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Valorization of wastewater from industrial hydroponic cultivations using the microalgal species *Chlorella vulgaris*

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ABSTRACT

The continuous increase in the world population is associated with a greater demand for food. This need has driven the development of new cultivation systems capable of producing large quantities of vegetable biomass in a small space, with precise and regulated control of the use of resources. Among these, vertical farms are systems developed in height, and they produce continuously throughout the year.

The hydroponic system is commonly used in vertical farming and entails the growth of plants in a solution rich in nutrients, easily assimilated by plants. However, hydroponic solutions still contain nutrient salts at the end of the productive cycle. Consequently, spent hydroponic solutions cannot be directly released into the environment because they would cause water pollution. Thus, they need to be appropriately treated, increasing production costs.

Microalgae represent a cost-effective solution for treating and valorizing hydroponic wastewater. They can easily consume the residual nutrients, generating valuable biomass that can be exploited as a biofertilizer, a biostimulant, or even as a valuable food supplement.

The present work showed the ability of the model eukaryotic microalga *Chlorella vulgaris* to use valuable resources derived from industrial cultivations of basil and tobacco in a hydroponic deep-water culture system. Although the use of spent hydroponic solutions slightly affected the microalgal biomass accumulation of more than ~40 % compared to the fresh solution, possibly due to the presence of root exudates that have an antagonistic effect toward microalgae, the phytoremediation activity of microalgae was achieved. The reported results described the consumption of more than 80 % and 70 % of P and N residual nutrients in hydroponic formulations, respectively. Moreover, more than 2 g/L of microalgal biomass was generated after 7 days of growth in air-lifted photobioreactors. The outcomes of this research provide new insights toward greater sustainability of vertical farming, following circular economy principles.

1. Introduction

The human population is rapidly growing, being estimated to reach 9 billion by the year 2050 [1,2]. This exponential growth is associated with a greater demand for food, which will be hard to satisfy with traditional farming methods, considering that 80 % of arable land is already exploited [3]. Moreover, soilless plant cultivation systems are required to produce food during space exploration and Moon/Mars colonization [4]. To tackle this problem, it is of utmost importance to develop innovative agricultural technologies that can guarantee higher yields of crops with greater quality and, at the same time, reduce the exploitation of natural resources. One solution that can satisfy these needs is vertical farming, hereafter referred to as VF [5,6]. VF is an innovative agricultural practice where crops are grown in vertically stacked layers, and it has been boosted in the last decades by advancements in artificial illumination and soilless cultivation. The main advantage of a VF system is that it allows control of many environmental parameters, ensuring stable productivity and uniform quality of the cultivation. In addition, it ensures lower water usage than open-field farming, and it can exclude the utilization of pesticides [7].

Abbreviations: VF, vertical farming; HS, hydroponic solution; frHS, fresh hydroponic solution; usHS, used hydroponic solution; EC, electrical conductivity; TAP, tris-acetate-phosphate medium.

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There are various types of soil-less cultivation [8]. Hydroponics is a technique used by many commercial greenhouses and vertical farms worldwide [2]. In a hydroponic system, plants grow without soil but are sustained by a neutral and inert substrate (sand, rocky wool, or vegetal fibers). The roots of the plants are immersed in a hydroponic nutrient solution, hereafter referred to as HS, enriched with minerals and salts necessary to support plant growth [9,10]. Currently, vertical farming systems mainly produce crops such as leafy greens (lettuce, arugula, basil), cherry tomatoes, strawberries, and species from the *Nicotiana* family.

One big challenge of hydroponic cultivation relies on the management of liquid wastes [11]. This is because, after the growth of plants, the remaining HSs are still rich in salts that can pollute water resources if freely released into the environment [12]. In addition, continuous recycling of used HSs (usHSs) is not recommended because of the changes in the level of their nutrient composition [13] and, most importantly, the accumulation of root exudates with phytotoxic activity [14,15]. Thus, wise management of hydroponic effluents is paramount to increase VF sustainability. Photoautotrophic microorganisms as microalgae represent a reliable solution for the valorization of hydroponic wastes since they can convert residual salts contained therein to usable biomass [16,17]. Microalgae require primary nutrients such as nitrogen, phosphorus, and carbon, with the first two dissolved as salts in the HS, and the latter easily supplied as bicarbonate or gaseous CO_2. In addition, traces of micronutrients such as calcium, potassium, magnesium, manganese, silicon, zinc, and iron are essential for photoautotrophic organisms and generally available in the hydroponic effluent as well [18].

Among the microalgae isolated and characterized up to now, *Chlorella* species and *Limnospira platensis* (Spirulina) represent 22 % and 53 %, respectively, of the microalgal biomass annually produced at the industrial level [19]. In particular, *Chlorella* has been recently granted the GRAS certification by the FDA and approved for food consumption [20], a factor that will further increase the industrial interest in these green algae. Other applications of *Chlorella* biomass or bioextracts range from being ingredients for livestock feed to being used as plant biostimulant or organic fertilizer or even in cosmetics or wastewater treatment [21–24].

In this work, *Nicotiana tabacum* var. New Virginia (tobacco) and *Ocimum basilicum* var. Genovese (basil) were grown in an innovative industrial vertical farming system. At the end of the plant growth cycles, the hydroponic effluents were collected and then tested for *Chlorella vulgaris* (*C. vulgaris*) cultivation. Nitrogen and phosphorus consumption by the microalgae was then evaluated.

The outcomes of this experimental effort provide new insights toward better exploitation of VF resources using photoautotrophic microorganisms for VF waste valorization.

2. Material and methods

2.1. Plant hydroponic cultivation

Plants were grown hydroponically using the formulation Idrofill Base (https://k-adriatica.it/eng/products/hydroponics/idrofill-base; K-Adriatica, Italy), ~2 g/L, supplemented with 50 mg/L Sequifill 6.0 T SS, a Fe-EDDHA-based product, as an iron source (https://k-adriatica.it/eng/products/meso-and-microelements/sequifill-6.0t-ss; K-Adriatica, Italy). The composition of Idrofill Base is available in Supplementary Table 1. The electrical Conductivity (EC) of the HS was set at 2.4 mS/cm and the pH was adjusted at ~5.5. These two parameters were respectively measured using a DiST4 EC tester and a Checker pHmeter, both from Hanna Instrument (USA). The nutrient solution of the plants kept in the growth chamber was refilled every two days and mild air bubbling was supplied to ensure oxygenation of the HS. In the industrial pilot plant (https://onoexponentialfarming.com/; ONO Exponential Farming, Italy), the pH and EC parameters of the HS were checked and adjusted daily.

Plant species that were cultivated in this work were *Ocimum basilicum cv. genovese* (Basil) and *Nicotiana tabacum* (New Virginia). Plants were grown at a humidity of ~60 % and temperature of ~23 °C. White-red-blue full spectrum LEDs were used to illuminate plants at an intensity of ~70 μmol/m^2/s. Seeds were placed in plastic plugs containing inert jute (Holland BioProducts, The Netherlands) to support plant rooting. Plugs were then put in specific metal grids and trays for hydroponic cultivation, manufactured by Ambrosi Srl (Italy). Seeds were kept in the dark and sprayed with water for the first 2 days after sowing to promote germination.

After germination, seedlings were placed under light, moisturizing the jute supports daily with nutrient solution to avoid drying. ~80 plants/m^2 were cultivated at lab-scale, whereas a plant density of ~200 plants/m^2 was used in the industrial hydroponic system. The hydroponic solution was added depending on the needs of the plants when the hydroponic cultivation was performed at lab-scale in a controlled chamber. The volume of nutrient solution in trays for lab-scale use was ~4 L. Upon reaching heights of ~25 cm and ~35 cm for basil and tobacco, respectively, the refills of the nutrient solution in lab-scale cultivations were stopped for three days before collecting them. In the case of industrial cultivation, plants were grown using a deep-water culture system [2] in 1.2 m^2 steel trays that contained the nutrient solution (~20 L). The refill of the nutrient solution was done daily to maximize biomass productivity, and the growth cycle was stopped when plants reached heights of ~25 cm and ~35 cm for basil and tobacco, respectively. The choice of these heights resulted from previous experiments conducted at the company aimed at optimizing both biomass production and space management inside the VF. At this stage, plants were harvested, and the residual nutrient solution was eventually collected.

2.2. Growth of microalgae

C. vulgaris 211/11P strain (SAG Culture Collection of Algae at Göttingen University) was used in this work. As control media, BG11 [25] and TAP [26] were used for testing autotrophic and mixotrophic growths, respectively. Cultures grown in flasks were kept at a temperature of ~24–26 °C, under 40 μmol/m^2/s of continuous white light. Cultures were inoculated at OD_{720} 0.1, and they all were supplied with 50 mM $NaHCO_3$, serving as a C source and, at the same time, equalizing the pH at ~8 thanks to its buffering capacity.

Growth experiments were performed in air-lifted photobioreactors (PBRs) comprised in a Multicultivator system (PSI, Czech Republic) where CO_2-enriched air (3 %) and high light intensities were supplied. In more detail, Multicultivator tests were conducted at a temperature of 25 °C, and PBRs were illuminated with 150 μmol/m^2/s of white light for the first 12 h. Then, light was increased to 500 μmol/m^2/s and kept for the following 12 h. After these initial steps, light was brought at 1500 μmol/m^2/s and maintained until the end of the growth cycle. Cultures were inoculated at OD_{720} 0.1 and supplied with 3 % CO_2 and 50 mM $NaHCO_3$. Two biological replicates were evaluated *per* each sample condition *per* each experiment. Cell growth experiments were repeated at least twice and cell densities were monitored daily by measuring OD_{720} with a V-730 spectrophotometer (Jasco, Japan) or by cell counting using Countess II-FL (Life Technologies, USA). In the latter, four cell counts per replicate were considered.

At the end of the growth cycle, *C. vulgaris* cells were pelleted down for the analysis of pigments and lyophilization. At the same time, the supernatant was recovered for measuring the pH and EC. Before lyophilization, pelleted cells from a 50 mL culture were washed once with purified water to remove residual salts. Lyophilized cells were eventually weighted to determine dry cell weight.

2.3. Pigments analysis

500 μL of microalgal culture was centrifuged and the pellet was

resuspended in 300 μL of DMSO, as in previous literature [27]. Tubes containing cells and solvent were loaded on a rotatory wheel for 1 h to complete pigment extraction. DMSO extracts were diluted with acetone 95 % to a final concentration of acetone 80 % before absorption spectra were taken in the visible light range (350-750 nm) using the Jasco V-730 spectrophotometer. Deconvolution analysis of spectra from microalgal acetonic extracts was conducted according to the literature [28].

2.4. Nitrogen and phosphorus quantitative analyses

The quantitative analysis of the nitrogen and phosphorous dissolved in the tested liquid solutions before and after microalgae growth, was conducted using a Multiparameter Photometer (Hanna Instruments, USA). Before element analysis, the liquids not used for microalgal growth were filtered (filter pore size 0.45 μm). Instead, the solutions used as microalgal media were centrifuged at 10,000*g* for 10 min to remove the microalgae, and then the supernatant was used for element analysis.

Elements of interest were phosphate and nitrate, analyzed with specific reagent kits, NI93717-01 and HI93766-50, respectively (Hanna Instrument, USA). Analyses were conducted following instructions from the manufacturer.

2.5. Statistical analysis

The statistical significance of cell counts measurements ($n = 2$ biological replicates, 4 counts per replicate) was evaluated comparing results obtained in the same experiment running Tukey-Kramer multiple comparison tests. Statistically significant variations with a p value <0.05 are marked with different letters.

3. Results and discussion

3.1. Growth of C. vulgaris in Erlenmeyer flasks with HS from lab-scale cultivations

C. vulgaris was chosen among different microalgae species for the present experimental effort because of its robustness, high growth rate, and GRAS certification, allowing for possible commercial applications of the biomass produced.

In this work, the compatibility of the salt formulation used for the hydroponic cultivation of tobacco and basil plants with *C. vulgaris* was

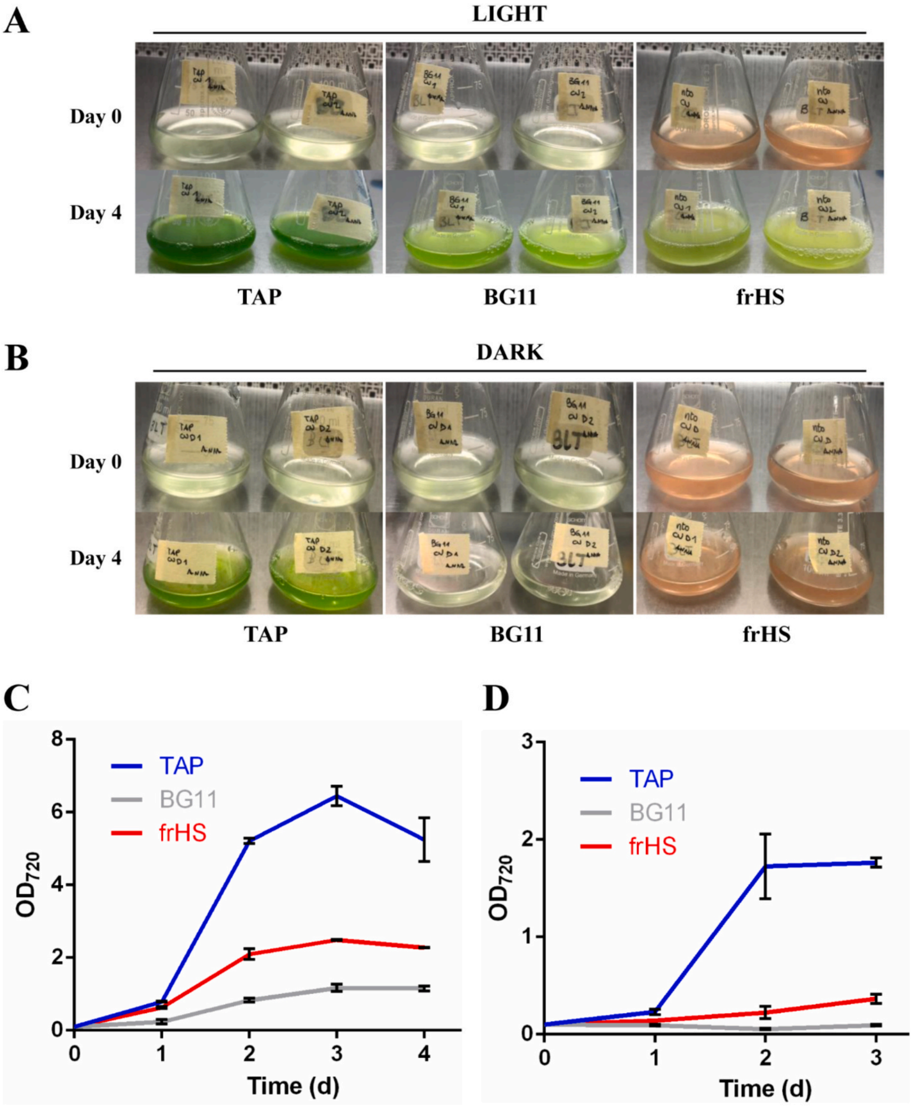

Fig. 1. growth of *C. vulgaris* in Erlenmeyer flasks in the presence (A) or absence of light (B). Microalgae were cultivated for 4 days in the media reported in the figure (TAP, BG11, frHS). Microalgal growths in the presence (C) or absence of light (D) were followed by absorption measurement at 720 nm. Error bars are reported as standard deviations ($n = 2$ biological replicates for OD_{720} analysis).

first evaluated. This robust microalgal strain can grow using nitrate [29] or ammonium [30] as N source. The HS formulation (Supplementary Table 1) contained nitrate, plus several other highly soluble elements, optimal for use in hydroponics. Thus, HS comprised all the required elements to grow photosynthetic organisms, either plants or microalgae. The high amount of calcium ions in HS (Supplementary Table 1) made *C. vulgaris* a better candidate than Spirulina, the most cultivated microalga [19], because the latter had a high optimum pH of ~9–10 [31]. This Spirulina feature was unsuitable for the growth in HS since calcium ions at high pH would have promoted the formation of recalcitrant calcium carbonate aggregates [32].

The freshly prepared commercial hydroponic solution, hereafter referred to as frHS, is the solution used at the hydroponic plant cultivation site of ONO-Exponential Farming (ONO-EF), and its composition is available in Section 2.1. Experimental controls were BG11 and TAP media, used for photoautotrophic and mixotrophic lab-scale cultivations of *C. vulgaris*, respectively [33]. Before algal inoculation, the media were supplemented with 50 mM $NaHCO_3$ to supply a carbon source for

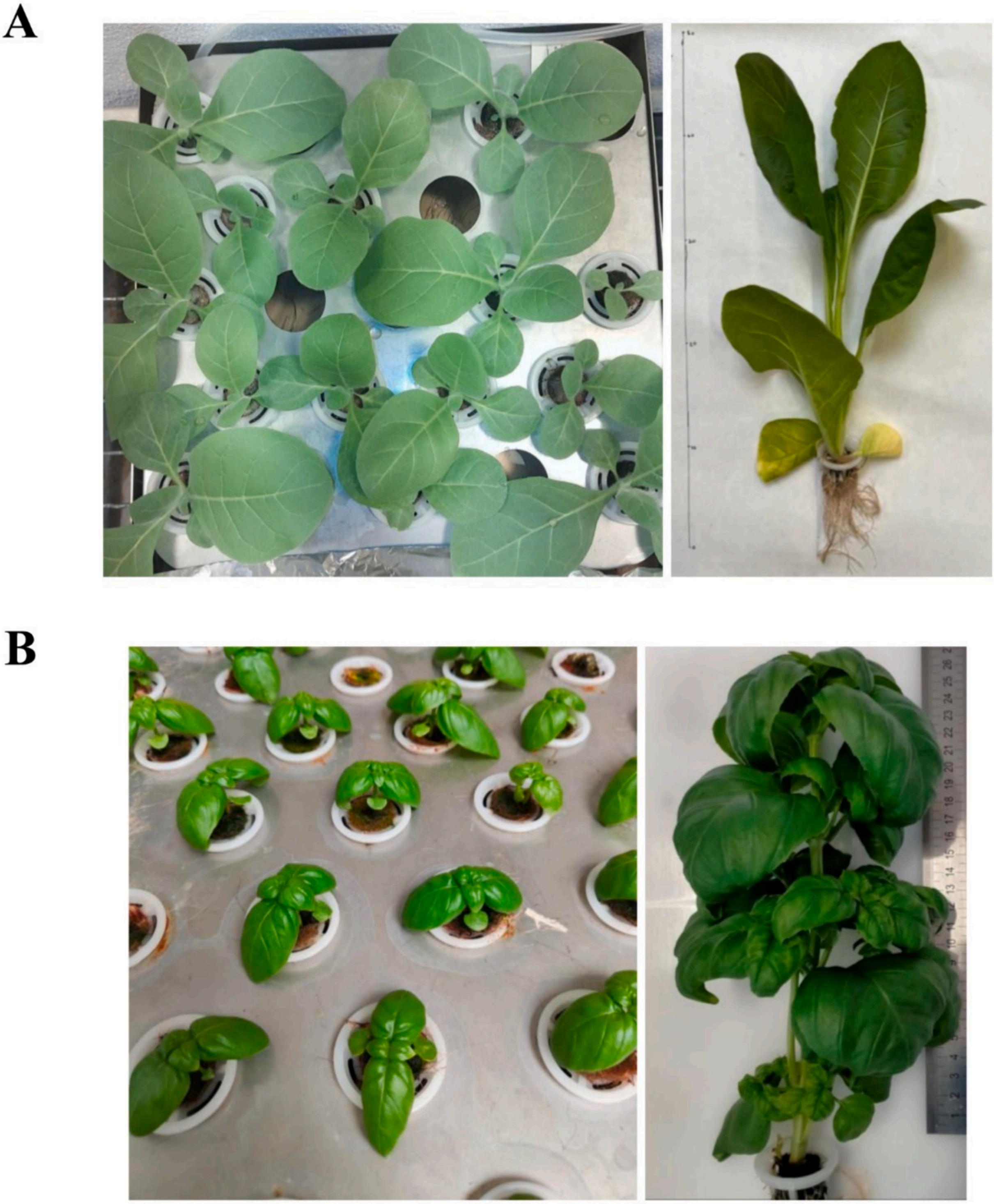

Fig. 2. Representative cultivations of tobacco (A) and basil (B) grown at lab-scale. Plants were grown on hydroponic trays (left) until the end of the growth cycle (right).

photoautotrophic metabolism and equalize the pH at ~8.

The first growth test was conducted in Erlenmeyer flasks, comparing cultures grown in the presence or absence of light (Fig. 1A and B, respectively) to evaluate the possible presence of organic nutrients in frHS.

If present, such nutrients would have sustained the heterotrophic growth of microalgae under dark conditions. Cell growth was followed for four days by daily measurements of OD_{720} with a spectrophotometer. Precise cell count was done only before the initial inoculum and at the closure of the growth cycle (Supplementary Table 2).

In the presence of light, *C. vulgaris* showed significant growth in both frHS and TAP, with the latter assuring the highest cell density based on the OD_{720} measurements (Fig. 1C). BG11, the minimal growth medium for *C. vulgaris*, showed the lowest growth in photoautotrophic conditions.

In the absence of light, microalgal growth was observed in TAP and, at a lower extent, in frHS, while it was absent in BG11 (Fig. 1D). These results suggested the presence of organic substances in frHS, possibly consumed by the microalgal heterotrophic metabolism, as in the case of TAP. Looking at the composition of the salt formulation (Supplementary Table 1), citrate ammonium was present as a stabilizer of phosphorus pentoxide (P_2O_5). Citrate could be the organic molecule in the HS exploited by the heterotrophic metabolism of *C. vulgaris* in dark conditions. The mixotrophic growth of *C. vulgaris* cells in the frHS medium was likely the reason for the enhanced cell growth compared to the BG11 case in light conditions (Fig. 1). However, the reduced carbon source in frHS did not saturate the heterotrophic metabolism of *C. vulgaris* cells, neither in the dark nor upon exposure to light. In fact, further enhanced growth was achieved using TAP medium, in which acetate was available to the cells as a reduced carbon source. Considering previous reports showing cultivation of *Chlorella* species in a mixotrophic condition to maximize biomass accumulation [17,33], the cultivation in frHS could be potentially further increased by adding additional reduced carbon sources such as acetate, sugars, or other C–rich waste products.

Because of the greater growth of microalgae observed in TAP compared to BG11 minimal medium, the first was used as a control in subsequent experiments.

A further development was the evaluation of used HS (usHSs) as cultivation media for *C. vulgaris*. In more detail, usHSs consisted of HSs already exploited by hydroponic plants for their growth, thus possibly depleted of nutrients and containing exudates from plants. To this aim, usHSs were obtained from lab-scale hydroponic cultivations of tobacco and basil, two well-known plant species (Fig. 2A-B). Both frHS and usHSs were characterized by low turbidity, and the contained salts were fully available to microalgae.

The growth of *C. vulgaris* in usHSs was first tested using flasks. TAP and frHS media were used as controls, and $NaHCO_3$ was added to all the media to align the pH to ~8. This step was necessary since HSs had an initial pH that spanned from ~5.5 in the case of frHS to ~6.5–6.8 in the case of usHSs. The EC of usHS from tobacco was ~2.3 mS/cm, thus similar to the EC of frHS corresponding to ~2.4 mS/cm. The high EC of usHSs suggested the presence of nutrients, possibly available for microalgae. Notably, the usHS from tobacco cultivation showed a stronger coloration, indicating an accumulation of iron ions in it. This evidence was likely due to the concentration of the HS occurring in the lab-scale cultivation, possibly caused by water consumption by plants and evaporation. In fact, plants were cultivated in a small steel tray containing a low volume of HS (see M&M), and such nutrient solution was rapidly consumed once plants reached maturity. Instead, usHS collected from basil cultures had a slightly elevated electroconductivity of ~3.2 mS/cm. Thus, the latter was diluted with osmotized water (0.33 volumes) to adjust the EC to 2.4 mS/cm, as in the cases of the other media. OD_{720} measurements and cell counts were conducted daily for

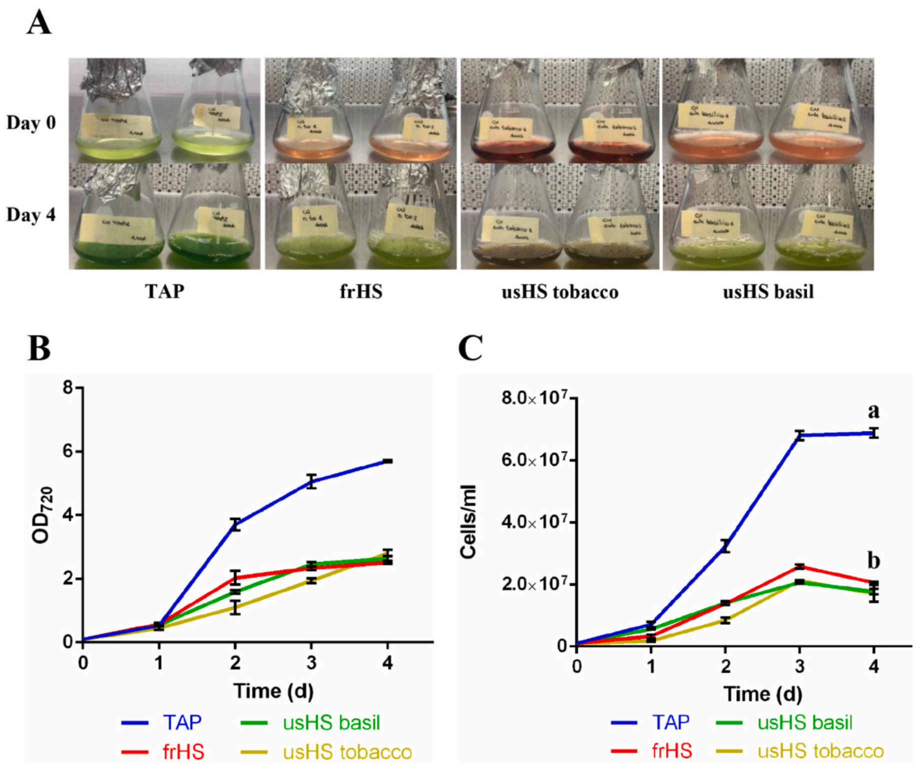

Fig. 3. Growth of *C. vulgaris* in spent hydroponic solutions from lab-scale cultivations (A). Microalgae were cultivated in flasks for 4 days in used solutions from cultivations of tobacco (usHS tobacco) and basil (usHS basil). TAP and frHS were used as control media. Microalgal growths were followed by absorption measurements at 720 nm (B) and by cell counts (C). Error bars are reported as standard deviations ($n = 2$ biological replicates for OD_{720} analysis; $n = 2$ biological replicates, 4 counts per replicate for cell counts, statistical significance is expressed by different letters according to Tukey-Kramer test).

four days in total. Results confirmed that usHSs could be used for growing *C. vulgaris* (Fig. 3A).

In more detail, the microalgal growth in usHSs after 4 days of cultivation did not significantly differ from the growth achieved in frHS (Fig. 3B-C). This evidence confirmed the presence of nutrients in usHSs, exploitable by microalgae.

3.2. *Growth of C. vulgaris in air-lifted PBRs with HS from lab-scale cultivations*

After successful growth in flasks, the same media were tested in air-lifted PBRs using a Multicultivator system (PSI, Czech Republic), in which non-limiting CO_2 and light were supplied to maximize the microalgal growth in the tested media (Fig. 4A).

Microalgal cultivations were carried out for four days and monitored by measuring OD_{720} (Fig. 4B). Cell counts were done on the first and the last day of growth (Table 1).

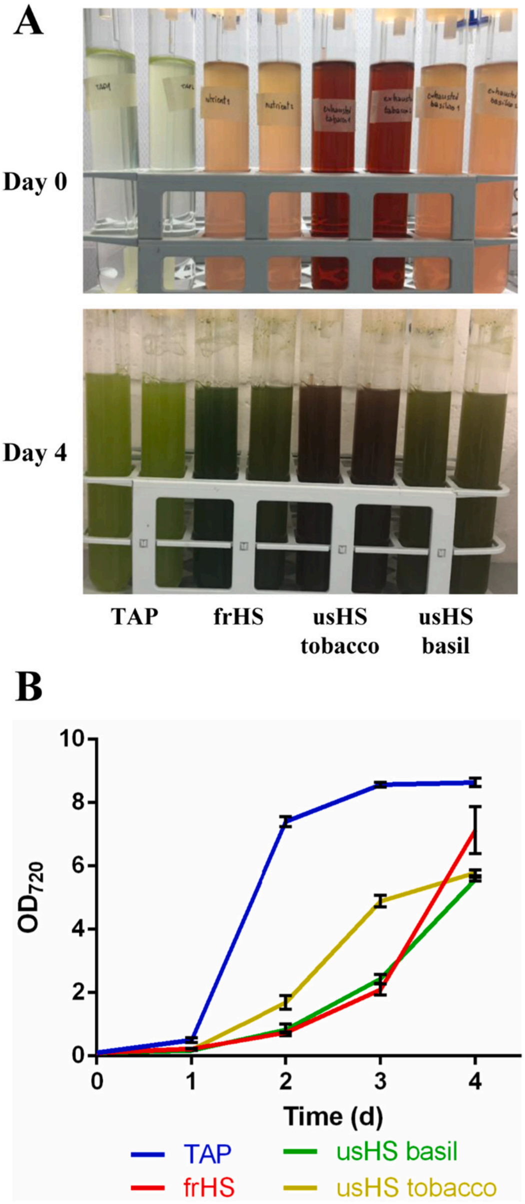

Fig. 4. Growth of *C. vulgaris* in air-lifted PBRs (A). Microalgae were cultivated for 4 days using media tested in Fig. 3. Microalgal growths were followed by absorption measurements at 720 nm (B). Error bars are reported as standard deviations ($n = 2$ biological replicates for OD_{720} analysis).

Table 1
Cell counts following the growth of *C. vulgaris* in air-lifted PBRs. For all conditions a initial inoculum of 1.68E+6 cells was used. Error is reported as standard deviation (n = 2 biological replicates; 4 counts per replicate). Statistical significance is expressed by different letters according to Tukey-Kramer test.

Day	TAP (Cells/mL ± SD)	frHS (Cells/mL ± SD)	usHS tobacco (Cells/mL ± SD)	usHS basil (Cells/mL ± SD)
4	1.27E+08^{a} ± 6.48E+06	8.43E+07^{b} ± 1.41E+07	6.98E+07^{b} ± 1.98E+06	7.59E+07^{b} ± 1.70E+07

As observed in Fig. 4A, microalgae grew significantly in all the tested conditions. In more detail, *C. vulgaris* showed a slightly faster growth induction in usHS from tobacco compared to usHS from basil or frHS. Anyway, cell density was greater on day 4 in frHS than in both usHSs. As expected, the cell accumulation was lower in flasks than in the Multicultivator, achieving OD_{720} of ~2.5 in frHS flask culture at its maximum, whereas it rose to ~7 in Multicultivator bioreactors.

The results described so far demonstrated the feasibility of growing *C. vulgaris* in HSs from lab-scale cultivations.

3.3. *Microalgal valorization of spent HSs from industrial cultivations*

The next step was the exploitation of *C. vulgaris* to valorize effluents from an industrial hydroponic pilot plant. To this aim, usHSs were gently provided by the pilot plant of the company ONO Exponential Farming (Santa Maria di Zevio, Italy), hereafter referred to as ONO-EF. At the ONO-EF production site, plants were grown at a high-density setting in steel trays containing the nutrient solution (Fig. 5A), until reaching heights of ~25 cm and ~35 cm for basil and tobacco, respectively. The daily and automated adjustments of both EC and pH of the nutrient solution were of utmost importance to boost biomass productivity, ensuring, at the same time, the quality of the final product.

At the end of the growth cycle, usHSs from basil and tobacco cultivations were collected (Fig. 5B) and tested for the cultivation of *C. vulgaris*. Growth in flasks was monitored on days 0 and 4 with OD_{720} measurements and cell counts (Supplementary Fig. 1). The greatest cell accumulation was observed in the TAP medium, followed by both usHSs. The lowest cell accumulation was observed in the frHS culture (Supplementary Table 3).

Subsequent tests were conducted in a Multicultivator system to maximize microalgal growth, providing higher irradiances (up to 1500 $\mu mol/m^2/s$) and 3 % CO_2 (Fig. 6A).

Cell density was followed for 7 days by measuring OD_{720} and counting cells daily (Fig. 6B-C). Growth of *C. vulgaris* significantly increased in the air-lifted PBRs compared to flasks (Fig. 6C). Microalgae grown in the TAP medium showed the fastest growth since they were in the exponential phase on day 2. Upon three days of cultivation, the growth rate diminished, and the culture reached the stationary phase. The greatest cell density in the 7 days cultivation was observed using frHS as a medium. This agreed with biomass accumulation, as shown in Supplementary Table 4. Microalgal cultures in usHSs grew similarly to the frHS culture during the first 3 days of cultivation, followed by an early stationary phase not observed in the latter (Fig. 6B-C). Absorption spectra of pigments extracted from microalgal cells were taken in the range of visible light (Fig. 6D). Spectra were normalized at the maximum absorption of chlorophyll *a* at ~663 nm. Evidence of different absorptions in the blue region (~450–500 nm) of normalized spectra indicated differential accumulation of carotenoids in the microalgal cells. The absorption spectrum showing the highest component attributable to carotenoids was obtained from TAP culture, followed by usHS cultures. This suggested the occurrence of stress conditions in such cultures. In parallel, the spectrum from frHS cells, representing the culture with the greatest cell density, showed the lowest carotenoid contribution among the growth conditions herein tested. Deconvolution

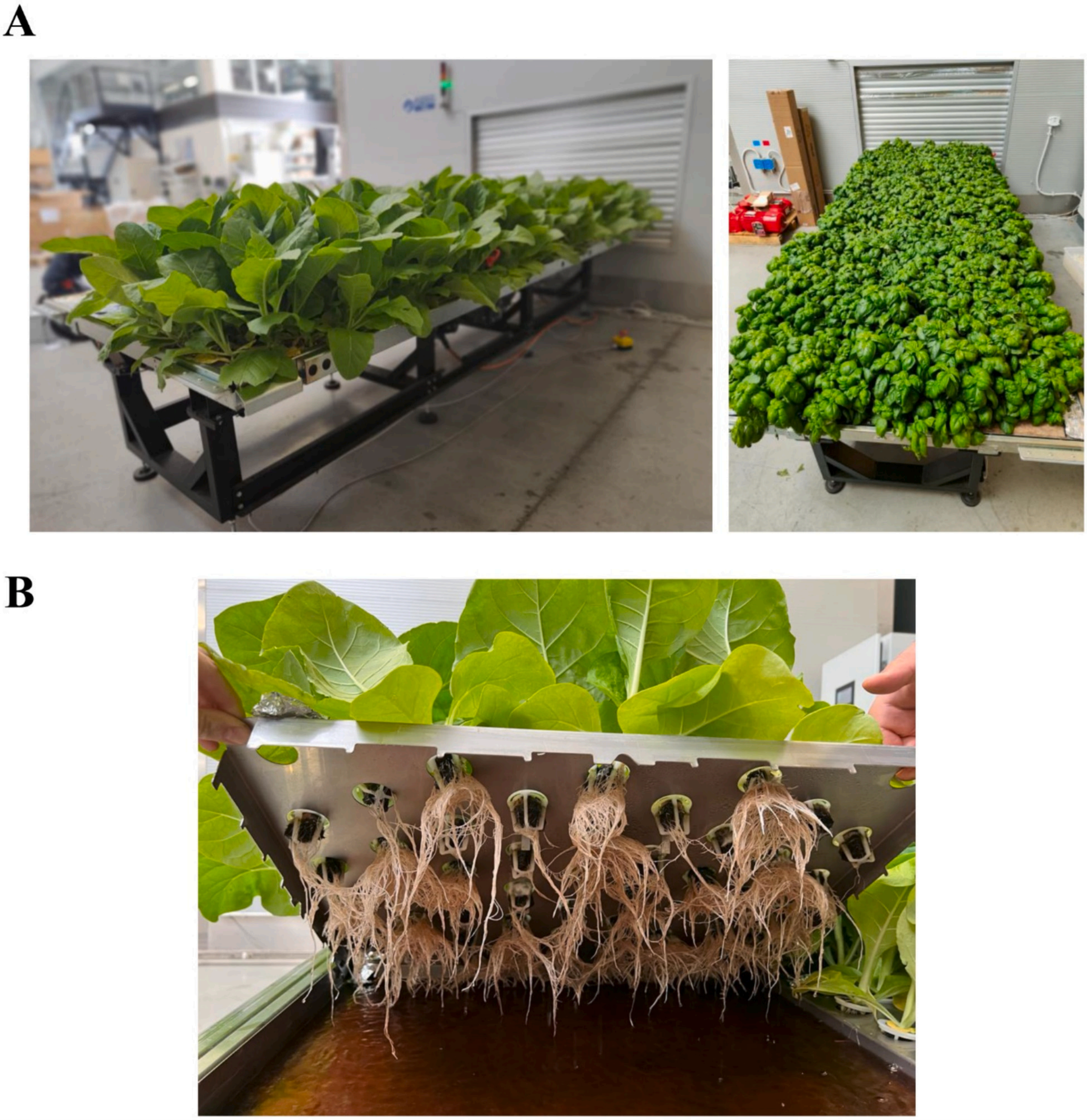

Fig. 5. Industrial cultivations of hydroponic tobacco (A) and basil (B). Plants were grown on hydroponic trays containing ~20 L of hydroponic solution (C).

analysis [28] of the available spectra confirmed the greater carotenoid content on a Chl basis of TAP and usHSs compared to frHS sample (Table 2). In addition, a slight increase of the Chl *a/b* ratio was observed in such stressed sample, suggesting a reduction of the Chl *b*-binding antenna complexes comprised in the photosynthetic apparatus [34]. Of interest was the evidence that the highest content of photosynthetic pigments (chlorophylls and carotenoids) per biomass was in frHS cultures while the lowest content was observed in mixotrophy conditions (TAP). These findings are also consistent with previous observations about decreased pigment content in *Chlorella* species grown in mixotrophic conditions compared to autotrophic growth [33]. The increased pigment content per biomass in frHS coltures corroborates previous indications of best photosynthetic performances in this condition (Supplementary Table 4).

It is worth mentioning that pH in all the treatments was ~8 upon 7 days of growth (Supplementary Table 5), suggesting that it was not the factor affecting microalgal biomass production in usHSs.

The lower growth of *C. vulgaris* observed in usHSs was likely due to factors other than macronutrient shortage since the EC of usHSs was adjusted daily in the industrial plant, as mentioned above. However, a possible specific depletion of some micronutrients in the usHSs cannot be excluded, which could partially affect cell growth. Similarly, the pH of the cultures was probably not affecting the microalgal growth as well since it remained ~8 after microalgal cultivation in all the treatments (Supplementary Tables 5–6). A critical factor impacting microalgal growth was probably the release of secondary metabolites by the plant root system with allelopathic effects on microalgae. Plants cultivated in hydroponic systems released high amounts of root exudates [14,35], and it was recently shown that benzoic acid could negatively affect the growth of *C. vulgaris* [36]. The hydroponic system developed at ONO-EF was a deep-water culture system [2], where plants were suspended on a defined volume of HS, with the roots fully submerged in it (Fig. 5, see

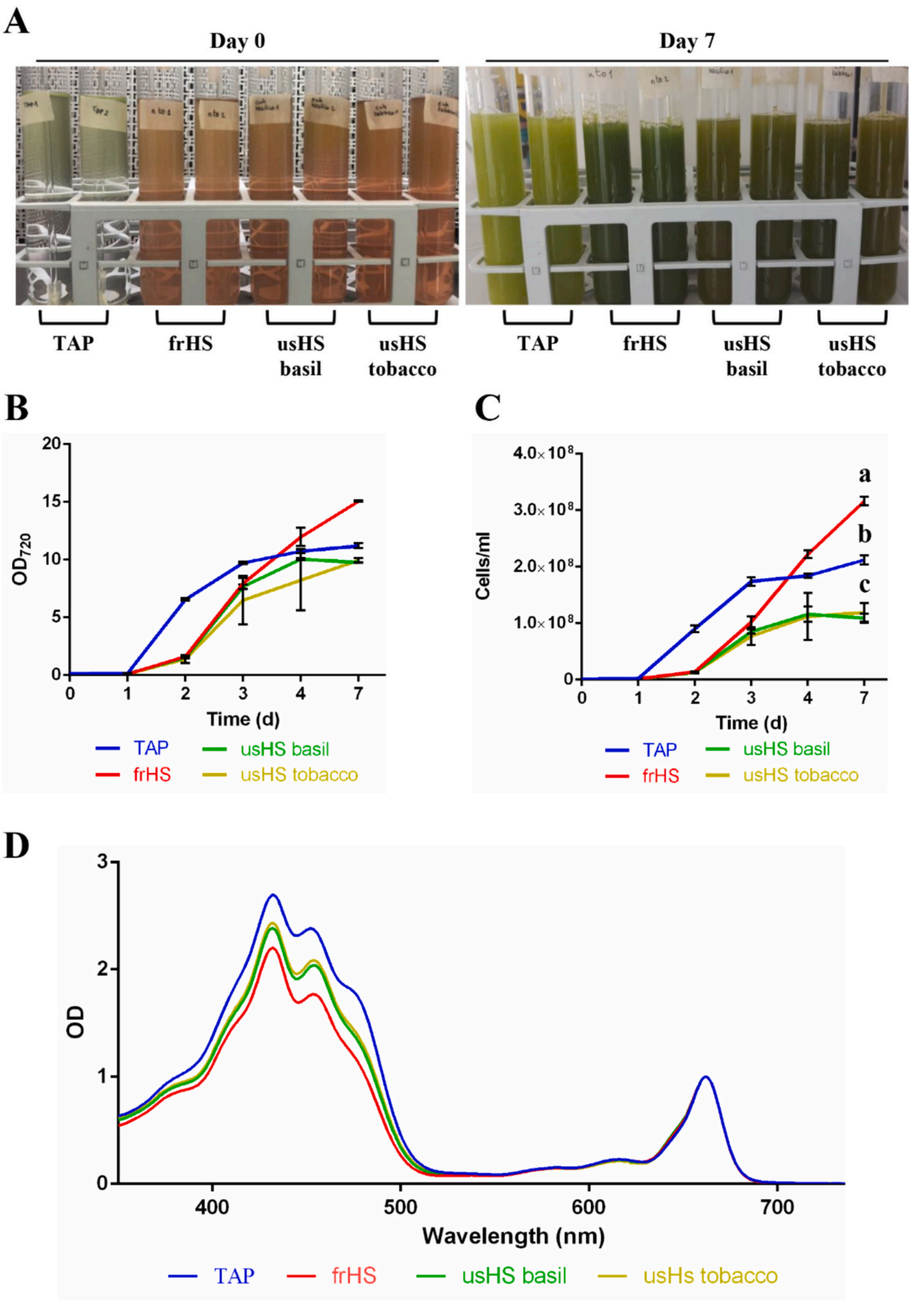

Fig. 6. 7 days cultivation of *C. vulgaris* in used hydroponic solutions from an industrial vertical farm (A). Microalgae were cultivated in a Multicultivator system using used solutions from cultivations of tobacco (usHS tobacco) and basil (usHS basil). TAP and frHS were used as control media. Microalgal growths were followed by absorption measurements at 720 nm (B) and by cell counts (C). Absorption spectra from extracted microalgal pigments were taken in the range of visible light (D). Error bars are reported as standard deviations ($n = 2$ biological replicates for OD_{720} analysis; n = 2 biological replicates, 4 counts per replicate for cell counts, statistical significance is expressed by different letters according to Tukey-Kramer test).

Table 2
Pigment analysis of microalgal extracts ($n = 2$ biological replicates). Biomass accumulations were taken from Supplementary Table 4.

	Chl *a*/*b* (Value ± SD)		Chl/Car (Value ± SD)		mgChls/g biomass (Value ± SD)		mgCar/g biomass (Value ± SD)	
TAP	2.79	±0.031	1.29	±0.020	7.85	±0.13	6.10	±0.12
frHS	2.50	±0.001	2.14	±0.031	20.23	±0.70	9.46	±0.36
usHS basil	2.53	±0.021	1.81	±0.055	16.44	±1.50	9.06	±0.83
usHS tobacco	2.56	±0.004	1.67	±0.002	9.64	±2.45	5.76	±1.47

M&M for more information). This cultivation system could have facilitated the accumulation of allelopathic exudates against microalgae. In previous literature, microalgal growth was not affected by using drainage solutions from greenhouses [37], probably because plant exudates had not accumulated. Such solutions were in contact only temporarily with the soil used for cultivation, possibly reducing the extraction of exudates from the latter. Thus, if the exudates in usHSs were under the toxicity threshold, the efficient growth of microalgae for the valorization of wastewater would still be possible, in agreement with recent literature [36].

At the end of the growth cycle, element analyses of the filtered media were performed to evaluate the consumption of nutrients. In particular, the contents of phosphates and nitrates were investigated because they were both abundant in the HS formulation, 5 % and 10 %, respectively. Such analyses were conducted using spectrophotometric assay kits not altered by the high heterogeneity of HSs (see M&M for more information). On the contrary, the reliability of the detection kits used for other elements was heavily affected (e.g. K, Mg, and Fe). In Table 3 it is shown that there were significant reductions in the amounts of nitrate and phosphate ions in media collected from 7-day-old cultures.

In more detail, phosphates were reduced by at least 77 %, as observed in TAP samples, surpassing 90 % consumption in usHS from basil. Regarding nitrate ions depletion, the most significant reduction was observed in the frHS sample, corresponding to 93 %, whereas both usHSs reached 70 % consumption. It is worth noting that the assay herein adopted revealed the presence of traces of nitrate in the TAP medium: because TAP contained ammonium and not nitrate, the values measured as nitrate content should be considered as a non-specific background signal.

To further increase nutrient depletion in usHSs, the cultivation of *C. vulgaris* in the air-lifted PBRs was prolonged to 10 days (Fig. 7A).

Microalgal cultures grown in frHS and usHSs showed a stable increase in cell density until 7 days of growth, but the cell density dropped in the following days (Fig. 7B-C). Cultures grown in TAP showed a faster growth induction than other samples, followed by a reduction in the growth rate. Surprisingly, cell density increased again in TAP cultures between 7 and 10 days of cultivation, differently from other evaluated samples. The cultures all showed pH values of ~8 upon 10 days of growth (Supplementary Table 6). This evidence corroborated the previous indication (Supplementary Table 5) that pH was not a critical factor affecting the different cultures. Absorption spectra analysis suggested that cultures accumulated similar amounts of carotenoids since the spectra in the blue region did not show relevant differences (Fig. 7D).

Concentrations of nitrate and phosphate ions were analyzed in the media after microalgae cultivation to assess the capacity of the photosynthetic cells to remove these nutrients/pollutants and convert them into organic biomass. Nitrate and phosphate ions were primarily removed from the investigated media, reaching residual concentrations of ~250–350 mg/L and ~30–40 mg/L, respectively. However, complete depletion was not achieved even with the prolonged cultivation from 7 to 10 days (Table 4).

Surprisingly, the contents of phosphates increased from day 7 to day 10 in some cases (e.g., TAP and frHS samples), possibly due to the release of metabolites from microalgae or even to cell lysis.

To summarize, it was observed that a significant consumption of two elements, N and P, both important for cell metabolism, in the media before and after the growth of *C. vulgaris* cells. This proved the activity of phytoremediation done by the microalgae (Table 3–4). Anyway, complete consumption of such nutrients contained in HSs by microalgae was not achieved. From the results obtained, the Italian legal limit (Decreto Legislativo n°152 del 03/04/2006, allegato 5, parte terza) needed for phosphates was secured, corresponding to 10 mg/L, in the case of usHS from tobacco (9 mg/L, Table 3). Conversely, further work is still necessary regarding the nitrates before freely discharging the usHSs. The current Italian limit corresponds to 30 mg/L, and both basil and tobacco usHSs still contained a relevant amount of nitrates, ~300 mg/L, after microalgal growth (Table 3). To achieve sufficient removal of nutrients from uHS solution, a possible solution could be the removal of the generated microalgal biomass to proceed with other cultivation cycles until complete consumption of nitrates and phosphates.

Further analyses are required to identify exudates released from the plants into the HSs, possibly identifying those impacting microalgae. However, a slow and controlled supply of nutrients to the microalgae in a continuous system could reduce the negative effect of plant exudates. Furthermore, exudates could be chemically removed upon treatment with UV/H_2O_2, detoxifying the HS [38]. These adjustments could allow a greater growth of microalgae and, consequently, a greater consumption of elements of interest to help usHS reach the legal threshold for levels of N and P to be discharged directly into the drainage system.

4. Conclusions

The development of innovative agricultural technologies is necessary to guarantee high yields and to save at the same time natural resources. Efficient cultivation methods in soilless systems based on the circular valorization of wastes are also required for future space explorations and colonization of the Moon or Mars [4]. VF can be part of the solution for satisfying these needs, but, as previously mentioned, wastewater management is one of the biggest challenges of VF systems, especially hydroponic VF. A solution could be the dilution of the HS before release, but it would require the consumption of additional precious freshwater [12]. Nutrients in used solutions could also be consumed directly by the plants growing in other trays inside the VF. However, it is worth considering that prolonged recycling of usHSs resulted detrimental in several cultures because of the imbalance of their nutrient composition [13] and the accumulation of root exudates with allelopathic activity [14,15]. A possible solution could be the sacrifice of some trays inside the VF, specifically designated to consume wastewater from plants grown on other trays. However, this approach could lead to the generation of low-quality products, and it would cause the reduction of trays exclusively used for production inside the VF. Thus, usHSs need to be wisely managed and, possibly, valorized.

As mentioned in previous literature [12,17,37,39,40], microalgae can valorize wastewater from hydroponic cultivations. This work aimed to evaluate the efficacy of microalgae in reducing the nutrient content of spent solutions from an industrial VF plant. The algal biomass of 2,16 g/L and 2,3 g/L (Supplementary Table 4) generated upon 7 days of growth in used solutions from industrial cultivations of basil and tobacco, respectively, could be further exploited. *Chlorella* species have been recently certified by the FDA to be used in the human diet [20], and, consequently, the value of its biomass is projected to increase because of

Table 3
Analysis of the consumption of phosphate and nitrate ions after 7 days cultivation of *C. vulgaris* in air-lifted PBRs. The change in the content of target ions between day 7 and day 0 (diff) is expressed in %. Nitrate content in TAP medium represents the background signal as TAP does not contain nitrate as N source.

Day	PO_4^{3-} (mg/L)				NO_3^- (mg/L)			
	TAP	frHs	usHS from basil	usHS from tobacco	TAP	frHS	usHS from basil	usHS from tobacco
0	113	130	92	130	68	1098	1186	1018
7	26	25	18	9	64	80	350	306
diff	−77 %	−81 %	−80 %	−93 %	−6 %	−93 %	−70 %	−70 %

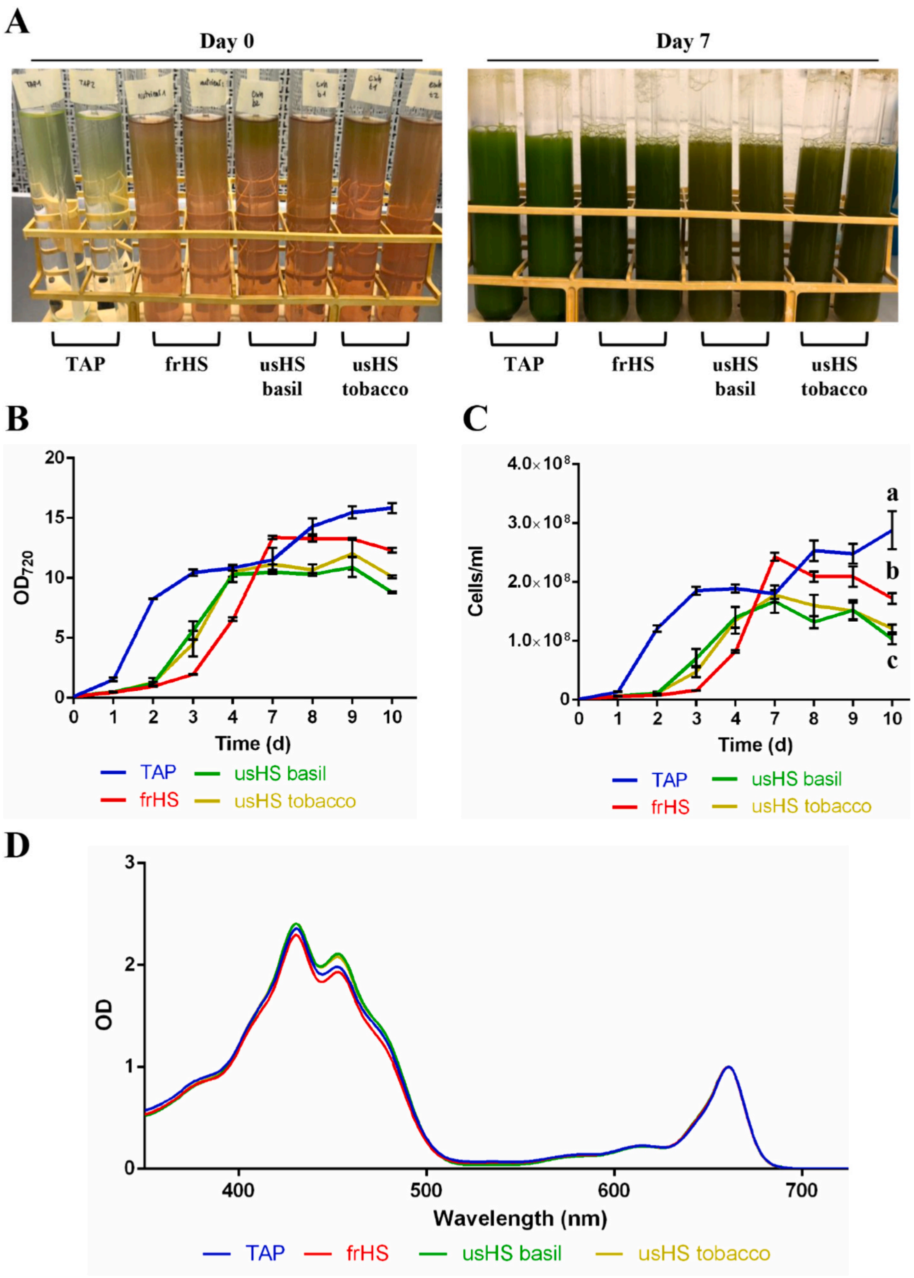

Fig. 7. 10 days cultivation of *C. vulgaris* in used hydroponic solutions from an industrial vertical farm (A). Microalgae were cultivated in air-lifted PBRs using solutions indicated in Fig. 6. Microalgal growths were followed by absorption measurements at 720 nm (B) and cell counts (C). Absorption spectra from extracted microalgal pigments were taken in the visible light range (D). Error bars are reported as standard deviations ($n = 2$ for OD_{720} measurements; n = 2 biological replicates, 4 counts per replicate for cell counts, statistical significance is expressed by different letters according to Tukey-Kramer test).

Table 4
Analysis of the consumption of phosphate and nitrate ions after 10 days of cultivation of *C. vulgaris* in air-lifted PBRs. The change in the content of target ions between day 7–8–9-10 and day 0 (diff) is expressed in %. Nitrate content in TAP medium represents background signal, as TAP does not contain nitrate as N source.

Day	PO_4^{3-} (mg/L)				NO_3^- (mg/L)			
	TAP	frHS	usHS from basil	usHS from tobacco	TAP	frHS	usHS from basil	usHS from tobacco
0	110	89	94	134	68	842	804	904
7	24	20	27	27	84	220	342	310
diff	−78 %	−78 %	−71 %	−80 %	24 %	−74 %	−57 %	−66 %
8	28	22	26	37	116	180	402	242
diff	−75 %	−75 %	−72 %	−72 %	71 %	−79 %	−50 %	−73 %
9	54	33	23	42	106	168	336	268
diff	−51 %	−63 %	−75 %	−69 %	56 %	−80 %	−58 %	−70 %
10	57	45	36	30	98	138	424	286
diff	−48 %	−49 %	−62 %	−78 %	44 %	−84 %	−47 %	−68 %

this. In addition, such microalga has other applications, such as supplements, cosmetics, and pharmaceuticals [22]. *Chlorella* can also have a biostimulant effect on crops [21,41]. Following principles of circular economy, the application of *Chlorella* as an organic fertilizer or a biostimulant would remarkably contribute to the environmental and economic sustainability of the VF [42].

In summary, the ability of *C. vulgaris* to exploit and valorize resources derived from industrial hydroponic cultivations of plants was shown. In particular:

- *C. vulgaris* can be efficiently cultivated using highly soluble salts for hydroponic application. In addition, the heterotrophic metabolism of the microalga exploited traces of organic carbon present in such salts (Figs. 1, 6).
- *C. vulgaris* consumed more than 70 % and 80 % of nitrate and phosphate salts in spent hydroponic solutions, respectively.
- The exploitation of wastewater from tobacco and basil hydroponic cultivations resulted in more than 2 g/L of microalgal biomass in 7 days.

Further work is required to assess the possible elements affecting microalgal growth in spent waters. Still, the reported results will contribute toward implementing more sustainable VF systems and a further boost of microalgal-based approaches.

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CRediT authorship contribution statement

Kristina Ljumović: Writing – review & editing, Investigation, Formal analysis. **Nico Betterle:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Conceptualization. **Anna Baietta:** Investigation, Formal analysis. **Matteo Ballottari:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matteo Ballottari reports financial support was provided by Cariverona Foundation. Matteo Ballottari reports financial support and equipment, drugs, or supplies were provided by ONO Exponential Farming. Matteo Ballottari reports a relationship with ONO Exponential Farming that includes: board membership.

Data availability statement

All relevant data have been included in the article.

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Appendix A. Supplementary data

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