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ENVIRONMENTAL BIOTECHNOLOGY

Mixotrophic metabolism of *Chlorella sorokiniana* and algal-bacterial consortia under extended dark-light periods and nutrient starvation

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Abstract Microalgae harbor a not fully exploited industrial and environmental potential due to their high metabolic plasticity. In this context, a better understanding of the metabolism of microalgae and microalgal-bacterial consortia under stress conditions is essential to optimize any waste-to-value approach for their mass cultivation. This work constitutes a fundamental study of the mixotrophic metabolism under stress conditions of an axenic culture of *Chlorella sorokiniana* and a microalgal-bacterial consortium using carbon, nitrogen, and phosphorous mass balances. The hydrolysis of glucose into volatile fatty acids (VFA) during dark periods occurred only in microalgal-bacterial cultures and resulted in organic carbon removals in the subsequent illuminated periods higher than in *C. sorokiniana* cultures, which highlighted the symbiotic role of bacterial metabolism. Acetic acid was preferentially assimilated over glucose and inorganic carbon by *C. sorokiniana* and by the microalgal-bacterial consortium during light periods. $N\text{-}NH_4^+$ and $P\text{-}PO_4^{-3}$ removals in the light stages decreased at decreasing duration of the dark stages, which suggested that N and P assimilation in microalgal-bacterial cultures was proportional to the carbon available as VFA to produce new biomass. Unlike microalgal-bacterial cultures, *C. sorokiniana* released $P\text{-}PO_4^{-3}$ under anaerobic conditions, but this excretion was not related to polyhydroxybutyrate accumulation. Finally, while no changes were observed in the carbohydrate, lipid and protein content during repeated extended dark-light periods, nutrient deprivation boosted both C-acetate and C-glucose assimilation and resulted in significantly high biomass productivities and carbohydrate contents in both *C. sorokiniana* and the microalgal-bacterial cultures.

Keywords Algal-bacterial consortium · Bioremediation · *C. sorokiniana* · Extended dark-light periods · Mass balances · Nutrient deprivation

Introduction

Microalgae can play a key role in the treatment of water pollution and support a green bioeconomy in this XXI century due to their high metabolic versatility and productivity rates. Microalgae are able to grow simultaneously under photoautotrophic and heterotrophic conditions (Pérez-García et al. 2010), and even nitrifying activity has been detected in *Chlorella* in the presence of ammonium (Kessler and Oesterheld 1970). Guieysse et al. (2013) confirmed the presence of the enzyme nitrate reductase (an enzyme involved in the bacterial denitrification pathway) in axenic cultures of *Chlorella vulgaris*. In addition, luxury P uptake in microalgae can result into structural P contents of up to 3 %, which could support a microalgae-based enhanced biological P removal (EBPR) and allow for nutrient recycling via fertilization with microalgae biomass (Powell et al. 2008, 2009; Arbib et al. 2014). In this context, microalgae cultivation as a platform technology for secondary or tertiary wastewater treatment can support a simultaneous C, N, and P removal via mixotrophic fixation and the heterotrophic degradation of persistent organic pollutant (Muñoz and Guieysse 2006; Abreu et al. 2012). Microalgae-based wastewater treatment results in a large production of residual biomass from which high-added-value products such as lipids, carbohydrates, amino acids, and polyunsaturated fatty

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acids or pigments could be extracted (Pérez-García et al. 2011a; Cea-Barcía et al. 2014).

The potential of microalgae to support a sustainable and economically profitable bioeconomy is based on the ability of microalgae to accumulate or biotransform biogenic and xenobiotic compounds when exposed to stress growth conditions using mechanisms similar to those found in bacterial metabolic routes (Prajapati et al. 2013; Markou and Nerantzis 2013). For instance, lipid accumulation occurs under nutrient deprivation conditions (Chu et al. 2013), salinity (Takagi et al. 2006), or heavy metal stress (Liu et al. 2008). Likewise, nutrient stress cultivation is used to enhance microalgal carbohydrate content (Kim et al. 2014). Recent studies have shown that certain cyanobacteria are capable of producing polyhydroxyalkanoates under extended dark-light periods or nutrient deprivation conditions similarly to polyphosphate accumulating bacteria (PAO) (Panda and Mallick 2007). In this context, Sharma and Mallick (2005) reported polyhydroxybutyrate (PHB) accumulation in *Nostoc muscorum* of 14, 20, and 35 % (*w*/*w*) when supplemented with 0.2 % acetate (585 g/m^3 C-acetate) and incubated under dark conditions for 3, 5, and 7 days, respectively. All these findings suggest that bacterial and algal metabolic pathways potentially involve similar precursors and enzymes (Subashchandrabose et al. 2011), which indicates that microalgal biotechnology harbors an industrial and environmental potential higher than that currently exploited. Thus, the elucidation of the influence of stress cultivation conditions on carbon and nutrient uptake, and biomass composition of microalgae and microalgae-bacteria consortia, is of key relevance in the optimization of any waste-to-value approach for microalgae mass cultivation and constitutes one of the most relevant knowledge gaps in this field. However, the lack of systematic empirical studies quantitatively evaluating the metabolism of microalgae and microalgae-bacteria consortia under stress conditions still hampers the exploitation of the full potential of these microorganisms. Therefore, the synergistic effects derived from the symbiosis between microalgae and bacteria in terms of metabolic plasticity and robustness represents a key niche for research in microalgal biotechnology.

This work constitutes a fundamental study using a carbon, nitrogen, and phosphorous mass balance approach of the mixotrophic metabolism under stress conditions of an axenic culture of *Chlorella sorokiniana* and a microalgal-bacterial consortium. Hence, the influence of repeated extended dark-light periods and nutrient deprivation on microbial growth, PHB and phosphorous accumulation, macroscopic biomass composition, and removal efficiencies of organic and inorganic carbon, nitrogen, and phosphorus was assessed.

Materials and methods

Microorganisms and inoculum cultivation conditions

The microalga *C. sorokiniana 211/8k* was obtained from the Culture Collection of Algae (SAG) of Göttingen University (Germany). The algal-bacterial consortium was obtained from a high-rate algal pond (HRAP) treating diluted centrates at the Department of Chemical Engineering and Environmental Technology of Valladolid University (Spain). This consortium was harvested from the HRAP broth by centrifugation for 10 min at 15,317×*g* (Sorvall, LEGEND RT+ centrifuge, Thermo Scientific) and resuspended in Sorokin-Krauss mineral salt medium (SK MSM). The microalgae/cyanobacteria population (from now on referred to as microalgae) was composed of (percentage of cells) *Limnothrix mirabilis (Böcher) Anagnostidis* (57.1 %), *Woronichinia sp.* (15.9 %), *Synechocystis aquatilis Sauvageau* (12.7 %), *Geitlerinema sp.* (11.4 %) and *Cyanosarcina sp.* (2.9 %). The composition of the bacterial population was not analyzed quantitatively by molecular tools but qualitatively assessed by microscopic observations, which confirmed the presence of bacteria in the algal-bacterial consortium used. *C. sorokiniana* and the microalgal-bacterial inocula were incubated in enriched SK MSM at 30 °C under magnetic agitation at 300 rpm and a photosynthetic active radiation (PAR) of 100±11 μE/m^2·s for 5 days. The SK medium was prepared according to Alcántara et al. (2013) and enriched with sterile solutions of glucose, CH_3COONa, peptone, and yeast extract to a final concentration of 1.25, 1.71, 0.0625, and 0.0625 g/dm^3, respectively.

Mixotrophic cultivation under extended dark-light periods

The first series of experiments consisted of cycles of extended dark stages (DS) under anaerobic conditions followed by illuminated stages (LS) at 100±11 μE/m^2·s. The duration of the dark stages in *C. sorokiniana* cultures was fixed at 7 days, while the illuminated stages lasted for 8, 14, and 30 days. On the other hand, the dark stages lasted for 7, 5, and 2.5 days in the tests conducted with the algal-bacterial consortium, while the duration of the subsequent illuminated stages was set at 14 days (Table S1 and Table S2 in "Supplementary Material"). The experiments were performed batchwise in 2.1 dm^3 glass bottles (five bottles for *C. sorokiniana* under sterile conditions and five bottles for the algal-bacterial consortium) containing 1.9 dm^3 of sterile modified BG-11 mineral salt medium (BG-11 MSM). This medium was composed of (per dm^3 of distilled water) the following: 0.1909 g NH_4Cl, 0.04 g K_2HPO_4, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.036 g $CaCl_2 \cdot 2H_2O$, 0.006 g citric acid, 0.006 g ferric ammonium citrate, 0.001 g Na_2EDTA, 0.02 g Na_2CO_3, and 1 cm^3 of a trace element solution containing (per dm^3 of distilled water) 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$,

0.39 g $Na_2MoO_4 \cdot 2H_2O$, and 0.0404 g $CoCl_2 \cdot 6H_2O$. The final pH of the medium was ≈7.2. The BG-11 MSM was supplemented with 0.375 g glucose/dm^3 and 0.513 g CH_3COONa/dm^3 as organic carbon source at the beginning of each dark period, which resulted in an initial total organic carbon (TOC) concentration of 300 g/m^3 (150 g/m^3 as C-CH_3COONa and 150 g/m^3 as C-glucose) (typical TOC concentrations in domestic wastewaters according to Asano et al. 2002). The initial nitrogen and phosphorus concentrations (50 g N-NH_4^+/m^3 and 7 g P-PO_4^{-3}/m^3, respectively) also mimicked the typical N and P concentrations in medium-strength domestic wastewater (Asano et al. 2002). In addition, *C. sorokiniana* and the algal-bacterial cultures were supplemented at the beginning of every dark stage with a sterile buffer solution of $NaHCO_3$ and $NaCO_3$ in order to increase the pH up to 9.0±0.3 to prevent acidification during the dark stage. The different buffer capacity observed in *C. sorokiniana* and the algal-bacterial broths resulted in average inorganic carbon (IC) concentrations of 105±2 and 147±17 g/m^3 at the beginning of each dark stage, respectively. These IC concentrations also corresponded to typical IC concentrations in domestic wastewater (≈100–150 g/m^3) (Asano et al. 2002). The term TIC represents here the total inorganic carbon in the system (gas C-CO_2 + dissolved IC). The bottles were always flushed with sterile nitrogen (N_2) for 15 min at the beginning of every cycle and allowed to equilibrate for 2 h prior to sampling (the renewal of the bottle's headspace was performed in a sterile bench by filtering the N_2 through 0.20-μm nylon filters previously autoclaved to maintain the sterility in *C. sorokiniana* cultures). The cultures were incubated at 30 °C under continuous magnetic agitation at 300 rpm.

Gas samples of 100 μL were drawn from the headspace of the bottles to measure the concentrations of CO_2, O_2, N_2, and CH_4 by GC-TCD. Liquid samples of 200 cm^3 were also drawn (under sterile conditions using sterile plastic syringes in an sterile bench in *C. sorokiniana* cultures) at the beginning and end of each dark and light stage in order to determine the concentrations of dissolved TOC, dissolved IC, dissolved N species (TN, N-NH_4^+, N-NO_2^-, N-NO_3^-, and $N_{organic}$), dissolved P (P-PO_4^{-3}), and biomass concentration as total (TSS) and volatile (VSS) suspended solid concentration. The concentration of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic, and heptanoic acids were quantified by GC-FID. The term VFA* stands here for the total carbon concentration of volatile fatty acids (C-VFA) except acetic acid. The liquid volume extracted was replaced by fresh BG-11 MSM (previously autoclaved for 20 min at 120 °C in *C. sorokiniana* cultures) before the beginning of each DS, in order to maintain the initial cultivation volume (1.9 dm^3). Likewise, the pH and TOC, N, and P concentrations were also adjusted at the beginning of each DS at a pH of 9, 300 g/m^3 of TOC, 50 g/m^3 of N-NH_4^+, and 7 g/m^3 of P-PO_4^{-3}. The C, N, and P contents of the algal and algal-bacterial biomass formed were also experimentally determined along with the PHB and lipid content. The protein and carbohydrate contents in the biomass were also determined.

Mixotrophic growth under nutrient deprivation conditions

A second series of experiments using *C. sorokiniana* and the microalgal-bacterial consortium was conducted in the presence of continuous irradiation (PAR of 100±11 μE/$m^2 \cdot s$) and initial C-CH_3COONa and C-glucose concentrations of 150 g C/m^3 (initial TOC concentration of 300 g/m^3) under N and P deprivation, where K_2HPO_4 was replaced by an equimolar concentration of KCl in the BG-11 MSM. The bottles were also initially flushed with sterile N_2 for 15 min to remove the O_2 from the headspace and allowed to equilibrate for 2 h prior to sampling. The test was monitored for 12 days as above-described sampling every 3 days until the N and P contents in the biomass remained constant (Table S3 in "Supplementary Material").

Mass balance calculation

A mass balance calculation was conducted for C, N, and P considering all their chemical species at the beginning and end of each dark and light stage. The validity of the experimentation carried out was assessed by means of recovery factors defined as follows (Alcántara et al. 2013):

$$\text{C mass recovery (\%)} = \frac{[\text{C-CO}_2 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{END POINT}}}{[\text{C-CO}_2 + \text{TOC} + \text{IC} + \text{C}_{\text{biomass}}]_{\text{START POINT}}} \times 100 \quad (1)$$

$$\text{N mass recovery (\%)} = \frac{[\text{N-NH}_4^+ + \text{N-NO}_2^- + \text{N-NO}_3^- + \text{N}_{\text{biomass}} + \text{N}_{\text{organic}}]_{\text{END POINT}}}{[\text{N-NH}_4^+ + \text{N-NO}_2^- + \text{N-NO}_3^- + \text{N}_{\text{biomass}} + \text{N}_{\text{organic}}]_{\text{START POINT}}} \times 100 \quad (2)$$

$$\text{P mass recovery (\%)} = \frac{[\text{P-PO}_4^{-3} + \text{P}_{\text{biomass}}]_{\text{END POINT}}}{[\text{P-PO}_4^{-3} + \text{P}_{\text{biomass}}]_{\text{START POINT}}} \times 100 \quad (3)$$

where C-CO_2 is the carbon concentration as gas CO_2 in the bottles' headspace, TOC is the total dissolved organic carbon concentration in the aqueous phase (C-CH_3COONa+

C-glucose+$C_{organic}$), IC is the dissolved inorganic carbon concentration in the aqueous phase in equilibrium with C-CO_2, $C_{biomass}$ is the particulate carbon concentration in the form of microalgal or microalgal-bacterial biomass, $C_{organic}$ is the dissolved organic carbon concentration from the particulate carbon hydrolyzed, and N-NH_4^+, N-NO_2^-, and N-NO_3^- represent the concentration of ammonium, nitrite, and nitrate, respectively, while $N_{biomass}$ and $N_{organic}$ account for the concentration of particulate organic nitrogen in the biomass and dissolved organic nitrogen from the particulate nitrogen hydrolyzed. P-PO_4^{-3} is the phosphorus concentration in the aqueous phase, and $P_{biomass}$ is the particulate phosphorus concentration in the form of biomass.

Analytical procedures

The irradiation was measured as PAR using a LI-250A light meter (LI-COR Biosciences, Germany). The pressure of the bottles' headspace was measured using a PN 5007 pressure sensor (IFM, Germany). The gas concentrations of CO_2, CH_4, O_2, and N_2 were determined using a CP-3800 gas chromatograph (Varian, USA) coupled with a thermal conductivity detector and equipped with a CP-Molsieve 5A (15 m×0.53 mm×15 μm) and a CP-Pora BOND Q (25 m×0.53 mm×15 μm) columns. The injector, detector, and oven temperatures were maintained at 150, 175, and 40 °C, respectively. Helium was used as the carrier gas at 13.7 cm^3/min. TOC, IC, and TN concentrations were determined using a TOC-V CSH analyzer equipped with a TNM-1 module (Shimadzu, Japan). N-NO_3^-, N-NO_2^-, and P-PO_4^{-3} were analyzed by HPLC-IC according to Alcántara et al. (2013). The soluble P concentration was also determined according to Eaton et al. (2005) using a spectrophotometer U-2000 (Hitachi, Japan). A Crison micropH 2002 (Crison instruments, Spain) was used for pH determination. Aliquots of 10 cm^3 of cultivation broth were filtered through 0.22 μm and acidified with H_2SO_4 to pH 2 prior to volatile fatty acid analysis in an Agilent 7820A GC-FID equipped with a G4513A autosampler and a Chromosorb WAW packed column (2 m×1/8″× 2.1 mm SS) (10 % SP 1000, 1 % H_3PO_4, WAW 100/120) (Teknokroma, Spain). The injector, oven, and detector temperatures were 375, 130, and 350 °C, respectively. N_2 was used as the carrier gas at 45 cm^3/min. The concentration of C-glucose was determined as the difference between the TOC and the sum of C-VFA and $C_{organic}$. The determination of the TSS and VSS concentrations of microalgal and microalgal-bacterial biomass was performed according to Eaton et al. (2005). The analysis of $C_{biomass}$ and $N_{biomass}$ was conducted using a LECO CHNS-932, while P biomass was measured using a 725-ICP Optical Emission Spectrophotometer (Agilent, USA) at 213.62 nm. The concentration of $C_{organic}$ and $N_{organic}$ released into the liquid phase was determined based on the percentage of structural C and N in the biomass and the decrease in TSS concentration in the dark stages. The identification, quantification, and biometry measurements of microalgae were carried out by microscopic examination (OLYMPUS IX70, USA) of microalgal samples (fixed with lugol acid at 5 % and stored at 4 °C prior to analysis) according to Sournia (1978). The determination of PHB was carried according to Zúñiga et al. (2011) using chloroform as extraction solvent in an Agilent 6890N GC-MS equipped with a DB-WAX column (30 m×0.250 mm× 0.25 μm) (J&W Scientific®, USA). The injector temperature was set at 250 °C. The oven temperature was initially maintained at 40 °C for 5 min, increased at 10 °C/min up to 200 °C and finally increased at 5 °C/min up to 240 °C (maintained for 2 min). Total lipid content in the biomass was quantified gravimetrically according to Gómez et al. (2013). Protein content in the biomass was estimated using a nitrogen to protein conversion factor of 4.44 (González et al. 2010). The carbohydrate content was estimated from the difference between the total biomass concentration and its content of lipids, proteins, and ashes.

Results

The results obtained, given as the average ± the error at 95 % confidence interval (n=5), were summarized in Tables S1, S2, and S3. The C, N, and P mass balances during dark cultivation showed recovery factors of 99.9± 1.7, 100.2±2.8, and 101.1±4.3 % in *C. sorokiniana* cultures, respectively, and 100.0±0.4, 99.8±0.4, and 99.8± 19.6 % in the algal-bacterial cultures (Tables S1 and S2). Similarly, the percentages of recovery obtained during illuminated cultivation were 105.7±12.5 % for C, 100.0 ±3.0 % for N, and 103.8±16.4 % for P in *C. sorokiniana* cultures and 100.3±0.5 % for C, 101.2±4.6 % for N, and 105.7±34.1 % for P in the algal-bacterial cultures (Tables S1 and S2). Finally, the C mass balance under nutrient deprivation conditions showed recovery factors of 99.8±5.3 and 99.6±1.7 % in *C. sorokiniana* and algal-bacterial cultures, respectively (Table S3).

Mixotrophic cultivation under extended dark-light periods

C. sorokiniana underwent a partial hydrolysis during the DS with a decrease in biomass concentration (as TSS) of 7.6±0.8, 15±6 and 17±9 % during DS I, DS II, and DS III, respectively. This hydrolysis entailed the built up of $C_{organic}$ and $N_{organic}$ in the liquid phase as a consequence of the solubilization of $C_{biomass}$ and $N_{biomass}$ (Fig. 1a and Table S1). However, biomass hydrolysis was negligible

in algal-bacterial consortium and acidogenesis from glucose occur in all DS, which resulted in increasing VFA concentrations and therefore in the acidification of the cultivation broth to neutral pH (Fig. 1b and Table S2). The fraction of glucose hydrolyzed to VFAs remained constant following 7 and 5 days of dark cultivation (63±2 % and a final pH of 7.2±0.2), while glucose biotransformation decreased to 39±6 % when the dark cultivation decreased to 2.5 days (final pH of 7.7±0.3). This acidogenesis from glucose in algal-bacterial cultures increased propionic acid concentrations at decreasing durations of the dark stage, with a maximum share of 60 % of the total VFA following 2.5 days of DS (Fig. 2). Neither isocaproic, hexanoic, nor heptanoic acid was detected at the end of the different DS evaluated (Fig. 2). On the other hand, an increase in the soluble $P\text{-}PO_4^{-3}$ concentration of 43 and 45 % was reported in *C. sorokiniana* cultures following DS I and DS II, respectively, while this value decreased to 20 % after DS III (Fig. 3a). Surprisingly, a negligible phosphorus release to the cultivation broth was recorded in the algal-bacterial consortium (Fig. 3b). The PHB (Fig. 3a), carbohydrate, protein, and lipid content in *C. sorokiniana* during dark cultivation remained constant at 0.1±0.0, 45±2, 43±2, and 0.7±0.1 %, along the three cycles tested. In the algal-bacterial cultures, an increase in the PHB concentration from 0.5±0.0 to 3.3±0.3 % occurred during the first 7 days of DS, but this value decreased during the first illuminated period and remained constant at 0.7±0.2 % along the entire experiment (Fig. 3b). The carbohydrate, protein, and lipid contents of the microalgal-bacterial biomass remained constant at 50±1, 32±1, and 2.5±0.1 %, respectively.

The biomass stoichiometric formulas experimentally determined following the LS I, II, and III for *C. sorokiniana*, and the algal-bacterial consortium were $CH_{1.73}O_{0.32}N_{0.17}S_{0.005}P_{0.007}$ and $CH_{1.74}O_{0.53}N_{0.15}S_{0.007}P_{0.009}$, respectively. *C. sorokiniana* presented biomass productivities of 70 g TSS/m^3·d, 19 g TSS/m^3·d, and 16 g TSS/m^3·d during the illuminated stages I, II, and III, respectively and a complete C-acetate assimilations (161±1, 150±3 and 160±5 g C-acetate/m^3, respectively) (Fig. 1a and Table S1). Surprisingly, *C. sorokiniana* only assimilated 26±5 % of the C-glucose during LS I (40±8 g C-glucose/m^3), while no glucose assimilation was recorded during LS II and III (Fig. 1a and Table S1). In the algal-bacterial cultures, both the initial C-acetate and the VFA formed from glucose biotransformation during DS were completely removed in LS I and LS II, with biomass productivities of 43 g TSS/m^3·d and 50 g TSS/m^3·d, respectively. These C eliminations corresponded with an assimilation of 191±17 g C-acetate/m^3 (136±25 g/m^3 from the initial C-acetate and 55±30 g/m^3 from glucose acidogenesis) and 46±5 g/m^3 corresponding to C-VFA* during LS I and 198±42 g C-acetate/m^3 (150±34 g/m^3 from the initial C-acetate and 48±55 g/m^3 from glucose acidogenesis) and 45±4 g/m^3 of C-VFA* during LS II (Fig. 1b and Table S2). Biomass productivity decreased to 24 g TSS/m^3·d in the illuminated stage III, which corresponded with an assimilation of 194±8 g C-acetate/m^3 and 17±9 g/m^3 of C-VFA*. C-glucose removal accounted for 0.2±0.0 % (3±10 g C-glucose/m^3 assimilated), 68±7 % (37±14 g C-glucose/m^3 assimilated), and 13±3 % (11±16 g C-glucose/m^3 assimilated) in LS I, LS II, and LS III, respectively (Fig. 1b and Table S2). TIC concentrations remained roughly constant during the illuminated stages, with an average TIC assimilation of 5.1±1.4 % in *C. sorokiniana* cultures and a TIC generation of 7.2±1.7 % (as a result of an intense respiratory release of CO_2) in the microalgal-bacterial broths (Fig. 1). *C. sorokiniana* supported $N\text{-}NH_4^+$ removals of 97±1, 59±12, and 67±13 % during the illuminated stages I, II, and III, respectively. Likewise, $N\text{-}NH_4^+$ removals of 97±7, 100±2, and 55±8 % were recorded in the algal-bacterial cultures during LS I, LS II, and LS III. Neither NO_2^- nor NO_3^- was produced by microalgae or by the algal-bacterial cultures (Tables S1 and S2). On the other hand, $P\text{-}PO_4^{-3}$ removal efficiencies of 81±21, 32±16, and 29±18 % were recorded in *C. sorokiniana* cultures in LS I, LS II, and LS III. Likewise, the algal-bacterial consortia supported $P\text{-}PO_4^{-3}$ removals of 84±18, 79±18, and 42±23 % in LS I, LS II, and LS III, respectively (Fig. 3).

Mixotrophic growth under nutrient deprivation conditions

Surprisingly, *C. sorokiniana* presented an average biomass productivity of 134 g TSS/m^3·d after 3 days of mixotrophic cultivation in the absence of N and P, while this productivity decreased sharply to 14 g TSS/m^3·d by day 6 and to 9 g TSS/m^3·d by day 12. Likewise, an average biomass productivity of 185 g TSS/m^3·d was recorded in the algal-bacterial cultures after 3 days of cultivation, which gradually decreased to 8 g TSS/m^3·d by day 12. A total C-acetate assimilation of 86±19 % (corresponding to 130±21 g C-acetate/m^3) was observed during *C. sorokiniana* cultivation, with removals of 43±15, 4.7±0.9, 19±8, and 20±8 % by day 3, 6, 9, and 12, respectively (Fig. 4a and Table S3). On the other hand, the algal-bacterial consortia presented a total C-acetate removal of 100 % (150±6 g C/m^3), with an assimilation of 91±7 % within the first 3 days (Fig. 4b and Table S3). Microalgae cultures assimilated 83±14 % of the C-glucose available (141±19 g C/m^3) within the first 3 days, and surprisingly glucose assimilation ceased afterwards (Fig. 4a and Table S3). Similarly, 93±8 % of the initial C-glucose (146±9 g C/m^3) was assimilated by the algal-bacterial cultures, with a removal of 70±9 % within the first 3 days of cultivation (Fig. 4b and Table S3). *C. sorokiniana* cultures underwent an increase in TIC from 7±1 to 11±1 % within the first 3 days likely associated with the aerobic biodegradation of C-

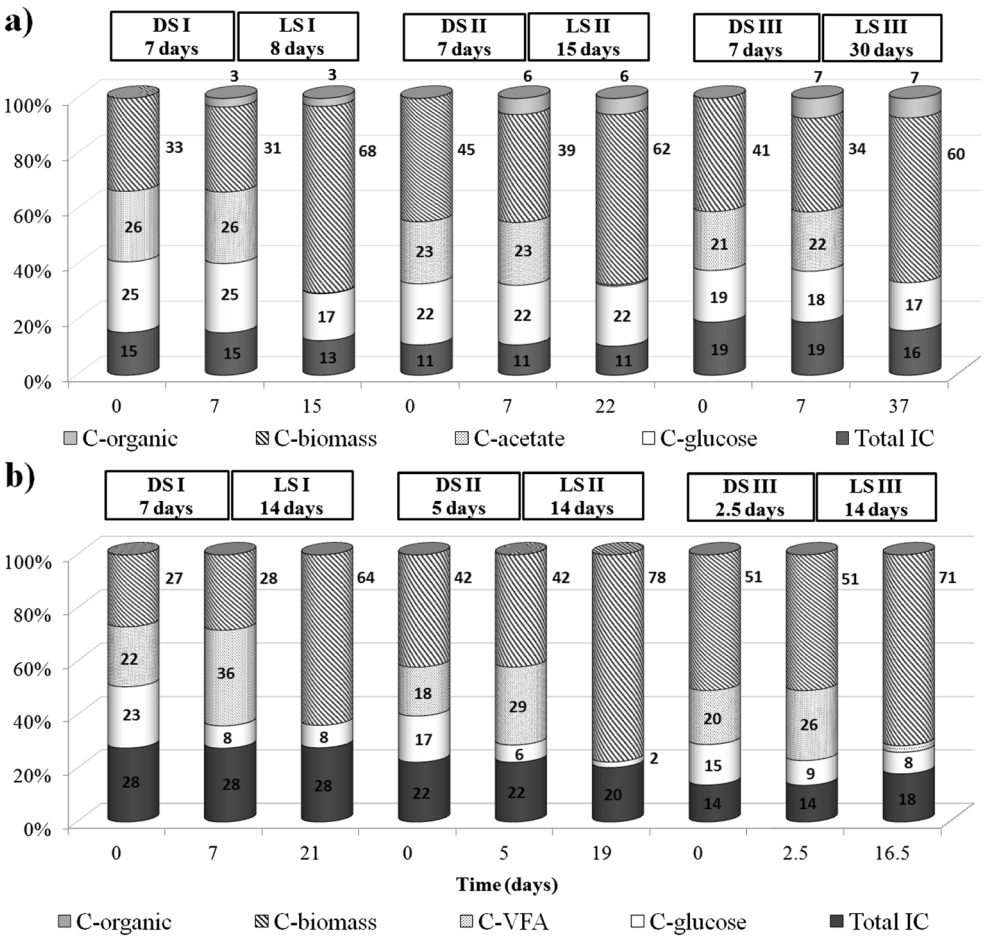

Fig. 1 Initial and final carbon distribution along the three sequential DS-LS cycles assessed in *C. sorokiniana* (**a**) and microalgal-bacterial (**b**) cultures

acetate and C-glucose. Afterwards, TIC concentration remained roughly constant during the entire cultivation (Fig. 4a and Table S3). In contrast, the algal-bacterial cultures showed a gradual TIC assimilation from 13±2 to 3±0 % (Fig. 4b and Table S3).

The carbohydrate content in *C. sorokiniana* increased from 48±1 to 72±1 %. This increase was concomitant with a decrease in the protein content from 40±1 to 16 ±1 %, while lipid and PHB contents remained constant at 0.8±0.1 and 0.4±0.0 %, respectively (Fig. 5a).

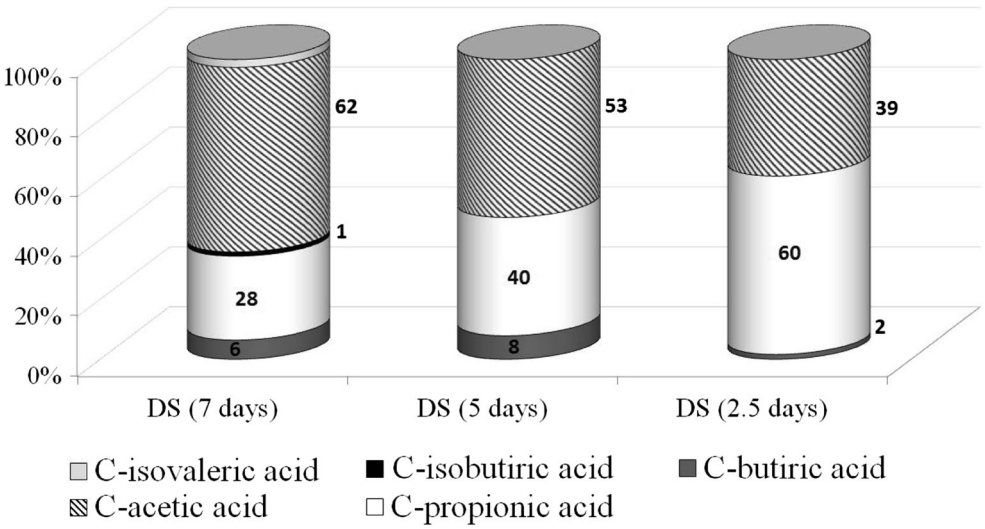

Fig. 2 VFA distribution at the end of the dark stages assessed in algal-bacterial cultures

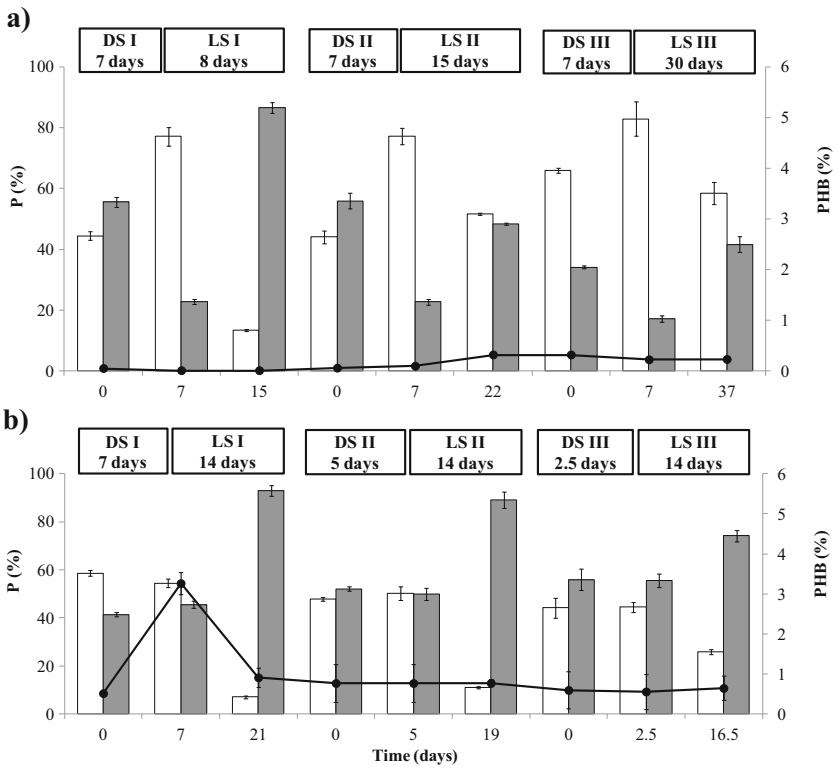

Fig. 3 Time course of the distribution of dissolved P-PO_4^{-3} (□) and $P_{biomass}$ (■) species and PHB content (–●–) in *C. sorokiniana* (**a**) and algal-bacterial (**b**) cultures

Similarly, the carbohydrate content in the algal-bacterial consortium increased from 49±3 to 65±2 %, along with a severe decrease in the protein content from 33±3 to 18±2 %. The lipid content remained constant at 2.3± 0.3 %, while PHB concentration initially increased from 2.9±0.2 to 3.9±0.1 % within the first 3 days under nutrient limitation but gradually decreased to 1.1± 0.2 % (Fig. 5b).

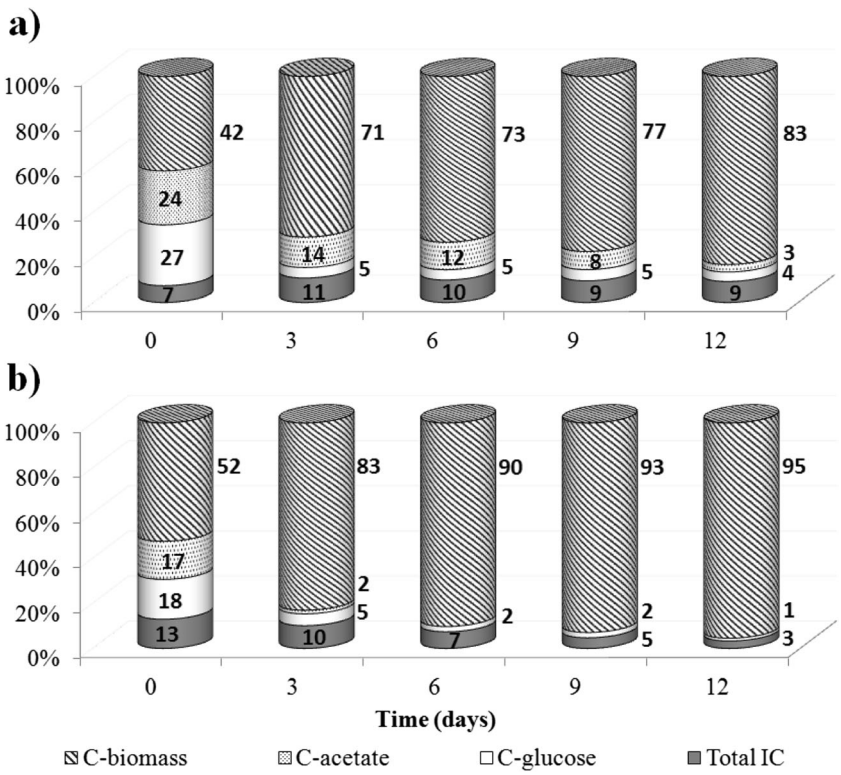

Fig. 4 Carbon distribution during *C. sorokiniana* (**a**) and algal-bacterial (**b**) mixotrophic cultivation under nutrient deprivation

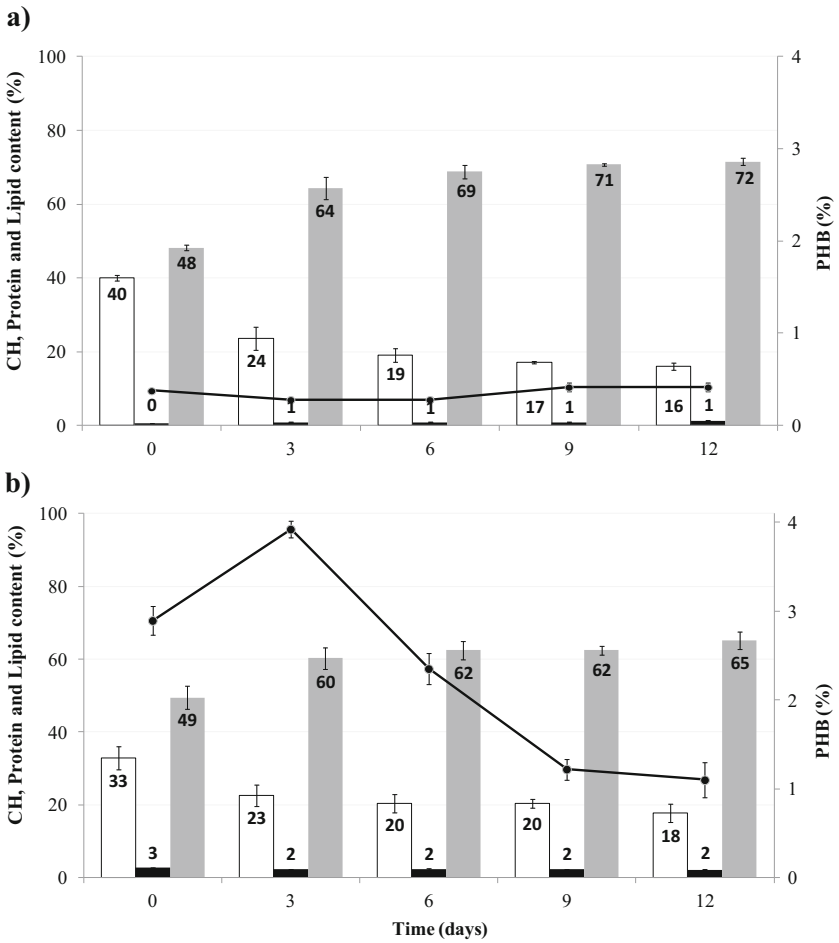

Fig. 5 Time course of carbohydrate (▩), protein (□), lipid (■), and PHB content (–●–) during the mixotrophic cultivation of *C. sorokiniana* (**a**) and the algal-bacterial consortium (**b**) under nutrient deprivation

Discussion

Carbon assimilation

The recovery factors of ≈100 % in all C mass balances validated both the analytical and instrumental methods used. *C. sorokiniana* clearly showed a higher affinity for acetate than for glucose or IC as a carbon source in both series of experiments, which suggest that C-acetate assimilation resulted more energetically favorable. It seems that as long as the level of acetate remains low (≤4000 g/m^3), some microalgae can use it as their preferential carbon source. This is important because acetate is a readily available and inexpensive substrate derived from many industrial applications and its use does not entail limitations during microalgae cultivation (Pérez-García et al. 2011a, b). In addition, the high pH recorded at the end of every LS (9.5±1.4 in *C. sorokiniana* and 10±0.7 in algal-bacterial cultures) as a result of photosynthetic activity, mediated an IC distribution mainly shifted towards bicarbonate and carbonate, which constitute IC species not readily available for *C. sorokiniana* growth (De Godos et al. 2010; Alcántara et al. 2013). Glucose assimilation by *C. sorokiniana* occurred only during LS I and resulted in a 26±5 % glucose removal, which was in agreement with the limited glucose assimilation (28±2 %) reported by Alcántara et al. (2013) during the mixotrophic cultivation of *C. sorokiniana* in a minimum MSM at initial N-NH_4^+ and P-PO_4^{-3} concentrations of 95±1 and 13±1 g/m^3, respectively. Although it is generally accepted that glucose can serve as a carbon source for the growth of certain microalgae, the effect of glucose on microalgae metabolism is species specific and influenced by the impinging irradiation (Kamiya and Kowallik 1987; Kamiya and Saitoh 2002). Interestingly, 83±14 % of C-glucose was assimilated by *C. sorokiniana* during the first 3 days of mixotrophic cultivation under nutrient starvation, which suggests that nutrient deprivation boosted both C-acetate and C-glucose assimilations. This additional TOC assimilation resulted in an increase of the dissolved IC within the first 3 days due to combined effect of an intensive respiratory release of CO_2 and a gradual increase in the pH of the cultivation broth (Fig. 4a and Table S3). These results confirm that microorganisms exhibiting a dual photoautotrophic and heterotrophic metabolism can shift their nutritional mode based on substrate and light availability (Abreu et al. 2012). Hence, when simple carbohydrates or organic acids are present in the medium, microalgae and cyanobacteria likely shift their

metabolism from an autotrophic to a mixotrophic nutrition mode to save energy (Venkata Mohan et al. 2014). The hydrolysis experienced by *C. sorokiniana* during the DS resulted in a decrease of TSS and consequently in increases in the concentration of dissolved $C_{organic}$ and $N_{organic}$ in the cultivation broth that were not further assimilated into biomass during the subsequent LS (Fig. 1a and Table S1). Based on the negligible hydrolysis of the algal-bacterial culture and its higher biomass productivity during LS II and III, the results suggest that *C. sorokiniana* was more sensitive to the absence of energy supply during the extended dark stages.

The results obtained in the cultivation of the algal-bacterial consortium suggested that the hydrolysis of glucose into VFAs was mediated by bacterial metabolism. The fact that the propionate to acetate ratio increased at decreasing duration of the dark stage indicated that glucose was firstly converted into propionic acid and finally into acetic acid. Thus, the ability of microalgal-bacterial consortia to hydrolyze and biotransform complex carbohydrates into simple organic molecules confirm the potential of these symbiotic consortia for wastewater treatment (He et al. 2013). The algal-bacterial biomass productivities and the negligible IC assimilation recorded also suggest that the extent of acidogenesis from glucose boosted TOC removal and consequently the superior algal-bacterial growth during the illuminated stages (Fig. 1b and Table S2). Nevertheless, the assimilation of the glucose not hydrolyzed was negligible. Similarly to *C. sorokiniana* cultures, nutrient starvation triggered C-glucose assimilation by the algal-bacterial consortium, which assimilated 93±8 % of the initial C-glucose (Fig. 4b and Table S3). Surprisingly, the 185 g TSS/m^3·d algal-bacterial productivity recorded during the first 3 days of cultivation in the absence of nutrients was higher than *C. sorokiniana* productivity (134 g TSS/m^3·d). This higher productivity was associated to the simultaneous occurrence of photoautotrophic microalgae growth, which entailed an assimilation of total IC of 78±7 % (165±14 mg of TIC consumed) during experiment II (Fig. 4b and Table S3).

Nitrogen and phosphorus removal

The recovery factors of ≈100 % in N and P mass balances also validated both the analytical and instrumental methods used during the evaluation of the fate of these nutrients. The absence of both N losses by stripping (closed bottles) and nitrification in both *C. sorokiniana* and algal-bacterial cultures confirm that the main mechanism for N-NH_4^+ removal was assimilation into biomass (as $N_{biomass}$) (Table S1 and Table S2). N-NH_4^+ removals of 97±1, 59±12, and 67±13 % and P-PO_4^{-3} removals of 81±7, 32±4, and 29±6 % were recorded in *C. sorokiniana* cultures during the illuminated periods I, II, and III, respectively (Table S1). Based on the fact that the microalgal N content experimentally determined in *C. sorokiniana* remained constant at 9.7±0.3 %, the decrease in N-NH_4^+ removal during LS II and LS III points out to a deterioration in *C. sorokiniana* metabolism mediated by the prolonged absence of light during DS II and III (Table S1). On the other hand, the release of P-PO_4^{-3} by *C. sorokiniana* during the dark stages together with the decrease in P-PO_4^{-3} assimilation during LS II and III induced a progressive decline in microalgal $P_{biomass}$. Hence, the P content of *C. sorokiniana* decreased from 1.5±0.2 % in LS I to 0.9±0.0 and 0.6±0.2 % by the end of LS II and LS III, respectively. These results confirmed that microalgae, similarly to PAOs (De-Bashan and Bashan 2004; Bajekal and Dharmadhikari 2008; Mesquita et al. 2013), can release P under anaerobic conditions in the absence of light, but P assimilation in the subsequent illuminated stages did not occur in a similar extent (e.g., structural P decreased from 1.9 to 0.8 % during DS I and increased again to 1.5 % at the end of LS I). Luxury P uptake in microalgae is influenced by the dissolved phosphate concentration, light intensity, and temperature during microalgae cultivation (Cade-Menun and Paytan 2010; Fanta et al. 2010) and might explain the highly variable phosphorus removal often reported in microalgae-based wastewater treatment. Indeed, the amount of $P_{biomass}$ is the result of the combined effects of a luxury uptake and a growth-associated P uptake (Powell et al. 2008, 2009). In this regard, the deterioration in the activity of *C. sorokiniana* caused by the cultivation under prolonged dark periods likely promote the preferential uptake of P-PO_4^{-3} for growth during LS periods.

N-NH_4^+ removals in the microalgal-bacterial cultures of 97±7, 100±2, and 55±8 % and P-PO_4^{-3} removals of 84±7, 79±11, and 42±6 % were recorded in the illuminated stages I, II, and III, respectively (Table S2). Despite similar N-NH_4^+ concentrations were initially present at the beginning of each cycle in both cultures, the algal-bacterial biomass exhibited lower $N_{biomass}$ contents than microalgae (7.3±0.2 % regardless of the operational stage). The absence of phosphorus release to the cultivation medium during the dark period along with the steady P-PO_4^{-3} removal rates during the illuminated stages can explain the constant $P_{biomass}$ content in the algal-bacterial biomass (1.1±0.1, 0.9±0.2, and 0.9±0.1 % at the end of LS I, LS II, and LS III, respectively). Finally, it must be stressed that both N-NH_4^+ and P-PO_4^{-3} removals surprisingly decreased at decreasing duration of the dark stages, which suggested that N and P assimilation in algal-bacterial cultures was influenced by the availability of VFAs, which itself was influenced by the duration of the dark stages.

Macroscopic biomass composition

While no changes were observed in the carbohydrate, lipid, and protein contents in the test series performed using extended dark-light cycles, nutrient starvation resulted in a steady increase in carbohydrate content in both *C. sorokiniana* and microalgal-bacterial cultures. In this context, phosphorus-starved microalgae and cyanobacteria can experience an

increased intracellular storage of carbohydrates, the extent of this accumulation being strain specific (González-Fernández and Ballesteros 2012). In our particular case, the carbohydrate/protein ratio in *C. sorokiniana* under N and P starvation increased from 1.2 to 2.7 within the first 3 days and up to 4.5 by day 12, which corresponded with an initial and final carbohydrate content of 48±1 and 72±1 %, respectively (Fig. 5a). These results were in agreement with the increase from 0.15 to 3.7 reported by Dean et al. (2008) in the carbohydrate/protein ratio during *Chlamydomonas reinhardtii* cultivation under P deficient conditions, while a lower increase in the carbohydrate/protein ratio from 0.4 to 1.0 was observed by Sigee et al. (2007) in the cultivation of P-starved *Scenedesmus subspicatus*. A slightly lower carbohydrate accumulation from 49±3 to 65±2 % was obtained in the algal-bacterial cultures (Fig. 5b), where the carbohydrate/protein ratio increased from 1.5 to 2.7 during the first 3 days and up to 3.7 by day 12. A negligible lipid accumulation concomitant with a decrease in the protein content was observed in both *C. sorokiniana* and algal-bacterial cultures under N and P deprivation (Fig. 5). While the fact that nitrogen starvation induces an increase in the biomass lipid content has been consistently proven (Li et al. 2012; Simionato et al. 2013), the presence of P plays a key role on lipid productivity under nitrogen deficient conditions in both microalgae (Feng et al. 2012; Chu et al. 2013) and bacteria (Harold 1966; Kulaev et al. 1999). For instance, Chu et al. (2013) reported that the P assimilated under nitrogen deficiency was utilized by the algal cells to metabolically synthesize enzymes for the lipid synthesis. Thus, the absence of phosphorous under nitrogen starvation in our cultivation medium was likely a suppression factor for lipid accumulation and promoted instead carbohydrates accumulation (Chu et al. 2013).

Cultivation under extended dark-light periods did not boost PHB accumulation neither in *C. sorokiniana* nor in the algal-bacterial consortium. In our particular study, PHB accumulation in *C. sorokiniana* cultures was negligible despite the release of P during the dark stages (which was hypothesized to be associated with the supply of the microbial energy demand in the absence of light) (Fig. 3a). On the other hand, a maximum accumulation of PHB of 3.3±0.3 % was observed at the end of the first 7 days of dark stage in the algal-bacterial cultures, which in fact was not associated with a $P\text{-}PO_4^{-3}$ release to the cultivation medium (Fig. 3b). PHB content decreased to 0.9±0.2 % during the first illuminated stage and remained roughly constant afterwards.

In the absence of N and P, *C. sorokiniana* possessed a constant PHB content of 0.4±0.0 % along the 12 days of cultivation (Fig. 5a), which was in agreement with the 0.7 % PHB content reported by De Philippis et al. (1992) during photoautotrophic cultivation of *Spirulina maxima* under N starvation. On the other hand, the PHB concentration in the microalgal-bacterial biomass increased during the first 3 days from 2.9±0.2 to 3.9±0.1 % but gradually decreased to 1.1±0.2 % afterwards (Fig. 5b). The results here obtained suggest that despite the ability of some cyanobacteria to accumulate significant amounts of PHB during mixotrophic cultivation under extended dark periods or nutrient deprivation (Sharma and Mallick 2005; Panda and Mallick 2007), PHB accumulation in *C. sorokiniana* and the algal-bacterial consortium here tested was not induced under N and P limiting conditions.

In brief, the ability of microalgal-bacterial consortia to hydrolyze and biotransform glucose into simple organic molecules under extended dark periods confirmed the potential of these symbiotic consortia for wastewater treatment. N and P assimilation in algal-bacterial cultures during the illuminated periods was influenced by the carbon available as VFAs, which itself was a function of the duration of the dark stages. C-VFA (initial C-acetate + C-VFA from glucose acidogenesis in the algal-bacterial cultures) was the preferred carbon source in *C. sorokiniana* and in the algal-bacterial consortium based on the low-glucose and inorganic carbon assimilations recorded. Hence, in the presence of simple carbohydrates or organic acids, microalgae and cyanobacteria can shift their metabolism from an autotrophic to a mixotrophic nutrition mode to save energy. Neither PHB nor lipid accumulation was induced in *C. sorokiniana* or in the algal-bacterial consortium under extended dark-light periods or N and P deprivation. Surprisingly, nutrient deprivation boosted an efficient C-acetate and C-glucose assimilation and resulted in a steady increase in carbohydrate content concomitant with a decrease in protein concentration in *C. sorokiniana* and microalgal-bacterial cultures. This work provided new insights on the potential of indigenous microalgae-bacteria symbiotic consortia as a platform technology to avoid the high cost and technical limitations associated with the axenic cultivation of microalgae in order to consolidate an industrial scale microalgae-to-biofuel technology based on wastewater treatment.

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