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Microalgal growth, nitrogen uptake and storage, and dissolved oxygen production in a polyculture based-open pond fed with municipal wastewater in northern Sweden

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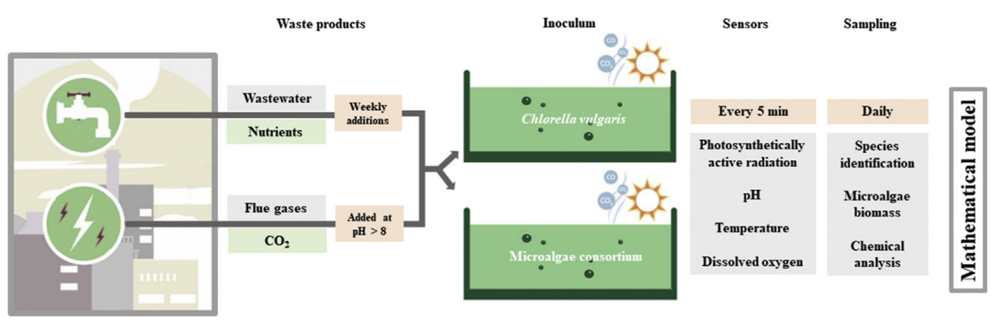
HIGHLIGHTS

- Nordic microalgae strains treated wastewater and sequestered CO_2 from flue gases.
- Microalgae consortium outcompeted *C. vulgaris* monoculture in outdoor open ponds.
- Microalgae consortium had higher biomass concentration and PO_4 removal than monoculture.
- Mathematical model on microalgae growth, N uptake/storage, and O_2 generation was made.
- Model simulated specific traits of microalgal behavior in complex growth conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgal-based wastewater treatment and CO_2 sequestration from flue gases with subsequent biomass production represent a low-cost, eco-friendly, and effective procedure of removing nutrients and other pollutants from wastewater and assists in the decrease of greenhouse gas emissions. Thus, it supports a circular economy model. This is based on the ability of microalgae to utilise inorganic nutrients, mainly nitrogen and phosphorous, as well as organic and inorganic carbon, for their growth, and simultaneously reduce these substances in the water. However, the production of microalgae biomass under outdoor cultivation is dependent on several abiotic and biotic factors, which impact its profitability and sustainability. Thus, this study's goal was to evaluate the factors affecting the production of microalgae biomass on pilot-scale open raceway ponds under Northern Sweden's summer conditions with the help of a mathematical model. For this purpose, a microalgae consortium and a monoculture of *Chlorella vulgaris* were used to inoculate outdoor open raceway ponds. In line with the literature, higher biomass concentrations and nutrient removals were observed in ponds inoculated with the microalgae consortium. Our model, based on Droop's concept of macronutrient quotas inside the cell, corresponded well

Abbreviations: a, specific interfacial area (1/m); *A*, algal biomass concentration (mg_{DW}/L); I_0, incident light intensity on a pond's external surface (W/m^2); I_{av}, average light intensity inside the pond (W/m^2); f_o, stoichiometry of net algal oxygen production (=1.24 mg/mg_{DW}); $k_{N,max}$, maximum nitrogen uptake rate (mg/mg_{DW}/day); $K_{L,O2}$, mass transfer coefficient (m/day); N_i, intracellular nitrogen (mg/L); N_e, extracellular nitrogen (mg/L); O_{2d}, dissolved oxygen concentration (mg/L); Q_N, nitrogen quota inside algae cells (mg/mg_{DW}); t_w, pond water temperature (°C); $\mu_{A,max}$, maximum algae growth rate (1/day); ρ_A, algae decay rate (1/day).

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to the experimental data and, thus, can successfully be applied to predict biomass production, nitrogen uptake and storage, and dissolved oxygen production in microalgae consortia.

1. Introduction

The development and intensification of human activities, such as agricultural practices, urbanisation, and industrialisation, has led to excessive production of wastewater and greenhouse gases (GHG). The continuous disposal of wastewater without adequate treatment into water bodies has resulted in severe water pollution (O'Neil et al., 2012). To tackle the negative effects of wastewater on water bodies, a combination of physical, chemical and biological methods, i.e. conventional wastewater treatment (CWWT), has been employed. The CWWT consists of three processes; (1) purification of raw material by removal of suspended solids, (2) removal of soluble and insoluble biodegradable organic matter, and (3) removal of inorganic and toxic pollutants (Gupta et al., 2012). However, CWWT is costly and produces a high sludge content (Boelee et al., 2011). The high concentrations of GHGs released into the atmosphere are the main cause of global warming and a major concern for many countries around the world. Since CO_2 represents approximately 77% of the total GHGs, a reduction in CO_2 levels would directly affect total GHG emissions (Cheah et al., 2015; López et al., 2013). Lately, several CO_2 sequestration strategies have been implemented, however, they are not environmentally sustainable and require considerable space and investment (Cheah et al., 2015; Lage et al., 2018; López et al., 2013).

For more than a half-century, extensive research has been done on the biological treatment of wastewater using microalgae (Caldwell, 1946; Ludwig et al., 1951; Oswald et al., 1957). Microalgal-based wastewater treatment has an effective uptake of nutrients (mainly, nitrogen and phosphorous), heavy metals and pharmaceuticals; thus, it represents a low-cost alternative to CWWT systems (Cai et al., 2013; Gentili and Fick, 2017; Rawat et al., 2013). This method has the added advantage of producing economically practical and environmentally sustainable biomass for bioenergy (Kothari et al., 2019; Lage et al., 2018; Rawat et al., 2013). Additionally, flue gases which may contain 6–15% (v/v) of CO_2 and 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 (Cheah et al., 2015; Praveenkumar et al., 2014). Microalgal-based wastewater treatment and CO_2 sequestration can offer an ecologically safer, cheaper, and efficient way of removing nutrients and other pollutants from wastewater and help in the reduction of GHG emissions, thus, having a key role in environmental restoration and a circular economy (Cheah et al., 2015; Kothari et al., 2019; Lage et al., 2018).

Most species used in microalgal-based wastewater treatment belong to the genus *Chlorella* and *Scenedesmus*. Although these microalgae have been successfully applied in microalgal-based wastewater treatment, their cultivation in non-sterile systems is susceptible to contamination by wild strains unless additional means of control are utilised (Christenson and Sims, 2011; Ferro et al., 2020). Alternatively, microalgae consortia can resist environmental fluctuations and invasion by other species (Padmaperuma et al., 2018); these consortia can naturally occur in the environment or be artificially engineered, by a combination of microorganisms that do not necessarily co-occur, for a specific purpose (Jagmann and Philipp, 2014; Novoveská et al., 2016). Nevertheless, locally isolated strains from wastewater ponds are more effective than strains from culture collections (Ferro et al., 2018b, 2020; Zhou et al., 2014).

Microalgal growth and, thus, microalgae-based wastewater treatment and CO_2 sequestration can be affected either positively or negatively by several biotic and abiotic factors. Biotic factors include the presence of bacteria, fungi, viruses, and other microalgae, while abiotic factors include light, temperature, pH, salinity, nutrient, dissolved oxygen concentration, and the presence of toxic compounds (Khan et al., 2018; Lage et al., 2018, 2019; Slegers et al., 2013). Some of these factors can be analysed with mathematical models. Several models have been proposed in the literature in past decades to assess the profitability and sustainability of the outdoor cultivation of microalgae on a large scale. Simpler models propose an archetype for the curve of algal biomass growth in time coupled with the consumption of nutrients (Droop, 1983). More complex models consider the transient conditions of temperature and light intensity, with different levels of detail (Béchet et al., 2013), as additional limiting factors to the algal growth rate. Model output curves must fit the time history of the experimental data to find the unknown model parameters that are specific to the cultivated microalgae (e.g., theoretical microalgal growth rate in absence of limiting factors, rate of nutrient uptake). Thus, the collection of experimental data about several aspects of the cultivated microalgae and the environment in which they are cultivated is of fundamental importance for a successful fitting process and an accurate estimation of the unknown model parameters. Moreover, additional information on other quantities not directly involved in the growth mechanism, such as dissolved oxygen concentration, may be helpful in the reconstruction of the growth curve.

The present study aimed to: (1) follow microalgal biomass growth, nitrogen uptake and storage, and dissolved oxygen production in a system combining microalgae biomass production with wastewater treatment and CO_2 sequestration from flue gases under Northern Sweden's summer conditions on a pilot-scale; and (2) analyse the factors affecting microalgae biomass production with a mathematical model, which could also be used in a predictive way at a later stage. For this purpose, a local microalgae consortium and monoculture of a local *Chlorella vulgaris* isolate were selected and used to inoculate open raceway ponds. The interpretation of the experimental data on microalgal growth, nitrogen uptake/storage, and oxygen generation in the ponds was assigned to a mathematical model, largely based on previous modelling efforts, developed in MATLAB/Simulink.

2. Materials and methods

2.1. Experimental setup

The experiment was conducted in two identical raceway systems 10 m long, 2 m wide, and about 0.3 m deep with a surface area of 19.14 m^2, a volume of ca 6 m^3, and equipped with paddle wheels with six blades located in Umeå, northern Sweden (63°86′ N) at Umeå Energy (Umeå, Sweden) combined heat and power plant (CHP-plant) during the 2017 summer season. The two raceway systems were placed outdoors. Municipal untreated wastewater influent was collected from the local wastewater treatment plant

(Vakin, Umeå, Sweden) and transported once a week to the CHP-plant. Flue gases from the CHP-plant (Umeå Energi, Umeå, Sweden), which burns both municipal and industrial solid wastes, were bubbled into the raceway systems using gas diffusers (Cole Parmer, USA) from the 28th of June until the 14th of August. The complete analysis of the flue gases measured every 30 min during the experiment ($n = 3916$) was CO_2 7.4 ± 1.9%, CO 4.4 ± 8.5 mg/Nm^3, NO_x 38 ± 24 mg/Nm^3 and SO_2 4.4 ± 4.7 mg/Nm^3. The flue gases (v/v) were added at pH values higher than 8.0 and stopped at pH values lower than 8.0. Temperature and light were not controlled and reflected those available in this area.

A consortium of local freshwater green-algae was used to inoculate one of the raceway systems (hereby named pond 1), while a local strain of *Chlorella vulgaris*, previously genetically identified (Ferro et al., 2018a), was inoculated in the second raceway system, (hereby named pond 2). Inoculation took place on the 18th of May 2017 with an inoculum/wastewater ratio of 1/240 (v/v). The biomass concentration of *C. vulgaris* in the inoculum was 0.5 g/L dry weight. The sampling period was between May 24th and August 14th of 2017.

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

Photosynthetically active radiation (PAR) was measured and recorded every 5 min using an 1400 datalogger connected to a LI 192 light sensor (LiCor, Lincoln, Nebraska, USA), which was placed above pond 2 and in close vicinity to pond 1.

Temperature, pH, and dissolved oxygen (optical dissolved oxygen) were also measured and recorded every 5 min using electrodes and sensors from Hach-Lange (Hach-Lange, Duesseldorf, Germany).

Climate conditions, i.e. temperature (°C), wind (m/s), precipitation (mm), relative humidity (%), atmospheric pressure (hPa), and solar radiation (W/m2) were measured by the Department of Applied Physics and Electronics, Umeå University at the TFE's weather station (Umeå, Sweden). The climate data is publically available at http://www8.tfe.umu.se/weather-new/historik.html.

2.3. Sampling

Water samples of the microalgae population from the two ponds (~100 mL) were collected daily (approx. at 1 pm) during the experiment duration, a few centimetres below the surface, and immediately frozen and kept at −20 °C until further processing. Aliquots of these samples were used for microalgae taxonomic identification, biomass quantification, and chemical analysis, i.e., phosphate, ammonium, and nitrate.

Microalgae biomass was recovered once a week by sedimentation for two days in 1 m^3 plastic containers to pre-concentrate the microalgae, followed by continuous centrifugation at 3949×*g* with a flow of approx. 1L/min (US Filtermaxx, Jacksonville, Florida, USA). The microalgal paste collected after centrifugation was kept at −20 °C until further processing.

2.4. Taxonomic identification of microalgae

Aliquots (50 μL) of water samples 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. Biomass quantification

Aliquots (50 mL) of water samples were used for biomass quantification. Samples were centrifuged (3580×g, 10 min), the supernatant was discarded, and pellets were dried at 70 °C for 24 h and then weighed. Biomass was expressed as g/L. The dry pellets were milled using a ball mill (MM200, Retsch, Haan, Germany) and used for total carbon and nitrogen analysis.

2.6. Chemical analysis

2.6.1. Phosphate, ammonium, and nitrate analysis

Water samples of ponds and municipal untreated wastewater influent collected as described above were filtered using 0.45 μm syringe filters (Sarstedt, Nümbrecht, Germany). Phosphate, ammonium, and nitrate (hereby named P-PO_4^{3-}, N—NH_4^+, and N—NO_3) were analysed in the filtered samples at the Department of Forest Ecology and Management, Swedish University of Agricultural Sciences (Umeå, Sweden). Analysis was done using standard colorimetric methods (Rice et al., 2012) with an AutoAnalyzer 3 Spectrophotometer (OmniProcess AB, Solna, Sweden).

2.6.2. Total C and total N analyses

Total carbon and nitrogen measurements in microalgae biomass samples 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 analysed by Elemental Analyzer - Isotope Ratio Mass Spectrometry (EA-IRMS). The instrumental setup consisted of an elemental analyser (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 analysed together with two *in-house* standards in several replicates. The accepted standard deviation of *in-house* laboratory standards is < 0.15‰. Data were corrected for drift and size before yielding the final results.

2.7. Statistic analyses

All statistical analyses, if not stated otherwise, were carried out in JMP® Version 14.0. SAS Institute Inc. (Cary, North Carolina, USA); the significance level was set to $\alpha = 0.05$.

First, the average pH, water temperature (TEMP), dissolved oxygen (DO), and photosynthetically active radiation (PAR) per sampling day was calculated, and these values were used for statistical comparisons. The percentage removal of P-PO_4^{3-}, N—NH_4^+, and N—NO_3 per sampling day was calculated.

Principal component analysis (PCA) on correlations were conducted to (1) explore the overall variability between the ponds; and (2) examine the relationships between the variables, i.e., biomass, total N and C, removal of P-PO_4^{3-} and N—NH_4^+, DO, TEMP, and pH. Considering that the PAR values were the same for both ponds, this variable was not used in PCA. After a first test, the variable removal of N—NO_3 was removed from PCA due to its low influence on the model's explanatory value. The explained proportion of the variance and the minimum number of components was determined by the broken stick model (Fig. S5). Pearson's correlation analysis was applied to further explore cross-correlations among all variables.

One-way analysis of variance (ANOVA) was used to evaluate whether pond as a categorical predictor (pond 1 versus pond 2) significantly influenced the biomass, total N and C, removal of P-PO_4^{3-}, N—NH_4^+, and N—NO_3, DO, TEMP, and pH. If the one-way ANOVA was significant, then the pond effect was evaluated for each variable using Tukey's honestly significant difference (HSD) test.

2.8. Mathematical model

A mathematical model was developed to simulate the dynamics of microalgae growth, nitrogen uptake/storage, and oxygen generation in the ponds. The model consisted of differential equations and analytic models that were taken and adapted from several other recently published models (Packer et al., 2011; Shriwastav et al., 2017; Wágner et al., 2016). At the core, the model expressed Droop's concept of macronutrient quotas inside the cells (Droop, 1983), which decouples microalgal growth from macronutrient uptake by considering the intracellular storage of the macronutrients.

2.8.1. Microalgal biomass accumulation

The balance of microalgal biomass was mainly governed by the rate of microalgal growth. This rate was calculated as a theoretical maximum microalgae growth rate $\mu_{A,max}$ (at infinite intracellular storage of nitrogen) reduced by limiting factors that depend on the nitrogen quota inside the cells, average light intensity, and water temperature in the pond. A microalgae decay rate ρ_A was also considered. The following balance is adapted from Packer et al. (2011); Shriwastav et al. (2017):

$$\frac{dA}{dt} = \left(\mu_{A,max} f(Q_N) f(I_{av}) f(t_w) - \rho_A\right) A \tag{1}$$

The limiting factor, depending on nitrogen quota, reduced the microalgal growth rate to zero when the internal quota of nitrogen Q_N was equal to the minimum/subsistence quota $Q_{N,min}$ (Droop, 1983):

$$f(Q_N) = 1 - \frac{Q_{N,min}}{Q_N} \tag{2}$$

The limiting factor, depending on the average light intensity I_{av}, in the pond was a Monod-like function:

$$f(I_{av}) = \frac{I_{av}}{I_{av} + K_{s,I}} \tag{3}$$

with half-saturation constant $K_{s,I} = 400$ W/m^2 (Béchet et al., 2013).

The average light intensity in the pond I_{av} was calculated by integrating over pond volume, the distribution of local light intensity $I(z)$ as a function of depth z inside the pond. This distribution was expressed by the Beer-Lambert law:

$$I(z) = I_0 \exp(-\sigma A z) \tag{4}$$

where I_0 is the incident light intensity on the pond external surface and σ is the coefficient of light attenuation due to the presence of the microalgal biomass itself, according to its concentration A.

Finally, the limiting factor, depending on pond water temperature t_w, was described by a continuous empirical function that was close to 1 for temperatures around 25 °C and near 0 for temperatures above 40 °C or below 0 °C (Béchet et al., 2013).

2.8.2. Nitrogen uptake and storage

The balance of intracellular and extracellular nitrogen was mainly governed by the rate of nitrogen uptake. This rate was calculated as a theoretical maximum uptake rate $k_{N,max}$ (at zero intracellular storage of nitrogen) reduced by limiting factors that depended on the nitrogen quota inside the cells and the concentration of nitrogen in pond water. Decayed biomass was also assumed to return its nitrogen content to pond water. The following balance is adapted from (Shriwastav et al., (2017); Wágner et al., 2016):

$$\frac{dN_i}{dt} = -\frac{dN_e}{dt} = \left(k_{N,max} f'(Q_N) f(N_e) - \rho_A Q_N\right) A \tag{5}$$

The limiting factor, depending on the nitrogen quota, reduced the uptake rate to zero when the internal quota of nitrogen Q_N was equal to its maximum value $Q_{N,max}$ (Wágner et al., 2016):

$$f'(Q_N) = 1 - \frac{Q_N}{Q_{N,max}} \tag{6}$$

and the limiting factor related to the concentration of nitrogen in pond water follows Michealis-Menten kinetics (Wágner et al., 2016):

$$f(N_e) = \frac{N_e}{N_e + K_{s,N}} \tag{7}$$

with half-saturation constant $K_{s,N} = 0.2$ mg/L (Packer et al., 2011; Shriwastav et al., 2017; Wágner et al., 2016).

The nitrogen quota inside the cells was by definition the ratio between intracellular nitrogen and microalgal biomass concentration:

$$Q_N = N_i / A \tag{8}$$

2.9. Oxygen generation

The balance of dissolved oxygen in the pond considered both the photosynthetic oxygen generation and the mass transfer of dissolved oxygen between the pond and atmosphere (Shriwastav et al., 2017):

$$\frac{dO_{2d}}{dt} = f_0 \frac{dA}{dt} - K_{L,O_2} a\left(O_{2d} - [O_2]^*\right) \tag{9}$$

where f_0 is the stoichiometry of net algal oxygen production, $K_{L,O2}$ is the mass transfer coefficient, a is the specific interfacial area for mass transfer, and $[O_2]^*$ is the saturation concentration of dissolved oxygen in water (this is considered to vary according to pond water temperature).

2.9.1. Model simulation

The differential equations of the model were solved in the MATLAB/Simulink environment with time steps of 5 min, providing predictions of the time history for all main quantities describing the cultivation of microalgae in the ponds.

3. Results and discussion

3.1. Community composition

Pond 1 inoculum was mainly constituted by *Chlorella* sp., *Scenedesmus dimorphus*, *Scenedesmus quadricauda,* and *Desmodesmus armatus*. Although the microalgae population suffered several changes through the sampling season (May–August), the inoculated species kept their dominance over the microalgae species introduced by the surrounding environment (Table S1). However, 'invasive' *D. opoliensis*, *S. acuminatus,* and *S. obliquus* had high frequencies. In pond 2, which was inoculated with a monoculture of a local strain of *C. vulgaris* on the 18th of May, had its microalgae population changed substantially due to contamination a few weeks later, i.e., 13th of June. At this point, and during the sampling season, *D. armatus* and *Coelastrum microporum* were the dominant species (Table S1). Thus, the pond inoculated with the local microalgae consortium (pond 1) kept its dominance through the season, while the pond inoculated with *C. vulgaris* monoculture (pond 2) was rapidly overtaken by other microalgae present in the environment. In agreement, Novoveská et al. (2016) and Ferro et al. (2020) observed a clear change in the microalgae community composition, from initial monocultures of *S. dimorphus* to a consortium of naturally occurring microalgal species after two months or an entire season, respectively, since the photobioreactor inoculations.

3.2. Seasonal and diurnal variations of abiotic factors

The light quality, intensity, and duration, as well as water temperature, strongly affects microalgae production (Huisman et al., 1999). In an outdoor cultivation system, solar radiation is the sole source of light and heat, which is, therefore, dependent on geographical location, climate, seasonality, and local weather. In this study, the pilot-scale raceway systems were located outdoors in northern Sweden during the summer months, which experiences more than 20 h of light per day with relatively high intensities. The local climate conditions measured at TFE's (Department of Applied Physics and Electronics, Umeå University, Umeå, Sweden) weather station during the experimental period are presented in Fig. S1 (Supplement material).

During the sampling season, PAR was on average 428.52 μmol/m^2/s and had 2331.50 μmol/m^2/s at its highest point (Fig. S2). Water temperature ranged between 7.21–23.87 °C and 7.35–23.79 °C; and was on average 16.19 ± 2.36 and 16.35 ± 3.17, in pond 1 and 2, respectively (Fig. S3). The pH was not regulated during the first month of cultivation, then, it was regulated by the addition of CO_2 via flue gases. The pH was 8.58 ± 0.91 and 8.48 ± 0.86, and its maximum values were 10.73 and 10.40, in ponds 1 and 2, respectively (Fig. S3). The bubbling of flue gases was controlled to have an average pH-value of 8.00. Considering that a pH-value of 8.00 minimizes NH_3 from volatilising and PO_4^{3-} from precipitating (Park et al., 2011). DO contents ranged from 0.00 to 21.00 mg/L (highest value measurable by the sensor) in both ponds (Fig. S3). A DO content of 0 mg/L exclusively represented times of biomass harvesting and new untreated wastewater being added to the ponds. The DO was 10.31 ± 4.15 and 11.57 ± 6.48 mg/L in pond 1 and 2, respectively (Fig. S3). The nutrient concentrations in of the different wastewater influents were not standardised and, therefore, varied with the shifts of municipality activities and population density. The average concentrations of P-PO_4^{3-} and N–NH_4^+ in the wastewater influent additions were 4.38 ± 2.06 and 46.26 ± 5.11 mg/L, respectively. The concentrations of N–NO_3 were negligible and varied between 0.04 and 0.99 mg/L. The hydraulic retention time (HRT) was on average 11.7 days for pond 1 and 16 days for pond 2.

3.3. Data visualisation – Principal Component Analysis and correlations

PCA identified two significant PCs based on the pH, TEMP, DO, biomass, total N and C, and removal of P-PO_4^{3-} and N–NH_4^+ (Fig. S5). The PCA biplot showed a separation between pond 1 and 2 data points, even though there was some overlap (Fig. 1). Therefore, the different inoculants and subsequent different biotic and abiotic conditions of the two ponds resulted in distinguishable biomass concentrations, nutrients uptake and storage, nutrient removal, and dissolved oxygen levels of the two ponds throughout the sampling period.

Overall, pond 1 data points had higher biomass concentrations, removals of P-PO_4^{3-} and N–NH_4^+, TEMP, and DO and lower pH, total N, and C than pond 2 (Fig. 1). The consortium pond (pond 1) had higher microalgae growth and nutrient removal than the monoculture pond (pond 2). Although a higher content of N and C was observed in the biomass from pond 2, considering that pond 1 had higher biomass concentration, then, in total, pond 1 had higher nitrogen and carbon uptake.

All variables had loadings ≥0.3 on either PC1 and/or PC2, and, therefore, influenced the grouping (Table S2). PC1 and PC2 had a cumulative explanatory contribution of 66.17% (Fig. 1 and Table S3). Hence, other factors not accounted for, e.g., biotic factors, such as the type of organisms present and their variability, i.e., bacteria and algae genus and species, might explain the remaining variance.

The PCA biplot suggested some cross-correlations between the variables (Fig. 1). The same ordination was found between the vectors of total N and C, and the removal of P-PO_4^{3-} and N–NH_4^+ (Fig. 1). In line with the PCA patterns, significantly strong positive correlations (Pearson's $r > 0.7$) were found between the former variable pairs (Table 1). Moderate (Pearson's $r > 0.5$) positive correlations were found between DO and removal of N–NH_4^+ (Table 1).

3.4. Microalgae growth – biomass concentration

The initial biomass concentration, i.e. biomass of the municipal untreated wastewater influent was 0.178 ± 0.016 g/L. The microalgae biomass concentration during the sampling season was 0.36 ± 0.13 and 0.23 ± 0.07 g/L in ponds 1 and 2, respectively (Fig. S4). These biomass concentrations are in line with a previous study performed in an open photobioreactor (surface area 2.72 m2, volume 650 L), situated in northern Sweden at 63°N. A consortium of native green-algae dominated by *Dictyosphaerium* sp. grown with municipal influent and flue gases, produced 0.22 ± 0.03 g/L total suspended solids after 7 days (Gentili and Fick, 2017).

The highest total biomass concentration measured was in pond 1 on August 1st with 0.601 g/L (Fig. S4A). The biomass in pond 1, inoculated with monoculture, was significantly higher than in pond 2, inoculated with a consortium of microalgae (Table S4 and S5).

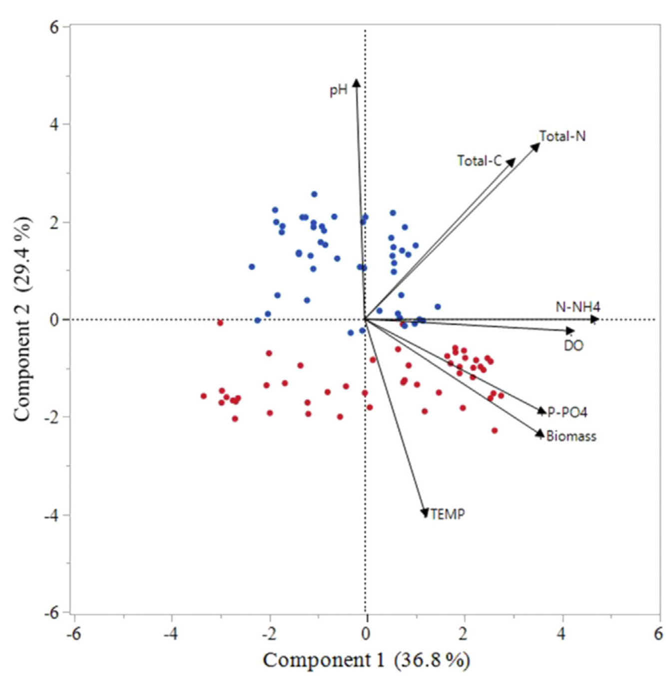

Fig. 1. Principal Component Analysis (PCA plots showing Component 1 and Component 2 with the variance explained) of pH, water temperature (TEMP), dissolved oxygen (DO), biomass, total nitrogen (N) and carbon (C), P-PO_4^{3-}, N–NH_4^+ removal. For pH, water temperature, dissolved oxygen the average value per day was used. Data points of pond 1 in red and pond 2 in blue, $n = 102$.

Table 1
Pearson's cross-correlations between biomass concentration, total nitrogen (N) and carbon (C), removal of P-PO_4^{3-}, N—NH_4^+ and N—NO_3, dissolved oxygen (DO), water temperature (TEMP), pH and photosynthetically active radiation (PAR). For DO, TEMP, pH and PAR the average per day was used. The colour intensity reflects the correlation coefficients value (r), with positive values in green and negative values in red, $n = 102$.

	Biomass	Total-N	Total-C	P-PO_4^{3-}	N-NH_4^+	N-NO_3	DO	TEMP	pH
Total-N	0.076								
Total-C	0.210	0.807							
P-$PO4^{3-}$	0.378	0.132	0.013						
N-$NH4^+$	0.462	0.427	0.315	0.698					
N-NO3	0.062	-0.128	0.002	-0.380	-0.261				
DO	0.487	0.442	0.223	0.360	0.584	-0.140			
TEMP	0.356	-0.127	-0.118	0.284	0.051	-0.018	0.127		
pH	-0.391	0.455	0.331	-0.195	0.047	-0.183	-0.056	-0.642	
PAR	0.223	-0.003	-0.047	0.087	0.096	0.019	0.315	0.105	0.043

Accordingly, Chinnasamy et al. (2010) shown that in comparison to monocultures, a consortium of green algae performed better in terms of biomass production potential in treated and untreated wastewater from carpet mills along with the sewage. While the monocultures had biomass concentrations between 0.16 ± 0.03 and 0.38 ± 0.03 and between 0.23 ± 0.04 and 0.34 ± 0.07 g/L (dependent on the microalgae species), the consortium had 0.41 ± 0.05 and 0.39 ± 0.09 g/L, in the treated and untreated wastewater, respectively.

3.5. Nutrient removal

The untreated municipal wastewater influent used in this study was rich in P-PO_4^{3-} and N—NH_4^+ but poor in N—NO_3 (Fig. 3). It has been previously suggested that the optimal N/P ratio is important for microalgal growth and removal of nutrients in municipal wastewater (Choi and Lee, 2015; Lee et al., 2015; Uggetti et al., 2014). The optimum N/P ratio for high biomass production and nutrient removal from municipal wastewater treatment using microalgae varies from 5 to 30, depending on the ecological conditions in the wastewater. The canonical Redfield N/P ratio of 16 is not a universal biochemical optimum but instead represents an average of species-specific N/P ratios (Klausmeier et al., 2004). In the current study, the overall N/P ratio of the untreated municipal wastewater influent was approximately 13. Due to the polyculture nature of this study, it is difficult to estimate the optimal conditions for the microalgae consortium used.

P-PO_4^{3-} was efficiently removed up to 90.16 and 89.42% in pond 1 and 2 respectively, in just 5 days after each new untreated wastewater addition. The removal of P-PO_4^{3-} was variable throughout the season, on average, 54.17 ± 22.99% and 45.03 ± 18.05% for pond 1 and 2, respectively (Fig. 3). Note that increases in P-PO_4^{3-} concentrations in the ponds are strictly due to additions of new batches of wastewater. In line with our results, two previous studies in Sweden reported P-PO_4^{3-} removal efficiencies up to 60—90% in summer conditions (Larsdotter et al., 2010) and on average 55.6 ± 10% in spring conditions (Gentili and Fick, 2017).

A significantly higher removal efficiency of P-PO_4^{3-} was determined for pond 1 (Table S4 and S5). In the literature, microalgae consortia have been shown to have higher P-PO_4^{3-} removal efficiencies than monocultures with most authors reporting P-PO_4^{3-} removal efficiencies above 90% for microalgae consortia. Different microalgae have different nutrient requirements, which result in the removal of multiple nutrients at the same time. Additionally, cooperative interactions between microalgae can result in increased removal efficiencies (Gonçalves et al., 2017; Lage et al., 2018; Zainith et al., 2021). Although pond 2 was inoculated with a monoculture; during most of the sampling season, it was constituted by a microalgae consortium due to contamination with local naturally occurring microalgae. The species composition of this pond most likely reflects the species with better adaptation to abiotic factor fluctuations, and thus, it does not necessarily comprise the species combination with higher biomass concentrations or nutrient removal.

The higher removal efficiency of P-PO_4^{3-} in pond 1 could also be due to its significantly higher biomass concentration (Table S4 and S5). However, since the correlation between P-PO_4^{3-} removal and biomass production was low, i.e. $r = 0.378$ (Table 1), it can be suggested that factors other than assimilation could be taking place. P-PO_4^{3-} uptake by microalgae is not always stoichiometric and can be altered by microalgal physiology, as well as the P-PO_4^{3-} concentration, its chemical forms, PAR, pH, and temperature. Some observations showed that the P-PO_4^{3-} uptake was inversely related to the internal P-PO_4^{3-} concentrations of the cell. Microalgae with low internal P-PO_4^{3-} concentrations showed higher uptake rates of P-PO_4^{3-} compared to algae with high internal P-PO_4^{3-} concentrations (Hernandez et al., 2006).

In both ponds, N—NH_4^+ was efficiently removed, up to 99%, 5 days after each new untreated wastewater addition (Fig. 3). The N—NH_4^+ removal efficiency was not significantly different between ponds; which was 64.47 ± 30.46% and 63.21 ± 28.74% in ponds 1 and 2, respectively (Fig. 3 and Table S4). In a previous study, with a similar setup and abiotic conditions, performed at 59°N Sweden, the nitrogen removal efficiencies were between 60 and 80% (Larsdotter et al., 2010). Higher N—NH_4^+ removal rates were observed just after new influent wastewater additions, when the N—NH_4^+ concentrations in the pond were at their highest, as previously reported (Jia and Yuan, 2018). It has been previously suggested that this could be due to the production of ammonia-oxidising bacteria, which are promoted by the high ammonium concentration (Chen et al., 2011). Although bacteria were not quantified in this study, due to the unsterile conditions typical of outdoor cultivation, bacteria were potentially present in both ponds. In pond 2, contamination with ammonia-oxidising and nitrite-oxidising bacteria might have occurred because an increase in N—NO_3 was observed in concurrence with a N—NH_4^+ decrease, even though the concentrations of N—NO_3 in the effluent wastewater were minor (Fig. 3).

The concentration of nutrients in water is one of the essential factors that directly affect microalgae growth and, subsequently, nutrient utilisation. A high abundance of microalgae will significantly increase nutrient removal and DO levels (Lee et al., 2015; Liu and Vyverman, 2015; Renuka et al., 2013; Uggetti et al., 2014). Accordingly, biomass production and DO was moderately correlated with N—NH_4^+ removal; which was strongly correlated with P-PO_4^{3-} removal (Table 1). Significant correlations, even though with lower Pearson's r, were also registered for the removal of $P-PO_4^{3-}$ with DO and biomass production (Table 1).

3.6. Total C and N

Carbon is the predominant element of green algae, which, in this study, represented 41.41 ± 2.94 and 43.80 ± 6.33% DW of the microalgae biomass of pond 1 and 2, respectively (Fig. 2). Although the carbon content difference among the biomass of the two ponds was limited, it was significantly higher in pond 2 biomass (Table S4 and S5).

The nitrogen content of the microalgae biomass of the ponds was within the 1–10% expected range (Wijffels et al., 2010); the microalgae biomass of pond 1 and 2 was 5.48 ± 0.96 and 6.08 ± 0.78% DW, respectively. The biomass of pond 2 also had significantly higher N than pond 1 (Tables S4 and S5). Notably, pond 1 had higher biomass concentration with lower N content than pond 2, which had less biomass production with a higher N content (Fig. 2, Tables S4 and S5, and Fig. S4). The total N in the biomass was positive correlated with $N-NH_4^+$ removal from the wastewater (Table 1). Only minor variations in the C/N ratio were determined. Nevertheless, higher C/N ratios matched time points of nitrogen depletion and, subsequently, declined in microalgae growth.

3.7. Estimation of model parameters from experimental data

The mathematical model described in section 2.9 was used to estimate the values of the five main model parameters ($\mu_{A,max}$, ρ_A, $Q_{N,min}$, $Q_{N,max}$, $k_{N,max}$, all specific features of the algae cultivated in the ponds) by fitting excerpts of the collected experimental data.

The following experimental measurements were provided as input to the model: incident light intensity on pond external surface (I_0) and pond water temperature (t_w). Then, MATLAB functions 'nlinfit' and 'nparci' were used to obtain the values and 95% confidence intervals of the five parameters, which were determined by minimising the sum of the squared error differences between the following measured quantities and the corresponding predictions of the model: algal biomass concentration (A); extracellular nitrogen concentration in the pond (N_e); nitrogen quota in the algal cells (Q_N); and dissolved oxygen concentration in the pond (O_{2d}).

Table 2 shows 3 multi-day periods that were selected as test cases and the corresponding values of the five model parameters as they were estimated using this procedure. The comparisons between experimental and simulated data are shown in Figs. 4–6. The agreement between the curves predicted by the model and the experimental data was generally good, and where the curves deviated more from the experimental data, the overall trend was captured.

As one might expect, the curves of nitrogen uptake and algal biomass growth followed the archetype resulting from Droop's equations, which link growth and nutrient uptake through the amount of nutrient (quota) stored inside the algal cells. Each selected simulated period began at 00:00h the day after the addition of a new batch of wastewater in the pond, which resulted in a sharp decrease in the concentration of algal biomass and dissolved oxygen and in the supply of additional nutrients (nitrogen) after the depletion of the nutrients from the previous batch. The algal biomass growth immediately started an exponential phase, limited by the day/night cycle of light intensity, algal decay, and, to a lesser extent, pond water temperature. During this phase, the nitrogen quota Q inside the cells was close to the maximum value (Q_{max}) that corresponded to a trade-off between nitrogen uptake rate, which is heavily limited when the quota is close to the maximum, and algal growth rate, which is higher for a higher Q. After the depletion of extracellular nitrogen in pond water, which occurred in roughly four days, the algal biomass continued to grow at a slower rate at the expense of the internal storage of nitrogen, so that Q started to decrease toward Q_{min}. The day/night cycle in the dissolved oxygen concentration was captured fairly well by the model, with algal oxygen production markedly prevailing during the first half of the day and diffusion of oxygen from pond water to the atmosphere prevailing during the second half of the day.

The model seemed less able to capture the day/night cycle in the consumption of the extracellular nitrogen. The experimental data showed that a large decrease of nitrogen concentration in pond water during the day was followed by a little growth during the darkest hours (it is probably not proper to use the word "night" in the period around the summer solstice in northern Sweden). The curves predicted by the model showed a change in their curvature but not as marked as that shown by the experimental

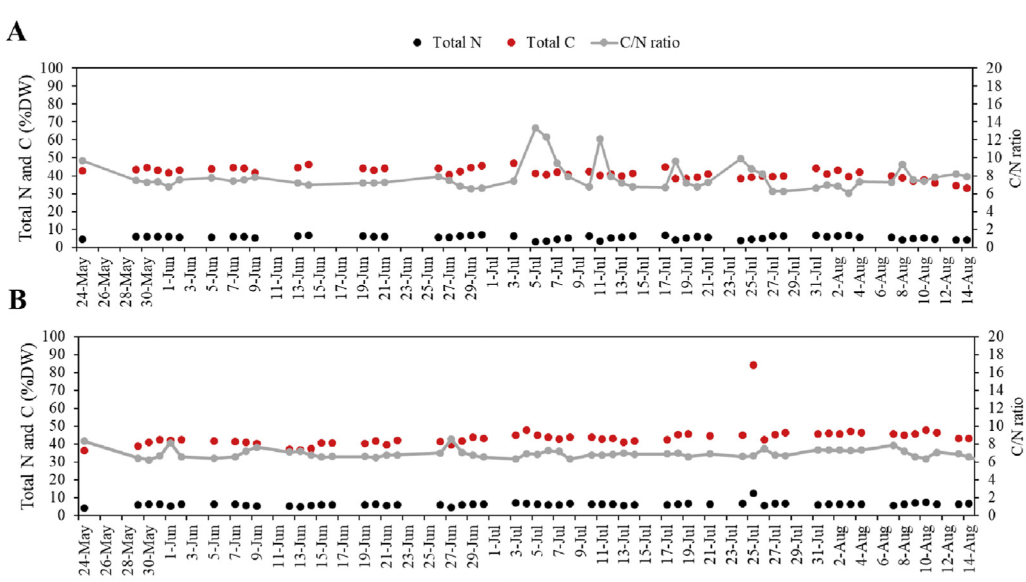

Fig. 2. Total nitrogen and carbon (N and C, % DW) and C/N ratio of microalgae biomass from pond 1 (A) and 2 (B), through the monitoring period.

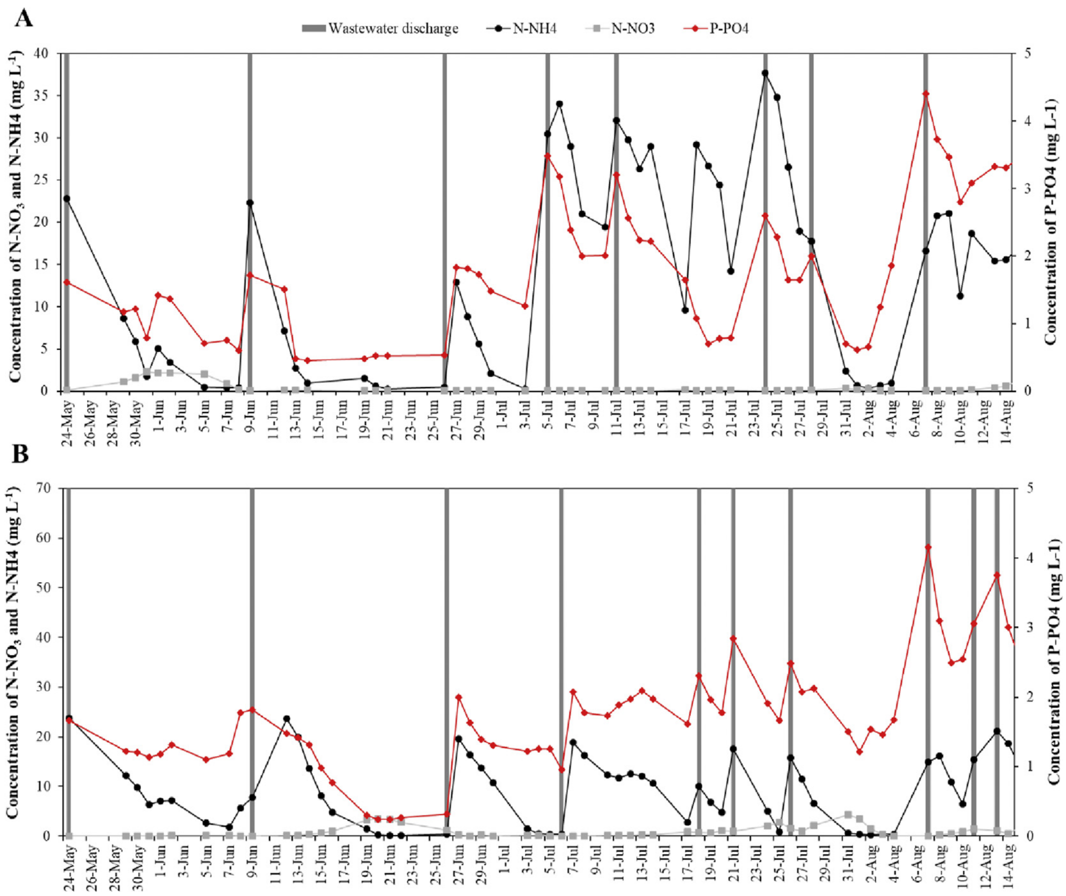

Fig. 3. Dates of wastewater influents addition and concentrations (mg/L) of phosphate ($P\text{-}PO_4^{3-}$), ammonium ($N{-}NH_4^+$) and nitrate ($N{-}NO_3$) in pond 1 (A) and 2 (B) during the monitoring period.

Table 2
The 3 multi-day periods that were selected as test cases and the corresponding values of the five model parameters as they were estimated using this procedure.

Pond	Start time	Period length	$\mu_{A,max}$ (1/day)	ρ_A (1/day)	$Q_{N,min}$ (mg/mg$_{DW}$)	$Q_{N,max}$ (mg/mg$_{DW}$)	$k_{N,max}$ (mg/mg$_{DW}$/day)
1	Jun 10, 00:00	6 days	1.34 ± 0.09	0.101 ± 0.027	0.0309 ± 0.0036	0.0784 ± 0.0015	0.176 ± 0.042
1	Jun 27, 00:00	7 days	1.19 ± 0.07	0.127 ± 0.016	0.0293 ± 0.0022	0.0778 ± 0.0013	0.226 ± 0.047
2	Jun 28, 00:00	8 days	1.48 ± 0.14	0.203 ± 0.027	0.0284 ± 0.0113	0.0760 ± 0.0035	0.208 ± 0.046

measurements of N_e.

The values estimated for the unknown algae properties were all reasonable and well within the ranges that can be found in the literature. Q_{max} had the narrowest confidence interval in relative terms and showed that the maximum quota of intracellular nitrogen storage was between 7.5 and 8%, on average, for the polyculture. Conversely, Q_{min} was around 3% but with larger confidence intervals in relative terms. The theoretical growth rate $\mu_{A,max}$, which was estimated with fair accuracy, showed somewhat different values in the three simulated periods, and this might depend on the presence of different shares of algal species in the polyculture in different periods (e.g. due to contamination, see Section 3.1). The two parameters that were estimated with the lowest accuracy were the maximum nitrogen uptake rate $k_{N,max,}$ and algal decay rate ρ_A. The first ($k_{N,max}$) had larger confidence intervals in relative terms because it appeared as a factor in a product with another unknown algal parameter in the differential equation describing nitrogen uptake. The differences in the estimated values were not large when the confidence intervals were considered, thus, algal species in the polyculture may not significantly affect $k_{N,max}$. The second (ρ_A) had a fundamental influence on the day/night cycles of both growth and nutrient uptake, and its larger confidence intervals may be related to the lesser ability of the model to capture the day/night cycle of the nutrient uptake, as mentioned above. The estimated values of ρ_A were significantly different, which may be explained by the different shares of algal species in the polyculture.

4. Implications

This study shows that it is not necessary to inoculate with a single strain because a natural polyculture better adapted to the local environmental conditions will take over and will outcompete the inoculated single strain. Moreover, the polyculture becomes stable over time as indicated by the fact that pond 1 inoculated with a polyculture performed better than pond 2 where the polyculture appeared during the season after it has outcompeted the inoculated strain. We consider that the interaction between bacteria and microalgae plays a pivotal role in the wastewater treatment process.

Future directions for developing the model and improving its

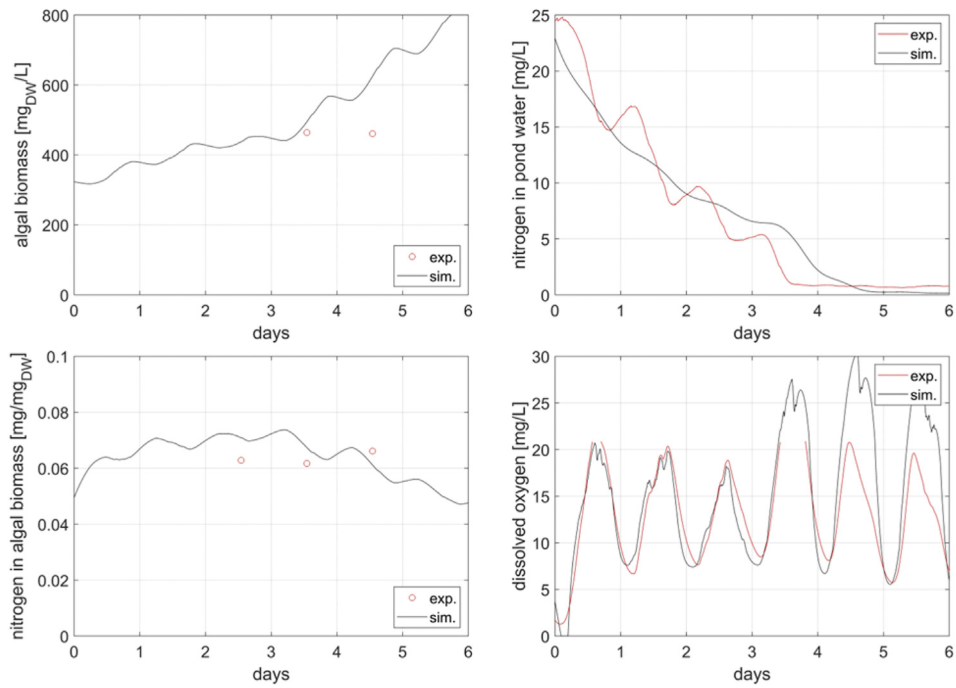

Fig. 4. Comparisons between experimental and simulated data of pond 1 (6 days starting from 10 June).

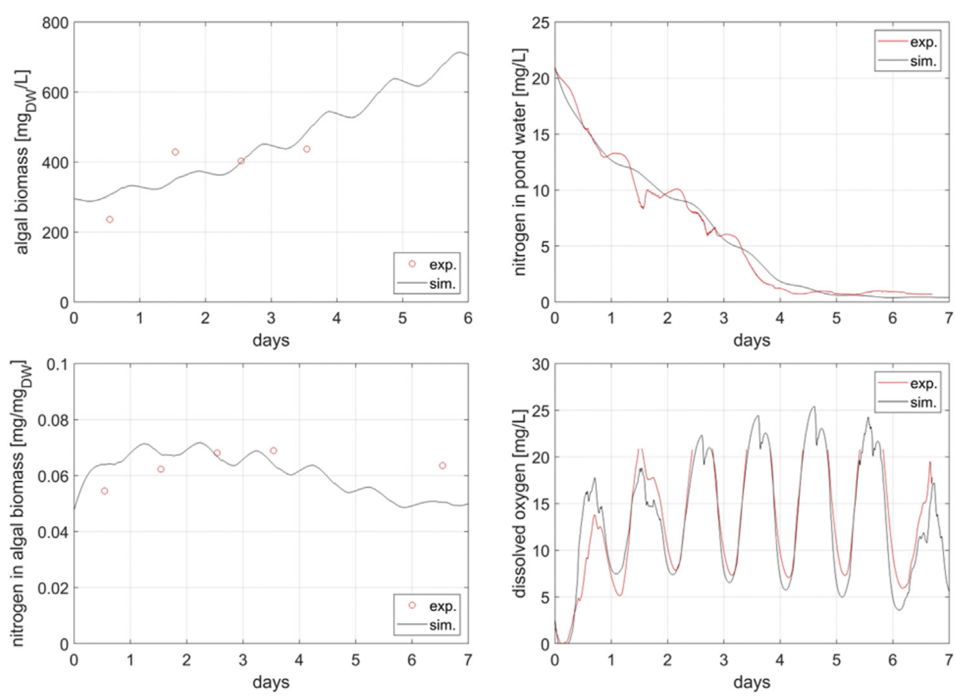

Fig. 5. Comparisons between experimental and simulated data of pond 1 (7 days starting from 27 June).

accuracy involve actions on both the numerical side (e.g. including equations that consider the presence of bacteria in the pond, or another aspect about microalgae composition, such as neutral lipid content) and the experimental side (e.g. increasing the frequency of sampling pond water to measure the concentration of both microalgae and bacteria, the nitrogen and neutral lipid content of the biomass), to have both more quantities to be validated and more data to support the validation.

5. Conclusions

A stable polyculture of microalgae was observed over the sampling period in the pond inoculated with the local microalgae consortium (pond 1) but not in the pond inoculated with *C. vulgaris* monoculture (pond 2). Pond 1 had significantly higher biomass concentrations and P-PO_4^{3-} removals than pond 2. Thus, inoculation of outdoor ponds with a local microalgae consortium has the potential to produce steady and high biomass concentrations and

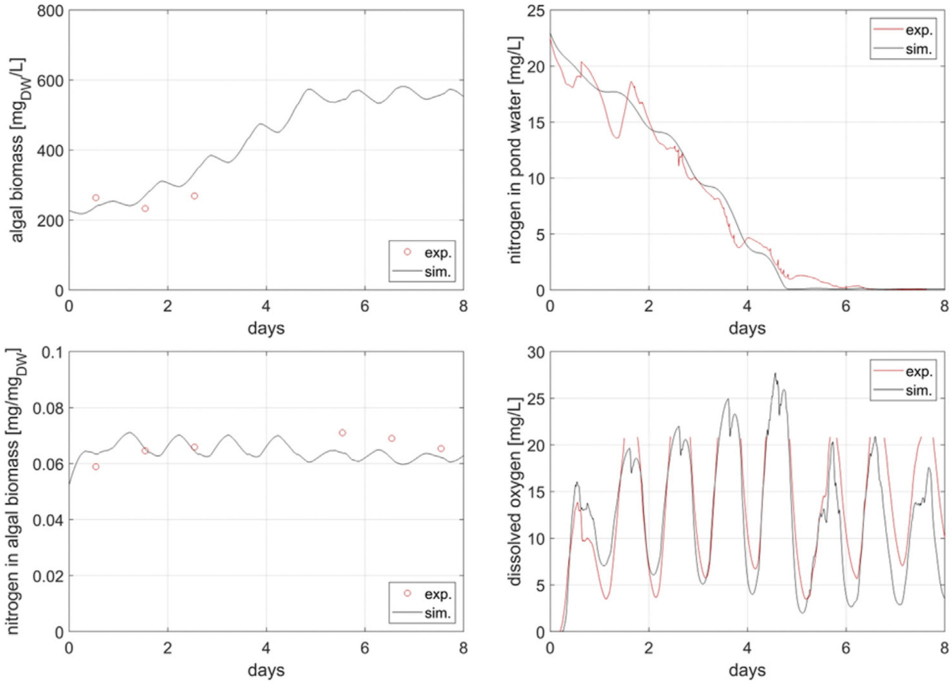

Fig. 6. Comparisons between experimental and simulated data of pond 2 (8 days starting from 28 June).

nutrient removals throughout the season. While inoculation with a monoculture (pond 2) can lead to high nutrient removal but with more variability throughout the season and significantly lower biomass concentrations. The taxonomic identification showed that the inoculated strain after colonizing the pond disappeared after few weeks outcompeted by better adapted wild microalgal strains. Future studies that follow the polyculture variation during several growing seasons are needed to investigate polyculture composition/stability over time.

In this study, we presented a comprehensive mathematical model for understanding the dynamics of microalgae growth, nitrogen uptake/storage, and oxygen generation in the outdoor ponds. The model was validated with experimental data and simulated with fairly good accuracy specific attributes of microalgal behaviour in complex growth conditions. This model has a potential application for gaining insights into the microalgae growth dynamics and accomplishing optimal system performance. For instance, this model allowed us to predict when to harvest the microalgae biomass depending on its future use, e.g., with a high level of N for the use as biofertilizer or the lowest level of N for use as biofuel. Thus, this mathematical formulation represents an important tool for improving the profitability and sustainability of microalgae-based wastewater treatment and CO_2 sequestration.

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.

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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.2021.130122.

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Author contributions

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

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