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Research Article

Oxygen Balanced Mixotrophy under Day−Night Cycles

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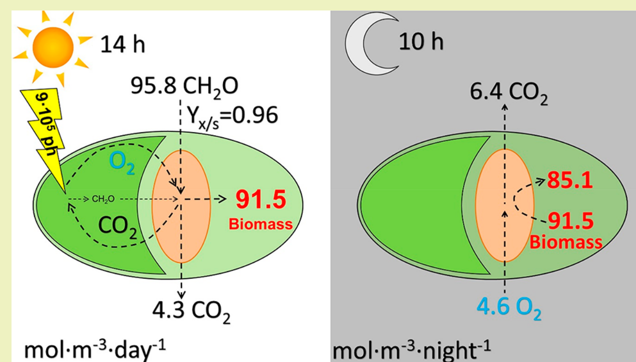

ABSTRACT: Using sunlight to fuel photosynthesis exposes microalgae to day−night cycles. Under day−night cycles microalgae tend to synchronize their metabolism by optimizing light utilization during daytime. During night storage compounds are consumed, leading to biomass losses and demand of O_2. We investigated "oxygen balanced" mixotrophy under 14:10 day/night cycles. In this mixotrophic setup, photosynthetic O_2 production was balanced by respiratory oxygen consumption and CO_2 required for photosynthesis was provided by aerobic conversion of acetic acid. This strategy allowed operation of the reactor without any gas−liquid exchange during daytime. Under these conditions *Chlorella sorokiniana* SAG 211/8K converted 96% of the substrate into biomass. Mixotrophic cultivation did not affect the photosystem II maximum quantum yield (Fv/Fm) or pigment contents of the microalgal cells. Mixotrophic biomass contained 50% w/w of protein and 7.3 mg g^{-1} of lutein. Acetic acid feeding was discontinued at night and aeration initiated. Respiration was monitored by online off-gas analysis and O_2 consumption and CO_2 production rates were determined. Biomass night losses were around 7% on carbon basis with no significant difference between mixotrophic and photoautotrophic cultures. Over 24 h, the mixotrophic culture required 61 times less gaseous substrate and its biomass productivity was doubled compared to the photoautotrophic counterpart.

KEYWORDS: *Circadian rhythms, Lutein, Microalgae productivity, Biomass yield on substrate, Photosynthetic efficiency, Gas−liquid transfer, Oxygen balance, Carbon balance*

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INTRODUCTION

The continuous growth of the human population is placing increasing pressure on our limited natural resources. Producers are facing more challenges to meet the growing food demand; there is competition for arable land, fresh water, and energy while simultaneously an urgent need to reduce the negative impact of agriculture on the environment.[1]

Microalgae are regarded as one of the most nutritious foods known to man.[2] Microalgae can provide a significant number of essential nutrients, such as vitamins, minerals, pigments, and essential fatty acids and amino acids, to support human health.[2,3] The high protein content (even >70%)[3] and quality, especially in relation to the composition and digestibility of amino acids[4] makes microalgae a promising novel source of proteins.

Microalgae can reach higher areal productivity than terrestrial plants, do not require arable land or fresh water,[5] and can use fertilizers with almost 100% efficiency.[6] Further research is needed to better understand the microalgal metabolic flexibility to be able to improve the production process aiming for a higher productivity, simpler reactor design, and lower energy requirement.

One of the strategies to decrease microalgae production costs is utilizing mixotrophic cultivation. In this trophic mode, light and organic carbons are simultaneously exploited, and both chemoheterotrophic (henceforth referred to as heterotrophic) and photoautotrophic (henceforth referred to as autotrophic) metabolisms operate concurrently within a single microalgal monoculture. We recently designed an "oxygen balanced" mixotrophic cultivation method which doubled microalgae productivity under continuous light and operation.[7] We demonstrated that dissolved oxygen concentration (DO) can be controlled by adjusting acetic acid supply rate with the rate of photosynthesis. In "oxygen balanced" mixotrophy the O_2 required for aerobic heterotrophic growth was supplied by oxygenic photosynthesis. Vice versa, the CO_2 needed to carry out photosynthesis was provided by the heterotrophic metabolism. This internal CO_2 recirculation converted 94% of substrate into biomass, making the process close to carbon neutrality. Due to internal gas recirculation the photobioreactor (PBR) was operated without any gas−liquid

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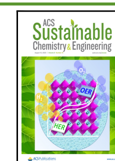

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exchange therefore saving the power otherwise needed for aeration. Moreover, the presence of two energy sources, light and reduced organic carbon, doubled biomass productivity and concentration.

The light energy needed to fuel photosynthesis can be provided by the sun or with the employment of lamps. Cultivation of microalgae on artificial light requires substantial energy input that increases production costs and decreases sustainability of the process.[8] Sunlight is free and abundant. However, the use of sunlight exposes microalgae to day−night cycles and seasonal change on the light pattern.

In most photosynthetic organisms, part of the carbon fixed during the light period is accumulated in storage compounds (e.g., carbohydrates or lipids). During the night, in the absence of light, storage compounds are used to support cell division.[9] The metabolic energy required is created by respiration and, for this reason, oxygen must be supplied in the night. Cell division is usually completed during the first hours after sunset,[9,10] after which energy is expected to be mainly consumed for nongrowth related processes defined as maintenance.[11] Thus, microalgal energy consumption, and with it microalgal oxygen demand, is not expected to be constant throughout the night. Furthermore, the consumption of cellular components leads to a decrease in cell weight, often referred to as biomass losses. Biomass losses in autotrophic culture are typically reported to be between 3 and 8%[12−14] of the biomass produced during the daytime, although losses up to 34% have been reported.[15]

No studies have been carried out to elucidate possible differences on night losses between autotrophic and mixotrophic culture. In a mixotrophic culture, when the organic substrate is completely consumed during the daytime, night biomass losses will lead to a decrease of the biomass yield on substrate. Furthermore, respiration requires O_2, and at nighttime aeration is needed to avoid anaerobic conditions. The amount of oxygen that needs to be provided to support nighttime metabolism is essential information for the scale-up of mixotrophic cultivation. The aim of this work therefore is to evaluate the effect of day−night cycles on the "oxygen balanced" mixotrophy. Specifically, we wanted to investigate the effects of these cycles on the biomass yield on light and the biomass yield on the organic substrate during daytime and the oxygen consumption and biomass losses during nighttime.

In order to achieve our goals, the model strain *C. sorokiniana* SAG211/8K was cultivated under day−night cycles, and a mixotrophic culture was compared to its autotrophic counterpart. The two cultures were grown in continuous mode with a fixed dilution rate (i.e., chemostat) where the culture was only diluted during daytime and not during the night (cyclostat). During daytime the dissolved oxygen concentration in the mixotrophic culture was controlled by tuning acetic acid supply rate to the rate of photosynthesis. During nighttime, no acetic acid was fed to the mixotrophic culture. In both the mixotrophic and autotrophic cultures the oxygen consumption related to night respiration was measured. Biomass productivity was assessed over the entire day and also the biomass loss during the night was measured. Finally, the protein and pigment contents of the mixotrophic and autotrophic cultures were compared.

■ MATERIALS AND METHODS

Organism, Media, and Cultivation Conditions. *Chlorella sorokiniana* SAG 211/8K was obtained from the algae culture collection of Göttingen University (SAG) and cultivated in modified M-8 medium[7] using ammonium as the nitrogen source. Axenic algal cultures were cryopreserved and stored in liquid nitrogen. Before reactor inoculation, cryopreserved cultures were used to inoculate 100 mL of medium in 250 mL flasks placed 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, U.S.A.) 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 PAR quantum sensor.

Photobioreactor Setup and Experiments. *Chlorella sorokiniana* SAG 211/8K was grown in a 3 L bioreactor (Applikon, The Netherlands) described in more detail in Abiusi et al.[7] This reactor had a working volume (V_{PBR}) of 1.946 L. The internal diameter was 0.130 m, while the liquid height was maintained at 0.166 m by a level sensor, resulting in a cylindrical illuminated area (A_{PBR}) of 0.068 m^2. The reactor was operated in cyclical steady state (cyclostat) under day−night cycle. At daytime the culture was diluted at fixed rate, while the cultures were not diluted during the night. During daytime we aimed to reproduce the dilution rate (D, day^{-1}) of our previous work.[7] We aimed for a D of 2 day^{-1} when considering only daylight hours, which is equivalent to 1.1 day^{-1} when referencing to the 24 h day−night period.

The reactor was illuminated from all sides creating a homogeneous light field over the cylindrical reactor surface. Light intensity on the reactor surface was measured at 16 fixed points inside the empty reactor obtaining an average PFD of 514 ± 17 μmol m^{-2} s^{-1}. Light was provided in day−night cycle of 14D:10N in "block" with constant illumination during the day. Previous work[10] indicated that light provided in "block" resulted in the same biomass yield on light ($Y_{x/ph}$, C-mol$_x$ C-mol$_s^{-1}$) as in "sine" waveform. The block approach was preferred over sine due to easier operation and comparison to our previous study.

The reactor was equipped with a dissolved oxygen (DO) sensor (VisiFerm DO ECS 225, Hamilton, U.S.A.). This DO sensor was calibrated inside the reactor filled with growth medium at operation temperature (37 °C) and pH (6.7). Calibration was done by sparging dinitrogen gas to obtain the 0% DO level, and sparging air to obtain the 100% DO level. A DO of 100% corresponds to 224 μmol L^{-1} at 37 °C. 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 with a marine impeller at 500 rpm was applied during all experiments. During the day, the autotrophic culture was aerated with compressed air enriched with 2% v/v carbon dioxide at a flow rate of 0.5 L L^{-1} min^{-1} using mass flow controllers (Smart TMF 5850S, Brooks Instruments, U.S.A.) while the mixotrophic culture was not aerated. During night both cultures were aerated with compressed air at a flow rate of 0.1 L L^{-1} min^{-1}. The CO_2 content of the compressed air was reduced below the detection limit by zeolite adsorption. The pH was controlled at 6.7 during the day by automatic base addition (1 M, NaOH) and at 6.8 during the night by automatic addition of acid (0.5 M, H_2SO_4).

The reactor was inoculated with an autotrophic culture at a density of 1.0 g$_x$ L^{-1}. A 5% w/w acetic acid solution was supplied at a fixed rate while gassing the reactor with CO_2 enriched air for 5 h. After this start-up phase the aeration was stopped during daytime resulting in a mixotrophic cultivation without gas exchange, where the supply rate of acetic acid was automatically adjusted to maintain a DO of 105%. At nighttime the feeding of acetic acid was stopped. The reactor was operated under these conditions for 9 consecutive days. For the last 4 days, a harvesting vessel was placed into an ice-cooled water bath. The harvesting vessel was changed daily at the end of the light phase. The harvested culture was mixed well, 10 mL of it was used for dry weight determination, while the remaining culture was collected for pigment analysis. During these 4 days, reactor samples were taken for off-line measurements multiple times a day.

After the first 9 days, acetic acid supply was stopped and aeration re-established, and the reactor was operated autotrophically. The autotrophic experiment also lasted for 9 days, and during the last 4

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days again samples were taken using the same procedure as for the mixotrophic experiment. Cultures were considered at cyclical steady state (cyclostat) when the daily change in biomass concentration over the day:night cycle was constant for at least 3 days. In our previous work[16] we demonstrated that a mixotrophic culture can switch to autotrophic metabolism with no effect on photosynthesis. This finding simplified our experimental design as we had no need to stop and restart the experiment to switch between trophic states.

The acid and base solutions, acetic acid solution, and the harvest bottle were placed on analytic balances. The balances, DO sensor, temperature probe, pH sensor, mass flow controllers, and gas analyzer (see next section) were connected to a data acquisition system interfaced via a computer by means of a virtual instrument (Lab View, National Instruments, U.S.A.) allowing for continuous data logging and process control. Culture samples for off-line measurements were taken aseptically from the reactor through a dedicated port. The complete setup, including all of the solutions, were sterilized prior the experiment by autoclaving for 60 min at 121°C.

Online Gas Analysis. Oxygen and carbon dioxide concentrations in the off-gas were measured online using a gas analyzer (Servomex 4100, The Netherlands). The gas analyzer was fitted with two sensor modules, a paramagnetic purity transducer to measure oxygen and an infrared 1500 transducer to measure carbon dioxide. Data from the gas analyzer and the mass flow controllers were collected every 4 s, and these data were stored per minute as moving average of 15 points.

Before the experiment, two wet and dry baselines were measured: one under nighttime conditions (0.1 L L^{-1} min^{-1} of air) and one under autotrophic daytime conditions (2/98% v/v CO_2/air at a flow rate of 0.5 L L^{-1} min^{-1}). The dry baseline was measured by leading the gas inlet directly over the gas analyzer. For the wet baseline the gas inlet was first sparged through the reactor filled with medium and maintained at the same temperature and pH as during the experiment. To minimize water vapor, the off-gas was passed through a condenser which was maintained at 2 °C. After passing the condenser the reactor off-gas was led through a membrane module (gas dryer model MD-110−24P, Perma Pure, U.S.A.) in which the reactor gas was further dried before being analyzed. The total gas flow leaving the reactor ($F_{g,out}$, mol min^{-1}) including remaining water vapor (∼0.5% v/v) was then calculated as follow:

$$F_{g,out} = F_{g,in}\left(\frac{X_{O2,db}}{X_{O2,wb}}\right) \quad (1)$$

where $F_{g,in}$ is the total gas inlet flow and $X_{O2,db}$ and $X_{O2,dw}$ are the molar fractions of O_2 respectively measured in the dry and wet baseline.

The total gas inlet flow ($F_{g,in}$, mol min^{-1}) was calculated by summing the air ($F_{air,in}$) and CO_2 inlet flow ($F_{CO2,in}$). The resulting $F_{g,out}$ was used to calculate the oxygen (r_{O2}, mol_{O2} L^{-1} min^{-1}) and carbon dioxide (r_{CO2}, mol_{O2} L^{-1} min^{-1}) production or consumption rate at a resolution of 1 min according to

$$r_{O2} = F_{g,out}(X_{O2,out} - X_{O2,wb}) \quad (2)$$

$$r_{CO2} = F_{g,out}(X_{CO2,out} - X_{CO2,wb}) \quad (3)$$

where $X_{O2,out}$ and $X_{CO2,out}$ are the molar fractions of O_2 and CO_2, respectively, measured during the experiment and $X_{O2,b}$ and $X_{CO2,wb}$ are the molar fractions of O_2 and CO_2 measured in the wet baseline.

Photobioreactor Calculations. The biomass production rate over 24 h (r_{x24}, g_x L^{-1} day^{-1}) was calculated multiplying the biomass concentration in the harvesting vessel (C_x, g_x L^{-1}), collected after a complete day:night cycle, times the dilution rate (D, day^{-1}). In the mixotrophic culture, we also calculated the r_x during daylight period (r_{x14}, g_x L^{-1} day^{-1}) by correcting for the night biomass loss.

$$r_{x14,mixo} = r_{x24,} + \frac{C_{x14} - C_{x0}}{t_{14} - t_0} \quad (4)$$

where C_{x0} and C_{x14} are the biomass concentrations at the beginning and the end of the day, respectively. 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 (MWx, g_x C mol_x^{-1}). MWx was determined in all off-line samples taken from the reactor and the average of those values was used to calculate the mixotrophic and the autotrophic MWx. In the autotrophic culture r_{c14} was calculated based on the CO_2 uptake rate (r_{CO2}, C mol_{CO2} L^{-1}) and both $r_{c,14}$ and $r_{c,24}$ were used to determine the biomass yield on light ($Y_{x/ph}$, C mol_x mol_{ph}^{-1}) over 24 h and during daytime only according to the formula:

$$Y_{x/ph} = \frac{r_{c,auto} V_{PBR}}{PFDA_{PBR}} \quad (5)$$

In the mixotrophic experiments, the volumetric substrate consumption rate (r_s, C mols L^{-1} day^{-1}) was calculated as follows:

$$r_s = \frac{F_{AA} C_{sAA} - D V_{PBR} C_s}{V_{PBR}} \quad (6)$$

where F_{AA} (L day^{-1}) and C_{sAA} (C mol_s L^{-1}) represent respectively the supply rate and the concentration of the acetic acid (AA) solution while Cs (C mol_s L^{-1}) is the acetic acid concentration in the reactor (C mol_s L^{-1}). The mixotrophic biomass yield on substrate ($Y_{x/s}^{mixo}$, C mol_x mol_s^{-1}) was calculated dividing r_c by r_s. The r_s was used also to estimate the fraction of biomass heterotrophically produced during the mixotrophic growth ($r_{c,het'}$, C molx L^{-1} day^{-1}). This was done by multiplying r_s for the heterotrophic biomass yield on substrate ($Y_{x/s}$, C mol_x C mol_s^{-1}). A $Y_{x/s}$ value of 0.5 C mol_x C mol_s^{-1} was used for this purpose.[7] The resulting $r_{c,het'}$ was subtracted from the overall mixotrophic r_c to estimate the fraction of biomass autotrophically produced during mixotrophic growth ($r_{c,auto'}$, C mol_x L^{-1} day^{-1}).

Night time losses were quantified measuring the difference in dry weight concentration (C_x, g_x L^{-1}) and the difference in total organic carbon content (TOC, g_c L^{-1}) between samples taken at the beginning and at the end of the night. The third method used to quantify nighttime losses was the CO_2 production rate (r_{CO2}, C mol_{CO2} L^{-1}) over the whole night, which was derived from the off-gas analysis.

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 was stored at −20 °C until analysis, while the pellet was washed twice with demineralized water and cooled to −20 °C, lyophilized, and stored at room temperature in the dark. Extra samples were taken from the reactor to quantify the dissolved inorganic carbon concentration (DIC, C mol L^{-1}) in the medium. This was done daily at the beginning and at the end of the night. To avoid CO_2 stripping, 950 μL of the supernatant fraction was alkalized immediately after centrifugation by the addition of 50 μL of base (2 M, NaOH). Alkalized samples were stored at −20 °C until analysis. During the last 4 days of the mixotrophic and the autotrophic experiment, 1 L of the harvested culture was centrifuged at 1200 RCF for 30 min. The supernatant was discharged while the pellet was washed twice with demineralized water and cooled to −20 °C, lyophilized, and stored.

Dry Weight Concentration. Culture growth was estimated by biomass dry weight (C_x, g_x L^{-1}) determination: aliquots of the culture (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 0.7 μm). The filters were washed with deionized water (25 mL) and dried at 105 °C until constant weight.

Cell Concentration. Cell concentration was measured using a Multisizer III (Beckman Coulter Inc., U.S.A.) 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 Absorption Cross Section. Average absorption cross section (a_x, m^2 kg^{-1}) in the PAR region (400−700 nm) of the spectrum was measured and calculated according to de Mooij et al.[17] The absorbance was measured in UV−vis/double beam spectropho-

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tometer (Shimadzu UV-2600, Japan) equipped with 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 (Photon Systems Instruments, Czech Republic). Prior to the measurement, samples were adapted to darkness for 15 min at room temperature and diluted to optical density at 750 nm between 0.3 and 0.5.

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

Pigment Analysis. Pigment extract were obtained by a sequence of mechanical cell disruption and solvent based (methanol) pigment extraction using 10 mg of lyophilized biomass. Cells were disrupted by bead beating (Precellys 24, Bertin Technologies, France) at 5000 rpm for 3 cycles of 60 s with 120 s breaks on ice between each cycle. The extraction was done through five washing steps with methanol. Separation, identification and quantification of pigments was performed using a Shimadzu (U)HPLC system (Nexera X2, Shimadzu, Japan), equipped with pump, degasser, oven (25 °C), cooled autosampler (4 °C), and photodiode array detector (PDA). Samples (20 uL) were quantitatively injected on a YMC Carotenoid C30 column (250 × 4.6 mm) coupled to a YMC C30 guard column (20 × 4 mm; YMC, Japan) at 25 °C, flow 1 mL min^{-1}. The mobile phases consisted of methanol (A), water/methanol (20/80 by volume) containing 0.2% ammonium acetate (B), and tert-methyl butyl ether (C). The gradient of elution used with this column was 95% A, 5% B isocratically for 12 min, a step to 80% A, 5% B, and 15% C at 12 min, followed by 18 min of linear gradient to 30% A, 5% B, and 65% C. A conditioning phase (30−40 min) was then used to return the column to the initial concentrations of A and B.

Total Organic and Total Inorganic Carbon and Nitrogen. The dissolved inorganic carbon (DIC) concentration was measured from the undiluted supernatant with a TOC-L analyzer (Shimadzu, Japan). The organic carbon and nitrogen content in the pellet were measured as total carbon (TOC, g_c L^{-1}) and total nitrogen (TON, g_N L^{-1}) respectively using the TOC-L analyzer. 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 kHz 40°C for 30 min. After this treatment samples were diluted ten times in demi water and immediately placed in the TOC-L analyzer. The biomass carbon content ($C_{\%}$, % w_c w_x^{-1}) and nitrogen content ($N_{\%}$, % w_N w_x^{-1}) were calculated by dividing the obtained total carbon and total nitrogen by the dry weight determined on the same sample. The $C_{\%}$ was used to determine the biomass molecular weight (MWx, g_x C mol_x^{-1}). MWx was determined by dividing the carbon molecular weight (12.011 g_c C mol^{-1}) by $C_{\%}$. The $N_{\%}$ was used to determine the biomass protein content using a protein-nitrogen fraction (0.168 g N g $protein^{-1}$).[18]

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

Statistical Analysis. Propagation of errors was calculated according to eqs 7 and 8 for sum and multiplication operations, respectively, to obtain the error.

$$\Delta z = \sqrt{\Delta x^2 + \Delta y^2 + ...} \tag{7}$$

$$\frac{\Delta z}{z} = \sqrt{\frac{\Delta x^2}{x} + \frac{\Delta y^2}{y} + ...} \tag{8}$$

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

In the comparison between the mixotrophic and the autotrophic cultures each day was considered as a replicate during the last 4 days of cyclostat. Figures and tables reports the standard deviation of these 4 replicates (n = 4). Significant differences between those two conditions were analyzed by one-way ANOVA. The significance level was $P < 0.05$.

■ RESULTS AND DISCUSSION

Oxygen Balanced Mixotrophy Under Day−Night Cycles. We previously demonstrated that a mixotrophic culture can operate without any gas−liquid transfer of oxygen or carbon dioxide.[7] We proposed to control respiratory oxygen consumption by tuning acetic acid supply. However, envisioning outdoor scale-up, this strategy needed to be tested under day−night cycles. In this study, a mixotrophic and an autotrophic culture grown under the same light-dark conditions were compared. First, we will describe the overall biomass productivity and biomass composition over a 24 h period. Next, we will zoom in on daytime and nighttime metabolisms.

Before going to the actual results, we will first discuss how off-gas analysis was applied in this study. Online off-gas analysis was used to calculate the oxygen (r_{O2}, mol_{O2} L^{-1} day^{-1}) and carbon dioxide (r_{CO2}, mol_{O2} L^{-1} day^{-1}) production or consumption rates. Day−night transitions, however, were followed by a change in the aeration rate and gas composition, which led to rapid changes in the chemical-physical equilibria of dissolved O_2 and CO_2. These chemical-physical artifacts necessitated further data treatment.

During the transition from day to night the r_{O2} was positive for a few minutes according to our raw data, meaning that oxygen was produced, which is impossible from a biological point of view (Supporting Information 1). This phenomenon is caused by the stepwise reduction in the aeration rate at the beginning of the night. In addition, especially in the autotrophic culture, the dissolved oxygen (DO, % air saturation) was higher than 100% during the day. When the night began, part of the oxygen dissolved in the liquid phase was stripped from the culture, giving an apparent positive r_{O2}. This experimental artifact was removed by recalculating the r_{O2} based on the dissolved oxygen (DO) and the general relations used to describe transfer of gaseous compounds between liquid and gas. The detailed procedure is explained in the Supporting Information 1. Following these procedures, we calculated the oxygen gas−liquid transfer coefficient (k_La, h^{-1}) adopting the steady state method (Supporting Information 1) while still using r_{O2} determined from off-gas analysis outside of the time with the day−night transition phenomena. More specifically, we calculated the k_La, during a long period at the end of the day, and at the end of the night, where gas analysis was not affected by transition events and where the system was in a steady state.

Similar to the r_{O2}, the carbon dioxide production or consumption rate (r_{CO2}) showed a peak during day−night transition (Supporting Information 2) which was too high to be merely due to biological activity. This overestimation is related to the fact that at the beginning of the day dissolved inorganic carbon (DIC, C mol L^{-1}) accumulates in the liquid phase until it reaches its chemical-physical equilibrium. This DIC is then stripped from the culture as CO_2 at the beginning of the night. DIC measured at the end of the day is reported in Table 1, and this DIC was completely removed by the end of the night. For this reason, to calculate the real r_{CO2}, the DIC was subtracted from the cumulative amount of the CO_2 exchange measured during the day and night (Table 2). Consequently, the r_{CO2} presents a nighttime average and we do

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Table 1. Overview of the Off-Line, DO, *D* Measurements on the Cultivation of *C. sorokiniana* SAG 211/8K under Mixotrophic and Autotrophic Conditions[a]

	unit	mixotrophic	autotrophic
DO (daytime)	air saturation %	98 ± 33	146 ± 5[b]
DIC_{out} (end of the day)	C mmol L^{-1}	3.75 ± 1.2	1.67 ± 0.6[b]
C_x (end of the day)	g_x L^{-1}	1.90 ± 0.02	0.88 ± 0.03[b]
C_x (harvesting)	g_x L^{-1}	1.82 ± 0.02	0.90 ± 0.01[b]
D	day^{-1}	1.12 ± 0.00	1.08 ± 0.00
r_x	g L^{-1} day^{-1}	2.03 ± 0.04	0.96 ± 0.03[b]
$C_{\%}$	% w_C w_x^{-1}	50.4 ± 0.6%	47.9 ± 0.8%[b]
$N_{\%}$	% w_N w_x^{-1}	8.9 ± 0.6%	8.0 ± 0.1%[b]
QY (end of the day)	Fv/Fm	0.77 ± 0.01	0.77 ± 0.01
a_x (end of the day)	m^2 kg^{-1}	258 ± 4	277 ± 17
protein (end of the day)	% w_P w_x^{-1}	50.1 ± 2.2%	45.1 ± 1.8%[b]
lutein (harvesting)	mg g_x^{-1}	7.3 ± 0.5	7.7 ± 0.5
chlrophyll a + b	mg g_x^{-1}	35.4 ± 1.7	37.1 ± 7.1

[a]The data presented are the average of 4 consecutive days at cyclostat (n = 4) and reported with the standard deviation of measurements. [b]Significant differences ($P > 0.05$).

not have insight of the dynamics of CO_2 production during the night.

Mixotrophic and Autotrophic Productivity and Composition over 24 h. The oxygen balanced mixotrophic strategy confirmed that microalgae productivity and concentration can be doubled (Table 1). Furthermore, we established that mixotrophic stoichiometry is the sum of the heterotrophic and autotrophic metabolism (Table 3). In fact, subtracting the fraction of biomass heterotrophically produced during the mixotrophic growth ($r_{c,het}$, C mol_x L^{-1} day^{-1}) from overall mixotrophic productivity ($r_{c,mixo}$, C mol_x L^{-1} day^{-1}), allowed us to calculate the fraction of biomass produced autotrophically ($r_{c,auto'}$). The $r_{c,auto'}$, and therefore the biomass yield on light ($Y_{x/ph}$, C mol_x mol_{ph}^{-1}), was not significantly ($P > 0.05$) different from the r_c of the autotrophic culture. Surprisingly, despite the 10 h of darkness, $Y_{x/ph}$ was identical to the 40.7 C $mmol_x$ mol_{ph}^{-1} reported in our previous study under continuous light.[7] Therefore, under day−night cycle, where some biomass is lost during the night, the daytime $Y_{x/ph}$ is expected to be higher than under continuous light, and this higher yield compensates for night biomass losses.[10] Those findings will be elucidated in more detail in the next sections. Thanks to the higher $Y_{x/ph}$ and despite the 10 h of darkness, the mixotrophic biomass yield on substrate under day−night cycle ($Y^{mixo}x/s$, C mol_x C mol_s^{-1}) was 0.88 C mol_x C mol_s^{-1} (Table 3) only 6% lower than previously reported under continuous light.[16]

Similar $Y_{x/ph}$ of the mixotrophic and the autotrophic cultures indicate that photosynthesis is not affected by the presence of organic substrate. In our experiment the effect of organic carbon on photosynthesis was assessed by measuring photosynthetic efficiency of PSII directly as quantum yield (QY), by measuring the average specific absorption cross section (a_x), and by measuring the total chlorophyll (a+b) and lutein contents (Table 1). These values did not vary between the mixotrophic and autotrophic cultures.

These results confirm our previous finding[7] but are in contrast with most of the existing literature where a decrease in pigment content is reported.[19−21] A possible explanation is that in order to balance oxygen production, the heterotrophic ($r_{c,het'}$) and the autotrophic ($r_{c,auto'}$) metabolisms are equally contributing to the overall mixotrophic growth (Table 3). Most of the previous work were conducted in batch or in repeated batch[17,18] with high initial substrate concentration and low light intensity, therefore the rate of heterotrophic metabolism was much higher than the rate of autotrophic metabolism. The dominance of heterotrophy in these studies might have resulted in a lower pigment content in comparison to our study.

A chlorophyll content between 20 and 40 mg g_x^{-1} is commonly found in this species[22−24] and our results are on the high side of this range (Table 1). The high chlorophyll content indicates that our cultures were photolimited. In our previous work we used a light model to estimate the attenuation of light intensity, caused by cellular light absorption, from the reactor surface toward the reactor center.[7] Applying this model to the present work, we estimated that 85% of mixotrophic and 71% of autotrophic cultures were experiencing a light level below 10 μmol m^{-2} s^{-1} which we assumed to be the compensation point of photosynthesis.[25] Microalgae acclimate to the light regime they experience. In case the algae are light limited they are known to increase their pigmentation.[26]

The lutein content found in our cultures was 7 to 8 mg g_x^{-1}, one of the highest ever reported for microalgae. Previous studies have reported lutein content commonly being in the range of 1−4.3 mg g_x^{-1} and values above this range are considered rare. In this strain, a maximum lutein content of 6 mg g_x^{-1} has been previously reported[27] while up to 15 mg g_x^{-1} has been obtained in *C. vulgaris*.[28] Understanding the biological reason behind this high lutein content might have important commercial applications. However, the scope of this work was primarily to compare mixotrophic and autotrophic cultures, and we can clearly conclude that pigments were not ill-affected by the presence of an organic substrate.

C. sorokiniana has been proposed as a sustainable source of food given its high protein content and nutritional value.[29] We measured a protein content of 50.1% ± 2.2 w/w in the mixotrophic and 45.1% ± 1.8 w/w in the autotrophic culture. These values are within the range reported for these species[23,30]. The higher protein content of the mixotrophic culture can partially explain its higher carbon content ($C_{\%}$;

Table 2. Average Mixotrophic and Autotrophic Specific Oxygen (q_{O2}) and Carbon Dioxide (q_{CO2}) Consumption/Production Rate over Day and Night[a]

	mixotrophic		autotrophic	
	day	night	day	night
q_{O2} (mol_{O2} C mol_x^{-1} day^{-1})	0	-40.5×10^{-3} ± 4.8	1.30 ± 0.04	-44.6×10^{-3} ± 2.4
q_{CO2} (mol_{CO2} C mol_x^{-1} day^{-1})	4.7×10^{-6} ± 0.3[b]	56.6×10^{-3} ± 6.8	−1.15 ± 0.04	73.2×10^{-3} ± 3.9

[a]The data presented are the average of 4 consecutive days at cyclostat (n = 4) and reported with the standard deviation of measurements. [b]Calculated according to DIC.

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Table 3. Carbon Mass Balance of *C. sorokiniana* SAG 211/8K Grown Mixotrophically over 14 h of Day-Light Period and over 24 h[a]

	unit	mixotrophic 24 h	mixotrophic 14 h	autotrophic 24 h	autotrophic 14 h
r_s	C mmol$_s$ L^{-1} day^{-1}	−95.8 ± 3.4	−95.8 ± 3.4	n.a	n.a
$r_{c,mixo}$	C mmol$_x$ L^{-1} day^{-1}	84.8 ± 2.7	91.5 ± 3.5	n.a	n.a
$Y_{x/s}^{mixo}$	C mol$_x$ C mol$_s$$^{-1}$	0.88 ± 0.04	0.96 ± 0.05	n.a	n.a
$r_{c,het'}$	mmol$_x$ L^{-1} day^{-1}	47.9 ± 4.2	47.9 ± 2.2	n.a	n.a
$r_{c,auto'}/r_{c,auto}$	C mmol$_x$ L^{-1} day^{-1}	36.9 ± 5.0	43.16 ± 5.5	39.5 ± 1.1	42.4 ± 0.8
$Y_{x/ph}$	C mol$_x$ C mol$_{ph}$$^{-1}$	40.9 ± 5.8	48.3 ± 6.4	43.8 ± 2.1	47.1 ± 2.0

[a]In the table the overall mixotrophic productivity ($r_{c,mixo}$) was split in the fraction of biomass heterotrophically produced ($r_{c,het'}$) and the fraction of biomass produced autotrophically ($r_{c,auto'}$). As comparison the autotrophic productivity ($r_{c,auto}$) is also reported. The data presented are the average of 4 consecutive days at cyclostat (n = 4) and reported with the standard deviation of measurements. Not applicable (n.a.).

Table 1). This hypothesis was confirmed by Kumar et al.[30] in another *C. sorokiniana* strain.

Daytime Metabolism in Mixotrophic and Autotrophic Cultures. Under day−night cycles, the application of automatic feeding of acetic acid to control DO proved to be more challenging than under continuous light (Figure 1) and some settings needed adjustment. In the initial configuration, the process was designed to provide acetic acid to the culture only if DO was exceeding a set point (DO 105%). Therefore, feeding of acetic acid would have started only after an initial oxygen production had begun. Surprisingly, without an initial addition of acetic acid, the culture did not start producing oxygen (data not shown). This phenomenon might have been caused by an insufficient level of dissolved inorganic carbon (DIC) present in the medium after the night, and without CO_2, photosynthesis could not start. For this reason, a small and constant acetic acid supply rate (F_{AA}, L min^{-1}) was maintained between 0.1 and 0.3 mL min^{-1}. Thus, the substrate was provided even when DO did not reach the set point yet. Introducing this basal F_{AA} led to a decrease in DO during the first 1.5 h, where the DO reached a minimum of 20%, after which DO rose again to the set point (DO 105%), which was reached after 3 h (Figure 1). Once the set point was reached, automatic feeding began to adjust F_{AA} based on the DO and succeeded in maintaining DO at the set point.

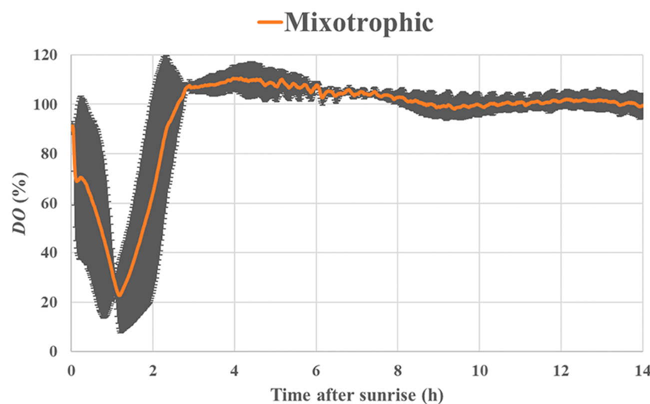

Figure 1. Daytime dissolved oxygen (DO) of *C. sorokiniana* SAG 211/8K grown mixotrophically without aeration. The data presented are the average of 4 consecutive days at cyclostat (n = 4) and the shaded area represents the standard deviation of measurements.

The autotrophic culture also needed about 3 h before reaching its full photosynthetic capacity corresponding to an r_{O2} of 62 μmol O_2 L^{-1} min^{-1} (Figure 2). It has been reported that in the first hours of the day algae need to restart photosynthesis and adjust the photosynthetic apparatus to the light intensity by increasing, or decreasing, their pigment content, among other things.[31] After this period, r_{O2} further increased reaching the maximum value of 63 μmol O_2 L^{-1} min^{-1} 5 h after the sunrise (Figure 2). Maximum r_{O2} was maintained for about 5 h, after which r_{O2} declined in the last 4 h of the daytime. Similar trends have been reported in other microalgal species[9,10,32] and although the precise mechanisms behind these circadian variations have not been discovered yet, it is well-known that photosynthesis is controlled by the circadian clock.[33] Cell division might reduce photosynthetic efficiency[10,32] which could have been the case in our culture at the end of the day (Figure 3) explaining the r_{O2} decline in the last 4 h of the daytime (Figure 2).

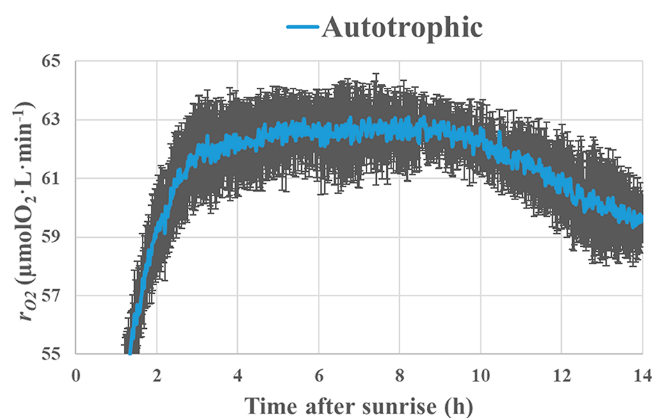

Figure 2. Daytime oxygen production rate (r_{O2}) of *C. sorokiniana* SAG 211/8K grown autotrophically. The data presented are the average of 4 consecutive days at cyclostat (n = 4) and the shaded area represents the standard deviation of measurements.

Synchronized cultures, where cell division occurs mainly at night, might have a higher daytime biomass yield on light ($Y_{x/ph}$, C mol$_x$ mol$_{ph}$$^{-1}$) compared with continuous light culture, where cell division occurs randomly.[9,10] This was the case in our experiment, where the average of daytime biomass yield on light for both the mixotrophic and autotrophic cultures was 48 C mol$_x$ mol$_{ph}$$^{-1}$ (Table 3), while in our previous experiment in continuous light culture it was 41 C mol$_x$ mol$_{ph}$$^{-1}$.[16] However, in order to confirm that the beginning of the cell division corresponded exactly with the decline in photosynthetic activity, cell counting should have been measured over 24 h, while our study we mainly focused on cell division at nighttime (see next section).

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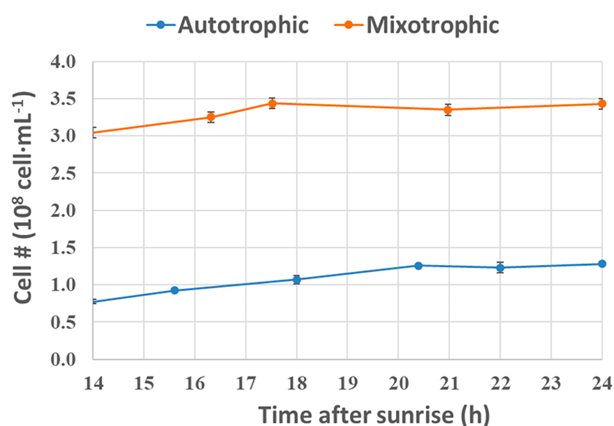

Figure 3. Nighttime cell number of *C. sorokiniana* SAG 211/8K that during the daytime was grown either mixotrophically (orange) or autotrophically (blue). The data presented are the average of 4 consecutive days at cyclostat (n = 4) and reported with the standard deviation of measurements.

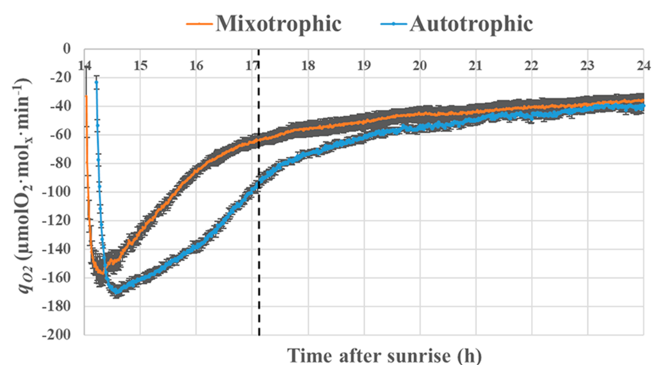

Figure 4. Nighttime specific oxygen consumption rate (q_{O2}) of C. sorokiniana SAG 211/8K that during the daytime was grown either mixotrophically (orange) or autotrophically (blue). The data presented are the average of 4 consecutive days at cyclostat (n = 4) and the shaded area represents the standard deviation of measurements. The dotted line indicates the time in which half of the total r_{O2} is reached.

In this study the autotrophic carbon uptake rate (r_{CO2}; Supporting Information 2) equals the biomass production rate r_C (r_C, C mol$_x$ L^{-1} day^{-1}; Table 3). The accuracy of this method was also confirmed by the ratio between r_{O2} and r_{CO2} that matched the value of 1.1 expected from autotrophic stoichiometry[7] using ammonium as nitrogen source. Off-gas analysis was also used to calculate the amount of CO_2 taken up from the reactor, on the total amount provided during the daytime (Supporting Information 3). Our results indicate that 90% of the ingoing CO_2 was lost in the autotrophic reference culture. Similar CO_2 losses are commonly reported[34,35] Low CO_2 uptake efficiency might have a dramatic impact on microalgae production cost and carbon footprint.[34,35] Several studies have been conducted to decrease CO_2 losses but even in optimized photobioreactors (PBRs), CO_2 losses are 25% at minimum in closed PBRs[36] and 50% in open ponds,[37] indicating that CO_2 uptake efficiency is one of the challenges in autotrophic cultivation of microalgae.

Nighttime Metabolism in Mixotrophic and Autotrophic Cultures. The average volumetric oxygen consumption rate (r_{O2}) was measured for 4 consecutive days and used to calculate the biomass specific oxygen consumption rate (q_{O2}, mol$_{O2}$ C mol$_x$ day^{-1}). We will use these specific rates because in the mixotrophic culture the biomass concentration was roughly double the concentration of the autotrophic culture (Table 1).

Mixotrophic and autotrophic cultures expressed a similar trend of q_{O2} in time (Figure 4) with higher oxygen consumption at the beginning of the night decreasing to a low and constant rate toward the end of the night. Calculations show that 50% of the oxygen was consumed within the first 3 h. A closer look at the graph reveals that q_{O2} decreased more rapidly in the mixotrophic culture compared to the autotrophic culture, while toward the end of the night the two cultures had a similar q_{O2}. As a consequence of the more rapid decline, the average q_{O2} during the night was slightly lower in the mixotrophic culture than in the autotrophic culture (Table 2).

Few studies have employed online off-gas analysis in microalgae to study dynamics in metabolism during the day—night cycle.[10,38,39] Most of these studies were conducted only during the day in an autotrophic culture, with the goal of quantifying biomass production rate based on r_{O2}. During the night, carbohydrate reserves are consumed to produce energy. In the case of aerobic respiration of sugar for energy production (catabolism), 1 mol of sugar (CH_2O) is respired, consuming 1 mol of O_2 and producing 1 mol CO_2. In a situation where part of the sugar is used as molecular building block for the formation of functional biomass (e.g., proteins, pigments) in anabolic pathways, the ratio between r_{O2} and r_{CO2} is lower than 1. Thus, the ratio between q_{O2} and q_{CO2} gives information on the relative contribution of catabolic and anabolic pathways. In our experiment this ratio was 0.71 for the mixotrophic culture during the night, and 0.61 for the autotrophic culture (Table 2). The difference suggests that in the autotrophic culture anabolic processes were more dominant.

The finding that anabolic processes were more dominant in the autotrophic culture than in the mixotrophic culture was confirmed by nighttime cell division (Figure 3). In the autotrophic culture cell number increased by 62% while in the mixotrophic culture only by 13%. However, neither of the cultures doubled their cell number during the night, indicating that cell division must have already started during the day as discussed in previous section. In the mixotrophic culture cell division was completed after 3.5 h while in the autotrophic culture it lasted for 6.5 h.

Not surprisingly q_{O2} declined after cell division (Figure 4) and this decline was faster in the mixotrophic culture than in the autotrophic culture. Cells need energy for growth related process, such as cell division, and less so for nongrowth related process defined as maintenance.[11] At night, after cell division, the cells enter in a metabolically quiescent stage of the cell cycle known as G_0.[31] In this stage energy is spent mainly for maintenance, and the energy for maintenance was constant in both cultures. The specific oxygen consumption for maintenance in this strain has been reported[40,41] to be 0.3 mmolO$_2$ g$_x$$^{-1}$ h^{-1}, which is in the same order the 0.1 mmolO$_2$ g$_x$$^{-1}$ h^{-1} measured in our study.

The most relevant question with respect to scale-up of mixotrophic cultivation is the amount of oxygen that needs to be provided to support night time aerobic heterotrophic metabolism (i.e., respiration). The amount of oxygen consumed during the night was similar between the two cultures, with mixotrophic culture requiring slightly less oxygen

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at night (Table 2). Moreover, most of the oxygen was consumed within the first hours of the night, so it is advisable to tune the aeration based on the DO, rather than aerate the culture at a constant rate. In fact, the oxygen requirement at night is only a minimal part of the overall daily gaseous substrate demand under autotrophy. Averaged over 24 h the mixotrophic culture required 61 times less gaseous substrates than the autotrophic culture (Table 3), confirming that the energy required for gassing under mixotrophy is almost negligible.

Another relevant question regarding scale-up is the amount of nighttime biomass losses. Nighttime losses were quantified using three methods: biomass dry weight concentration (C_x, g_x L^{-1}), total organic carbon content (TOC, C mol_s L^{-1}), and CO_2 production rate (r_{CO2}, C mol_s L^{-1}). The results obtained with these three different methods are reported in Table 4. The three methods did not show any significant difference ($P < 0.05$) with the exception of C_x of the autotrophic culture. The C_x based method failed to quantify nighttime losses of the autotrophic culture, probably because it was not sensitive enough, and for this reason the night losses of the autotrophic culture were calculated using the other two methods. Excluding the C_x and making an average of r_{CO2}, and TOC, no significant difference in the nighttime losses were found between the two cultures. Nighttime losses were around 7% on carbon basis This value is within the typical range of 3−8% reported for autotrophic cultures.[12−14] Previous studies[12,13] indicated that nighttime losses depend on the growth rate of the day. In our experiment the cultures were grown in a cyclostat at a constant dilution rate during the day, and therefore expressed the same specific growth rate. This equivalent specific growth rate might explain the similar nighttime losses for both cultures.

Table 4. Nighttime Losses in a Mixotrophic and an Autotrophic Culture, According to Biomass Dry Weight Concentration (C_x, g_x L^{-1}), Total Organic Carbon Content (TOC, C mol_s L^{-1}), and CO_2 Production Rate (r_{CO2}, C mol_s L^{-1})[a]

method	mixotrophic	autotrophic
C_x	−8.7% ± 1.5%	0.7% ± 7.0%[b]
TOC	−6.8% ± 1.6%	−6.9% ± 2.9%
r_{CO2}	−5.4% ± 0.1%	−7.9% ± 0.5%

[a]The data presented are the average of 4 consecutive days at cyclostat (n = 4) and reported with the standard deviation of measurements. [b]Significant differences ($P > 0.05$).

Total organic nitrogen content (TON, g_N L^{-1}) did not change significantly along the night (Supporting Information 4). Absence of nitrogen uptake has been previously reported in other green algae[42,43] and is consistent with the hypothesis of a quiescent stage.[31] The protein fraction contains 90% of microalgal nitrogen,[44] therefore an absence of nitrogen uptake might be associated with a lack of protein synthesis. Other studies however reported that part of the carbon accumulated during the day in the form of starch or lipids, is consumed during the night for protein synthesis.[13,45] Even so, whether or not protein synthesis occurs during the night is unclear and goes beyond the scope of this study. Neither mixotrophic or autotrophic cultures expressed significant nitrogen uptake during the night, and we can safely conclude that night time nitrogen uptake is not affected by mixotrophy.

Practical Application of Oxygen Balanced Mixotrophy. In this study we demonstrated that oxygen balanced mixotrophy allows for complete removal of day-time gas−liquid exchange and that the oxygenation required in the night period is very low. In several photobioreactor (PBR) designs, however, gassing is an integral part of the mixing of the microalgal culture. In vertical panel or column type PBRs mixing is exclusively provided by gassing, but in tubular PBRs mixing and gassing are separated. In tubular PBRs mixing is ensured via a liquid pump, while oxygen and carbon dioxide gas−liquid exchange is supported by a dedicated unit usually in the form of a bubble column. In tubular PBRs the energy for gassing is 25% of the operational energy cost.[36] Our process might allow for the complete removal of the bubble column saving the related energy consumption and dramatically decreasing the complexity of the system. Also the rate of mixing (liquid circulation through the tubes) potentially can be decreased as no accumulation or depletion of oxygen or carbon dioxide is expected.

One of the major challenges of mixotrophic outdoor cultivation is the undesired contamination by heterotrophic microorganisms, mainly bacteria and fungi,[46] that compete with microalgae for the assimilation of organic carbon. Bacteria have a growth rate that is an order of magnitude higher than microalgae and they can easily outcompete microalgae for organic carbon uptake. However, since the start of commercial production of Chlorella in 1964 the pioneers involved already replaced CO_2 by acetic acid in open ponds,[47] without serious contamination of the culture. A similar approach has been recently embraced by Heliae Development LLC.[48]

In search of strategy to prevent bacterial contamination, Deschênes et al.[49] demonstrated the possibility to control bacterial contamination under mixotrophic conditions by preventing the simultaneous presence of nitrogen and organic carbon in the culture medium. The main idea behind this cultivation strategy was that microalgae can grow when either nitrogen or organic carbon are not present in the culture medium by consuming the internal quota of nitrogen and by photosynthesis, respectively, whereas most bacteria can grow only if all nutrients are simultaneously present in the culture medium. A similar strategy has been successfully adopted for microalgae heterotrophic growth in nonaxenic condition.[50]

Another possible solution to avoid bacteria contamination is to employ acidophilic microalgae that have a pH optima below 3, where most of the bacteria cannot grow. This strategy has been used to cultivate *Galdieria sulphuraria* in unsterilized primary effluent.[51] The authors reported that at pH 2 the initial bacterial population was reduced by 98% and lowering the pH resulted in complete removal of pathogen.

We strongly believe that although it might be technically feasible to run a closed photobioreactor, without aeration, with minimal infection risk, contaminations can be further controlled by employing one of the above-mentioned strategies.

■ CONCLUSIONS

In the present work, a mixotrophic microalgal monoculture was grown without gas exchange during the day, and with minimal aeration during the night. In mixotrophy biomass productivity and concentration doubled compared to an autotrophic reference culture. In the mixotrophic culture, due to efficient light utilization, 88% of the substrate was converted into biomass, making the process close to carbon

neutrality. Mixotrophic and autotrophic cultures had similar nighttime oxygen consumption patterns, with most of the oxygen consumed within the first 3 h of the night. Overall, mixotrophy required 61 times less gaseous substrates compared to autotrophy. Thus, mixotrophy is an effective strategy for reducing the requirement for gassing by at least 98%. Biomass nighttime losses were about 7% regardless of the trophic mode. The mixotrophic culture had 5% more protein and the same lutein content as the autotrophic culture. Our results indicate that mixotrophy is a successful strategy for producing protein and lutein, while still maintaining the same efficiency of light utilization as an autotrophic culture.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c03216.

O_2 production/consumption rate (section S1), raw data of CO_2 production/consumption rate (section S2), CO_2 fraction in the gas inlet and outlet (section S3), and total organic nitrogen content at nighttime (section S4) (PDF)

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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 photo active radiation, 400−700 nm
TOC total organic carbon (g_c L^{-1})
DIC dissolved inorganic carbon (C mol L^{-1})
TON total organic nitrogen (g_N L^{-1})

Symbols

V_{PBR} photobioreactor working volume (L)
A_{PBR} photobioreactor illuminated area (m^2)
D dilution rate (day^{-1})
$Y_{x/ph}$ biomass yield on light (C mol_x mol_{ph}^{-1})
F flow (mol min^{-1})
r volumetric production/consumption rate (mol L^{-1} min^{-1})
X gas molar fraction (%)
C concentration (mol L^{-1})
$Y_{x/s}$ biomass yield on substrate (C mol_x C mol_s^{-1})
MW molecular weight (g C mol^{-1})
a_x average absorption cross section (m^2 kg^{1-})
QY quantum yield (Fv/Fm)
$C_{\%}$ biomass carbon content (% w_c w_x^{-1})
$N_{\%}$ biomass nitrogen content (% w_N w_x^{-1})
K_La O_2 gas−liquid transfer coefficient (h^{-1})
q biomass specific production/consumption rate (mol C mol_x day^{-1})

Sub/super script

mixo mixotrophic
auto autotrophic
auto' autotrophic fraction of the mixotrophic biomass
het' heterotrophic fraction of the mixotrophic biomass
g gas
in/out inlet/outlet
ph PAR photons
x biomass
c carbon based biomass
s substrate
AA acetic acid

■ REFERENCES

(1) Godfray, H. C. J.; Beddington, J. R.; Crute, I. R.; Haddad, L.; Lawrence, D.; Muir, J. F.; Pretty, J.; Robinson, S.; Thomas, S. M.; Toulmin, C. Food Security: The Challenge of Feeding 9 Billion People. *Science* **2010**, *327* (5967), 812−818.

(2) García, J. L.; de Vicente, M.; Galán, B. Microalgae, old sustainable food and fashion nutraceuticals. *Microb. Biotechnol.* **2017**, *10* (5), 1017−1024.

(3) Lupatini, A. L.; Colla, L. M.; Canan, C.; Colla, E. Potential application of microalga Spirulina platensis as a protein source. *J. Sci. Food Agric.* **2017**, *97* (3), 724−732.

(4) Becker, E. W. Micro-algae as a source of protein. *Biotechnol. Adv.* **2007**, *25* (2), 207−210.

(5) Wijffels, R. H.; Barbosa, M. J. An outlook on microalgal biofuels. *Science* **2010**, *329* (5993), 796−799.

(6) Tredici, M. R. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. *Biofuels* **2010**, *1* (1), 143−162.

(7) Abiusi, F.; Wijffels, R. H.; Janssen, M. Doubling of microalgae productivity by oxygen balanced mixotrophy. *ACS Sustainable Chem. Eng.* **2020**, *8*, 6065.

(8) Blanken, W.; Cuaresma, M.; Wijffels, R. H.; Janssen, M. Cultivation of microalgae on artificial light comes at a cost. *Algal Res.* **2013**, *2* (4), 333−340.

(9) León-Saiki, G. M.; Remmers, I. M.; Martens, D. E.; Lamers, P. P.; Wijffels, R. H.; van der Veen, D. The role of starch as transient energy buffer in synchronized microalgal growth in Acutodesmus obliquus. *Algal Res.* **2017**, *25*, 160−167.

(10) de Winter, L.; Cabanelas, I. T. D.; Martens, D. E.; Wijffels, R. H.; Barbosa, M. J. The influence of day/night cycles on biomass yield and composition of Neochloris oleoabundans. *Biotechnol. Biofuels* **2017**, *10* (1), 104.

(11) Pirt, S. J.; Hinshelwood, C. N. The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. London, Ser. B* **1965**, *163* (991), 224−231.

(12) Michels, M. H. A.; Camacho-Rodríguez, J.; Vermuë, M. H.; Wijffels, R. H. Effect of cooling in the night on the productivity and biochemical composition of Tetraselmis suecica. *Algal Res.* **2014**, *6*, 145−151.

(13) Ogbonna, J. C.; Tanaka, H. Night biomass loss and changes in biochemical composition of cells during light/dark cyclic culture of Chlorella pyrenoidosa. *J. Ferment. Bioeng.* **1996**, *82* (6), 558−564.

https://dx.doi.org/10.1021/acssuschemeng.0c03216
ACS Sustainable Chem. Eng. 2020, 8, 11682−11691

(14) Edmundson, S. J.; Huesemann, M. H. The dark side of algae cultivation: Characterizing night biomass loss in three photosynthetic algae, Chlorella sorokiniana, Nannochloropsis salina and Picochlorum sp. *Algal Res.* **2015**, *12*, 470−476.
(15) Torzillo, G.; Sacchi, A.; Materassi, R.; Richmond, A. Effect of temperature on yield and night biomass loss in Spirulina platensis grown outdoors in tubular photobioreactors. *J. Appl. Phycol.* **1991**, *3* (2), 103−109.
(16) Abiusi, F.; Wijffels, R. H.; Janssen, M. Doubling of Microalgae Productivity by Oxygen Balanced Mixotrophy. *ACS Sustainable Chem. Eng.* **2020**, *8* (15), 6065−6074.
(17) de Mooij, T.; Janssen, M.; Cerezo-Chinarro, O.; Mussgnug, J. H.; Kruse, O.; Ballottari, M.; Bassi, R.; Bujaldon, S.; Wollman, F.-A.; Wijffels, R. H. Antenna size reduction as a strategy to increase biomass productivity: a great potential not yet realized. *J. Appl. Phycol.* **2015**, *27* (3), 1063−1077.
(18) Kliphuis, A. M. J.; Klok, A. J.; Martens, D. E.; Lamers, P. P.; Janssen, M.; Wijffels, R. H. Metabolic modeling of Chlamydomonas reinhardtii: energy requirements for photoautotrophic growth and maintenance. *J. Appl. Phycol.* **2012**, *24* (2), 253−266.
(19) Yang, C.; Hua, Q.; Shimizu, K. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. *Biochem. Eng. J.* **2000**, *6* (2), 87−102.
(20) Grama, B. S.; Agathos, S. N.; Jeffryes, C. S. Balancing photosynthesis and respiration increases microalgal biomass productivity during photoheterotrophy on glycerol. *ACS Sustainable Chem. Eng.* **2016**, *4* (3), 1611−1618.
(21) Wilken, S.; Schuurmans, J. M.; Matthijs, H. C. Do mixotrophs grow as photoheterotrophs? Photophysiological acclimation of the chrysophyte Ochromonas danica after feeding. *New Phytol.* **2014**, *204* (4), 882−889.
(22) Myers, J.; Graham, J.-R. The Photosynthetic Unit in Chlorella Measured by Repetitive Short Flashes. *Plant Physiol.* **1971**, *48* (3), 282.
(23) Mandalam, R. K.; Palsson, B. Elemental balancing of biomass and medium composition enhances growth capacity in high-density Chlorella vulgaris cultures. *Biotechnol. Bioeng.* **1998**, *59* (5), 605−611.
(24) Van Wagenen, J.; De Francisci, D.; Angelidaki, I. Comparison of mixotrophic to cyclic autotrophic/heterotrophic growth strategies to optimize productivity of Chlorella sorokiniana. *J. Appl. Phycol.* **2015**, *27* (5), 1775−1782.
(25) Blanken, W.; Postma, P. R.; de Winter, L.; Wijffels, R. H.; Janssen, M. Predicting microalgae growth. *Algal Res.* **2016**, *14*, 28−38.
(26) Dubinsky, Z.; Stambler, N. Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications. *Aquat. Microb. Ecol.* **2009**, *56* (2−3), 163−176.
(27) Cuaresma, M. *Cultivation of microalgae in a high irradiance area*; Wageningen University & Research, 2011.
(28) Gong, M.; Li, X.; Bassi, A. Investigation of simultaneous lutein and lipid extraction from wet microalgae using Nile Red as solvatochromic shift probe. *J. Appl. Phycol.* **2018**, *30* (3), 1617−1627.
(29) Safi, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. *Renewable Sustainable Energy Rev.* **2014**, *35*, 265−278.
(30) Kumar, K.; Dasgupta, C. N.; Das, D. Cell growth kinetics of Chlorella sorokiniana and nutritional values of its biomass. *Bioresour. Technol.* **2014**, *167*, 358−366.
(31) Strenkert, D.; Schmollinger, S.; Gallaher, S. D.; Salomé, P. A.; Purvine, S. O.; Nicora, C. D.; Mettler-Altmann, T.; Soubeyrand, E.; Weber, A. P. M.; Lipton, M. S.; Basset, G. J.; Merchant, S. S. Multiomics resolution of molecular events during a day in the life of Chlamydomonas. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (6), 2374−2383.
(32) de Winter, L.; Cabanelas, I. T. D.; Órfão, A. N.; Vaessen, E.; Martens, D. E.; Wijffels, R. H.; Barbosa, M. J. The influence of day length on circadian rhythms of Neochloris oleoabundans. *Algal Res.* **2017**, *22*, 31−38.
(33) Post, A. F.; Eijgenraam, F.; Mur, L. R. Influence of light period length on photosynthesis and synchronous growth of the green alga Scenedesmus protuberans. *Brit Phycol J.* **1985**, *20* (4), 391−397.
(34) Smith, R. T.; Bangert, K.; Wilkinson, S. J.; Gilmour, D. J. Synergistic carbon metabolism in a fast growing mixotrophic freshwater microalgal species Micractinium inermum. *Biomass Bioenergy* **2015**, *82*, 73−86.
(35) Kim, G.-Y.; Roh, K.; Han, J.-I. The use of bicarbonate for microalgae cultivation and its carbon footprint analysis. *Green Chem.* **2019**, *21* (18), 5053−5062.
(36) Acién, F. G.; Fernández, J. M.; Magán, J. J.; Molina, E. Production cost of a real microalgae production plant and strategies to reduce it. *Biotechnol. Adv.* **2012**, *30* (6), 1344−1353.
(37) Doucha, J.; Straka, F.; Lívanský, K. Utilization of flue gas for cultivation of microalgae Chlorella sp.) in an outdoor open thin-layer photobioreactor. *J. Appl. Phycol.* **2005**, *17* (5), 403−412.
(38) Cuaresma, M.; Janssen, M.; Vílchez, C.; Wijffels, R. H. Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency. *Bioresour. Technol.* **2011**, *102* (8), 5129−5137.
(39) Norsker, N. H.; Michiels, M.; Slegers, P. M.; Swinkels, G. L. A. M.; Barbosa, M. J.; Wijffels, R. H. Productivity of Nannochloropsis oceanica in an industrial closely spaced flat panel photobioreactor. *Algal Res.* **2019**, *43*, 101632.
(40) Kliphuis, A. M. J.; Janssen, M.; van den End, E. J.; Martens, D. E.; Wijffels, R. H. Light respiration in Chlorella sorokiniana. *J. Appl. Phycol.* **2011**, *23* (6), 935−947.
(41) Zijffers, J.-W. F.; Schippers, K. J.; Zheng, K.; Janssen, M.; Tramper, J.; Wijffels, R. H. Maximum Photosynthetic Yield of Green Microalgae in Photobioreactors. *Mar. Biotechnol.* **2010**, *12* (6), 708−718.
(42) Voltolina, D.; Gómez-Villa, H.; Correa, G. Nitrogen removal and recycling by Scenedesmus obliquus in semicontinuous cultures using artificial wastewater and a simulated light and temperature cycle. *Bioresour. Technol.* **2005**, *96* (3), 359−362.
(43) Boelee, N. C.; Janssen, M.; Temmink, H.; Shrestha, R.; Buisman, C. J. N.; Wijffels, R. H. Nutrient Removal and Biomass Production in an Outdoor Pilot-Scale Phototrophic Biofilm Reactor for Effluent Polishing. *Appl. Biochem. Biotechnol.* **2014**, *172* (1), 405−422.
(44) Becker, E. W. Microalgae in human and animal nutrition. *Handbook of microalgal culture: biotechnology and applied phycology*; Blackwell Science, Oxford: 2004; pp 312−351.
(45) Blanken, W.; Magalhães, A.; Sebestyén, P.; Rinzema, A.; Wijffels, R. H.; Janssen, M. Microalgal biofilm growth under day-night cycles. *Algal Res.* **2017**, *21*, 16−26.
(46) Unnithan, V. V.; Unc, A.; Smith, G. B. Mini-review: A priori considerations for bacteria−algae interactions in algal biofuel systems receiving municipal wastewaters. *Algal Res.* **2014**, *4*, 35−40.
(47) Iwamoto, H. 11 Industrial Production of Microalgal Cell-mass and Secondary Products−Major Industrial Species. *Handbook of microalgal culture: biotechnology and applied phycology* **2004**, 255−263.
(48) Ganuza, E.; Tonkovich, A. L. An Industrial Approach to Mixotrophy in Microalgae. In *Industrial Biorenewables*; Heliae Development, LLC, 2016; pp 323−339.
(49) Deschênes, J.-S.; Boudreau, A.; Tremblay, R. Mixotrophic production of microalgae in pilot-scale photobioreactors: Practicability and process considerations. *Algal Res.* **2015**, *10*, 80−86.
(50) Di Caprio, F.; Altimari, P.; Iaquaniello, G.; Toro, L.; Pagnanelli, F. Heterotrophic cultivation of T. obliquus under non-axenic conditions by uncoupled supply of nitrogen and glucose. *Biochem. Eng. J.* **2019**, *145*, 127−136.
(51) Delanka-Pedige, H. M. *Algal-based wastewater treatment: assessment of pathogen removal and bacterial community profile of effluent*; New Mexico State University, 2018.