

Microalgae-based wastewater treatment for circular economy: Efficiency of indigenous green microalgae for nutrient removal and resource recovery

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Abstract

Microalgae biotechnology can strengthen circular economy concepts in the wastewater treatment sector. This study demonstrated the potential of the Norwegian microalgae strains of *Tetradesmus wisconsinensis*, *Lobochlamys segnis*, and *Klebsormidium flaccidum* for wastewater nutrient removal and valuable biomass generation. In the laboratory batch-experiment with synthetic municipal wastewater, all strains accomplished total removal of nitrogen and phosphorus. *L. segnis* removed all NH_4^+ and PO_4^{3-} (initial concentration of 28 and 15 mg L^{-1} , respectively) earliest among others (within 4 days) and the harvested biomass had the highest total fatty acid (TFA) content ($193 \pm 12 \text{ mg g}^{-1}$ dry cells). *T. wisconsinensis* biomass on the other hand was superior in total carbohydrates content (40%) while *K. flaccidum* produced a protein-rich (53%) cells and accumulated fatty acids that consisted largely of polyunsaturated fatty acids (82% of TFA). Observed variations in biomass components can be used for a strategic production of targeted compound in resource recovery scenarios for the studied strains.

Introduction

The use of microalgae-based technology has been recognized as an attractive option for wastewater treatment [1–3]. Microalgae recycle nutrients from wastewaters and generate biomass, which is a renewable resource e.g., for high-value products. Thus, the microalgae-based wastewater treatment not only supports the sustainable management of wastes but also fulfills the concept of circular economy – “reduce, reuse and recycle” [2]. To prevent possible nutrient pollution, i.e., eutrophication in the environment, wastewaters should be treated to minimize the nutrient contents before discharging into recipient waters. Conventional wastewater treatment technologies are commonly operated with multiple-step-processes including mechanical and chemical treatments to reduce the dissolved nitrogen (N) and

phosphorus (P) to specified concentrations in the effluent [4]. In addition, anaerobic digestion is combined to decrease organic loading and to recover the energy content, however, the effluent of this process still needs to be treated to reduce its high N content [1]. During the process, the excess N and P are transformed into products without or with limited values, e.g., N_2 and biosolids, that lead an eventual nutrient loss [3]. Overall, there still are some challenges to overcome for the current wastewater management such as high-energy demand, massive sludge generation and greenhouse gas emissions [5].

The advantage of integrating a microalgae-based technology is an upgrade for the reuse-and-recycle-concept in conventional wastewater treatment. It will facilitate a direct recovery of the nutrients, especially N and P into microalgae biomass. This nutrient removal process can be completed in a single-step; therefore, this technology will contribute to enhance the operation efficiency [4]. Moreover, a reduction of CO_2 emission can be achieved by exploiting the inorganic carbon assimilation of photosynthetic microalgae for their metabolism, i.e., a direct CO_2 capture into biomass [3]. Wastewaters are generally rich in N and P compounds that can be assimilated by microalgae, as well as most of the essential trace elements. Several microalgae species can tolerate various types of wastewaters and adapt to the different levels of nutrients in terms of quality and quantity [5, 2]. Although most of the microalgae-based wastewater treatment studies focus mostly on the uptake of nutrients, the potential of the bio-products must be taken into a consideration based on the characteristic of microalgal cell compositions. Depending on the share and quality of carbohydrates and lipids, microalgae can be used as a feedstock for biohydrogen and bioethanol by carbohydrate fermentation, biodiesel and other transportation fuels by direct combustion of the crude oil, and/or anaerobic digestion of biomass for biogas/methane [6]. Moreover, the protein content in microalgae can be used for further exploitation as an alternative source of proteins [7–10].

So far, rather a small number of microalgae species have been studied for wastewater treatment purposes. In most of the previous studies, different species of genus *Chlorella* (such as *C. vulgaris* and *C. sorokiniana*), genus *Tetradismus/ Scenedesmus* (typically *T. obliquus*), and genus *Desmodesmus* were investigated [11, 1, 12–14]. The limited scope of evaluated microalgae species indicates a need for the evaluation of different species. Several studies have explored the appealing potential of applying indigenous species for wastewater treatment [15–19].

This study aimed to evaluate the feasibility of selected native Norwegian microalgae strains for nutrient removal from municipal wastewater, and to characterize their biomass for the composition of proteins, carbohydrates, and fatty acids. Three freshwater chlorophyte strains; *Tetradismus wisconsinensis*, *Lobochlamys segnis*, and *Klebsormidium flaccidum* were chosen for this study. To the best of our knowledge, these species were not previously used for wastewater treatment purposes.

Material And Methods

Selected microalgae strains and cultivation conditions

Tetradasmus wisconsinensis H1 and *Lobochlamys segnis* F12 were originally isolated from field samples of Norsjø and Tårntjernet, respectively, in Telemark County, Norway and they had been maintained as mono-species cultures. Species were identified based on 18S rDNA phylogeny and morphology [20]. The sequence data for *T. wisconsinensis* H1 and *L. segnis* F12 are available in GenBank with the accession number MT968755 and MT973497/MT968751, respectively. *T. wisconsinensis* is a colonial (forming coenobia), non-motile alga in the family Scenedesmaceae [21]. *L. segnis* is a unicellular flagellate, which is a close relative of the largest green algal genera *Chlamydomonas* [22]. *Klebsormidium flaccidum* NIVA-CHL80 was obtained from the Norwegian Culture Collection of Algae (NORCCA). *K. flaccidum* is an unbranched filamentous alga [23].

The experiment cultures were grown in a 1 L Erlenmeyer flask containing 700 mL medium under a continuous illumination providing $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiation in the incubator at 24°C (Infors HT Multitron Pro, infors AG, Botternigen, Switzerland). The cultures received aeration with filtered (pore size of $0.2 \mu\text{m}$) air supplemented with 2% (v/v) CO_2 gas.

Experimental set-up

The microalgae were grown in batch cultivation (triplicates for each species) using a synthetic municipal wastewater for 14 days. All experiment cultures were started with a comparable cell density (optical density 0.005 ± 0.001 , measured at 750 nm). Sampling was conducted every 2 days. Sterilized synthetic wastewater was used because it would provide a consistent nutrient composition for biological mechanisms of the microalgae under the experimental condition. The municipal wastewater was prepared based on previous studies [24, 25] using NH_4Cl : 89 mg L^{-1} , K_2HPO_4 : 28 mg L^{-1} , CaCl_2 : 40 mg L^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 75 mg L^{-1} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 5 mg L^{-1} , $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$: 2.5 mg L^{-1} , H_3BO_3 : $50 \mu\text{g L}^{-1}$, ZnCl_2 : $50 \mu\text{g L}^{-1}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: $30 \mu\text{g L}^{-1}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$: $39 \mu\text{g L}^{-1}$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$: $50 \mu\text{g L}^{-1}$, $\text{KAl}(\text{SO}_4)_2$: $178 \mu\text{g L}^{-1}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$: $50 \mu\text{g L}^{-1}$, $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$: $101 \mu\text{g L}^{-1}$, sodium acetate: 200 mg L^{-1} and NaHCO_3 : 750 mg L^{-1} . All medium components were dissolved in dH_2O and autoclaved, except for NaHCO_3 solution, which was filter-sterilized and added to the autoclaved medium.

Determination of biomass accumulation and growth rate

The biomass accumulation was determined by measuring the increase in cell density and dry cell weight. The change in cell density was monitored by recording optical density (OD) at the wavelength of 750 nm using a microplate reader (Spark, Techan, Männedorf, Switzerland). For cell dry weight, the sample was filtered using glass fiber filters (Whatman GF/A) and was washed with dH_2O . The sample was subsequently dried at 105°C for 24 h. The correlation between OD and dry cell weight measurements was confirmed in all strains (Supplementary information Fig. S1a-c), and the specific growth rate (μ) was determined using the following equation:

$$\mu = (\ln N_2 - \ln N_1) / t_2 - t_1$$

where N_1 and N_2 represents the OD values at times (days) t_1 and t_2 .

Determination of nutrient removal efficiency

The sample was centrifuged (3500 rpm for 10 min), and filtered (cellulose acetate membrane, 0.20 μ m). The concentrations of anions (NH_4^+ , K^+ , Mg^{2+} , Ca^{2+}) and cations (PO_4^{3-} , NO_2^- , NO_3^-) were measured using an ion chromatography (940 Professional IC Vario, Metrohm, Herisau, Switzerland) equipped with the anion column Metrosep A Supp 5–150/4.0 (Metrohm), and the cation column Metrosep C6–150/4.0 (Metrohm). It was operated using an anion eluent containing 64 mM Na_2CO_3 and 20mM NaHCO_3 with a flow rate of 0.7 mL min⁻¹, and a cation eluent containing 1.7 mM 2,6- pyridinedicarboxylic acid and 1.7 mM HNO_3 with a flow rate of 0.9 mL min⁻¹.

Characterization of microalgae cell composition

The cell compositions of total protein and total carbohydrate were measured 4 times over the experimental period (on days 6, 10, 12, and 14). The cell samples for the analyses were prepared by centrifugation at 3500 rpm for 10 min at 4°C and pelletized by centrifuge at 15000 rpm for 2 min. The samples were freeze-dried and stored at – 80°C.

The total protein concentration was determined by a colorimetric assay using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories) by the manufacturer's protocol. The total carbohydrate content was estimated using the Anthrone method [26]. The sample was treated with 2M HCl and heated in a water bath at 100°C for 1 h. The anthrone reagent (0.2% anthrone in concentrated H_2SO_4) was added to the sample to the ratio of 4:1. The sample mixture was placed in the water bath at 70°C for 15 min. The absorbance of the sample was measured at 620 nm using the microplate reader (Spark, Tecan). The total carbohydrate content was quantified using the standard curve for the absorbance measurement of glucose concentration ($R^2 \geq 0.99$).

Fatty acids analysis was conducted using the samples collected at the end of the experiment. Fatty acids extraction and quantification were performed using a method described by Breuer et al. (2013) [27]. The freeze-dried sample was mixed with internal standard C19:0, and the cells were disrupted using a bead beater (FastPrep-24, MP Biomedicals, OH, USA) and an ultrasonic bath (VWR, PA, USA).

Chloroform/methanol solution (1:2) was added, and the liquid phase was collected after centrifugation. Tris buffer (50 mM Tris and 1 M NaCl, pH 7.0) was added, and the lipid was extracted while rotating for 10 min. The chloroform phase was evaporated with nitrogen gas. For the transesterification to fatty acid methyl esters (FAMES), H_2SO_4 (5% in methanol) was added to the lipid extracts and incubated for 30 min at 70°C. The sample was mixed with water and hexane to extract the fatty acid methyl esters (FAMES) in the hexane phase. The FAMES were identified and quantified using a gas chromatography-mass spectrometry system (HP 6890 GC system and 5973 Mass Selective Detector, Hewlett Packard, California, USA together with 6890 Injector, Agilent Technology, CA, USA). The system was fitted with a GC column (AG121-2323, DB-23, 20 m, 0.18 mm, 0.2 μ m) operated by a constant pressure of 11.19 psi and linear velocity of 31 cm s⁻¹ with helium as the carrier gas. The FAMES were identified using Supelco 37 Component FAME Mix standard.

Results

Nutrients and pH

The NH_4^+ was removed completely from the culture medium by day 4 in the case of *L. segnis*, day 6 in the case of *T. wisconsinensis*, and by day 10 in *K. flaccidum* cultures (Fig. 1a). No detection of NO_3^- and NO_2^- was recorded in the media indicating absence of microbial nitrification activity during the experiment. The PO_4^{3-} concentration decreased rapidly to depletion by day 4 in *T. wisconsinensis* and *L. segnis* cultures, while it remained until day 13 in *K. flaccidum* culture (Fig. 1b). None of the strains used up all K^+ provided (Fig. 1c). The removal of K^+ was in total 5.3 ± 0.3 , 5.8 ± 0.4 and $4.1 \pm 0.3 \text{ mg L}^{-1}$ in *T. wisconsinensis*, *L. segnis* and *K. flaccidum*, respectively.

The pH fluctuated slightly in all cultures (Fig. 2). In the *L. segnis* culture, the pH varied between 7.3 ± 0.1 and 8.2 ± 0.2 . The pH of the other strains did not exceed this range.

Microalgae Growth

The highest biomass accumulation of $1.28 \pm 0.02 \text{ g cell dry weight (g dw) L}^{-1}$ was recorded for *T. wisconsinensis*, followed by $1.02 \pm 0.20 \text{ g dw L}^{-1}$ for *L. segnis* and $0.63 \pm 0.08 \text{ g dw L}^{-1}$ for *K. flaccidum* (Fig. 3).

L. segnis and *T. wisconsinensis* showed the highest growth rate of 1.12 ± 0.06 at day 4 and 1.10 ± 0.19 at day 2, respectively (Fig. 4a and b). The growth rate of *K. flaccidum* was the lowest of all. This strain had two growth rate peaks, the first peak at day 2 (0.55 ± 0.14) and the second at day 12 (0.50 ± 0.24) (Fig. 4c).

Cell Composition

Total proteins

The strain with the highest total proteins content was *K. flaccidum* ($53.2 \pm 3.5\%$ of dry biomass weight (% dw)) followed by *L. segnis* ($33.7 \pm 0.5\%$ dw) and *T. wisconsinensis* ($30.9 \pm 4.8\%$ dw). In all strains, the total protein share of the cell was the highest at the earliest stage (day 6) and was decreased over the experiment period (Fig. 5a). A considerable reduction of the protein content (42% reduction) was observed between day 6 and 10 for *T. wisconsinensis* (Fig. 5a). The reduction led to the lowest protein content ($16.1 \pm 0.5\%$ dw) among all tested strains. *K. flaccidum* showed a similar decrease between days 10 and 12 (40% reduction). The change of protein content in *L. segnis* was moderate over time and it was limited to a 28% reduction from the maximum to the minimum.

Total carbohydrates

T. wisconsinensis had the highest total carbohydrates content of $40.4 \pm 6.0\%$ dw. When compared to the others, this strain showed a higher carbohydrate share in their cells at all sampling points (Fig. 5b). The lowest share of total carbohydrate was recorded for *K. flaccidum* at day 6 ($11.6 \pm 1.3\%$ dw), however later, the total carbohydrate content increased remarkably (191% increase from day 6 to 12). Other strains also increased their carbohydrate content over time (Fig. 5b). There was a noticeable decrease from day 12 to 14, except for *K. flaccidum* that did not show any change during this period (Fig. 5b).

Total fatty acids contents and profiles

The total fatty acid (TFA) content was compared at the end of the experiment. It was the highest in *L. segnis* ($192.8 \pm 11.7 \text{ mg g}^{-1}$ dry cells), followed closely by *K. flaccidum* ($183.6 \pm 5.9 \text{ mg g}^{-1}$ dry cells) (Fig. 6). *T. wisconsinensis* contained the lowest TFA ($129.1 \pm 2.2 \text{ mg g}^{-1}$ dry cells) (Fig. 6).

There were 15 detectable fatty acids with the carbon-chain length longer than C14 (Table 1). Each strain had a different profile and abundance of the individual fatty acids (Fig. 7). The fatty acid profile of *K. flaccidum* was characterized by a remarkable proportion ($73.3 \pm 0.3\%$ of TFA) of linoleic acid (C18:2 *n*-6) (Fig. 7). The dominance of this fatty acid made their profile particularly high in polyunsaturated acids (PUFA) ($82.2 \pm 0.1\%$ of TFA), and low in the *n*6 to *n*3 ratio (0.11 ± 0.0) (Table 1). In comparison, the PUFA content of *L. segnis* was slightly lower ($67.2 \pm 0.3\%$). Nevertheless, this strain contained higher amount of *n*3 fatty acids, especially α -linolenic acid (C18:3 *n*-3), that made a higher *n*6 to *n*3 ratio (1.3 ± 0.1) (Table 1). The profile of *T. wisconsinensis* was represented by a large proportion of oleic acid (18:1 *n*-9) ($37.6 \pm 0.7\%$ of TFA) (Fig. 7), and a prominent share of monounsaturated fatty acids (MUFA) compared to the other strains (Fig. 7).

Table 1

Fatty acid composition (mg g⁻¹ dry algae cells) of *Tetradasmus wisconsinensis*, *Lobochlamys segnis* and *Klebsormidium flaccidum*. Mean value ± standard deviation is given (3 replicate cultures for each strain, 3 measurements from each replicate)

Fatty acid composition (mg g ⁻¹ dry algae cells)	Microalgae strain		
	<i>Tetradasmus wisconsinensis</i> H1	<i>Lobochlamys segnis</i> F12	<i>Klebsormidium flaccidum</i> NIVA-CHL80
C14:0	1.08 ± 0.03	0.27 ± 0.01	0.41 ± 0.01
C16:0	21.08 ± 0.35	25.35 ± 0.24	13.87 ± 0.07
C16:1 <i>n</i> 7 (E*)	0.83 ± 0.07	0.45 ± 0.04	0.18 ± 0.01
C16:1 <i>n</i> 7 (Z*)	0.32 ± 0.10	0.33 ± 0.04	0.49 ± 0.04
C16:2 <i>n</i> 6	1.35 ± 0.11	1.66 ± 0.20	0.59 ± 0.03
C16:3 <i>n</i> 3	1.40 ± 0.08	3.16 ± 0.09	1.35 ± 0.09
C16:4 <i>n</i> 3	2.74 ± 0.05	7.21 ± 0.27	N.D.
C18:0	2.82 ± 0.12	0.71 ± 0.09	1.16 ± 0.06
C18:1 <i>n</i> 9 (Z*)	35.42 ± 0.80	2.93 ± 0.08	1.37 ± 0.04
C18:1 <i>n</i> 7 (Z*)	N.D.	2.74 ± 0.13	0.33 ± 0.01
C18:2 <i>n</i> 6	14.07 ± 0.32	22.03 ± 0.86	73.32 ± 0.29
C18:3 <i>n</i> 6	N.D.	5.51 ± 0.11	0.29 ± 0.03
C18:3 <i>n</i> 3	15.39 ± 0.38	25.24 ± 0.67	6.65 ± 0.12
C18:4 <i>n</i> 3	2.50 ± 0.13	2.41 ± 0.05	N.D.
C20:1 <i>n</i> 9	1.0 ± 0.04	N.D.	N.D.
Total <i>n</i> 6	15.42 ± 0.42	29.21 ± 0.94	74.20 ± 0.30
Total <i>n</i> 3	22.03 ± 0.61	38.02 ± 0.84	8.00 ± 0.21
<i>n</i> 3: <i>n</i> 6 ratio	1.43 ± 0.06	1.30 ± 0.07	0.11 ± 0.00

*E: trans isomer, Z: cis isomer. N.D.: not detected.

Discussion

Nutrients removal efficiency

L. segnis achieved the best performance in terms of nutrient removal by showing the fastest uptake of the N and P in the wastewater medium. Regarding NH_4^+ removal, *L. segnis* completed total removal within 4 days (Fig. 1a). In comparison, *T. wisconsinensis* took slightly longer time (6 days) to take up all NH_4^+ (Fig. 1a). The difference in the N removal between these two strains was assumed to be related to their growth performance. *L. segnis* and *T. wisconsinensis* showed a similarly high maximum growth rate at the early stage of the experiment (1.10 ± 0.19 and 1.12 ± 0.06 , respectively). However, the maximum growth rate was recorded on day 2 for *L. segnis* (Fig. 4a), and on day 4 for *T. wisconsinensis* (Fig. 4b). The NH_4^+ was depleted faster in *L. segnis* presumably because it was converted to their biomass at higher rate in the earlier stage than *T. wisconsinensis*. Therefore, our result confirms that microalgae strains with a short lag phase, i.e., with an ability for a rapid acceleration of growth are advantageous for N removal.

L. segnis and *T. wisconsinensis* used the PO_4^{3-} equally fast (Fig. 1b), despite the differences in the N uptake rate. The result indicates that the P consumption rate would not be understood solely by a comparison of their growth rate. The result might, for example, be explained by the luxury P uptake mechanism known for microalgae [28]. Some microalgal species take up P more than their immediate need for metabolism, and the excess P will be stored until they endure a shortage of P supply in the environment [28, 29]. Considering the observation of the fast P removal before reaching the highest growth phase in *T. wisconsinensis* (Fig. 1b and 4b), this mechanism might be involved in their large P uptake and storage. However, this assumption needs a further study of their cell metabolism to confirm.

Another possible explanation is a variation in their metabolic needs for P in relation to N. Assimilation of these two macronutrients in microalgae has been shown to be correlated, and the correlation is described as N:P ratio. It was described originally by Redfield (1958) proposing a constant elemental ratio of carbon (C): N: P in marine plankton (so-called Redfield ratio) [30], then it was further debated by various research fields related to microalgae [31]. Most importantly, the correlation between the N and P uptake is associated with the crucial linkage of protein and ribosomal RNA syntheses that are the major regulation for the N and P demands, respectively [32, 31]. The importance of the N:P ratio has been suggested in the screening studies of microalgae species for wastewater nutrient remediation [33, 34]. Whitton et al. (2016) studied the relationship between the internal N:P ratio in association with nutrient uptake among freshwater microalgal species. Interestingly, this study showed that the internal N:P ratio was flexible in accordance with the “nutrient environment”, and their nutrient uptake was rather controlled by species specific demands for cell metabolism and storage [33]. When compared to *L. segnis*, *T. wisconsinensis* might have assimilated more PO_4^{3-} per amount of NH_4^+ . Therefore, the P removal was similarly fast but the N removal was slower in *T. wisconsinensis*. Conversely, *K. flaccidum* showed a different characteristic regarding the N:P ratio requirement. Despite the slow nutrient removal, this strain consumed all NH_4^+ before taking up all PO_4^{3-} . Thus, they required more NH_4^+ per amount of PO_4^{3-} compared to the former two strains. This result suggests that this strain might be suitable for nutrient removal purposes for other types of wastewaters that have low P in relation to N contents.

Our result also suggested that the studied strains required only a low concentration of K^+ for their growth. K is an essential nutrient for cell metabolism, such as for enzymes involving photosynthesis and respiration, and for protein and carbohydrate synthesis [35]. A study of various waste streams showed that K^+ concentrations in municipal wastewater were relatively low and that the concentrations ranged between 13–20 $mg\ L^{-1}$ [36]. The wastewater used in this study contained 11 $mg\ L^{-1}$ K^+ which remained until the end of the experiment. Therefore, we could assume that K would not primarily be the growth limiting factor when using municipal wastewater as a growth medium for the studied strains. This assumption might be applicable essentially to chlorophytes. Tokuşoglu and üUnal (2003) reported that the internal element compositions of K in *Chlorella vulgaris* and *Haematococcus pluvialis* were low (0.45–0.98%) when compared to those of a cyanobacterium, *Spirulina* (2.58%) and of a haptophyte *Isochrysis* (1.19%) [37]. Thus, the nutritional requirement for K varies among different groups of microalgae, and it would be necessary to study further about K uptake of other microalgae species.

Resource Recovery Potential

Concerning the potential of using the harvested biomass for protein production, the most promising strain was *K. flaccidum*, which contained up to 53% protein in the cells. A recent review addressed microalgae protein showed that the protein contents varied from 6 to 71% and the typical value was 40 to 60% [38]. The protein content of *K. flaccidum* shown in this study was, therefore, comparative to those previously reported values of the microalgae species. However, their biomass productivity was low. Especially when the *K. flaccidum* cells contained the highest protein content (day 6) (Fig. 5a), the biomass accumulation was limited to $0.078 \pm 0.015\ g\ dw\ L^{-1}$, which was only 15% of the highest-grown *L. segnis*. Thus, when considering protein production using this strain, there will be a need for further optimization of biomass productivity.

The total carbohydrate production was the highest in *T. wisconsinensis*. The advantage of this strain is its high biomass generation. At its highest value, this strain contained 40% total carbohydrate (Fig. 5b). The biomass accumulation at the time was $975 (\pm 66)\ mg\ L^{-1}$. This gives their total carbohydrate productivity of $390\ mg\ L^{-1}$. An interesting use of microalgae rich in carbohydrates is their potential for generation of biofuel products (e.g., bioethanol biohydrogen, biobutanol) [39, 40]. In addition, a biorefinery concept can be adapted to microalgae-based biofuel production where both carbohydrates and lipids fractions in biomass are utilized [39]. Total carbohydrate could be a target product for an operation of microalgae-based wastewater treatment using *T. wisconsinensis*.

For the use of the recovered biomass for fatty acid production, *L. segnis* and *K. flaccidum* were favorable over *T. wisconsinensis*. The TFA content of these strains was 20%. It was lower than the average content of oleaginous green microalgae, which was reported as 25.5% [41]. However, the abundance of PUFA in their total fatty acid was noteworthy. The contents of PUFAs accounted for 82% and 67% of the total fatty acids in *K. flaccidum* and *L. segnis*, respectively. These values are markedly higher than those of the many other strains reported previously from the screening studies for PUFA production [42]. The FA of *K.*

flaccidum had high purity (more than 70%) of linoleic acid (C18:2 *n*-6). This strain will be suitable as an alternative source of this compound. The FA of *L. segnis* was mainly of C18:3 *n*-3 and C18:2 *n*-6, which made the ratio of *n*-3 to *n*-6 up to 1.3, which was much higher than that of *K. flaccidum* (Table 1). The *n*-3/*n*-6 ratio is another quality indicator for the nutritional application of FAs [43]. The examined strains did not contain very long-chain (VLC) omega-3 PUFA. Generally, C20 and C22 PUFAs are seldom found among chlorophyte species [44]. The C18 PUFAs produced by microalgae are essential for freshwater food chain [45] and freshwater fish can utilize C18:3 *n*-3 and C18:2 *n*-6 for elongation and desaturation to synthesize VLC omega-3 PUFA more efficiently than marine fish [46]. Therefore, the application of *L. segnis* containing these C18 PUFA for freshwater aquaculture feeds can be an interesting research topic to pursue.

Optimizing Biomass Recovery And Cell Compositions

Our study also illustrated how harvest timing would affect differences in the biomass recovery. The highest amount of biomass among all at the end of the experiment was accumulated by *T. wisconsinensis* ($1.28 \pm 0.02 \text{ g dw L}^{-1}$) (Fig. 3). In comparison, the final biomass of *L. segnis* was lower ($1.02 \pm 0.20 \text{ g dw L}^{-1}$), although, its biomass accumulation had been superior to *T. wisconsinensis* until day 10 (Fig. 3). Supposedly, *L. segnis* could not sustain the high rate of biomass increase because of a lack of nutrient at the late experimental stage. Our experiment was conducted under a batch operation. Under this condition, the production of microalgae can be strategically controlled by harvesting their biomass when the exponential growth ceases. This harvesting practice can shorten the production cycle, and thus, it can reduce the energy input for biomass production for each cycle. When focusing on the microalgae-based technology for nutrient removal purposes, this strategic biomass production also enhances the wastewater treatment capacity, by increasing flow rate of influent (untreated wastewater) into the system. Another strategy to effectively recover the microalgae biomass is a semi-continuous operation, where a part of the culture is regularly harvested and replaced by untreated wastewater to supply nutrients for further microalgae growth. In general, a high biomass accumulation is problematic for the operation of conventional biological wastewater treatments because the biomass value would not compensate the energy cost for separation of biomass from the treated water. In such operations, the main purpose of the wastewater treatment is to remove excess nutrients, not to recover the generated biomass. However, in the microalgae-based approach, the enhancement of high-value biomass generation is as important as the removal of nutrients from the waste streams. For this purpose, the growth characteristics of the microalgae strains and best practices for cultivation/harvesting should be defined for the most beneficial strategy for the operation of biomass recovery.

Moreover, based on the results we expect that the composition of the recovered biomass can be manipulated by choosing a harvesting time when the proportion of component of interest reaches a peak. Our result showed that the proportions of proteins and carbohydrates in the biomass changes over time according to nutrient availability. Especially, the protein content in the cells was related to NH_4^+ availability. *T. wisconsinensi* consumed all NH_4^+ by day 6 (Fig. 1a), and the event of largest reduction of

the protein content happened when they started to starve for N (during the time between day 6 and day 10, Fig. 5a). In the meantime, *L. segnis* used up all NH_4^+ by day 4 (Fig. 1a) and had already starved for NH_4^+ at least for 2 days at the first analysis of protein content (day 6). The reduction of the protein content of this strain was rather gradual between the sampling times (Fig. 1a). For *K. flaccidum*, day 10 was when they used up all NH_4^+ (Fig. 1a), and the largest reduction of the protein content was observed from day 10 to day 12. Accordingly, these results suggested that the protein content was principally related to the N availability, and the largest reduction happened just after the depletion of the N source. The considerable loss of protein contents might be related to a degradation of the protein compounds to continue their growth after the N-depletion event. When the N source in the surroundings reduces, microalgae may recycle internal N by degrading the protein compounds for the maintenance of their cells and further cell division [47]. Additionally, N-limitations induce redirection of metabolism to the production of storage compounds that do not contain N, i.e. carbohydrates and lipids [47]. Therefore, as the N-depletion is sustained, the redirection of metabolism could be evident in the increase in the total carbohydrate. Indeed, phosphorus availability also influences carbohydrate contents in microalgae. Carbohydrate accumulation starts when the intracellular phosphorus concentration reduces to below a threshold level [40]. Our result showed that the total carbohydrate was reduced from day 12 to day 14 in *T. wisconsinensis* and *L. segnis* (Fig. 5b). These strains had experienced a longer N- and P-depletion period at this stage (Fig. 1a and b). It might indicate that the metabolism changed the course again towards the production of more stable storage compounds, triacylglycerides [41].

Taken together, resource recovery scenarios for the studied strains could be further optimized by targeting specific cell component production with a specified cultivation period combined with the efficiency for nutrient removal from wastewater.

Conclusions

In this study, three Norwegian strains of chlorophyte microalgae were studied regarding their potential use for wastewater treatment combined with resources recovery in the context of a circular economy. Their capacity for nutrient removal, biomass production, and cell compositions (total proteins and carbohydrates) were assessed. In addition, the fatty acids were analyzed at the end of the study. Among all, *L. segnis* F12 was the best for nutrient removal performance. This strain accomplished total removal of N and P (the initial concentration of $28 \text{ mg L}^{-1} \text{ NH}_4^+$ and $15 \text{ mg L}^{-1} \text{ PO}_4^{3-}$) before other strains by growing strongly (a maximum specific growth rate of 1.12 ± 0.06). The biomass of this strain was favorable for the total fatty acid (TFA) content ($193 \pm 12 \text{ mg g}^{-1}$ dry cells). *T. wisconsinensis* H1 generated biomass with a high share of total carbohydrates content (40% dw). *K. flaccidum* NIVA-CHL80 showed rather slow nutrient removal and growth. However, this strain was superior for the protein content ($53 \pm 4\%$) and for the TFA production ($184 \pm 6 \text{ mg g}^{-1}$ dry cells) with a remarkable proportion of polyunsaturated fatty acid (82% of TFA) that consisted mainly of C18:2 *n*-6 (73% of TFA). Each strain showed variations in the share of the proteins and carbohydrates content over time according to nutrient

availability. Therefore, resource recovery scenarios could be further optimized for a specific cell component production combined with an appropriate strategy for nutrient removal from wastewater.

Declarations

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Competing interest

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the conception and design of this study. I. Umetani and M. Sposób performed material preparation, data collection and analysis. The draft of the manuscript was written by I. Umetani, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

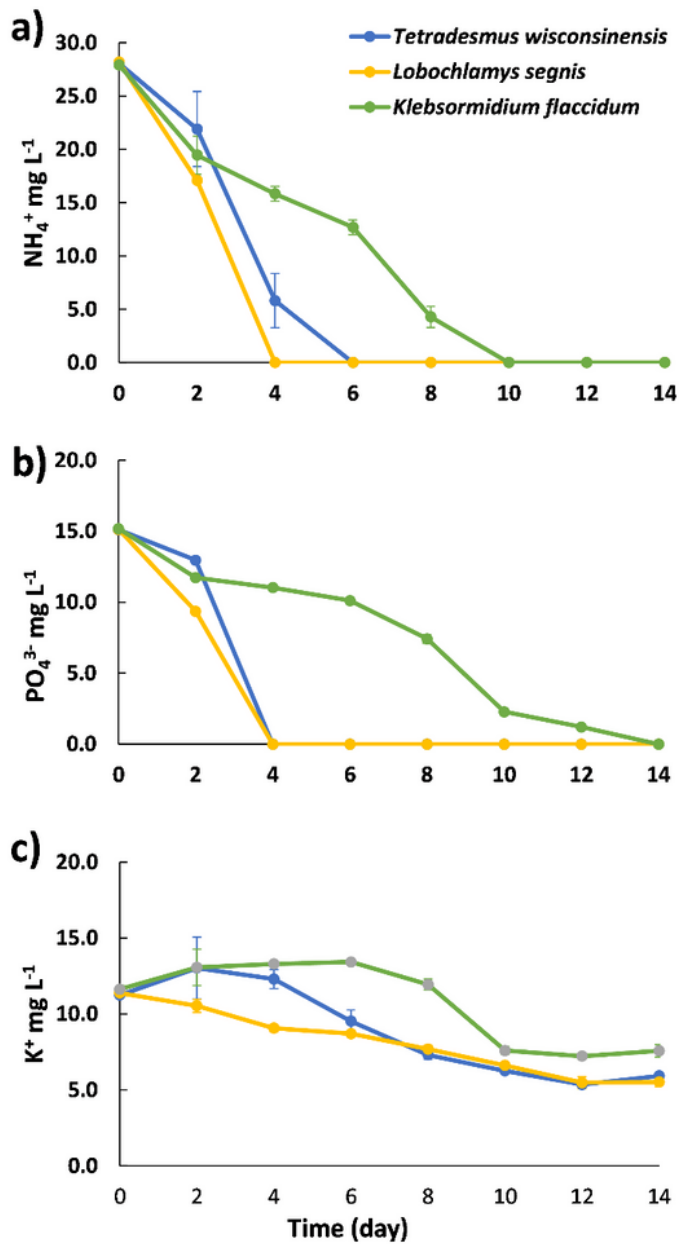


Fig. 1 Nutrient removal rate for a). NH_4^+ , b) PO_4^{3-} and c) K^+ in *Tetradismus wisconsinensis* (●), *Lobochlamys segnis* (●), and *Klebsormidium flaccidum* (●) cultures during the experiment. Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 1 measurement from each replicate culture)

Figure 1

See image above for figure legend

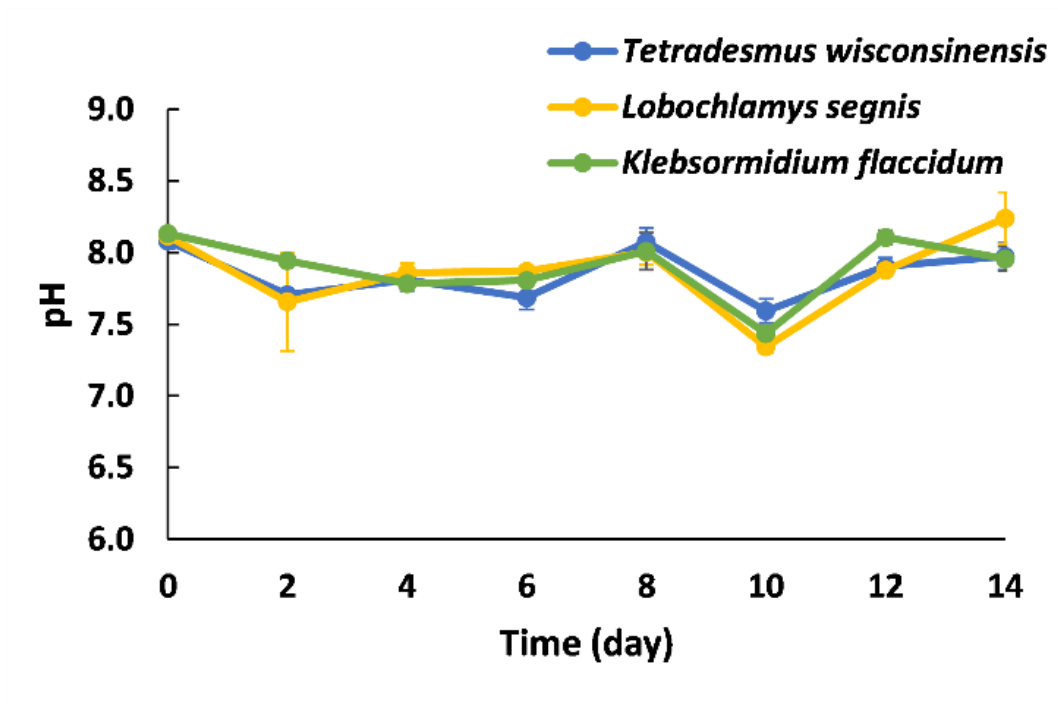


Fig. 2 Changes of pH in the culture medium of *Tetradesmus wisconsinensis* (●), *Lobocholamys segnis* (●), and *Klebsormidium flaccidum* (●) during the experiment. Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 1 measurement from each replicate culture)

Figure 2

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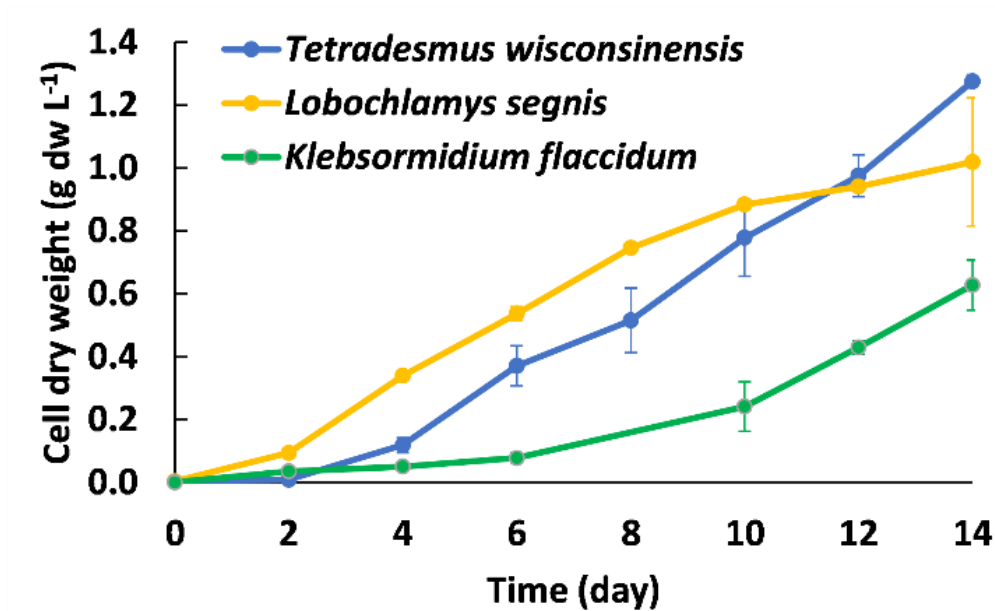


Fig. 3 Biomass accumulation of *Tetradesmus wisconsinensis* (●), *Lobocholamys segnis* (●), and *Klebsormidium flaccidum* (●) measured by cell dry weight (g dw L⁻¹). Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture)

Figure 3

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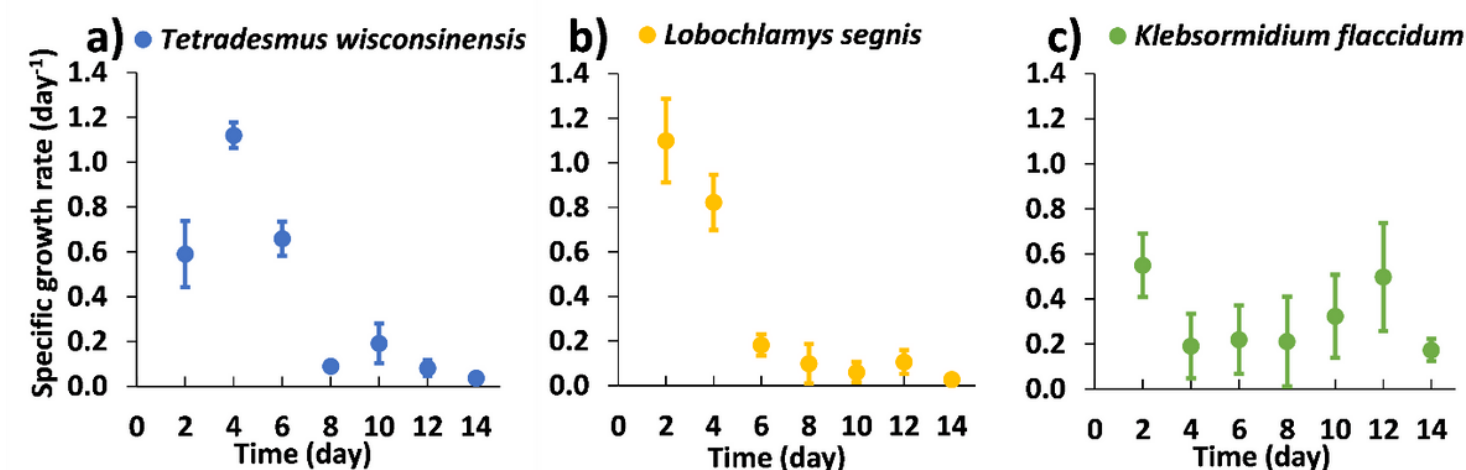


Figure 4

Specific growth rate (day^{-1}) for a) *Tetradasmus wisconsinensis*, b) *Lobochlamys segnis* and c) *Klebsormidium flaccidum* based on the optical density measurements at 750 nm. Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture)

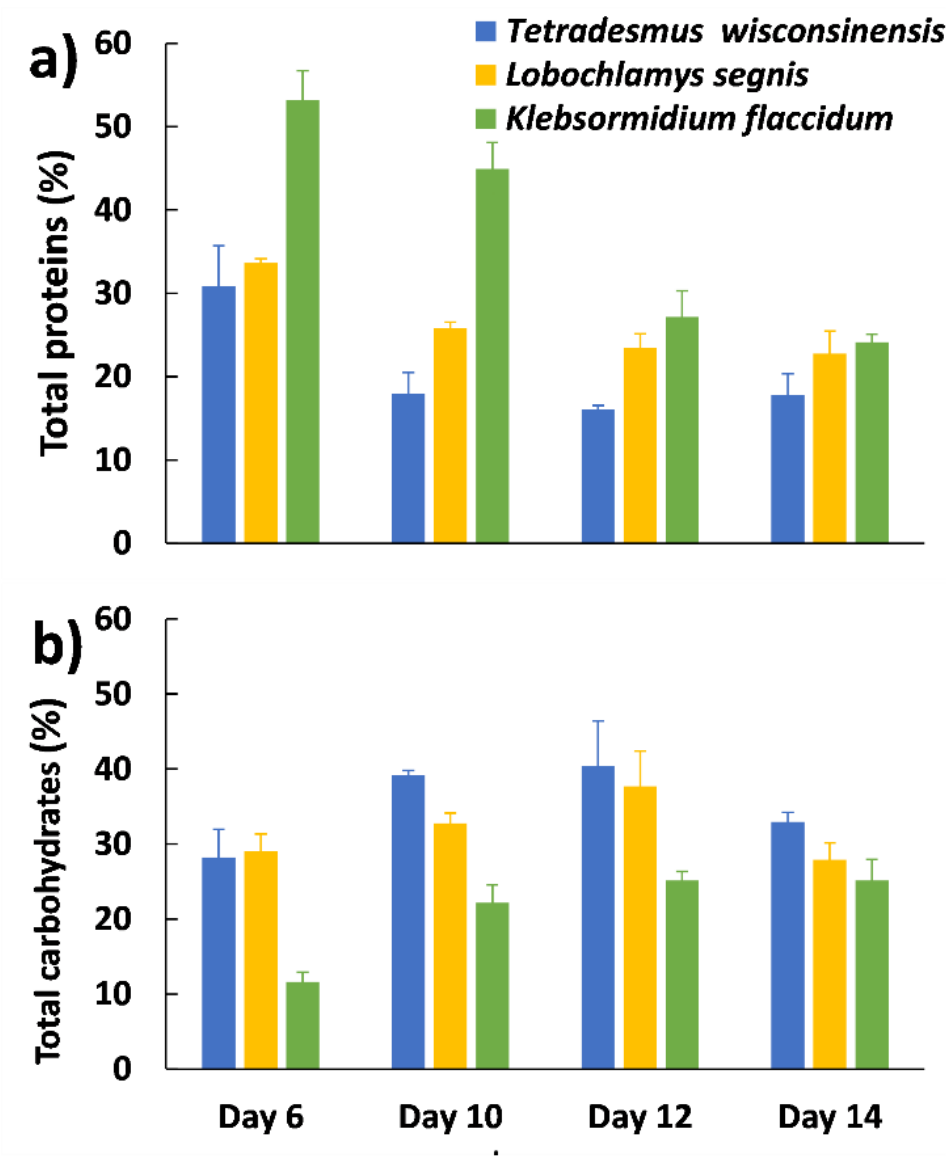


Fig. 5 Cell contents (% of dry cell weight) of a) total proteins and b) total carbohydrates in *Tetradasmus wisconsinensis* (■), *Lobochlamys segnis* (■), and *Klebsormidium flaccidum* (■). Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture)

Figure 5

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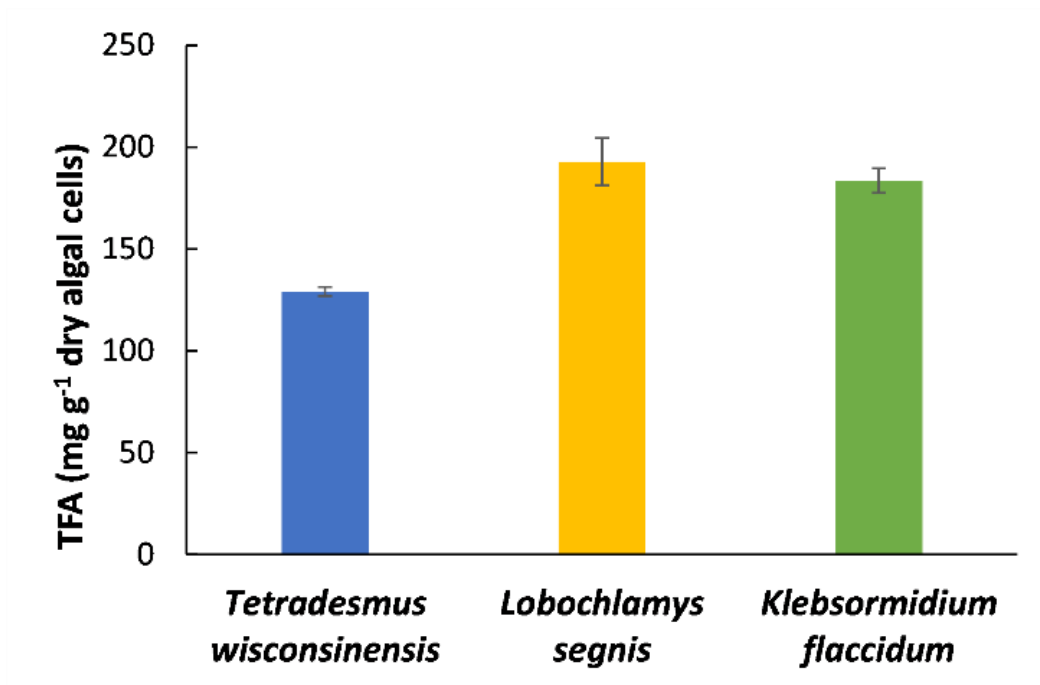


Fig. 6 Total fatty acid (TFA) contents (mg g⁻¹ dry cells) measured in *Tetradesmus wisconsinensis* (■) *Lobochlamys segnis* (■) and *Klebsormidium flaccidum* (■) at the end of a 14-day batch cultivation. Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate)

Figure 6

See image above for figure legend

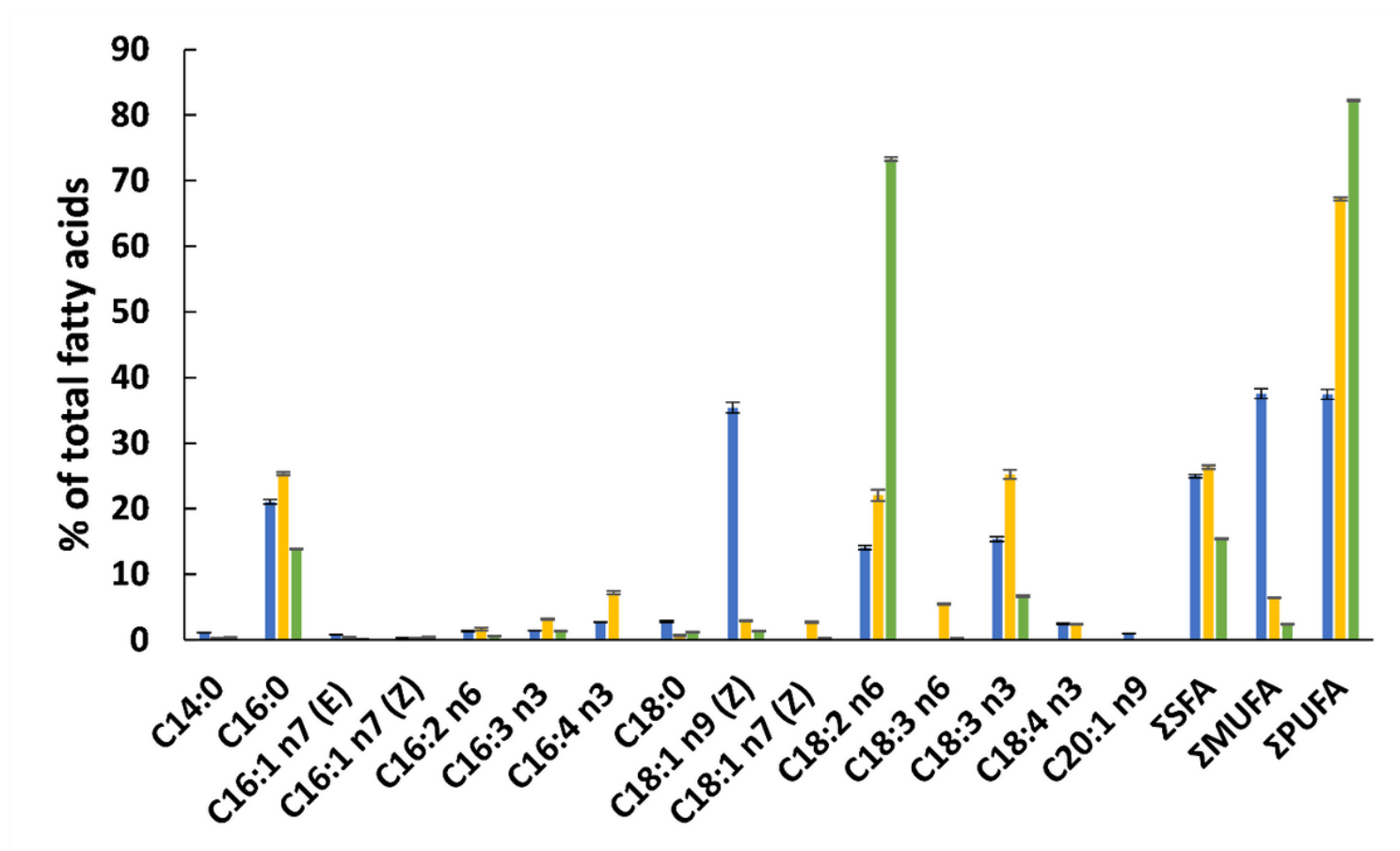


Fig. 7 Fatty acid profiles that shown by proportions of fatty acids (% of total fatty acids) in *Tetradesmus wisconsinensis* (•), *Lobocholamys segnis* (•), and *Klebsormidium flaccidum* (•) at the end of a 14-day batch cultivation. Error bars represent mean $\pm$ 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate)

Figure 7

See image above for figure legend

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