75 (day 7–9)
T	No	55	21	63[a)]	10.2 ± 2.2	0.88 (day 4–7)
Tp	Yes	55	21	126[a)]	10.8 ± 1.2	1.02 (day 9–12)

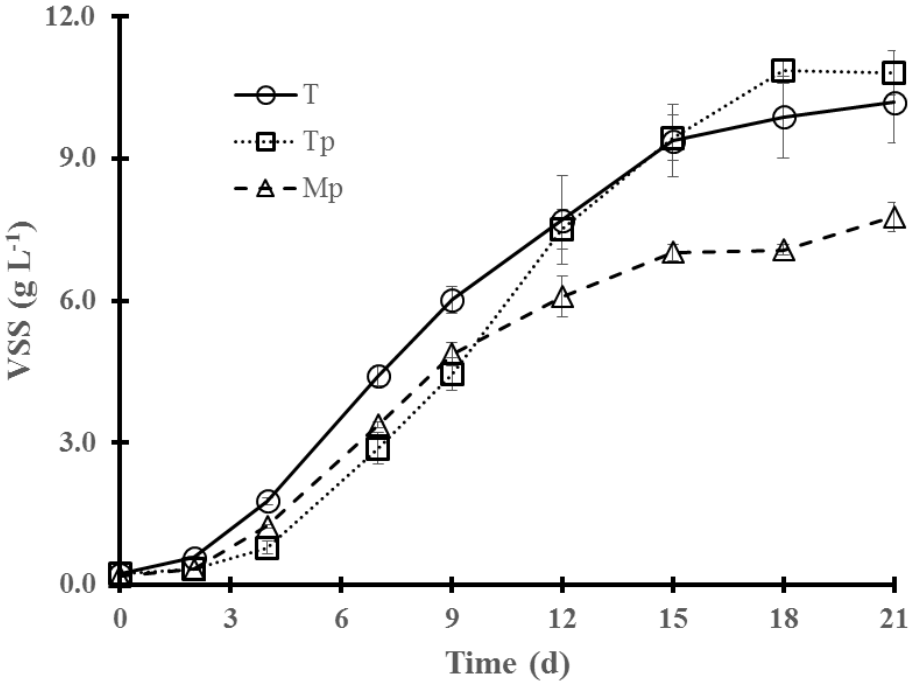
588 a) data originated from Asunis (2015)

589 b) microalgae were cultivated in 1.5-times diluted digestate (Tao et al., 2017)

590

591 **Figures**

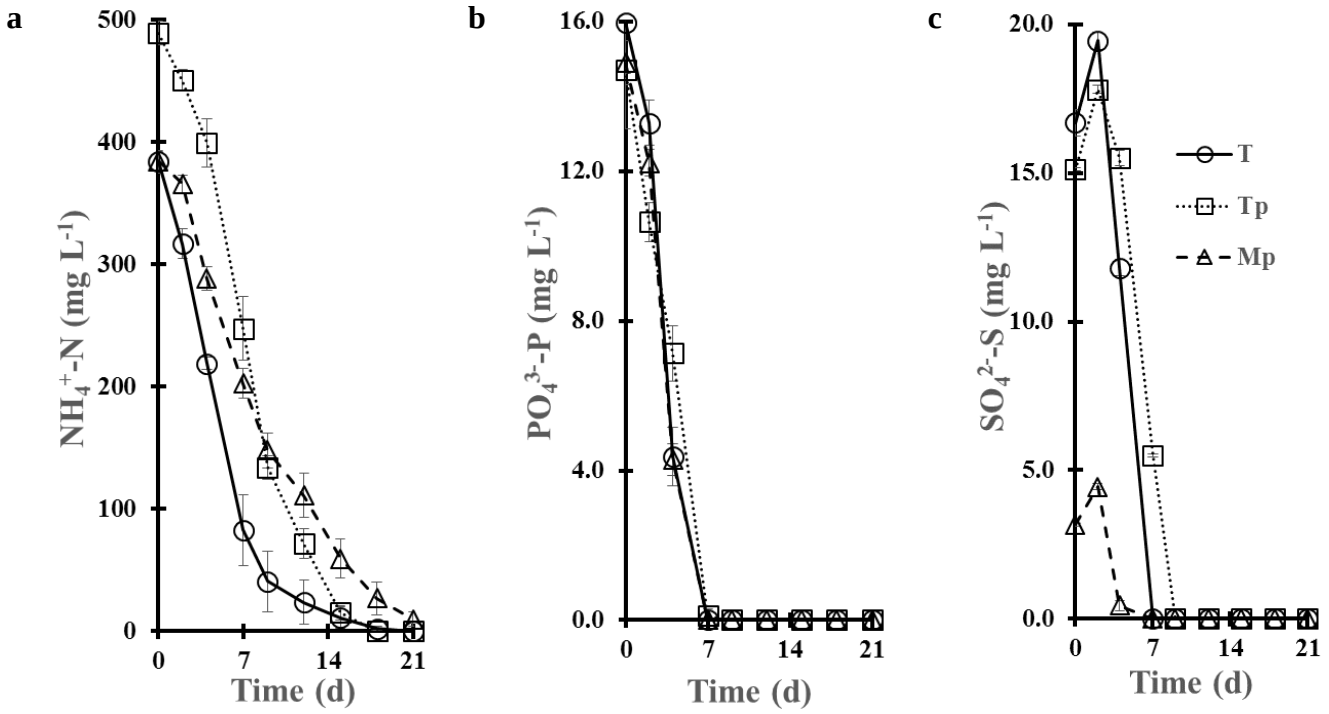
592 **Fig. 1**



593

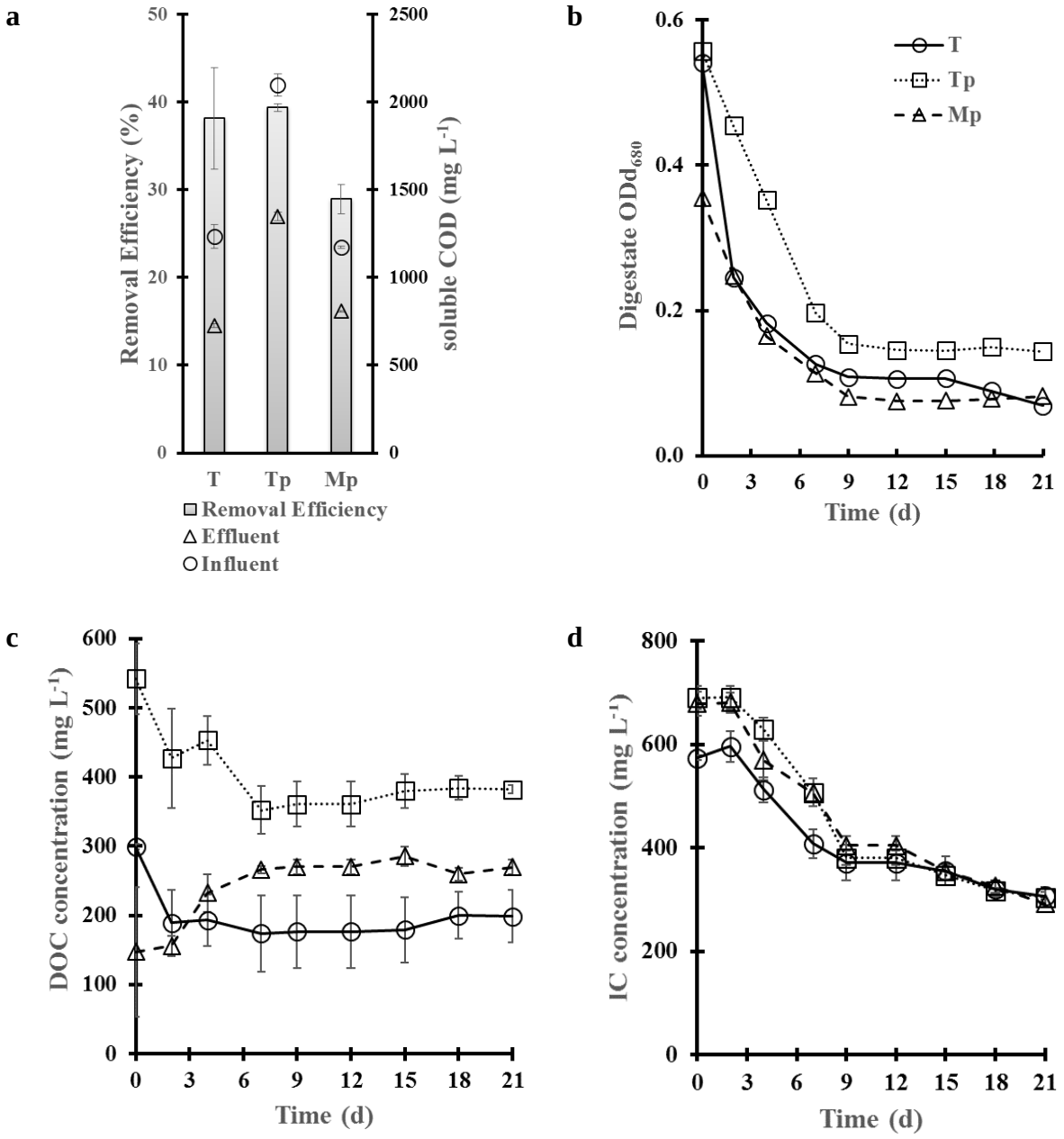
594

595 **Fig. 2**



596

597 **Fig. 3**


a
Removal Efficiency (%)
soluble COD (mg L-1)
T
Tp
Mp
Removal Efficiency
Effluent
Influent
b
Digestate ODd680
Time (d)
c
DOC concentration (mg L-1)
Time (d)
d
IC concentration (mg L-1)
Time (d)

598