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Kinetic Modelling of Starch and Lipid Formation during Mixotrophic, Nutrient-limited Microalgal Growth

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ABSTRACT

Microalgal starch and lipids, carbon-based storage molecules, are useful as potential biofuel feedstocks. In this work, cultivation strategies maximising starch and lipid formation were established by developing a multi-parameter kinetic model describing microalgal growth as well as starch and lipid formation, in conjunction with laboratory-scale experiments. Growth dynamics are driven by nitrogen-limited mixotrophic conditions, known to increase cellular starch and lipid contents whilst enhancing biomass growth. Model parameters were computed by fitting model outputs to a range of experimental datasets from batch cultures of \textit{Chlamydomonas reinhardtii}. Predictive capabilities of the model were established against different experimental data. The model was subsequently used to compute optimal nutrient-based cultivation strategies in terms of initial nitrogen and carbon concentrations. Model-based optimal strategies yielded a significant increase of 261\% for starch (0.065 gC L\textsuperscript{-1}) and 66\% for lipid (0.08 gC L\textsuperscript{-1}) production compared to base-case conditions (0.018 gC L\textsuperscript{-1} starch, 0.048 gC L\textsuperscript{-1} lipids).

**Keywords:** Biofuels, microalgal dynamics, kinetic modelling, starch and lipids optimisation, \textit{Chlamydomonas reinhardtii}
1. Introduction.

Our current dependence on fossil fuels raises two major concerns: the overexploitation of finite crude oil resources and the associated emissions of greenhouse gases (GHG) leading to global warming (Scaife et al., 2015). About 60% of the fossil fuels directed annually towards primary energy consumption are taken up by the transportation sector (Escobar et al., 2009). Although biofuels have emerged as a suitable and renewable replacement for transport-associated fuels such as gasoline and diesel, sustainable and cost-effective biofuel production systems must first be developed (Escobar et al., 2009; Scaife et al., 2015).

Due to exhibiting faster growth rates than terrestrial plants, microalgal biomass has recently been considered as a potential biofuel feedstock (Brennan and Owende, 2010), as opposed to traditional food-based or lignocellulosic substrates which compete for food or arable land (Scaife et al., 2015). Microalgae are photosynthetic organisms which synthesize biologically important compounds such as carbohydrates, lipids, proteins and nucleic acids, acting as storage or functional elements (Brennan and Owende, 2010; Choix et al., 2012). In particular, their internal pool of carbohydrates and lipids has directed the attention towards the use of microalgae as a renewable feedstock for sugar and lipid-based fuels.

In green microalgae, the main storage carbohydrate synthesized by cells is starch, which is located within the chloroplast in the form of granules (Choix et al., 2012; Markou et al., 2012). Meanwhile, oil bodies are found in the cytosol and chloroplast (Ball and Deschamps, 2009; Goodson et al., 2011). Cellular contents of starch and lipids (triacylglycerol, TAG) have been shown to increase under nitrogen-starved conditions (Bajhaiya et al., 2016). Nevertheless, enhanced starch and lipid accumulation rates
come hand-in-hand with a decrease in biomass growth (Markou et al., 2012), suggesting the need to identify an optimal balance between microalgal growth and starch and lipid accumulation.

Microalgal cells can be phototrophic or heterotrophic depending on whether their carbon fixation route requires the presence of an inorganic or an organic carbon source (Brennan and Owende, 2010). Some strains are able to grow mixotrophically by utilizing both inorganic and organic carbon sources. In most cases, mixotrophic cultivation leads to improved growth rates against standard phototrophic conditions (Chapman et al., 2015; Johnson and Alric, 2013), causing substantial increases in biomass productivities (Moon et al., 2013). Consequently, quantifying starch and lipid accumulation during nitrogen-limited mixotrophic growth is of great relevance for microalgae-based biofuels.

*Chlamydomonas reinhardtii*, the chosen microalgal strain in this work, has been widely studied (e.g. Goodenough et al., 2014; Bajhaiya et al., 2016). Its carbon metabolism is well known (Johnson and Alric, 2013), making it suitable for the analysis of the carbon assimilation and its distribution between starch and lipid reserves. In particular, a clear increase in starch and lipid accumulation for mixotrophic growth under nutrient stress has been observed for this strain (Bajhaiya et al., 2016; Bekirogullari et al., 2017).

Nevertheless, despite nutrient stress being regarded as a simple and cost-effective strategy to enhance starch and lipid formation, optimisation of the process is required for viable large-scale cultivation.

Robust kinetic models capable of simultaneously predicting starch and lipid formation can significantly aid in the establishment of optimal cultivation strategies. Models that
take into account the structured and segregated (i.e. each cell behaves as an individual unit with dynamic composition) nature of cells, and/or even the stochastic nature of cell growth (Alonso et al., 2014) can realistically predict the formation of multiple intracellular components as well as cells’ response to cultivation conditions. Such models can provide useful insights about the algal metabolic networks and intracellular fluxes (Chapman et al., 2015; Rügen et al., 2012), but are usually highly complex and computationally expensive (Shuler and Kargi, 1992). Unstructured non-segregated models, on the other hand, have a simpler formulation (assume all cells in culture are identical), but have been shown to be applicable to practical algal cultivation systems.

Most of the existing unstructured models for microalgae, however, have focused solely on the simulation of lipid production (Bekirogullari et al., 2017; Packer et al., 2011). These models assume that lipid formation is a consequence of excess carbon (between the amount fixated and the amount required for cell growth) directed towards synthesis of lipids rather than other carbon-based elements, such as cellular organelles or proteins. Although this assumption is in agreement with the carbon pathways of microalgae, any excess of assimilated carbon is also directed towards formation of starch reserves (Johnson and Alric, 2013). Models for nutrient-limited algal growth considering both sugar and lipids dynamics have been proposed by Mairet et al., 2011b and Kumar et al., 2016, but with a focus on lipid production during phototrophic growth. Mixotrophic dynamics were proposed by Adesanya et al., 2014, but encompassing sugar and lipids into one single storage molecule, preventing the identification of each individual profile.

Thus, the aim of this work is to develop a predictive multi-parameter model for the simultaneous optimization of starch and lipid formation during nitrogen-limited mixotrophic microalgal growth. Our proposed model couples both carbon (C) and
nitrogen (N) substrates. The model is fitted and validated against datasets obtained from lab-scale culture experiments under different N and C regimes. The validated model can then be used with confidence for the identification of optimal cultivation conditions for maximum starch and lipid production.


2.1. Strain and cultivation.

All experiments were carried out with the wild-type strain *C. reinhardtii* CCAP 11/32C, obtained from the Culture Collection of Algae and Protozoa, UK. The strain was maintained under batch mixotrophic conditions in Tris-Acetate-Phosphate (TAP) medium (Harris, 1989) at a temperature of 25°C. Prior to lab-scale experimentation, an initial algal inoculum was propagated in 150 mL of TAP medium up to the late exponential phase (5-7 days). This inoculum was placed in an orbital shaker at 150 rpm and constant illumination of 125 µmol m⁻² s⁻¹ (from above) in a light/dark cycle of 16/8 hours. All further lab-scale experimental tests were carried out at the same environmental growth conditions in vessels containing 500 mL of sterile culture medium and 1 mL of algal inoculum.

2.2. Lab-scale culture experiments.

In order to evaluate nitrogen and carbon effects on microalgal growth as well as starch and lipid accumulation, lab-scale cultures of *C. reinhardtii* were grown under different nitrogen and acetate concentrations. Given that TAP medium is the most routinely used growth medium for this strain and allows results to be compared with other studies, a “control” culture was grown in TAP medium, in which $N_o = 0.3824 \text{ gN L}^{-1}$ and $A_o = 0.42 \text{ gC L}^{-1}$. Subsequently, and whilst keeping constant the concentration of all the
remaining TAP components, acetate-dependent cultures were grown in: $A_0 = 0.21 \text{ gC L}^{-1}$ (A-), 0.75 gC L$^{-1}$ (A+), 1.26 gC L$^{-1}$ (A++), and 2.52 gC L$^{-1}$ (High A). Similarly, nitrogen-dependent cultures were grown in: $N_0 = 0.3350 \text{ gN L}^{-1}$ (N--), 0.3568 gN L$^{-1}$ (N-), and 0.7430 gN L$^{-1}$ (High N). Two additional cultures were grown by simultaneously changing the initial concentrations of nitrogen and acetate: one in 2.52 gC L$^{-1}$ and 0.7430 gN L$^{-1}$ (High A-N), and another in 1.16 gC L$^{-1}$ and 0.3151 gN L$^{-1}$ (A'-N'). Samples were taken daily during the cultivation period, until cells attained the stationary phase after 8 days. Sufficient identical culture vessels were prepared to allow for duplicate samples to be fully harvested (sacrificed) at each sampling time. For the nitrogen-dependent cultures, media was prepared by modifying exclusively the initial concentration of ammonium chloride (NH$_4$Cl) in the TAP medium, which contains two other nitrogen sources: i) Tris-base buffer (H$_2$NC(CH$_2$OH)$_3$), and ii) ammonium molybdate tetrahydrate, a smaller trace element ($\left(\text{NH}_4\right)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$). The total concentration of these two nitrogen sources amounted to 0.2844 gN L$^{-1}$ and was kept constant in all experiments. For acetate-dependent cultures, media was prepared simply by increasing or decreasing accordingly the volume of acetic acid. When necessary, the pH was adjusted to a starting value of 7, using potassium hydroxide (KOH) 3M or hydrochloric acid (HCl) 3M. Experimental data was statistically analysed by the two-way ANOVA test in GraphPad Prism 7 (version 7.02).

2.3. Analytical Methods.

2.3.1. Cell growth.

Microalgal growth was measured in terms of dry cell weight (DCW), quantified by harvesting all 500 mL cultures for 3.5 min at 3,000 g in an Eppendorf Centrifuge 5424. The residual pellet was separated from the supernatant and allowed to dry for 24 hr at
70°C. The DCW was then measured gravimetrically in an analytical balance (Sartorius M-Pact AX124, Germany). Samples of the supernatant were stored in 50 mL Falcon tubes and frozen at -20°C for further analysis of the acetate and nitrogen concentrations.

2.3.2. Starch and lipid quantification.

The starch content of cells was quantified according to a Total Starch Assay kit (Megazyme International, Ireland). Briefly, this assay consists of high temperature, two-stage ($\alpha$-amylase and $\beta$-amyloglucosidase) enzymatic hydrolysis which solubilises starch and releases free D-glucose. The concentration of free D-glucose was determined colourimetrically by measuring sample absorbance values at 508 nm against a D-glucose standard curve. Total starch concentration was then calculated by multiplying D-glucose concentration by 0.9 (162/180, a factor adjusting free D-glucose to anhydrous D-glucose). Quantification of the lipid content was determined by solvent extraction in a SOXTEC Unit 1043 over a triple-stage procedure involving: extraction, rinsing, and solvent recovery (Bekirogullari et al., 2017). Hexane (ACS spectrophotometric grade, $\geq$ 98.5 %, Sigma Aldrich, UK) was used as extracting solvent since it has shown to perform well as an extracting agent of neutral lipids (TAGs) induced under nitrogen-deprived conditions (McNichol et al., 2012). Prior to extraction, dried cell pellets were pulverised by a double-cycle of liquid nitrogen immersion and manual grinding with mortar and pestle. Pulverized cells were then placed in cellulose extraction thimbles (26 x 60 mm, thickness 1.5 mm, Whatman, UK) and positioned in the SOXTEC unit. Extracted lipids were then measured gravimetrically. Starch and lipid concentration is reported in volumetric terms (g L$^{-1}$), calculated by relating the storage content (%) of each sample with the corresponding total DCW medium concentration.
2.3.3. Acetate concentration.

The residual acetate concentration was measured by High Pressure Liquid Chromatography (HPLC) in a Hi-Plex 8 µm 300x7.7mm column using sulphuric acid (H$_2$SO$_4$) 5 mM as mobile phase at a flow rate of 0.6 mL min$^{-1}$ and a temperature of 50 °C. Acetate was identified by a UV detector at a wavelength of 210 nm. Prior to analysis, all supernatant and calibration samples were filtered in 0.45 µm nitrocellulose membranes (Millipore Ltd.) and diluted appropriately in HPLC grade water.

2.3.4. Total nitrogen and nitrogen quota.

The residual concentration of total nitrogen was measured in a Total Organic Carbon/Total Nitrogen measuring unit (TOC-VCSH/TNM-1 Shimadzu). Prior to analysis, calibration standards were prepared with ammonium chloride as the sole nitrogen source. All experimental samples were diluted appropriately in distilled water. The nitrogen quota, $q_N$, at each sampling time point was calculated according to Eq. (1), which is equivalent to the one employed by Bougaran, Bernard, & Sciandra (2010) to quantify phosphorus cell quotas:

$$q_N = \frac{N_o - N}{X}$$  \hspace{1cm} (1)

Here $N_o$ is the initial concentration of total nitrogen in the medium, and $N$ and $X$ are the residual concentrations of total nitrogen and biomass (DCW), respectively.

2.3.5. Active biomass and carbon equivalent concentrations.

The active biomass (or starch- and lipid-free biomass) concentration was determined by subtracting the concentration of storage molecules from the total biomass (DCW)
concentration. The elemental composition of the active biomass fraction was assumed to be constant in all experiments regardless of the nutrient regime in which the cultures were grown, taken as CH$_{1.75}$O$_{0.56}$N$_{0.08}$, reported for *C. reinhardtii* by Eriksen et al. (2007). In all computations, the concentration of each carbon-based compound was expressed in terms of their specific carbon content, for which the following conversion factors were employed (gC g$^{-1}$): 0.444 for starch, 0.77 for lipids (C$_{55}$H$_{98}$O$_{6}$), 0.40 for acetate, and 0.504 for the active biomass fraction.

### 3. Model construction.

A multi-parameter kinetic model was developed to predict *C. reinhardtii* growth and formation of starch and lipid under mixotrophic conditions. The model includes 8 state variables: total biomass ($X$, gC L$^{-1}$), total nitrogen ($N$, gN L$^{-1}$), nitrogen quota ($q_{N}$, gN gC$^{-1}$), acetate ($A$, gC L$^{-1}$), starch ($S$, gC L$^{-1}$), lipids ($L$, gC L$^{-1}$), active biomass ($x^*$, gC L$^{-1}$), and pH ($H$). Total biomass is equivalent to the sum of the two major carbon-based compartments: the storage pool made up of starch and lipids, and the active biomass. Microalgal growth and the formation of each cellular component are regulated by the flows shown in Figure 6.

Carbon flows were based on those presented by Mairet et al. (2011) for the microalgal strain *Isochrysis aff. Galbana*, in which the carbon source was assumed to be directed initially towards sugar synthesis. In the present model carbon assimilation is initially directed towards the formation of active biomass so as to follow more closely the central carbon metabolism of *C. reinhardtii*. A detailed diagrammatic representation of this metabolism is provided by Johnson and Alric (2013), which shows that assimilation of acetate is not only used for starch formation, but also for other important functions.
such as cellular respiration, flagellar motion, and formation of acetyl-CoA, a precursor
of numerous biochemical reactions.

The cellular flows for carbon assimilation as well as for nitrogen uptake, as depicted in
Figure 6, are regulated by six governing equations: the specific growth rate, $\mu$, the
nitrogen uptake rate, $\rho_N$, and the intracellular reaction rates $R_1, R_2, R_3,$ and $R_4$. These
equations are described below. All definitions and corresponding units of the kinetic
parameters used in the model are listed in Table 1.

3.1. Specific growth rate, $\mu$

One of the most widespread equations for microalgal growth is the Droop model
(Eq. (2)), where the growth rate, $\mu$, is linked to the internal quota, $q$, of a limiting
nutrient ($q_N$, for nitrogen-limited growth) rather than to its external concentration.

$$\mu = \mu_{\text{max}} \cdot \left(1 - \frac{q_{N,0}}{q_N}\right)$$

In Eq. (2), $\mu_{\text{max}}$ is the hypothetical maximum growth rate and $q_{N,0}$ is the minimum
nitrogen quota required for growth (Droop, 1968). This simple yet effective model has
been used successfully to predict microalgae growth with additional terms accounting
for multiple-nutrient limitation or the self-shading effects observed at high cell densities
(Adesanya et al., 2014; Bernard, 2011; Bougaran et al., 2010; Mairet et al., 2011b;
Packer et al., 2011). Microalgal cells can be autotrophic, heterotrophic, or mixotrophic,
but most kinetic models describe solely autotrophic or heterotrophic growth. Adesanya
et al. (2014) described the kinetics of a mixotrophically growing culture by expressing
the hypothetical maximum growth rate as the sum of the autotrophic and heterotrophic
growth rates. This approach was adapted into the present model with the inclusion of
weighting functions controlling the extent of each rate on overall growth. The specific growth rate was thus expressed as:

$$\mu = \bar{\mu}_{M,max}(A, I) \cdot \left(1 - \frac{q_{N,0}}{q_N}\right)$$  \hspace{1cm} (3)

Here $\bar{\mu}_{M,max}(A, I)$ is the maximum specific growth rate under mixotrophic conditions, as shown in Eq. (4), and is proportional to the sum of the heterotrophic and phototrophic rates, $\mu_H(A)$ and $\mu_I(I)$, respectively.

$$\bar{\mu}_{M,max}(A, I) = \mu_{max} \cdot [w_H \cdot \mu_H(A) + w_I \cdot \mu_I(I)]$$  \hspace{1cm} (4)

In order to account for photoinhibition and substrate inhibition, $\mu_H(A)$ and $\mu_I(I)$ were expressed as Andrews functions (Andrews, 1968) as shown in Eq. (5):

$$\bar{\mu}_{M,max}(A, I) = \mu_{max} \cdot \left[w_H \cdot \frac{A}{A + K_{s,A} + A^2/k_{i,A}} + w_I \cdot \frac{I}{I + K_{s,I} + I^2/k_{i,I}}\right]$$  \hspace{1cm} (5)

Here, $K_{s,A}$ and $k_{i,A}$ are the acetate-associated saturation and inhibition constants for growth, whereas $K_{s,I}$ and $k_{i,I}$ are the light-associated saturation and inhibition constants, respectively. The weighting functions, $w_H$ and $w_I$, shown in Eq. (6), were defined in terms of the saturation constants in a similar fashion to those presented in Shuler and Kargi (1992):

$$w_H = \frac{A/K_{s,A}}{A/K_{s,A} + I/K_{s,I}}; \quad w_I = \frac{I/K_{s,I}}{A/K_{s,A} + I/K_{s,I}}$$  \hspace{1cm} (6)

Light distribution $(I)$ throughout the culture vessel was represented by the Beer-Lambert law shown in Eq. (7), where $I_0$ (\(\mu\)mol m$^{-2}$s$^{-1}$) is the incident light intensity, $\sigma$ is the light attenuation coefficient, and $z$ (m) is the culture depth within the vessel.
\[ I = I_0 \cdot e^{-\sigma X z} \quad (7) \]

3.2. Nitrogen uptake rate, \( \rho_N \)

The expression for nitrogen uptake rate is a crucial element of the model, since nitrogen entering the cells is directly linked to the nitrogen quota, which regulates cell growth (Eq. (3)). The uptake rate of nitrogen, shown in Eq.(8), was expressed as Andrews-type kinetics to account for the growth inhibition of \textit{C. reinhardtii} observed at high external nitrogen concentrations. Since analogous observations were made in the cultures subject to high acetate treatments, acetate inhibition was similarly considered.

\[
\rho_N = \bar{\rho}_{N, \text{max}}(N_0, X) \cdot \frac{N}{N + K_{s,N} + N^2 / K_{i,N}} \cdot \frac{A}{A + K_{s,A:N} + A^2 / K_{i,A:N}} \quad (8)
\]

Here, \( K_{s,N} \) and \( k_{i,N} \) are the nitrogen-associated saturation and inhibition constants for nitrogen uptake. Similarly, \( K_{s,A:N} \) and \( k_{i,A:N} \) are the acetate-associated saturation and inhibition constants for nitrogen uptake. The maximum nitrogen uptake rate, \( \bar{\rho}_{N, \text{max}}(N_0, X) \), depends on the initial nitrogen concentration (\( N_0 \)) under which cultures were grown and on the current biomass concentration (\( X \)):

\[
\bar{\rho}_{N, \text{max}}(N_0, X) = \rho_{N, \text{max}} \cdot \frac{N_0^n}{N_0^n + K^n} \cdot e^{-\phi N X} \quad (9)
\]

The maximum nitrogen uptake rate was built under the concept of “luxury consumption”, used to describe the abrupt uptake of a nutrient from the cultivation medium (Droop, 1983). \( k \) is a saturation constant, \( n \) is a shape-controlling parameter, and \( \phi \) is a regulation coefficient. A detailed explanation is included in section 4.2.

3.3. Rates of formation of cellular compartments (\( R_1, R_2, R_3, R_4 \)).
The synthesis rates for starch and lipids, \( R_1 \) and \( R_3 \), respectively, were assumed to be controlled by the specific growth rate and the active biomass, as shown in Eq. (10) and Eq. (11). Both sets of reactions include: i) an Andrews-type term dependent on the internal nitrogen concentration, \( N_i = q_N X \), and ii) an exponential term dependent on the internal carbon concentration, \( A_{int} = A_{o} + A \). The exponential term accounts for the higher formation of storage molecules in the cultures grown in high acetate media, which was observed to take place even after biomass had reached stationary phase (Figure 8). This increase was thus assumed to be uncoupled from cellular growth and only a consequence of excess in the internal carbon pool.

\[
R_1 = r_1 \cdot \frac{N_i^n}{N_i^n + K_{s,s}^{n_s} + (N_i^{n_s}/k_{l,s})^{n_s}} \cdot \frac{k_1}{k_1 + N/N_0} \cdot \left[ 1 + \frac{1}{\mu} \cdot e^{\Phi_S A_{int}} \right] \cdot \mu \cdot X^* 
\]

\[
R_3 = r_3 \cdot \frac{N_i^n}{N_i^n + K_{s,l}^{n_l} + (N_i^{n_l}/k_{l,l})^{n_l}} \cdot \frac{k_2}{k_2 + N/N_0} \cdot \left[ 1 + \frac{1}{\mu} \cdot e^{\Phi_L A_{int}} \right] \cdot \mu \cdot X^* 
\]

Here, \( K_{s,s} \) and \( K_{s,l} \) are saturation constants, \( k_{i,s} \) and \( k_{i,l} \) are inhibition constants, \( n_s \) and \( n_L \) are shape-controlling exponents, \( \Phi_S \) and \( \Phi_L \) are regulation coefficients, and \( k_1 \) and \( k_2 \) regulate synthesis rates with respect to nitrogen consumption. Active biomass formation was also linked with starch and lipid degradation (\( R_2 \) and \( R_4 \)). These rates, shown in Eq. (12), were defined as functions of the nitrogen quota since cellular components such as proteins or nucleic acids, depend on nitrogen availability.

\[
R_2 = \frac{r_2}{q_N} \cdot X; \quad R_4 = \frac{r_4}{q_N} \cdot X
\]

### 3.4. Time-dependent kinetic expressions.
The dynamics for active biomass ($x^*$), starch ($S$), lipids ($L$), and total biomass ($X$), were obtained from the corresponding mass conservation equations as follows:

$$\frac{dx^*}{dt} = \mu \cdot X + R_2 + R_4 - (R_1 + R_3) \quad (13)$$

$$\frac{dS}{dt} = R_1 - R_2 \quad (14)$$

$$\frac{dL}{dt} = R_3 - R_4 \quad (15)$$

Total biomass (i.e. $X = x^* + S + L$) conservation simplifies thus to:

$$\frac{dX}{dt} = \mu \cdot X \quad (16)$$

Acetate consumption was expressed by means of the acetate to biomass yield coefficient, $Y_{X/A}$, multiplied by a time-varying fraction accounting for the carbon used heterotrophically:

$$\frac{dA}{dt} = -\frac{1}{Y_{X/A}} \cdot \frac{\mu_H(A)}{\mu_H(A) + \mu_I(I)} \cdot \frac{dX}{dt} \quad (17)$$

By considering that the removal of $H^+$ ions from the medium is a direct consequence of microalgal growth (e.g. acetate consumption), pH evolution was represented as:

$$\frac{dH}{dt} = K_H \cdot \frac{dx^*}{dt} \quad (18)$$

Here, $K_H$ is a pH coefficient. The rate of nitrogen consumption was expressed as:
$$\frac{dN}{dt} = -\rho_N \cdot X$$

(19)

Differentiation of the nitrogen quota with respect to time yields:

$$\frac{dq_N}{dt} = \frac{d(N_i/X)}{dt} = \frac{d(N_i/X) \cdot dx}{X^2} = \frac{1}{X} \cdot \frac{dN_i}{dt} - \frac{N_i}{X} \cdot \left(1 \cdot \frac{dX}{dt}\right)$$

(20)

where \(N_i\) is the internal nitrogen concentration, and its accumulation rate is given by:

$$\frac{dN_i}{dt} = -\frac{dN}{dt} = \rho_N \cdot X$$

(21)

By substituting Eq. (16) and Eq. (21) in Eq. (20), the time-dependent equation for the nitrogen quota simplifies thus to:

$$\frac{dq_N}{dt} = \rho_N - \mu \cdot q_N$$

(22)

3.5. Parameter estimation.

The proposed model, given by Eq.(13) - Eq.(19) and Eq.(22), consists of 8 state variables and 31 kinetic parameters (Table 1). Sensitivity analysis was carried out by estimating sensitivities (gradients of each state variable with respect to each of the parameters) numerically using central finite differences for a 10% change in each parameter. The results can be found in the Supplementary material. We noticed that sensitivities above value of 0.02 denoted that the corresponding variable was sensitive to changes in the parameter. Through this sensitivity analysis, 4 parameters were deemed insensitive, \(\sigma\), \(K_{s,b}\), \(K_{s,S}\) and \(\Phi_L\). \(K_{s,S}\) and \(\Phi_L\) were neglected from the final model as it was noticed that setting them to zero did not affect results. \(K_{s,l}\) was set to 1.4 as in
the literature (Mairet et al., 2011b) and $\sigma$ was set equal to 1. Hence, Eq. (10) and (11) become:

$$R_1 = r_1 \cdot \frac{N_i^{ns}}{N_i^{ns} + (N_i^2/k_{i,L})^{ns}} \cdot \frac{k_1}{k_1 + N/N_0} \cdot \left[ 1 + \frac{1}{\mu} \cdot e^{\Phi_s A_{int}} \right] \cdot \mu \cdot x^*$$

(23)

$$R_3 = r_3 \cdot \frac{N_i^{nl}}{N_i^{nl} + K_{s,L}^{nl} + (N_i^2/k_{i,L})^{nl}} \cdot \frac{k_2}{k_2 + N/N_0} \cdot [\mu + 1] \cdot x^*$$

(24)

Estimation of the remaining 27 kinetic parameters was carried out by minimizing an objective function defined as the sum of the squared relative error between the model predictions and the experimental data, as shown in (Vlysidis et al., 2011):

$$\min G(P) = \sum_{h=1}^{nh} \sum_{i=1}^{ni} \sum_{k=1}^{nk} \left( \frac{Z_{hik}^{pred}(P) - Z_{hik}^{Exp}}{Z_{hik}^{Exp}} \right)^2$$

(25)

where $G(P)$ is the objective function dependent on a vector $P$ containing all kinetic parameters and $Z$ is a vector containing all state variables, $nk$ is the number of experimental datasets used for parameter fitting, $ni$ is the number of state variables ($ni = 8$), and $nh$ is the number of data points in time ($nh = 7$). Minimization of the objective function was performed by first employing Simulated Annealing (SA), a stochastic optimisation algorithm which is capable of approximating the solution set around a global minimum. Then, using the solution obtained by SA as initial guess, a refined and final solution set of parameters were computed by using Successive Quadratic Programming (SQP) (Vlysidis et al., 2011). Both techniques were coded in-house and implemented in MatLab®.

The value of each kinetic parameter was restricted to specified bounds according to data found in literature (for those cases in which available data existed) or relevant
experimental analysis. Initial values for the model ODEs were equal to those implemented in each of the five experimental datasets used for parameter fitting ($nk = 5$): TAP, N-, A++, High N, and High A-N. These datasets were selected so as to cover scenarios representative of the cultivation stage under both low and high concentrations of nitrogen and acetate. The remaining datasets (N-, A+, and A'-N') were used for model validation.

4. Results and discussion.

4.1. Effect of nitrogen and acetate in biomass, starch, and lipid formation.

All of the cultures analysed under the conditions established in section 2.2 reached early stationary phase after 150 h, but cultures were allowed to grow for a further period of 48 h to ensure they had all reached stationary phase and were accumulating carbon storage products. Experimental results for biomass growth as well as for starch and lipid formation are shown in Figure 7, which are representative of the cultures during the stationary stage (192 h).

Results showed (Figure 7.b) that the cellular contents of starch and lipids increased significantly ($p < 0.0001$, two-way ANOVA) as the initial nitrogen concentration in the culture medium was reduced from 0.3824 gN L$^{-1}$ (TAP), to both 0.3568 gN L$^{-1}$ and 0.335 gN L$^{-1}$. Specifically, starch concentration increased from 6% (at $N_o$=0.3824 gN L$^{-1}$) to 17% (at $N_o$=0.3350 gN L$^{-1}$), whereas lipid increased from 14% to 21%, respectively. This enhanced accumulation observed under nitrogen limitation is in agreement with previous analysis of C. reinhardtii (Bajhaiya et al., 2016) and with findings reported for other microalgae strains, such as Chlorella vulgaris P12 (Brányiková et al., 2010) or Tetraselmis subcordiformis (Yao et al., 2012). However,
this increase is at the expense of biomass growth (Figure 7.a), which was observed to decrease significantly under nitrogen-limited conditions (p = 0.0006 between 0.3824 gN L$^{-1}$ and 0.335 gN L$^{-1}$, two-way ANOVA). The magnitude of this negative trade-off in biomass growth ultimately controls starch and lipid formation in terms of volumetric yields, and should be considered in any nutrient-based cultivation strategy.

As per the ANOVA test, increases in starch and lipid contents in the culture grown at a high nitrogen concentration ($N_o$=0.7426 gN L$^{-1}$) were not statistically significant with respect to the culture grown under standard TAP concentrations. However, biomass concentration decreased significantly (p < 0.0001, two-way ANOVA), indicating that a high nitrogen concentration inhibited biomass growth. Nitrogen has been widely reported as a limiting nutrient suitable for increased accumulation of lipid (Cakmak et al., 2012; Rodolfi et al., 2009; Xin et al., 2010) and carbohydrate (Behrens et al., 1989; Dragone et al., 2011). Nitrogen is a vital component of important biomolecules like proteins and DNA, and it is estimated to represent 7-20% of the cellular mass. When cells are exposed to a nitrogen depleted environment, the protein synthesis pathway is negatively affected, which results in the carbon fixation mechanism being instead redirected towards the production of carbohydrates or lipids (Juneja et al., 2013; Markou et al., 2012).

In addition, experimental results (Figure 7.c) showed that when compared to the culture grown in TAP ($A_o$=0.42 gC L$^{-1}$), an increase in the initial acetate concentration had a significant effect on *C. reinhardtii* growth (p < 0.0001 for $A_o$=0.75 gC L$^{-1}$ and $A_o$=1.26 gC L$^{-1}$; p = 0.0024 for $A_o$=0.21 gC L$^{-1}$). Specifically, the biomass concentration (192 h) rose from 0.25 gC L$^{-1}$ to 0.41 gC L$^{-1}$ as the initial acetate concentration increased from $A_o$=0.21 gC L$^{-1}$ to $A_o$=1.26 gC L$^{-1}$. The presence of an additional organic carbon source
(such as acetate) has been shown to: i) boost microalgal biomass growth (Chapman et al., 2015), and ii) increase starch and lipid accumulation, caused possibly by either the greater cell sizes of acetate-enhanced cultures (Goodson et al., 2011) or the larger availability of the carbon pool which shifts or lengthens the biosynthetic pathways (Fan et al., 2012; Goodenough et al., 2014). Although the cellular contents of the storage molecules increased slightly as a result of acetate addition (Figure 7.d), the extent of this accumulation was less noticeable (p > 0.05, between 0.42 gC L\(^{-1}\) and all carbon treatments) than the nitrogen-driven accumulation.

Similar to nitrogen-limited growth observations, biomass concentration decreased at a high acetate concentration of \(A_o=2.52\) gC L\(^{-1}\). The combined inhibitory effects posed by high nitrogen and acetate concentrations were further verified experimentally in the HIGH A-N culture (\(A_o=2.52\) gC L\(^{-1}\) and \(N_o=0.7430\) gN L\(^{-1}\)), which attained a biomass concentration of 0.21 gC L\(^{-1}\) (Figure 7.e). Thus, the expected increase in costs for such a high-nutrient strategy, coupled with the growth inhibition, undermines its potential use for \(C.\ reinhardtii\) cultivation.

4.2. Predictive performance of the kinetic model.

The microalgae-based model developed in this work consists of 8 ODEs and 31 kinetic parameters. The estimated values of each kinetic parameter computed by the methodology described in Section 3.5 are presented in Table 1, which also provides reference values available in the open literature. The resulting concentration profiles of each state variable, as predicted by the model, are shown in Figure 8 against their corresponding experimental values. The model was capable of predicting accurately all 8 state variables, as shown by the good agreement obtained between predicted and
experimental values in both datasets that were used in the fitting process (e.g. TAP and
N-) and datasets obtained at different conditions (e.g. A++ and N’-A’) for validation.
Additional details are available as Supplementary Information.

The model was able to compute accurate dynamic concentration profiles for all species
involved under different nitrogen and carbon concentration regimes (Figure 8),
including total biomass, \( X \), indicating that the Droop-based expression used for the
specific growth rate (Eq.(3)) can adequately describe microalgal growth dynamics under
nitrogen-limited mixotrophic conditions. Although both the classic Monod and Droop’s
model have been widely used to model microalgal growth, an added advantage of the
Droop’s model is its dependence on internal nutrient availability, which allows to
capture the observed ability of microalgae to grow even after complete exhaustion of a
limiting nutrient (Lee et al., 2015).

Small disagreements between predictions and experimental data can be seen for pH
nitrogen dynamics (Figure 8.b, Figure 8.h). The variation in pH predictions might be
the result of using a rather simple expression (Eq.(18)) that does not take into account
the formation of other organic acids produced in small quantities by \( C. \) reinhardtii, such
as formic acid or glycolic acid (Bekirogullari et al., 2017). Another potential cause for
these disagreements could be related to the presence of tris-base in the cultivation
medium. Tris-base acts as a biochemical buffer, but its concentration profile is not
predicted by the kinetic model. Instead, tris-base is only implicitly included within
nitrogen dynamics due to its high contribution to total nitrogen concentration: almost
70% of the total nitrogen present in standard TAP medium originates from tris-base.
The latter might also explain the slight discrepancies observed between the predicted
and experimentally obtained dynamics of nitrogen uptake. However, rather than
incorporating individual uptake expressions for each nitrogen source, which would increase complexity and computational time, the kinetic model was built with one single expression for nitrogen uptake (Eq. (8)).

Models in which microalgal growth is limited by an internal nutrient pool generally assume Michaelis-Menten (MM) uptake kinetics, as in Droop’s original approach (Droop, 1983). Under this assumption, nutrient uptake is assumed to be dependent on a single enzyme system that controls the uptake of extracellular substrates (Shuler and Kargi, 1992). In the current model, however, Andrews-type kinetics were employed to effectively predict *C. reinhardtii*’s growth inhibition at high nitrogen concentrations. Inhibited uptake dynamics then cause less nitrogen to enter the cells, which translates into smaller nitrogen quota and, consequently, decreased growth.

Although MM-type kinetics have been successfully implemented in microalgae-oriented models (Adesanya et al., 2014; Mairet et al., 2011b; Packer et al., 2011), it was suggested (Bonachela et al., 2011) that the use of this rather static model is not capable of capturing the ability of microalgae to adapt their “uptake machinery” to a changing environment. It follows from the same logic that inhibited-kinetics (as employed in this model) might suffer from the same weakness. This flaw is potentially a result of treating the maximum uptake rate, $\rho_{N,\text{Max}}$, as a constant rather than as a dynamic variable (Morel, 1987), preventing an organism’s uptake kinetics to respond to environmental changes (Bonachela et al., 2011). In microalgae, an abrupt increase in nutrient availability might lead to the phenomenon of *luxury* consumption, which refers to the sudden drop in the maximum uptake rate, $\rho_{N,\text{Max}}$, as a constant rather than as a dynamic variable (Morel, 1987), preventing an organism’s uptake kinetics to respond to environmental changes (Bonachela et al., 2011). In microalgae, an abrupt increase in nutrient availability might lead to the phenomenon of *luxury* consumption, which refers to the sudden drop (uptake) of a nutrient from the surrounding medium (Droop, 1983). This phenomenon was observed in all our lab-scale experiments, where nitrogen concentration decreased rapidly in the first 48 hours following inoculation (Figure 8.b). Since the degree of
luxury consumption was expected to be dependent on the current cell density and the nutrient concentration of the fresh medium (Droop, 1983), the maximum uptake rate (Eq. (9)) was expressed as a decreasing function of biomass (Zhang et al., 2008) coupled with a Monod-like function of the initial nitrogen concentration. Moreover, the model was able to predict the higher nitrogen consumption yields observed in those cultures grown under high acetate concentrations. From the datasets shown in Figure 8.b, for example, it was estimated that the culture grown in $A_o=1.26$ gC L$^{-1}$ (A++) consumed about 93% of the total nitrogen supplied, whereas the culture grown in $A_o=0.42$ gC L$^{-1}$ (TAP) consumed 77% (both cultures grown in $N_o=0.382$ gN L$^{-1}$). This is because cells require a large supply of nitrogen to compensate for acetate-enhanced growth rates, as observed in this work and that of Chapman et al. (2015), where *C. reinhardtii* cells showed a higher growth rate under mixotrophic rather than phototrophic conditions.

As observed in Figure 8.e, Figure 8.f, the proposed model was also able to predict adequately the simultaneous concentration profile of starch and lipids under a wide range of initial nitrogen and acetate concentrations. The high predictive behaviour shown by the model proposed in this study, particularly for starch and lipid formation during nitrogen-limited mixotrophic growth conditions, confirms its potential as a robust tool in the development of optimal nutrient-based microalgal cultivation strategies. An optimisation study was thus undertaken and is presented next.

### 4.3. Optimal nutrient-based strategies for starch and lipid formation.

In order to profit from the accurate predictions obtained, the model was subsequently used to establish the optimal initial conditions to attain maximum concentrations of the
two valuable biofuel feedstocks: starch and lipids. This procedure was carried out by identifying the maximum in a contour plot of each variable, as computed by the validated model, at a time equivalent to the point of highest storage molecule formation ($t=192$ h). The resulting contour plots can be seen in Figure 9.

The maxima of the contour plots shown in Figure 9.b and Figure 9.c allow to identify optimal nitrogen and acetate concentrations that maximise lipid (lipid-enhanced scenario) or starch (starch-enhanced scenario). These optimal sets are: i) $\text{OPT}_{\text{Starch}} = (A_o=1.06 \text{ gC L}^{-1}$ and $N_o=0.336 \text{ gN L}^{-1})$ for the starch-enhanced scenario, producing a starch concentration of 0.065 gC L$^{-1}$ with corresponding lipid concentration of 0.069 gC L$^{-1}$; and ii) $\text{OPT}_{\text{Lipids}} = (A_o=1.15 \text{ gC L}^{-1}$ and $N_o=0.378 \text{ gN L}^{-1})$ for the lipid-enhanced scenario producing a lipid concentrations of 0.08 gC L$^{-1}$ with corresponding starch concentration of 0.043 gC L$^{-1}$, respectively.

When compared to the base case, $\text{TAP} = (A_o=0.42 \text{ gC L}^{-1}$ and $N_o=0.3824 \text{ gN L}^{-1})$, which predicts a starch concentration of 0.018 gC L$^{-1}$ and a lipid concentration of 0.048 gC L$^{-1}$, the starch-enhanced scenario accounts for a drastic increase in starch of 261% (and corresponding 44% increase in lipids), whereas the lipid-enhanced scenario accounts for an increase in lipids of 66% (and 139% increase in starch). In each optimised case both starch and lipid concentrations are maximised with respect to the base case, due in part by the acetate boost (enhanced mixotrophic conditions), which was previously shown to increase biomass growth. Indeed, the model predicted that the highest microalgal concentration (Figure 9.a) could be achieved at $A_o = 1.1 \text{ gC L}^{-1}$ and $N_o = 0.415 \text{ gN L}^{-1}$.
The starch and lipid enhanced-scenarios were further validated experimentally by growing two additional microalgal cultures under the initial concentrations $\text{OPT}_{\text{Starch}}$ and $\text{OPT}_{\text{Lipids}}$, respectively. Predicted concentration profiles and data obtained experimentally from these enhanced scenarios are presented in Figure 10. For comparison, microalgal dynamics obtained by standard TAP concentrations are also plotted. As observed, the model performance proved once more its ability to capture adequately the trade-off between starch and lipid formation under nitrogen-limited mixotrophic growth. It could be argued that the magnitude of the predicted increases in biomass, starch, and lipids would not justify the required increase in acetate inputs. The good predictive performance of the model, however, allows carrying out alternate optimization strategies in which other factors are taken into account as per cultivation requirements (e.g. productivity, nutrient consumption yields, etc.). The outcome of such model-based optimised scenarios will undoubtedly aid in the development of microalgae as a biofuel feedstock by tackling challenges faced during the cultivation stage, such as reducing the nutrient-associated costs whilst simultaneously increasing starch and lipid productivities.

5. Conclusions.

A multi-parameter kinetic model was developed to predict nitrogen-limited mixotrophic microalgal growth coupled with simultaneous starch and lipid formation. All kinetic parameters were accurately computed by minimising the squared relative error between experimental values and model predictions. The predicted time-profiles of the model’s state variables were then validated against additional experimental datasets obtained under different nutrient concentration regimes. Model-based optimised cultivation strategies, maximising starch (261% increase with respect to base case) and lipid (66%
increase with respect to base case) production, were subsequently computed, and further experimentally validated.

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**References.**


11. Brányiková, I., Maršílková, B., Doucha, J., Brányik, T., Bǐšová, K., Zachleder, V., Vitová,


Figure 1. Schematic representation of the cellular compartments and flows used in the kinetic model. $X$, total biomass; $\mu$, specific growth rate; $\rho_N$, nitrogen uptake rate; $R_1$, starch synthetic rate; $R_3$, lipid synthetic rate; $R_2$, starch degradation rate; $R_4$, lipid degradation rate.

Figure 2. Biomass production and corresponding distribution of carbon compartments at $t=192$ h (8th day of cultivation) for: a) b) N-dependent cultures (starting $A_o = 0.42$ gC L$^{-1}$), c) d) A–dependent cultures (starting $N_o = 0.3824$ gN L$^{-1}$), and e) d) a high A-N culture. Treatments that do not share uppercase letters are significantly different ($p < 0.05$), as determined by two-way ANOVA.

Figure 3. Comparison between the predicted time-profile (lines) and experimental data (points) for the cultures grown in: TAP ($A_o=0.42$ gC L$^{-1}$, $N_o=0.3824$ gN L$^{-1}$), N- ($A_o=0.42$ gC L$^{-1}$, $N_o=0.356$ gN L$^{-1}$), A++ ($A_o=1.26$ gC L$^{-1}$, $N_o=0.3824$ gN L$^{-1}$), and N’- A’ ($A_o=1.16$ gC L$^{-1}$, $N_o=0.3151$ gN L$^{-1}$). Fitting datasets: TAP and A++; Validating datasets: N- and N’-A’. Data and standard deviation are the mean of 2 experimental replicates.

Figure 4. Contour plots generated from model predictions for: a) biomass, b) starch, and c) lipid formation (at $t=190$h) during C. reinhardtii cultivation.

Figure 5. Comparison between the predicted time-profile (lines) and experimental data (points) for the cultures grown in: TAP ($A_o=0.42$ gC L$^{-1}$, $N_o=0.3824$ gN L$^{-1}$), OPT$_{Starch}$ ($A_o=1.06$ gC L$^{-1}$ and $N_o=0.336$ gN L$^{-1}$), and OPT$_{Lipids}$ ($A_o=1.15$ gC L$^{-3}$ and $N_o=0.378$ gN L$^{-1}$). Data and standard deviation are the mean of 2 experimental replicates.
Table 1. Kinetic parameters used in our proposed model.

<table>
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<th>Reference</th>
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^a Reported values have been converted to gC L^{-1}.
Figure 6.

\[ X = x^* + (S + L) \]
Figure 7.
Figure 8.
Figure 9.
Figure 10.