Impact of different nitrogen sources on the growth of *Arthrospira* sp. PCC 8005 under batch and continuous cultivation – A biochemical, transcriptomic and proteomic profile

Frédéric Deschoenmaeker a, Guillaume Bayon-Vicente a, Neha Sachdeva a, Orily Depraetere b, Juan Carlos Cabrera Pino c, Baptiste Leroy a, Koenraad Muylaert b, Ruddy Wattiez a,⇑

⇑ Corresponding author.

E-mail address: Ruddy.Wattiez@umons.ac.be (R. Wattiez).

http://dx.doi.org/10.1016/j.biortech.2017.03.145

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Cyanobacteria such as *Arthrospira platensis* are biotechnologically important due to their high nutritional value and have been used for so many years as food, functional food, dietary supplement, nutraceutical, and animal feed (Yao et al., 2016; Borowitzka, 2013; Wijffels and Barbosa, 2010). The ability of these cyanobacteria to use different nitrogen sources including urea, to fix carbon dioxide and produce oxygen make them a potential candidate as nutritional resource for future long-haul manned space missions to lunar bases or flights to Mars. The high costs associated with bringing food and oxygen from Earth into space and waste management on space stations limit these explorations. To counter these obstacles, the European Space Agency (ESA) has designed the concept of a self-sufficient artificial ecosystem termed as MELiSSA (Micro-Ecological Life Support System Alternative) loop. The MELiSSA loop based on four microbiological compartments connected to higher plants has been developed to produce part of food, oxygen and to contribute to water and waste recycling in harsh environment such as space conditions (Hendrickx et al., 2006).

In the MELiSSA loop context, nitrogen (N) is provided by human waste as either nitrate (NO₃⁻) ammonium (NH₄⁺) or urea. The metabolic robustness of oxygenic filamentous cyanobacteria such as *Arthrospira* sp. and their ability to assimilate both organic (e.g. urea, glutamine etc.) and inorganic N sources (e.g. nitrate, nitrite (NO₂⁻), ammonium, etc.), individually as well as simultaneously
make them eligible and cost effective candidates for the MELiSSA loop and for other industrial applications including wastewater harvesting (Markou, 2015; Morocho-Jácome et al., 2015; Avila-Leon et al., 2012; Yuan et al., 2011; Rodrigues et al., 2010; Bothe et al., 2010).

The recent advances in understanding the genomic sequences of the *Arthrospira* sp. (Janssen et al., 2010) have provided opportunities to understand the regulation and mechanism of N metabolic pathways at the genomic levels. In contrast to other cyanobacteria (e.g. *Anabaena*, *Trichodesmium*, *Nostoc calcicola*,..), *Arthrospira* sp. is not able to fix the N₂ (Bothe et al., 2010). Basically, the ions NO₃⁻/NO₂⁻ and NH₄⁺ require specific membrane transporters like nitrate (NRTs) and ammonium (Amt) transporters (Flores et al., 2005; Muro-Pastor et al., 2005; Muro-Pastor and Florencio, 2003; Ohashi et al., 2011). Once inside the cells, nitrate is reduced to nitrite and subsequently to ammonium by the assimilatory enzymes nitrate reductase (Nar) and nitrite reductase (Nir), respectively (Flores et al., 2005). Then, ammonium is incorporated into carbon skeletons mainly via the successive chemical reactions catalysed by the glutamine synthetase (GS) and the glutamate oxoglutarate aminotransferase (GOGAT) throughout the GS-GOGAT cycle (Flores et al., 2005; Muro-Pastor et al., 2005).

The nitrogen metabolic pathways are subjected to a strict regulation which depends on the carbon-to-nitrogen (C:N) ratio (Flores et al., 2005; Muro-Pastor et al., 2005). In general, ammonium reversibly inhibits the assimilation of alternative N sources (e.g. nitrate ions) through the repression of specific transports (e.g. NrtABCD and NrtP) and N assimilation enzymes (e.g. Nar and Nir). This regulation loop involves several molecular actors, such as the transcription factor NtcA. NtcA regulates the expression of the most of the genes involved in the nitrogen metabolism (e.g. *amt* and *nir* operon) (Muro-Pastor et al., 2001). The presence of ammonium has been reported to reversibly inhibit the assimilation of other N sources by the repression of respective N transporters and N assimilation enzymes. Additionally, ammonium has often been known to be associated with toxicity phenomena at concentrations greater than 3 mM (Li et al., 2012). Later studies linked this toxicity to the working pH of the cultivation system and established that at pH > 9.25 most of NH₄⁺ are converted to gaseous ammonia (NH₃) (Markou et al., 2014; Belkin and Boussiba, 1991), which inhibits the photosynthetic system of the cyanobacterial cells, thus creating toxic conditions. Furthermore, the toxicity could also be amplified under higher light (Markou and Muylasert, 2016).

Clearly, the chemistry and the concentration of N source impact directly the nitrogen metabolism and thus the quality of the biomass of *Arthrospira* sp. PCC 8005, making it important to investigate these modifications at a molecular level during N source fluctuations and/or transitions. The present study makes the first attempt to investigate the degree of freedom in terms of use of the different N sources (both individually or in combination) for the cultivation of oxygenic photosynthetic cyanobacteria *Arthrospira* sp. PCC 8005 and evaluate the changes at proteomic and transcriptomic levels brought by N sources, nitrogen concentration and mode of cultivation (batch or continuous mode), as well as on the growth profile and the biomass composition (pigments, lipids, proteins, exopolysaccharides).
2. Materials and methods

2.1. Cyanobacterial strain and culture conditions

Arthrospira sp. strain PCC 8005 was generously provided by the Expert Group for Molecular and Cellular Biology (MCB), SCK•CEN, Belgium. The axenic stock culture was photoautotrophically maintained in 250 mL Erlenmeyer flasks in Zarrour medium (pH adjusted to 8.5) at 30 °C, on rotary shaker (140 rpm) under continuous illumination of 43 μmol photons m⁻² s⁻¹ as previously described (Deschoenmaeker et al., 2014).

2.1.1. Batch cultivation

Batch experiments were conducted in 250 mL Erlenmeyer flasks to evaluate the effects of chemistry and concentration of three different N sources (NaNO₃, urea and NH₄Cl) on the growth profile, biomass composition, transcriptome and proteome of Arthrospira strain PCC 8005 (Deschoenmaeker et al., 2014). Briefly, inocula were washed twice with sterile N free Zarrour medium and resuspended in Zarrour medium adjusted at pH 8.5 and containing NaNO₃, NH₄Cl or urea at various concentrations (2 mM, 6 mM or 12 mM in term of total N). Experiments were repeated three times. Samples were collected through filtration and lyophilised biomass was kept frozen till further analyses.

2.1.2. Photo-bioreactor cultivation

For the continuous photo-bioreactor (PBR) experiments, cyanobacteria were incubated in a 2-L glass vessel (Biotast®, Sartorius AG, Germany) at 30 °C under constant illumination of ±140 μmol photon m⁻² s⁻¹ (Li-193SA; Li-Cor BioSciences, USA) with the agitation settled at 100 rpm. The pH (Metler Toledo, InPro 3250) was automatically maintained at 8.5 with HCl (0.5 N), whereas the dilution rate was adjusted between 0.1 and 0.3 per day with fresh Zarrour medium (28 mM NaNO₃ or NH₄Cl, pH adjusted at 7.00) to maintain an optical density (OD₇₅₀) about 1.2. The pO₂ probe (InPro 6800, Metler Toledo, USA) was calibrated with sensor checking gel (InPro 3030/120, Metler Toledo, USA) for the current zero and in the air for the 100% signal. Experiments were repeated three times. Samples were collected through filtration and lyophilised biomass was kept frozen until further analyses.

2.2. Growth profile and biomass compositional analysis

2.2.1. Growth profile and biomass yield

Optical density at 750 nm (OD₇₅₀) and percentage sedimentation index (SI%) were used to monitor the growth and stress profile of cyanobacteria cells (Deschoenmaeker et al., 2014). The %SI of the sample was calculated as the following equation (Deschoenmaeker et al., 2014):

$$\%SI = \frac{OD_{750}(t_0) - OD_{750}(t_{16})}{OD_{750}(t_0)}$$

where OD (t₀) is the OD of the sample taken immediately at the time of sample collection and OD (t₁₆) is the OD taken at the end of 16 min without disturbances. Dry cell weight (DCW) was determined by the weighing the residual dried biomass obtained by centrifuging (5810 R, Eppendorf, Germany) 10 mL of sample for 15 min (1 × 10⁴ rpm, 4 °C) and the biomass was dried to determine the DCW. At the time of analysis, the biomass was rehydrated in phosphate buffer saline (BupH, Thermo Fisher Scientific) and subjected to a freeze/thaw step (liquid nitrogen) combined to an enzymatic lysis treatment (0.4% (w/v) lysozyme in 50 mM phosphate buffer, pH 7.0) at 37 °C for two hours. Phycobiliproteins (APC and PC) were recovered by centrifugation (15 min, 1 × 10⁴ rpm, 4 °C) and OD were measured at 650 and 620 nm for APC and PC, respectively. Chlorophyll a (Chla) was then extracted with methanol (100%) by sonication 10 min and incubated overnight at 4 °C in the dark. The recovered supernatants (15 min, 1 × 10³ rpm, room temperature) were used to measure the OD₆₅₅. All measures were normalized to dry weight of each sample.

2.2.2. Pigment analyses

Chlorophyll a (Chla), Allophycocyanin (APC) and Phycocyanin (PC) were quantified by the differential extraction of photosynthetic pigments as previously described (Deschoenmaeker et al., 2014). Briefly, 10 mL culture was centrifuged (15 min, 1 × 10⁴ rpm, 4 °C) and the biomass was dried to determine the DCW. At the time of analysis, the biomass was rehydrated in phosphate buffer saline (BupH, Thermo Fisher Scientific) and subjected to a freeze/thaw step (liquid nitrogen) combined to an enzymatic lysis treatment (0.4% (w/v) lysozyme in 50 mM phosphate buffer, pH 7.0) at 37 °C for two hours. Phycobiliproteins (APC and PC) were recovered by centrifugation (15 min, 1 × 10⁴ rpm, 4 °C) and OD were measured at 650 and 620 nm for APC and PC, respectively. Chlorophyll a (Chla) was then extracted with methanol (100%) by sonication 10 min and incubated overnight at 4 °C in the dark. The recovered supernatants (15 min, 1 × 10³ rpm, room temperature) were used to measure the OD₆₅₅. All measures were normalized to dry weight of each sample.

2.2.3. Total lipid, exopolysaccharide (EPS) and cyanophycin quantification

For total lipid quantification, 5 mg lyophilised biomass was treated with ethanol 1:20 (w/v) at 60 °C. The soluble fraction was used to quantify total lipid contents through GC–MS analysis (GCMS-QP2010, Shimadzu, Japan). Samples were silylated with 10 μL of pyridine and 50 μL of BSTFA for 30 min at 60 °C. Derivatives were then separated through a 0.25 mm × 30 m optima 5.0 MS capillary column (0.25 μm film thickness, Macherey Nagel, Germany) with He (0.75 ml/min). The electron impact mass data of the derivatives (70 eV) was identified under the following GLC conditions: initial column temperature 100 °C for six minutes, heated (30 °C min⁻¹) to 320 °C for eight minutes; injector temperature 310 °C, split ratio 20:1.

The left over residue from total lipid extraction was used for total EPS quantification. The residue was resuspended in distilled water and centrifuged (4800 g, five minutes, Room Temperature) post two hours incubation at 100 °C. Thereafter, 1% cetyltrimethylammonium bromide was used to precipitate total EPS recovered through another centrifugation (1 × 10^4 g, 10 min, Room temperature), washed with saturated sodium acetate in 95% ethanol and absolute ethanol. Changes in the composition of exopolysaccharides were also characterized (i.e., total sugar by the phenol/sulphuric method, total uronic acids by the MHDF method (Filisetti-Cozzi and Carpita, 1991) and sulphate groups (Jaques et al., 1968).

For cyanophycin quantification, 20 mL of the culture was subjected to high-pressure cellular disruption (French press, Thermo) and centrifuged (15 min, 2 × 10^4 rpm, 4 °C) using a 50 Ti rotor (Beckman L7 Ultracentrifuge). The resulting pellet was then incubated (twice) in two ml 0.1 M HCl for 30 min at room temperature. The supernatants were pooled and used for the quantification of cyanophycin content using the assay as previously reported (Allen et al., 2005).

2.2.4. Ammonium, nitrate, urea assays

Colorimetric spectrophotometric assays were used to quantify changes in the concentration of the different N sources used for cyanobacterial cultivation. Indophenol-Blue assay was used for the quantification of ammonium (Ivancic and Degobbis, 1984). Urea concentration from samples was quantitated by the O-phthalaldehyde colorimetric assay (Jung et al., 1975) or using a higher sensitivity commercial colorimetric assay kit (Biovision). Commercial spectroscopic assay kit (Cayman) was used for the determination of nitrate/nitrite concentrations.

The N uptake and the N assimilation rates (calculated at the time of total exhaustion of N source) were defined as the time required for the exhaustion of the respective N sources and as the total production of biomass according to the amount of the nitrogen source usable in the medium, respectively.

2.3. Targeted transcriptomic analysis

2.3.1. RNA extraction and preparation

The filtered cyanobacterial cells from 20 mL exponentially growing culture (OD_750 around 0.8) were washed twice with N-free Zarrouk medium to remove any residual N source. Post 30 s suspension in N free media, the cell pellet was resuspended the sterile Zarrouk media with either a single N source or two different N sources resulting in following five combinations: a) Subset A: 20 mM NaNO_3 (control); b) Subset B: 4 mM NH_4Cl; c) Subset C: 20 mM NaNO_3 and 4 mM NH_4Cl; d) Subset D: 2 mM Urea and e) Subset E: 20 mM NaNO_3 and 2 mM Urea. The resultant cultures were incubated under same culture conditions as described under...
Section 2.1.1. Aliquots from each treatment were taken at end of 2 h and 10 h and subjected to total RNA extraction using hot phenol and LiCl treatment as previously reported (Pathak and Lochab, 2010).

2.3.2. qRT-PCR

qRT-PCR technique was employed to evaluate the relative abundances of mRNA of the genes (involved in N metabolism) ntcA, nrtA, nirA, narB and glnA (Lochab et al., 2014). The RNA extracts (Section 2.3.1) were treated with DNAse enzyme (Thermo Scientific, USA) and used as templates for the synthesis of cDNA (AccuScript High Fidelity 1st Strand cDNA Synthesis Kit, Agilent, USA). The gene-specific primers for narB, glnA and nrtA were designed (in-house) with SnapGene Viewer (version 2.8.2) as reported in Table S1. The primers for 16S rRNA, nirA and ntcA were designed as described by Lochab et al. (2014). Strict checks were performed to confirm the efficiency, specificity (and any possible DNA contamination) of each primer by employing a negative control (i.e., no DNAse treatment). The generated cDNA were subjected to qRT-PCR using brilliant III SYBR Green Master Mix (Agilent, USA) under the following conditions: an initial denaturation at 95 °C for 10 min, an amplification (40 cycles) with a denaturation step (30 s, 95 °C), annealing step at 59 °C for 30 s and an extension step at 72 °C for 30 s. The relative abundance of each mRNA level was calculated based on the 16S rRNA amplicon (control).

2.4. Proteomic analysis

2.4.1. Protein extraction and preparation

The protein preparation for the differential label-free proteomic analyses was performed as described previously (Deschoenmaeker et al., 2014). The biomass was treated with a lysis buffer (6 M (w/v) guanidine HCl, 50 mM K2HPO4/KH2PO4, pH 8.5) followed by ultrasonication (3 × 10 s, 20% amplitude; U50 IKA Technik). 50 μg of the extracted proteins were then reduced and alkylated using the ICPLTM kit reagents (Serva, Germany) according to the manufacturer's instructions. The proteins were finally recovered by acetone precipitation and digested with 0.005% (w/v) trypsin (Promega V5111) in 25 mM (w/v) NH4HCO3 (pH 8.5). The trypsin treatment was stopped by adding 0.1% formic acid (v/v).

2.4.2. Separation of peptides

Prior to the mass spectrometry (MS) analysis, reverse phase chromatography was used to separate the extracted. The reverse-phase column (length 25 cm, diameter 75 μm, flow 300 nl/min; PepMap C18, Dionex) was equilibrated with 4% (v/v) acetonitrile for 20 min and peptide elution was carried out over an acetonitrile gradient from 4% to 35% (v/v) from 120 min. The separated peptides were then analysed online by TripleTOF 5600 mass spectrometer (AB Sciex, USA).

Fig. 4. Changes in the relative abundance of glnA (A), ntcA (B), nrtA (C), nirA (D) and narB transcripts (E) of Arthrospira sp. PCC 8005 cultivated in: Subset A 20 mM nitrate (NaNO3) only; Subset D 2 mM urea only and Subset E 20 mM nitrate (NaNO3) and 2 mM urea, at the end of 2 and 10 h incubation. Means were taken from three biologically independent replicates and error bars indicate the SEM (n = 3, ANOVA, P value <0.05*, <0.001***).
Fig. 5. Schematic mapping of the main modifications at mRNA and protein levels when ammonium (A) or urea (B) was present in the medium. Arthrospira sp. PCC 8005 was cultivated (A) with 4 mM NH₄Cl (continuous lines) or, with 4 mM NH₄Cl + 20 mM NaNO₃ (stippled lines) and (B) with urea (continuous lines) or, with urea and nitrate (stippled lines). Proteins and genes are represented by squares and triangles, respectively. The first symbol represents changes occurred after 2 h and the second symbol represents changes after 10 h of incubation. When the expression level of genes under NH₄Cl or urea conditions was significantly higher than the expression under control condition (20 mM NaNO₃), corresponding symbols were filled with red. When the expression level of genes under NH₄Cl or urea conditions was significantly lower than the expression under control condition (20 mM NaNO₃), corresponding symbols were filled with green. Proteins changes were represented as fold change calculated as 4 mM NH₄Cl/2 mM urea (or 4 mM NH₄Cl/2 mM urea + 20 mM NaNO₃) VS 20 mM NaNO₃. Means are taken from 3 biological replicates.
Fig. 6. Changes in the growth rate (A), the sedimentation index (B), N consumption profile (C), partial pO₂ pressure (D), phycocyanin (OD620), allophycocyanin (OD650), chlorophyll a (OD665) content (E) and cyanophycin content (F) of Arthrospira sp. PCC 8005 under transition from nitrate to ammonium in continuous cultivation mode. The lower-case letters a, b, c and d (“) correspond to the sampling time points for the proteomic analyses. The ammonium (•) and nitrate levels (○) represent the residual values of respective N source and the ammonium (▼) and nitrate levels (▲) represent the expected residual concentrations of the N respective N sources based on the dilution rate and feeding concentration. Each curve is representative of three biologically independent replicates.

Table 1
Relative changes in the exopolysaccharides and lipids (total and individual) during continuous cultivation of Arthrospira sp. PCC 8005 under transition of N source form nitrate to ammonia. Means were taken from three independent replicates (P value <0.05 *).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>16</th>
<th>21</th>
<th>33</th>
<th>38</th>
<th>42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exopolysaccharide (EPS) Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total EPS Content</td>
<td></td>
<td>2.6 ± 0.1</td>
<td>2.0 ± 1.3</td>
<td>4.27 ± 0.2</td>
<td>2.0 ± 0.7</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Total Sugar¹</td>
<td></td>
<td>68.9 ± 12.9</td>
<td>63.6 ± 11.1</td>
<td>50.7 ± 1.1</td>
<td>38.7 ± 10.2</td>
<td>36.7 ± 6.7</td>
</tr>
<tr>
<td>Total Uronic Acid²</td>
<td></td>
<td>6.6 ± 1.5</td>
<td>7.4 ± 1.7</td>
<td>4.0 ± 0.3</td>
<td>2.8 ± 0.7</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Sulphate Groups²</td>
<td></td>
<td>4.5 ± 0.4</td>
<td>6.1 ± 1.7</td>
<td>3.1 ± 0.01</td>
<td>3.9 ± 0.5</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Lipid Content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Lipid Content</td>
<td></td>
<td>16.1 ± 1.3</td>
<td>16.5 ± 0.8</td>
<td>15.8 ± 2.2</td>
<td>18.2 ± 4.2</td>
<td>14.5 ± 3.4</td>
</tr>
<tr>
<td>Eicosane Content</td>
<td></td>
<td>5.91 ± 1.7</td>
<td>4.82 ± 0.5</td>
<td>2.75 ± 0.5¹</td>
<td>3.01 ± 1.6</td>
<td>3.92 ± 2.0</td>
</tr>
<tr>
<td>Phytol Content</td>
<td></td>
<td>1.48 ± 0.9</td>
<td>0.76 ± 0.8</td>
<td>0.85 ± 0.4¹</td>
<td>2.29 ± 0.2</td>
<td>1.88 ± 0.2</td>
</tr>
<tr>
<td>Palmitic Acid¹</td>
<td></td>
<td>9.75 ± 1.6</td>
<td>8.85 ± 1.7</td>
<td>6.98 ± 0.5</td>
<td>6.30 ± 1.1</td>
<td>6.10 ± 0.6</td>
</tr>
<tr>
<td>Linoleic Acid¹</td>
<td></td>
<td>3.41 ± 0.7</td>
<td>3.30 ± 1.8</td>
<td>3.15 ± 0.3</td>
<td>2.58 ± 0.5</td>
<td>2.54 ± 1.3</td>
</tr>
<tr>
<td>Glycerol glycosides⁴</td>
<td></td>
<td>79.45 ± 3.4</td>
<td>82.26 ± 4.5</td>
<td>86.26 ± 0.9³</td>
<td>85.81 ± 0.9⁶</td>
<td>85.56 ± 0.07³</td>
</tr>
</tbody>
</table>

¹ Expressed as percentage of Dry cell weight.
² Expressed as relative content of Total EPS content.
³ Expressed as relative content of Total lipid content.
⁴ Glycerol glycosides are sugar extracted in the methanol fraction containing lipids. Expressed as peak Area% relative to the total detectable constituents in the lipid extract.

2.4.3. SWATH acquisition

Peptide spectra were acquired in a data-dependent (DDA) and data-independent (DIA) acquisitions modes. The MS/MS library was acquired in the DDA mode and analysed by ProteinPilot software (version 4.5, AB Sciex, USA) using the algorithm Paragon (version 4.5.0.0, AB Sciex, USA). Briefly, the trypsin was chosen as the cleavage specificity and alkylation (C) set to iodoacetamide, carbamidomethylation as fixed modifications, oxidation (M) and deamination (N, Q) as variable modifications were set. All biological modifications and amino acid substitutions were considered and a thorough ID search was applied with a peptide confidence set at 0.99 (Shilov et al., 2007). The raw spectral data obtained served as the input for ProteinPilot against the Arthrospira sp. PCC 8005 protein database (version 5 provided by the Genoscope) obtained the Arthrospira sp. PCC 8005 genome sequence (Janssen et al., 2010). For the database search, the cut-off peptide confidence limit was set at 95%. ProteinPilot provided the global false discovery rate of 1%, whereas the local false discovery rate of 5%. Accumulation time was set to 0.1 s for MS1 scan and 65 ms for MS2 scan, with total cycle time being approximately 3.8 s.

For the SWATH analysis (DIA, AB Sciex), 32 incremental steps defined as windows of 25 m/z containing 1 m/z for the overlap of window was passed over the full mass range (400–1250 m/z).

Peak intensity method was used for the quantitation of peptides. The ion chromatogram of top six fragmented peptides was extracted and their area was integrated over the 15 min on six transitions. The tolerance was set at 100 ppm. The SWATH data were processed with PeakView software (version 2.1.0.11041, AB Sciex, USA). The retention time (RT) was calculated manually from a group of 15 selected peptides having RT in the range of 20–100 min. Software MarkerView (version 1.2.1, AB Sciex, USA) was used for the analysis of the relative abundance of the peptides. For the batch experiments, relative abundance (fold changes) of genes in the respective subsets (as described under Section 2.3.1) was evaluated by comparison to the control (20 mM NaNO3).

For PBX experiment, the sampling was done on Day 17 (which acted the baseline/control), Day 31, Day 38 and Day 42. Relative abundance (fold changes) of genes was evaluated by comparison to the control (Day 17).

For all experiments, proteins identified with one peptide were rejected for interpretation.

2.5. Statistical analyses

ANOVA and t-tests were performed with a threshold limit of 0.05. Prior to this, application conditions were checked and tested. If the distribution of residuals did not follow a normal distribution, the data were transformed to normalize them (square root or logit function). In order to analyse the pairwise differences, a Tukey’s tests (post hoc test) was performed. These analyses were performed by R software (version 2.2.1).

3. Results and discussion

3.1. Batch cultivation: physiological changes

Firstly, the impact of the chemistry and the concentration of NH4Cl, urea and NaNO3 on the growth profile (OD750) and nitrogen assimilation rates was evaluated in a batch cultivation mode (Figs. 1 and 2).

Although no significant differences were observed between the three N sources at 2 mM (Fig. 1A), a longer lag phase was observed under 6 mM NH4Cl (Fig. 1D). This is consistent with previous results that showed 6 mM NH4Cl (without pH control) corresponds to the inhibitory concentration of ammonium for the cultivation of Arthrospira platensis (Belkin and Boussiba, 1991; Carvalho et al., 2004). Moreover, it has been also demonstrated that a lower biomass density is more susceptible to ammonia inhibition at higher pH (pH > 9), while higher biomass densities assimilate NH3 quicker then mitigating the inhibitory effect (Markou et al., 2014). This could explain the longer lag phase observed under 6 mM NH4Cl. As previously described, no growth was detected at 12 mM NH4Cl (Fig. 1G), suggesting a toxicity and a possible cellular death (Carvalho et al., 2004; Jha et al., 2007).

Interestingly, an early onset of SI (around 72 h) was observed for 2 mM (in term of N) urea subset vs 96 h for NH4Cl and NaNO3 (Fig. 1B) indicated a possible nutrient stress. Indeed, nitrogen starvation has been shown to induce an accumulation of glycogen accompanied with an increase in the cellular density and sedimentation (Depraetere et al., 2015b). Therefore, this observation could be attributed to an early exhaustion of urea in the media (Fig. 1C) at around 48 h compared to NH4Cl and NaNO3. In contrast, there were no statistical variations observed for the % SI for all three N sources at 6 mM (in term of N) (Fig. 1E). Moreover, the rate of consumption of urea was the fastest followed by ammonium and nitrate as observed at 2 mM (in term of N) (Fig. 1C). As shown on Fig. 2, higher N uptake and assimilation rates for urea at 2 and 6 mM (in term of N), further indicated that urea is a better nitrogen source compared to others for cultivation of Arthrospira sp. PCC 8005. At 12 mM (in term of N), nitrate and urea showed the same exhaustion profile and the corresponding rates were not significantly different. Urea has been also observed to promote the growth of non-heterocystous cyanobacteria (Donald et al., 2011) and cyanobacterial blooms (Belisle et al., 2016).

3.2. Batch cultivation: transcriptomic and proteomic analysis

As reported by Lochab et al. (2014), 4 mM NH4Cl was enough to induce early changes in the relative abundance of the gene set involved to the pathway of nitrogen assimilation (Fig. 3). Indeed, NH4Cl induced a significant decrease in the relative abundance of the glnA transcript after 2 h of incubation (Fig. 3A), whereas ntcA, nrtA and narB transcripts showed a significant decrease after 10 h of incubation (Fig. 3B, C, E). As the exception, the relative level of nirA mRNA showed a significant decrease after both 2 and 10 h of incubation (Fig. 3D). Ammonium thus induced a repression in the expression of specific genes, which could ultimately results in lower nitrate assimilation if both nitrogen sources are simultaneously presents. Another study showed similar results for Synechococcus sp. PCC 7002 cultivated in presence of 10 mM NH4Cl (Ludwig and Bryant, 2012).

As Arthrospira sp. PCC 8005 is able to use urea, the influence of this nitrogen source on the mRNA level of different genes involved to the pathway of nitrogen assimilation was also considered. As shown in Fig. 4, urea induced a decrease in the relative abundance of nrtA and nirA transcripts after 2 h of incubation similar to NH4Cl. In contrast to the NH4+-promoted repression, an increase in the relative abundance of the ntcA transcripts was observed after 10 h of incubation (Fig. 4B) in presence of urea as the sole usable nitrogen source in the medium. Additionally, no significant changes were detected for glnA and narB transcripts (Fig. 4A, E). It should be noted that urea was totally uptaken from the medium over 2 h of culture (data not shown), and that could rapidly resulted in a nitrogen starvation. A N deficiency could ultimately enhance the transcription of ntcA and the ntcA-dependent genes, such as the nir operon (Herrero et al., 2004). Consequently, it could explain this increase between 2 h and 10 h of culture under the presence of urea.

Complementarily to the transcriptomic analysis, a large-scale characterization of cellular proteome was performed at 2 and 10 h of incubation in presence of ammonium or urea as sole source in the medium. Additionally, no significant changes were detected for glnA and narB transcripts (Fig. 4A, E). It should be noted that urea was totally uptaken from the medium over 2 h of culture (data not shown), and that could rapidly resulted in a nitrogen starvation. A N deficiency could ultimately enhance the transcription of ntcA and the ntcA-dependent genes, such as the nir operon (Herrero et al., 2004). Consequently, it could explain this increase between 2 h and 10 h of culture under the presence of urea.
nitrogen source or in combination with nitrate. In this context, a proteomic analysis based on a SWATH method (Gillet et al., 2012) was performed. Overall, after 2 h of incubation, 986 and 940 proteins were identified and quantified respectively for the ammonium (4 mM NH₄Cl) and urea (2 mM urea) experiments, whereas 992 and 987 proteins were respectively identified and quantified after 10 h of culture. In presence of the two simultaneous nitrogen sources (4 mM NH₄Cl + 20 mM NaNO₃) and (2 mM urea + 20 mM NaNO₃), 986 and 940 proteins were respectively identified and quantified after 2 h of incubation, whereas 992 and 987 polypeptides were respectively analysed after 10 h of bacterial culture. A summary of the major proteome modifications in parallel to transcriptomic data are mapped in the Fig. 5.

3.2.1. Ammonium-promoted changes in proteome

Results obtained for both conditions 4 mM NH₄Cl and 4 mM NH₄Cl + 20 mM NaNO₃ were very similar. Interestingly, we observed a decrease of the relative abundance of NirA enzyme after 2 and 10 h of cultivation under both conditions of ammonium. This is in accordance with the results obtained at the mRNA level and consistent with the NH₄-promoted repression (Muro-Pastor et al., 2005). Upstream to NirA, the enzyme NarB (i.e., reduces NO₃⁻ to NO₂⁻) was not identified in our data. However, a lower activity of this enzyme has been previously observed in Arthrospira platensis after the addition of 5 mM NH₄Cl (Jha et al., 2007). Moreover, Ohashi et al. (2011) showed that the ammonium-promoted repression disrupts the interaction between the reduced ferredoxin (red-Fd) associated with the PSI and NarB, which is required for the nitrate reduction into nitrates. Taken together, these results clearly indicated that the NH₄+-promoted repression in the nitrate assimilation pathway occurred in Arthrospira sp. PCC 8005.

Additionally, after 2 h ammonium (whether or not with NO₃⁻) seemed to induce an alteration of light-harvesting antennae and photosynthetic electron transfer chain (Fig. 5). This observation was emphasized by the significant decrease in the relative abundance of the c-phycocyanin alpha (0.66 fold change) and beta subunits (0.35 fold change). This decrease contrasts with the suggestion that NH₄+/NH₃⁺ growing Synecochoccus sp. PCC 7002 probably have a higher content of phycobiliproteins than nitrate growing cells (Ludwig and Bryant, 2012). Moreover, the quantum yield in presence of ammonium significantly decreased over time, when the concentration of NH₄Cl increases from 0.625 to 10 mM (data not shown), which could support the hypothesis of a decrease in the photosynthetic activity. A link between the ammonium toxicity and photodamage has been revealed in Synecochystis sp. PCC 6803 (Drath et al., 2008) and Nostoc sp. (Dai et al., 2008). Ammonium ions seem to act as photosensitizer of the oxygen-evolving complex (OEC) by binding and inhibiting the Mn cluster of the OEC (Oyala et al., 2015). This toxicity is amplified when it over pass the capacity of the photosystem II repair cycle (Dai et al., 2014). An increase in the relative abundance of PsbA and Psb27 was observed, this increase could most likely balance these alterations in phycobilisomes and/or optimize the energy transfer to make cells less sensitive to photodamage as it occurs during heat stress (e.g. Synecochystis sp. PCC 6803 (Rowland et al., 2010; Slabas et al., 2006)). As consistent with this hypothesis, our data showed an increase in the relative abundance of ChlH, ChlP (i.e., chlorophyll synthesis) and PetC (i.e., PETC). After 10 h of cultivation under NH₄+, an increase in the relative abundance of PetC and a phycobilisome linker was still observed.

Taken together, these results indicated early changes in the cellular proteome of Arthrospira sp. PCC 8005 in presence of 4 mM ammonium. This reduced N source seemed to perturb the photosynthesis, and induced cellular stress and toxicity. Interestingly, a significant increase of the relative level of many stress-related proteins (e.g. ClpP4 (1.31 fold change), CtpA1 (1.38 fold change), Peptidase C14 (1.67 fold change) and ClpA-ClpP (1.59 fold change) were observed in presence of (whether or not with NO₃⁻) after 2 and 10 h of incubation.

3.2.2. Urea-promoted changes in proteome

When Arthrospira sp. PCC 8005 was cultivated 2 h in presence of urea as the sole usable nitrogen source in the medium, a significant increase in the relative abundance of proteins involved in the photosynthesis (e.g. PsbB (1.63 fold change), PsbA2 (1.53 fold change) and AcsF (1.21 fold change)) was observed (Fig. 5B). Concomitantly, the relative level of N-metabolism related protein GlnA (1.22 fold change) also showed an increase, which could represent an increase in the assimilation of the NH₄⁺ released during the degradation by urease. After 10 h of incubation in the same conditions, a decrease in the abundance of the constitutive protein of phycobilisomes ApCe (0.84 fold change) and enzymes involved in the synthesis of Chla (e.g., Por (0.86 fold change), ChlP (ARTTHRO05_11327, 0.81 fold change), Chlb (0.64 fold change) and AcS (0.65 fold change)) was observed. As mentioned before, the urea was exhausted from the medium after 2 h of culture and then could result in an N starvation. A deficient of nitrogen into cells is well known to induce a decrease in the cellular content of pigments in Arthrospira sp. PCC 8005 (Depraetere et al., 2015a; Deschoenmaeker et al., 2014) as well as other cyanobacteria (e.g. Gloeothece sp. PCC 6909 (Pereira et al., 2011)).

In contrast to these results, the culture condition 2 mM urea + 20 mM NaNO₃ for 2 h (Supplementary File SF4) resulted in a decrease of the relative abundance of several proteins involved in the photosynthesis (e.g., CpcC2, PsbO, PsAF, PsAD and PetC), whereas an increase of some proteins constitutive of PSI and PSII was observed (e.g. Psb27 and PsAF) after 10 h of cultivation. These latter increases could occur to balance the decrease observed at 2 h of the bacterial culture, most likely to maintain the photosynthesis. In contrast to ammonium-promoted changes, stress-related proteins did not shown changes except for ClpC (0.35 fold change) and peptidase C14 (1.52 fold change) after 2 and 10 h under 2 mM Urea and 2 mM urea + 20 mM NaNO₃, respectively.

3.3. Continuous cultivation: physiological and proteomic analyses

Transitions between nitrate and ammonium (NO₃⁻ to NH₄⁺ and NH₄⁺ to NO₃⁻) in a pH-controlled continuous PBR (Section 2.1.2) was performed to assess the inhibitory effect of high light intensity and ammonium on the growth of Arthrospira sp. (Markou and Muylaert, 2016; Ogbonda et al., 2007; Markou et al., 2014). Our results clearly showed (Fig. 6A) that Arthrospira sp. PCC 8005 could survive in presence of NH₄⁺ at concentration higher than 12 mM (pH < 9.25 and light intensity < 150 μmol m⁻² s⁻¹). However, the increase in the%SI of the cells (Fig. 6B) in presence of ammonium indicated the onset of a nutritional stress. This is in accordance with that the N consumption profiles (Fig. 6C) showed an accumulation of NH₄⁺ known to have an inhibitory effect as demonstrated in batch cultivation (Sections 3.2 and 3.3). The proteomic analysis highlighted a significant decrease in the relative abundance of the Amt1 (0.237 fold change) and GlnA (0.584 fold change) (Supplementary File SF5), which could explain the build-up of NH₄⁺ in the media. To further evaluate the impact of the chemistry and concentration of the N source on cyanobacterial cells, physiological characterization (pigments, lipids, exopolysaccharides and cyanophycin content) of the biomass was also performed (Colla et al., 2007).

As shown in the Fig. 6E, the phycocyanin (PC) content was seen to rapidly increase throughout the PBR run, whereas allophycocyanin (APC) content slightly decreased during the first few days
and stabilised when the cells were being fed with NH₄⁺. In contrast, the Chlorophyll (Chla) content firstly increased and then decreased slightly before stabilising (Fig. 6E). These observations were supported by the proteomic analyses, which indicated an increase in various photosynthesis-related proteins (e.g. Pb27, PetA and CpcC1), further indicative of build of NH₄⁺ in the vessel. Nitrogen chemistry and N depletion have been recently reported to affect the pigment content of the photosynthetic organisms (Negi et al., 2016).

Cyanobacterial (CP) is the N accumulation polymer present in the cyanobacterial cells (Deschoenmaeker et al., 2014). The CP content, which increased in presence of NO₃⁻ (Day 0 to Day 12), was observed to drastically decrease when the cells were fed with NH₄⁺ (Day 12 to Day 28) and recovered when the second transition was made to NO₃⁻ (Day 30 and later) (Fig. 6F). In parallel, the proteomic analysis indicated the repression of CphA (cyanophycin synthase) and CphB (cyanoephycinses) genes in presence of NH₄⁺ and an upregulation on further transition of NO₃⁻. These results further confirmed the inhibitory effect of NH₄⁺ on the N metabolism of Arthrospira sp. PCC 8005.

The lipid content and composition of the fatty acid are known to change under nutrient stress in all microalgae (Negi et al., 2016). No significant variations were observed in the total lipid content of the Arthrospira sp. PCC 8005 under both NO₃⁻ and NH₄⁺ (Table 1). However, the close examination of the individual fatty acid content indicated at the onset of nutrient stress in the cells (Table 1). The content of the fatty acid eicosane (C₂₀H₄₂) and phytol (3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, C₁₆H₃₂O₂) were observed to markedly decrease after 33 days of cultivation (under NH₄⁺) but started to recover on transition to NO₃⁻ (Day 38 onwards). Similar trends (though statistically less quantifiable) were observed for palmitic acid (C₁₆H₃₂O₂) and linoleic acid (cis-9,12C₁₈H₃₂O₂). These changes in the fatty acid content further pointed at the onset of nutrient deficient and inhibitory conditions created in presence of NH₄⁺.

The metabolic pathways in cyanobacteria are driven by their C: N ratios. Any deviations (excess or depletion) in the ratio cause deviations/changes in the pathways (Hagemann, 2011; Wever et al., 2008). In this context, the exopolysaccharides (EPS) content was characterized and quantified (Table 1). The percentage EPS content was shown to be higher around Day 33 (NH₄⁺ accumulation) compared to Day 16, 21, 38, 42 (NO₃⁻) indicating at an onset of nutritional stress. While no statistically-supported changes were detected in the composition of exopolysaccharides, an increase in the glycerol-glycosides, a carbon precursor involved in cell wall synthesis (Li et al., 2002) content was observed. In parallel, the proteomic analysis indicated a 10.63 fold increase of the relative abundance of enzyme UDP-N-acetyl-D-mannosamine-6-dehydrogenase. These results further confirmed the onset of N deplete conditions in cells leading to increase in the overall carbon content and eventually increasing the EPS synthesis.

4. Conclusion

Ammonium repressed N assimilation genes of Arthrospira sp., while urea exhibited improved growth profile, N uptake and assimilation rates (even at 12 mM concentration) making it a better N source. Our results established importance of nutrient concentration and chemistry on physiology, metabolism and by-product synthesis. The continuous PBR run further established that inhibitory effects of the nutrient chemistry and stress could be avoided by pulse feeding (instead of continuous) of N source under a lower light intensity (<150 μmol m⁻² s⁻¹); further paving the way of use of ammonium and urea in MELISSA loop for Arthrospira sp. PCC 8005 cultivation.

Acknowledgements

The authors acknowledge the BELSPO and European Space Agency (GST ‘Melgen-3’, BIORAT 2, ArtEMISS projects and ELIPS4) along with FNSR (Grant number 2877824) and Fund for Research Training in Industry and Agriculture (FRIA) for providing financial support and funding.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.03.145.

References


