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Indigenous Green Microalgae for Wastewater Treatment: Nutrient Removal and Resource Recovery for Biofuels and Bioproducts

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Abstract
Microalgae biotechnology can strengthen circular economy concepts in the wastewater treatment sector. This study investigated the Norwegian microalgae strains of *Tetradesmus wisconsinensis*, *Lobochlamys segnis*, and *Klebsormidium flaccidum* for their efficiency in nutrient removal. Their biomass productivity and compositions were evaluated for bioenergy and bioproducts development. 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, respectively) earliest among others. *T. wisconsinensis* biomass was superior in total carbohydrates content (40%) and fatty acid profile that imply biorefinery potential. The fatty acid (TFA) content was the highest in *L. segnis* (193 ± 12 mg/g dry cells), while *K. flaccidum* accumulated fatty acids that consisted largely of polyunsaturated fatty acids (82% of TFA). The highest protein level was measured in *K. flaccidum* (53%). Observed variations in biomass components can be used for a strategic production of targeted compound in resource recovery scenarios for biofuel generation.

Keywords Nutrient recycling · Microalgae biomass composition · Biorefinery · Total proteins · Total carbohydrates · Fatty acid profile

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 can be utilized as a renewable resource for value-added products such as biofuels, animal feeds, and biostimulants [2, 4, 5]. Thus, the microalgae-based wastewater treatment not only supports the sustainable management of wastewater 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 [6]. 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 to an eventual nutrient loss [3].

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 and reduce the overall energy demand [6]. 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

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tolerate various types of wastewaters and adapt to the different levels of nutrients in terms of quality and quantity [2, 7].

Benefit of microalgae-based wastewater treatment will not be limited to the nutrient removal effect. But it should consider potential bioproduct development for the harvested biomass. A suitable biomass utilization should be evaluated based on the characteristic microalgal cell compositions. There are currently legal constraints on the biomass that is produced using wastewaters for the food-related applications [8]. Therefore, biofuels are an attractive marketable product from such biomass. 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 [5]. Green microalgae are known to produce starch as storage compound, while glucose, xylose, mannose, and galactose have been reported as their major monosaccharides [9]. The microalgae starch can be efficiently converted into sugars that can be further processed to produce bioalcohols by fermentation technology [9]. In addition, their non-polar lipids (mono-, di-, and triglycerides) are suitable for biodiesel production [5]. Despite its promise in quality, the microalgae biofuel has not been relevant for the market because of the high capital demand and production costs [5]. Biofuel production combined with the microalgae-based wastewater treatment will significantly reduce the costs for nutrients, CO_2, and water [8]. Consequently, it will advance a sustainable and carbon-neutral feedstock generation for biofuel industries. Moreover, the protein content in microalgae can be used for further exploitation of the biomass as an alternative protein source for feed manufacturing [10]. Essentially, the economic vitality of biofuel production in the microalgae-based wastewater treatment can be strengthened by a biorefinery approach of converting the harvested biomass into several commodities [8]. Altogether, characterization of the macro-components of microalgae is an essential step to determine the most desirable target product.

So far, a rather small number of microalgae species have been studied for wastewater treatment purposes. Different species of the genus *Chlorella* (such as *C. vulgaris* and *C. sorokiniana*), genus *Tetradesmus/Scenedesmus* (typically *T. obliquus*), and genus *Desmodesmus* have been investigated for their applicability in the bioremediation of wastewaters, such as urban wastewater [11] and liquid digestate [1, 12]. The limited scope of evaluated microalgae species indicates a need for the evaluation of different species. Recent studies have explored the appealing potential of applying indigenous species for wastewater treatment [13–15]. The advantages of utilizing indigenous strains would be (1) their physiological characteristics that are naturally suitable for the cultivation in the local climate (temperature, light intensity, etc.), (2) utilization of unexploited domestic biological resources, and (3) prevention of biological contamination of foreign species when utilized for large-scale operations.

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, i.e., *Tetradesmus wisconsinensis*, *Lobochlamys segnis*, and *Klebsormidium flaccidum*, were chosen for this study. To the best of knowledge, these species were not previously used for wastewater treatment purposes.

Material and Methods

Selected Microalgae Strains and Cultivation Conditions

Tetradesmus 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 using Bold's Basal Medium [16]. Species were identified based on 18S rDNA phylogeny and morphology [17]. 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 [18]. *L. segnis* is a unicellular flagellate, which is a close relative of the largest green algal genera *Chlamydomonas* [19]. *Klebsormidium flaccidum* NIVA-CHL80 was obtained from the Norwegian Culture Collection of Algae, and the stock culture was maintained in the Z8 medium [20]. *K. flaccidum* is an unbranched filamentous alga [21].

The experiment cultures were grown in a 1-L Erlenmeyer flask under a continuous illumination providing 70 µmol photons/m^2/s irradiation in the incubator at 24 °C (Infors HT Multitron Pro, infors AG, Botterningen, Switzerland). The cultures received aeration with filtered (pore size of 0.2 µm) air supplemented with 2% (v/v) CO_2 gas. The culture vessel contained 700-mL synthetic municipal wastewater medium, which was prepared based on previous studies [22, 23]. The medium consisted of NH_4Cl, 89 mg/L; K_2HPO_4, 28 mg/L; $CaCl_2$, 40 mg/L; $MgSO_4{\cdot}7H_2O$, 75 mg/L; $FeSO_4{\cdot}7H_2O$, 5 mg/L; $Na_2EDTA{\cdot}2H_2O$, 2.5 mg/L; H_3BO_3, 50 µg/L; $ZnCl_2$, 50 µg/L; $CuSO_4{\cdot}5H_2O$, 30 µg/L; $MnCl_2{\cdot}4H_2O$, 39 µg/L; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 50 µg/L; $KAl(SO_4)_2$, 178 µg/L; $CoCl_2{\cdot}6H_2O$, 50 µg/L; $NiSO_4{\cdot}6H_2O$, 101 µg/L; sodium acetate, 200 mg/L; and $NaHCO_3$, 750 mg/L. 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. Sterilized synthetic wastewater was used because it would provide a consistent nutrient composition for biological mechanisms of the microalgae under the experimental condition.

Experimental Set-up and Statistical Analysis

All experimental cultures were conducted in triplicate ($n = 3$), and they were randomly allocated in the incubator. The microalgae were grown in batch cultivation for 14 days. All cultures were started with a comparable cell density (optical density 0.005 ± 0.001, measured at 750 nm). Sampling was conducted every 2 days.

The biomass production, total protein and carbohydrate contents, and total fatty acid accumulation were compared using one-way analysis of variance (ANOVA). Significant differences found by ANOVA ($p < 0.05$) were further analyzed by multiple pair-wise comparison using Tukey's honest significant differences (HSD) and compact letter displays. The statistical analyses were conducted using R software (version 4.2.2).

Determination of Biomass Production 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 cell dry weight measurements was confirmed in all strains (Supplementary information Fig. SI1a-c). The specific growth rate (μ) was determined using Eq. (1) [24], where $N1$ and $N2$ represents the OD values at times $t1$ and $t2$ (days).

$$\mu = (\ln N2 - \ln N1)/t2 - t1 \quad (1)$$

Determination of Nutrient Removal Efficiency

The sample was centrifuged (3500 rpm for 10 min), and filtered (cellulose acetate membrane, 0.20 µm). 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 chromatograph (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, 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.

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 15,000 rpm for 2 min. The samples were freeze-dried and stored at −80°C.

The total protein concentration was determined by a colorimetric Lowry assay [25] 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, Techan). The total carbohydrate content was quantified using the standard curve for the absorbance measurement of glucose concentration ($R^2 \geq 0.99$).

Fatty acid analysis was conducted using the samples collected at the end of the experiment. Fatty acid extraction and quantification were performed using a method described by Breuer et al. [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 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 µm) operated by a constant pressure of 11.19 psi and linear velocity of 31 cm/s 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 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 ± 0.3 mg/L 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 Biomass Production and Growth Rates

The highest biomass yield of 1.28 ± 0.02 g cell dry weight (g dw/L) was recorded for *T. wisconsinensis*, followed by 1.02 ± 0.20 g dw/L for *L. segnis* and 0.63 ± 0.08 g dw/L for *K. flaccidum*. The biomass production was higher in *L. segnis* than *T. wisconsinensis* until day 8 (Fig. 3). The biomass increase was not observed before day 10 in *K. flaccidum* (Fig. 3).

L. segnis and *T. wisconsinensis* showed the highest growth rate of 1.12 ± 0.06 at day 2 and 1.10 ± 0.19 at day 4, respectively (Fig. 4a, 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 ± 3.5% of dry biomass weight (% dw)) followed by *L. segnis* (33.7 ± 0.5 % dw) and *T. wisconsinensis* (30.9 ± 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 ± 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.

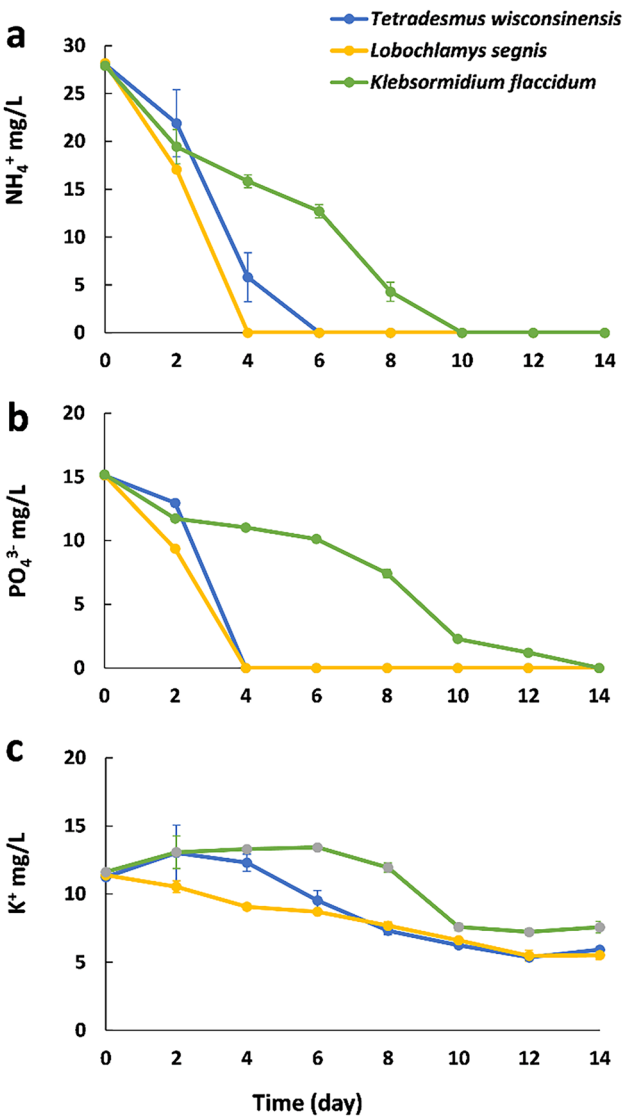


Fig. 1 Nutrient removal rate for **a** NH_4^+, **b** PO_4^{3-}, and **c** K^+ in *Tetradesmus wisconsinensis* (blue circle), *Lobochlamys segnis* (yellow circle), and *Klebsormidium flaccidum* (green circle) cultures during the experiment. Error bars represent mean ± 1 SD (3 replicate cultures for each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

Total Carbohydrates

T. wisconsinensis had the highest total carbohydrates content of 40.4 ± 6.0% dw. When compared to the others, this strain showed a relatively 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 ± 1.3% dw); however, later, the total carbohydrate content increased remarkably (191% increase from day 6 to 12). Also *T. wisconsinensis* and *L. segnis* showed tendencies of

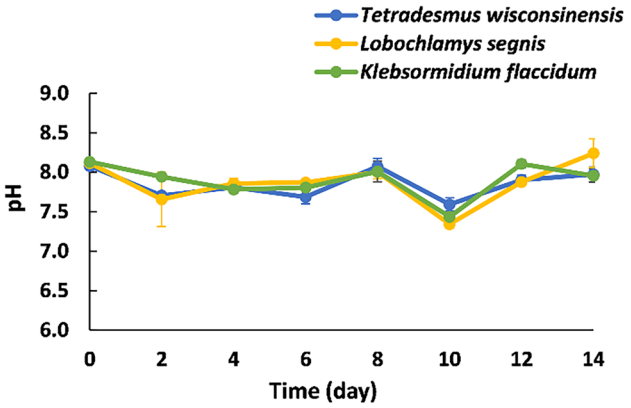


Fig. 2 Changes of pH in the culture medium of *Tetradesmus wisconsinensis* (blue circle), *Lobochlamys segnis* (yellow circle), and *Klebsormidium flaccidum* (green circle) during the experiment. Error bars represent mean ± 1 SD (3 replicate cultures for each strain, 1 measurement from each replicate culture)

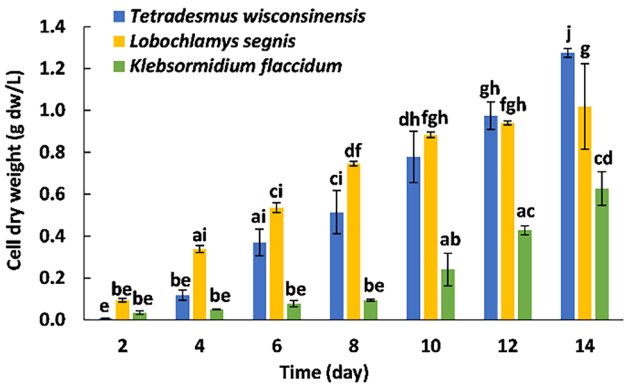


Fig. 3 Biomass production of *Tetradesmus wisconsinensis* (blue bar), *Lobochlamys segnis* (yellow bar), and *Klebsormidium flaccidum* (green bar) measured by cell dry weight (g dw/L). Error bars represent mean ± 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture). Significant differences are shown by different letters (one-way ANOVA with Tukey's HSD, $p < 0.05$)

increase in the carbohydrate content over time, and slight decreases from day 12 to day 14 (Fig. 5b).

Total Fatty Acid 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 ± 11.7 mg/g dw), followed closely by *K. flaccidum* (183.6 ± 5.9 mg/g dw) (Fig. 6). *T. wisconsinensis* contained the lowest TFA (129.1 ± 2.2 mg/g dw) (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 (Table 1). The fatty acid profile of *K. flaccidum* was characterized by a remarkable proportion (73.3 ± 0.3% of TFA) of linoleic acid (C18:2 *n*-6). The dominance of this fatty acid made their profile particularly high in polyunsaturated acids (PUFAs) (82.2 ± 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 ± 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 ± 0.7% of TFA), and a prominent share of monounsaturated fatty acids (MUFAs) (37.57 ± 0.73% of TFA) compared to the other strains (Table 1, Supplementary information Fig. SI2).

Discussion

Nutrient Removal Efficiency

L. segnis achieved the best performance in terms of nutrient removal by showing the fastest uptake of the N and P in

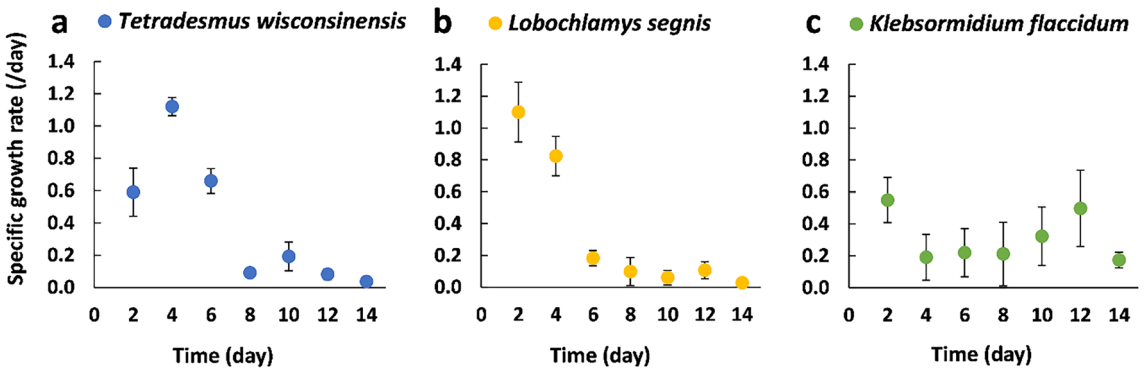


Fig. 4 Specific growth rate (/day) for **a** *Tetradesmus wisconsinensis*, **b** *Lobochlamys segnis*, and **c** *Klebsormidium flaccidum* based on the optical density measurements at 750 nm. Error bars represent mean ± 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture)

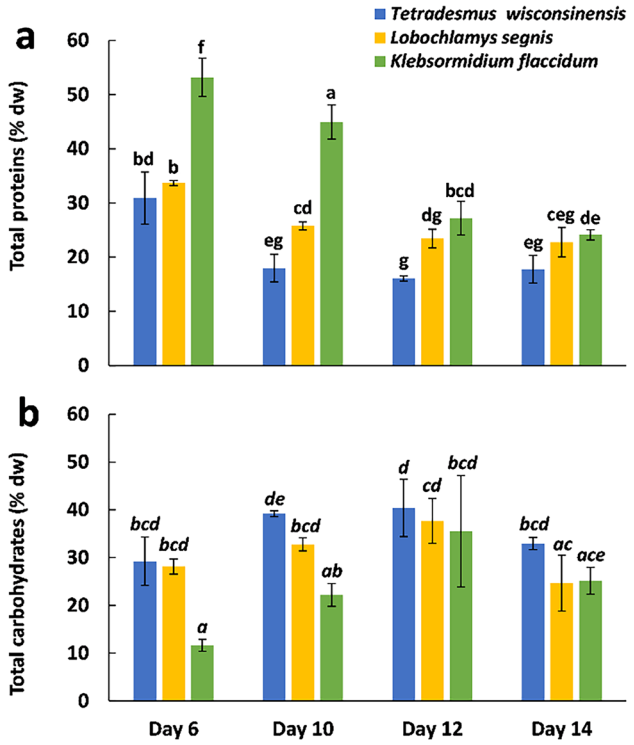


Fig. 5 Cell contents (% of dry cell weight) of **a** total proteins and **b** total carbohydrates in *Tetradesmus wisconsinensis* (blue bar), *Lobochlamys segnis* (yellow bar), and *Klebsormidium flaccidum* (green bar). Error bars represent mean ± 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate culture). Significant differences are shown by different letters (one-way ANOVA with Tukey's HSD, $p < 0.05$)

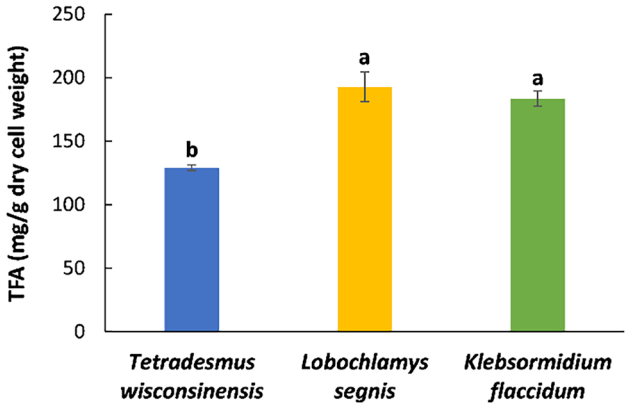


Fig. 6 Total fatty acid (TFA) contents (mg/g dry cell weight) measured in *Tetradesmus wisconsinensis* (blue bar), *Lobochlamys segnis* (yellow bar), and *Klebsormidium flaccidum* (green bar) at the end of a 14-day batch cultivation. Error bars represent mean ± 1 SD (3 replicate cultures for each strain, 3 measurements from each replicate). Significant differences are shown by different letters (one-way ANOVA with Tukey's HSD, $p < 0.05$)

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, the results confirm 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 results indicate 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* (Figs. 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 [30] proposing a constant elemental ratio of carbon (C):N:P in marine plankton (so-called Redfield ratio), 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 [31, 32]. 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. [33] 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. The wastewater medium used in this study had a N:P ratio of 1.9. 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

Table 1 Fatty acid composition (mg/g dry cell weight) of *Tetradesmus wisconsinensis*, *Lobochlamys segnis*, and *Klebsormidium flaccidum*. Mean value ± 1 standard deviation is given (3 replicate cultures for each strain, 3 measurements from each replicate)

Fatty acid composition (mg/g dry cell weight)	Microalgae strain		
	Tetradesmus wisconsinensis H1	*Lobochlamys segnis* F12	*Klebsormidium flaccidum* 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 *n*7 (E*)	0.83 ± 0.07	0.45 ± 0.04	0.18 ± 0.01
C16:1 *n*7 (Z*)	0.32 ± 0.10	0.33 ± 0.04	0.49 ± 0.04
C16:2 *n*6	1.35 ± 0.11	1.66 ± 0.20	0.59 ± 0.03
C16:3 *n*3	1.40 ± 0.08	3,16 ± 0.09	1.35 ± 0.09
C16:4 *n*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 *n*9 (Z*)	35.42 ± 0.80	2.93 ± 0.08	1.37 ± 0.04
C18:1 *n*7 (Z*)	N.D.	2.74 ± 0.13	0.33 ± 0.01
C18:2 *n*6	14.07 ± 0.32	22.03 ± 0.86	73.32 ± 0.29
C18:3 *n*6	N.D.	5.51 ± 0.11	0.29 ± 0.03
C18:3 *n*3	15.39 ± 0.38	25.24 ± 0.67	6.65 ± 0.12
C18:4 *n*3	2.50 ± 0.13	2.41 ± 0.05	N.D.
C20:1 *n*9	1.0 ± 0.04	N.D.	N.D.
Total *n*6	15.42 ± 0.42	29.21 ± 0.94	74.20 ± 0.30
Total *n*3	22.03 ± 0.61	38.02 ± 0.84	8.00 ± 0.21
*n*3:*n*6 ratio	1.43 ± 0.06	1.30 ± 0.07	0.11 ± 0.00
ΣSFA (%)	24.98 ± 0.24	26.33 ± 0.31	15.43 ± 0.07
ΣMUFA (%)	37.57 ± 0.73	6.44 ± 0.06	2.37 ± 0.05
ΣPUFA (%)	37.45 ± 0.75	67.22 ± 0.27	82.20 ± 0.11

**E* trans isomer, *Z* cis isomer, *N.D.* not detected. ΣSFA, ΣMUFA, and ΣPUFA: the sum of the saturated, mono unsaturated, and polyunsaturated fatty acids in the total fatty acids (wt%)

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.

The results 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 and 20 mg/L [36]. The wastewater used in this study contained 11 mg/L K^+ which remained until the end of the experiment. Therefore, it could be assumed 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 Ünal [37] 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%). 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

Regarding the usage of harvested biomass for bioenergy production, *T. wisconsinensis* could be a favorable candidate. The total carbohydrate production was constantly the highest among all strains. 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 (± 66) mg/L. This gives their total carbohydrate productivity of 390 mg/L. An interesting use of microalgae rich in carbohydrates is their potential for bioenergy production. Microalgae carbohydrates can be utilized for both liquid (bioethanol, and biobutanol) and gaseous (biomethane and biohydrogen) biofuels [9]. Qu et al. [15] examined an indigenous green microalga, *Parachlorella kessleri*, for carbohydrate production using swine wastewater.

The carbohydrate content of this strain was optimized to more than 50% by light and temperature manipulations, and it had a suitable profile (70% glucose and 25% xylose) for bio-alcohol production. Therefore, a further optimization of carbohydrate production of *T. wisconsinensis* could extend the potential of this strain for biofuel production. In addition, a biorefinery concept can be adapted to microalgae-based biofuel production where both carbohydrates and lipids fractions in biomass are utilized [9]. Previously, *T. wisconsinensis* H1 showed unsuitable biodiesel fuel properties mainly because of its high share of PUFAs (69%) [17]. Under the experiment condition in this study, this strain contained much lower PUFAs (37%) and high MUFAs (38%), although the total fatty acid accumulation was low. Fatty acid accumulation and profiles are known to be influenced by culture conditions, including nutrients and physical status in microalgae [38]. Therefore, a further investigation for monitoring the fatty acid profile in parallel to the changes in the carbohydrate accumulation will be needed to propose the best biorefinery approach. Overall, feedstock for biofuel could be a target product for an operation of microalgae-based wastewater treatment using *T. wisconsinensis*.

The total fatty acid contents of 20% in *L. segnis* and *K. flaccidum* were favorable over *T. wisconsinensis*, although it was lower than the average content of oleaginous green microalgae, which was reported as 25.5% [38]. The high shares of PUFAs in *L. segnis* and *K. flaccidum* (82% and 67% of TFA, respectively) indicate that their fatty acids would not be suitable for biofuel applications. This is because their PUFA contents are likely to cause instability of products upon storage [38]. Nevertheless, these PUFA values are markedly higher than those of the many other strains reported previously from the screening studies for PUFA production [39]. 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 with the ratio of *n*-3 to *n*-6 up to 1.3, which was much higher than that of *K. flaccidum* (Table 1). Principally, FA with a *n*-3:*n*-6 ratio higher than 1:1 has suitable quality for fish-feed manufacturing [40]. The examined strains did not contain very long-chain (VLC) omega-3 PUFA. The C18 PUFAs produced by microalgae are essential for freshwater food chain [41] 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 [42]. Jin et al. [43] have tested supplementation of microalgal C18 LC-PUFAs in a feed for black seabream, *Acanthopagrus schlegelii*. Their results pointed out the importance of low dietary *n*-6 for the growth and lipid metabolism, while showing 0.7 as the best *n*-3:*n*-6 ratio in the total sum of C18, C20, and C22 PUFAs. Therefore, the application of *L. segnis* containing these C18 PUFA for freshwater aquaculture feeds can be an interesting research topic to pursue.

Concerning protein productions, 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%, with the typical value in the range 40 to 60% [44]. The protein content of *K. flaccidum* shown in this study was, therefore, comparative to those previously reported values of the microalgae species. Therefore, the *K. flaccidum* protein produced using wastewater could be applicable for feed manufacturing [1]. Microalgae proteins and amino acids can also be utilized for biofertilizer and biostimulant, and this application is appealing for biorefinery approach [4]. However, the biomass productivity of *K. flaccidum* was low. Especially when the cells contained the highest protein content (day 6) (Fig. 5a), the biomass accumulation was limited to 0.078 ± 0.015 g dw/L, 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.

Optimizing Biomass Recovery and Cell Compositions

This 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 ± 0.02 g dw/L) (Fig. 3). In comparison, the final biomass of *L. segnis* was lower (1.02 ± 0.20 g dw/L), 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. This study 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 biomass generation, i.e., profitable output of the system, 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, it is expected 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. Generally, the macro-components of microalgae change according to nutrient availability, and the accumulation of storage metabolites, i.e., starch and lipids, is enhanced especially by nitrogen depletion [45]. This study demonstrated that the proportions of proteins and carbohydrates in the biomass changed over time, and these changes appeared to be influenced by 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 [46]. Additionally, N-limitations induce redirection of metabolism to the production of storage compounds that do not contain N, i.e., carbohydrates and lipids [46]. 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 [47]. The 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, b). It could be speculated that the metabolism changed the course again towards the production of more stable storage compounds, triacylglycerides [38]; however, a further study to monitor changes in the fatty acid accumulation is needed to confirm this.

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

Promising performance of indigenous freshwater green microalgae was demonstrated for their usage in wastewater treatment combined with biomass production. *L. segnis* showed the best ability for the nutrient removal from the wastewater. A favorable characteristic of total carbohydrate productivity and fatty acid profile for biofuel application was found in *T. wisconsinensis*. *K. flaccidum* biomass was superior for protein contents. The fatty acids of *K. flaccidum* and *L. segnis* showed PUFA-rich profiles. These strains can be beneficial for other applications than biofuels such as feed or biofertilizer. The results provided strain-specific resource recovery scenarios with a potential for biorefinery approach.

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Declarations

Competing Interests The authors declare no competing interests.

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