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Metabolic composition of the cyanobacterium *Nostoc muscorum* as a function of culture time: A ^{1}H NMR metabolomics study

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ABSTRACT

Cyanobacteria are considered a factory of added-value compounds. However, knowledge about the array of interesting compounds that could be extracted from these prokaryotic organisms is still very limited. Nuclear Magnetic Resonance (NMR) spectroscopy is a widely used technique for metabolic profiling that allows an overview of the main metabolites present in complex biological matrices. In this work, high resolution ^{1}H NMR was employed to screen the metabolic composition of the freshwater cyanobacterium *Nostoc muscorum*. This species showed high longevity, being able to grow for more than one hundred days without any medium supplementation. During the period of study, several interesting metabolites were detected, such as several sugars and oligosaccharides, lipids (*e.g.*, glycolipids, ω-3 and ω-6 fatty acids), amino acids, including mycosporin-like, peptides, and pigments (*e.g.*, chlorophyll *a* and carotenoids). Owing to the long-term monitoring implemented in this study, the production of these compounds could be associated to specific moments of the growth of *N. muscorum*, providing new insights into the most appropriate harvesting time points for the biotechnological exploitation of specific molecules.

1. Introduction

The search for natural compounds with biological activity is an active and growing area of research. While bacterial and fungal microorganisms (*Actinomycetes* and *Hyphomycetes*) have been the most widely explored sources of bioactive compounds for a long time, in recent years, the focus largely shifted to microalgae and cyanobacteria [1]. Cyanobacteria are prokaryotic organisms, which synthesize a plethora of secondary metabolites in response to environmental conditions. Many of these metabolites can be harvested and exploited for a multitude of applications, which supports the biotechnological potential of these microorganisms. Recent reviews on cyanobacteria metabolites highlight their use as chelating agents, biofuels, biocides, cosmetics, pharmaceuticals, fertilizers, foods, and others (*e.g.*, [2–7]). The molecules identified so far belong to various chemical groups, including fatty acids, terpenoids, phenolics, phytohormones, alkaloids, isoprenoids, polysaccharides and photoprotective compounds [8]. However, from >10,000 cyanobacteria species, only a thousand were analysed for some kind of metabolite characterization and a much smaller number of species is currently cultured at the industrial scale for commercial exploitation [9]. This means that current knowledge about the biotechnological potential of these organisms is still very limited, configuring a new avenue that is worth exploring.

Nostoc is a genus of filamentous nitrogen-fixing cyanobacteria (*i.e.*, diazotrophic species). It can be found in terrestrial and aquatic environments, often forming macroscopic or microscopic colonies [10]. Specialized cells like heterocysts (N_2-fixing cells) and akinetes (resistance cells) are present in its filaments or trichomes [11]. Several species of the *Nostoc* genus (*e.g.*, *Nostoc flagelliforme*, *Nostoc commune*) have been used as edible food items in China, other Asian countries and in South America [12,13]. These species of *Nostoc* are also known to produce interesting compounds with a wide range of biological activities. Cyanovirin-N (CV-N) is a protein discovered in *Nostoc ellipsosporum* able to inactivate some primary strains of HIV-1 [14]. Borophycin is a metabolite produced by the marine strains *Nostoc linckia* and *Nostoc spongiaeforme* var. *tenue*, which shows cytotoxicity against human epidermoid carcinoma and human colorectal adenocarcinoma cell lines [14]. Cryptophycin, isolated from some strains of *Nostoc*, showed potent

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fungicide activity and was cytotoxic against human tumour cell lines [14]. These examples, among many others, support the interest on further studying this genus for potential biotechnological exploitation.

The biological activity of both documented and novel cyanobacteria metabolites is of significant relevance to various applications, including in the medical field. Thus, there is growing interest in detecting these molecules in different sample species and assessing the dynamics of their production. Metabolomics methods are powerful for that purpose, as they enable the detection, identification, and quantification of a multitude of metabolites in complex biological matrices. The most popular analytical techniques employed in metabolomics are Mass Spectrometry (MS), typically preceded by some kind of chromatographic separation, and Nuclear Magnetic Resonance (NMR) spectroscopy [15]. Although the relatively low sensitivity of ^{1}H NMR generally precludes the detection of compounds in sub-micromolar concentrations, this technique readily provides structural and quantitative information on a wide range of metabolites, from different chemical families, in a rapid and non-destructive way [16,17]. Accordingly, NMR-based metabolomics has proven useful in the metabolic characterization of several algal species [18–22] and cyanobacteria [23–25].

The present study reports the first NMR metabolomics investigation of freshwater cyanobacteria from the *Nostoc* genus, with two main goals. The first was to prospect the presence of compounds that can be worthy of further biotechnological exploitation. Secondly, knowing that the growth dynamics of these organisms is constrained by abiotic (*e.g.*, accumulation of metabolic products) and biotic factors (*e.g.*, light, density-dependent competition for nutrients), this study aimed to assess the changes in the cells' metabolic composition during long-term growth. Other works have observed differences in lipid classes [26], as well as on the metabolites secreted [27] throughout the span of cyanobacteria life. Moreover, different growth conditions have been shown to influence the production of important compounds, such as pigments (*e.g.*, light and temperature; [28]), heterocyst glycolipids (*e.g.*, nitrates; [29]), and fatty acids (*e.g.*, temperature; [30]). To address these goals, changes in the metabolome of *Nostoc muscorum* were monitored throughout a growth period of more than one hundred days, with no medium renewal or nutrient supplementation.

2. Material and methods

2.1. Cyanobacteria culture and growth monitoring

The filamentous cyanobacterium *N. muscorum* UTAD_N213 [31] was cultured in triplicate, in 5-L Schott flasks with 5 L of Woods Hole MBL culture medium [32]. A 9-days inoculum was used to onset the cultures, which achieved an initial optical density (440 nm) of 0.08 ± 0.01 (UV–Vis spectrophotometer, Schimadzu UV 1800). The cultures were incubated with constant aeration at 26 ± 2 °C under a 16 h-light/8 h-dark photoperiod cycle; light intensity during the day period was set to 37 μmol photon m^{-2} s^{-1} (Quantum meter MQ-200, Apogee Instruments, Logan, Utah, USA) provided by cool white fluorescent tubes. The cyanobacteria were kept under these conditions for a period of 112 days with no medium renewal or supplementation. For growth kinetics assessment, samples were collected three times *per* week, and the optical density measured at 440 nm. Although the culture was not axenic at the start of the experiment, these measurements were performed in a flow chamber, using sterile material to prevent additional bacterial contamination. A total of 270 mL was collected from each batch (2 mL per sample × 3 replicates). Due to the frequent sampling (for growth kinetics and metabolic profiling), appropriate volumes were not available for analysis after 112 days.

2.2. Sample collection and treatment for NMR analysis

Two technical sample replicates from each replicated culture were collected every two weeks for NMR analysis (see Section 2.3), in a total of 6 replicates *per* timepoint. Samples (15–20 mL) of each homogenized culture were harvested under sterile conditions and vacuum-filtered through a nylon membrane (0.45 μm pore size, 47 mm diameter; Whatman). A washing step with 10 mL NaCl 0.9 % was run at room temperature, then the membrane was scrapped to recover the retained cells into a pre-washed (MeOH) glass tube containing ~100 mg of cooled pre-washed (MeOH) glass beads (0.5 mm; Scientific Industries). Solvents (MeOH:$CHCl_3$:H_2O; 1:1:0.7) were added to the biomass (indicative volumes are given for 20 mg of biomass) or to the blank samples (filter with no cells washed with NaCl) set in parallel, and extractions were run inside an icebox. Briefly, cold MeOH 80 % (−20 °C; 800 μL) was added to the biomass, vortexed, cooled down on ice, and vortexed again (for some samples, an extra step of ultrasounds bath with ice for samples to dissolve was considered). Each tube was then added with cold $CHCl_3$ (−20 °C; 320 μL) twice and cold ultrapure water (4 °C; 288 μL), with 2-min intermediate vortexing steps. The tubes were placed on ice for 15 min and centrifuged at 4111*g* (4 °C; 15 min). The upper aqueous phase was then transferred to a microtube and dried in a speedvac (Eppendorf concentrator with UNIJET II vacuum pump), while the lower organic phase was placed into an amber glass vial and dried under nitrogen. All samples were stored at −80 °C. For NMR analysis, dried aqueous and organic phases were reconstituted with deuterated phosphate buffer (pH 7.4 containing 0.1 mM TSP-d_4; 600 μL) or deuterated dimethyl sulfoxide (DMSO-d_6 containing 0.01 % TMS; 600 μL), respectively. The samples were then vortexed, homogenized (2 min at 2500 rpm; Digital Disruptor Genie, Scientific Industries), placed in a cold ultrasound bath for 180 s for complete dissolution, and transferred to 5 mm NMR tubes (550 μL).

2.3. NMR spectra acquisition and processing

NMR spectra were acquired on a Bruker Avance III HD 500 spectrometer (University of Aveiro, Portuguese NMR Network) operating at 500.13 MHz for ^{1}H observation, at 298 K, using a 5 mm TXI probe. Standard 1D spectra (Bruker pulse programs 'noesypr1d', with water suppression, for aqueous extracts, and 'zg' for organic extracts) were recorded with a 7002.8 Hz spectral width, 32 k data points, a 2 s relaxation delay and 512 scans. Spectral processing (TopSpin 4.0) comprised exponential multiplication with 0.3 Hz line broadening, zero filling to 64 k data points, manual phasing, baseline correction, and chemical shift calibration to the TSP or TMS signal at 0 ppm. To assist spectral assignment, 2D experiments were also recorded for selected samples, namely: i) ^{1}H—^{1}H total correlation (TOCSY) spectra, which show the spin systems corresponding to scalar coupled protons in a molecule, giving a unique 2D pattern; ii) ^{1}H—^{13}C heteronuclear single quantum correlation (HSQC) spectra, which shows proton-carbon single bond correlations, being especially useful for assigning singlets that have no TOCSY correlations; iii) *J*-resolved spectra where the ^{1}H signals multiplicity and coupling constants are made explicit along the F1 axis. Additionally, some assignments were further confirmed through in-house NMR analysis of standard solutions and spiking experiments (addition of standard to sample to confirm a specific assignment based on signal increments).

2.4. Data analysis

The first stage of data analysis consisted of assigning the signals detected in the spectra of aqueous and organic phases of extracted biomass. Compound annotation was based on matching 1D and 2D spectral information to several literature reports [33–38] and reference spectra available in Chenomx (Edmonton, Canada), BBIOREFCODE-2–0–0 (Bruker Biospin, Rheinstetten, Germany), and HMDB [39]. Then, representative signals of assigned compounds, free of major overlap, were selected for integration in Amix-Viewer 3.9.15. The areas of selected signals were then normalized by the total spectral area (excluding residual solvent signals) and used to assess quantitative

changes over time, through multivariate analysis (performed in Metaboanalyst 5.0, https://www.metaboanalyst.ca) and univariate statistics. Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were applied to normalized and unit variance-scaled data to assess, respectively, grouping trends (as visualized in the scores scatter plot), and the most important variables accounting for sample discrimination across time of growth (variable importance to the projection, VIP ≥ 1). Additionally, Spearman rank correlation analysis was employed to search for the most time-correlated variables ($|r| > 0.6$; $p < 0.05$). One-way ANOVA with post-hoc Tukey tests was applied (Systat SigmaPlot®) to assess the effect of time in the production of each metabolite with a VIP score ≥ 1 and/or $|r| > 0.6$. An alpha level of 0.05 was considered in all analyses.

3. Results

3.1. Growth of the N. muscorum culture in the long-term

The growth of the *N. muscorum* was monitored through 112 days using the optical density as a proxy of biomass. An adjusted exponential equation described accurately the increase in absorbance as denoted by the coefficient of determination higher than 0.98 (Fig. 1). These data reflect a continuous increase in absorbance throughout the entire experiment, with higher standard deviations being recorded for later time points (after day 80), which likely relates to the increased turbidity caused by high sample concentrations. Although there was no nutrient reinforcement throughout the study period, the cultures never reached a clear stationary phase.

3.2. Metabolic profile of N. muscorum

The 1D ^{1}H NMR spectra of aqueous and organic extracts (Fig. 2) revealed complementary information on the biomass constituents, which were identified based on the sets of 2D spectra collected for each sample type, as well as on spiking experiments, as detailed in the experimental section. The compounds detected in aqueous extracts comprised amino acids and small peptides, monosaccharides, disaccharides and oligosaccharides, organic acids, nucleotides and compatible solutes (betaines, glucosylglycerol). The organic extracts contained mainly lipids and photosynthetic pigments (chlorophyll and carotenoids). The list of assigned resonances is shown in Supplementary Table S1. In some cases, the information retrieved from spectral analysis was insufficient to provide definite assignments, hence, some compounds were identified in more general terms, namely: i) peptides 1, 2 and 3 (NMR profile consistent with bound amino acids, but specific identity not determined); ii) AXP (adenosine mono/di/tri-phosphate) and UXP (uridine mono/di/tri-phosphate); iii) glycolipids (galactosyl, glucosyl and glyceryl moieties, as in [36,38]); iv) sucrose-containing oligosaccharides, as in [37]; v) betaines 1, 2 and 3 (3 singlets with ^{1}H and ^{13}C chemical shifts characteristic of these amino acid derivatives). Overall, 36 signals were integrated and their areas, which are proportional to compound concentrations, were used as input for multivariate analysis.

As a first approach to identify grouping trends within the samples, unsupervised PCA was applied to signal areas (representative of the different metabolites identified). The scores scatter plot relating PC1 and PC3 (Fig. 3A) showed a clear time-related gradient distribution of samples along PC1, which explained 54.3 % of the data variance. This is particularly evident up to day 56, after which the samples from different time points were less well separated, indicating higher similarity between their metabolic profiles. This observation was further corroborated by the dendrogram obtained by HCA (Fig. 3B), where samples from days 84 and 112 were clustered closely together and included in a larger cluster also comprising the later time points (from day 56 onwards), which separated from another branch comprised of days 14, 28 and 43.

The heatmap representation of the 36 signal areas measured for all samples (Fig. 4) also showed two main metabolic signatures corresponding to the first and second half of the growth period. In days 14, 28 and 43, cyanobacteria were characterized by higher relative abundances of malto-oligosaccharides, amino acids, nucleotides, chlorophyll *a*, and some lipids, mainly polyunsaturated fatty acids and glycolipids. In the second half of the growth period (from day 56 onwards), the levels of these compounds were lower, while the levels of sucrose-derived oligosaccharides, nucleosides and peptides, among others, were relatively higher. Metabolites such as carotenoids, valine, isoleucine and trehalose did not follow any of the patterns described above, being produced heterogeneously during the growth of *N. muscorum*.

To determine the compounds with greater importance for time-related sample separation, PLS-DA was applied to the 36-variables matrix. The resulting model discriminated the sample groups with high robustness (Q^2 0.96 for 2 components, Fig. 5A) and showed a trajectory along the first latent variable (LV) according to culture time. The compounds contributing the most to the observed LV1 scores distribution (VIP $\geq$ 1) are highlighted in Fig. 5B. These results were further confirmed by correlation analysis, since all the compounds with VIP ≥ 1 were also significantly correlated with time ($|r| > 0.6$; $p < 0.05$), as shown in Fig. 5C.

The relative levels of compounds with VIP ≥ 1 and with significant correlation coefficients, at different culture times, are displayed in Fig. 6. A number of amino acids, namely glutamate, aspartate, ornithine, alanine and a mycosporin-like amino acid, decreased consistently over time, especially from 14 to 56 days. The levels of peptides increased, especially in the second half of the growth period. There were also marked changes in carbohydrates. Maltose-derived oligosaccharides showed a decreasing trend through time, whereas sucrose-derived oligosaccharides were very low in early time points and steadily

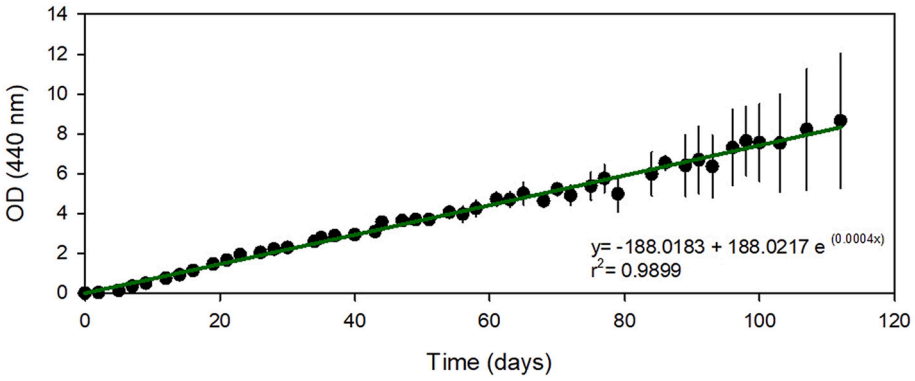

Fig. 1. Growth kinetics of *N. muscorum* throughout a monitoring period of 112 days. Marks represent the mean of 3 replicates and the error bars represent the standard deviation. The best fitted exponential model was added as a solid green curve. Note that OD measurements were always made using controlled sample dilutions resulting in readings up to a threshold of 1.000. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

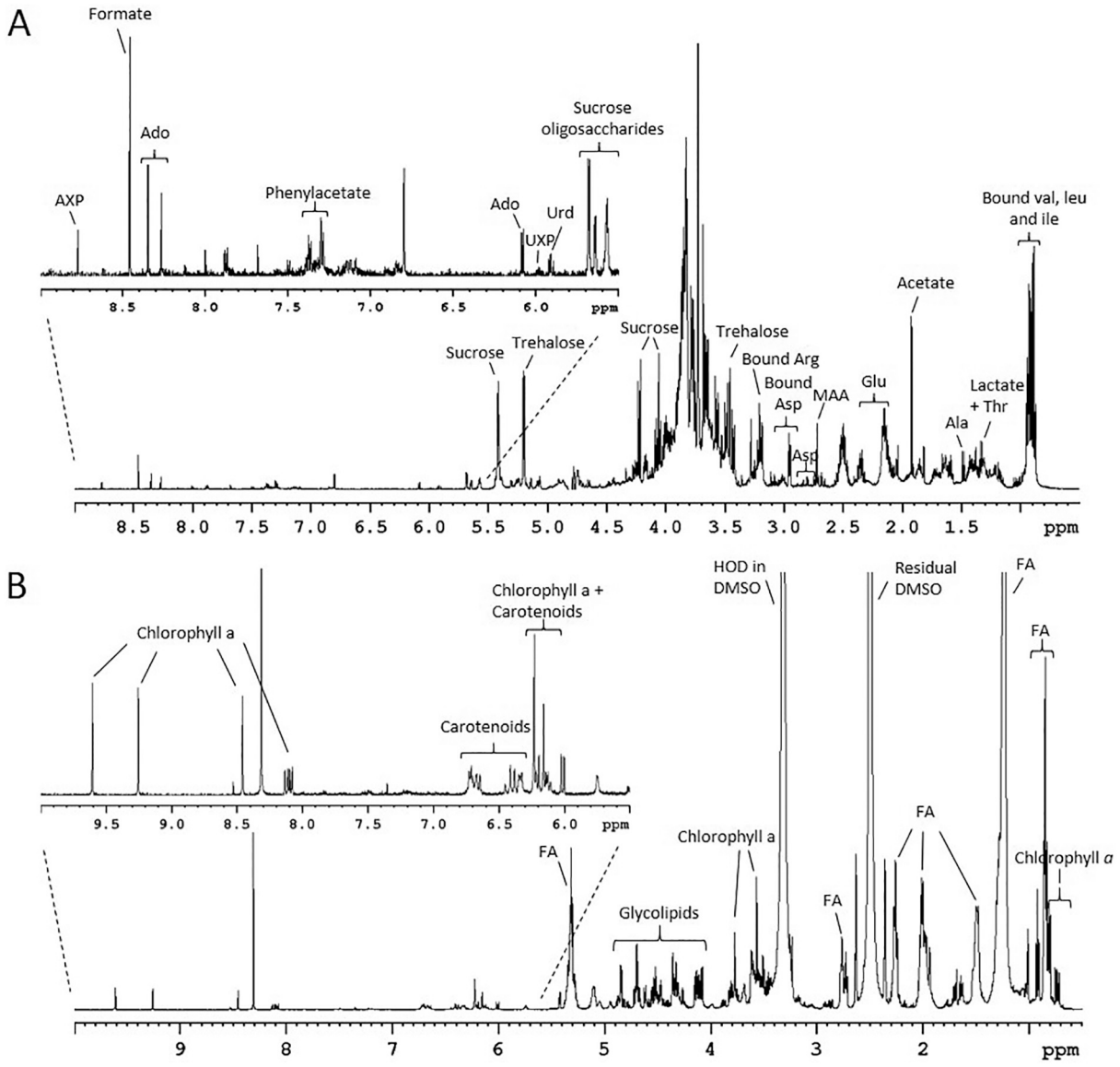

Fig. 2. Representative 500 MHz ^{1}H NMR spectra of the aqueous (A) and organic phase (B) of *N. muscorum* culture at day 70. Some assignments are indicated, while a full list of identified compounds is provided in Supplementary Table S1. Three-letter code used for amino acids; MAA, mycosporine-like amino acid; Ado, adenosine; AXP, adenosine mono/di/tri-phosphate; UXP, uridine mono/di/tri-phosphate; FA, fatty acyl chains in lipids.

accumulated over time. As for compatible solutes, glucosylglycerol and one betaine increased through culture growth, whereas two other betaines decreased. Lipid-related compounds also displayed diverse variation patterns. While polyunsaturated fatty acids (PUFA), including linolenic acid, decreased with time, those with a single unsaturation (MUFA) were accumulated. As for the levels of total fatty acids (first row in the heatmap of Fig. 4), there was greater heterogeneity between samples, although an increasing trend could be seen towards the end of the growth period. Moreover, the ratio of linoleic (ω-6) to linolenic (ω-3) acid increased significantly from early to late time points (Fig. 6). Other compounds with VIP ≥ 1 and/or $|r| > 0.6$ included 3-hydroxybutyrate, acetate, nucleotides, and chlorophyll *a*. They all had higher relative levels in younger cultures and decreased over the second half of the growth period.

4. Discussion

Cyanobacteria are a promising source of natural compounds with multiple biotechnological applications. Among explored species, those from the *Arthrospira* genus are the most well-known and studied, mainly due to their edible properties and suitability for large-scale culture. The *Nostoc* genus has also been used as a food source for many years, but is far less characterized, and underexplored at the industrial level [12,13]. The present study demonstrated that *N. muscorum* can grow for over 100 days without nutrient supplementation, which underscores its potential for long-term maintenance at reduced cost, a key feature in sustainable industrial exploitation.

Given the large variability in cyanobacteria's eco-physiological features (*e.g.*, [40]), characterizing the metabolic composition of each species is an important component of its biotechnological value assessment. The use of NMR spectroscopy for screening cyanobacteria metabolites has proven useful before, although it has only been applied to a single or a limited number of time points (*e.g.*, [23,41,42]). To the best of our knowledge, this is the first study employing untargeted metabolic profiling to monitor long-term variations in cyanobacteria's metabolome. Such knowledge not only improves current understanding of these organisms' physiology, but is also useful from an applied perspective, namely, to identify the most appropriate growth stages for

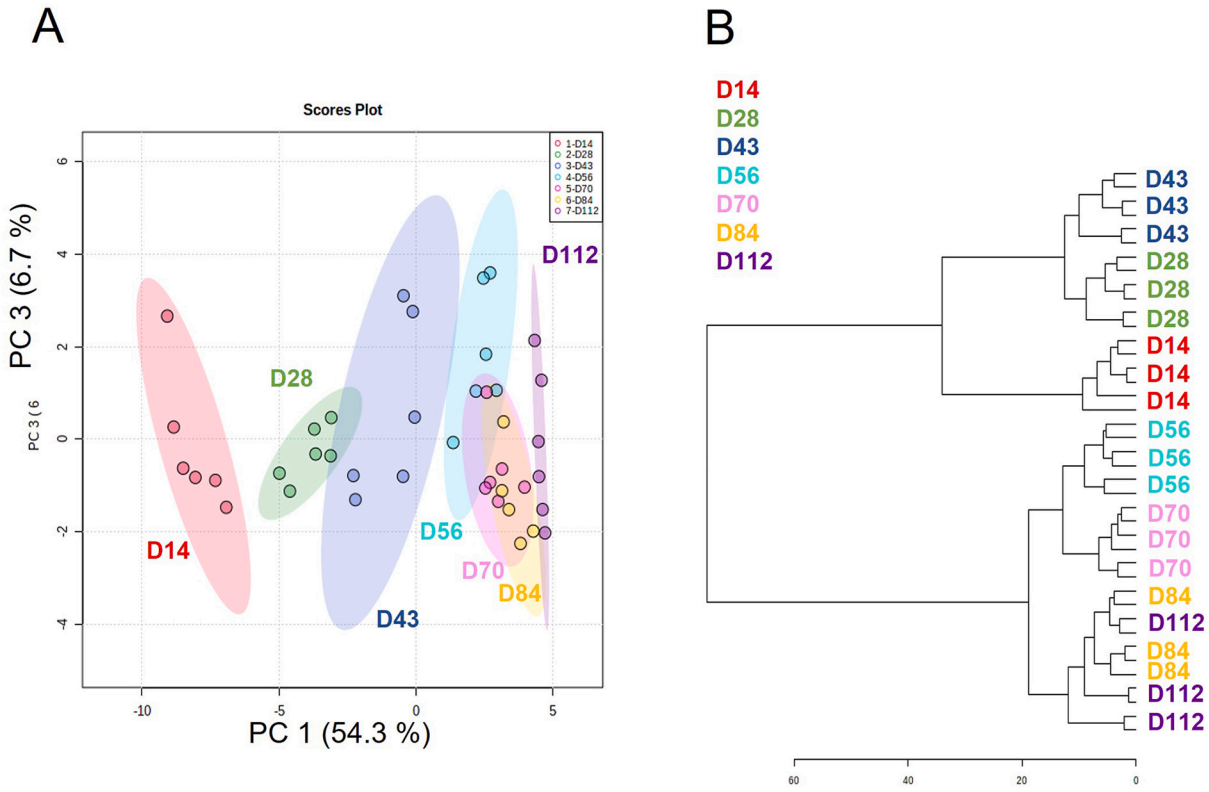

Fig. 3. A) Principal component analysis (PCA) scores scatter plot and B) hierarchical cluster analysis (HCA) dendrogram (Euclidean distance, ward clustering algorithm), evidencing the changes in the metabolome of *N. muscorum* through a 112-days monitoring period. Samples are color-coded by sampling timepoint (days 14, 28, 43, 56, 70, 84 and 112). In the PC1 *vs* PC3 scores scatter plot (best representing the separation between sample groups), the shadowed areas represent 95 % confidence regions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracting specific metabolites. Along with assuring that the stock cultures grow at an optimal, stable pace in the long term, this is key for the industrial exploitation of target compounds, so that harvesting can be scheduled to the growth phase where their maximal production occurs.

In this work, NMR profiling of cell extracts enabled a multitude of metabolites to be identified, confirming the first hypothesis that *N. muscorum* is a rich source of compounds with biotechnological value. Also, the metabolic profile of *N. muscorum* was not homogeneous over the growth period, with some key metabolites being preferentially produced in specific culture phases. Thus, the second hypothesis was verified, evidencing that it is possible to optimize the best harvesting period for the extraction of a desired metabolite. Analysis of the production patterns suggests a pronounced shift in metabolism at approximately half term of the studied period (from day 43 to 56). This could possibly reflect some age-related metabolic adaptations, as the number of older cells, relative to total cell numbers, naturally increases with time. Moreover, as the culture medium was not supplemented during the growth period, consumption of certain nutrients and release of metabolic waste products are also expected to influence the cells metabolic activity. In future studies, a parallel analysis of extracellular medium is warranted to verify this hypothesis.

Several compounds such as amino acids showed higher production during earlier stages of the growth period (up to 43 days). Within the amino acids identified in *N. muscorum*, mycosporin-like amino acids (MAAs) are particularly interesting. So far, >30 MAAs, present in several biological groups, such as corals, micro and macroalgae, dinoflagelates, fungi, lichens and cyanobacteria [43], have been identified [4]. Specifically for *Nostoc* species, several MAAs were reported (*e.g.*, MAA-312, MAA-330, shinorine, glycosylated MAAs, porphyra-334 and extracellular MAAs) [44]. The biosynthesis of MAAs is thought to include intermediates from different metabolic pathways, namely the shikimate pathway, used for the biosynthesis of aromatic amino acids, and the pentose-phosphate pathway [45]. Moreover, MAAs biosynthesis is known to be non-species-specific and to depend on abiotic conditions, especially the spectral distribution and intensity of radiation [46]. In cyanobacteria, UV-B is the most important factor constraining the accumulation of MAAs, but nitrogen availability also constrains its composition [47]. In the present work, no radiation stress was induced, suggesting that MAAs production is naturally high in the *Nostoc* species studied, and has the potential to be even further stimulated by specific conditions. The recognized roles of MAAs in photoprotection, antioxidant protection, osmotic regulation, reproduction control, and as nitrogen reservoirs [48], justify their wide application as anti-photoaging agents [49], wound healing agents [50], and anti-cancer agents [51,52].

While the levels of amino acids decreased with growth, the production of peptides increased. Peptides are a class of attractive compounds for the pharmaceutical industry, namely as a starting point for the development of novel drugs [53]. They bind selectively to cellular targets, which reduces the risk of side effects [54], and can present biological activity (*e.g.*, antitumor and antifungal; [53]). While some peptides are common to several cyanobacteria genera, like the toxin microcystin, others have only been identified in species of the genus *Nostoc* (*e.g.*, nostocyclopeptides, cryptophycins, nostopeptolides) [55]. Hence, a targeted analysis should be conducted in the future to unequivocally identify the peptides found herein to accumulate in *N. muscorum*.

Sugars and other metabolites are produced by cyanobacteria (and by most microorganisms) as compatible solutes involved in the response against osmotic stress [56]. Sucrose, trehalose, glucosylglycerol, glucosylglycerate, and glycine betaine are typical in cyanobacteria [57]. Freshwater cyanobacteria species with low halotolerance mostly accumulate sucrose and/or trehalose, while moderately halotolerant

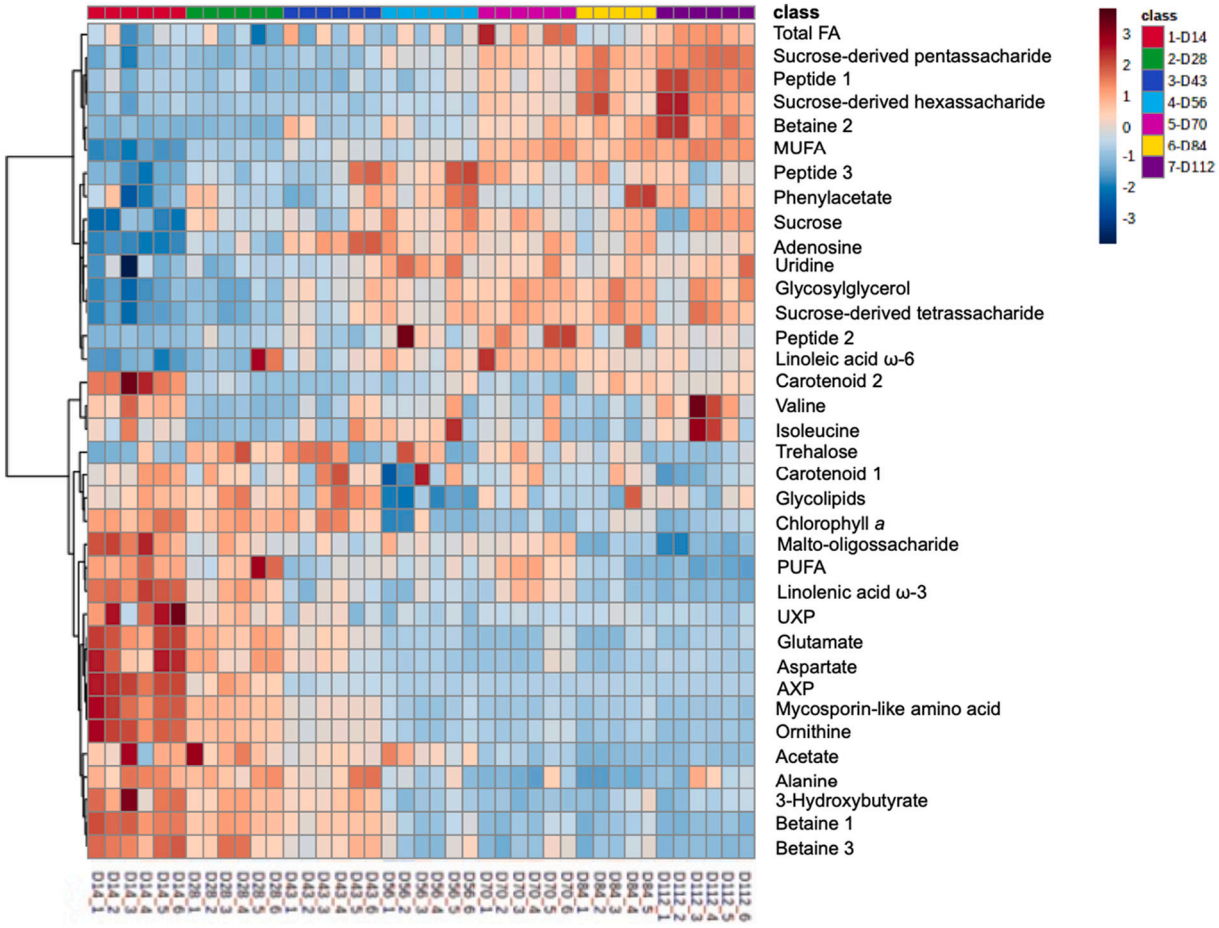

Fig. 4. Heatmap representation of relative abundances (from low, in blue, to high, in red) of the 36 metabolites (or metabolite groups) detected in *N. muscorum* extracts at different culture time points. The left-hand cladogram evidences the similarities between compound production patterns through time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(marine) species mostly use glucosylglycerol as the main compatible solute and glucosylglycerate as a secondary compatible solute; glycine-betaine or glutamate betaine are normally used by halophilic species [56]. The *Nostoc* strain used in this study was collected in a freshwater habitat. However, along with sucrose and trehalose, glucosylglycerol was also produced by the species, which suggests a higher halotolerance than expected for a freshwater species. Some *Nostoc* species are known to be tolerant to desiccation and to accumulate trehalose [58]. Glucosylglycerol was also reported for other freshwater species [59], indicating higher salt resistance than the sucrose- and/or trehalose-accumulating species [60]. The production of sucrose and glucosylglycerol, mostly at later culture phases, may indicate disturbance of the osmotic balance caused by changes in medium composition. To deal with this challenge, most organisms use a "salt-out" strategy, *i.e.*, they produce these compatible solutes to lower internal water potential in the presence of high external salinity, allowing osmotic regulation [61]. Photoautotrophic cyanobacteria normally use *de novo* synthesis instead of taking up compatible solutes, which are normally not available in their environment [56].

Contrarily to several carbohydrates, lipids like linolenic acid (ω-3), 3-hydroxybutyrate, and polyunsaturated fatty acids (PUFA) slightly decreased with time. Lipids are essential molecules in primary metabolism, especially in light reactions of the photosynthesis [62]. Lipids from cyanobacteria, mostly located in the thylakoid membranes [63], are mainly composed of diacylglycerols [64]. In photosynthesis, lipids can play different functions, such as the mediation of protein-protein interactions, the oligomerization or protein-cofactor interactions, and as providers for lipophilic regions within protein complexes [62]. Thus, the observed decrease in the production of some lipids is consistent with the reduction in the production of chlorophyll *a*. This relationship was also described for *Synechocystis* sp. PCC 6803, where an increase in lipid content was reported during the phases of faster cell division and concomitant higher photosynthetic demand [65]. While the production of total MUFA showed a slight increase with time, total PUFA decreased slightly. In cyanobacteria, and also in higher plants, changes in the unsaturation of fatty acids could affect the membrane fluidity and the photochemical and electron-transport reactions that occur in thylakoid and other membranes [66]. Lipid composition is also dependent on growth conditions [65], thus medium changes caused by nutrients depletion and/or by the excretion of metabolites can support the present results. This is also a class of compounds with high market value. For instance, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-linolenic acid (GLA) and arachidonic acid (ARA) are important PUFA which can be used in pharmaceutical and therapeutic applications [67–71]. Among cyanobacteria species, *Arthrospira* is considered a good produced of linolenic acid, being able to accumulate 1 % of this fatty acid in dry cell mass [72]. However, the *Nostoc* species also produces linolenic acid, as demonstrated in the present study and in other reports [73].

Glycolipids also bear a range of interesting bioactivities with applications in biomedical, pharmaceutical and cosmetic sectors [74], as their amphiphilic nature provides good surfactant capacity. Microbial glycolipids perform better compared to petroleum-derived or plant-based synthetic surfactants by bearing higher surface activity, higher

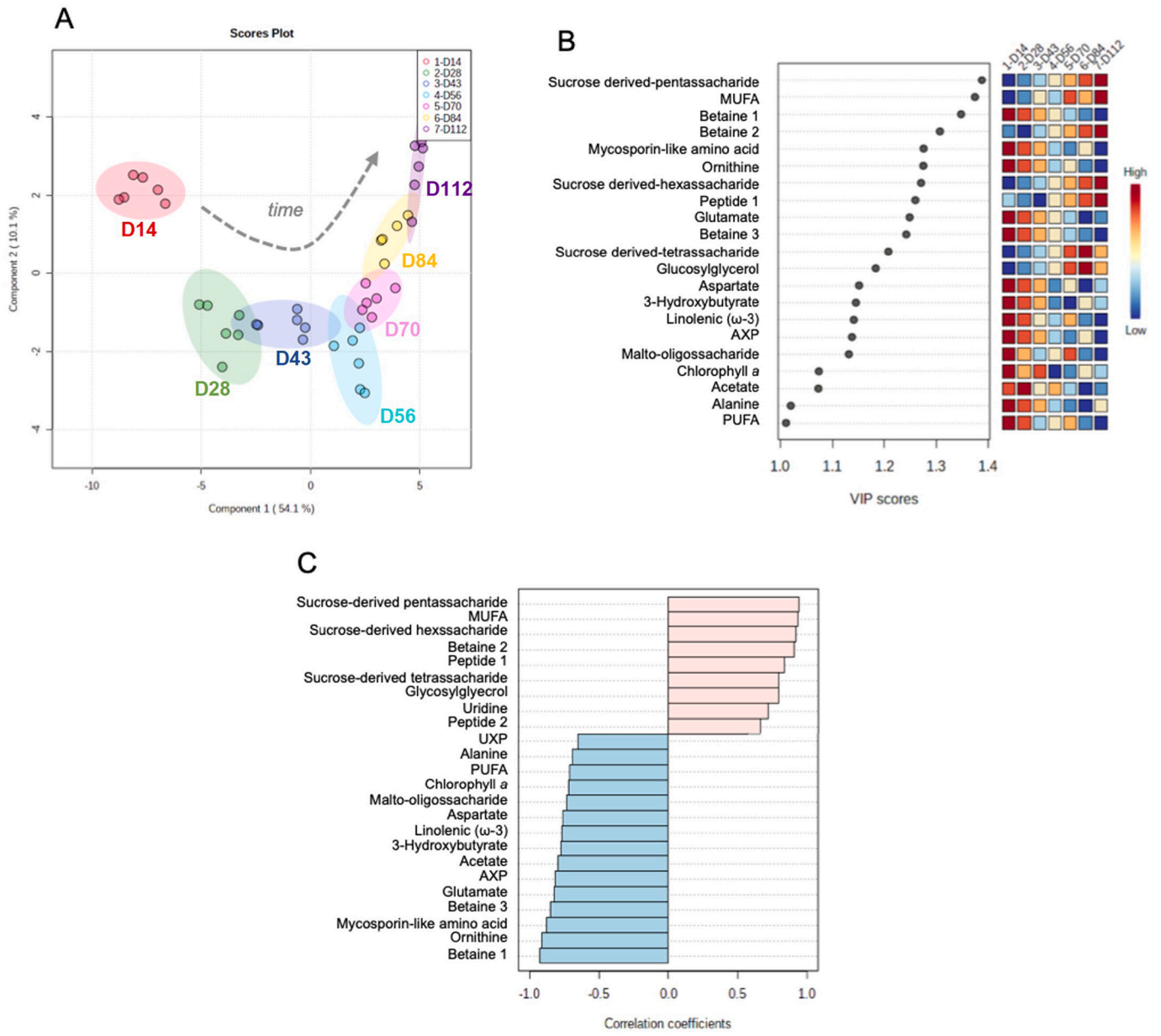

Fig. 5. A) Partial Least Squares Regression with Discriminant Analysis (PLS-DA) scores scatter plot and B) the variables (metabolites) most important (VIP $\geq$ 1) for sample distribution along the first PLS-DA component; C) top 24 metabolites correlated with time, from day 14 to day 112 (Spearman rank correlation).

emulsifying power, lower critical micelle concentrations, higher biodegradability (compared to petroleum-derived surfactants), and lower ecotoxicity [75]. The major constraint to a wider exploitation of microbial glycolipids is their low yield and high production cost, especially for pharmaceutical applications, where high purity is required [76]. In this context, cyanobacteria represent an under looked alternative, as glycolipids represent approximately 90 mol% of total lipids in the thylakoid membranes [77]. According to our results, *N. muscorum* is a promising candidate for glycolipids production.

Metabolites related to energy production (*i.e.*, UXP and AXP) and the pigment chlorophyll *a* tend to be less produced throughout time. This suggests growth deceleration, which is not consistent with the optical density results showing constant biomass increase, although with high variability during the last period. However, it is important to recognize that spectrophotometry is blind to whether the cells are alive or dead, and that the increased turbidity caused by high cell concentration, as well as cell morphology (*i.e.*, presence of filaments), can interfere with these measurements. The decrease in the content of chlorophyll *a* may have been caused by deficiencies in nutrients which are modulators of photosynthetic activity. For instance, iron and molybdenum play a major role in cyanobacterial growth and physiology, being involved in photosynthesis and in nitrogen fixation [78]. The biosynthesis of chlorophyll and phycobilin is also iron-dependent, although neither contains iron [79]. Iron limitation causes a reduction in the synthesis of pigments, hence possibly explaining the lower levels of chlorophyll *a* found after the 43th day. Photosynthesis is not the single process affected by limitations in specific nutrients. The metabolism involved in nitrogen fixation also requires the synthesis of iron and molybdenum subunits. Iron limitation decreases the levels of nitrite reductase [80], which is essential for the reduction of nitrate from di-nitrogen fixation or from an external source. In the case of diazotrophic cyanobacteria, as *N. muscorum*, the iron demand is higher due to the abundance of iron-containing enzymes necessary to nitrogen-fixation [78]. Molybdenum is also involved in nitrogen metabolism, working as a cofactor in nitrate reductase and nitrogenase [81]. Therefore, its scarcity leads to lower nitrogen fixation rates [81], to the increase in number of heterocysts,

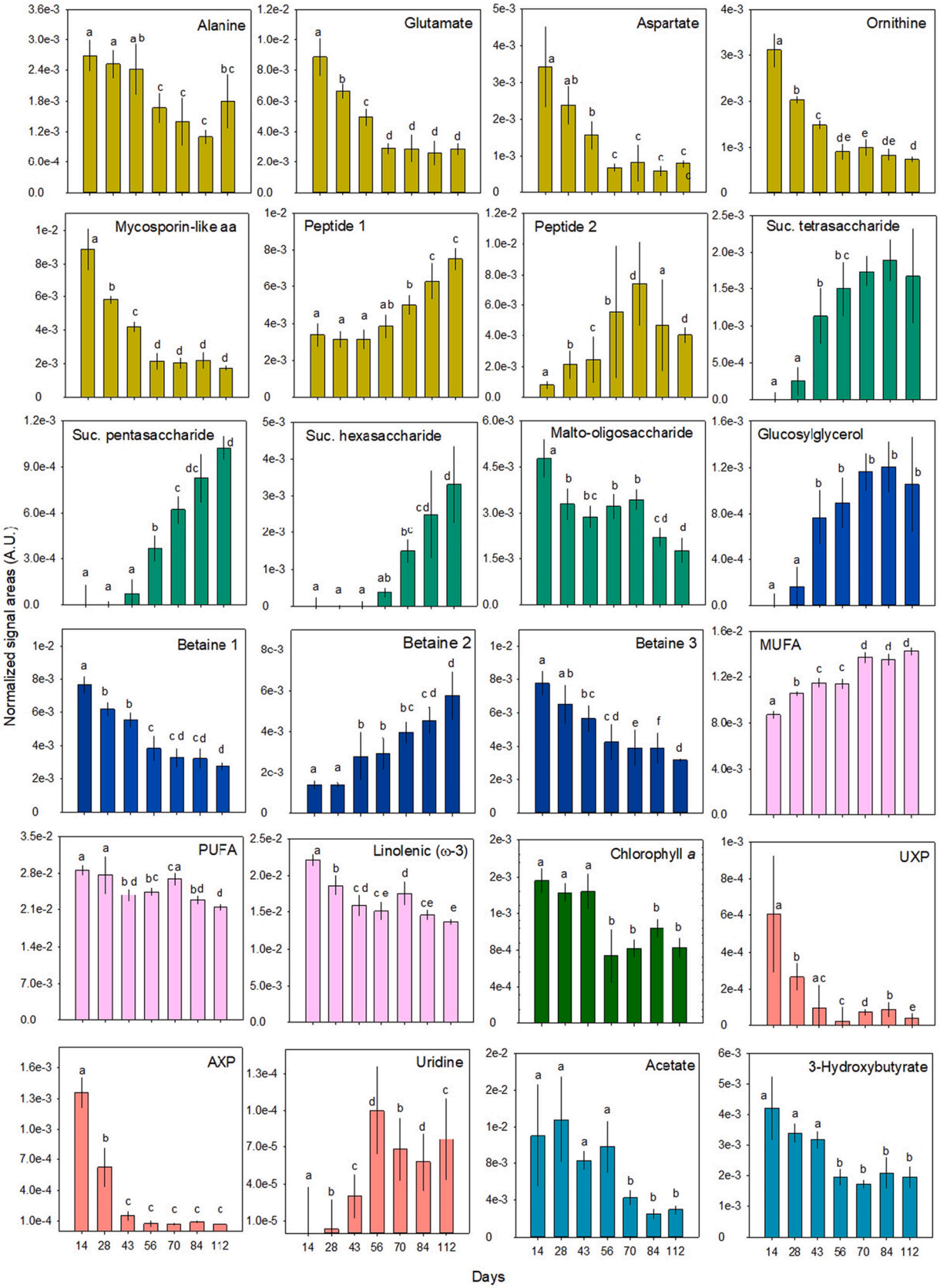

Fig. 6. Relative levels of compounds with VIP ≥ 1 and significant correlation with time, at different culture time points. Results are expressed as the mean (bars) $\pm$ standard deviation (error bars) of six replicates. Different letters indicate statistically significant differences among sampling time-points (Tukey test; $p < 0.05$ following one-way ANOVA - statistical summary in supplemental Table S2). Graphic colors represent different classes of metabolites: olive green for amino acids and peptides, green for carbohydrates, dark blue for compatible solutes, purple for lipids, dark green for chlorophyll *a*, light red for nucleotides/nucleosides, and light blue for others. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and to a decrease in pigment content [82].

Pigments like carotenoids and phycobilins are among the compounds currently extracted from cyanobacteria with the highest commercial value [83]. These natural antioxidants protect the organisms from reactive oxygen species (ROS) produced during photosynthesis and respiration. Common carotenoids in cyanobacteria include β-carotene, myxoxanthophyl, nostoxanthin, caloxanthin, zeaxanthin, canthaxanthin, and echinenone [84,85]. The examined *Nostoc* culture produced two detectable carotenoids, one of which (carotenoid 1) likely being β-carotene. Carotenoids cannot be synthesized by humans, who have to acquire them through diet or direct absorption, hence these pigments have great biotechnological relevance in the food, nutraceutical and cosmetic industries [86]. Although most carotenoids with cosmetic and/or pharmaceutical applications are produced synthetically [83], the increasing consumers' demand for natural products raises the relative value of natural carotenoids [86]. Their market size reached more than USD 200 million in 2015 and is likely to exceed USD 300 million by 2024 (https://www.gminsights.com, assessed May 2021). Among the available carotenoids, β-carotene, lycopene, astaxanthin, zeaxanthin, and lutein are the most demanded in this context [87].

5. Conclusion

N. muscorum was shown to be a very interesting cyanobacteria species, worthy of being further explored. It was able to grow for 112 days, without medium replacement or supplementation, suggesting low nutritional requirements, which has a positive implication for its industrial exploitation and long-term maintenance. NMR successfully monitored 36 compounds (or compound groups), accumulated in the cyanobacterium over an extended growth period. Several compounds with interesting bioactivities and potential biotechnological applications were identified, including mycosporin-like amino acids, carotenoids, sugars, and important lipids (*e.g.*, ω-3 and ω-6 fatty acids and glycolipids). A detailed analysis of the metabolic profile throughout the growth period allowed the patterns of production of these compounds to be characterized. Several metabolites showed higher production during earlier stages of the growth period (up to 43 days), including maltooligosaccharides, some amino acids, polyunsaturated fatty acids (namely linolenic acid), glycolipids, nucleotides and chlorophyll *a*. Cells from later time points (from day 56 onwards) were relatively richer in sucrose and sucrose-derived oligosaccharides, glucosylglycerol, peptides, fatty acids (including linoleic acid), and nucleosides. The production of compounds like carotenoids and trehalose did not follow clear time-dependent patterns. The metabolic profile changed through the growth period, and this knowledge is critical to define the most appropriate harvesting moments, thus allowing the biotechnological exploitation of different molecules of interest to be optimized. This study demonstrates that understanding the metabolic dynamics of cultures to optimize culturing conditions and harvesting schedules is an efficient path towards sustainable exploitation protocols.

CRediT authorship contribution statement

Macário, I.P.E.: Conceptualization, Methodology, Data treatment, Writing; **Veloso, T.**: Methodology; Data treatment; **Romão, J.**: Data treatment; **Gonçalves, F.J.M.**: Supervision, Writing - review and editing; **Ventura, S.P.M.**: Conceptualization, Writing - review and editing, Supervision; **Pereira, J.L.**: Conceptualization, Writing - review and editing, Supervision; **Duarte, I.F.**: Conceptualization, Methodology, Data treatment, Writing - review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102792.

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