

CyanoFactory - Executative summary

CyanoFactory - Design, construction and demonstration of solar biofuel production using novel (photo)synthetic cell factories (www.cyanofactory.eu) – brought together together ten leading European partners (six Universities, two research organisations and two SMEs) to address the need for significant advances in both new science and technologies to convert solar energy into a solar fuel. Custom designed photosynthetic microbial hydrogen producing cells (chosen as one example of a future solar fuel) was used as a model system to advance genetic engineering tools and techniques - Synthetic biology – in combination with novel and unique devices and systems to generate a future energy carrier - Advanced photobioreactors. The clear **aims** of CyanoFactory were further European developments of the important, new and emerging technologies **Synthetic biology** and **Advanced photobioreactors**.

The **scientific progress** of CyanoFactory involved the: Development of a ToolBox for cyanobacterial synthetic biology, Built up of a DataWarehouse/Bioinformatics web-based capacities and functions, Improvement of chassis growth, functionality and robustness, Introduction of custom designed hydrogen producing units, Improvement of photosynthetic efficiency towards hydrogen production, Analyses of the designed cyanobacterial cells to identify bottlenecks with suggestion on further improvements, Metabolic modelling of the engineered cells, Development of an efficient laboratory scale photobioreactor unit, and Assembly and experimental performance assessment during two seasons of a larger (1350 liter) flat panel photobioreactor system outdoors. For synthetic biology to be successful and accepted in the society, the engineered cyanobacterial cells should be controllable and able to be switched off, thus the project addressed biosafety design mechanisms (Biosafety). Obtained results were published in internationally recognised journals. Efficient Management and active Dissemination made sure that progress and results of CyanoFactory were assessed regularly, and that the achievements within the project were an are visible in the European community and internationally.

The project partners **trained many students and young researchers** (at Master, PhD and PostDoc levels) in this important R&D field of microbial solar fuel production, with a clear focus on innovative synthetic biology and advanced photobioreactors as well as how to handle, store and analyse larger data sets. In addition, CyanoFactory brought significant scientific training to all partners localised throughout Europe. Two dedicated **workshops** with invited internationally leading scientist covering the R&D field of CyanoFactory further positioned CyanoFactory internationally as well as were excellent opportunities for students and young researchers to follow and to in be involved in the development of Synthetic biology and Advanced photobioreactors. The two SMEs participating in CyanoFactory developed links with all partners in the project. Two publically available **Web-aggregates** were set up and maintained regularly, covering Algal biofuels and Synthetic biology, respectively. There was considerable interest in Synthetic biology, with close to 5,000 views and 2,000 unique visitors.

A **provisional patent application** was submitted covering construction and method to target molecule in a cyanobacterium. From **societal perspective** was "Making-of Synthetic Biology: The European CyanoFactory Research Consortium" published as a book chapter in "Ambivalences of Creating Life: Societal and Philosophical Dimensions of Synthetic Biology", a contribution towards the emerging discussion about Synthetic biology and how it will change our societies.

CyanoFactory – Description of the project context and main objectives

CyanoFactory "Design, construction and demonstration of solar biofuel production using novel (photo)synthetic cell factories" was developed in direct response to the FP7-ENERGY-2012-1 call, topic ENERGY 2012.10.2.1. "Future Emerging Technologies" and the need for significant advances in both new science and technologies to convert solar energy into a solar fuel. Custom designed photosynthetic microbial H₂ producing cells is being constructed using groundbreaking, and still under development, standardised genetic engineering tools and techniques - Synthetic biology - in combination with novel and unique devices and systems to generate a future energy carrier - Advanced photobioreactors.

CyanoFactory is an example of "purpose driven" R&D with identified scientific goals which also creates a new basic technology. This emerging field - Custom design and purpose construction of microbial cells for the production of desired products using synthetic biology – goes well beyond conventional paths, is highly innovative, very ambitious, and has the potential for really high impact.

CyanoFactory brought together ten leading European partners (six Universities, two research organisations and two SMEs) with a common goal – To develop the future technologies Synthetic biology and Advanced photobioreactors. The scientific content is organised into ten workpackages.

List of partners

- 1 - Peter Lindblad, Coordinator, Uppsala University (UU) Sweden, SE, Higher education
- 2 - Röbbé Wünschiers University of Applied Sciences, Mittweida (UM) Germany, DE, Higher education
- 3 - Paula Tamagnini Instituto de Biologia Molecular e Celular (IBMC), Portugal, PT, Research organisation
- 4 - Matthias Rögner Ruhr-University Bochum (RUB), Germany, DE, Higher education
- 5 - Marko Dolinar University of Ljubljana (UL) ,Slovenia, SI, Higher education
- 6 - Phillip C. Wright University of Sheffield (USFD), United Kingdom, UK, Higher education
- 7 - Javier Urchueguia Universidad Politécnica de Valencia (UPVLC), Spain, SP, Higher education
- 8 - Hans-Jürgen Schmitz KSD Innovation GmbH (KSD) Germany, DE, SME
- 9 - Giuseppe Torzillo CNR-ISE (CNR-ISE) Italy, IT, Research Institute
- 10 - Marcello M. Diano M2M Engineering sas (M2M) Italy, IT, SME

The scientific content of CyanoFactory was organised into 10 Workpackages (respective lead partner indicated):

- WP1 ToolBox for cyanobacterial synthetic biology (UU)
- WP2 DataWarehouse/Bioinformatics (UM)
- WP3 Improvement of chassis growth, functionality and robustness (IBMC)
- WP4 Introduction of custom designed hydrogen producing units (UU)
- WP5 Improvement of photosynthetic efficiency towards H₂-production (RUB)
- WP6 Biosafety (UL)
- WP7 Analyses of the purpose designed cyanobacterial cells to identify bottlenecks and suggest further improvements (USFD)

WP8 Metabolic modelling of the engineered cells (UPVLC)

WP9 Development of an efficient photobioreactor unit (KSD)

WP10 Assembly and performance assessment of a larger prototype photobioreactor System (CNR-ISE)

Short description of aims within each workpackage

WP1 aimed to develop a repository of standard genetic parts for the use in cyanobacterial synthetic biology – A dedicated Cyanobacterial Toolbox. Specifically there are needs to develop techniques to introduce genetic circuits, and to develop regulatory elements for protein expression (promoters, RBS, terminators, inducible systems etc).

WP2. The different WPs of CyanoFactory have generated and required different types of data, i.e. mainly annotated sequence, physiological performance data and different –omics data. These data are the basis for the generation of custom designed vectors, regulatory elements and protein coding sequences for the development and improvement of synthetic biology tool, parts and modules, the chassis and bioreactors – all stored and processed in a Data Warehouse developed in WP2 and made available via CyanoWeb and CyanoDesign.

WP3 aimed to address robust cyanobacterial chassis, a cell suitable for the production of a selected solar fuel/product e.g. hydrogen; therefore constituting a “cell factory” for efficient and safe, engineering biotechnological applications. The construction of a robust photoautotrophic chassis able to function under different environmental conditions is of fundamental importance for the application in larger scale photobioreactors.

WP4 aimed to use an artificial, synthetic biology approach to introduce a synthetic genetic construct(s) encoding purpose designed functional non-native hydrogenases into a cyanobacterial cell.

In **WP5** the cyanobacterial metabolism of electron acquisition and distribution were to be engineered to ensure an efficient flow towards production formation. Key players directly involved in this process are photosystem II, ferredoxin-NADP⁺ reductase, and ATPase.

WP6. For synthetic biology to be successful and accepted by society, the engineered cyanobacterial cells should be controllable and able to be switched off. Thus, the project addressed biosafety, the genetic design mechanisms controlling growth (Biosafety).

In **WP7**, wild type and engineered cyanobacterial cells were to be analyzed using different omic techniques in order to detail metabolism, identify bottlenecks for desired performance and suggest further improvements. By means of bioinformatics integration and mathematical modelling of available metabolomics, transcriptomic, proteomic and fluxomic data, **WP8** aimed to identify the experimental conditions for an increased production of hydrogen using a variety of mathematical modelling approaches.

In **WP9** addressed the need to develop a smaller in-door laboratory scale photobioreactor while in **WP10** a larger >1,000 liter outdoor flat panel photobioreactor system was to be designed, built and operated with cyanobacteria.

Organised dissemination (**WP 12**) were planned to include e.g. a publically available web-site (www.cyanofactory.eu) with two integrated publically available Web-aggregates

covering Algal biofuels and Synthetic biology, respectively, and two dedicated CyanoFactory workshops with two full days of CyanoFactory science by the partners followed by one day of presentations and discussions by invited internationally leading scientists in the field of CyanoFactory science (in addition to more regular CyanoFactory project meetings).

CyanoFactory

Design, construction and demonstration of solar biofuel production using novel (photo)synthetic cell factories

Grant agreement no: 308518

**Main Science & Technology
Results**

CyanoFactory - Main Science & Technology results

All partners were highly active in the project, all work-packages generated results and knowledge reaching preset milestones, significant progresses were made in Synthetic biology and Advanced photobioreactors further positioning Europe in these new and emerging technologies. Already existing interactions between partners were further developed and many new interactions and cooperations between two or more partners were initiated and established during the time of the project. However, as with all new and emerging technologies, some unexpected difficulties occurred, planned R&D did not function or was shown not be a preferred pathway forward. Unexpected findings include the realisation of the fundamental importance of the translation process, in addition to transcription, when expressing non-native proteins in cyanobacteria highlighting not only promoters and "classical" ribosome binding sites but also dual Shine-Delgarno motifs/Bicistronic design. On the unexpected positive side is the submitted provisional patent application covering construction and method to target molecule in a cyanobacterium. From societal perspective was "Making-of Synthetic Biology: The European CyanoFactory Research Consortium" published as a book chapter in "Ambivalences of Creating Life: Societal and Philosophical Dimensions of Synthetic Biology", a contribution towards the emerging discussion about Synthetic biology and how it will change our societies.

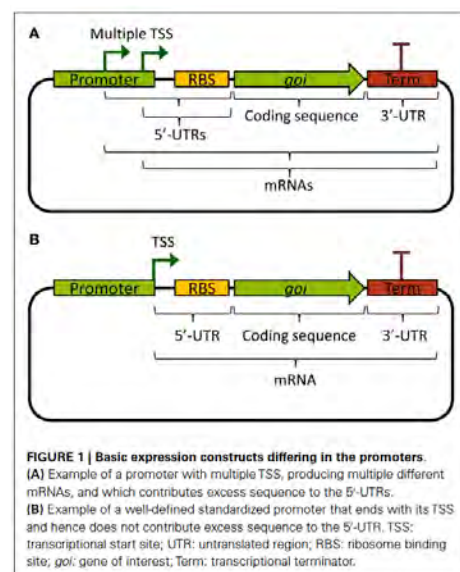
WP1 – Toolbox for cyanobacterial synthetic biology

The objectives when developing the toolbox for cyanobacterial synthetic biology were (1) Development of optimized techniques for the introduction of genetic circuits into cyanobacterial cells, (2) Development of genetic tools for the regulation of protein expression in cyanobacteria, and (3) Increase of the efficiency of introduced enzymatic pathways by protein scaffolding and/or directed localisation within the cell.

Techniques for the introduction of genetic circuits have been identified and described and a high number of promoters with different strengths have developed and characterised, Figure 1. A specific review "Engineered transcriptional systems for cyanobacterial biotechnology" summarised present knowledge and suggested good promoters to use when engineering cyanobacteria (Camsund and Lindblad 2014). Together with the development of e.g. Ribosomal binding sites (RBS) and terminators an internationally leading and recognised ToolBox for cyanobacterial synthetic biology has been established.

A number of scientific papers covering the development of a ToolBox for cyanobacterial synthetic biology have been published:

- 1 - High throughput system for promoter characterisation (Huang & Lindblad 2013, Camsund et al 2014),
- 2 - List with characteristics of promoters, terminators and RBS (Huang & Lindblad 2013, Camsund et al 2014, Camsund & Lindblad 2014, Huang et al 2015), and
- 3 - Cyanobacterial strains with inserted genetic circuit (Huang & Lindblad 2013, Camsund et al 2014).



Alltogether the work resulted in two PhD students successfully presenting, discussing, defending and finishing with their PhDs at Uppsala University.

- Promoter Engineering for Cyanobacteria. An Essential Step (2013) Hsin-Ho Huang.
- Engineering Transcriptional Systems for Cyanobacterial Biotechnology (2014) Daniel Camsund.

The third objective, Increase of the efficiency of introduced enzymatic pathways by protein scaffolding and/or directed localisation within the cell, was not planned to involve any experimental work. Planed work involved theoretical identification of mechanisms – where is the forefront in the respective research field and where do we scientifically stand in cyanobacterial synthetic biology? However, eventhough we developed a scientifically very advanced toolbox and knowledge about techniques for the introduction of genetic circuits into cyanobacterial cells, cyanobacterial promoters terminators, RSBs, protein degradation tags etc for expression of introduced non-native genetic material we, or others, had no prior knowledge of any potential problems with translation in cyanobacteria.

When introducing foreign genetic information, custom designed gene(s) of interest, we could experimentally visualise the presence of corresponding transcript(s) (transcription functioned). However, sometimes we were unable, and consistently when expressing *hydA* (non-native, custom designed gene encoding a FeFe-hydrogenase), to detect any protein (or marker of the protein), i.e. translation did not occur. In addition, this was specific for cyanobacteria since it worked ok in *Escherichia coli*. This was unexpected but we realised with time (after informal discussions) that this was not uncommon internationally in other laboratories doing similar experiments. So, instead of increasing the efficiency of introduced genetic constructs and/or identify mechanisms for direct localisation of protein we focussed and redirected our resources to theoretical and experimental approaches to overcome the problem of ”efficient and functional transcription but no further translation into a protein”. Through inspiration from the literature (Mutalik et al 2013) a so called Bicistronic design (BCD) was introduced, two Shine-Dalgarno motifs resulting in more precise and reliable transcription and translation in the cyanobacterial cells. This further development of the synthetic biology toolbox, specifically around RBS and Shine-Delgarno motifs, for cyanobacterial biotechnology, was unexpected and not planned. In the end we managed to identify the theoretical bottleneck and to experimetically demonstrate successful translation into a functional HyDA.

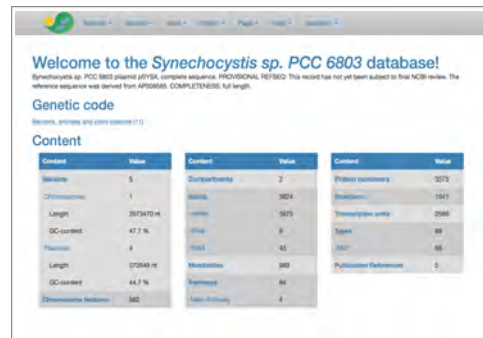
WP2 - DataWarehouse/Bioinformatics

The objective was the implementation of a knowledge base that incorporates all experimental data from the research consortium and makes them available to all partners and the public for data visualisation and modelling.

Synthetic Biology is strongly dependent on input from both systems biology and molecular biology. The former delivers the solution space within which devices and parts, e.g. promoters, ribosomal binding sites, protein coding sequences or terminators, are recombined. For defining this solution space by mathematical modelling, solid molecular biological and biochemical descriptions of the parts are required. These data was collected, integrated and made available to all other WPs and the public.

This activity is entirely computational. Thus, the scientific result comes as computer source code: e.g. ”<http://github.com/CyanoFactory/CyanoFactoryKB>” and the accessible web page: ”<http://cyanofactory.hs-mittweida.de/warehouse/>”

A - Data Warehouse. The data warehouse CyanoFactory KB (knowledge base) is a knowledge base that was intended to embrace all data produced by and relevant to the CyanoFactory project partners. It was designed to enable comprehensive simulations of entire cells and organisms with the incorporated CyanoDesign tool. CyanoFactory KB is currently centered around *Synechocystis* PCC 6803, the gram-negative model cyanobacterium of the consortium that is capable of photosynthesis and provides comprehensive, quantitative descriptions of individual engineered strains.



The data warehouse front- and backend has been established and is available for all project partners at <http://cyanofactory.hs-mittweida.de/warehouse/>. Accounts for accessing the warehouse are available upon request. Major developments included: user and right management; upload and download formats, visualisation of annotations; development of a data structure to deal with mutants, i.e. inheritance of annotations and history tracking. The interface has a barrier-free design, a.k.a. web accessibility 2.0.

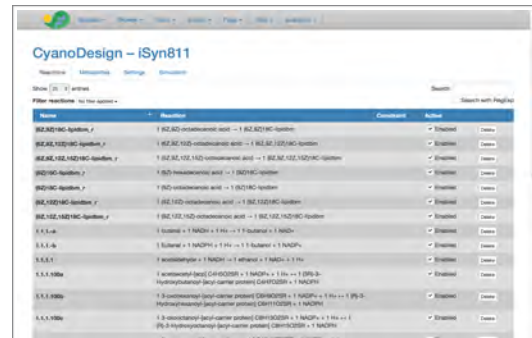
B - CyanoMaps - Web-based graphical representation of cyanobacterial hydrogen metabolism with the possibility to identify metabolic hubs and regulatory bottlenecks. The establishment of CyanoMaps proved to be more difficult than anticipated. The initial idea to use SBGN (systems biology graphical notation) as a graphical representation for hydrogen metabolism proved to be unpractical for the experimentalists. This is the result of personal communications and a poll performed within the context of CyanoFactory. Nevertheless, it is present in the warehouse: <http://cyanofactory.hs-mittweida.de/warehouse/sbgn/>). Due to the difficulties encountered in practical use of SBGN maps, we started to implement data visualisation on Boehringer and KEGG maps (<http://cyanofactory.hs-mittweida.de/warehouse/boehringer/> and <http://cyanofactory.hs-mittweida.de/warehouse/kegg/>). Both are fully accessible at the knowledge base.



CyanoMaps harbours tools to display metabolic and regulatory network data. It can be regarded as a collection of generic stand-alone tools under the umbrella of the CyanoFactory KB. Boehringer, KEGG Pathways and SBGN of *Synechocystis* are linked to the knowledgebase. The intention of developing CyanoMaps was the generation of a platform for highlighting selected enzymes or metabolites in metabolic maps. This has currently been implemented for KEGG maps (Kyoto Encyclopedia of Genes and Genomes) and the Boehringer Map, originally developed by Gerhard Michal from the Boehringer Company. The SBGN maps were developed as part of CyanoFactory as an attempt to bring all regulatory and metabolic information about hydrogen metabolism of *Synechocystis* PCC 6803 together. The SBGN

visualization itself is an attempt to visualize biological processes in a human and computer readable way.

C - CyanoDesign - Web-based software that allows studies on flux and growth experiments and simulations of engineered cells. The most demanding task was the implementation of CyanoDesign. However, combined work in WP2 and WP7 managed to get it running (see <http://cyanofactory.hs-mittweida.de/warehouse/design/>). CyanoDesign is an interface to the PyNetMet software tools. The large network of *Synechocystis* PCC 6803 and metabolic models have been incorporated into the knowledge base.



CyanoDesign is designed to perform genome-scale metabolic modeling. By default, two models are pre-installed in the CyanoFactory KB: iSyn811 for *Synechocystis* PCC 6803 and a much smaller toy model. The latter is used to introduce new users to the functionality.

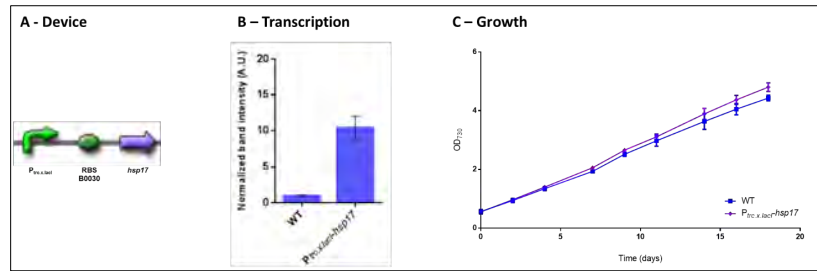
WP3 - Improvement of chassis growth, functionality and robustness

The aim was to improve a *Synechocystis* based chassis in terms of growth/robustness and functionality. For this purpose, the tolerance to environmental factors was assessed, namely temperatures below optimal (~30 °C) and salinity, foreseeing the outdoors cultivation of *Synechocystis* using seawater. The results showed that the growth rate decreases with temperature, being 10 °C the tolerance limit observed for non-acclimated cells. Additionally, a light and temperature profile mimicking a summer day in Florence, where the 1300 L outdoor bioreactor is implemented, was defined based on the measurements performed by M2M: temperature ranging from 20 to 41 °C and a 14 h light/10 h dark regimen (Tvar Florence). Under these conditions the growth of *Synechocystis* was found to be similar to that observed for cells grown at continuous temperature of 20 °C at a 12 h light / 12 h dark regimen, suggesting that growth impairment is related to cell exposure to temperatures above 35 °C for 6 hours. Regarding halotolerance, NaCl concentrations between 0-7% (w/v) were tested and it was observed that *Synechocystis* growth rate decreases as the concentration of salt increases, being 6% the tolerance limit for non-acclimated cells. Considering the data obtained and envisaging the improvement of the chassis robustness, a set of candidate genes/pathways (targets) was identified, leading to the design of strategies focusing on heat shock response proteins (HSP) and compatible solutes (CS) synthesis. Several synthetic devices were constructed and implemented in *Synechocystis* using replicative vectors, and some of the mutants exhibited growth improvement under temperature fluctuations or higher compatible solute production:

- (i) the HSP device leading to an increased *Synechocystis* fitness under temperature fluctuations (Tvar Florence) was comprised by the native *hsp17* gene preceded by a strong RBS (BBa_B0030) and under the control of a strong constitutive synthetic promoter ($P_{trc.xlacI}$). The *Synechocystis* mutant harboring this device exhibited a 10-fold increase in *hsp17* transcript levels and a 7% improvement in growth compared to the wildtype (Figure 2);

Figure 2. Characterization of the *Synechocystis* mutant harboring the $P_{trc.xlacI}$ -*hsp17* device. (A) Specifications of the synthetic device. (B) Analysis of *hsp17* transcription by RT-PCR.

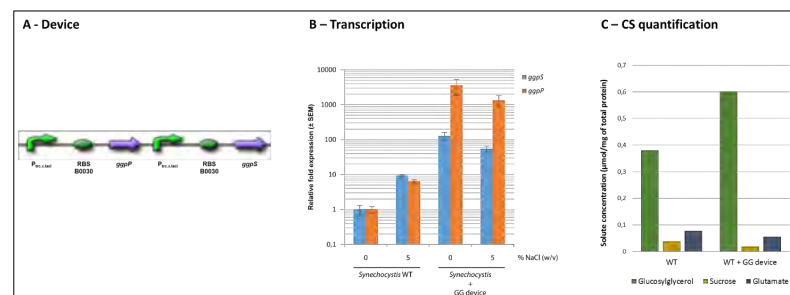
Synechocystis wild-type (WT) and *Synechocystis* harboring the device were grown under the Tvar Florence profile, temperature ranging from 20 to 41 °C and a 14 h light / 10 h dark regimen.



The bars represent the mean intensity of the replicates normalized to the levels of *Synechocystis* wildtype and error bars represent \pm SD. (C) Effect of *P_{trc.x.lacI}-hsp17* device in growth. Cells were grown under the same conditions as (B) and growth was monitored measuring the OD₇₃₀.

(ii) the CS device leading to an increased production of the native compatible solute glucosylglycerol was comprised by the native *ggpS* and *ggpP* genes preceded by BBA_B0030 RBSs and under the control of *P_{trc.x.lacI}* promoter. The mutant containing this device showed a remarkable increase in *ggpS* and *ggpP* transcription (even without NaCl supplementation) and produces approximately 50% more glucosylglycerol than the wildtype (Figure 3). In addition, iTRAQ and RNA-seq studies were performed and data analysis is being performed (IBMC in collaboration with USFD).

Figure 3. Characterization of the *Synechocystis* mutant harboring the GG device. (A) Specifications of the synthetic device. (B) RT-qPCR analysis of *ggpS* and *ggpP* transcripts levels. *Synechocystis* wild-type (WT) and mutant harboring



the GG device were cultivated in BG11 medium supplemented with 0 or 5% (w/v) NaCl. The relative fold expression is normalized for the wild-type at 0% (w/v) NaCl. Data represents mean \pm SEM. (C) NMR quantification of the compatible solutes (CS) - glucosylglycerol, sucrose and glutamate. *Synechocystis* wild-type (WT) and the mutant harboring the GG device were grown in BG11 supplemented with 3% (w/v) NaCl.

To improve *Synechocystis* functionality, several neutral sites were identified for the stable integration of synthetic devices in the chromosome through systematic genome mapping. Disruption mutants in those loci were generated and extensively characterized in terms of fitness, transcription and proteomics (IBMC in collaboration with USFD and UPVLC), validating the neutrality and functionality of the sites. Furthermore, the constructed integrative vectors include a BioBrick-compatible multiple cloning site insulated by transcription terminators, that revealed to be robust and insulated cloning interfaces. Finally, the mutants and vectors generated are available and can be used for synthetic biology approaches (Pinto et al 2015; Portuguese Provisional Patent, Process #108564).

For the modulation of the intracellular oxygen concentration and foreseeing the introduction into the chassis of synthetic modules encoding O₂-sensitive enzymes (such as hydrogenases), several Oxygen Consuming Devices (OCD) based on the CueO (*Escherichia coli*'s native laccase) were assembled. These devices were characterized *in vitro* in *E. coli* (protein crude

extracts), assessing the specific laccase activity and oxygen consumption rate, revealing that the OCDs are functional in all conditions tested. Moreover, characterization of the OCDs *in vivo* using *E. coli* cells confirmed the consumption of O₂ in cells harboring the devices. The assessment of the laccase-based OCDs performance is also being carried out in *Synechocystis*. For this purpose; the *E. coli* devices were reassembled changing the regulatory elements and a device with a bicistronic design was also assembled by UU.

Furthermore, the chassis functionality and robustness was also assessed by inactivating the gene *slr1270*, which encodes a TolC homologue. TolC is an outer membrane protein associated to biomolecules secretion, including proteins and endo- and/ or exogenous metabolites. This work highlights the marked physiological fitness that the TolC-like Slr1270 bestows to *Synechocystis* and presents a valuable model for studying OMVs formation and release (Oliveira et al, in press). In the future, OMVs and optimized secretion system(s) can be used as tools to increase the functionality of a chassis based on *Synechocystis*. In line with these findings, we also evaluated the functional role of the extracellular protein (Alr0267) transported via the TolC-dependent secretion system, using the cyanobacterium *Anabaena* sp. PCC 7120 as model organism (Oliveira et al 2015). The protein (Alr0267) named HesF was found in the extracellular milieu when cells were cultivated in diazotrophy and the Δ *tolC* mutant was shown to be impaired in HesF secretion. It was proposed that HesF is a carbohydrate-binding exoprotein that plays a role in maintaining the heterocyst cell wall structure. A combination of and possibly interaction between HesF and heterocyst-specific polysaccharides seems to be responsible for filament adhesion and culture aggregation in heterocyst-forming cyanobacteria (Oliveira et al 2015).

WP4 - Introduction of custom designed hydrogen producing units

The work summarised in part of CyanoFactory focused on to design, construct and introduce a hydrogen producing unit into cyanobacterial cells, and to demonstrate hydrogen production from such an engineered cell. For this purpose, the structural gene, *hydA*, encoding the [FeFe] hydrogenase from *Chlamydomonas reinhardtii*, and the maturation genes *hydEFG* from *Clostridium acetobutylicum* were selected. *Chlamydomonas hydA* codon optimized for *E. coli* and *Synechocystis* was introduced onto a self-replicating pPMQAK1 vector while the maturation cassette was integrated into the chromosome to, in parallel, knockout the native [NiFe] *hox* hydrogenase of *Synechocystis*. This ensured no background hydrogen activity. A strong *trcO1* core promoter and RBS star were used to express both, the hydrogenase and the accessory genes. While it was possible to show stable translation of these genes in *E. coli*, their translation in *Synechocystis* PCC 6803 appeared to be transient and unstable. Concurrently, reverse transcript (RT) PCR for both the *hydA* and the accessory genes showed stable transcripts.

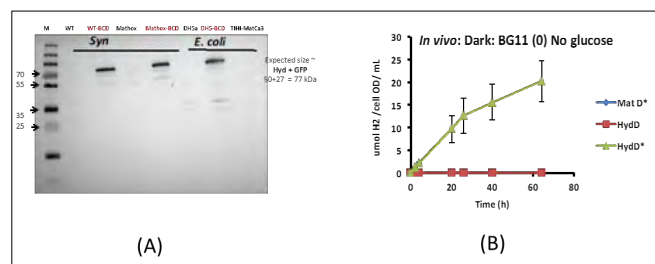
To order to obtain stable translations, the constructs were redesigned to introduce a ‘bicistronic device’ (BCD) that incorporated a leader peptide with an encrypted Shine Dalgarno sequence. This was based on a recent study conducted by Mutalik et al (2013) that showed predictable translations in *E. coli* based on usage of this device. These new constructs were conjugated into *Synechocystis* strain harbouring the accessory *hydEFG* to develop the BCD-hyd *Synechocystis* Mathox strain. Translation of HydA was confirmed with Western immunoblots (Figure 4A); however, *in vivo* hydrogen production from these strains under anaerobic conditions produced no measurable hydrogen. From these results it was deduced that the accessory genes *hydEFG* as well were transcribed but not translated. Thereafter, the maturation construct was redesigned to incorporate the BCD. Whole cell

protein extracted from the *Synechocystis* strain harbouring this new construct was sent to our collaborators in USFD. iTRAQ analysis confirmed the presence of all the three proteins HydE, HydF and HydG. Unfortunately, numerous repeated attempts to create a strain harbouring both the structural gene *hydA* and the accessory gene *hydEFG* incorporating the BCD failed.

Therefore, to test the functionality of HydA we decided to use a synthetic variant of the [2Fe] sub site. The [2Fe] subsite is dependent on a specific subset of maturation enzymes (HydEFG) for its biosynthesis and incorporation into HydA. FeFe hydrogenase carrying cells assemble a pre-catalyst of unknown structure on HydF through the activities of HydE and HydG. The ‘primed’ hydF is then known to carry the active site to the apo-enzyme, where it is assembled. Synthetic chemistry has made the synthesis of analogues of that subsite possible (Berggren et al 2014). Elementary *in vivo* trials were attempted in *E. coli*. Activation of apo-HydA could be readily assessed by monitoring the increase of H₂ gas released by the cell cultures, and indeed, treating early log-phase cultures expressing apo-HydA with complex 1 prior to the incubation in enriched media resulted in significantly increased H₂ production. Addition of 100 µg and 1 mg of complex per 100 mL cell culture resulted in a 15 ($0.04 \pm 25\% \mu\text{M H}_2 \cdot \text{h}^{-1} \cdot \text{mL}^{-1} \cdot \text{O.D.}^{-1}$) and 25-fold ($0.06 \pm 10\% \mu\text{M H}_2 \cdot \text{h}^{-1} \cdot \text{mL}^{-1} \cdot \text{O.D.}^{-1}$) increase over the background activity respectively. Thereafter, similar trials were conducted with *Synechocystis* cells expressing apo-HydA (Figure 4B). Hydrogen production from the selected strain was tested *in vivo* both under light and in darkness. Under the selected experimental conditions, the *Synechocystis* strain outperformed *E. coli* in terms of hydrogen production rate and yield. This is the first example of *in vivo* activation of apo-hydrogenases and indeed of any enzyme using synthetic analogues of the active site.

We were thus further interested in determining how such a synthetically activated enzyme interacted with the metabolic circuit of the cell. BCD-hyd *Synechocystis* samples incubated anaerobically with the synthetic active site analogue for four days were sent to our collaborators in USFD. iTRAQ analysis confirmed that indeed such an artificially activated enzyme, produced detectable metabolic flux changes that resulted in hydrogen production. The study thus demonstrates a powerful new tool for activating hydrogenases under *in vivo* conditions, in the absence of the still only incompletely characterized maturation machinery.

Figure 4. Expression and functionality of the expressed apo-HydA in *Synechocystis* (A) Immunoblot analysis of BCD-hyd GFP fusion proteins in *Synechocystis* and *E. coli* (B) *In vivo* analysis of the functionality of the expressed apo-HydA in a delta Hox background using a synthetic variant of the [2Fe] subsite.



As discussed above we were able to demonstrate the functionality of the apo-HydA expressed in the *Synechocystis* cells using the synthetic active site analogue. However,, we did not “reach all the way” designing and constructing a cyanobacterial cell harbouring a functional and hydrogen producing non-native FeFe-hydrogenase based on introducing the gene encoding the structural apoprotein (HydA) and the genes encoding the maturation machinery/proteins (HydEFG) - We did manage to design and construct a cyanobacterial cell harbouring a functional and hydrogen producing non-native FeFehydrogenase based on introducing the

gene encoding the structural apoprotein (HydA) and adding a synthetic complex mimicking the catalytic site. Moreover, these cells/cell cultures were functional >24 hours. This had consequences for the progress of some of the work in other parts of CyanoFactory. However, as detailed elsewhere, most significant and relevant novel advances were still made within all these activities.

WP5 - Improvement of photosynthetic efficiency towards hydrogen production

The overall objective was to provide the tools and techniques for an efficient hydrogen production via a hydrogenase incorporated into the cyanobacterial design organism. The work was subdivided in the development of the photobioreactor setup, enabling the controlled transition from aerobic growth to anaerobic production conditions, the engineering and improvement of the cyanobacterial metabolism, the increase of the linear photosynthetic electron flow and the rerouting of electrons, and the establishment of the hydrogenase in the production organism.

For successful application of photosynthetic energy production the interplay between the engineered photobioreactors and the cyanobacterial organism with the designed metabolism is of major importance. Eight different engineered cyanobacterial strains were investigated. The strains were cultured in flat-panel photobioreactors applying a turbidostatic process control. This enables the cultivation of the cultures at a steady state with constant media composition and at a constant cell density. In general, the mutants with truncated light-harvesting antenna tolerated higher light intensities and reach higher cell densities in the photobioreactors.

As CyanoFactory aims to route photosynthetic electrons primarily towards the new introduced electron sink (hydrogenase) instead of the carbon fixing metabolism, design cells may accumulate lower amounts of storage compounds during the day resulting in lower resistance towards extended dark periods. Different light profiles – continuous illumination, rectangular, and natural light profiles – were applied to characterize the effects of the diurnal cycle on the cells. The total amount of carbon assimilated depended mainly on the total amount of light quanta supplied and the strain used, but is rather independent from the profile of the light/dark cycle. The experiments achieved a controlled transition to anaerobiosis, Figure 5.

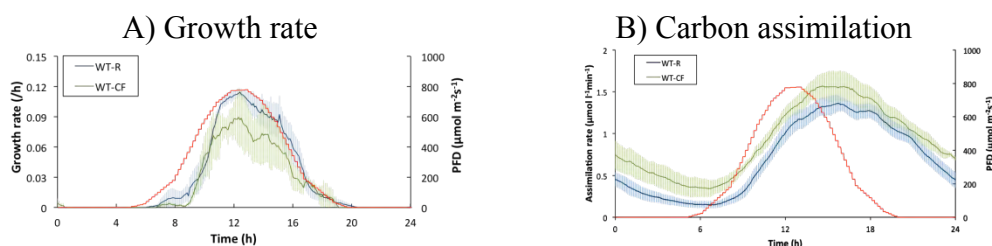


Figure 5. Different light profiles used to characterise light/dark cycles. Growth rate (A) and carbon assimilation (B) are monitored for two different wild type strains of *Synechocystis* PCC 6803 in dependence of different light profiles (red). Day to day variations are symbolised by green and blue bars for the two strains, respectively.

Next to truncation of the light-harvesting antenna, partial decoupling of the ATPase was identified as a route to increase the photosynthetic electron transport (D5.2, D5.3). For this purpose, the deletion of the C-terminal domain of ATPase subunit ϵ , which was shown to accelerate photosynthetic electron transport, was introduced into the Olive mutant. The

resulting Olive- $\Delta C\epsilon$ mutant displayed a fourfold increased electron transport rate in comparison with the wild type. However, only 25% of the electrons produced in the Olive- ΔC mutant are transferred into the cyanobacterial metabolism, while 75 % are transferred to O_2 in the Mehler reaction catalyzed by the flavodiiron protein Flv1 and Flv3. This indicates, that introduction of a hydrogenase into this system as a new "sink", combined with rerouting of the electrons, would not result in a competition of future hydrogen production with the demands of the metabolism.

Tuning of the FNR-Ferredoxin interaction. Based on the structural information (Liauw et al 2012), by rational design variants of the FNR with reduced ferredoxin affinity were created and screened for their activity by two independent *in vitro* assays (Figure 6, Table 1). FNR variants K78D, K75A-K78D and K75D-K78D show significantly lower electron transfer towards NADPH. Further improvements were obtained by designing rational ferredoxin variants. The FNR variant FNR_S-D71K was integrated into the chassis of the Olive mutant leading to a slightly reduced CO₂ fixation.

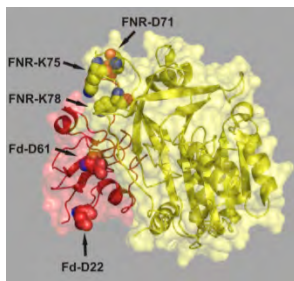


Figure 6. Structure of the FNR:Fd complex. The sites targeted for reducing the complex stability are labelled within the structure

Table 1: List of FNR and Fd variant ordered by decreasing affinity

FNR variants	Fd variants
FNR _{L/S}	Fd
FNR _S -D71K	Fd-D22A
FNR _S -K75A	Fd-D61A
FNR _S -K75D	Fd-D22A-D61A
FNR _S -K78A	
FNR _S -K78D	
FNR _S -K78A-K78D	
FNR _S -K75D-K78D	

WP6 - Biosafety

Although biosafety issues can have a fundamental impact on public opinion and acceptance of cyanobacterial biotechnology, biosafety of genetically modified cyanobacteria has remained unexplored. In CyanoFactory, we have addressed this important topic through development and incorporation of intrinsic biosafety mechanisms (kill switches) in cyanobacterium *Synechocystis* PCC 6803.

Two strategies were employed. In the first, conditional lethality was dependent on intracellular degradation of DNA, to accomplish not only regulated killing of cells but also destruction of cellular genetic material, which could otherwise be available for horizontal gene transfer. To that end, we made use of non-specific DNA/RNA nuclease NucA and its inhibitor NuiA from the cyanobacterium *Anabaena* PCC 7120. While *Synechocystis* sp. PCC6803 does not contain a NucA homolog, nucleases of this type are present in many bacteria and are believed to have evolved to serve for nutritional purposes and sometimes as bacteriocides. We envisioned that by rewiring the nuclease/inhibitor pair for conditional expression, cell survival could be achieved specifically in the photobioreactor, while upon accidental release into the environment, the rewired nuclease would prevail over the inhibitor, thereby killing the cells. The nuclease gene was placed under an inducible promoter to allow induction upon exposure to environmental inducer (Figure 7, graph). To protect cells from possible leaky nuclease production in the bioreactor, the nuclease inhibitor gene was

fused to a weak constitutive promoter. By using metal-ion inducible promoters to trigger nuclease expression, we were able to elicit efficient cell killing upon inducer addition. The most efficient promoter was a variant of *PcopM*. Under native conditions, *PcopM* promoter regulates the *copMRS* operon involved in *Synechocystis* metal ion homeostasis (Giner-Lamia et al., 2015). Thus, addition of 12 mM Zn^{2+} ions to *Synechocystis* carrying the *PcopM*-driven suicide switch (construct $KS_{PcopM195-BCD-nucA}$) triggered complete killing of bacteria (Figure 7A & B). Translation efficiency of the suicide construct was optimized by using the bicistronic design (BCD) (Mutalik et al 2013). The $KS_{PcopM195-BCD-nucA}$ suicide switch was also analyzed under simulated environmental conditions, including seasonal changes.

In the second approach, native *Synechocystis* toxin-antitoxin (TA) systems were rewired for conditional lethality by using metal-ion inducible promoters. TA systems are widespread prokaryotic genetic elements comprising a stable toxic protein and a less stable neutralizing antidote involved in various cellular functions ranging from plasmid addiction to stress response. At the start of CyanoFactory, almost nothing was known about TAs in cyanobacteria. In order to employ TA pairs in suicide switch construction, we first needed to identify and evaluate possible *Synechocystis* TA systems. Following that, we created variants of TA-based suicide switches. For that, we selected two *Synechocystis* toxin/antitoxin pairs: the pair *slr6101/slr6100* that we identified and evaluated in *E. coli*, and the pair *ssr1114/slr0664* that had previously been shown to be toxic to *E. coli* (Ye & Ning 2010; Ning et al 2011). Interestingly, unlike the nuclease-based safeguards, the suicide constructs with toxins Slr0664 or Slr6100 (which encode RelE-like ribonucleases) caused reduced growth of bacteria rather than efficient cell killing, suggesting that bacteria were able to cope with the cellular damage inflicted by the toxins (Figure 8A & B).

The choice of promoters used for construction of biocontainment safeguards was crucial for the killing efficiency. To achieve an optimal promoter combination for the kill switches, we tested different highly inducible metal-ion responsive promoters (together with various metal-ion inducers) and two constitutive promoters. Adding to the existing cyanobacterial toolbox, we evaluated activity of selected cyanobacterial promoters by using the beta-galactosidase reporter assay. Our results indicated that current understanding of cyanobacterial promoters is still incomplete. We summarized our findings in a paper sent for publication to Biology Open (Čelešnik et al, submitted).

Figure 7. Induced autokilling of *Synechocystis* PCC 6803 carrying nuclease-based suicide switch. (A) Growth curves of cyanobacterial cells harboring plasmid-encoded kill switch

(KS), in which the *nucA* gene is driven by inducible *PcopM*-promoter variant and *nucA* gene is driven by low constitutive *PrnpB-RBS* promoter (diagram). After induction of bacteria (10^6 cells/ml) with 12 mM Zn^{2+} , no growth is observed for cells harboring the suicide switch (graph). WT: wild type.

(B) Cultures from (A) were plated on solid media at day 13. Complete killing was observed for suicide-switch cells (KS triplicates 1-3) at 12-14 mM Zn^{2+} induction, while wild type (WT triplicates 1-3) remained viable. At 11 mM Zn^{2+} , some survival was observed for KS cells.

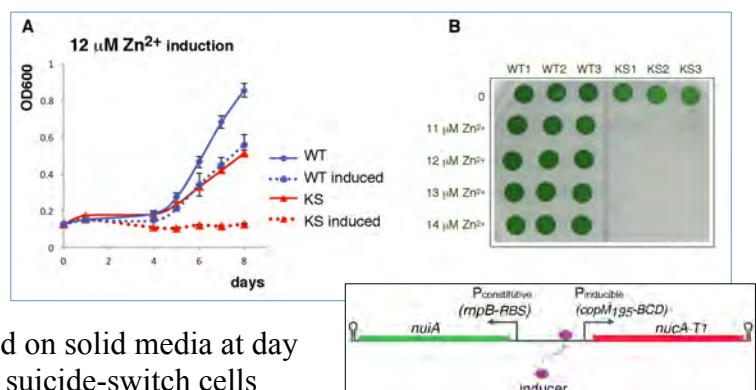
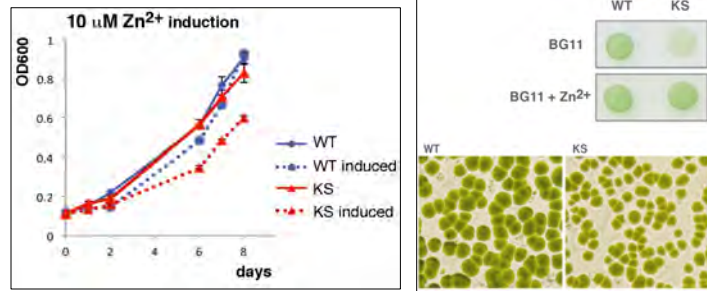


Figure 8. Regulatable autotoxicity of *Synechocystis* PCC 6803 carrying toxin-based suicide devices. (left) Growth curves of cyanobacterial cells harboring plasmid-encoded suicide switch (KS), in which the toxin *slr0664* gene is driven by the inducible



PcopB promoter and the antitoxin *ssr1114* gene is driven by low constitutive *PrnpB* promoter. After induction with 10 mM Zn²⁺, growth retardation is observed, indicating induced toxin activity. WT: wild type. (right) Regulatable autotoxicity of kill-switch cells carrying constitutively expressed *slr0664* toxin gene (driven by *PrnpB* promoter) and inducible *ssr1114* antitoxin gene (driven by *PcopB* promoter). (upper) Toxic effect is seen on BG11 agar plates without added metal ions, while addition of 4 mM Zn²⁺, which induces antitoxin production, turns off the biosafety switch. (lower) Colony size on BG11 agar plates without added metal ions observed under stereomicroscope (25x magnification).

In addition to examining toxin/antitoxin and nuclease/inhibitor modules for increased biosafety, we explored a cyanobacterial enzyme named ‘orthocaspase’, homologous to programmed cell death enzymes in higher organisms (Klemenčič et al 2015). It is proposed that there might be a link between orthocaspases and toxin/antitoxins in cyanobacteria (Klemenčič & Dolinar 2016).

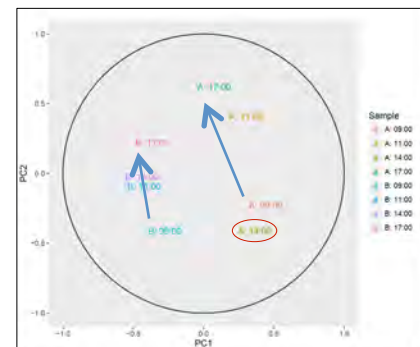
WP7 - Analyses of the purpose designed cyanobacterial cells to identify bottlenecks and suggest further improvements

In CyanoFactory, USFD produced preliminary proteomics data investigating the metabolic differences in *Synechocystis* PCC 6803 between hydrogen-producing and standard laboratory growth conditions. This data was summarised with cellular pathway diagrams, and was used to highlight key features that changed between these two conditions. The findings discussing the improvements USFD made to the proteomics pipeline, contribute collectively towards the delivery of quantitative proteomics data together with recommendations for forward cellular engineering. They achieve this by facilitating the work where new chassis-circuits have been designed by UU and IBMC and are analysed with proteomic methods, systems-level effects are analysed using quantitative proteomics. Clearly improved protein identification and quantification on both a localised and systems level analysis is key to generating the high quality data required for computational modelling work. Following these developments to the pipeline, USFD improved the number of confident protein quantifications from a single iTRAQ from 200 to over 2000 by the end of the CyanoFactory project.

Beyond self-driven work, USFD collaborated with most of the other partners in the consortium to produce datasets that will eventually be key to developing more comprehensive models of the entire system. The data have been collected and will be transferred to the consortium repository for public interaction once the key papers have been published and any potential IP issues have been allayed. The work completed in collaboration with CNR-ISE, provides key proteomic datasets that feed into the best-case PBR design scenario. The findings from this form the basis for the final recommendations to the consortium. Output from the subsequent sections feeds back into it, to keep the large-scale industrial applicability context of the CyanoFactory work in prime importance. The key

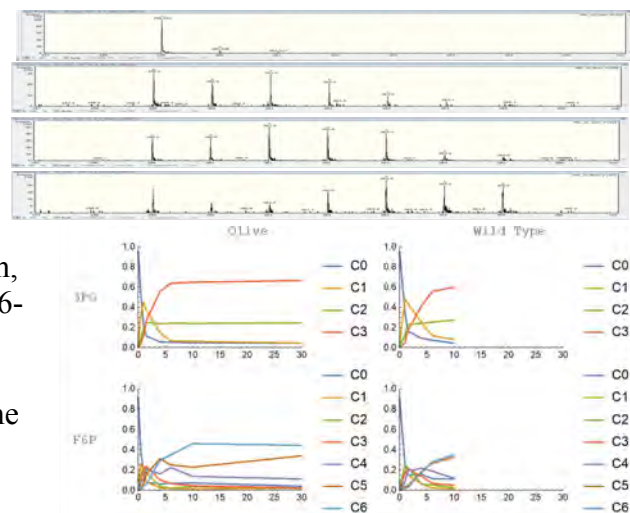
finding from the USFD CNR-ISE collaboration was identification of systematic changes over the course of a typical operating day, where the solar stress effects not only provide biomass production but also contribute to ideal hydrogen operating conditions. These findings are summarised in the Principal Component Analysis (PCA), Figure 9.

Figure 9: PCA plot: Almost parallel vectors can be plotted running from 0900 for each sample through to 1700 (blue arrows). This is to be expected as systematic changes in proteome response to increased light and heat build-up in the system.



The work completed in collaboration with RUB characterising a key chassis modification with metabolic flux analysis and proteomic investigation. The experimentation was carried out in the 5 L photo-bioreactor produced by KSD, as it was determined to be a suitable bioreactor system produced from the consortium. This work demonstrated a novel pipeline developed at USFD, with photo-autotrophic metabolic fluxes being experimentally measured of the first time on an industrially relevant strain (*Synechocystis* PCC 6803 Olive), Figure 10.

Figure 10. Top: Isolated data for Fructose-6-Phosphate at 4 time-points in WT *Synechocystis*, shown as an example of raw data from the mass spectrometer. Bottom: the distribution of metabolites shown over the time series for 2 identified key metabolites in the central carbon metabolism, 3-phosphoglyceric acid (3PG) and fructose-6-phosphate (F6P).



Collaborations investigating the effects of the metabolic engineering of an external hydrogenase were performed with UU. This highlights work that demonstrates a clear progression from the "analysing samples" to repeatedly give feedback on the engineering work performed by UU. It is a key example of the iterative improvements developed in CyanoFactory.

USFD had extensive collaborations with IBMC, resulting in a joint ms highlighting key technical details for more stable genetic engineering in *Synechocystis*; and the production of an RNA-seq transcriptomic dataset investigating genetic and environmental changes simultaneously. The chassis development fed into consortium work, providing key details for UU and IBMC to perform more stable genetic engineering. The transcriptome analysis was combined with a proteomic analysis, to generate the integrated -omic analysis. All of the RNAseq samples were combined into a single, multi-condition comparative experiment covering both an investigation into genetic modifications to the chassis, as well as environmental changes to the bioreactor by adding NaCl. The RNA analysis identified 2214 genes, with 4264 isoforms, 4188 transcription start sites, 3562 coding sequences and 61992 promoters within the genome over the 2 replicates of 8 conditions.

Summary of final recommendations to the consortium:

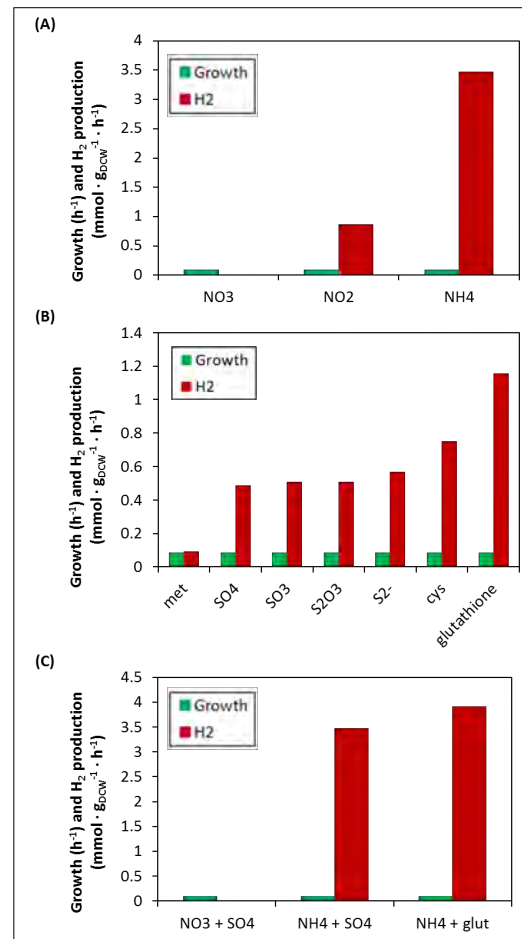
- 1 - Exposure to the sun over the course of the day builds up reductive stress and activates the proteomic profile for hydrogenase expression.
- 2 - Collected data suggests that timing the O₂ removal for after 5 pm following an aerobic growth phase during a sunny day should trigger significantly increased H₂ production.
- 3 - Additionally, the cells have naturally lower levels of nitrogen as they are repairing the photosystems which coincides with a wealth of established literature on H₂ production.
- 4 - It would also be interesting to measure what levels of intracellular oxygen might be present with biological modelling of light vs dark phase growth.
- 5 - USFD recommends running the 1,350 L PBR time course experiment with the *olive* strain by RUB to compare against WT and to provide important data on the scaled up growth of *olive* in outdoor conditions.
- 6 - The UU synthetic site doesn't appear to generate a systematic effect on *delta hox Synechocystis* without a hydrogenase apo-enzyme.
- 7 - The UU synthetic site produces a large number of systematic changes in *delta hox Synechocystis* with a hydrogenase apo-enzyme, suggesting the possibility of a phenotypic rescue. This suggests that the synthetic site may act as a suitable positive control for structurally accurate genetically engineered expression of the active site.

WP8 - Metabolic modelling of the engineered cells

Rational design of living organisms for biotechnological purposes is a challenging interdisciplinary effort which can be leveraged by *in silico* analysis. Modelling strategies serve as a basis for unravelling the underlying mechanisms responsible of cell behaviour and allow theoretical assessment of environmental and genetic variations, whereas laboratory experiments are often expensive, time demanding or shed light on just a specific process of the system. Engineering cyanobacteria for producing metabolites of interest, such as hydrogen, implies an overall knowledge of metabolism but also a detailed comprehension of photosynthesis in particular. Additionally, the application at industrial-scale requires further research on optimal growing conditions in designed photobioreactor framework.

In order to understand the biochemical processes occurring within a *Synechocystis*' cell metabolic models of the strains of interest are needed. The departing point to build an accurate, up-to-date, genome-scale metabolic model of *Synechocystis* sp. PCC 6803 was a model previously developed by our group: iSyn811 (Montagud et al 2010 & 2011). More than 200 reactions were added and/or updated leading to important improvements in simulations, such as greater plasticity, greater accuracy at electron consuming pathways and more realistic energy needs, that yield more precise metabolic characterization of the strains and enhance the predictability of the model. This model can be considered as the "reference" model of *Synechocystis* PCC 6803 wild type, however other wild type or mutant strains have also shown interesting properties so that derivative models are required to study them. We have developed a pipeline that aims at the elucidation of how differences in sequence might affect the metabolic function of these strains, allowing the construction of models for the new strains based on the original one.

Figure 11. Results of the simulation of hydrogen production at a given growth rate with different sources of inorganic nutrients obtained by using the Synechocystis metabolic model. Simulations were carried out under phototrophic conditions, with fixed amount of photons and CO₂, at a fixed growth ratio (normal growth for this carbon intake), and allowing the input of different (A) nitrogen sources: nitrate (NO₃), nitrite (NO₂) and ammonia (NH₄); (B) sulphur sources: methionine (met), sulphate (SO₄), sulphite (SO₃), thiosulphate (S₂O₃), sulphide (S₂-), cysteine (cys) and glutathione; and (C) combination of nitrogen and sulphur sources leading at best maximum optimal hydrogen production.

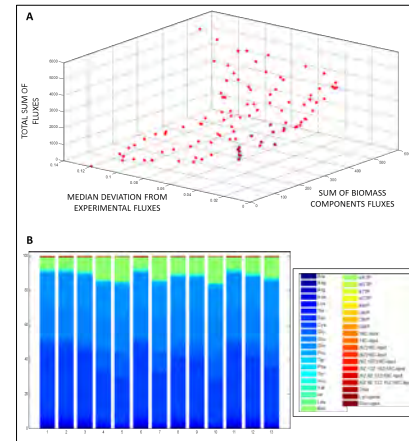


These metabolic models must serve to guide strain design by assessing how different modifications in media or genomes can improve growth or productivity of desired metabolites. In that sense, a substrate study was performed to determine the best sources of inorganic nitrogen and sulphur in terms of hydrogen production. In the case of nitrogen, the results obtained from the simulations qualitative matched previous experimental observation (Guttham et al 2007; Baebprasert et al 2011), while regarding sulphur, this is, to our knowledge, the first work in which different sources have been tested to improve hydrogen production. Altogether, the results of this study showed that by choosing the appropriate source of these inorganic substrates, maximum optimal H₂ production can be increased substantially (Figure 11). Also genetic changes were analysed, in collaboration with IBMC, in order to choose the best option between some candidates for the synthesis of compatible solutes. The results of this study showed that in terms of metabolic cost, glycine betaine is the less resource-consuming and therefore most optimal between the proposed options, as greater production of the solute is possible at equal growth rates. So, several analyses have been carried out by means of metabolic simulations to give support to experimentalist in the selection of the best environmental and genetic conditions enabling enhanced H₂ production.

Although classic metabolic simulations solve appropriately some biotechnological problems, nowadays with the advent of high-throughput technologies, the field of systems biology has amassed plentiful omics data that can be combined with metabolic models to heighten the predictive capabilities of computational simulations and their plasticity when dealing with perturbed conditions. In this field, we have applied a previously described algorithm, IOMA (Yizhak et al 2010), in which proteomics, metabolomics and kinetic parameters, are integrated into metabolic simulations, what qualitatively improves the simulations' response to environmental changes, even when still some adjustments must be done to improve its accuracy when working with genome-wide simulations. Besides, we have developed META-MODE (ms), an algorithm that performs multi-objective optimizations of a genome-scale metabolic model allowing the use of biologically-relevant non-linear constraints and that, with the inclusion of experimental flux data, permits researchers to have realistic metabolic

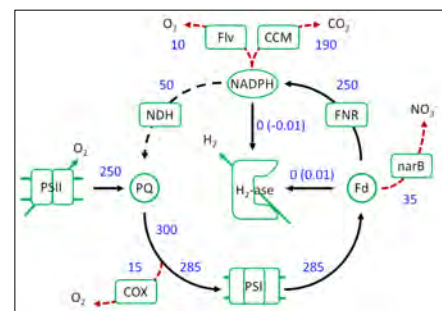
simulations without having to impose constraints that are tiresome to find and even sometimes cryptic to understand. Furthermore, with META-MODE, simulations can be driven maximizing growth without stating any biomass equation, so that different biomass compositions will be obtained depending on the conditions (Figure 12). The final goal is to incorporate different algorithms into META-MODE, leading to a comprehensive tool prepared for different omics integration that performs advanced metabolic simulations.

Figure 12. (A) Pareto front obtained with Met-spMODE by multi-objective heuristic optimization of three objectives: growth (as maximization of sum of biomass components), closeness to experimental fluxes (as minimization of median deviation) and parsimonious character (as minimization of total sum of fluxes). Selected solutions are marked with blue squares. (B) Biomass composition (molar %) of the selected solutions. With this tool biomass composition doesn't have to be imposed and slightly different compositions are obtained depending on the trade-off between objectives



On the other side, as modelling whole metabolism in a fully mechanistic manner is a demanding task that requires a huge amount of experimental information, alternative strategies can be applied to reach useful conclusions. One option is to construct more detailed mathematical models on relevant cellular pathways. Particularly, as photosynthetic microorganisms are to be used to profit from solar energy, photosynthesis is a key answer for proper modelling of metabolism as this process drives main energetic pools in autotrophic conditions. In this regard, the construction of a photosynthesis dynamic model was considered critical for this purpose. Such an *in silico* tool provides the opportunity to estimate electron fluxes and their dynamics in the transport chain by using several sets of data including reaction stoichiometry, flux rates, kinetic and thermodynamic constraints. Consequently, a photosynthesis model of the main electron flow chain reactions consisting on around 50 ordinary differential equations was developed. It delivers dynamic fluxes that allow a better understanding on several processes such as respiration, carbon fixation or hydrogen production. For the sake of simplicity, main results of fluxes in steady-state are depicted in Figure 13. Regarding hydrogen evolution, the model could describe the recognised impossibility of generating such gas in the wild-type strain in normal conditions due to thermodynamic limitations as NADPH acts as an electron acceptor rather than donor (Gutythann et al 2007). Besides, oxygen at cytoplasmic concentrations blocks the hydrogenase activity and moreover inhibitory levels of oxygen can be reached within seconds after the onset of light. Interestingly, these conditions were numerically predicted by the model giving not just a qualitatively well-known description of the phenomenon but also shedding light on the process by means of quantitative predictions expressed in real units.

Figure 13. Simplified scheme of the photosynthetic electron chain of *Synechocystis* PCC 6803 wild-type grown at saturating light intensity. Fluxes are given in $e^- \cdot s^{-1} \text{ PSII}^{-1}$ after onset of light transitory phase. In brackets transient fluxes are depicted.



Another objective achieved within the modelling work package has consisted in creating a photobioreactor scale model that is able to predict physiological properties of photosynthetic cultures. Cells grown in such reactors experience complex events: irregular trajectories, inhomogeneity of dissolved carbon and salt concentrations, shear and light stress, among others. However, if cells are grown with an appropriate culture medium and are mixed in a homogeneous and random manner, it can be assumed that they are constantly moving from the reactor surface to the interior part of the vessel. Further, in dense cultures light is not normally leading to photoinhibition but is a limiting variable. In such cases, which are typical for large-scale photobioreactor facilities, it is reasonable to assume that there are two main factors to consider for optimal photosynthesis yields: light as energy input and CO₂ as carbon source. Additionally, in most of the studies performed on photosynthesis research, the Photosynthetically Active Radiation (PAR) value is indicated as reference input for the experiment. Though this is a practical way to show the influence of light on the studied biological mechanism, it does not allow a proper benchmarking with analogous experiments. This is due to the fact that not just the total incident light intensity but also the lamp emission characteristics, the cell concentration, the shape, the depth of the reactor and its previous photoacclimation (Kwon et al 2013) affect the real irradiance inside the culture vessel and henceforth photosynthesis, too. In addition, all photosynthetic processes are directly regulated by certain wavelengths: pigment light absorption, non-photochemical quenching or state transitions among other mechanisms are controlled by the spectral composition of light and consequently treating light as a simple value is not sufficient for unravelling the underlying mechanisms that control photosynthetic responses. For all these reasons, it was decided to apply the Inherent Optical Properties methodology to predict light quantity and quality in the cell suspension. The model uses optical properties obtained from experiments carried out by Ruhr-Universität Bochum research group, has been validated with literature data (Lea-Smith et al 2014) of wild-type and Olive strain and delivers reliable results on PAR and spectral light attenuation at different cell densities and LED lamps of several colours (ms). Finally, the light model was coupled with CO₂ gas-liquid mass transfer and cyanobacterial carbon fixation and concentration mechanisms to build up a simplified PBR model that predicts growth with light and carbon as main variables.

Future *in silico* work will seek the integration of detailed photosynthesis mechanisms as input to genome-scale metabolic models in order to further restrict the solution space towards more realistic metabolic landscapes. Special focus will be done on the impact of light on the cell then this is a challenge not solved yet in most modelling approaches and moreover, this effect is at the same time directly coupled with culture-level conditions. The advances in data gathering and statistical analysis are making possible rather complex mathematical models which will be able to predict the physiological evolution of main metabolic reporters, understand the resource allocation at any moment of the day and hence suggest operation guidelines for improving overall culture performance.

WP9 - Development of an efficient photobioreactor unit

The goal was the production of 5L and 100L flat panel photo bio reactors (FPBR) using an industrial standard, associated automated control of these reactors, as well as the evaluation of the up and down-stream process.

Development of the bioreactors. A flat panel photo bioreactor (FPBR) design was selected, since this type of reactor system offers a uniform illumination of all cells. Due to the used material (polymers) and the possibility to produce most of the parts by milling, the reactor

can be produced at a low cost in comparison to handmade or other saleable variants. 1L, 5L and 100L versions of the reactor were designed and constructed. The reactors are equipped with industrial standard connectors (G1/8", G1/4", Luer-Lock) to fit the need of most users and to be suitable for various applications (Figure 14).

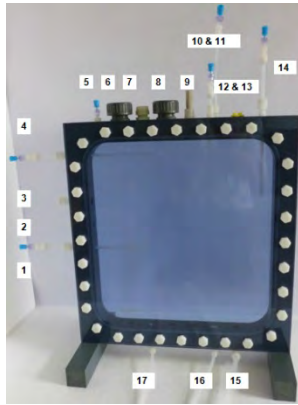


Table 2. Connectors at the 5 l FPBR

Pos.	Description	Pos.	Description
1	Sampling port G1/8"	10	Optional (antifoam)
2	OD-probe	11	Optional (feed)
3	Optional	12	Acid
4	Overflow	13	Base
5	Gas out port	14	Media feed
6	Optional	15	Gas inlet
7	pH probe	16	Drain port
8	Heater	17	Gas inlet
9	Additional probe		

Figure 14. Example of the 5L FPBR.

Development of the online control system. The development of an online control system to automate the process of cultivation was established by creating a computer program based on the LabView development environment in cooperation with the RUB. The program is designed to be freely adjustable to various application setups. Different parameters can be measured and several actors can be controlled by the Software (e.g. gas flow rate and mixture). This enables an automated continuous cultivation and can keep the culture in a constant state (e.g. constant growth rate) and therefore is a facilitator for reproducible experiments. The measured results are saved and can later be used for further evaluations. The software can also be used to simulate changes in the culture conditions (e.g. light intensity, temperature).

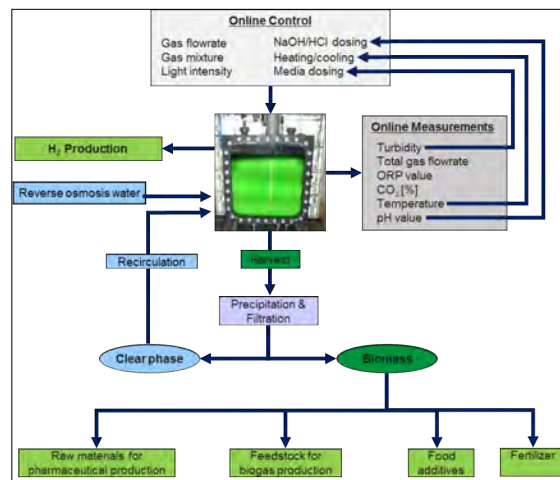
Sterilization and Neutralization. A new process for the chemical sterilization of the reactors has been developed in this project, after the sterilization process the liquid will be neutralized and remains in the reactor. After the neutralization process is finished the medium and culture are added directly into this solution. This new process uses organic acid to neutralize the peroxide solution, which is less harmful for the environment than previously used EDTA.

Down-Stream processing and recirculation. To reduce the cost and the amount of waste water during the cultivation process, a method to separate the biomass from the clear phase of the produced culture and recirculate the clear phase into the process was established. Similarly, up to 70% of the cost (www.nachhaltige-produktion.de) can be saved. For the separation process the precipitation by a precipitation agent was chosen, since this method was the most effective and energy efficient. It could be show that the recirculated clear phase does not negatively influence the cell growth.

Analysis of the clear phase. To evaluate the amounts of nutrients required to restore the original composition of the culture medium the concentrations of Iron, Phosphate, Zinc, Nitrate and Potassium were measured in the clear phase after precipitation. It could be show that Nitrate, Potassium and Phosphate have to be added after operation. Iron and Zinc stay approximately the same.

Figure 15. An overview of the complete process of cultivation and recirculation in the FPBR.

Evaluation of FPBR system on the basis of economical and ecological parameters. A cost analysis was conducted on the basis of economical and ecological parameters using a 1000L photo bioreactor as a pilot plant situated in a Germany.



Considering the prices for the needed consumables the costs for the preparation phase add up to 2,750 €. The nutrient solution represents the major cost factor in this stage of the operation process. Water savings amounting to 800 € could be realized with our neutralization process.

The costs for the operation phase add up to 48,000 € for a year with a total harvest of 87,500L. Major cost factors are the sterilization and neutralization processes. The highest costs are caused by the ingredients Peroxide and HEPES. Nevertheless, the neutralization process reduces wastewater and hence costs for wastewater treatment. On the basis of a 1000 litres FPBR the recirculation of the clear phase saves water costs of 31,500 € per year.

WP10 Assembly and performance assessment of a larger prototype photobioreactor system

Within the project *Cyanofactory*, a multiplate photobioreactor (hereafter MPL-PBR) has been designed, assembled and installed in the outdoor experimental area at CNR-ISE (Florence). The PBR system (working volume 1350 L) is composed of 20 vertical parallel plates (5-cm light path) in direct communication with each other. The inlet of the culture is achieved from the bottom part of the panel through two manifolds (i.d. 9 cm), while the outlet by two manifolds located in the upper part of the panels through which the cultures flow back to the degasser (Figure 16). Therefore, the cyanobacterial culture is circulated in a parallel mode (i.e., parallel independent compartments). In this way the culture mixing time (T_{mix}) of the reactor has been strongly reduced (2-min), and pH control, CO₂, nutrient supply, and O₂ degassing of the culture strongly improved.



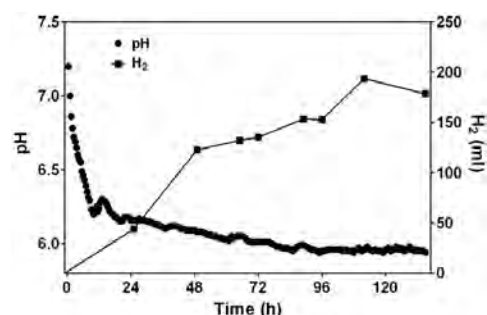
Figure 16. The 1350L MPL-PBR installed at the CNR-ISE outdoor experimental area (Florence, Italy).

1. Light Conversion Efficiency (LCE) attained with the Multiplate-Photobioreactor. The MPL- PBR set up with plate spacing of 0.5m, was able to intercept 96.7% of the light falling on the horizontal surface (10 m^2) in summer, while with a plate spacing of 1 m it captures 57% of the light reaching the horizontal surface. The corresponding biomass yield calculated on the basis of the ground area occupied by the reactor (10 m^2) was $25.2 \text{ g m}^{-2} \text{ day}^{-1}$ and $8.8 \text{ g m}^{-2} \text{ day}^{-1}$, respectively. The light conversion efficiency (LCE) was significantly higher in the configuration with plate spacing of 0.5 m, reaching 5,08 % on PAR basis (i.e., 2.2% on solar basis). However, in both cases the LCE was still almost 50% lower compared to that measured in the laboratory cultures. Most likely the cultures, especially the one grown in the PBR in 1m panel spacing configuration, may be been subjected to excessive light exposure. This hypothesis seems supported by the lower F_v/F_m and photosynthesis rate recorded during the day.

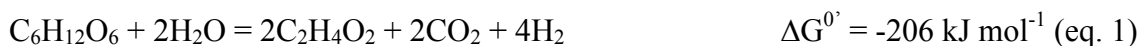
2. Hydrogen production outdoors. The simplest and most effective way to produce hydrogen photobiologically is the so-called direct biophotolysis, which involves direct transfer of electrons from water to hydrogenase. Unfortunately, oxygen is a strong inhibitor of hydrogenase, therefore *Synechocystis* mutants with oxygen-tolerant enzymes would be required, which were not available at this point of the project. Therefore, we followed the alternative approach, i.e. the indirect biophotolysis, which involves separation between water splitting reaction (aerobic phase) and hydrogen production (anaerobic phase). During the first phase, photosynthesis enables carbon dioxide fixation and accumulation of carbohydrates that will be the source of reducing power for hydrogen production.

Hydrogen production by *Synechocystis* PCC 6803, was carried out under natural light in a 50 litres tubular photobioreactor. The 50L PBR was chosen because its culture volume is the same as one single plate from the 1350L MPL-PBR. Experiments consisted of two steps, the first one involving carbohydrates accumulation under illumination (aerobic phase), and the second one involving hydrogen production in the dark (anaerobic phase). In order to achieve anaerobic conditions at the end of the carbohydrate accumulation, the culture was bubbled with pure N_2 gas for 30 min, and covered with a black sheet to prevent light penetration. Hydrogen release started immediately after degassing and continued steadily for five days (Figure 17). A maximum total output of $193.4 \text{ mL PBR}^{-1}$ was obtained. The highest hydrogen production rate achieved was $0.05 \text{ mL H}_2 \text{ L}^{-1} \text{ h}^{-1}$. Carbohydrate content decreased from 49.9 to 44.0% (of DW), while dry weight declined from 1.51 to 1.31 g L^{-1} , indicating that only a small amount (0.18 g L^{-1}) of carbohydrates were fermented (i.e., 8.93 g per 50 L reactor).

Figure 17. Hydrogen production (squares) and pH (circles) measurements of *Synechocystis* culture in a 50L PBR under dark anaerobic phase.



A mass balance between carbohydrate consumption and H_2 production indicated that during the anaerobiosis phase there was a decrease in concentration of these compounds of about 0.18 g L^{-1} , which for 50 litres of culture (i.e. the volume of the PBR) corresponded to a total amount of fermented carbohydrate of 8.93g. Fermentation of carbohydrates and hydrogen and acetic acid production occurs according the following reaction;



According to this reaction one should expect about 4.4 litres of hydrogen from the fermentation of 8.93g of carbohydrates, while the total amount produced was just 193.4ml (i.e., 178.3ml in the headspace plus 15.0mL dissolved in the culture medium). Therefore, only about 4.4% of the theoretical yield was attained. Several factors may account for this large gap. One of the most important factors is the existence of a bidirectional [NiFe]-hydrogenase which is expected to be more active as the dissolved H₂ concentration in the medium increases. In our case the partial pressure of hydrogen in the PBR reached 0.018 atm, although a large headspace was available. The rate achieved when the process was scaled up to 50L was lower, reaching 0.05mL H₂ L⁻¹ h⁻¹. However, in another experiment the maximum total amount of H₂ produced per PBR was 312ml (i.e., 285ml in the headspace plus 27mL dissolved in the culture medium). The maximum hydrogen production rate achieved in our experiments was 0.78mL H₂ L⁻¹ h⁻¹. This amount compares well with those produced by *Chalmydomonas reinhardtii* under sulfur starvation (Torzillo et al., 2014). This fact indicate that there is enough room to increase the output by optimizing the process.

Potential hydrogen production with the 1350 litre photobioreactor. With the data on hydrogen production gathered with the 50L PBR, we can predict the hydrogen output potential of this novel MPL-PBR design with reasonable accuracy. Using the indirect biophotolysis process, the hydrogen produced per cycle in the 50L PBR ranged between 193 ml and 312 ml; consequently, with the 1350 litres PBR (10 m² ground area), the H₂ produced should, *coeteris paribus*, range between 5.02 and 8.1 litres per cycle (usually 120 hours long). It must be noted that theoretically, according to eq. 1, from the fermentation of 234 g of carbohydrate (0.18 g L⁻¹ x 1300 L) in the flat-plate PBR, at least 115 litres of hydrogen should be produced. It is also possible to calculate the corresponding light conversion efficiency (LCE), assuming that production of carbohydrates was attained within 3 days of culture during which the average light amount was 16 MJ/m²/day, and assuming a 12.94 J/ml gross calorific value of H₂ (upper combustion value): LCE = [(12.94 J/ml 115.000 ml)/ 16.000.000 J/m²/day x 3days x 10 m²] x100 = 0.3% (solar basis).

The above calculation shows that the indirect process for hydrogen production in the same large 1350 litres MPL-PBR could, theoretically, attain a LCE only 7-fold lower than that reached experimentally and calculated on the production of biomass (LCE= 2.2 %, solar basis).

Several factors may account for such a low LCE. First of all, the amount of unfermented carbohydrates at the end of the process was extremely high. Indeed, the percentage of carbohydrates only went from 50% of the biomass at the start of the dark phase to 44% at the end of it. Since it has been frequently observed that the amount of carbohydrates in a the biochemical composition of cells grown in normal conditions ranges between 10-13 %%, it is reasonable to suppose that the carbohydrate content could drop to at least 13 % (i.e., from 50% to 13% of dry weight). This would correspond to a total amount of carbohydrates fermented of 0.642 g L⁻¹, that is, 832 g of carbohydrates in the whole PBR (i.e., 0.642 x 1350), for a corresponding 410 litres of H₂.

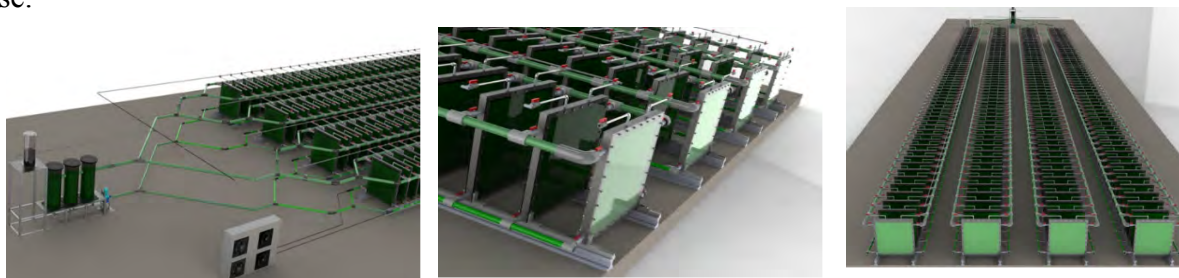
Light conversion efficiency on solar basis would then be: LCE=[(410 ml x 1000 x 12.94J/ml)]/[(16.000.000 J m⁻²/day x 3day x10 m²)] x100 = 1.1%.

Furthermore, in our experiments, reaching 50% of carbohydrate in the biomass required 3 days, in cultures with a starting residual concentration of nitrogen of about 100 mg/l, but a steep raise in carbohydrate content was observed after the nitrogen content dropped to zero. Therefore, if the accumulation of carbohydrate could begin in a nitrogen-depleted medium and as a result be achieved within 1-2 days of light exposure, the LCE would raise to 2.2% - 3.3% of solar light. Among the other factors which may still limit the carbohydrate fermentation process and the hydrogen production, two must be mentioned: the drop of pH due to the high amount of acetate released and the raise of the hydrogen concentration which is likely to activate the reverse reaction, leading to consumption of hydrogen produced.

In conclusion, although indirect biophotolysis is less effective than direct photolysis for hydrogen production, the possibility to achieve 3% of LCE should prompt to study the process in more detail and improve its overall yield and efficiency, employing more suitable strains as well as further optimizing the culture conditions.

Multiplate photobioreactor scale-up. An industrial plant of 100 kW was designed in a modular and scalable subdivision of autonomous sub-plants-modules optimized called "Large Scale Unit". The theoretical calculation of the plant 100 kW also indicated in the proposal was based on a theoretical yield of direct photolysis with photosynthetic efficiency PE= 5%, and therefore for a power of 100 kW the plant has a volume of 450.000 litres. The plant is modular and scalable to 450,000 litres divided into "Large Scale Unit" autonomous of 14,000 litres each, composed of 240 MPL-PBRs. Considering an efficiency of direct photolysis of 5% and the large scale unit from 14,000 litres (240 MPL-PBRs), the plant by 100Kw therefore requires NR.32 large scale unit (14,000x32 = 450,000 litres), then for a total number of MPL-PBRs 240x32=7,680 MPL-PBRs, and occupying an area of about 4500-5000m². However, considering the experiments carried out in the field which reported 3% LCE (using indirect photolysis), the volume will raise to 750,000 litres, the number of Large Scale Unit to 53, and the number MPL-PBRs: 240x53 = 12,720 MPL-PBRs, occupying an area: 7,500 m².

Figure 18. A 3D technical design representation of the scaled up MPL-PBR for commercial use.



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CyanoFactory - Potential impact

CyanoFactory is an example of “purpose driven” fundamental R&D with identified scientific goals which also creates a new basic technology. The emerging field - Custom design and purpose construction of microbial cells for the production of desired products using synthetic biology – goes well beyond conventional paths, is highly innovative, very ambitious, and has the potential for really high impact. A new technology that may well have a socio-economic impact on the European societies.

CyanoFactory addressed the **TECHNOLOGIES** for this science, specifically Synthetic biology (the technologies to design and construct microorganisms to produce a molecule/product of your choice, and to analyse and understand the engineered strain including modelling and predictions) and Advanced photobioreactors (the technology to grow engineered organisms efficiently and economically at large scale).

SYNTHETIC BIOLOGY HAS SOCIETAL IMPLICATIONS. One of the partners of CyanoFactory (Wünschiers, partner 2) recently published a book-chapter with the title “Making-of Synthetic Biology: The European CyanoFactory Research Consortium” in the book “Ambivalences of Creating Life: Societal and Philosophical Dimensions of Synthetic Biology”, a contribution towards the emerging discussion about Synthetic biology and how it will change our societies (full reference below). The author presents and discusses: CyanoFactory, Hydrogen as an energy carrier, Hydrogen production, Hydrogen from cyanobacteria, Challenges for the CyanoFactory work (Biological: DNA parts, Genome integration and Biosafety, and Metabolic design), and Conventional versus Synthetic biology;

Wünschiers R (2016) Making-of Synthetic Biology: The European CyanoFactory Research Consortium. In: Hagen K, Engelhard M & Toepfer G. Ambivalences of Creating Life: Societal and Philosophical Dimensions of Synthetic Biology, pages 55-72. Springer-Verlag Berlin Heidelberg. doi: 10.1007/978-3-319-21088-9_3.

PATENT. A Portuguese Provisional Patent Application (Process #PT108564) with the title “A construction, a vector and a method production of a target molecule in a cyanobacterium” has been filed (partner 3, IBMC, Portugal) as result of CyanoFactory science. In popular words the patent covers the identification and characterization of so called neutral sites, physical places in the cyanobacterial chromosome where genetic elements/constructs (DNA) preferentially can be introduced.

Within almost all workpackages there are possible to identify achievements and/or **RESULTS THAT MAY BE EXPLOITED:**

WP1 ToolBox for cyanobacterial synthetic biology. Exploitable items: Genetic parts developed for advanced genetic engineering (synthetic biology) and/or techniques used to implement them may be patented. See also the provisional patent application (PT108564) described above.

WP2 DataWarehouse/Bioinformatics. Exploitable items: Web-based software developed to e.g. model a cyanobacterial cell under different conditions, cyanobacterial metabolism and/or used to predict changes and outcomes when modifications are introduced may be provided free of charge or for a fee.

WP3 Improvement of chassis growth, functionality and robustness. Exploitable items: Purpose designed and engineered strains for functionality and robustness may be patented. See also the provisional patent application (PT108564) described above.

WP4 Introduction of custom designed hydrogen producing units. Exploitable items: Novel enzymes/hydrogenases and purpose designed and engineered strains producing a specific product may be patented.

WP5 Improvement of photosynthetic efficiency towards H₂-production. Exploitable items: Novel, engineered catalysts/proteins with improved efficiency may be patented.

WP6 Biosafety. Exploitable items: Developed genetic constructs encoding a biosafety mechanisms (kill-switches) may be patented.

WP8 Metabolic modelling of the engineered cells. Exploitable items: Web-based software developed to e.g. model a cyanobacterial cell under different conditions, cyanobacterial metabolism and/or used to predict changes and outcomes when modifications are introduced may be provided free of charge or for a fee.

WP9 Development of an efficient photobioreactor (PBR) unit. Exploitable items: Development of a PBR unit, or parts thereof, or methods/techniques to run the system may be patented. PBR units may be commercially sold. In fact, KSD (in charge of WP9) has already manufactured a number PBR units and distributed them for a very low cost to different partners of CyanoFactory, and to some external buyers to a much higher price.

WP10 Assembly and performance assessment of a larger prototype flat panel photobioreactor System. Exploitable items: The novel design of the photobioreactor designed and constructed within the CyanoFactory will be used for further international and national projects dealing with the production of bioenergy from microalgae, and for exploiting them for the production of molecules for pharma, nutraceutical, food additives and cosmetic. The photobioreactor can also be used for microalgal biomass production on demand by private and public institutions. In addition, the development of a flat panel photobioreactor system, or parts thereof, or methods/techniques to run the system may be patented.

PROGRESSION. Several of the PIs scientific activities and achievements have been noticed by others internationally and based on their scientific skills within synthetic biology and/or advanced photobioreactors they been invited to participate as partners in applications including EU supported projects. For instance, the coordinator is a partner in the recently started Horizon2020-Energy project Photofuel, coordinated by VW, where the synthetic biology tools developed in CyanoFactory are used to engineer cyanobacteria to produce future transportation fuels.

WEB-SITE AND WEB-AGGREGATES. Dissemination included a publically available web-site (www.cyanofactory) with two integrated publically available, updated and maintained Web-aggregates covering Algal biofuels (www.scoop.it/t/algal-biofuels) and Synthetic biology (www.scoop.it/t/synthetic-biology), respectively. The statistics for the Algal biofuels aggregate is - as of November 30, 2015 - 382 scoops (news posts) with 51 followers, 39 reactions (like 'thank you' replies, forwards etc.) and 965 unique visitors. The number of views was 1900 (on average, each visitor opened ~2 posts). For Synthetic biology aggregate we had in total 382 scoops, 274 followers, 207 reactions and 1947 unique visitors. The total number of views was 4900 (average 2.5 reads per visitor). All together, including readers of re-scoops (i.e. somebody else copied/pasted this news on his/her site), we had 8200 views (number of views is rounded to the nearest 100). Visits continue to grow, for Synthetic biology, the number today is 2042 unique visitors with 5000 views while for Algal biofuels it is 985 unique visitors and 2000 views.

TWO WORKSHOPS. Two dedicated CyanoFactory workhops with two full days of CyanoFactory science by the partners, followed by one day of presentations and discussions by invited internationally leading scientists in the field of CyanoFactory science were organised in Porto (Portugal; November 18-20, 2013; www.ibmc.up.pt/cyanofactory/) and Ljubljana (Slovenia; December 8-10, 2014; http://wiki.fkkt.uni-lj.si/index.php/CyanoFactory2014_programme), respectively.

The first CyanoFactory workshop in Porto included lecturers Koji Sode (Japan) coordinator of the project Cyanofactory in Japan, Patrik Jones (Finland) coordinator of the EU project DirectFuel, Klaas Hellingwerf (The Netherlands) member of the management team of a major Dutch initiative in research in solar energy: "Towards BioSolar Cells", and had the participation from young researchers from all partners and students from Master courses.

The second CyanoFactory workshop in Ljubljana included lecturers Koji Sode (Japan) coordinator of the project Cyanofactory in Japan, Kenneth Reardon (USA) coordinator of US project on Integrated design of cyanobacterial biorefineries, Koichi Abe (Japan) expert on cyanobacterial riboregulators, Lei Pei (Austria) addressing novel approaches to improve biocontainment, and had the participation from young researchers from all partners and students from Master courses.

Additionally, all PIs and CyanoFactory involved younger researchers met regularly for CyanoFactory project meetings.

TWO CYANOFACORY PROJECTS ! The coordinator, together with some additional CyanoFactory PIs, were invited and participated in the yearly meetings of the Japanese Cyanofactory project (similar content as the European CyanoFactory). The coordinator of the Japanese Cyanofactory project (Professor Koji Sode, and colleagues, at Tokyo University of Agriculture and Technology) participated in the workshops and some project meetings of the European CyanoFactory project.

MID-TERM REVIEW. A mid-term review was introduced by the commission and was held September 30 – October 1 (2014), in Firenze (Italy), the site for the larger (1350 liter) outdoor flat panel photobioreactor system. The outcome was a very fruitful discussion about the progress, problems and pathways forward.

In addition, all senior scientists presented and discussed CyanoFactory science at numerous national and international meetings and published results in internationally recognised journals.

INVITED LECTURES / DISSEMINATION TOWARDS A SCIENTIFIC AUDIANCE

Oral communications

Tamagnini P (2015) “Tools for the use of *Synechocystis* sp. PCC 6803 as a photoautotrophic chassis.” 2nd International Workshop of Cyanofactory, 5-6/3/2015, Tóquio, Japão.

Tamagnini P (2014) “Strategies to improve the robustness and functionality of a cyanobacterial chassis based on *Synechocystis* sp. PCC 6803.” 1st International Workshop of Cyanofactory, 6-7/3/2014, Tóquio, Japão.

Tamagnini P (2013) “Preparation of a photoautotrophic chassis based on the cyanobacterium *Synechocystis* sp. PCC 6803 and construction of synthetic devices.” 25/10/2013. Workshop on cyanobacteria as a biotechnological model of autotrophic organisms, Department of Agrifood Production and Environmental Sciences, Universidade de Florença, Itália.

BioH₂ 2013 August 5th – 7th, 2013 Montreal Canada. Recent Advances in the Biotechnology of Hydrogen Production with the Microalga *Chlamydomonas reinhardtii*. Giuseppe Torzillo.

5th Congress of the International Society for Applied Phycology 2014- Australian Technology Park, Sydney, June 22 - 27, 2014. G Torzillo.

2nd International Workshop of *Cyanofactory* March 5-6, 2015, Tokyo, Japan. “Biotechnology of a mass cultivation of *Synechocystis* PCC 6803 in photobioreactors”. G Torzillo.

1st International Solar Fuels Conference. April 26-May 1, 2015, Uppsala, Sweden. “Biological constraints in the scale-up of photobiological hydrogen production”. G Torzillo.

Siurana, M., Reynoso-Meza, G., Montagud, A., Conejero, J.A., Gamermann, D., Sanchis, J., Fernández de Córdoba, P. & Urchueguia, J.F. *Multiobjective evolutionary algorithm for genome-scale flux simulation*. 1st International Solar Fuels Conference (ISF1). Uppsala (Sweden), 2015. <http://www.solarfuel.se/meetings/>

Fuente, D. & Urchueguia J.F. *Study of CO₂ availability in photobioreactors on *Synechocystis* cultures carbon fixation and hydrogen production*. 1st International Solar Fuels Conference (ISF1). Uppsala (Sweden), 2015. <http://www.solarfuel.se/meetings/>

Metabolic engineering of cyanobacteria for the production of hydrogen from water. 03'14. Tokyo University. M. Rögner. Seminar (invited talk)

Using photosynthetic efficiency for custom-designed reactions. 10'14. DENSO München. Rögner. Invited talk.

Metabolic engineering of cyanobacteria for the production of hydrogen from water. 11'14. KAIST Daejeon/Korea. Rögner. AOAI 2014 (invited talk)

Design of photosynthetic light energy transformation in cyanobacteria: Balance between survival and benefit. 01'15. Zürich/Switzerland. Rögner. Univ. Zürich (invited talk)

Engineering of light harvesting in cyanobacterial design cells and metabolic impact. 03'15. Atami/Japan. Rögner. Seminar (invited talk)

Engineering of photosynthesis-based hydrogen production. 03'15. Univ. Kobe/Japan. Rögner. Seminar (invited talk)

Rational design of cyanobacteria for hydrogen production. Berlin. 03'15. Rexroth. Annual Meeting of DPG (invited talk)

Rational design of cyanobacteria for hydrogen production. Uppsala/Sweden. 05'15. ISF-1 conference Rexroth. Annual Meeting of DPG (invited talk)

Engineering cyanobact. photosynthesis for biotechnological applications: Design cells & model systems. 10'15. Nara, Japan. Yamada Conf. Rögner. Invited talk

Engineering cyanobact. photosynthesis for biotechnological applications: Design cells & model systems. 10'15. Gyeongsang Natl. Univ., Jinju/Korea. Rögner. Seminar (invited talk)

Parameters of cyanobacterial photosynthetic electron transport and their potential applications. 10'15. Evang. Akademie Tutzing. German-chinese PS-meeting (invited talk).

Design, engineering, and construction of photosynthetic microbial cell factories for direct solar fuel production. Korea – Sweden Excellence seminar, 2015. Seoul, Korea. April 22, 2015. Keynote speaker.

CyanoFactory: A European project to design, construct and demonstrate solar biofuel production using novel (photo)synthetic cell factories. International cyanobacteria meeting. Tokyo University of Agriculture and Technology, Tokyo (Japan). March 5, 2015. Invited lecture.

Engineering of cyanobacteria for the direct production of solar fuels. Departmental seminar. January 9, 2015. Chulalongkorn University, Bangkok (Thailand). Invited speaker.

Design, engineering, and construction of photosynthetic microbial cell factories for direct solar fuel production. Departmental seminar. October 28, 2014. Key Laboratory of Biofuels, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao (PR China). Invited speaker.

Design, engineering, and construction of photosynthetic microbial cell factories for direct solar fuel production. International Symposium on Synthetic biology. October 27, 2014. Beijing University of Chemical technology (PR China). Invited speaker.

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. Departmental seminar. October 23, 2014. Swedish University of Agricultural Sciences, Uppsala (Sweden). Invited oral presentation.

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. Departmental seminar. October 14, 2014. Wageningen University (The Netherlands). Invited oral presentation.

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. Workshop on algal biotechnology. September 22, 2014. Bielefeld (Germany). Invited oral presentation.

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. European Workshop on the Molecular Biology of Cyanobacteria. September 10, 2014. Den Helder (The Netherlands). Invited speaker.

Promising technologies that will decouple transport fuels from biomass and produce new alternative fuels. June 27, 2014. EU Industry grouping on Biomass. Brussels (Belgium). Invited speaker.

Design, engineering, and construction of photosynthetic microbial cell factories for direct solar fuel production. June 9, 2014. KAUST, Tuwal (Saudi Arabia). Invited key-note speaker. CyanoFactory Europe, Present status and future perspectives. International Workshop of *CyanoFactory*. March 6-7, 2014. Tokyo University of Agriculture, Japan. Invited speaker.

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production. International BioH₂ Conference. Montreal (Canada), 2013-08-06. Oral presentation

Metabolic and genetic engineering of cyanobacteria for enhanced hydrogen production – A promising technology that will decouple transport fuels from biomass and produce new alternative fuel. 10th International Hydrogenase Conference. Szeged (Hungary). 2013-07-12. Invited key-note lecture

Promising technologies that will decouple transport fuels from biomass and produce new alternative fuels. European Industrial Bioenergy Initiative (EIBI) Conference as part of the European Union Sustainable Energy Week. Brussels (Belgium). 2013-06-26. Invited speaker

Design, Engineering, and Construction of Photosynthetic Microbial Cell Factories for Renewable Solar Fuel Production. PhD Research School. Ljubljana (Slovenia). 2013-03-06. Invited lecture

Pinto F (2015) “Hydrogen production using cyanobacteria” in the course Environmental Biotechnology of the Master in Ecology, Environment and Territory (FCUP) and Master in Bioengineering (FEUP) and at the Department of Biology, Faculty of Sciences, University of Porto. March 19th.

Pinto F (2014) “Hydrogen production using cyanobacteria” in the course Environmental Biotechnology of the Master in Ecology, Environment and Territory (FCUP) and Master in Bioengineering (FEUP) and at the Department of Biology, Faculty of Sciences, University of Porto. May 19th.

Pinto F (2013) “Hydrogen production using cyanobacteria” in the course Environmental Biotechnology of the Master in Biology and the Master in Environmental Sciences and

Technologies and Master in Ecology, Environment and Territory at the Department of Biology, Faculty of Sciences, University of Porto. May 3rd.

Posters

Oliveira P, Martins NM, Santos M, Pinto F, Büttel Z, Couto NAS, Wright PC & Tamagnini P "From protein secretion mechanisms to membrane vesicles in cyanobacteria." 1st Iberian Meeting on Extracellular Vesicles, Porto, Portugal, 30th September 2015.

Oliveira P, Martins NM, Santos M, Pinto F, Büttel Z, Couto NAS, Wright PC & Tamagnini P "Protein secretion mechanisms in the cyanobacterium *Synechocystis* sp. PCC 6803." 15th International Symposium on Photosynthetic Prokaryotes, Tübingen, Germany, 2nd-6th August 2015.

Pacheco CC, Pinto F, Oliveira P, Ferreira E, Pereira J & Tamagnini P "Tools and devices for the use of *Synechocystis* sp. PCC 6803 as a photoautotrophic chassis" 15th International Symposium on Photosynthetic Prokaryotes, Tübingen, Germany, 2nd-6th August 2015.

Pinto F, Pacheco CC, Oliveira P, Montagud A, Landels A, Couto N, Wright PC, Urchueguía JF & Tamagnini P "Improving a *Synechocystis*-based photoautotrophic chassis through systematic genome mapping and validation of neutral sites" Gordon Research Conference - Synthetic Biology Advancing Biosystems Design, Sunday River, ME, USA, 28th June-3rd July 2015.

Pinto F, Pacheco CC, Oliveira P, Ferreira E & Tamagnini P "Improving *Synechocystis* sp. PCC 6803 towards a more functional and robust chassis" I3S 4th Annual Meeting, Póvoa de Varzim, Portugal, 30th-31th October 2014.

Pinto F, Pacheco CC, Oliveira P, Ferreira E, Tamagnini P "Improving *Synechocystis* sp. PCC 6803 towards a more functional and robust photoautotrophic chassis" 9th European Workshop on Molecular Biology of Cyanobacteria, Texel, Netherlands, 7th-11th September 2014.

Oliveira P, Pinto F, Pacheco CC, Mota R, Tamagnini P "HepF, an exoprotein required for *Anabaena* sp. PCC 7120 filaments adhesion and aggregation" 9th European Workshop on Molecular Biology of Cyanobacteria, Texel, Netherlands, 7th-11th September 2014.

Pinto F, Pacheco CC, Oliveira P, Montagud A, Urchueguía JF, Tamagnini P "Characterization of neutral sites to use *Synechocystis* sp. PCC 6803 as a photoautotrophic chassis." SB6.0 The 6th International meeting on Synthetic Biology, London, UK, 9th-11th July 2013.

Pinto F, Pacheco CC, Büttel Z, Oliveira P, Montagud A, Urchueguía JF, Tamagnini P "Exploiting *Synechocystis* sp. PCC 6803 as a photoautotrophic chassis for Synthetic Biology applications". Gordon Research Conference - Synthetic Biology (Re-)constructing and Re-programming Life. West Dover, VT, USA, 9th-14th June 2013.

1st International Solar Fuels Conference. April 26-May 1, 2015, Uppsala, Sweden. "A bioenergetic assessment of photosynthetic growth of *Synechocystis* PCC 6803 in continuous cultures". E Touloupakis, B Cicchi, G Torzillo.

Phototech 2015: Towards a photosynthesis-biobased economy, October 7-9, 2015 Rome, Italy. “Scale-up of cyanobacterial cultures for photobiological hydrogen production” E. Touloupakis and Giuseppe Torzillo.

Kind, G., Montagud, A., Siurana, M., Nina, V. M., Zuchantke, E., Fuente, D., Conejero, J.A., Triana, J., Fernández de Córdoba, P., Urchueguia, J.F. & Wünschiers R. *CyanoFactoryKB – An open-source web-based software program for constructing model organism databases for Synechocystis sp. PCC 6803*. XII Symposium on Bioinformatics. Sevilla (Spain), 2014. <http://www.bioinformaticsconference2014.org/>

Reyes, R., Siurana, M., Gamermann, D., Montagud, A., Triana, J., Jaime, R., Nina, V.M., Fuente, D., Pacheco, Y., Urchueguía, J. F. & Fernández de Córdoba, P. *COPABI: a Computational Platform for Automation on the Genome-Scale Metabolic Models Reconstruction*. XII Symposium on Bioinformatics. Sevilla (Spain), 2014. <http://www.bioinformaticsconference2014.org/>

Siurana, M., Montagud, A., Urchueguia, J.F. *Synechocystis metabolic modelling and production strategies assessment*. 1st International Solar Fuels Conference (ISF1). Uppsala (Sweden), 2015. <http://www.solarfuel.se/meetings/>

Kind, G., Montagud, A., Zuchantke, E., Siurana, M., Conejero, J.A., Fernández de Córdoba, P., Wünschiers & R., Urchueguia, J.F. *CyanoDesign - A web-based tool for the generation and analysis of in silico mutants for Synechocystis sp. PCC 6803*. 1st International Solar Fuels Conference (ISF1). Uppsala (Sweden), 2015. <http://www.solarfuel.se/meetings/>

Kind, G., Zuchantke, E., Siurana, M., Conejero, J.A., Urchueguia, J.F. & Wünschiers R. *CyanoFactory KB - An open-source web-based software for constructing model organism databases for Synechocystis sp. PCC 6803*. 1st International Solar Fuels Conference (ISF1). Uppsala (Sweden), 2015. <http://www.solarfuel.se/meetings/>

CyanoDesign – A web-based tool for the generation and analysis of in silico mutants for Synechocystis sp. PCC 6803. G Kind, A Montagud, E Zuchantke, M Siurana, JA Conejero, P Fernández de Córdoba, R Wünschiers & JF Urchueguía. 1st International Solar Fuels Conference. Uppsala, Sweden, April 26 to Mai 1, 2015

Protein Interaction Network Analysis and Visualization of Synechocystis sp. PCC 6803. E Zuchantke, G Kind & R Wünschiers. 16. Nachwuchswissenschaftlerkonferenz. Berlin, Germany, April 16, 2015

A Web-Based Knowledge, Modelling & Visualization Base. G Kind, E Zuchantke & R Wünschiers. Joint Conference of the Association for General and Applied Microbiology (VAAM) and the Society of Hygiene and Microbiology (DGHM). Dresden, Germany, October 05-08, 2014

CyanoFactoryKB – An open-source web-based software program for constructing model organism databases for Synechocystis sp. PCC 6803 G Kind, A Montagud, M Siurana, VM Nina, E Zuchantke, D Fuente, JA Conejero, J Triana, P Fernández de Córdoba, JF

Urchueguia, R Wünschiers. XII Symposium on Bioinformatics. Sevilla, Spain, September 21-24, 2014

CyanoFactory Knowledge WebBase – A Modelling & Visualization Tool. G Kind, E Zuchantke & R Wünschiers. 11th Horizons in Molecular Biology. Göttingen, Germany, September 15-18, 2014

CyanoFactory: An Emerging Technology for Solar Fuel: The Knowledge Base. G Kind, E Zuchantke & R Wünschiers. Saxon Biotechnology Symposium 2014. Dresden, Germany, March 19th 2014

Participation in Round tables discussions

Tamagnini P (2013) I AEICBAS Biomedical Congress, participação na mesa redonda *Uso e Abuso de Recursos Naturais* – tema Biologia Sintética 5-7/4/2013, Porto, Portugal.

DISSEMINATION TOWARDS THE GENERAL PUBLIC

In 2015, CC Pacheco was invited speaker in the course “Biotecnologia às cores” meant for high school teachers.

CC Pacheco and F Pinto are also part of “Embaixadores da Ciência”, a group of IBMC/INEB researchers engaged in the dissemination of science in schools.

Alger, framtidens producenter av bränslen. Svenska Naturskyddsföreningen. Uppsala (Sweden). 2013-01-16. Lecture

FAIRS where CyanoFactory was presented and discussed:

Biotechnica 2015 (Hannover, Germany, October 6-8, 2015) Solargestützte Erzeugung von Wasserstoff und Biogas (PI: Wünschiers, partner 2)

Analytica 2014 (Munich, Germany, April 1-4, 2014)
Solargestützte Erzeugung von Wasserstoff und Biogas (PI: Wünschiers)

Biotechnica 2013 (Hannover, Germany, October 8-11, 2013)
Solargestützte Erzeugung von Wasserstoff und Biogas (PI: Wünschiers)