

Executive Summary:

The development of Synthetic Biology (SB) in Europe faces three major obstacles that have, up to date, hampered the development of an SB-based framework in our continent: [i] The new field is still missing a comprehensive language and a shared conceptual framework for the description of minimally functional biological; [ii] scientists and technicians, particularly in Europe, have so far failed to recognize their latent capacity to shape a brand-new discipline within their very scope; and [iii] promotion of SB might touch upon social sensitivities related to recreating "life-in-the-test-tube", which threatens a re-enactment of the controversy stirred up by GMOs. TARPOL led by the Universitat de València and composed of a total of 18 partners (<http://www.sb-tarpol.eu/>), has developed a dynamic two-year program of activities, run by a large collection of European stakeholders in the field and aimed at coordinating the so-far fragmented efforts to channel this emerging discipline into the most industrially beneficial and socially viable directions. TARPOL has been successful in recruiting the necessary environmental competences from neighbouring disciplines and developing numerous material and computational resources for advanced refactoring of biological systems. Furthermore, TARPOL has progressed in laying the foundations of SB, particularly on genome reduction strategies including a novel view of living organisms as information traps and contributed with dozens of basic, conceptual and applied publications to the development of this new field. The TARPOL consortium has paid particular attention to education, and a range of conferences, dissemination and training activities have been organized, including the support to a successful iGEM team and the organization of two intensive SB Summer Schools in Valencia (Spain) and Basel (Switzerland) in Spring and Summer 2010, respectively.

Mobilization, interdisciplinary collaboration and training: the objectives of TARPOL concerning SB converge into a single goal: to set up this brand-new discipline which may have revolutionary effects on our lives, in Europe. TARPOL has significantly contributed to such objectives as a key step to implementing a powerful, sustainable knowledge-based bio-economy in our old continent.

Project Context and Objectives:

The basic premise of SB is that methods commonly used to design and construct non-biological systems, such as those employed in the computational sciences and the engineering disciplines, could also be used to model and program novel synthetic biological systems. SB is thus intrinsically transdisciplinary and draws expertise from Biology, Chemistry, Physics, Computer Science, Mathematics and Engineering. Synthetic biologists are attempting to develop 'artificial life', for both its tremendous applications in biotechnology and as a proxy for shedding light into the question of the origins of life. This is attempted by following two separate and competing routes: the 'top-down' and 'bottom-up' approaches to minimal cells. In the former, a primordial or minimal cell is generated by systematically reducing a biological cell's genome until it no longer functions (Glass et al., 2006; Lartigue et al., 2007). The bottom-up methodology, on the other hand, seeks to assemble from scratch components or information units until an aspect of life emerges (Bedau, 2003). The overall intellectual and experimental challenges of implementing artificial life remain relatively long-term goals. However, along the way, guiding principles, experimental methodologies and theoretical insights from Biomimetic Chemistry and SB can be adopted in new ways for practical applications on a realistic, yet not necessarily immediate, time-frame.

Based on system biology (SB) achievements, there is a possibility of building a living being, or a part of it, which either already exists or is a non-natural entity. This particular view on SB represents a very promising applied field to areas as different as biomedicine, bioenergy, environment, etc. This type of program requires a serious reflection on the acquired responsibilities, as a consequence of the new natures that we, humans, may create. In addition, synthetic biology demands a philosophical thinking on the panoply of futures that, more than ever, are close to man-made reality.

The main objective of TARPOL is to catalyze the shaping of the European SB Community for translating this new conceptual and technical field into relevant environmental applications. This general goal comprises several specific objectives, including:

- To foster the transfer of SB conceptual fields into technical applications directed to solve environmental problems.
- To brand the conceptual and material interfaces between the various disciplines required for the surfacing and establishment of a European community on SB focused on tackling, monitoring and preventing environmental problems.
- To empower the SB-Environmental Biotechnology field with material, technical and web based resources.
- To ensure that all research areas of SB are coordinated at the European level, so there is a maximum exchange of knowledge.
- To enable the development of a conceptual frame in which ethical and safety aspects of the novel SB-related technologies, especially those related with environmental applications, can be optimally integrated into an emerging community.
- To promote the scientific training and specialization on SB, especially among scientists in early stages of their careers.

- To identify European R&D needs and priorities on SB and to make recommendations for future cooperation areas and innovative research activities to be launched in the EU.
- To create awareness at the academic, industry, social, policy and decision-makers level on the economic and scientific potential of SB for environmental applications.

Project Results:

We strongly believe that TARPOL project has led to important advances in the conceptual field of SB. For instance, the state of the art of cell engineering in the context of genome research has been reviewed by UVEG during the first year, paying particular attention to what has been learned on naturally reduced genomes from either symbiotic or free living bacteria. Also by UVEG, the identification of a genomic core for a cyanobacterium (*Synechococcus elongatus*) was accomplished. This analysis will set the basis to designing a minimal photoautotrophic system suitable for a plethora of biotechnological applications. During the whole project, as the leading partner, UVEG focused on the general coordination of the project, management, dissemination activities, and conceptual frame and consensual language definitions. The latter task has been broadly covered with a range of peer-reviewed contributions on the definition of the minimal genetic array for life to exist; the evolution of prokaryote-animal symbiosis; and theoretical limitations of synthetic life. Examples of this production are a review paper dealing with the milestones and challenges of synthetic biologists, coauthored by several TARPOL partners and coordinated by UVEG, which is currently under revision, several book chapters on minimal cells, and a myriad of publications on the origin of life, ethics of teaching evolution and a range of conceptual aspects of SB (see publications).

Dissemination and training activities organized by UVEG were key points of the project, with activities including a Summer Course on SB held in Valencia on April 2010 and the international Meeting on Synthetic and Streamlined Genomes. Thinking of Synthetic Biology (SB) as a new field of technology in the intersection of biology and engineering, the importance of attracting and training new researchers in this discipline becomes evident. With this objective, the Cavanilles Institute (UVEG) and the Intertech Group (UPVLC), co-organized the above-mentioned Summer Course on Synthetic Biology, with several TARPOL partners as speakers, as a pioneer international SB course with a clear focus on young researchers. This activity was developed from two sides: training and motivational. For this reason, the program offered the combined expertise of the organizers to train scientists from an interdisciplinary perspective, in order to offer a range of activities for young researchers from a wide range of fields. The Summer Course on Synthetic Biology also included lectures by leading scientists of this new discipline on the constitutive principles underlying SB, which allowed attendees to see and understand the great possibilities of this new scientific area.

Regarding the Workshop on Streamlined and Synthetic Genomes, it was organized as part of the TARPOL and EMERGENCE Programs to promote Synthetic Biology in Europe, and was held in Valencia, Spain, in November 16-17, 2009. The workshop joined world-wide recognized experts, including Nobel laureate Hamilton Smith or Luis Serrano. The objectives of this two-day workshop were to assess the status, identify constraints, and discuss the potential of research on minimal/streamlined genomes from different perspectives. This included the minimal and sufficient features of life, the study of naturally evolved reduced genomes, the engineering of minimal cells from bottom-up and top-down approaches, as well as various practical applications derived from research on minimal living systems. This event was supported, among others, by the Cavanilles Institute on Biodiversity and Evolutionary Biology (University of València,

www.uv.es/~biodiver/e/index.htm), and Centre for Public Health Research CSISP (València, www.csisp.gva.es/web/csisp/home). The workshop consisted of four sessions:

Synthetic and Digital Biology, chaired by V. Martins dos Santos; Streamlining Genomes, chaired by I. Economidis; Building Genomes, chaired by G. Posfai; and Circuit Design and Evolution, chaired by V. de Lorenzo.

A Satellite Meeting on Insect Symbiosis in the Era of Systems and Synthetic Biology was held on November 18, 2009, also in Valencia, and allowed many of the workshop attendants to join the community of synthetic biologists and discuss topics at the very interphase between these closely related disciplines.

Besides meetings, formation and training efforts, UVEG-led initiatives also served as a forum on which TARPOL partners had the opportunity to discuss and prepare further research projects. For example, members of UVEG and CSIC attended the BBSRC meeting of the UK networks on Synthetic Biology at Costwold (UK). We also participated in a course held at Spetses (Greece) on September 2010 focusing on how Bioscience will generate major advances in our understanding of how molecular systems can support all of the properties associated with living organisms. The key to progress here was concluded to be to increase the number of significant inputs from the physical sciences, engineering, computation and mathematics, leading to powerful new quantitative and precise methods of analysis and far deeper insight into the fundamental principles of living systems. In the introductory parts of the course, it was explored how one can build up a comprehensive picture of a living system starting with principles of macromolecular structure and function, how molecules in living cells self-organise, how macromolecules assemble to form complexes, pathways and subcellular structures, how they function in pathways, how these are all networked together, and how they are controlled and regulated. Emphasis was placed on the full range of quantitative techniques required to study biosystems. Different approaches to capturing the kinetics of pathways using mathematical representations such as biochemical systems theory and metabolic control analysis were presented, and the analysis of rate control and regulation discussed. Particular consideration was given to the challenges for modelling posed by gene expression systems and macromolecular assembly pathways. Following on from this, in the sessions on synthetic biology, it was shown how the design, modelling, construction and testing of man-made biomolecular systems can be developed from a thorough understanding of naturally evolved biomolecular systems. This unique course was of value to talented young PhD students and postdocs who are keen to engage with the exciting opportunities provided by these burgeoning interdisciplinary areas of research. We like to think that the course was informative, challenging, exciting and thought-provoking. More information is available here: <http://www.mib.ac.uk/spetses2010/index.html>.

In addition to courses and meetings, the interaction between TARPOL members also resulted in the germ of -hopefully- new European Research Projects. Organizational meetings took place in Kolymbari (Greece) and Vienna in order to set-up a promising SB-based engineering of endosymbionts with medical applications.

And finally, in the intersection among research, collaboration, training and dissemination, a TARPOL-linked iGEM team (supervised by UVEG and UPVLC members), the Valencia team, attended during 2009 and 2010 the international Genetic Engineered Machine competition and recently got, in 2010, a Gold medal with an innovative project on the implementation of a prion-based control circuit for tuning the Martian climate. The project was entitled "Mad yeasts on Mars?" and under this curious title it is hidden the ambitious Project of the Valencia iGEM team in which the students presented an intermediate scenario in the pathway towards the terraforming of Mars (i. e., modifying the atmosphere and temperature of Mars in order to get the appropriate conditions to make it habitable for Terran living organisms). The idea is that, after preliminary changes devoted to make Mars conditions more suitable for life, it can be colonized by microorganisms that will accelerate some changes to make the planet conditions acceptable for plant life which, then, will be able to generate enough oxygen to eventually allow the colonization by animals, including humans. The success of the Valencia team in 2009 was even more important, with Best New Application, Best experimental Measurement and Second-Runner Up awards. The project also included a Human Practices report with the largest inquiry on SB ever made, with more than 1000 interviews (http://2009.igem.org/wiki/images/0/0d/Sins_Ethics_and_Biology.pdf). The iGEM 2009 research project, describing the first biological lightning display with aequorin-expressing yeasts as living pixels reported (<http://2009.igem.org/Team:Valencia>; <http://en.wikipedia.org/wiki/iLCD>) had, besides the recognition of the iGEM organization Committee, an unprecedented impact on international media, including many broadcast and TV news, and a plethora of articles on general journals, among which a mention in The New York Times Magazine as "a real breakthrough" (<http://www.nytimes.com/2010/02/14/magazine/14Biology-t.html?ref=global-home>). Besides the Valencia team contribution, it has to be noted the overwhelming success of European teams in the competition (5 out of six finalists and 6/6 in 2010 and 2009, respectively, were European), which demonstrates that the combined effort of European Universities and Research Centres may be able to change the fate of the geographic location of the excellence core on Synthetic Biology.

iGEM might be called THE major educational success in the area of synthetic biology. In our estimate, the following elements in iGEM together provide an essentially "irresistible" attraction to students (as can be witnessed by the appr. 130 teams which participated in iGEM2010, again a substantial increase over the number of teams of the year before. Appr. one third of the teams were from Europe). Given the fact that iGEM projects are a rather expensive form of education (lab work, overseas travel and accommodation for entire teams) that does not (yet) mix well with more traditional educational programs in the life sciences, the TARPOL consortium suggests that the EU commission takes the funding of iGEM projects under consideration under the auspices of its research or education funding schemes.

As a partner, UVEG has focused on training and theoretical development. But it is obvious that, in addition to the theoretical framework aiming at defining how a basic artificial cell should be, a real chassis is needed. In order to achieve this goal, BCR-HAS has carried out an applied work by developing core-genome E. coli strains to serve as host cells ("chassis and engine") for various synthetic biology applications. Reduced complexity of the cells allows for more precise

control/programming of cellular processes and for increased stability of engineered, synthetic genetic circuits. A set of bacterial strains is now available for use. In order to construct streamlined genome *E. coli* strains, up to 75 deletions were combined in a single genome, representing all together a 25% genome reduction. This reduced genome approaches the core genome size predicted by comparative genomics for intestinal *E. coli* strains. The strain series was characterized for growth, transformation efficiency, mutation rate and other practical parameters. Milestone strains with best phenotypic characteristics were further modified for specific tasks. These include stable maintenance of normally unstable DNA constructs, and inducible, high efficiency recombinant protein expression. Strains with enhanced mutagenic capability, serving as host for in vivo mutagenesis, are also available. Specific attention was paid to reduced-evolvability variants. Evolvability is an intrinsic feature of all living cells. However, newly emerging, evolved features can be undesirable when genetic circuits, designed and fabricated by rational, synthetic biological approaches, are installed in the cell. BCR-HAS has shown that delayed genetic adaptation of reduced-genome host cells, devoid of all mutation-generating mobile genetic elements, improve maintenance of unstable genetic constructs. To further reduce the mutability of the host, point-mutation rates were significantly reduced by specific gene deletions. It was shown that various stress conditions, including recombinant protein expression, induce mutation-generating mechanisms to a high level in regular *E. coli* hosts. In contrast, the modified multi-deletional strains display low mutation rates, even under stress conditions. These minimalized, genetically stabilized strains are suggested to be beneficial hosts (SB chassis/engine) in both laboratory and industrial settings.

With the aim of defining the limits of construction of a cell factory, through re-sequencing and re-annotating *Bacillus subtilis* a better comprehension of the limits between the paleome and the cenome has been achieved by IP. A genomic database for *B. subtilis* has been created by IP partner (BacilluScope), and another one is in process (SubtiliCyc).

Regarding advances in molecular assets, CSIC partner, has develop, in the first year, a fully synthetic mini-Tn5 delivery vector (Figure 1), and has also developed an application of one *Pseudomonas putida* strain for detection of 2,4 DNT in soil. During the second year, CSCIC has focused on synthetic genetic and molecular tools. Indeed, the functioning of complex regulatory networks, or even a single gene, is revealed only when perturbations are entered in the corresponding dynamic systems and the outcome monitored. These endeavours rely on the availability of genetic tools to successfully modify à la carte the chromosome of target bacteria. Key aspects to this end include the removal of undesired genomic segments, systems for production of directed mutants and allelic replacements, random mutant libraries to discover new functions, and means to stably implant larger genetic networks into the genome of specific hosts. The list of Gram-negative species that are appealing for such genetic refactoring operations is growingly expanding. However, the repertoire of available molecular techniques to do so is very limited beyond *Escherichia coli*. In this Report, utilization of novel tools is described (exemplified in two plasmids systems: pBAM1 and pEMG) tailored for facilitating chromosomal engineering procedures in a wide variety of Gram-negative microorganisms. The way that goes from Genetically Engineered Microorganisms to synthetic, or at least heavily refactored counterparts involves the stepwise replacement of growing portions or their naturally

occurring genomes by rationally designed and chemically manufactured DNA. Although the current ability to synthesize long genomic segments is now in the range of 1 Mb, the contemporary level of knowledge does not allow assembling new activities or genetic circuits involving more than 20-30 kb of engineered DNA. It is thus likely that still for some time most Synthetic Biology endeavors of this sort will focus on handling a relatively short range of DNA sizes, whether for deletions from existing chromosomes, for genomic replacements of alleles by designed variants, or by straight implantation of new sequences. Numerous genetic tools exist in *E. coli* to this end but the state of affairs for other biotechnologically relevant Gram-negative bacteria such as *Pseudomonas putida* is far less satisfactory. In this Chapter two strategies are described for implementing a large number of genetic manipulations in the chromosome of a large variety of Gram-negative microorganisms. For this purpose, *P. putida* was used as the target bacterium, and the constructs named pBAM1 and pEMG (Fig. 1) adopted to give details of the underlying concepts and their practical application. As explained below, these plasmids are tailored for either implantation/insertion of heterologous DNA segments in the genome of the targeted strain as well as for directed mutagenesis or deletion or pre-specified chromosomal regions. Even though the procedures are different for each plasmid system, their utilization share a good deal of the biological materials listed in the corresponding section below. The organization and properties of pBAM1 (Fig. 1A) make it suitable to be used either for creating saturated random transposon mutant libraries or for stably introducing gene networks or functional cassettes into the genome of a specific bacterial host. Both of these properties are due to the special characteristics of mini-transposons. The pBAM1 plasmid is composed of 4 blocks (Fig. 1A).

The first segment corresponds to the plasmid selectable marker, ampicillin. Next, an R6K origin of replication that makes its maintenance dependent of the trans supply of the p protein (pir gene). Thus, pBAM1 must be replicated in specialized *E. coli* strains, which expresses the p protein from a lysogenic phage, such as *E. coli* CC118Ipir. A Tn5 transposase borne by the same plasmid (tnpA) recognizes the end sequences of the mini-Tn5 transposon module (ME-I and ME-O) and catalyzes the random motion of the mini-Tn5 cassette the target genome. All of these features have been individually optimized by CSIC, cured of the most common restriction sites present within its sequence, and then assembled and chemically synthesized de novo. The pBAM1 frequencies of transposon insertions when applied to *P. putida* are in the range of 10^{-3} when the plasmid is delivered to the recipient by mating (see procedure below), or 10^{-7} when electroporation is used as an alternative method of suicide donation. On the other hand, the pEMG plasmid (Fig. 1B) is used to generate directed scar-less deletions, as well as allelic replacements in the genome (Martínez-García and de Lorenzo in preparation). This genetic system is a recreation of the method developed before for the same purpose in *E. coli*. The procedure is based on the homologous recombination forced by the appearance of double strand breaks (DSB) in the genome of the target bacterium upon cleavage in vivo by I-SceI, a homing endonuclease from *Saccharomyces cerevisiae* that recognizes an 18-bp DNA sequence.

The I-SceI recognition sequence is not present in any of the microbial genomes sequenced so far, as revealed by blastn search against the 1379 completed (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). In this way, integration of pEMG into the chromosome of choice

endows flanking I-SceI target sequences to the extremes of a lacZ-alpha pUC19-based poylinker. Intracellular expression of the I-SceI enzyme in live bacteria is brought about in vivo by the cognate pSW plasmid (Fig. 1C) Transient expression of the nuclease is tightly controlled in pSW by means of the 3-methylbenzoate-inducible promoter Pm. The steps of the deletion strategy process include [i] cloning regions homologous to those flanking the desired deletion/replacement into pEMG, [ii] cointegrating the resulting plasmid into the genome of the target host, [iii] introduction of pSW into cells bearing the cointegrate, [iv] induction of the DSBs, [v] selection of the deleted/replaced strain and [vi] pSW curation.

For this deletion/replacement process, the I-SceI expressing plasmid (pSW) can be introduced before (preferred option if multiple deletions within the same strain are desired) or after obtaining the co-integrate (if one plans a single deletion). Only the second option is described here. To facilitate a specific example of the deletion protocol one example of the procedure is detailed below. To this end a chromosomal region of *P. putida* KT2440 between the genome coordinates 5680657-5690333 was chosen. This region comprises 7 genes that resemble a type IV pili operon.

The delivery of the pEMG plasmid could be done either by mating or electroporation. Here, only the electroporation technique is discussed (for mating just follow the same procedure described before but using *E. coli* DH5 α harboring pEMG-Ts and finally plate onto M9 citrate Km 50 μ g ml⁻¹). To prepare electrocompetent cells of *P. putida* KT2440 (hereafter KT2440) follow the same steps described earlier.

To summarize the CSIC's work, a synthetic plasmid composed of multiple formatted and optimized functional parts that behave as predicted -both individually and as an integrated system- has been created. To the best of our knowledge, since the 90s this is the first report that describes a fully edited genetic tool optimized and streamlined for its final applications -rather than relying on cutting and pasting naturally occurring sequences⁵². In a nutshell, non-functional DNA sequences were trimmed-off, common restriction sites present outside the multiple cloning site inside the mobile element were eliminated and the plasmid was designed following a modular pattern in which each business sequence was flanked by non-frequent restriction sites. In this respect, the key features of pBAM1 include not only the removal of many bottlenecks that flaw utilization of many of its predecessors, but also the incorporation of a fixed standard for physical assembly and exchange, where required, of new DNA pieces while maintaining its overall layout. The modularity of the design and the origin of the parts (in mobile elements which considerable orthogonality) enables pBAM1 for two specific applications. The first is the exploitation of the cargo site (Fig. 1 and 2) to place a whole collection of extra genetic gadgets for expression of heterologous genes, reporter systems and environmental markers at user's will. The second is the possibility of cloning large DNA fragments inside the mobile element for a final implantation of new traits into the chromosome of the target strain. Given the randomness and the high frequencies of such insertions, one can then select the insertion out of a large collection, which adjusts expression of the desired feature to the right level under the desired operation conditions^{53,54}. Furthermore, the ease of replacement of the antibiotic

resistance marker (or any other functional part) allows the same transposition/delivery system to be reused for subsequent insertions. In sum, this work shows the value of DNA synthesis and standardization of functional modules for combining in a single genetic tool many valuable properties that are otherwise scattered in various vectors and rendered useless for the lack of fixed assembly formats. We anticipate pBAM1 to become one frame of reference for the construction of a large number of vectors aimed at deployment of heavily engineered genetic and metabolic circuits

UMIL partner also focused on *P. putida*, particularly on optimizing the genetic background of these bacteria through the abolishment of physiological bottlenecks to the expression of the desired phenotypes. Moreover, UNIL is developing a site-specific integration system that will enable to insert large DNA fragments into the genomes of re-engineered bacterial strains with synthetic constructs. UNIL has already been successful in producing artificial integration sites, which are targeted with a higher efficiency than the natural ones. Nowadays, these target sites carry a conditional switch that let the cell produce green fluorescent protein when the DNA is integrated in the correct site.

It is remarkable the substantial increase of knowledge obtained during the last years about the genetics, regulatory processes and metabolism of microbial forms of life. Such information obtained by means of different technologies of increasing power and efficiency is being included in large databases, many of them of free access. Once combined with the vast number of scientific publications a huge volume of data is deposited in the hands of researchers. Synthetic biology aims the partial design of organisms with specific functionalities by a rational usage of such information. It is well known that there is a large room for improvement concerning the available computational tools and the limitation in storage capacity, coherence and interoperability of the existing databases. This is a main target of the international efforts in bringing S.B to a mature state capable of producing highly reliable organism capable of performing all kinds of useful tasks. The work carried out during the duration of this project by our group has the potential of modifying substantially the state of the art in this realm.

One of the fundamental principles of synthetic biology is the construction of biological standardized parts and devices, which are interchangeable. A proper characterization of these parts and devices appears as a key issue in order to make them reusable in a predictive way. In the recent past scientists have witnessed several initiatives towards the design and fabrication of synthetic biological components and systems as a promising way to explore, understand and obtain beneficial applications from live. For instance, in the post genomic era one of the most fascinating challenges scientists are facing is to understand how the phenotypic behaviour of living cells arise out of the properties of their complex network of signalling proteins. While the interacting biomolecules perform many essential functions in these systems, the underlying design principles behind the functioning of such intracellular networks still remain poorly understood. Several initiatives have been reported in this line of thought to uncover some key working principles of such genetic regulatory networks via quantitative analysis of some relatively simple, experimentally well characterized, artificial genetic circuits.

The desired performance of these synthetic networks and in turn the resultant phenotype is strongly dependent on the expression level of the corresponding genes, which is further controlled by several factors such as promoter strength, cis- and trans-acting factors, cell growth stage, the expression level of various RNA polymerase-associated factors and other gene-level regulation characteristics. Thus, one important ingredient to elucidate gene function and genetic control on phenotype would be to have access to well-characterized promoter libraries. These promoter libraries could be in turn useful for the design and construction of novel biological systems.

Recently a methodology (<http://partsregistry.org/Measurement>) has been reported to characterize the activity of promoters in the Registry of Standard Biological Parts (<http://partsregistry.org>) by using two different cell strains. As a part of our work UPVLC proposed the use of a synthetic gene regulatory network as a framework to characterize different promoter specifications by using a single-cell strategy. In this context characterization stands for evaluating the parameters of a query promoter as compared to a standard promoter acting as a "scale".

A proper promoter characterization is an essential step towards a realistic standardization. Once this step is accomplished S.B will arrive to a new stage where simplicity will allow the massive design of organisms. This desired transition will provide society a powerful tools that could be employed to address several important goals such as the massive production of biofuels or the reduction of atmospheric pollution.

HZI developed ToBiN, which is a collection of computational tools for the genome-wide study of microbial physiology. Currently the platform ToBiN contains several modules articulated in a way that allows them to go far beyond the exposure of annotation flaws and to reach a transversal view of the interaction's hierarchy, from regulatory circuits to host-pathogen relationships. Among the several components supplied by the platform, the one most-likely to be used by the highest number of modules is a Visualization Engine able to render a representation of the genome-wide physiological organization of the cell. This visual component can be panned and zoomed in a Google-Maps⁸⁴⁸² fashion and, whenever connected to modules generating data-sets with values mappable to a particular compound, reaction or gene, is able to overlay graphical representations (e.g. heatmaps) of selected quantitative and/or qualitative data-sets. An example of the utility of the Visualization Engine would be on visually-aiding the perception of the correspondence between a metabolic flux distribution and transcription levels for the various pathways.

Developing automatic design tools was the goal of CNRS partner, who have to aid in the engineering of gene and RNA circuits from modular components. Their modules will be an important component in the development of a language for SB. They have further developed computational tools to be added as part of the SynBio Toolbox (design gene networks: Genetdes software; design gene networks from assemblies of SBML parts: Asmparts software). CNRS also set a Working Group and a Workshop Series on Computational Frameworks and Tools for SB, and contributed to the SynBio

Toolbox, a dynamic repository for modelling and design tools in Synthetic Biology, with the PROTDES, Genetdes and Asmparts tools. Finally, they contributed with the ad-hoc developed tool Desharky to automatically design biodegradation pathways using a database of enzymes. The Kegg database has been used as a proof-of-concept, but the tool could be adapted to any database.

The Imperial College group participates in terms of setting up a repository of modelling frameworks for SB and developing an open-source system for collaborative tool development and problem solving in SB. Their study shows that the genetic circuit model effectively describes accurately the function of the system and its dynamics providing a solid basis for a systems understanding of the metabolism of important pollutants, such as toluene and xylenes. Also, in the side of modeling, GA contributed with the development of foundational tools and concepts in accordance with its firm technical expertise in DNA synthesis methodology. Within this task a number of straight forward developments and applications have been initiated, and existing processes and tools have been expanded and adjusted to the requirements of environmental and general SB (methods, vectors, computer programs, etc.), which have immediately been implemented into the company's technology platform to expand the service portfolio. Further, partner GA's tightened strategic gene synthesis market position provided access to many industrial, academic and governmental SB stakeholders, opening opportunities for a continuous dialogue with different experts from diverse disciplines. As illustrated by the comprehensive list of dissemination activities, addressing the scientific community, the industry sectors, civil society, policy makers, and students, this dialog has been maintained and is further continued in order to address and influence social, ethical and regulatory issues, as well as to trace potential market opportunities in environmental SB. The dissemination activities were mainly related to inform scientific and industrial researchers and developers about technological opportunities and applications (large scale gene synthesis, gene assembly, directed evolution, enzyme evolution, etc.), to discuss regulatory (e.g. Biosecurity/Biosafety) and financial (funding) issues and for teaching purposes. In addition, the engagement in synthetic biology resulted in participation in and even foundation of interest groups (IGSC, SBIA, BioM-WB, DECHEMA work group) involved in regulation, cooperation, information, funding and other related activities on national and international levels. The topics addressed within this project and the activities initiated and concluded represent an integral part of the ongoing development of SB in Europe that is far from being finalized. The development of SB not only provides new and exciting opportunities and research potential for science but it also represents a very promising economical prospect. The results of this project directly contribute to the technological basis required to take advantage of this prospect, although the actual demand and market for commercial SB projects is limited.

Databases are an integral part SB. CNIO has developed Bionemo database (Figure 2), which stores manually curated information about proteins and genes directly implicated in the Biodegradation metabolism. When possible, the database also includes information on sequence, domains and structures for proteins, as well as regulatory elements and transcription units for genes.

CNIO accomplished the proposed objectives on WP4, leading to an important advance on the development of SB-related databases. First, the creation of a corpus of document relevant to Biodegradation metabolism and regulation. It will be necessary to generate a set of documents enriched in the desired information. This task, which was completed in the previous reporting period, was extended for the generation of a bibliome (literature collection) consisting in all the articles relevant for any given bacterium. This methodology will be tested in the contexts of the MICROME project. Second, a database containing all the knowledge on biodegradation reactions was created and the Bionemo database created. Bionemo can be accessed via its web site (<http://bionemo.bioinfo.cnio.es>). The web server implements a simple search interface that allows simultaneously querying all the biological entities described above. The results are shown categorized by tabs representing classes containing the entity types (reactions, complexes, etc.). From the results page, the user can easily access entity-specific pages, in which all information available is summarized. Links to external databases including the original UM-BBD metabolic information, GenBank and Uniprot, the NCBI Taxonomy database for microbial species, and the PubMed references to the original information sources, are provided. In addition to the Web site access described above, currently Bionemo can be downloaded as a SQL dump and installed locally. A Perl API (application program interface) is also provided (<http://bionemo.bioinfo.cnio.es/api.html>). A REST service (a key design idiom that embraces a stateless client-server architecture in which the web services are viewed as resources and can be identified by their URLs) is currently integrated in the Biological Web service Proxy (<http://code.google.com/p/bwsproxy/>). This is a free resource developed by CNIO which main goal is to speedup the responses from different web services related with biology, bioinformatics and synthetic biology. The proxy catches several operations that highly demand computational resources. Finally, a MaDAS system was implemented. The main goal of MaDAS (<http://madas2.bioinfo.cnio.es>) is to allow users to add their own annotations. A project is a unit that typically stores different annotations related to one genome or stores annotations related to a particular issue across several genomes. This is the case of the TARPOL project where biodegradation related annotations were collected in several microbial species. A Bionemo plug-in that connect MaDAS with the Bionemo database was created. Through this plug-in the annotations stored in Bionemo are now also available in MaDAS and can be retrieved in DAS format using the embed MaDAS DAS server.

The design of metabolic pathways for "Biochemical Building Blocks" has been identified as foreseen applications in SB by IDC. Instead of searching for particular biomaterials or compounds, IDC has suggested to look for useful biochemical "building blocks" and their potential to serve as a starting material for a larger group of derivatives. Many of those building blocks have excellent potential to compete with petrochemical equivalents, as many new products are possible with novel functionality or new applications.

TARPOL is also contributing to the exchange of knowledge among European researchers working on SB through the organization of several workshops and conferences. Among these activities CNRS-IHPST organised a two-day workshop in Paris, in the Ecole Normale Supérieure, gathering historians, philosophers and biologists to evaluate the place of SB among other biological disciplines, and its

novelty; HZI organized a special session on the topic Computational Design Tool for Synthetic Biology within the BioPathways meeting in Stockholm; and UPVLC organized a symposium on SB titled "III Jornadas Internacionales de Biología Sintética"; least but not last, UVEG in coordination with HZI is organizing a workshop to foster the discussion on minimal cells and its applications to take place next November. CSIC will organize the 5th Meeting of the Spanish Network of Systems Biology in December 2009 on the general topic "Fostering Systems and Synthetic Biology in Southern Europe."

Potential Impact:

Training and dissemination activities on SB are also an important subject of TARPOL project. In this vein, UMIL has scheduled a course entitled "Evolution and Design of Biomolecular Systems: Concepts and strategies for systems and synthetic biology", which took place in Illetes-Mallorca (Spain) with an outstanding panel of speakers. Many dissemination activities have been carried on by the diverse partners of TARPOL. These include participation in scientific meetings, peer-reviewed articles, conferences to the general public, and science popularization articles, among others (Figure 3). A web site for the TARPOL Project (<http://www.sb-tarpol.eu/>) has been designed, and is under continuous update. It contains public and private sections.

UPVLC has also participated intensively in the organization of dissemination activities. Institute Cavanilles (UVEG) and Intertech Group of UPVLC co-organized the Summer Course on Synthetic Biology. This activity was developed from two sides : training and motivational. For this reason, the program combined the expertise of the organizers to train scientists from an interdisciplinary perspective, in order to offer a range of activities for young researchers from a wide range of fields. The Summer Course on Synthetic Biology conference also included lectures by the leading scientists of this new discipline on the constitutive principles underlying SB, which allowed attendees to see and understand the great possibilities of this new scientific area. The laboratory practice of SB - "wetlab" - aimed at researchers from technological branches who had no expertise, with the working principles of a molecular biology laboratory. Practices of "drylab" aimed at students from Life Sciences, with the intent to convey the basics of computer design in SB. The result of such and initiative will hopefully be a motivated group of young researchers in the field leading to its desired expansion. The participation in dissemination activities not directly supported by TARPOL consisted on the preparation of two courses on synthetic Biology (2008 and 2009) and the organization of two days conferences on the same topic during the three years of the project.

Finally, the ethical, human practices and safety issues have been approached in an unprecedented way. The US Presidential Commission for the Study of Bioethical Issues held its first meeting on July 8-9 in Washington, DC. The primary topic was synthetic biology. Speakers included Craig Venter, Drew Endy, George Church, and other leaders in synthetic biology, as well as experts in ethics, policy, regulation and government. TARPOL partner Markus Schmidt from IDC was be the only European speaker at this meeting, giving an overview of completed, ongoing and planed activities about societal implications of synthetic biology in Europe. (See agenda: <http://www.bioethics.gov/meetings/070810/>). The work performed on economic, environmental and ethical implications of synthetic biology applications in environmental biotechnology has been reviewed in a comprehensive report, which is one of the most complete reviews on the societal and environmental implications of a novel technology.

The report deserves the following exhaustive summary:

The report led by IDC and prepared in collaboration with a group of partners (BU, UVEG, UNIVE, CNRS-ENS, IP and CEA), tries to give a glimpse into the future of synthetic biology (SB) and its potential applications in the area of environmental biotechnology. There are a number of applications where SB could well make a difference in order to transfer our society to become more economical and environmental sustainable. In this report we have highlighted 4 major areas (biofuels, bioremediation, biomaterials and novel developments in SB) with a total of 20 specific applications where SB has a great likelihood to improve currently available technologies. Each of the 20 applications has been assessed in detail in order to find out (1) to what extent SB could improve current technologies; (2) what the economic impact of SB could be; (3) what the environmental benefits and downsides could be and (4) whether any social or ethical problems would be created, exacerbated or improved. This assessment is intended to support national and international funding agencies in their decisions to allocate resources to SB-based biotech applications while taking into account any foreseeable economic, environmental and social/ethical issues. Our outlook is based on the current scientific state-of-the-art, however, there is of course a notable degree of uncertainty about future development paths which we have to acknowledge when giving recommendations for what we see as the most promising directions for SB in environmental biotech.

Biofuels

We are convinced that synthetic biology can help to produce state-of-the-art and next generation biofuels. Current efforts are mainly targeted towards an improved production of bio-ethanol from agricultural products, although we see significant problems with this approach as ethanol exhibits some technical problems (miscible with water, limited use in existing engines). Other non-ethanol biofuels such as bio-butanol or biodiesel are much better suited to replace petroleum-based gasoline, as their chemical properties resemble it much closer. Synthetic biology could help to overcome current impasses in the production of butanol and other non-ethanol fuels, namely poor fermentation yield and toxicity to butanol-producing microorganisms. One problem that is faced by most biofuels produced from plant material is the limitation of the use of hemi- and lignocellulosic material. Any improvement in that area would definitely increase economic feasibility of biofuel production. One important problem will arise, should synthetic biology be able to provide a solution to the technical problems just mentioned, namely that more and more agricultural land will be devoted to plant energy-crops instead of food crops. In order to avoid this competition for food, we suggest to use also non-food-competing biological resources such as perennial plants grown on degraded lands abandoned from agricultural use, crop residues, sustainably harvested wood and forest residues, double crops and mixed cropping systems, municipal and industrial wastes.

In contrast to agricultural based ethanol, biodiesel and butanol, there is also algae based biofuels and biohydrogen. Current concepts foresee a significant advantage of algae-based biofuels over agriculture-based biofuels, because of higher yield per area and the independence of arable land, and clean water. First calculations predict, however, that future algae production systems will only be economically feasible if the price for one barrel oil is constantly above 70US\$ and if the production

systems entails at least an area of 200ha. The capital costs of such large production facilities will probably lead to an exclusion of SMEs and play in favour of "big oil". Still, algae production systems could be a highly promising avenue of future fuel production, once major obstacles are solved dealing with algae genomics, metabolism and harvesting. Although bio-hydrogen has been praised as an extremely promising fuel by many scientists, our assessment is more cautious. Hydrogen is only useful as fuel if large changes in infrastructure take place (distribution and storage system, new fuel cell engines), and point to a more distant future beyond 2050, also termed as the hydrogen economy. Although synthetic biology could well contribute to improve yield of hydrogen producing cyanobacteria, the actual impact of hydrogen in society and economy depends much more on other areas such as infrastructure. Finally we analysed the prospects of microbial fuel cells (MFC), as energy converter. Although we see MFCs as extremely promising and an area where synthetic biology could contribute a lot, it will most likely be applied in some niche markets and areas of application, rather than large scale deployment due to the limited energy production.

Bioremediation

Bioremediation is an area with a great potential of benefits provided by Synthetic Biology. Bioremediation is usually applied on materials with a massive occurrence such as solid (organic) wastes, sewage, industrial waste water, contaminated soil or contaminated ground water, any of them measured in millions of tons or cubic meters. We believe that SB has the potential to create tools to improve the treatment methods, saving costs and environmental resources. Moreover, it can provide methods to produce energy or valuable goods from waste or wastewater. It can also provide tools for making up fresh or drinking water either from contaminated water or seawater. Another possible field of application is the production of biosensors to monitor environmental goods and hazards. At a differentiated evaluation, we have concluded that biosensors provided by SB-tools would have a great positive effect on the environment since they will help to survey environmental hazards more precisely and effectively. However, their economic and social impact is rather low because they can be considered as niche products.

Synthetic Biology based approaches may provide a way of capturing, storing and recycling carbon dioxide. This may be through the re-engineering of existing organisms or the creation of novel carbon processes especially using bottom up approaches where inorganic chemistry is linked to living processes through agents such as the emerging protocell technology.

Synthetic Biology based carbon capture may not be able to sink carbon dioxide to completely remediate the current escalating levels that are being released through fossil fuel consumption as geoengineering scale approaches are necessary but they can offer the possibility of carbon capture and recycling which current industrial scale processes cannot do.

It is recommended that because of the scale of the problem with carbon dioxide emissions and the urgent need for remediation that Synthetic Biology approaches are supported in order to develop the next generations of carbon capture technologies which will do more than store the carbon dioxide but recycling it into fuels and biopolymers with positive environmental impact.

Another positive impact, particularly to the environment, can be expected for soil and ground water remediation, especially with regard to the enhancement of the clean-up efficiency and to the development of new methods. On this field, the economical and social impacts are rather moderate, since it is a specific field with a limited scope of time.

The strongest impact we expect is for solid waste and wastewater treatment and for water desalination. The importance of the latter cannot be overstated in a world, where billions of people have no access to clean drinking water or to fresh water for agricultural use. Solid waste and wastewater treatment also bear a great potential for improvement by Synthetic Biology due to their sheer amount and to their considerable organic content. We therefore strongly recommend to support the development of these 3 issues. However, a possible constraint should be mentioned: solid waste and waste water can not be treated in sealed vessels or rooms, simply due to their huge amount. They have to be treated openly in piles or basins. Therefore, the use of engineered cells may create a problem of interaction with the environment, which has to be kept in mind. But we expect no limitations for the use of non-proliferative systems like enzymes or protocells created with the aid of Synthetic Biology.

Biomaterials

Synthetic Biology will have a significant impact on the biomaterials market particularly in the areas of fine chemicals and bioplastics. A tool box of products that will act as biodegradable materials is recommended. The bulk chemicals industry will also be significantly affected by the Synthetic Biology based technology but uptake and therefore environmental impacts will be slower although when new practices are adopted changes will last longer and take place on a much larger scale. In fine chemicals industry the incentives for investment relate to the economic potential of the end product (in contrast to bulk chemical manufacturing). The payoffs could predominantly have environmental impacts although these may be significantly limited to more efficient use of energy since the core manufacturing practice relies on petrochemicals. There is also limited potential for Synthetic Biology based techniques to have an impact on avoiding recalcitrant molecules in the production process. Nonetheless investment in Synthetic Biology based processes in bulk chemicals is likely to have a positive overall impact on the manufacturing systems used in the running of the plant such as, use of biodiesel and less overall chemical waste and because of the scale at which these processes take place, small changes may have significant positive environmental impacts. For both fine and bulk chemical production we recommend the deployment of the "Chemical Building Block System" as designed (or similar to) the USDoE.

The field of biopolymers and bioplastics most urgently needs revisiting in terms of its current labeling for recycling purposes since categorization of the various products is extremely complex with negative economic consequences because of this (bioplastics are not necessarily biodegradable). We recommend application of a method through which those bioplastics that need recycling and those that can be composted are clearly recognizable, before large scale use of Synthetic Biology for production of bioplastics take place. An urgent need to develop completely biodegradable plastics exists which would benefit from focused Synthetic Biology research and development into this area. Additionally there is also a pressing need for high performance structural bioplastics for manufacturing coupled with completely biodegradable additives. Both of these significant growth areas in the bioplastics industry could be greatly improved by Synthetic Biology based research.

Investment is particularly needed in research and development for new methods and products that will expand and develop tools and manufacturing processes with reduced environmental impact compared with the current manufacturing approaches although adequate biosafety issues on large scale manufacturing units need to be established. Cellulosomes (complex molecules that degrade hemi- and lignocellulosic material) possess high economic potential for biofuels, paper and waste processing. Synthetic Biology has the potential to design more efficient and completely new cellulosome complexes to make new, efficient cellulose digesting proteins. Open sourcing of the cellulosome technology is recommended owing to the justice of distribution issues involved in the technology.

Novel developments in SB

Protocell technology represents a bottom up approach to Synthetic Biology bridging inorganic and organic processes. Protocell technology enables better understanding of Synthetic Biology as a whole to develop new technologies. Although the research is in an early stage, the development of potentially radically novel and significant environmental interventions e.g. for remediation of carbon emissions and alternative biofuels technology seem feasible. Investment in basic science to underpin the research and support it whilst imminent private investment is happening is strongly recommended.

Protocell technology has a huge potential to offer radically different tools and methods than previously encountered with Synthetic Biology based approaches because of its bottom up nature and because of its overlaps with basic chemistry. A toolbox of potential products and investigation of issues related to open sourcing the technology should also be looked into. Xenobiology (also known as chemical synthetic biology) is another bottom up approach to design and construct radically new biological systems with properties not found in nature. Using e.g. non-canonical amino acids, alternative base pairs to enlarge the genetic alphabet, or different chemical backbones in a xenonucleic acid, these chemically modified organisms and systems will enable a much higher level of biosafety when using engineered biosystems for or in the environment. For example novel enzymes (such as amylase) with non-canonical amino acids can be used to reduce the optimal

temperature for breaking starch into glucose, thus saving enormous amounts of energy, contributing to a decrease in green house gas emissions. Organisms with an enlarged genetic alphabet or a DNA with a different chemical backbone could be designed by Synthetic Biology in order to impede horizontal gene transfer and genetic pollution between engineered and natural organisms. Similar to protocells, xenobiology is in a very early stage of development and requires increased support for basic research in order to be able to achieve radically new concepts and applications. See attached Table for details.

List of Websites:

<http://sb-tarpol.org>