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Chemical composition and species identification of microalgal biomass grown at pilot-scale with municipal wastewater and CO_2 from flue gases

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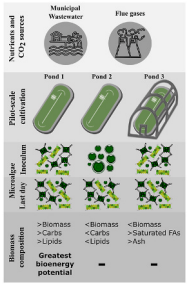
HIGHLIGHTS

- A local algae consortium, a locally isolated strain, wastewater and flue gases.
- Higher potential of the consortium/outdoor biomass for biofuels production.
- Biomass from the greenhouse had the highest monounsaturated fatty acids content.
- Outdoor ponds biomass had higher heating value than biomass from the greenhouse.

GRAPHICAL ABSTRACT



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ABSTRACT

The production potential of a locally isolated *Chlorella vulgaris* strain and a local green-algae consortium, used in municipal wastewater treatment combined with CO_2 sequestration from flue gases, was evaluated for the first time by comparing the elemental and biochemical composition and heating value of the biomass produced. The microalgae were grown in outdoor pilot-scale ponds under subarctic summer conditions. The impact of cultivation in a greenhouse climate was also tested for the green-algae consortium; additionally, the variation in species composition over time in the three ponds was investigated. Our results showed that the biomass produced in the consortium/outdoor pond had the greatest potential for bioenergy production because both its carbohydrates and lipids contents were significantly higher than the biomasses from the consortium/greenhouse and *C. vulgaris*/outdoor ponds. Although greenhouse conditions significantly increased the consortium biomass's monounsaturated fatty acid content, which is ideal for biodiesel production, an undesirable increase in ash and chemical elements, as well as a reduction in heating value, were also observed. Thus, the placement of the pond inside a greenhouse did not improve the production potential of the green-algae consortium biomass in the current study infrastructure and climate conditions.

1. Introduction

Increasing concerns about climate change and declining fossil fuel resources have put the feasibility of microalgal biofuel into account, mainly due to their higher areal productivity over traditional biomasses and the possibility of microalgae being cultivated in low-quality water.

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However, the microalgae production cost is still one major obstacle to the commercialization of microalgae-derived biofuels (Chisti, 2007; Lage et al., 2018). An alternative is to concurrently produce multiple biofuels and/or biofuel with value-added co-products (Rawat et al., 2013). For instance, microalgal biomass contains lipids that can be transesterified to generate biodiesel (Abinandan and Shanthakumar, 2015) and carbohydrates that can be fermented to produce bioethanol or anaerobically digested to produce biogas (Jones and Mayfield, 2012; Lage et al., 2021b). Furthermore, the coupling of microalgae cultivation with industrial, farm, municipal, or agricultural wastewater treatment, can lead to the production of environmentally and commercially viable biofuels (Lage et al., 2018). These, by saving the costs of nitrogen and phosphorus fertilizers required for microalgae growth, and simultaneously removing the above-mentioned nutrients as well as decreasing the values of chemical oxygen demand, biological oxygen demand, heavy metals, and other hazardous compounds in the wastewater (Christenson and Sims, 2011; Cai et al., 2013; Rawat et al., 2013; Lindberg et al., 2021). Thus, potentially allowing the treated wastewater to be used for other purposes, such as irrigation. Moreover, flue gases with high concentrations of CO_2 (~15%, v/v) that are released from different industrial sectors including thermal power, cement, steel, and incineration, can be used as an economical CO_2 source for microalgae biomass production, which benefits the overall process economy (Chiu et al., 2011; Praveenkumar et al., 2014; Lage et al., 2021a). However, sulfur oxides, nitrogen oxides, hydrogen sulfide, and particulate matter in flue gases can cause acidification of the medium and pose environmental stress to the microalgae (Chiu et al., 2011; Jiang et al., 2013; Kumar et al., 2014). The economic viability of this microalgae biorefinery approach is dependent on the quality of the microalgal biomass generated and on its suitability for further transformation to biofuels and/or high-value products, which is subsequently dependent on the selection of appropriate microalgal strain/consortium able to grow in specific wastewaters and flue gases (Ferro et al., 2018b; Lage et al., 2018; Lage et al., 2021a,b).

The majority of the microalgal species used in wastewater treatment are from the genus *Chlorella* and *Scenedesmus*. Even though these microalgae have been successfully used in microalgal-based wastewater treatment, their cultivation in non-sterile systems is vulnerable to contamination by wild strains unless additional control measures are implemented (Christenson and Sims, 2011; Ferro et al., 2020). Locally isolated strains from wastewater ponds have been suggested to outperform strains from culture collections (Zhou et al., 2014; Ferro et al., 2018b, 2020). The local isolate *Chlorella vulgaris* showed to be very efficient to remove nutrients from municipal wastewater and to produce large amount of biomass when grown in laboratory conditions both at 25 °C and at 5 °C (Ferro et al., 2018b). Moreover, microalgae consortia, locally isolated or artificially engineered, have been shown to withstand environmental fluctuations and invasion by wild strains (Jagmann and Philipp, 2014; Novoveská et al., 2016; Padmaperuma et al., 2018). However, the bioenergy feedstock production potential of locally isolated strains and local microalgae consortia grown in pilot-scale open ponds with wastewater treatment and flue gases have rarely been investigated (Chinnasamy et al., 2010; Lage et al., 2018).Apart from the selected species, microalgae biomass concentration and composition depend on several factors, including existing climatic conditions, i.e., light intensity and cycle, temperature, wastewater and flue gases composition, pH, and infrastructure (Christenson and Sims, 2011; Lage et al., 2018). Thus, to better predict the microalgal biomass bioenergy feedstock production of a strain versus a consortium; microalgae should be grown simultaneously, with the same type of pond, climate conditions, wastewater and flue gases composition.

The aim of the study herein was to evaluate, for the first time, the microalgal biomass production potential of a locally isolated strain of *Chlorella vulgaris* and a local green-algae consortium, grown in our integrated system combining microalgae wastewater treatment and CO_2 sequestration from flue gases under subarctic summer conditions at a pilot scale. The local strain and consortium were inoculated in identical open raceway pond reactors (RPRs) located outdoors. The impact of cultivation under a greenhouse on the consortium biomass production potential and the variation in species composition over time in the three ponds, was also tested. The quantity and quality of the biomass produced in, lipids each pond was assessed in terms of elemental composition, i.e., nitrogen (N), carbon (C), and other 23 elements, biochemical composition, i.e., lipids (polar and neutral), carbohydrates, proteins, ash, and heating value.

2. Materials and methods

2.1. Experimental setup

The experiment was performed in three identical pilot-scale RPRs placed in close connection to Umeå Energi combined heat and power plant (CHP-plant) in Umeå, Northern Sweden (63°87 N, 20°80E), during the 2017 summer season as previously described by Lage et al. (2021a). The RPRs had a surface area and volume of ca. 19.14 m^2 and 6 m^3, respectively; the dimensions were 10 m long, 2 m wide, ca. 0.3 m deep. The RPRs were equipped with paddle wheels with six blades. Two RPRs were placed outdoors, hereby named ponds 1 and 2, and the other one was placed in a non-illuminated greenhouse, hereby named pond 3. The greenhouse was 13 m long and 8 m wide and was covered with two layers of polyethylene film sheets of 0.2 mm thickness.

Ponds 1 and 3 were inoculated with a mixed population of local freshwater green algal species lying on the bottom of the ponds after the winter. These two ponds had an inoculum/wastewater ratio of 1/60 (v/v). Pond 2 was inoculated with a local strain of *Chlorella vulgaris* with an inoculum/wastewater ratio of 1/240 (v/v), previously genetically identified (Ferro et al., 2018a). All inoculations took place in May. The sampling period was between June and August for ponds 1 and 3 and between June and September for pond 2.

The microalgae were grown in municipal untreated wastewater influent from the local wastewater treatment plant (Vakin, Umeå, Sweden). The nutrient concentrations in the various wastewater influents were not standardized and thus varied with changes in municipal activity and population density. The average P-PO_4^{3-} and N–NH_4^+ concentrations in the wastewater influent additions were 4.38 ± 2.06 and 46.26 ± 5.11 mg/L, respectively. N–NO_3^- concentrations were negligible, ranging from 0.04 to 0.99 mg/L. The microalgae culture was bubbled with flue gases from the CHP-plant (Umeå Energi, Umeå, Sweden), which combusts both municipal and industrial solid wastes. The flue gases were bubbled into the RPRs using gas diffusers (Cole Parmer, USA) from the 28th of June until the 31st of August at pH values higher than 8.0 and stopped at pH values lower than 8.0 (Lage et al., 2021a). The flue gases were composed by 7.4 ± 1.9% of CO_2, and 4.4 ± 8.5, 38 ± 24 and 4.4 ± 4.7 mg/Nm^3 of CO, NO_x, and SO_2, respectively. Temperature and light were not controlled and reflected those available in this area.

2.2. Photosynthetically active radiation, pH, temperature, and dissolved oxygen measurements

Photosynthetically active radiation (PAR) was measured and recorded every 5 min using a LiCor 1400 datalogger connected to two LI 192 light sensors (LiCor, Lincoln, Nebraska, USA). One sensor was placed outdoors just above pond 2 and in close vicinity to pond 1 and the other sensor was inside the greenhouse just above pond 3. The water temperature, pH, and dissolved oxygen (optical dissolved oxygen) were measured and recorded every 5 min using electrodes and sensors from Hach-Lange (Hach-Lange, Germany).

2.3. Sampling

Water samples of the microalgae population from the three ponds

(~100 mL) were collected daily during the experiment duration, a few centimeters below the surface. Aliquots of these samples were used for microalgae taxonomic identification, cell counting, biomass concentration.

Sedimentation was used for harvesting microalgae biomass; once a week part of the algal culture was pumped in 1 m^3 plastic containers and left to settle for 2 days to pre-concentrate (ca. 100 times) the microalgae. The obtained pre-concentrate was centrifuged in a continuous process at 3949×*g* with a flow of approx. 1 L/min (US Filtermaxx, Jacksonville, Florida, USA). The microalgae paste collected after centrifugation was kept at −20 °C until further processing. Subsequently, the microalgae paste was lyophilized in a freeze dryer (Edwards high vacuum international, Crawley, England) at −40 °C overnight and milled in a ball mill (Retsch MM200, Retsch Germany). Lyophilized samples were used for the analysis of biomass composition (i.e., lipids, fatty acid methyl esters (FAMEs), proteins, carbohydrates, and ash), heating value, total N and C, and 23 chemical elements.

2.4. Taxonomic identification of microalgae

Water samples (50 μL) were used for the morphological identification of microalgae species. Species were identified by a light microscope (B-353 LD2, Optika, Ponteranica, Italy) according to the Bellinger and Sigee (2010) taxonomic key.

2.5. Microalgae abundance

Water samples (50 μL) were diluted to 1/200 (v/v) with physiological saline solution and sonicated for 2 min in an ultrasound bath at room temperature, to break microalgae colonies. The microalgae cells were counted, in triplicate, in four size classes (i.e., 2–6, 6–10, 10–20, and 20–40 μm), given as equivalent sphere diameter, with a Coulter Counter Multisizer 3 and calculated using MS-Multisizer 3 software (Beckman Coulter, High Wycombe, UK). Microalgae cell abundance was expressed as cells/mL.

2.6. Chemical analysis

2.6.1. Total N and C analyses

Total N and C analysis of freeze-dried and milled microalgae biomass samples (about 1 mg) were performed at the Department of Forest Ecology and Management, Swedish University of Agricultural Sciences (Umeå, Sweden) as described by Werner et al. (1999). The samples were analyzed by Elemental Analyzer - Isotope Ratio Mass Spectrometry (EA-IRMS). The instrumental setup consisted of an elemental analyzer (Flash EA, 2000) connected to a continuous flow isotope ratio mass spectrometer (DeltaV), both from Thermo Fisher Scientific (Bremen, Germany). Each sequence of samples was analyzed together with two *in*-house standards in several replicates. The accepted standard deviation of *in*-house laboratory standards was <0.15‰. Data were corrected for drift and size before yielding the results.

2.6.2. Elemental analysis

The elemental analysis of freeze-dried and milled microalgae biomass samples (1g) was performed at ALS Scandinavia AB (Luleå, Sweden) (EPA, 1994; ISO, 2016). The following elements were analyzed: Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, V, Zn.

2.7. Analytical methods

2.7.1. Total lipids

The lipids extraction was performed, in triplicate, according to Axelsson and Gentili (2014) and based on Folch et al. (1957). Briefly, freeze-dried and milled microalgae biomass samples (10–20 mg) were resuspended in a 2:1 (v/v) chloroform:methanol (MeOH) mixture, followed by addition of a 0.73% NaCl solution to reach a ratio of 2:1:0.8 (v/v/v) chloroform:MeOH:NaCl. The chloroform phase was recovered after centrifugation, and the aqueous phase was washed twice with chloroform. After extraction, solvents were evaporated in a vacuum multi-evaporator (Syncore® Polyvap, Büchi Labortechnik AG, Flawil, Switzerland) at 40 °C, under the pressure of approx. 270 mbar, with shaking at 100 rpm overnight. Total lipids were estimated gravimetrically and expressed in % dry weight (DW).

2.7.2. Fatty acid methyl esters quantification and characterization

Solid-phase extraction (SPE) was used to purify and separate neutral and polar fatty acid contained in the triplicate crude lipid samples, obtained as described above. Hypersep SI SPE columns with a capacity of 3 mL (Thermo Scientific, Waltham, Massachusetts, USA) were used. Afterward, the fatty acids were transmethylated into FAMEs as previously described by Lage and Gentili (2018) and based on Christie and Han (2010). The extracted FAMEs were re-suspended with heptane and analyzed using a TRACE™ 1310 (Thermo Fisher Scientific, Hägersten, Sweden) GC system equipped with a flame ionization detector and a 30 m FAMEWAX column (Restek Corporation, Bellefonte, Pennsylvania, USA) (Lage and Gentili, 2018). FAMEs were identified by comparing their retention times with those of authentic standards (Larodan, Solna, Sweden). For the determination of FAMEs concentrations real response factors were used. The internal standard methylated heptadecanoic acid (C17:0-Me) was used to normalize the data.

2.7.3. Total protein

Freeze-dried and milled microalgae biomass samples (2–3 mg) were extracted and analyzed for free and bound amino acids with Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) at the Swedish Metabolomics Centre (Umeå, Sweden) (Benavente et al., 2022). The sum of all amino acids (free and bound) was used as a proxy of total protein content.

2.7.4. Total carbohydrates

Total carbohydrate was determined by Saemann hydrolysis followed by Anthrone assay, with minor modifications (Scott Jr and Melvin, 1953; Updegraff, 1969). Primarily, 175 μl of 72% (v/v) sulfuric acid was added to triplicate samples (1 mg) of freeze-dried and milled microalgae biomass and of positive controls, i.e., microcrystalline cellulose (Avicel® PH101, Sigma-Aldrich, St. Louis, MO, USA). The resulting suspensions were incubated at 60 °C for 30 min, with vortex-mixing every 5 min to ensure full contact between the sample and the sulfuric acid solution, and therefore guaranty that all carbohydrates were being hydrolyzed. Afterward, 425 μl of Milli-Q water (Millipore, Burlington, MA, USA) were added to the samples, which were subsequently vortex-mixed and centrifuged (10,000×*g*, 1 min). To 5 μL aliquots of each hydrolysate (and glucose solutions, to generate a calibration curve) were added 195 μl of Milli-Q water. In all following steps, the samples were kept in the dark by covering them with aluminum foil. Anthrone reagent (400 μl) was added to the samples, which were subsequently incubated at 100 °C for 5 min, cooled on ice for 3 min, and vortex mixed. Finally, 200 μl of the supernatants were placed in wells of an Elisa plate and their absorbance at 620 nm was measured using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Hägersten, Sweden).

2.7.5. Ash content

The ash content of freeze-dried and milled microalgae samples (0.5 g) was determined in triplicate using a standard ash test at 550 °C (ISO, 2015).

2.8. Heating value

The freeze-dried and milled samples of microalgae biomass were pressed into pills with a hydraulic press. The energy content of the biomass was calculated by its heating value, which was determined in a

bomb calorimeter (Parr 6400, Moline, IL, USA) (ISO, 2010). Energy Grass (*Phalaris arundinaceae* L.) certified reference material (NJV 94-4) was used as a control.

2.9. Statistics

All statistical analyses were carried out in JMP® Version 14.0. SAS Institute Inc. (Cary, North Carolina, USA); the significance level was set to an $\alpha = 0.05$. One-way analysis of variance (ANOVA) was used to evaluate whether pond as a categorical predictor significantly influenced microalgae biomass and cell abundance (2–6, 6–10, 10–20, and 20–40 μm), microalgae composition (ash, carbohydrates, lipids, FA, proteins, total N and C, C/N ratio, protein/N ratio, and other elements) and microalgae heating value. One-way ANOVA was also applied to test if PAR, pH, water temperature, DO were significantly different in the three ponds. Normal distribution was confirmed by Q-Q plot analyses. The variables PAR, cell abundance, total N and C, C/N ratio, and chemical elements were log-transformed to achieve normal distribution. If the one-way ANOVA was significant, the significant difference of each pond was tested using Tukey's honestly significant difference (HSD).

3. Results and discussion

3.1. Microalgae community composition

The local green algae consortium inoculum of ponds 1 and 3, was mainly constituted by *Scenedesmus dimorphus*, *S. quadricauda*, *Desmodesmus armatus* and *Chlorella* sp. (Lage et al., 2021a). The frequency of these species in ponds 1 and 3 suffered several changes through the sampling season (Table S1). In pond 1, the inoculated species kept their dominance over the microalgae species introduced by the surrounding environment, even though the introduced *D. opoliensis*, *S. acuminatus,* and *S. obliquus* had high frequencies (Table S1). In pond 3, only the inoculated *S. dimorphus* and *Chlorella* sp. were present in all samples analyzed, concurrent with either other introduced *Scenedesmus* species or cyanobacteria (Table S1). Pond 2, which was inoculated with a monoculture of the local strain *Chlorella vulgaris*, during May, had its microalgae population changed substantially, due to contamination, by the start of the sampling season (June 13th). At this point, and during the sampling season, *D. armatus* and *Coelastrum microporum* were the dominant species, Table S1 (Lage et al., 2021a). The susceptibility of monocultures cultivated in open non-sterile systems, like the RPRs used in the present study, to be overtaken by other algae present in the environment is well described in the literature (Novoveská et al., 2016; Ferro et al., 2018a; Lage et al., 2018). Consequently, the lower species diversity in pond 3 in comparison to ponds 1 and 2 is probably caused by pond 3 being in the greenhouse, therefore less susceptible to contamination by the surrounding environment.

3.2. Environmental parameters and microalgae biomass

During the experimental period, PAR varied due to seasonal variability of the environmental conditions (Fig. S1). In ponds 1 and 2, the monthly average of the PAR daily mean varied between 202.18 ± 118.08 μmol/m^2/s (in September) and 463.27 ± 164.63 μmol/m^2/s (in June), while the daily maximum varied between 1042.71 ± 394.37 μmol/m^2/s (in September) and 1865.20 ± 352.61 μmol/m^2/s (in June) (Table S2). In pond 3, the pond located inside a greenhouse, the monthly average of the PAR daily mean and maximum were between 137.86 ± 86.83 and 279.77 ± 103.54 μmol/m^2/s and between 800.68 ± 325.82 and 1336.44 ± 309.73 μmol/m^2/s, in September and June, respectively (Table S2).The polyethylene film sheets of the greenhouse cover resulted in a significant decrease in the measured PAR in pond 3 in comparison with ponds 1 and 2 which were placed outdoors (Tables S4 and S5).

Considerable variations in the pH, water temperature, and DO were recorded in the course of the experimental time (Fig. S2). The water temperature daily minimum and maximum per month ranged from 14.91 ± 1.79–21.1 3 ± 2.70 °C, 12.12 ± 1.42–21.18 ± 2.57 °C, and 18.14 ± 1.60–24.07 ± 2.98 °C, in ponds 1, 2 and 3, respectively (Table S2). The highest water temperature daily mean per month was measured in June in pond 3, i.e., 21.37 ± 2.41 °C (Table S3). The water temperature in pond 3 was significantly higher than in the outdoor ponds, while there was no significant difference in the water temperature between ponds 1 and 2 (Tables S4 and S5). In the first month of cultivation, the pH of the ponds was not regulated. However, from June 28th onwards, CO_2 was added via flue gases, at pH values higher than 8.0 and stopped at pH values lower than 8.0. Therefore, pH in ponds remained constant throughout the season (Fig. S2). The monthly average of the pH daily mean varied between 7.02 ± 0.18 (August/pond 3) and 9.16 ± 0.87 (June/pond 1)(Table S3). Nevertheless, pond 3 had a significantly lower pH than the outdoor ponds and pond 1 had a significantly higher pH than pond 2 (Tables S4 and S5). DO contents ranged from 0.00 to 21.00 mg/L (the range of values measurable by the sensor) (Fig. S2).DO contents of 0 mg/L represent exclusively times of microalgae biomass harvesting and new untreated wastewater being filled into the ponds. The DO average daily minimum and maximum per month was 0.68 ± 1.43 (July/pond 3) and 19.47 ± 3.42 mg/L (July/pond 2) (Table S3).

It should be noted that the locally isolated *C. vulgaris* and the local green-algae consortium were used as a green alternative for municipal wastewater treatment. When the N–NH_4^+ levels in the ponds fell below 10 mg/L, treated wastewater was discharged and new untreated wastewater was added, resulting in a reduction of up to 99% in the concentrations of N–NH_4^+ in the ponds every 5 days. While, during the same period, P-PO_4^{3-} was removed up to 90% and 89% by *C. vulgaris* and the consortium, respectively (Lage et al., 2021a).

The average microalgae biomass concentration was 0.349 ± 0.111, 0.209 ± 0.071, and 0.229 ± 0.065 g/L in ponds 1, 2, and 3, respectively (Table 1 and Fig. S3), and the highest biomass concentration was measured in pond 1 on July 24th, i.e., 0.73 ± 0.07 g/L. The microalgae biomass was significantly higher in pond 1 than in the other two ponds, and no significant difference was found between ponds 2 and 3 (Tables S4 and S5). Therefore, agreeing with the literature that consortia of local microalgae perform better in terms of biomass production than monocultures when cultivated with wastewater (Chinnasamy et al., 2010; Bhatnagar et al., 2011; Lage et al., 2018). In a previous lab-scale study, after 7 days, the biomass concentrations of four consortia (1:1 ratio of *Chlamydomonas globosa/Scenedesmus bijuga*, *C. globosa/Chlorella minutissima*, *C. minutissima/S. bijuga*, and 1:1:1 ratio of *C. globosa/C. minutissima/S. bijuga*) were 0.279, 0.309, 0.349, and 0.338 g/L, respectively. Meanwhile, the monocultures of *C. globosa*, *C. minutissima*, and *S. bijuga* had biomass concentrations of 0.217, 0.292, and 0.342 g/L, respectively (Bhatnagar et al., 2011). Moreover, Chinnasamy et al. (2010) shown, that after 10 days of growth under pilot-scale conditions, in untreated wastewater from carpet mills and sewage, monocultures had biomass concentrations ranging from 0.23 ± 0.04 to 0.34 ± 0.07 g/L (depending on the microalgae species, i.e., *Botryococcus braunii*, *Chlorella saccharophila*, *Dunaliellatertiolecta* and *Pleurochrysis carterae*), while the microalgae consortium had 0.39 ± 0.09 g/L. This superiority of consortia in biomass production may be attributed to a fatal loss of one algae population in the consortia being compensated by other, as well as a complementarity of nutrient requirements between species (Lage et al., 2018) (see Table 2).

Although both ponds 1 and 3 were inoculated with the same local-isolated consortium of green-algae, pond 3 had lower microalgae biomass. This was most likely due to the greenhouse cover of pond 3, which resulted in a significantly higher water temperature and lower light intensity, and negatively affected the growth of the inoculated cold-adapted microalgae consortium. Strains of *Chlorella* can tolerate a wide range of temperatures ranging from 4 to 38 °C, however, cold-adapted *Chlorella* strains have been shown to have the highest growth at 20 °C (Teoh et al., 2013). Moreover, a higher temperature during the

Table 1
Biomass, percentage of total nitrogen (N) and total carbon (C) per DW, C/N ratio and Protein/N ratio of the microalgae cultivated in the three ponds. Values are expressed as mean ± standard deviation, Pond 1 ($n = 24$), Pond 2 ($n = 33$), and Pond 3 ($n = 27$), except for Protein/N which Pond 1, 2 and 3 have n of 8, 12 and 8, respectively.

	Pond 1			2			3		
Biomass	0.349	±	0.111	0.209	±	0.071	0.229	±	0.065
N	6.390	±	0.963	6.805	±	1.342	5.689	±	0.537
C	43.799	±	2.224	44.521	±	7.217	38.739	±	2.456
C/N	6.980	±	0.903	6.612	±	0.634	6.836	±	0.315
Protein/N	3.592	±	0.794	4.216	±	0.636	3.354	±	0.582

Table 2
Elemental composition of the algae cultivated in the three ponds. Values are expressed as mean ± standard deviation, Pond 1 ($n = 9$), Pond 2 ($n = 12$), and Pond 3 ($n = 7$).

Element (mg/Kg)	Pond 1			2			3		
Al	5070.000	±	1663.979	4265.000	±	1968.454	12892.857	±	1799.173
As	1.600	±	0.385	1.462	±	0.289	2.424	±	0.301
B	5.222	±	2.130	3.271	±	0.964	11.733	±	1.758
Ca	15943.333	±	7128.408	11832.500	±	4523.798	21128.571	±	4171.217
Cd	2.076	±	1.211	1.485	±	0.749	2.886	±	0.878
Co	3.774	±	1.548	2.863	±	0.863	7.921	±	0.807
Cr	12.470	±	3.801	7.524	±	2.208	23.000	±	4.244
Cu	118.867	±	54.461	244.583	±	119.990	640.229	±	291.635
Fe	13780.000	±	4228.191	12236.667	±	3978.358	21528.571	±	2252.935
Hg	0.165	±	0.041	0.172	±	0.051	0.360	±	0.087
K	8803.333	±	1888.154	5847.500	±	648.370	4452.857	±	315.104
Mg	5308.889	±	2156.296	3280.000	±	1439.438	2932.857	±	625.985
Mn	472.333	±	62.514	508.667	±	150.205	663.143	±	110.497
Mo	0.859	±	0.203	0.743	±	0.270	2.417	±	0.497
Na	1072.444	±	274.098	776.333	±	224.521	1352.857	±	148.965
Ni	11.711	±	4.442	8.452	±	2.329	18.171	±	2.258
P	16433.333	±	1101.136	12640.833	±	2280.393	17971.429	±	2335.747
Pb	14.101	±	5.980	9.924	±	3.502	23.186	±	3.166
S	3412.222	±	302.770	5597.500	±	889.434	5575.714	±	755.333
Se	0.538	±	0.152	0.369	±	0.128	0.716	±	0.048
Si	5896.667	±	594.706	4660.833	±	724.022	2574.286	±	547.627
V	4.369	±	1.584	2.220	±	1.068	5.714	±	0.903
Zn	386.000	±	126.272	305.750	±	98.258	500.429	±	52.893

light and dark period of the day can result in a higher specific rate of biomass loss (Huesemann et al., 2016). Likely, this significantly lower microalgae biomass in pond 3 resulted in a significantly lower DO and significantly lower pH in pond 3 than in the outdoor ponds (Tables S4 and S5). Additionally, the formation of microalgae flocs might have contributed to the sedimentation of the microalgae and reduction of the DO (Jimoh et al., 2019). High temperature combined with reduced light hours induced flocculation in the same strain of *C. vulgaris*, previously isolated in Northern Sweden from wastewater collected at the same wastewater treatment plant of the present study (Ferro et al., 2018a, 2018b). Microalgal flocs were observed in pond 3 during mid-to-late July, coincident in time with the lowest DO values measured in pond 3 (Fig. S2). Although not observed, the potential presence of grazers in pond 3 cannot be ruled out (Day et al., 2017).

3.3. Microalgae cell abundance

Out of the four size classes of microalgae (2–6, 6–10, 10–20, and 20–40 μm) investigated, the smaller size class was dominant in all three ponds, which contribute up to 67.04, 89.23, and 94.76% of the total cell density in ponds 1, 2 and 3, respectively (Fig. S4). The highest frequency of *Chlorella* spp. in pond 3 compared to the other two ponds is a confirmation of the high number of small algal cells in pond 3 (Table S1). In agreement with the microalgae biomass concentration results, the cell abundance of pond 1 was significantly higher than in the other two ponds in all size classes, except for the largest size class, which had no significant difference between pond 1 and 2 (Tables S4 and S5). The cell abundance of pond 2 was significantly higher than of pond 3 in all size classes, except for the smallest size class in which no significant difference was observed (Tables S4 and S5).

The 2–6 μm size class is constituted by microalgae cells of *Chlorella* strains, which have an average size of 3–4 μm, although *C. vulgaris* often reach sizes up to 5–7 μm. Single cells of *S. obliquus* and/or *D. armatus* can also be found in this size class (Ferro et al., 2018b). Although bacteria were not quantified in this study, due to the unsterile conditions typical of open-pond cultivation with wastewater, it is likely that bacteria were present and dominated the cell counts of this size class. Bacteria groups are ubiquitously detected in wastewater treatment plants and consequently in algae ponds for bioconversion of wastewater (Ibekwe et al., 2017; Zhang et al., 2018). Moreover, non-axenic microalgae strains have an associated microbiome that can facilitate microalgae nutrient acquisition, produce/catabolize phytohormones that impact on microalgae growth, or compete with pathogens and protect microalgae from microbial pathogen attack (Fuentes et al., 2016). Previously a high diversity of bacteria was found in a photobioreactor inoculated with an axenic culture of *S. dimorphus* UTEX 417 cultivated with wastewater from the same wastewater treatment plant as the present study and placed in a greenhouse (Ferro et al., 2020). However, controlling the culture pH below 8, as carried out in the present study, has been shown to lead to preferential growth of microalgae (i.e., low bacterial co-existence or contamination) (Yu et al., 2022).

The size classes of 6–10 μm and 10–20 μm had single cells, 2–4 cells colonies and ≥4 cells colonies of both *Scenedesmus* (~2–10 μm as a single cell) and *Desmodesmus* (from ~2 to 7 μm to ~ 6–16 μm as a single cell) strains, similarly to what is commonly found (Bold, 1978; Olenina, 2006). *Coelastrum microporum* as a single cell (2–30 μm) were also

counted in the former size classes. However, the coenobia (colonies) of *Coelastrum* strains can reach up to 100 μm and thus were counted in the largest size class (Hegewald et al., 2010). The coenobia of *Scenedesmus* and *Desmodesmus* with ≥4 cells, were also counted in the size class 20–40 μm. Although samples were sonicated before cell counting, a short sonication cycle was preferred over a long one, to prevent the disruption of microalgae cell walls. Consequently, microalgae cell colonies were visible in the 10–20 and 20–40 μm size classes.

The microalgae cell sizes (equivalent sphere diameter) of *Scenedesmus*, *Desmodesmus,* and *Chlorella* species in pond 3 were approximately half the size of the outdoor ponds. Thus, in pond 3, *Scenedesmus* and *Desmodesmus* 2–4 cells colonies might also be present in the size class 2–6 μm (data not shown). These observed microalgae cell size difference between ponds is likely due to light intensity and temperature, which are two key natural factors that affect microalgae growth and cell size. With increasing light intensity under constant temperature, the size of the mother cells increases significantly, which is accompanied by an increase in the number of daughter cells (Zachleder, 1995; Bišová and Zachleder, 2014). Accordingly, PAR outdoors (ponds 1 and 2) was significantly higher than in pond 3, inside the greenhouse (Tables S4 and S5). Additionally, it has been previously observed that the lower the temperature, the larger the cell size (Morimura, 1959; Teoh et al., 2013). Accordingly, in pond 3 the water temperature was significantly higher than in the outdoors ponds (Tables S4 and S5). Previously, Ferro et al. (2018b) also observed an increase of *Scenedesmus* and Desmodesmus strains cell volume at low temperature, however the same was not observed for *C. vulgaris*, suggesting the temperature-size rule to be species-specific.

3.4. Microalgae biomass chemical composition and heating value

There was a statistically significant difference in the biomass chemical composition (carbohydrates, lipids, proteins, and ash) of the microalgae grown in the three ponds (Fig. 1, Table S4). The microalgae biomass from pond 1 had a significantly higher carbohydrate content than the other two ponds, with no significant difference between ponds 2 and 3 (Table S5). The carbohydrate content of the outdoor ponds varied greatly during the season with a much higher concentration during the first half of the growing period compared to the second half (Fig. 1). The carbohydrate content varied from 17.90 ± 2.30 to 41.60 ± 0.83% of DW in pond 1 and 15.11 ± 2.96 to 29.24 ± 0.09% of DW in pond 2 (Fig. 1A and B). While in pond 3 the microalgae carbohydrate content was constant through the season; on average 20.95 ± 2.36% of DW (Fig. 1C). The lipid content was also significantly higher in pond 1 compared to the other two ponds and no significant difference was found between ponds 2 and 3 (Table S5). Moreover, the biomass lipid content had a low variation throughout the season, which was on average 18.16 ± 1.19, 17.29 ± 2.55, and 16.11 ± 3.48% DW on ponds

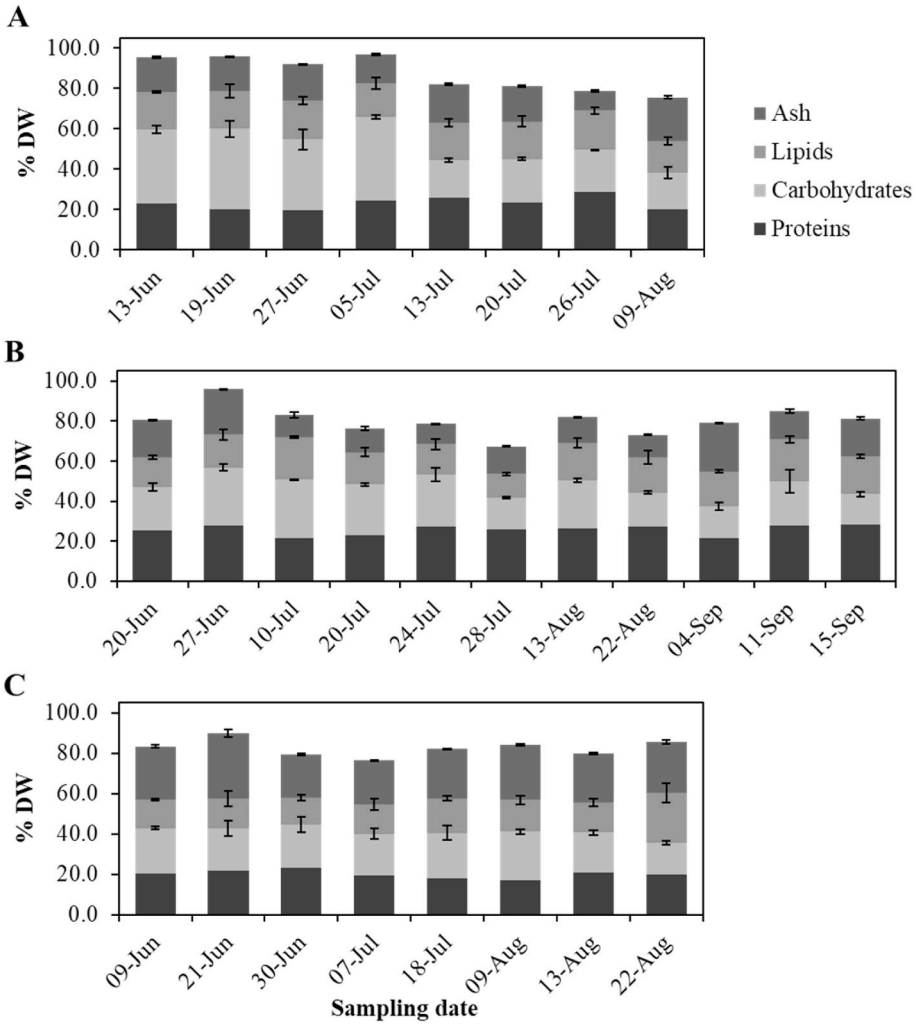

Fig. 1. Biomass composition; total lipids, total carbohydrates, total proteins and ash content of algae biomass samples harvested in pond 1 (A), 2 (B) and 3 (C), during the sampling period. Ash, lipids and carbohydrates are expressed as % DW mean ± standard deviation, $n = 3$. Proteins are calculated as the sum of total amino acids, $n = 1$.

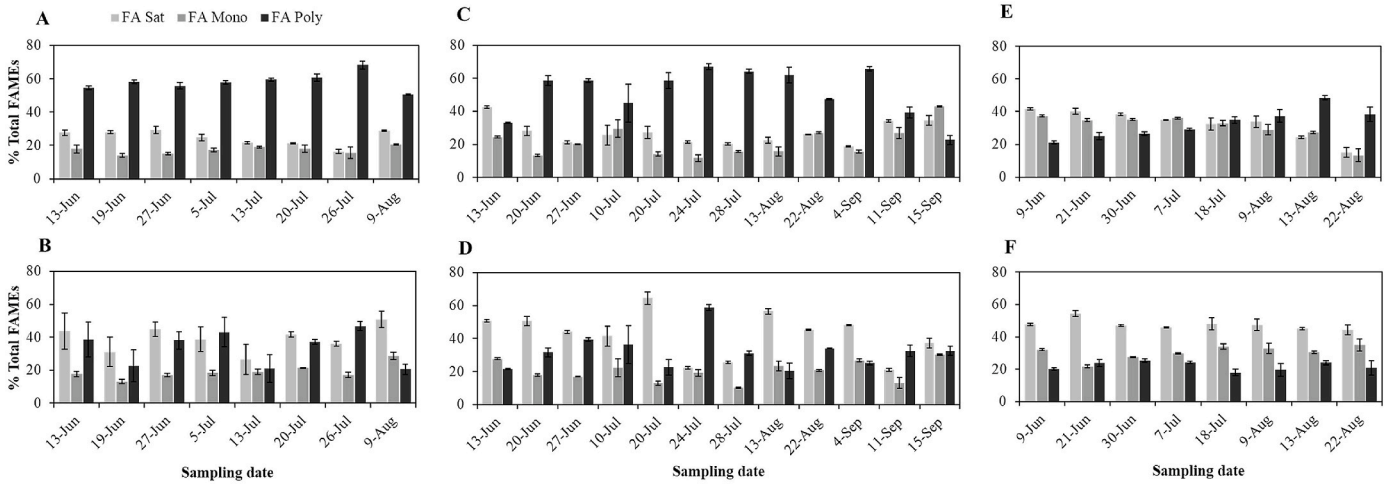

Fig. 2. Percentage of saturated (FA Sat), monounsaturated (FA Mono) and polyunsaturated (FA Poly) fatty acids (%Total FAMEs) of neutral and polar lipids from algae biomass samples harvested at pond 1 (A and B), 2 (C and D) and 3 (E and F), during the experimental period. Values are expressed as mean ± standard deviation, n=3.

1, 2, and 3, respectively (Fig. 1). These results agree with previous studies, in which *Desmodesmus*, *Scenedesmus,* and *Chlorella* species had a lipid content between 12.8 and 24.7% DW (Pan et al., 2011; Kaur et al., 2012; van den Broek et al., 2018); and *C. vulgaris* P12 and *Chlamydomonas reinhardtii* UTEX 90, which had up to 41 and 35% DW carbohydrates, respectively (Dragone et al., 2011). The protein content of the biomass from pond 2 was significantly higher than in pond 3, while the protein content of pond 1 biomass was higher than in pond 3 but not significantly (Fig. 1, Table S5). In the outdoors ponds there is a seasonal variation in the biomass protein content, with the lowest (ca. 17% of DW) concentrations measured in mid-June in both ponds and the highest (ca. 29% of DW) in late-July in pond 1 and in September in pond 2 (Fig. 1).

Although regulated in most of the season, the significantly lower pH in pond 3 (ca. pH 7) might have been responsible for the significantly lower carbohydrate content of the microalgae biomass of this pond in comparison to the outdoors ponds (ca. pH 8) (Fig. S2, Table S5). Although most microalgae prefer neutral pH, as measured in pond 3, various microalgal species have been reported to flourish in moderately alkaline (pH > 9) environments, and generate higher carbohydrate contents (Gatamaneni et al., 2018). *Dunaliella bardawil* and *Chlorella ellipsoidea*, have been shown to have their maximum carbohydrate accumulation at pH 7.5 and 9.0, respectively (Khalil et al., 2010). A slightly alkaline pH (above 7) was also shown to be favourable for *C. vulgaris* carbohydrate and protein production (Yadav and Singh, 2021). *Skeletonema costatum* showed a carbohydrate production increase up to 17.7% (pH 9.4) with increasing pH (Taraldsvik and Myklestad, 2000). The same rationale regarding water temperature and pH effect on microalgae biochemical composition is applied to the lower protein but higher carbohydrate and lipid content of pond 1 biomass in comparison with pond 2.

Under nitrogen-depletion conditions, many microalgal strains can transform proteins or peptides into lipids or carbohydrates as energy reserve components (Huo et al., 2011). For instance, previously a 55% carbohydrate content was achieved when cultivating *C. vulgaris* on a medium containing low N–NO_3^- content (37.5 mg/L) (Illman et al., 2000). Nevertheless, there is a competition between lipid and carbohydrate synthesis because the metabolic pathways associated with the synthesis and degradation of these energy-rich compounds are closely linked (Siaut et al., 2011; Ho et al., 2012).

The microalgae biomass fatty acids profile (neutral and polar) of the outdoor ponds was significantly different from pond 3 under the greenhouse, but there was no significant difference among ponds 1 and 2 (Fig. 2; Tables S4 and S5). Specifically, the neutral saturated fatty acids and the neutral and polar monounsaturated fatty acids were significantly lower in the outdoor ponds than in pond 3; while the polar and neutral polyunsaturated fatty acids were significantly higher in the outdoor ponds than in pond 3 (Table S4). In the microalgae biomass of the outdoor ponds, linolenic acid (C18:3) was the major constituent (ca. 43%) of the neutral fatty acid profile; while palmitic acid (C16:0) and C18:3 dominated the polar fatty acid profile (Fig. S5). In microalgae biomass of pond 3, C16:0 and palmitoleic acid (C16:1) had the highest percentages for most of the growing period in both neutral and polar fatty acid profiles (Fig. S5).

The fatty acid composition of members of Chlorophyceae (most of the species present in the ponds belong to this class) consists predominately of C16:0, oleic acid (C18:1), linoleic acid (C18:2), and C18:3 (Knothe, 2008). In the outdoor ponds, *Desmodesmus* species were part of the dominant species, interestingly, high amounts of C18:3 have been previously detected in *Desmodesmus elegans* under late exponential growth phase (Kaur et al., 2012). High values of C18:3 were also previously detected in RPRs with a microalgae community dominated by *Desmodesmus* species (van den Broek et al., 2018), as in the current study. The neutral fatty acids are the lipids of interest for biodiesel production (Olmstead et al., 2013). However, high amounts of neutral polyunsaturated fatty acids, i.e., C18:3, as measured in the microalgae of the outdoor ponds are detrimental for application as biodiesel due to the poor oxidative stability (Ramos et al., 2009). Instead, C18:1 is one of the most desirable oil components for biodiesel since it gives a good balance between cold flow property and oxidative stability (Hoekman et al., 2012). Additionally, microalgae rich in monounsaturated fatty acid, like the microalgae in pond 3, and saturated fatty acid are good for biodiesel production (Hoekman et al., 2012; Stansell et al., 2012). In general, good quality biodiesel should have a 5:4:1 mass fatty acid ratio of C16:1, C18:1, and myristic acid (C14:0), as recommended by Schenk et al. (2008).

The ash content was significantly higher in the pond inside the greenhouse than in the outdoor ponds, with no significant difference between the outdoor ponds (Table S5). The lowest ash content was found in pond 1 with 9.43 ± 0.49% of DW while pond 3 had the highest ash content of 32.45 ± 2.05% of DW (Fig. 1). Moreover, ponds 1, 2, and 3 had a mean ash content for the entire growing period of 16.75 ± 3.36, 16.04 ± 4.91, and 25.40 ± 3.25% of DW respectively (Fig. 1). These values of ash even though high (Zhu et al., 2015; Lage et al., 2018), are lower than what was previously found for microalgae grown on wastewater at the same facilities (Liu et al., 2020). It is reasonable to consider

that the higher temperature of pond 3, compared to the outdoor ponds, has generated higher water evaporation, hence a higher concentration of nutrients and salts in the water.

Microalgae biomass heating value varied from 16.33 ± 0.01 MJ/kg of pond 3 to 22.18 ± 0.01 and 22.11 ± 0.03 MJ/kg of ponds 1 and 2, respectively (Fig. 3). The heating value of the microalgae biomass from the outdoor ponds was significantly higher than pond 3, and there was no significant difference between ponds 1 and 2 (Table S5). The heating value of the biomass produced in the three ponds was higher than what was previously found for microalgal cultivated in high-rate algal ponds receiving domestic effluent, where the biomass produce had an energy content of 13.2–14.5 MJ/kg (Costa et al., 2017).

3.5. Total N and C

The total N content of the microalgae biomass varied between 3.81 and 8.57% (both in pond 2), which is within the 1–10% range expected for microalgae (Wijffels et al., 2010). The microalgae biomass N content of pond 2 was significantly higher than of pond 3, and there was no significant difference in the total N content of the microalgae from the former ponds and pond 1 (Table 1 and S5). The total C content of the microalgae biomass varied between 24.43 and 52.55% (both in pond 2). The microalgae of the outdoor ponds had a C content significantly higher than the biomass from pond 3; while the C content of pond 1 and 2 microalgae was not significantly different (Table 1 and S5). The C/N ratio of the microalgae biomass varied between 5.69 in pond 2 and 8.99 in pond 1, and it was not significantly different between ponds (Table 1 and S4). The protein/N ratio of the microalgae biomass of pond 2 was significantly higher than that of pond 1 (Table 1, Tables S4and S5); a possible explanation of this difference is the different composition of the microalgae consortium. For the three ponds, the average protein/N ratios were lower than the traditional factor of 6.25 (Table 1), which assumes that protein contains 16% nitrogen and a negligible concentration of non-proteinaceous nitrogen (Jones, 1931). However, this default value may not be an appropriate conversion factor for all protein sources; for instance Lourenço et al. (2004) observed that microalgae have larger contents of non-protein N-containing compounds. Moreover Slocombe et al. (2013) showed that N-conversion factors of seven species of marine microalgae ranged from 2.7 to 5.4, which agrees with our results (Table 1). Moreover, when N is insufficient to support protein synthesis,

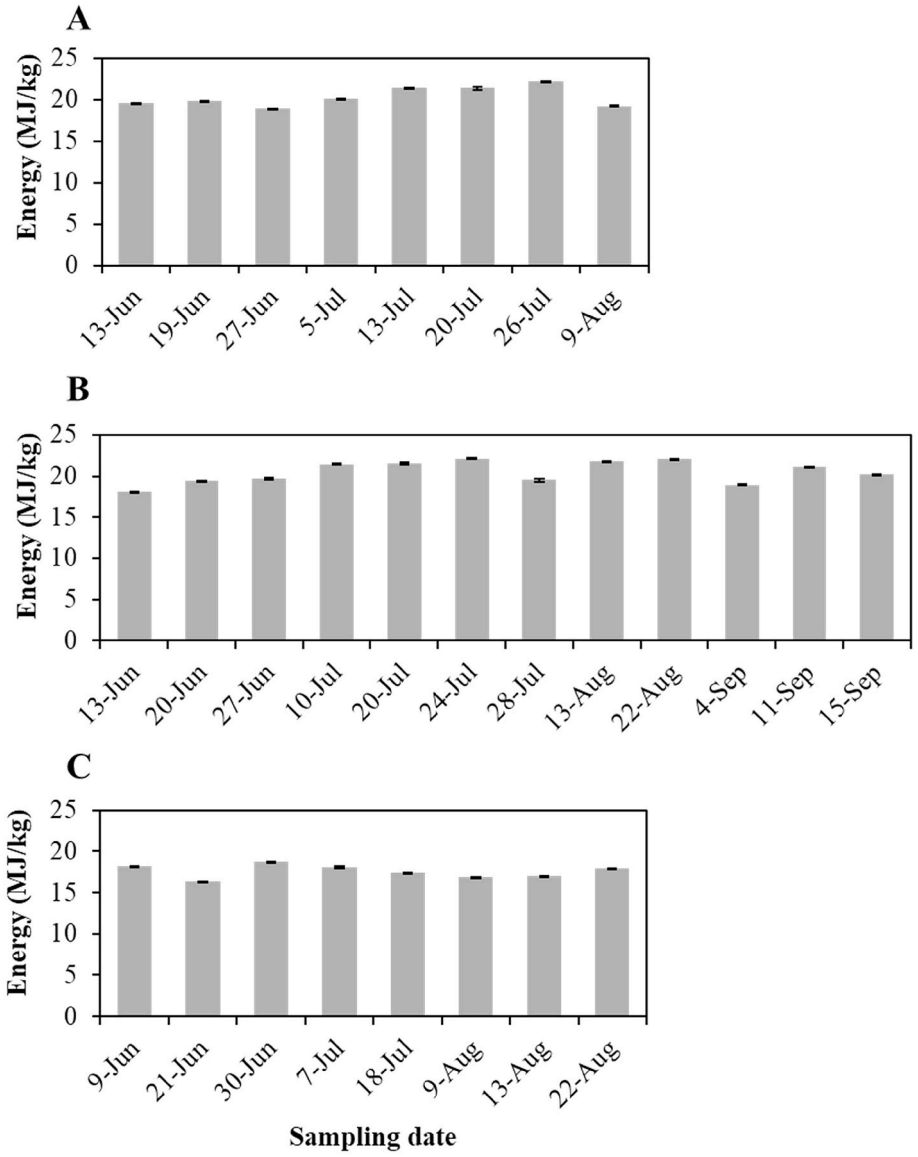

Fig. 3. Energy content (MJ/kg) of algae biomass samples harvested in pond 1 (A), 2 (B) and 3 (C), during the sampling period. Values are expressed as % DW, mean ± standard deviation, n=3.

excess C from photosynthesis is diverted into storage molecules such as lipids and carbohydrates, and thus cells with higher contents of N have lower lipids and carbohydrates contents, and vice-versa, as observed in the present study (Table S5) (Scott et al., 2010).

3.6. Elemental composition

The concentration of 19 (i.e., Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Na, Ni, P, Pb, Se, V, and Zn) out of 23 elements analyzed was significantly higher in pond 3 than both outdoor ponds (Table 2, Tables S4and S5), except for pond 1 concentrations of K, Mg, Si and for pond 2 concentration of S. This was also indicated by the significantly higher ash content of the microalgae biomass of pond 3 than of the outdoors ponds (Fig.1, Tables S4 and S5). The ponds received the same type of wastewater, however, pond 3 inside the greenhouse was exposed to higher temperature, hence higher evaporation that led to a higher concentration of the elements in the wastewater. Nevertheless, the concentration of K, and Si was significantly lower in pond 3 than in the outdoor ponds (Tables S4 and S5). The concentration of S in pond 3 was significantly higher than in pond 1, but not of pond 2 (Tables S4 and S5). The higher Si in the ponds without cover (ponds 1 and 2) was most probably due to dust and soil deposition. The macronutrients Ca, Mg, K, Na, P, and S concentration of the algae biomass produced in the three ponds fell in the range of what previously found in microalgae from different phyla grown in artificial media (Tibbetts et al., 2015). However, the algae produced in the present study had a higher concentration of trace elements than the microalgae of different phyla grown in artificial media (Tibbetts et al., 2015; Di Lena et al., 2020). A possible explanation of the higher trace elements and metals in the algae produced in the present study is the use of untreated municipal wastewater and even though treated of flue gases from a combined heat and power plant. It has been previously found that high level of Cu, Fe and Zn could inhibit the activity of enzymes involved in the anaerobic digestion (Tawfik et al., 2022). Hence, it is possible that the level found in the present study and especially in pond 3 could inhibit enzymes activity during anaerobic digestion resulting in a lower biogas production.

4. Conclusions

The ponds inoculated with the local consortium of green-algae had a constant microalgae community, throughout the experimental period; while the pond inoculated with the locally isolated strain of *Chlorella vulgaris*, had its microalgae population changed substantially in few weeks after inoculation. Pond 1 (consortium/outdoors) had a significantly higher biomass concentration, carbohydrate, and lipid content than the other ponds; thus, it had the greatest production potential. Pond 3 (consortium/greenhouse) had a significantly higher neutral saturated and monounsaturated fatty acids and a significantly lower neutral polyunsaturated fatty acid than the outdoors ponds, making its biomass ideal for biodiesel production. However, pond 3 biomass also had a significantly higher ash, elements content and significantly lower heating value. Moreover, due to the polyethylene film sheets of the greenhouse cover, pond 3 had a significantly lower PAR and higher water temperature than the outdoor ponds. These, consequently might have contributed to a significantly lower photosynthetic activity in this pond, a significantly lower DO and biomass than the outdoor ponds. Thus, overall, the positioning of the pond inside a greenhouse did not improve the production potential of the green-algae consortium.

Credit author statement

FG acquired the funding. **FG** conceptualized the project. **SL** performed the experimental work, data collection, and analysis, under the supervision of **FG**. All authors were involved in data interpretation and manuscript preparation and reviewing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.137344.

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