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Synthetic Biology

Latest developments, biosafety considerations and regulatory challenges

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Picture cover page: Strains of *Escherichia coli* have been developed to produce lycopene, an antioxidant found in tomatoes. Source: (Baker 2011).

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SUMMARY

Synthetic Biology (SB) is a multidisciplinary and rapidly evolving field. It can be summarized as the rational design and construction of new biological parts, devices and systems with predictable and reliable functional behavior that do not exist in nature, and the re-design of existing, natural biological systems for basic research and useful purposes.

Four major SB approaches have been distinguished in this document: (i) Engineering DNA-based biological circuits; (ii) Defining a minimal genome/minimal life (top-down approach); (iii) Constructing protocells or synthetic cells from scratch (bottom-up approach); and (iv) Developing orthogonal biological systems (Xenobiology).

There is currently no internationally agreed consensus about a definition of synthetic biology. Although having such a definition could facilitate enabling a rational discussion of this issue, we do not see the adoption of a definition as key for discussing the potential regulatory and risk assessment challenges of SB.

It is expected that on the short term activities in SB will focus on research and development or on commercial production of substances in contained facilities. It should take some time before a 'synthetic organism' is introduced into the environment or before commercial environmental applications become available. In consequence we can expect that for the moment work on synthetic organisms will remain restricted to facilities where potential risks for human health and the environment can be controlled more easily.

Current developments in SB mainly involve the use of well-characterized micro-organisms and genetic material, for which sufficient knowledge and appropriate comparators are available to assess the potential risks. We think that it is hardly conceivable that in a near future micro-organisms or entities will be generated that are far different from existing organisms. Therefore the manipulation of synthetic organisms in the laboratory or their accidental release in the environment would not represent additional risk.

In the future synthetic organisms could be developed that will differ more fundamentally from naturally occurring ones. In such cases it could be more difficult to identify an appropriate comparator, to gather relevant information to perform characterization of the potential hazards and/or to predict the behavior of such engineered organisms in case of accidental release in the environment. In case of high complexity and uncertainty, a precautionary approach should be adopted. Containment and confinement measures should be applied in a realistic, risk-proportionate and case-by-case manner in order not to excessively hamper research and developments initiatives. Containment and confinement measures should be adapted progressively when more data become available to feed the risk assessment.

While the first commercial application of SB (the production of an anti-malarial drug) is expected to be available by 2013, environmental applications of SB are not expected to materialize before several years. Today it is therefore premature to determine whether such applications will lead to potential challenges in environmental risk assessment. However when such applications will concern micro-organisms, it is important to note that risk assessors and regulators have had relatively little experience when considering the potential risks posed by the intentional release of micro-organisms, including genetically modified micro-organisms.

At the regulatory level, the main conclusion that can be drawn to date is that current activities involving the development and use of synthetic organisms make use of techniques that fall within the scope of Directives 2009/41/EC and 2001/18/EC. In consequence the European GMO regulatory framework is for the moment adequate to support risk assessment of these activities. This applies also to cases where pathogenic micro-organisms are manipulated or reconstituted, since the scope of the Belgian legislation on contained use of GMOs also covers pathogens. However, it should be noted that some developments of SB (e.g. protocells or orthogonal systems) could raise potential issues as regard the regulatory status of the resulting organisms as these approaches could be considered as not leading to GMO or not meeting the definition of an organism in the meaning of the EU legislation.

SB involves the combination of different scientific disciplines that go beyond biology, including engineering, chemistry, physics, computer sciences and bioinformatics. This multidisciplinary also characterizes the iGEM initiative, a worldwide competition where students are challenging each other in designing and building synthetic systems. SB also led to the 'do-it-yourself' (DIY) biology, a movement where activities are performed outside biological laboratories by people without a traditional education in biology. These aspects can make the awareness of potential biological risks and the application of biosafety measures more challenging, and appear to circumvent regulatory provisions in some way. Although a certain level of self-governance has been implemented the question remains whether such activities should be subject to more government-led oversight and control.

In conclusion, we are of the opinion that sufficient knowledge is available to adequately assess and manage short-term SB applications. The current risk assessment principles and methodology, and the GMO regulatory framework, seem robust enough to deal with these applications. It is difficult to make a judgment whether this will still be the case on the long run.

Science and technology developments in the field of SB should be reviewed regularly and action taken if voluntary codes or current regulatory procedures appear insufficient. In this regards, exchange between the research community, risk assessors and policy makers will be key to expand scientific and technical knowledge and to fill potential gaps in risk assessment and regulation of evolving developments.

Further approaches to reconsider effective risk governance should also be taken in a global perspective, allowing international coordination and dialogue. It is therefore important for the European Union to advance further in defining a harmonized view about safety, security and regulatory oversight of SB.

CONTENTS

1. OBJECTIVES OF THIS DOCUMENT

This document is a background paper aiming at providing general information and considerations about techniques, applications and safety aspects of Synthetic Biology (SB). It is not a position paper. It should be considered as food for thought to sustain and stimulate further discussion on this topic amongst interested stakeholders.
It is important to note that SB is a rapidly evolving field encompassing many different topics. It is therefore worth mentioning that this document does not aim at providing an exhaustive overview of all SB applications and is based on currently available scientific evidence. Its descriptive and analytic parts could be subject to review and change if new important information becomes available.

For the purpose of this document and according to (Schmidt et al. 2009) we will distinguish four major approaches in SB: (i) Engineering DNA-based biological circuits; (ii) Defining a minimal genome/minimal life (top-down approach); (iii) Constructing protocells or synthetic cells from scratch (bottom-up approach); and (iv) Developing orthogonal biological systems (Xenobiology).

Each of these approaches will be shortly described in chapter 3 (Developments within synthetic biology) to illustrate how they are used in basic research (to study for example the organization, function and expression of the genes in naturally occurring organisms), but also in the development of commercial applications in fields such as health, energy generation and environmental remediation or protection.

Chapter 3 will also focus on risk assessment aspects associated with the use of SB. Indeed, while several of the current SB applications involve genetic engineering that is basically anchored in recombinant DNA technology, SB potentially also aims at the *de novo* design and synthesis of organisms that may be radically different from those constructed by the insertion of naturally occurring foreign (GMO) or mutated DNA sequences. This development raises questions as to whether the evaluation of biological risks associated with the manipulation and/or environmental release of such organisms may be adequately addressed by the current GMO risk assessment principles and procedures.

Chapter 4 (Synthetic Biology: Risk Management Strategies) will briefly address management measures that can be applied to mitigate potential risks for human health and the environment identified as a result of the risk assessment or to address uncertainties resulting from the risk assessment..

Chapter 5 (Synthetic Biology: from Science to Governance) will give an overview of several official statements and recommendations as well as options of self-governance, thereby reflecting on some aspects of the current debate of the governance of SB.

The developments made in the field of SB and the activities involving organisms developed through SB have also fueled concerns related to biosecurity issues. This aspect will be discussed in Chapter 6 (Biosecurity issues associated with Synthetic Biology).

The European GMO regulatory framework

In the European Union, activities involving the contained use of genetically modified micro-organisms (GMMs) and the deliberate release of genetically modified organisms (GMOs) are regulated by the European Directives 2009/41/EC (EC 2009b) and 2001/18/EC (EC 2001) respectively. According to these Directives, a case-by-case biological risk assessment of these activities is required before authorization in order to limit their possible negative impact on human health and the environment. The general risk assessment procedure for 'conventional' GMOs as defined in European Directives 2009/41/EC and 2001/18/EC is partly based on the comparative analysis between the GMO and its non-genetically modified counterpart. This could make the assessment of associated risks more challenging in case no natural organisms could be taken as reference, because data required for comparative risk assessment may be lacking.

2. INTRODUCTION

The synthetic, engineered-based approach to life, serving both the understanding of biology and the modification of organisms to meet human needs, has been a recurring theme in history. The term 'Synthetic Biology', which already appeared in 1912 (Leduc 1912), has undergone several terminological shifts thereby capturing for example recombinant DNA techniques in the 1970s and disappearing from usage as the term 'genetic engineering' was rising. Therefore, while this document will further deal with the contemporary significance of 'synthetic biology' as used from the early 2000s, it could be pointed out that there were attempts to name this field differently (e.g. 'constructive biology' or 'intentional biology') and that the term 'synthetic biology' has many roots in terms of history, fields of research and context.

Synthetic Biology (SB) is a heterogeneous and complex developing field, which results from the combination of different scientific disciplines: molecular biology, engineering, genetics, chemistry, computer sciences and bioinformatics. Diverse definitions of SB can be found in the scientific community, such as the one provided by the Synthetic Biology community (http://syntheticbiology.org) which defines SB as "*the design and construction of new biological parts, devices and systems, and the re-design of existing, natural biological systems for useful purposes*" (Schmidt and Pei 2011).

SB receives considerable attention within expert circles, but the notion of 'synthetic biology' has hardly entered public awareness (Pauwels 2009). This could be partly explained by the fact that according to some opinions in the public, the perceived usefulness of applications from SB on the one hand and those from genetic engineering (based on recombinant DNA technology) on the other hand hardly differs. However, owing to the crosstalk between different scientific disciplines, a distinct feature of SB is that this field opens the way for the rational and intentional design, building and engineering of biological systems at all levels of biological structures, from individual molecules to whole cells, tissues and organisms, involving whole interacting genetic networks, genomes and ultimately entire organisms.

The field of SB is also rapidly expanding as it relies to a large extent to techniques such as synthesis and sequencing of nucleic acids and analogs. These techniques have considerably increased in cost-effectiveness, performance and efficiency over the last years (see figure 1), and have taken benefit from novel tools in computational modeling, as detailed in the textbox below. This technical evolution has allowed SB to grow into what it is today with its diverse applications (Liang et al. 2011). It has also led to initiatives aiming at promoting public dialogue on SB to enable articulating some important questions on this developing field (e.g. SB public dialogue initiated by two research councils in UK, http://www.bbsrc.ac.uk/society/dialogue/activities/synthetic-biology/findings-recommendations.aspx).

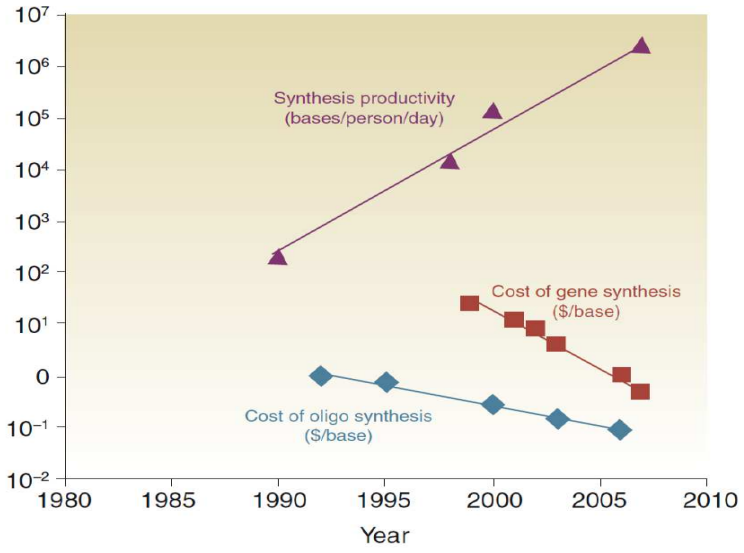

Figure 1: The price of oligo/gene synthesis has fallen down while the synthesis productivity improved considerably. Source : (Carlson 2009).

Techniques that have enabled advances in synthetic biology have also been employed for the study of behavior of complex biological organization and processes in terms of the molecular constituents, a discipline which is called "systems biology". Because the key to successfully engineering, or reengineering, biological systems, is through understanding their complexity, both synthetic biology and systems biology are closely interlinked and the distinction sometimes seems blurred. However, an important distinction between these two approaches is that systems biology attempts to obtain a quantitative understanding of existing biological systems while synthetic biology is focused on the rational (re-)engineering of these systems" (Serrano 2007).

Techniques in synthetic biology

Some important tools have contributed to the design, building and optimalization of cellular processes in the field of SB and have also largely facilitated the study and use of complex biological systems as a whole (systems biology).

- *DNA synthesis tools* are used in most SB applications and are essential in the creation of optimized synthetic sequences, genomes and pathways. Today, almost every sequence can be mail-ordered simply by sending the desired sequence to a DNA synthesis company.

- De novo *protein synthesis tools*: The two best known protein engineering approaches are 'rational design' and 'directed evolution' (Dougherty and Arnold 2009). The rational design is hampered by knowledge gaps in proteomics, while the latter overcomes this struggle by using Nature's optimization algorithm, 'evolution'. In order to overcome limitations related to the randomness, directed evolution is often combined with other approaches that focus on genes of interest, e.g. Iterative Saturation Mutagenesis (ISM) (Reetz and Carballeira 2007), Incorporating synthetic oligonucleotides via Gene Reassembly (ISOR) (Herman and Tawfik 2007), one-pot, simple methodology for cassette randomization and recombination for focused directed evolution (OSCARR) (Hidalgo et al. 2008), overlap-primer-walk polymerase chain reaction (OPW-PCR) (Kumar and Rajagopal 2008) or Site Directed Domain Scanning Mutagenesis (SDDSM) (Deng et al. 2007).

- Multiplex Automated Genome Engineering (MAGE) enables rapid and continuous generation of sequence diversity at many targeted chromosomal locations across a large population of cells through the repeated introduction of synthetic DNA approach (Wang et al. 2009; Wang et al. 2012).

- 'Omics' refers to profiling techniques to analyze biological systems at the genomic, transcriptomic, proteomic and/or metabolomic level, such as Micro-array, and ChIP-analyses for genomic studies, mass spectrometry and activity or reporter-studies for proteomics.

Hundreds of *computational tools* are available to facilitate the design of synthetic systems and the possibility for a user to add, edit, combine and play with biological elements (DNA, RNA, proteins) (see http://openwetware.org/wiki/Synthetic_Biology:Tools). Concrete examples are
(i) The database of essential genes (DEG) (http://tubic.tju.edu.cn/deg or http://www.essentialgene.org) which is an online library of the most important genes from different species (bacteria, yeasts, humans, mice, worms, fruit flies, zebra fishes, frogs, plants...)
(ii) The Standard Virtual Biological Parts (SVBPs) database (Cooling et al. 2010) which offers a collection of standardized models that can readily be recombined in own SB projects and that can be compared with the 'Registry of Standard Biological Parts' of the Massachusetts Institute of Technology (http://partsregistry.org/Main_Page), the best known example of library of biological parts
(iii) The FMM (From Metabolite to Metabolite) which is a web-tool for metabolic pathway reconstruction and comparative analysis (http://fmm.mbc.nctu.edu.tw/) (Chou et al. 2009)
(iv) More general informatics tools aiming at facilitating the design of synthetic systems and the possibility for a user to add, edit, combine and experiment with biological elements (DNA, RNA, proteins). These include e.g. VectorNTI by Informax, inc; GeneDesigner by DNA2.0; or mFold by Michael Zuker.

3. DEVELOPMENTS WITHIN SYNTHETIC BIOLOGY

3.1. DNA circuits - Pathway engineering

The rational design of biological circuits with predictable function is a dominating field in SB. It consists in the redesigning or optimization of metabolic pathways and the fine-tuning of transgene expression in response to specific exogenous cues or endogenous metabolites. This approach is mainly based on the fact that systems or synthetic circuits can be developed from a small number of basic modular elements that can be easily chemically synthesized. Synthetic biologists have developed standardized and interchangeable biological parts, also called “bioparts” or "biobricks" that are aimed at performing desired functions in a predictable manner.
Several local registries of such biological parts have been developed such as “BrickIt” (http://brickit.wiki.sourceforge.net), “TinySeq” (http://www.bioperl.org/wiki/Tinyseq_sequence_format), Clotho (http://www.clothocad.org/) or “BioMortar” (http://igem.uwaterloo.ca/biomortar). “The Registry of standardized parts” developed at MIT is however the best known example of library of biological parts that is available on Internet (http://www.parts.mit.edu, http://partsregistry.org). Noteworthy the MIT organizes every year since 2004 the international Genetically Engineered Machine (iGEM) competition, which is a student competition where teams design and build synthetic systems that operate in living cells using a defined registry of standard biological parts.

The creation of novel functional genetic networks with increasing size and complexity not only relies on registries of biological parts, it also demands computational tools for the design of biological parts and software aimed to design systems of parts. These computational tools have made possible multiple sequence alignments, the creation of databases (e.g. those that can integrate available genomic, protein structure and metabolic data), the protein design by two distinctive and complementary approaches (rational design and directed evolution methods), the elimination of DNA methylation sites and/or the optimal codon usage to achieve for example optimal translation rates (Suarez et al. 2010).

In cases where synthetic biological parts are assembled together through a series of restriction and ligation processes to enable “pathway engineering”, the approach could be considered as an advanced extension of classical recombinant DNA techniques. However the SB approach is different in the sense that instead of single parts, whole systems can be potentially built using more than hundred of traits (genes/parts) from different donor organisms. One of the purposes of synthetic circuitry is also the engineering of complex, high precision control devices that couple sensing and delivery mechanisms.

Systems that are developed are generally transferred into bacteria or yeast to evaluate their functionality, but some genetics systems have also been applied to mammalian cells (Greber and Fussenegger 2007; Wieland and Fussenegger 2012) and crops (Misawa 2011). The approaches are for example used to query the principles underlying complex signaling or transcriptional networks or to uncover complex dynamics underlying symbiotic relationships. More recently, spatial optimization of metabolism has brought metabolic engineering beyond the designing of linear metabolic pathways and channeling of metabolic flux by engineering pathways through isolation and organization of multicell and multienzyme complexes (Agapakis et al. 2012). A concrete example is the exploration of the behavior of engineered photosynthetic bacteria, like *Synchococcus elongatus*, inside eukaryotic cells with the aim of mutualistic relationships between photosynthetic bacteria and mammalian cells and thereby creating artificial, engineerable animal chloroplasts (Agapakis et al. 2011).

Along with the gain in fundamental insights and tools in a variety of biological processes, the approach of pathway engineering leads to expectations in many fields, such as the development of new therapeutics, vaccines, environmental biosensors and methods for food, drugs or energy production. Some of these applications are reviewed below.

Health applications

SB offers advanced strategies for the development of diagnostic immunoassays (the ability to express any ‘open reading frame’ (ORF) reduces time and effort to generate recombinant proteins for testing) and drug production (reviewed by Weber and Fussenegger 2011). By improving the production

capacity of the natural host, or by transferring the desired genes or pathways into an industrial/typical laboratory host, such as *Escherichia coli* and *Saccharomyces cerevisiae* (Neumann and Neumann-Staubitz 2010), SB allows for the development of drug screening platforms (e.g. the identification of a food additive as a defense of multi-drug-resistant tuberculosis) (Weber et al. 2008) or the reduction of drug manufacturing processes. Other examples of product synthesis via SB platform include the production of novel antibiotics (Chandran et al. 2006; Menzella et al. 2005), benzylisoquinoline alkaloids, a diverse class of plant secondary metabolites that exhibit a broad range of pharmacological activities (Hawkins and Smolke 2008) or the anticancer drug Taxol (Ajikumar et al. 2010). The production of artemisinin, which is an anti-malarial drug, is expected to be the first commercial application available by 2013 (http://www.amyris.com/en/markets/artemisinin) (Hale et al. 2007; Martin et al. 2003; Ro et al. 2006).

Another development in SB that could potentially lead to a multitude of health applications but still is at a research stage is the building of therapeutic sensor-effector devices. These systems are designed to sense disturbances such as changes in the micro-environment of tumor cells (Anderson et al. 2006; Liu et al. 2002; Ramachandra et al. 2001), or to seek out pathological conditions and restore functions e.g. by fine-tuning the expression of desired genes in space and time and/or by the controlled release of synthetic drugs such as controlled administration of synthetic hormones to patients with glucocorticoid-responsive disease (Stavreva et al. 2009). Interestingly recent work in animal models of human diseases demonstrates that the use of sensor-effector devices and the reprogramming of mammalian cells may soon pave the way for gene- and cell- based therapies (Gitzinger et al. 2012; Kemmer et al. 2010; Ye et al. 2011).

In the field of complex and dynamic biological phenomena such as the mammalian circadian clock, SB approaches also enable the development of systems exhibiting oscillatory behavior, which could provide a deeper understanding of the biological clock and of clinical problems (e.g. sleep disorders, depression, cancer and dementia) associated with it (reviewed in (Susaki et al. 2010)).

Approaches with therapeutic application also consist in engineering bacteriophages to target bacterial biofilms involved in clinically important infections or to enhance bacterial killing by antibiotics (Lu and Collins 2007; Lu and Collins 2009).

Last but not least, in the domain of vector delivery for vaccination, SB could contribute to the identification and design of relevant immunogen candidates (Kindsmüller and Wagner 2011) or to the development of safer vaccines. The “Synthetically Attenuated Virus Engineering” (SAVE) approach is an attempt to provide a universal way to develop safe vaccine candidates for many pathogens (Burbelo et al. 2010). It is based on codon deoptimization, which aims at weakening microbes by rendering them unable to efficiently grow and replicate. In an initial application of this approach, poliovirus expressing deoptimized coding sequences was made relatively innocuous in mice (Coleman et al. 2008). SAVE has also been used to attenuate influenza viruses for vaccination and provided protection from subsequent challenge with wild type influenza virus (Mueller et al. 2010).

Renewable energy applications

Various research institutes and companies are currently working on the development of micro-organisms with optimized synthetic metabolic pathways for the production of biofuels (Savage et al. 2008) and commercial applications are under way. SB is being used for the development of synthetic enzymes that can break down biomass (e.g. cellulose or hemicellulose) into sugars (Dien et al. 2003) or for the development of “synthetic” micro-organisms that produce fuels such as 1-propanol or 1-butanol from glucose (Atsumi et al. 2008; Fortman et al. 2008; Shen and Liao 2008) or hydrogen (de Oliveira and Krassnig 2007; Waks and Silver 2009).
Most recently, a proof-of-concept was provided for the production of fuel substitutes for precursors suitable for three engine types (gasoline, diesel, jet) by using cellulolytic and hemicellulolytic *E.coli* strains (Bokinsky et al. 2011) thereby consolidating two steps – depolymerizing cellulose and hemicellulose into sugars, and fermenting the sugars into fuels – into a single step. The engineering of cyanobacterial species and eukaryotic algae has also recently been explored for the production of valuable bioindustrial compounds such as biofuels and other commodity chemicals (Ducat et al. 2011; Ducat et al. 2012; Radakovits et al. 2010).

Environmental applications

Potential environmental applications of SB cover biosensing systems which convert environmental signals into specific cellular events, like for example the detection of arsenic in water by E. coli displaying a detectable pH change (see one of the iGEM project in 2006 at (http://2006.igem.org/wiki/index.php/Edinburgh_summary_page). Further examples have been reviewed by (Marchisio and Rudolf 2011).

Other research and development efforts have focused on the engineering of micro-organisms to remediate (degradation or accumulation) some of the most hazardous environmental contaminants, such as heavy metals (arsenic) and pesticides (e.g. herbicide atrazine), by taking advantage of natural biodegradative pathways (Chopra and Kamma 2006; de la Pena et al. 2006; Sinha et al. 2010; Yang et al. 2005).

Interest has also recently emerged in engineering microbial consortia, which are multiple interacting microbial populations. Consortia can perform more complex functions compared to individual populations e.g. increased resistance to environmental conditions. The development of synthetic microbial consortia are explored for their potential health, environmental, and industrial applications and are also used as a tool for studying microbial interactions and evolution (Brenner *et al.*, 2008). In that respect, synthetic ecosystem communication between mammalian and bacterial cells (cross-kingdom communication) has been designed with the ultimate aim of studying and mimicking fundamental coexistence patterns in nature such as symbiosis, parasitism or predator-prey interactions (Weber et al. 2007).

Other applications: biomaterials

SB could also allow the optimization of the production of some existing biomaterials in 'synthetic' biological systems, which are easy-to-handle and to produce *in vitro*. One example is the development of various synthetic pathways enabling the production of spider dragline silk in *Salmonella* strains for textile application.

Regulatory and biosafety issues associated with DNA circuits - pathway engineering

DNA circuits or pathway engineering can be considered as a refinement of the recombinant DNA technology potentially leading in some cases to a higher order of complexity. The GMO regulatory framework seems adequate to offer the basic elements of risk assessment to deal with these applications of SB.

The robust engineering of efficient and functional micro-organisms through the combination of multiple parts/circuits should not be expected in the short term given the current gaps of fundamental knowledge on how molecular networks operate in single cells. Indeed, the creation and engineering of operable pathways and networks with desired features requires a good knowledge of the genes being introduced, the characteristics of the receiving organism and the interactions involved. It has been suggested that population dynamics and physiological control of gene expression will prevail over most, if not all artificial attempts to engineer and optimize performance of the wanted catalytic activities. Therefore, one can expect that the vast majority of synthetic biological systems developed for a certain application will be engineered firstly by transferring a limited number of well characterized genetic circuits (cfr MIT's registry of Standard Biological Parts') into well-known and non-pathogenic microbial hosts (Canton et al. 2008). Potential hazards (such as disease to humans, animal or plants and deleterious effects due to establishment or dissemination in the environment) associated with the recipient, the donor micro-organism, the vector or inserted material should therefore be limited and the corresponding risk evaluation should rely on the experience and familiarity gained in the risk assessment of "classical" GMOs and pathogens.

However, in the future, applications might involve the insertion of an increased number of parts/traits such as encountered in the building of complex synthetic DNA circuits. The possibility that unintended and unexpected properties emerge upon this higher-order of combination of parts

cannot be ruled out and therefore make risk assessment more difficult. Even if the source of all of the parts of a synthetic micro-organism are known, and every new genetic circuit understood, it could be difficult to assess the interactions between all these parts/circuits and to predict in advance whether the organism would have any unexpected emergent properties. At some point, the more a genetically engineered organism departs from a known host or donor organism or genetic sequence, the more difficult it will be for risk assessors to predict the characteristics of the engineered organism on the basis of the characteristics of the different single parts. Given that the risk for human health and the environment associated with the accidental release of such an organism from a contained use installation would be difficult to assess in advance, a precautionary approach should be adopted: high biosafety requirements should be implemented until a reasonable level of safety could be demonstrated.

Other challenges in the risk assessment could be associated with some environmental applications, in particular those involving organisms which can easily spread in the environment and/or organisms developed to live under specific biotic conditions or stresses (e.g. bioremediation). Relevant information will be needed to assess the survival, multiplication and dispersal capacities of these organisms in all potentially receiving environments.

3.2. Top-down approach : Genome Minimization

The “top-down approach” aims at simplifying an existing micro-organism by determining the smallest number of genes required to survive under laboratory conditions (Schmidt and Pei 2011). Taking benefit of the fact that minimal genomes require less energy to perform specific tasks and produce less undesired waste products, one of the goals is to develop a genetically stable cellular platform or “chassis” with still robust metabolic performance.

Genome minimization is generally used as a first step to engineer new biological circuits or pathways (see previous chapter) for instance by transferring different metabolic pathways or BioBricks in order to genetically programming the micro-organism to carry out specific functions (Gil et al. 2004; Luisi 2002). Genome minimization also aims at providing insights into the regulations of metabolism and the origin of life of micro-organisms and more complex organisms. Genome reduction by about 20% has been obtained for *Escherichia coli* (Kolisnychenko et al. 2002; Posfai et al. 2006; Sharma et al. 2007), *Bacillus subtilis* (Morimoto et al. 2008) and *Mycoplasma genitalium* (Glass et al. 2006). Genome minimization is also currently explored within the eukaryotic model system *Saccharomyces cerevisiae* within the frame of the synthetic yeast genome project *Sc2.0*, that progressively replaces native yeast DNA with synthetic sequences (Dymond and Boeke 2012). Another Synthetic Biology project consists in the construction of *Mycoplasma laboratorium* by injecting a reduced version of the *Mycoplasma genitalium* genome in an existing *M. genitalium* whose genetic material has been removed (US patent application: 20070122826).

Several approaches have been used to identify the minimum genome under certain conditions (laboratory, industrial large scale processes). This includes *in silico* sequence comparison of distinct ancient lineages (Koonin et al. 2002; Mushegian 1999) or large scale gene inactivation to identify non-essential genes via global transposition or single-gene deletions or knockout deletions (Gerdes et al. 2003; Giaever et al. 2002). Researchers have developed a variety of deletion methods, including homologous recombination using suicide plasmids, linear DNA recombination using the phage Red system, site-specific recombination system, and random deletion by double transposition, enabling targeted and/or scarless (i.e. devoid of all exogenous sequences) deletion (Fehér et al. 2007; Pennisi 2005; Sung et al. 2009; Zhang et al. 2010).
More recently the approach of genome minimization has led researchers to develop a mathematical model of a hypothetical cell with the minimum number of genes necessary to grow and divide in an optimally supportive culture environment, with the aim of allowing the explicit connection of genomic sequence information to physiological predictions (Shuler et al. 2012).

Regulatory and biosafety issues associated with the top-down approach

In this approach, the size of the genome of known micro-organisms is reduced to a minimum.
From a safety point of view, this approach should not result in micro-organisms that raise other concerns than those associated with their wild-type counterpart. Moreover, the minimal cells will only be able to perform a limited set of physiological but "desired/required" functions. It is expected that most of these cells will only survive under specific laboratory conditions and will have a limited persistence in the environment compared to the original counterpart. Hence research and development will most probably be restricted to contained activities while accidental release in the environment will represent reduced, if not negligible, risks.
Of course, further engineering of these minimal cells by integration of biological circuits would require additional risk assessment on a case-by-case basis (see previous chapter).

From the regulatory point of view, a potential issue relates to the regulatory status of the technique used to generate minimal cells. Indeed certain techniques mentioned above are not considered as techniques leading to GMOs in the meaning of the current European GMO regulatory framework or are exempted from this framework.

3.3. Bottom-up approach : Synthetic genomics and protocells

Contrary to the "top-down" approach, the creation of new kinds of minimal cellular life are an illustration of how a "bottom-up" approach can result in the engineering of novel minimal biological systems with desired purposes. To this end scratch, raw elements, are used that are not necessarily natural but mimic the properties of natural molecules and that were never alive before (Kuruma et al. 2009; Luisi 2002; Rasmussen 2008; Solé et al. 2007; Walde 2010).

Recent advances in DNA synthesis and computational methods (see previous textbox: techniques in synthetic biology) have led to the reconstitution of organisms with complete synthetic genomes. Technical improvements in the assemblage of large pieces of DNA have led to several milestones including the recreation of RNA Poliovirus by mail-ordered DNA sequences (Cello et al. 2002a; Cello et al. 2002b), the first *de novo* synthesis of a DNA virus (bacteriophage phiX174) (Smith et al. 2003), the recovery of the '1918' influenza virus from preserved tissues of victims (Basler et al. 2001;Tumpey et al. 2005), or the reassembling of chemically synthesized DNA segments into bacterial genome (e.g. *Mycoplasma genitalium* and *M. mycoides*) (Gibson et al. 2008; Gibson et al. 2010).
Synthetic genomics is also applied in the frame of the synthetic yeast genome project Sc2.0 mentioned in the previous chapter. In this case, a complete synthetic 'eukaryotic' chromosome arm has been synthesized, a first step towards the design and construction of an entirely synthetic version of the *Saccharomyces cerevisiae* genome (Dymond et al. 2011; Muller et al. 2012). It shows that today it is possible to reconstitute life chemically based on (biological) knowledge mostly combined with the help of appropriate intermediate organisms.

The ultimate bottom-up creation is "the protocell". A protocell is a self-organized, membrane-like structure of polypeptides which separates the inner from the outer world and, depending on the level of complexity, contains components to perform desired cell functions within a stabilized internal cytoplasm-like environment. Protocells have properties of self-reproduction, self-maintenance and evolution.
Recently, protocell-like or protocellular systems with self-reproduction and self-maintenance properties but not yet fully autonomous have been obtained, thereby beginning to narrow the gap between a once radical vision and experimental reality (Budin and Szostak 2010). Hereby it seems that also "protocell-like creations" will be applicable in the near future (Liu et al. 2009; Xu et al. 2010; Zepik et al. 2008).

Regulatory and biosafety issues associated with the bottom-up approach

From a regulatory point of view, synthetic genomics makes use in general of molecular and recombinant DNA techniques which are not inherently different from those used to develop "traditional" (familiar or well described) GMOs. The current biosafety legislation provides therefore a good framework for supporting the risk assessment of such organisms. This applies also to the case where pathogenic micro-organisms are reconstituted, since the scope of the Belgian legislation on contained use of GMOs also includes pathogens. In consequence, the risk assessment criteria, procedures and risk management measures (adequate biosafety level, protective equipment and laboratory practices) in place for the contained use of GMOs and pathogens seem adequate to address the potential biological risks associated with this approach of SB.

From a safety point of view, the reconstitution of already existing micro-organisms (pathogenic or not) should not lead to main challenges in the risk assessment, as natural comparators will be available. However, the reconstitution in laboratories of controlled or eradicated deadly viruses merits special attention given the harm these organisms could confer to human health and the environment in case of their possible accidental release into the environment. In that respect, some additional measures could be taken during their construction to mitigate the risk. It is not expected for the time being that recreated micro-organisms will be developed for deliberate release in the environment. However, if this would be the case in the future, we can expect that the GMO regulatory framework will be adequate to support the evaluation of the potential biological risks, although a case-by-case and precautionary approach should be taken before making a definitive judgment on the suitability of the current risk assessment procedure.

Current risk assessment criteria and methodologies could be far more challenging in case unknown artificial (e.g. via Directed evolution or MAGE) sequences or complex combinations are used, giving rise to new organisms which are very different from those found in nature. In such cases, identification of an appropriate comparator could be difficult which might complicate the gathering of relevant information related e.g. to pathogenicity of the new organisms, possible toxic or allergenic effects or capacities for survival, multiplication and dispersal in potentially receiving environments. Nevertheless, since protocellular systems have not yet reached all properties of natural forms of life (self-reproduction, self-maintenance, evolution) the persistence of protocells outside laboratory condition (if accidental release would occur) should be very low. Moreover, it is expected that applications of this technology in the near future will be restricted to contained uses.
One could nevertheless point to a potential legal issue related to the regulatory status of protocellular systems. These are entities that are not able to replicate and therefore could not meet the definition of an organism in the meaning of Directives 2001/18/EC and 2009/41/EC. In this case, protocellular systems and bottom-up derivatives without all properties of life would fall outside the scope of both Directives.

3.4. Developing orthogonal biological systems (Xenobiology)

The three SB approaches described so far rely on the genetic manipulation of microbial or eukaryotic forms of life involving nucleic acids and proteins found in nature. This explains why these approaches are considered sharing similarities with recombinant DNA techniques.
The development of orthogonal biological systems represents a totally different area where the objective is to create other forms of life based on chemical manipulation for the laboratory synthesis of biological structures that do not exist in nature. Within this subfield of SB, sometimes called *Xenobiology*[1] (Schmidt 2010a) or *Chemical Synthetic Biology* (Chiarabelli et al. 2012), some scientists, mainly chemists, are applying the orthogonality principle to biology.

[1] Xenobiology (composed of *xenos*- from the Greek meaning "foreign" and biology) has at least 3 acceptations: (1) As used in this document and referring to the development of orthogonal systems Xenobiology is defined as a “new form of life”; (2) A sub-discipline of astrobiology designed to advance our understanding of life’s origin, evolution, and distribution in the universe (covering *e.g.*. exobiology, the study of extremophiles since 1970 or even prebiotic chemistry); and (3) A terminology related to xenotransplantation (transplantation of living cells, tissues or organs from one species to another). It is worth mentioning that the distinction between Synthetic Biology and Xenobiology is not always done in the scientific literature.

Fully orthogonal systems contain biochemical pathways that cannot interfere with naturally occurring ones at all.. It is sometimes also presented as the 'genetic firewall of Xenobiology' (Schmidt 2010a). Described approaches consist in the development of alternative basic biochemical building blocks (*e.g.* nucleic acids or base pairs) or orthogonal ribosomal populations with the aim of creating biological systems that are different both in metabolism and on the genetic information level. Currently, no living organisms based on such unnatural biochemistry exist. The main interests of the research in Xenobiology are to better understand and define life and Darwinian evolution (basic research), but also to develop new biotechnology and pharmaceutical applications.

Before giving more details about Xenobiology an important distinction has to be made between:

- Nucleic acid analogs designed to be used ("recognized") by natural DNA or RNA polymerases and resist nuclease degradation. The chemical properties of these synthetic nucleic acids fit with some applications of genetic engineering and SB described in the previous chapters (*e.g.* therapeutic approaches) but are not suitable if the orthogonal principle is desired (Appella 2009).
- Xeno Nucleic Acids[2] (XNA) designed not to be recognized by natural DNA or RNA polymerases, meeting in principle the requirements of the orthogonality principle.

This chapter will not provide an exhaustive list of all the approaches at present under development but will give some representative examples and show the extent of this explorative field. Some current trends are presented hereunder pinpointing some developed molecules. The following vocabulary is used in Xenobiology: XNA, xenosome, xenoprotein and xeno-organism.

Nucleic acid analogs and expansion of the genetic alphabet

Broadly speaking, the use of XNAs can be summarized in three approaches: (i) to try defining what was the first genetic element of life on earth; (ii) to use XNAs as antisense agents to inhibit for example naturally occurring RNAs; and (iii) to build new genetic systems based on XNAs that may ultimately make possible the synthesis of new forms of life. For this last approach the XNA must be able to catalyze its own replication (*i.e.* to undergo evolution on a self-sustained manner).

One important chemical property of these nucleic acid analogs is that the genetic information is still stored in the four canonical base pairs but, since the backbone is different, natural DNA polymerases cannot read and duplicate the genetic information (Schmidt 2010a). Several xenonucleotides have already been produced, such as the acrylic analogs Glycol Nucleic Acid (GNA) and Flexible Nucleic Acid (FNA) based on formyl glycerol (Orgel 2004). Peptide Nucleic Acid (PNA) is another polymer analog of DNA or RNA synthesized for the first time in 1991. PNA is made from the same chemical bases as DNA but the molecule's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds (Nielsen et al. 1991;Wittung et al. 1994). Recently developed thioester PNA (t-PNA) in which the nucleobases were shown to self-assemble onto the peptide backbone in the presence of an oligonucleotide template. This mechanism could mimic replication in synthetic biology construct (Appella 2009).
Other unnatural nucleic acids such as α-L-threofuranosyl Nucleic Acid (TNA) (Schoning et al. 2000) and Hexitol Nucleic Acid (HNA) (Herdewijn and Marliere 2009) can form a Watson-Crick type double helix. Locked Nucleic Acid (LNA) is another example. LNA™ are a class of high-affinity RNA analogs in which the ribose ring is "locked" in the ideal conformation for Watson-Crick binding, substantially increasing affinity for its complementary strand compared to traditional DNA or RNA oligonucleotides. They form more stable duplex than DNA-RNA duplex and are used for antisense inhibition of RNA *in vitro*. LNA™ presents a high *in vitro* and *in vivo* stability envisaged for therapeutic approaches (Petersen et al. 2003).

Intrasystem and intersystem cross pairing
The ability for a nucleic acid to form hydrogen-bonded duplexes to a complementary nucleic acid is called intrasystem cross pairing. On the other hand, intersystem cross pairing is the capacity for an unnatural nucleic acid to interact with complementary sequences of DNA or RNA (formation of XNA-DNA or XNA-RNA duplexes). With these properties in mind it is worth mentioning that, for instance, TNA, HNA and LNA engage in both intra- and intersystem cross pairing. Another property of these XNAs is the resistance to biological nucleases.

[2] XNA: "NA" stands for nucleic acid and "X" refers to the sugar moiety or its substitute. XNA backbone motifs would differ from deoxyribose and ribose and polymerisation will not interfere with DNA and RNA synthesis.

Some scientists have enlarged the genetic alphabet of DNA with synthesized base pairs, leading to genetic codes of more than 4 bases. Benner's team created almost 2 decades ago DNA containing two artificial nucleotide bases "K" and "X" (Sismour and Benner 2005). In a more recent study 3,600 candidate unnatural base pairs recognized by natural DNA polymerase were tested. An optimized base pair (dSICS:dMMO2) was selected and analyzed as a candidate for expansion of the genetic alphabet (Leconte et al. 2008).

One step further in the development of orthogonal systems: polymerization of XNAs
Generally, nucleic acid analogs are difficult to produce in large quantities, and the non-enzymatic polymerization of synthetic polymers is quite inefficient. The solution to overcome this problem has been to design polymerases that are able to replicate XNAs. Artificial genes resistant to biodegradation have been produced[3]. This work demonstrates that synthetic nucleic acids (six alternative genetic polymers not found in nature) have the ability to sustain heredity and evolution. The important step of polymerase engineering to reach the encoded synthesis of unnatural biopolymers is now reached (Pinheiro et al. 2012).

Orthogonal RNA-RNA / translation interactions

This experimental approach uses the specificity of the interaction between ribosome binding site (RBS) on the mRNA and the 16S rRNA on the ribosome to create by directed evolution, orthogonal ribosome populations that do not translate natural mRNAs but only those mRNAs containing an orthogonal RBS (Chin 2006).

'Xeno' amino acid sequences

Another area of Xenobiology is the identification of amino acid sequences (peptides or proteins) that do not occur in nature (Schmidt 2010a). While the number of known natural protein sequences is quite large, it is infinitely small compared to the number of proteins theoretically possible using the 20 natural amino acids. Thus, a huge number of protein sequences potentially exist that have never been observed in nature, the so-called "never born proteins". Several attempts have been made in this area, such as:

- The generation of random amino acid sequences (Luisi et al. 2006);
- The generation of a unique transfer RNA (tRNA)/aminoacyl-tRNA synthetase pair that expands the number of genetically encoded amino acids in *E.coli* (Wang et al. 2001);
- The development of an approach that allows unnatural amino acids with diverse physicochemical and biological properties to be genetically encoded in mammalian cells and the incorporation into proteins of non canonical amino acids (Liu et al. 2007);
- Protein engineering strategies based on the theories of molecular evolution (Yoshikuni et al. 2008).

Regulatory and biosafety issues associated with orthogonal systems

At the current stage of development of orthogonal systems (chemical synthetic biology) main approaches are dedicated to basic research and to dig in the unknown. The main challenge here is rather to define the complex phenomenon called "life" than take potential biorisks into consideration. Other research works are developing new drugs, biotech applications or even nanotechnologies. For the time being, there are still many hurdles to achieve the building of fully operational orthogonal biological systems.

Moreover, it can be expected that first concrete developments in this field will result in systems that are totally dependent on specific culture conditions in a laboratory for replication and/or (xeno-)protein synthesis and consequently highly unlikely to persist in the natural environment. These systems will be handled in laboratories only and the risks should be assessed under the current regulatory framework for contained use of GMOs and pathogens. Scientific uncertainties

[3] A variant of replicative polymerases of *Thermococcus gorgonarius* that can synthesise XNA from DNA template and the engineering of polymerases that can reverse transcribe XNA back into DNA.

should be taken into account to assign these activities to the appropriate containment level according to the precautionary principle.
A potential regulatory challenge may nevertheless be pointed out: as XNAs / orthogonal systems are different from conventional nucleic acids as originally meant in the GMO Directives, it may be questioned whether organisms developed through this approach may still fall under the scope of the existing GMO regulatory framework.

It is unclear whether and when further developments of xenobiology could lead to the engineering of "xeno-organisms" (also called *chemically modified organisms*) that are capable of producing unnatural nucleotides and passing them on to future generations. In any case, they can only be expected to occur in the long term. For this reason it seems premature to draw any conclusion as regards the suitability and applicability of the GMO regulatory framework, current risk assessment procedures and principles for the safety evaluation of such approaches. When more knowledge becomes available, the assessment of potential risks may become easier.

Xenobiology approach is presented by some teams as the key to many biosafety issues as orthogonality is aimed at preventing any exchange of genetic information with the natural world ("genetic firewall"). This statement remains questionable in the current state of knowledge of this technology as it seems indeed difficult to guarantee that orthogonal systems will not interact with organisms already found on earth. In any case, if future applications of Xenobiology lead to the generation of xeno-organisms designed to be released into the environment, necessary precautions will have to be taken. The following specifications have been proposed as a basis of a precautionary approach aiming at ensuring that xeno-organisms would not be able to interact with natural organisms already present into the environment (adapted from (Schmidt 2010a):

- Xeno-organisms should not loose their auxotrophic character;
- Natural organisms should also not be able to produce these essential biochemicals, to avoid a symbiotic relationship with XNA;
- Natural DNA polymerase should not be able to transcribe XNA to DNA;
- Natural RNA polymerase should not be able to transcribe XNA to RNA;
- Artificial polymerase should not be able to transcribe NA to XNA
- XNA genes be taken up by DNA organisms should not be recognized by natural transcription factors;
- Preferably, single stranded XNA should not interfere with the transcription process in natural cells (like iRNA);
- Symbiogenesis between XNA and DNA should not take place;
- XNA should not be a recalcitrant chemical, but should act as “food” for natural organisms after its death/destruction.
- Preferably, additional layers of orthogonality such as non canonical base pairs, rearranged codon assignment etc. should be used to increase the safety mechanism even further.

Some important research groups active in the field of Synthetic Biology in Belgium

- The **Laboratory of Medicinal Chemistry, Rega Institute for Medical Research**, Katholieke Universiteit Leuven. This research group has largely contributed to the development of xeno nucleic acids (XNA). (http://www.kuleuven.be/research/researchdatabase/researchteam/50000720.htm)

- The **Department of Plant Systems Biology, VIB-Ghent**, research department integrating genetics, genomics and biocomputing with the aim of unraveling the biology and to further explore the potential of plants. One of the research projects paves the way for the understanding and further development of drugs with plant-derived natural products by expressing plant biosynthesis pathways in heterologous hosts such as *E. coli* or yeast.

The two following research groups have also played a major role in leading a multidisciplinary group of students in the frame of the iGEM competitions.

- The **Leuven Center for Bio-Science, Bio-Engineering and Bio-Technology** (BioSCENTer): a research center of the Group Science and Technology of the Katholieke Universiteit Leuven KULeuven (http://www.kuleuven.be/bioscenter/igem/) that emerged from 4 original clusters: the research cluster on bioinformatics and systems biology, the cluster on virtual life, the cluster on biomolecular interaction (Biomint), and the cluster on evolutionary biology (iCEB). This research center has successfully participated in the iGEM competitions of 2008, 2009 and 2011 with projects named "Dr. Coli", a *E. coli* that produces a drug to meet patient's needs (http://2008.igem.org/Team:KULeuven), "Essencia Coli", a vanillin producing bacterium equipped with a control system that maintains the concentration of vanillin at a constant level (http://2009.igem.org/Team:KULeuven) and "E.D.Frosti", an engineered bacterium that is able to induce or inhibit ice crystal formation upon demand (http://2011.igem.org/Team:KULeuven).

- The **Laboratoire de Bioinformatique des Génomes et des Réseaux (BiGRe), Faculté des Sciences, Université Libre de Bruxelles** (http://www.ucmb.ulb.ac.be/index.shtml). Their projects involved the engineering of a synthetic *Escherichia coli* strain which synthesizes an adhesive material (http://2009.igem.org/Team:ULB-Brussels), the design of a genetically engineered *E. Coli* with an improved natural hydrogen production pathway, using the organic compounds found in waste waters as substrate (http://2010.igem.org/Team:ULB-Brussels), and the one-step gene insertion or deletion system conferring to *E. coli* the useful properties of yeasts (http://2011.igem.org/Team:ULB-Brussels).

Since Synthetic Biology encompasses multitude of disciplines, some research groups have not always their affiliation in departments that have a long history of genetic engineering. Examples include:

- **The Department of Electrical Engineering and Computer Science, Systems and Modeling, University of Liège**, which is involved in the elucidation of the topology of genetic regulatory networks (GRNs) by computational systems biology.

- **The Laboratory for Ecophysiology, Biochemistry and Toxicology, Department of Biology, University of Antwerp**, which focuses on the selection and characterization of PCB-binding DNA aptamers.

4. SYNTHETIC BIOLOGY: RISK MANAGEMENT STRATEGIES

As for any GMOs, management measures shall be applied for any activities involving the use of synthetic organisms to mitigate potential risks for human health and the environment identified as a result of the risk assessment or to address uncertainties resulting from the risk assessment.

Considering that most organisms developed so far through SB approaches are used in contained facilities, traditional risk management measures (working practices, primary safety equipments, physical containment) used for contained use activities involving GMOs or pathogens can be applied. As suggested by COGEM (COGEM 2008), if the risks cannot be properly assessed due to scientific uncertainties, the activities involving such organisms should be assigned to a sufficiently high level of containment.

When potential risks associated to intentional or unintentional release in the environment of the synthetic organisms have been identified, the implementation of biological confinement strategies or monitoring measures should be envisaged. In this context, it should be noted that some applications of SB will result in the development of organisms which have inherent very poor or even absent growth and/or replication features. This could be the case e.g. for organisms containing minimized genomes, or for protocellular systems.
Moreover, several approaches can be implemented to create organisms with reduced or absent capacities of surviving and replicating outside the laboratory. One option consists in designing synthetic organisms that have competitive disadvantage (e.g. by changing metabolic pathways), or whose growth is dependent on a form of energy or raw material that is unavailable in the environment (auxotrophy). Another approach consists in engineering the organisms to make them sensitive to external signals (such as magnetic fields (Stikeman 2002)) to turn relevant genes on and off. As mentioned above, some applications of SB (like orthogonal biological systems based on alternative biochemical structures) are also presented by some authors as a powerful biocontainment strategy preventing any exchange of genetic information with natural biological systems (Marliere 2009;Schmidt 2010b). One should keep in mind however, that most of these biological confinement strategies have not been fully tested yet to assess how effective they are in achieving full protection of the environment.

In addition to physical containment and/or biological confinement, post-release management strategies could also be applied such as case-specific monitoring and general surveillance measures foreseen by the GMO legislation. Other strategies have also been proposed, such as the inclusion of DNA watermarks (a genetic “bar code”) or morphological genetic markers in synthetic organisms to allow straightforward screening and traceability of their appearance in the environment or in food and feed (Chopra and Kamma 2006).

5. SYNTHETIC BIOLOGY: FROM SCIENCE TO GOVERNANCE

In the past years, several official governmental bodies and national academies have published official statements or recommendations addressing in particular safety or regulatory aspects of SB. These aspects have also been reviewed in a few recent papers (Bar-Yam et al. 2012; de Lorenzo 2010; Schmidt 2010b; Zhang et al. 2011).

Official views in the European Union

In the European Union, statements or recommendations have been expressed for example by the following official bodies:

- The Zentrale Kommission für die Biologische Sicherheit (ZKBS, Central Committee on Biological Safety, Germany).
- The Swiss Academy of Sciences (www.geneticresearch.ch/f/themen/Synthetic_Biology/index.php)
- The Royal Netherlands Academy of Arts and Sciences, together with the Health Council of the Netherlands and the Advisory Council on Health Research (Health Council of the Netherlands et al. 2008).
- The German Academy of Sciences Leopoldina, together with the German Academy of Science and Engineering and the German Research Foundation (DFG 2009).
- The Netherlands Commission on Genetic Modification (COGEM 2008).
- The Royal Academy of Engineering in the UK (Royal Academy of Engineering 2009).
- The European Group on Ethics in Science and New Technologies who presented in 2009 a comprehensive opinion with several recommendations on the ethical, legal and social implications of SB (EGE 2009).
- The European Academies Science Advisory Council, formed by the national science academies of the EU Member States, who issued in 2010 a report on scientific opportunities and good governance in the field of Synthetic Biology (EASAC 2010).

The main conclusions emerging from these recommendations are as follows:

- Choice of terminology is key to enabling a rational discussion of this issue. In particular, a clear definition of SB should be established. However, in practice, this precondition of having a universally agreed definition has been questioned by some authors as it may hamper rather than help the development of regulations, as shown with controversies surrounding the GMO definition.
- Current applications of SB such as metabolic pathway engineering, the use of "biobricks" or the synthesis of a minimal genome organism use well-defined pieces of hereditary material whose functions are known and which are manipulated according to a predetermined plan. This means that sufficient knowledge is available to adequately assess and manage the potential risks of working with synthetic organisms over the short term using the current risk analysis method under the current risk policy.
- Regulation governing GMOs is fully applicable to the majority of SB research and applications (which represent an extension of recombinant DNA technology). In addition, SB products will have to comply with the existing specific regulations depending on the use to which the products of SB might be put. This means that there is no need for new safety legislation on the short term.
- Existing legislation may need to be re-considered, if there are significant advances in modifying the basic chemistry underpinning genetic information machinery and processes, or for some areas of SB that do not necessarily fall within the scope of the GMO legislation.
- On the long term, considering the possibility that more complex applications could be developed, it is very difficult at the moment to make a judgment on the suitability of the current risk analysis at that time.
- In that context, the European Commission should compile information on current risk assessment procedures in the EU to determine if there might be gaps in regulation that need to be addressed in preparation for the advent of novel products developed using the methods of SB.
- A step-by-step approach should be used as for GMOs. This means that the first experiments should be carried out on a small scale in contained facilities until sufficient data have been obtained. Once these data are available, the activities can be carried out on a slightly larger scale. The experiment is thus increased in scale at every step and at each subsequent step more data become available.

- Until a synthetic organism is demonstrated to be harmless, it should be handled with high safety requirements adopted from those already in place for other research and subject to the well-established systems of regulation in place at EU and national levels.
- For synthetically produced organisms, the precautionary principle is an important part of sound ethical debate and of legal, regulatory and political decisions.
- An open dialogue on safety-relevant issues should be installed between the regulators and the researchers to allow innovations in the field to be covered by the regulations.
- The European Commission should take the initiative to have a code of conduct for research on synthetic micro-organisms. The Code should, for example, assure that SB organisms are manufactured in a way that they cannot autonomously survive if accidental release into the environment would take place.

In March 2010, the European Commission's DG SANCO organized a workshop on Synthetic Biology with the aim to provide an overview of the science and its applications, and to discuss the challenges and opportunities of SB in terms of governance, social, ethical and legal issues (EC 2010). The major conclusions of this workshop in relation to biosafety issues where: (i) the need to establish a clear definition for SB and its products (through the European standardization bodies); (ii) the need to undertake a review of the EU regulatory framework to determine whether all potential applications of SB are adequately covered; (iii) the need to include a systematic consideration of the relevant safety and ethical aspects in EU research projects on SB, and (iv) the need for early public engagement, as SB is likely to raise public concerns similar to those of GMOs.

More recently, the European Commission published a report drafted by a New Technique Working Group (NTWG) established at EU level, and considering regulatory and safety aspects of techniques for which it was unclear whether they would result in a GMO and whether the resulting products fall under the scope of the existing GMO/GMM legislation. Synthetic genomics was one of the techniques considered. The NTWG concluded that the application of this technique and its potential for the development of novel synthetic organisms may lead to some challenges as regard to certain steps in the risk assessment, such as the difficulty of identifying suitable or appropriate comparator. The NTWG also observed that there were different possible interpretations as regard to how the technique should be covered by the GMO legislation and questioned whether the GMO legislation would be the most appropriate place to deal with this technique, and with SB in general.

Official views in the United States

In the United States, the dominant idea is also that the existing policy and regulatory framework for biotechnology applies, with minor adaptations, to cover synthetic organisms. For example, the NIH Recombinant DNA Advisory Committee came to the conclusion that in most cases, research with synthetic nucleic acids presents biosafety risks that are comparable to recombinant DNA research and that the current risk assessment framework can be used to evaluate synthetically produced nucleic acids with attention to the unique aspects of this technology. Recently, the NIH clarified the scope of the Guidelines for research involving recombinant DNA molecules to specifically cover synthetic nucleic acid molecules in order to provide principles and procedures for risk assessment and management of research involving such synthetic nucleic acids. According to the latest version of the NIH Guidelines, synthetic DNA segments which are likely to yield a potentially harmful polynucleotide or polypeptide (e.g., a toxin or a pharmacologically active agent) are regulated as their natural DNA counterpart. If the synthetic DNA segment is not expressed *in vivo* as a biologically active polynucleotide or polypeptide product, it is exempt from the NIH Guidelines (see http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.htm). For intended environmental releases of synthetic organisms, the coordinated framework for the regulation of biotechnology products involving EPA, USDA and FDA would be also appropriate (Rodemeyer 2009).

In 2010, the US Presidential Commission for the Study of Bioethical Issues published a report recommending the adoption of a system of "prudent vigilance that carefully monitors, identifies and mitigates potential harms over time" (PCSBI 2010). Five ethical principles and 18 recommendations were highlighted in this report, including mandatory ethics training for engineers working in the area, identification of gaps in the risk assessment practices, adoption of measures that would limit the survival/lifespan of synthetic organisms in the event of inadvertent/accidental release in the environment, continuous assessment of specific security and safety risks of SB research activities in both institutional and non-institutional settings as the field progresses.

Initiatives at international level

Another important point that was repeatedly stated by the above-mentioned bodies is that any approach to regulating SB should be global, rather than involving only the EU and US. Initiatives in that way have already been taken such as such the Joint Conference of the OECD, the UK Royal Society and the US National Academies of Science on ‘‘Opportunities and Challenges in the Emerging Field of Synthetic Biology’’ held in July 2009 in Washington DC (OECD 2009). Discussions on safety issues related to SB have also started in the frame of the Convention on Biological Diversity (see http://www.cbd.int/emerging/). The current work focuses on compiling and synthesizing available scientific information and experiences on the possible impacts of SB techniques and products on biodiversity, on definitions and understandings of SB relevant to the Convention on Biological Diversity and explores and identifies possible gaps and overlaps with the provisions of the Cartagena Protocol on Biosafety.

Self-governance and risk governance

In addition to government-led oversight and control, options for self-governance have also been proposed, mainly coming from the SB community itself. One example is the self-regulation initiative put forward at the SB2.0 conference, which modeled itself on an Asilomar mode[4] of regulation. This proposal was withdrawn due to criticisms from civil society organizations. Another example of self-governance initiative comes from the Craig Venter Institute (Balmer and Martin 2008; Garfinkel et al. 2007) which formulated in 2007 several options for good governance applying to three major stakeholders: commercial firms that sell synthetic DNA to users, owners of laboratory “bench-top” DNA synthesizers, and the users of synthetic DNA themselves and the institutions that support and oversee their work. Although these options focus on synthetic genomics, they can be considered relevant for SB as a whole. In relation with biosafety aspects, the proposed options include the education of potential users of synthetic DNA, the compilation of a manual for biosafety in SB laboratories, and a broad role for Institutional Biosafety Committee to identify and review experiments for both safety and security concerns, plus enhance enforcement of compliance with biosafety guidelines.

In many cases, biosafety considerations related to SB have been part of more general risk governance recommendations encompassing issues such as innovation, risks to people and the environment, and interests and values of all relevant stakeholders. These recommendations suggest that any effective approach to risk governance of SB must be capable of evolving as scientific and technical knowledge expands, requiring flexibility in the face of uncertainties about the eventual nature of products, processes, benefits and risks (see some of the official bodies mentioned above and e.g. Guidelines from the International Risk Governance Council (IRGC 2010). Governance should also recognize that international coordination and dialogue is essential for safety (PCSBI 2010).

In a recent working paper (Zhang et al. 2011) made a critical analysis of the way the current debate about the governance of SB is framed. They argue that instead of trying to format the problem to fit readily available solutions (e.g. by saying that applications of SB will be covered by existing regulations for GMOs), effective governance of SB should employ proactive, open-ended regulatory styles able to work with uncertainty and change, to make links across borders, and to adapt to evolving relations among changing stakeholders, including researchers, research funders, industry, and multiple publics. Effective global governance of SB may be promoted through transnational joint production of "orientational questions".

Research projects addressing safety issues related to synthetic biology

Developments in synthetic biology, including safety, ethical and societal issues, are or have also been addressed in a number of projects. Some of them (the list is far to be exhaustive) are presented below for information.

[4] The Asilomar Conference on Recombinant DNA was an influential conference organized by the scientific community itself (primarily biologists, but also including lawyers and physicians) to discuss the potential risks linked to recombinant DNA techniques. During the conference, held in February 1975 at a conference center at Asilomar State Beach, the principles guiding the recommendations on how to conduct experiments using this technology safely were established.

In the EU, based on a reference document on Synthetic Biology established in 2003 by the European Commission (EC 2003) and as a follow-up of discussions in a NEST (New and Emerging Science and Technology) high-level expert group in 2005 (EC 2005), an important funding effort was initiated by the European Commission under the 6th framework programme. It resulted in the implementation of 18 Synthetic Biology projects, with a total budget of over €32 million (de Oliveira and Krassnig 2007;Pei et al. 2011). These projects include application-specific work in areas ranging from energy to healthcare, as well as horizontal projects looking at SB from a European perspective and analyzing the safety and ethics of synthetic life.
Amongst these projects, SYNBIOSAFE was the first project in Europe addressing safety and ethical aspects of Synthetic Biology (http://www.synbiosafe.eu/). The SYNBIOSAFE report identified three different types of techniques and applications that necessitate a review and adaptation of current risk assessment practices (Schmidt et al. 2009): (1) DNA-based biocircuits consisting of a larger number of different DNA circuits; (2) Novel minimal organisms used as platform/chassis for DNA based biocircuits; and (3) Biological systems based on an alternative biochemical structure, e.g. genetic code based on novel types of nucleotides, or an enlarged number of base pairs. Other projects relevant for biosafety includes TESSY (Towards a European Strategy for Synthetic Biology - http://www.tessy-europe.eu/) (Gaisser et al. 2008;Gaisser and Reiss 2009) and SYNBIOLOGY (http://www2.spi.pt/synbiology/index.asp).
Funds for Synthetic Biology have also been delivered under the EU's Seventh Framework Programme under the heading of Nutrition, Agriculture and Biotechnology. Other EU initiatives are also taking place in the context of the EU-funded Knowledge-Based Bio-Economy (KBBE) programme, of an ERA-NET (European Research Area Network) for SynBio (aiming at providing the basis for a successful forum for the exchange of information between EU member states), or in other projects such as EpiGeneSys (a FP7 European Community-funded Network of Excellence) or EuroSYNBIO (a Collaborative Research Project funded by the European Science Foundation).

In the US, synthetic biology, including risk assessment and regulatory aspects, is addressed in a number of projects, such as:

- The Synthetic Biology Project, established as an initiative of the Foresight & Governance Program of the Woodrow Wilson International Center for Scholars (http://www.synbioproject.org/)
- The Synthetic Biology Engineering Research Center (SynBERC) (http://www.synberc.org/).

6. BIOSECURITY ISSUES ASSOCIATED WITH SYNTHETIC BIOLOGY

In the 90s there was little awareness of the consequence of misuse of the life sciences or the power of Synthetic Biology. Today biosecurity[5] is a matter of concern and has been increasingly recognized as a national and international priority (Inglesby and Henderson 2012). Very often the emergence of biosecurity is associated in occidental countries with the episode of the "Anthrax letters" (*i.e.* letters containing spores of *B. anthracis*) sent after the attack of the World Trade Centre in 2001 (Jernigan 2001). These events have revived fears about the use of biological agents as bioweapons in terrorism activities. Furthermore the growing number of laboratories handling biological agents is likely to increase the risk of laboratory accidents, the accidental release of agents responsible for infectious and/or contagious diseases and the use of biological material for malicious purposes. In theory, those terrorist activities could be directed against humans, animals or even cultivated crops.

Biosafety and biosecurity

Whereas biosafety aims at protecting public health and environment from accidental exposure to GMOs and/or pathogens, biosecurity deals with the prevention of misuse through loss, theft, diversion or intentional release of pathogens, toxins and any other biological materials. It is worth mentioning that the prevention goals of biosecurity are defined independently of the origin of the biological material. Biosafety and biosecurity are complementary to address biorisk issues. With time biosecurity has become associated with biosafety to form the contemporary approach of "biorisk management".

The major fear of the biotech sector, the policy makers and the public comes from the fact that SB gathers various technologies potentially able to design and create "*from scratch*" entirely new organisms that can be harmful. Human pathogenic organisms can cause moderate (incapacitating weapon) to severe (lethal weapon) infectious diseases. Indeed, research intended for legitimate purposes (*e.g*. medical or veterinary applications) may have a potential to be misused in the development of bioweapons. This is what is called "dual-use"[6] research referring to technologies which can be used for both peaceful and harmful aims.

The awareness about the necessity to reinforce biorisk management appeared *inter alia* when some members of the scientific community (including the biosafety community) and policy makers clearly acknowledged that gene sequencing and synthesis (today in the Megabase range in less than a week) became cheaper and easier than never before and that genome and coding sequences of many organisms were publically available on line, *e.g*. GeneBank (http://www.ncbi.nlm.nih.gov/) or the Ensembl project (http://www.ensembl.org/index.html). For example, the 3,215 base pairs of the Hepatitis B virus (HBV) genome could be synthesized for less than € 100 (Wimmer et al. 2009).

Furthermore it was also noticed that there was a growing interest (i) in academic and bio-industry worlds to develop applied research in SB and (ii) in the biohackers community[7] to conduct their own experiments apparently outside of the "traditional" safety oversight associated with the biosafety regulatory framework (see textbox on *Do It Yourself biology community*). It worth mentioning that, as it is of upmost importance for academic and the private sector to publish scientific data in *peer reviewed* journals, to protect intellectual property and to patent inventions, it's quite usual for biohackers to freely exchange information through websites, personal blogs or to share their experiments on videos on YouTube©.

[5] Biosecurity definition adopted by the World Health Organization is the following: "*The protection, control and accountability for biological agents and toxins (defined as Valuable Biological Material) within laboratories, in order to prevent their loss, theft, misuse, diversion of, unauthorized access or intentional unauthorized release*" (WHO/CDS/EPR/2006.6).

[6] Initially, used to refer to the aspects of certain material, information, and technology that are useful in both military and civilian spheres. It is increasingly being used to refer not only to military and civilian purposes, but also to criminal and terrorist activities. Source: OECD Biosecurity codes, http://www.biosecuritycodes.org/gloss.htm.

[7] A biohacker is an amateur biologist and should not be confused with a bioterrorist.

Do-it-yourself biology community

The ease of access to genetic information and to molecular biology techniques is increasing the accessibility of biological research to biohackers or "garage scientists" of all experience levels and backgrounds (de Vriend 2006). With the movement known as do-it-yourself biology[8], biological research is no longer contained in laboratories of universities and private companies.
In the U.S, compliance with the NIH guidelines for research involving recombinant DNA molecules is mandatory for projects receiving NIH funds and are also widely accepted and voluntary followed by public or private organizations. In Europe, activities involving GMOs must comply with the provisions of Directives 2009/41/EC and 2001/18/EC, and those involving pathogens are regulated by Directive 2000/54/EC.
However, activities performed by "garage scientists" are not regulated, which appears in contradiction with the GMO and pathogens legislations and all the measures taken by scientists working in recognized institutions that must comply with these regulations. One can suppose that most of the people of the DIYbio community are driven by scientific curiosity and not by malicious intent. However, even in the hands of well-intentioned researchers, unintended risks could always occur. Biosafety resources and web links referencing to a code of conduct are available on the DIYBio website (http://diybio.org/blog/category/safety). Anyway, these codes of conduct when available are only applied on a voluntary basis.

Production of biological agents "from scratch"

In the last ten years several research teams have published results showing the feasibility to produce functional biological agents *in vitro* through synthetic biology. The published data by the Craig Venter team particularly propagated the expression "from scratch" meaning literally "*ex nihilo*" (Gibson et al. 2008). As already shown in the previous chapters the "bricks" are available and the technologies to assemble those genetic elements are mastered. Nowadays the idea that pathogenic organisms that are no longer found in nature (*e.g.* Smallpox virus) can be "reconstructed" or that existing pathogens can be quite easily synthesized to increase their pathogenicity becomes quite realistic.

As we will see later on in this chapter, this recognized feasibility raised concerns and boosted public and private initiatives to develop code of conducts and other recommendations to mitigate the biorisks potentially associated with, but not only, Synthetic Biology. On the one hand the risk of creating new pathogenic organisms "*from scratch*" should not be overestimated. In the current state of development of biotechnology, it is certainly easier and cheaper to isolate natural pathogenic micro-organisms rather than manufacturing them via a SB approach and deliberately releasing them in a efficient way (Serrano 2007). On the other hand the recreation of known biological agents becomes easier with time. For instance the *in vitro* production of poliovirus[9] from mail-ordered DNA sequences in 2002 (Cello et al. 2002a; Cello et al. 2002b) and the reconstruction of the 1918 Spanish Influenza strain in 2005 (Tumpey et al. 2005) are good examples of research projects that raise important biosecurity concerns. Figure 2 illustrates how fast the *in vitro* production of gene sequences and micro-organisms has been growing in the past years.

[8] See the website http://DIYbio.org. DIY biology represents a movement with an informal network of 2,000 members apparently mainly active in North America and Europe.
[9] In 1985 the team of Racaniello already published a paper demonstrating the feasibility of the *in vitro* synthesis of infectious poliovirus RNA (Columbia University College of Physicians and Surgeons, NY).

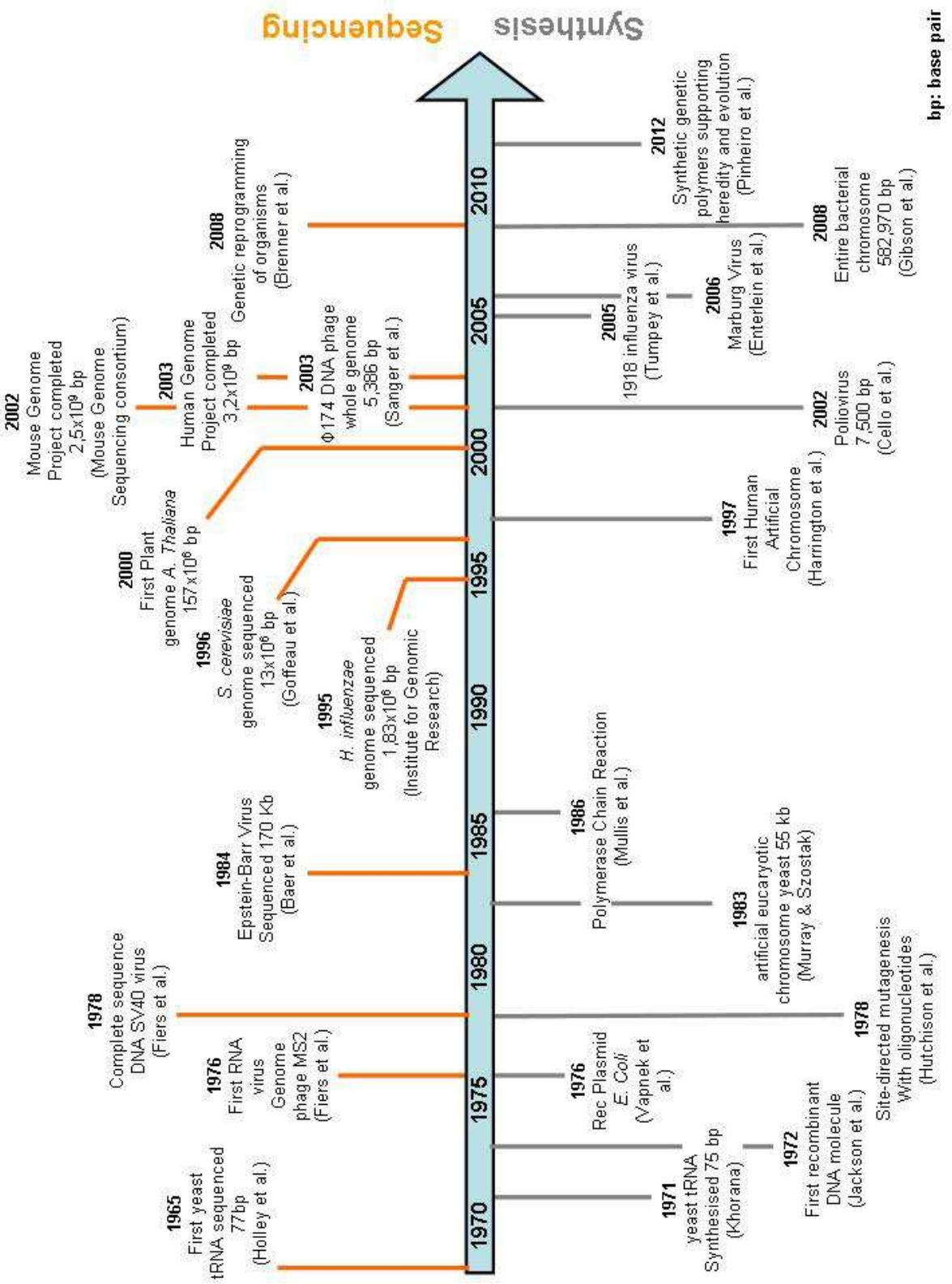

Figure 2: Timeline showing the increasing "feasibility" of gene sequences and micro-organisms reconstruction *in vitro*, and the parallel increase in size of genome synthesized

A recent controversy, although not directly related to SB, has also demonstrated that the publication in the scientific community of experimental results involving certain pathogenic micro-organisms is sometimes sensitive enough to generate heated debate on "dual use" research and the possible production of biological agents "from scratch". This happened after the request for publication in the scientific journals *Nature* and *Science* of details about experiments with the H5N1 bird flu, showing in particular transmission of mutant avian influenza H5N1 strains from ferret to ferret[10] (see textbox).
This controversy is notably a result of the new rules adopted since 2003 by several scientific journals (including *Biochemistry, Biosecurity and Bioterrorism, Journal of Virology, Nature, PNAS, Science*...) to select papers that can potentially lead to dual-use of biological agents (Atlas et al. 2003).

Highly pathogenic H5N1 experiments: an example of the current debate on "dual use" research

In December 2011 an intense debate started within the scientific community about the possible public release of experimental data obtained concomitantly by two research teams[11], related to the *in vitro* production of H5N1 bird flu virus transformed into mutant forms falling into the category of highly pathogenic avian influenza (HPAI)[12]. The fact that these influenza viruses were able to spread among mammals has revived the controversy about the opportunity to publish or not such data.
In a first instance the US National Science Advisory Board for Biosecurity (NSABB) asked the scientific journals *Nature* and *Science* to withhold details about experiments with the H5N1 bird flu. As a result, a 60-day moratorium was agreed by the scientific community on research on mutant avian influenza H5N1 strains. A meeting was subsequently organized by the WHO about virus research with the conclusion that the detailed results of the above-mentioned researches could be published. As a result, the NSABB agreed on the full publication of the two papers (Yong 2012).
The scientific community remains divided on this issue. On the one hand, some scientists argue that these data are of upmost importance to better understand the properties of the avian H5N1 influenza virus, including public health protection goals (*e.g.* virulence study, vaccine development). On the other hand, other scientists disagree with the WHO recommendations, being convinced that when these data are available, it will allow *Biohackers,* or worse (bio)-terrorists, to reproduce the experiments and to obtain mutant H5N1 strains "from scratch". They are of the opinion that without any oversight this type of experimentation could represent a potential risk for human population in the form of a pandemic.

Addressing biosecurity issues

There are two main types of initiatives related to biosecurity concerns: those developed specifically for SB and those that take into consideration all types of biorisks including SB products (mainly focusing on pathogenic organisms). Because biosafety and biosecurity are entangled, some initiatives are not repeated here, as they were already listed in chapter 5 above.

Initiatives addressing specifically Synthetic Biology
Various governmental or private initiatives have been implemented at the international level in order to mitigate risks associated with potential misuse of SB. These include the screening and checking orders by firms supplying synthetic DNA for potential harmful DNA sequences using specific software, the introduction of biosafety and biosecurity background in the educational system, the set-up of a professional society for Synthetic Biology, or the development of a Biosafety manual for SB laboratories (EC 2005; Garfinkel et al. 2007). Another example is the adoption of commitments in security by the iGEM community (http://2011.igem.org/Security).
In 2007, a report entitled "Synthetic Biology & Biosecurity Awareness in Europe" was published in the frame of the Synbiosafe European project (Kelle 2007). This report lists biosecurity and other regulations applicable to SB in Europe, including the terms of the Biological Toxins Weapon Convention (BTWC) [13].

[10] Ferret is the experimental animal model to study human-to-human transmission.
[11] The paper of the Fouchier's team in the Netherlands (Erasmus Medical Centre) was submitted to *Science* and the paper of the Kawaoka's team in the USA (University of Wisconsin-Madison) was submitted to *Nature*.
[12] From a biosafety point of view, HPAI strains currently require a Biosafety containment Level 3.
[13] The convention on the prohibition of the development, production and stockpiling of biological and toxin weapons on their destruction entered into force on 26 March 1975.

The Industry Association Synthetic Biology[14] issued in 2008 a report entitled "Technical solutions for Biosecurity in synthetic biology" (Bernauer et al. 2008). The same association established also in November 2009 a global code of conduct and developed norms for best biosecurity practices in gene synthesis (http://www.ia-sb.eu). Among the IASB recommendations, a strengthened cooperation with the Goldman School of Public Policy in building the infrastructure for a Virulence factor database was agreed. The resource called the "Virulence Factor Information Repository" (VIREP) is a web-based database containing the annotate genomes of select pathogenic organisms. In principle, the use of this database allows *inter alia* semi-automated identification of virulence factor sequences.

In a report published in 2004 the US National Research Council highlighted the fact that it was the scientist's responsibility to consider the "dual-use" potential of its research and to take the precaution needed to minimize the misuse of their data (NRC 2004). This report recommended a system of voluntary self-governance by the scientific community. Following this report, the U.S. government established the National Science Advisory Board for Biosecurity (NSABB), a federal advisory committee providing advice and guidance regarding biosecurity related to "dual use" research. In April 2010 the NSABB published a report (NSABB 2010) providing the following main recommendations: (i) SB should be subject to institutional review and oversight since some aspects of this field pose biosecurity risks; (ii) Oversight of "dual-use" research should extend beyond the boundaries of life sciences and academia. SB is just one example of an area of science that may pose some dual-use research concerns and whose practitioners span multiple scientific disciplines; (iii) Outreach and education strategies should be developed that address "dual-use" research issues and engage the research communities that are most likely to undertake work under the umbrella of SB; (iv) The US Government should include advances in SB and understanding of virulence / pathogenicity in efforts to monitor new scientific findings and technologies.

Particular security issues related to research with dual-use potential has been addressed again by the National Research Council in 2010 (NRC 2010) in the US and very recently in UK networks in Synthetic Biology. One of the conclusion of this last report was that a more systematic dual-use education effort was needed within the scientific community at large (Edwards and Kelle 2012).

Initiatives covering all kind of biorisks including Synthetic Biology

Some representative biosecurity laws, regulations and guidelines that may be related to SB have been listed in a recent publication (Bubela et al. 2012).

At international level, the WHO has published a Biorisk Management document entitled "Laboratory Biosecurity Guidance" as a complement to its Laboratory Biosafety manual. The third issue was released in 2006 (WHO 2006).

Since several years now, biosecurity issues are also taken into consideration at the United Nations level through the *Biological and Toxin Weapons Convention* (BTWC). This international agreement covers biological agents and toxins whatever their origin or method of production when they have no justification for prophylactic, protective or other peaceful purposes. The concerns are that SB techniques could generate artificial pathogenic organisms and therefore facilitate the preparation of bioweapons. In 2007, a code of conduct for biosecurity has been proposed by the Royal Netherlands Academy of Arts and Sciences to the scientific community as required by the BTWC (Royal Netherlands Academy of Arts and Sciences 2007).

In the US, relevant documents include the *Select Agents Regulations* (SAR)[15] which regulates the possession, use and transfer of select agents, and the *Screening Framework Guidance for Producers of Double Stranded DNA*[16] which aims to cover potential harmful synthetic biological agents and toxins that are not covered by the SAR.

At EU level, the European Commission has adopted in 2007 a Green paper on bio-preparedness (EC 2007). The aim of this paper was to stimulate a debate and launch a process of consultation at European level on how to reduce biological risks, and to enhance preparedness and response capabilities. The Green paper did not encompass the SB issue but mainly focused on the possible occurrence of a bio-terror attack in the EU and a list of identified pathogenic organisms.

[14] IASB is a consortium of gene-synthesis companies located mainly in Europe proposing some actions to counter bioterrorism.
[15] http://www.selectagents.gov/Legislation.html
[16] http://www.phe.gov/Preparedness/legal/guidance/syndna/Documents/syndna-guidance.pdf

So far, no EU legislation specifically addressing biosecurity has been developed except the dual-use regulation. In 2009 the European Commission has indeed adopted a Regulation significantly amending the Council Regulation (EC) No 1334/2000 of 22 June 2000 setting up a Community regime for the control of exports of dual-use items and technology (EC 2009a). This Regulation includes biological agents when developed as bioweapons as defined in the BTWC.

In May 2010 the European Union launched another 'risk mitigation' initiative, called the EU CBRN Risk Mitigation Centres of Excellence network project or CBRN CoE (http://www.cbrn-coe.eu/#). This network project aims at implementing a coordinated strategy to mitigate the risk of different potential dangerous materials at the international, regional and national levels. The objective is to protect the world and the EU against intentional (proliferation, theft, sabotage and illicit trafficking) and non-intentional (man-made or natural) incidents with chemical, biological, radiological or nuclear (CBRN) material. This network is implemented jointly by the European Commission's Joint Research Centre (JRC) and the United Nations Interregional Crime and Justice Research Institute (UNICRI).

Other recent examples of initiative addressing biorisks including those posed by Synthetic Biology include:

- The invitation to adopt the *Laboratory Biorisk Management CWA 15793:2011* to mitigate the production of bioweapons. This document set up in 2008 and revised in 2011, describes the technical, behavioral and management measures based on biological risk assessment (CEN 2011).
- A collective work entitled "Setting a standard for stakeholdership: Industry contribution to a strengthened BTWC" published as a result of a one-day seminar entitled "The Biological Weapons Convention, Biosecurity and the Industry" organized by the Belgian Foreign Ministry in Brussels on 20 June 2011 (Zanders 2011).
- The Virtual Biosecurity Center (VBC)[17]. This global multi-organizational initiative, founded in 2011 and spearheaded by the Federation of American Scientists, focuses on SB but not only. It is committed to countering the threat posed by the development or use of bioweapons and the responsible use of science and technology. The VBC is a valuable source for biosecurity information, education, best practices, and collaboration.

Some reflections on biosecurity issues associated with Synthetic Biology

The upcoming results of SB should not mask the possible risks associated with it, especially in terms of biorisks. The (micro)-organisms developed through techniques similar to classical DNA, RNA and protein engineering will be comparable to already known "natural" organisms (pathogenic or not) or GMOs. When xeno-organisms (cfr. Chapter 3.4) and related chemical components (XNA, xenosomes, xenoproteins) will be available on a large extent and considered as "Valuable Biological Materials", they will eventually be covered by the biosecurity principles under the Biorisk management framework. There is an internationally converging trend since several years to take into account not only the biosafety of SB but also the biosecurity aspects.

If organisms obtained thanks to the various technologies of Synthetic Biology do not pose immediate risk necessitating reinforced biosecurity measures[18], the question is raised for the near future. Indeed, it is difficult to predict to what extent the discipline will evolve and how dangerous the applications of SB could be. Self-regulation approach proposed by the Industry sector for SB companies and biohackers (*e.g.* IASB initiative) or more generally, the implementation of the Laboratory Management CWA 15793:2011 by private and public research institutions and the adoption of a code of conduct by scientists may be seen as important steps to reinforce global security. However, these initiatives are taken on a voluntary basis and do not perhaps give all the necessary biological security warranties. According to some stakeholders, an independent oversight would still be needed (Anonymous 2008).

[17] http://virtualbiosecuritycenter.org/
[18] Taking into account that appropriate biosafety measures are already applied.

7. DISCUSSION

Synthetic Biology is a heterogeneous, complex and rapidly evolving field. Some features of the products, tools and approaches involved may pose challenges at the regulatory and risk assessment levels.

A first challenge relates to the fact that there is currently no internationally agreed consensus about a definition of Synthetic Biology. On the one hand, having such a definition could facilitate enabling a rational discussion of this issue. On the other hand, working on this definition could polarize controversies and hamper rather than foster the debate on this topic, as shown with controversies surrounding the GMO definition. We do not see the adoption of a definition as key for enabling discussion on the potential regulatory and risk assessment challenges of SB. In particular it should be stressed that the majority of current SB researches and developments involve well-known and characterized techniques, in particular recombinant DNA technology. This is important from the regulatory viewpoint but also from the risk assessment viewpoint. Some authors pointed out that placing a new name on an old technology does not necessarily create a new hazard (Synthetic Biology is a "Buzzword"). We think indeed that we should avoid assigning 'new' hazards to all approaches of SB but we are recognizing that the combination of various disciplines may give rise to an additional level of emerging and unintended hazards.

When addressing potential challenges in risk assessment, it is also important to note that though SB paves the way for various applications (medicine, renewable energy strategies or environmental remediation), for the short term (within 5 years) we may expect that activities in this field will focus on research and development. It should take some time before a 'synthetic organism' is introduced into the environment or before commercial applications become available. In consequence, we can expect that for the moment work on synthetic organisms will remain restricted to laboratories where potential risks for human health and the environment can be controlled more easily provided that appropriate biosafety measures are in place.

Considering the current and short-term developments in this field, it is reasonable to assume that the risk assessment criteria and methodology and risk management systems established for GMOs and pathogens can serve as a good basis for addressing potential risks associated with SB. For instance, one should not expect the comparative approach to be challenged in case of genome minimization, insertion of a (limited number) of well-characterized genetic circuits using isolated and characterized 'standard biological parts', or bottom-up approach for reconstituting known micro-organisms. Moreover, current developments mainly involve the use of well-characterized micro-organisms and genetic material, for which sufficient knowledge is available to assess the potential risks. Last but not least, in the near future we don't think that above-mentioned approaches or other developments such as the engineering of protocells or orthogonal systems will generate micro-organisms or entities that are far different from existing organisms. It can therefore be reasonably expected that their manipulation in the laboratory or their accidental release in the environment would not represent additional risks.

In the future, synthetic organisms could be developed that will differ more fundamentally from naturally occurring organisms, e.g. by increasing the number of introduced biological parts, by using sequences that are unknown or by using orthogonal systems. In such cases, it could be more difficult to identify an appropriate comparator, to gather relevant information to perform characterization of the potential hazards and/or to predict the behavioral knowledge of such engineered organisms in case of accidental release in the environment. Within this regard, uncertainties in the risk assessment of activities involving the use of these organisms in confined area could be managed by adopting a sufficient high level of containment, while potential risks associated to unintentional release in the environment could be addressed by the implementation of biological confinement strategies[19] and monitoring measures. However, while we agree that a precautionary approach is important in cases of high complexity and uncertainty, we are of the opinion that application of containment and confinement measures should be realistic, proportionate to risk and adopted on a case-by-case basis to allow sufficient flexibility and not excessively hamper research and developments initiatives.

[19] Xenobiology is presented by some scientists as a powerful tool in the development of biological confinement strategies. We are of the opinion that such a statement is premature in the current state of knowledge of this technology.

Increased containment only brings increased safety when applied appropriately. In this regard, a step-by-step approach should be adopted aiming at increasing knowledge during the development phases. In this manner, containment and confinement measures could be adapted progressively when more data become available to feed the risk assessment.

As mentioned in this document some developments in the field of SB could lead to environmental applications in case organisms are deliberately released into the environment. Addressing potential environmental risks in case of deliberate release adds a layer of complexity to the risk assessment as one has to deal with the complexity of biological and physico-chemical interactions. For applications involving micro-organisms it should be noted that risk assessors and regulators have relatively little experience considering the potential risks posed by the intentional release of micro-organisms, including GMOs. Indeed the experience gained in the environmental risk assessment of GMOs comes almost exclusively from GM plants. Compared to environmental plant biology the environmental microbiology is more complex and there is still little knowledge about the natural baseline of soil functioning (the nature of the soil microbiota, its dynamics, activities, interactions and response upon a disturbance), a key element to perform an environmental risk assessment. Keeping this in mind, we are of the opinion that addressing potential challenges in environmental risk assessment is premature since environmental applications of SB are not expected to materialize before several years.

At the regulatory level, the main conclusion that can be drawn to date is that current activities involving the development and use of synthetic organisms make use of techniques that fall within the scope of Directives 2009/41/EC and 2001/18/EC. In consequence the European GMO regulatory framework is for the moment adequate to support risk assessment of these activities. This applies also to cases where pathogenic micro-organisms are manipulated or reconstituted, since the scope of the Belgian legislation on contained use of GMOs also covers pathogens. However, it should be noticed that the generation of minimal cells, the use of protocells or the development of orthogonal systems (modification of the basic chemistry on which genetic information machinery and processes is relying) could raise potential issues as regard the regulatory status of the resulting organisms as these approaches could be considered as not leading to GMO or not meeting the definition of an organism in the meaning of the EU legislation.

A specific aspect of SB is that practitioners have diverse academic and professional backgrounds that go beyond biology, including physicists, computer scientists or electrical engineers. This multidisciplinary also characterizes the iGEM initiative, where students are challenging each other in designing and building synthetic systems. These people do not necessarily have experience with microbiological safety, which can make the awareness of potential biological risks and the application of biosafety measures more challenging. Moreover, the increased cost-effectiveness, performance, efficiency of DNA synthesis and sequencing and the increased accessibility and availability of computational tools and molecular biology techniques have lead to movements known as ‘do-it-yourself’ (DIY) biology. The corresponding activities are performed outside laboratories by people without a traditional education in biology and without any supervision, and appear to circumvent regulatory provisions. In the recent years, several initiatives have been taken in the DIY community to increase awareness and implement a certain level of self-governance. This includes the development of code of conducts provided on the website of the DIY community or established by the industry or service providers manufacturing custom proteins, DNA fragments and genes on demand. These elements can contribute to reinforce biosafety as well as biosecurity management. They could also enhance transparency and communication of further research. Self-governance has also been recommended by some official governmental bodies as regards biosecurity and dual-uses aspects of Synthetic Biology. As mentioned earlier in this document, risks associated with DIY biology should not be overestimated. The question remains however whether such activities should be subject to more government-led oversight and control. It is indeed a paradox that activities performed by DIY biologists during “garage biology” sessions escape the strict regulatory and safety requirements of the GMO legislation, while these apply to the same kind of activities performed by trained scientists in recognized institutions.

In conclusion, we are of the opinion that sufficient knowledge is available to adequately assess and manage short-term applications of Synthetic Biology. The current risk assessment principles and methodology, and the GMO regulatory framework, seem robust enough to deal with these applications. It is difficult to make a judgment whether this will still be the case on the long run. Science and technology developments in the field of SB evolve rapidly and should be reviewed

regularly. Action should be taken if voluntary codes or current regulatory procedures appear insufficient. In this regards, exchange between the research community, risk assessors and policy makers will be key to expand scientific and technical knowledge and to fill the potential gaps in risk assessment and regulation of evolving developments. Further approaches to reconsider effective risk governance should also be taken in a global perspective, allowing international coordination and dialogue. It is therefore important for the European Union to advance further in defining a harmonized view about safety and regulatory oversight of Synthetic Biology.

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