Journal of Structural Biology 196 (2016) 385–393

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Nitrogen depletion in *Arthrospira* sp. PCC 8005, an ultrastructural point of view

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ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 30 August 2016
Accepted 31 August 2016
Available online 2 September 2016

Keywords:
Cyanobacteria
Nitrogen
Starvation
Glycogen
Polyhydroxyalkanoates
Exopolysaccharides

ABSTRACT

In cyanobacteria, the nitrogen and carbon metabolisms are functionally bridged and consequently respond to the carbon-to-nitrogen ratio. Consequently, a nitrogen deficiency results in carbon excess. For the first time, the biological adaptation of *Arthrospira* sp. PCC 8005 to nitrogen starvation has been deeply characterized at the cellular structure scale. The results indicated that the carbon excess is rerouted into carbon storage granules, such as the polyhydroxyalkanoate and glycogen granules corroborating existing data. Additionally, this photosynthetic organism hugely secreted exopolysaccharides, which could constitute another biological carbon reservoir. It has been reported that few cells in trichomes of *Arthrospira* sp. PCC 8005 still display a high level of fluorescence after a long-term nitrogen starvation. The transmission electron microscopy showed that some cells still contained thylakoids and phycobilisomes after this long-term nitrogen starvation, which could explain the remaining fluorescence.

1. Introduction

Among the most widely spread microorganisms on Earth, cyanobacteria play a dominant role in the global nitrogen and carbon cycles. They constitute a phylum of organisms with an oxygenic-photosynthesis based metabolism, showing a great plasticity of metabolic abilities. The metabolic abilities of cyanobacteria also make them useful for industrial applications (*e.g.* production of biodiesel), and therefore, they have been extensively characterized (Garcia-Pichel, 2009; Schirrmeister et al., 2015). In fact, cyanobacteria appeared 3 billion years ago and contributed to the oxidative shift of the primary reductive atmosphere (Garcia-Pichel, 2009).

The main limiting factor in cyanobacterial growth is the availability of nutrients and particularly deprivations of essential nutrients, for example nitrogen (N) starvation (Schwarz and Forchhammer, 2005). The N starvation was widely investigated at large scale in the phylum Cyanobacteria (*e.g.*, *Synechocystis* sp. PCC 6803 (Spat et al., 2015), *Synechococcus elongatus* (Klotz et al., 2015), *Arthrospira* sp. PCC 8005 (Depraetere et al., 2015a; Deschoenmaeker et al., 2014) and *Microcystis aeruginosa* (Harke and Gobler, 2013)). Acclimation to N starvation is probably the best example of complexity in molecular responses. The biological response varies from metabolic changes to cellular differentiation. During N starvation, the central metabolism is often reprogrammed, including energy production and conversion, amino acid metabolism, and carbohydrate transport and metabolism. Indeed, two simultaneous phenomena have been described: (i) optimization and use of intracellular nitrogenous components (*e.g.*, cyanophycin and phycobilisomes) and (ii) redraft of C metabolism to direct excess carbon into C reservoir (Depraetere et al., 2015a; Deschoenmaeker et al., 2014; Hasunuma et al., 2013; Sandh et al., 2011; Wegener et al., 2010; Yue et al., 2015). The N deficiency induces the degradation of phycobilisomes (PBS) by the Nbl system appearing as a colour shift of culture turning from blue-green to yellow-green (Kato et al., 2014; Sendersky et al.,

Abbreviations: CP, cyanophycin; EPS, extracellular polymeric substance; FT-IR, Fourier transform-infrared spectroscopy; HFN^-, high fluorescent $nitrogen^-$ cell; PHA, polyhydroxyalkanoate; RPS, released polysaccharide; TGA, thermogravimetric analysis.

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http://dx.doi.org/10.1016/j.jsb.2016.08.007
1047-8477/

2015). Amino acids released by the degradation of phycobilisomes, cyanophycin and proteolysis are used for the synthesis of glycogen (Deschoenmaeker et al., 2014; Hasunuma et al., 2013). Some cyanobacteria exhibit buoyancy to move in the water column in order to harvest an optimal light energy quantity for promoting efficient photosynthetic activities. This buoyancy involves the gas vesicle proteins (Gvp), such as GvpA and GvpC, which form the protein shell of gas vesicles (Miklaszewska et al., 2012; Mlouka et al., 2004). Under nutrient deficiency such as nitrogen starvation, some cyanobacteria (*e.g.*, *Arthrospira* sp. PCC 8005) are subjected to bio-flocculation and sedimentation. This could be explained by an increase of cellular density issued from an accumulation of glycogen (Chu et al., 2007; Depraetere et al., 2015b; Deschoenmaeker et al., 2014).

Among the order Oscillatoriales, *Arthrospira* sp. PCC 8005 appears as open helical colonies named trichomes unable to fix dinitrogen (Janssen et al., 2010). This strain PCC 8005 has been selected by the European Space Agency to be one of the key members for the Micro-Ecological Life Support System Alternative (referred as MELiSSA loop), where it will provide oxygen and biomass rich in protein, for instance (Hendrickx et al., 2006).

Here, we report the alterations in the cellular ultrastructure and composition of the non-heterocystous *Arthrospira* sp. PCC 8005 subjected to nitrogen starvation conditions. Because cyanobacteria respond to the C-to-N ratio, N starvation ultimately results in carbon excess. It has been shown that N starvation induced an accumulation of carbon-storage compounds such as polyhydroxyalkanoate and glycogen granules, whereas lipids did not seem to play the role of carbon reservoir. Additionally, the trichomes secreted huge amount of exopolysaccharides (*i.e.*, component of extracellular polymeric substance) surrounding each cells.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The strain PCC 8005 of the genus *Arthrospira* sp. was kindly provided by the Expert Group for Molecular and Cellular Biology MCB (SCK-CEN, Belgium). The strain was incubated at 30 °C in a modified Zarrouk medium on a rotary shaker (160 rpm), under illumination of ±43 μmol s^{-1} m^{-2} for the photosynthetic photon flux density (Li-193SA; Li-Cor BioSciences) as previously reported (Deschoenmaeker et al., 2014). When $OD_{750\ nm}$ reached the value of ±0.8, cyanobacteria were washed twice with nitrate-free Zarrouk medium (N^-) and incubated under the same incubation conditions as previously mentioned. Four biological replicates were assessed and the control cultures were treated in the same way, except the washing and incubation steps were carried out in nitrate-Zarrouk medium (N^+).

2.2. Large-scale biomass characterization

The cyanobacterial biomass was harvested and three times washed with phosphate-buffered saline (BupH, Thermo Fisher Scientific) through filtration (Sartorius, cut off 0.2 μm) at day 30 of culture. Thermogravimetric analysis (Thermo-gravimetric analyser, TGAQ500, TA instruments) was used to examine the thermal stability of the bacterial biomass against temperature. Analyses were performed using around 5 mg sample and performed in a nitrogen gas atmosphere in platinum crucibles. After a first step at 110 °C to evaporate the residual water, the biomass was equilibrated at 40 °C. Samples were finally heated from 40 to 600 °C at a heating rate of 3 °C.min^{-1}. This low heating rate was applied to reach a good resolution about the derivative weight percent. Fourier transform-infrared analyses were carried out by the potassium bromide pellet method with a Perkin-Elmer Spectrum One FT-IR spectrometer (Norwalk, USA) in the range 400–4000 cm^{-1}.

Afterward, the dried biomass was subjected to a lipid (expressed as % of the dried weight) and pigment extraction using the procedures reported (Chaiklahan et al., 2008). Briefly, the extraction was carried out in six-stages (20 min per stage) with a biomass–ethanol ratio of 1:20 (w/v) at 60 °C. The ethanol soluble fraction was used to identify the lipid contents by GC–MS analysis and to characterize pigments by visible spectroscopy. Visible absorption spectra (400–700 nm) were determined on a Spectronic Genesys 10 Bio (Thermo) UV–vis Spectrophotometer, and GC–MS analysis was carried out as follow. Dried samples were silylated by adding 10 μl of pyridine and 50 μl of BSTFA and heating for 30 min at 60 °C in sealed vials. The trimethylsilylated derivatives were separated by GC (GCMS-QP2010, Shimadzu) using a 0.25 mm × 30 m optima 5 MS capillary column (0.25 μm film thickness) (Macherey Nagel, Germany) and identified by their electron impact (70 eV) mass data. Helium (0.75 ml/min^{-1}) was used as carrier gas. GC conditions were as follows: initial column temperature 100 °C, held for 6 min, ramped at 30 °C/min^{-1} to 320 °C and held for 8 min; injector temperature 310 °C, split ratio 20:3.

2.3. Fluorescent PHA assessment

Since Nile Red is commonly used to screen PHA granules (Hauf et al., 2015; Schlebusch and Forchhammer, 2010; Tyo et al., 2006), it was used to observe and quantify the PHA granule content in *Arthrospira* sp. PCC 8005 using a modified method (Wase et al., 2014). Briefly, the biomass from 1 mL of bacterial culture was harvested (18,000 rcf, 15 min, room temperature), and washed using fresh Zarrouk. Biomass was then centrifuged (18,000 rcf, 15 min, room temperature) and the recovered pellets were suspended in mix (1:1) of fresh medium and 30 μM Nile Red dissolved in DMSO. Finally, the biomass was washed once again and resuspended in fresh medium prior to analysis.

Fluorescence quantification was assayed using a microplate reader (FLUOstar Optima, BMG Labtech) with 520 nm and 560 nm as excitation and emission filters, respectively. Fresh medium was used as blank and fluorescence was normalized with dry weight.

2.4. Transmission electron microscopy

Cells were pelleted and washed twice with a washing buffer (0.05% red ruthenium (w/v), 17 mM NaCl, 0.1 M cacodylate buffer at pH 7.8). Pellets were then fixed in fixation solution (glutaraldehyde (3% final, v/v) in washing buffer) for 1 h 30 at room temperature. The biomass was then washed twice with 10-min changes of washing buffer, and post-fixed in post-fixation solution (OsO4 (1% final, v/v) in washing buffer) for 1 h 30 at room temperature. Samples were subsequently dehydrated in a graded ethanol series (25, 50, 75, 90 and 100%), and the dehydrated biomass was embedded in Spurr resin (TAAB Laboratories Equipment, Berks, England). Ultrathin sections (70–90 nm) were cut with a Leica Ultracut UCT ultramicrotome equipped with a diamond knife and transfered to copper grids (Spi). Then, sections were stained with uranyl acetate and lead citrate.

Samples were observed with a Zeiss LEO 906E transmission electron microscope (60 kV, 6–7 μA emission current), and images were acquired with analySIS (Soft Imaging System, Switzerland).

2.5. Extracellular polymeric substance and protein assay

The extraction and purification of exopolysaccharides from extracellular polymeric substances (EPSs) associated to the biomass is based on a method described by Filali Mouhim et al.

(1993). Cells were pelleted and suspended in 1 mL of Suspending buffer (0.025 M Na_2EDTA, 0.05 M TAPS at pH 8.15). Then pellets were incubated at 100 °C for 20 min in order to separate and solubilize the extracellular sheath of trichomes. After centrifugation at 20,000*g* for 20 min at 30 °C, the supernatant was recovered and anionic exopolysaccharides of EPS were precipitated using 3% cetyltrimethylammonium bromide (w/v) dissolved in concentrated H_2SO_4. Precipitated exopolysaccharides were purified by 4 successive cycles of re-solubilisation in a graded KCl series (1.5 M, 750 mM, 300 mM and H_2O), re-precipitation in 50% (v/v) ethanol-H_2O and centrifugation at room temperature. Finally, the purified EPS corresponding to the last pellet were then lyophilized for colorimetric assays. Uronic acids were determined by a colorimetric assay through $OD_{520\,nm}$ measurements according to Blumenkrantz and Asboe-Hansen (1973), using galacturonic acid as a standard. Total soluble carbohydrate content was determined through $OD_{580\,nm}$ measurements according to Yemm and Willis (1954), using D-glucose as a standard. In parallel, proteins were quantified according to the Bradford's method (Bradford, 1976).

2.6. Soluble released polysaccharides

The polysaccharides released in the medium (*i.e.*, non associated with the biomass) were quantified as follows. Briefly, a 20 mL sample of culture medium was filtered in vacuum through a 0.2 µm filter (Sartorius). The resulting filtrate was purified by ultrafiltration using a Vivaspin ultrafiltration device with molecular weight cut off 100 kDa in order to eliminate compounds of low molecular weight and salts (centrifugation at 3000*g* during 1 h). The concentrate containing the released polysaccharides (RPS) was diluted in deionised water and the centrifugation step was repeated twice. The extensively ultrafiltrated and concentrated polysaccharide fraction was dissolved in a final volume of 2 mL and total sugar content was determined by the phenol/sulfuric method (DuBois et al., 1956).

2.7. Statistical analysis

To evaluate the significance of changes, *t*-test and ANOVA were performed with a threshold set at 0.05. Prior to this, application conditions were checked and tested (Shapiro-Wilk and Bartlet's test). If the distribution of residuals did not follow a normal distribution, the data were transformed to normalize them (square root or logit(x) function). In order to analyse the pairwise differences, a Tukey's (post hoc) test was performed. These analyses were performed by R software (2.2.1 version) coupled to the SciViews R console (0.9.2 version).

3. Results and discussion

In order to highlight changes induced by the nitrogen starvation, *Arthrospira* sp. PCC 8005 was cultivated with (N^+) or without (N^-) nitrate as the sole usable nitrogen source. A preliminary characterization of the strain PCC 8005 by thermogravimetric and Fourier transform-infrared analyses was performed after 30 days of bacterial culture (Fig. 1).

The TGAs of both type of biomass showed two main decomposition steps corresponding to dehydration (50–170 °C) and devolatilization (180–550 °C) of the bacterial biomass. Weight losses in each of these steps are clearly different for non-starved and N-starved biomass. In the TGA of non-starved biomass, the second weight loss is significantly higher. The step of de-volatilization (180–550 °C) is assigned to the decomposition of proteins, lipids and polysaccharides (Marcilla et al., 2009). In this case, the difference between N^+ and N^- biomasses could be attributed to the higher content of proteins in N^+ cells. Indeed, higher protein content was observed after 30 days of cultivation under non-starved condition (Fig. 6C).

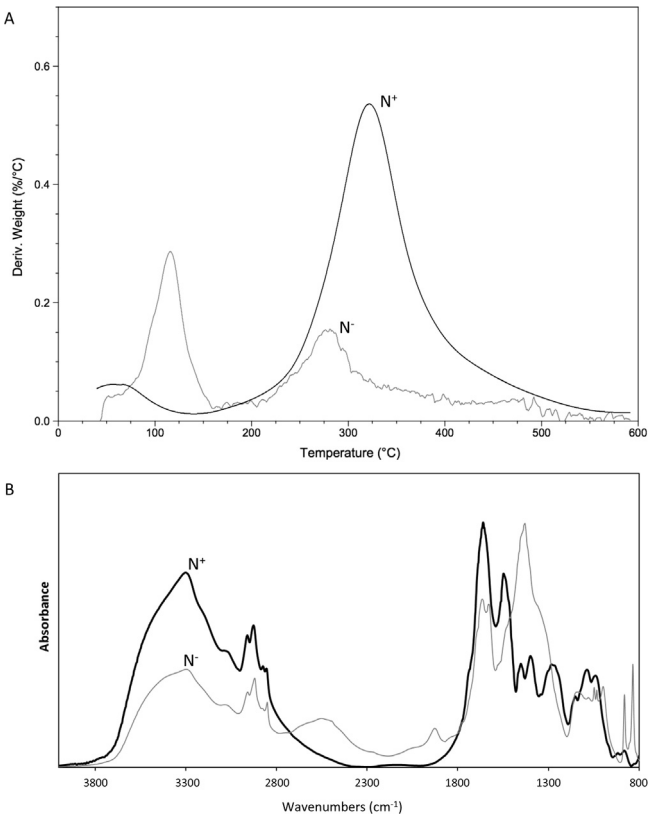

Fig. 1. Thermogravimetric (A) and Fourier transform-infrared (B) analyses of 30 days N-starved *Arthrospira* sp. PCC 8005. Cyanobacteria were cultivated on an orbital shaker (160 rpm) with (N^+) or without (N^-) nitrate as the sole usable nitrogen source (30 °C and 43 µmol s^{-1} m^{-2}). In each case, 5 mg dry weight was subjected to the TGA and FT-IR analyses. The curves represent the average of 3 independent biological replicates.

The general shape of the FT-IR spectra for non-starved and N-starved biomass also showed different bands and peak intensities at particular wavenumbers depending on the growth conditions. Strong absorption bands at approximately 1080, 1540, 1660, 2920 and 3290 cm^{-1} dominate spectra for non-starved growth conditions. These correspond to the P=O bond of nucleic acids, the N—H bending vibration of amide II of protein, the C=O stretching vibration of amide I, the asymmetrical stretching CH_2 group and the OH vibration, respectively. The spectral region between 1200 and 1800 cm^{-1} showed the major differences between samples, thus the N-starved biomass spectrum showed the strongest absorption bands at 1430 cm^{-1} (bending of methyl from proteins) and 1660 cm^{-1} (the C=O stretching vibration of Amide I).

Both analyses clearly showed that the non-starved and N-starved biomass were different in their biochemical composition. Additionally, the visible spectrum scanning indicated drastic differences between the 2 types of biomass, including the disappearance of pigments (Fig. S1). To decipher the origin of these qualitative changes in *Arthrospira* sp. PCC 8005, the biomass was subjected to electron microscopy and several biochemical assays.

3.1. Cytoplasmic space

At day 0, the samples issued from both conditions (N^+ & N^-) exhibited the same ultrastructural characteristics (Fig. 2). Cells contained 2–5 thylakoids that bear phycobilisomes and are closely

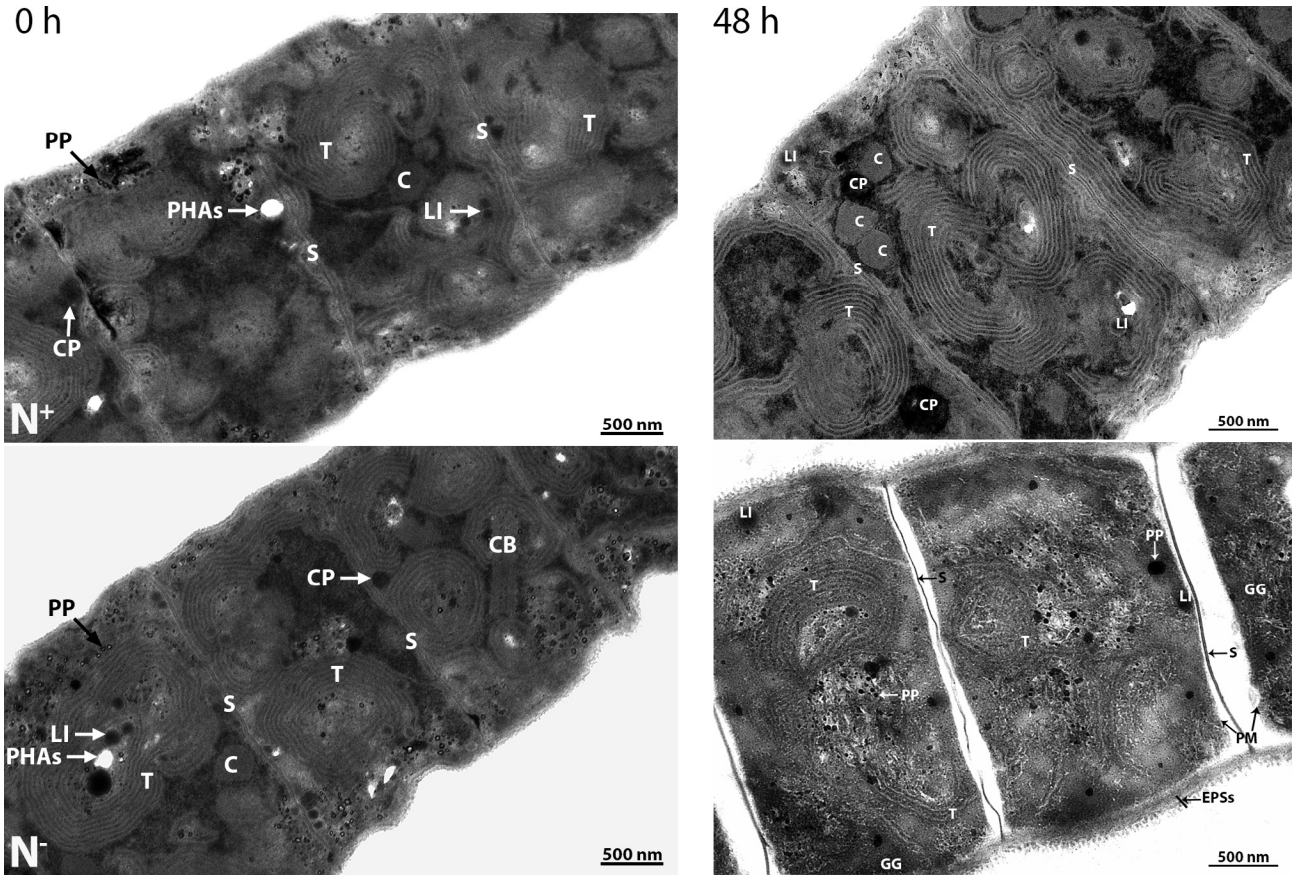

Fig. 2. Alterations of the cellular structure of *Arthrospira* sp. PCC 8055. Cyanobacteria were cultivated with (N^+) or without (N^-) nitrate and incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}). Cyanobacterial colonies were harvested after 0 h and 48 h after their shifting in nitrate-free medium (n = 4) and visualized by TEM: Polyphosphate granule (PP), Polyhydroxyalkanoates (PHAs), Cyanophycin granule (CP), Septum (S), Thylakoid (T), Carboxysome (C), Lipid inclusion (LI), Cylindrical body (CB), Glycogen granule (GG), Plasma membrane (PM), Extracellular polymeric substances (EPSs).

associated with up to 6 carboxysomes. Closely to the nuclear components, polyphosphate granules were observed in the chromatoplasm (*i.e.*, peripheral cytoplasm). The cytoplasm contained cyanophycin and polyhydroxyalkanoate granules, which constitute internal N and/or C storage. In addition to these intracellular components, lipid inclusions as well as cylindrical bodies were randomly localized in the cyanobacterial cells (Fig. 2).

3.1.1. Disappearance of photosynthetic apparatus

After 48 h of N starvation (Fig. 2), the chromatoplasm appeared clearer than in non-starved cells, whereas the nucleoplasm (*i.e.*, central cytoplasm) was still electron-dense as previously observed. The numbers of thylakoids seemed to decrease (N^+: 2–5 per cell, N^-: 2 per cell) and these organelles even totally disappeared in some cells. In addition, the remaining thylakoids in N^- cells lost their cylindrical shape and their lamellae were loosely attached to each other. Moreover, the putative thylakoid formation protein Thf1-like has been reported to decrease after 8 h of nitrate depletion in *Arthrospira* sp. PCC 8005 (Deschoenmaeker et al., 2014). The remaining thylakoid membranes did not exhibit PBSs anymore, this disappearance of PBSs is most likely issued from the bleaching process (*e.g.*, *Oscillatoria willei* BDU 130511 (Kumar Saha et al., 2003), *Gloeothece* sp. PCC 6909 (Pereira et al., 2011), *Synechocystis* sp. PCC 6803 (Krasikov et al., 2012) and *Arthrospira* sp. PCC 8005 (Depraetere et al., 2015a; Deschoenmaeker et al., 2014)). Additionally, the carboxysomes seemed less abundant after 1 day of N-starved growth (N^+: 2–3 per cell, N^-: 0–1 per cell) until disappearing in most of the cells after 2 days of growth in the absence of nitrate. The constitutive protein of carboxysome RbcL has been already mentioned to decrease in N-starved *Arthrospira* sp. PCC 8005 (Depraetere et al., 2015a; Deschoenmaeker et al., 2014). Consistently, the down-regulation of the CO_2 fixation machinery has been already mentioned for N-starved cyanobacteria, such as *Synechocystis* sp. PCC 6803 (Krasikov et al., 2012) and *Oscillatoria willei* BDU 130511 (Kumar Saha et al., 2003).

3.1.2. Mobilization of cyanophycin

When extracellular nitrogen is limiting, the cyanophycin granules (CP) are degraded in order to supply N metabolic demands (Deschoenmaeker et al., 2014; Kolodny et al., 2006; Liu and Yang, 2014). No CP granule was observed over 48 h of cultivation under nitrogen depletion (Fig. 2), which is consistent with a previous study (Deschoenmaeker et al., 2014). Moreover, a proteomic analysis has shown that the abundance of cyanophycinase is higher under N-stress conditions, thereby providing a large quantity of arginine and aspartate (Wegener et al., 2010) most likely to balance the intracellular N deficiency.

3.1.3. Intracellular C storage granules: glycogen and PHA

As N metabolism in cyanobacteria responds to the carbon-to-nitrogen ratio (C-to-N) (Luque and Forchhammer, 2008), the N starvation induces an imbalance of this ratio with carbon in excess. This disequilibrium of the C-to-N ratio results in the accumulation of reserve C-polymers (De Philippis and Vincenzini, 1998). As previously described, the N starvation is known to enhance glycogen synthesis. Indeed, an intracellular accumulation of glycogen granules appeared in N-stressed cells and the glycogen filled the cytoplasm of these N^- cells after 48 h of culture (Figs. 2 and S2).

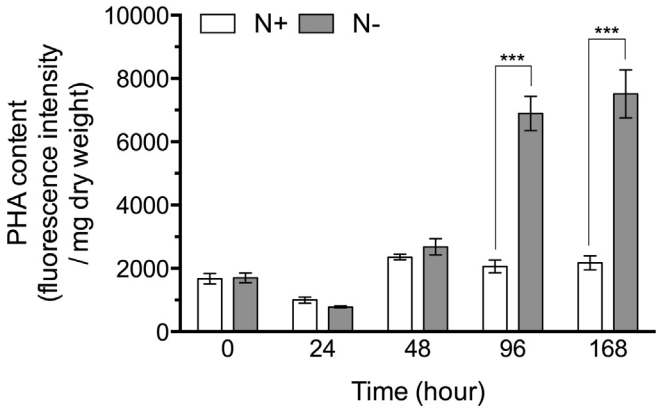

Fig. 3. Content of PHAs within N-stressed *Arthrospira* sp. PCC 8005. Cyanobacteria were cultivated with (N^+) or without (N^-) nitrate and incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}). PHA granules were quantified through the Nile Red fluorescence (excitation wavelength = 520 nm and emission wavelength = 560 nm) according to Wase et al. (2014). The averages correspond to 4 independent biological replicates and the error bars indicate the SEM (ANOVA, *P* value <0.001***).

Ultimately, the glycogen granules serve as an endogenous carbon and energy reservoir to rapidly reinitiate growth when N is resupplied (Gründel et al., 2012). These authors also emphasized that a deficiency in glycogen synthesis renders *Synechocystis* sp. PCC 6803 unable to switch its metabolism under N starvation (*i.e.*, absence of the chlorotic response) and those cells were not viable. These observations clearly highlight glycogen granules as indispensable storage polymers to efficiently acclimate to such stress condition.

Cyanobacteria are also known to store energy and carbon in form of polyhydroxyalkanoates (Kaewbai-ngam et al., 2016). The N depletion affected the levels of polyhydroxyalkanoates (PHAs) as it is well known in *Synechocystis* sp. PCC 6803 (Nakaya et al., 2015). In comparison to non-starved cells, PHA granules were more abundant as well as larger (N^+: up to 55 nm, N^-: 350 nm) in N^- cells at day 1 of culture. After 240 h of N^- cultivation, the size of PHAs greatly increased (N^+: ±45 nm, N^-: ±500–1000 nm) (Fig. S3). These results were corroborated by a PHA assay using the Nile Red dye. Indeed, Nile Red fluorescence increased up to almost 4 fold after 168 h of culture under N stress, suggesting an accumulation of PHAs within N-stressed cells over time (Fig. 3). Moreover, Depraetere et al. (2015) reported that the beta-ketothiolase (*phbA*) was up-regulated under similar conditions, and this enzyme catalyses the first step of PHA synthesis. It has been also proposed that anabolic enzymes of PHAs are regulated through post-translational modifications (Georg and Hess, 2011), which could explain why all enzymes did not display a higher abundance. Taken together, it is reasonable to include PHAs in *Arthrospira* sp. PCC 8005 as a C storage granule during the N starvation as it is the case in *Synechocystis* sp. PCC 6803 (Schlebusch and Forchhammer, 2010).

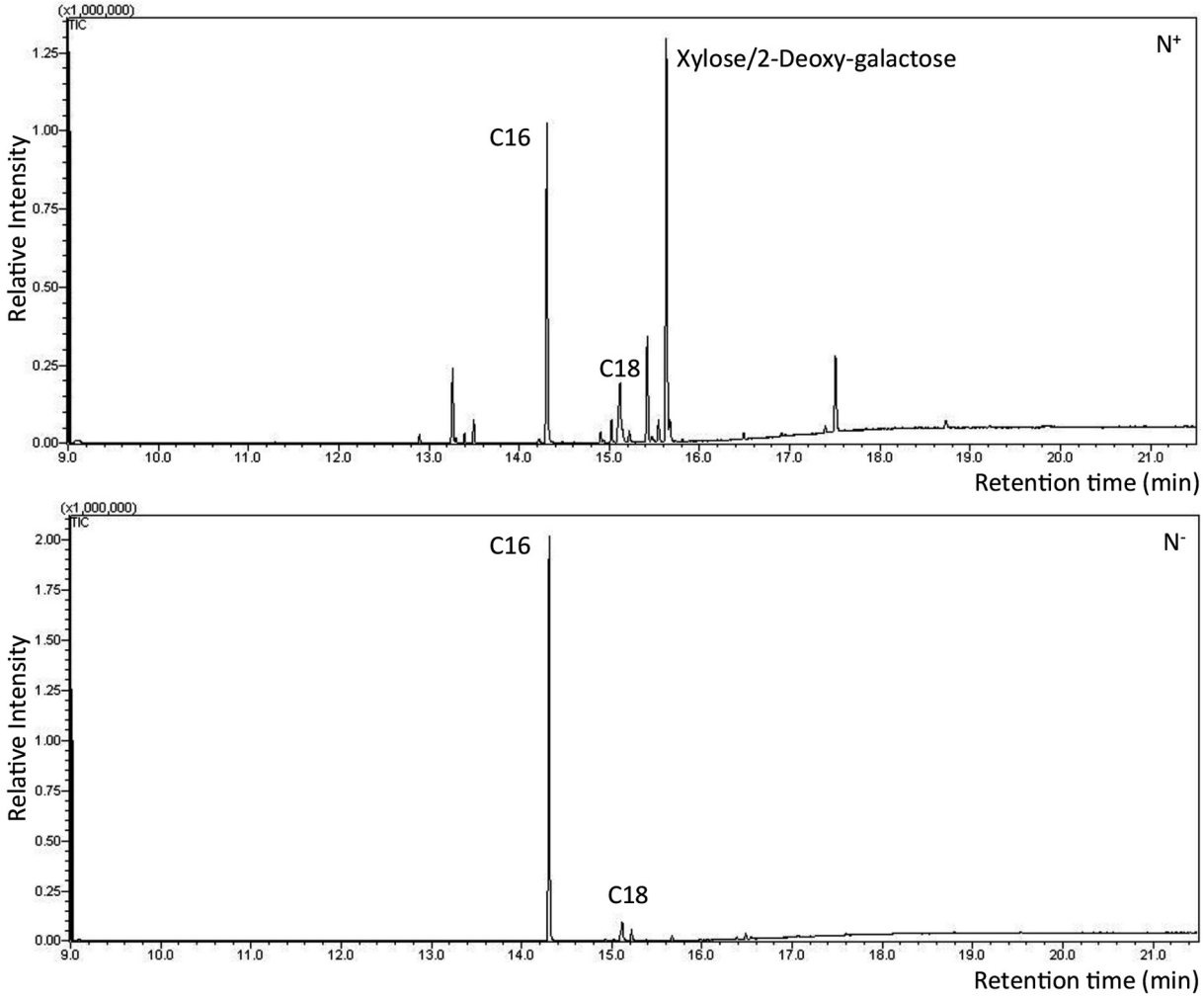

Fig. 4. Investigations of fatty acid from *Arthrospira* sp. PCC 8005 by GC–MS. Cyanobacteria were cultivated with (N^+) or without (N^-) nitrate (n = 3). 5 mg of dried biomass was subjected to extraction and GC–MS analysis. Cyanobacteria were incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}).

3.2. Extracellular space

3.2.1. Cell wall and membranes

Under N^+ condition, the cyanobacterial cells exhibited a cell wall (thickness: ±35–40 nm) with a four-layered structure (Fig. S4): (1) L-I, the electron-transparent internal layer closely beside the cytoplasmic membrane (thickness: ±5–10 nm); (2) L-II, an electron-dense layer (thickness: ±10–15 nm); (3) L-III, an electron-transparent layer (thickness: ±5–10 nm); (4) L-IV, the outermost layer adjoining to the extracellular polymeric substances (thickness: ±10–15 nm). After 1 day of nitrogen starvation (Fig. S5), the cell wall was thicker in comparison with those of N^+ cells (N^+: ±45 nm, N^-: 55–60 nm).

The N-stressed cells exhibited septa (*i.e.*, invaginations of L-II flanked by L-I) that swelled over time (24 h, N^+: 40 nm and N^-: up to 100 nm; 48 h, N^+: 40 nm and N^-: up to 200 nm). However, a decrease was then observed, with thickness stabilization after 10 days (N^+: 40 nm, N^-: 50–100 nm). Lipids are also known to be involved in several abiotic stresses, such as the heat stress (Horváth et al., 2012) and salt stress (Qiao et al., 2013). The analysis of total lipid content by ethanol extraction after 30 days of cultivation indicated a slightly lower lipid amount in the N-starved biomass (N^+: 14.7 ± 0.53% of dry weight, N^-: 12.2 ± 1.4% of dry weight). Additionally, the GS-MS analysis indicated an increase in C16 content after 30 days of N starvation (Fig. 4). Although the N-starved *Oscillatoria willei* BDU 130511 showed modifications in the fatty acid pattern with some that appeared new (*e.g.*, C15 and C22), the C16 content has been described to decrease (Kumar Saha et al., 2003). In contrast, an increase in palmitic acid has been reported in *Scytonema geitleri* under water stress (Singh et al., 2014). These observations emphasized that lipids and their properties change when a cellular stress is applied. Here, no significant increase in the total amount of lipids was observed suggesting that lipids did not seem to act as carbon storage in order to balance the N-to-C ratio. Nevertheless, changes in the fatty acid pattern (*i.e.*, palmitic and stearic acids) were detected during the nitrogen starvation.

3.2.2. Extracellular C storage compound, EPSs

On the external side of the cell wall, the extracellular polymeric substances (EPS) give rise to the cyanobacterial sheath of about 50 nm thick. This EPS sheath was thicker (50–200 nm) after several days of N-stressed culture. After 240 h, it was possible to discriminate a continuous layer (cEPS) closely associated with the cell wall and a fibrillar layer (fEPS), which is more external and thicker (Fig. 5). Some cells were surrounded by a fEPS measuring up to 300 nm, without including the fEPS with a thickness of several µm in certain locations. The changes in the exopolysaccharide abundance (*i.e.*, main component of the extracellular polymeric substances) were monitored under N^+ and N^- conditions over time. The assay of uronic acids showed a significant increase over time under the N-stress condition Fig. 6A). Simultaneously, the total carbohydrates of EPSs showed a significant increased over time (Fig. 6B). These results clearly indicated that *Arthrospira* sp. PCC 8005 abundantly synthesized and secreted exopolysaccharides under N-starvation resulting in the thickening of the sheath as occurred in *Cyanothece* sp. (De Philippis et al., 1993), *Nostoc* sp. BTA97 and *Anabaena* sp. BTA990 (Tiwari et al., 2015). Moreover, the released exopolysaccharide fraction (RPS) showed an increase after 30 days of cultivation under N-starved conditions (Fig. 7). These secreted exopolysaccharides could also represent a significant C-amount rerouted out of the cells to balance C-to-N ratio (De Philippis and Vincenzini, 1998) and could affect the cell adhesion (Pereira et al., 2011). In parallel to the evaluation of EPSs, the measurements of protein contents indicated a decrease under nitrogen starvation over time, whereas it increased under N^+ conditions. The decrease in protein content could be issued from the intracellular degradation of proteins that occurred to balance the N-deficient entry into cells (Depraetere et al., 2015a; Deschoenmaeker et al., 2014; Doyle et al., 2015; Sandh et al., 2011).

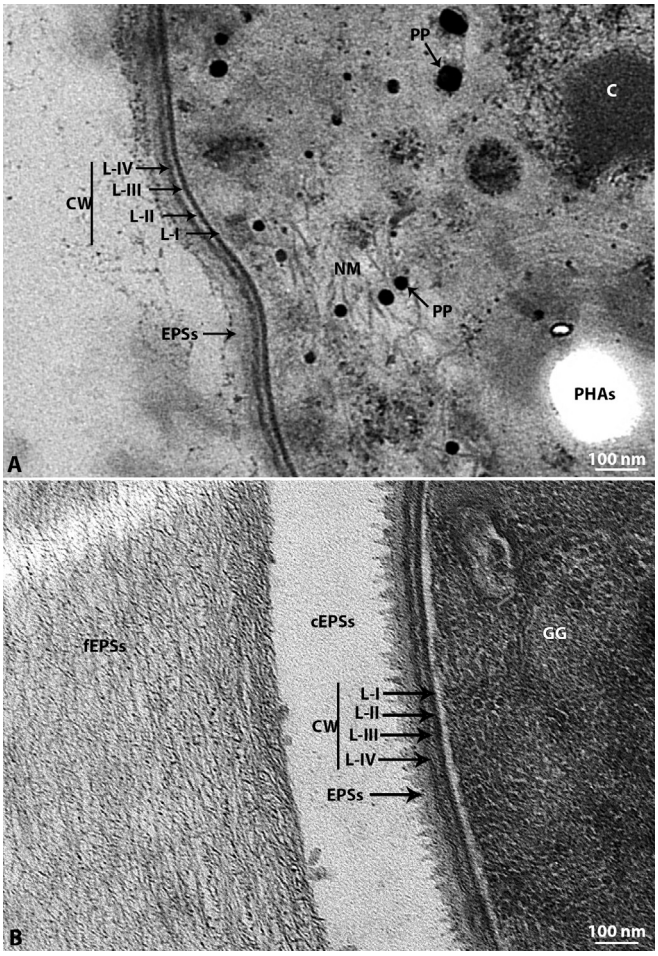

Fig. 5. Accumulation of EPSs surrounding *Arthrospira* sp. PCC 8005 after 240 h of cultivation with (N^+) or without (N^-) nitrate (n = 4). Cyanobacteria were incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}) and visualized by TEM: Cell wall (CW), Layers from the cell wall (L-I, L-II, L-III, L-IV), Extracellular polymeric substances in direct association with the cells (EPSs), Continuous layer of EPSs (cEPSs), Fibrillar layer of EPSs (fEPSs), Nuclear material (NM), Polyphosphate granules (PP), Polyhydroxyalkanoates (PHAs).

Taken together, these results showed that *Arthrospira* sp. PCC 8005 synthesize and secreted a huge amount of exopolysaccharides resulting into an increase in the surrounding sugar matrix (*i.e.*, sheath and released sugars).

3.3. Description of HFN^- cells

Recently, *Arthrospira* sp. PCC 8005 showed a survival capacity during long-term N starvation (30 days) most likely by maintaining a basic activity in few cells that probably sustain the viability of colonies. These cells named "HFN^-" (*i.e.*, highly fluorescent $nitrogen^-$) still exhibited a high fluorescence level after 30 days under N depletion (Deschoenmaeker et al., 2014). When a nitrogen source is available, all cells in trichomes exhibit a red autofluorescence thanks to their photosynthetic pigments. Under N^- conditions, this red fluorescence gradually disappeared, and only a few cells in the trichomes still kept a substantial red fluorescence despite the long-term nitrogen starvation (Deschoenmaeker et al., 2014).

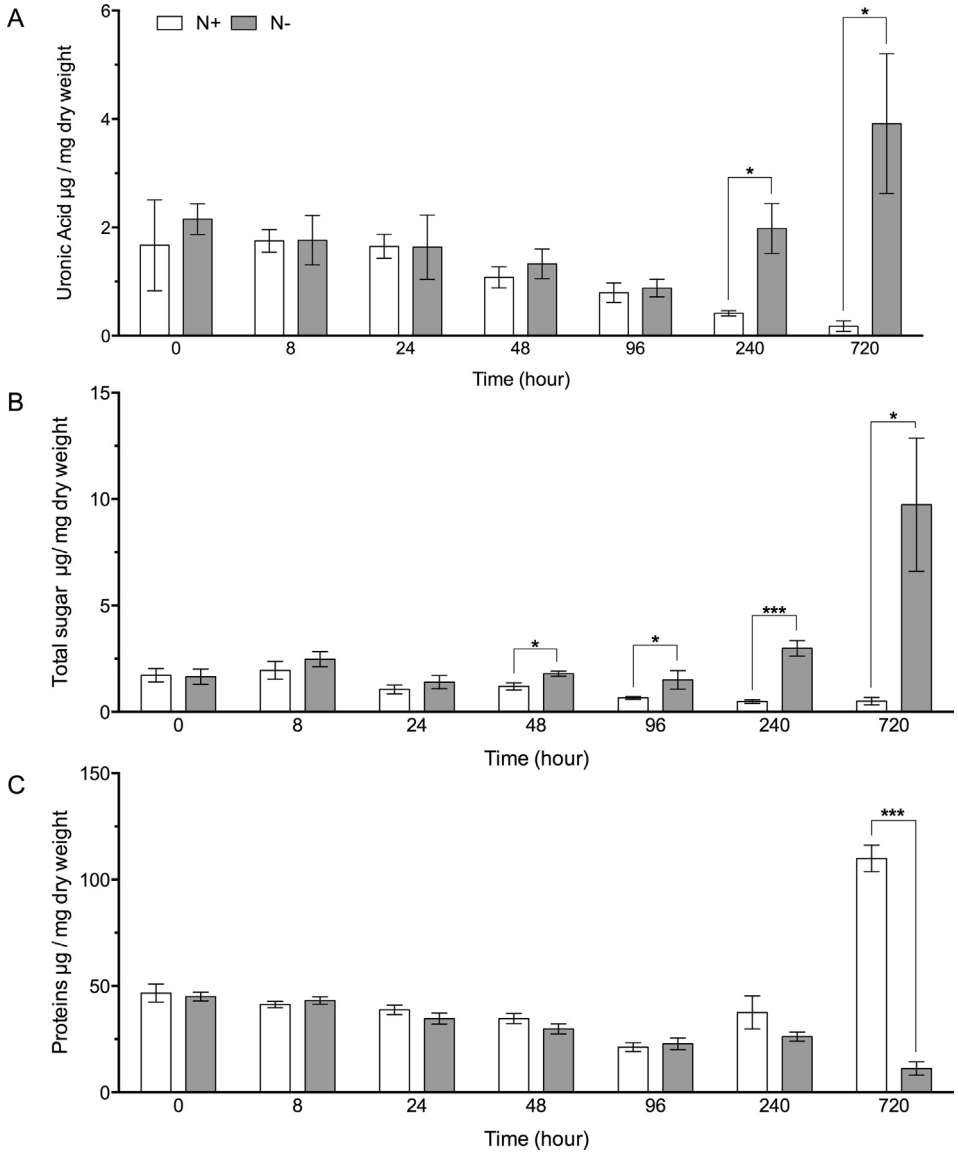

Fig. 6. Changes in uronic acid (A), total sugars (B) of EPS and protein (C) contents of *Arthrospira* sp. PCC 8005. Cyanobacteria were cultivated with (N^+) or without (N^-) nitrate and incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}). Uronic acid and total sugars were assayed over time according to Blumenkrantz and Asboe-Hansen (1973) and Yemm and Willis (1954), respectively. The proteins were quantified according to the Bradford's method (1976). The averages correspond to 4 independent biological replicates and the error bars indicate the SEM (ANOVA, *P* value <0.05*, <0.001***).

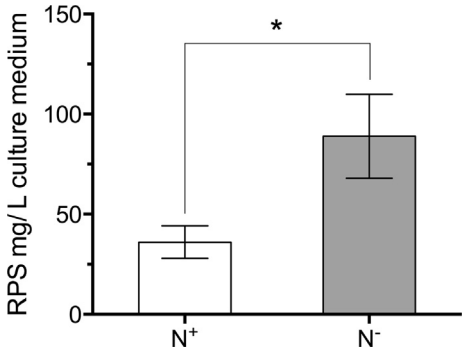

Fig. 7. Evaluation of EPSs released by *Arthrospira* sp. PCC 8005 as soluble polysaccharides in the medium after 30 days of cultivation with (N^+) or without (N^-) nitrate. Cyanobacteria were incubated at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}). The RPSs were assayed according to Dubois et al. (1956). The averages correspond to 4 independent biological replicates and the error bars indicate the SEM (*t*-test, *P* value < 0.05*).

TEM investigations highlighted two phenotypes of cells in the trichomes of *Arthrospira* sp. PCC 8005 after 30 days of culture under nitrogen starvation. It was possible to observe cyanobacterial cells with and without photosynthetic apparatus (*i.e.*, thylakoid membranes with apparent phycobilisomes) (Fig. 8). These few cells containing a conserved photosynthetic apparatus (Fig. S6) could correspond to HFN^- cells. Indeed, the observed fluorescence in HFN^- cells is based on the presence of phycobilisomes associated with the PSII of the photosynthetic apparatus (Deschoenmaeker et al., 2014). Thereby, the presence of thylakoid membranes (and, in certain locations, phycobilisomes) might coincide with the reported red autofluorescence. Moreover, it should be noted that these presumed HFN^- cells seemed to contain cylindrical body which may be involved in the biosynthesis of thylakoids (Baalen and Brown, 1969).

When cells still contained thylakoids, their photosynthetic apparatus was surrounded by huge amounts of glycogen granules. Therefore, presumed HFN^- cells could also accumulate large

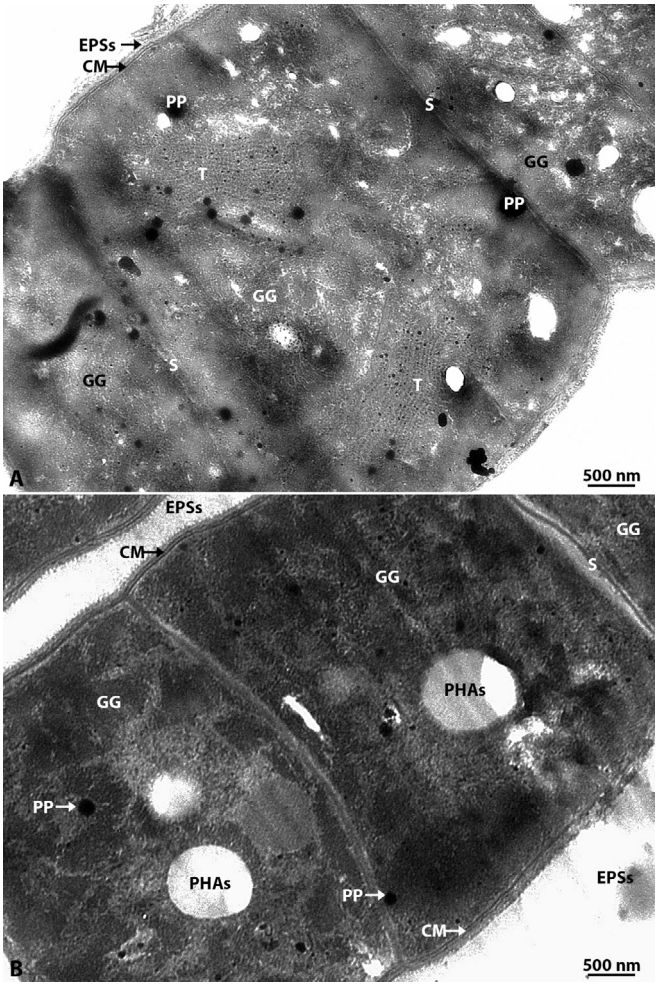

Fig. 8. Electron microscopy investigations of *Arthrospira* sp. PCC 8005 under 30 days of N-starvation. Cyanobacteria were cultivated without nitrate at 30 °C on an orbital shaker under artificial light (43 µmol s^{-1} m^{-2}). Cyanobacterial colonies were harvested 30 days after shifting in nitrate-free medium (n = 4), and visualized by TEM, potential HFN^- cell (A) and a classical N-starved cell (B): Polyphosphate granule (PP), Polyhydroxyalkanoates (PHAs), Septum (S), Thylakoid (T), Carboxysome (C), Glycogen granule (GG), Extracellular polymeric substances (EPSs), Cytoplasmic membrane (CM).

amounts of storage polysaccharides. However, it should be noted that no carboxysome was observed in association with these remaining thylakoids. The thylakoid-free phenotype, assumed HFN^- cells, seemed to possess lipid inclusions and PHAs (with diameters ranging from 500 nm to 1 µm). However, this part of the results is still speculative, as further analyses are required to specifically characterize these particular cells.

4. Conclusion

To summarize, nitrogen starvation induced an imbalance of the carbon-to-nitrogen ratio with carbon present in excess. Therefore, this carbon excess is rerouted in *Arthrospira* sp. PCC 8005 towards C storage compounds, such as glycogen, polyhydroxyalkanoates and extracellular polymeric substance. Consequently, cells are (i) surrounded by a thick EPS layer and (ii) overwhelmingly filled with glycogen granules and to lesser extent with PHAs. The previously described HFN^- phenotype could derive from cells that kept intact some remaining thylakoids flanked by PBSs in order to maintain the viability of the trichomes.

Acknowledgements

This research was supported by the European Space Agency and BELSPO (GSTP 'Melgen-3' and ArtEMISS projects). Frédéric Deschoenmaeker is a PhD student awarded by the Fund for Research Training in Industry and Agriculture (FNRS-FRIA grants). Patrick Flammang is Research Director of the Fund for Scientific Research of Belgium (F.R.S.-FNRS).

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.jsb.2016.08.007.

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