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Growth, biostimulant and biopesticide activity of the MACC-1 *Chlorella* strain cultivated outdoors in inorganic medium and wastewater

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ABSTRACT

The use of wastewater (WW) for cultivation contributes to the sustainability of microalgae production due to the reduced costs of cultivation. The main objective of this work was to study growth, physiological performance and bioactivity of the microalgae strain *Chlorella vulgaris* MACC-1 grown in two nutrient sources – inorganic BG-11 medium and centrate from municipal WW. For the comparison, two thin-layer cultivation units – thin-layer cascade and a novel, thin-layer raceway pond – were used. The cultures grew well in both units showing good photosynthetic activity. The germination index of watercress seeds, as well as the auxin-like activity in mung bean and cytokinin-like activity in wheat growth tests were used to evaluate the biostimulant potential. The slight increase on the germination index was determined in *C. vulgaris* cultures grown in BG-11, but the biomass revealed no biostimulant activity when cultivated in WW. On the other hand, the antibacterial and antifungal activities determined by antagonism bioassay using dual cultures were significantly higher when grown in WW. We expect that the antimicrobial activities may be induced by WW-microbes and the biostimulating effect could probably be suppressed by the presence of some inhibiting substances. The results revealed a clear interplay among ambient irradiance intensity, growth rate, maximum quantum yield of PSII, F_v/F_m and oxygen production/respiration.

1. Introduction

Alternative technologies to increase productivity in sustainable agricultural systems need to be found [1] and microalgae represent one of them [2]. A number of microalgae strains produce biologically active compounds, such as plant hormones (responsible for biostimulating activity) and antimicrobial compounds (responsible for biopesticide activity). The biomass (extracts) can be applied as an alternative to chemical pesticides and fertilizers causing pollution [3,4] as it can enhance the plant growth and protect the agricultural crops [2,5–8].

Microalgae can be cultivated either in open reservoirs (mixed ponds, raceways or cascades) with direct contact of the microalgae culture with the environment or in closed or semi-closed vessels – photobioreactors (PBRs) [9–12]. The biomass production in open systems is cheaper than that in closed PBRs as the former are easier to clean and require lower operational costs [13]. In microalgae culturing, the biomass composition may be affected by modulation of various environmental factors and conditions [14]. Microalgae cultivation requires major nutrients such as nitrogen, phosphorus and carbon. Urban wastewaters contain high amounts of nitrogen (mainly in the form of ammonium) and phosphorus which can replace these expensive mineral fertilizers used for microalgae cultivation thereby reducing the cultivation costs

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[5,6,15–18]. *C. vulgaris* is often used in wastewater treatments as it possess a great tolerance to high concentrations of ammonia [5,19,20].

In order to optimize growth regime for successful microalgae cultivation, monitoring of physicochemical variables (pH, temperature, dissolved oxygen concentration, nutrient status, etc.) and photosynthetic activity are required. Chlorophyll fluorescence measurement has become one of the most frequently used techniques for monitoring microalgae mass cultures [21–25]. The most frequently used variable, the maximum quantum yield of PSII (F_v/F_m) has been used to correlate photosynthesis and growth [21,25–30]. Measurements of photosynthetic oxygen production give similar information as measurement of F_v/F_m (using saturation pulse analysis of fluorescence quenching), but in the former one important advantage concerns the possibility to distinguish between photosynthetic oxygen production (POE) and dark respiration (R) [31].

Light availability is the most crucial prerequisite of microalgae growth. By choosing the correct cultivation unit, an optimum number of photons can access each microalgae cell [10]. In the present experiments we cultured the microalga *C. vulgaris* outdoor in two thin-layer units – a thin-layer cascade and a novel, thin-layer raceway pond, as these are more suitable for light utilization. The growth and physiological performance of the microalgae cultivated in two different nutrient sources – inorganic BG-11 medium and centrate from the municipal WW, were evaluated to find out whether the centrate can be used for cultivation, reducing the need for clean water and expensive nutrients. The biopesticide and biostimulant activity of all *C. vulgaris* biomass samples were analyzed using various bioassays: the determination of inhibition index (biopesticide effect), germination index and root induction (auxin-like effect) and the stimulation of chlorophyll production (cytokinin-like effect).

2. Materials and methods

2.1. Strain and inoculum preparation

For the trials the unicellular green microalga *Chlorella vulgaris* MACC-1 (further abbreviated as *C. vulgaris*) was selected due its high growth rate [32] and potential biostimulating and biopesticide activity (V. Ördög, unpublished data). It was obtained from the Algal Culture Collection of the Széchenyi István University in Mosonmagyaróvár, Hungary. A seed culture (inoculum) of *C. vulgaris* was grown in the laboratory at 28–30 °C in 10 L Pyrex glass bottles using the inorganic BG-11 medium [33,34] which was bubbled with air +1% CO_2 (*v*/v) until the stationary phase. Photosynthetically active radiation (PAR) of about 120 μmol photons m^{-2} s^{-1} was provided continuously by a panel of dimmable warm-white tubes (55 W, Dulux L, Osram, Germany) placed vertically behind the cultivation unit.

2.2. Wastewater preparation

In the municipal wastewater treatment plant (WWTP) in Třeboň, an automatic addition of flocculant was used for the separation of liquid (centrate) and solid parts from the activated sludge after the secondary aerobic digestion. As the centrate caused cell aggregation during microalgae cultivation, this problem was overcome by centrifugation of the sludge without added flocculant in the laboratory. It was taken from the WWTP and centrifuged at 3000*g* for 5 min (centrifuge Sigma 8KS) to separate the liquid centrate (WW) from the solid matter. Then, the brownish-color WW (see the composition in Table 1) was collected and used undiluted for growth in outdoor cultivation units.

2.3. Outdoor trials

Once the stationary growth phase in the laboratory PBRs was reached, the cultures were mixed in a plastic tank with 4 parts of either the BG-11 medium (Trial 1) or wastewater centrate (Trail 2); then they were transferred to outdoor cultivation units. The *C. vulgaris* cultures were grown in parallel in a thin-layer cascade (TLC) and a novel, thin-layer raceway pond (TL-RWP) placed in polycarbonate greenhouses with controlled environment (Fig. 1) [35]. Both cultivation units, TLC and TL-RWP are characterized by the microalgae growth in very thin layer (the maximum culture layer of 20 mm) and hence, these units are more adequate for the light utilization during cultivation. The TLC was circulated by a pump only during the day (between 7:30 a.m. and 7:30 p.m.); at night the culture was stored in a retention tank and mixed by an air pump [35]. The TL-RWP was operated continuously. In both units an automatic addition of CO_2 maintained the pH value at 8.0 ± 0.2 (pH-stat).

The initial biomass density in outdoor trials was set to 0.7–0.8 g DW L^{-1}; the cultures in WW had a brown-green color due to the wastewater addition. The dark color of cultivation media or dense culture could cause substantial light absorption when cultivated in a unit with a thick culture layer; the thin-layer systems used in these trials were advantageous for these purposes.

C. vulgaris was firstly grown in a batch mode for 7 d to set-up a well-growing, dense culture and then a semi-continuous growth regime was operated for 4 d at a dilution rate of 0.25 d^{-1}. Every morning at 0800 h, one-quarter of the culture volume was harvested from each unit and replaced by fresh BG-11 medium or WW centrate. At the end of the semi-continuous cultivation regime, 20 L of microalgae culture was collected in the morning (0800 h) and the same amount in the afternoon (1300*h*), centrifuged and freeze-dried for bioassays. In these trials we used a model of large-scale production where microalgae were grown in semi-continuous/continuous regime replacing daily a part of the culture with fresh medium/WW. Thus, samples for bioassays were taken in semi-continuous culturing regimes in the morning and in the afternoon when physiological status and photosynthetic activity of *C. vulgaris* cultures may differ due to temperature and light variations.

Table 1
Averaged composition (± SD, $n = 3$) of the centrate of municipal wastewater (taken from the Wastewater Treatment Plant in Třeboň) which was used for cultivation of *C. vulgaris* in outdoor units. Total nitrogen and phosphorus content in the BG-11 medium are shown for comparison.

	Centrate [mg L^{-1}]	BG-11 [mg L^{-1}]
BOD[a]	197 ± 38	–
COD[b]	1367 ± 208	–
TOC[c]	607 ± 130	–
N-NO_3	0.11 ± 0.14	250
N-NO_2	0.005 ± 0.002	–
N-NH_4	207 ± 6	–
Total N	**273 ± 32**	**250**
P-PO_4	163 ± 12	7
Total P	**183 ± 21**	**7**

The most important macroelements for microalgae growth are shown in bold.
[a] Biological oxygen demand.
[b] Chemical oxygen demand.
[c] Total organic carbon.

2.4. Location and weather conditions

The outdoor trials were carried out in the Centre Algatech (48°59′16.6"N, 14°39.9″E) in August and September in 2019. In both units the morning temperatures ranged between 12 and 20 °C (TL-RWP) and 11–20 °C (TLC) while at midday the temperature varied between 23 and 37 °C (TL-RWP) and 25–37 °C (TLC) in Trial 1 and 2, respectively. This range of temperatures is not limiting for *C. vulgaris*. The evaporation was compensated every morning by the addition of tap water.

Photosynthetically active radiation (PAR) inside the greenhouses was recorded by a weather station (IP Warioweather, model ME 13). During Trial 1 with the BG-11 medium the weather conditions were fair; daily irradiance maxima inside the greenhouses were between 600 and 800 μmol photons m^{-2} s^{-1} during the first week and around

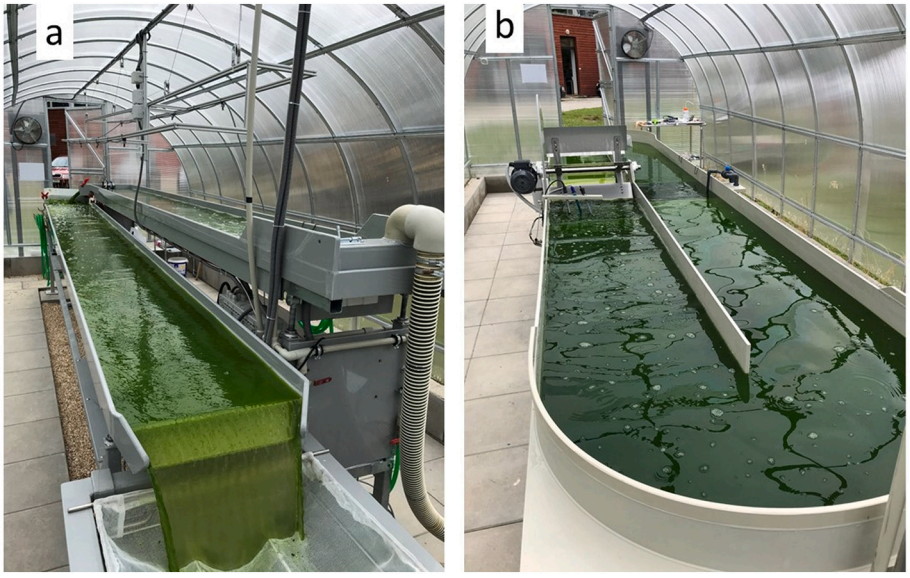

Fig. 1. Outdoor cultivation units used in trials were placed in polycarbonate double-layer greenhouses to protect cultures from cross-contamination and unfavorable outdoor conditions: a) thin-layer cascade (TLC; surface of 5 m^2, working volume of 70 L, culture depth of 8–10 mm, flow speed of 0.5 m s^{-1}) with the ratio of total surface to volume about 80 m^{-1} and b) thin-layer raceway pond (TL-RWP; surface of 5 m^2; work volume of 120 L at a culture depth of 15–20 mm, flow speed of 0.2 m s^{-1}) with the ratio of total surface to volume about 40 m^{-1}.

400–500 µmol photons m^{-2} s^{-1} during the second week (Fig. 2a). The light intensity inside the greenhouses was much higher during Trial 2 when the cultivation of *C. vulgaris* in undiluted WW was performed. During the first week daily irradiance maxima were between 1000 and 1700 µmol photons m^{-2} s^{-1} and between 1100 and 1500 µmol photons m^{-2} s^{-1} during the second week (Fig. 2b).

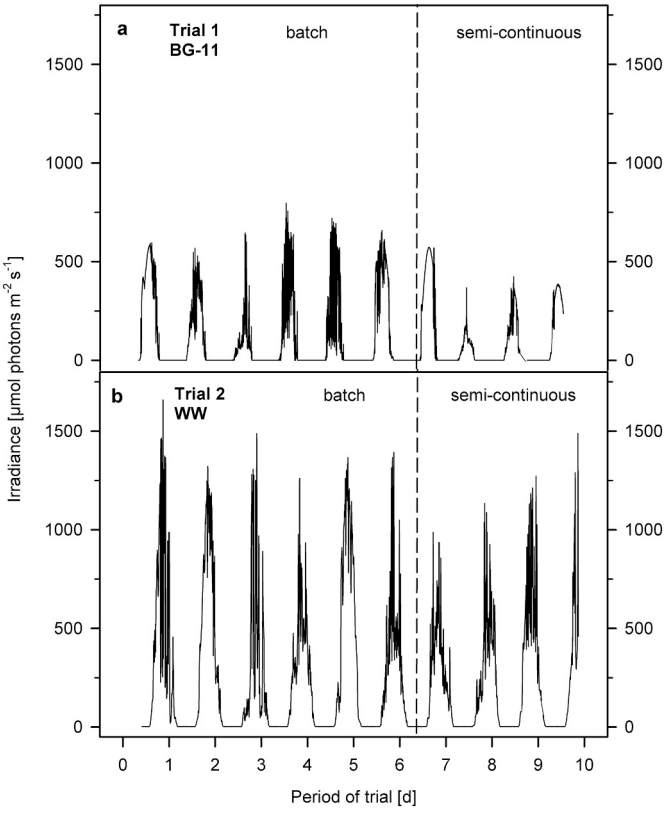

Fig. 2. Light intensity measured inside the greenhouses during the growth of *C. vulgaris:* a) Trial 1 with BG-11 medium; and b) Trial 2 with centrate (WW) in batch and semi-continuous regime.

2.5. Dry weight determination

The measurement of biomass density was performed as previously described [24,36]. The biomass content was measured in triplicate once a day in the morning (0800 h after the compensation of evaporation) when cultivation was performed in batch regime and twice a day (in the morning after compensation of evaporation and after dilution) during the semi-continuous regime. The specific growth rate $\mu = (\ln X_2 - \ln X_1) / \Delta t$ [d^{-1}] of microalgae cultures was calculated over the period of linear phase in batch regime.

2.6. Nutrient analysis

The centrate (25 mL) was filtered using a syringe filter and then kept at −20 °C until analysis (provided by the company Povodí Vltavy Ltd., České Budějovice). The following components and variables were determined (Table 1): BOD (Biological Oxygen Demand) by suppression of nitrification, COD (Chemical Oxygen Demand) using an analytical commercial kit (Merck), TOC (Total Organic Carbon) by thermal decomposition with Pt catalyst, nitrite content (NO_2-N) by automatic discrete photometry, ammonium nitrogen (NH_4-N) using acidimetrically after distillation, total nitrogen content (N_{tot}) by thermal oxidation with electrochemical detection and nitrate concentration (NO_3-N) by addition to the total nitrogen content. The content of orthophosphate-phosphorus (PO_4-P) was assayed by automatic discrete photometry and the content of total phosphorus (P_{tot}) by photometry after mineralization.

2.7. Photosynthesis measurements

Photosynthetic activity of the cultures was estimated using two techniques: by Chl fluorescence to follow changes in maximum photochemical yield F_v/F_m and by measurement of POE and R to construct steady-state light-response curves. These techniques have already been described in detail previously [24,27,36,37].

2.7.1. Rapid light-response curves

Rapid light-response curves (RLCs) of relative electron transport (rETR) of microalgae samples were measured in a light-protected measuring chamber with mixing (3 mL glass cuvette, light path of 10 mm) using a pulse-amplitude-modulation fluorimeter (PAM-2500, H. Walz, Germany). Before measurements the culture was diluted to 0.2–0.3 g DW L^{-1} to avoid re-absorption problems in dense suspension

and the sample was dark adapted for 10 min.

The minimum and maximal fluorescence level (F_0, F_m) were determined using a weak modulated light [<0.15 μmol photons $m^{-2} s^{-1}$, frequency of 0.5–1 kHz] in the dark-adapted samples (actinic irradiance = 0; first step of RLC). A stepwise increase of irradiance intensity (between 0 and 2500 μmol photons $m^{-2} s^{-1}$) was applied in 20-s intervals to obtain the steady state fluorescence level. The maximal PSII photochemical efficiency was calculated as the ratio of variable and maximal fluorescence, $F_v/F_m = (F_m - F_o) / F_m$. It is frequently used as a convenient estimate of the photochemical performance as it indicates the maximum quantum efficiency of primary photochemistry [38].

2.7.2. Measurement of photosynthetic oxygen evolution and respiration rates

POE and R measurements were carried out in a temperature-controlled chamber with adjustable illumination and mixing (DW2/2, Hansatech, UK) connected to temperature-controlled bath and control unit (Oxygen Monitoring System Oxylab+, Hansatech, UK). The dark-adapted samples were diluted as above and measured at temperature corresponding to the outdoor unit. Steady-state light-response curves were recorded using a stepwise increasing light intensity of 0, 200, 400, 600, 1200 and 1800 μmol photons $m^{-2} s^{-1}$, when each step lasted 2 min. The first step (in the dark) provided a value of the dark R. POE and R were calculated in pmol O_2 $cell^{-1} h^{-1}$.

2.8. Bioactivity determination

The antimicrobial (biopesticide) activity of the *C. vulgaris* biomass samples were detected by antagonism bioassay in the freeze-dried samples collected at the end of trials in the morning (0800 h) and in the afternoon (1300 h). The plant biostimulating activity was detected by three different bioassays to measure the auxin-like (seed germination, mung bean rooting) and cytokinin-like (chlorophyll retention) activities.

2.8.1. Antagonism bioassay by dual culture

The protocol followed in this work was described previously [39,40]. The amount of 10 mg DW mL^{-1} of lyophilized *C. vulgaris* biomass was sonicated (Branson sonicator 150, amplitude 40%, 3 min) and subsequently the extracts were tested against the growth of phytopathogenic fungi and bacteria in vitro using the dual culture technique according to the protocol [39,40]. The activity of all extracts was tested against two fungi – *Fusarium oxysporum* f.sp. *melonis* and *Rhizoctonia solani* (further as *Fusarium, Rhizoctonia*), two oomycetes – *Phytophthora capsici* and *Pythium ultimum* (further as *Phytophthora* and *Pythium*) and four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas campestris* pv. *vesicatoria, Pseudomonas syringae* pv. *tomato,* and *Pectobacterium carotovorum* (further as *Clavibacter, Xanthomonas, Pseudomonas* and *Pectobacterium*). All strains were provided by the Spanish Type Culture Collection (CECT) at the Science Park of the University of Valencia. In the case of bacteria, the growth inhibition clear zones were measured in the presence of *C. vulgaris* extract. The inhibition of the growth of phytopathogenic fungi was calculated according to the diameter of the inhibition clear zones in the presence of *C. vulgaris* extract.

The inhibition index was calculated according 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 in the absence of microalgae extract (mm) and T is the diameter of the zone of the pathogen in the presence of algal extract (mm). In all cases, control bioassays were prepared using distilled water.

2.8.2. Seed germination bioassays

The biostimulant activity was tested on 100 cress (*Lepidium sativum*) seeds using extracts of 0.5 and 2 mg DW mL^{-1} of microalgae biomass according to the procedure previously described [41]. To carry out this bioassay, samples were taken from lyophilized *C. vulgaris* biomass and prepared as mentioned above. The percentage of seed germination as well as the radicle elongation were taken into account for the calculation of the Germination Indexes, based on the following formula:

$$GI = (G_S\%*L_S)/(G_w\%*L_w)$$

where GI is Germination Index in %, G_S% is a percentage of germinated seeds in the presence of microalgae extract, G_w% is a percentage of germinated seeds in the presence of distilled water, L_S is a mean of radicle elongation (mm) in the presence of microalgae extract and L_w is a mean of radicle elongation (mm) in the presence of distilled water.

2.8.3. Determination of auxin-like activity

The bioassay was performed according to reference [42]. Mung bean (*Vigna radiata* (L.) Wilczek) seeds were soaked for 4 min in 0.33% sodium hypochlorite solution and rinsed under running tap water for 24 h. The seeds were planted at 1 cm depth in moistened perlite in plastic trays. The trays were placed in the growth chamber maintained at 27 °C and about 60 to 65% relative humidity, illuminated with fluorescent lamps (120 μmol photons $m^{-2} s^{-1}$) for 7 to 10 d. The seedlings are then cut with a clean razor 12 cm below the two leaves. Five uniform cuttings were placed in vials of 25 × 90 mm (three vials per treatment) containing 10 mL of distilled water or algal nutrient solution, as controls, and algal suspensions (0.5, 1.0, 2.0 and 3 g DW L^{-1}), as treatment. Freeze-dried *C. vulgaris* biomass was suspended in water and sonicated for 3 min and used in the bioassays. A standard curve to each bioassay was also prepared by using indol-3-butyric acid (IBA) at concentrations of 0, 0.3, 0.5, 0.7 and 1 mg DW L^{-1}. The vials were placed in the growth chamber in the original growth conditions for one day. The plants were then removed from the vials, washed thoroughly with distilled water and putted back to the growth chamber for 8 d. The solution (lost by transpiration) was brought up to its original level with distilled water daily. After the incubation period, the number of roots (longer than 1 mm) were recorded on each hypocotyl. The mean number of roots were compared to IBA which was used as a standard auxin. The results are given in IBA equivalent concentrations.

2.8.4. Determination of cytokinin-like activity

Cytokinins accelerate chloroplast differentiation as well as regulate and stimulate chlorophyll production. The use of wheat leaves as a specific bioassay for cytokinins was already reported [43]. Seeds of a wheat cultivar (*Triticum aestivum* L.) were soaked in sodium hypochlorite solution (2%) for 5 min, then removed and rinsed under running tap water for 24 h. The seeds were planted at 1 cm depth in moistened perlite in plastic trays. The trays were placed in a growth chamber maintained at 25 °C and about 60–65% relative humidity, illuminated with a fluorescent lamp (120 μmol photons $m^{-2} s^{-1}$) for 7 d. Leaves from seedlings (about 10 cm height) were collected and then cut 35 mm below their apical tip into 10 mm segments. The fresh weight of ten cuttings were weighed with an analytical grade balance and placed in glass vials of 25 × 90 mm size (four vials per treatment) containing 10 mL of distilled water or nutrient medium as controls, and algae extracts (0.5, 1.0, 2.0 and 3.0 g DW L^{-1}) as treatments. A calibration curve to each bioassay was prepared by using kinetin (KIN) at the concentration of 0, 0.3, 0.5, 0.7 and 1 mg DW L^{-1}. The vials were placed in the dark growth chamber for 4 d. After the incubation period the leaves were blotted dry and transferred into centrifuge tubes containing 8 mL of 80% ethanol. The tubes were kept in a water bath (warmed up to 80–90 °C) for 10 min than cooled down to room temperature and completed with 80% ethanol up to 10 mL. The chlorophyll extract was then carefully poured, excluding debris, into spectrophotometer cuvettes. The optical density was measured at 645 nm and compared to the KIN. The results are given in KIN equivalent.

2.9. Statistical analysis

Data were measured in triplicate. Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and Holm-Sidak test was conducted for every binary combination of data. *P* values lower than 0.05 were considered significantly different.

3. Results

3.1. Growth

The *C. vulgaris* cultures were grown in the outdoor cultivation units – TLC and TL-RWP either in inorganic BG-11 medium or in undiluted WW. During the first week of cultivation in batch regime in BG-11, both cultures grew well, almost linearly between Day 1 and Day 7, only a short lag phase was observed between Day 0 and Day 1 as they were transferred from laboratory to higher irradiance outdoors (Fig. 3). Nevertheless, the *C. vulgaris* culture grown in TLC showed by about 35% higher growth ($\mu = 0.19\ d^{-1}$) as compared to the culture grown in TL-RWP ($\mu = 0.14\ d^{-1}$). The slower growth in TL-RWP can be caused by deeper culture layer causing lower average cell irradiance compared to that in TLC. When *C. vulgaris* culture was grown in WW, the lag phase was observed between Day 0 and Day 2. The specific growth rate in TLC was higher by about 21% ($\mu = 0.23\ d^{-1}$; Fig. 3b) compared to that in BG-11 medium (Fig. 3a). Here, we consider that as a result of better light conditions during cultivation in WW (Fig. 2). At the end of the batch regime the culture grown in BG-11 reached the biomass density of 2.6 and 2.1 g DW L^{-1} in TLC and TL-RWP, respectively while in WW it was 3.1 and 2.4 g DW L^{-1} (Fig. 3). Yet, it is important to note that the WW used as a source of nutrients did not have any negative effects on growth, but rather positive.

During the second week starting from Day 7 afternoon, the cultures were grown in semi-continuous regime, removing 25% (dilution rate $DR = 0.25\ d^{-1}$) of culture volume every day and it was replaced by either BG-11 medium or undiluted WW. On the next day the cultures were almost able to reach the biomass density measured before dilution indicating that the DR can be reduced to 0.2 d^{-1} in the future. We have to consider that the higher growth of *C. vulgaris* culture in both units using WW (Fig. 3b) was probably caused by higher irradiance intensity in this period (Fig. 2b). During the cultivation of *C. vulgaris* cultures in WW, the concentrations of total N and P decreased during the batch regime (Day 0–Day 7) in both units (Fig. 4a). From Day 0 until Day 7 the concentration of total N decreased from 290 mg DW L^{-1} to 170 mg DW L^{-1} in TLC and from 190 to 140 mg DW L^{-1} in TL-RWP (Fig. 4). The uptake of P during the batch regime was not so efficient (Fig. 4a–b). This could be caused by fast N-NH_4 exhaustion [44]. The concentration of N-NH_4 in both cultivation units dropped during the first two days almost to zero while the uptake of N-NO_3 was efficient only in TLC. The concentration of N-NO_3 in TL-RWP was still 3.4 mg DW L^{-1} at the end of the batch regime.

3.2. Photosynthesis performance

The changes in photosynthetic activity were estimated as the maximum PSII photochemical rate, F_v/F_m by Chl fluorescence (Fig. 5). The data was measured twice a day, in the morning (0800 h) and at midday (1300 h). The lower F_v/F_m values were observed in Trial 1 (in BG-11 medium) in both units during the Day 1 which indicated that laboratory cultures were partially photo-stressed after the exposure to outdoor irradiance and needed some time for acclimation. This corresponded to the lag phase also seen in growth curves for the first two days (Fig. 3).

The variable F_v/F_m did not change much during both trials, it mostly ranged between 0.70 and 0.79. In green microalgae such values show that the cultures are in good physiological condition [45]. Starting from Day 7 after the transfer to the semi-continuous regime, the measurements were carried out before the dilution to avoid any problems of culture disturbance. Compared to the cultures grown in Trial 1 (Fig. 5a-b), the F_v/F_m values of cultures grown in Trial 2 (in WW) ranged from 0.70 to 0.77 and dropped significantly to 0.3–0.5 on the last day of cultivation which indicated a higher degree of stress (e.g. shade adaptation in dense cultures, accumulation of some inhibiting substances).

3.3. Photosynthetic oxygen production and respiration

When the *C. vulgaris* cultures were grown in BG-11 the maximum of POE activity in both units after inoculation was between 60 and 100 pmol O_2 $cell^{-1}\ h^{-1}$ (Day 0) as the cultures were acclimating to the high light compared to the light intensity in the laboratory. The maximum POE up to 150 pmol O_2 $cell^{-1}\ h^{-1}$ was reached (Day 3) after the acclimation during the batch regime (Fig. 6a-b). In the semi-continuous regime, the POE activity was higher in TLC as compared to that in TL-RWP. In TLC, the respiration R rates were between 50 and 80 pmol O_2 $cell^{-1}\ h^{-1}$ in the batch regime; in semi-continuous regime these increased from 80 to 200 pmol O_2 $cell^{-1}\ h^{-1}$. In semi-continuous regime the R rate even increased due to higher biomass density and lower ambient irradiance. The same trends were observed in TL-RWP culture, only the photosynthesis rate was lower and R rate higher which is probably due to the growth in deeper-layer culture (Fig. 6b). It is important to note, that the R rate in both cultures was quite high; in semi-continuous regime usually higher than photosynthesis, most probably due to the growth in high biomass density and relatively low

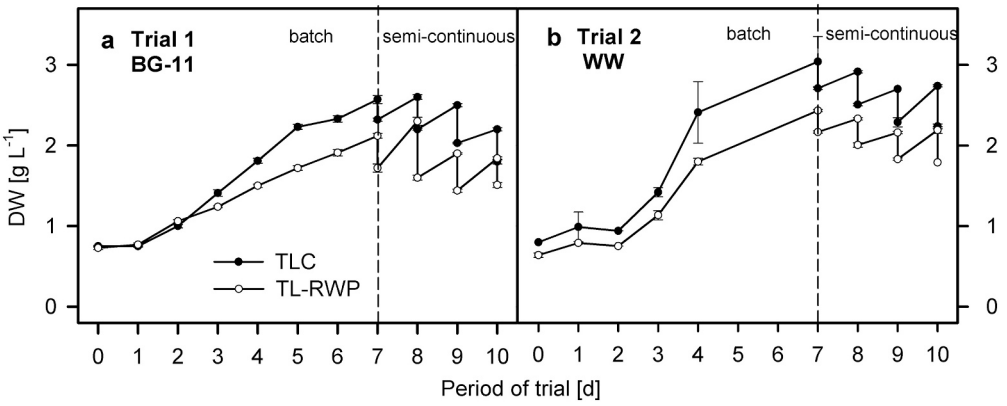

Fig. 3. Changes in the biomass density (DW) of *C. vulgaris* MACC-1 cultures grown in batch and semi-continuous regime in thin-layer cascade (TLC; black circle) and thin-layer raceway pond (TL-RWP; white circle) grown in: a) BG-11 medium (Trial 1) and b) centrate of municipal wastewater (Trial 2). Error bars represent analytical standard deviation as the DW was determined in triplicate.

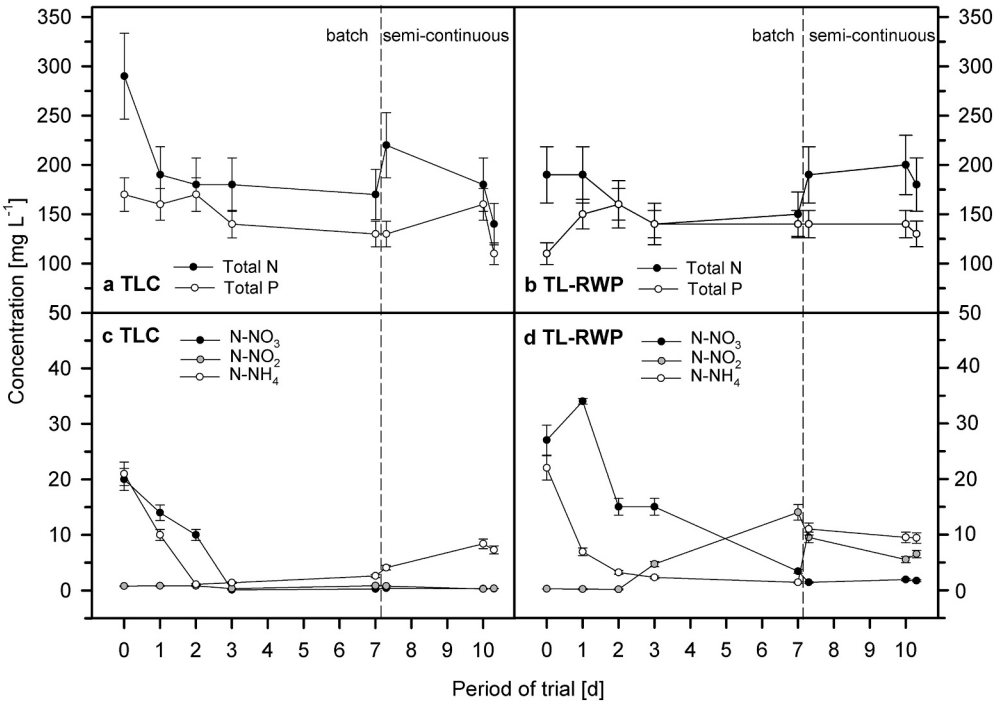

Fig. 4. Nutrient concentration during the growth of *C. vulgaris* in municipal wastewater: a) total nitrogen (black circle) and phosphorus (white circle) during the cultivation in thin-layer cascade (TLC); b) total nitrogen (black circle) and phosphorus (white circle) during the cultivation in thin-layer raceway pond (TL-RWP); c) nitrate-nitrogen ($N\text{-}NO_3$; black circle), nitrite-nitrogen ($N\text{-}NO_2$; grey circle) and ammonium-nitrogen ($N\text{-}NH_4$; white circle) amount during the cultivation in TLC; d) nitrate-nitrogen ($N\text{-}NO_3$ black circle), nitrite-nitrogen ($N\text{-}NO_2$; grey circle) and ammonium-nitrogen ($N\text{-}NH_4$; white circle) amount during the cultivation in TL-RWP. Error bars represent analytical standard deviation as the DW was determined in triplicate.

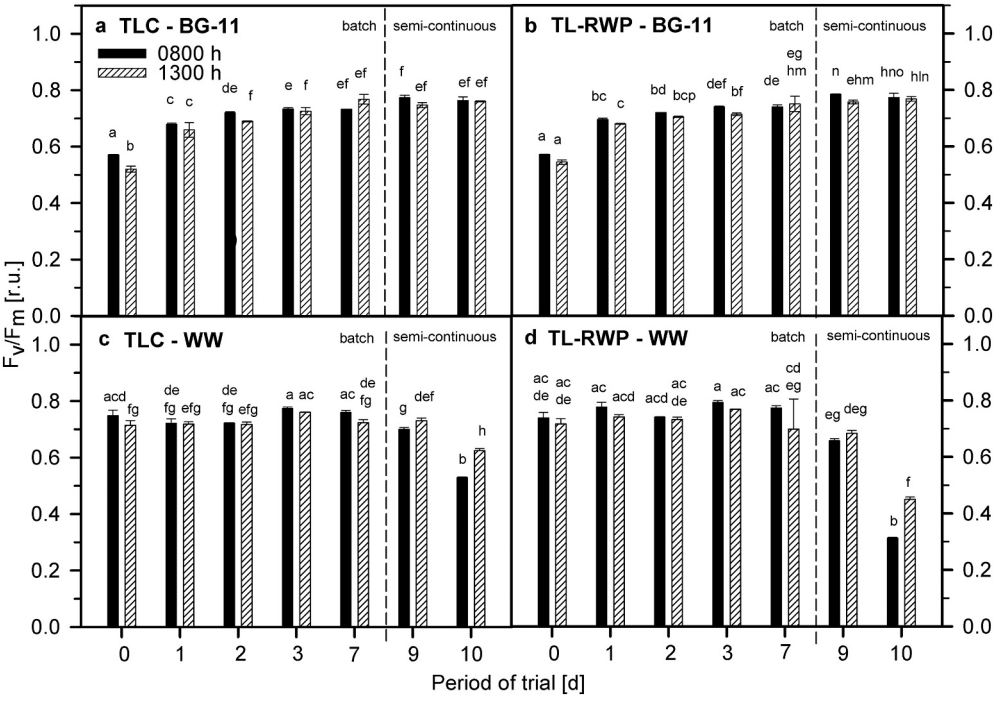

Fig. 5. Changes in the maximum effective quantum yield of PSII (F_v/F_m) during the cultivation of *C. vulgaris* cultures in batch and semi-continuous regime: a) cultures grown in TLC in BG-11 media; b) cultures grown in TL-RWP in BG-11 media; c) cultures grown in TLC in WW; d)-cultures grown in TL-RWP in WW. The black columns represent the measurement at 0800 h and dashed columns represent the measurement at 1300 h. Error bars represent analytical standard deviation as the DW was determined in triplicate. Values with the same symbol did not differ significantly from each other ($P > 0.05$).

ambient irradiance.

When the *C. vulgaris* culture was cultivated in WW in batch regime, higher activity of POE was observed in both units – up to 340 pmol O_2 $cell^{-1}$ h^{-1} on Day 3 compared to Trial 1 performed in BG-11 media. When the units were operated in semi-continuous regime, POE was still high in both units and then it was decreasing. The R rate was high, especially in TL-RWP which could be caused by the deeper culture layer and less cell exposure to the light as mentioned above.

If we compared the growth rate (Fig. 3), maximum photochemical PSII yield (Fig. 5), POE and R rate (Fig. 6), we found the correlation as the trends are similar. In batch regime we observed relatively good growth rate in both units although TLC was performing better. There was not much difference in F_v/F_m and POE between both units, only R was higher in TL-RWP. In semi-continuous regime when the *C. vulgaris* culture was grown in WW, we could find a clear interplay among ambient irradiance intensity, growth rate, PSII photochemical efficiency F_v/F_m and POE/R. In both units the growth rate was quite high due to sufficient ambient irradiance, increasing or decreasing stepwise according to dilution, but still the growth rate was 10–15% higher in TLC compared to that in TL-RWP. At the end of Trial 2, F_v/F_m significantly

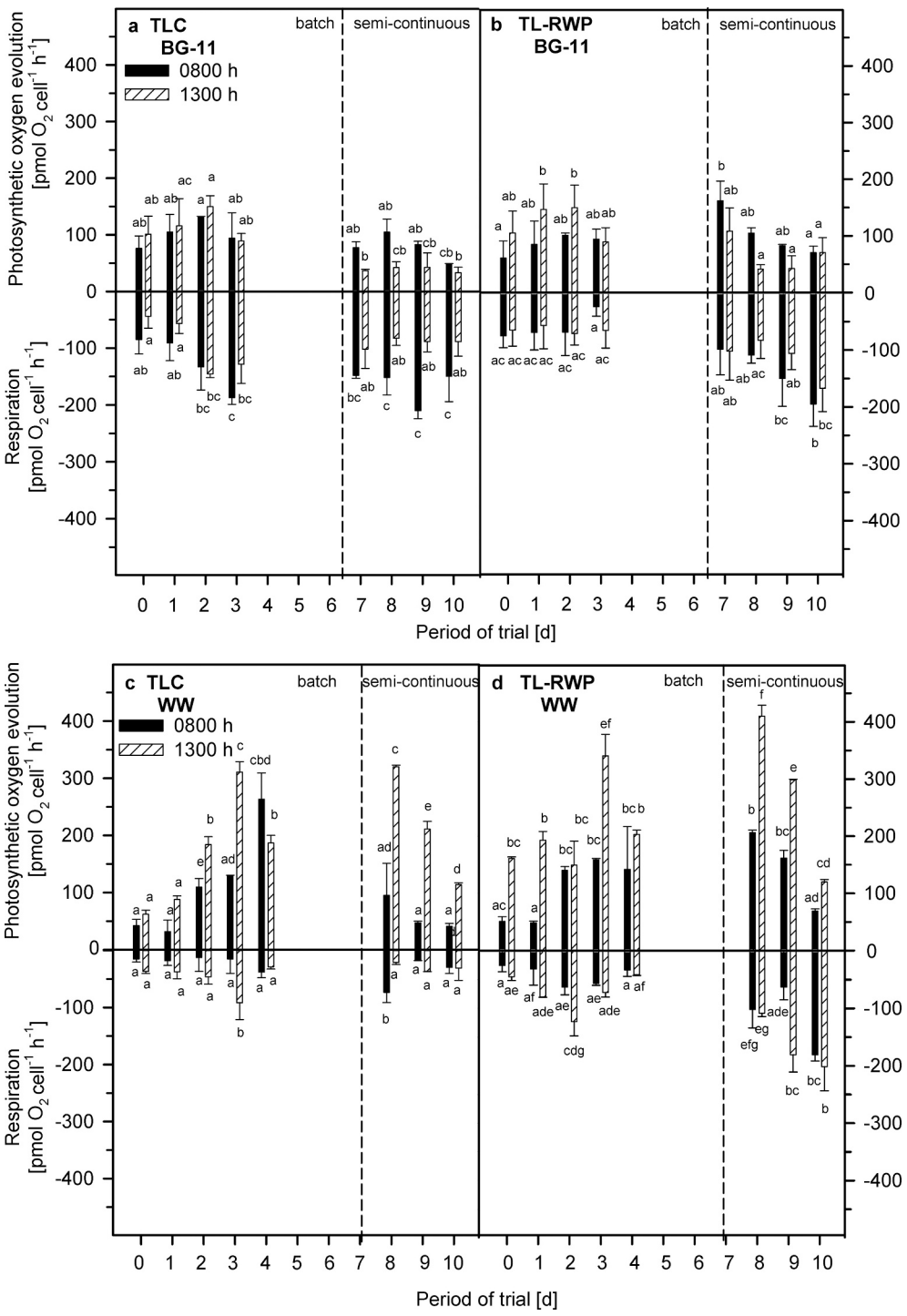

Fig. 6. Changes of photosynthetic oxygen evolution and respiration measured in *C. vulgaris* cultures in two outdoors cultivation units in batch (Day 0 – Day 6) and semi-continuous cultivation regime (Day 7–Day 10): a) culture grown in TLC in BG-11; b) culture grown in TL-RWP in BG-11; c) culture grown in TLC in WW centrate; d) culture grown in TL-RWP in WW centrate. The black columns represent the measurement at 0800 h and dashed columns show the measurements at 1300 h. Error bars represent analytical standard deviation as the DW was determined in triplicate. Values with the same symbol did not differ significantly from each other ($P > 0.05$).

decreased in both units as well as POE; on the contrary R significantly increased compared to the batch regime.

3.4. Bioactivity determination

Results of the dual culture assay for determination of biopesticide activity were varying as the extracts from biomass samples at different daytimes were active against individual bacteria and fungi (Table 2). Out of the total number of 8 microorganisms, the biomass extracts of *C. vulgaris* cultivated in BG-11 medium were active only against one bacterial strain of *Clavibacter* and three fungal strains, *Fusarium, Rhizoctonia* and *Phytopthora.* Antifungal activity up to 37.5% against *Fusarium* was determined for the biomass harvested from TLC. Generally, the antibacterial and antifungal activity was higher when *C. vulgaris* cultures were grown in WW as compared to those grown in BG-11 (Table 2). It is important to note that antifungal activity of the *C. vulgaris* cultures grown in WW was about twice higher compared to antibacterial activity. Antifungal activity against all fungal pathogens was almost always higher when *C. vulgaris* biomass was harvested at midday. The highest antifungal activity of *C. vulgaris* was 41.4% (0800 h) and 50.4% (1300 h) was observed against *Phytopthora* when grown in WW. The results obtained in these experiments show clearly that the cultivation in WW has a positive effect on the accumulation of bioactive compounds responsible for antibacterial and even more for

Table 2
Antibacterial and antifungal activity of *C. vulgaris* extracts. Cultivation was performed in thin-layer cascade (TLC) and thin-layer raceway pond (TL-RWP) using BG-11 medium and wastewater (WW) as well and the biomass was harvested at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h). The values represented inhibition index in % (±SD, $n = 3$) against four bacteria strains – *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato* and *Pectobacterium carotovorum*, two funghi – *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *melonis*, and two oomytes – *Phytopthora capsici* and *Pythium ultimum*, considering distilled water as the control ($I = 0\%$). Statistical analysis was performed between the rows. Values with the same letter did not differ significantly from each other ($P > 0.05$).

	Inhibition index [%]							
	Trial 1 (BG-11)				Trial 2 (WW)			
Pathogen	TLC		TL-RWP		TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h	0800 h	1300 h
Bacteria								
C. michiganensis	25.6 ± 0.6^{a}	21.7 ± 0.3^{a}	22.8 ± 0.3^{a}	22.8 ± 0.3^{a}	0^{b}	20.8 ± 3.3^{a}	0^{b}	23.1 ± 7.3^{a}
X. campestris	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	18.3 ± 5.3^{b}	16.9 ± 5.3^{b}	21.9 ± 5.3^{b}
P. syringae	0^{a}	0^{a}	0^{a}	0^{a}	12.2 ± 5.3^{b}	16.7 ± 0.1^{bc}	0^{a}	17.8 ± 2.0^{c}
P. carotovorum	0^{a}	0^{a}	0^{a}	0^{a}	14.7 ± 4.7^{b}	15.8 ± 6.7^{b}	15.6 ± 4.0^{b}	17.3 ± 7.5^{b}
Fungi								
R. solani	11.3 ± 3.0^{a}	23.8 ± 4.8^{b}	0^{c}	10.1 ± 1.8^{a}	27.2 ± 0.2^{b}	42.3 ± 4.0^{d}	28.8 ± 0.2^{b}	44.2 ± 0.2^{d}
F. oxysporum	37.5 ± 8.9^{a}	33.3 ± 7.1^{a}	1.2 ± 1.2^{b}	32.1 ± 8.3^{a}	28.7 ± 1.3^{a}	26.8 ± 2.0^{a}	31.6 ± 2.0^{a}	32.0 ± 2.0^{a}
Oomycetes								
P. capsici	19.6 ± 2.6^{a}	34.6 ± 2.5^{ab}	0^{c}	3.7 ± 3.7^{c}	41.4 ± 0.7^{b}	50.4 ± 0.1^{b}	46.9 ± 6.7^{b}	41.4 ± 17.3^{b}
P. ultimum	0^{a}	0^{a}	0^{a}	0^{a}	25.7 ± 2.7^{b}	34.3 ± 1.3^{c}	33.3 ± 4.0^{c}	35.6 ± 1.3^{c}

antifungal activity (Table 2).

The biostimulant activity determined by seed germination was found only in the biomass obtained from the cultivation in BG-11 medium. The higher activity was observed when a less concentrated biomass extract (0.5 mg DW mL^{-1}) was applied. It ranged from 108.5% to 115.7% as compared to the maximum of 105% when the extract of 2 mg DW L^{-1} was used. As concerns daytime, the higher biostimulant activity of all extracts was observed when the *C. vulgaris* biomass was harvested in the morning (0800 h) using less concentrated biomass extract (Table 3). Surprisingly, no biostimulant activity was found in the *C. vulgaris* cultures grown in WW. This could be caused by the presence of inhibiting substance in WW which remain in the freeze-dried biomass sample inhibiting seed germination [46].

The biostimulant activity of all samples determined by auxin- and cytokinin-like bioassay was correlated to the concentration equivalent of IBA and KIN, respectively. The most promising auxin-like activity equivalent to 0.3 mg DW L^{-1} of IBA was found when 2.0 g DW L^{-1} *C. vulgaris* biomass harvested from TLC at 0800 h was used. No activity was observed for the same sample harvested in the afternoon (at 1200 h). Both auxin- and cytokinin-like activities were detected in 2.0 and 3.0 g DW L^{-1} *C. vulgaris* samples harvested from TL-RWP at 1300 h. In this case the auxin-like activity was equivalent to 0.5 mg DW L^{-1} of IBA, cytokinin-like activity was equivalent to 0.3 mg DW L^{-1} of KIN. This was the only sample demonstrating cytokininlike activity (Table 4).

Table 3
Biostimulant activity of *C. vulgaris* was tested using the two different biomass extracts (in mg DW mL^{-1}). The biomass was harvested from TLC and TL-RWP at the end of semi-continuous regime in the morning (0800 h) and in the afternoon (1300 h) when cultivated in BG-11 medium, and lyophilized. Water extracts were prepared using 0.5 and 2 g biomass per liter. Results are expressed in a percentage as means ± standard deviation ($n = 3$) considering distilled water as the control (100%). No activity of biomass harvested at the end of Trial 2 (WW) was found. Values with the same letter did not differ significantly from each other ($P > 0.05$).

Biomass extract [mg mL^{-1}]	Germination index [%]			
	Trial 1 (BG-11)			
	TLC		TL-RWP	
	0800 h	1300 h	0800 h	1300 h
0.5	**108.5 ± 0.2^{a}**	108.5 ± 0.1^{a}	**115.7 ± 0.5^{b}**	108.6 ± 1.2^{a}
2	**100.8 ± 0.2^{d}**	85.5 ± 0.2^{e}	**105.4 ± 0.5^{f}**	86.0 ± 2.1^{e}

The highest biostimulant activity is always highlighted in bold.

The results obtained in these experiments show clearly that the cultivation in WW has a positive effect on the accumulation of bioactive compounds responsible for antibacterial and even more for antifungal activity (Table 2) but no biostimulating effects were detected in the seed germination bioassay (Table 3) and in the bioassays for detection of auxin- and cytokinin-like activities (Table 4).

4. Discussion

Microalgae can be grown under controlled conditions in various constructed cultivation units where they can reach a higher production of biomass per unit area when compared to higher plants [47,48]. In outdoor, deep-culture systems such as open ponds, low turbulence results in low biomass productivity [17]. Shallow, thin-layer systems like sloping cascades or cascade raceways are more advantageous due to cell turbulence which are more suitable for the photosynthetic efficiency due to high intermittency [10,49–51]. In order to increase cultivation sustainability, microalgae can be grown using alternative sources of nutrients [52]. By the utilization of wastewater as a nutrient medium the production costs can be reduced to below 5 € per kg [52,53].

In this study we aimed to the cultivation of *C. vulgaris* strain in WW and to the evaluation of bioactivity. Two open, thin-layer cultivation systems – TLC and TL-RWP were tested whose design is suitable for efficient light utilization inducing high biomass productivity. These systems are also quite suitable for fast-growing *Chlorophyta* as they can tolerate high average cell irradiance [35]. Moreover, the thin-layer systems are advantageous when working with the dark-colored WW as to avoid substantial light absorption. In our experiments, the TLC was performing slightly better than the TL-RWP due to lower culture depth, but on the other hand, the latter is more universal as it can be operated at higher volume per area and the paddle wheel is more gentle for fragile or filamentous strains. [10,50,51]. Based on our findings, the freshwater *C. vulgaris* can be grown in this type of undiluted centrate from municipal WWTP as the only nutrient source. It is evident that *C. vulgaris* used in this study represents a robust, well-growing species that can assimilate the high content of ammonium (about 180 mg DW L^{-1}) and organic carbon (TOC, about 490 mg DW L^{-1}). The *C. vulgaris* culture in our trials was growing better in WW as compared to that in BG-11, presumably due to higher ambient irradiance during the cultivation in this trial. These results are supported by previous reports [54,55] although diluted centrate after anaerobic digestion was used there for outdoor culturing of *Scenedesmus*. Data in the literature and our results have confirmed that microalgae can be grown in WW, recovering nutrients and recycling

Table 4
Auxin- and cytokinin-like activity (equivalent to IBA and KIN concentration in mg L^{-1}) of *C. vulgaris* samples detected by mung bean root development and wheat chlorophyll retention test. Water extracts were prepared using 0.5–3 g biomass per liter. No activity of biomass harvested at the end of Trial 2 (WW) was found.

Sample	Time of harvesting	Auxin- and cytokinin-like activity							
		Extraction of microalgae biomass [g L^{-1}]							
		0.5		1.0		2.0		3.0	
		IBA	KIN	IBA	KIN	IBA	KIN	IBA	KIN
TLC	0800 h	0	0	0	0	0.3	0	0.5	0
	1300 h	0	0	0	0	0	0	0	0
TL-RWP	0800 h	0	0	0	0	0	0	0	0
	1300 h	0	0	0.5	0	0.5	0.3	0.5	0.3

water for further use [5,16,52,56,57].

To survive adverse conditions (e.g. microbial contaminants in this study) some microalgae strains are able to synthetize various compounds [58], some of them showing antibacterial and antifungal activity [59,60]. These include unsaturated lactones, glycosides, sulphur containing compounds, phenols and phenolic glycosides, saponins and phytoalexins as well as fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing heterocyclic compounds, carbohydrates and phenols [58]. Few studies have been related to the antibacterial and antifungal activity of *C. vulgaris* [59,61–63]. The early reporting of compounds with biopesticide activity from microalgae show a bioactive compound from the green alga *C. vulgaris*, the so-called chlorellin that inhibited the growth of Gram-negative and Gram-positive bacteria [64]. Later, more extracts of green algae, diatoms, and dinoflagellates were reported [63,65], for example, antifungal activity of *C. vulgaris* against human [59] and plant pathogens [61,62]. In our trials we have found that cultivation in WW induced the production of the compounds responsible for antimicrobial activity that was well active against the fungus *Phytopthora*. The inhibition of this pathogen reached up to 50%. Our data on antimicrobial activity of *C. vulgaris* against phytopathogens used in our experiments were unique as we were not able to find any equivalent experiments in the literature.

For the plant biostimulating effect, several amino acids, vitamins and polyamines can act as growth-promoting substances [1,47,66–68]. Also, extracts and hydrolysates of high protein containing microalgae biomass (up to 50–60% in dry weight) contain the active substances, mainly amino acids and small peptides which can act as plant biostimulants. Microalgae also contain polysaccharides (such as α-glucan) that may also be involved in the improvement of plant growth [69,70]. Only some of our *C. vulgaris* biomass samples obtained from the cultivation in BG-11 medium showed biostimulating activity, but not from those grown in WW. This could probably be caused by inhibiting substances present in WW which accumulated in *C. vulgaris* biomass and inhibited the potential activity [46]. On the other hand, the biostimulant activity of the biomass sample can be increased by choosing a proper source of waste nutrients for microalgae cultivation, which was confirmed by the cultivation of *Scenedesmus obliquus* in brewery wastewater, which achieved biostimulating activity of up to 150–200% [53].

From all *C. vulgaris* samples obtained from the cultivation in BG-11 medium, the highest auxin-like activity equivalent to 0.3 mg DW L^{-1} of IBA was detected for *C. vulgaris* (2.0 g DW L^{-1}) when cultivated in TLC and harvested in the morning (0800 h). Both auxin- and cytokinin-like activities (in the range between 0.3 and 0.5 mg DW L^{-1} of IBA or KIN) have been found for the biomass harvested from TL-RWP in the afternoon (1300 h).

In out trials, auxin-like activity (with the equivalent of 0.5 mg L^{-1} of IBA was found in *C. vulgaris* biomass grown in BG-11 harvested at midday. The cytokinin-like activity was determined only in the biomass extracts at higher concentrations of 2 and 3 mg L^{-1}, where the KIN equivalent was 0.3 mg L^{-1} at midday. This is in accordance with the previous findings of Ördög et al. (2004) [71] where KIN equivalent of 0.1 mg L^{-1} was found in *C. vulgaris* sp. and *C. vulgaris minutissima* samples. We have to take into account, that they used 3 times more concentrated extracts for testing compared to our experiment. The auxin- and cytokinin-like activity of *Arthrospira platensis* with 0.3 mg L^{-1} equivalent of the standards were studied by Mógor et al. (2018) [1]. No effect on mung bean rooting and mild stimulation of chlorophyll formation were observed when *Arthrospira* samples were hydrolyzed.

5. Conclusions

To conclude our trials, the microalga *C. vulgaris* grew well in both nutrient sources – inorganic medium and undiluted centrate from municipal wastewater when cultured in thin-layer culture units, TLC and TL-RWP. In semi-continuous regime when the *C. vulgaris* culture was grown in BG-11 medium or WW, we could find a clear interplay among ambient irradiance intensity and growth rate vs. photochemical efficiency of PSII measured as $F_v/_{fm}$ and POE/R. Nevertheless, the growth rate in TLC was 10–15% higher compared to that in TL-RWP.

Under selected cultivation regime *C. vulgaris* MACC-1 can be grown to obtain biomass either with biostimulating or antimicrobial properties. Biostimulating, i.e. auxin- and cytokinin-like activity was found when the culture was grown in inorganic BG-11 medium at midday; no biostimulant activity was observed when grown in WW which can be caused by the presence of some inhibitors. This might be related/confirmed by low photosynthetic activity that was measured at harvesting time. On the other hand, the presence of microorganisms in WW probably stimulated the production of antimicrobial compounds as they were generally higher compared to the biomass grown in BG-11. In both cultivation units antimicrobial activity was usually higher when the biomass was harvested at midday compared to the morning sampling. It is important to mention that antifungal activity of *C. vulgaris* cultures grown in WW was about twice higher in comparison to antibacterial activity.

CRediT authorship contribution statement

Karolína Ranglová: Writing – Original Draft, Formal analysis, Visualization **Gergely Ernö Lakatos:** Investigation **João Artur Câmara Manoel:** Investigation **Tomáš Grivalský:** Investigation **Francisca Suárez Estrella:** Investigation, Writing – Review & Editing **Francisco Gabriel Acién Fernández:** Writing – Review & Editing, Funding acquisition **Zoltán Molnár**: Investigation **Vince Ördög:** Writing – Review & Editing **Jiří Masojídek:** Writing – Review & Editing.

Ethical statements

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

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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References

[1] Á.F. Mógor, V. Ördög, G.P.P. Lima, Z. Molnár, G. Mógor, Biostimulant properties of cyanobacterial hydrolysate related to polyamines, J. Appl. Phycol. 30 (2018) 453–460, https://doi.org/10.1007/s10811-017-1242-z.

[2] J. Garcia-Gonzalez, M. Sommerfeld, Biofertilizer and biostimulant properties of the microalga *Acutodesmus dimorphus*, J. Appl. Phycol. 28 (2016) 1051–1061, https://doi.org/10.1007/s10811-015-0625-2.

[3] N.C.P. de Albuquerque, D.B. Carrão, M.D. Habenschus, A.R.M. de Oliveira, Metabolism studies of chiral pesticides: a critical review, J. Pharm. Biomed. Anal. 147 (2018) 89–109, https://doi.org/10.1016/j.jpba.2017.08.011.

[4] T. Gomiero, Food quality assessment in organic vs. conventional agricultural produce: findings and issues, Appl. Soil Ecol. 123 (2018) 714–728, https://doi.org/10.1016/j.apsoil.2017.10.014.

[5] D. Ronga, E. Biazzi, K. Parati, D. Carminati, E. Carminati, A. Tava, Microalgal biostimulants and biofertilisers in crop productions, Agronomy 9 (2019), https://doi.org/10.3390/agronomy9040192.

[6] M.M. Kulik, The potential for using cyanobacteria (blue-green algae) and algae in the biological control of plant pathogenic bacteria and fungi, Eur. J. Plant Pathol. 101 (1995) 585–599, https://doi.org/10.1007/BF01874863.

[7] V. Ördög, W.A. Stirk, J. Van Staden, O. Novák, M. Strnad, Endegenous cytokinins in three genera of microalgae from Chlorophyta, J. Phycol. 40 (2004) 88–95, https://doi.org/10.1046/j.1529-8817.2004.03046.x.

[8] C. Ledda, G.I. Romero Villegas, F. Adani, F.G. Acién Fernández, E. Molina Grima, Utilization of centrate from wastewater treatment for the outdoor production of *Nannochloropsis gaditana* biomass at pilot-scale, Algal Res. 12 (2015) 17–25, https://doi.org/10.1016/j.algal.2015.08.002.

[9] G.C. Zittelli, N. Biondi, L. Rodolfi, M.R. Tredici, Photobioreactors for mass production of microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult. Appl. Phycol. Biotechnol, Second, Wiley-Blackwell, Oxford, UK, 2013, pp. 225–266, https://doi.org/10.1002/9781118567166.ch13.

[10] J. Masojídek, M. Sergejevová, J.R. Malapascua, J. Kopecký, Thin-Layer Aystems for Mass Cultivation of Microalgae: Flat Panels and Sloping Cascades, in: Algal Biorefineries, Springer International Publishing, Cham, 2015, pp. 237–261, https://doi.org/10.1007/978-3-319-20200-6_7.

[11] M. Sergejevová, J.R. Malapascua, J. Kopecký, J. Masojídek, Photobioreactors with Internal Illumination, in: Algal Biorefineries, Springer International Publishing, Cham, 2015, pp. 213–236, https://doi.org/10.1007/978-3-319-20200-6_6.

[12] F.G. Acién, E. Molina, A. Reis, G. Torzillo, G.C. Zittelli, C. Sepúlveda, J. Masojídek, Photobioreactors for the production of microalgae, microalgae-based biofuels bioprod, From Feed. Cultiv. To End-Products. (2017) 1–44, https://doi.org/10.1016/B978-0-08-101023-5.00001-7.

[13] C.U. Ugwu, H. Aoyagi, H. Uchiyama, Photobioreactors for mass cultivation of algae, Bioresour. Technol. 99 (2008) 4021–4028. https://doi.org/10.1016/j.biortech.2007.01.046.

[14] G. Lakatos, K. Ranglova, J. Câmara Manoel, T. Grivalský, J. Kopecký, J. Masojidek, Bioethanol production from microalgae polysaccharides, Folia Microbiol. (Praha). 64 (2019), https://doi.org/10.1007/s12223-019-00732-0.

[15] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel production using wastewater resources, Bioresour. Technol. 102 (2011) 17–25, https://doi.org/10.1016/j.biortech.2010.06.035.

[16] N. Renuka, R. Prasanna, A. Sood, A.S. Ahluwalia, R. Bansal, S. Babu, R. Singh, Y. S. Shivay, L. Nain, Exploring the efficacy of wastewater-grown microalgal biomass as a biofertilizer for wheat, Environ. Sci. Pollut. Res. 23 (2016) 6608–6620, https://doi.org/10.1007/s11356-015-5884-6.

[17] G.I. Romero-Villegas, M. Fiamengo, F.G. Acién-Fernández, E. Molina-Grima, Utilization of centrate for the outdoor production of marine microalgae at the pilot-scale in raceway photobioreactors, J. Environ. Manag. 228 (2018) 506–516, https://doi.org/10.1016/j.jenvman.2018.08.020.

[18] O. Tsolcha, A. Tekerlekopoulou, C. Akratos, G. Aggelis, S. Genitsaris, M. Moustaka-Gouni, D.D. Vayenas, Agroindustrial wastewater treatment with simultaneous biodiesel production in attached growth systems using a mixed microbial culture, Water. 10 (2018) 1693, https://doi.org/10.3390/w10111693.

[19] E. Krichen, A. Rapaport, E. Le Floc'h, E. Fouilland, Demonstration of facilitation between microalgae to face environmental stress, Sci. Rep. 9 (2019) 16076, https://doi.org/10.1038/s41598-019-52450-9.

[20] I. Krustok, J. Truu, M. Odlare, M. Truu, T. Ligi, K. Tiirik, E. Nehrenheim, Effect of lake water on algal biomass and microbial community structure in municipal wastewater-based lab-scale photobioreactors, Appl. Microbiol. Biotechnol. 99 (2015) 6537–6549, https://doi.org/10.1007/s00253-015-6580-7.

[21] G. Torzillo, P. Accolla, E. Pinzani, J. Masojidek, In situ monitoring of chlorophyll fluorescence to assess the synergistic effect of low temperature and high irradiance stresses in *Spirulina* cultures grown outdoors in photobioreactors, J. Appl. Phycol. 8 (1996) 283–291, https://doi.org/10.1007/BF02178571.

[22] K. Maxwell, G.N. Johnson, Chlorophyll fluorescence - a practical guide, J. Exp. Bot. 51 (2000) 659–668, https://doi.org/10.1093/jxb/51.345.659.

[23] N.R. Baker, Chlorophyll fluorescence: a probe of photosynthesis in vivo, Annu. Rev. Plant Biol. 59 (2008) 89–113, https://doi.org/10.1146/annurev.arplant.59.032607.092759.

[24] K. Ranglová, G.E. Lakatos, J.A.C. Manoel, T. Grivalský, J. Masojídek, Rapid screening test to estimate temperature optima for microalgae growth using photosynthesis activity measurements, Folia Microbiol. (Praha). (2019), https://doi.org/10.1007/s12223-019-00738-8.

[25] J.R.F. Malapascua, C.G. Jerez, M. Sergejevová, F.L. Figueroa, J. Masojídek, Photosynthesis monitoring to optimize growth of microalgal mass cultures: application of chlorophyll fluorescence techniques, Aquat. Biol. 22 (2014) 123–140, https://doi.org/10.3354/ab00597.

[26] G. Torzillo, P. Bernardini, J. Masojídek, On-line monitoring of fluorescence to access the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (Cyanobacteria), J. Phycol. 34 (1998) 504–510, https://doi.org/10.1046/j.1529-8817.1998.340504.x.

[27] A.M. Silva Benavides, K. Ranglová, J.R. Malapascua, J. Masojídek, G. Torzillo, Diurnal changes of photosynthesis and growth of *Arthrospira platensis* cultured in a thin-layer cascade and an open pond, Algal Res. 28 (2017) 48–56, https://doi.org/10.1016/j.algal.2017.10.007.

[28] J.R. Malapascua, K. Ranglová, J. Masojídek, Photosynthesis and growth kinetics of *Chlorella vulgaris* R-117 cultured in an internally LED-illuminated photobioreactor, Photosynthetica. (2018), https://doi.org/10.32615/ps.2019.031.

[29] P.J. Ralph, R. Gademann, Rapid light curves: a powerful tool to assess photosynthetic activity, Aquat. Bot. 82 (2005) 222–237, https://doi.org/10.1016/j.aquabot.2005.02.006.

[30] J. Masojídek, A. Vonshak, G. Torzillo, Chlorophyll fluorescence applications in microalgal mass cultures, in: D.J. Suggett, O. Prášil, M.A. Borowitzka (Eds.), Chlorophyll *a* Fluoresc. Aquat. Sci. Methods Appl, Springer Netherlands, Dordrecht, 2010, pp. 277–292, https://doi.org/10.1007/978-90-481-9268-7_13.

[31] F.L. Figueroa, C.G. Jerez, N. Korbee, Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems, Lat. Am. J. Aquat. Res. 41 (2013) 801–819, doi:103856/vol41-issue5-fulltext-1.

[32] V. Ördög, W.A. Stirk, P. Bálint, C. Lovász, O. Pulz, J. van Staden, Lipid productivity and fatty acid composition in *Chlorella* and *Scenedesmus* strains grown in nitrogen-stressed conditions, J. Appl. Phycol. 25 (2013) 233–243, https://doi.org/10.1007/s10811-012-9857-6.

[33] M.M. Allen, R.Y. Stanier, Growth and division of some unicellular blue-green algae, J. Gen. Microbiol. 51 (1968) 199–202, https://doi.org/10.1099/00221287-51-2-199.

[34] R.Y. Stanier, J. Deruelles, R. Rippka, M. Herdman, J.B. Waterbury, Generic sssignments, strain histories and properties of pure cultures of cyanobacteria, Microbiology. 111 (1979) 1–61, https://doi.org/10.1099/00221287-111-1-1.

[35] T. Grivalský, K. Ranglová, J.A. da Câmara Manoel, G.E. Lakatos, R. Lhotský, J. Masojídek, Development of thin-layer cascades for microalgae cultivation: milestones (review), Folia Microbiol. (Praha). 64 (2019) 603–614, https://doi.org/10.1007/s12223-019-00739-7.

[36] A. Babaei, K. Ranglová, J.R. Malapascua, J. Masojídek, The synergistic effect of selenium (selenite, $-SeO_3^{2-}$) dose and irradiance intensity in *Chlorella* cultures, AMB Express. 7 (2017), https://doi.org/10.1186/s13568-017-0348-7.

[37] A. Babaei, K. Ranglová, J.R. Malapascua, G. Torzillo, J. Shayegan, A.M. Silva Benavides, J. Masojídek, Photobiochemical changes in *Chlorella* g120 culture during trophic conversion (metabolic pathway shift) from heterotrophic to phototrophic growth regime, J. Appl. Phycol. (2020), https://doi.org/10.1007/s10811-020-02137-w.

[38] Analysis of the chlorophyll a fluorescence transient, in: R.J. Strasser, M. Tsimilli-Michael, A. Srivastava, G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll *a* Fluoresc, A Signat. Photosynth, Springer Netherlands, Dordrecht, 2004, pp. 321–362, https://doi.org/10.1007/978-1-4020-3218-9_12.

[39] F. Suárez-Estrella, M. Ros, M. Vargas García, M. López, J. Moreno, Control of *Xanthomonas campestris* pv. *vesicatoria* using agroindustrial waste-based compost, J. Plant Pathol. 96 (2014) 243–248, https://doi.org/10.4454/JPP.V96I2.028.

[40] N. Sánchez San Fulgencio, F. Suárez-Estrella, M.J.J. López, M.M.M. Jurado, J.A. A. López-González, J. Moreno, Biotic aspects involved in the control of damping-off producing agents: the role of the thermotolerant microbiota isolated from composting of plant waste, Biol. Control. 124 (2018) 82–91, https://doi.org/10.1016/j.biocontrol.2018.04.015.

[41] F. Zucconi, A. Pera, M. Forte, M. de Bertoldi, Evaluating toxicity of immature compost, Biocycle. 22 (1981) 54–57.

[42] C. Hess, Mung bean bioassay for the detection of root promoting substances, Plant Physiol. 36 (1961) 21.

[43] J.A. Kuhnle, G. Fuller, J. Corse, B.E. Mackey, Antisenescent activity of natural cytokinins, Plant Physiol. 41 (1977) 14–21, https://doi.org/10.1111/j.1399-3054.1977.tb01514.x.

[44] A. Galès, A. Bonnafous, C. Carré, V. Jauzein, E. Lanouguère, E. Le Floc'h, J. Pinoit, C. Poullain, C. Roques, B. Sialve, M. Simier, J.-P. Steyer, E. Fouilland, Importance of ecological interactions during wastewater treatment using high rate algal ponds under different temperate climates, Algal Res. 40 (2019) 101508, https://doi.org/10.1016/j.algal.2019.101508.

[45] J. Masojídek, G. Torzillo, M. Koblížek, Photosynthesis in microalgae, in: A. Richmond, Q. Hu (Eds.), Handb. Microalgal Cult, John Wiley & Sons, Ltd, 2013, pp. 21–36, https://doi.org/10.1002/9781118567166.ch2.
[46] C. Garcia, B. Ceccanti, G. Masciandaro, T. Hernandez, Fractionation and characterization of humic substance fractions with different molecular weights, obtained from animal wastes, Soil Sci. Plant Nutr. 41 (1995) 649–658, https://doi.org/10.1080/00380768.1995.10417015.
[47] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96. https://doi.org/10.1263/jbb.101.87.
[48] S.P. Cuellar-Bermudez, G.S. Aleman-Nava, R. Chandra, J.S. Garcia-Perez, J. R. Contreras-Angulo, G. Markou, K. Muylaert, B.E. Rittmann, R. Parra-Saldivar, Nutrients utilization and contaminants removal. A review of two approaches of algae and cyanobacteria in wastewater, Algal Res. 24 (2017) 438–449, https://doi.org/10.1016/j.algal.2016.08.018.
[49] E.W. Becker, Microalgae: Biotechnology and Microbiology, Cambridge University Press, 1994. https://books.google.cz/books?id=KAKx4I7NWEYC.
[50] A. Richmond, Growth characteristics of ultrahigh-density microalgal cultures, Biotechnol. Bioprocess Eng. 8 (2003) 349–353, https://doi.org/10.1007/BF02949278.
[51] J. Masojídek, J. Kopecký, L. Giannelli, G. Torzillo, Productivity correlated to photobiochemical performance of *Chlorella* mass cultures grown outdoors in thin-layer cascades, J. Ind. Microbiol. Biotechnol. 38 (2011) 307–317, https://doi.org/10.1007/s10295-010-0774-x.
[52] F.G. Acién, C. Gómez-Serrano, M.M. Morales-Amaral, J.M. Fernández-Sevilla, E. Molina-Grima, Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment? Appl. Microbiol. Biotechnol. 100 (2016) 9013–9022, https://doi.org/10.1007/s00253-016-7835-7.
[53] E. Navarro-López, A. Ruíz-Nieto, A. Ferreira, F.G. Acién, L. Gouveia, Biostimulant potential of *Scenedesmus obliquus* grown in brewery wastewater, Molecules. 25 (2020) 664, https://doi.org/10.3390/molecules25030664.
[54] M. del Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Production of microalgae using centrate from anaerobic digestion as the nutrient source, Algal Res. 9 (2015) 297–305, https://doi.org/10.1016/j.algal.2015.03.018.
[55] M. del Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Outdoor production of *Scenedesmus* sp. in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source, Algal Res. 12 (2015) 99–108, https://doi.org/10.1016/j.algal.2015.08.020.
[56] I.T.D. Cabanelas, Z. Arbib, F.A. Chinalia, C.O. Souza, J.A. Perales, P.F. Almeida, J. I. Druzian, I.A. Nascimento, From waste to energy: Microalgae production in wastewater and glycerol, Appl. Energy. 109 (2013) 283–290, https://doi.org/10.1016/j.apenergy.2013.04.023.
[57] C. Gómez-Serrano, M.M. Morales-Amaral, F.G. Acién, R. Escudero, J.M. Fernández-Sevilla, E. Molina-Grima, Utilization of secondary-treated wastewater for the production of freshwater microalgae, Appl. Microbiol. Biotechnol. 99 (2015) 6931–6944, https://doi.org/10.1007/s00253-015-6694-y.
[58] O. Skulberg, Bioactive chemicals in microalgae, Microalgal Cult. (2007) 485–512, https://doi.org/10.1002/9780470995280.ch30.
[59] Y. Ghasemi, A. Moradian, A. Mohagheghzadeh, S. Shokravi, M. Hossein Morowvat, Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: characterization of antimicrobial activity of *Chroococcus dispersus*, J. Biol. Sci. 7 (2007) 904–910, https://doi.org/10.3923/jbs.2007.904.910.
[60] S. Singh, B. Kate, U.C. Banerjee, Bioactive compounds from cyanobacteria and microalgae: an overview, Crit. Rev. Biotechnol. 25 (2005) 73–95, https://doi.org/10.1080/07388550500248498.
[61] M. Hussien, A. Abd El-All, S. Mostafa, Bioactivity of Algal Extracellular Byproducts on Cercospora Leaf Spot Disease, Growth Performance and Quality of Sugar Beet, in: 4th Conf, Recent Technol. Agric., 2009.
[62] M. Vehapi, A.T. Koçer, A. Yılmaz, D. Özçimen, Investigation of the antifungal effects of algal extracts on apple-infecting fungi, Arch. Microbiol. 202 (2020) 455–471, https://doi.org/10.1007/s00203-019-01760-7.
[63] A. Renu, Antibacterial activities of freshwater algae *Chlorella ellipsoidea*, J Basic Appl Biol. 4 (2010) 22–26.
[64] R. Pratt, T.C. Daniels, J.B. Eiler, J.J. Unnison, W.D. Kumler, J.F. Oneto, L.A. Strait, H.A. Spoehr, G.J. Hardin, H.W. MIilner, J.H.C. Smith, H.H. Strain, Chlorellin, an antibacterial substance from *Chlorella*, Science 99 (1944) 351–352, https://doi.org/10.1126/science.99.2574.351.
[65] J. Moreau, D. Pesando, P. Bernard, B. Caram, J.C. Pionnat, Seasonal variations in the production of antifungal substances by some dictyotales (brown algae) from the french mediterranean coast, Hydrobiologia. 162 (1988) 157–162, https://doi.org/10.1007/BF00014538.
[66] W.A. Stirk, P. Bálint, D. Tarkowská, O. Novák, M. Strnad, V. Ördög, J. van Staden, Hormone profiles in microalgae: gibberellins and brassinosteroids, Plant Physiol. Biochem. 70 (2013) 348–353, https://doi.org/10.1016/j.plaphy.2013.05.037.
[67] J.J. Tate, M.T. Gutierrez-Wing, K.A. Rusch, M.G. Benton, The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae *Chlorella* sp.: a review, J. Plant Growth Regul. 32 (2013) 417–428, https://doi.org/10.1007/s00344-012-9302-8.
[68] B.M. Plaza, C. Gómez-Serrano, F.G. Acién-Fernández, S. Jimenez-Becker, Effect of microalgae hydrolysate foliar application (*Arthrospira platensis* and *Scenedesmus* sp.) on Petunia × hybrida growth, J. Appl. Phycol. 30 (2018) 2359–2365, https://doi.org/10.1007/s10811-018-1427-0.
[69] H.E.L. Arroussi, Microalgae polysaccharides a promising plant growth biostimulant, J. Algal Biomass Util. 7 (4) (2016) 55–63, eISSN: 222.
[70] P. Calvo, L. Nelson, J.W. Kloepper, Agricultural uses of plant biostimulants, Plant Soil 383 (2014) 3–41, https://doi.org/10.1007/s11104-014-2131-8.
[71] V. Ördög, W.A. Stirk, J. Van Staden, O. Novák, M. Strnad, Endogenous cytokikins in three genera of microalgae from the Chlorophyta, J. Phycol. 40 (2004) 88–95, https://doi.org/10.1046/j.1529-8817.2004.03046.x.