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Growth and bioactivity of two chlorophyte (*Chlorella* and *Scenedesmus*) strains co-cultured outdoors in two different thin-layer units using municipal wastewater as a nutrient source

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ABSTRACT

The application of microalgae in wastewater treatment has recently been at the forefront of interest due to the increasing concern about environmental protection and economic sustainability. This work aimed to study two chlorophyte species, *Chlorella vulgaris* and *Scenedesmus acutus*, co-cultured outdoors in centrate of municipal wastewater as a nutrient source. Two different thin-layer units were used in these trials — thin-layer cascade (TLC) and thin-layer raceway pond (TL-RWP), suitable for this purpose due to their high biomass productivity and better culture transparency when using muddy wastewater. The units were operated in batch, and subsequently in semi-continuous growth regime — and monitored in terms of photosynthetic performance, growth, nutrient removal rate, and bioactivity. The results showed that the co-cultures grew well in the centrate, achieving the maximum biomass densities of 1.3 and 2.1 g DW L^{-1} in TLC and TL-RWP, respectively, by the end of the batch regime and 1.9 and 2.0 g DW L^{-1} by the end of the semi-continuous regime. Although TL-RWP grown cultures showed faster growth, the TLC-one revealed better nutrient removal efficiencies batch wise than the culture grown in TL-RWP — removing up to 48% of total nitrogen and 43% of total phosphorus. Conversely, the latter was more efficient under the semi-continuous regime (54% and 42% consumption of total nitrogen and phosphorus, respectively). In the harvested biomass, an important antimicrobial activity (specifically antifungal) was detected. In this sense, the *in-vitro* growth of the oomycete *Pythium ultimum* was inhibited by up to 45% with regard to the control. However, no biostimulating activity was observed. The present findings confirm the possibility of using these two species for biomass production in municipal wastewater centrate using highly

Abbreviations: TLC, thin-layer cascade; TL-RWP, thin-layer raceway pond; TL, thin-layer; TN, total nitrogen; TP, total phosphorus.

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productive thin-layer systems. This technology can be a valuable contribution to circular economy since the produced biomass can be re-applied for agricultural purposes.

1. Introduction

The introduction of microalgae in wastewater treatment is currently under the spotlight due to the integration of circular economy applications in the EU Water Framework Directive goals through re-application of the phytoremediated microalgal biomass obtained in other activities. The application of microalgae in wastewater treatment helps to remove excess nutrients, heavy metals, and other organic contaminants while simultaneously benefiting from a reduction in biomass production costs [1–4]. Selected microalgal species suitable for this purpose have a biological profile characterized by a high growth rate and tolerance to high nutrient concentrations (such as ammonium and carbon dioxide). *Chlorella* and *Scenedesmus* are well-established genera, utilized in wastewater treatments that are robust and possess the added benefit of producing metabolites with applications for low (biofertilizers) and high-value (biostimulants and biopesticides) products [5–7]. The added ability to synthesize valuable compounds is also necessary to render a positive economic balance to the whole process. Still, there are other several challenges that also need to be considered for a successful production. Another relevant and difficult variable in wastewater treatment pertains to the evolution of natural, or selected, populations as mono- or co-cultures. The use of microalgae in co-cultures as opposed to monocultures in wastewater treatment has been reported to yield more stable cultures (less susceptible to crashes) as well as improve nutrient uptake [8,9]. Previous studies have explored the microalgal co-cultures of *Chlorella* and *Scenedesmus*, naturally assembled in high rate algal ponds of wastewater treatment plants with success [5,10–12]. An additional, growth-limiting factor that needs addressing in microalga-based wastewater treatments relates to light availability, which is greatly affected by the type of photobioreactor used and the cultivation regime applied (i.e., batch, semi-continuous). Open cultivation systems guarantee low construction and maintenance costs compared to closed photobioreactors. Due to the presence of suspended solids and other particles in the wastewater that can interfere with light penetration, the units with a short light path can be expedient, especially in climates with less sunlight.

To our knowledge, few studies have addressed the use of co-cultures grown in wastewater in thin-layer systems [13] with bioactivity assessment. In this work, the behavior of co-cultures of two green microalgae, *Chlorella vulgaris* and *Scenedesmus acutus*, were studied when grown outdoors in two different thin-layer (TL) outdoor units — a thin-layer cascade (TLC) and a thin-layer raceway pond (TL-RWP). Centrate from municipal wastewater after secondary aerobic treatment was employed as the sole nutrient source. Photosynthetic activity, growth, and nutrient removal efficiency were compared in both units, as well as biostimulant and antimicrobial activity of biomass extracts.

2. Materials and methods

2.1. Strains and laboratory culture condition

Two green microalgal strains, *Chlorella vulgaris* MACC-1 (hereafter abbreviated to *C. vulgaris*) and *Scenedesmus acutus* MACC-677 (hereafter abbreviated to *S. acutus*) obtained from the Algal Culture Collection of the Széchényi István University, Mosonmagyaróvár, Hungary, were used in these trials. The strains were selected for their fast growth [14], as well as biostimulating and biopesticide activities (V. Ördög, unpublished data). The cultures were initially grown as monocultures in BG-11 medium [15,16] in 10-L Pyrex bottles in the laboratory at 28–30 °C under an irradiance of 200 μmol photons m^{-2} s^{-1} and mixed by bubbling a mixture of air with 1% CO_2 (v/v).

2.2. Centrate preparation

The centrate used in the cultivation was collected directly from the municipal wastewater treatment plant (WWTP) in Třeboň (Czech Republic). To avoid cell aggregation, due to the automatic addition of flocculant in the process, the activated sludge was taken from WWTP just after secondary aerobic digestion (secondary-treated wastewater) and centrifuged at 3000*g* for 5 min (centrifuge Sigma 8KS) to separate liquid centrate from solid sludge (similar procedure as in the WWTP). The supernatant (centrate) of brownish color was collected and used (non-diluted) as nutrient medium (see the composition in Table 1). The total nitrogen (TN) to total phosphorus (TP) ratio in the centrate was about 1.5. However, comparing the centrate to the inorganic medium BG-11, commonly used for cultivation, total content of nitrogen was similar (250 mg L^{-1} in BG-11), while phosphorus was more than 20 times higher (7 mg L^{-1} in BG-11).

2.3. Cultivation trials in outdoor units

Two outdoor cultivation units – TLC and TL-RWP – were used to grow the selected microalgal strains. These two units differed in the circulation device used for moving the culture — paddle wheel versus centrifugal pump [17]. Each of the 5 m^2 cultivation units was placed in a separate east-west oriented greenhouse which were placed side by side; they protected cultures from cross-contamination and unfavorable outdoor conditions. The units were described in detail elsewhere [18]. Trials were carried out in July 2019 at Centre Algatech, Třeboň (N 48°59′, E 14°46′).

The TL-RWP was operated continuously, with a volume of 100 L and a culture thickness of 18 mm; the flow speed was about 0.2 m s^{-1}. In both units, the automatic regulation of CO_2 supply kept pH between 7.8 and 8.2. The TLC was operated with a working volume of 70 L, using a culture depth of 10 mm; and the flow speed was about 0.5 m s^{-1}. The culture was circulated only during the daytime, and stored in a retention tank during the nighttime, where it was mixed via air bubbling (light/dark regime about 12/12 h). The evaporation was compensated every morning by addition of tap water. Weather conditions (culture temperature and irradiance) were recorded using a meteorological station (modular control system ADiS-AMiT) with a solar radiation sensor located by the units and temperature sensors submerged in the cultures (Supplementary Fig. S1 and S2 for temperature and radiation, respectively).

Each unit was inoculated to the same biomass density, ca. 0.7 g of dry weight (DW) L^{-1}, using laboratory-grown cultures (TLC inoculated one day later than TL-RWP because of the technological complexity). The samples for various measurements were taken daily at 08:00 h. The microalgae were grown in the batch regime for seven days to get a dense culture in the late logarithmic-phase. Afterward, the semi-continuous growth regime was operated for another five days by harvesting 25%

Table 1
Minimum and maximum measured values ($n = 3$) of the wastewater centrate used in the cultivation trials concerning biochemical and chemical O_2 demand (BOD and COD respectively), total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP).

Measured variable	Concentration (mg L^{-1})
Biochemical O_2 demand (BOD)	180
Chemical O_2 demand (COD)	1000–1100
Total organic carbon (TOC)	310–560
Total nitrogen (TN)	230–260
Total phosphorous (TP)	150–170

of the cultures daily at 09:00 h and replacing it with fresh centrate — so as to mimic large-scale semi-continuous biomass production.

2.4. Photosynthesis measurements

The experimental techniques were described in detail in previous articles [19–22]. Microalgal samples were taken from the outdoor cultures and analyzed *off-situ* after dilution to 0.2–0.3 g DW L^{-1} with tap water and dark-adapted for 10 min in the water bath, kept at the same temperature as in the outdoor units. Photosynthetic activity of the cultures was measured using a saturation pulse analysis of fluorescence quenching (PAM-2500, H. Walz, Germany) to construct rapid light-response curves (RLC). Data were recorded in triplicates. Analysis of RLCs was used to estimate changes in the actual photochemical yield through PSII, Y_{II}, in terms of dependence on light intensity. The relative electron transport rate (rETR) was calculated by multiplying the actual PSII photochemical yield by the corresponding PAR value (E_{PAR}) [23–25]. The RLC were fitted by non-linear least-squares regression [26], using PamWin_3 software to estimate maximum rETR ($rETR_{max}$) and saturating irradiance.

2.5. Analytical procedures

The measurement of biomass density was performed as described previously [19,20]. Biomass content (presented as g of DW L^{-1}) was measured as DW in triplicate by filtering 5 mL of culture samples on pre-weighed glass microfiber filters (GC-50). The cells were washed twice with deionized water, dried in an oven at 105 °C for 8 h, and finally transferred to a desiccator and weighed (precision of ±0.01 mg).

2.6. Nutrient analysis

Both centrate and samples from the co-cultures (25 mL) were centrifuged (12,000 *g*, 5 min) before syringe filtering (0.45 μm) and stored at −20 °C until further analysis (performed by the laboratory Povodí Vltavy, České Budějovice). The parameters and nutrient content were determined according to specific protocols: biological oxygen demand (BOD) by suppression of nitrification, chemical oxygen demand (COD) using a commercial analytical kit (Merck), and total organic carbon (TOC) by thermal decomposition with Pt catalyst. Nitrite content (NO_2-N) was determined by automatic discrete photometry, ammonium nitrogen (NH_4-N) by acidimetry after distillation, and nitrate concentration (NO_3-N) was calculated as the sum to make up to TN content — which was, in turn, determined by thermal oxidation with electrochemical detection. Both TP and orthophosphate-phosphorus (PO_4-P) were assayed after mineralization using automatic discrete photometry.

Nutrient removal was calculated as the difference between the supplied nutrient (TN and TP) content found in the wastewater and the one found in the respective cultures by the end of each culture regime (batch and semi-continuous). Results were expressed in g m^{-2} d^{-1} and as percentage of nutrients supplied in the wastewater.

2.7. Bioactivity tests

The resulting freeze-dried biomass harvested by the end of the semi-continuous regime was suspended in distilled water (10 mg DW L^{-1}), and sonicated (Branson sonicator 150, amplitude 40%, 3 min) before being tested for two different agricultural applications: antimicrobial and biostimulating activities. The antimicrobial (biopesticide) activities of the samples were evaluated with an antagonism bioassay. Three different bioassays were used to detect plant biostimulating activities: the cress seed germination, the mung bean rooting and the wheat leaf chlorophyll retention. All bioassays were performed in triplicate.

2.7.1. Bioassays of Germination Index

Freeze-dried microalgal biomass (100 mg) was suspended in 10 mL of distilled water (10 g DW L^{-1}) for the germination index assay. The suspension was sonicated and then incubated with stirring and heating for 2 h. The biostimulant activity was tested on 100 cress (*Lepidium sativum*) seeds using sonicated aqueous extracts of 0.5 and 2 g DW L^{-1} of microalgal biomass, as described elsewhere [27,28]. The percentage of seed germination, as well as radicle elongation were taken for the germination index (GI) calculation according to the following formula: $GI\ (\%) = (G_S \times L_S) / (G_w \times L_w)$, where G_S is the percentage of germinated seeds in the presence of the microalgal extract, G_w is the percentage of germinated seeds in the presence of distilled water, L_S is the mean of radicle elongation (mm) in the presence of the microalgal extract, and L_w is the mean of radicle elongation (mm) in the presence of distilled water.

2.7.2. Determination of auxin-like activity

The bioassay of auxin-like activity was performed according to Hess [29]. The algal suspensions at concentrations of 0.5, 1.0, 2.0, and 3.0 g DW L^{-1} were used for treatment of seedling cuttings of mung bean (*Vigna radiata* (L.) Wilczek) in the rooting tests. The number of roots (longer than 1 mm) was recorded after application of the biomass extract. A standard curve of indol-3-butyric acid (IBA) at concentrations of 0, 0.3, 0.5, 0.7, and 1 mg DW L^{-1}, was prepared for comparison.

2.7.3. Determination of cytokinin-like activity

The bioassay of cytokinin-like activity was performed according to Kuhnle et al. [30]. Leaves from wheat (*Triticum aestivum* L.) seedlings (about 10 cm height) were collected and then cut 35 mm below their apical tip into 10 mm segments. The algal suspensions at concentrations of 0.5, 1.0, 2.0, and 3.0 g DW L^{-1} were applied to the detached leaf segments. After incubation for four days, the chlorophyll content of the leaf segments was measured. A standard curve of kinetin (KIN) at the concentrations of 0, 0.3, 0.5, 0.7, and 1 mg DW L^{-1}, was used for comparison.

2.7.4. Antagonism bioassays by dual culture

The aqueous extracts were tested against the growth of phytopathogenic fungi, bacteria, and oomycetes *in-vitro* using the dual culture technique according to published protocols [31,32]. The activity of the extracts was tested against two fungi – *Fusarium oxysporum* f. sp. *melonis* and *Rhizoctonia solani* (further abbreviated as *F. oxysporum* and *R. solani*), two oomycetes – *Phytophthora capsici* and *Pythium ultimum* (further abbreviated as *P. capsici* and *P. ultimum*) and four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato*, *Pectobacterium carotovorum* (further abbreviated as *C. michiganensis*, *X. campestris*, *P. syringae*, *P. carotovorum*). All strains were provided by the Spanish Type Culture Collection (STCC). The inhibition index was calculated according to the following formula: $I = [(C - T) / C] \times 100$, where I is the inhibition index in %, C is the diameter of the zone of the pathogen patches in the absence of microalgal extract (mm), and T is the diameter of the zone of the pathogen patches in the presence of microalgal extract (mm). In all cases, control bioassays were performed using distilled water.

2.8. Statistical analysis

One biological replicate was performed for each unit with several technical replicates performed under batch and semi-continuous growth until steady-state was achieved. A paired sample non-parametric test (Wilcoxon test) was used to check for significant differences between the cultures grown in the different units (TLC and TL-RWP). Only results with $p < 0.05$ were considered statistically different. All tests and graphs were produced using R software.

3. Results and discussion

3.1. Growth

In the present study, two different species, *C. vulgaris* and *S. acutus* were grown as a co-culture in two different cultivation units – TLC and TL-RWP – in batch and subsequently in semi-continuous regime, using centrate from municipal wastewater as the only nutrient source. The culture grew faster in TL-RWP, reaching about 2.1 ± 0.0 g DW L^{-1} ($\mu = 0.15\ d^{-1}$) after one week of the batch regime while it was only 1.3 ± 0.0 g DW L^{-1} ($\mu = 0.10\ d^{-1}$) in TLC. A more extended lag phase was observed in TLC's culture, probably due to the lower thickness of the culture layer (Fig. 1). In any case, both TLC and TL-RWP cultures were characterized by a steeply increasing biomass density in the batch regime. According to our previous experience, when the biomass density reached about 1 g DW L^{-1}, both cultures in outdoor TL units became photoadapted and started to grow faster. However, the semi-continuous regime promoted a higher specific growth rate of the TLC culture (1.9 g DW L^{-1}; $\mu = 0.32\ d^{-1}$), that reached a similar biomass density as that in TL-RWP (2.0 g DW L^{-1}; $\mu = 0.19\ d^{-1}$) with a lower specific growth rate. The thickness of the culture layer in TL-RWP and high culture density at the end of the batch regime probably promoted photolimitation, allowing to achieve higher growth rates in the TLC culture under the semi-continuous regime.

After surpassing 0.8 g DW L^{-1}, the highest growth promoted in the TLC unit can also be attributed to a lower oxygen build-up, as this gas is released when the culture falls into the collection tank through a net that promotes degassing. In both units, pH was controlled and kept between 7.8 and 8.2, where even though the dominant form in the NH_4^+/NH_3 buffer system is NH_4^+ (ion dissociation constant, pKa, is about 9.25 at 25 °C), at this set point values of pH, NH_4^+ conversion to NH_3 is already ongoing [33]. As so, degassing can probably promote ammonia stripping and thus prevent algal growth inhibition [34]. Nonetheless, all situations unfolded the possibility of using aerobic centrate as the sole nutrient source, which provided suitable growth conditions.

The biomass concentrations achieved in this study using TL systems surpassed the usual biomass density of 1 g DW L^{-1} achieved in high rate algal ponds or conventional RWP — characterized by deep light paths [35,36]. The short optical path resulted in higher biomass density of the co-culture grown in both TL units, showing lower light absorption when the brownish centrate was used as nutrient source.

By the end of the trial, microscopic observation showed that the co-culture was dominated by *S. acutus* cells (Supplementary Fig. S3). It has already been reported [5] that *Chlorella* is one of the first microalgae to colonize wastewater environments since it is a more stress-tolerant genus. Later on being overgrown by *S. acutus*, which can be more sensitive to high concentrations of ammonium [5]. However, this balance can also be influenced by seasonality [37].

3.2. Photosynthetic performance

Changes in the photosynthetic activity can reflect adaptation mechanisms to higher irradiances as to minimize photo-stress. In this study, these changes were estimated as $rETR_{max}$ by chlorophyll fluorescence (Fig. 2). Supplementary data estimated from the RLCs including the initial slope of the curves (α) and non-photochemical quenching (NPQ) are shown in the Supplementary Table S1. The $rETR_{max}$ values of the TLC culture increased at the beginning of the cultivation trial and remained relatively stable with only a slight decrease at the end of batch regime, reaching a maximum value of 349 ± 15 μmol e^- m^{-2} s^{-1} under the semi-continuous regime. On the other hand, $rETR_{max}$ in the TL-RWP cultures increased at the beginning of the cultivation trial reaching the maximum of 373 ± 3 μmol e^- m^{-2} s^{-1}; and started to decrease reaching a minimum of 188 ± 0 μmol e^- m^{-2} s^{-1} at the end of batch regime. The recovery was seen in semi-continuous mode, although $rETR_{max}$ of the TL-RWP culture remained lower than that in the TLC cultures.

An initial photoacclimation was observed in the cultures grown in both units, although the lower initial values during the first days can also

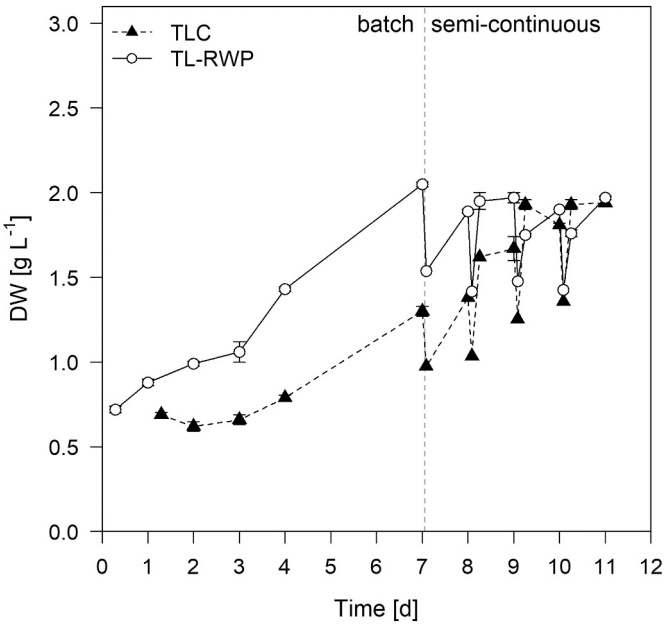

Fig. 1. Growth in terms of g dry weight (DW) L^{-1} of co-cultures of *C. vulgaris* and *S. acutus* cultivated in a thin-layer cascade (TLC; black triangle with dashed line) and in a thin-layer raceway pond (TL-RWP; white circles with solid line) under batch and semi-continuous regime (25% dilution rate) using centrate as a nutrient source ($n = 1$). The culture in TLC was inoculated one day later than that in TL-RWP. Error bars represent analytical standard deviation, as DW was determined in triplicate.

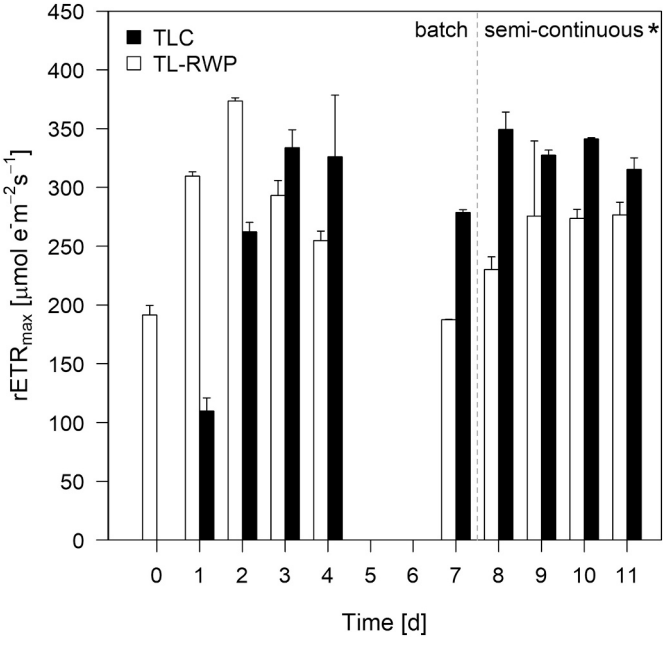

Fig. 2. Maximum relative electron transport rate ($rETR_{max}$) of the co-culture of *C. vulgaris* and *S. acutus* cultivated in TLC (black bars) and TL-RWP (white bars) in batch and semi-continuous (25% dilution rate) regime using centrate as nutrient source ($n = 1$). Missing values represent non-sampling days. Error bars represent analytical standard deviation as the measurements were performed in triplicate. Growth regime marked with an asterisk (*) indicates statistically significant differences ($p < 0.05$) between TLC and TL-RWP units.

be attributed to ammonium inhibition (see Fig. 3) that constrains electron transport in PSII [33,38]. While higher $rETR_{max}$ were found in TL-RWP during the first three days; overall, the TLC culture revealed significantly higher ($p < 0.05$) $rETR_{max}$ values. This was probably caused by higher averaged irradiance per cell in the thinner layer of the TLC culture (7–10 mm) compared to that in TL-RWP (15–25 mm). The slightly higher culture density (see Fig. 1), as well as culture depth, in the TL-RWP unit (compared to TLC) promoted a lower average cell irradiance, resulting in lower $rETR_{max}$ values at the end of batch regime. The lower $rETR_{max}$ values of both cultures at the last day of the batch regime can respond to nutrient deficiency in nitrogen-limited media (see Fig. 3) [25]. This is supported by the later increase of $rETR_{max}$ in the semi-continuous regime when part of the culture was replaced by fresh centrate and N availability was again higher, and photolimitation was reduced.

3.3. Nutrient removal

The evolution of TN, TP and TOC removal efficiencies of *C. vulgaris* and *S. acutus* co-culture, when using centrate as nutrient source, was assessed during the experiment. Results are shown in Table 2.

The removal efficiencies of TN and TP by the end of the batch regime were higher in the TLC grown culture reaching 48 and 43%, respectively, as opposed to the TL-RWP cultures that reached only 34 and 0%. Under the semi-continuous regime, the TN uptake was increased in both TLC (53%) and TL-RWP (54%), while the TP removal efficiency decreased in TLC (29%) and improved in the TL-RWP (42%) cultures. The inability of the co-culture in TL-RWP to uptake P in the batch regime is somehow surprising and points to another limiting element. As such, further experiments should be performed in order to establish the limiting factor to increase nutrient removal efficiency. Nonetheless, neither combination could reach the admissible values for wastewater discharge in sensitive areas of 10–15 mg L^{-1} of TN and 1–2 mg L^{-1} of TP [39]. Still, the TN and TP removal efficiencies were improved by the use of a co-culture (*Chlorella* and *Scenedesmus*) when compared to a similar experiment performed with *Chlorella* monocultures [18]. However, a longer residence time could provide the improved results [11].

Table 2
Nutrient removal efficiency in g m^{-2} d^{-1} (in %) of total nitrogen (TN), total phosphorus (TP) and total organic carbon (TOC) in the co-cultures of *C. vulgaris* and *S. acutus* grown in a thin-layer cascade (TLC) and a thin-layer raceway pond (TL-RWP) in batch and semi-continuous cultivation regime (25% dilution rate). The centrate of municipal wastewater was used as a nutrient source ($n = 1$).

Unit	Cultivation mode	Removal efficiency in g m^{-2} d^{-1} (%)		
		TN	TP	TOC
TLC	Batch	0.35 (48%)	0.19 (43%)	−2.54 (−287%)
	Semi-continuous	0.89 (53%)	0.32 (29%)	1.15 (17%)
TL-RWP	Batch	0.22 (34%)	0.00 (0%)	−2.98 (−330%)
	Semi-continuous	1.13 (54%)	0.71 (42%)	2.76 (33%)

The TOC concentrations increased in both cultures grown in batch mode which resulted in negative removal efficiencies (−287 and −330 in TLC and TL-RWP, respectively). This increase in TOC was probably caused by the decomposition of natural organic matter present in the centrate (e.g. humic acid, amines, urea, etc.) as it is evident also by the decrease of NH_4 during the lag phase at the beginning of the trial — see Fig. 3) [40] and by the presence of starch in a gradually increasing number of microalgae cells during the batch regime. This resulted in a negative TOC removal efficiency. The TOC left in the culture during the batch regime was higher than the one consumed by heterotrophic organisms, such as bacteria [10]. The higher growth rate of the culture grown in TLC during semi-continuous regime resulted in higher density of the cells containing starch and thus, the TOC removal efficiency was lower (17%) as compared to that in TL-RWP (33%). As the centrate was added every day, the bacteria population proliferated quicker than microalgae, being able to uptake more TOC which resulted in lower concentrations at the end of the semi-continuous regime.

The specific nutrient uptake was more efficient in both cultivation units in the batch regime, whereas it was not fast enough in the semi-continuous one, resulting in the accumulation of some nutrients in the cultivation medium, e.g., phosphate (Fig. 3). Phosphate removal can be limited when $N\text{-}NH_4$ is exhausted [11]. It is understandable as the TN/TP ratio (about 1.6) in the centrate (Table 1) was relatively low, considering the content of nitrogen and phosphorus in microalgal

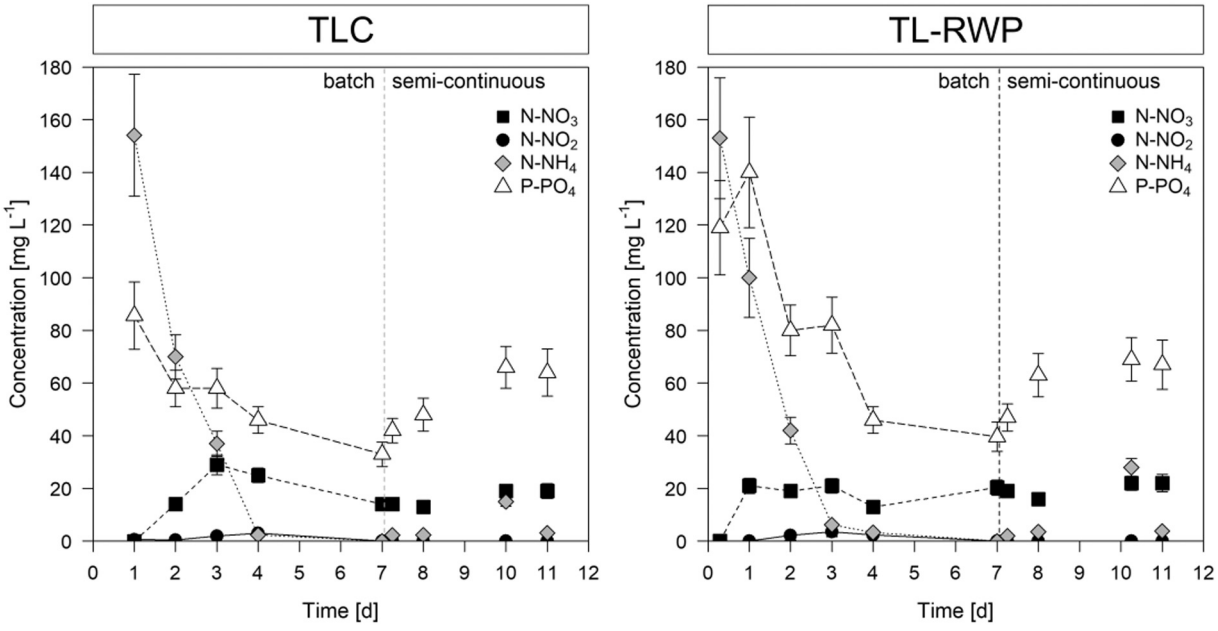

Fig. 3. Concentration (mg L^{-1}) of nitrate-nitrogen ($N\text{-}NO_3$; black square), nitrite-nitrogen ($N\text{-}NO_2$; black circle), ammonium-nitrogen ($N\text{-}NH_4$; grey diamond), and phosphate-phosphorus ($P\text{-}PO_4$; white triangle) in the co-culture of *C. vulgaris* and *S. acutus* grown in a thin-layer cascade (TLC; left panel) and a thin-layer raceway pond (TL-RWP; right panel) in batch and semi-continuous (25% dilution rate) growth regime using centrate as nutrient source ($n = 1$). Error bars represent analytical standard deviation as the measurement was performed in triplicate.

biomass is usually reported up to a maximum of 12% and 3% (ratio N/P of 4) of ash-free DW, respectively [41]. The daily addition of tap water to compensate for the evaporation contributed to the total nutrient with only 20 μg L^{-1} of NO_3^- and 28 μg L^{-1} of PO_4^{3-} (maximum), which are rather negligible amounts, and therefore, were not accounted for.

Nitrite-nitrogen was efficiently removed by the co-culture grown in both units, reaching values below 0.3 mg L^{-1}, which is the maximum admissible threshold in drinking water [42]. After three days of cultivation, ammonium-nitrogen concentration dropped significantly in both cultivation units achieving the value of 2.4 mg L^{-1} in TLC compared to 6.2 mg L^{-1} in TL-RWP. Being the preferred nitrogen source, ammonium-nitrogen concentrations dropped first under the batch regime, thus reflecting the opposite trend of nitrate-nitrogen. The decrease in ammonium concentration was accompanied by the increased nitrate during the batch regime, probably due to bacterial nitrification. Moreover, some ammonium may have been lost due to stripping to the atmosphere in gaseous form [34].

3.4. Biological activity

3.4.1. Determination of auxin- and cytokinin-like activity

No biostimulating activities were detected when two different concentrations of the microalgal extracts (0.5 and 2 g DW L^{-1}) were used for seed germination (data not shown). The mung bean rooting tests (data not shown) demonstrated no auxin-like activity. No chlorophyll retention was detected when the samples were tested for cytokinin-like activity (data not shown). Some inhibiting substances in the centrate could explain the absence of biostimulating activity in the freeze-dried biomass sample [43]. Another explanation for the absence of biostimulating activity could be due to the type of nutrient source used since previous studies were able to find such activities in *Chlorella* [18].

3.4.2. Antimicrobial activity

The biomass obtained from the co-cultures of microalgae grown in the centrate showed antagonistic activity against various phytopathogenic agents (Fig. 4). Antifungal activities were the most noticeable in the co-cultures developed in both units. Similar inhibition of fungal activities of at least 43, 31, and 42% against *R. solani*, *F. oxysporum*, and *P. capsici*, respectively, were detected regardless of the cultivation unit. These results are similar to those obtained for monocultures of *Chlorella* MACC-1 [18] and *Scenedesmus* MACC-677 (unpublished). The antifungal activity of 45% against *P. ultimum* was found only when the biomass was cultivated in TLC. This could indicate the predominant presence of *Chlorella* MACC-1 in the co-culture of TLC as no bioactivity of *Scenedesmus* MACC-677 against this pathogen was observed (unpublished). On the other hand, antibacterial activity against one microbial species, *C. michiganensis,* was detected only in the biomass harvested from TL-RWP.

One reason for the loss of multi-antibacterial capacity, which has been previously reported in *Chlorella* cultures [7,18] could be caused by the presence of a higher concentration of *S. acutus* cells found at the end of the trial. However, since culture conditions can influence antimicrobial agents' production, it would be interesting to pursue a better understanding of the co-culturing influence on the biological activity of the microalgae, since keeping a monoculture in open systems can be a difficult task [44]. Our results indicated that the resulting biomass had a high action capacity against several pathogens commonly found in crops, helping to fight several fungal and oomycete infections, thus preventing wilting, rotting, and ultimately host death [45,46]. Therefore, the agricultural application of microalgal biomass after wastewater treatment has added value, as it implies the potential use of a natural biopesticide that provides protection to crops, while concomitantly contributing to the implementation of a circular economy.

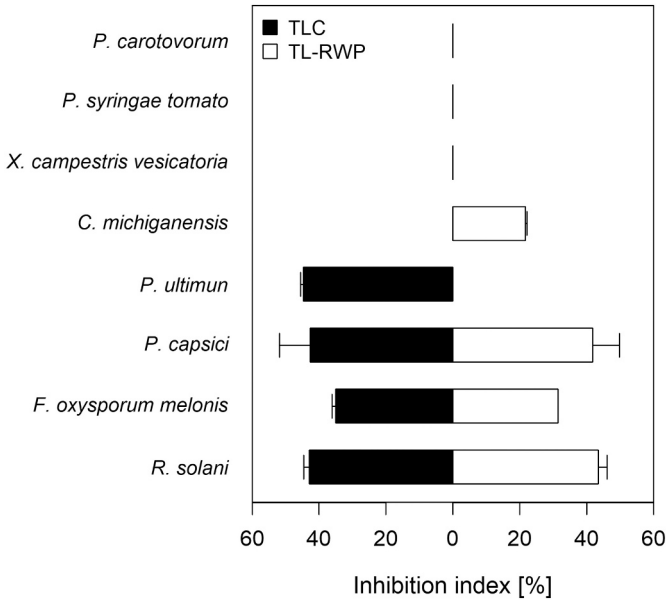

Fig. 4. Antibacterial and antifungal activities of the resulting biomass of co-cultures of *C. vulgaris* and *S. acutus* grown in a thin-layer cascade (TLC; black bars) and in a thin-layer raceway pond (TL-RWP; white bars) in centrate. Results are expressed as inhibition index (%) against two fungi – *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *melonis*, two oomycetes – *Phytophthora capsici* and *Pythium ultimum* and four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis*, *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato* and *Pectobacterium carotovorum* ($n = 3$). Error bars represent standard deviation.

4. Conclusion

The use of municipal wastewater centrate as the sole nutrient source to grow a co-culture of two chlorophytes (*Chlorella* and *Scenedesmus*), in two different outdoor TL units, was overall feasible. After a short adaptation period, the cultures started to grow linearly and were transferred to a semi-continuous regime. Although a longer residence time can probably provide better results, nutrient removal of macronutrients from the wastewater centrate was effective. Even though microalgae's co-culturing benefits depend on population succession dynamics, the resulting biomass revealed a potential application in agriculture as antimicrobial agents, specifically against common agriculture fungi and oomycetes. Both TL systems are suitable for biomass production as well-mixed cultures are exposed to ambient light resulting in high average cell irradiance. TL-RWP use provided very similar results to that of TLC, as they are operated at similar culture layer depth. However, the TLC unit provided a higher productivity (and slightly better nutrient removal) under the semi-continuous regime compared to the culture grown in TL-RWP. In addition, the co-culture of *Chlorella* and *Scenedesmus* yielded better nutrient removals than the use of *Chlorella* monocultures in a similar experimental design, reaffirming the usefulness of using consortiums in wastewater treatment. This study confirms that the centrate separated from municipal wastewater can be used as a nutrient medium for chlorophyte cultivation, while effectively stripping nutrients to reduce eutrophication. Moreover, a final product with agricultural application can be obtained, thus contributing to a circular economy.

Compliance with ethical statements

This article does not contain any studies with human participants or animals performed by any of the authors.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102299.

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