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RESEARCH PAPER

Respirometry as a tool to quantify kinetic parameters of microalgal mixotrophic growth

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Abstract

Modeling microalgal mixotrophy is challenging, as the regulation of algal metabolism is affected by many environmental factors. A reliable tool to simulate microalgal behavior in complex systems, such as wastewaters, may help in setting the proper values of operative variables, provided that model parameters have been properly evaluated. In this work, a new respirometric protocol is proposed to quickly obtain the half-saturation constant values for several nutrients. The protocol was first verified for autotrophic exploitation of ammonium and phosphorus (Monod kinetics), as well as of light intensity (Haldane model), further elaborated on specific light supply basis. It was then applied to measure the kinetic parameters of heterotrophic growth. The half-saturation constants for nitrogen and phosphorus resulted comparable with autotrophic ones. The dependence on acetate and dissolved oxygen concentration was assessed. Mixotrophy was modeled as the combination of autotrophic/heterotrophic reactions, implemented in AQUASIM, and validated on batch curves with/without bubbling, under nutrient limitation, and different light intensities. It was shown that the reliability of the proposed respirometric protocol is useful to measure kinetic parameters for nutrients, and therefore to perform bioprocess simulation.

Keywords *Chlorella protothecoides* · Oxygen · Acetate · Monod · Growth modeling · AQUASIM

Abbreviations

$C_{C_{org}}$	Concentration of organic carbon (mg C L^{-1})
C_{CO_2}	Concentration of dissolved CO_2 (mg C L^{-1})
C_{O_2}	Concentration of dissolved oxygen (mg O_2 L^{-1})
$C^*_{O_2}$	Saturation concentration of oxygen in the liquid (mg O_2 L^{-1})
$C^*_{CO_2}$	Saturation concentration of CO_2 in the liquid (mg C L^{-1})
C_N	Concentration of ammonium nitrogen (mg N L^{-1})
C_P	Phosphorus concentration (mg P L^{-1})
C_x	Biomass concentration (mg L^{-1})
D_{CO_2}	Carbon dioxide diffusion coefficients (m^2 s^{-1})
D_{O_2}	Oxygen diffusion coefficients (m^2 s^{-1})
I_0	Incident light intensities (μmol photons m^{-2} s^{-1})
I_{sp}	Specific light supply rate (mmol photons g^{-1} day^{-1})
K_{CO}	Half-saturation constant for organic carbon (mg C L^{-1})
K_{CO_2}	Half-saturation constant CO_2 (mg C L^{-1})
K_I	Inhibition constant of the specific light supply rate (mmol photons g^{-1} day^{-1})
K'_I	Light inhibition constant (μmol photons m^{-2} s^{-1})
K_L	Half-saturation constant of the specific light supply rate (mmol photons g^{-1} day^{-1})
K'_L	Light half-saturation constant (μmol photons m^{-2} s^{-1})
K_N	Nitrogen half-saturation constant (mg N L^{-1})
K_{O_2}	Oxygen half-saturation constant (mg O_2 L^{-1})
K_P	Phosphorus half-saturation constant (mg P L^{-1})
$Y_{i/x}$	Yield of nutrient i on biomass (mg_i mg_X^{-1})
k_La	Oxygen mass transfer coefficient (min^{-1})
$r_{O_2,A}$	Autotrophic oxygen production rate (mg O_2 L^{-1} day^{-1})
$r_{O_2,H}$	Heterotrophic oxygen production rate (mg O_2 L^{-1} day^{-1})
$r_{X,A}$	Autotrophic biomass growth rate (mg L^{-1} day^{-1})

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$r_{X,H}$	Heterotrophic biomass growth rate (mg L^{-1} day^{-1})
$\mu_{APP,1}$	Autotrophic apparent growth rate (day^{-1})
$\mu_{APP,2}$	Heterotrophic apparent growth rate (day^{-1})
$\mu_{MAX,A}$	Autotrophic maximum specific growth rate (day^{-1})
$\mu_{MAX,H}$	Heterotrophic maximum specific growth rate (day^{-1})
H	Reactor thickness (m)
I	Light intensity (μmol photons m^{-2} s^{-1})

Introduction

In the last years, mixotrophic microalgae metabolism has gained researchers' interest thanks to its potential applicative advantages. Mixotrophy may solve issues linked to traditional microalgae cultivation, either autotrophic or heterotrophic, by allowing higher growth rate [1–5], prolonged exponential growth phase [5], resistance-gaining with respect to photo-oxidative stress, and reduction of photoinhibitory effects [6]. Moreover, mixotrophic cultures are cheaper to operate, as they require relatively lower light intensity with respect to autotrophic ones [7], and less nutrients than heterotrophic ones to reach the same productivity [2]. This metabolism can be exploited in several industrial applications, such as wastewater treatment and bioremediation [4, 7, 8], bio-refinery [1, 5, 8] and production of valuable secondary products such as lipids, pigments or food complements [1, 7]. Describing and simulating algal behavior is the only way to improve bioreactor design, process control and optimization of industrial bioprocesses of this type.

Mixotrophic metabolic pathways were characterized at both biochemical and genetic levels [9], but their regulation as a response to environmental conditions are still largely unknown, as well as their kinetic description. In fact, only a few attempts to describe mixotrophic growth with simple models able to fit experimental data at macroscale level were proposed so far. The easiest way to address this problem is to assume that mixotrophic growth is the sum of autotrophic and heterotrophic growths [10, 11]. However, a complex combination of factors, such as light intensity [12, 13], C/N ratio, temperature and the type of organic carbon sources [1, 7, 8], can drive the metabolism toward autotrophy rather than heterotrophy, or *vice versa*. Other studies account for some of these external conditions by adding an inhibitory factor in their model [6].

Modeling algal growth in wastewaters [14, 15] is aimed at optimizing and controlling the performance of cultivation systems, which are applied to specific conditions. On the other hand, only a few attempts were made to account for mixotrophy in the models, and these usually focused on carbon source dependence [8].

In the field of wastewater treatment, respirometry is a technique traditionally applied for activated sludge bacteria characterization [16–18]. The fastest and easiest protocol consists in measuring the oxygen consumed by bacteria due to substrate oxidation (OUR, oxygen uptake rate) under controlled condition. The respiration rate obtained is related to the aerobic biodegradation process, which allows the estimation of kinetic parameters of a Monod-type equation [19]. In general, this basic protocol has been used to evaluate kinetic parameters of nitrifying bacteria in activated sludge [20].

The determination of microalgae growth kinetics from dissolved oxygen evolution measurements, in analogy with bacterial respirometry experiments, can be a versatile method to derive the values of kinetic parameters related to different nutrient substrates. In fact, depending on their metabolism, microalgae growth is related to an oxygen production rate (OPR) through photosynthesis when light is available, while in dark conditions oxygen is consumed due to endogenous respiration/organic carbon consumption. A number of studies evidenced that chlorophyll concentration and dissolved oxygen production rate in water are correlated in the case of microalgae [21], suggesting that OPR can be used to determine the autotrophic biomass activity [3, 6, 22]. Some authors [17] showed that respirometry could in fact be used to derive microalgal growth kinetics, under autotrophic conditions. However, the limitation effects caused by substrate (nutrients and light) availability were not taken into account in their model, where only the maximum growth rate was considered.

Overall, respirometric assays are rarely applied on microalgae [22, 23]. Conversely, algal growth kinetics and substrate limitation effects are generally studied by means of batch experiments, which are often time-consuming and may be affected by the formation of biomass debris [22].

In this work, a new approach of respirometric method was used to measure kinetic parameters (i.e., nutrients half-saturation constants) of *Chlorella protothecoides*, first under autotrophic conditions, to verify the validity of the protocol by comparing the results with available literature data. The same protocol was then used to assess heterotrophic and mixotrophic behavior, and evaluate the model parameters, by analyzing the growth rate dependence on oxygen, organic carbon as well as nitrogen and phosphorus concentration.

To validate the parameter values experimentally measured, the kinetic model was implemented in AQUASIM simulation software. The simulation results were hence compared with experimental ones of batch growth curves carried out under different external conditions of nutrient replete/depleted levels, various light intensities, air–CO_2 bubbling/no bubbling, and the presence/absence of organic carbon. The effect of CO_2 and O_2 production/consumption on the autotrophic/heterotrophic rates was also evaluated.

Materials and methods

Microalgal species, growth medium and experimental condition of preinocula

Chlorella protothecoides 33.80 (obtained from SAG Goettingen, Germany) was axenically maintained in liquid BG11 medium at 24 °C for the inoculum, and renewed weekly with fresh medium. In the respirometric tests, BG11 composition was modified to study the effect of different nutrients on the oxygen production rate. Details are reported in the corresponding sections. All media and materials were sterilized by autoclaving.

The preinocula were cultivated in batch Drechsel bottles (50 mm diameter, with a total volume of 250 ml) under CO_2–Air (5% v/v) supply and axenic conditions (checked by plating samples in LB Petri dishes). The reactors were placed in a thermostatic incubator at 24 °C, and the culture mixing in each reactor was ensured by a magnetic stirrer. Continuous artificial light was provided from one side of the bottles by a LED panel located inside the incubator, with a constant intensity of 150 µmol photons m^{-2} s^{-1}, measured by a photo-radiometer Delta OHM HD 2101.1 (with a LPPAR probe, that measures irradiance in the range between 400 and 700 nm, Delta OHM). Light intensity was measured at the reactor surface, and averaged on at least 4 measurement points. Preinocula were fed with fresh medium 4 days before the respirometric test, to obtain an active biomass, sampled in late exponential phase, so to avoid high internal content of nutrients due to possible luxury uptake phenomena that may occur at the beginning of the lag–log phases [24].

The biomass concentration was measured as dry weight (DW) in terms of g L^{-1}. This was done gravimetrically on biomass which was filtered (0.22 µm, cellulose nitrate filters, Sartorius) and dried at 100 °C in a laboratory oven for 2 h.

Model description

The biomass composition of *C. protothecoides* and the corresponding autotrophic growth stoichiometry can be written as [25]:

$$CO_2 + 0.22NH_4^+ + 0.013H_2PO_4^- + 0.59H_2O \rightarrow 1.171O_2 + 0.207H^+ + C_1H_{1.88}N_{0.22}O_{0.3}P_{0.013} \quad (1)$$

Mixotrophy is commonly described as the combination of autotrophic and heterotrophic growths [6, 11]. Accordingly, the organic material exploitation reaction can be written as [6]:

$$0.635CH_3COO^- + 0.013H_2PO_4^- + 0.22NH_4^+ + 0.1O_2 + 0.428H^+ \rightarrow CH_{1.88}N_{0.22}O_{0.3}P_{0.013} + 0.27CO_2 + 0.680H_2O \quad (2)$$

In particular, the stoichiometric equation was balanced based on the biomass/carbon yield measured experimentally in Sforza et al. [25], corresponding to 0.78 mg of carbon in the biomass per mg C of acetate (comparable to values reported by Barros et al. [26]). This value accounts for the ratio of organic carbon which is actually fixed in the biomass. The equation describing the autotrophic microalgal growth rate ($r_{X,A}$), based on Monod model for microbial growth, can be written as a function of biomass (C_x) and nutrients (C_i) concentrations.

$$r_{X,A} = \mu_{MAX,A} C_x \cdot \frac{C_{CO_2}}{K_{CO2} + C_{CO_2}} \cdot \frac{C_N}{K_N + C_N} \cdot \frac{C_P}{K_P + C_P} \cdot \frac{I}{K_L' + I + \frac{I^2}{K_I'}} \quad (3)$$

where $\mu_{MAX,A}$ is the maximum specific autotrophic growth rate (day^{-1}), and K_i are the half-saturation constants (mg_i L^{-1}). To model the effect of light intensity (I), a substrate inhibition model was used (Haldane equation, with $K_L^{'}$ and $K_I^{'}$ being the light half-saturation and inhibition constants, respectively). However, to account for self-shading effects due to increasing biomass concentration, the light variable was described in terms of specific light supply rate (I_{sp}, mmol photons g^{-1} day^{-1}), i.e., the light intensity normalized on biomass concentration:

$$I_{sp} = \frac{I_0}{C_x H}, \quad (4)$$

where I_0 is the incident light intensity (µmol m^{-2} s^{-1}) and H is the thickness (m) of the respirometric device or growth reactor, with planar frontal surface and rectangular geometry (see "Respirometric apparatus" and "Model validation and simulations").

Accordingly, the Haldane function can be rewritten as:

$$f(I_{sp}) = \frac{I_{sp}}{K_L + I_{sp} + \frac{I_{sp}^2}{K_I}}. \quad (5)$$

For each component, the production (positive) or consumption (negative) rate can be linked to the biomass growth rate by means of the yield term:

$$Y_{i/x} = \frac{dC_i}{dC_x}. \quad (6)$$

Accordingly, reactant consumption rate (r_r) and product formation rate (r_P) can be, respectively, written as:

$$r_r = -\frac{r_X}{Y_{X/r}}, \quad (7)$$

$$r_P = \frac{Y_{P/r}}{Y_{X/r}} r_X. \quad (8)$$

If all nutrients, except one, are not limiting, the biomass growth Eq. (3) can be simplified as:

$$r_{X,A} = \mu_{MAX,A} C_X \frac{C_i}{K_i + C_i} \tag{9}$$

with i being the limiting nutrient. Thus, as a common procedure, the dependence of the growth kinetics can be specifically studied for each nutrient i, if all the others are supplied in excess.

In particular, in the case of autotrophic metabolism, oxygen is a product of the reaction, and its production rate (with a single limiting nutrient i) can be written as:

$$r_{O_2,A} = \frac{Y_{O_2/i}}{Y_{X/i}} r_{X,A} = \frac{Y_{O_2/i}}{Y_{X/i}} \mu_{MAX,A} C_X \frac{C_i}{K_i + C_i}. \tag{10}$$

By defining an apparent-specific growth rate

$$\mu_{APP,1} = \mu_{MAX,A} Y_{O_2/i} \Big/ Y_{X/i} \tag{11}$$

at fixed biomass concentration, the oxygen production rate can be measured as a function of the limiting nutrient provided:

$$\frac{r_{O_2,A}}{C_X} = \mu_{APP,1} \frac{C_i}{K_i + C_i}. \tag{12}$$

Similarly, the heterotrophic biomass growth rate can be written as:

$$r_{X,H} = \mu_{MAX,H} C_X \frac{C_{O_2}}{K_{O_2} + C_{O_2}} \cdot \frac{C_N}{K_N + C_N} \cdot \frac{C_P}{K_P + C_P} \cdot \frac{C_{C_{org}}}{K_{CO} + C_{C_{org}}}. \tag{13}$$

In this case, oxygen is a reactant consumed during the reaction, and its consumption rate can be written as:

$$-r_{O_2,H} = \frac{r_{X,H}}{Y_{X/O_2}} = \frac{\mu_{MAX,H} C_X}{Y_{X/O_2}} \frac{C_i}{K_i + C_i}. \tag{14}$$

Again, by considering that

$$\mu_{APP,2} = \frac{\mu_{MAX,H}}{Y_{X/O_2}} \tag{15}$$

at fixed biomass concentration, the oxygen consumption rate can be measured as a function of the limiting nutrient:

$$\frac{-r_{O_2,H}}{C_X} = \mu_{APP,2} \frac{C_i}{K_i + C_i}. \tag{16}$$

According to the equations here reported, it is possible to evaluate the Monod half-saturation constants for each nutrient by measuring the oxygen production or consumption rates under variable nutrient concentrations.

Respirometric apparatus

To evaluate the oxygen production/consumption rates of *C. protothecoides* as a function of light and nutrients, tests based on respirometry were carried out [25]. A similar technique was previously reported by Decostere et al. [17], under autotrophic conditions only, and not considering the dark respiration phase. In addition, respirometry was recently applied as a tool to evaluate microalgal performances in wastewater [23].

Dissolved oxygen (DO) concentration was continuously measured by means of a HD2109.1 DELTA OHM oximeter connected to a PC using Deltalog9 provided by DELTA OHM. The oxygen measurement was carried out in airtight glass flasks of 100 mL, with square section (4 cm of depth and about 6 cm of working volume height) to prevent high oxygen transfer between the liquid and the external air. In addition, minimum gas headspace was left to avoid gas losses in the gas phases. The liquid sample was mixed by a magnetic stirrer and the temperature was maintained constant at 25 °C using a thermostatic water bath. Accordingly, the function on temperature was neglected in the growth kinetic model.

The oxygen mass transfer coefficient in the system was experimentally measured according to

$$\frac{dC_{O_2}}{dt} = k_L a \left(C^*_{O_2} - C_{O_2} \right), \tag{17}$$

where dC_{O_2}/dt is the derivative of C_{O_2} ($mg_{O_2}\ L^{-1}$) over time ($mg_{O_2}\ L^{-1}\ min^{-1}$), $k_L a$ is the global oxygen mass transfer coefficient (min^{-1}), and $C^*_{O_2}$ is the oxygen saturation concentration in the liquid, determined according to the Henry's law:

$$C^*_{O_2} = H_{O_2} \cdot P_{O_2}, \tag{18}$$

where H_{O_2} is the Henry's constant for oxygen in water (equal to 42.15 $mg_{O_2}\ atm^{-1}\ L^{-1}$ at 25 °C [27]), and P_{O_2} is oxygen partial pressure in air (= 0.21 atm). The $k_L a$ coefficient was evaluated carrying out abiotic tests in distilled water, implementing the log-deficit procedure as described in ASCE [23]. This method is based on measuring the oxygen re-aeration rate in the liquid after total stripping by nitrogen bubbling. In particular, the measurement was carried out three times for at least 18 h and a final averaged value of 0.0033 min^{-1} was obtained, which was used in the following respiration measurements.

Respirometric protocol

The protocol proposed relies on the measurement of oxygen produced or consumed by microalgal biomass, which is actually related to the auto/mixotrophic growth, through yields factors. The variables investigated, which represent the main factors involved in auto/mixotrophic metabolisms, were: CO_2, O_2, N, P, sodium acetate concentration and light intensity. The procedure proposed is based on the oxygen production/consumption under cycles of light and dark, as a function of one single variable at time, keeping the other ones constant.

Each respirometric test started with fresh culture medium (BG11), in which a constant biomass inoculum (about 0.2 g L^{-1} of DW) was resuspended. The pH of the medium was buffered at 7.5 with HEPES 1M, and its value was checked during the entire duration of the test. To ensure a constant microalgal biomass composition and acclimation, biomass was always sampled from preinocula cultivated for 4 days, as reported in "Microalgal species, growth medium and experimental condition of preinocula", after checking axenic conditions by LB (Luria broth) plating.

Each test lasted about 3 h and consisted in alternating cycles of light and dark (5:5 min), obtained by means of a digital controller connected to a LED lamp. Illumination was provided frontally, and the light intensity was measured with the photoradiometer both at the front and at the back of the respirometric device. The duration of the light:dark cycle was chosen as sufficient to obtain enough DO measurements (one acquisition every 30 s) to fit the model. Moreover, it was verified that the biomass concentration C_X can be considered constant throughout the duration of the test, as the growth dynamics are slower than the duration of the experimental run. Each run started in the dark phase, and the first 10 min of data acquisition were discarded to allow the acclimation of the microorganisms to the environmental conditions applied. This protocol resulted in DO profiles along with time, an example of which is reported in the Online Resources (Fig S1). When light was on, a positive increase of dissolved oxygen was measured, while a negative one was observed under dark.

Experimental DO data were fitted by a model including the simultaneous occurrence of oxygen mass transfer, due to mixing, and the biological DO consumption/production, according to:

$$\frac{dC_{O_2}}{dt} = k_L a\left(C^*_{O_2} - C_{O_2}\right) + \text{OPR}. \quad (19)$$

Each OPR value was estimated as the average of at least four measurements (i.e., four dark–light cycles of 5:5 min each). The specific oxygen production and consumption rates ($mg_{O_2}\ mg_X^{-1}\ day^{-1}$) were obtained by normalization of OPR ($mg_{O_2}\ L^{-1}\ day^{-1}$) with respect to the initial biomass concentration measured as DW ($mg_X\ L^{-1}$).

Different environmental conditions were applied during respirometric tests, depending on the variable (nutrient or light) and metabolism (autotrophy, mixotrophy, heterotrophy) under investigation. For each variable, the tests were carried out at least in double biological replicate, by repeating the entire protocol starting from different preinocula. The basic procedure here proposed is explained in detail in the following sections, for each specific variable studied.

Autotrophic growth

Concerning autotrophic growth, three tests were carried out, namely as a function of light intensity, NH_4–N concentration and PO_4–P concentration, respectively. For all autotrophic tests, preinocula were diluted to about 0.2 g L^{-1} of biomass in the respirometric apparatus, in BG11 medium with the addition of 1 g L^{-1} of $NaCO_3$ (as a pH buffer). Pure CO_2 was bubbled in the device prior to the respirometric run, so to avoid carbon limitation and simultaneously reduce to about 4 mg L^{-1} the dissolved oxygen. The pH was measured at the beginning and during the tests, and it was equal to 7.5.

When studying the effect of light intensity, all nutrients were supplied in excess (standard concentration of BG11: N = 247 mg L^{-1}, P = 5.4 mg L^{-1}), and after the bubbling step, oxygen measurements were carried out under progressively increasing lights (in the range between 20 and 1500 µmol photons $m^{-2}\ s^{-1}$), which were changed after at least three cycles of light–dark phases.

To study the effect of nitrogen, preinocula were first filtered (0.22 µm, syringe filter), washed with sterile water and then resuspended in modified BG11 medium, without nitrogen salts. This was then used to carry out the respirometric protocol and considered as the point at zero external concentration of the nutrient under investigation. During light cycles, light intensity was 150 µmol photons $m^{-2}\ s^{-1}$. After that, subsequent additions of nutrient (NH_4Cl, from a concentrated, sterilized stock) were carried out, about every 30 min, and the positive (light) and negative (dark) OPR values were measured as a function of increasing external nutrient concentrations. A similar protocol was applied also to phosphorus, using a BG11 modified without PO_4, and with progressive additions of K_2HPO_4.

For all the tests in autotrophic condition, the gross oxygen rate (gOPR) was considered according to Rossi et al. [23]:

$$\text{gOPR} = \text{OPR}_L - \text{OPR}_D, \quad (20)$$

where L and D subscripts refer to light and dark conditions, respectively. In fact, OPR_D represents the basal respiration that occurs also during the light phase.

Hetero/mixotrophic growth

Four tests were then carried out to provide information about hetero/mixotrophic metabolisms, specifically as a function of NH_4–N, PO_4–P, organic carbon and O_2 concentration.

For all the hetero/mixotrophic tests, preinocula were diluted in BG11 medium to about 0.2 g L^{-1} of biomass in the respirometric apparatus, and an initial bubbling with N_2 was carried out to reduce the oxygen concentration in the liquid down to about 4 mg L^{-1}. Organic carbon was then added in the form of sodium acetate, with a final concentration corresponding to 0.2 g L^{-1} of C, which can be considered a non-limiting carbon concentration. A procedure of sequential addition of nutrients was applied to study the effect of NH_4–N and PO_4–P, starting from a medium lacking the specific nutrient, similar to autotrophic tests ("Autotrophic growth"). Light intensity was 50 µmol photons m^{-2} s^{-1}, lower than that used in the autotrophic tests, based on previous observations that high light intensity may reduce the organic carbon exploitation [14, 22].

For the kinetic study of organic carbon exploitation, different aliquots of concentrated stock of sodium acetate were sequentially added, every 30–40 min, and the corresponding OPR_L and OPR_D trends were measured.

To study the effect of O_2 concentration in the liquid, a complete initial stripping with N_2 was carried out on the sample, and after an initial injection of pure CO_2, the oxygen production and consumption were measured. All the nutrients (including acetate) were in excess. After the first four light:dark cycles, a prolonged light phase was used to increase the oxygen in the liquid, thanks to photosynthetic production, up to the desired DO concentration, at which OPR trends were then measured. The valid concentration range was assumed between 0 and 11 mg_{O_2} L^{-1}. Over this point, supersaturation phenomena lead to bubble formation, thus affecting the OPR measures.

For each nutrient investigated, average values of OPR_L and OPR_D were reported as a function of external nutrient concentration. Basal photosynthetic and respiration activities, measured at zero concentration of external nutrients, were used to normalize the data obtained under increasing concentration (see the Results section for details). The data of gross oxygen rate (gOPR) were used to study the mixotrophic growth, while OPR_D were specifically used to get information about heterotrophy.

The net oxygen production as a function of external nutrient concentration was used to fit the values of Monod kinetic parameters, taking into account both standard deviations and the covariance matrix.

Model validation and simulations

To verify the validity of the measured kinetic parameter values, the proposed mixotrophic growth model was implemented in the simulation software AQUASIM [28]. Specifically, the microalgal biomass autotrophic and heterotrophic growths have been considered as separate dynamic processes, according to Eq. (3) and Eq. (13), respectively, hence also the corresponding growth rate values are evaluated separately in the simulation. The stoichiometric reactions are those reported in Eq. (1) and Eq. (2). The overall biomass production is therefore determined by the combination of the two separate contributions.

In addition, the mass transfer of gaseous compounds (O_2 and CO_2) has been taken into account. Oxygen mass transfer was calculated according to Eq. (17). Similarly, CO_2 mass transfer was determined according to Decostere et al. [17]:

$$\frac{dC_{CO_2}}{dt} = k_L a \left(\frac{D_{CO_2}}{D_{O_2}}\right)^{0.5} \left(C^*_{CO_2} - C_{CO_2}\right) \quad (21)$$

with D_{CO_2} and D_{O_2} being the carbon dioxide and oxygen diffusion coefficients in the liquid phase (m^2 s^{-1}). The saturation concentrations of O_2 and CO_2 in the liquid ($C^*_{O_2}$ and $C^*_{CO_2}$) are evaluated by the Henry's law.

Three sets of conditions were simulated, to verify the results in comparison with the experimental ones. All simulations have been referred to a batch-flattened glass photobioreactor of about 200 mL of volume and a thickness $H = 0.04$ m, and starting biomass concentration $C_{x,in}$ of about 100 mg L^{-1}, for a duration of 3–7 days, depending on the condition applied and the corresponding duration of the growth curve.

First, autotrophic growth with bubbling of 5% v/v CO_2–air mixture ($k_L a = 172$ day^{-1}) and excess nutrients was simulated at different incident light intensities of 15, 75 and 130 µmol m^{-2} s^{-1}, in BG11 with no nutrient limitation ($C_{N,in}$ = 247 mg L^{-1} and $C_{P,in}$ = 50 mg L^{-1}).

Second, the effect of nutrients (N and P) limitation, was checked by lowering their initial concentration down to $C_{N,in}$ = 30 mg L^{-1} and $C_{P,in}$ = 3 mg L^{-1}, under the same experimental conditions ($I_0 = 130$ µmol m^{-2} s^{-1}) and with no limitation of CO_2.

Finally, autotrophic, heterotrophic, and mixotrophic growth curves were simulated for a system with excess nutrients and no bubbling ($k_L a = 2.0$ day^{-1}). More specifically, the incident light intensity in the case of autotrophic/mixotrophic conditions was equal to 130 µmol m^{-2} s^{-1}, while the initial concentration of organic carbon in heterotrophic and mixotrophic growth was equal to 200 mg C L^{-1} (as sodium acetate). Preinocula used for mixo/heterotrophic growth experiments were pre-acclimated for 3 days to sodium

acetate. The validation experiments were carried out in the experimental system described in "Model description", at least in biological duplicate.

Results and discussion

Determination of parameters for autotrophic growth

Effect of light intensity

The use of oxygen production to quantify the response of microalgae to light intensity is broadly recognized in the PI curves [29–32]. In this work, the PI test was used as a validation of the protocol described above, to assess the reliability of the currently proposed method. Eight incident irradiances were used, sequentially. The results of the gross specific oxygen production obtained are reported in Fig. 1a. By fitting these data with the Haldane model, the values of K_L and K_I resulted equal to 151 ± 20 and 1899 ± 399 µmol photons m^{-2} s^{-1}, respectively, which are similar to values commonly reported in the literature [33]. Thus, the protocol proposed can be considered reliable to study the overall phototrophic metabolism by measuring the gross oxygen rate.

To better quantify the effect of specific light on growth, light data were normalized on biomass concentration, and expressed in terms of I_{sp}, as defined in Eq. (4). The kinetic model as a function of light intensity was rewritten considering this new term, according to Eq. (5):

$$\frac{r_{O_2,A}}{C_X} = \mu_{APP,1} \frac{I_{sp}}{K_L + I_{sp} + \frac{I_{sp}^2}{K_I}} \quad (22)$$

As shown in Fig. 1b, data were again fitted by the Haldane model. Thus, new values of half-saturation and inhibition constants could be derived. Specifically, values of $K_L = 955 \pm 178$ and $K_I = 10{,}130 \pm 2859$ mmol photons g^{-1} day^{-1} were found. This light function and the corresponding parameters were hence implemented in the simulation model developed in AQUASIM (see "Validation of model parameters").

Ammonia and phosphorus

Respirometric tests were also carried out as a method to easily and quickly determine the half-saturation constants of the Monod terms containing nitrogen and phosphorus concentrations.

Similar to what reported in Fig. 1a, an example of results obtained from a single respirometric test is reported in Online Resources, Fig. S2, as a function of increasing

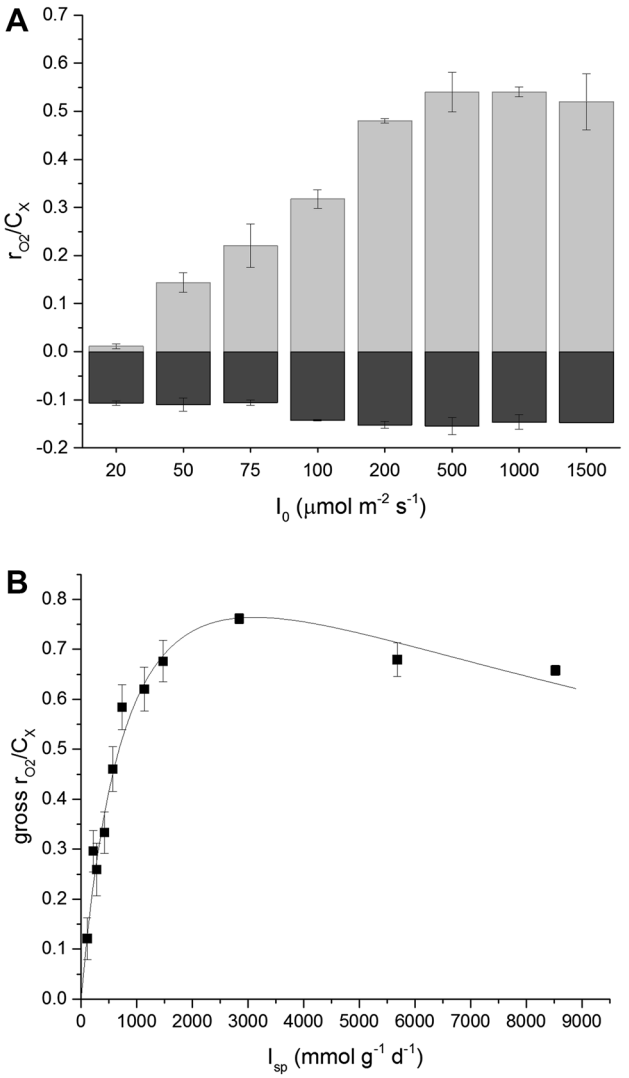

Fig. 1 In **a** an example of specific OPR_L (light gray) and OPR_D (dark gray) normalized on biomass concentration is reported as a function of incident light intensity. The protocol was repeated starting from three different preinocula, and the resulting gross-specific OPR (from the three independent biological replicates) is reported in **b** as a function of specific light supply rate. Data fitting (straight line) was obtained based on the minimization of sum of squared errors adjusting the parameters from Haldane equation (Eq. 22)

nitrogen concentrations. Concerning the first point tested (external N concentration equal to zero), even though possible nutrient residuals from the starting preinoculum medium were completely removed thanks to filtration (which was verified by colorimetric tests), basal oxygen production/consumption was measured. This is likely due to the internal nutrient concentration in the biomass, which is exploited for the usual metabolism by microalgal cells. On the other hand, adding the nutrient during the test resulted in an increased oxygen production, suggesting that the overall metabolism is enhanced under increasing concentration of external

nutrient. Data processing was then carried out, to take into account only the net oxygen production increase due to nutrient addition. Accordingly, specific gross OPR data were normalized by subtracting the initial basal value. Results are reported in Fig. 2a and follow a typical Monod trend, according to Eq. (12). These data were fitted and the half-saturation constant resulted $K_N = 14.23 \pm 2.17$ mg N L^{-1}. Interestingly, the value of half-saturation constant is similar to that obtained with the same species by growth experiments, reported in our previous work [34], but in agreement also with other values reported in the literature [33, 35, 36]. These parameters are usually determined by multiple and lengthy batch tests carried out at different initial nutrient concentrations, which require time-consuming procedures of biomass growth and nutrient consumption measurements.

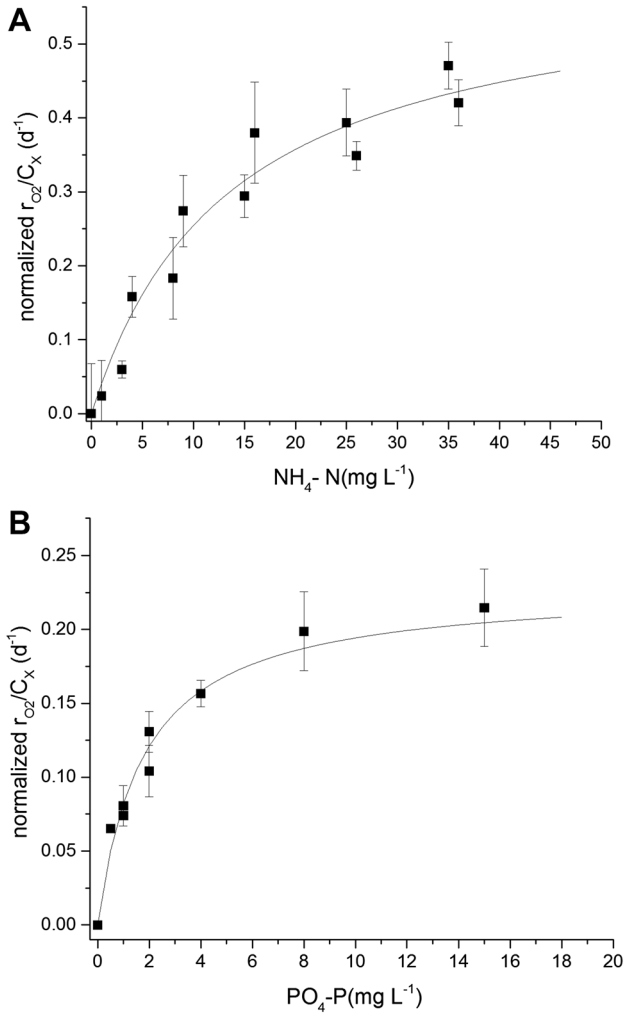

Fig. 2 Specific gross OPR data normalized on biomass concentration as a function of nitrogen (**a**) and phosphorus (**b**) external concentrations. Fitting (straight line) was based on the minimization of the sum of squared errors adjusting the parameters of the Monod equation (Eq. 12)

The same protocol was used to determine the half-saturation constant of phosphorus. Data, normalized on the basal value at $c_P = 0$, are reported in Fig. 2b. The half-saturation constant resulted to be $K_P = 1.8 \pm 0.28$ mg P L^{-1}, consistent with that reported by Rowley et al. [33]. On the other hand, the values found in the literature for P are extremely variable, as complex phenomena such as luxury uptake by microalgae may affect the quantitative determination of this parameter. The dependence of growth on P concentration is actually a complex phenomenon, as microalgae have a high capability to fast accumulate internal P as polyphosphate, which is then used for growth when the external concentration decreases [24, 37, 38] a phenomenon depending also on temperature. In our case, as an increased production of oxygen was measured in a timeframe of minutes, it can be concluded that the increase in OPR is related to the P actually incorporated in the biomass and possibly used for metabolism.

In summary, the results obtained for light and nutrients under autotrophic conditions suggest that the protocol is reliable to determine the kinetic parameters related to the growth dependence on nutrient availability in a rather quick way. On the opposite, this protocol appears to be not applicable for μ_{max} assessment, as the values of μ_{app} depend on nutrient/oxygen yields, and in addition, they should be specifically studied as a function of both light and temperature, which are the variables mostly affecting this parameter.

Determination of parameters for heterotrophic and mixotrophic growth

Ammonia and phosphorus

To study heterotrophic and mixotrophic metabolisms, a slightly different experimental procedure was used, as reported in "Respirometric apparatus". In particular, light intensity was reduced to 50 µmol photons m^{-2} s^{-1}, as previous papers reported that high light intensity may reduce the organic carbon exploitation [11, 12, 39]. In addition, since also the excess of CO_2 was found to inhibit mixotrophy [40], initial bubbling was carried out with N_2 gas, so to reduce both CO_2 and O_2 concentrations to about 4.5 mg L^{-1}. Organic carbon was added in excess, at the beginning of the test, at a concentration of 0.2 g L^{-1}. The first dark and light periods were discarded to allow the microalgal biomass to acclimate to the new conditions. An example of the oxygen production and consumption rate is reported in Online Resources, Fig. S3. In this case, an increased oxygen consumption rate (OPR_D) was observed after each nutrient addition to the respirometric apparatus, while the oxygen production rate remained almost the same. Also in this case, a basal OPR_D was observed at the zero initial external nutrient concentration, as a result of internal nutrient reserves.

Accordingly, data were normalized by subtracting this initial point. Both the specific OPR_D and gOPR are reported in Fig. 3a, b for nitrogen and phosphorus, respectively, so to account for the effect of nutrient addition on heterotrophic (OPR_D) as well as mixotrophic (gOPR) growth. Both trends were fitted with Monod equation, and interestingly, the resulting values of half-saturation constant were similar, suggesting that the overall metabolism is influenced to the same extent by the external nutrient addition. Specifically, the values obtained for nitrogen were 15 ± 3.2 and 13 ± 3.7 mg N L^{-1} for hetero- and mixotrophic metabolism, respectively, while the corresponding values for P were 1.83 ± 0.35 and 1.80 ± 0.38 mg P L^{-1}. Even more interestingly, the values of half-saturation constants measured under heterotrophic and mixotrophic conditions resulted to be not significantly different from those obtained under autotrophic conditions, which are reported in "Ammonia and phosphorus" above. Therefore, it is suggested that the effect of macronutrients such as N and P on *C. protothecoides* metabolism is not affected by the carbon source. This actually allows to simplify the kinetic expression of mixotrophic growth rate, as the N and P terms are equal in both autotrophic and heterotrophic conditions.

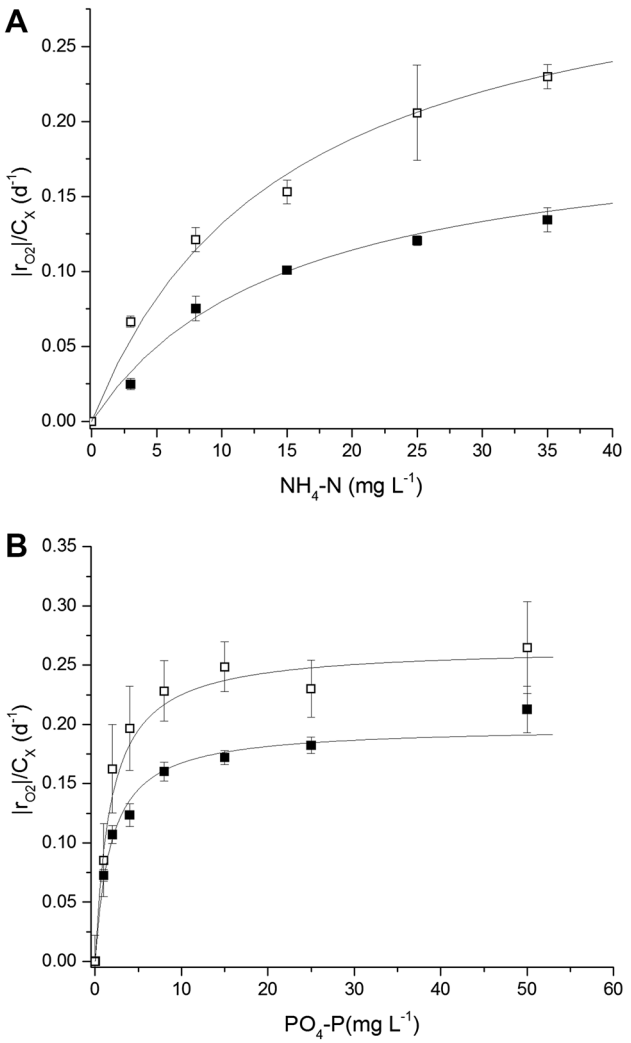

Fig. 3 Specific gOPR (open squares) and $|OPR_D|$ (full squares) data normalized on biomass concentration as a function of nitrogen (**a**) and phosphorus (**b**) external concentrations. Fittings (straight line) were obtained based on the minimization of the sum of squared errors by adjusting the parameters of Monod equation (Eq. 12)

Oxygen and organic carbon

Based on the protocol described in the previous paragraphs, the dependence on organic carbon concentration was addressed by means of sequential addition of sodium acetate during the respirometric test. The measured trend is reported in Fig. 4A, and the resulting half-saturation constant resulted equal to 3.89 ± 0.8 mg_C L^{-1}. Wagner et al. [15] found a value of 6.3 g_{COD} m^{-3} in the case of mixotrophic and heterotrophic growth, based on data of acetate consumption from batch experiments carried out with mixed green microalgal consortia.

To study the effect of dissolved oxygen on heterotrophic metabolism, a different protocol was used, starting with a concentration close to zero achieved thanks to prolonged stripping with N_2, followed by a bubbling of CO_2. Then, the oxygen produced by mixotrophy during light cycles was exploited to increase its concentration in the medium to the desired values. An example of the procedure is reported in the Online Resources (Fig. S4).

Data of specific $|OPR_D|$ shown in Fig. 4b allowed to determine a half-saturation constant of 2.06 ± 0.48 mg_{O_2} L^{-1}. In this case, no data were found in the literature for the heterotrophic dependence on oxygen availability of microalgae.

Validation of model parameters

The values of half-saturation parameters determined in this work were implemented in AQUASIM according to the kinetic model described in "Materials and methods", i.e., considering mixotrophy as the simple linear combination of autotrophic and heterotrophic contributions. The outputs of the simulations were compared to ad hoc independent experiments carried out in different conditions of nutrients supply, including gas, and light intensity.

The values of the parameters used are summarized in Table 1, and results of simulations are displayed in Fig. 5.

In the first set of experiments, autotrophic conditions were applied to assess the capability of the model to simulate microalgal growth under different light intensities, based on the specific light supply rate (I_{sp}) function. In particular, three incident lights were simulated, namely 15, 70 and 130 µmol photons m^{-2} s^{-1}. As reported in Fig. 5a, the simulation results display a good correlation with experimental

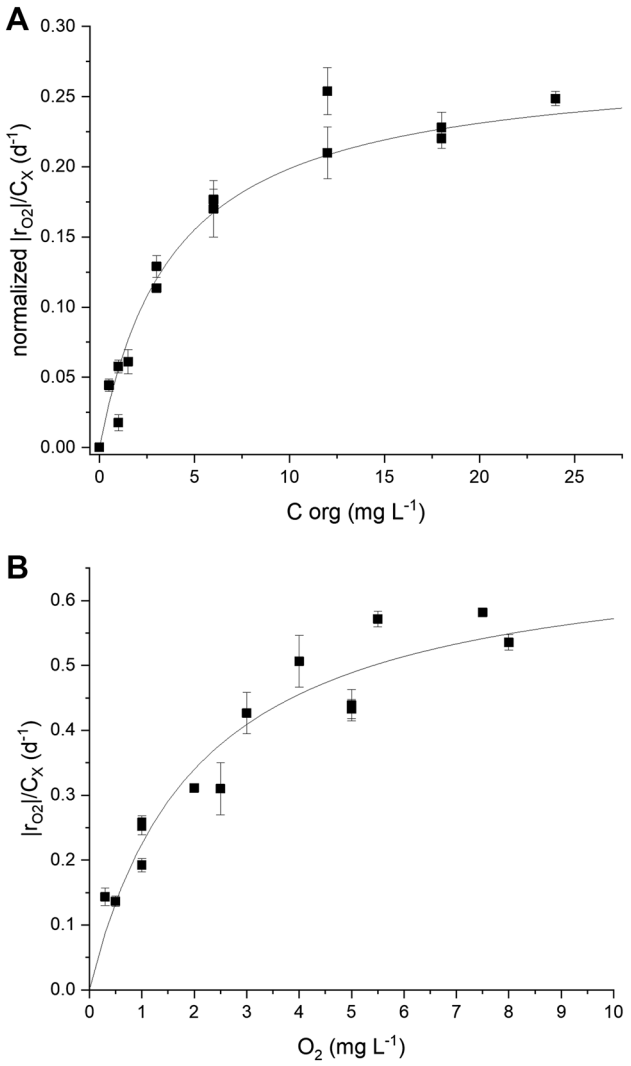

Fig. 4 Specific $|OPR_D|$ data normalized on biomass concentration as a function of organic carbon (**a**), In **b** data of specific $|OPR_D|$ are reported as a function of dissolved oxygen. Organic carbon was provided as sodium acetate. Fitting was obtained based on the minimization of the sum of squared errors by adjusting the parameters of Monod equation (Eq. 12)

data for all the three light conditions investigated. In these tests, the cultivation system was prepared with non-limiting nutrient concentrations and CO_2 was supplied in excess (a mixture of CO_2–air 5% v:v), so to address the effect of light only. In Fig. 5a, the effect of N and P limitation is also shown. In this case, carried out under an incident light intensity of 130 µmol photons m^{-2} s^{-1} and without CO_2 limitation, the initial N and P concentrations were reduced to 30 mg N L^{-1} and 3 mg P L^{-1}. A much lower growth was observed, which is also well predicted by the model, demonstrating the reliability of the measured parameters by the respirometric tests in describing the autotrophic growth of *C. protothecoides*.

A second set of experiments was aimed at understanding if the mixotrophy model proposed is able to correctly describe microalgal growth, and it can therefore be applied to design real cultivation systems for wastewater treatment. In particular, three conditions (autotrophic, heterotrophic and mixotrophic growth) were studied under gas limitation (no bubbling), to assess the hypothesis that the ratio of CO_2/O_2 produced or consumed by hetero/autotrophic rates can play a role in the regulation of the two metabolic pathways. The absence of an external bubbling will certainly result in gas limitation, so that it is possible to evaluate the effect of oxygen and carbon dioxide production/consumption on the mass balance of the system itself. In fact, as the Monod terms of CO_2 and O_2 are present in the auto- and heterotrophic kinetic equations, and the overall growth results from the sum of the two rates, if one of the two gases is limiting in the medium, it will limit the corresponding rate consequently. This aspect may be seminal in the case of application of microalgae and bacteria to wastewater treatment, showing that the gas mass transfer is an operating variable that may strongly influence the photosynthetic pathways of microalgae, and consequently, the net oxygen production [25].

As shown in Fig. 5B, the simulations reproduced satisfactorily the experimental biomass concentration profiles over

Table 1 Values of kinetic parameters implemented in AQUASIM simulation software to model microalgal growth

	Autotrophy	Heterotrophy
Growth rate	$\mu_{MAX,A} C_x \cdot \frac{C_{CO_2}}{K_{CO_2}+C_{CO_2}} \cdot \frac{C_N}{K_N+C_N} \frac{C_P}{K_P+C_P} \cdot \frac{I_{sp}}{K_L+I_{sp}+\frac{I_{sp}^2}{K_I}}$	$\mu_{MAX,H} C_X \frac{C_{O_2}}{K_{O_2}+C_{O_2}} \cdot \frac{C_N}{K_N+C_N} \cdot \frac{C_P}{K_P+C_P} \cdot \frac{C_{C_{org}}}{K_{CO}+C_{C_{org}}}$
μ_{MAX}	1.9 day^{-1}[a]	0.6 day^{-1}[a]
K_N	14 mg N L^{-1}	14 mg N L^{-1}
K_P	1.8 mg P L^{-1}	1.8 mg P L^{-1}
K_L	960 mmol photons g^{-1} day^{-1}	–
K_I	10,130 mmol photons g^{-1} day^{-1}	–
K_{CO_2}	1.3 mg C L^{-1}	–
K_{O_2}	–	2 mg O_2 L^{-1}
K_{CO}	–	3.89 mg C L^{-1}

[a]Parameters were obtained from batch growth curves reported as Online Resources

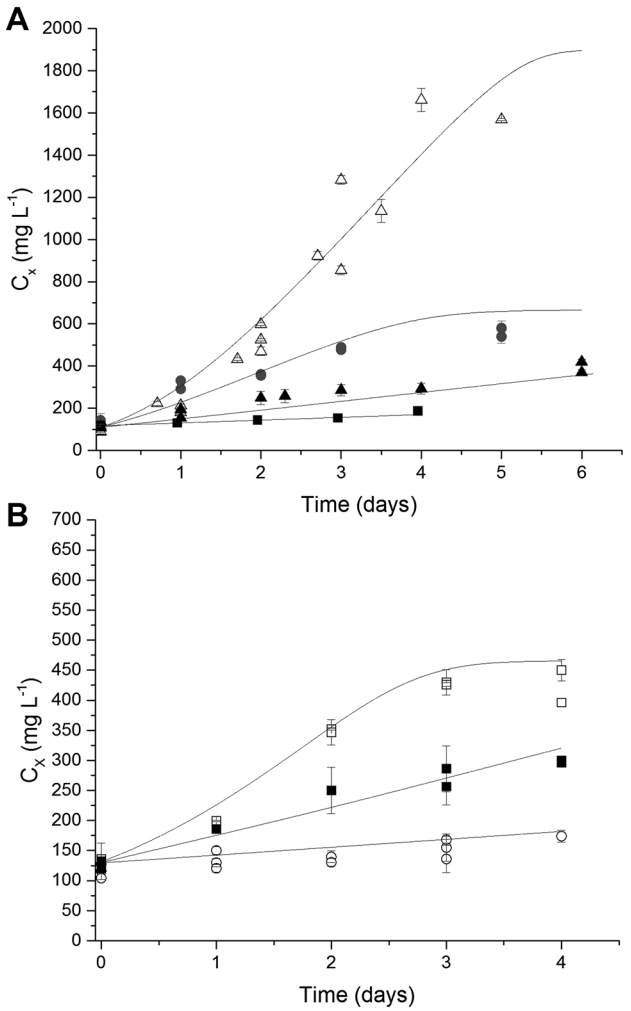

Fig. 5 Experimental (dots) and simulated (straight line) biomass growth curves under different external conditions. **a** Autotrophic growth at different values of incident light intensity: 15 (black triangles), 70 (dark circles) and 130 (open triangles) µmol photons m^{-2} s^{-1}; dark squares represent autotrophic growth at 130 µmol photons m^{-2} s^{-1} under limiting N and P conditions. **b** Autotrophic (open circles), heterotrophic (dark squares) and mixotrophic (open squares) growth with no air–CO_2 bubbling

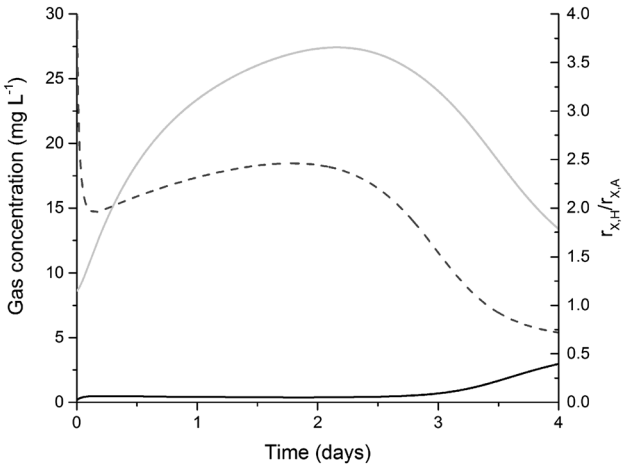

Fig. 6 Continuous lines represent oxygen (light gray) and carbon dioxide (black) concentration profiles in the liquid; dashed line represents the ratio between heterotrophic and autotrophic growth rates

time for all the three metabolisms, confirming the validity of the approach proposed as well as that of the measured parameters values.

Eventually, the simulated concentration profiles of oxygen and carbon dioxide in the liquid under mixotrophic conditions are reported in Fig. 6, together with the ratio between heterotrophic and autotrophic growth rates. It appears that the latter approximately follows the same trend of the gas availability, with the heterotrophic contribution being more relevant at lower CO_2 concentrations, and then decreasing when O_2 concentration drops and CO_2 increases. It suggests that O_2 and CO_2 concentrations are indeed strongly relevant for the regulation of the two metabolic pathways from a mass balance point of view. Certainly, the physiological regulation of mixotrophic pathways is more complex than a simple gas mass balance, but the simulation reported in this work suggests that the mass transfer of the cultivation system may play a role itself in the regulation of algal metabolism. This is corroborated by other experimental reports, where a reduction of organic carbon mixotrophic exploitation was observed under CO_2 bubbling [40] as well as under high light intensity [14, 22]. In this scenario, the gas exchange and mass balance of O_2 and CO_2 may itself play a role in the regulation of the metabolism, a fact that should be carefully accounted for when cultivating microalgae under mixotrophic conditions.

Conclusions

In this work, respirometry was proposed as a tool to develop a method to quickly measure nutrients half-saturation constants of *C. protothecoides*, under autotrophic, heterotrophic and mixotrophic conditions. The proposed protocol was first verified in autotrophic conditions, demonstrating that the value of kinetic parameters measured for nitrogen and light intensity is comparable to those obtained by conventional batch growth experiments. The protocol was then applied to assess heterotrophic and mixotrophic parameters based on acetate, analyzing the dependence on oxygen, organic carbon, as well as nitrogen and phosphorus concentration. Half-saturation constants of N and P were found not to be influenced by the carbon source and the corresponding metabolism. Eventually, mixotrophic growth was modeled as the combination of autotrophic and heterotrophic reactions. Using the measured kinetic parameters, the model was implemented in AQUASIM and validated by comparing

the simulation outputs with independent experimental batch growth curves, obtained with different external conditions. The results proved the reliability of the proposed respirometric protocol to derive kinetic parameters for nutrients.

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