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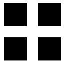
COGEM TOPIC REPORT
CGM/130117-01

SYNTHETIC BIOLOGY – UPDATE 2013

ANTICIPATING DEVELOPMENTS IN SYNTHETIC BIOLOGY

INDEPENDENT SCIENTIFIC ADVICE AND INFORMATION FOR THE DUTCH GOVERNMENT



COGEM TOPIC REPORT
CGM/130117-01

SYNTHETIC BIOLOGY – UPDATE 2013

ANTICIPATING DEVELOPMENTS IN SYNTHETIC BIOLOGY

COGEM
January 2013

Colofon

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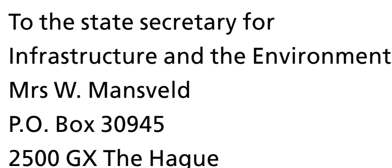
COGEM provides scientific advice to the government on the risks to human health and the environment of the production and use of GMO's and informs the government of ethical and societal issues linked to genetic modification. (Environmental Management Act §2.3).



To the state secretary for
Infrastructure and the Environment
Mrs W. Mansveld
P.O. Box 30945
2500 GX The Hague

DATE 17 January 2013
REFERENCE CGM/130117-01
SUBJECT Topic Report 'Synthetic Biology – Update 2013'

Dear Mrs Mansveld,

I am pleased to present the topic report 'Synthetic Biology – Update 2013: Anticipating developments in synthetic biology' (CGM/130117-01).

SUMMARY

Synthetic biology is a nascent research field and has attracted considerable interest in recent years. On the one hand, it is seen as a technology that offers new possibilities for research and biotechnological applications; the first applications, including the production of biofuels, bioplastics, medicines and vaccines, are expected to come onto the market within a few years. On the other hand, there are concerns about the ability to control the potential risks associated with this technology.

COGEM has previously issued topic reports on synthetic biology, the first in 2006, and has developed activities within the Netherlands and internationally to monitor this field of research. Over the past four years several developments have taken place that have prompted COGEM to produce an update of its last report, which was published in 2008. This present report reviews recent developments during the past four years (2008–2012) and examines the potentials and challenges in each of the various sub-fields of synthetic biology. It explores how possible future difficulties with the risk assessment may be resolved.

COGEM concludes that the current risk assessment method is still adequate for the research being conducted in the field of synthetic biology. Should current trends continue, the risk assessment may in future be made more difficult by an increase in the complexity of the interactions, a blurring of the distinction between donor and host, and the absence of a natural reference as a framework for assessing the risks and risk perceptions.

These developments may in future lead to situations in which:

- **there is no known and characterised host organism as a reference framework, or;**
- **the expression of the introduced characteristics and interactions with the host organism are unpredictable.**

Moreover, in the short term the increasing scale and speed at which DNA molecules can be assembled may lead to:

- **practical and organisational problems with the current case-by-case approach.**

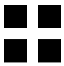
Researchers must provide the data needed to carry out the risk assessment when permit applications are made. As the issues under study become more complex, two-way learning processes between researchers and risk assessors will become increasingly important. Risk assessors and licensing authorities should indicate in advance the type of information that must be provided for the environmental risk assessment. The tensions between the need for practicable safety measures for research and safe working practices for genetic modification may therefore require more intensive contacts between licensing authorities, risk assessors and applicants or researchers. The government can play a facilitating role in this process by taking account of the requirements of the risk assessment methodology when drawing up research programmes.

In addition, attention has been drawn to the possible administrative difficulties that may arise as a consequence of the increasing scale and speed of new developments, leading to problems with the case-by-case assessment method currently in use. Further studies, for example into how other European or international licensing authorities deal with this issue, may throw up ideas and solutions for minimising the administrative burden of the case-by-case approach.

The full text of the report is attached.

Yours sincerely,

Professor Bastiaan C.J. Zoeteman
Chair of COGEM



SUMMARY

This topic report reviews recent developments in synthetic biology during the period 2008–2012 and examines the potentials and challenges in the five technological fields within synthetic biology. In addition, it explores whether problems can be expected with the risk assessment in future and how solutions to these problems can be found and implemented.

Synthetic biology is a nascent research field and has attracted considerable interest in recent years. On the one hand, it is seen as a technology that offers new possibilities for research and biotechnological applications; the first applications, including the production of biofuels, bioplastics, medicines and vaccines, are expected to come onto the market within a few years. On the other hand, there are concerns about the ability to control the potential risks associated with this technology.

In recent years, COGEM has issued a number of topic reports on synthetic biology and has developed activities within the Netherlands and internationally to monitor this field of research. Over the past four years several developments have taken place that have prompted COGEM to produce an update of its last report, which was published in 2008. This present report focuses on possible problems that could arise with the environmental risk assessment as a result of these developments.

Chapter 2 gives an update on the potentials and challenges of the developments in the main technological areas or sub-fields of synthetic biology. These sub-fields are synthetic genomics, metabolic pathway engineering, minimal genome organism, protocells and xenobiology.

- **Synthetic genomics** is the synthesis of artificial DNA to make genes or a complete genome. This technology opens up possibilities for various developments within other sub-fields of synthetic biology; it is an 'enabling technology'. Recent developments include the large-scale production of mutants, such as multiplex automated genome engineering, and applications in the development and production of vaccines. The challenges in this sub-field have for some time concerned the ability to faultlessly synthesise longer strands of DNA.

- **Metabolic pathway engineering** is the practice of producing high-value chemicals, plastics, fuels, pharmaceutical components, and odorants and flavourings in modified organisms. The products of greatest interest are those that are produced only in small quantities in their natural form or are difficult to process. Key activities in this sub-field are designing and inserting specific functions. Because this sub-field is so diverse, it attracts professionals from various disciplines, including biochemis-

try, nanotechnology and ICT. Recent research in metabolic pathway engineering has focused on the production of biofuels and the bioremediation of harmful substances in the environment. The challenges in this sub-field arise from the complexity of the interactions within metabolic networks and the difficulty of making organisms that can be produced on an industrial scale.

- A **minimal genome organism** is a model organism that possesses only the most essential genes for survival. Research in this field investigates both the fundamental questions about the emergence of life as well as the development of an ideal production organism. Over the past few years researchers have been able to remove about 20% of the genomes of *Mycoplasma genitalium* and *Escherichia coli* without damaging the essential functions of these organisms. In a few cases the removal of the genes even had a beneficial effect on the growth of the bacteria. One of the challenges in this field is that there is no known set of genes that are universally essential.

- A **protocell** is the simplest artificial chemical model of a living cell, consisting of organic and/or inorganic elements that mimic the function of some, but not necessarily all, natural cell components and molecules. Because these cells are constructed 'from scratch', they are included within the bottom-up approach to synthetic biology. Most research in this area is fundamental research into the functioning of cells; other research aims to develop applications for drug delivery. Many hurdles have yet to be overcome before it will be possible to develop autonomously functioning protocells that resemble natural cells. Recently researchers successfully replicated an information carrier (DNA) and a cell membrane as the first steps towards creating a replicating protocell.

- **Xenobiology** is the chemical alteration of existing genetic code by changing the chemical composition of nucleic acids or incorporating non-natural amino acids into proteins. Research seeks to answer fundamental questions as well as to create artificial systems and develop medical applications, such as proteins with unique pharmacological properties. Most xenobiological research is still in the experimental stage. In 2011 researchers succeeded in replicating an altered form of DNA (XNA) in vitro for the first time.

In Chapter 3 the principles underlying the risk assessment of genetically modified organisms (GMOs) are taken as the basis for exploring the problems that could arise from current and future developments in synthetic biology. Besides the recent developments described in Chapter 2, consideration is also given to the results of a workshop organised by COGEM and the Rathenau Institute in 2011. During this workshop, scientists and risk assessors examined a number of cases studies to identify problems that could be encountered in the environmental risk assessment and the data that would be needed to resolve such problems.

The current environmental risk assessment method is a case-by-case assessment that depends on knowledge of the host/donor system that is used. The resulting GMO is compared with the wild-type host organism (the starting organism as the 'natural' reference). The risk assessment consists of several steps in which possible hazards and the probability of those hazards occurring are estimated (together these make up the risk) and the possibilities for containing the hazards by taking specific measures are assessed. The methodology of the risk assessment of GMOs therefore consists of combining and weighing up information about the GMO (host and donor sequence) and the nature of the activities.

At the moment there do not appear to be any major problems in the environmental risk assessment of synthetic biology applications. For the current applications sufficient information is available about both the starting organism and the introduced characters. Recent research is indicative of the future direction of research and the applications that can be expected in future. Should current trends continue, the risk assessment may in future be made more difficult by an increase in the complexity of the interactions (metabolic pathway engineering), a blurring of the distinction between donor and host (minimal organism) and the absence of a natural reference (protocells and xenobiology).

These developments may in future lead to situations in which:
- there is no known and characterised host organism as a reference framework (minimal organism, protocells and xenobiology), or;
- the expression of the introduced characters and interactions with the host organism are unpredictable (metabolic pathway engineering).

Moreover, in the short term the increasing scale and speed at which DNA molecules can be assembled (synthetic genomics) may lead to:
- practical and organisational problems with the current case-by-case approach.

Chapter 4 examines the more prominent developments and trends in the public debate about synthetic biology. It is noted that the scope of the debate about synthetic biology as a whole has widened in recent years as the topics under discussion have shifted from more the fundamental issues to broader issues of sustainability and justice. The chapter also discusses the framing of developments within synthetic biology by researchers and the media. COGEM notes that framing a technology as revolutionary and spectacular not only generates media interest, but may also lead to disproportionate reactions, measures and regulations in the social and ethical sphere. This underlines the continuing importance of accuracy and realism in information provision in all areas of research, and certainly in synthetic biology.

In the final chapter, Chapter 5, COGEM concludes that over the past four years great strides have been made in the field of synthetic biology. Important breakthroughs

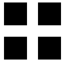
include the first 'synthetic' cell (2010), the creation of a replicating semi-synthetic protocell (2011) and the creation of an alternative genetic alphabet (xenobiology) capable of in vitro replication (2011).

COGEM concludes that the current risk assessment method is still adequate for the research being conducted in the field of synthetic biology, in line with various other reports published in recent years by sister organisations of COGEM in other European countries. Continued monitoring of developments will improve our ability to identify with more accuracy where problems may arise in future should current trends and the current research effort continue.

COGEM notes that many researchers no longer categorise their research as synthetic biology, but rather as falling within one of a number of more specific sub-fields. Whereas synthetic biology has never been a well-defined and clearly demarcated research field, this ambiguity does not affect the various sub-fields. However, as the synthetic biology label gradually falls into disuse, developments will become less visible to outsiders, with the risk that attention to solving future problems in environmental risk assessment may wane. Also, the public may be caught unaware by the appearance on the market of unexpected applications and products, which may lead to resistance or objections.

COGEM calls attention to the importance of good communication and the exchange of information and ideas between scientists and risk assessors. When permit applications are made, researchers must provide the data needed to carry out the risk assessment, and as the issues under study become more complex, two-way learning processes between researchers and risk assessors will become increasingly important. Risk assessors and licensing authorities should indicate in advance the type of information that must be provided in order to carry out an environmental risk assessment. In connection with the three problems mentioned above, risk assessors could state more explicitly what information about a new organism and its characteristics are required for the risk assessment when there is no reference organism. The tensions between the need for practicable safety measures for research and safe working practices for genetic modification may therefore require more intensive contacts between licensing authorities, risk assessors and applicants or researchers. The government can play a facilitating role in this process by taking account of the requirements of the risk assessment methodology when drawing up research programmes.

In addition, an increase in the scale and speed of new developments may lead to administrative problems with the case-by-case assessment method currently in use. Further research may throw up ideas and solutions for minimising the administrative burden of the case-by-case approach, for example by examining how other European or international licensing authorities deal with this issue.



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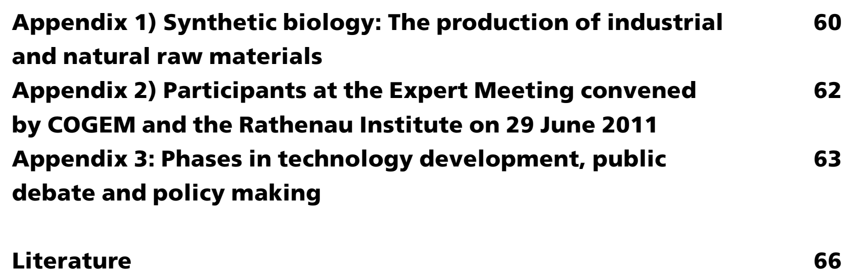
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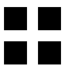


1

INTRODUCTION

From the moment the term synthetic biology was applied to specific sub-fields of research, a discussion arose about the definition of this technology. What is synthetic biology and what techniques and applications does it encompass? One definition of synthetic biology is the synthesis and alteration of complex artificial biological systems for the purpose of studying natural biological phenomena and using them in various applications.[1,2,3,4,5,6] Some years ago a large group of scientists considered their work to be part of the field of synthetic biology, but the trend now appears to be in the opposite direction and many researchers say their work falls within a more specific field of study. Whatever the case, we can state that expectations of synthetic biology research are high. Synthetic biology is seen as a technology that offers new possibilities for biotechnological applications and research. It seeks to modify existing organisms and to design and synthesise new organisms. In general, we can identify the following sub-fields in which synthetic biology plays a key role: synthetic genomics, metabolic pathway engineering, minimal genome organism, protocells and xenobiology. Because wholly or partially synthetic organisms are created by means that are not possible through reproduction or natural recombination, the technology falls under the legislation on genetically modified organisms (GMOs). Right from the start, though, there were doubts whether the existing risk assessment methodology used for GMOs would also be adequate for synthetic biology. Furthermore, aspects like ethical issues and biosecurity are of greater concern in the synthetic biology debate than in discussions about genetic modification. The developments in synthetic biology have led to a public debate about the creation of new forms of life and the possible risks and uncertainties this may entail. The Commission on Genetic Modification (COGEM) has previously published topic reports on synthetic biology in 2006 and 2008.

1.1 PREVIOUS COGEM REPORTS PUBLISHED IN 2006 AND 2008

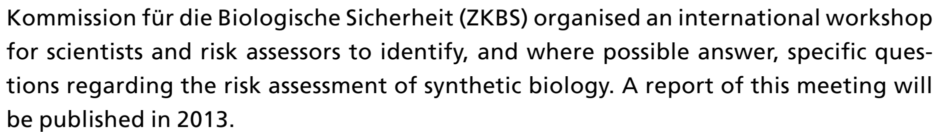
In 2006 COGEM published its first topic report on synthetic biology titled '**Synthetic Biology: A research field with far-reaching consequences**' (in Dutch) (CGM/060228-03). The research field was relatively new and was limited largely to the United States. At that time, little research in this area had been done in the Netherlands. COGEM noted that synthetic biology as a technology could eventually exceed certain boundaries, making it more difficult to estimate potential risks using the existing risk assessment methodology. If a risk assessment cannot be carried out, the activities have to take place at

the highest containment level, which could be a stumbling block to carrying out such research in the Netherlands. Drawing up an adequate risk assessment methodology in advance will prevent unforeseen difficulties at a later stage, ensure safety and prevent unnecessary constraints on scientific progress. In this report, therefore, COGEM highlighted the complex issues raised by this field of research.

Between 2006 and 2008 media interest in synthetic biology increased and the number of scientific papers on the topic continued to grow. Scientists and others speculated in the media about future developments, claiming almost limitless possibilities, while others warned of the possible consequences of this new and groundbreaking technology. In 2008 COGEM prepared another topic report on synthetic biology at the request of the then environment minister. The minister asked COGEM, among other things, whether the current risk assessment method and the assessment framework for GMOs would also be suitable for assessing the expected future developments in synthetic biology and at what point this would no longer be the case. The minister also enquired about how government could best facilitate the public debate on synthetic biology. COGEM answered these questions in its topic report '**Biological Machines? Anticipating developments in synthetic biology**' (CGM/080925-01), concluding that the current risk assessment methodology for GMOs could also be applied to developments in synthetic biology for some years to come. This conclusion was based, among other things, on the expectation that in the near future work would presumably be restricted to biologically contained or apathogenic organisms and that the function of the genes used would be known. COGEM expected that a release into the environment of a synthetic or semi-synthetic organism would not occur for some time. Furthermore, COGEM argued that scientific knowledge would increase as developments progressed so that in most cases it would remain possible to carry out a risk assessment. However, COGEM also observed that the insertion of multiple complex metabolic pathways into organisms or the use of genes with partially or entirely unknown functions would make it more difficult to carry out risk assessments. At that time, it was not possible to foresee where any specific problems would arise.

1.2 DEVELOPMENTS SINCE 2008

Since 2008 developments in synthetic biology have progressed on many fronts and the first applications have appeared on the market. COGEM has followed these developments closely over the past few years. In 2011 COGEM and the Rathenau Institute organised an expert meeting to review recent developments and obtain a more detailed picture of any potential problems these may cause for the environmental risk assessment. In addition, COGEM contacted several of its sister organisations in Europe to set up arrangements for joint monitoring of developments in the field of synthetic biology. At the end of 2012, COGEM and the French Haut Conseil des Biotechnologies (HCB), the Belgian Biosafety and Biotechnology Unit (SBB) and the German Zentrale

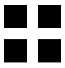


Kommission für die Biologische Sicherheit (ZKBS) organised an international workshop for scientists and risk assessors to identify, and where possible answer, specific questions regarding the risk assessment of synthetic biology. A report of this meeting will be published in 2013.

1.3 STRUCTURE OF THE REPORT

In its first topic report, COGEM posed several questions about the way in which synthetic biology could develop and the issues this would raise. These questions were partly answered in the second topic report (2008). This third topic report contains an update on developments and elaborates further on the following questions:

- What developments can we expect in the field of synthetic biology?
- Will use of the current GMO risk assessment method for new developments in synthetic biology run up against difficulties, and what might these be?
- What knowledge do we need to resolve these difficulties?
- Are there practical and workable measures to manage these difficulties?
- What possibilities are there for structural monitoring of this policy field in future?



2

SYNTHETIC BIOLOGY – STATE OF THE ART

This chapter provides a non-exhaustive review of the most important developments since 2008. It is expected that synthetic biology will find increasing application in the industrial sector (biofuels and bioplastics), the food sector (odorants and flavourings) and the pharmaceutical sector (medicines and vaccines).[7] Moreover, in the medical field synthetic biology is expected to enable advances in the characterisation of pathogens, analysis of clinical presentation, diagnostics and screening. Scientists expect that the application of synthetic biology will lead to shorter development times, increased precision and falling production costs.[8] Although the main research effort in synthetic biology is taking place in the US and to a lesser extent in Europe, progress is also being made in other parts of the world (such as China).[9] Over the past few years various collaborative ventures and spin-off companies have been launched and the first applications have appeared on the market (see text box: Examples of synthetic biology applications). Despite extensive R&D programmes, especially in the industrial sector, relatively few synthetic biology products have as yet become commercially available on the market.

Examples of synthetic biology applications

- **DuPont is collaborating with sugar producer Tate & Lyle on the production of a bioplastic. In this process synthetic/GM-yeast converts maize sugars into a raw material for the production of the bioplastic. This plastic is sold under the brand name Sorona and is used in carpets and other products.[10],[11] DuPont is also working on the production of vanilla, stevia (a sweetener) and palm oil.**
- **Genencor (DuPont) is using synthetic biology techniques to insert a metabolic pathway into Escherichia coli for the production of isoprene (BioIsoprene), a raw material for the production of synthetic rubber. The company is working with tyre manufacturer Goodyear to make BioIsoprene suitable for commercial production.[12]**
- **Solazyme is using synthetic biology techniques to produce biofuels. The company claims to have created algae that convert sugar into biodiesel until it makes up more than 80% of the dry cell weight. In 2010 Solazyme produced more than 80,000 litres of algae-produced biodiesel for the US navy, which has already used this biodiesel as an aircraft fuel.[13]**
- **Amyris Biotechnologies, co-founded by the UC Berkeley biotechnologist Jay Keasling, is working on the production of artemisinin (the precursor of antimalarial drugs) and the production of** >

chemical products and biofuels. The company is also working on the production of farnesene, the building block for a variety of chemical products, detergents, cosmetics, perfumes and industrial lubricants. [14]

- **Synthetic Genomics Vaccines Inc. was established in 2010 by synthetic biology guru Craig Venter (US), who signed a contract with the pharmaceutical company Novartis to create viruses for the development of vaccines.**[15]

It is expected that sooner or later synthetic biology will deliver substitutes for various industrial and natural raw materials (*see Appendix 1: Synthetic Biology: Production of industrial and natural raw materials*). A recent review by the Woodrow Wilson Institute (US) confirms that the production of various raw materials by synthetic organisms is already in the pipeline. [16] In addition, synthetic biology is used to test and optimise the possibilities for using the technology in existing areas of research.

Although there are still differences of opinion on the definition of synthetic biology, we can identify the following main sub-fields in which synthetic biology plays a key role (*see Table 1: Overview of the sub-fields of synthetic biology*).[9,17]

TABLE 1: OVERVIEW OF THE SUB-FIELDS OF SYNTHETIC BIOLOGY
DNA synthesis / Synthetic genomics
The synthesis of artificial DNA (oligos, genes or a complete genome). **Application:** *facilitating other applications, such as metabolic pathway engineering.*
Metabolic pathway engineering
Inserting combinations of genes into an organism to introduce a new or altered function. **Application:** *fundamental research, production of high-value chemicals, biofuels, pharmaceutical products (e.g. artemisinin), bioremediation*.
Minimal genome
Making a model organism which only performs the most essential functions (top-down). **Application:** *creating model organisms, fundamental research.*
Protocells
Making a partially or entirely synthetic cell (bottom-up). **Application:** *fundamental research, developing drug delivery systems.*
Xenobiology
Chemical synthetic biology, or the introduction of an alternative genetic alphabet. **Application:** *fundamental research, production of molecules with specific properties.*

Synthetic biology techniques are used in a broad spectrum of applications. It is therefore impossible to give an exhaustive overview of all the research that has been carried out in recent years. In the following sections we give an impression of the possibilities and challenges in each field of activity, based on a review of the literature and some concrete examples.

2.1 DNA SYNTHESIS (SYNTHETIC GENOMICS)

Synthetic genomics is the design and synthesis of large pieces of DNA (genes or complete genomes). Synthetic DNA constructs are used for fundamental research and have potential for the development of drugs and for industrial and agronomic applications. Synthetic genomics researchers work mainly with single-celled organisms (prokaryotes), but also on the synthesis of 'mini-chromosomes' to alter multicellular eukaryotic organisms.[18,19,20]

It has been possible to synthesise pieces of DNA (oligonucleotides) using specialised machines for almost 25 years, but the capacity, accuracy and speed of synthesis have increased exponentially in recent years. The techniques and machines for synthesising DNA and assembling large fragments have developed rapidly since the first pieces of DNA were synthesised without the use of a natural template.[21,22,23] Long strands of DNA can be synthesised increasingly quickly and inexpensively (*see Figure 1: Productivity of oligonucleotide synthesis and the cost of oligos and genes*)[24,25] and it is now possible to order almost any gene or piece of DNA from commercial companies. Not only the copying of existing genes, but also the alteration and introduction of new pieces of DNA and the rearrangement of genetic information has been made easier by the advances in the field of DNA synthesis.

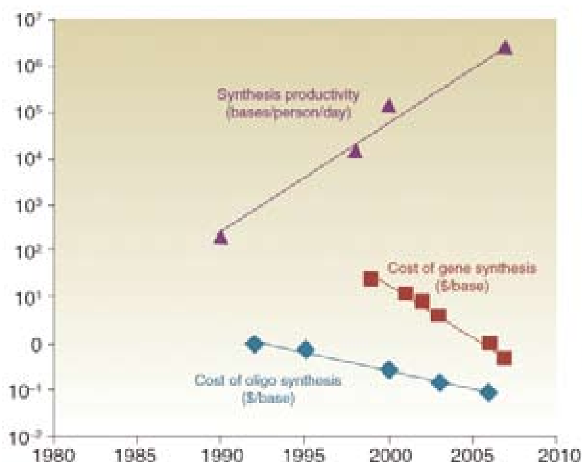


Figure 1: Productivity of oligonucleotide synthesis and the cost of oligos and genes (Figure: Carlson R, 2009. The changing economics of DNA synthesis. *Nature Biotechnology Commentary 27:12, 1091–1094*).[26]

It is not yet possible to synthesise very long strands of DNA in an uninterrupted process without any errors. For this reason shorter pieces of DNA are produced and then joined together. Various techniques have been developed to do this. The most commonly use techniques for DNA synthesis are (from 'small to large'):[27]

- chemical coupling of nucleotides (synthesis of small fragments up to 200 bases);
- use of DNA ligases (joining small fragments with overlapping sequences);
- polymerisation (based on polymerase chain reaction (PCR)) for fragments up to 15 kilo base pairs (kbp);
- recombination (joining large DNA fragments (from 100 kbp) or constructing a complete genome in vitro or in vivo (e.g. in yeast cells)).

In 2012 the maximum length of a continuous piece of DNA that could be synthesised by direct chemical synthesis was around 230 kbp.[28] Between 1970 and 2008 the longest possible piece of DNA that could be synthesised increased from 75 bases to 582,970 base pairs (bp) (*see Table 2: Length of de novo synthesised DNA 1955–2010*). The longest strand of DNA that has been synthesised to date is the complete genome of *Mycoplasma mycoides*, which was assembled in 2010. This piece of DNA consists of 1.08 mega base pairs (Mbp) (1,080,000 bp). In the same year, this genome was used to create the first 'synthetic organism' (*see text box: Synthia, the first 'synthetic organism'*).[29]

TABLE 2: LENGTH OF DE NOVO SYNTHESISED DNA 1955–2010

Year	Oligo/gene target	length (bases / bp)
1955	Dinucleotide dTdT	2
1962	Tetranucleotides TTTT	4
1965	Dodecanucleotides	12
1970	Yeast alanine tRNA	77
1976	*Escherichia coli* tyrosine suppressor tRNA	207
1981	Human interferon alpha	514
1986	Bovine rhodopsin	1,057
1990	Redesigned pUC19	2,050
1995	pUC182Sfi	2,703
2002	Poliovirus type 1 Mahoney 9PV1(M)	7,501
2004	DEBS gene cluster	31,656
2008	*Mycoplasma genitalium*	582,970
2010	*Mycoplasma mycoides*	1,080,000

Source: Carr PA, Church GM, 2009. Genome Engineering. Nature Biotechnology Review 27, 12 December 2009 (supplementary table).

Synthia, the first 'synthetic organism'

In July 2010 researchers at the J. Craig Venter Institute published a report of their successful attempt to create a cell with a completely synthesised genome.[30,31] It took a team of 25 researchers 15 years to complete the project, which cost more than 40 million dollars. DNA synthesis was used to make 1078 DNA cassettes (1080 bp per fragment), which were then cloned in *E. coli.* These cassettes were joined together in three steps in yeast sells (*Saccharomyces cerevisiae*) to reconstruct the entire genome of *Mycoplasma mycoides*. The assembled genome of *M. mycoides* (1.08 Mbp) was then inserted into an empty cell (the chassis) of the related bacterium *M. capricolum*. The resulting organism proved capable of maintaining and reproducing itself under laboratory conditions. The sequence of the synthetic genome contains several 'watermarks' of the J. Craig Venter Institute and was labelled *M. mycoides JCVI-syn1.0*.

Possibilities

Synthesising large pieces of DNA, genes or even complete genomes is an enabling technology, which makes other developments in synthetic biology possible. For example, it enables the synthesis and screening of large numbers of mutants (*see text box: High throughput synthesis of mutants*) and the generation of synthetic viruses (*see text box: Synthetic viruses and vaccine development*). Synthetic DNA can be used, for example, in genetic pathway engineering and for the development of minimal organisms or protocells (*see sections 2.2, 2.3 and 2.4*). The synthesis of DNA is mostly contracted out to specialised companies. The accurate synthesis of DNA in any desired sequence is the technological basis for the design capabilities of synthetic biology. It is expected that the focus of DNA synthesis will in future shift from the reconstruction of copies of existing genes (as in genetic modification) to the design of new genetic circuits and functions (a characteristic feature of synthetic biology).[26] Other facilitating technologies for synthetic biology include protein synthesis, 'omics' techniques and information technology.[32]

High-throughput synthesis of mutants

Multiplex automated genome engineering (MAGE) is a technique for simultaneously inserting large numbers of DNA sequences at various locations in a chromosome.[33,34] This occurs in fully automated cycles and across evolving cell populations. At the moment about ten of these cycles can be completed in a single day. The cells are then tested to determine their genotype and/or phenotype and the cycle can be repeated using the subset of cells that possess the genomic sequences of interest. To demonstrate a possible application of MAGE, a strain of E. coli was 'fed' with >

twenty endogenous genes involved in the 1-deoxy-D-xylulose-6-phosphate (DXP) pathway for the production of lycopene, an antioxidant, and four genes were inserted to influence or switch off secondary pathways in the bacteria. MAGE was thus used to create and screen a total of 24 genes in 4.3 billion variations of E. coli each day. Fifteen billion genetic variants were produced in 35 cycles. After screening, some of the bacteria were found to produce much higher quantities of lycopene (up to 5 times the normal amount).[35] MAGE has potential for the production and optimisation of industrial and chemical raw materials, biofuels and pharmaceutical components.

Challenges

The field of DNA synthesis throws up several challenges. The error margins in the chemical synthesis of DNA are still relatively large compared with natural replication. The error margin in the current chemical synthesis process is around 10^{-2} to 10^{-3} (or 1–10 errors per kbp).[36] In comparison, the error margin of natural DNA replication in prokaryotic and eukaryotic cells lies between 10^{-7} and 10^{-8} thanks to the various proofreading and mismatch repair systems.[37,38,39,40,41,42,43] These scan the DNA sequence during replication and replace any incorrectly placed bases. A general rule in chemical synthesis is that the larger the piece of synthetic DNA produced, the more errors it will contain. It is proving difficult to further reduce the error margin in the initial synthesis process, but various methods have been developed to detect and eradicate errors. For example, size-exclusion techniques can be used to detect and remove erroneous insertions and deletions by separating the DNA molecules according to size and molecular weight. However, these methods do not work for errors that do not result in any change in the size or weight of the DNA molecules (or when there are the same number of deletions and insertions). When synthesising smaller pieces of DNA or genes, clones that contain no errors can be selected by means of nucleotide sequence determination, gene expression tests and functional screening. However, functional screening is only effective for protein coding regions in DNA sequences and it does not detect ‘silent’ mutations.[37]

When synthesising small numbers of large pieces of DNA it is not always possible or even desirable (for reasons of cost) to select pieces that contain no errors, and so attempts are made to repair errors instead.[27] As long as a template is available, this can be done using DNA mismatch recognition proteins and techniques such as nuclease excision and polymerase resynthesis, or site-directed mutagenesis.[27,33]

Synthetic viruses and vaccine development

The rapid advances made in DNA sequencing and synthesis open up opportunities for synthesising viruses and developing vaccines.[23] The poliovirus was synthesised in 2002 and a few years >

later the no longer circulating 1918 strain of the influenza virus was recreated.[21] The universal goal of this strategy is to learn more about the properties of these viruses and to use the knowledge thus obtained to protect humans and animals against viral infections in future. Expectations in this field are high,[23] although at the same time critical questions have been raised about the possible bioterrorism implications of these developments. The ability to synthesise genomes without the use of a natural template makes it possible to recreate viruses no longer in circulation and easier to alter the structure and function of the genetic information of a virus. The complete sequences of more than 3000 viruses are known and can be obtained for research purposes from the Viral Genome Resource Database.[44] Synthesised viruses, or parts of viruses, are used not only for research purposes, but also to develop vaccines. Researchers are now able to make an effective non-replicating subunit vaccine for Ebola in mice by synthesising part of the virus's DNA.[45] Evolva, a company in Switzerland, uses DNA synthesis for the mass insertion of synthetic plant, animal and fungal chromosomes, or combinations of these, into yeast.[46] From about a billion combinations they have obtained around 10,000 interesting yeast varieties worth investigating further. The Swiss company Novartis is working with the American company Synthetic Genomics Vaccines Inc. on a three year project to develop vaccines using synthetic genomics techniques (*see text box: Examples of applications of synthetic biology*).[47]

2.2 METABOLIC PATHWAY ENGINEERING

Metabolic pathway engineering takes genetic modification a step further by introducing entirely new or multiple pathways into an organism. Genetic modification has so far been restricted to altering or introducing just one or a few genes. Metabolic pathway engineering, as its name suggests, takes a more engineering approach and seeks to produce high-value chemicals, plastics, fuels, pharmaceutical components, and odorants and flavourings. The products of greatest interest are those that are produced only in small quantities in their natural form or are difficult to process.

The diversity of metabolic pathway engineering attracts professionals from a wide range of disciplines, such as biochemistry, nanotechnology and information technology. Key techniques in this sub-field are DNA synthesis and standardising specific DNA constructs. One of the first initiatives for registering and standardising synthetic genetic circuits was the development of 'BioBricks',[48] an open source database where anyone can submit their 'genetic components'.[49] These components are DNA sequences with specific (gene) functions, such as promoters, functional genes and terminators. The main users of this database are the participants in the annual iGEM[a] competition (*see text box: Bacterial sticker indicates when meat has spoiled*). A criticism of BioBricks is that many of the registered components have not been properly characterised and do not always work in the ways for which they were designed.[17,50] The International Open Facility Advancing Biotechnology (BioFab) was established in 2009 to address

this problem. The objective of this publicly financed platform is the professional optimisation of existing components/BioBricks.[51]

Bacterial sticker indicates when meat has spoiled

In 2012 the annual International Genetically Engineered Machine (iGEM) competition was won by a team of students at the University of Groningen.[52,53] The team developed a bacterium that changes colour when meat products start to spoil. Meat decay is caused by bacteria, which release certain substances, tiny amounts of which can be detected by the bacterium *Bacillus subtilis*. The students found out which of this bacterium's own genes become active as soon as it 'smells' the spoiling meat. In front of this gene in the DNA is a promoter, a piece of DNA that switches on the gene behind it. If a gene for a yellow pigment is inserted immediately behind this promoter, the bacterium only makes the pigment when it detects the decaying meat. Because it is not advisable to place meat for sale in supermarkets into direct contact with genetically modified bacteria, the students designed a plastic envelope to contain the bacteria (a 'sticker') that allows the odour from the meat to permeate through it, but not the genetically altered *B. subtilis* bacteria. As soon as the meat begins to decay, the sticker turns bright yellow.

To be suitable for standardisation and use in metabolic pathway engineering, BioBricks must possess a large number of specific properties, such as specificity, orthogonality, sensitivity, robustness, compatibility and adjustability. In future, computer models will play an important part in the standardisation and rational design of synthetic organisms with new functions. The results of a study to develop the first computer model of a complete bacterium were recently published. The scientists made a computer model of the bacterium *Mycoplasma genitalium* which maps a considerable number of molecular interactions in the organism based on 1900 experimentally determined parameters.[54]

Possibilities

Besides fundamental research, the main purpose of metabolic pathway engineering is to produce high-value substances and biofuels (*see text box: Production of biofuels in bacteria and algae*), fine chemicals (e.g. riboflavin, succinate) and medicines.[55,56,57,58] In addition, metabolic pathway engineering can be used to detect and break down harm-

[a] The International Genetically Engineered Machine (iGEM) is a synthetic biology competition for talented young scientists. Student teams are given a standard kit of DNA constructs, or BioBricks, which they use to design and build an innovative biological machine. Projects developed under the iGEM flag include biosensors, bioremediators and light-controlled cells.

ful substances (*see text box: Bacteria detect and break down herbicide in the environment*). A large number of companies throughout the world are aware of the potentials of metabolic pathway engineering, according to a recent survey by the Woodrow Wilson Institute, which reviewed progress in the development of techniques for the production of various biological substances in synthetic organisms, including biofuels (biodiesel, ethanol, butanol), chemicals (nylon, plastic), foodstuffs (vanilla, citrus, stevia) and pharmaceutical products (vaccines, antibiotics, antimalarial drugs, diabetes medication).[16] This field of synthetic biology is highly diverse and is illustrated by the different examples given in this section.

Production of biofuels in bacteria and algae

A considerable amount of research is being done on the production of biofuels in bacteria and algae.[59] American researchers at Harvard University are working on the production of biobutanol in cyanobacteria.[60] Isobutanol (called biobutanol when it is produced in a biological system) is an alcohol used in the chemical industry as a solvent and a fuel. Gregory Bukanski and his research team have produced a genetically altered form of *E. coli* that can convert cellulose and hemicellulose from plant material into precursors for biofuels for use as diesel, aviation fuel and other fuels.[61] In the introduction to this chapter it was stated that for the first time an aeroplane has flown on biofuel derived from algae, which was produced by a company called Solazyme. In 2010 Solazyme produced more than 80,000 litres of algae-based biodiesel for the US navy.[13] Other companies, such as Amyris, are also working on the production of biodiesel and aviation fuel.[62]

Bacteria detect and break down herbicide in the environment

American researchers used synthetic biology techniques to alter a strain of *E. coli* to give it the capacity to detect the herbicide Atrazine in the environment and metabolise it.[63,17] Atrazine is a pollutant and can be harmful to animals. The researchers used a synthetic switch (an Atrazine-binding riboswitch) which can bind to the Atrazine molecule and change its form. This leads to a change in gene expression, which is followed by the expression of an Atrazine degrading gene derived from another bacterium. This application has proved successful in detecting and breaking down Atrazine in Petri dishes under laboratory conditions.

Reprogramming genes can also be considered a type of pathway engineering. Examples of the possibilities of these applications include new possibilities for vaccine research (*see text box: Synthetic attenuated virus engineering*).

Synthetic attenuated virus engineering

Synthetic attenuated virus engineering (SAVE) opens up new possibilities for the production of vaccine candidates for a broad range of virus species. The wild-type viruses are attenuated (weakened) by reprogramming parts of the genome so that codon pairs are suboptimised, but still code for the amino acid sequence of the wild-type virus. These sequences contain hundreds of nucleotide alterations compared with the wild type and are generated by chemical synthesis. The resulting viruses are not able to reproduce efficiently.[64] Research into polio and influenza vaccines has so far delivered promising results.[65,66]

Challenges

Standardising components for use as ready-made building blocks in organisms for the purpose of obtaining desired products is often portrayed in the popular scientific literature as being easier than it actually is. The standardisation of DNA constructs is still in its infancy and most of the applications of metabolic pathway engineering developed so far are custom made.

Since the synthesis of genes has become easier, increasing emphasis has been placed on the design aspect (also the most difficult). It is not enough to insert individual building blocks; these components have to be optimised and their functioning coordinated to prevent bottlenecks caused by the accumulation of intermediate products in the organism.[67] This is because designed genetic circuits are not isolated components, but function in the context of the whole genome. In 2011 the highest level of complexity of introduced metabolic pathways with combinations of genes was around 10 to 15 introduced genes.[17]

There are other challenges than the production of interesting components. To make a product suitable for commercialisation, the efficiency and cost/benefit balance of the production process have to be better than those of the existing production process. Various chemical and natural raw materials have passed the proof of principle stage for production in synthetic/GM microorganisms, but are not yet marketable because the cost/benefit balance cannot compete with the existing production process. One of the most cited successes of metabolic pathway engineering is probably the antimalarial drug precursor artemisinic acid in engineered yeast cells. However, the fact that about 150 person years have been invested in this project is an indication of the complexity of such processes.[68] The antimalarial drug developed by Amyris will be manufactured by the pharmaceutical company Sanofi-Aventis. The product has been announced several times over the years and is now expected to go to market in 2013.[69]

2.3 MINIMAL GENOME (TOP-DOWN)

A minimal genome organism is an organism that possesses only the most essential hereditary material needed to survive in a nutrient medium. As many non-essential genes as possible are removed in the laboratory (top-down approach), reducing the organism's hereditary material to such a minimal level that it requires a specific incubation medium to function and reproduce. These minimal organisms are therefore highly biologically contained and only able to survive under laboratory conditions.

The question of how many genes are essential for an organism to function, and which genes these are, has been studied for many years. In 1989 André Goffeau drew up a list of several minimal functions a cell needs to be able to survive. He estimated that a minimal genome containing genes for replication, transcription, translation, metabolism, transport and a cell membrane would consist of about 500 kb and contain about 250 genes.[70] This is possibly an underestimate for a fully autonomously functioning organism. Studies of the minimal genome work with existing organisms with a small genome. The smallest known fully autonomously functioning bacterium is *Pelagiabacter ubique* (1.3 Mbp / 1389 genes).[71] There are several other bacteria from different habitats with about the same number of genes. From this it is estimated that the minimal set of genes for prokaryotic cells lies around the 1400 mark.[72,73] The only species with a smaller genome are organisms like endocellular symbionts and parasitic single-celled organisms (such as *M. genitalium*), which depend for some of their functions on a host cell. The number of genes essential for the survival of these bacteria is estimated to be about 300.[74]

Several strategies are available for research into minimal organisms, such as comparing the sequences of related species of microorganisms and activating or deleting genes to identify non-essential genes. Mathematical models are also being developed to predict which genes are the minimum required for a cell to be able to function under laboratory conditions. The results of a computer model of a minimal cell with 241 essential genes, based on the bacterium *E. coli*, were published in 2012.[75]

Possibilities

The main aim of research on the minimal cell is to determine which genes are essential for survival. Removing genes makes it possible to study the fundamental processes in an organism and answer questions such as What are the functions of individual genes and what interactions are there between the gene products? and What are the minimum requirements for 'life'? Much of the research in this area is on the parasitic bacterium *M. genitalium* (*see text box: Mycoplasma genitalium minimal genome*).

Mycoplasma genitalium minimal genome

For some time various research groups have put considerable effort into finding ways to turn the parasitic bacterium *Mycoplasma genitalium* into a functioning minimal cell. The genome of the bacterium consists of 582,970 bp and contains 482 protein coding genes (524 genes in total). Although the bacterium is less suitable as a production platform (because of its relatively slow growth rate and weak cell membrane (plasma membrane)), its small genome makes it a suitable starting point for creating a minimal cell. Researchers at the J. Craig Venter Institute have identified about 100 genes in the genome of *M. genitalium* that are most probably non-essential.[74] A minimal *M. genitalium* would therefore theoretically contain 381 genes. Since then work has progressed on developing computer models and simulations to further define the gene content of a minimal version of *M. genitalium.*[76] In 2012 researchers at Stanford University and the J. Craig Venter Institute published the results of a successful computer simulation of the life cycle of the bacterium. The model contains 28 categories of molecular interactions, including DNA, RNA, proteins and metabolites. The simulation of a single cell division takes about 10 hours (about the same time it takes a real M. genitalium cell to divide) and generates 0.5 gigabytes of data.[54]

Besides research into the ultimate minimal organism, work is also underway on minimal organisms that can serve as model organisms or be used as a chassis for inserting new functions. It should be noted that a minimal organism is not by definition an ideal model organism. A model organism must be capable of robust and efficient production of a desired molecule, whereas a minimal organism simply has to contain the genes essential for its functioning. A model organism can function as an optimal chassis organism for 'plugging in' natural or synthetic genes and metabolic pathways for the production of high-value substances.[77] Potential host cells for use as a chassis or production platform include *E. coli, B. subtilis, S. cerevisiae and Pseudomonas putida*.[78] Researchers have succeeded in removing about 15% to 30% of the genome of *E. coli* (*see text box: Escherichia coli minimal genome*),[79] and are working on a eukaryotic model system for a synthetic version of the yeast *S. cerevisiae*.[80] In the Synthetic Yeast Genome Project 2.0, researchers are systematically replacing the natural chromosomes with fully synthetic genomes.[81,80]

Escherichia coli minimal genome

The bacterium Escherichia coli (4640 kbp; 4434 genes) is considered to be a suitable candidate for a minimal cell that can also function as a production system. *E. coli* is also known as the >

workhorse of genetic modification because researchers have been working on it for a long time. The bacterium has a robust cell wall, an efficient replication system and a relatively simple cell division mechanism. Moreover, much is known about the functions of the individual genes in the genome of *E. coli*. In 2005, Japanese researchers succeeded in creating a strain of *E. coli* with just 70% of the parent genome.[82] However, the resulting bacteria displayed a deviant cell morphology and an increased cell doubling time. Japanese researchers later succeeded in reducing the total size of the E. coli genome by 22% without any significant effects on the development of the bacterium.[83] In 2006, Hungarian researchers removed more than 700 genes from the *E. coli* genome (about 15%),[79] without significantly altering growth and protein production. Moreover, the removal of the genes appeared to actually have some positive effects, such as reducing the mutation rate, increasing electroporation efficiency and improving gene stability. American researchers have investigated the suitability of one of the Hungarian deletion mutants for use as an industrial production organism.[84] They concluded that both the growth curve of the deletion mutant and its production of recombinant proteins differ little from the parent strain. In 2008 Japanese researchers made a deletion mutant in which 1080 of the 4396 coding regions (approx. 25%) had been removed.[85] The resulting cell (MGF-01) grew for longer than the wild type under laboratory conditions and was less quick to enter a stationary growth phase as a result of a high cell density.

Challenges

The aim of fundamental research on the minimal cell is to determine which genes are essential for survival. However, there is no consensus on the question of whether or not there are universal genes (genes present in all known bacteria).[86] Attempts have been made to identify these genes using comparative genomics to examine genes shared by different species that may be universal. However, the more species that were examined, the smaller the number of agreements became.[87] Besides *M. genitalium*, various intracellular symbiotic bacteria are known with even smaller genomes (e.g. *Candidatus hodgkinia cicadicola*, 144 kbp, 188 genes) that not only throw up new insights but also raise questions about the function and operation of essential genes.[88] These bacteria miss some of the genes needed for cell wall synthesis (making them dependent on a host cell) or genes involved in DNA repair, but these smallest genomes do contain genes responsible for replication, transcription and translation.

Research has shown that relatively large parts of the hereditary material can be removed without demonstrable consequences for the bacterial cell under laboratory conditions. On the other hand, cases are known in which a small modification or deletion has consequences for the cell's fitness, and even a point mutation can be fatal. Moreover, many fundamental functions are carried out in different ways by different genes in different organisms, whereas certain virtually universal genes can be removed without any apparent fatal consequences.[89]

2.4 PROTOCELLS (BOTTOM-UP)

Cells are the smallest living units in nature. They are complicated structures consisting of millions of molecules that work together to make the cell function.[90] A protocell is the simplest artificial chemical model of a living cell and consists of organic and/or inorganic elements that mimic the function of some, but not necessarily all, natural cell components and molecules.[91] Because these cells are constructed 'from scratch', they are included within the bottom-up approach to synthetic biology.
An ideal protocell model contains all the essential components needed to provide the minimum requirements for a cellular life form. A functional definition of a cell is that it consists of a number of self-organising subsystems: a metabolic system, a form of hereditary information for reproduction, and a shell to enclose and contain the system. This enables cells to maintain themselves, grow, replicate and evolve.[91,92,93] No protocell has yet been developed that satisfies all these criteria. Protocells are currently defined as chemical entities and not as living organisms. Protocell models are constructed by combining a membrane-like structure with one or more components that mimic cell functions in a cytoplasm-like environment. The models may consist of various components, such as fatty acids (e.g. phospholipids, which are also the building blocks of natural cell membranes),[90,92] organic components (DNA, RNA, polymerases)[94] or nanotechnological components.[95] The creation of models for the membrane of a protocell make widespread use of the mechanism by which fat molecules spontaneously form cell-like spheres (micelles or liposomes) when dissolved in water[96] (*see text box: Protocell models: Cell membrane*).

Protocell models: Cell membrane

The membranes of protocell models usually consist of a combination of hydrophobic and hydrophilic structures placed in an aqueous solution, where they arrange themselves to form membranes and spherical structures, or primitive 'cells'.[97] Protocell research on mimicking the cell membrane involves building both relatively simple models (micelles) using drops of oil in water[98,99] as well as more complex double membrane structures (liposomes).[100] Liposomes form a double membrane structure of hydrophilic and hydrophobic components (e.g. phospholipids). Micelles consist of a lipid monolayer with a hydrophilic head and a hydrophobic tail or core (e.g. a fatty acid). The formation of new micelles can be induced by changes in pH,[101] the composition of the solution,[102] the application of an electric field across the solution[103] and by the formation of covalent bonds. When a protocell is not able to form new membrane structures during cell division, it will divide itself into two and become increasingly smaller. Research into the propagation or replication of protocells focuses on the synthesis of new phospholipids within a liposome using membrane enzymes. Methods for synthesising proteins include in vitro transcription and translation systems (e.g. PURE).[b]

[b] *Protein synthesis Using Recombinant Elements (PURE) is a commercially available system based on isolated and purified components from E. coli that contain the minimal components for the in vitro translation of proteins.*

Besides their use in research on membrane models, protocells are also constructed to mimic replication and evolution.

Possibilities

Protocell models are used to learn about the structure, function, dynamics and evolution of cells. Protocells are also potential platforms for the production of chemical components and the development of drug delivery systems in the medical sector.[105,106]

Various groups around the world are taking different approaches to constructing protocells (chemical, biotechnological and nanoscience). Scientists have succeeded in mimicking individual cell components or bringing together a few components within a liposome.[92,98,107,108] For example, it is possible to induce the polymerase-catalysed replication of a small single-stranded DNA molecule contained within a liposome and at the same time induce replication of the protocell itself (*see text box: Protocell models: Replication*),[109] to induce the expression of proteins embedded in the lipid membrane and thus improve nutrient uptake,[110] and to replicate an RNA sequence using proteins coded for in the same RNA molecule.[111]

Protocell models: Replication

In the autumn of 2011, Tadashi Sugawara's research group (Japan) published the results of their research into the production of a self-replicating artificial cell.[112] It is the first partially artificial cell capable of a form of replication in which both the information-carrying component (DNA, RNA) and the membrane compartment are copied. Previously these processes had only been achieved separately in protocell models. The cell built by the Japanese researchers consists of short pieces of DNA surrounded by an artificial cell wall consisting of phospholipids. Each piece of DNA is charged and is attracted to the oppositely charged (hydrophobic) phospholipids that make up the membrane. A polymerase chain reaction (PCR) is then initiated to generate additional copies of the DNA in the 'cell', and these are also attracted to the cell wall. At the same time, the replication of the DNA initiates the formation of additional phospholipids from an available precursor, prompting the formation of daughter cells around the new DNA, which eventually detach themselves from the 'mother cell'. The result is an identical copy of the original cell. It should be noted here that there is a difference between replication and reproduction. Replication is the construction of an exact copy or clone, whereas reproduction is an evolutionary process leading to changes in the resulting organism.

Challenges

Despite recent developments and the successes achieved, no truly autonomous cell possessing all the desired properties and functions has yet been created. In the previous section about the minimal cell we have already described the various challenges in

identifying essential genes. The ideal protocell must be less complex than existing cells to make it easier to alter, control and maintain. To be able to function effectively, a protocell must possess an effective mechanism for cell replication and have a low mutation rate. A robust cell structure is also important, especially for protocells intended for production purposes. In such protocells, the cell wall/membrane must be capable of functioning in conditions of high cell densities and withstand production conditions without a reduction in the growth rate or any lysis occurring. This can be a problem in certain situations, for example in the production of alcohol or hydrocarbonates. The cell wall or cell membrane must be sturdy, but also semipermeable in order to allow the passage of substance in and out of the cell.[92,113] For this reason, many membrane models resemble natural cells, for example in their composition, size and ability to change their form (division and fusion).[91,90] In 'natural' cells, proteins with specific functions are responsible for transporting nutrients and waste products in and out of the cell. However, artificial lipid membranes do not possess the advanced and variable permeability of natural cells, which is why a protocell in which DNA was replicated stopped functioning when the incorporated nucleic acids were exhausted.[110] Other systems are capable of replication, but do not share the available molecules so that the daughter cells contain increasingly few elements ('death by dilution').[114] Other challenges in creating the ideal protocell as a production organism include measuring and controlling interactions and obtaining predictable system kinetics. The computer models of cells mentioned earlier in this report may in future help to overcome these challenges.[54,75]

2.5 CHEMICAL SYNTHETIC BIOLOGY (XENOBIOLOGY)

This sub-field of synthetic biology involves the chemical alteration of the existing genetic code by changing the chemical composition of nucleic acids or by replacing them.

The nucleic acid DNA (deoxyribonucleic acid) consists of chains of nucleotides. Each nucleotide consists of a phosphate group, a sugar group and one of the four bases: adenine (A), thymine (T), cytosine (C) and guanine (G). The unique sequence of these nucleotides in the DNA encodes the information that determines the hereditary characters. DNA is double-stranded, the opposing chains being complementary. The genetic code in DNA is transcribed into messenger RNA by RNA polymerase. RNA consists of a single chain of nucleotides (single-stranded) in which the base thymine is replaced by its demethylated form, uracil (U). The ribosomes read the base sequence in the RNA and translate this into a sequence of amino acids. A chain of amino acids forms a peptide, which is folded to form a protein with a specific function. The proteins occurring in nature are built up from a pool of 20 different amino acids. A combination of three bases (called a triplet or codon) codes for a single amino acid. Several different codons can code for the same amino acid, because with four bases (ATCG or AUCG) in the DNA/RNA there are 64 (4^3) possible codons of three letters, which code for the 20 different amino acids in total.
The development of orthogonal biological systems is called chemical synthetic biol-

ogy, or xenobiology. A fully orthogonal system is based on biochemical reactions that cannot interfere with the natural DNA system, which is why some researchers consider these systems to be the ultimate biosafety tool.[115,116,117] No living organisms based on an alternative genetic alphabet have yet been created. The xenobiological systems that have so far been developed are partially orthogonal.

The developments in xenobiology can be divided into various different categories. For example, a distinction can be made between the development of alternative nucleic acids that are still recognised by natural DNA and RNA polymerases, and the development of nucleic acids that are not compatible with the existing natural system. Another division can be made according to the type of alteration made to the DNA:

- **Modifying the structure and composition of DNA:** creating xDNA (expanded DNA) and yDNA (wide DNA), for example by adding an extra benzene ring or fluorescent molecule.[118]
- **Replacing the backbone:** creating xeno-nucleic acids (XNAs), for example glycol nucleic acid (GNA) or threose nucleic acid (TNA).[119]
- **Expanding the codons:** for example, from four bases (ATGC) to six bases (ATGCPZ),[120] and codons consisting of four bases instead of three bases.[121]

These possibilities are discussed further in the following sections, drawing on a number of examples.

Possibilities

One aim of xenobiological research is to find answers to some fundamental questions (*What was the first nucleic acid? Why do nucleic acids contain ribose and deoxyribose and not glucose? Why are there 20 amino acids and not 10 or 15?*).[122] A second aim of the research is to create artificial systems and develop medical applications, such as proteins with unique pharmacological properties (*see text box: Growth hormone with an unnatural amino acid*).

Growth hormone with an unnatural amino acid

People that cannot make any growth hormone (hGH) are treated with a recombinant growth hormone. This hormone molecule is very small and so it is soon excreted from the body, which makes it necessary to inject the patients daily. One way of extending the half-life of the hormone is to attach it to polyethylene glycol (PEG), making the molecule too big to be directly filtered out by the kidneys. Attaching PEG to the hormone is a complex chemical process. In 2011, scientists succeeded in incorporating an unnatural amino acid (p-acetylphenylalanine >

(pAcF)) that binds well with PEG into the growth hormone, making it much easier to attach the two molecules to each other.[123] This growth hormone has already been tested in clinical trials in which patients only had to receive injections of the hormone once a week instead of every day. The researchers expect that the technology can be used to develop new drugs and optimise existing drugs.

Most xenobiological research aims to answer fundamental questions and is still in the experimental stage. Three approaches to creating alternative forms of DNA are discussed below: modifying the sugar groups in DNA, expanding the set of bases in DNA, and inserting 'unnatural' amino acids into DNA.[124,125]

Expanding the genetic alphabet: modified sugar groups (XNAs)
The backbone of DNA consists of the deoxyribose sugar group (the 'D' in DNA). In RNA this is the ribose sugar group (the 'R' in RNA). XNA research seeks to replace these sugar groups with other components. These new nucleic acids are called xeno-nucleic acids (XNAs). The 'X' in XNA indicates the unnatural component that replaces the sugar group. Examples are glycerol nucleic acid (GNA), threose nucleic acid (TNA), peptide nucleic acid (PNA), hexitol nucleic acid (HNA) and cyclohexenyl nucleic acid (CNA).[122] The XNA molecules still contain the conventional base pairs A, C, T and G and therefore retain their ability to pair with DNA and RNA. In 2011 it was demonstrated for the first time that polymerases can convert XNA into DNA and back to XNA (see text box: Replication of XNA molecules).[119,94] XNA molecules are not recognised or only poorly recognised by natural nucleases (the enzymes that break down DNA and RNA), which means these molecules have potential for the development of medicines that can only be broken down slowly.

Replication of XNA molecules

In 2012 scientists succeeded in using mutant polymerases to generate six different XNA molecules from a DNA template. They mixed XNA nucleotides in a solution with thousands of mutant DNA polymerases.[94] Some of these polymerases were able to synthesise XNA molecules using the DNA template. These polymerases were then filtered and purified. Scientists have also been able to create polymerases that convert XNA into DNA. This research shows that it is possible to store genetic material in unnatural nucleic acids and pass it on to the next generation via an XNA–DNA–XNA pathway. In this system, XNA is first converted into DNA, which is then copied via PCR and converted back into XNA. The next challenge is to find a way to efficiently generate XNA molecules without the intervention of DNA.

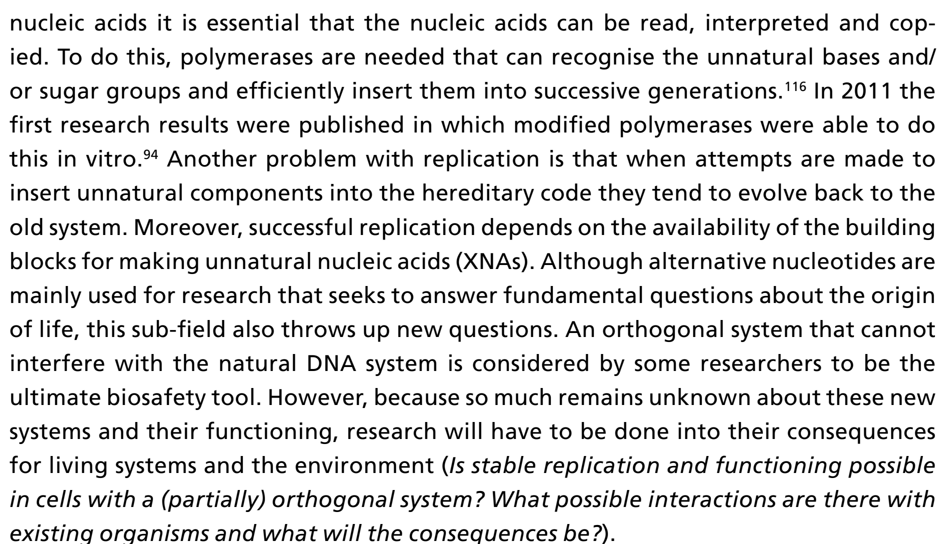
Expanding the genetic alphabet: extra bases (ATGCPZ)
Another line of research seeks to expand the genetic alphabet by inserting new bases. The resulting nucleic acids contain not only the conventional base pairs A, T, C and G, but also an additional base pair (e.g. P and Z) (see text box: Unnatural base pairs).[120,126]

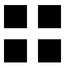
Unnatural base pairs

The conventional base pairs A–T and C–G are joined together by hydrogen bonds. It has always been thought that these bonds are necessary for efficient DNA replication, but recent research indicates that this is not the case.[126] Scientists have succeeded in making an unnatural base pair bonded by hydrophobic forces. Yang and his research team (US) have published the results of a study in which they made two artificial nucleotides that could be efficiently replicated in vitro. The new code consists of six bases (ATGCPZ) instead of the standard four (ATCG). The researchers were able to insert two synthetic DNA nucleotides (with the bases P and Z) next to the standard four bases and replicate them. This study shows that in in vitro experiments the new base pair is functionally equivalent to the natural base pairs. In a further study, Yang et al. want to modify a strain of *E. coli* that will accept plasmids with P–Z bases.

Inserting unnatural amino acids
In addition to altering the backbone of DNA (sugar groups and bases), the codon triplet can be expanded and used to insert unnatural amino acids (UAAs) into proteins.[127] A codon is a sequence of three bases (A, C, G or T for DNA and A, C, G or U for RNA) that code for one of the amino acids that make up proteins. There are 61 codons, which code for 20 amino acids (some codons code for the same amino acid). In addition, there are three stop codons that end the translation process. Theoretically, just one stop codon is sufficient and so 63 (64 minus one stop codon) different amino acids could be created by modifying the codons. The number of known natural protein sequences is relatively small (about 6.5 million) compared with the theoretically possible number of protein sequences based on the average length of 500 amino acids in proteins.[116] Scientists have succeeded in incorporating more than 40 new amino acids not normally found in proteins into E. coli, yeast and animal cells.[128,129,130,131] Moreover, functional codons consisting of four instead of three bases have been created,[17,121] further increasing the possibilities for creating new proteins. Researchers claim that the introduction of unknown amino acids into proteins opens up possibilities for creating proteins with certain properties, such as hyperstability and protease-resistance (proteases are enzymes that break down proteins).

Challenges
XNAs are difficult to produce in large quantities and are usually not replicated by natural polymerases. To obtain a biologically functional system based on unnatural


nucleic acids it is essential that the nucleic acids can be read, interpreted and copied. To do this, polymerases are needed that can recognise the unnatural bases and/or sugar groups and efficiently insert them into successive generations.[116] In 2011 the first research results were published in which modified polymerases were able to do this in vitro.[94] Another problem with replication is that when attempts are made to insert unnatural components into the hereditary code they tend to evolve back to the old system. Moreover, successful replication depends on the availability of the building blocks for making unnatural nucleic acids (XNAs). Although alternative nucleotides are mainly used for research that seeks to answer fundamental questions about the origin of life, this sub-field also throws up new questions. An orthogonal system that cannot interfere with the natural DNA system is considered by some researchers to be the ultimate biosafety tool. However, because so much remains unknown about these new systems and their functioning, research will have to be done into their consequences for living systems and the environment (*Is stable replication and functioning possible in cells with a (partially) orthogonal system? What possible interactions are there with existing organisms and what will the consequences be?*).



3

RISK ASSESSMENT OF SYNTHETIC BIOLOGY

In 2008 the then minister of housing, spatial planning and the environment, Jacqueline Cramer, asked COGEM whether the existing risk assessment for GMOs could also be used to assess the risks associated with future synthetic organisms. In its reply, COGEM concluded at that time that synthetic biology applications could be assessed under the current legislation on genetic modification and that the precautionary principle provided a sufficient guarantee. The safety measures employed for GMOs and wild-type pathogens (safe working practices and containment regulations) would also be adequate for containing synthetic organisms. COGEM indicated that the current risk assessment methodology for activities involving synthetic organisms would be adequate for some years to come. It was expected that in the short term work would be restricted to biologically contained and apathogenic organisms. However, COGEM observed that in the longer term (about ten years) developments may lead to situations in which the current risk assessment methodology would no longer be adequate.

In recent years various articles have appeared in the literature that raise questions about the risk assessment and the data needed to make a reasonable estimation of the risks.[132] Based on the developments described in Chapter 2 and the results of an expert meeting held in 2011, this chapter identifies possible situations in which the current risk assessment method will no longer be up to the task. The following key questions apply to the different sub-fields of synthetic biology:

- Can the risks be estimated?
- What data are needed to assess the risks?
- Can technical safety measures be taken to control or manage potential risks?

3.1 RISK ASSESSMENT METHODOLOGY FOR GMOS AS THE STARTING POINT

The GMO regulations and accompanying (environmental) risk assessment are taken as the starting point for the risk assessment of synthetic biology. The risk assessment consists of several steps in which the possible hazards and the probability of these hazards occurring are estimated (together these make up the risk), and the possibilities for containing/controlling the hazards by taking specific measures are assessed. Broadly speaking, the

risk assessment methodology for GMOs consists of a process of weighing up combined information about the GMO, the host or host cells if used, and the nature of the activities. The following elements play a key role in the environmental risk assessment:

Genetically modified organism (GMO)
Information is needed about the various components of the GMO. These are:
- the acceptor organism (the recipient or host);
- the insert (the 'foreign' DNA inserted into the organism);
- the vector sequences used to introduce the insert into the recipient organism.

As a rule, when the insert does not code for a harmful gene product, the activities involving the GMO can be carried out under the containment level that applies to the starting organism (wild type).

Recipient
The recipient is the host organism into which the gene or genes are inserted. For the risk assessment it is important to know what the characteristics of this organism are. For example, is the host organism pathogenic to humans, animals or plants, and to what degree? Organisms are classified according to their degree of pathogenicity. This classification runs from pathogenicity class 1, which includes apathogenic microorganisms, to pathogenicity class 4, the group of highly pathogenic microorganisms. Activities involving pathogens have to be carried out under the level of containment appropriate for their pathogenicity class and the nature of the activities.

Insert (donor)
The insert is the gene (or genes) introduced into the recipient organism. A distinction is made between uncharacterised sequences and characterised sequences. For uncharacterised sequences it is essential to have information about their origin. If the origin (donor organism) is unknown, it is impossible to determine whether any potentially harmful sequences can be passed on, making it difficult to complete a full risk assessment. If the donor organism has been specified, there will still be a difference between uncharacterised and characterised sequences. When uncharacterised sequences are used, it must be assumed that these sequences are harmful. In such cases the safety measures or containment measures must be based on a worst-case scenario. If the insert is a characterised sequence, it can be assessed for any potentially harmful effects. Clear criteria can be given for several harmful sequences. For a toxin, for example, this is the determination of its LD50. Criteria for other sequences are less obvious and expert opinions will have to be obtained to determine whether the use of certain sequences may involve a risk to humans and the environment and therefore require stricter safety measures.

Vector
The vector is the system that is used to construct a GMO by transferring the insert into the recipient. Vectors may consist of a piece of bacterial DNA, a plasmid or a virus. For

the environmental risk assessment it is important that the vector is fully described and is not capable of independently transferring genetic material to other organisms. The assessment should determine whether the insert is capable of altering the relevant characteristics of the vector.

Reference

The reference, or comparator, is the organism against which the GMO or synthetic organism is compared. This is an important aspect of the final assessment of environmental risks because it determines whether the new organism involves more, equivalent or fewer risks than its natural reference organism. In the Guidance documents published by the European Food Safety Authority (EFSA), the position of the comparator in the risk assessment is described as follows: *'The overall Environmental Risk Assessment (ERA) strategy for genetically modified (GM) organisms seeks to deploy appropriate methods and approaches to compare the GM organism and by-products with their non-GM comparators and with other wild types with some history of familiarity in order to determine environmental effects.'*

Nature of the use

Equally essential for the risk assessment is how the GMO will be used. Various different types of activities involving GMOs can be distinguished and these activities can take place in different environments (e.g. in a laboratory, in greenhouses and in the open field). The nature of the activities may require placing them in a different containment category or the use of different control measures. Special activities or large-scale production require specific or additional procedures or conditions. The same applies to laboratory use as opposed to applications in which new or modified organism are released into the environment.

3.2 EXPERT MEETING ON RISK ASSESSMENT OF SYNTHETIC BIOLOGY

In 2011 COGEM and the Rathenau Institute organised an expert meeting to critically examine issues surrounding the risk assessment of synthetic biology. The meeting had two aims. First, COGEM wanted to review the validity of its conclusion of 2008, that over the short term synthetic biology applications can be adequately assessed using the current assessment framework for GMOs. Second, COGEM wanted to identify more precisely the sorts of problems than may occur with the risk assessment of synthetic biology applications in future.

3.2.1 Structure of the workshop

The meeting was organised and chaired by an external moderator (Huib de Vriend, LIS Consult). Ten Dutch experts in the field of synthetic biology and risk assessment and a few

staff members from COGEM and the Rathenau Institute took part in the workshop (*see Appendix 2: Participants in the expert meeting held on 29 June 2011*). During the meeting six cases were discussed, based on fact sheets and underlying literature. The selected cases were representative of the current trends in the field of synthetic biology:

1. High-throughput synthesis of mutants (MAGE)[34,35]
2. Metabolic pathway engineering: production of butanol in *E. coli*[133,134]
3. Minimal genome organism[30,135]
4. Xenobiology[136,116]
5. Optimising natural processes: photosynthesis in algae[137,138]
6. Symbiosis: zebrafish with photosynthetic capability[139,140]

The first four cases are examples that directly reflect the sub-fields of synthetic biology described in Chapter 2. Two cases (5 and 6) could not be classified exclusively within any one of these sub-fields. Increasing the productivity of photosynthesis in algae is closely related to applications in genetic modification and is therefore a realistic example of the type of research currently taking place which may lead to applications in the environment in the foreseeable future. The case of the symbiotic relation between a zebrafish and GM cyanobacteria with a photosynthetic capability is a curious and somewhat futuristic application. This case was included because of its potentially far-reaching implications.

Protocells were not discussed in the workshop because at the time the workshop was held research in this area was focused primarily on protocells containing chemical, non-replicating components and was therefore less relevant for the risk assessment. However, various articles published in late 2011 and in 2012 describe semi-synthetic cells that are capable of replication. For this reason, the developments in this subfield are also discussed in this report.

3.2.2 Results of the workshop

For each case the participants discussed whether a risk assessment would be possible and what data on the organism concerned would be needed for the risk assessment. The general conclusion was that the existing assessment framework would be adequate as long as the host organism used in each case was well characterised.[141] This condition is generally met in a number of the cases discussed (e.g. 2 and 3). For research in which lesser known organisms or genes may be used, as in the case of high-throughput synthesis or xenobiology (cases 1 and 4), it is important that the host organism is well characterised.

It was noted that the conclusion that the current risk assessment framework is adequate was based largely on applications involving contained use (laboratory activities), for which various containment measures are possible. When activities involve release

into the environment, new, more complex issues will arise regarding the organism and the interactions with the recipient ecosystem. Interactions at the system level will also present major challenges for risk assessment when multiple or less targeted large-scale modifications are involved, and when sequences are used for which there is no known comparator. However, as the possibilities of synthetic biology increase, knowledge in this area will also increase, thus facilitating the risk assessment.

Two of the cases discussed (3 and 6) involve a situation in which distinguishing between the host and the donor may present problems in the risk assessment. The first case is the creation of a symbiotic relation between zebrafish embryos and GM cyanobacteria. This case prompted the question of how to deal with future situations in which two systems are combined in (radical) new ways and in modified forms. Also mentioned were other possibilities that could present a challenge to the environmental risk assessment if used in similar research, such as the use of endophytes [c] to make new combinations in plants or the use of endosymbionts [d] in insects. The second case involved the creation of a minimal genome organism as a chassis for constructing an organism containing large numbers of inserted new characteristics (combination with metabolic pathway engineering). This raises the question of whether the risk assessment should be based in the first instance on the properties of the host organism or the inserted genes.

It should be noted here that the conclusion of the workshop is a direct outcome of the discussion of existing cases. The risks involved in such cases can be estimated because the research has already been carried out. Using existing cases was a conscious decision to make it possible 1) to verify whether developments over the short term are still in line with the existing environmental risk assessment method, and 2) to keep the discussion as concrete as possible by basing it on existing and available data. However, extrapolating current research trends highlighted several questions about the risk assessment in future, which in turn can point the way to identifying potential difficulties with greater precision.

3.3 FUTURE CHALLENGES IN THE RISK ASSESSMENT OF SYNTHETIC BIOLOGY

This section draws on the recent developments outlined in Chapter 2 and the results of the expert meeting to consider the possible future difficulties with risk assessment in more depth. As explained in the first section of this chapter, the current environmental risk assessment method takes a case-by-case approach and is based on a host/donor system for which full or partial knowledge about both the host and the donor is available. The resulting GMO is compared with the wild type host organism.

[c] *A symbiotic fungus occurring in plants which may protect them against insect pests.*

[d] *An organism that lives symbiotically in the cells or in the body of a host organism.*

Until now it has been possible to properly evaluate synthetic biology applications using the environmental risk assessment method. Recent research is indicative of the applications that can be expected in future and this section examines whether and where the risk assessment may run into difficulties if the current trends continue. If a risk assessment is not possible, this will not by definition mean there will be risks to human health and the environment, but it will present an obstacle to the further development of the field of synthetic biology, because the activities will have to be placed in the highest containment level. The experiments will then be subject to numerous restrictions and will only be possible at great expense.

3.3.1 The case-by-case approach

The increasing scale and speed at which variations of an organism or organisms with new characteristics can be produced may make it difficult to assess these organisms on a case-by-case basis under the existing regulations and within the statutory periods. This may be the case, for example, for the high-throughput synthesis of mutants (see text box: High-throughput synthesis of mutants (MAGE), in Chapter 2). This need not present a problem when variations are introduced in a single gene or in a single functional area, because the scope of the risk assessment can be kept within certain margins. However, some studies involve the random insertion of vast numbers of sequences from plants, animals, bacteria, viruses and fungi into microorganisms in an attempt to produce interesting material.[46,142] A combination of the scale of such operations and unknown sequences from multiple donor organisms can strain the case-by-case approach to breaking point if those responsible for assessing applications are overwhelmed with complex issues. This raises the question of whether it would be possible to assess all the variations and whether it would actually be necessary to investigate all the interactions.

3.3.2 Complex interaction

Questions about the interaction between the products of these genes are highly relevant in metabolic pathway engineering in which multiple genes or pathways are inserted into an organism. This is further complicated if these multiple genes come from multiple donors. If the resulting organisms are intended for release into the environment, the environmental risk assessment rapidly becomes much more complex, because release into the environment involves more parameters than contained use. Moreover, the more an organism is altered, the more complex the number of possible effects on the environment will become. In the case of multiple modifications, it is not only the modifications themselves that need to be assessed, but also the interactions between these modifications and any effects these may have on the organism and the environment or ecosystem. It is expected that the increasing understanding of metabolomics will in future play a major part in the analysis of these interactions.

3.3.3 Boundary between donor and recipient

A minimal organism can only carry out the most essential physiological functions itself and depends on artificial laboratory conditions for all other functions. From the safety point of view, a minimal organism will in principle present little risk and be highly biologically contained. Research has confirmed that this will be true in the majority of cases. However, the results of some studies with minimal organisms show that under laboratory conditions they functioned more efficiently for specific characteristics (such as rate of growth) when certain genes were removed (*see text boxes in section 2.3*). It should be noted that such effects are not limited to synthetic biology or genetic modification, and neither do they by definition pose an increased risk. However, if the deletions affect the genes involved in pathogenicity or virulence, this should be taken into account in the risk assessment. Other research has shown that removing specific genes (e.g. anti-virulence genes in plant-pathogenic fungi) can lead to increased pathogenicity.[143] Another example is the evolution of the pathogenic organism Yersinia pestis (bubonic plague), in which deletions played an important role in its growing pathogenicity.[144,145]

Research on minimal organisms also aims to create a model organism, or chassis, for use as a production platform. Most of these studies make use of organisms in the lowest pathogenicity class, which therefore pose a negligible risk to humans and the environment. If multiple metabolic pathways are inserted into a minimal organism, the risk assessment may become more complex, to the point that it may be questioned whether the starting organism can still be used as a reference in the risk assessment.

3.3.4 Natural reference

In the current environmental risk assessment, the GMO is compared with the starting or host organism (the 'natural' comparator). The envisaged applications of metabolic pathway engineering include the production of biofuels in algae (in the environment) and detecting and breaking down environmental pollutants. When multiple new (not present in nature) metabolic pathways are inserted into plants and microorganisms, the absence of a comparator or reference organism may present a problem in the risk assessment, unless the applicant can provide convincing experimental evidence that the new organism has no adverse effects on humans and the environment.

Natural references are also not available for xenobiological applications in which the composition of the nucleotides has been altered. In the literature, xenobiology is mentioned as a potentially suitable way of biologically containing synthetic organisms ('genetic firewall'),[124] which may suggest that the application itself entails no risks. Most research in this area is currently being conducted under contained conditions in the laboratory and no organisms that have an entirely alternative form of DNA have yet been created. However, any future application in a living organism or release into the environment would raise new questions about the risk assessment. *How can the interactions with the environment be tested and what information needs to be col-*

lected to do this? What should these data be compared with? Because of the increase in the number and type of biomolecules, such as XNAs, existing databases of allergens and toxins may not provide a broad enough basis for comparison. Little is known about how cells based on other nucleotides function and how they interact with the environment. *How is it possible to detect whether the building blocks for the orthogonal organism are present in the environment, thus enabling it to survive? If there is no interaction between the organism and other 'natural' organisms (i.e. no biological containment), will this increase the risk of the organism spreading unchecked?*

For protocells, too, there are no natural references or data on interaction with other organisms and the environment. A truly autonomous protocell capable of growing, reproducing and evolving has not yet been created and it is expected that this will not be possible for many years. Developments in protocell engineering will only become interesting from an environmental risk perspective when such a protocell has been made.

3.3.5 Legislation: GMO or not?

Dutch GMO regulations are designed specifically for the production and use of GMOs and not for working with wild-type pathogenic organisms. In other European countries both these types of organisms are covered by the same legislation. In some of the sub-fields of synthetic biology it is not immediately obvious whether the objects of study (e.g. protocells and xenobiology) should be defined as GMOs or genetic material. As discussed above, protocell research seeks to create completely synthetic cells and semi-synthetic cells containing DNA. This raises the question of when these cells can be considered to be living organisms and when they cannot. In xenobiology, for example, there is a question regarding the status of XNA/orthogonal systems in relation to conventional nucleic acids (DNA, RNA) as described in the GMO regulations.

3.4 DATA/INFORMATION REQUIRED FOR THE ENVIRONMENTAL RISK ASSESSMENT OF SYNTHETIC BIOLOGY

Besides identifying the possible difficulties that may arise in the risk assessment of synthetic biology, there still remains the question of what information and knowledge is required to resolve these problems and what possible measures can be taken to control the risks. When experiments are conducted in the laboratory, various measures can be taken to biologically or physically contain the organisms. Moreover, in the absence of certain information, additional measures can be taken as a precaution. It should be noted, though, that such precautionary measures could hamper research progress and are in principle unwelcome. Furthermore, when a synthetic organism is introduced into the environment the possibilities for containment are limited. For these applications, therefore, it is important to have enough information to carry out an adequate

risk assessment. An article recently published in Nature (Dana G.V. et al., 2011) sets out several lines of research that could generate at least some of the data needed for the risk assessment of synthetic biology.[132]

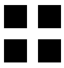
- Research into the fundamental differences between natural and synthetic organisms and how they interact with the environment, such as the possible production of new toxic substances or harmful metabolites.

- Research into possible changes in habitat, food chains and biodiversity caused by the release (intentional or accidental) of synthetic organisms into the environment. It is expected that the first synthetic organisms to be introduced into the environment will be for bioremediation. Will they be able to compete with the existing organisms, and if so, how, and what will the effects be?

- Because synthetic organisms can evolve and adapt, they may occupy new ecological niches. It is therefore important to investigate how easily and how quickly synthetic organisms and their genetic material can adapt to the environment. In addition, research is needed to determine whether these organisms can persist, disperse or adapt their behaviour to the natural environment.

- Finally, the possibilities of gene transfer from synthetic organisms should be investigated. In specific situations, microorganisms are able to exchange genetic information or ingest free DNA from the environment. As new or different forms of DNA are introduced, it is important to have information about possible compatibility with natural organisms or the presence of foreign DNA in the environment. Greater understanding about this process will benefit the risk assessment.

From the analysis in this chapter, we can add to this list the identification of a reference organism or function against which to compare the synthetic organism or cell. If this is not available, it could be difficult to make a final assessment of the possible risks and to determine which containment and control measures will be required before the research can be carried out. For new developments in synthetic biology, the applicants themselves will more often than not be required to provide evidence showing that the environmental risks are negligible. Besides the research lines described above, and combinations of these, such evidence may consist of specific experimental data demonstrating effective biological containment or the development of new tools to ensure biological containment.



4

THE PUBLIC DEBATE ON SYNTHETIC BIOLOGY

Over the years that COGEM has been monitoring developments in the field of synthetic biology, the public debate on this subject has broadened. This is reviewed below:

2006 In 2006 COGEM reported on synthetic biology as a new development within the field of biotechnology. This technology was identified as a potential source of public objections comparable with or even stronger than those raised against genetic modification. Questions about the acceptability of creating new life forms ('playing God') and the fundamental question of what life is were expected to play a more prominent part in the debate about synthetic biology. COGEM also raised the issue of the possible misuse of the technology (bioterrorism).

2008 At the request of the then environment minister, COGEM issued a report on the possible course of the public debate on synthetic biology. The report analysed the debate from the perspective of the technological hype, the phases of an ethical debate and the policy cycle (*see Appendix 3: Phases in technology development, public debate and policy making*). It concluded that at that time the world was in a hype phase in which synthetic biology seemed to offer unlimited possibilities. The public debate focused on fundamental questions about the differences between man and machine and what constitutes life. In addition, questions were emerging about social relations, health and wellbeing, and freedom of choice. COGEM indicated that in this phase there is little point in actively participating in or organising a public debate. It proposed monitoring developments and waiting for the main issues to emerge more clearly.

2012 Recent reports indicate that in 2012 synthetic biology is still a topic of debate, although it appears less frequently in the popular media. A number of non-governmental organisations (NGOs) are still actively engaged in the subject. In 2011 various NGOs called for a moratorium.[146] They claim that because too little is known about the functioning and effects of the products the commercialisation of synthetic biology is premature, and are calling for specific legislation and regulations. Not only NGOs, but also the scientific community and government organisations, and therefore COGEM too, are reconsidering whether the existing legislative framework is still adequate for the task at hand. This chapter examines the broadening of the public debate about synthetic biology in recent years, the importance of communication and people's perceptions of synthetic biology, and the status of relevant governance initiatives.

4.1 THE WIDENING PUBLIC DEBATE ON SYNTHETIC BIOLOGY

Initially, the debate on synthetic biology was concerned primarily with fundamental questions about what life is and how far experiments on the building blocks of life should be permitted to go. Questions about safety and bioterrorism were also prominent in the debate. In recent years the scope of the debate about synthetic biology has widened. Rather than the earlier focus on more fundamental questions (*What is life? What is the difference between humans and machines? Are we playing God and should this be allowed?*) and the potential risks of synthetic biology, the debate now addresses the broader issues of justice, sustainability and freedom of choice. The discussion is not limited to the risks and advantages alone, but also explores the question of who benefits, how the risks are perceived and who bears the risks.[147] Recurring topics are:

Justice: Replacing natural raw materials with materials produced by synthetic organisms would deny farmers in developing countries the opportunity to develop or sustain markets for the conventional cultivation of these products, such as vanilla, artemisinin, palm oil and natural rubber.[146] Appendix 1 contains a list of the compounds that in future could be produced by synthetic organisms. It should be noted that this argument has two sides to it. For example, synthetic rubber and vanilla have been produced for a long time and the natural production of palm oil is subject to considerable criticism on the grounds of sustainability and loss of biodiversity.

Sustainability: There are also positive sustainability aspects in the debate about synthetic biology. New organisms are proposed that can produce valuable substances more cheaply, efficiently and with less environmental impact.

Freedom of choice: Replacing the production of natural raw materials by crop plants with production by synthetic organisms raises questions of how these substances should be evaluated and labelled when they are used in foods and medicines.[148] For example, in Europe substances made by GM microorganisms but which no longer contain any part of these organisms do not have to be labelled as GM products. This is also expected to be the case for organisms modified by synthetic biological techniques to produce specific substances. Although synthetic biology does fall under the GMO legislation, like nanotechnology it is considered by many people to be a ‘novel technology’, again opening up the discussion about labelling and freedom of choice.

Risk perception: Risks can be perceived in broader terms than the purely technical and scientific risks described in Chapter 3. These are known as the ‘soft impacts’ of technological innovation[149] and include the effects on individuals or society when consumers are faced with synthetic biology products. At the moment there are too few commercial synthetic biology products on the market to get to grips with these risks. When the time

comes, it will be important to study how these new techniques interact with the social environment for which they are intended, because the success of synthetic biology will also depend on its acceptance by the public. How are the possible risks of a new technology perceived, and does this depend on the technology itself or on specific applications? The perceived risks of the application of synthetic biology for biofuels will probably be different from those associated with the application of synthetic biology for vaccines or foods. The current debate about these topics may also affect the perception of risks, such as the attitudes people have towards national vaccination programmes.

Instrumentalisation: When different fields of scientific and technological expertise are combined, such as biotechnology, chemistry, ICT and robotics, the results can overstep certain conceptual boundaries. Whereas the focus of biotechnology used to be on mimicking and combining natural systems, it is now shifting to the functioning of an object or organism through the application of expertise from a wide range of scientific disciplines and technologies. This increasing emphasis on the design and engineering aspect is characteristic of synthetic biology. Developments in the field of protocells or the combination of biotechnology and nanotechnology, for example, can lead to a blurring of the distinction between organism and machine and between life and non-life. Developments in synthetic biology also raise both fundamental and concrete issues that are not limited to synthetic biology or genetic modification. Whereas in the past questions were raised about 'playing God' and 'creating' new life, now non-religious questions are also being asked about the instrumentalisation and moral status of 'novel' organisms or objects, which may, for example, contain a combination of living and non-living material (*see text box in section 4.2*). The above examples show that in recent years the questions and issues surrounding developments in synthetic biology and its applications have, as expected, have come into sharper focus and become more concrete.

4.2 COMMUNICATION & FRAMING

Media reports on synthetic biology often contain sensational or provocative headlines to attract the reader's attention. Recurring themes in these articles are creation (playing God), tinkering/randomness, artificiality and hybrid life forms (organic/inorganic).

- **'Playing God' is vital if we are to create a better future for all** (The Guardian, 27 July 2012)
- **'Robo-mosquitoes' in Margaritaville** (Miami Herald, 13 August 2012)
- **Enzymes grow artificial DNA** (Nature, 19 April 2012)
- **Synthetic biology: genetic engineering on steroids** (TechCentral, 16 March 2012)
- **Synthetic biology and the rise of the 'spider-goats'** (The Guardian, 14 January 2012)
- **Life's code rewritten in four letter words** (New Scientist, 17 February 2010)
- **What to make with DNA origami?** (Nature, 10 March 2010)

Although many readers are indeed attracted by such eye-catching titles, this form of framing can have disadvantages in communication. Framing is the reformulation of a subject or development to render it comprehensible to the interested non-professional, making it an essential tool in the communication of complicated subjects. The type of frame that is chosen, either consciously or unconsciously, can have a big impact on how the wider public reacts and the impressions people form about a certain technology or development.[148] An example:

Artificial jellyfish with rat heart cells

In July 2012 researchers at the California Institute of technology (US) published the results of a study in which animal cells were used on a synthetic template to create a system that pulses when placed in an electric field.[150] A silicon template in the shape of a jellyfish was covered with heart cells from a rat and placed in a liquid. The researchers call these semi-synthetic objects 'medusoids'. When the medusoids are placed in an electric field they can swim like a jellyfish by making two movements or strokes. In the first stroke, the muscle cells rapidly contract so that the medusoid is pulled into the characteristic bell shape of a jellyfish. In the second stroke, the cells relax and the silicon pulls the medusoid back to its original flat shape.[151] The approach taken by the researchers is a form of reverse bioengineering based on the form and function of an organic component, rather than mimicking an existing system. The study is a proof of concept involving input from various disciplines, such as design and architecture, robotics, computer modelling, chemistry and biotechnology. The intended application of this groundbreaking research is to test medicines, such as heart medicines. The framing of the researchers ('We Took a Rat Apart and Rebuilt It as a Jellyfish', *The Atlantic*, 22 July 2012) was used by the media to emphasise the creation of a living entity and the hybrid aspect of the medusoid. However, the main effect of the media interest this created was that the lasting, and misguided, impression of this breakthrough was the creation aspect, rather than an understanding of the real implications and aim of the research, such as the testing of heart medicines and reducing the use of laboratory animals. The impression thus created reinforces the negative connotations of the 'mad professor' conducting bizarre experiments in his lab, like creating monsters.

Framing can be used consciously or unconsciously and is virtually unavoidable: just about every statement is framed by a set of assumptions. Researchers need to be aware of this aspect of their communication with the outside world before popularising their scientific results. Framing a technology as revolutionary and spectacular not only generates media interest, but can also lead to disproportionate reactions, measures and regulations in the social and ethical sphere. This effect can be heightened for issues considered to be controversial by some sections of society. Some researchers believe

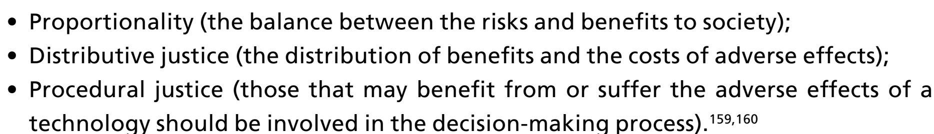
that hypes and exaggerated claims can be counterproductive for the development of ethical regulations that stakeholders can work with now and in the future.[4] Accurate information provision is therefore a key consideration in all research, and certainly in synthetic biology.

4.3 GOVERNANCE AND THE CONTROL OF TECHNOLOGY

In recent years European and international meetings have been held to shape the development and social embedding of synthetic biology and find a satisfactory form of governance.[152] This has led to many publications on finding effective responses to rapidly changing technology, knowledge and understanding.[153,155,154] Over the past seven years about 40 reports on the governance of synthetic biology have been published worldwide.[156] COGEM also addressed this issue some years ago in a research report. In response to early indications from the scientific community and the relevant agencies, actions have been taken in recent years to monitor biosafety issues associated with synthetic biology.[157,158] A few examples of European organisations that have reported on biosafety, ethics and governance issues relating to synthetic biology in recent years are listed below:

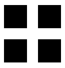
2012	Wetenschappelijk Instituut Volksgezondheid, Biosafety and Biotechnology Unit (ISP-WIV)
2012	Zentrale Kommission für die Biologische Sicherheit (ZKBS)
2010	The European Academies of Science Advisory Council (EASAC)
2010	Schweizerischen Akademie der Technischen Wissenschaften (SATW)
2009	The European Group on Ethics and New Technologies (EGE)
2009	Royal Acadmy of Engineering (UK)
2009	Deutsche Forschungsgemeinschaft (DFG), Deutsche Akademie der Technikwissenschaften, Deutsche Akademie der Naturforscher Leopoldina – Nationale Akademie der Wissenschaften
2008	Koninklijke Nederlandse Academie van Wetenschappen (KNAW), Gezondheidsraad (GR) en Raad voor Gezondheid Onderzoek (RGO)

However, it has proved difficult to come up with any clear-cut answers to questions about the appropriate form of governance, how responsibilities should be allocated, and when legislation and regulations should be amended or restrictions imposed. One thing is clear, though: an integrated approach to converging technologies like synthetic biology is desirable. The development, introduction and social embedding of a new technology like synthetic biology is an interactive and partly unpredictable process that may only be possible in a step-by-step approach. Besides the precautionary principle, studies on the governance of synthetic biology identify the following key elements that have to be addressed:



- Proportionality (the balance between the risks and benefits to society);
- Distributive justice (the distribution of benefits and the costs of adverse effects);
- Procedural justice (those that may benefit from or suffer the adverse effects of a technology should be involved in the decision-making process).[159,160]

In recent years, the public debate about synthetic biology has widened and several concrete issues have emerged. However, this widening of the scope of the debate may lead to such a disparate discourse that specific aspects are overlooked or not given the attention they deserve. The framing and popularisation of scientific results in the popular media have a considerable influence on public perceptions, but may divert public attention from the real developments and implications. Accurate and clear reporting on the developments in and applications of synthetic biology are essential for giving direction to the development of an accountable form of governance.



5

CONCLUSION & DISCUSSION

In its first topic report on synthetic biology (2006), COGEM posed several questions about the way in which the field of synthetic biology could develop and the associated biosafety and ethical issues. In 2008 the then environment minister asked COGEM to investigate several such questions. Although some of these questions were answered at that time, COGEM stated that others could not be answered with any precision because it was not possible to foresee how certain developments would unfold. A number of these questions were again raised during the expert meeting held by COGEM and the Rathenau Institute in 2011. This concluding chapter draws on the results of this workshop and a review of the latest developments in synthetic biology to summarise the key questions and, where possible, provide some answers.

What is synthetic biology?
From the moment the term synthetic biology was given to specific sub-fields of research, a discussion arose about the precise definition of this technology: What is synthetic biology, and what techniques and applications does it encompass? At the moment there is no consensus on a definition, and this may well be due to another development. Whereas just a few years ago large groups of scientists took up the banner of synthetic biology, the trend now seems to be in the other direction and many researchers no longer say they work in the field of synthetic biology, but rather within one of a number of specific sub-fields, such as metabolic engineering or protocell research. As a result, developments in the field may go largely unnoticed by the general public until applications appear on the market.

5.1 DEVELOPMENTS

What developments can we expect in the field of synthetic biology and when are they likely to occur?

Over the past four years developments in the field of synthetic biology have made great strides. Some of the major breakthroughs were:

- the first 'synthetic' cell (2010);
- the creation of a replicating semi-synthetic protocell (2011);

- the creation of an alternative genetic alphabet (xenobiology) capable of in vitro replication (2011);
- the introduction of more complex pathways into organisms (2008–2012);
- the appearance on the market of the first applications (e.g. the bioplastic Sorona, see Chapter 2).

Despite a number of recent breakthroughs in the field of xenobiology and protocells, practical applications are not expected for many years to come. The developments over the past four years reviewed here are in line with what was expected at the time of COGEM's last report on this topic in 2008. The hype phase appears to have cooled down now that the introduction of many concrete applications on the professional and consumer markets does not appear to be as imminent as previously thought. This has much to do with the challenges discussed in Chapter 2. Although steady scientific progress continues to be made, we have entered a phase in which interest in synthetic biology in the popular media is gradually waning.

5.2 RISK ASSESSMENT

What difficulties will arise with the use of the current risk assessment method for GMOs?

From the outcome of the expert meeting held by COGEM and the Rathenau Institute in 2011, it can be concluded that the current risk assessment method is still adequate for the research being conducted in the field of synthetic biology. This conclusion is in line with various other reports published in recent years by sister organisations of COGEM in other European countries (*see section 4.3*). Monitoring recent developments will improve our ability to identify with more accuracy where problems may arise in future should current trends and research efforts continue. These include:

- the blurring of the boundaries between donor and recipient (metabolic pathway engineering / minimal genome);
- the absence of a natural comparator (metabolic pathway engineering / xenobiology / protocell);
- the characterisation of inserts and sequences (metabolic pathway engineering);
- the growing strain being put on the case-by-case approach by the increasing scale and speed of activities (high throughput).

In addition, there are a number of questions relating to the suitability of the current legislation. Are the cells or organisms produced by synthetic biology always GMOs? Do products made from or by (partially) synthetic organisms fall under the current regula-

tions (e.g. on labelling)?[17] Examples of such products are foods, fuels, and flavourings and odorants,[148] as well as the production of traditional medicinal plant extracts (e.g. artemisinin).[161]

What knowledge do we need to resolve these difficulties?

Section 3.4 indicates several lines of research that could generate data needed for the risk assessment of synthetic biology:

- research into the fundamental differences between natural and synthetic organisms and how they interact with the environment;
- research into possible changes in habitat, food chains and biodiversity caused by the release (intentional or accidental) of synthetic organisms into the environment;
- research into the possible adaptation of synthetic or semi-synthetic organisms to their environment (persistence, dispersal and behaviour);
- research into the possibilities of gene transfer from synthetic organisms.

Identifying a reference organism or function against which to compare the synthetic organism or cell is also important for the risk assessment. For new developments in synthetic biology, the applicants themselves will more often than not be required to provide evidence showing that the environmental risks are negligible. Besides the research lines described above, and combinations of these, such evidence may consist of experimental data demonstrating effective biological containment or the development of new tools to ensure biological containment.

5.3 RISK MANAGEMENT

Are there any possible practical and workable control measures to manage these difficulties?

The existing risk management measures for GMOs can also be used in the assessment of developments in synthetic biology described in this report. These include:

- assigning an appropriate containment category;
- biological and physical containment measures;
- a cautious approach to release into the environment.

In other words, an appropriate safety level should be chosen, and if there is any uncertainty about the specific risks, the precautionary principle should be applied. Risks can also be controlled by biological and physical containment measures. COGEM observes that some researchers working in the field of synthetic biology consider an alternative alphabet for DNA/RNA to be an ideal containment method. However, caution will have to be exercised when considering this application as a containment method because as yet, little is known about these alternative genetic building blocks and how they will behave in the environment.

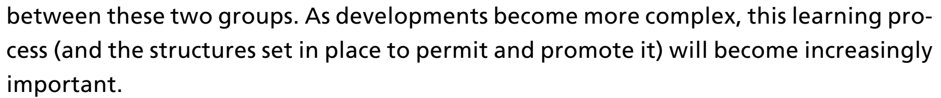
COGEM has noted that the increase in the scale and speed of activities may lead to problems with the case-by-case approach to assessing permit applications. With respect to this, COGEM points out the importance of monitoring international developments and reviewing how other countries and organisations are responding to this issue.

5.4 POLICY

Will it be necessary to maintain a close watch on this area of policy making in future, and how can this be done?

So far, the growth in our knowledge and understanding has kept pace with developments in synthetic biology. Because synthetic biology brings together several modern technologies (chemistry, information science and biotechnology) and will in future generate new applications, it will remain important to monitor developments to identify possible problems that could arise with the environmental risk assessment.

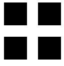
Keeping up to date with international developments will be crucial. Several European advisory bodies (the French Haut Conseil des Biotechnologies (HCB), the Belgian Biosafety and Biotechnology Unit (SBB) and the German Zentrale Kommission für die Biologische Sicherheit (ZKBS)) and COGEM have established a network to keep track of developments within Europe and where possible outside Europe as well. The aspects that could cause difficulties for the environmental risk assessment mentioned in section 3.3 were identified in the European workshop organised by these organisations in December 2012. A report of this meeting will be published in 2013. In addition, participants at the Dutch expert meeting held in 2011 raised the importance of the free exchange of information and views between researchers and risk assessors. Researchers must provide the data needed to carry out the risk assessment when permit applications are made. This raises the question of the capacity of researchers in the field to estimate in advance which information about new developments risk assessors will need and emphasises the need for good communication and an open dialogue



between these two groups. As developments become more complex, this learning process (and the structures set in place to permit and promote it) will become increasingly important.

Risk assessors and licensing authorities should indicate in advance the type of information that must be provided for the environmental risk assessment. In connection with the three problems mentioned above, risk assessors can state more explicitly what information and characteristics of the new organism are required for the risk assessment in the absence of a reference organism. The tensions between the need for practicable safety measures for research and safe working practices for genetic modification may therefore require more intensive contacts between licensing authorities, risk assessors and applicants or researchers. The government can play a facilitating role in these processes by taking account of the requirements of the risk assessment methodology when drawing up research programmes.

In addition, attention has been drawn to the possible administrative difficulties that may arise as a consequence of the increasing scale and speed of new developments, leading to problems with the case-by-case assessment method currently in use. Further research may throw up ideas and solutions for minimising the administrative burden of the case-by-case approach, for example by examining how other European or international licensing authorities deal with this issue.



GLOSSARY

BioBricks
BioBricks are standardised and interchangeable DNA sequences of defined structure and function. These DNA parts are designed to be incorporated into living cells, such as bacteria, to carry out a specific function (a biological system).

Biological containment
The characteristics of an organism that restrict its survival or dispersal in the environment. These include the characteristics of a host/vector system that restrict the transmission of the vector.

Chassis / minimal genome organism
A minimal genome organism is a model organism that possesses only the most essential genes for its functioning under laboratory conditions. Research in this field is concerned both with the fundamental questions about the emergence of life and with the development of an ideal production organism. A minimal genome organism intended for use as a production organism is also called a 'chassis genome'. Additional genomes can be added to the chassis to make it perform a specific function.

De novo
Latin for 'from the beginning'. In the context of this report, de novo refers to DNA synthesis in which a series of chemically synthesised bases or base pairs are linked together one by one. Another method is to link together isolated pieces of DNA from organisms. This is not a form of de novo synthesis.

Donor organism
The organism from which the genetic information introduced into a host organism was originally obtained.

Framing
Framing is the conscious or unconscious use of conceptual frameworks in communication. Just about every statement is framed by a set of assumptions. Instead of using arguments as a rational means of persuasion, framing makes use of the associations elicited by an image or idea. Words and images are chosen in such a way that a number of aspects of the topic being described are implicitly highlighted.

Host organism
In genetic modification, the host is the organism into which genetic material from a donor organism is inserted.

Governance
Governance is the action or manner of governing, a code of conduct or the oversight and supervision of organisations. The World Bank defines governance as 'the exercise of political power and the use of institutional resources to manage society's problems and affairs'.

In vitro
Experiments and techniques carried out under laboratory conditions that use biological material outside a living organism. In vitro means 'in glass' and dates from the time that glass test tubes and similar equipment were commonly used.

In vivo
Experiments conducted in a living system.

Laboratory conditions
When an organism is held under laboratory conditions it lives in a fully controlled and managed environment in which the provision of the elements required for its survival (nutrients, oxygen) is regulated. The organism is not exposed to external influences, such as competition or threats from other organisms.

LD50
LD50 stands for 'median lethal dose' and is the amount of a toxin or other substance that is required to kill half the members of a population. As the substance is administered in a single dose, LD50 is an indicator of its acute toxicity and says nothing about long-term toxicity. LD50 is usually expressed in µg or mg per kg of living tissue.

Metabolic pathway engineering
Metabolic pathway engineering is the design and insertion of specific functions into an existing organism. This sub-field concerns the production of high-value chemicals, plastics, fuels, pharmaceutical components, and odorants and flavourings in modified organisms.

Omics
-omics is a suffix often used for research fields in biology, such as genomics (the quantitative study of genes, regulatory and coding sequences), transcriptomics (RNA and gene expression), proteomics (protein expression) and metabolomics (metabolites and metabolic networks). Research data from various -omics fields are used, among other things, to study intended and unintended differences at the composition level between a GMO and its natural reference.

Orthogonal biological system / xenobiology
In mathematics, two objects are orthogonal if they are at right angles to each other. In statistics, the term refers to the complete absence of a correlation between two variables. The development of orthogonal systems aims to create different or novel life

forms by altering the basis of living cells (the DNA). This is also called xenobiology. The functioning of a fully orthogonal system would be based on biochemical reactions that cannot interfere with natural systems.

Physical containment
Equipment, appliances and structures fitted to working areas to prevent the dispersal of organisms, including genetically modified organisms. Containment measures are classified into four levels of safety.

Precautionary principle
This principle was established in the Rio Declaration. The precautionary principle states that new technologies may not be used without taking precautionary measures if they are likely to involve risks to the environment or human health, even if those risks have not (yet) been established without doubt by scientific research.

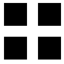
Protocell
A protocell is the simplest artificial chemical model of a living cell, consisting of organic and/or inorganic elements, that mimics the function of some, but not necessarily all, natural cell components and molecules. Because these cells are constructed 'from scratch', they are included within the bottom-up approach to synthetic biology.

Synthetic biology
Synthetic biology is a research field that seeks to modify existing organisms to perform useful functions and to design and synthesise artificial genes and complete biological systems.

Synthetic genomics
Synthetic genomics is the chemical synthesis of artificial DNA to make genes or a complete genome.

Wild type
The natural genotype or phenotype of a certain organism (or gene).

Xenobiology (see orthogonal biological system)



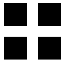
APPENDIX 1

SYNTHETIC BIOLOGY: THE PRODUCTION OF INDUSTRIAL AND NATURAL RAW MATERIALS

Source: International Civil Society Working Group on Synthetic Biology (2011). A submission to the convention on biological diversity's subsidiary body on scientific, technical and technological advice (SBSTTA) on the potential impacts of synthetic biology on the conservation and sustainable use of biodiversity.

Natural compound	Institution/Firm developing synthetic biology production	Stage of development	Natural product sourced from	Synthetic biology production based in	Market size (estimates)
Artemisinin (Artemisia annua)	Amyris/Sanofi Aventis; Riken Institute	To be commercialized 2012 by Sanofi	China, Vietnam, Cameroon, Ethiopia, Kenya, Mozambique, Tanzania, Uganda and Zambia	USA, Czech Republic, South Africa, Japan	Global supply and demand for artemisinin ~ 120-140 MT
Jojoba Oil (Simmondsia chinensis)	LS9 Inc.	Pre-commercial	Argentina, Australia, Chile, Egypt, India, Israel, Mexico, Peru, South Africa, USA	USA	~5,000 tons of jojoba is used in personal care products worldwide
Liqourice (Glycyrrhiza glabra)	RIKEN Institute, Tokiwa Phytochemical Co.	Proof of principal	India, Spain, Iraq, Iran, Turkey, Russia, China, Mongolia, Kazakhstan	Japan	20,839 tons of liquorice dried extract (2004)
Plm Oil (Elaeis species)	Solazyme/Unilever, Sunthetix Genomics Inc./ Genting group	R&D	Malaysia, Indonesia, Thailand, Colombia, Benin, Kenya, Ghana	USA	48 million tons of palm oil (accounts for 30% of global production of oils and fats)
Natural Rubber (Hevea brasiliensis)	Amyris/Michelin; Genencor/Dupont/ Goodyear Tire & Rubber Co.; GlycosBio/ Bio-XCell Sdn BHd (Malaysia)	To be commercialized 2013 (Genencor) or 2014 (GlycosBio)	Thailand, Malaysia, Indonesia, India, Vietnam, China, Sri Lanka, Cambodia, Papua New Guinea, Philippines	USA,	8,9 million metric tons (demand for isoprene per annum)
Pyrethrin (Tanacetum cinerariae-folium)	Wageningen University	R&D	Kenya, Tanzania, Australia, Japan, Dalmatia, Ecuador, Rwanda, Uganda, Papua New Guinea	Netherlands	2850 tons of pyrethrum flowers harvested worldwide (2000)

Natural compound	Institution/Firm developing synthetic biology production	Stage of development	Natural product sourced from	Synthetic biology production based in	Market size (estimates)
Stevia (Stevia rebaudiana)	Evolva Inc., Vineland Research	Pre-commercial, R&D	Paraguay, Brazil, Argentina, USA, Uruguay, Israel, China, Thailand	Switzerland, USA, Canada	worldwide sales of stevia extract 3,500 tons (2010)
Taxol (Taxus brevifolia)	University of California Berkely	Proof of principal	USA/Canada	USA	N/A
Vanilla (Vanilla planifolia)	Evolva Inc.	Scale-up. To be commercialized 2014.	Madagascar, Comoros, Reunion, Indonesia, French	Denmark, Switzerland	Approx. US $ 200 million

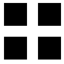


APPENDIX 2

PARTICIPANTS AT THE EXPERT MEETING CONVENED BY COGEM AND THE RATHENAU INSTITUTE ON 29 JUNE 2011

Name	**Institution**
Dr Hans Bergmans	Bureau for Genetically Modified Organisms
Prof. Roel Bovenberg	University of Groningen
Prof. Dr. Jeroen Cornelissen	University of Twente
Prof. Gerrit Eggink	Wageningen UR
Prof. Ron Fouchier	Erasmus MC
Prof. Vitor Martins dos Santos	Wageningen UR
Dr Ben Peeters	Central Veterinary Institute
Prof. Jos van Putten	Utrecht University
Prof. Rob Verpoorte	Leiden University
Drs. G. van Willigen	Leiden University Medical Center

Organisation	
Dr Frank van der Wilk	COGEM
Ruth Mampuys M.Sc	COGEM
Dr Dirk Stemerding	Rathenau Institute
Mr. Drs. Virgil Rerimassie	Rathenau Institute
Ir. Huib de Vriend	LIS Consult

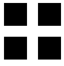


APPENDIX 3

PHASES IN TECHNOLOGY DEVELOPMENT,PUBLIC DEBATE AND POLICY MAKING

Technology (Technology Hype Cycle – Gartner)	**Technology trigger**	**Peak of inflated expectations**	**Trough of disillusionment**	**Slope of enlightenment**	**Plateau of productivity**
Ethical discussion (Parliamentary paper 21 319 – Ritzen)	**Identification**	**Articulation**	**Authoritative value assignment**		**Analysis and underpinning of values**
Policy formulation (Policy cycle – Winsemius)	**Recognition**		**Policy formulation**	**Solution**	**Management**
Description	First reports appear in the scientific literature, popular science magazines and the media. The actual developments and future technological possibilities cannot be foreseen.	More is known about the course of developments and the technical possibilities – explosion of media interest. The issues are discussed in news-papers and on TV and the radio. Stakeholders are asked to give their expert/lay opinions. The public and interest groups articulate their expectations of possible applications or express their concerns.	Media interest declines; neither the dream scenarios nor the doom scenarios turn out to be right. Although no applications are on the market yet, they are taking on concrete shape beyond the gaze of the media.	The first applications of the technology come onto the market and receive media coverage. Media interest grows again.	Like a well-oiled machine, the technology delivers new applications which are taken up by society.
Scientists / Companies	Inform government and the media about developments. Reflect on the possible implications, risks and ethical and societal aspects.	Inform government and the media about the current state of developments. Take responsibility for issues of public safety and the desirability of applications and also communicate these.	The scientific and business communities draw closer as more concrete applications come into view. These parties should inform government and the media.	Inform government and the media about the current developments. Evaluate suppliers of applications: do these applications meet previously agreed statements, promises and expectations about the potentials?	Inform and evaluate
NGOs	Keep abreast of reports in the media and make further enquiries. Where possible put the topic on the agenda.	Articulate possibilities and impossibilities and issues that can be put on the political agenda for debate. Possible interaction with the media to highlight these points.	Interest in the topic declines, but does not disappear. Interest groups continue to air their opinions.	Interest in the topic grows again. Issues raised for debate are focused more on concrete applications.	Interest in the topic continues. Specific viewpoints are still expressed, but meet with less response in the media and among stakeholders.

Technology (Technology Hype Cycle – Gartner)	**Technology trigger**	**Peak of inflated expectations**	**Trough of disillusionment**	**Slope of enlightenment**	**Plateau of productivity**
Ethical discussion (Parliamentary paper 21 319 – Ritzen)	**Identification**	**Articulation**	**Authoritative value assignment**		**Analysis and underpinning of values**
Policy formulation (Policy cycle – Winsemius)	**Recognition**		**Policy formulation**	**Solution**	**Management**
Media	Inform the public via attention-grabbing articles				
Citizens	The public sees the first reports in the media. The possible applications put forward seem to be a long way off, but appeal to the imagination.	The public are confronted with a wide range of possible applications – dream and doom scenarios. They can use them to brainstorm about the desirability of applications – ethical and societal questions are articulated. Citizens form interest groups to convey their views more forcefully.	Interest among the wider public declines and few or no new questions are added. The lack of concrete applications creates the impression that things will not happen very quickly.	The first applications are made available to the public and interest grows again. The public and/or interest groups articulate their views on these applications.	The technology has been embraced by society; applications are put on the market with no complaints from the public. Some people continue to oppose the technology as a matter of principle using arguments put forward during the identification and articulation phases.
Government	Gather information with a view to identifying and affirming whether a new develop-ment is emerging. In this phase questions of safety and risks may arise. Government must take action and obtain information and advice in order to answer these questions.	Can provide information about developments and how the government is guaranteeing the safety of its citizens. Based on the information obtain-ed, government articulates opportu-nities and problems that may be thrown up by the new technology, for example in the areas of risk management and legal, economic, ethical and societal issues. The govern-ment must decide whether or not to encourage the developments by providing subsidies or through research programmes.	Realisation that the first applications begin to take shape in this stage. Remain informed about the latest developments. Which scenarios are realistic, and when? The ethical and societal questions of practical relevance to the technology have been clearly defined. Which parties could have a judgement to make on these, formulate new policy or amend existing policy?	The safety issue has been defined. Implementation of the policies made. Key activities in this phase are evaluation and learning. Government must keep the situation under scrutiny to check whether the policies formulated respond properly to the identified problems. The appearance of new (unexpected) developments may make it necessary to amend some aspects of the risk assessment.	Monitor the situation and make policy amendments should unexpected situations arise. Remain informed about public expectations and concerns.



LITERATURE

1. Bedau MA *et al.* (2009). Social and ethical checkpoints for bottom-up synthetic biology, or protocells. Syst Synth Biol (2009) 3:65–75.
2. Andrianantoandro E, Basu S, Karig DK *et al.* (2006). Synthetic biology: new engineering rules for an emerging discipline. Mol Syst Biol 2:0028.
3. Endy D (2005). Foundations for engineering biology. Nature 438:449–453.
4. European Commission (2005). Synthetic biology: Applying engineering to biology. Report of a NEST High-Level Expert Group. Project report EUR 21796.
5. Serrano L (2007). Synthetic biology: promises and challenges. Mol Syst Biol 3:158.
6. Purnick PEM, Weiss R (2009). The second wave of synthetic biology: from modules to systems. Nat Rev Mol Cell Biol 10:410–422.
7. The International civil society working Group on synthetic biology (2011). A submission to the convention on biological diversity's subsidiary body on scientific, technical and technological advice (SBSTTA) on the potential impacts of synthetic biology on the conservation and sustainable use of biodiversity.
8. Weber W, Fussenegger M (2011). Emerging biomedical applications of synthetic biology. Nature Genetics reviews vol 13 Jan. 2012.
9. Pei L, Schmidt M, Wei W (2011). Synthetic biology: An emerging research field in China. Biotechnol Adv. 2011 November; 29(6-3): 804–814.
10. Iles A, Martin AN (2012). Expanding bioplastics production: sustainable business innovation in the chemical industry. Journal of Cleaner production – first online may 2012.
11. Dupont (2012). DuPont™ Sorona® Renewably Sourced Fiber in Mohawk's SmartStrand® Silk™ Carpet. DuPont News, February 27, 2012.
12. Dupont (2009). Goodyear Research Collaboration. Internet: http://biosciences.dupont.com/about-us/collaborations/goodyear/ (accessed 10 December 2012).
13. Solazyme website. Internet: http://solazyme.com/fuels (accessed 10 December 2012).
14. Bullis K (2012). Why Amyris is Focusing on Moisturizers, Not Fuel, for Now. Technology review news 9 May 2012.
15. Synthetic Genomics (2010). Synthetic Genomics Inc. and J. Craig Venter Institute Form New Company, Synthetic Genomics Vaccines Inc. (SGVI), to Develop Next Generation Vaccines. Synthetic Genomics press release 7 October 2010.
16. Synthetic Biology Project / Woodrow Wilson Institute (2012). Inventory of synthetic biology products – existing and possible. Draft; 27 July 2012).
17. Schmidt M, Pei L (2011). Synthetic Toxicology: Where Engineering Meets Biology and Toxicology. Toxicol. Sci. (2011) 120 (suppl 1): S204-S224.
18. Macnab S, Whitehouse A (2009). Progress and prospects: human artificial chromosomes. Gene Therapy 16. 1180–1188.
19. Carlson SR *et al.* (2007). Meiotic transmission of an in vitro assembled autonomous maize minichromosome. Plos genet. 3, 1965-1974.
20. Kazuki Y *et al.* (2010). Refined human artificial chromosome vectors for gene therapy and animal transgenesis. Gene Therapy (2011) 18, 384–393.

21. Cello J, Paul AV, Wimmer E (2002). Chemical synthesis of poliovirus cDNA, generation of infectious virus in the absence of natural template. Science 297, 1016-1018.
22. Tumpey TM *et al.* (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 310, 77-80.
23. Wimmer E *et al.* (2009). Synthetic viruses: a new opportunity to understand and prevent viral disease. Nat. Biotechnology. 27, 1163-1172.
24. GenScript USA Inc. (2012). GenScript Launches a Premier Gene Synthesis Service "Gene-Bricktm" for Building Large DNA FragmentsTM. Press release 14 June 2012.
25. GenScript USA Inc. (2012). GenScript Sponsors iGEM – The Largest Competition in Synthetic Biology Field. Press release 28 May 2012.
26. Carlson R (2009). The changing economics of DNA synthesis. Nature biotechnology commentary. vol. 27:12 1091 – 1094.
27. Carr PA, Church G (2009). Genome engineering. Nature Biotechnology 27, 1151 - 1162 (2009).
28. DNA2.0. Internet: https://www.dna20.com/index.php?pageID=17 (accessed 10 December 2012).
29. Gibson DG *et al.* (2008). Complete Chemical Synthesis, Assembly, and Cloning of a Mycoplasma genitalium Genome. Science 29 February 2008: Vol. 319. no. 5867, pp. 1215 – 1220.
30. Gibson DG *et al.* (2010). Creation of a Bacterial Cell Controlled by a Chemically Synthesized genome. Science 329, 52 (2010).
31. J. Craig Venter Institute website. Overview first self-replicating synthetic bacterial cell. Internet: http://www.jcvi.org/cms/research/projects/first-self-replicating-synthetic-bacterial-cell/ (accessed 10 December 2012).
32. Pauwels K. *et al.* (2012). Synthetic Biology, latest developments, biosafety considerations and regulatory challenges. Publicatie van Wetenschappelijk Instituut Volksgezondheid; biosafety and biotechnology unit. September 2012.
33. Tian J, Ma K, Saaem I (2009). Advancing high-throughput gene synthesis technology. Mol Biosyst. 2009 Jul;5(7):714-22.
34. Wang HH *et al.* (2012). Genome Scale promoter engineering by coselection MAGE. Nat. Methods. 9 591 – 593.
35. Wang HH *et al.* (2009). Programming cells by multiplex genome engineering and accelerated evolution. Nature 460, 894-898 (13 August 2009).
36. Matzas M *et al.* (2010). High-fidelity gene synthesis by retrieval of sequence verified DNA identified using high-throughput pyrosequencing. Nature Biotechnology 28 November 2010.
37. Ma S, Saaem I, Tian J (2012). Error correction in gene synthesis technology. Trends in Biotechnology, mart 2012 vol. 30, no 3.
38. Schofield MJ, Hsieh P (2003). DNA mismatch repair: molecular mechanisms and biological function. Annu. Rev. Microbiol. 57, 579-608.
39. Li GM (2008). Mechanisms and functions of DNA mismatch repair. Cell Res. 18, 85-98.
40. Tian J *et al.* (2004). Accurate multiplex gene synthesis from programmable DNA microchips. Nature 432, 1050-1054.
41. Carr PA *et al.* (2004). Protein-mediated error correction for de novo DNA synthesis. Nucleic Acids Res. 32, e162.
42. Hoover DM, Lubkowski J (2002). DNA Works: an automated method for designing oligonucleotides for PCR based gene synthesis. Nucleic Acids Res. 30, e43.

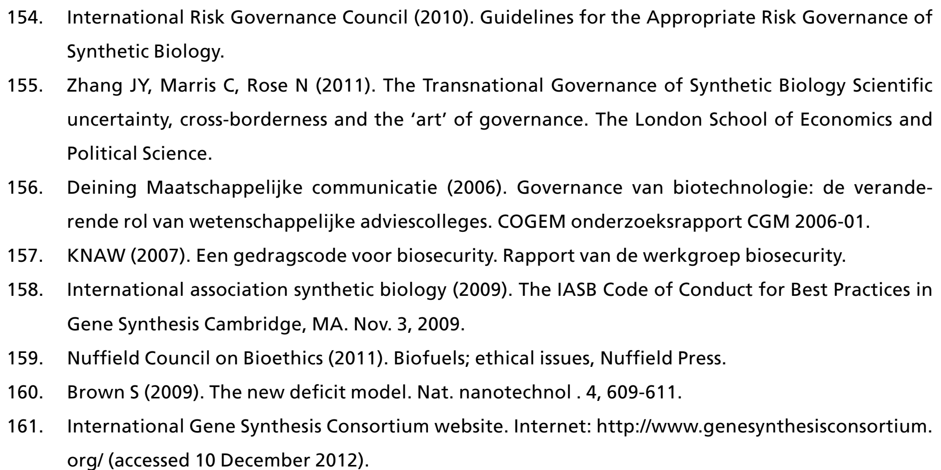
43. Xiong AS *et al.* (2004). A simple, rapid, high-fidelity and cost-effective PCR based two-step DNA synthesis method for long gene sequences. Nucleic Acids Res. 32, e98.
44. NCBI virus genoom database. Internet: http://www.ncbi.nlm.nih.gov/genomes/GenomesHome.cgi?taxid=10239 (accessed 10 December 2012).
45. Phoolcharoen W *et al.* (2011). A nonreplicating subunit vaccine protects mice against lethal ebola virus challenge. PNAS published online before print December 5, 2011.
46. Hawthorne F (2012). Bioterrorist battles. A Swiss-based firm may have a back-door way to thwart a bioterrorist attack—by fighting the flu. The scientist January 2012.
47. Gen News (2010). Novartis Teams with Synthetic Genomics Vaccines to Develop Flu Seed Virus Banks. News 7 October 2010.
48. Biobricks Foundation website. Internet: http://biobricks.org/about-foundation/ (accessed 10 December 2012).
49. Registry of Standard Biological Parts website. Internet: http://partsregistry.org/Main_Page (accessed 10 december 2012).
50. Kean S (2011). A lab of their own. Science News Vol. 333 no. 6047 pp. 1240-1241.
51. International Open Facility Advancing Biotechnology (BIOFAB) website. Internet: http://biofab.org/about (accessed 10 December 2012).
52. IGEM competitie Team Groningen. Internet: http://2012.igem.org/Team:Groningen/Project (accessed 10 December 2012).
53. Jaspers A (2012). Bacterie waarschuwt voor bedorven vlees. Wetenschap 24 nieuws November 2012.
54. Karr JR *et al.* (2012). A Whole-Cell Computational Model Predicts Phenotype from Genotype. Cell, Volume 150, Issue 2, 389-401, 20 July 2012.
55. Rude MA, Schirmer A (2009) New microbial fuels: a biotech perspective. Curr. Opin. Microbiol. 12, 274–281.
56. Baker D *et al.* (2006). Engineering life: building a fab for biology. Sci. Am. 294, 44–51 (2006).
57. Curran KA, Alper HS (2012). Expanding the chemical palate of cells by combining systems biology and metaboli cengineering, Metabolic Engineering, vol. 14, no. 4, 289-297, 2012.
58. Planson AG, Carbonell, P, Grigoras I *et al.* (2012). A retrosynthetic biology approach to therapeutics: from conception to delivery. Current Opinion in Biotechnology, 23:948-956, 2012.
59. Zhang F, Carothers JM, Keasling J (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nature Biotechnology 30:354-359, 2012.
60. Ducat DC, Way JC, Silver PA (2011). Engineering cyanobacteria to generate high-value products. Trends in Biotechnology Volume 29, Issue 2, February 2011, p. 95-103.
61. Bokinsky *et al.* (2011). Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered Escherichia coli. PNAS early edition. PNAS November 28, 2011.
62. Amyris website - biofuels. Internet: http://www.amyris.com/en/markets/fuels (accessed 10 December 2012).
63. Sinha J, Reyes SJ, Gallivan JP (2010). Reprogramming bacteria to seek and destroy an herbicide. Nat. Chem. Biol. 6, 464–470.
64. Mordechai M, Mueller S, Wimmer E (2011). Synthetic Attenuated Virus Engineering (SAVE) A Novel Strategy to Generate Viral Vaccine Candidates. GIT laboratory journal, 18 January 2011.
65. Coleman JR (2008). Virus attenuation by genome-scale changes in codon pair bias. Science 320, 1784-1787).

66. Mueller S *et al.* (2010). Live attenuated influenza virus vaccines by computer aided rational design. Nat. biotechnol. 28, 723-726.
67. Liang J, Luo Y, Zhao H (2011). Synthetic biology: putting synthesis into biology. Wiley Interdiscip. Rev. Syst. Biol Med. 2011 Jan-Feb; 3(1):7-20.
68. Kwok R (2010) Five hard truths for synthetic biology. Nature 2010 463: 288-290.
69. Amyris website - artemisinine. Internet: http://www.amyris.com/en/markets/artemisinin (accessed 10 December 2012).
70. Danchin A (1989). Complete Genome sequencing: future and prospects in: BAP 1988-1989 (Goffeau A., ed.) pp.1-24, Commission of the European Communities, Brussels.
71. Internet: http://en.wikipedia.org/wiki/Pelagibacter_ubique (accessed 10 December 2012).
72. Porcar M *et al.* (2011). The Ten Grand challenges of synthetic life. Syst Synth Biol. 2011 June; 5(1-2): 1–9.
73. Podar M *et al.* (2008). A genomic analysis of the archaeal system Ignicoccus hospitalis-Nanoarchaeum equitans.Genome Biol. 2008; 9(11):R158.
74. Glass JI *et al.* (2006). Essential genes of a minimal bacterium. P Natl Acad Sci USA. 2006;103(2):425–430.
75. Shuler ML, Foley P, Atlas J (2012). Modeling a minimal cell. Methods Mol. Biol. 881, 573 – 610.
76. Suthers PF *et al.* (2009). A Genome-Scale Metabolic Reconstruction of Mycoplasma genitalium, iPS189. PLoS Comput Biol 5(2): e1000285.
77. Baker M (2011). Synthetic Genomes: The next step for the synthetic genome. Nature vol. 473 P403-408, 19 May 2011.
78. Royal Academy of Engineering (2009). Synthetic biology: scope, applications and implications. London.
79. Pósfai G *et al.* (2006). Emergent Properties of Reduced-Genome Escherichia coli. Science 312, 1044 (2006).
80. Dymond JS *et al.* (2011). Synthetic chromosome arms function in yeast and generate phenotypic diversity by design. Nature 477,471–476(22 September 2011).
81. Dymond J, Boeke J (2012). The Saccharomyces cerevisiae SCRaMbLE system and genome minimization. Bioengineered bugs 3, 168-171.
82. Hashimoto M *et al.* (2005). Cell size and nucleoid organisation of engineered Escherichia coli cells with a reduced genome. Molecular Biology (2005) 55(1), 137-149.
83. Mizoguchi H, Mori H, Fuji T (2007). Escherichia Coli minimum genome factory. Biotechnol. Appl. Biochem. 46, 157-167.
84. Sharma SS (2007). Recombinant protein production in an Escherichia coli reduced genome strain. Metabolic Engineering Volume 9, Issue 2, March 2007, Pages 133–141.
85. Mizoguchi H *et al.* (2008). Superpositioning of deletions promotes growth of Escherichia coli with a reduced genome. DNA Res 2008;15:277–284.
86. Danchin A (2012). Scaling up synthetic biology: do not forget the chassis. FEBS letters. Volume 586, Issue 15, 16 July 2012, Pages 2129–2137.
87. Juhas M, Eberl L, Glass JL (2011). Essence of life: essential genes of minimal genomes. Trends in cell biology October 2011, vol. 21 no. 10.
88. McCutcheon JP, McDonald BR, Moran NP (2009). Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. PLoS Genet. 5, e1000565 (2009).

89. McCutcheon, JP, Moran NP (2012). Extreme genome reduction in symbiotic bacteria. Nature Microbiology reviews vol. 10 January 2012.
90. Walde P (2010). Building artificial cells and protocell models: experimental approaches with lipid vesicles. Bioessays. 2010 Apr; 32(4):296-303.
91. Rasmussen S, Bedau MA, Chen L *et al.* (2009). Protocells: Bridging nonliving and living matter. Cambridge: The MIT Press.
92. Dzieciol AJ, Mann S (2012). Designs for life: protocell models in the laboratory. Chem Soc Rev. 41:79-85...
93. Jewett MC, Forster AC (2010). Update on designing and building minimal cells. Curr Opin Biotechnol. 21: 697–703.
94. Pinheiro VB *et al.* (2011). Synthetic genetic polymers capable of heredity and evolution. Science 336, 341-344.
95. Bertrand OJN *et al.* (2012). Active, motor-driven mechanics in a DNA gel. PNAS vol. 109 no. 43.
96. Schrum JP, Zhu TF, Szostak JW (2010). The Origins of Cellular Life. Cold Spring Harb Perspect Biol. 2010 Sep;2(9).
97. Budin I, Devaraj NK (2012). Membrane Assembly Driven by a biomimetic coupling reaction. Journal of the American Chemical Society 134, 751-753.
98. Caschera F, Rasmussen S, Hanczyc MM (2012). An Oil Droplet Division-Fusion Cycle. ChemPlusChem, DOI: 10.1002/cplu.201200275.
99. Hanczyc M (2011). The line between life and not-life. TED talk may 2011. TEDSalon London Spring 2011.
100. Kuruma Y (2009). A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. Biochimica et Biophysica Acta (BBA) – Biomembranes. Volume 1788, Issue 2, February 2009, Pages 567–574.
101. Walde P *et al.* (1994). Autopoietic self-reproduction of fatty acid vesicles. Am. Chem. Soc. 116, 11649-11654.
102. Pautot S *et al.* (2003). Spontaneous Formation of Lipid Structures at Oil/Water/Lipid Interfaces. Langmuir 19, 10281-10287.
103. Angelova M, Dimitrov DS (1986). Liposome electro formation. Faraday Discuss. Chem. Soc. 81, 303-311.
104. Xu J, Sigwordth FJ, Lavan DA (2010). Synthetic protocells to mimic and test cell function. Adv. Mater. 22, 120 – 127.
105. Liu *et al.* (2009). Porous nanoparticle supported lipid bilayers (protocells) as delivery vehicles. J. Am. Chem. Soc. 131, 1354 – 1355.
106. Zepik HH *et al.* (2008). Lipid vesicles as membrane models for toxicological assessment of xenobiotics. Crit. Rev. Toxicol. 38, 1-11.
107. Stano P *et al.* (2011). Compartmentalized reactions as a case of soft-matter biotechnology: synthesis of proteins and nucleic acids inside lipid vesicles. J. Mater. Chem., 2011,21, 18887-18902.
108. V. Noireaux, Y. T. Maeda, A. Libchaber (2011). Development of an artificial cell, from self-organization to computation and self-reproduction. Proc. Natl. Acad. Sci. USA., 2011, 108, 3473-3480.
109. Shohda K, Sugawara T (2006). DNA polymerization on the inner surface of a giant liposome for synthesizing an artificial cell model. Soft Matter 2: 402-408.

110. Noireaux V, Libchaber A (2004). A vesicle bioreactor as a step toward an artificial cell assembly. Proc Natl Acad Sci USA 101: 17669-17674.
111. Kita H *et al.* (2008). Replication of genetic information with self-encoded replicase in liposomes. Chem Bio Chem 9:2403-2410.
112. Kurihara K *et al.* (2011). Self-reproduction of supramolecular giant vesicles combined with the amplification of encapsulated DNA. Nature Chemistry 3, 775–781.
113. Foley PL, Shuler ML (2010). Considerations for the design and Construction of a synthetic platform cell for biotechnological applications. Biotechnol Bioeng. 2010 Jan 1;105(1):26-36.
114. Luisi PL (2006). (Book). The Emergence of Life. From Chemical Origins to Synthetic Biology. Cambridge University Press.
115. Herdewijn P, Marlière P (2009). Toward safe genetically modified organisms through the chemical diversification of nucleic acids. Chem Biodivers. 2009 Jun; 6(6):791-808.
116. Schmidt M (2010). Xenobiology: a new form of life as the ultimate biosafety tool. Bioessays 32: 322–331.
117. Acevedo-Rocha CG, Budisa N (2011). On the Road towards Chemically Modified Organisms Endowed with a Genetic Firewall. Angewandte Chemie International Edition, Volume 50, Issue 31.
118. Krueger *et al.* (2007). Synthesis and Properties of Size-expanded DNAs: Toward Designed, Functional Genetic Systems. Acc Chem Res. 2007 February ; 40(2): 141–150.
119. Pinheiro VB, Holliger P (2012). The XNA world: progress towards replication and evolution of synthetic genetic polymers. Curr Opin Chem Biol. 2012 16:245-52.
120. Yang Z *et al.* (2011). Amplification, mutation and sequencing of a six-letter synthetic genetic system. J Am Chem Soc. 133: 15105-15112.
121. Neumann H *et al.* (2010). Encoding multiple unnatural amino acids via evolution of a quadruplet decoding ribosome. Nature Letter Nature 464, 441-444.
122. Joyce GF (2012). Towards an alternative biology. Science Vol. 336 no. 6079 pp. 307-308.
123. Cho H *et al.* (2011). Optimized clinical performance of growth hormone with an expanded genetic code. Proc Natl Acad Sci U S A. 108: 9060-5.
124. Schmidt M, De Lorenzo V (2012). Synthetic constructs in/for the environment: Managing the interplay between natural and engineered Biology. FEBS letters. FEBS Letters 586 (2012) 2199–2206.
125. Marlière P *et al.* (2011). Chemical Evolution of a Bacteriums Genome. Angew. Chem. Int. Ed. 2011, 50, 7109 –7114.
126. Malyshev DA *et al.* (2012). Efficient and sequence-independent replication of DNA containing a third base pair establishes a functional six-letter genetic alphabet. Proc Natl Acad Sci U S A. 2012 Jul 24;109(30): 12005-10.
127. Voloshchuk N, Montclare JK (2009). Incorporation of unnatural amino acids for synthetic biology. Mol. BioSyst., 2010, 6, 65–80.
128. Wang Q *et al.* (2009). Expanding the genetic code for biological studies. Chem. Boil. 16, 323-336.
129. Young TS, Schultz PG (2010). Beyond the canonical 20 amino acids: expanding the genetic lexicon. The Journal of Biological Chemistry, 285, 11039-11044.
130. Minniham EC (2009). Unnatural amino acids: better than the real thing? F1000 Biol Rep. 2009; 1: 88.
131. Kazane S *et al.* (2012). Site-specific DNA-antibody conjugates for specific and sensitive immuno-PCR Proceedings of the National Academy of Sciences, 109 (10), 3731-3736.

132. Dana GV *et al.* (2011). Synthetic biology: Four steps to avoid a synthetic-biology disaster. Nature 483,29.
133. Atsumi S, Taizo H, Liao JC (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature Vol. 451, p. 86-90, 3 January 2008.
134. Atsumi S, Hegashide W, Liao JC (2009). Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde, Nature Biotechnology 27, p. 1177-1180.
135. Zhang L,, Chang S, Wang J (2010). How to make a minimal genome for synthetic minimal cell. Protein Cell 2010, 1(5): 427–434, DOI 10.1007/s13238-010-0064-4.
136. Joyce, GF (2002). The antiquity of RNA-based evolution, Nature 4 18, 214-221 (11 July 2002).
137. Beer, LL. et.al. (2009). Engineering algae for biohydrogen and biofuel production. Current Opinion in Biotechnology 2009:20, p. 264-271.
138. Ort DR, Zhu X, Melis A (2011). Optimizing Antenna Size to Maximize Photosynthetic Efficiency. Plant Physiology, January 2011, Vol. 155, pp. 79–85.
139. Agapakis, CM *et al.* (2011). Towards a Synthetic Chloroplast. PLoS ONE, April 2011, Vol. 6 (4), pp. 1-8.
140. Silver P *et al.* (2011). Synthetic Biology and Light-Dependent Systems. Lecture at the 5th International Synthetic Biology Conference, 15 June 2011, Stanford, Cal.
141. Stemerding D. (2012). Verslag expertbijeenkomst biosafety synthetische biologie op 29 June 2011. 12 January 2012 (not published).
142. Naesby M *et al.* (2009). Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in Saccharomyces cerevisiae. Microbial Cell factories 8:45.
143. Marmeisse R *et al.* (1993). Disruption of the Avirulence Gene avr9 in two races of the tomato pathogen cladosporium fulvum causes virulence on tomato genotypes with the complementary resistance gene CF9. Molecular plant-microbe interactions vol. 6 no 4, 412-417.
144. Gillen A, Sherwin F (2006). The origin of the bubonic plague. Journal of Creation vol. 20.
145. Wren BW (2003). The Yersiniae – a model genus to study the rapid evolution of bacterial pathogens. Nature Reviews Microbiology vol. 1.
146. ETC Group (2012). The principles for the oversight of synthetic biology.
147. Gaskell G *et al.* (2011). The 2010 Eurobarometer on the Life Sciences. Nat. biotechnol. 29, 113-114.
148. Bubela T, Hugen G, Einsiedel E (2012). Synthetic biology confronts publics and policy makers: challenges for communication, regulation and commercialization. Trends in Biotechnology, march 2012, vol, 30, no 3.
149. Van der Burg S (2009). Taking the “Soft Impacts” of Technology into Account: Broadening the Discourse in Research Practice. Social Epistemology: A Journal of Knowledge, Culture and Policy. Volume 23, Issue 3-4.
150. Nawroth JC *et al.* (2012). A tissue engineered jellyfish with biomimetic propulsion. Nature biotechnology 30: 8.
151. Brouwers L (2012). Wetenschappers maken zwemmende kunstkwal van rattenhartcellen en rubber. NRC wetenschap 24 July.
152. European Commission (2010). Workshop on Synthetic Biology: From Science to governance. 18-19 March 2010, Brussels.
153. The Presidential Commission for the Study of Bioethical Issues (2010). New Directions: The Ethics of Synthetic Biology and Emerging Technologies.



154. International Risk Governance Council (2010). Guidelines for the Appropriate Risk Governance of Synthetic Biology.
155. Zhang JY, Marris C, Rose N (2011). The Transnational Governance of Synthetic Biology Scientific uncertainty, cross-borderness and the ‘art’ of governance. The London School of Economics and Political Science.
156. Deining Maatschappelijke communicatie (2006). Governance van biotechnologie: de veranderende rol van wetenschappelijke adviescolleges. COGEM onderzoeksrapport CGM 2006-01.
157. KNAW (2007). Een gedragscode voor biosecurity. Rapport van de werkgroep biosecurity.
158. International association synthetic biology (2009). The IASB Code of Conduct for Best Practices in Gene Synthesis Cambridge, MA. Nov. 3, 2009.
159. Nuffield Council on Bioethics (2011). Biofuels; ethical issues, Nuffield Press.
160. Brown S (2009). The new deficit model. Nat. nanotechnol . 4, 609-611.
161. International Gene Synthesis Consortium website. Internet: http://www.genesynthesisconsortium.org/ (accessed 10 December 2012).

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