Systematic formulation of brewery effluent for high-efficiency epuration and food-grade microalgae production

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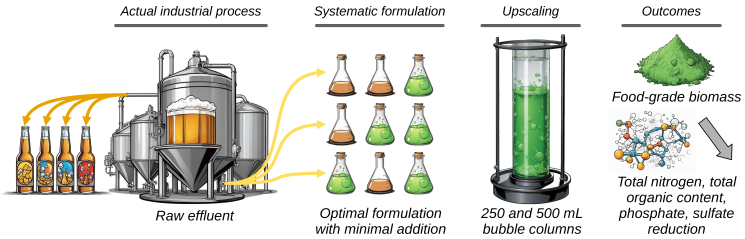
Actual (vs. synthetic) effluents were recovered, cost-effectively settled, augmented, and inoculated with *Chlorella vulgaris*. These minimal modifications ensure ease of process scale-up and economical viability while preserving the food-grade status of the stream. After successful shake flask runs, the process was transferred to 100 and then 500-ml working volume photobioreactors. The achieved performances (biomass production from 0.29 to 0.49 g/L/d - light dose dependent - and removal rates, 24.5 $\pm$ 4.3 mg/L/d for PO_4^{3-}, 6.8 $\pm$ 1.6 mg/L/d for SO_4^{2-}, 107 $\pm$ 32 mg/L/d for Total Nitrogen, and 1.30 $\pm$ 0.32 mg/L/d for Total Organic Content) are twice as high as the ones reported by other scholars. Cell productivity and illumination were almost perfectly correlated, demonstrating that the process can be easily controlled via adjustment of the illumination. Finally, the produced microalgae exhibit a balanced macronutrient profile and can be deemed food-grade, opening the way to biomass valorization as feed.



Graphical abstract

Microalgae | Brewery | Phycoremediation | Upscaling | Food-grade

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1. Introduction

Since the Second World War, the human population has dramatically increased. Despite technological progress, this growth and the associated quality of life improvement have put great stress on our ecosystem (1). The most striking examples are pressures on resources such as fossil fuels, water, arable lands, and biodiversity. A global indicator of this trend is the overshoot day, which arrives sooner every year (2). In this context, microalgae are considered a potent tool as they are capable of producing many molecules with applications ranging from food and feed to advanced compounds used in the cosmetic and pharmaceutical industries (3, 4), while delivering ecosystemic benefits (CO_2 fixation (5), phosphate fixation (6), nitrogen fixation (7), . . .). Still, before they realize the full extent of their promises, their production cost has to be considerably lowered.

The fact that microalgae can grow in harsh environments (piggery (8), paper mill (9)) has sparked the idea that it should be possible to grow microalgae in industrial side streams. Ideally, a successful culture would have three benefits: remediate part of the pollution potential of the side stream, produce microalgal biomass to be later valorized, and reduce the cost of the whole process. This triad is challenging to achieve, especially from a financial point of view, as the biomass produced may be of low quality, limiting its use to biofuel (10), or biofertilization applications (11, 12).

Acknowledging this complex situation, authors have proposed to focus on the food industry side streams to develop this type of approach (13, 14). Indeed, if the side stream is deemed food-grade, then the produced microorganisms would be food-grade, opening the way to, at least, a valorization as feed (15). Furthermore, food industry side streams, such as the dairy industry (16), can be rich in numerous macro and micronutrients required for microalgal growth.

In this view, brewery effluents are of peculiar interest. Indeed, they represent a high-eutrophication power stream as it was initially intended to grow other microorganisms, *i.e.*, yeast. Furthermore, they are the only co-products of beer brewing that is not yet valorized, while they represent a sizable stream with 3 to 10 liters being generated per liter of final product. Consequently, several authors have therefore tackled the question of brewery effluent phycoremediation. The most prolific, while sometimes redundant, author might be Mata, whose investigations aimed at producing microalgal biofuel using brewery effluents. By growing *Scenedesmus obliquus* on synthetic autoclaved effluents - which represent an optimistic avenue -, the authors showed the cost of micralgal biooil could be brought down to 2 €/L (17–19).

In the line of this pioneering work, other scholars' endeavors are to be reported. They can be categorized by the type of raw material they use. Synthetic effluents were also used to demonstrate that *Scenedesmus* and *Chlorella* sp. deliver similar performances on most indicators (growth, lipid productivity, pollutant removal), except COD removal, for which *Scenedesmus* sp. is about twice as efficient as *Chlorella* sp. (20). Those strains can also be used in a consortium to try to obtain improved performance (21).

Better approaching actual effluent, Zibarev *et al.* worked

with centrifugation supernatant (22). This pretreatment allows the removal of the solid fraction as well as part of the endogenous flora. Furthermore, the supernatant was used with different dilutions with nitrogen-poor culture medium to favor light penetration at a reduced cost (3:7, 5:5, and 7:3). Interestingly, the use of actual effluent, even though in part, allowed to discriminate culture performance based on their color. 3:7 allowed a vibrant green microalgae culture to thrive, 5:5 led to a green culture, and 7:3 dilution led to a culture crash with a brown color. Using the same dilution approach, Raposo *et al.* (23) used *Chlorella vulgaris* to epurate effluent under low illumination and deliver data on removal performances. The authors exhibited that the use of brewery effluent modulated fatty acid and protein amino acid profiles, compared to conventional culture medium.

Actual undiluted effluents were used in two studies. Choi *et al.* investigated the influence of aeration and light in epuration performances (24). They concluded that light and aeration led to improved performance. Yet, they also evidenced microalgal (*Chlorella vulgaris*) heterotrophic growth. Finally, in an investigation centered on bacteria/microalgae interaction, He *et al.* documented bacterial population dynamic, *i.e.*, an early rise and a fall, and *Chlorella sorokiniana* large increase in mass (25).

In most configurations, the authors reported high removal of nitrogen and phosphorous (17, 18, 20, 23, 24) by microalgae while achieving final biomass concentration around 1 gDW/L (17, 18, 21, 24). In a nutshell, they demonstrated the relevance of microalgae as a tool to remediate brewery side stream pollution while producing biomass. This work aims at bringing this research to the next step. Indeed, autoclaving, centrifuging, or diluting with fresh medium represent costly pretreatments. For example, harvesting biomass by centrifugation accounts for a third of the final product cost (26). Therefore, using it as pretreatment is prohibitive. Hence, this work aims to bring forward the phycoremediation of brewery effluent. To do so, actual (vs. synthetic) brewery effluents were recovered from an operating plant. Liquid and solid were separated affordably using food-grade chitosan-assisted settling, which can easily scale to the industrial level. Then, the key chemical species lacking in biomass growth are added to augment the culture medium and avoid a costly dilution by a fully formulated microalgal culture medium. Finally, flask-scale findings were upscaled to 500 mL photobioreactor. All in all, this represents the first proposal of an easily scalable microalgae cultivation process retaining the food-grade status of the initial side stream.

2. Materials and methods

2 1. Brewery effluent

Brewery effluent was provided by Brasserie du Pays Flamand (Merville, France) in several batches. As the brewery produces various types of beer, batch-to-batch variability was observed. To avoid a potential intra-experiment bias, each batch was dedicated to an experiment. The liquid part of the effluent was recovered by chitosan-assisted (10 mg/L final concentration (27, 28)) decantation for 48 hours at room temperature. Chitosan was chosen for three reasons. First, it bounds to negatively charged particles and flocculates best when pH is raised at a level compatible with microalgal culture (about 7). Second, it can be food-grade and is used in food and medical industries. Third, it is relatively inexpensive (about 2 USD/kg) (27). During this process, residual fermentation by endogenous flora was observed. Ultimately, a light brown liquid was recovered and used for cell cultivation.

2 2. Microalga strain, subculture medium, and procedure

The strain used in the study was *Chlorella vulgaris* (species SAG 211-11b, purchased from the Culture Collection of Algae at Göttingen University, Göttingen, Germany). This strain was chosen as it is Generally Recognized As Safe (GRAS status) by the US FDA and considered safe food by the European Food Safety Authority. Therefore, by deploying a food-grade production process, the cells could be valorized at least as feed.

The cells were maintained, amplified, and cultivated in suspension. The medium used for cell maintenance was a Bold Basal Medium with three times the nitrogen load (referred to as B3N medium (29)). This medium was chosen as it is chemically defined and particularly rich in nitrogen while not inducing substrate inhibition. Finally, cell culture was conducted in an orbitally shaking incubator (Infors HT Minitron, 100 rpm, 30 µmolPhotonPAR/m^2/s, 25 °C, under air with 1 % CO_2).

2 3. Cell cultivation on brewery effluent

2.3.1. Culture medium development. While it was demonstrated by other authors that brewery effluent could be used as a culture medium for microalgae, each brewery has different inputs and transformation processes. It is sometimes reported that at least a pH adjustment is required. Furthermore, scholars used dilution with culture media either to supplement the culture lacking micronutrients or to increase cell light access.

For all these reasons, a design of experiments approach was deployed to screen the effect of these parameters in a systematic manner. Two pHs deemed compatible with microalgae growth were tested: 6.5 and 7.5. To test for the need for micronutrient supplementation, dilution with medium B3N or water was chosen as the second factor of this design of experiments. Finally, the dilution rate (5 or 10) was chosen as the third factor. This setup leads to eight runs with a full-factorial approach.

The eight runs were carried out simultaneously in the same orbital shaker (MaxQ8000, 50 mL medium in 250 mL Erlenmeyer, 150 rpm), under continuous illumination of 100 µmolPhoton/m²/s, at 25 °C, in an atmosphere containing 2.5 %vol of CO_2. The run lasted seven days. Positive control (B3N inoculated with *Chlorella vulgaris*) and negative controls (concentrated effluent, no inoculation, with and without light) were carried out as preliminary tests. The positive control showed thriving microalgal cells. The negative controls showed mild signs of fermentation (with and without light)

allegedly because of residual sugar (confirmed by the media analysis, see below).

2.3.2. Culture medium augmentation. Following the results of the first systematic analysis, a second one was carried out to pinpoint the components of the B3N medium that were effectively lacking in the brewery effluent. The best conditions for the first experiments were retained (pH of 6.5 and a 5-fold dilution). The three factors were chosen as key components of the B3N medium (29): acidified iron solution (presence or absence), EDTA solution (presence or absence), and boron & trace solution (presence or absence). A full-factorial approach was also retained, leading to eight runs.

In the same way as the first design of experiments, the eight runs were carried out simultaneously in the same orbital shaker (MaxQ8000, 50 mL medium in 250 mL Erlenmeyer, 150 rpm), under continuous illumination of 100 µmolPhoton/m²/s, at 25 °C, in an atmosphere containing 2.5 %vol of CO_2. The run lasted seven days.

2 4. Mini-bubble column photobioreactors

Once the successful formulation of the effluent has been achieved, the next step was to upscale the process. Two objectives were in sight: validating that the results obtained in shake flasks were reproducible at a larger scale, producing enough biomass to allow daily monitoring of the cultures.In this step of the process, the main change does not lie in the volume culture volume by itself but in the shift in technology (continuous bubbling vs. diffusive gas transfer, mechanical stirring vs. orbital shaking). Therefore, three 250-ml bubble columns (3.6 cm diameter, 100 ml working volume) were used. They were lit on one side with 390 µmolPhoton/m²/s and mixed using pre-moist air with 2.5 %vol of CO_2 at 1 vvm. The three runs were led in parallel, in three identical mini-bubble column photobioreactors.

A first run with pure B3N medium was led to validate that the cell could grow at a nominal rate in the culture vessel (results not presented). Afterward, runs were led in semi-continuous mode with augmented brewery effluents. The inoculum consisted of 80 ml of cells grown in B3N medium and 20 ml of pure augmented effluent (the equivalent of a 5-fold dilution). Every seven days, about 20 ml of pure augmented effluent was added to feed the culture and compensate for the volume withdrawn for sampling.

The cultures were followed daily by withdrawing 3 ml samples. The samples were used for cell concentration measurement on filters (0.22 µm pore size), optical density monitoring (at 750 and 680 nm), and medium composition analysis. The tests were carried out in triplicate and lasted 21 days. At the end of the run, the best-performing culture was upscaled to 500 ml.

2 5. 500-ml bubble column upscaling

The best-performing culture was transferred to a 500-ml bubble column (5.0 cm diameter, 420 ml working volume) and expanded by adding augmented effluent (perpetuating the 5-fold dilution of fresh effluent). It was first brought from 100 ml to 150 ml and maintained for 14 days. Once stability had been ensured, the operating volume was brought to 420 ml and maintained for 70 days. Finally, to maintain the light dose, illumination was doubled after 21 days by turning on twice the initial amount of LEDs. The two runs were led in parallel, in two identical 500-mL bubble column photobioreactors.

Operational parameters were kept identical to the ones in the mini-bubble photobioreactors. Runs were carried out in duplicate.

2 6. Medium composition analysis

Anions and cations were monitored over the course of the upscaled cultures. Samples were filtered (0.22 µm filters) and diluted before being presented to an ICS-5000+ Ion Chromatography system (Thermo Fisher Scientific) coupled with a conductivity detector. All ions were identified by comparison to their retention time with standard solutions. Quantification was achieved by using the area of the peak in external calibration. The range of concentrations was from 0.05 to 5 mg/L for cations, and the range was from 0.2 to 20 mg/L for anions. All standards were purchased from Sigma-Aldrich with a TraceCert quality.

Total Nitrogen was monitored throughout the cultures. Samples were filtered (0.22 µm filters) and diluted before being presented to a TOC-L CSH analyzer (Shimadzu). The analyzer oxidized, at high temperature (750 °C), all nitrogen contained in the samples into nitrogen dioxide, which was then quantified by chemiluminescence. Quantification was achieved by comparison of the peak area obtained with the standard solution's calibration curve. The range of concentrations was from 20 to 1000 mg/L.

To determine sample Total Organic Carbon (TOC) content, the samples were filtered (0.22 µm filters) and diluted before being presented to a TOC-L CSH analyzer (Shimadzu). The analyzer oxidized all carbon contained in the samples into carbon dioxide, which was then quantified. Quantification was achieved by comparison of the peak area obtained with the standard solution's calibration curve. The range of concentrations was from 20 to 1000 mg/L. TOC measurements were preferred to COD measurements as they are faster to perform and do not generate harmful molecules (*e.g.*, hexavalent chromium, mercury, ...). Furthermore, a linear correlation exists between the values, as COD = 3.00 (pm 0.71) × TOC + 49.2 (in mg/L) (30).

2 7. Biomass analysis

Lipids were quantified gravimetrically. First, 100 mg of freeze-dried microalgae powder was resuspended in 10 mL of milliQ. Then, cells were homogenized using MP Biomedicals FastPrep42 bead miller. Lipids were extracted from lyzed cells following Bligh and Dyer protocol (31). The chloroform phase containing the lipids was then left to evaporate, and the solid residues were weighed.

For determination of the cells' protein content, 5 mg of freeze-dried microalgae was resuspended into 20 ml of water which was then analyzed by TOC-L CSH analyzer (Shimadzu) for total nitrogen quantification. Protein content was

pH	COD	Total Nitrogen	Total Phosphorus	Total Organic Acid	Glucose	Ethanol	Ref.
6.9	5.650 10^3	43.77	11.85	-	-	-	(22)
7.3	1.250 10^3	543	6.4	-	-	-	(24)
-	565 - 7.837 10^3	-	-	-	-	-	(23)
7.8	2.064 10^3	22.78	1.18	-	-	-	(25)
4.5	107.6 10^3 *	2.788 10^3	-	9.578 10^3	2.043 10^3	27.81 10^3	This work – Batch 1
4.5	94.7 10^3 *	1.939 10^3	-	8.488 10^3	905.7	27.34 10^3	This work – Batch 2

Table 1. Composition of the different effluent batches - macroscopic species. Total organic acids account for lactic acid, acetic acid, succinic acid, and propionic acid. * computed based on TOC to COD conversion factor. Concentrations in mg/L

NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Ref.
20.85	0.022	1.6	25.86	207.2	(22)
-	-	-	-	-	(24)
3.07 - 106.44	-	1.86 - 11.16	56.98 - 325.75	-	(23)
18.3	0.08	0.66	0.23	-	(25)
417.4	N.D.	N.D.	1.360 10^3	143.9	This work – Batch 1
276.6	N.D.	N.D.	1.345 10^3	153.9	This work – Batch 2

Table 2. Composition of the different effluent batches - ionic species. Concentrations in mg/L. N.D. - Not Detected (< 0.2 mg/L)

calculated from total nitrogen content using a correction factor derived for *Chlorella* sp., yielding the following equation (32):

$$Protein\ (mg/L) = 4.57 \times TN\ (mg/L) \quad \textbf{(1)}$$

5 mg of freeze-dried cells were homogenized before total carbohydrates determination using the anthrone blue method (33) (calibration curve realized twice at 630 nm using glucose, linearity range 0.05 g/L to 0.5 g/L, 5 points, $R^2 = 0.999$, with control to nullify potential chlorophyll contribution at 630 nm).

2 8. Removal rates and biomass productivity computations

Removal rates were obtained in two steps. First, every downward section of ion concentrations was fitted using the ordinary least square method. The slopes (*i.e.*, individual removal rates, in mg/L/d) were then averaged and presented with their 95 % confidence interval.

Biomass productivities were computed for the bubble-column tests. To do so, a mass balance was drawn over the system. Between two sampling points, one has to account for cell concentration variation, culture medium volume increase by medium addition (not containing biomass), and culture medium decrease by sampling (containing biomass). Therefore, the overall mass production (Δm_i) between to time points (t_i and t_{i+1}) can be written as:

$$\Delta m_i = V_{i+1}C_{i+1} - V_iC_i - V_{sample,i}C_i \quad \textbf{(2)}$$

Where V is the culture medium volume, C is the cell concentration and the last term accounts for the biomass withdrawn by sampling. From the mass variation, the average productivity (Π) of a given duration (T) can be computed as:

$$\Pi = \frac{1}{T}\sum_i \frac{\Delta m_i}{\frac{1}{2}(V_{i+1}+V_i)}(t_{i+1}-t_i) \quad \textbf{(3)}$$

3. Results and discussion

The first comment on the conduct of the microalgae culture on brewery effluent is that the overall culture color (vibrant green, green, or brown) is a good qualitative proxy of the microalgae condition. In a consistent manner, unsuccessful culture quickly turned brown. This observation echoes the comment of Zibarev *et al.* (22), who reported the same behavior.

3 1. Effluent analysis

The compositions of the effluent used in this work are presented in Table 1 and Table 2. The two batches were not characterized to the same extent (yet, differences will appear to be minimal), as the first one was used to focus on cell growth (tests in shake flasks), and the second one was used to deploy a combined cell production and effluent remediation process, in which performances were documented in depth (tests in bubble columns). The second batch used in this work is somewhat less rich than the first one (two-thirds nitrogen content, 1 g/L glucose versus 2 g/L). This type of batch-to-batch variation was to be expected as actual industrial side streams were used. Nevertheless, their compositions can be deemed quite similar. Indeed, even though glucose concentration is halved in the second case, the absolute values remain low. Together, they contrast with other authors' reports. Using raw effluents dramatically increases the amount of organic carbon available (20 to 50 fold), nitrogen (50 to 100 fold), and phosphorous (20 to 50 fold). While those figures seem quite high, the different analyses converge. Indeed, the high ethanol (30 g/L) and organic acid (around 10 g/L) correlate well with high COD values.

Ultimately, the observed discrepancies show that pretreatments chosen by authors have had a sizable impact on the medium composition. It can be concluded that, while it may not be as efficient as centrifugation to clarify the medium, chitosan-assisted settling preserves better the nutrients available in the raw effluent. Finally, the observed rich composi-

tion further emphasizes the need to manage this effluent because of its very eutrophication potential, as well as the opportunity it represents as a candidate medium for microalgae cultivation.

3 2. Design of experiment - Culture medium development

The effect of three factors (pH, dilution medium, and dilution rate) was tested in a systematic manner. The first comment is that from a visual perspective, runs diluted with water did not turn green but remained with a translucent brown color. Then, from a qualitative perspective, these showed an increase in NH_4^+ (up to +140 %) and a low overall PO_4^{3-}, and SO_4^{2-} final removal (between 3.3 and 37 % and 0 and 12 %, respectively). The increase in NH_4^+ can be explained by the degradation of complex nitrogen compounds by bacteria (34), while the low removal of PO_4^{3-}, and SO_4^{2-} can be attributed to endogenous bacteria activity. Such behavior was already observed by He *et al.* (25).

On the contrary, the use of B3N as a dilution medium led to microalgae proliferation associated with sizable pollution removal performances. For example, the two runs were B3N with used with a 10-fold dilution, lead to 100 % removal of SO_4^{2-}, 98 % removal of PO_4^{3-}, and 100 % removal of NH_4^+, for both pH 6.5 and 7.5 runs.

Finally, using a design of experiments approach allows to go beyond simple observations and assess the magnitude of each effect and their interactions. Figure 1 illustrates the influence of the three factors and their combinations. As one can see, the medium used for the dilution accounts for the overwhelming majority of the removal of NH_4^+, PO_4^{3-}, and SO_4^{2-}. In addition, the fact that the dilution rate only has a minimal effect suggests that access to light (fostered by a high dilution rate) was not a significant limitation in this configuration.

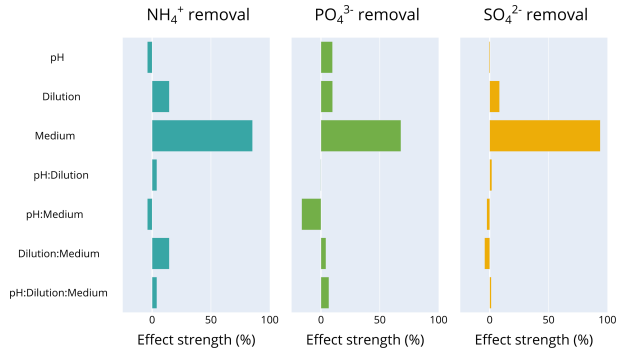

Fig. 1. Effect strength of the three tested parameters (pH, dilution rate, and dilution medium) on NH_4^+, PO_4^{3-}, and SO_4^{2-} removal (in %). For NH_4^+, release by endogenous flora led to negative removal (*i.e.*, net production), which were culled to zero for the analysis of the results

3 3. Design of experiment - Culture medium augmentation

The previous investigation suggested that some components of the culture medium were key to ensuring adequate growth of the cells. Therefore, it was chosen to operate at an initial pH of 6.5 with a 5-fold dilution and test the different components of the medium (acidified iron solution, EDTA solution, and boron & trace solution).

The first observation is that the run with EDTA supplementation alone turned brown and released NH_4^+, suggesting a strong bacterial activity but no growth of the microalgal cells. From a quantitative perspective, the most successful combinations were in the presence of acidified iron solution and boron & trace solution. They achieved 97 % removal of SO_4^{2-}, between 95 and 99 % removal of PO_4^{3-}, and between 98 and 100 % removal of NH_4^+. As one can conclude, the presence of EDTA only marginally influenced the results.

These observations are supported by the effects analysis based on the design of experiment data (Fig. 2). The plot of the effects confirms that the acidified iron, the boron & trace solution, and their combination are the most potent effects for pollutant removal. On the contrary, EDTA addition has an unclear effect.

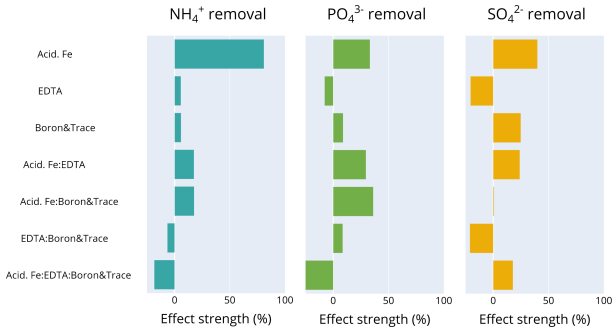

Fig. 2. Effect strength of the three tested parameters (acidified iron solution, EDTA solution, and boron & trace solution) on NH_4^+, PO_4^{3-}, and SO_4^{2-} removal (in %). For NH_4^+, release by endogenous flora led to negative removal (*i.e.*, net production), which were culled to zero for the analysis of the results

It can, therefore, be concluded that turning raw brewery effluent into a culture medium requires adjusting the pH to around 6.5, diluting it 5 times with water supplemented with acidified iron and the boron & trace solution. Yet, adding iron to a phosphate-containing medium raises the question of compound precipitation. In order to validate that the addition of iron did not lead to precipitation, phosphate ions were quantified before and after the addition of the supplement and two days of settling (with chitosan). The solution showed mild signs of fermentation by the endogenous flora and an almost stable phosphate concentration (-3.12 %). Hence, it can be concluded that the observed removal of phosphorus originates from a biological phenomenon and not a chemical one.

3 4. Mini-bubble column test

The first comment is that, out of the three runs, one crashed (referred to as run B). After the second feeding, the culture turned brown, and the amount of microalgae observed by the microscope quickly decreased. Despite careful investigations, it was not possible to pinpoint a convincing explanation. The second comment is that runs A and C led to vibrant green and regular green-colored cultures with a microbial population dominated by *Chlorella vulgaris* cells. The

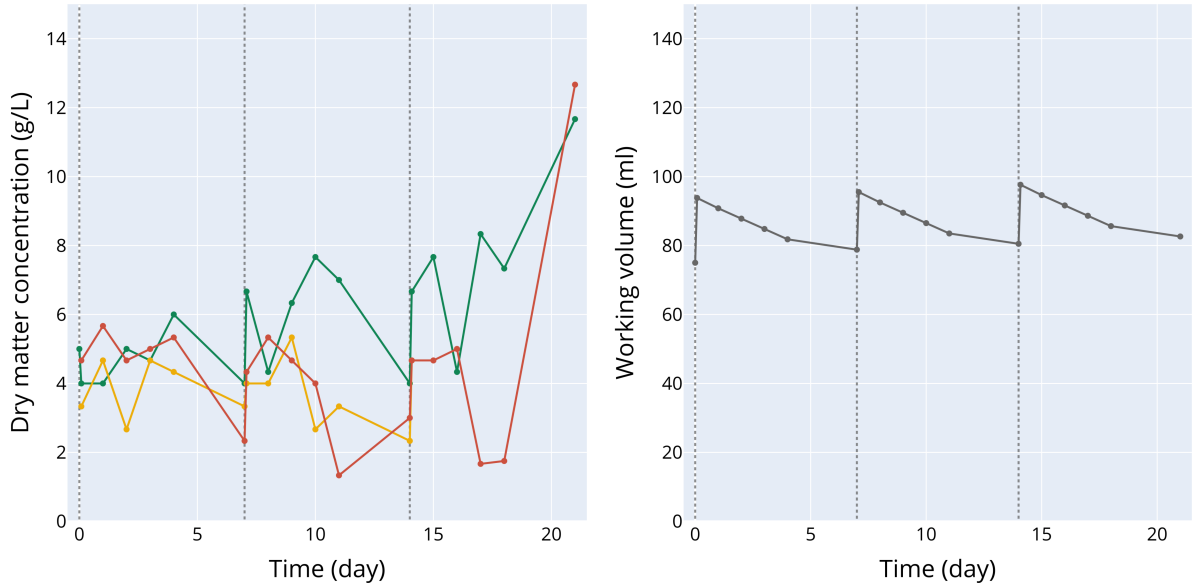

Fig. 3. Dry matter concentration (left) and working volume (right) in the 250 ml bubble column photobioreactors. Green line - photobioreactor A, Yellow line - photobioreactor B, Red line - photobioreactor C. Dashed gray lines - new effluent addition

third comment is that the cultures exhibited a regular amount of foaming. Yet, given the extended duration of the cultures, it led to the formation of sizable biofilm above the free surface.

Moving on to quantitative analyses, Figure 3 presents the dry matter concentration within the photobioreactors over time. As one can see, the measurements exhibit a high level of noise. This can be explained by the presence of cell aggregates originating from biofilm, which fell back into the culture medium over time. Nevertheless, dry matter concentration can be estimated between 4 and 5 gDW/L at the beginning and around 12 gDW/L at the end of the process. While it would be surprisingly high for culture led on chemically defined media, the observed level of noise is corroborated by other scholars' works on brewery effluents (17, 22, 23). Furthermore, the dry matter content is much higher than the one previously reported by other authors (around 1 gDW/L (17, 18, 21, 24)). This can be explained by the high surface-to-volume ratio of the photobioreactor, the relatively high illumination, and the high phosphorous content of the effluent used in this study. Nevertheless, it also shows that brewery effluent contains enough nutrients to support high-density cultures. Finally, from a quantitative perspective, the two photobioreactors that did not experience a crash achieved averaged microalgal productivities of 0.47 and 0.49 g/L/d, respectively.

Figure 4 shows PO_4^{3-}, NO_3^-, NH_4^+, SO_4^{2-} concentrations as well as Total Organic Carbon and Total Nitrogen over the run. The first comment is that NO_3^- concentration drops quickly and never repletes. This shows that the potential remains of the initial medium swiftly disappear and do not modify the culture behavior. SO_4^{2-} is also removed efficiently by the culture as its concentration steadily decreases at an average rate of 7.04 $\pm$ 2.41 mg/L/d, sometimes even reaching 0 mg/L. A similar comment can be made for PO_4^{3-} (removal rate of 26.0 $\pm$ 6.51 mg/L/d), while its concentration never reaches 0 mg/L. The impossibility of the culture to fully remove PO_4^{3-} can be explained by the imbalance between nitrogen and phosphorous in the effluent. As a comparison point, the molar composition of plankton complied by Redflied from surveys all around the world is $C_{140}N_{20}P_1$, leading to an N/P ratio of 20 (35) (or 22 for in a recent survey (36)). In the present case, the batch shows a value of 8.6, less than two times less, further pointing out toward nitrogen limitation.

In this regard, NH_4^+ concentrations are more difficult to interpret. The trends can be either upward or downward. Upward trends can be explained by the proteolysis that can be led by some bacteria (34). The downward trend could be attributed to two mechanisms: biological fixation by microalgae and stripping. While the first one is obvious, the second is to be discussed. NH_4^+/NH_3 couple pKa is 9.25, meaning that from a pH of 8.25 and on, nitrogen could leave the culture as ammonia (37). Still, in the present case, the pH remained between 7.5 and 8. Therefore, the ammonium ion concentration evolution can be attributed to a complex interaction between microalgae and bacteria population (25), which was already indirectly observed by other authors (22). Consequently, it is easier to analyze the Total Nitrogen content of the culture medium to track the fate of extracellular nitrogen. The trend is bi-phasic, with a sharp decrease over the two to three days following the medium addition, to which a plateau succeeds. The first phase reaches a removal rate of 100.8 $\pm$ 26.3 mg/L/d, while the plateau phase stagnates around 100 mg/L of Total Nitrogen. The other macroscopic performance indicator, the Total Organic Carbon concentration, follows the same trend: a removal rate of 1.564 $\pm$ 0.453 g/L/d and a plateau of around 1 g/L.

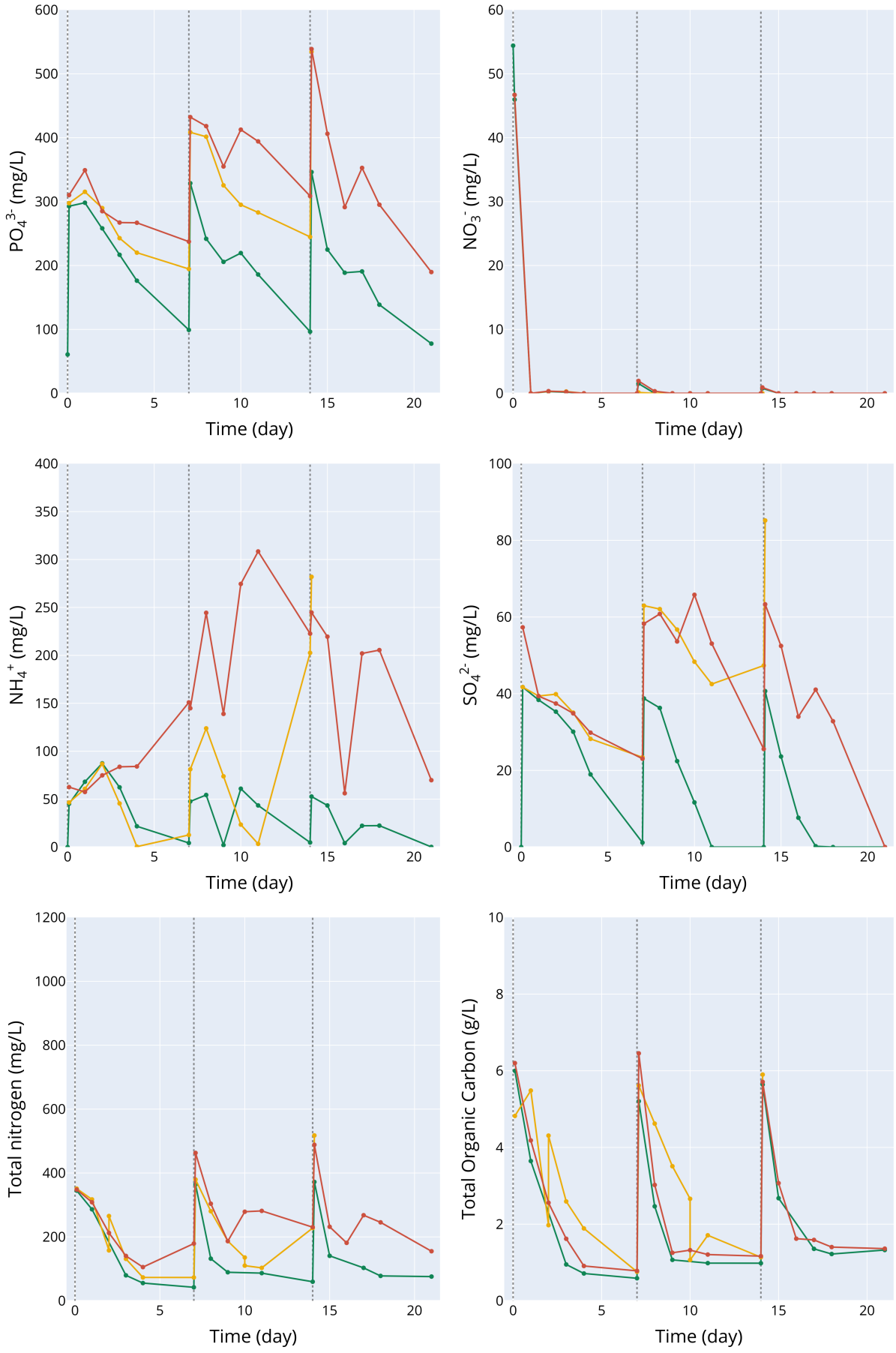

Fig. 4. PO_4^{3-}, NO_3^-, NH_4^+ and SO_4^{2-} concentrations and Total Nitrogen and Total Organic Carbon in the 250 ml bubble column photobioreactors. Green line - photobioreactor A, Yellow line - photobioreactor B, Red line - photobioreactor C. Dashed gray lines - new effluent addition

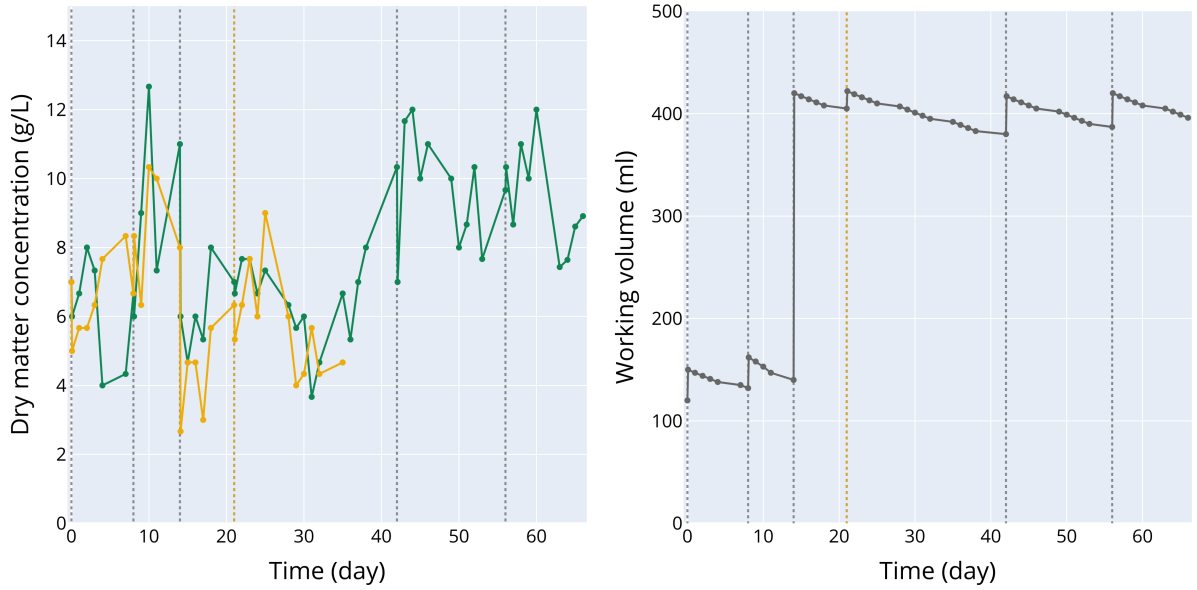

Fig. 5. Dry matter concentration (left) and working volume (right) in the 500 ml bubble column photobioreactors. Green line - photobioreactor A, Yellow line - photobioreactor B. Dashed gray lines - new effluent addition. Dashed yellow line - new effluent addition and illumination increase

3 5. 500-ml bubble column upscaling

Figures 5 and 6 present dry matter, volume, ions, Total Nitrogen, and Total Organic Content concentrations over time. The first comment is that the runs were very similar until the second photobioreactor experienced a failure of the aeration over a weekend. Following this event, it quickly turned brown. Despite the reactivation of the aeration, the crash of the culture could not be avoided.

Dry matter concentration increased from 6 to 10 gDW/L. The two photobioreactors achieved averaged productivities of 0.29 and 0.43 g/L/d, respectively (before crashing for photobioreactor B). The difference between photobioreactor A and the other runs originates from its higher working volume (420 ml), which is only partially compensated by the increase in light collection surface and light intensity. Indeed, despite the increase in illumination following the increase in working volume, the light dose was divided by 1.68, which is extremely close to the 1.62 factor in the reduction of productivity.

Nevertheless, monitored ionic species exhibited a similar behavior in the previous runs. SO_4^{2-} removal rate was 6.60 $\pm$ 2.11 mg/L/d, with concentrations reaching 0 mg/L before medium addition. PO_4^{3-} removal rate was 23.0 $\pm$ 4.9 mg/L/d, with a non-zero concentration before dilution (about 200 mg/L). In the same manner, Total Nitrogen and Total Organic Carbon concentrations exhibited trends close to the one observed in a lower volume, with removal rates of 112.1 $\pm$ 52.0 g/L/d and 1.075 $\pm$ 0.366 g/L/respectively, and plateau values around 100 mg/L and 1 g/L, respectively.

The fact that epuration performances are close between the 250 mL and the 500 mL bubble columns systems is to be commented on. Indeed, as microalgal growth drives the epuration, the fact that biomass productivity is reduced in the larger system could lead one to expect that removal rates should also be reduced by the same extent. Yet, microalgae are capable of making extra reserves of valuable components by a mechanism called luxury uptake. For example, in the case of phosphorous, they can triple their inner content under phosphate-replete conditions in case they face dire conditions later on (6). This mechanism, combined with semi-continuous operation, is thought to explain this observation.

Obtaining similar results after expanding the culture volume from 100 to 420 mL underlines the scalability of the process and further emphasizes that potential remains of the initial medium swiftly disappear and do not modify the culture behavior. Still, as in any photoautotrophic microalgal cultivation process, the light dose is key, and care has to be taken to maintain it throughout the upscaling process.

3 6. Biomass composition

Biomass lipid, carbohydrate, and protein contents were characterized twice using biomass coming from the 500-ml bubble columns runs after process stabilization at a high volume (day 42 and day 56). The microalgal cells contained 24 and 27 % of lipids, 36 and 26 % of carbohydrates, and 32 and 34 % of proteins. All these fractions add up to 92 and 87 %, which is coherent as about 6 to 16 % of ash can be found in microalgal cells (38). Furthermore, the fact that the compositions are similar between the two time points reinforces the observation that the process entered a quasi-steady state. As a point of comparison, the protein content is higher than the one reported by He *et al.* (20 % (25)) and somewhat closer to what is expected for *Chlorella vulgaris* (about 50 % (38)). As the production process preserves the food-grade condition of the stream, the obtained protein content opens the way to biomass valorization as feed with a balanced macronutrient profile.

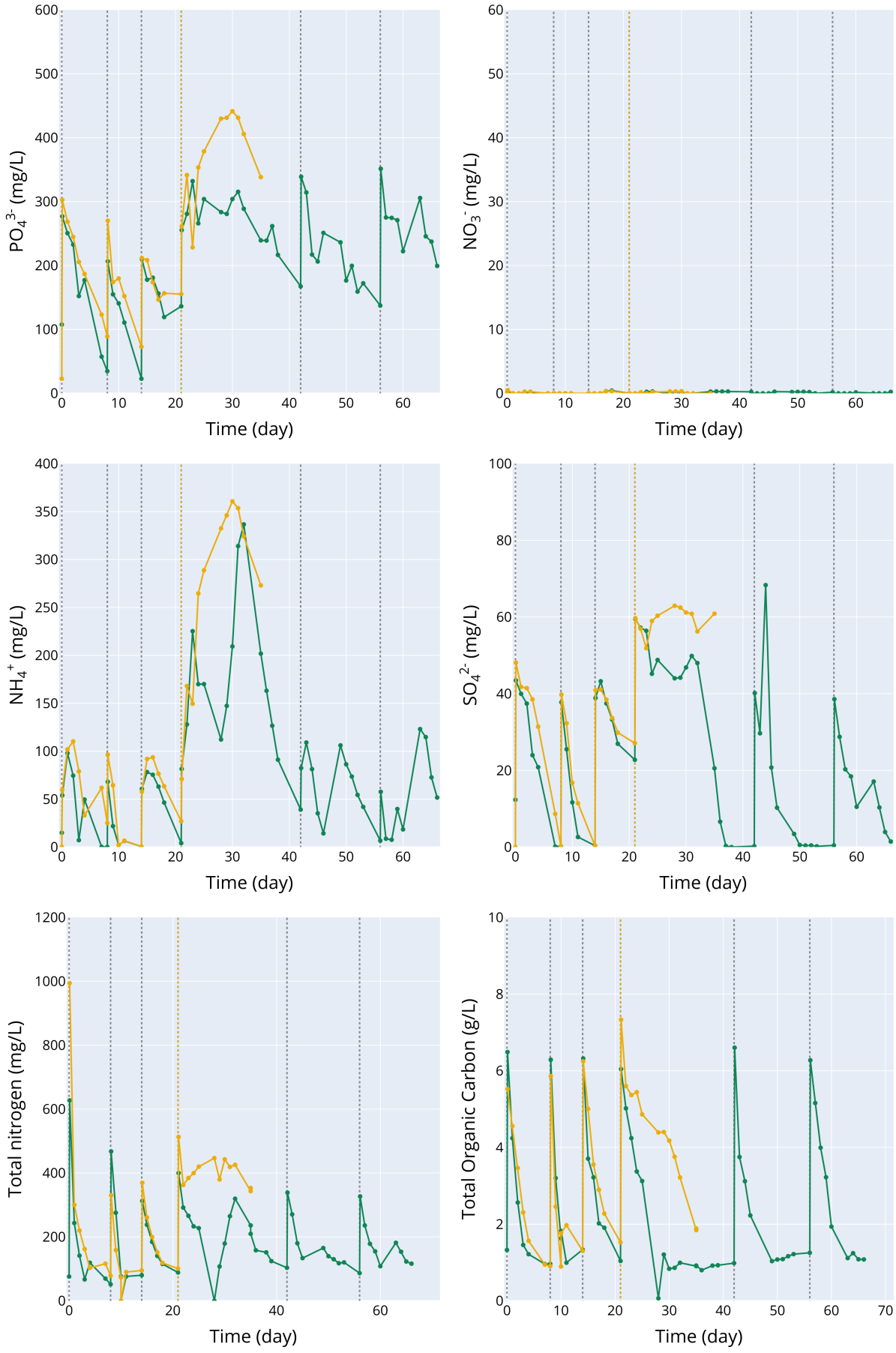

Fig. 6. PO_4^{3-}, NO_3^-, NH_4^+ and SO_4^{2-} concentrations and Total Nitrogen and Total Organic Carbon in the 500 ml bubble column photobioreactors. Green line - photobioreactor A, Yellow line - photobioreactor B. Dashed gray lines - new effluent addition. Dashed yellow line - new effluent addition and illumination increase

Medium	Removal rate (mg/L/d)						Biomass productivity (g/L/d)	Strain	Ref.
	PO_4^{3-}	SO_4^{2-}	NH_4^+	COD	Total Nitrogen	Total Phosphorus			
Raw effluent	1.35†	-	-	117.19	62.06	0.44	0.08	*Chlorella vulgaris*	(24)
Raw effluent	-1.53†	-	8.65	107	8.39	-0.5	0.2	*Chlorella* spp. & *Scenedesmus* sp	(25)
Centrifugated effluent	-	-	-	847.50	5.11	-	-	*Chlorella sorokiniana*	(22)
Synthetic effluent	10.35†	-	3.88	179.91	17.50	3.38	0.2	*Scenedesmus* sp.	(20)
Synthetic effluent	-	-	-	221.35	1.01	-	0.1	*Scenedesmus obliquus*	(17, 18)
Synthetic effluent	8.51†	-	4.75	300	10	2.775	0.175	*Chlorella sorokiniana*	(21)
Raw effluent	24.5	6.82	-	4007.7*	106.45	-	0.46	*Chlorella vulgaris*	Present work

Table 3. Cell productivity and removal rates. * computed based on TOC to COD conversion factor. † computed based on PO_4^{3-} / P molar mass ratio

3 7. Comparison with other processes

Table 3 compares the performances obtained with the proposed process to the ones obtained by other scholars. Cell production is about twice as high as the best-reported performance. The same comment can be made for all the removal rates. This is not surprising, as biomass growth drives nutrient removal. Still, COD removal value and absolute values (Tab. Tab. 1 and 3) can be questioned. Indeed, in present case, they were obtained using a correlation linking TOC and COD. This correlation may lead to an overestimation by 50 % (as COD removal rate is 3 times higher while biomass production is twice higher). Still, this does not change the overall trend of the results.

4. Conclusion

Chlorella vulgaris was grown using actual brewery effluents. The effluent was formulated minimally in order to ensure ease of process scale-up, economical viability, and preserve the food-grade status of the stream. After successful shake flask runs, the process was transferred to 100 and then 500-ml photobioreactors. The achieved performances (biomass production and removal rates) are twice as high as the ones reported by other scholars. Finally, the produced microalgae exhibit a balanced macronutrient profile and can be deemed food-grade, opening the way to biomass valorization as feed.

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Data availability

Data will be made available upon request to the corresponding author.

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