Evaluation of *Chlorella vulgaris* and *Scenedesmus obliquus* growth on pretreated organic solid waste digestate

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Abstract

In this research *Scenedesmus obliquus* and *Chlorella vulgaris* growth was tested on digestate sludge obtained from the anaerobic co-digestion treatment of the organic fraction of municipal solid waste (OFMSW) together with waste activated sludge (WAS). Digestate was diluted 1:10 and tested in three batch experimental conditions: with no pre-treatments (noPT), after centrifugation (AC) and after filtration (AUF), in order to evaluate microalgae limiting growth factors. The best growth was obtained by *C. vulgaris* on digestate AC compared to *S. obliquus,* reaching 479 ± 31 cell million ml^{-1} and 131 ± 12 cell million ml^{-1} respectively. Ammonia removal evaluated in *C. vulgaris* and *S. obliquus* cultures was 99.2% ± 0.3 and 98.146% ± 0.008 in AC condition, respectively. Considering that AUF showed similar microalgae growth values, the digestate pretreatment for microalgae growth, could be limited to centrifugation.

Keywords: microalgae, anaerobic digestion, *Scenedesmus obliquus*, *Chlorella vulgaris*, organic waste, digestate.

Acronyms list:

AD: Anaerobic Digestion

OFMSW: Organic Fraction of Municipal Solid Waste

noPT: none Pre-Treatment

AC: After Centrifugation

AUF: After centrifugation and Filtration

TS: Total Solid

TVS: Total Volatile Solid

TKN: Total Kjeldahl Nitrogen

P_{org}: Organic phosphorus

VFA: Volatile Fatty Acids

OLR: Organic Loading Rate

SGP: Specific Gas Production

sCOD: soluble Chemical Oxygen Demand

Ch a: Chlorophyll a

Ch b: Chlorophyll b

1. Introduction

Anaerobic Digestion (AD) is considered the most sustainable method to produce energy (biogas) treating organic waste, in particular the Organic Fraction of Municipal Solid Waste (OFMSW) or industrial food waste. During the last ten years, several wastewater treatment plants (WWTP) implemented OFMSW-AD in order to increase both wastewater treatment efficiency and energy recovery. The AD effluents are usually characterized by high nitrogen and phosphorus content; digestate undergoes solid/liquid separation and the liquid fraction is sent back to the WWTP where these pollutants are biologically removed (Fdez.-Güelfo et al., 2011). Recently, lots of research studies are focused on digestate treatment aimed to remove/recover nutrients, such as ammonia and phosphorus. Among the proposed technologies, microalgae culture using digestate as medium is of growing interest in fact, it could be used to face up to the expensive process of microalgae biofuels production (Zhu, 2015). Microalgae needs large quantities of phosphorus and nitrogen to grow and to stock by-products, which is, from an economic and environmental point of view, unsustainable. A possible strategy is to use digestate nutrients integrating AD and microalgae processes, thus using digestate (usually as liquid fraction after solid/liquid separation) as substrate for microalgae growth (Olguin EJ, Sànchez G, 2000; Phang et al., 2000). The application of this strategy could therefore decrease the operating cost and close the loop in a circular economy view (digestate remediation and secondary high value product production) (Stiles et al., 2018, Toledo-Cervantes et al., 2016). Scientific literature showed several research studies on microalgae proliferation and phytoremediation using digestate, typically obtained by animal manure, agro-industrial waste and municipal waste AD treatment (Cicci and Bravi, 2014; Meng et al., 2017; Uggetti et al., 2014; Xia and Murphy, 2015). Among these wastes, OFMSW is of increased interest due to its high production and improved separate collection efficiency (Cai et al., 2013b); moreover, the AD treatment of the OFMSW represent a goal for biomethane production (Fernández et al., 2008). Most of the European member states national legislation forbid the direct spread of digestate obtained by OFMSW and

69 wastewater in the environment, due to possible heavy metals content (Trzcinski and Stuckey, 2011),
70 high concentration of ammonia nitrogen (from 400 ppm to 6000 ppm) and phosphorous (from 0,2 to
71 0,8 g/kg) (Da Ros et al., 2017) and presence of recalcitrant compounds (Cesaro and Belgiorno, 2014).
72 The scientific literature reports few papers about microalgae cultivation on OFMSW digestate or co-
73 digestion OFMSW and sludge (Massa et al., 2017; Zuliani et al., 2016a) and on municipal sludge
74 digestate (Cai et al., 2013c, 2013a; Cho et al., 2013, 2011; Dickinson et al., 2014; Uggetti et al., 2014;
75 Veronesi et al., 2015b; Yun et al., 2015). Most of these digestate sludges were pretreated to allow
76 sterilization (autoclavation or ultrafiltration) and used with dilution. All studies highlighted the
77 necessity of dilution to avoid ammonia toxic effect, in fact 160 mg l^{-1} of ammonia in digestate was
78 reported as threshold inhibition value of microalgae growth (Cho et al., 2013; Uggetti et al., 2014).
79 Dilution allows microalgae proliferation and consequent digestate treatment, with a total removal of
80 ammonia and phosphorous (phytoremediation effect) that permit the subsequent discharge of the
81 treated effluent into the environment (Bjornsson et al., 2013); however, dilutions must be carried out
82 in such a way that the overall water footprint of the remediation process is minimized.

83 *Chlorella* sp. is one of the most studied and used microalgae in biotechnological processes, from the
84 pharmaceutical to the food and biomaterials industry. In fact, this microalga contains polysaccharides,
85 antioxidants, vitamins, lipids and its storage capacity of these fractions is associated to specific
86 environmental conditions (i.e. pH, salinity, light intensity, and temperature) (Falkowski et al., 1985).
87 On the other hand, *Scenedesmus obliquus* is able to accumulate lipids or other secondary high-value
88 products under stress condition (as nitrogen deficit) (Arbib et al., 2013). For these reasons, *Chlorella*
89 spp. and *Scenedesmus* spp. were studied and proposed as good candidates for wastewater treatments
90 (Mandal and Mallick, 2009).

91 In this study *Scenedesmus obliquus* and *Chlorella vulgaris* microalgae were cultivated on digestate
92 obtained from the anaerobic co-digestion of OFMSW with waste activated sludge (WAS) and tested
93 in three different conditions: without pre-treatment (no PT), after centrifugation (AC) and after

94 filtration (AUF). The main objectives were i) to select the microalgae species with the best growth
95 capacity on OFMWS derived digestate and ii) to assess the different digestate pretreatment effects on
96 microalgae growth and nutrient uptake. The experimental test was aimed to overcome the bottlenecks
97 related to digestate exploitation as microalgae nutrient source; a reduction of digestate pretreatment
98 step will increase the economic and environmental sustainability of its application compared to other
99 treatment (e. g. autoclave).

100

101 ## 2. Material and methods

102 *2.1 Anaerobic digestate characterization*

103 The anaerobic digestate was collected in a wastewater treatment plant located in the north-east of
104 Italy, in which the anaerobic co-digestion of the OFMSW with waste activated sludge (WAS) has
105 been implemented (2000 m^3 reactor volume, 37±2 °C working temperature, 1.8 $kgTVSm^3d^{-1}$ average
106 organic loading rate (OLR) and 0.6±0.1 m^3kgTVS^{-1} specific gas production (SGP)).. The digestate
107 was characterized in terms of total solids and total volatile solids (TS, TVS), pH, alkalinity, ammonia
108 nitrogen, volatile fatty acids (VFA) and soluble chemical oxygen demand (sCOD) (Table 1). All
109 analyses were performed according to the APAT, IRSA-CNR (APAT-IRSA/CNR, 2003) and APHA,
110 AWWA, WET methods (APHA/AWWA/WEF, 2012)**.**

111 **Table 1.**

112 *2.2 Microalgae strains and experimental setup*

113 *C. vulgaris* and *S. obliquus* wild type were supplied by Federico II University of Naples (Naples,
114 Italy), and maintained on ISO 8692 ("INTERNATIONAL STANDARD ISO inhibition test with
115 unicellular green algae," 2012) medium with continuous light irradiation at 3000 lux, air bubbling at
116 2.3 vvm and mechanical magnetic agitation at 330 rpm in a 300 ml Erlenmeyer flask. Temperature

was controlled at 20 ± 1 °C. Optical density (OD_{680} and OD_{750}), and cell count analysis were performed daily in order to monitor cellular growth and identify the exponential growth phase.

OD_{680} (max adsorption of chlorophyll *a*) and OD_{750} (adsorption pick of cells, both bacteria and microalgae) were measured spectrophotometrically (spectrophotometer UV4 100 Heλos ϒ , United Kingdom) (Zuliani et al., 2016); the cellular count was evaluated using a Leika DMIL microscope equipped with a Bürker chamber using 10 µl sample of the cell suspension. Every analysis was performed in duplicate or triplicate.

During the exponential growth phase *C. vulgaris* and *S. obliquus* were inoculated in digestate diluted 1:10 with ISO 8692 medium. 300 ml cultures were grown in mixotrophic conditions (with applied irradiance) and heterotrophic conditions (without applied irradiance). *S. obliquus* and *C. vulgaris*' initial cell density was 7 ± 1 cell million ml^{-1} and 2.9 ± 0.5 cell million ml^{-1} respectively. The digestate was tested in three different condition: i) digestate without pretreatment (noPT), ii) digestate after centrifugation at 5,000 rpm for 5 minutes (AC) and iii) digestate after filtration (0.45 µm) with acetate cellulose filters (AUF). All tests were performed in duplicate using continuous air bubbling (137.5 lh-1 vvm) and magnetic agitation (300 rpm). In mixotrophic cultures, the required metabolic condition was maintained by uniform irradiation of the flask at 2010 lux (He et al., 2015); in heterotrophic cultures, heterotrophy was enforced by total shielding of the flask with aluminum foil. Some initial tests carried out to assign culturing time by monitoring cell count showed that 8 days are sufficient to reach steady state and this culturing time was adopted in all subsequent test runs. All flasks conditions were tested in duplicate. The optical density (OD at 680 nm and 750 nm wavelenght) (Griffiths et al., 2011) and cellular count (millions of cells per ml) were analyzed daily. The photoperiod applied was of 24:0 h (i.e., continuous irradiance). Temperature was controlled at 20 ± 1 °C. Every experimental condition was evenly tested in autotrophic, mixotrophic (1 gl^{-1} glucose) and heterotrophic (1 gl^{-1} glucose) controls for both strains, as reported in Di Caprio's study (Di Caprio et al., 2018). Glucose was added as an easily assimilable substrate at 1 gl^{-1} (by weight) in the control

condition because this concentration can be used to assess whether a microalgae growth inhibition correlated to microalgae capacity to use glucose in their mixotrophic and heterotrophic metabolic condition (Pentose Phosphate Pathway and Embden-Meyerhof Pathway respectively) exists (Yeh et al., 2012). Specific growth rate (μ, d^{-1}) was calculated for every experimental condition as reported by Dickinson (2014), by the equation 1:

$$(\mu, d^{-1}) = (\ln(X)) - (\ln(X_0)) / (t_f - t_i) \quad \text{Eq. 1}$$

The use of OD analysis for specific growth rate was avoided for growth rate quantification (as reported by Cai et al. (2013c) due to the presence of particulate matter in digestate and yellow-brown coloring. At the end of each test the supernatant was sampled and ammonia removal was measured using an ammonia probe (Hanna Instrument); chlorophyll *a*, *b* (Ch *a* and Ch *b*) and carotenoids accumulation ($\mu g\ ml^{-1}\ cell^{-1}$) were also evaluated using Jalal at al. (2013) and Linschitz and Sarkanen (1958) methods. Chlorophylls and carotenoids were quantified adopting Dere et al. (1998) equation 2, 3 and 4:

$$Ch\ a = 15.65 * OD_{666} - 7.340 * OD_{653} \quad \text{Eq 2}$$

$$Ch\ b = 27.05 * OD_{653} - 11.21 * OD_{666} \quad \text{Eq. 3}$$

$$Carotenoids\ total = (1000 * OD_{470} - 2.860\ Ch\ a - 129.2\ Ch\ b)/245 \quad \text{Eq. 4}$$

All the biological tests were performed in duplicate and for each test all the measurements were performed in duplicate. Cell count and OD analysis were performed daily. Data elaboration was based on calculating the average and standard deviation of 4 replicates.

3. Results and discussion

3.1 Evaluation of microalgae growth

Although both optical density and cell count was recorded regularly, and despite the wide use of OD to estimate specific growth rate in many general purpose experiments, it was found that only cell

166 counts were suitable to discriminate between the different tested culturing conditions and actual
167 growth in this type of microalgal culturing application. Indeed, the presence of particulate and the
168 yellowish color of the cultures did not warrant an accurate discrimination of the contribution of cell
169 mass to the total absorbance. It should be noted that centrifugation of the sample would have not been
170 viable prior to OD determination, as this would have evenly abated both suspended contaminants and
171 the microalgae. Cell count analysis (Table 2) was therefore adopted to evaluate biomass growth.

172 **Table 2**

173 The results obtained in the control condition show that neither of the two tested microalgal strains
174 use glucose under prevailing irradiance conditions; in fact, the mixotrophic and autotrophic cultures
175 showed the same growth trend, while a ultimate higher biomass would be expected if glucose were
176 also uptaken and used for biomass growth (Chiranjeevi et al., 2016). As reported by Yeh's study (Yeh
177 and Chang, 2012) *C. vulgaris* ESP-31 could growth on 1% glucose but the capability of using glucose,
178 in microalgae, is strain-specific and is typically associated to lack of lactate dehydrogenase enzyme
179 or other enzymes that are used in glucose assimilation. In test condition with digestate, results
180 obtained highlight *C. vulgaris* capacity to use digestate as substrate better than *S. obliquus* on AC and
181 AUF mixotrophic conditions. Cellular proliferation in the noPT mixotrophic growth condition
182 showed a limit in microalgae proliferation, probably due to light limitation in the flasks, in turn due
183 to significant absorbance of the digestate, which also contains suspended solids. Thus, the main
184 parameters that affect microalgae growth in noPT was the culture medium turbidity related to high
185 suspended solid in digestate that cause a reduced light penetration. No heterotrophic cultures in the
186 diluted digestate show any significant cell growth. From these results, the higher *C. vulgaris* growth,
187 compared to *S. obliquus*, could be associated with suspended solid removal which permits the
188 activation of the mixotrophic metabolism and use the digestate as a substrate with value comparable
189 between AC and AUF condition. *C. vulgaris*' best growth observed in AC mixotrophic condition
190 compared to *S. obliquus* was even reported by the study of Zuliani et al. (Zuliani et al., 2016b). Zuliani

et al. tested *C. vulgaris* and *S. obliquus I* on OFMSW digestate centrifuged and diluted 1:5 (550 cell million ml^{-1} and 150 cell million ml^{-1} respectively).

The growth rate (μ) was measured both in control and test conditions (Table 2), based on cellular density obtained with cellular direct count on optical microscope. The specific growth rates estimated for *C. vulgaris* in noPT mixotrophic condition are less reliable because of cell morphology (round) and size (smaller than that of suspended microparticulate) that makes cells assessment difficult at the optical microscope. *C. vulgaris*' growth rates in AC and AUF mixotrophic conditions were higher than *S. obliquus* while in noPT, AC and AUF heterotrophic conditions no significant cell growth was detected for both strains. Growth rate value obtained in AC and AUF mixotrophic and control conditions were similar to those obtained by Uggetti et al. (2014) and Khanh (2016) on *S. obliquus* and *C. vulgaris* on digestate, where they observed a wide μ values variation, ranged between 0.2 and 1 d^{-1} on digestate and control associated with several parameters such as light intensity, inoculum size, digestate turbidity and composition. As reported by Bouterfas et al. (Bouterfas et al., 2002) a possible growth rate increase was linked to higher light irradiation used (400-420 μmol m^{-2} s^{-1} or 29.6-31 klux) and the temperature stable at 35 °C. *C. vulgaris*'s growth rate obtained in AC and AUF mixotrophic conditions showed values higher than controls. *S. obliquus*'s μ in mixotrophic growth showed a decrease when the digestate was more treated (AUF), in fact μ value is higher in AC than in AUF condition, probably it was correlated to molecular or micro-organisms removal after filtration that limited *S. obliquus* proliferation. *S. obliquus*'s μ value in heterotrophic conditions showed the same trend of *C. vulgaris*, probably correlated to light absence. *S. obliquus* lower growth rate in AC and AUF mixotrophic condition, compared to *C. vulgaris*, could be associated with micro-organisms removal after pretreatment that have a syntrophic effect with microalgae. Digestate was not autoclaved, so some bacteria or fungi could grow together with the microalgae, with a low concentration, and they could give a syntrophic association on digestate degradation, releasing simple

molecules that were used by microalgae, or gave CO_2 releasing in medium that could be use by microalgae (Chiranjeevi et al., 2016).

Table 3

OD analysis results (680nm and 750nm wavelength) are reported in Table 3. OD analysis is typically used to measure bacteria and unicellular microorganisms. It is a rapid and non-destructive method but not all times absorbed light could be directly associated with cell number or cell mass because the particle size, morphology change and other variation could give an inaccurate estimation (Clesceri et al., 1998). 680nm wavelength is typically correlated with pigment maximal absorbance and 750 nm with minimum absorbance that could be associated with "cellular turbidity" and do not have a large discrepancy with dry weight in axenic culture in specific standard medium (Griffiths et al., 2011). In this study, the presence of digestate with or without pre-treatment could not permit the OD use for quantifying biomass production, so OD analysis was only applied to compared data obtained with cellular count in AC and AUF mixotrophic and heterotrophic conditions to looking at similar growth trend. OD_{680} analysis showed 0.68 ± 0.09, 0.147 ± 0.002 and 0.67 ± 0.008 absorbance by noPT, AC and AUF medium without microalgae before test respectively. The noPT absorbance decrease detected in heterotrophic condition after 8 days, could be associated with suspended solid fragmentation increase linked to mechanical agitation. In noPT mixotrophic condition after 8 day, the increase of OD value was linked to microbial proliferation, but it was impossible to separate the microalgae biomass from suspended solid. On the other hand, OD_{680} analysis showed absorbance variation in digestate linked with AC and AUF pretreatment where OD_{680} decreased with increase of pre-treatment. This decrease of OD_{680} is associated with endogenous microorganisms and macromolecules removal filtration could lessen microalgae proliferation, than latter because they can provide small molecules supporting microalgal growth, and the former because they can provide exocellular enzymmes capable of accelerating degradation of the materials itself. As showed for growth rate data obtained, *S. obliquus* probably suffered this endogenous microorganisms removal

240 and the growth capacity is limited compared to *C. vulgaris*. In heterotrophic condition the suspended
241 solid effect on OD analysis was clearer, in fact noPT condition showed value higher than AC and
242 AUF condition where cellular growth was near zero. AC and AUF mixotrophic's OD_{680} analysis
243 obtained were closed to Zuliani et al. results with *C. vulgaris* and *Scenedesmus I* (Zuliani et al.,
244 2016b), with values of 2 and 1.9 respectively. Moreover, in this case, OD analysis for both strains in
245 AC and AUF mixotrophic conditions gave similar trend curve than cellular count analysis (graphs
246 not reported). It was possible to deduce that both strains could grow on digestate just after
247 centrifugation and this could decrease the cost of biomass production compared with filtration
248 pretreatment. Centrifugation and filtration pretreatment, as in the downstream of the processes, could
249 be a problem in processes cost evaluation, in fact downstream treatment represent 20-30% of the total
250 production cost. As for biomass recovery, centrifugation was typically applied for its feasibility and
251 capacity to treat large volume rapidly even if it is energy intensive (Molina Grima et al., 2003).

252 *3.2 Ammonia removal*

253 Final ammonia removal (%) was measured in those test conditions where it was detected a cellular
254 growth (digestate no PT, AC and AUF mixotrophic metabolic conditions) (Table 4).

255 **Table 4**

256 After eight days in *C. vulgaris* and *S. obliquus* the ammonia removal was higher than 96% in all
257 mixotrophic conditions, with the 50% of ammonia removed after 24h due to air stripping effect. Initial
258 ammonia concentration was 50 mg $N\text{-}NH_4^+$ l^{-1}, a no-toxic concentration compatible with *C. vulgaris*
259 and *S. obliquus* survival. Franchino et al. (Franchino et al., 2013) tested *C. vulgaris* and *S. obliquus*
260 on agro-zootechnical digestate 1:10 diluted and they obtained, after 20 days, an ammonia removal of
261 99.9% and 83.7% respectively starting from an initial ammonia concentration of 163.4 mg $N\text{-}NH_4^+$ l^{-1}
262 . Ledda et al. (2015) tested *C. vulgaris* on digestate after pre-treatment (no PT, AC and ultrafiltration
263 at 0.2 μm) and they obtained an ammonia removal of 95%-98% after 14 days (with an initial ammonia

264 of 124 mg $N\text{-}NH_4^+$ l^{-1}). Kumar et al. (Jeevan Kumar et al., 2017) tested *C. vulgaris* on digested from
265 piggery effluent and they obtained an ammonia removal of 54% after 10 days (with an initial ammonia
266 concentration of 20.6 mg $N\text{-}NH_4^+$ l^{-1}); pH value in their study was between 8.6 and 9. An ammonia
267 reduction of 63-88% was detected by Ji et al. (Ji et al., 2015) after 6 days; they tested *S. obliquus* on
268 municipal wastewater with a low N concentration (21 mg l^{-1}) with air bubbling. Cicci et al. (Cicci and
269 Bravi, 2014) obtained an ammonia removal efficiency of 30% with *Scenedesmus dimorphus* growth
270 on cattle digestate 1:10 diluted with 82 mg $N\text{-}NH_4^+$ l^{-1} initial ammonia concentration. Massa et al.
271 (Massa et al., 2017) tested *S. obliquus* on zootechnical and vegetable digestate and they obtained an
272 ammonia removal of 99.8% and 99.2% (after 14 days) starting from 466.6 mg $N\text{-}NH_4^+$ l^{-1} and 666.6
273 mg $N\text{-}NH_4^+$ l^{-1} of initial ammonia concentration respectively. When ammonia removal is quantified,
274 both microalgae ammonia removal and ammonia stripping by air bubbling should be take into
275 account. Kim et al. (Kim et al., 2016) and Ruiz-Martinez et al. (Ruiz-Martinez et al., 2012) showed
276 that at pH value between 8.5-9.5 caused by photosynthetic activity, the ammonia removal by stripping
277 mechanism increase. Ledda et al. (Ledda et al., 2015) and Nuñez et al. (Nuñez et al., 2001) observed
278 that the nitrogen uptake by microalgae biomass was about 25%-35% of the total nitrogen of the
279 growth medium.

280 *3.3 Pigment characterization*

281 Pigment quantification is a typical analysis aimed to identify stress or unstress microalgae culture
282 condition, correlated with high or low light intensity or nutrient depletion, that influence biomass
283 composition in term of proteins or lipids storage, respiratory and photosynthetic rate and
284 photochemistry efficiency (He et al., 2015). Chlorophyll *a*, *b* (Ch *a* and Ch *b*) and carotenoids
285 analyses were performed at the end of cellular growth and it was also considered the
286 chlorophyll/carotenoids ratio in all mixotrophic conditions (Figure 1 and Table 5).

287 **Figure 1**

288 **Table 5**

289 For autotrophic organisms, the abundance of sunlight is an essential factor to produce organic
290 molecules using inorganic carbon (photosynthesis) (Carvalho and Monteiro, 2009). He et al. (2015)
291 and Ferreira et al. (2015) studied the effect of incident of light irradiation on chlorophyll accumulation
292 in microalgae biomass: they observed that in *S. obliquus* growth under low light irradiation 2.9 klux
293 and 1.25 klux respectively, gave a 128% increase of intracellular chlorophyll content compared to
294 control condition while in *Chlorella sp.* an increase of light intensity gave a decrease of chlorophyll
295 accumulation. This typical change of chlorophyll content in microalgae cells is correlated by
296 adaptations to light/dark change to improve light energy utilization (Ferreira and Sant, 2017). There
297 are other factors that could influence chlorophyll content in microalgae cells, for example: nitrogen,
298 phosphorus, zinc starvation, mixotrophic and heterotrophic cultivation, strong agitation and non-
299 axenic cultivation (Ferreira and Sant, 2017).

300 In this study, Ch *a*, Ch *b* and carotenoids analysis in autotrophic control condition gave an
301 accumulation of Ch *a* and Ch *b* in *C. vulgaris*, 26 ± 3 μg ml^{-1} $cell^{-1}$ and 42 ± 10 μg ml^{-1} $cell^{-1}$
302 respectively, and *S. obliquus*'s Ch *a* and Ch *b* of 2.3 ± 0.8 μg ml^{-1} $cell^{-1}$ and 4 ± 1 μg ml^{-1} $cell^{-1}$
303 respectively, in both strains no carotenoids accumulation was detected. Both strains in noPT, AC and
304 AUF mixotrophic conditions had a reduction of Ch *a* and Ch *b* storage compared to control; contrary
305 it was observed a smallest carotenoid increase (Figure 1). Ch *a* and Ch *b* high accumulation in control
306 conditions could be associated at general low irradiance, determined by low light intensity, high
307 cellular density or brown medium; this phenomenon is known as photoacclimation (Deng et al.,
308 2017). Results obtained for noPT, AC and AUF conditions in this study disagree with Yu et al. study
309 (Yu et al., 2017) where they tested *Chlorella* SDEC-18, *Scenedesmus* SDEC-8 and *Scenedesmus*
310 SDEC-13 in anaerobic digestate from kitchen waste (KWADE) and they obtained that the presence
311 of digestate inside medium increase chlorophyll accumulation in microalgae biomass compared to

312 control condition, highlighting that the presence of NH_4^+ in KWADE than NO_3^- in control medium
313 could influence the faster chlorophyll synthesis in these strains.

314 Total chlorophyll-carotenoids ratio showed that there was an increase of this value in all tested
315 mixotrophic conditions compared to controls (0 ± 0.01 and 0 ± 0.3 in *C. vulgaris* and *S. obliquus*
316 respectively): 3.77 ± 0.06, 3.6 ± 0.1 and 3.8 ± 0.2 in *C. vulgaris* and 4.3 ± 0.2, 4.3 ± 0.1, 4.1 ± 0.1 in
317 *S. obliquus* growth on noPT, AC and AUF respectively. Zuliani et al. (Zuliani et al., 2016b) obtained
318 a different result studying *C. vulgaris* and *S. obliquus I* growth on digestate from municipal and
319 agricultural wastes, where they obtained Ch $_{tot}$/Carotenoids $_{tot}$ ratio of 1.87 and 2.62 for *C. vulgaris*
320 and *S. obliquus I* growth on OFMSW digestate 1:5 diluted respectively. In this study, the presence of
321 digestate gave an increase of Ch_{tot}/$Carotenoids_{tot}$ ratio for both strains that suggest a carotenoids
322 accumulation with a corresponding chlorophyll *a* core complex degradation associated with stress
323 growth condition.

324 Also, the Ch *a*-carotenoids ratio (Table 5) was calculated to observe if there was an effective strains
325 response to nitrogen starvation at the end of test. As it was showed before, the ammonia at the end of
326 the test was totally removed and nitrogen starvation gave a chlorophyll decrease and carotenoid
327 accumulation, detected with as discoloration of cells (Becker, 1994). Hooks (1988) showed that the
328 normal range of Ch *a*-carotenoids ratio was between 2 and 7; this is an indicator of the physiological
329 condition of the culture and it is correlated with the medium composition (N starvation). In this study
330 Ch *a*-carotenoids ratio values (Table 5) obtained in digestate no PT, AC and AUF mixotrophic
331 conditions were in the range indicated by Hooks and it is even close to Hodaifa et al. (2009) value
332 obtained with *S. obliquus* growth on olive oil mill wastewater (OMW) (between 1.30 and 2.07),
333 observed an increase of carotenoids storage in test conditions that could be associate with an increase
334 of carotenogenesis determinate by nitrogen limitation or light stress condition (Zuliani et al., 2016b).

335 Ch_{tot}/carotenoids and Ch *a*/carotenoids ratio show an effective and similar stress condition for *C.*
336 *vulgaris* and *S. obliquus* on digestate noPT, AC and AUF correlated with Ch *a* and *b* degradation and

carotenoids accumulation. But *C. vulgaris* could grow better in AC and AUF conditions than controls; probably this strain could use the substrate in digestate also after pre-treatment. Quite the opposite *S. obliquus* underwent a negative growth effect if digestate was pre-treated, probably associated with microorganism removal, and this strain did not have the capacity to break down substrate without syntrophic cooperation.

Although the present study has a preliminary character, it shows that both tested microalgal strains can be cultivated on digestate after limited preliminary treatment or no pre-treatment at all, to produce new biomass that can be recycled back to anaerobic digestion in order to increase the overall Bio-Methane Potential of the feed. However, the *Chlorella* appeared to be more robust toward toxic components that can be found in digestate than the Scenedesmus strain and, ultimately, more productive in terms of new biomass produced out of the digestate organic load. During the described experiments the digestate was diluted with a 'sufficient' medium to highlight toxic effects while avoiding any potential nutrient limitation effect arising from the feed. If the tested process were to be deployed at a commercial scale, however, dilution would be carried out with water obtained from whatever water source is available at a low or nil cost, possibly from upstream or downstream processes. Indeed, an end-of-pipe treatment should be applied to processed digestate before any discharge of the treated water (such as an active sludge process), which suggests that this water could probably be used in the upstream microalgal process and that such a process choice would lower the design standards and cost of the remediation facility quite a bit. Recycling would probably cause nutritional limitations in the microalgal process to appear, and these might require the process operator to compensate them by feeding synthetic micro- or macronutrients.

4. Conclusions

Digestate and its liquid fraction, after centrifugation and filtration pretreatment, were tested for *C. vulgaris* and *S. obliquus* growth, comparing mixotrophic and heterotrophic metabolisms. The results obtained indicate that for both strains mixotrophic metabolisms is the only way to use this substrate for cellular growth. *C. vulgaris* showed similar growth performance in presence of AC and AUF compared to control conditions (0.6 ± 0.0 d^{-1}, 0.6 ± 0.0 d^{-1} and 0.5 ± 0.0 d^{-1} respectively), *S. obliquus* showed a decrease of growth capacity with the increase of digestate treatment (0.5 ± 0.1 d^{-1} and 0.4 ± 0.0 d^{-1} in AC and AUF conditions respectively). Ammonia removal in all mixotrophic conditions for both strains was more than 90% and mostly associated to ammonia stripping mechanism. Ch *a*, *b* and carotenoids analysis showed that both strains were in stress condition, but *C. vulgaris* preserved its growth capacity in AC and AUF condition, so it was detected as the best strain on this kind of substrate. Future test will be focus on scale up system of *C. vulgaris* with low digestate dilution and only AC pre-treatment.

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Table 1: Anaerobic OFMSW digestate physical-chemical characteristics.

Parameter	
TS digestate noPT (gl^{-1}) *	1.8±0.2
TVS digestate noPT, (%TVS,TS)	65.2±0.3
TS digestate AC (mgl^{-1}) *	103.0±4.0
TS digestate AUF (mgl^{-1}) *	17.0±4.0
pH	7.6±0.3
P_{org} (gP $kgTS^{-1}$)	13.7±3.7
TKN (gN $kgTS^{-1}$)	40.0±8.1
Total alkalinity ($gCaCO_3$ l^{-1})	2.2±0.5
Partial alkalinity ($gCaCO_3$ l^{-1})	1.4±0.4
$N\text{-}NH_4^+$ (gN l^{-1})	0.6±0.1
VFA (g l^{-1})	0.2±0.2
sCOD (g l^{-1})	0.3±0.1

Note: Variability is shown as standard deviations, n=2; * value obtained after 1:10 dilution.

603 **Table 2:** *C. vulgaris* and *S. obliquus* cell density (cell million * ml) and growth rate (d^{-1}) in
604 autotrophic, mixotrophic and heterotrophic control and experimental condition: without pretreatment
605 (noPT), after centrifugation (AC) and after filtration (AUF) in mixotrophic and heterotrophic
606 metabolic conditions. Variability is shown as standard deviations, n=4.

	*Cell count (cell million * ml)*		*Growth rate (d^{-1})*	
	C. vulgaris	*S. obliquus*	*C. vulgaris*	*S. obliquus*
Autotrophic	138.1± 4.0	44.0±1.0	0.41±0.02	0.3±0.0
Mixotrophic (1 gl^{-1} glucose)	140.0±12.0	27.0±2.0	0.41±0.02	0.3±0.0
Heterotrophic (1 gl^{-1} glucose)	44.2±11.0	23.1±0.8	0.27±0.20	0.2±0.1
Mixotrophic Digestate noPT	98.0±10.0	92.1±27.0	0.44±0.01	0.4±0.1
Mixotrophic Digestate AC	479.0±31.0	131.0±12.0	0.60±0.00	0.5±0.1
Mixotrophic Digestate AUF	539.0±11.0	123.0±20.0	0.60±0.00	0.4±0.0
Heterotrophic Digestate noPT	15.0±1.0	11.6±0.1	0.20±0.00	0.2±0.1
Heterotrophic Digestate AC	4.2±0.0	7.1±0.0	0.04±0.00	0.1±0.1
Heterotrophic Digestate AUF	3.2±0.4	6.0±0.9	0.01±0.01	0.1±0.1

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615 **Table 3:** *C. vulgaris* and *S. obliquus*' OD analysis on λ 680 nm and 750 nm in autotrophic,
616 mixotrophic and heterotrophic control and experimental condition (without pretreatment (noPT),
617 after centrifugation (AC) and after filtration (AUF)). Variability is shown as standard deviations,
618 n=4.

	OD 680 nm		OD 750 nm	
	C. vulgaris	*S. obliquus*	*C. vulgaris*	*S. obliquus*
Autotrophic	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0
Mixotrophic (1 gl^{-1} glucose)	0.5±0.0	0.4±0.0	0.5±0.0	0.4±0.0
Heterotrophic (1 gl^{-1} glucose)	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Mixotrophic Digestate noPT	1.6±0.0	1.6±0.4	1.3± 0.0	1.3±0.3
Mixotrphic Digestate AC	1.4±0.5	1.5±0.2	1.2±0.4	1.3±0.2
Mixotrophic Digestate AUF	1.8±0.1	1.7±0.0	1.6±0.1	1.5±0.0
Heterotrophic Digestate noPT	0.4±0.0	0.9±0.4	0.3±0.1	0.8±0.3
Heterotrophic Digestate AC	0±0.0	0.0±0.0	0±0.0	0.0±0.0
Heterotrophic Digestate AUF	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

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627 **Table 4:** Ammonia removal (%) of *C. vulgaris* and *S. obliquus* in tested conditions.

Ammonia removal (%)	*C. vulgaris*	*S. obliquus*
Mixotrophic Digestate no PT	96.0±2.0	96.0±3.0
Mixotrophic Digestate AC	99.2±0.3	98.1±0.0
Mixotrophic Digestate AUF	99.4±0.0	97.9±0.4

628 Note: no pre-treatment (PT), after centrifugation (AC) and after filtration (AUF). Variability is shown
629 as standard deviations, n=4.

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643 **Table 5:** *C. vulgaris* and *S. obliquus* Ch *a* and carotenoids ratio in autotrophic control, digestate no
644 PT, AC and AUF mixotrophic conditions.

Ch *a*/carotenoids	*C. vulgaris*	*S. obliquus*
Autotrophic	0±0.0	0±0.1
Mixotrophic Digestate no PT	2.5±0.0	2.6±0.1
Mixotrophic Digestate AC	2.4±0.1	2.3±0.1
Mixotrophic Digestate AUF	2.5±0.0	2.2±0.2

645 Note: no pre-treatment (PT), after centrifugation (AC) and after filtration (AUF). Variability is shown
646 as standard deviations, n=4.

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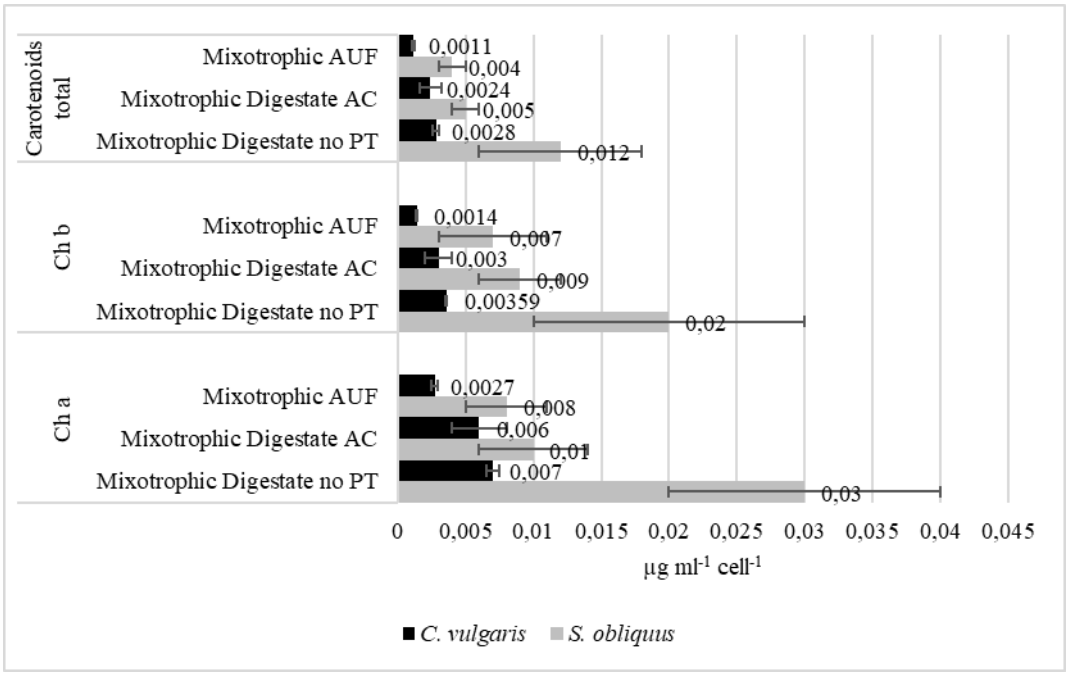

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657 **Figure 1:** *C. vulgaris* and *S. obliquus* chlorophyll *a* (Ch *a*), chlorophyll *b* (Ch *b*) and carotenoids
658 accumulation at the end of cellular growth in digestate no pre-treatment (PT), after centrifugation
659 (AC) and after filtration (AUF) mixotrophic conditions. Variability is shown as standard deviations,
660 n=4.