# Metabolic Modeling of *Arthrospira* sp. PCC 8005 - Network Definition and Experimental Validation

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**Abstract:** Metabolic modeling is a valuable tool for studying microbial metabolism and has broad applications across fields like biotechnology, medicine, and environmental science. The construction of metabolic networks is crucial in this process, though their development presents significant challenges. While genome-scale networks offer detailed insights, they are computationally demanding, and smaller networks are often too simplified. This study discusses a methodology to derive a metabolic network of intermediate size by combining biological knowledge, experimental data, and mathematical tools to refine the network definition. The present study focuses on the modeling of photosynthetic cyanobacteria *Arthrospira* sp. PCC 8005 and experimental validation is achieved using cultures in continuous mode. The procedure is effective, yielding promising results, and metabolic analyses show predictive capabilities that are in agreement with existing studies while studying the impact of different nitrogen sources on the growth of cyanobacteria.

*Keywords:* Metabolic modeling, metabolic flux analysis, metabolic network, photosynthetic organisms, nitrogen metabolism, cyanobacteria

## 1. INTRODUCTION

Photosynthetic cells are important both ecologically and economically and play key roles in energy production, oxygen generation, carbon cycling, and supporting biodiversity. In particular, cyanobacteria such as *Arthrospira platensis* have significant biotechnological value due to their high nutritional value (rich in proteins, vitamins, minerals, and essential fatty acids) and their ability to assimilate various nitrogen sources, to fix carbon dioxide and produce oxygen. With such impressive features, these cyanobacteria appear to be the best nutritional resource for long-haul space exploration missions. In that context, the European Space Agency has developed the concept of a self-sufficient artificial ecosystem, known as the MELiSSA loop, with the goal of producing part of food and oxygen while contributing to water and waste recycling in harsh environments such as space conditions (Hendrickx et al., 2006). To fully harness the biotechnological potential of these organisms, a deeper understanding of their metabolic capabilities is essential.

Among the realm of possible methods to study microbial metabolism, metabolic modeling is a powerful tool to study the flow of metabolites through different biochemical pathways, identify metabolic bottlenecks, and predict responses to genetic modification or environmental changes (Stephanopoulos et al., 1998). In the case of *A. platensis*, which has a complex metabolism involving both photosynthetic and heterotrophic processes, metabolic modeling can provide insight into optimizing its growth conditions, enhancing biomass production, or maximizing metabolite yields. The latter are important metabolic objectives, especially for bioregenerative life-support systems.

However, the construction of a comprehensive metabolic network is a complex task that requires integrating genomic, proteomic, and metabolomic data. On the one hand, genome-scale metabolic reconstructions (GEMs) are widely used despite multiple challenges (Covert et al., 2001; Price et al., 2003; Reed and Palsson, 2003). As a matter of fact, the completeness and accuracy of genome annotations can vary, leading to gaps in metabolic pathways that need to be filled using computational tools or experimental evidence. Also, to ensure the consistency and robustness of such networks, the use of methods based on convex analyses is required (Schuster and Hilgetag, 1994; Schilling et al., 2000; Klamt et al., 2003; Orth et al., 2010) and even though the latter methods are effective, they can become computationally costly with the size of the network. On the other hand, smaller metabolic networks have been deduced to facilitate the analyses and the application of control strategies (Provost and Bastin, 2004; Zamorano et al., 2010; Fernandes de Sousa et al., 2016). Still, they are simplified and omit parts of the metabolic interactions. In any case, organisms like *A. platensis* have unique metabolic traits, such as specialized pathways for photosynthesis and nitrogen fixation, which may not be well-documented in existing databases. Furthermore, the stoichiometry and thermodynamics of biochemical reactions must be carefully curated to avoid inconsistencies in the network. These challenges underscore the importance of

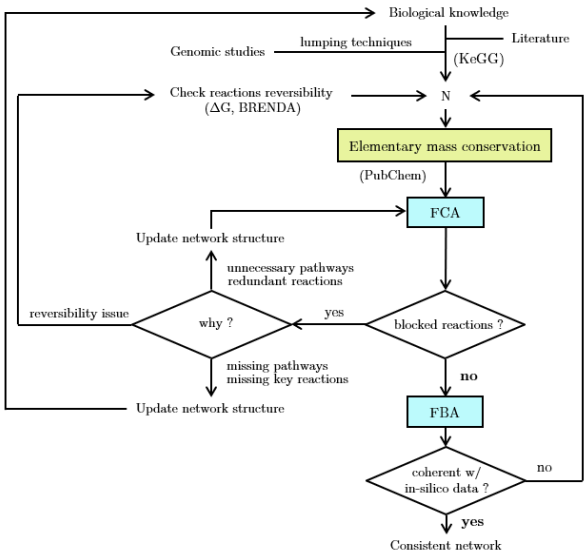


Fig. 1. Methodology and algorithmic scheme of the mathematical methods to get mid-size metabolic networks

systematically refining and validating the metabolic model to ensure it accurately reflects the organism's metabolic potential. In the case of *A. platensis*, experimental validation is particularly valuable given the organism's metabolic flexibility and its ability to adapt to varying environmental conditions, such as light intensity and nutrient availability. For this purpose, this paper discusses a procedure to derive a mid-size metabolic network structure and refine the network definition using experimental data. The modeling methodology is iterative and combines biological knowledge and mathematical methods, leading to a metabolic network comprised of a couple hundred reactions, which is detailed enough to capture the complexity of the cellular metabolism yet still simple to analyze and use at the process level. Experimental validation is then achieved considering the cultivation of *Arthrospira* sp. PCC 8005 in continuous mode and techniques of metabolic flux analysis (MFA).

The paper is organized as follows. Sec. 2 describes the modeling procedure and focuses on the modeling of *Arthrospira* sp. PCC 8005. Sec. 3 covers the experimental validation of the metabolic network to obtain a robust model, discusses the results, and particularly the impact of the different nitrogen sources on the growth of the organisms. Finally, conclusions are drawn in Sec. 4.

## 2. MODELING PROCEDURE

The modeling procedure is depicted in Fig. 1 but is not the main focus of this article. The main steps of the algorithmic scheme are outlined for the sake of clarity and are applied to the modeling of prokaryotic microorganisms. The original aspect of the procedure lies in the use of mathematical methods sequentially and iteratively while ensuring the consistency of the network.

First, a preliminary set of metabolic reactions is selected (bottom-up approach). The core metabolic pathways are common to most microorganisms and can be found in literature and biochemistry textbooks (Nelson and Cox, 2008). At this stage, genomic studies can be useful in identifying specific metabolic functions and deriving the corresponding intracellular reactions. To simplify the network and reduce its dimensionality, lumping techniques should be employed. In this case, lumping refers to the process of grouping similar chemical species or reactions into a single entry to facilitate the mathematical modeling (Martinez, 1990).

Additionally, it is essential to assess the reversibility of chemical reactions to ensure the network's consistency. Determining whether a reaction is reversible involves several factors, such as thermodynamics, kinetics, and physiological context. In this study, the standard free energy change $\Delta$G is used as a criterion, as well as enzyme regulation, i.e., the enzyme's capability to catalyze a reaction in both directions depending on cellular conditions.

Mathematically, the metabolic reactions are organized into a matrix representation $N$, which defines the stoichiometric matrix of the network. It is a $m \times n$ matrix, where $m$ is the number of metabolites, $n$ is the number of chemical reactions, and the entry $N_{ij}$ reflects the stoichiometry of the metabolite $i$ involved in the $j^{th}$ reaction. From this matrix representation, a series of mathematical methods can be applied.

For network consistency, a test ensuring elementary mass conservation is required. It consists of verifying that each reaction in the network balances the atoms of each chemical element between reactants and products. It is performed by analyzing the stoichiometric matrix to confirm that the number of atoms (especially of carbon, nitrogen, and phosphorus) remains consistent across all reactions. It is important to note that an average atomic composition is considered for the mass balance of biomass and macromolecules. Then, constraint-based methods are exploited to limit the possible solutions and explore the solution space. The basis of such methods lies in the addition of constraints that govern the operation of the network in a steady state. By applying the pseudo-steady state assumption on the general equation of internal metabolite dynamics and considering that network fluxes $\underline{v}$ are subject to positivity constraints, the following convex analysis problem is obtained:

$$\{N\underline{v} = 0 \ ; \ \underline{v} \geq 0\} \tag{1}$$

However, most large-scale metabolic networks are underdetermined, i.e., there are more biological reactions than metabolites, and no unique solution exists. In that context, additional constraints can be applied to reduce the solution space further. For instance, flux coupling analysis (FCA) is a framework particularly useful for studying the topological and flux connectivity features of large networks. This method involves solving a series of linear programming (LP) problems and helps to reduce the network's dimensionality by identifying pairs of metabolic fluxes. As introduced in (Burgard et al., 2004), three types of couplings are commonly defined: directional, partial, and full couplings. Pairs not belonging to one of these categories are considered uncoupled, which defines blocked reactions, i.e., reactions that cannot carry a flux under steady-state conditions for a given uptake scenario. In this study, finding blocked reactions helps to identify incomplete pathways, highlight errors or omissions in metabolic reconstructions, and potentially reveal issues regarding reactions reversibility. Mathematically, it consists of solving the following LP problem once for every flux:

$$maximize \quad v_j \tag{2}$$

$$subject\ to \quad \sum_{j=1}^{N} N_{ij} v_j = 0,\ \forall i \in M \tag{3}$$

$$v_j^{uptake} \leq v_j^{uptake^{max}},\ \forall j \in N_{transport} \tag{4}$$

$$v_j \geq 0,\ \forall j \in N \tag{5}$$

In this formulation, reversible reactions are represented as two irreversible reactions in opposite directions, meaning that if the maximum flux value is zero, then the reaction is unusable or blocked, given the uptake-secretion scenario. Similarly, flux balance analysis (FBA) is another popular constraint-based method. This approach assumes optimal cell behavior and involves calculating an optimal flux distribution $\underline{v}$ that either maximizes or minimizes a specific objective function $Z = \underline{c}^T \underline{v}$ such that:

$$\underline{v}^{opt} = \max_{\underline{v}} \left( Z \right) \ s.t.\ \{ N\underline{v} = 0\ ;\ \underline{v} \geq 0\ ;\ \underline{v}_l \leq \underline{v} \leq \underline{v}_u \} \tag{6}$$

where $\underline{c}$ represents weights that quantify the contribution of each reaction to the objective function, while $\underline{v}_l$ and $\underline{v}_u$ are vectors defining the lower and upper bounds.

Using in-silico data to quantify intracellular mechanisms and applying the iterative modeling procedure depicted in Fig. 1 lead to a metabolic network composed of 171 metabolites and 198 reactions, including energy aspects such as the proton motive force (PMF) barely outlined in existing studies. Details are indicated in Table 1.

Table 1. Information related to the metabolic network using the iterative procedure

| iter. | # met. | # rnx | # blocked rnx |
|---|---|---|---|
| 1 | 157 | 167 | 39 |
| 2 | 170 | 191 | 16 |
| 3 | 161 | 182 | 7 |
| 4 | 161 | 179 | 7 |
| 5 | 154 | 172 | 0 |
| 6 | 155 | 182 | 0 |
| 7 | 171 | 198 | 0 |

## 3. EXPERIMENTAL VALIDATION

This section is devoted to the experimental validation of the metabolic network established in Sec. 2. Data were provided by the Department of Proteomics and Microbiology of the University of Mons. Information relative to the cell line, the media, the bioreactor mode operation and the analysis methods can be found in (Deschoenmaeker et al., 2017). Methods for experimental validation are shown in Fig. 2.

### *3.1 Experimental Conditions and Data Processing*

Photo-bioreactor (PBR) started under batch mode and continuous feeding started after 7 days. For the continuous PBR experiments, cyanobacteria were incubated in a radially illuminated 2L cylindrical double jacket reactor under a constant light power density of 125 $W.m^{-2}$ with the agitation settled at 150 rpm. The pH was automatically maintained at 8.5 with HCl (0.5 M) whereas the dilution rate was kept around 0.2 per day with fresh Zarrouk medium

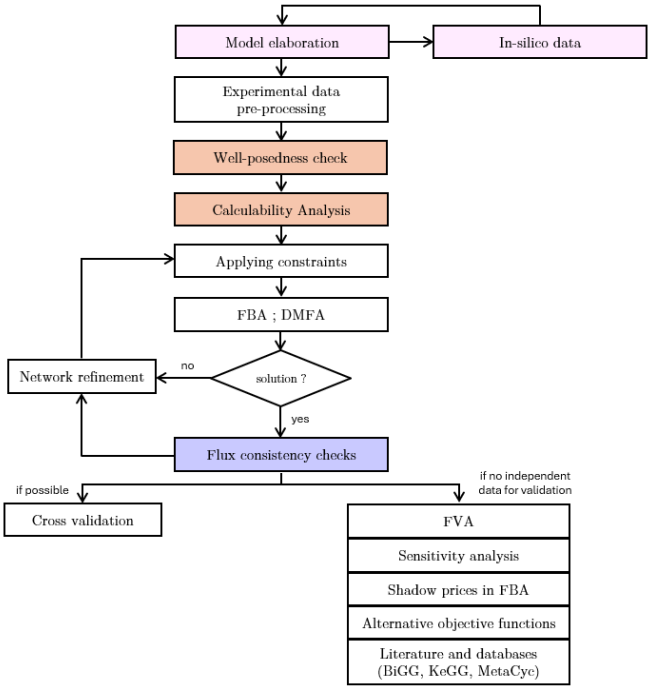


Fig. 2. Scheme of methods for experimental validation

(30mM-N $NaNO_3$ or a mixture of nitrogen sources). Experimental data include biomass productivity and oxygen productivity (in $g.L^{-1}.d^{-1}$) ; residual nitrogen concentration (i.e., $NO_2^-$, $NO_3^-$, $NH_4^+$ and urea in mM) ; pigments concentration (i.e., $chlorophyll_a$ in $g.L^{-1}$) ; TC, TOC, TIC and TN in biomass and supernatant (in $mg.L^{-1}$) and protein, lipid, and carbohydrate content in biomass. $O_2$ is monitored in exhaust gas (purging with air to prevent an increase in the oxygen saturation of the medium).

In metabolic flux analyses conducted in Sec. 3, the light intensity is expressed in a number of moles of photons per square meter per second (or Einstein per square meter per second: $E.m^{-2}.s^{-1}$) by assuming an average wavelength $\lambda = 600\ n$m, expressing the energy of a single photon with Planck's equation and using Avogadro's number. Experimental uptake and secretion rates are expressed in $mmol.g_{DW}^{-1}.h^{-1}$ using smoothing splines and differentiation methods. Biomass concentration X (DCW, $g.L^{-1}$) is retrieved from the biomass productivity and oxygen concentration is computed by numerical integration when the oxygen productivity is known at different time instants (cumulative integration).

### *3.2 Mathematical Analysis*

Because the network identified in Sec. 2 is highly underdetermined and the number of experimental measurements is limited, a network reduction is suggested using FCA. As noticed in (Burgard et al., 2004), flux coupling analysis is also particularly useful to simplify over-detailed networks. Doing so, the metabolic network is reduced to 175 biochemical reactions and 152 metabolites by simplifying the metabolism of tetrahydrofolate and the cofactors' metabolism.

Before proceeding to metabolic flux analyses, mathematical tools turn out to be interesting for assessing the consistency of the network. In this regard, ensuring the formulation of a well-posed problem is essential. Specifically, it involves determining whether certain pathways within the metabolic network connect non-measured inputs to non-measured outputs. If such pathways exist, they can lead to an unbounded set of solutions for the corresponding reaction rates, making the system's behavior difficult to interpret and predict. In that respect, taking into account experimental measurements, Eq. (1) becomes:

$$\begin{pmatrix} N & 0 \\ N_m & -\underline{\nu}_m \end{pmatrix} \cdot \begin{pmatrix} \underline{v} \\ 1 \end{pmatrix} = 0 \tag{7}$$

where $\underline{\nu}_m$ is the vector of specific uptake and excretion rates of the measured external species, and $N_m$ is the stoichiometric matrix of extracellular measurements. Therefore, checking if the system is well-posed is equivalent to computing the elementary flux modes collected in a matrix $E$ of the matrix in Eq. (7). The system is said to be well-posed if there is no null column in E. In this case, after using METATOOL (Pfeiffer et al., 1999), a matrix containing 303583 EFMs is obtained. After analysis, no null column is identified, meaning the problem is well-posed.
Furthermore, calculability/observability analysis is also relevant to examine underdetermined networks, as discussed in (Klamt et al., 2002), and consists of identifying fluxes that can be uniquely determined. In this context, it enables an improved interpretation of flux distributions, avoiding misleading conclusions from non-unique solutions, but it can also guide experimental efforts if additional experimental measurements are needed. After computation, it was determined that only 32 fluxes are calculable when the growth rate is considered a well-known flux.

### *3.3 Direct Validation*

This study focuses on the quasi-steady state under constant light regimes, using the full spectrum of metabolic analysis tools. Several tests have been made to validate the network on the basis of the iterative procedure suggested in Fig. 2. However, inconsistencies have been observed after a detailed analysis of the intracellular flux values. An example is related to the chemical reactions associated with nitrogen assimilation, where the reaction rates adjust to maintain a balance between $Fe_{red}$ and $Fe_{ox}$, making it impossible to impose experimental constraints on more than one nitrogen source. To solve the problem, one approach is to disregard $Fe$, i.e., iron-sulfur proteins that function as electron carriers, in the network definition or to add the corresponding transport reactions. This inconsistency issue highlights (i) the importance of validating the network with experimental data in addition to in-silico validation, (ii) the necessity of performing flux consistency checks, and (iii) the necessity of an iterative strategy both for network definition and experimental validation.

Experimental validation of a metabolic network is quite challenging because all available data (uptake and secretion rates, metabolite concentrations, and flux measurements if applicable) are used to constrain the system. Nevertheless, several strategies can be applied to evaluate

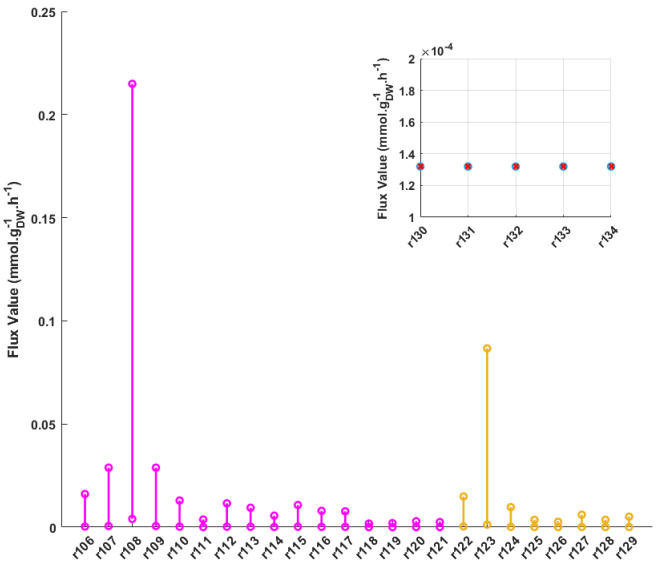


Fig. 3. Results of flux variability analysis

the validity and reliability of the model, as depicted in Fig. 2. For direct validation, it is proposed to perform flux variability analyses (FVA), flux consistency checks via the analysis of a flux map and to compare the outcomes with published data from literature and databases. Initially, a series of fundamental tests is conducted to verify the coherence of the network. For instance, flux variability analysis is achieved by imposing only experimental uptake rates and ensuring experimental secretion rates belong to the predicted intervals. Also, FBA with biomass optimization is performed by imposing both experimental uptake and secretion rates and ensuring that the predicted optimal value for the growth rate is higher than the measured growth rate. Doing these analyses is a prudent approach to enhance confidence in analyses and verify the model accurately reflects the organism's growth potential and efficiency. Subsequently, all experimental constraints are applied, and FVA is conducted to explore the range of possible fluxes through each reaction within the network. Fig. 3 displays the outcomes of FVA performed at a specific time instant for the 30mM-N nitrate feeding case. Reactions $r_{106}$ to $r_{121}$ are related to the synthesis of lipid, reactions $r_{122}$ to $r_{129}$ are related to the synthesis of carbohydrates and reactions $r_{130}$ to $r_{134}$ are related to the biosynthesis of chlorophyll. Narrow intervals indicate that the predictions are more reliable, whereas broader intervals suggest that the predictions are less significant, indicating that the model could benefit from additional constraints. Furthermore, when the interval narrows to a single value, it indicates that the corresponding flux is calculable. These results are compared with an analysis conducted on a genome-scale network of 875 reactions in (Klanchui et al., 2012) and roughly similar observations are noticed, i.e., larger intervals for reactions related to the central carbon metabolism and smaller intervals for anabolic pathways.

FBA is also conducted to analyze the flux distribution of the model. It is performed by imposing the experimental uptake and secretion rates and the growth rate while maximizing ATP production. After analysis of the flux map, the results are in agreement with existing studies (Baroukh et al., 2015). As depicted in Fig. 4, the regime

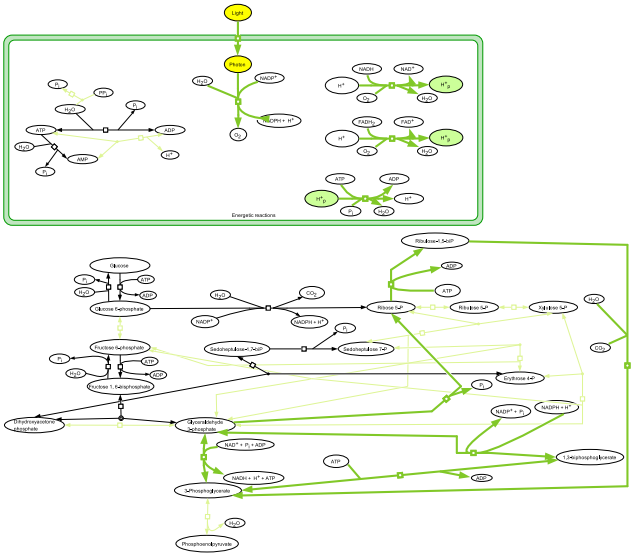

Fig. 4. Part of the flux map at a specific time instant for the 30mM-N nitrate feeding case

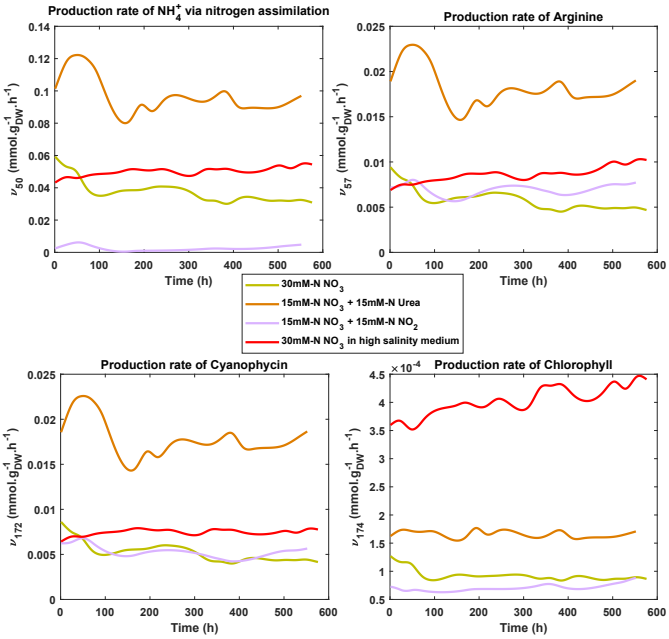


Fig. 5. Time evolution of some intracellular fluxes for different feeding strategies

is characterized by high fluxes in the photosynthetic pathways and the activation of the Calvin-Benson cycle. Upper glycolysis operates in the glyconeogenic direction to produce carbohydrates and sugar precursors metabolites (PEP, G6P and R5P), essential for growth. The pentose phosphate pathway (PPP) is in the reductive mode and ATP synthesis is driven by the PMF and is synthesized via photophosphorylation and oxidative phosphorylation (in addition to substrate-level phosphorylation). Also, it is interesting to note that glyceraldehyde-3-phosphate (G3P) is mainly produced via the Calvin-Benson cycle. The reductive PPP contributes a little, but glycolysis (i.e., the breakdown of fructose-1,6-bisphosphate) is not a major pathway for G3P production, particularly when the organism is in a photosynthetic state. Besides, the analysis of the whole map shows the activation of the citric acid cycle and the GS-GOGAT pathway and the urea cycle are involved for nitrogen assimilation.

Another objective of this study is to assess the impact of different nitrogen sources on the metabolism of *Arthrospira* sp. For this purpose, FBA are performed using experimental data for different feeding strategies (30mM-N nitrate ; 15mM-N nitrate + 15mM-N nitrite ; 15mM-N nitrate + 15mM-N urea ; 30mM-N nitrate in high salinity medium). The results of these analyses will also allow the validation of the network structure by comparing the predicted intracellular fluxes with known biological principles. The time evolution of some intracellular fluxes is shown in Fig. 5.
First, it is noticed that the production rates of cyanophycin and arginine are greater when urea is the nitrogen source because of the faster production of ammonia and then the rapid synthesis of glutamine and glutamate. The production rate of chlorophyll is also larger because urea can be hydrolyzed into $CO_2$ thus increasing the photosynthetic activity. In contrast, when nitrate or nitrite are the main nitrogen sources, more ATP and reducing power are needed for nitrogen assimilation, potentially reducing growth efficiency in certain conditions. Besides, cultures grown on nitrate or nitrite may produce lower pigment levels than urea-fed cultures, as more energy is diverted towards nitrogen reduction. A deeper analysis of the intracellular fluxes shows that it increases the metabolic burden on glycolysis, the acid citric cycle and the PPP, as these pathways provide ATP, NADH and NADPH necessary for nitrogen assimilation. These results agree with the study led by (Deschoenmaeker et al., 2017) on the same strain. Under high salinity, a larger demand for ATP and NADPH is required to maintain osmotic balance and repair oxidative damage. An increased production of ammonia is noticed, increasing the activity of ornithine cycle (impacting the production rate of arginine) as a way to manage the nitrogen overload that can occur when the GS-GOGAT pathway efficiency is reduced. Furthermore, to protect cellular structures from osmotic stress, the cell can increase the synthesis of glycine and proline that act as osmoprotectant solutes in high-salinity conditions. Therefore, the predicted fluxes are biologically coherent and align with established biological knowledge, definitely validating the metabolic network.

### *3.4 Cross-Validation*

Cross-validation is quite complex when applied to metabolic networks because all available data are typically used to constrain the model. Most cross-validation strategies in MFA focus on comparing predicted fluxes with experimental fluxes when $^{13}$C-labeled flux data are available as a ground truth. Such data are not available for the present study. However, three experiments have been conducted using the same feeding strategy, i.e., 30mM-N nitrate feeding. Consequently, this study suggests performing a form of cross-validation by computing mean exchange fluxes from two datasets (training phase) to predict intracellular fluxes via FBA, which will be then compared against the results from the third dataset (test phase) using flux similarity metrics. The idea is to test how well the general trends in flux distributions generalize when faced to a completely new set of constraints. Results are summarized in Table

2 for all folds, and relative flux error (RFE) is used as a metric (see (Kim et al., 2016) for more information). It measures the relative difference between two flux distributions and is normalized such that it is comprised between 0 (i.e., perfect similarity) and 1 (i.e., complete dissimilarity). The proposed indicator is defined as follows:

$$RFE = \frac{1}{I}\sum_{i=1}^{I}\left(\frac{1}{J}\sum_{j=1}^{J}\frac{|f_{train,i,j} - f_{test,i,j}|}{max(|f_{train,i,j}|, |f_{test,i,j}|, \epsilon)}\right) \quad (8)$$

where $f_{train,i,j}$ and $f_{test,i,j}$ are the $i^{th}$ flux at time instant $j$ from the training set or the test set, respectively. $\epsilon$ is a small constant to avoid division by zero (typically, $\epsilon = 1e^{-6}$). Therefore, the analysis of the RFE shows a high similarity between fluxes, highlighting a robust network.

Table 2. Relative flux error for all folds

| | FOLD (1-2) VS 3 | FOLD (1-3) VS 2 | FOLD (2-3) VS 1 |
|---|---|---|---|
| RFE | 0.0518 | 0.0538 | 0.0581 |

## 4. CONCLUSION

This work validates the definition of a mid-size metabolic network for *Arthrospira* sp. PCC 8005 using experimental data and two iterative strategies. First, a modeling procedure that combines biological knowledge and a series of constraint-based methods is applied to derive a first draft of the network consistent with in-silico data. Then, experimental validation is achieved with a refinement of the metabolic network if needed. The results are promising, and metabolic analyses show predictive capabilities that concord with existing studies. Furthermore, the impact of different nitrogen sources on the growth of cyanobacteria has been addressed, highlighting that urea is, overall, a better nitrogen source compared to nitrate and nitrite and that ammonia shows a repressive effect on the nitrate-assimilation pathway.

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