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Microalgal cultivation on ricotta cheese whey: Modulating nutrient supply during cultivation to improve culture growth

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ABSTRACT

Dairy industry generates significant effluent streams, comprising wastewaters and nutrient-rich by-products such as cheese whey, which pose environmental challenges for disposal, but also offer valorisation opportunities. Microalgae cultivation on whey represents a sustainable biotechnological approach that couples biomass production with effluent treatment. This study aimed at optimizing microalgal cultivation using ricotta cheese whey as substrate. Eighteen microalgal strains, mainly belonging to *Scenedesmus* and *Chlorella*, were initially screened for their ability to grow on whey. Selected strains were further tested in 300-mL photobioreactors under different management options: Low Whey Load (LWL), High Whey Load (HWL), Salinity and Micronutrient (S&M), and Moderate Whey Load (MWL) trials. Results showed that most of the tested strains grew better when cultivated on diluted whey than on standard synthetic media, with increases from 56 to 590 % during the first days, and with high removal efficiencies. Several issues were identified during the trials, including nutrient shortage, salinity increase, micronutrients deficiency and potential inhibitory effects of whey compounds, which have hindered long term cultivation of microalgae on whey. In MWL trial, optimization of whey concentration (12 % v/v), replenishment strategy (30 % of the culture volume every 3-days), and micronutrient supplementation overcame these issues extending the cultivation period and improving biomass yield (203.6 billion cells L^{-1} $whey^{-1}$ with the best strain). Overall, this work underscores the importance of carefully optimize microalgal cultivation management to maximise exploitation of dairy by-products and minimize growth-limiting effects of whey accumulation, thus offering a promising starting point for large-scale applications of whey in microalgae cultivation.

1. Introduction

Food-processing activity generally requires a remarkable amount of water in every step of the process. In the dairy industry, from 6 to 10 litres of effluents are generated per litre of processed milk and per kilogram of cheese produced [1,2], comprising wastewaters and by-products such as milk whey. Ricotta, with about 1 million tons per year, is one of the main typical Italian and Mediterranean spread cheese produced [3]. Whey is the liquid by-product remaining after the precipitation of milk proteins during ricotta production. The high amount of ricotta cheese whey (scotta) generated by this production process can represent both an environmental challenge for its disposal and an economic opportunity [3]. Today, most of the cheese whey produced is used as animal feed and fertiliser, which are low-value products [4], and still approximately 40 % of the whey generated globally is discharged in water streams, leading to ecosystem damages such as salinization and eutrophication, and to loss of valuable nutrients (N, P, minerals, vitamins, proteins, sugars and bioactive compounds) [4,5]. Over the years, several alternatives have been explored to reuse and valorise this by-product of the dairy industry [6]. For instance, it has been used as additive for alfalfa silage improving its fermentation characteristics [7], as an ingredient to develop electrolyte replenishment beverages [8], as a substrate in the production of lactic acid, bioethanol and bioplastic [9,10] and as a component for artisanal beer production [11].

The increasing demand for sustainable biotechnological solutions and circular economy approaches has drawn considerable attention toward microalgae ability to efficiently utilize agro-industrial wastewaters and by-products, such as cheese whey, for their growth [12–14]. Considering that microalgae, due the variable and versatile composition of the biomasses, may be considered among the most promising biorefinery feedstock, providing alternatives for different sectors, such as food, agriculture, feed, plastics, cosmetics and health industries, as well

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as for biofuels [13], their cultivation in dairy by-products can significantly increase the added value generated [15].

The advantages of cultivating microalgae in cheese whey are several, such as *i*) production of valuable biomass using organic and inorganic carbon, nitrogen, phosphorus and minerals contained in whey, without the need for additional synthetic nutrients; *ii*) treatment of potentially harmful effluents; *iii*) CO_2 sequestration which is more efficient than that of terrestrial plants; and *iv*) possibility of extracting high value products (lipids, proteins, carbohydrates, bioactive compounds) from the produced biomass [13,14,16]. Moreover, cultivating microalgae in whey might exploit the ability of some strains to utilise organic carbon in mixotrophic conditions showing an enhanced and faster growth compared to the cultivation in photoautotrophic condition [12,14,17–19]. Therefore, developing efficient, cost-effective microalgal cultivation strategies using cheese whey can provide a significant contribution to the advancement of circular economy models. However, although microalgal cultivation on dairy effluents is well-documented [20–23], one of the main bottlenecks is maintaining a consistent and high microalgal growth and productivity over time, which is often achieved by adding inorganic synthetic nutrients [24]. Efficient and less-expensive nutrient supply strategies, as well as a rational use of whey during growth, could enhance productivity and meet the growing demand for circularity and sustainability in resource use.

In many works the capability of hydrolysing lactose in dairy effluents was examined, which may suggest a potential for the utilization of sugars in whey [20,21,25–27] in addition to organic and inorganic compounds present. Among the microalgae investigated, species belonging to the genera *Chlorella*, *Scenedesmus*, and *Tetradesmus obliquus* and *Auxenochlorella protothecoides* have demonstrated high growth rates and biomass productivity when cultivated phototrophically on dairy effluents [20–22,25,28–35]. In the vast majority of these studies cultivation experiments were performed in batch and in many of those diluted dairy effluents were supplemented with inorganic synthetic nutrients to reach the desired nitrogen and phosphorous concentration and provide other nutrients that may lack in effluents [20,21,29,33]. The use of diluted cheese whey supplemented solely with trace elements solution is little reported [25]. Therefore, to the best of our knowledge, limited information is available on the use of diluted dairy effluents for microalgal cultivation as the sole substrate without the addition of external macronutrients.

In this work different culture management options were tested, including different initial nitrogen concentrations and different strategies of nutrient replenishment as fresh whey during the trial, to explore the potential of microalgae to grow on ricotta cheese whey (scotta). The investigation focused on biomass production and nutrient (N and P) removal capabilities of selected microalgal strains trying to optimize operative procedures and whey management in order to provide the basis for a future application in an industrial scale process.

2. Materials and methods

2.1. Strains, photobioreactors and cultivation parameters

In the first part of the work (i.e. the screening phase) 18 microalgal strains were tested for their ability to grow on diluted ricotta cheese whey. The 18 strains were chosen based on their isolation environment and on our previous unpublished data on cultivation in municipal and livestock wastewaters or with glucose as carbon source, and for some of them, on results on bioactivity (in vitro test with *Arabidopsis thaliana*). The selection was also supported by existing literature on mixotrophic ability, with particular focus on dairy effluents [25–35], and bioactivity of the genera considered [36–39] in view of the potential application of the produced biomass. Strains were provided by Fotosintetica & Microbiologica Microalgae and Cyanobacteria Culture Collection (Florence, Italy) and were routinely sub-cultured throughout the experimental period to maintain actively growing cultures. Fifteen strains were of freshwater origin, mainly belonging to *Scenedesmus* (F&M-M267, F&M-M274, F&M-M526, F&M-M269, F&M-M16, F&M-M497, F&M-M263, F&M-M259, F&M-M405) genus. The other freshwater strains belonged to *Chlorella* (F&M-M151, F&M-M262, F&M-M523), *Chlorococcum* (F&M-M150), *Trebouxia* (F&M-M155) and *Nannochloris* (F&M-M14) genera. Three strains were of marine origin and belonged to *Chlorella* (F&M-M180 and F&M-M467) and *Tetraselmis* (F&M-M33) genera.

Trials were performed under controlled laboratory conditions. The Screening Phase (SP) was performed in 100 mL Erlenmeyer flasks with 35 mL working volume kept in an orbital shaker incubator (INR-401, Gallenkamp, Cambridge, UK) at 75 rpm, under continuous illumination (50 μmol photons m^{-2} s^{-1}) and at a constant temperature of 30°C. Pure CO_2 was continuously injected in the cultivation chamber to enhance growth and regulate pH between 7.50 and 8.50. To help keeping the pH in that range, a 1 M $NaHCO_3$ solution was added to the growing media as buffer. In all the other trials, cultivation was performed in 300-mL glass bubble tube photobioreactors, 350 mm high, with an inner diameter of 36 mm and a working volume of 280 mL. Light was supplied by fluorescent lamps (Lumilux Cool daylight, Osram, Germany), with a continuous illumination at an average intensity of 120 μmol photons m^{-2} s^{-1}. Culture mixing was provided by continuous injection of an air: CO_2 mixture (2 % CO_2) filtered at 0.22 μm (Nylon Syringe Filter, VWR, USA) to regulate pH between 7.40 and 8.50 (also by addition of 1 M $NaHCO_3$ solution), and temperature was kept at about 25°C.

Maintenance cultures and inocula for the trials were grown in modified BG11 medium [40] for freshwater strains and in F medium [41] for marine strains. In modified BG11, K_2HPO_4, $MgSO_4*7H_2O$, $CaCl_2*2H_2O$ and ferric ammonium citrate concentrations were increased assuming the content of the key element in the biomass as 1 % P, 0.5 % Mg, 0.5 % Ca and 0.04 % Fe to avoid nutrient limitations that might compromise optimal growth and lead to underestimation of biomass production in synthetic media. The inocula for the trials were grown in 300-mL bubble tubes photobioreactors under the same cultivation conditions described above.

2.2. Dairy whey pretreatment and characterization

The whey used in this work was a by-product from the ricotta cheese production (scotta) provided by a local dairy in Italy. In total, 5 different batches of whey were used in the trials.

The whey was pretreated with microfiltration based on the findings reported in Casà et al. [28] in which this technique was preferred in comparison with other treatments to eliminate suspended solids, coarse particles and to reduce turbidity and indigenous bacterial load. This technique is scalable at industrial level adopting various microfiltration systems already applied in dairy industry. Initially pH was neutralised with NaOH 6 M, then the whey was centrifuged at 3600 *g* for 10 min (Neya 8, Remi Elektrotechnik Ltd., India). The supernatant was then filtered sequentially through glass microfibre filters, 47 mm diameter, 1.2 μm and 1.0 μm pore sizes (MFV3, Filter-Lab, Spain and VWR International, Italy), followed by filtration in sterile conditions using 0.45 μm pore size cellulose nitrate sterile membrane filters (⌀ 47 mm, Whatman®, England). The recovery efficiency following this pretreatment process ranged from 70 % to 85 % in the different batches. The final filtrate was then transferred in sterile bottles and stored at −20°C until use Table 1.

A partial characterisation of the pre-treated whey was carried out prior to its utilisation. pH (PH20 pH-meter, VWR international, Leuven, Germany), conductivity (HCO304 conductivity-meter, VWR international, Leuven, Germany) and salinity (portable refractometer) were determined. These measurements were performed also on culture supernatants during the trials. Chemical Oxygen Demand (COD), total carbohydrates and total proteins were respectively determined according to the APAT CNR IRSA 5130 method, phenol-sulphuric acid method [42] and to the Folin-Ciocalteu reagent method [43].

Table 1
Partial characterisation of pre-treated ricotta cheese whey. Values are expressed as means and standard deviations of the different batches collected in different time periods. COD, total carbohydrates and proteins are referred to the last batch of whey used in S&M and MWL trials.

Parameters	Values
pH	4.9 ± 0.4
EC (mS cm^{-1})	15.2 ± 2.5
Salinity (‰)	117.3 ± 20.5
N-NO_3^- (mg L^{-1})	344.1 ± 66.9
N-NH_4^+ (mg L^{-1})	214.3 ± 103.9
P-PO_4^{3-} (mg L^{-1})	828.0 ± 351.4
COD (g O_2 L^{-1})	155.1
Total Carbohydrates (g L^{-1})	163.2
Total Proteins (g L^{-1})	27.2
Colour	Yellowish

2.2.1. Determination of nitrate concentration

Nitrate (N-NO_3^-) concentration was assessed by second derivative UV spectroscopy, a validated method for fresh- and wastewater samples [44,45]. Direct absorbance spectra were recorded with Cary 60 UV-Vis spectrophotometer (Agilent Technologies Inc., USA) using Optech quartz cells (Exacta+Optech GmbH, Germany) diluting samples with H_2O Milli-Q 1:100 v/v according to the sensitivity instrumental limit and second derivate was calculated with Agilent Cary WinUV Software (Agilent Technologies Inc., USA). Each sample was determined in six replicates.

2.2.2. Determination of ammonium concentration

Ammonium (N-NH_4^+) concentration was determined using a modified version of phenol-hypochlorite method [46]. Four reagent solutions were prepared: 4 g phenol (>99 %, ThermoFisher, Germany) in 500 mL ethanol (96°, Carlo Erba, Italy) called solution A; 0.75 g L^{-1} Na-Nitroprusside Dihydrate (VWR Chemicals, Belgium) called solution B; 15 g L^{-1} trisodium citrate (99 %, ThermoFisher, Germany) dissolved in 0.8 g L^{-1} NaOH (≥98 %, Honeywell Fulka, Sweden) called solution C; 4 % NaClO (<5 % v/v) dissolved in solution C, called oxidising solution D. For each analysis, samples (1 mL) were diluted with H_2O as needed within the sensitivity detection limit of 3.5 mg N-NH_4^+ L^{-1} and water was used as blank. Then, the reagents were added sequentially to the tubes containing the samples, previously washed with HCl (10 % v/v) and rinsed with H_2O: 1 mL solution A, 1 mL solution B and 2 mL solution D. After a 2-hour of incubation, absorbance at 640 nm was measured using the Cary 60 UV-Vis spectrophotometer and the N-NH_4^+ concentration was calculated integrating the absorbance value in the calibration curve equation. Each sample was determined in duplicate.

2.2.3. Determination of phosphate concentration

Orthophosphate (P-PO_4^{3-}) concentration was determined using the kit Spectroquant® Phosphate Test, Supelco 1.1.14848.0001 (Supelco, USA). The protocol adopted was modified: briefly, 3 mL samples were mixed with 3 drops of reagent 1 and a 2/3-full micro spoon of reagent 2 and vigorously vortexed. After few minutes, the sample absorbance was measured spectrophotometrically at 690 nm. The value obtained was used in the calibration curve equation to determine the P-PO_4^{3-} concentration. Each analysis was carried out using water as a blank. Each sample was determined in duplicate.

2.3. Experimental conditions

A comprehensive summary of the different trials, their nutritional conditions and cultures management are reported in Table 2.

During the Screening Phase (SP), whey diluted at 10 % (v/v) in H_2O (10WY) or at 10 % (v/v) in artificial seawater (Adriatic Sea Equipment, Italy) with a salinity of 30 ‰ was used as substrate for the growth of treatment cultures for the freshwater and marine strains (18 strains, listed in paragraph 2.1), respectively. For control cultures, modified BG11 and F media [40,41] were used. In all trials where controls were used, the culture medium (modified BG11 or F) was diluted to match the inorganic nitrogen concentration to that of the diluted whey of the respective treatment cultures. Moreover, in all the trials performed the initial cell concentration, calculated as g dry weight L^{-1}, was the same for each condition and strain tested. Cultivation lasted 9 days and on day 3 and 6, 10 % (v/v) of initial culture volume (3.5 mL) was removed and replenished with 10 % (v/v) diluted whey in treatment cultures and fresh media in control cultures. In all the trials each cultivation condition was tested in duplicate. In the first trial, the whey load was chosen based on the expected microalgal growth and considering an average elemental nitrogen content in the biomass of 10 % (on dry weight).

During the Low Whey Load (LWL) trial, F&M-M274, F&M-M526, F&M-M497 and F&M-M151 strains were inoculated in 10 % (v/v) whey diluted in H_2O (10WY) as substrate for treatment cultures and in modified (as in SP trial) BG11 for the control cultures. With respect to the previous trial, whey/medium replenishment was more frequent and at a higher load, as a higher growth was expected with changing culture system and conditions. Cultivation lasted 7 days and on day 2 and 4, 15 % (v/v) of the initial culture volume (42 mL) was replenished with 10 % (v/v) diluted whey in treatment cultures and fresh media in control cultures. In each of the subsequent trials whey load was adjusted based on the results of the respective previous trial.

For the High Whey Load (HWL) trial, 22 % (v/v) whey diluted in H_2O (22WY) and modified BG11 were used as treatment and control substrates, respectively, for strains F&M-M526 and F&M-M497. Cultivation lasted 6 days and every two days (day 2 and 4) 20 % (v/v) of initial culture volume (56 mL) was replenished with fresh undiluted (100 %) whey and modified BG11 for treatments and controls, respectively.

In the Salinity and Micronutrients (S&M) trial, inocula used of the strains F&M-M526 and F&M-M497 were previously adapted to salinity by cultivating them on BG11 medium with the addition of 15 ‰ NaCl. During the trial, 10 % (v/v) whey diluted in H_2O (final salinity concentration 15 ‰) without and with micronutrient supplementation was used as substrate for the treatment cultures with no control cultures. The

Table 2
Summary of the nutritional conditions of the different trials and culture management. Replenished volume is the percentage of the total culture volume removed (and media/whey replenished) in the photobioreactor. Concentration is the dilution rate of whey used in the replenishment. Interval indicates the day of replenishment.

Trial	Initial N and P concentration					Whey replenishment management			Trial duration (days)
	Initial whey dilution (%)	N-NO_3^- (mg L^{-1})	N-NH_4^+ (mg L^{-1})	P-PO_4^{3-} (mg L^{-1})	Inorganic N (mg L^{-1})	Volume (% of the total culture volume)	Concentration (%)	Interval	
SP	10	39.3	15.9	113.8	55.2	10	10	Three days	9
LWL	10	43.5	37.7	93.4	81.2	15	10	Two days	7
HWL	22	67.0	22.1	191.2	89.1	20	100	Two days	6
S&M+	10	27.5	23.7	22.5	51.2	50	10	Daily	7
MWL+	12	33.0	28.5	27.0	61.4	30	12	Three days	13

+ indicates the addition of micronutrients in the treatment substrate during the trial.

micronutrient concentration in the treatment substrate was the same as in the modified BG11. Cultivation lasted 7 days and 50 % (v/v) of the initial culture volume (140 mL) was replenished daily with 10 % (v/v) diluted whey with and without micronutrient supplementation.

All the previous protocol optimisations lead to the final Moderate Whey Load (MWL) trial, in which 12 % (v/v) whey (12WY) diluted in H_2O (final salinity concentration 15 ‰) with micronutrient supplementation, as previously described, was used for cultivation of the freshwater strain F&M-M526, whereas 12 % whey (12WY) diluted in artificial seawater (final salinity 30 ‰) with micronutrient supplementation was used for cultivation of the seawater strain F&M-M180. The micronutrient concentration in the treatment substrate was the same as in the modified BG11. Cultivation lasted 13 days and 30 % (v/v) of the initial culture volume (84 mL) was replenished on day 3, 6, 9 and 13 with 12 % (v/v) diluted whey added with micronutrients.

2.4. Culture growth and productivity determination

Culture growth was evaluated by measurement of cell number using a Thoma counting chamber (Hirschmann EM Techcolor, Hirschmann Laborgeräte GmbH & Co. KG, Germany). Measurements were carried out at the start of the trial, at each replenishment and at the end of the trial. In all the trials, samples (500 μL) were collected from the photobioreactors, then diluted differently (1:2 – 1:25) with H_2O to obtain countable cell numbers and counted using an optical microscope (Eclipse 50i, Nikon, Japan). Cell productivity was determined for the interval period between the start of the trial and the first replenishment, between two subsequent replenishments, and between the final replenishment and the end of the trial by calculating the difference in cell number between each replenishment and dividing it for the time interval in days. The total cell yield was determined as total cells produced per liter of undiluted whey and per mg of inorganic nitrogen (sum of the nitric and ammoniacal forms) and expressed as cells $(L\ WY)^{-1}$ and cells $mg^{-1}\ N^{-1}$ respectively.

2.5. Nutrient removal quantification

As regards the analytical quantification of nitric and ammoniacal nitrogen ($N\text{-}NO_3^-$ and $N\text{-}NH_4^+$) and phosphate ($P\text{-}PO_4^{3-}$), the methods used are reported in paragraphs 2.2.1–2.2.3. The concentration of these nutrients in the whey was determined at the beginning of the trials and before each whey replenishment. The analyses for nutrient quantification in the microalgal cultures were done in the supernatants obtained after centrifugation at 3600 *g* for 10 min (Neya 8, Remi Elektrotechnik Ltd., India). Quantification of nutrients in the microalgal cultures was performed at the beginning, at each replenishment and at the end of the trials in order to evaluate their removal. Nutrient removal efficiency, expressed as percentage, was calculated as the ratio between final nutrient concentration and nutrient concentration added at the beginning and during the trial minus nutrients withdrawn by dilution in the time interval considered.

2.6. Gross biomass composition

Biomass harvested in MWL trial was analyzed prior freeze-drying for carbohydrate, protein and lipid content according to Dubois et al. [42], Lowry et al. [43] and Marsh & Weinstein [47], respectively.

2.7. Statistical analysis

The statistical analyses and graphical representations of data were performed using Prism program version 8.0.2. (GraphPad Software, Inc®, San Diego, CA, USA). Productivities and nutrient removal efficiencies data were statistically analysed with mixed models 1-way and 2-way analysis of variance (ANOVA) and Tukey's multiple comparison test to assess significant differences. Significance level was 95 % ($p < 0.05$).

3. Results and discussions

3.1. Screening Phase

During the Screening Phase (SP), 15 strains successfully grew in the 10WY substrate and only 3 (*Chlorococcum* sp. F&M-M150, *Neochloris* sp. F&M-M14 and *Trebouxia* sp. F&M-M155) died after 6 days of cultivation. The trial lasted nine days and, for most of the strains, cell productivity recorded in the first three days of growth was markedly higher compared to that recorded in the following days (Fig. 1). This trend was also observed in the screening performed by Liu et al. [48] in which the majority of strains exhibited higher growth in 10 % swine wastewater during the first three-four days of cultivation. In the present work, a remarkable growth decrement was observed for most of the strains from day 6 to day 9, for which productivities were not reported).

As shown in Fig. 1a, cell productivities after 3 days of cultivation were significantly different between 10WY treatment and the relative control for the different strains tested. In most of them growth was significantly higher in 10WY treatment ($p < 0.002$) except for F&M-M274, F&M-M16, F&M-M405, F&M-M33 and F&M-M523 in which it was similar to that of the control and for F&M-M467 that showed a general higher growth in the control than in the treatments. In particular, F&M-M267 presented the highest productivity in 10WY ($16.3{\cdot}10^6$ cells $mL^{-1}\ d^{-1}$), followed by F&M-M526 ($12.3{\cdot}10^6$ cells $mL^{-1}\ d^{-1}$). On the opposite, F&M-M16, F&M-M405 and F&M-M33 showed the lowest cell productivities in 10WY. Among the *Chlorella* strains, F&M-M151 resulted the best performing in 10WY ($3.8{\cdot}10^7$ cells $mL^{-1}\ d^{-1}$ obtained in the first three days) and, conversely, F&M-M523 and F&M-M467 had the lowest productivities.

During the cultivation period from day 3 to day 6 (Fig. 1b), most strains showed no significant differences in productivity between the 10WY treatment and the relative controls, except for strains F&M-M263, F&M-M180 and F&M-M467, for which a significant reduction in 10WY compared to the control ($p < 0.05$) was measured. Despite the generally reduced productivity compared to that recorded in the first three days of cultivation, some of the tested strains, namely F&M-M274, F&M-M526, F&M-M497 and F&M-M405, maintained good growth performances with productivities ranging between 3.3 and $5.1{\cdot}10^6$ cells $mL^{-1}\ d^{-1}$. On the contrary, productivity for F&M-M267 dropped markedly (from $16.3{\cdot}10^6$ in the first three days to $2.3{\cdot}10^6$ cells $mL^{-1}\ d^{-1}$ in the second three days). As regards *Chlorella* strains, the same trend of productivity reduction was observed. F&M-M151 showed a decrease in productivity from $3.8{\cdot}10^7$ (day 0–3) to $2.7{\cdot}10^7$ cells $mL^{-1}\ d^{-1}$ (day 3–6) but remained the best performing *Chlorella* strain among those tested.

Although *Chlorella* and *Scenedesmus* are generally recognized as the most effective genera for microalgae wastewater treatment due to their capacity to adapt to various waters and salinity levels [34,49–53], different species and strains within these genera have been reported to grow and consequently remove nutrients differently [32,48] as may have diverse nutritional and environmental preferences [54]. Accordingly, data obtained in this work indicate that growth performances on whey diluted at 10 % v/v was strain-specific, with considerable variations within the same genus.

Nutrient removal efficiencies in 10WY assessed at the end of cultivation (day nine) are reported in Fig. 1c. Ammonium removal efficiency was consistently high and generally showed no significant differences among strains. In contrast, results showed strain-specific removal efficiencies for nitrate ($N\text{-}NO_3^-$) and phosphate ($P\text{-}PO_4^{3-}$). Nitrate removal efficiency varied significantly among the strains with F&M-M151 showing the highest efficiency (92.2 %) and F&M-M263 showing the lowest (56.8 %). Besides F&M-M151, F&M-M497 and F&M-M274 were the best strains for nitrate removal, with 86.5 % and 70.4 % removal efficiencies, respectively. Phosphate showed the most variable removal efficiency among the strains, ranging from 31.1 % in F&M-M263 to

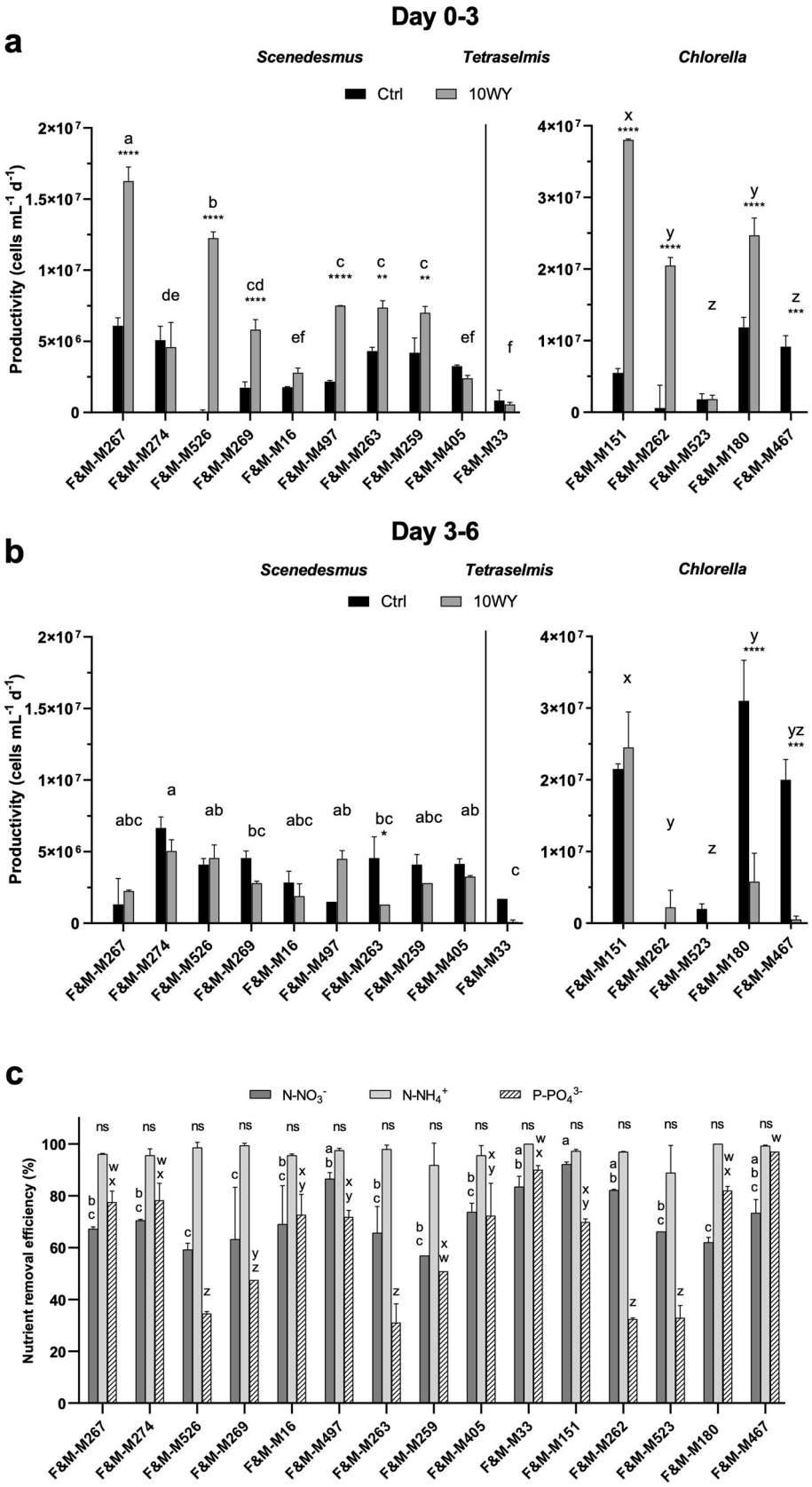

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Fig. 1. Daily cell productivity of strains tested during the Screening Phase (SP) in the first three days of the trial (a) and from day three to day six of the trial (b). For each figure, the left graph reports the productivity of the *Scenedesmus* and *Tetraselmis* strains, that on the right the productivity of *Chlorella* strains. Data are reported as average ± standard deviation of two replicates. Different letters indicate significant differences ($p < 0.05$) among productivities within the same time point and the same group of strains; * indicate statistical differences between each treatment and its relative control ($p = 0.0332$ *, $p = 0.0021$ **, $p = 0.0002$ ***, $p < 0.0001$ ****). Nutrient removal efficiency (c) of the strains tested in the Screening Phase in the 10WY treatment, measured at the end of the trial. Error bars indicate standard deviations. Different letters indicate significant differences ($p < 0.05$): a, b, c letters refer to nitric nitrogen ($N\text{-}NO_3^-$) removal efficiency; w, x, y letters refer to phosphate phosphorus ($P\text{-}PO_4^{3-}$) removal efficiency. ns, not significant, refer to ammoniacal ($N\text{-}NH_4^+$) nitrogen removal efficiency.

97.1 % in F&M-M467. In addition to F&M-M467, good removal rates were also observed for F&M-M180 (82.1 %), F&M-M274 (78.3 %), F&M-M267 (77.6 %), F&M-M497 (71.8 %) and F&M-M151 (69.9 %).

Similar to what was assessed for growth performance, nutrient removals also showed strain-specific variability. Nitrogen is an abundant element in microalgae being around 10 % of biomass dry weight [55], thus its supply for microalgal production is one of the main nutrient issues [56]. Nitrogen can be taken up in different forms, but the favourite among inorganic nitrogen sources is ammonium, since its uptake requires very little energy [57] and, in some cases, can diffuse passively into the cell if in the form of gaseous ammonia [56]. In our trial, ammonium removal was almost totally achieved for all the strains tested, thus resulting in the highest average removal efficiency and in the lowest variability (96.7 ± 3.1 %) among the nutrients monitored. However, it must be considered that some ammonia stripping may occur with aeration already at pH around 7 [57,58]. Instead, nitrate generally requires a more energy-demanding process to be absorbed and used by cells [57] and usually its consumption does not occur until ammonium is almost completely used [49]. Phosphorus is mainly taken up actively as orthophosphate (PO_4^{3-}) and its uptake is strongly influenced by light, pH, temperature, salinity, dissolved oxygen, and N/P ratio [49,56,59,60]. Moreover, the presence of some ions (especially Ca^{2+}) may enhance phosphate uptake or lead to its precipitation [61,62]. This might explain the variable phosphate removal ability detected in the different strains tested in the present work, as shown by the high variability of the removal efficiencies (62.8 ± 22.5 %) compared to the other nutrients. Craggs et al. [63] highlighted that 30 microalgal strains out of 84 able of growing on a sewage effluent, removed most of ammonium (>84 %) within the first days of cultivation, while phosphorous removal was less constant, with efficiencies ranging from 53 % to 85 %, as observed in the present work for most of the strains.

Considering both growth on whey and nutrient removal efficiency in SP, four strains (F&M-M274, F&M-M526, F&M-M497, and F&M-M151) emerged as the best candidates for cultivation on dairy whey and were further tested in Low Whey Load (LWL) trial. In fact, F&M-M526, F&M-M497 and F&M-M151 were among the strains with the highest cell productivity, with values in the first three days of cultivation significantly higher than the control. In addition, F&M-M497 and F&M-M151 displayed the highest nitrate removal efficiencies and good phosphate removal efficiencies. Despite the lower productivity recorded compared to the other selected strains, F&M-M274 maintained constant productivity over the six days of cultivation and a high nutrient removal efficiency, indicating stability and reliability of performances. Interestingly, despite the highest initial productivity, F&M-M267 was excluded from the selection due to major cell morphological changes (smaller size) during cultivation on whey, as also observed by Mercado *et al.* [29] for whey-grown *Scenedesmus*.

3.2. Low Whey Load trial

The Low Whey Load (LWL) trial was performed in a scaled-up photobioreactor (300-mL glass tube) with similar whey concentration (10 % whey) in the substrate and similar whey replenishment with respect to the previous Screening Phase. Three of the selected strains belonged to *Scenedesmus* (F&M-M274, F&M-M526 and F&M-M497) and one belonged to a freshwater *Chlorella* (F&M-M151).

Results show that F&M-M274 exhibited an initial rapid growth in 10WY treatment which then slowed down on day four with a productivity reduced by almost 9 times at the end of the trial. The relative control instead exhibited continuous growth up to day seven, exceeding the cell concentration of the treatment already after two days of cultivation (Fig. 2a). Strains F&M-M526 and F&M-M497 showed a consistently higher growth in 10WY compared to the control, although for F&M-M526 growth ceased from day 4. The control culture of F&M-M526 reached approximatively the same cell concentration of its relative treatment at the end of the trial. The control culture of F&M-M151, instead, showed no growth throughout the experimental period (Fig. 2). The difference observed between this result and that of the screening trial can be explained by the higher average light intensity (more than double) provided to the cultures of the LWL trial which may have caused light stress, considering that the starting cell concentration was the same in the two trials.

Overall, results of the LWL trial, confirmed for most of the strains a higher growth in the first two days of cultivation in 10WY compared to the control (modified BG11).

The nutrient removal efficiencies in LWL trial assessed on day seven for whey-grown strains demonstrated significant strain-specific differences, except for $N\text{-}NH_4^+$ in which removal efficiency was similar for all strains (around 95 %) (Fig. 3). Although nitrate removal efficiencies were slightly variable, with F&M-M151 showing significantly higher removal efficiency (91.7 %) compared to F&M-M526 (83.6 %) and comparable to F&M-M274 and F&M-M497 (88.4 % and 86.3 % respectively), they were on average improved by 18.7 % compared to SP. Phosphate removal efficiencies, comparable to that obtained during SP, exhibited the greatest variability, with F&M-M274 showing a significantly higher efficiency (79.7 %) than that of the other strains tested (67.8 %, 64 % and 57.2 % for F&M-M526, F&M-M151 and F&M-M497, respectively).

Analogous nutrient removal results were reported for *Chlorella sorokiniana* and *Scenedesmus acuminatus* in cheese whey [30,64] and for *Chlorella pyrenoidosa* and *Chlorella spp*. cultivated on dairy wastewaters [31,65]. Together, these findings show the effectiveness of the selected *Chlorella* and *Scenedesmus* strains to exploit the nutrients present in dairy wastewater for their growth. Moreover, the decreasing growth trend observed throughout this trial, coupled with the high nutrient removal efficiencies, as also observed by Girard *et al.* [21] reporting the end of exponential growth concomitantly with nitrogen depletion, suggests that the supply of essential macronutrients by means of whey addition during the trial was not sufficient to sustain growth for periods longer than two or four days, depending on the strain.

3.3. High Whey Load trial

Based on the results of LWL trial and of preliminary biostimulant assays carried out on *A. thaliana* with biomass extracts of the strains tested in the previous trial (data not shown), two strains, F&M-M526 and F&M-M497, were selected for the High Whey Load (HWL) trial. In HWL trial, the whey concentration in the substrate and the replenishments during cultivation were increased compared to the previous trial to avoid nutrient depletion and extend the cultivation period. Initial whey concentration was set at 22 % and every two days 20 % of the culture volume was replaced with undiluted whey in order to ensure nitrogen and phosphorus availability.

During the six days of trial the two strains exhibited similar growth curves (Fig. 4). In particular, in 22WY treatments, both strains grew better than the control in the first four days, but subsequently the cell

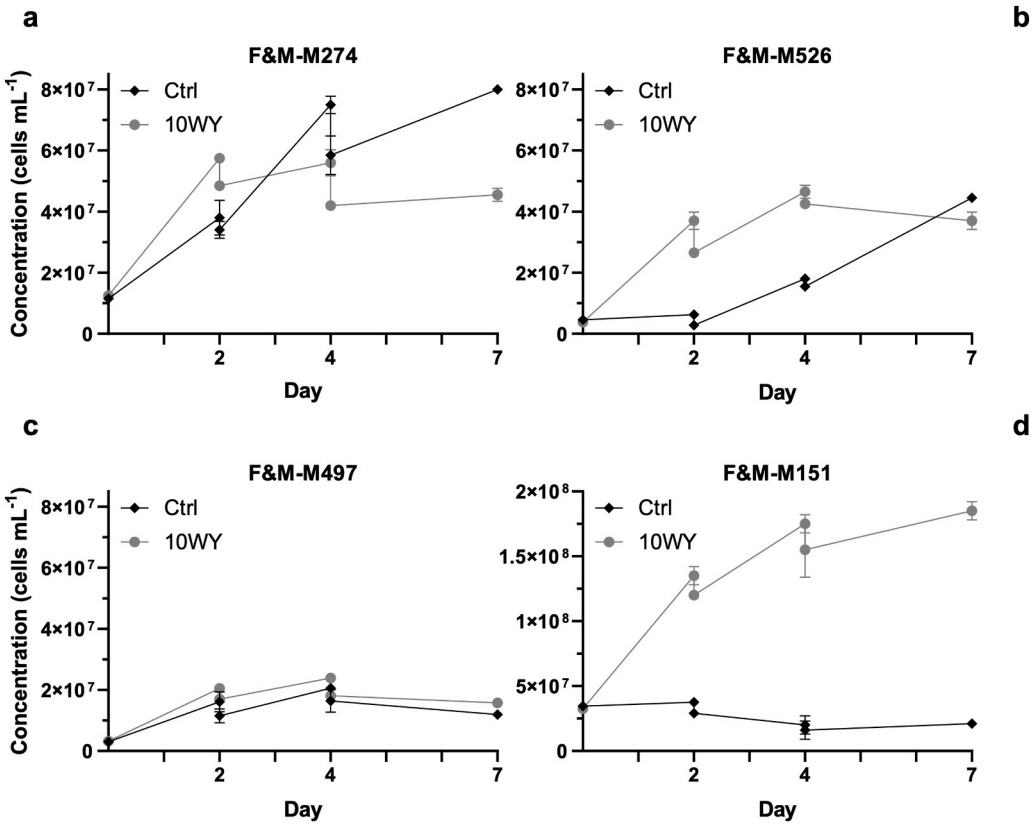

Fig. 2. Growth curves of the strains tested in Low Whey Load (LWL) trial in the seven days of cultivation: *Scenedesmus* F&M-M274 (a), *Scenedesmus* F&M-M526 (b), *Scenedesmus* F&M-M497 (c) and *Chlorella* F&M-M151 (d). Data are reported as average ± standard deviation of two replicate cultures for each treatment.

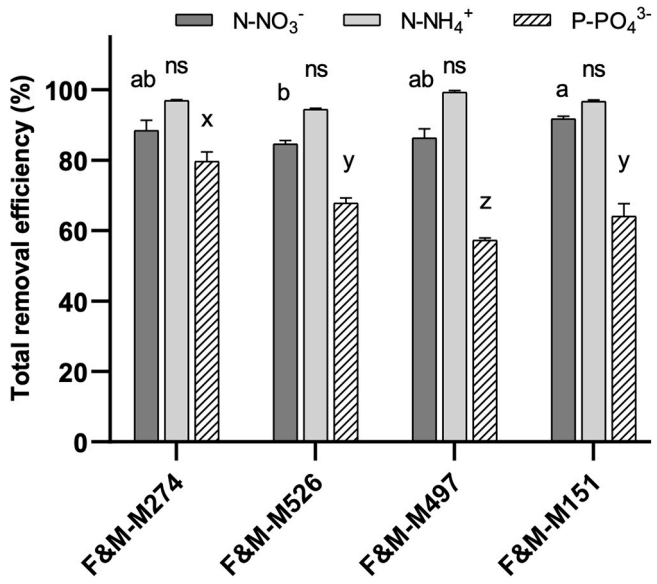

Fig. 3. Nutrient removal efficiency for strains tested in Low Whey Load (LWL) trial in 10WY treatment recorded at the end of the trial. Data are reported as average ± standard deviation of two replicates. Different letters indicate significant differences ($p < 0.05$): a, b, are referred to nitric nitrogen ($N\text{-}NO_3$) removal efficiency; ns, not significant, is referred to ammoniacal ($N\text{-}NH_4^+$) nitrogen removal efficiency; x, y, z are referred to phosphate ($P\text{-}PO_4^{3-}$) removal efficiency.

number of both strains declined (Fig. 4). During the trial, an increase in conductivity and salinity in 22WY treatment cultures was recorded, changing from 3.34 mS cm^{-1} and 22 ‰ to about 8.5 mS cm^{-1} and 45 ‰ in the first four days of the trial, after which growth decline and these values, at the end of the trial, reached 11 mS cm^{-1} and 60 ‰. It is to be considered also that organic soluble solids might influence these measurements [66,67].

Productivity comparison between the 22WY treatment cultures of the two strains did not show significant differences ($p > 0.05$) in any of the time periods considered except for day 4–6 where negative and significatively different values from day 0–2 were recorded. However, significant differences for the same strain among different time periods were observed (Fig. 4c). On day two, the two strains exhibited relatively high productivities ($12.8 \cdot 10^6$ cells mL^{-1} d^{-1} and $13.5 \cdot 10^6$ cells mL^{-1} d^{-1} for F&M-M526 and F&M-M497, respectively). In the next days (day 2–4), productivity declined for F&M-M526 ($4.7 \cdot 10^6$ cells mL^{-1} d^{-1}), but it remained relatively stable for F&M-M497 ($11.1 \cdot 10^6$ cells mL^{-1} d^{-1}). No significant increase in productivity of 22WY treatments compared to control cultures was found, except for F&M-M497 on day 0–2 ($p < 0.05$) (Fig. 4c).

Nutrient concentrations in 22WY treatments were periodically monitored allowing the assessment of removal efficiencies during the trial. Removal efficiency varied greatly over time, especially as regards $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ (Fig. 5). F&M-M526 demonstrated high nitrate removal efficiency (74.8 %) on day 0–2, which decreased substantially to about 34.7 % on day 2–4 and reached almost null removal in the last two days (Fig. 5). In F&M-M497 similar nitrate removal efficiencies (69.6 %) were detected which however, unlike in F&M-M526, remained stable on day 2–4 (69.2 %) and then sharply dropped on day 4–6.

Differently, ammonium removal efficiency was consistently high for both strains throughout the trial, especially in the first four days of cultivation in which it ranged from 96.6 ± 0.7 % and 95.1 ± 0.2 % for F&M-M526 and F&M-M497, respectively. During day 4–6 of the trial, ammonium removal efficiencies were significantly lower with values of 85.2 % for F&M-M526 and 81.4 % for F&M-M497. As observed for

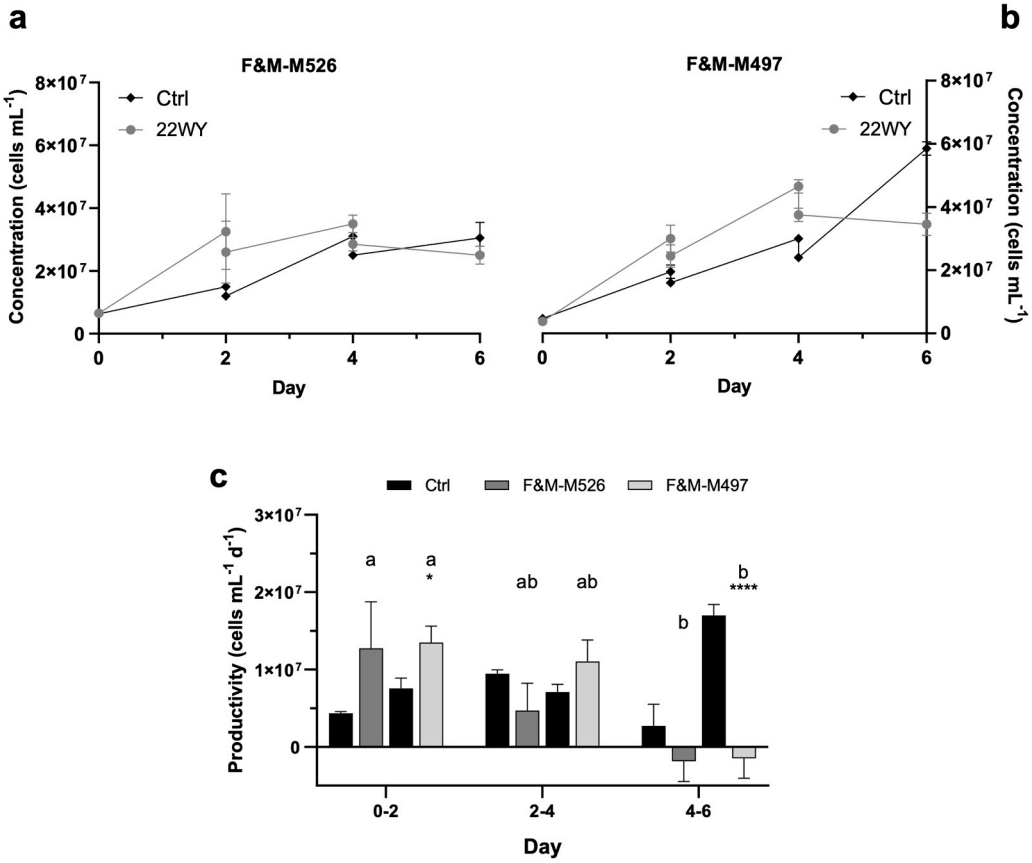

Fig. 4. Growth curves of strains tested in High Whey Load (HWL) trial in the six days of cultivation: *Scenedesmus* F&M-M526 (a) and *Scenedesmus* F&M-M497 (b). Cell productivity (c) of strains tested in High Whey Load trial on 22WY treatment. Data are reported as average ± standard deviation of two replicate cultures for each treatment. Different letters indicate significant differences ($p < 0.05$) across different days for each strain in the treatment conditions. * indicates statistical differences between each treatment and its relative control ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****).

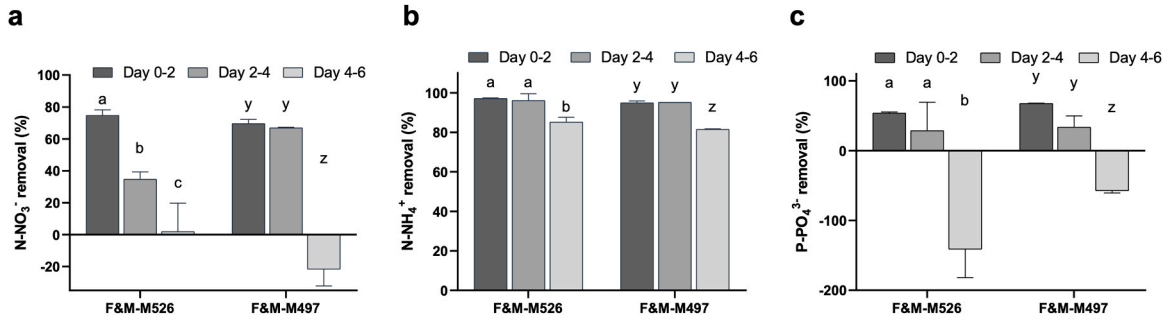

Fig. 5. Nutrient removal efficiencies for strains tested in High Whey Load (HWL) trial on 22 % whey. Nitric nitrogen ($N\text{-}NO_3^-$) removal efficiencies (a), ammoniacal ($N\text{-}NH_4^+$) nitrogen removal efficiencies (b), phosphate ($P\text{-}PO_4^{3-}$) removal efficiencies (c. Data are reported as average ± standard deviation of two replicates, different letters indicate significant differences ($p < 0.05$) across different days for each strain: a, b, c refer to *Scenedesmus* F&M-M526; y, z refer to *Scenedesmus* F&M-M497.

previous trials, phosphate removal is characterised by substantial differences between time periods, but it was similar in the two strains tested. During the trial phosphate removal efficiency gradually decreased and an accumulation of phosphate occurred in the culture medium. In F&M-M526 it decreased from 53.9 % (on day 0–2) to 28.7 % (on day 2–4), although difference was not significant due to the wide standard deviation. Similarly, in F&M-M497 it decreased from 67.6 % to 33.8 % in the same time points.

Since in this trial the deflection in cell productivity could not be attributed to nitrogen and phosphorus limitation, as their removal was not complete during the trial and the productivity determined were generally lower compared to the LWL trial, we hypothesized that treatment cultures might have suffered from an increase in salinity. In fact, frequent replenishment with undiluted whey led to an increase in salinity which gradually rose in the trial until reaching 45 ‰ (about 0.77 M) at the end of the trial, value that might have inhibited cell growth of strains of freshwater origin. Salt tolerance may represent a critical factor in wastewaters treatment as high salt concentrations in many effluents can limit microalgal growth. This behaviour can differ among strains and species of the same genus and is likely related to the different biochemical strategies adopted to cope with salinity, which may also be linked to the environment of the strain isolation habitat [53]. Stratigakis *et al.* [25] demonstrated that cultivating *Chlorella* in dairy whey with NaCl concentrations higher than 0.4 M inhibited

microalgal growth altering cellular processes and metabolic activities, such as decrease in chlorophyll content, damage to PSII, reduction in photosynthetic efficiency and ROS generation [68–70]. On the other hand, the maximum cell concentration reported by Mercado *et al.* [29] after ten days of cultivation of *Scenedesmus* sp. on 100 % dairy industry wastewaters was similar to that recorded in our work after 4 days for F&M-M526 in LWL and F&M-M497 in HWL, despite the 3.5 times lower starting concentration.

Taking into account the results of the first three trials (SP, LWL and HWL trials) in most of the strains tested an enhancement in growth and productivity was observed in cultures with whey supplementation compared to controls grown in photoautotrophic conditions, especially in the first two to four days of cultivation. Considering that experiments were set up to equalize inorganic nitrogen and phosphates in treatment cultures and controls, organic substances present in the whey must have played a key role in stimulating cultures growth. Many works reported consistently higher productivities in *Chlorella* and *Scenedesmus* strains grown mixotrophically on dairy whey compared to their photoautotrophic counterparts. For instance, Abreu *et al.* [12], Amouri *et al.* [13], de Andrade *et al.* [14], reported biomass concentrations and productivity for *C. vulgaris* cultivated on cheese whey, from 1.8 to 3–5 times higher compared to purely autotrophic conditions. Other studies found in literature report even higher biomass concentrations in mixotrophy conditions; for instance, Dhull *et al.* [18] and Brar *et al.* [17] showed 8 and 54 times higher biomass produced with strains of *Chlorella* grown on whey compared to the autotrophic counterparts and increments of 247 times for *Scenedesmus* strains. All these data align with the results presented in this work for *Scenedesmus*, with increments from about 3–21 times in HWL and LWL trials (*Scenedesmus* F&M-M497 and *Scenedesmus* F&M-M526, respectively) and up to almost 300 times in SP (*Scenedesmus* F&M-M526), while for *Chlorella* (F&M-M151) the highest increase was of almost 30 times in LWL.

Since lactose is the main compound in whey constituting 70–72 % of total solids [26] and up to 79 % in ricotta whey (scotta) [11], the typology of whey used in this work, it could have been used by the microalgae to support a mixotrophic growth, simultaneously utilizing inorganic carbon (CO_2) and organic carbon [12,14]. In fact, it is documented that some microalgae, included strains belonging to *Chlorella* and *Scenedesmus* are able to produce the extracellular enzyme β-galactosidase that catalyses the hydrolysis of lactose into glucose and galactose, making them available to the cell [26,27]. These readily metabolizable organic carbon sources in whey, including lactose, glucose, and galactose [26], may facilitate rapid cellular growth and reduce the metabolic energy required for carbon assimilation.

3.4. Salinity and Micronutrient trial

Since results of the HWL trial highlighted the need of a salinity optimization strategy to improve and extend strain cultivation on whey, in the Salinity and Micronutrient (S&M) trial, inocula of the same two strains (F&M-M526 and F&M-M497) were adapted to salinity before the starting of the trial.

In particular, inocula were cultivated in BG11 medium with the addition of 15 ‰ NaCl, which was the salinity value that resulted to be the most suitable according to preliminary tests (data not shown). Then, in the S&M trial, they were transferred in 10 % whey (10WY treatment) and cultivated adopting a daily replenishment of 50 % of the initial culture volume, to keep salinity constant at 15 ‰. Moreover, the impact of micronutrient supplementation on growth performance in cultures (indicated with "+") was also evaluated to provide a deeper insight into the factors influencing microalgal growth in whey-based cultivation processes.

Growth curves obtained during the S&M trial revealed strain-specific and micronutrient-dependent responses. Both strains demonstrated rapid initial growth until day 3 (Fig. 6), although higher cell concentrations were recorded for F&M-M526 compared to F&M-M497 (on average $2.5{\cdot}10^7$ cells mL^{-1} and $2.2{\cdot}10^7$ cells mL^{-1}, respectively). After day three, the growth of F&M-M526 culture gradually decline, whereas F&M-M526 + cultures showed a slightly higher and more stable growth from day four to day six (Fig. 6, left). Also, for F&M-M497 growth performances diminished constantly over the trial period, especially after day three of the trial. However, in this case both conditions (F&M-M497 and F&M-M497 +) exhibited a similar decline in cell concentration over the seven days of the trial (Fig. 6, right). At day seven in both strains and conditions cell concentration was strongly reduced.

Although maintaining a constant salinity and previous acclimation of the inocula to salinity allowed to extend the cultivation period by a few days compared to the previous trials, beyond the sixth day all the cultures, regardless of strain and type of treatment, stopped growing. This suggests that inhibitory conditions, that cannot be attributed exclusively to salinity or to macro- and micro-nutrient deficiency, might progressively develop or accumulate in the culture medium. Other compounds contained in large amounts in whey, such as organic matter, may influence long-term microalgal productivity in whey-based media. As a result, frequent input of organic-rich substrates may lead to the accumulation of extracellular organic matter (EOM), as also reported by de Andrade *et al.* [14] and Mamami Condori *et al.* [71] as well as of other inhibitory metabolites that negatively impact microalgal growth [72,73]. The comparison between the two strains showed overall more constant growth for *Scenedesmus* F&M-M526 with micronutrient supplementation than for *Scenedesmus* F&M-M497.

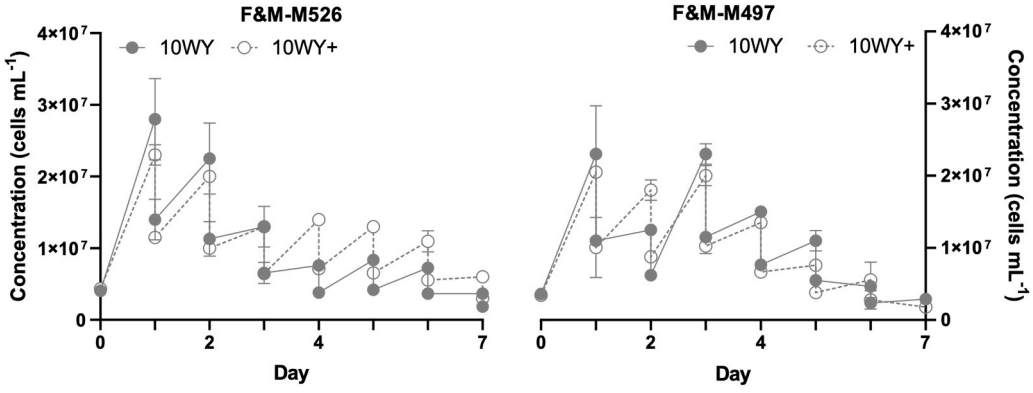

Fig. 6. Growth curves of the strains tested in Salinity and Micronutrient (S&M) trial in the seven days of cultivation: *Scenedesmus* F&M-M526 (left) without (10WY) and with (10WY+) micronutrients supplementation and *Scenedesmus* F&M-M497 (right) without (10WY) and with (10WY+) micronutrients supplementation. Data are reported as average cell number per mL ± standard deviation of two replicate cultures for each treatment.

3.5. Moderate Whey Load trial

The Moderate Whey Load (MWL) trial was performed by modulating the culture management to optimize the amount of whey used in the culture medium, while avoiding the previously identified negative effects (increased salinity, micronutrient deficiency and accumulation of inhibitory substances), with the aim of further extending the cultivation period. To this end, all conditions tested were micronutrient-supplemented, dilution rates were reduced from 50 % to 30 % of culture volume and replenishment frequencies from daily to every three days. Considering the relevance of salinity adaptation for microalgae cultivation on whey, together with the salinity-adapted F&M-M526 strain, the seawater *Chlorella* F&M-M180 strain was also tested as the better performing among the seawater strains in the SP.

Growth curves showed in Fig. 7 (left) indicate distinct patterns for the two strains. Both strains exhibited a rapid initial growth (cells about 5 times higher than the initial concentration after three days of cultivation). Then, F&M-M180 + demonstrated a constant growth throughout the cultivation period and after each replenishment until day nine after which no further growth was observed. F&M-M526 + displayed a higher growth than F&M-M180 + at all time points considered, with a peak in cell concentration at day six and a decrease in growth emerging at day nine.

Productivity comparisons revealed significant variations ($p < 0.05$) for each strain throughout the cultivation period (Fig. 7 right). Compared to F&M-M180, F&M-M526 + showed a similar ($p > 0.05$) and a higher productivity in the day 0–3 and day 3–6, with values of $6.8{\cdot}10^6$ and $5.1{\cdot}10^6$ cells mL^{-1} d^{-1}, respectively. However, F&M-M526 + productivity strongly decreased to $2{\cdot}10^6$ and $1.5{\cdot}10^6$ cells mL^{-1} d^{-1} in the day 6–9 and day 9–13, respectively. Similarly, F&M-M180 + showed the highest productivity on day 0–3 ($5.5{\cdot}10^6$ cells mL^{-1} d^{-1}), but in this case the significant reduction ($p < 0.05$) occurred already by the sixth day of cultivation ($1.9{\cdot}10^6$, $2.5{\cdot}10^6$ and $0.3{\cdot}10^6$ cells mL^{-1} d^{-1} in the day 3–6, day 6–9 and day 9–13, respectively).

Nutrient concentrations were monitored throughout the MWL trial as shown in Fig. 8. Nitrate removal efficiency (Fig. 8a) demonstrated significant temporal fluctuations. F&M-M526 + achieved the highest removal efficiency (93 %) in the period day 0–3, followed by a gradual decrease (77.5 % and 59.1 % respectively in the periods day 3–6 and day 6–9), and a stabilization at about 69.4 % in the period day 9–13. F&M-M180 + exhibited a lower, but more constant nitrate removal (on average 55.6 ± 6 %) throughout the cultivation period compared to F&M-M526. The different trend was evidenced also by the variation of nitrate concentration (Fig. 8d) in which both strains showed a rapid initial decrease in nitrate concentration, from the initial concentration of 32.9 mg L^{-1} to 2.3, for F&M-M526 + , and 13.2 for F&M-M180 + , mg L^{-1} at day three. Then, a quite regular removal pattern was measured.

As regards ammonium, both strains showed high and constant removal efficiencies (94 ± 5.9 % for F&M-M526 + and 98.7 ± 0.9 % for F&M-M180 +) during the trial (Fig. 8b). Ammonium concentrations in both cultures dropped from the initial concentration of 28.5 mg L^{-1} to values close to zero during the three days between two consecutive replenishments (Fig. 8e).

Finally, as regards phosphate removal efficiencies (Fig. 8c), F&M-M180 + showed significantly higher ($p < 0.05$) efficiencies than F&M-M526 + , except for day nine. For F&M-M180 + the removal peaks were reached on day 0–3 and on day 9–13, with 83.1 ± 5.0 % removal efficiency; whereas F&M-M526 + showed less variability, with similar removals obtained on day 0–3, 3–6 and 9–13 (on average 40.6 ± 4.5 %). The concentration kinetics (Fig. 8f) highlighted these patterns, with phosphate concentrations sharply decreasing to low levels (5.8 ± 2.3 mg L^{-1}) for F&M-M180 + , while for F&M-M526 + exhibiting slower and partial phosphate removal, stabilizing at higher residual phosphate concentrations (16.2 ± 1.8 mg L^{-1}) until the end of the trial. The determination of COD, performed at the end of the trial, revealed relatively high and similar removal efficiencies for both strains, with 68.4 ± 2.4 % for F&M-M526 + and 73.3 ± 2.3 % for F&M-M180 + removal compared to the initial values.

Generally, these removal efficiencies, especially that of nitrate (72.0 ± 17.9 %), were markedly higher compared to those obtained with the same strain in the HWL trial (54.7 ± 28.3 % for nitrate) without considering the last days with negative removal efficiencies. This suggests that optimization in whey supply during the trial could improve the efficiency of microalgae treatment. This trial suggests that optimizing operational conditions such as lowering whey load and dilution frequency and gradually adapt microalgae cultures to the growth inhibiting conditions (salinity) should allow to significantly extend the cultivation period. The general reduction in COD observed during this trial may indicate active uptake of organic carbon compounds, indicating the occurring of mixotrophic metabolism in the tested microalgal strains. As bacteria concentration increased significantly during the first three days of cultivation and then remained stable until the end of the trial (data not shown), we can assume their contribution to COD reduction and nutrients removal especially at the beginning of the trial.

The management options adopted in the MWL trial represents an advancement to achieve extended, productive, and sustainable microalgal cultivation. In fact, a well-defined substrate replenishment strategy is essential to ensure nutrient supply to sustain microalgal growth thus avoiding external supplementation with synthetic inorganic nutrients consequently lowering biomass production costs. In fact, the cost of whey pre-treatment at industrial scale using cross flow filtration system amount to about 0.03 or 0.13 € m^{-3} [74]. Instead, if the amounts of inorganic N and P present in one cubic meter of whey were supplied as synthetic inorganic nutrients, the cost would be much higher (1.35 € m^{-3} for $NaNO_3$ and 4.81 € m^{-3} for NaH_2PO_4 assuming a cost of 0.4 €

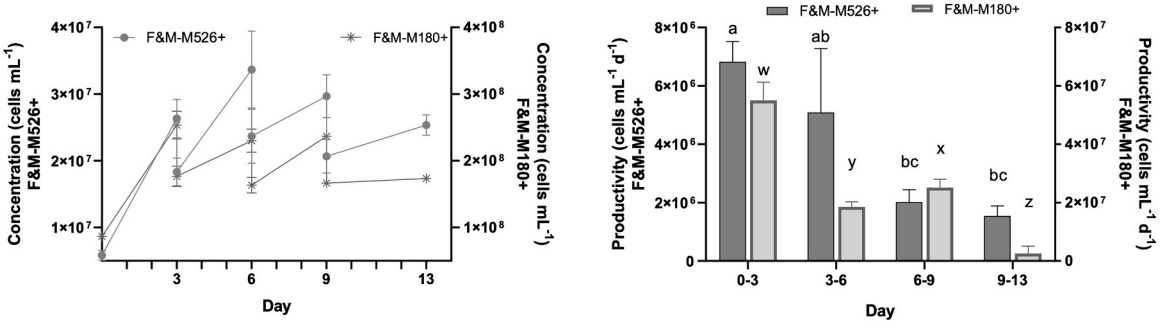

Fig. 7. Growth curves (left) and cell productivity (right) of the strains tested in Moderate Whey Load (MWL) trial in the 13 days of cultivation. Data for *Scenedesmus* F&M-M526 with micronutrients supplementation (F&M-M526 +) is shown on the left y-axis, data for *Chlorella* F&M-M180 with micronutrients supplementation (F&M-M180 +) on the right y-axis. Data are reported as average ± standard deviation of two replicate cultures for each treatment. Different letters indicate significant differences ($p < 0.05$) across different days for each strain: a, b, c refer to *Scendesmus* F&M-M526; w, x, y, z refer to *Chlorella* F&M-M180.

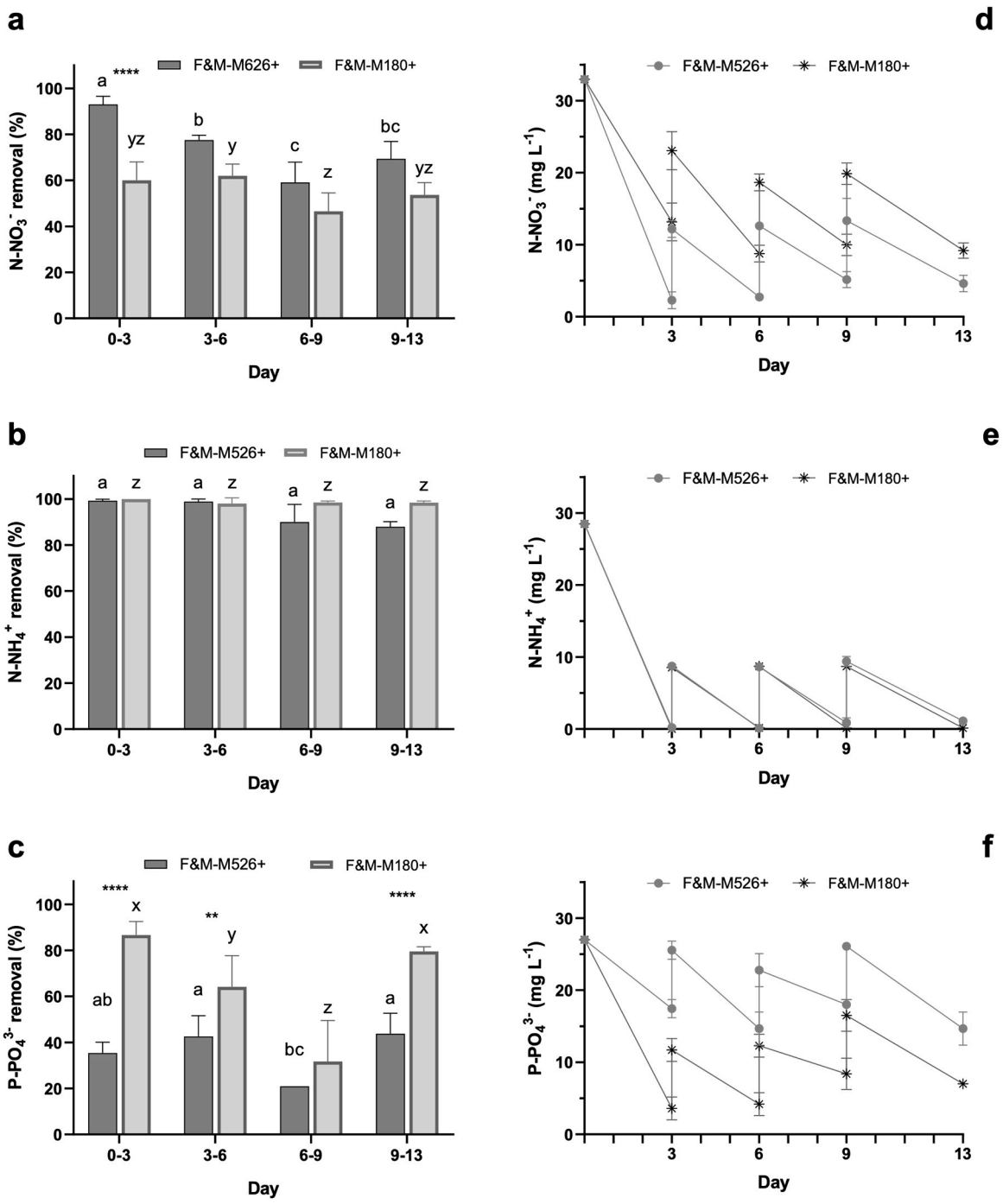

Fig. 8. Nutrient removal efficiencies (left) and nutrient concentration over time (right) for strains tested in Moderate Whey Load (MWL) trial on 12 % whey with micronutrients supplementation (+). Nitric nitrogen ($N\text{-}NO_3$) removal efficiencies (a) and concentration (d); ammoniacal ($N\text{-}NH_4^+$) nitrogen removal efficiencies (b) and concentration (e); phosphate ($P\text{-}PO_4^{3-}$) removal efficiencies (c) and concentration (f). Data are reported as average ± standard deviation of two replicates, different letters indicate significant differences ($p < 0.05$) across different days for each strain: a, b, c refer to *Scendesmus* F&M-M526; x, y, z refer to *Chlorella* F&M-M180.

kg^{-1} and of 1.5 € kg^{-1} respectively [75]).

In view of the exploitation of the whey-grown biomass, gross biochemical composition was evaluated, revealing a protein and carbohydrate content in line with the literature, both in photoautotrophy and mixotrophy [12,76,77], for F&M-M180 + (38.0 ± 3.6 and 34.1 ± 0.8 %, respectively) and for F&M-M526 + as regards protein content (30.0 ± 7.4 %) [78]. Carbohydrate content of F&M-M526 + (44.0 ± 1.8 %) was higher than those reported in literature for strains grown in photoautotrophy [79]. It is however to be considered that mixotrophic cultivation has been reported to stimulate accumulation of carbohydrates in *Scenedesmus* compared to photoautotrophic cultivation [80], thus supporting our data. Lipids were rather low (about 14 % for both strains). Considering that whey is a by-product of the food industry and that several *Chlorella* species are included in the EU Novel Food status Catalogue, its biomass could be valorised for the food and feed sector, especially for its protein content [81]. The high amounts of carbohydrates in the biomasses of both strains configure the application in several sectors such as cosmetics, pharmaceutics, food industry (e.g.

as substrates for fermentation processes), for biomaterials and in agriculture [82–84], depending on their composition. In fact, extracts of several species of the genus *Chlorella* and *Scenedesmus* have been shown to be exploitable in agriculture as biostimulants and antimicrobials, without showing cytotoxicity in vitro, thanks to the presence of various bioactive compounds including phytohormones, polyphenols and carbohydrates [82,85–87]. Since some bioactivities, as antimicrobial bioactivity, may vary among strains of the same genus and other factors (e.g. harvest time) [88], the potential of the whey-grown biomasses of specific strains should be evaluated in dedicated experiments to assess the best uses among the various possibilities.

3.6. Total cell yield

Table 3 groups the total cells produced per liter of undiluted whey (WY) and per mg of inorganic N used in the four experimental trials for the two best-performing strains among those tested, F&M-M526 and F&M-M497. F&M-M526 exhibited significant variability among trials, achieving the highest number of cells produced per liter in the LWL trial with 369.6 billion cells L^{-1} WY, significantly surpassing results of all the other trials performed with the same strain. The second highest result was obtained in the MWL trial, of 203.6 billion cells L^{-1} WY, which was significantly higher than HWL and S&M trials in which inhibitory conditions occurred by combination of high salinity and accumulation of other inhibitory compounds. This highlights the positive effect of optimized whey load and replenishment strategies. Supplementation with micronutrients in the S&M trial slightly improved cell yield for F&M-M526 (103.6 billion cells L^{-1} WY) compared to the no supplementation condition (85.3 billion cells L^{-1} WY) although no statistical difference was found between the two conditions ($p > 0.05$).

F&M-M497 showed less variability between trials; however, even in this case the highest, statistically significant, result was obtained in LWL trial, with 168.5 billion cells L^{-1} WY. Approximatively half of the yield was obtained in HWL compared to LWL trial and similar values were obtained in S&M trial. In this case, a slight lower yield with micronutrients supplementation was obtained.

As regards total cells produced per mg of inorganic nitrogen, F&M-M526 showed no differences ($p > 0.05$) between the LWL and MWL trials. This can be attributed to the higher concentration of inorganic nitrogen in the batch used for LWL trial compared to the batch used in MWL trial which resulted in a similar nitrogen content (31.1 ± 2.2 mg N) for the two trials considered.

Comparing total cells produced per inorganic nitrogen supplied to *Chlorella* F&M-M180 in the MWL trial, Hamidian & Zamami [22] obtained within the same time period (13 days) about 4 times lower total cells per mg of N cultivating *Chlorella sorokiniana* on dairy effluents. This could be due to the different cultivation vessel (flasks vs tubes), cultivation regimen (batch vs semicontinuous), mixing system (manually four times per day vs continuous air bubbling) and light intensity (80 vs 120 μmol photons $m^{-2}s^{-1}$). Similarly, Mercado et al. [29] obtained $2.8{\cdot}10^7$ total cells per mg of total N with *Scenedesmus* sp. in 11 days batch cultivation in dairy wastewater, a value lower than that obtained in 7 days for *Scenedesmus* F&M-M497 in the LWL trial ($0.2{\cdot}10^9$ cells mg^{-1} N^{-1}), and markedly reduced if compared to what obtained with *Scenedesmus* F&M-M526 ($0.46{\cdot}10^9$ cells mg^{-1} N^{-1} in LWL trial in 7 days and $0.4{\cdot}10^9$ cells mg^{-1} N^{-1} in MWL trial in 13 days) in this work (Table 3). The lower yield can be attributed to the different photoperiod (12:12 h light:dark vs 24 h light) at the same light intensity than our work, and despite the addition of fertilizers.

In the work of Caporgno et al. [89], in which a *Chlorella* strain were grown on centrate diluted in municipal wastewater to reach inorganic nitrogen concentration similar to those used in our work, total cell produced did not exceed $400{\cdot}10^8$ per liter of municipal wastewater added with centrate and $12{\cdot}10^{11}$ per liter of centrate; the latter result being higher than that obtained in the present work ($2–4 \cdot 10^{11}$ total cells L^{-1} WY). Despite the different type of substrate for cell growth, the main difference in yield can be due to the shorter light path of the photobioreactor used by Caporgno et al. [89]. Total nitrogen removals from municipal wastewaters and other wastewaters containing ammonium as the main form of inorganic nitrogen (e.g. brewery wastewaters) are generally high (> 92 %) [89–91]. With the dairy whey used in the present work, in which nitrate and ammonium nitrogen are both present (ratio 1.6:1), high ammonium removal (> 94 %) was also observed, while nitrate removal was lower (about 72 % for the best performing strains).

Table 3
Comparative results of total cells ± standard deviations (SD) produced per liter of undiluted whey (billion cells L^{-1} WY) and per mg of nitrogen (billion cells mg^{-1} N) used in the treatment substrates for the *Scenedesmus* F&M-M526 and *Scenedesmus* F&M-M497 selected strains in the various trials (LWL, HWL, S&M, MWL). (+) indicates results for micronutrient-supplemented condition, when tested. Different letters indicate statistical difference ($p < 0.05$) among different trials: a, b, c, d, e refer to cells produced per L of WY; y, z refer to cells produced per mg of N.

Trial	Total cells produced per L of WY		Total cells produced per mg of N	
	F&M-M526	F&M-M497	F&M-M526	F&M-M497
LWL	369.6 ± 3.2^{a}	168.5 ± 5.8^{c}	0.46 ± 0.00^{z}	0.21 ± 0.00^{y}
HWL	50.4 ± 1.1^{e}	73.7 ± 6.2^{de}	0.12 ± 0.00^{x}	0.18 ± 0.02^{xy}
S&M	85.3 ± 10.9^{de}	97.9 ± 2.6^{de}	0.17 ± 0.02^{xy}	0.19 ± 0.01^{y}
S&M+	103.6 ± 7.0^{d}	91.1 ± 18.2^{d}	0.20 ± 0.01^{xy}	0.18 ± 0.04^{xy}
MWL+	203.6 ± 15.8^{b}	-	0.40 ± 0.03^{z}	-

4. Conclusions

This study shows that microalgae cultivation using diluted ricotta cheese whey (scotta) as a source of nutrients may significantly increase biomass production compared to cultivation on standard synthetic medium, thus suggesting a promising and sustainable alternative approach to exploit dairy byproducts and generate added value.

The initial screening phase identified the most suitable strains for cultivation on whey, which belonged to *Scenedesmus* and *Chlorella* genera. Tests with these strains during subsequent trials allowed us to highlight salinity as the main issue that needs to be addressed, and to study the impact of micronutrient availability in order to extend the cultivation period of the selected strains on whey.

However, the use of salinity-adapted and marine strains, and of cultivation management aiming to maintain constant salinity levels in the cultures did not completely overcome the problem of growth reduction of the cultures, which occurred after the first days of the trial in all cultures, regardless of micronutrients supply. This suggests that inhibitory conditions, that cannot be attributed exclusively to salinity or to micronutrient deficiency, and which need to be addressed in future targeted experiments using metabolomic and metagenomic analyses, progressively develops in the culture medium.

In the last trial, optimization of cultivation management allowed us to enhance productivity and extend the cultivation period by selecting appropriate whey concentration in the culture medium and by lowering replenishments amount and frequency compared to the S&M trial.

Overall, this work underscores the necessity of carefully tailored cultivation management strategies to achieve high biomass yields and nutrient removal efficiencies for microalgae cultivation on whey. In particular, our results suggest the importance of finding a balance between the need to provide sufficient whey to support growth, avoiding the addition of inorganic nutrients, and the need to minimize the growth-limiting effects of whey accumulation in the culture media. Overcoming these critical challenges is essential to extend the cultivation period and enable pre-industrial scale photobioreactor trials to assess the long-term reliability of the process.

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CRediT authorship contribution statement

RODOLFI Liliana: Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Giacomo Sampietro:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Natascia Biondi:** Writing – review & editing, Conceptualization. **Gaia Santini:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Paolo Capano:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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Data availability

Data will be made available on request.

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