Research Article

Doubling of Microalgae Productivity by Oxygen Balanced Mixotrophy

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ABSTRACT: Microalgae productivity was doubled by designing an innovative mixotrophic cultivation strategy that does not require gas–liquid transfer of oxygen or carbon dioxide. *Chlorella sorokiniana* SAG 211/8K was cultivated under continuous operation in a 2 L stirred-tank photobioreactor redesigned so that respiratory oxygen consumption was controlled by tuning the acetic acid supply. In this mixotrophic setup, the reactor was first operated with aeration and no net oxygen production was measured at a fixed acetic acid supply rate. Then, the aeration was stopped and the acetic acid supply rate was automatically regulated to maintain a constant dissolved oxygen level using process control software. Respiratory oxygen consumption was balanced by phototrophic oxygen production, and the reactor was operated without any gas–liquid exchange. The carbon dioxide required for photosynthesis was completely provided by the aerobic conversion of acetic acid. Under this condition, the biomass/substrate yield was 0.94 C-$mol_x \cdot C\text{-}mol_S^{-1}$. Under chemostat conditions, both reactor productivity and algal biomass concentration were doubled in comparison to a photoautotrophic reference culture. Mixotrophic cultivation did not affect the photosystem II maximum quantum yield (Fv/Fm) and the average-dry-weight-specific optical cross section of the microalgal cells. Only light absorption by chlorophylls over carotenoids decreased by 9% in the mixotrophic culture in comparison to the photoautotrophic reference. Our results demonstrate that photoautotrophic and chemoorganotrophic metabolism operate concurrently and that the overall yield is the sum of the two metabolic modes. At the expense of supplying an organic carbon source, photobioreactor productivity can be doubled while avoiding energy intensive aeration.

KEYWORDS: *Microalgae productivity, Biomass yield on substrate, Oxygen balance, Carbon balance, Mixotrophic cultivation*
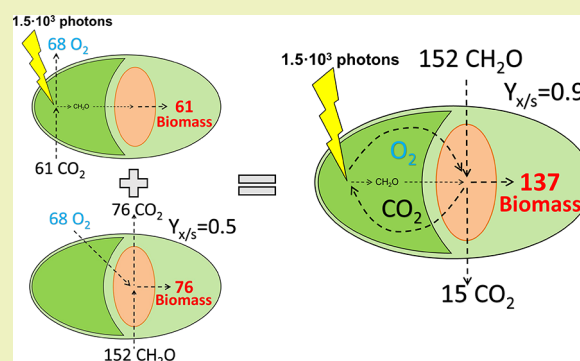
INTRODUCTION

The growing demand for food and fossil-derived products is placing increasing pressure on our current resources. This has resulted in an ongoing search for renewable resources and more environmentally friendly production processes.[1] In this scenario, microalgae are regarded as a high potential renewable feedstock.[2,3] The most common procedure for cultivating microalgae is photoautotrophic culture (henceforth referred to as autotrophic),[4] in which cells harvest light energy, use carbon dioxide (CO_2) as a carbon source, and release oxygen (O_2) as a byproduct.

Despite the advantage of CO_2 mitigation and use of solar energy, autotrophic cultures have limitations. In autotrophic cultures, light availability is the main growth limiting factor. Cellular self-shading hinders light availability limiting biomass production. To overcome this problem, low biomass concentrations are generally maintained in autotrophic cultures, reducing the volumetric productivity. Another limitation of autotrophic culture is the need for gassing demanding substantial energy. Gas–liquid transfer is necessary to avoid O_2 accumulation in the liquid culture and to provide the CO_2 required to run photosynthesis.

CO_2 supply is a frequently overlooked challenge in microalgae commercialization. Atmospheric CO_2 levels (~0.04% v/v) are not sufficient to support high biomass productivities because the driving force for gas–liquid transfer is too small and too high gas flows would be required.[5] For this reason, CO_2-enriched gas streams are provided to achieve high biomass productivity. Not all CO_2 provided is taken up, and in open ponds, up to 97% of the provided CO_2 might be lost to the atmosphere.[6] Even in optimized photobioreactors, CO_2 losses minimally are 25% in closed photobioreactors (PBRs)[7] and 50% in open ponds.[8] Anthropogenic CO_2-enriched gas (e.g., flue gas with 10–15% CO_2) is envisioned to meet the

requirement for large-scale production. However, considering the high CO_2 demand of a large-scale facility, without an extensive and costly infrastructure of CO_2 capture and transportation, only a limited amount of areas are suitable for large-scale production.[9]

An alternative to autotrophic cultures are chemoorganotrophic (henceforth referred to as heterotrophic) cultures in which organic carbons, such as sugars and organic acids, are used as carbon sources in the absence of light. In contrast to autotrophic cultures, heterotrophic cultures can be performed in conventional fermenters, requiring O_2 by intensive aeration, reaching higher concentration and productivity. Despite the high productivity, heterotrophic growth has been observed in a few microalgal species only. Moreover, darkness can lead to reduced pigmentation, limiting the potential of heterotrophic cultivation for the large-scale production of these phytochemicals.[10]

Autotrophic and heterotrophic cultivation of microalgae can be combined in mixotrophic cultivation. In this trophic mode, light and organic carbons are simultaneously exploited and both heterotrophic and autotrophic metabolism operate concurrently within a single microalgal monoculture. Mixotrophic cultivation offers several advantages that can overcome both autotrophic and heterotrophic limitations. In mixotrophic cultivation, the simultaneous presence of two energy sources (light and reduced organic carbon) can significantly increase biomass productivity.[11,12] Moreover, higher biomass concentration can be reached at a given light intensity, reducing downstream processing cost.[13,14] Recent studies also indicated that mixotrophic cultivation has the potential to drastically reduce the need of gas–liquid exchange,[9,15] since the O_2 required by aerobic heterotrophic growth can be covered by oxygenic photosynthesis. Vice versa, the CO_2 needed to carry on photosynthesis can be provided by the heterotrophic metabolism. This internal CO_2 recirculation will maximize the biomass yield on substrate,[12] making the process close to carbon neutrality. As a comparison, in a heterotrophic culture, typically 40–60% of the carbon is lost.[16] Moreover, preventing any gas–liquid exchange of oxygen and carbon dioxide greatly reduces the power required for the mixotrophic production process, as compared to either an autotrophic or a heterotrophic production process.

In order to minimize gas exchange, the heterotrophic and autotrophic contributions to the overall mixotrophic growth need to be equilibrated. Such balanced mixotrophic growth can only be obtained if the organic carbon supply rate is controlled. Unfortunately, batch experiments are dominant in the literature on mixotrophic cultivation of microalgae. In such dynamic batch processes, the dominance of the autotrophic and heterotrophic metabolism changes over time,[9] making balanced mixotrophic cultivation impossible. These batch dynamics might be a reason for contradictory conclusions in previous studies. Part of the studies agree on the fact that, during mixotrophic cultivation, autotrophic and heterotrophic metabolism can proceed noncompetitively and that the overall growth is the sum of the two metabolisms.[7,10,11] In other studies, both positive[15,17] and negative[18,19] interactions between the two metabolisms are reported.

The aim of the study is to design an oxygen balanced mixotrophic process that does not require any gas exchange. To this end, the model strain *C. sorokiniana* SAG211/8K was cultivated in a closed PBR, under continuous operation, trying to maintain constant dissolved oxygen concentration (DO) by tuning the acetic acid supply rate to the rate of photosynthesis. Special attention was given to the carbon balance to investigate the hypothesis that the mixotrophic metabolism is the sum of the heterotrophic and autotrophic metabolisms.

■ MATERIALS AND METHODS

Organism, Media, and Cultivation Conditions. *Chlorella sorokiniana* SAG 211/8k was obtained from the algae culture collection at Göttingen University (SAG) and cultivated in modified M-8 medium.[20] Medium composition can be found in Supporting Information 1. Axenic algal cultures were cryopreserved and stored in liquid nitrogen. Before reactor inoculation, cryopreserved cultures were defrosted and used to inoculate 250 mL flasks with 100 mL volume in an incubator operated at 37 °C, 4.5% v/v CO_2, and stirring at 100 rpm with a magnetic rod. In this incubator, the flasks were illuminated 24/24 from below with a warm-white LED (BXRAW1200, Bridgelux, USA) at a photon flux density (PFD, μmol m^{-2} s^{-1}) of 500 μmol m^{-2} s^{-1}. The PFD was measured with a LI-COR 190-SA 2π sensor (PAR-range: 400–700 nm). Based on this procedure, two inocula were prepared: an autotrophic inoculum on M8a medium and a mixotrophic inoculum on M8a supplemented with 3.41 g·L^{-1} of sodium acetate.

In the heterotrophic flask experiments, glucose, acetate, and glycerol were supplemented to the modified M8a medium. The heterotrophic experiments were started by using an autotrophic inoculum, and this culture was adapted to heterotrophic growth for at least 2 weeks using the three different substrates. Flasks were incubated at 37 °C in the darkness while being shaken at 250 rpm. The acclimated heterotrophic cultures were diluted the day before the experiment using the same M8a medium with organic carbon resulting in exponentially growing cultures, which were used as inoculum for the experiment.

Heterotrophic Flask Experiments. The heterotrophic biomass yield on substrate ($Y^{het}_{x/s}$, C-mol$_x$·C-mol$_s^{-1}$) was determined in dark batch experiments. Glucose, acetate, and glycerol were supplemented to the M8a medium based on their carbon molarity (C-mol·L^{-1}). In order to provide 83.3 C-mmol·L^{-1}, 2.75 g·L^{-1}, 3.4 g·L^{-1}, and 2.56 g·L^{-1} of glucose monohydrate, sodium acetate, and glycerol were used. Sodium acetate was tested also at double concentration (167 C-mmol·L^{-1}). Glucose and glycerol were sterilized by autoclaving, while acetate was sterilized by filtration. The pH was stabilized by adding 0.1 mol·L^{-1} of HEPES. The experiments were started at an optical density at 750 nm (OD$_{750}$) between 0.3 and 0.5.

During the experiments, samples were taken every 2 h until steady values were reached, indicating substrate depletion. The microalgae concentration was quantified measuring OD$_{750}$. For each sample, first OD$_{750}$ was measured and then 1 mL was centrifuged, and the supernatant was extracted and stored at −20 °C prior to analysis of substrate concentration by (U)HPLC.

The OD$_{750}$ was converted into dry weight (C_x, g$_x$·L^{-1}) using a linear regression (see analytical methods). At the end of each experiment, C_x was measured to verify that the correlation was still valid. The heterotrophic biomass yield on substrate $Y^{het}_{x/s}$ was calculated as follows

$$Y^{het}_{x/s} = -\frac{(C_{x_e} - C_{x_0})}{MW_x \cdot (S_e - S_0)} \tag{1}$$

where C_{x_0}/C_{x_e} and S_0/S_e are respectively the biomass and the substrate concentrations (C-mol·L^{-1}) at the start and end of the exponential phase, while MW$_x$ (g$_x$·mol$_x^{-1}$) is the weight of 1 carbon mole of biomass. MW$_x$ was determined at the end of the experiment, and it was assumed to be constant during the batch.

The specific growth rate (μ, h^{-1}) during exponential growth was calculated according to

$$\mu = \frac{\ln(C_{x_e}) - \ln(C_{x_0})}{t_e - t_0} \tag{2}$$

where t_0/t_e and C_{x_0}/C_{x_e} are, respectively, the time and the biomass concentration at the start and the end of the exponential phase. Experiments were performed in biological duplicates. The averages and standard deviations will be shown in graphs and tables.

Photobioreactor Setup and Experiments. *Chlorella sorokiniana* SAG 211/8k was grown in chemostat mode in a 3 L bioreactor (Applikon, The Netherlands) depicted in Figure 1. The fermenter had
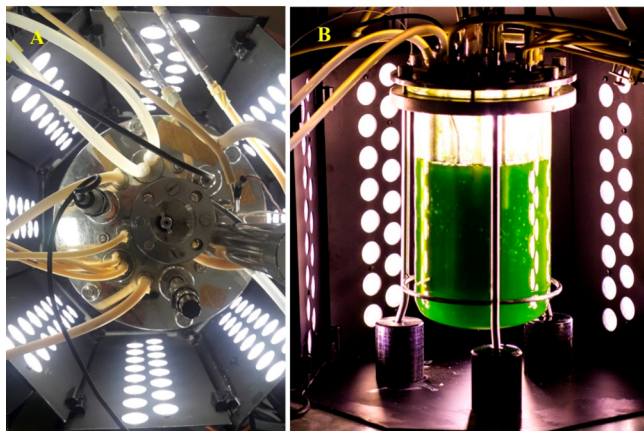

Figure 1. Top view (A) and side view (B) of the photobioreactor used in the study.

a working volume (V_{PBR}) of 1.923 L when aeration was provided and 1.975 L without aeration. The internal diameter was 0.130 m, while the culture height was maintained at 0.165 m by a level probe, resulting in an illuminated area (A_{PBR}) of 0.067 m^2. The cylindrical reactor was illuminated from all sides, resulting in a homogeneous light field over the vertical reactor surface. More specifically, a circular light source was constructed, consisting of eight vertical light panels placed around the fermenter as an octagon. Each panel was composed of 16 warm-white 3 W LEDs (Avago ASMT-MY22-NMP00, Broadcom, USA) equipped with a plastic lens with FHWM of 25.5° (Part no. 10393, Carclo-optics, UK). Light intensity on the reactor surface was measured at 16 fixed points inside the empty reactor prior to each experiment. The measured light intensities at all 16 points were averaged obtaining an average PFD of 498 ± 17 μmol m^{-2} s^{-1}.

The reactor was equipped with a dissolved oxygen (DO) sensor (VisiFerm DO ECS 225, Hamilton, USA). This DO sensor was calibrated inside the reactor filled with growth medium and sparged with dinitrogen gas, or air, to give a DO level of, respectively, 0 and 100%. The reactor was kept at 37 °C by a heat exchanger inside the reactor vessel. To prevent evaporation, the reactor was equipped with a condenser connected to a cryostat feeding cold water of 2 °C. Continuous stirring at 500 rpm was applied during all experiments. When aerated, air enriched with 2% v/v carbon dioxide was provided at a flow rate of 1 L·min^{-1} using mass flow controllers (Smart TMF 5850S, Brooks Instruments, USA). The pH was continuously measured and controlled at 6.7 by automatic base addition (1 M, NaOH).

Two reactor experiments were performed. In the first experiment, the reactor was operated mixotrophically inoculated at a density of 0.3 g$_x$·L^{-1} with a mixture of autotrophic and mixotrophic cultures. A 5% w/w acetic acid solution was supplied at a fixed rate while gassing the reactor with air enriched with CO$_2$ ("mixo with gas exchange"). After this phase, the aeration was stopped, resulting in a mixotrophic cultivation without gas exchange ("mixo without gas exchange") where the supply rate of acetic acid was automatically adjusted to maintain a DO of 135%. Before ending this first experiment, the acetic acid supply was stopped, the aeration was re-established, and the reactor was operated autotrophically ("autotrophic 1"). In the second experiment, the reactor was operated autotrophically ("autotrophic 2") inoculated with an autotrophic culture at a density of 0.3 g$_x$·L^{-1}.

The base solution, the acetic acid solution, and the harvest bottles were placed on analytic balances. The balances, DO sensor, temperature, pH sensor, and mass flow controllers were connected to a data acquisition system interfaced via a computer by means of a virtual instrument (Lab View, National Instruments, USA), allowing for continuous data logging and process control. Culture samples for off-line measurements were taken aseptically from the middle of the reactor through a dedicated port. The complete setup, including all of the solutions, was sterilized by autoclaving for 60 min at 121 °C. After inoculation, the reactor was operated in batch until a biomass density of about 1.5 g$_x$·L^{-1} was reached, and then, it was operated as a chemostat at a dilution rate of about 2 day^{-1}. Once the steady state was obtained, it was maintained for at least 4 consecutive days during which samples were taken for off-line measurements.

Photobioreactor Calculations. In the chemostat experiments, the volumetric biomass production rate (r_x, g$_x$·L^{-1}·day^{-1}) was calculated multiplying the measured biomass concentration (C_x, g$_x$·L^{-1}) with the measured dilution rate (D, day^{-1}). The r_x was also converted into its carbon equivalent (r_c, C-mol$_x$·L^{-1}·day^{-1}) by dividing r_x by the molecular weight of 1 C-mol of biomass (MW$_x$, g$_x$·C-mol$_x^{-1}$). The MW$_x$ was determined in each sample taken from the reactor. In the two autotrophic experiments, r_c was used to determine the biomass yield on light ($Y_{x/ph}$, C-mol$_x$·mol$_{ph}^{-1}$) according to the formula

$$Y_{x/ph} = \frac{r_{c,auto} \cdot V_{PBR}}{PFD \cdot A_{PBR}} \tag{3}$$

In the mixotrophic experiments, the volumetric substrate consumption rate (r_s, C-mol$_s$·L^{-1}·day^{-1}) was calculated as follows, assuming ideal mixing

$$r_s = \frac{F_{AA} \cdot C_{s,AA} - D \cdot V_{PBR} \cdot C_s}{V_{PBR}} \tag{4}$$

where F_{AA} (L·day^{-1}) and $C_{s,AA}$ (C-mol$_s$·L^{-1}) represent, respectively, the supply rate of the acetic acid solution and the concentration of the acetic acid solution, while C_s (C-mol$_s$·L^{-1}) is the acetic acid concentration in the reactor (C-mol$_s$·L^{-1}).

The mixotrophic yield on substrate ($Y^{mixo}_{x/s}$, C-mol$_x$·mol$_s^{-1}$) was calculated dividing r_c by r_s.

■ ANALYTICAL METHODS

Culture Sampling and Off-Line Measurements. Samples were taken aseptically multiple times per day for off-line measurements. Two 1 mL aliquots were centrifuged at 20238 RCF for 10 min. The supernatant fractions were stored at −20 °C until analysis, while the pellet was washed with demineralized water and cooled to −20 °C, lyophilized, and stored. Extra aliquots of sample were taken from the reactor to quantify the total inorganic carbon concentration (TIC) in the medium. To avoid CO$_2$ stripping, immediately after centrifugation, 950 μL of the supernatant fraction was alkalized by addition of 50 μL of base (2 M, NaOH). Alkalized samples were stored at −20 °C until analysis.

Dry Weight Concentration. Culture growth was estimated by biomass dry weight (C_x, g$_x$·L^{-1}) determination: aliquots of the culture (2.5−5 mL) were diluted to 25 mL with demineralized water and filtered over preweighed Whatman GF/F glass microfiber filters (diameter of 55 mm, pore size of 0.7 μm). The filters were washed with deionized water (25 mL) and dried at 105 °C until constant weight.

Optical Density. The optical density was measured in duplicate on a spectrophotometer (DR6000, Hach-Lange, USA) at 680 and 750 nm. The relationship between C_x and OD$_{750}$ was determined with biomass grown heterotrophically in a range of 0.1−2 g$_x$·L^{-1} by filtering at least 5 mg of algal dry biomass onto preweighed glass fiber filters (Whatman GF/F, GE Healthcare UK Ltd., UK) which were dried overnight at 105 °C until constant weight. This resulted in the following correlation:

C_x (g$_x$·L^{-1}) = 0.48·OD$_{750}$ (R^2 = 0.98)

Cell Concentration and Biovolume. Cell biovolume and concentration were measured using the Multisizer III (Beckman Coulter Inc., USA) with a 50 μm aperture tube. Samples were diluted in ISOTON II diluent. The measured cellular biovolume was converted to cell diameter assuming spherical cells.

Average-Dry-Weight-Specific Optical Cross Section. The average-dry-weight-specific optical cross section (a_x, $m^2 \cdot kg^{-1}$) was measured and calculated according to de Mooij et al.[21] using the absorbance from 400 to 750 nm with a step size of 1 nm. The absorbance was measured in a UV−vis/double beam spectrophotometer (Shimadzu UV-2600, Japan) equipped with an integrating sphere (ISR-2600). Cuvettes with an optical path of 2 mm were used.

Photosystem II Quantum Yield. The photosystem II maximum quantum yield (QY, Fv/Fm) was measured at 455 nm with an AquaPen-C AP-C 100 instrument (Photon Systems Instruments, Czech Republic). Prior to the measurement, samples were adapted to darkness for 15 min at room temperature and diluted to an OD750 between 0.3 and 0.5.

Acetic Acid and Glucose Determination. Acetic acid and glucose concentrations were determined using an Agilent 1290 Infinity (U)HPLC equipped with a guard column (Security Guard Cartridge System, Phenomenex, USA). The compounds were separated on an organic acid column (Rezex ROA-Organic acid H+ 8% column, Phenomenex, USA) at 55 °C with a flow of 0.5 mL·min^{-1} 0.005 M H_2SO_4 as eluent. A final concentration of 50 mM propionic acid was used as the internal standard.

Total Organic and Total Inorganic Carbon. The organic carbon content in the pellet was measured as total carbon ($g_c \cdot L^{-1}$) using a TOC-L analyzer (Shimadzu, Japan). Possible traces of inorganic carbon in the lyophilized pellet were removed by resuspending the pellet in 1 mL of HCl (1 M) and sonicating the solution at 80 kHzQ 40 °C for 30 min. After this treatment, samples were diluted 10 times in demi water and immediately placed in the TOC-L analyzer. The biomass carbon content ($C_\%$, % $w_c \cdot w_x^{-1}$) was calculated by dividing the obtained total carbon by the C_x determined on the same sample. The $C_\%$ was used to determine the biomass molecular weight (MW_x, $g_x \cdot C$-mol_x^{-1}). MW_x was determined by dividing the carbon molecular weight (12.011 $g_c \cdot C$-mol^{-1}) by $C_\%$. The TIC was measured in the undiluted supernatant with the TOC-L analyzer.

Assessment of Bacterial Contaminant. During the experiment, axenicity was checked daily by DNA staining of culture samples with SYBER Green I (Sigma-Aldrich, USA) and fluorescence microscopy (EVOS FL auto, Thermo Fisher Scientific, USA).

Statistical Analysis. Propagation of errors was calculated according to eq 5 and eq 6 for sum and multiplication operations, respectively, to obtain the error

$$\Delta z = \sqrt{\Delta x^2 + \Delta y^2 + ...} \tag{5}$$

$$\frac{\Delta z}{x} = \sqrt{\frac{\Delta x^2}{x} + \frac{\Delta y^2}{y} + ...} \tag{6}$$

where Δx is the absolute error associated with the value x and so on.

Reproducibility of duplicates was performed by t test. Significant differences between different conditions were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test, using the software Graph Pad Prism 5.00 (GraphPad Prism Software, San Diego, USA). The significance level was $P < 0.05$.

RESULTS AND DISCUSSION

Heterotrophic Reference Experiments. Strict heterotrophic and autotrophic reference experiments were conducted to determine the heterotrophic biomass yield on substrate ($Y^{het}_{x/s}$) and the biomass yield on photons ($Y_{x/ph}$).

The $Y^{het}_{x/s}$ was determined in heterotrophic batch experiments where *C. sorokiniana* was grown in darkness in modified M8a medium supplemented with three organic substrates:

glucose, glycerol, and acetate. Since the inoculum was obtained from an autotrophic culture, the culture expressed a lag phase of about 48 h (data not shown). After this lag phase, the cultures on glucose or acetate grew exponentially, while no growth was observed on glycerol (data not shown).

Heterotrophic biomass production and substrate consumption are reported in Figure 2. At 100 C-$mmol_s \cdot L^{-1}$, cultures
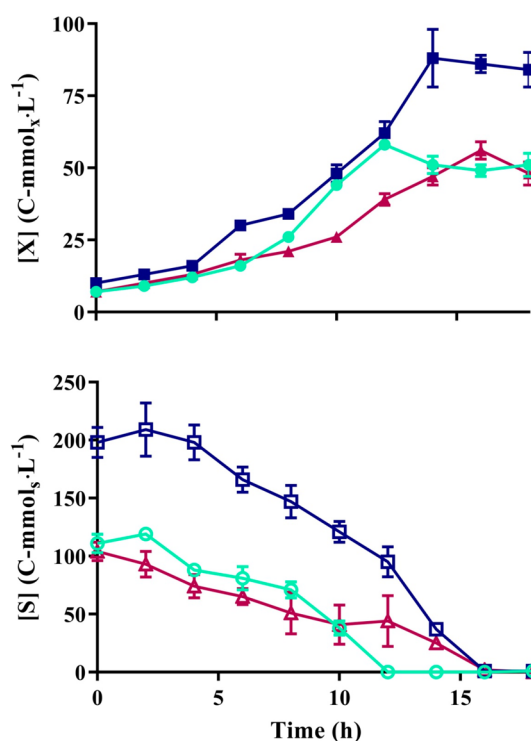
Figure 2. Heterotrophic biomass production (dark) and substrate consumption (open) of *C. sorokiniana* SAG 211/8K cultivated in M8a medium with 100 C-mmol·L^{-1} sodium acetate (circles), 200 C-mmol·L^{-1} sodium acetate (squares), and 100 C-mmol·L^{-1} glucose (triangles).

grew exponentially until the substrate was completely consumed. Both substrates, glucose and acetate, resulted in a biomass concentration of 57 C-$mmol_x \cdot L^{-1}$ corresponding to a $Y_{x/s}$ value of 0.49 ± 0.06 (C-$mol_s \cdot C$-mol_s^{-1}). A significant ($P < 0.05$) difference in the specific growth rate (μ) was found for growth on glucose and acetate: on glucose, μ was 0.14 ± 0.00 h^{-1}, whereas, on acetate, it was 0.18 ± 0.00 h^{-1}. Given its better performance, acetate was also tested at 200 C-mmol·L^{-1} (Figure 2). At this concentration, the culture grew exponentially for 14 h and a slightly higher $Y_{x/s}$ was found (0.51 ± 0.05 C-$mol_x \cdot C$-mol_s^{-1}), although it was not statistically significant ($P > 0.05$). The specific growth rate μ (0.15 ± 0.00 h^{-1}) was comparable with the μ obtained using glucose but significantly lower than the one obtained at 100 C-$mmol_s \cdot L^{-1}$ of acetate ($P < 0.05$). In summary, a $Y_{x/s}$ value of 0.50 ± 0.04 C-$mol_x \cdot C$-mol_s^{-1} was obtained on both glucose and acetate. This value falls in the middle of the range 0.40−0.63 C-$mol_x \cdot C$-mol_s^{-1} reported for this microalgal species,[16] and it will be used for further calculations. The maximal $Y^{het}_{x/s}$ for aerobic heterotrophic organisms is 0.7 mol_x mol_s^{-1}, and it is bound by thermodynamic constraints.[22] A yield of 0.7 C-$mol_x \cdot C$-mol_s^{-1} has also been found in *Chlamydomonas reinhardtii*[23] and in *Scenedesmus acuminatus*,[24] indicating that microalgae are as

Table 1. Overview of the Off-Line, DO, D Measurements on the Cultivation of *C. sorokiniana* SAG 211/8K under Autotrophic Conditions and under Mixotrophic Conditions with and without Gas Exchange[a]

	unit	mixo with gas exchange	mixo without gas exchange	autotrophic 1	autotrophic 2
DO	air saturation %	92.3 ± 1.9[a]	141.8 ± 5.4[b]	135.7 ± 2.6[c]	132.7 ± 1.6[c]
TIC_{in}	C-mmol L^{-1}	n.d.	0.04 ± 0.02	n.d.	n.d.
TIC_{out}	C-mmol L^{-1}	n.d.	3.73 ± 0.45	n.d.	n.d.
C_x	g$_x$·L^{-1}	1.41 ± 0.07[a]	1.16 ± 0.06[b]	0.70 ± 0.07[c]	0.70 ± 0.06[c]
D	day^{-1}	1.94 ± 0.00[a]	2.44 ± 0.00[b]	2.07 ± 0.00[a]	2.11 ± 0.00[a]
r_x	g·L^{-1}·day^{-1}	2.74 ± 0.07[a]	2.78 ± 0.06[a]	1.41 ± 0.07[b]	1.48 ± 0.06[b]
$C_{\%}$	% w$_c$·w$_x^{-1}$	51.0 ± 2.8[a]	54.2 ± 2.5[b]	51.1 ± 1.4[a]	50.8 ± 2.4[a]
cell volume	μm^3·cell^{-1}	23.6 ± 1.1[a]	22.8 ± 3.3[a]	39.5 ± 5.7[b]	33.5 ± 3.0[b]
a_x	m^2·kg^{-1}	249 ± 11[a]	271 ± 13[a,b]	272 ± 11[b]	323 ± 30[c]
Chl/Car		0.53 ± 0.00[a]	0.55 ± 0.00[b]	0.60 ± 0.00[c]	0.60 ± 0.00[c]
QY	Fv/Fm	0.70 ± 0.00[a]	0.77 ± 0.01[b]	0.77 ± 0.01[b]	0.77 ± 0.01[b]

[a]Along the rows, the same letter indicates no significant differences ($P > 0.05$). Not determined (n.d.).

efficient as other heterotrophic organisms (bacteria and yeasts) in the aerobiotic conversion of substrate into biomass.

Autotrophic Reference Experiments. $Y_{x/ph}$ was determined in two strictly autotrophic cultures. The first autotrophic culture was performed after 2 weeks of mixotrophic cultivation, called autotrophic 1, while the second experiment, called autotrophic 2, was performed in an independent experiment. All of the experiments, including the mixotrophic cultures, were conducted in a chemostat at the same dilution rate and light regime.

The two autotrophic cultures reached a stable and identical biomass concentration (C_x) of 0.7 g·L^{-1} and a volumetric biomass production rate (r_c) of 61.4 C-mmol$_x$·L^{-1}·day^{-1} (Figure 2, Table 1). Likewise, none of the other parameters measured (Table 1) showed any statistical difference ($P < 0.05$) between the two cultures. These results indicate that the autotrophic 1 was not affected by the previous 2 weeks of mixotrophic cultivation and that the experimental setup and routines were reproducible. The only exception was the average-dry-weight-specific optical cross section (a_x) that was 16% higher in the autotrophic 2 compared to the autotrophic 1. *C. sorokiniana* is known to adjust its a_x in a few hours in response to changes in the light regimes;[25] for this reason, the difference in a_x between the two autotrophic cultures might have been caused by unknown batch to batch variation rather than the shift between mixotrophic and autotrophic metabolism.

In summary, a $Y_{x/ph}$ value of 40.7 ± 0.0 C-mmol$_x$·mol$_{ph}^{-1}$ was obtained in the two autotrophic experiments, which is equivalent to 0.98 g$_x$·mol$_{ph}^{-1}$. This $Y_{x/ph}$ is 33% lower than the maximum reported value (54.2 C-mmol$_x$·C-mol$_{ph}^{-1}$) for this strain.[26] We used the light model developed by Evers for cylindrical vessels[27] to estimate the attenuation of the light intensity, caused by cellular light absorption, from the reactor surface toward the reactor center. Details of this calculation are presented in Supporting Information 2. The model requires as input the biomass concentration (C_x) and a_x. The values reported in Table 1 were used to calculate the light gradient for each condition tested. The model clearly indicates that, under all of the conditions tested, over 64% of the culture volume was experiencing a light level below 10 μmol m^{-2} s^{-1} which we assumed to be the compensation point of photosynthesis.[16] Under this light regime, suboptimal $Y_{x/ph}$ is expected, since the culture spends a relevant part of the light absorbed for maintenance purposes rather than for growth, lowering the overall biomass yield on light. Despite being suboptimal, this

light regime is within the range of light conditions prevalent within outdoor PBRs. For example, tubular PBRs have diameters between 5 and 9 cm and are operated at biomass concentrations of 1.3–2.1 g$_x$·L^{-1}.[3,7] In PBRs, yields of 0.6–0.8 g$_x$·mol$_{ph}^{-1}$ have been obtained, similar to the one reported in this study. Furthermore, outdoor vertical PBRs, experience maximal incident light intensities close to the light intensity tested in our study,[26] making our study comparable to outdoor microalgae production. Nevertheless, a direct extrapolation of the present work to large-scale outdoor production is complicated and is beyond the scope of this study.

Mixotrophic Growth and Oxygen Balance. In the two mixotrophic experiments, the carbon based volumetric biomass production rate (r_c) was the double of the autotrophic references (Figure 3, Table 1). We indeed expected that in a mixotrophic culture the presence of two energy sources (light and reduced organic carbon) would lead to a significant increase of productivity.[11,12] The extent of this increase can be quantified assuming that, in mixotrophy, the autotrophic and heterotrophic metabolism can proceed noncompetitively and
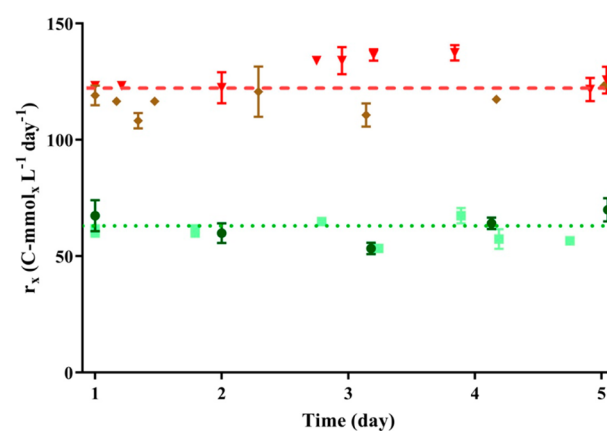

Figure 3. Volumetric carbon based biomass production rate (r_c) of *C. sorokiniana* SAG 211/8K grown mixotrophically with gas exchange (diamonds) and without gas exchange (triangles). Also included are two autotrophic reference cultures: autotrophic 1 (squares), which was carried out immediately after 2 weeks of mixotrophic growth, and autotrophic 2 (circles), which was carried out as a second independent experiment. The dashed line indicates the average r_c in mixotrophic cultivation (top) and in the two autotrophic cultures (bottom).

that the overall growth is the sum of the two metabolisms. In the next section, we will elucidate in detail this hypothesis.

The volumetric biomass production rate in mass units (r_x) also doubled under mixotrophy in comparison to autotrophy because the biomass carbon content was constant under all conditions tested (51−54% $w_c \cdot w_x^{-1}$, Table 1). Biomass productivity is the product of biomass concentration and dilution rate. This implies that, at steady state and constant dilution rate, biomass productivity can only be doubled by doubling the biomass concentration. The mixotrophic experiment with gas exchange and the two autotrophic experiments were performed at an identical dilution rate, and as expected, the biomass concentration of the mixotrophic experiment with gas exchange was about double the autotrophic cultures. Unfortunately, this was not the case in the mixotrophic culture without gas exchange where the biomass productivity was doubled but the biomass concentration was only 46% higher than the autotrophic reference. This discrepancy can be explained having a close look to the dilution rate (D). D was comparable among the cultures with gas exchange, while it was 22% higher in the mixotrophic culture without gas exchange (Table 1). The different D has been caused by the outlet pump that operated at constant rpm. In the culture with gas exchange, the outlet pump was removing a mixture of liquid and gas. Instead, without gas exchange, only the liquid phase was pumped out of the reactor, increasing the volume removed from the reactor per rpm of the outlet pump. Dissolved oxygen and biomass concentration over the entire experiments 1 and 2 are reported in Supporting Information 3.

A surprising result was that in the two mixotrophic experiments all of the substrate was completely converted into biomass, resulting in a mixotrophic biomass yield on substrate ($Y^{mixo}_{x/s}$) of 1 (Table 2). This finding implies the

Table 2. Carbon Mass Balance of *C. sorokiniana* SAG 211/8K Grown Mixotrophically with and without Gas Exchange[a]

	mixo with gas exchange	mixo without gas exchange	prediction
$r_{c,mixo}$	116.3 ± 0.0	127.9 ± 0.0	136.9
$C_s \cdot D$	3.2 ± 0.2	0.00 ± 0.00	
r_s	−111.5 ± 0.2	−126.5 ± 3.3	−151.8
$r_{c,het'}$	54.8 ± 0.0	66.5 ± 0.0	75.9
$Y^{het'}_{x/s}$	0.46 ± 0.00	0.53 ± 0.00	0.5
r_{CO_2}	n.d.	9.0 ± 0.01	14.9
$Y^{mixo}_{x/s}$	1.04 ± 0.00	1.01 ± 0.00	0.90
$Y^{mixo'}_{x/s}$	n.d.	0.94 ± 0.00	

[a]The values predicted by the summation of autotrophic and heterotrophic stoichiometry assuming oxygen balance are reported as a comparison. Yields are expressed as $C\text{-mol}_x \cdot C\text{-mol}_s^{-1}$, while all of the other parameters are expressed as $C\text{-mmol} \cdot L^{-1} \cdot day^{-1}$. Not determined (n.d.).

absence of CO_2 production. Such a hypothesis was verified in the mixotrophic experiment without gas exchange, where the CO_2 production rate (r_{CO_2}, $C\text{-mol} \cdot L^{-1} \cdot day^{-1}$) can be estimated by measuring the total inorganic carbon concentration (TIC, $C\text{-mol} \cdot L^{-1}$) in the liquid phase

$$r_{CO_2} = (TIC_{out} - TIC_{in}) \cdot D \tag{7}$$

where TIC_{in} and TIC_{out} are the total inorganic carbon concentration in the inlet and outlet medium. The TIC_{in} was measured once in medium in equilibrium with air and assumed constant over the whole experiment, while the TIC_{out} was

measured daily. The TIC_{in} was about 2 orders of magnitude smaller than the TIC_{out} (Table 1), clearly indicating CO_2 production (Table 2). The r_{CO_2} calculated was used to correct the mixotrophic yield on substrate ($Y^{mixo*}_{x/s}$, $C\text{-mol}_x \cdot C\text{-mol}_s^{-1}$) according to the following formula:

$$Y^{mixo*}_{x/s} = \frac{P_C - P_{CO_2}}{P_s} \tag{8}$$

Using this correction, $Y^{mixo}_{x/s}$ in the mixotrophic culture without gas exchange decreased from 1 to 0.94 $C\text{-mol}_x \cdot C\text{-mol}_s^{-1}$, which fits with our expectations, as explained later.

Complete substrate conversion cannot be excluded in the mixotrophic experiment with gas exchange. A close look at the volumetric substrate consumption rate (r_s) (Table 2) reveals that in the mixotrophic experiment with gas exchange r_s was 16% lower than in the mixotrophic without gas exchange. In the experiment with gas exchange, r_s was empirically adjusted to maintain the DO constant around the value measured under the same conditions with only medium. In this empirical approach, the provided feeding rate might not have been enough to balance the photosynthetic oxygen production rate, but instead, it might have balanced photosynthetic carbon dioxide consumption. Using the stochiometric eqs 9, 10, and 11 that will be presented in the next section, it is possible to predict the net oxygen production rate in the scenario case of CO_2 balance. We calculated a rate of 13.3 mmol of O_2 L^{-1} day^{-1}. Although this is a plausible scenario, oxygen production did not result in a significant increase of the DO. Possibly the increase in DO was too low to be detected.

In our mixotrophic experiments, we were able to reach almost complete substrate to biomass conversion. This is much higher than the biomass yields on substrate ($Y^{mixo}_{x/s}$) of 0.5−0.7 $C\text{-mol}_x \cdot C\text{-mol}_s^{-1}$ that are generally reported in mixotrophic experiments operated in batch.[28,29] Our higher performance can be explained looking at the mixotrophic stoichiometry (see next section). In an oxygen balanced mixotrophic culture, the autotrophic and heterotrophic metabolisms are operating concurrently almost at the same rate, equally contributing to the overall energetics of the cell. Previous studies on mixotrophic cultivation[28,29] were often unbalanced with a larger heterotrophic contribution to the mixotrophic growth in comparison to autotrophic metabolism. Under this condition, the heterotrophic metabolism dominates, and the culture produces CO_2 at a higher rate compared to the photosynthetic needs. In contrast to these batch approaches, Barros et al.[30] applied a fed-batch and reached a higher $Y^{mixo}_{x/s}$. In this work, the acetic acid supply was added stepwise and coupled to the incident solar radiation to ensure that excess organic carbon substrate did not accumulate in the culture medium. Using this approach, the authors obtained a $Y^{mixo}_{x/s}$ value of 0.94 $C\text{-mmol}_x \cdot C\text{-mol}_s^{-1}$, which is equal to the yield found in our mixotrophic culture without gas exchange.

In the mixotrophic experiment without gas exchange, we demonstrated that is possible to regulate and maintain constant dissolved oxygen (DO) levels by automatically regulating substrate feeding. Using this approach, the reactor was operated for several days without any gas exchange. Furthermore, in automated feeding, the substrate was completely consumed, while when the substrate was fed at a constant rate 3% of the supplied substrate was not consumed (Table 2). The better performance of the automatic feeding can be explained by looking at the regular fluctuations of the

DO around the preset value (Supporting Information 3). These fluctuations were caused by the variation in the substrate feeding rate (r_s), that increased when the DO exceeded the preset value. Most likely, this increase in DO was a sign of complete substrate consumption.

The DO is often used to dose the substrate supply in heterotrophic fed-batch cultivations,[31] but this feeding strategy is not commonly used in mixotrophic cultivation of microalgae. Ganuza et al. in a patent application[32] claimed to automatically control DO and pH by adjusting the acetic acid and the CO_2 feeding rate. The authors claimed to couple DO and pH control by feeding acetic acid when DO and pH simultaneously exceeded the set point, while CO_2 was provided to lower the pH when only the pH exceeded the set pH. Although the DO can be successfully controlled using this strategy, coupling pH and DO control has some limitations. For example, this strategy can only be applied when the overall stoichiometry of the process consumes protons (e.g., when nitrate is used as a nitrogen source). In addition, the substrate must be an organic acid with a pK_a lower than the pH of the culture (e.g., acetic acid to control the pH around neutrality). In our process, by decoupling pH and DO control, these limitations are solved, allowing any type of substrate (e.g., glucose) to be used and any type of mixotrophic microalgae (e.g., acidophilic strains) to be cultivated.

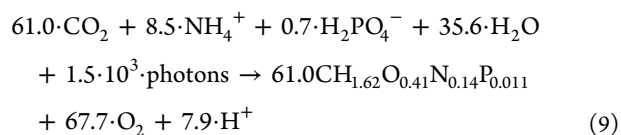**Mixotrophic Growth Stoichiometry and Interaction between Heterotrophic and Autotrophic Metabolism.** In this section, we will describe mixotrophic growth stoichiometry and investigate the hypothesis that mixotrophic metabolism is the sum of heterotrophic and autotrophic metabolisms. According to this hypothesis, mixotrophic stoichiometry can be split into two components:

(1) A heterotrophic component in which an organic substrate is partly oxidized to derive energy to support biomass growth (catabolism) and partly used as a building block for growth (anabolism). In the overall reaction, CO_2 is produced and O_2 consumed.

(2) An autotrophic component in which light is used as an energy source, CO_2 is consumed and used as a building block, and O_2 is produced as a waste product.
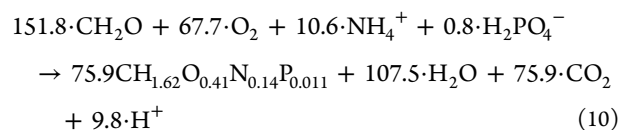
To describe the stoichiometry of autotrophic and heterotrophic cultivations, the biomass elemental composition, the heterotrophic biomass yield on substrate ($Y^{het}_{x/s}$), and the autotrophic biomass yield on light ($Y_{x/ph}$) need to be known. If the mixotrophic metabolism is the sum of the heterotrophic and autotrophic metabolisms, the yield factors can be assumed to be constant regardless of the trophic mode. The elemental biomass composition of $CH_{1.62}O_{0.41}N_{0.14}P_{0.01}$ reported by Kliphuis et al.[33] was used as a reference. $Y^{het}_{x/s}$ and $Y_{x/ph}$ were measured in this study and were, respectively, 0.50 ± 0.04 C-mol$_x$·C-mol$_s^{-1}$ and 40.7 ± 0.0 C-mmol$_x$·mol$_{ph}^{-1}$.

It has to be stressed that these numbers reflect nutrient replete growth conditions using ammonia as a nitrogen source. Under nitrogen limited conditions, storage compounds such as carbohydrates and lipids accumulate and the relative contribution of proteins in algal biomass decreases. Consequently, the elemental composition and the growth stoichiometry will change. At the same time, algal growth rates decline. An analysis of mixotrophy under these specific conditions was outside of the scope of our study.
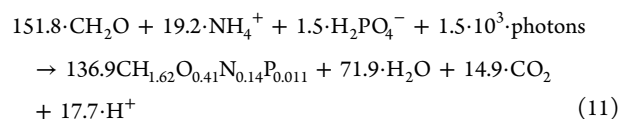
The autotrophic stoichiometry, given a light input of 1.5 mol$_{ph}$·L^{-1}·day^{-1}, can be written as

$$61.0 \cdot CO_2 + 8.5 \cdot NH_4^+ + 0.7 \cdot H_2PO_4^- + 35.6 \cdot H_2O$$
$$+ 1.5 \cdot 10^3 \cdot photons \rightarrow 61.0 CH_{1.62}O_{0.41}N_{0.14}P_{0.011}$$
$$+ 67.7 \cdot O_2 + 7.9 \cdot H^+ \tag{9}$$

where all stoichiometric coefficients reflect volumetric rates in mmol·L^{-1}·day^{-1} observed in the photobioreactor (PBR). Similarly, the heterotrophic stoichiometry can be set up where the oxygen consumption is set equal to the oxygen production according to the autotrophic part of the metabolism shown above:

$$151.8 \cdot CH_2O + 67.7 \cdot O_2 + 10.6 \cdot NH_4^+ + 0.8 \cdot H_2PO_4^-$$
$$\rightarrow 75.9 CH_{1.62}O_{0.41}N_{0.14}P_{0.011} + 107.5 \cdot H_2O + 75.9 \cdot CO_2$$
$$+ 9.8 \cdot H^+ \tag{10}$$

Adding up the autotrophic and heterotrophic stoichiometry, the following overall reaction equation is obtained reflecting mixotrophic growth under O_2 balance:

$$151.8 \cdot CH_2O + 19.2 \cdot NH_4^+ + 1.5 \cdot H_2PO_4^- + 1.5 \cdot 10^3 \cdot photons$$
$$\rightarrow 136.9 CH_{1.62}O_{0.41}N_{0.14}P_{0.011} + 71.9 \cdot H_2O + 14.9 \cdot CO_2$$
$$+ 17.7 \cdot H^+ \tag{11}$$

Also, in this equation, the stoichiometric coefficients reflect the volumetric rates in mmol·L^{-1}·day^{-1} expected in the PBR.

According to the mixotrophic stoichiometry, the volumetric biomass production rate ($r_{c,mixo}$) was expected to be 136.9 mmol·L^{-1}·day^{-1}. This value closely matches the $r_{c,mixo}$ of 127.9 mmol·L^{-1}·day^{-1} found in the mixotrophic culture without gas exchange. In the mixotrophic with gas exchange, the biomass production rate was 116.3 mmol·L^{-1}·day^{-1}, which is 15% lower than the expected value (Table 2). The lower performance of the mixotrophic with gas exchange can be explained by a lower substrate consumption rate (r_s), which can be attributed to the empirical approach used to set the feeding rate, as discussed in the previous section.

In order to correct for the different r_s, we calculated the fraction of biomass heterotrophically produced during the mixotrophic growth ($r_{c,het'}$, C-mol$_x$·L^{-1}·day^{-1}). The $r_{c,het'}$ value was calculated by subtracting the autotrophic biomass productivity ($r_{c,auto}$) to $r_{c,mixo}$. The $r_{c,het'}$ was then used to calculate the heterotrophic yield on substrate occurring in mixotrophy ($Y^{het'}_{x/s}$) according to the equation

$$Y^{het'}_{x/s} = \frac{r_{C,het}}{S_c} \tag{12}$$

A close look at the numbers in Table 2 reveals that $Y^{het'}_{x/s}$ in both of the mixotrophic experiments was equal to the biomass yield on substrate found in the heterotrophic reference experiment, supporting our hypothesis that the mixotrophic stoichiometry is the sum of the heterotrophic and autotrophic metabolism.

The finding that mixotrophy can be described as the sum of heterotrophic and autotrophic metabolisms implies that photosynthesis is not affected by the presence of organic substrate. In our experiments, the effect of organic carbon on photosynthesis was assessed by measuring the photosynthetic efficiency of PSII directly as the quantum yield (QY) and by measuring the average-dry-weight-specific optical cross section (a_x). In the mixotrophic culture without gas exchange, the QY was 0.77, the same value found in the two autotrophic cultures

(Table 1), indicating that photosynthesis is not affected by the presence of organic substrate. In the mixotrophic culture with gas exchange, the QY was 0.70. Despite that this value is lower than the QY under the other conditions, a QY of 0.70 still indicates optimal PSII performance.[25]

The average-dry-weight-specific optical cross section (a_x) obtained in all of the experiments falls in the range that is normally observed in mass culture for this strain.[25] Also, a_x was constant along all experiments regardless of the trophic mode, a further indication that the heterotrophic and autotrophic metabolism can operate concurrently without affecting each other.

The absorption spectra recorded to calculate the average-dry-weight-specific optical cross section were also used to detect possible changes in pigment ratios when shifting from autotrophy to mixotrophy. Chlorophylls have an absorption maximum at both 400−500 and 600−700 nm, while carotenoids only have an absorption maximum between 400 and 500 nm. Thus, the ratio between light absorbed at 600−700 nm (chlorophylls) and at 400−500 nm (carotenoids) (Chl/Car) can be used as a proxy for the relative chlorophyll abundance within the pigment pool. The mixotrophic cultures had a lower Chl/Car than the autotrophic cultures; this might indicate either a lower chlorophyll abundance in total pigments or a higher carotenoid content. In conclusion, we do not exclude that the presence of organic carbon might have some effect on pigment composition as reported in other studies,[15,34,35] but according to our results, these changes are not affecting the overall photosynthetic activity.

The finding that mixotrophy can be described as the sum of the heterotrophic and autotrophic metabolism allowed us to conduct a sensitivity analysis on the effect of the elemental biomass composition, $Y_{x/ph}$ and $Y^{het}_{x/s}$, on the oxygen balanced mixotrophic productivity. The effect on an increase of $Y_{x/ph}$ and $Y^{het}_{x/s}$ on the volumetric biomass productivity (r_C) is reported in Figure 4. The results indicate that an increase in $Y_{x/ph}$ is not affecting the ratio between $r_{c,auto}$ and $r_{c,het}$ and leads to a linear increase of $r_{c,mixo}$. On the contrary, an increment in $Y^{het}_{x/s}$ changes the ratio between $r_{c,auto}$ and $r_{c,het}$ and dramatically increases the contribution of $r_{c,het}$ on the overall oxygen balanced mixotrophic growth, leading to an exponential increase of $r_{c,mixo}$. According to our sensitivity analysis, the cultivation of a microalgal strain with high $Y^{het}_{x/s}$ might lead to quadruplicate $r_{c,mixo}$ that is the maximum biomass increase expected in an oxygen balanced mixotrophic culture.

CONCLUSIONS

In the present work and to the best of our knowledge, a mixotrophic microalgae monoculture was grown for the first time for several days in a closed PBR without net oxygen production or CO_2 consumption, allowing the system to operate without any gas exchange. Under this condition, mixotrophic stoichiometry could be described as the sum of heterotrophic and autotrophic stoichiometry and the overall biomass productivity was the exact sum of the two metabolisms. The presence of two complementary growth modes within a microalgal monoculture led to doubled biomass productivity and doubled biomass concentration in comparison to an autotrophic reference. Furthermore, 94% of the substrate was converted into biomass, making the process close to carbon neutral. Our results indicate that mixotrophy is a successful strategy to increase microalgae biomass concentration.
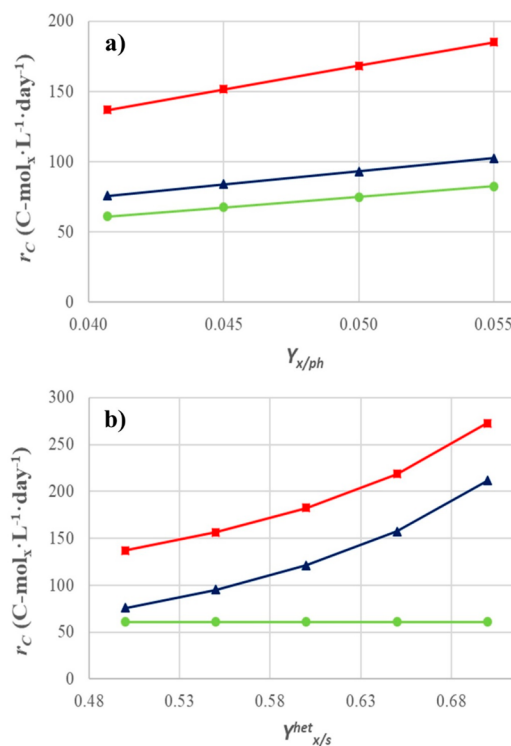

Figure 4. Sensitivity analysis on the effect of (a) the biomass yield on photons ($Y_{x/ph}$) and (b) the heterotrophic biomass yield on substrate ($Y^{het}_{x/s}$) on the oxygen balanced mixotrophic biomass productivity ($r_{c,mixo}$, squares). The contributions of autotrophic ($r_{c,auto}$, circles) and heterotrophic ($r_{c,het}$, triangles) metabolisms to the mixotrophic volumetric biomass productivity are reported separately. A constant $Y^{het}_{x/s}$ was assumed in the simulation of the effect on an increase in $Y_{x/ph}$. Vice versa, a constant $Y_{x/ph}$ was assumed in the simulation of the effect on an increase in $Y^{het}_{x/s}$. As a starting point, the $Y^{het}_{x/s}$ and $Y_{x/ph}$ found in this work were used.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c00990.

> Medium composition (section S1), calculation of the light gradient in the photobioreactor (section S2), and dissolved oxygen and biomass concentration over the entire experiment 1 and 2 (section S3) (PDF)

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https://pubs.acs.org/10.1021/acssuschemeng.0c00990

Notes

The authors declare no competing financial interest.

■ NOMENCLATURE

Abbreviations

DO Dissolved oxygen concentration (% air saturation)
PBR Photobioreactor
PFD Photon flux density (μmol·m^{-2}·s^{-1})
PAR Photoactive radiation, 400–700 nm
TIC Total inorganic carbon (C-mol·L^{-1})

Symbols

$Y_{x/s}$ Biomass yield on substrate (C-mol$_x$·C-mol$_s^{-1}$)
OD_{750} Optical density at 750 nm
C_x Biomass dry weight concentration (g$_x$·L^{-1})
MW_x Biomass molecular weight (g$_x$·C-mol$_x^{-1}$)
μ Specific growth rate (h^{-1})
V_{PBR} Photobioreactor working volume (L)
A_{PBR} Photobioreactor illuminated area (m^2)
r_x Volumetric biomass production rate (g$_x$·L^{-1}·day^{-1})
r_c Carbon based volumetric biomass production rate (C-mol$_x$·L^{-1}·day^{-1})
D Dilution rate (day^{-1})
$Y_{x/ph}$ Biomass yield on light (C-mol$_x$·mol$_{ph}^{-1}$)
r_s Substrate consumption rate (C-mol$_s$·L^{-1}·day^{-1})
F_{AA} Acetic acid supply rate (L·day^{-1})
$C_{s,AA}$ Acetic acid concentration in the stock solution (C-mol$_s$·L^{-1})
C_s Acetic acid concentration in the reactor (C-mol$_s$·L^{-1})
a_x Average-dry-weight-specific optical cross section (m^2·g^{-1})
$C_\%$ Biomass carbon content (% w$_c$·w$_x^{-1}$)
r_{CO_2} CO$_2$ production rate (C-mol·L^{-1}·day^{-1})
Chl/Car Ratio between light absorbed at 600–700 nm (chlorophylls) and at 400–500 nm (carotenoids)

Sub/Superscript

auto Autotrophic
het Heterotrophic
het' Heterotrophic fraction of the mixotrophic biomass
mixo Mixotrophic
ph PAR photons
x Biomass
s Substrate

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