Light intensity affects the mixotrophic carbon exploitation in Chlorella protothecoides: consequences on microalgae-bacteria based wastewater treatment

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ABSTRACT

Microalgal-bacteria consortia application on wastewater treatment has been widely studied, but a deeper comprehension of consortium interactions is still lacking. In particular, mixotrophic exploitation of organic compounds by microalgae affects gas (CO\textsubscript{2} and O\textsubscript{2}) exchange between microalgae and bacteria, but it is not clear how environmental conditions may regulate algal metabolism. Using a respirometric-based protocol, we evaluated the combined effect of organic carbon and light intensity on oxygen production and consumption by \textit{C. protothecoides}, and found that the chemical oxygen demand (COD) was not consumed when incident light increased. Batch experiments under different incident lights, with \textit{C. protothecoides} alone and in consortium with activated sludge bacteria, confirmed the results obtained by respirometry. Continuous system experiments testing the combined effects of light intensity and residence time confirmed that, under limiting light, mixotrophy is preferred by \textit{C. protothecoides}, and the nutrient (COD, N, P) removal capability of the consortium is enhanced.

Key words | activated sludge, COD, continuous system, nitrogen, phosphorus, respirometry

INTRODUCTION

According to the increasing demand for fresh water and for green and cost-effective water remediation technologies, biological wastewater treatment may play a crucial role in the future. In particular, integration of wastewater treatment plants (WWTP), based on activated sludge technology, with microalgae technology seems promising (Karya \textit{et al.} 2013; Anbalagan \textit{et al.} 2016; Krustok \textit{et al.} 2016). Despite the strong reliability and constant improvements of conventional WWTP, some criticisms, such as sludge aeration costs (Longo \textit{et al.} 2016) as well as nitrogen and phosphorus recovery, have not been completely solved yet. Namely, nutrient removal often requires additional steps involving chemical treatments, thus increasing costs and environmental impact. For these reasons, many authors are focusing on microalgal-bacteria consortia, recognising them as an environmentally friendly technology (Boeele \textit{et al.} 2014; Gupta \textit{et al.} 2016; Ibehwe \textit{et al.} 2017). In principle, gas exchange between microalgae and bacteria may act as a growth promoting agent for both cultures (Boeele \textit{et al.} 2014) and the nitrogen and phosphorus removal capability of microalgae may be exploited in a simpler, single step treatment process (Beuckels \textit{et al.} 2015; Beltrán-Rocha \textit{et al.} 2017). In addition, from a circular economy perspective, microalgae store the nitrogen and phosphorus present in wastewaters, with clear impact on nutrient recycling strategies. Microalgae also synthesize potentially commercially valuable byproducts, such as pigments, fatty acid and biofertilizers (Beuckels \textit{et al.} 2015; Beltrán-Rocha \textit{et al.} 2017).

However, applying consortium technology to water remediation still has some unsolved issues. It is well known that microalgal metabolism is extremely flexible, as it is affected by temperature, photoperiod (Delgadillo-Mirquez \textit{et al.} 2016), and also by light intensity (Kim \textit{et al.} 2013) and wastewater source (municipal or industrial), thus potentially affecting the pollutants’ removal efficiency (Collos \textit{et al.} 2005; Wu \textit{et al.} 2014; Abinandan & Shanthakumar 2015). Light availability is a major factor determining microalgal photosynthesis and thus productivity, but when organic carbon is present, complex interactions may occur (Moya \textit{et al.} 1997).
Moreover, the algal mixotrophic metabolism plays a critical role in consortium interactions (Sforza et al. 2018). The regulation of mixotrophic metabolism in complex environments is still a matter of discussion.

Our work addresses the effect of light intensity on the mixotrophic capability of *C. protothecoides*, with particular attention to effects on oxygen production, pollutant removal and consequences on *C. protothecoides* and the activated sludge bacteria consortium. *C. protothecoides* is a suitable species to study mixotrophy and application to wastewater treatment, as the genus *Chlorella* is commonly found in high-rate algal ponds (HRAP) (Beuckels et al. 2015; Acién et al. 2016). To properly compare the role of light on mixotrophy and consortia interactions, respirometric tests were carried out to quantify the effect of light on oxygen production by photosynthesis in autotrophic and mixotrophic conditions. Batch experiments with the consortium under different light intensities were compared to controls with *C. protothecoides* alone, to confirm the results obtained by respirometric tests. To better assess the effect of light on the consortium, continuous experiments under different light intensities and residence times were carried out. Finally, the aim of this work is to provide useful information, obtained under controlled laboratory conditions, which can be used to portray complex phenomena that may occur in real systems.

**MATERIALS AND METHODS**

**Microalgal, bacterial species, growth medium and wastewater**

*Chlorella protothecoides* 33.80 (SAG Goettingen, Germany) was axenically cultivated and maintained in liquid BG11 medium for the pre-inoculum, at 24 ± 1 °C, and 100 μmol m⁻² s⁻¹.

Bacteria inoculum, obtained from the activated sludge sampled at the full scale municipal wastewater treatment plant of Montebello Vicentino (Vicenza, Italy; latitude 45°27′26″N, longitude 11°23′4″E) was maintained in exponential phase in liquid flasks with synthetic wastewater (recipe reported in Table S1 of Supplementary materials, available with the online version of this paper), and inoculated in fresh medium at 30 °C, one day before each experiment to minimize the inert content of biomass, and inoculate actively growing cells.

A standard synthetic wastewater (OECD 2001) was slightly modified in order to have a composition similar to local urban wastewater. Chemicals used for preparing the synthetic wastewater are reported in Table S1 (Supplementary materials), while the final characterization is the following: NH₄⁺-N 13 mg L⁻¹, total nitrogen (TN) 29 mg L⁻¹, PO₄³⁻-P 7 mg L⁻¹, chemical oxygen demand (COD) 313 mg L⁻¹, which is similar to the real one of the Montebello plant (Sforza et al. 2018).

**Respirometric assays**

In order to evaluate the oxygen production rate of *C. protothecoides* at different light intensities, in the presence of both inorganic and organic carbon substrates, some tests based on respirometry were carried out. Decostere et al. (2013), Sforza et al. (2018) and Rossi et al. (2018) previously reported a similar approach.

Dissolved oxygen (DO) concentration was continuously measured by means of a Handylab Ox 12 SCHOTT® oximeter connected to a PC by using Multi/ACHAT II software provided by WTW. The oxygen measurement was carried out in airtight flasks of 100 mL, in order to prevent oxygen transfer between the liquid and the external air. In addition, minimal gas headspace was left to avoid gas losses. The liquid was continuously mixed by a magnetic stirrer and the temperature was maintained constant at 25 °C by using a thermostatic water bath.

Each respirometric test was started with fresh culture medium (BG11), where a constant and axenic microalgal biomass inoculum (about 0.44 g L⁻¹ of DW), previously centrifuged, was resuspended. To check the axenicity of the preinoculum, a sample of the culture was plated on LB (Luria Broth medium) one day before each respirometric test. An inorganic carbon source, to stimulate autotrophy, was added as sodium bicarbonate, while peptone was used as organic substrate. Both substrates were supplied at a concentration of about 200 mg L⁻¹ of C, which can be considered non-limiting, based on the mass balance of carbon in microalgae growth and growth kinetic. In particular, carbon was supplied in an amount sufficient for at least one duplication of the biomass, which however occurs in a longer time than that needed for the respirometric test. Each test lasted about 60 min and consisted of alternating cycles of light and dark (7.7 min), obtained thanks to a digital controller connected to a LED lamp at irradiation of 10, 70 or 130 μmol m⁻² s⁻¹.

The average light intensities (*I*ₐᵥ, μmol m⁻² s⁻¹) in the reactor were calculated by means of Lambert Beer’s law, reported as follows (Grima et al. 1997):

\[
I_{av} = \frac{I_0}{H \cdot K_a C_M} \cdot (1 - \exp(-H K_a C_M))
\]
Experimental set-up and procedures

**Batch experiments**

In order to verify the results obtained in respirometric tests, and possibly provide information that can be used to understand complex phenomena in real systems, batch experiments with *C. protothecoides* and activated sludge bacteria grown in synthetic wastewater were carried out under controlled conditions. This was meant to investigate their growth and their nutrient removal capacity under continuous light at different intensities. Each experiment started with an initial biomass inoculation of OD750 with an initial biomass inoculation of OD750. Each run started with a dark phase, and the first 14 min of data acquisition were discarded to allow the acclimation of the microorganisms to the applied operating conditions. The pH of the medium was buffered at 8 with HEPES 1M. This protocol resulted in DO trends, of which an example is reported in Sforza et al. (2018) and Figure S1 (Supplementary materials, available online).

When the DO values increase (light on) or decrease (light off), oxygen production (OPR, mgO2 L⁻¹ min⁻¹) and consumption rate (OCR, mgO2 L⁻¹ min⁻¹) can be calculated from the slope. Each OPR and OCR were estimated as the average of at least three measurements. The specific oxygen production and consumption rates (mgO2 min⁻¹ gM⁻¹) were obtained, dividing by the initial microalgal biomass concentration measured as DW (gM L⁻¹).

**Continuous experiments**

Continuous experiments were carried out in a flattened glass tank, of 6.5 cm thickness. A scheme of the continuous system is reported in the Supplementary materials (Figure S2, available online). The reactor was initially inoculated with the consortium and it was firstly run in batch mode, allowing cells to enter into an exponential phase of growth and to reach a sufficient biomass concentration to avoid washout occurring. The photobioreactor was then switched to continuous mode, and fresh medium was continuously supplied from an external sterilized stirred bottle by means of a peristaltic pump (Sci-Q 400, Watson Marlow, USA), at a constant volumetric flow-rate Q (mL d⁻¹). The culture volume was kept constant by the presence of an overflow tube, placed at the opposite side of the fresh medium inlet, from which biomass was continuously collected at the same flow-rate. The hydraulic residence time (HRT, τ) of the reactor is calculated as:

\[
\tau = \frac{V_R}{Q}
\]

where \( V_R \) is the reactor volume (mL). The flow-rate was regulated in order to obtain the desired residence time. Two series of experiments were carried out at 1.6, 1.9, 2.5, 3.2 and 4 days of HRT, under continuous incident lights of 50 or 150 \( \mu \)mol m⁻² s⁻¹. Biomass and nutrient concentrations were measured daily in the outlet and averaged for at least 4–5 points at steady state. Steady state was reached within 3.2 and 4 days of HRT.
assumed when biomass concentration was found to be stable for at least 5 days.

Analytical methods

The overall consortium concentration was monitored by spectrophotometric analysis of the optical density, measured at 750 nm, to avoid specific absorption of photosynthetic pigments (Chlorophyll), by double beam spectrophotometer UV-Visible UV 500 from Spectronic Unicam, UK. In order to measure microalgal cell concentration, specifically, cells were counted with a Bürker Chamber (HBG, Germany).

pH was checked and manually adjusted, when needed, in both continuous and batch experiments. The concentration of total biomass of each experiment was also gravimetrically measured as dry weight (DW) in terms of g L⁻¹, on biomass filtered (0.22 μm) and dried at 105 °C in a laboratory oven for 2 h. As the medium was synthetically formulated, and biomass was sampled in the exponential phase of growth, the inert content can be considered negligible. Nitrate (NO₃⁻N), nitrates (NO₂⁻N), ammonium (NH₄⁺-N), N₉ₒ₉₆, orthophosphate (PO₄³⁻-P) (Hydrocheck Spectratest kits by Reasol®) and COD (Spectroquant® solutions by Merck KGaA) were determined for both batch and continuous systems, in order to verify the consumption by microalgae or the consortium, after biomass removal by filtration, to measure dissolved compounds. TN was measured as nitrate, after persulfate digestion in autoclave for 1 h.

Statistical analysis

One way analysis of variance (ANOVA) was applied on normally distributed data, to ascertain meaningful differences among specific oxygen production and consumption rates, followed by a post hoc Tukey HSD test, performed by a MatLab® script. The same approach was also applied for nutrient consumption in batch experiments and for results of the continuous system. Shapiro-Wilk tests for normality were applied to data (p > 0.05), followed by a graphical test (QQ-plot) to confirm the first test. In batch growth curves, final biomass concentration between the consortium and microalgae-alone control was assessed by applying regular Student’s t-tests. The level of statistical significance was assumed for P < 0.05, and significantly different results are highlighted with different letters in the figures. More details are also reported in the corresponding captions.

RESULTS AND DISCUSSION

Respirometric measurements

The oxygen production rate of axenic microalgae increased with light intensity under both autotrophic and mixotrophic conditions (Figure 1), as expected. At high light intensity, the source of carbon (organic or inorganic) had no effect on oxygen production by C. protothecoides under auto- or mixotrophy (0.45 and 0.53 mg O₂ L⁻¹ g⁻¹, respectively), while oxygen production was lower under mixotrophic conditions at low light intensity (Figure 1). At dark, under mixotrophic conditions, the consumption rate was generally twice that under autotrophy, confirming that the organic carbon is actually consumed. In addition, the light intensity provided during the light phase did not influence the respiration at dark. In the presence of organic carbon, a correlation between light intensity and the prevalence of metabolic pathway is also evidenced (Figure 1(c)): at high light values, photosynthetic metabolism is preferred (i.e. there is no reduction in O₂ production between the two cases), even though organic carbon is present in the medium. Instead, at lower light, consumption of organic carbon occurred, reducing the net oxygen production with respect to the autotrophic case. In particular, at 10 μmol m⁻² s⁻¹, a reduction of almost 100% in net oxygen production was found when organic carbon was supplied.

This result suggests that low irradiation may be beneficial for nutrient removal by microalgae from wastewater containing organic carbon and nitrogen, an outcome that looks to be in contrast with the general idea of mutual gas exchange between algae and bacteria in wastewater treatment. The acclimation capability of microalgal metabolism to different environmental conditions is relevant and widely reported in the literature (Abinandan & Shanthakumar 2015). Microalgal species can switch from autotrophic to mixotrophic metabolism according to the medium nutrients’ composition (Wu et al. 2014; Abinandan & Shanthakumar 2015; Sforza et al. 2018), while only a few papers have reported the effect of light intensity (Moya et al. 1997).

Batch growth curves

Batch growth curves of C. protothecoides alone (M) and in consortium with bacteria (M + B) are reported in Figure 2 as cell concentration, while optical density measurements, which take into account bacteria, are available in the
Figure 1 | Specific oxygen production and consumption rate in autotrophic (a) and mixotrophic conditions (b) of Chlorella protothecoides (axenic culture). Grey bars represent average oxygen production, black bars are oxygen consumption (average ± standard error, n = 4 for each). The reduction of O₂ production was also correlated (y = -40.5ln(x) + 167.23, R-square = 0.998) to lₐᵥ (c), calculated as reported in Equation (1). Letters refer to statistically significant differences, based on ANOVA one way test (a) Fₚₐₚₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜ¢...
Supplementary materials (Figure S3, available with the online version of this paper).

A different behaviour was observed between low (10 μmol m⁻² s⁻¹) and high light intensity (70 and 130 μmol m⁻² s⁻¹). Details are reported in Table S2 (Supplementary materials, available online). At 10 μmol photons m⁻² s⁻¹, microalgae alone grew better than in co-cultivation with bacteria, and also the specific microalgal growth rate constant was higher. Usually, under autotrophic conditions (Eriksen et al. 2007; Sforza et al. 2015), the growth rate is linearly correlated with light intensity below saturation. On the other hand, when mixotrophy occurred, low light corresponded to the highest microalgal growth. This is in agreement with the results of respirometric tests, suggesting that low light may be beneficial for mixotrophic growth, and increases the specific growth rate of microalgal biomass.

For C. protothecoides, the capability to carry out mixotrophy in wastewater was already demonstrated (Sforza et al. 2018). The autotrophic-mixotrophic metabolisms seem to have a more complex regulation when both light and organic carbon are provided, also considering the acclimation flexibility of microalgae to the available nutrients (Collos et al. 2005). This hypothesis is confirmed by looking at the nitrogen consumption (Figure 3(a)). In both conditions tested (M and M+B), nitrogen removal efficiency is inversely related to the light intensity, as the organic nitrogen consumption decreases with increasing light, confirming the hypothesis of a competition of mixotrophic-autotrophic pathways when both light and organic compounds are provided in excess.

The final nitrogen concentration at 70 and 130 μmol m⁻² s⁻¹ in microalgae cultivated alone is similar (13.4 and 14.2 mg L⁻¹, respectively), while the consortium reaches concentrations of about 15 and 18 mg L⁻¹, respectively. No significant presence of nitrates and nitrites was found, probably due to low activity of nitrifying bacteria. Since ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) have lower growth rates (0.54 and 0.67 day⁻¹, respectively, as reported in Blackburne et al. 2007) than an averaged value of 0.9–1.3 day⁻¹ for C. protothecoides (see Supplementary materials), it is reasonable that all the ammonia available in the wastewater was exploited more quickly by microalgae. This supports the hypothesis by Craine et al. (2005) that nutrient competition among vegetal species is ruled more by the net availability than the uptake rate of these compounds. In addition, the oxygen request by nitrifying bacteria is probably not fully supplied by photosynthesis, thus limiting their growth.

P removal was also assessed, and the corresponding results and discussion are reported in the Supplementary materials.

COD reduction was similar in the two conditions (99 and 96 mg L⁻¹ for M and M+B, respectively) at low light intensity, while under higher light the final COD concentration increased. In particular, in the presence of bacteria, the final COD was lower than in cultures with microalgae cultivated alone. This suggests there might be competition for nutrients (e.g. nitrogen) between algae and bacteria, which would reduce growth and trigger the production of organic carbon compounds by microalgae, with the overall result of increasing the COD concentration (Natrah et al. 2014; Wu et al. 2014). Consistent with literature data, an
excretion of compounds by microalgae (such as EPS) is also possible, when they coexist with bacteria (Natrah et al. 2014), or the release of cell materials due to growth decay in the late stationary phase.

In summary, respirometry and batch experiments highlighted that low light may be beneficial for triggering mixotrophic exploitation of organic compounds by microalgae, resulting in an improvement of the overall performance of the consortium. On the other hand, besides the total consumption of nutrients, the interaction between light and carbon availability may strongly affect the kinetics of the process, in particular in co-cultivation of microalgae with bacteria.

Continuous experiments

The different growth kinetics of microalgae and bacteria may affect the nutrient removal in a continuous system, which is commonly preferred in real operating WWT systems. Results of cell and biomass (DW) concentrations of continuous experiments are shown in Figure 4 (data of OD and biomass productivity are reported in the Supplementary materials, Figures S4 and S5, available online). These experiments were focused on understanding the behaviour of a microalgal-bacteria consortium in a continuous reactor and the effect of light and residence time on mixotrophic exploitation of organic carbon.

For both of the irradiances tested, we observed that the biomass concentration increased along with residence time (HRT), which is a common response of continuous biological reactors. At the lowest HRT value (1.6 d), washout of microalgal biomass occurred, while a bacterial concentration was always measured. At 1.9 d, we obtained a value close to the washout, under 50 μmol m⁻² s⁻¹. The most interesting outcome was found at higher residence times: at lower irradiances (50 μmol m⁻² s⁻¹) the biomass concentration increased more than under 150 μmol m⁻² s⁻¹, which is the opposite of what usually happens when microalgae are cultivated alone, and under autotrophic conditions (Eriksen et al. 2007; Sforza et al. 2015). In fact, at high light intensities, an increase of biomass concentration is generally encountered, except for very strong irradiations that may cause severe photoinhibition (Sforza et al. 2015). We found an opposite trend: the higher the light intensity (even not inhibiting), the lower the growth performance of the consortium. This is particularly evident in the case of HRT = 4 d, not only as far as the dry weight is concerned (0.36 g L⁻¹ and 0.29 g L⁻¹ under 50 and 150 μmol m⁻² s⁻¹, respectively), but also in terms of microalgal cell concentrations, as shown in Figure 4 (about 48 and 22 million cells mL⁻¹ under 50 and 150 μmol m⁻² s⁻¹, respectively).

In agreement with both respirometric and batch growth results, it can be concluded that the availability of light decreases the microalgal mixotrophic capability of exploiting organic substrates. Apparently, when light is supplied to the culture, microalgae do not efficiently consume the organic matter, and growth is limited also by other nutrients, including CO₂. Conversely, at 50 μmol m⁻² s⁻¹, the mixotrophic metabolism is preferred, allowing the exploitation of organic carbon and nitrogen, which enhanced the microalgal growth.

While microalgal cells can be easily counted by a microscope, the current techniques do not allow separation and quantification of the bacterial biomass. On the other hand, a rough method can be applied to have a preliminary...
estimation of the ratio between the two populations, based on the correlation of microalgal cell concentrations with dry weight, as suggested by Pastore & Sforza (2018). Thus, as shown in Figure S6 of the Supplementary materials (available online), the microalgae:bacteria biomass ratio was generally higher at lower irradiances, as a result of the increased capability of microalgae to exploit organic carbon. This result should be confirmed with a method able to discriminate among different populations.

N and COD concentrations were also analysed as a function of both residence time and light intensity. Results under 50 μmol m\(^{-2}\) s\(^{-1}\) are reported in Figure 5, where a clear difference is found between low and high residence times, due to the washout of microalgae below 1.9 d of HRT. At lower residence time, bacteria completely converted nitrogen to ammonia, which is only consumed when microalgae are present. At higher residence times, not all the organic nitrogen was consumed, probably because of some competition between microalgae and bacteria, as already observed in Sforza et al. (2018) and in the batch experiments of Figure 3(a).

Interesting results were also obtained for COD, with very low values in the outlet stream for all the HRT tested. A slight increase of final COD occurred at higher residence times, as a result of two possible phenomena: the already mentioned competition between populations, or an excretion of organic molecules by microalgae, according to what is reported in the batch section and in other papers (Natrah et al. 2014).

Both organic nitrogen and COD removal were lower under high (150 μmol m\(^{-2}\) s\(^{-1}\)) than under low light (Figure 6). Such results confirm that the higher the light irradiance, the lower the organic matter consumed by microalgae, leading to the lower biomass concentration reported in Figure 4 for the same light intensity. Details of P removal are reported in the Supplementary materials.

In summary, it is not clear yet which is the role of mixotrophic metabolism in the interactions with the bacterial population. As a general comment, based on respirometric tests, when mixotrophy is preferred, lower oxygen is available in the culture for heterotrophic bacteria. Besides the ratio between microalgal and bacterial populations, lower light seems to increase the nutrient removal, a result which could be beneficial in terms of surface requirement of a microalgal:bacteria based wastewater treatment system. In fact, treating wastewater by autotrophic growth alone would require an extension of land which is incompatible with economical applications of this type.

**CONCLUSIONS**

In this work, the effect of light on growth performances of microalgal-bacteria consortia was analysed, in order to better ascertain the complex interaction that may occur in a complex medium such as a wastewater. In particular, low light was found to be beneficial to obtain a higher nutrient removal from urban wastewater thanks to the mixotrophic metabolism of *C. protothecoides*. The respirometric test showed a reduction of organic carbon exploitation under high light and a correlation between
the reduction of O₂ production and light reaching the cells was proposed. The co-presence of bacteria in consortium increased the microalgal growth rate. However, microalgal metabolism seems to be more affected by light availability, which influences algal capability to mixotrophically exploit organic carbon and nitrogen. Results of batch experiments were validated in a continuous system, where it was found that hydraulic residence time (HRT) affected the biomass concentration, but lower light stimulated a higher organic nutrient consumption. Also, HRT lower than 2.5 d caused a washout of microalgae from the system, and a consequent reduction of N and P removal. In conclusion, low light seems to be potentially beneficial for an urban wastewater treatment plant where microalgae are applied together with bacteria. The cultivation of photosynthetic organisms alone usually requires large areas, but thanks to the occurrence of mixotrophic metabolisms, less surface is necessary with consortia, ensuring an efficient one-step simultaneous removal of nitrogen, phosphorus and organic carbon, in a possibly economically acceptable solution.

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