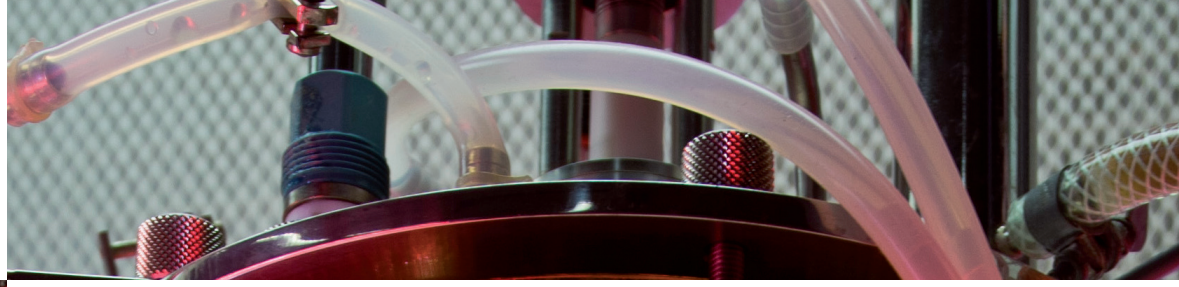

Algae and genetic modification

Research, production and risks

CGM 2012-05

ONDERZOEKSRAPPORT

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FOOD & BIOBASED RESEARCH
WAGENINGEN UR

Algae and genetic modification

Research, production and risks

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May 2012

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Preface

Algae are a hot issue these days. The large and diverse group of simple, typically autotrophic (self-feeding) organisms is increasingly seen as a solution for sustainable production of food, feed and above all bio-fuel.

The search for economic feasible production goes together with improvement of production systems and genetic modification (GM). Genetic modification of algae aims at increasing the productivity or enhancing the composition of the anticipated products in the GM-algae.

The Netherlands Commission on Genetic Modification (COGEM) expects an increase in GM-algae research in the Netherlands. Recently COGEM issued two advises regarding GM-algae, however, we feel that the knowledge on the risks of GM-algae is still rather limited.

COGEM wants to be prepared for the near future and has commissioned a project that must provide an overview of the developments in research and production of genetically modified algae, the potential risks of GM-algae and the knowledge already available and required.

The project, including a desk research and a workshop was performed by Christien Enzing (project leader) and Anke Nooijen of Technopolis Group The Netherlands and Gerrit Eggink, Jan Springer and Rene Wijffels of Food and Biobased Research Wageningen UR.

The desk research, inspiring workshop and constructive discussions between the board and the researchers resulted in an report in which al issues are intensively discussed. The report is a solid base for future risk assessment in the COGEM but also of interest for the algae research population.

The Supervisory Board wants to thank the performers and all participants in the project.

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Executive summary

The potential of algae for bio-fuel production has accelerated the development of algae-based production systems. Genetic modification (GM) of algae is now being investigated with the aim of increasing the productivity or enhancing the composition of the anticipated products in the GM-algae. GM-algae are been researched outside Europe for quite some time. Recently it has become also part of research projects in Europe and the Netherlands. Recently the Netherlands Commission on Genetic Modification (COGEM) issued two advises on the contained use of GM-algae. However, as the knowledge on the risks of GM-algae is still rather limited the COGEM has commissioned a project with the aim to provide an overview of the developments in research and production and to identify potential risks of GM-algae.

A team of researchers of the Wageningen University and Research Centre and the Technopolis BV Amsterdam has performed the project, of which the results are being presented in this report. Based on a desk study in the second chapter of the report an overview is given of the different types of products that are made or can be made from algae, nowadays and in the future, and of the algae production systems that are being used indoors and outdoors. This second chapter also provides a description of the current developments in algae research. The chapter closes with an overview of Dutch algae research and production.

The taxonomy of algae is an on-going process as new species of algae are found and identified. Genetic analysis has shown that some known species have to be re-classified. As the taxonomy of algae is an important aspect for environmental risk assessment, the report spends a separate chapter (Chapter 3) on the available definitions and classifications of algae and provides an overview of the life cycle of algae. Also an overview of pathogenic and toxin producing algae is given in the last section of the third chapter of the report.

The fourth chapter describes the state of the art on transgenic research on algae. It starts with an overview of the genetically transformed algae strains followed by overviews of the DNA delivery methods and of the targets of genetic modification of algae.

The risks of GM-algae are first addressed in the desk research (Chapter 5) with a description of the Dutch law concerning GMOs and overviews of what is already known about the risks related to production systems of (GM-)algae. Both natural locations and open ponds are considered as deliberate release into the environment with no effective protective measurements to prevent the algae from entering the surrounding environment. Closed systems (PBRs) could be considered contained when placed inside a building. Cultivation of a GM-algae in a closed system which is placed outside may be considered under the regulation of contained use when ‘specific containment measures are used to limit their contact with the general population and the environment (Directive 2009/41/EC, Article 2c)”.

The overview of potential risks of GM-algae focuses on the harmful properties of the algae for the recipient organism, of the insert, the vector and the resulting GM-algae with respect to human health and the environment. The report concludes that in general the genetic modification of algae aimed at modifying either photosynthesis, carotenoid biosynthesis or lipid biosynthesis is not expected to generate harmful strains with respect to human health. None of the genes used encode toxins or are suspected to lead to toxin production through enhanced metabolic steps or metabolic pathways, especially when they are expressed in “safe” algae hosts. Effects of introducing genes encoding enzymes not found naturally in the host may have phenotypic effects which should be analysed and monitored over time. When expressing (pharmaceutical) proteins, potential effects of these proteins on humans have to be addressed in the risk assessment.

The random type integration - which is currently the most observed type in algae genetic modification - could have an adverse effect on the recipient algae; here a careful analysis and monitoring of the fate of the inserted DNA and the effect on the phenotype is necessary. Only *Chlamydomonas reinhardtii* has a history of stable genetic modifications and subsequent cultivation of the GM-strains. Stability of other GM-algae (which is mainly an issue in the production using these algae) still has to be confirmed especially under non-selective conditions since stability will most likely be gene and integration dependent. The methodology of risk assessment used for GMOs can be applied to cyanobacteria without major modifications.

An important aspect to be addressed in an environmental risk assessment (ERA) is the transfer of inserted genetic material to other organisms. Therefore horizontal gene transfer (HGT) - the transfer of genetic material from one organism to another which is a natural mechanism and has played an important role in evolution - is a point of concern. In cyanobacteria HGT is a mechanism in real time adaptation and for that reason it is part of the risk assessment of GM-bacteria. In eukaryotic algae HGT poses no additional risk in GMOs. Vertical gene transfer uses reproduction as a means of gene transfer through generations and may be a risk with GM-algae when the species used has a sexual reproduction cycle and wild type partners are present in the environment.

The results of the desk research were discussed in a workshop which was attended by a wide range of experts both from the Netherlands and abroad. The workshop provided an overview of issues that are relevant when taking into consideration the risks of GM-algae for human and the environment, how they could be assessed and more important, contained and prohibited.

Based on both the desk research and the workshop, the following conclusions have been drawn on the (potential) risks of GM-algae:

- Strain identity is an important parameter for determining the potential risk of mass cultivation of industrial GM-algae.
- The ‘history of safe use‘ of algae is only valid in case the identity of the algae strain is known.
- A few algae species are known pathogens in humans or animals; they belong to the Prototheca or Chaetoceros or are mentioned on the IOC-Unesco list of harmful algae. A number of algal species, especially belonging to the dinoflagellates and the diatoms, produce toxins that impact humans, animals and birds. Also some cyanobacteria produce harmful toxins. So if the identity of the strain is established potential pathogenicity or toxicity can be evaluated.
- The fitness of the GM-strain in relation to wild types in the environment should be an important aspect of an environmental risk assessment. Insight in the fitness of the GM-algae to exist and survive in native environment is needed.
- Effects of introducing genes encoding enzymes not found naturally in the host may have phenotypic effects. These effects should be analysed and monitored over time.
- Using a mitigation technology could be an approach to reduce the survival of the GM-algae in the environment.
- Horizontal gene transfer is a point of concern with cyanobacteria. The focus must be on the likelihood of potential adverse effects of horizontal gene transfer such as toxicity, pathogenicity, antibiotic resistance competitive advantage, utilization of novel substrates, in the environment.
- A careful analysis of the life cycle of the GM-algae used and of the ecological niche in which the GM-algae might be released will provide

indications of the risk of sexual interaction and thereby the risk of gene transfer from the GM-algae to wild type algae.

1. Introduction

1.1 Background of the project

Algae are a large and diverse group of simple, typically autotrophic (self-feeding) organisms, ranging from unicellular to multi-cellular forms, such as the giant kelps that grow to 65 meters in length. In this report we address both the eukaryotic algae and the cyanobacteria that are commonly referred to as blue-green algae or sometimes as prokaryotic algae.

Already since a long period algae have been used for producing food, food ingredients and ingredients for cosmetics. In the last decades research on algae for the production of bio-fuel, food, feed or chemicals has expanded rapidly. Recently, their potential for bio-fuel production has accelerated the development of algae-based production systems. One development in algae research is genetic modification (GM), often with the aim of increasing the productivity or enhancing the composition of the anticipated products in the GM-algae. Genetic modification of algae has already been researched outside Europe for some time but is currently also part of research projects in Europe and the Netherlands.

To date the Netherlands Commission on Genetic Modification (COGEM) has gained much experience and knowledge in the field of genetically modified plants and microorganisms, and has issued many advises in this field already since the beginning of the 80's. Only recently COGEM issued two advises regarding GM-algae (one on eukaryotic algae and one on cyanobacteria)[1]. However, the knowledge on the risks of GM-algae is still rather limited.

For that reason COGEM has commissioned a project that must provide an overview of the developments in research and production of genetically modified algae and on the potential risks of GM-algae and the knowledge already available and knowledge that is required.

Based on this overview, COGEM is better able to determine - based on the current environmental risk assessment system - what knowledge and data is missing (knowledge gaps) that are required for performing an environmental risk assessment. The COGEM aims to develop a sound knowledge base to prevent unnecessary delays in the granting of permits for the use of GM-algae.

1.2 Two parts of the project: desk study and workshop

This project has the aim to provide such an overview. The project consisted of two parts: a desk research and a workshop (including preparatory interviews).

The desk study aimed at getting an overview of the used algae strains, their characteristics, the state of the art concerning genetically modified algae, the different types of production systems, the associated potential risks to the environment based on the expertise available in the project team, literature and reports. The results of the study have been written down in a report. Two experts in the field have reviewed the report[2]. The desk study was done by researchers of the Wageningen University and Research Centre (Wageningen UR).

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[1] Classification of nine species of eukaryotic algae. The applicant wants to genetically modify these species of algae by introducing genes involved in glycolysis and fatty acid metabolism. (CGM/110706-01)
Large-scale production of lactic acid by GM cyanobacteria in a culture system for single use (CGM/110418-03).

[2] Prof.dr. Steve.P. Mayfield (UC San Diego, US) and Prof. dr. Alison. Smith (University of Cambridge, UK)

The second part of the project included first of all a workshop. The aim of the workshop was to discuss the report with a number of important stakeholders (in research and production) and to come to a first overview of the relevant risk aspects related to GM-algae research and production. The workshop's aim was to answer the following questions:

1. On which risk aspects of GM-algae is **sufficient** knowledge available in order to make the risk assessments that are necessary for applications made in the framework of environmental regulation of GM-algae in the Netherlands/Europe?

2a. On which risk aspects of GM-algae is **no or insufficient** knowledge available in order to make the environmental risk assessments that are necessary for judging applications in the framework of environmental regulation of GM-algae in the Netherlands/Europe?

2b. Given the above: to what new research questions in GM-algae and risk research (addressing the knowledge needs) does this lead?

Participants in the workshop that took place on March 19, 2012, in Wageningen were international and Dutch experts in the field related to the subject of the study, representatives of Dutch companies active in algae production and a number of other persons interested in the subject of the workshop. See Appendix Hfor the program of the workshop and Appendix I for the list of participants.

In addition to the workshop the second part of the project included three interviews[3] for preparing the workshop.

Finally Part 2 also included the writing of this report (in which the report of Part 1 was integrated). Technopolis BV (Amsterdam) was responsible for this part of the project and the overall coordination.

1.3 This report

The following four chapters present the results of the desk study. Chapter 2 provides an overview of the algae-based products, the algae production systems, new developments in algae research and Dutch algae research and production. Chapter 3 deals with several taxonomic aspects of algae: definitions and classifications of algae, the different life cycles of algae and the overview of pathogenic and toxin producing algae. Chapter 4 presents the genetically transformed algae strains and their stability, followed by an overview of the DNA delivery methods and of the targets of genetic modification of algae. Chapter 5 provides an overview of the risks related to production systems of (GM-)algae and of GM-algae for human health and the environment. Chapter 6 presents the results of the workshop and the interviews. Chapter 7 holds the conclusions of both the desk study and the workshop.

[3] Prof. dr. O. Kruse (Bielefeld University, Germany), Prof.dr.J. Huisman (Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam) and Dr. P. Bruinenberg (AVEBE).

2. An introduction to algae: products, production and new research themes

2.1 Introduction

This chapter gives an overview of the different types of products made from algae, nowadays as well as in the near future, and of the algae production systems that are being used indoors and outdoors. In addition it describes current developments in algae research. The chapter closes with an overview of Dutch algae research and production.

2.2 Algae-based products

The past decades have shown a growing interest in algae as production organisms. Algae, especially marine algae, have been used as food, feed and fertilizer for centuries. Commercial farming of macro-algae (seaweed) for instance has a long history, especially in Asia. In the 1950's algae were considered a candidate for protein supply for the increasing world population. From the 1950's on a search for biologically active substances from algae began; also wastewater treatment with algae was implemented. Commercial large scale cultures of Chlorella were started in the early 1960's followed by Arthrospira in the 1970's. The energy crisis in the 1970's caused an increased interest in the use of algae for renewable energy production and research programs were initiated (Spolaore, Joannis-Cassan et al. 2006). By 1980 large-scale algae production facilities were established in Asia, India, the USA, Israel and Australia. Nowadays approximately 200 species of algae are used worldwide. About 10 species are intensively cultivated, such as the brown algae *Laminaria japonica* and *Undaria pinnatifida*, the red algae *Porphyra*, *Eucheuma*, *Kappaphycus* and *Gracilaria*, and the green algae *Monostroma* and *Enteromorpha*. *Laminaria japonica*, a brown alga also known as kelp, is the most important with 4.2 million tonnes cultivated mainly in China. Seaweed as such is used as food e.g. Nori the Japanese name for various edible seaweed strains of the red alga *Porphyra*, while components of seaweeds (agar, carrageenans, alginates) are used in the production of food, feed, chemicals, cosmetics and pharmaceuticals (Luning and Pang 2003).

Food supplements from micro-algae (mostly growing unicellular) comprise an important market in which compounds such as beta-carotene, astaxanthin, polyunsaturated fatty acid (PUFA) such as DHA and EPA and polysaccharides such as beta-glucan dominate (Pulz and Gross 2004; Spolaore, Joannis-Cassan et al. 2006).

Currently, the micro-algae biomass market has a size of about 5,000 tonnes/year of dry matter and generates a turnover of ca. US$ 1.25 billion per year (Pulz and Gross 2004). Figure 1 shows some examples of micro-algae that are used in commercial applications.

Figure 1 Examples of algal species used in commercial applications

Application	**Examples of algal species used**
Human nutrition	*Arthrospira, Chlorella, Dunaliella, Aphanizomenon*
Animal nutrition (aquaculture)	*Arthrospira, Chlorella, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema, Thalassiosira, Haematococcus*
Animal nutrition (other)	*Chlorella, Arthrospira*
Cosmetics	*Chlorella, Arthrospira, Nannochloropsis, Dunaliella*
High value chemicals for various applications (PUFA's, pigments)	*Crypthecondium, Ulkenia, Dunaliella, Haematococcus, Spirulina*

In recent years innovative processes and products have been introduced in both macro- and micro-algal biotechnology. Figure 2 shows an overview of recent (health) products from algae.

Figure 2 Examples of health products from algae

Product	Algae	Company
DHA	Crypthecodinium	Martek/Omegatec
ASTA (Astaxanthin)	Haematococcus	Cyanotec
ASTA (Astaxanthin)	Haematococcus	MERA
Carbohydrate extract	Chlorella	OceanNutrition
EPA	Odontella	InnovalG
VitaminB12	Spirulina	Panmol/Madaus
DHA	Ulkenia	Nutrinova/Celanese
Carrageenan	Kappaphycus	Gates Foundation
Macrolides	Lobophora	R&D
Biomass	Rhodophyta	BSV
Hexose oxidase	Macroalgae	Danisco

Source: Pulz and Gross 2004

While the use of algae in functional food and animal feed has reached or will soon reach the level of mass production, their use in chemical, energy, pharmaceutical and industrial (chemical, energy, waste water treatment) applications is subject of research now and is likely to lead to new products in the near future (Dufossé, Galaup et al. 2005; Anders S Carlsson 2007).

Potential pharmaceutical products produced by algae and in the near future by GM-algae are[4]:

- Antimicrobials, Antivirals & Antifungals: Some strains of both micro-algae and macro-algae exhibit antimicrobial activity, which finds use in various pharmaceutical industries. Examples are metabolites and toxins from cyanobacteria;
- Neuroprotective Products: Some strains from both micro-algae and macro-algae contain neuroprotective agents that promote nerve cell survival. Examples are *Spirulina* and *Ulva conglobata;*
- Human Therapeutic Proteins: Pharmaceutical companies could substantially reduce the expense of costly treatments for cancer and other diseases produced from mammalian or bacterial cells by growing human therapeutic proteins in algae;
- Drugs: Examples are cryptophycin 1 which has been isolated from blue-green algae and alkaloids from macro-algae. Both components are considered promising anti-cancer drugs.

In the report of Carlsson (2007) the utility of algae for industrial applications products from algae are discussed: see Figure 3 for an overview of potential products from algae.

Figure 3 Possible industrial products from algae

Product group	Product	Subcategory
Energy	Biomass	Biomethane
	Biofuel	Bio-oil

[4] http://www.articlecity.com/articles/environment_and_going_green/article_790.shtml

		Biodiesel
		Biohydrogen
CO2	CO2 mitigation	
	CO2 sequestration	
	Carbon trading	
High-value products	Small molecules	Chemicals
	Polymers	Hydrocolloids
		Elicitors
	Pharmaceuticals and cosmetics	
	High value oils	
	Colorants	
Materials	Silica structures	
Waste water treatment	Removal of nutrients	
	Removal of organic pollutants	
	Removal of heavy metals	

In the EU FP7 project Aquafuels an inventory was made of algae species based on the literature concerning biofuel production, and on already commercially produced algae[5].

The table in Appendix Blists the industrially relevant eukaryotic algae and cyanobacteria with respect to biofuel production mentioned in the inventory.

2.3 Algae production systems

A distinction is made between indoor and outdoor production systems. Since the focus of this report is on environmental release of GM-algae and not on contained use of GM-algae indoor production systems will be described briefly while outdoor production systems will be described in more detail.

2.3.1 Indoor production systems

For small-scale algae production - mostly for research purposes - a wide range of systems is used. Algae can be cultivated in simple erlenmeyers, in fermenters (photo-bioreactors) and in so called flat panel reactors, among other things. Figure 4 shows examples of these systems.

Figure 4 Three examples of contained production of algae: erlenmeyers, fermenters and flat panel reactors

Steel fermenters are used for large scale production with heterotrophic algae; depending on the size, they are placed indoor or outdoor (figure 5). Fermenters are for instance used for the production of long chain unsaturated fatty acids by the heterotrophic alga *Crypthecodinium cohnii*.

[5] For the complete inventory see http://www.aquafuels.eu/attachments/079_D%201.2%20Taxonomy.pdf

Figure 5 A 1500 litre steel fermenter

2.3.2 Outdoor production systems

Because of the need for light for most algae a lot of research on large- scale production is performed on outdoor production systems. Outdoor production systems for algae can be divided into open and closed systems.

2.3.2.1 Open systems

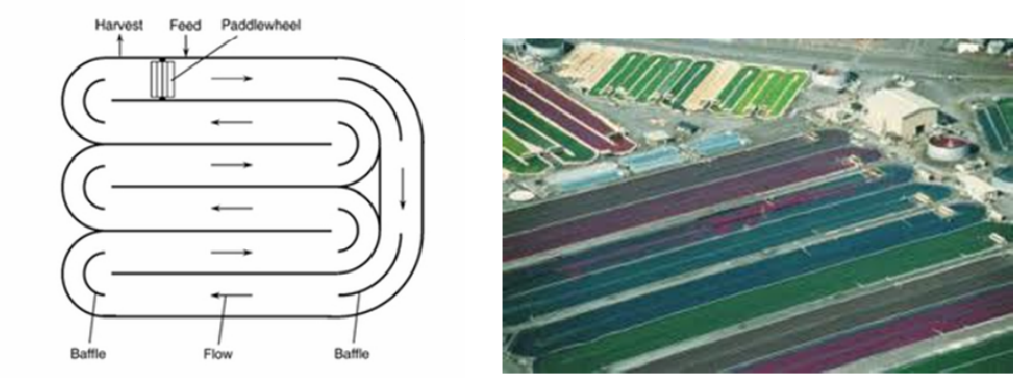
Open systems for algae cultivation include so-called natural locations and open pond systems.

Cultivation of alga at natural locations includes both micro and macro-algae (seaweed). E.g. the micro-algae *Dunaliella salina* and *Haematococcus pluvialis* are cultivated under high salt conditions in ponds in coastal areas. China, Japan and the Philippines are the world's largest producers of traditional macro-algae which are used for food, fertilizer, or for the extraction of alginate, agar and carrageenan as food ingredients. Seaweed is currently under consideration as a potential source of bioethanol.

In the Netherlands research on this type of algae cultivation is done by Wageningen-UR in the Oosterschelde. Species grown there are *Ulva lactuca, Laminaria digitata, Laminaria saccharina and Palmaria palmata.*

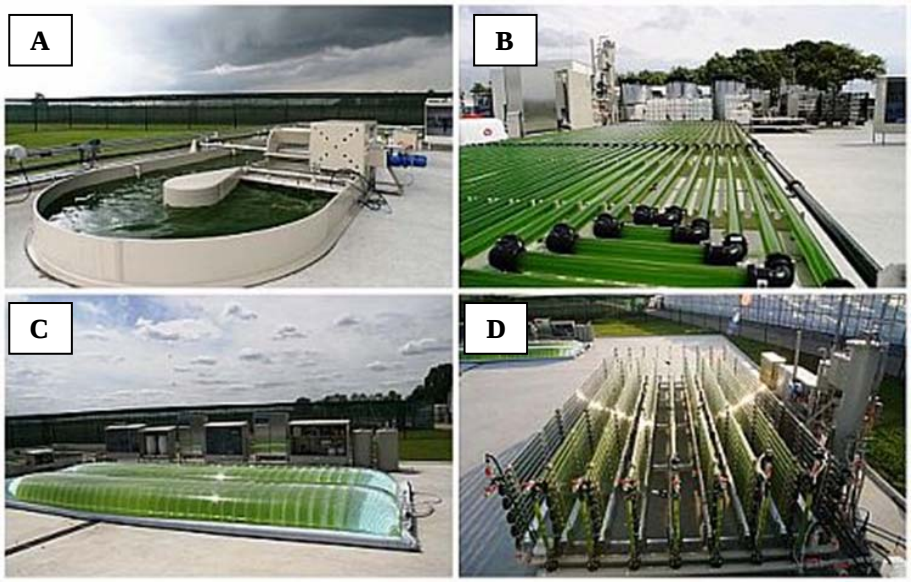
Open ponds systems are shallow artificial pools often constructed as an oval with a paddlewheel to circulate the content. Open pond systems are used for commercial algae cultivation for decades already. On Hawaii for instance Cyanotech is growing *Spirulina* and *Haematococcus* on 90 acres (3.6 hectare): see Figure 6. The advantages of open ponds are the relative low costs of construction and maintenance. A disadvantage is the open surface that allows other airborne micro-organisms to enter the pond.

Figure 6 Algae cultivation in an open pond system



In the Netherlands open pond systems are used by Ingrepro at Borculo, by AquaPhyto at Zeewolde and Schiphol Airport, and by Kellstein Greencircle at Hallum. In addition an open pond system is present at the AlgaeParc research facility of Wageningen UR (Figure 7).

Figure 7 Algae production systems at AlgaeParc



A. Open pond system. B. Horizontal tubular system. C. Polyethylene bag system. D. Vertical tubular system.

2.3.2.2 Closed systems

There is a large variety of closed systems used for the production of algae. These closed systems prevent contact between the enclosed algae and the environment. For sampling and harvesting precautions can be taken that limit the contact with the environment. Tubular systems of different sizes (either vertical or horizontal placed), polyethylene sleeves or bags are most commonly used, but there are also designs like biodomes and even floating bags on ocean waters. However, the containment of these systems may be breached by accidents or careless handling.

Closed systems are typically referred to as photo bioreactors (PBR's). They can be placed outdoors; in some cases they are placed inside greenhouses to allow more controlled conditions. The major advantage of using these systems is the increased surface area for a certain culture volume. Other advantages of PBR's are lower contamination risk, easier mixing which improves mass transfer, and easier control of

temperature, pH and nutrient supply. However, the cost of installation and operation is much higher than those of open pond systems. Figure 7 (B, C and D) shows three types of PBR's used at AlgaeParc.

2.3.3 Growth conditions

The growth conditions in algae production systems are diverse and depend very much on the specific natural conditions. Eukaryotic algae are found just about everywhere on earth: in the sea, in rivers and lakes, on soils and wall, and in animals and plants as symbionts. Well known symbionts of algae are lichens, coral, sea sponges and hydra. Algae are prominent in bodies of water, common in terrestrial environments and are found in unusual environments, such as on snow and on ice where they may be actively growing[6]. Also cyanobacteria can be found in almost every terrestrial and aquatic habitat, from oceans to fresh water to bare rock to soil. Cyanobacteria are able to perform oxygenic photosynthesis.

Most algae are autotrophic (using energy from light by photosynthesis), some are heterotrophic (get energy from non-photosynthetic origin also). Mixotrophic algae can use sunlight or organic carbon.

The growth of algae for industrial productions also shows a wide range: from dark in steel fermenters (heterotrophic) to light in glass or plastic growth systems (phototrophic or mixotrophic), from salt (seawater), brackish to fresh water. Other factors like pH, temperature, nutrients and aeration are of importance for optimal growth. Optimization of culture conditions is an important issue in algae research.

Figure 8 presents a number of general parameters dealing with conditions for culturing micro-algae, based on the FAO "Manual on the Production and Use of Live Food for Aquaculture"[7].

Figure 8 General parameters for algal cultivation

Parameters	**Range**	**Optima**
Temperature (°C)	16-27	18-24
Salinity ($g.l^{-1}$)	12-40	20-24
Light intensity ($mmol/m^2/s$	15-135 (depends on volume and density)	40-70
Photoperiod (light: dark, hours)		16:8 (minimum) 24:0 (maximum)
pH	7-9	8.2-8.7

Source: Manual on the Production and Use of Live Food for Aquaculture, FAO

2.3.4 Harvesting and processing

During or at the end of cultivation algae are harvested in order to analyse or extract the products. Current procedures used are centrifugation and filtration often aided by flocculation. In the Netherlands research is also conducted on harvesting and extraction of algae. The AlgaePARC biorefinery program is developing research at laboratory and pilot scale on continuous and scalable technology and processes to fractionate micro algal biomass into different components (e.g. lipids, proteins and carbohydrates)[8].

[6] http://www.antarctica.gov.au/media/news/?a=7909

[7] http://www.fao.org/DOCREP/003/W3732E/w3732e06.htm

[8] http://ispt.eu/news_and_press/news/ISPTandAlgaePARCpartnersstartnewprojectonAlgaebiorefinery for a more details on this program.

2.4 New developments in algae research

2.4.1 Research topics

As described in the previous paragraph algae have been exploited for the benefit of man and animals for a long time already, but in the past decades the interest in algae has increased substantially. Especially since the oil crisis in the 1970s large research programs have been initiated developing micro algal energy production systems. Apart from the boost in algal research generated by the oil crisis and environmental motives (climate change, CO2 issues and land use) also technical developments like the introduction of new generation DNA sequencers, improvements in algal genetic modification, and the rise of systems biology have contributed to the expanding of research on algae.

Nowadays, two main fields of research can be distinguished:

- Technological productivity improvement: this includes reactor design, process control, harvesting and extraction.
- Strain improvement: this includes strain selection, mutagenesis and genetic modification.

Research on traditional algae products is mostly focussed on improving productivity by testing new production systems, new strains, optimize growth conditions, optimize extraction procedure etc.

Recent research on algae is also focussing on new production systems and on improving production of traditional products but also on new algae products like biodiesel, bio-ethanol, bioplastics and new pharmaceuticals. With the advances in genome and transcriptome analysis and in genetic modification of algae new approaches in algal research come within reach. Metabolic pathways can be introduced, deleted or changed (Scott, Davey et al. 2010, Radakovits, Jinkerson et al. 2010, Hallman 2007).

More complex issues like optimisation of photosynthesis are also addressed for instance in the Dutch research programme Towards Biosolar Cells[9] that aims at developing background knowledge regarding solar cells that are based on the primary steps in photosynthesis.

2.4.2 Investments in algae research

Despite the long history of algae for food and feed and the promising products from algae in the nutraceutical and pharmaceutical field the big boost in algal research in the past decade has been on developing micro-algal energy production systems. Micro-algae are considered as one of the most promising feedstock for biofuels. The productivity of these photosynthetic microorganisms in converting carbon dioxide into carbon-rich lipids, only a step or two away from biodiesel, greatly exceeds that of agricultural oleaginous crops, without competing for arable land. Worldwide, research and demonstration programs are being carried out to develop the technology needed to expand algal lipid production from a craft to a major industrial process (Waltz 2009; Barbosa 2010; Michael Hannon 2010; Scott, Davey et al. 2010; Singh, Nigam et al. 2011).

Current large investments in algae research and development mostly focus on bio-fuel production. A quick scan on newsflashes resulted in the figure below showing some of the major investments in research on microalgae by companies and governmental departments in the US (Figure 9).

[9] http://www.fom.nl/live/nieuws/artikel.pag?objectnumber=140992

Figure 9 Major investments in algae research for energy production in the US

Organisation	Investment in algae research
Defence Advanced Research Projects Agency (public)	$ 85 million
US Department of Energy (public)	$ 85 million
Exxon Mobil. Corp	$ 600 million
Sapphire Energy Inc.	$ 300 million
Solazyme	$ 200 million
Algenol	$ 50 million
Aurora biofuels	$ 65 million

Research on algae is also a major topic in Europe and several algae R&D projects are on-going. In the Aquafuels project[10] an inventory was made on algae research projects within the EU. The inventory identified approximately 50 on-going algae-related research projects funded by the EU or by national or regional governments, often with involvement of industry. In Appendix C Figure 22 shows the summary of EU-funded projects and Figure 23 shows the projects that are currently running in European countries and that are being funded by national or regional public funding programmes[11].

2.5 Dutch algae research and production

In the Netherlands research on algae is also increasing. At least 17 out of the aforementioned 49 projects from the Aquafuels inventory are Dutch projects or projects with Dutch participation.

Part of the Dutch research on algae is clustered in a number of large research initiatives that have started in 2010 and 2011. These include Towards BioSolar Cells, AlgaePARC and Wetsus.

Towards BioSolar Cells is a five years research project (started in 2011) in which universities, research institutes and companies cooperate in research on optimisation of photosynthesis in plants and algae (including cyanobacteria). The budget of BioSolar Cells is € 42 million. With respect to microalgae three research topics have been identified: 1) Systems biology in order to improve the performance of cyanobacteria and algae for production of bio-fuels and other products; 2) Synthetic biology for improvement of the performance of cyanobacteria and algae for optimized production of bio-fuels and other products; and 3) Optimized production of biofuel and other products in culture systems for algae[12].

AlgaePARC is the first research centre in the world that allows comparison of different outdoor photo bioreactor designs. The pilot facility comprises four large (24 m^2) and three small (2.4 m^2) photo bioreactors. The systems will run in parallel and will be compared on technical, economic and sustainability performance. The results will be used to build up knowledge required for commercial production of microalgae for bulk products. The facilities of AlgaePARC are financed by the ministry of Economic Affairs, Agriculture and Innovation (EL&I), the province of Gelderland and Wageningen University and Research centre[13].

Wetsus is the Dutch centre of excellence of sustainable water technology. One of the research themes is 'biofuels from microalgae' that started in 2008. The objective of

[10] The project is aimed at establishing the state of the art on research, technological development and demonstration activities regarding the exploitation of various algal and other suitable non-food aquatic biomasses for 2nd generation biofuels production.

[11] The complete inventory can be found at: http://www.aquafuels.eu/attachments/079_D%204.3%20Report%20on%20ongoing%20RD%20Projects%20FINAL.pdf.

[12] www.biosolarcells.nl/onderzoek

[13] www.algaeparc.nl

this research program is to realise breakthroughs leading to the successful commercialization of an algal production process for biofuels feedstock. Thirteen companies support the research theme[14].

Large Dutch companies involved in algal research are Unilever, DSM and AKZO-Nobel. A number of smaller companies are also involved in algae research often with a focus on the development of production systems (Figure 10).

Figure 10 An overview of small and young Dutch companies involved in algae research

Dutch company	Activity	Link
Photanol	GM-Cyanobacterial production	www.photanol.nl/
Ingrepro	Open pond algae production	www.ingrepro.nl/
Lgem	Closed tubular algae production	www.lgem.nl/
Algaelink	Photo bioreactor systems	www.algaelink.com/
Aquaphyto	Open pond production of algae	www.aquaphyto.com/
Maris projects	Development of open pond systems	www.maris-projects.nl/

[14] www.wetsus.nl

3. Characteristics of algae

As will be discussed later in this report, the taxonomy[15] of algae is an important aspect for environmental risk assessment. The taxonomy of algae is an on-going process as new species of algae are found and identified. Also genetic analysis show that some known species have to be re-classified.

In this chapter the available definitions and classifications of algae (3.1) and an overview of the life cycle of algae (3.2) is provided. As a very important aspect of the risk assessment is the pathogenicity and the toxin production of some algae, an overview of pathogenic and toxin producing algae is given in the last section of this chapter (3.3).

3.1 Taxonomy

3.1.1 Definition of algae

The “Tree of life” defines algae (sometimes called protists with chloroplasts) to be the photosynthetic organisms excepting plants[16]. Algae are photosynthetic like plants, and considered "simple" because their tissues are not organised into the many distinct organs found in higher plants. The largest and most complex marine forms are called seaweeds or macro-algae.

Though the prokaryotic cyanobacteria (commonly referred to as blue-green algae or sometimes as prokaryotic algae) were traditionally included as "algae", many modern sources regard this as outdated as they are now considered to be bacteria. The term algae is now restricted to eukaryotic organisms. All true algae therefore have a nucleus enclosed within a membrane and plastids bound by two, three or four membranes. The definition “algae” is more a traditional and practical naming and should not be considered as a group of organisms of common ancestry.

However in this report we will include cyanobacteria because the research on cyanobacteria is close to algae research with respect to aims and cultivation techniques. Cyanobacteria and microalgae together are defined as phytoplankton.

The estimated number of algal strains varies but it is safe to say that the group of algae is large. AlgaeBase[17] for instance contains 128,162 strains and intra-specific names in the database, while the algal collection of the U.S. National Herbarium[18] has 219,548 accessioned and inventoried specimens.

3.1.2 Classification of algae

Algae are mostly photosynthetic eukaryotes, found in all fresh-water and marine environments. The group of algae is extremely large and also extremely diverse; algae can be very different from another. For that reason defining the taxonomic position of these organisms is rather difficult.

[15] Taxonomy is the science of identifying and naming species, and arranging them into a classification

[16] http://tolweb.org/tree/

[17] http://www.algaebase.org/

[18] http://tolweb.org/tree/

The "Tree of life" distinguishes algae on a number of different characteristics. The most important characteristics are[19]:

- The combination of photosynthetic pigments that are present in the plastid;
- The presence of flagella (and if so how many, how do they insert in the cell and how do they beat);
- Is the cell surrounded by extracellular material? If so, what is that material - organic or inorganic, a continuous wall or a layer of scales);
- Are the cells motile or not?
- Do they occur singly, in colonies, filaments or exhibit differentiation that would allow them to satisfy the criterion of multi cellularity?

Recent molecular genetic studies confirmed that algae belong to genetically widely diverse groups of organisms often closer related to non-photosynthetic organisms than to more distant algal clades and this can be considered as the result of different and independent events of secondary endosymbiosis.

The most recent results on algal taxonomy are summarized in detail by the Tree of Life project[20], AlgaeBase[21] and NCBI[22]. All databases provide up to date taxonomic information concerning classification of algal strains that is continuously being updated and revised in light of newest results obtained by molecular genetic approaches such as DNA sequence comparisons (Ben Ali, De Baere et al. 2001).

In this way nine phyla of algae have been identified plus the *Cyanophyta* (cyanobacteria)[23]: see Figure 11 for an overview.

Figure 11 Phyla of algae with some characteristics.

Phylum	Endosymbiont	Organization	Major pigments
Chlorophyta Green algae	Cyanobacterium	single celled, colonial and multicellular, free-living	Chlorophyll b
Rhodophyta Red algae	Cyanobacterium	free-living and parasitic, single celled, and multicellular	Phycobilins
Glaucophyta	Cyanobacterium	flagellated and non-flagellated cells	Phycobilin
Chlorarachniophyta	Green algae	syncytial, free-living	Chlorophyll b
Euglenophyta	Green algae	single cells	Chlorophyll b
Dinophyta	Red algae	unicellular, colonial, syncytial; free-living, symbiotic and parasitic	chlorophylls a and c, some symbionts
Cryptophyta	Red algae	single cells, rarely forming colonies, some are endobiotic	Chlorophylls a and c, phycobilins
Haptophyta	Red algae	single cells	Chlorophylls a and c
Heterokontophyta	Red algae	single celled, colonial and multicellular, free-living and parasitic	Chlorophylls a and c

Classification of cyanobacteria[24] is presented in Figure 12.

[19] http://tolweb.org/tree

[20] http://tolweb.org/tree

[21] www.algaebase.org

[22] www.ncbi.nlm.nih.gov/Taxonomy/Browser

[23] http://www.eolss.net/Sample-Chapters/C17/E6-58-03-03.pdf

Figure 12 Classification of cyanobacteria

Cyanobacteria (blue-green algae)
Chroococcales
Gloeobacteria
Nostocales
Oscillatoriales
Pleurocapsales
Prochlorales (prochlorophytes)
Stigonematales

Source: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi

A ribosomal RNA database for all three domains of life (Bacteria, Archaea and Eukarya) is available at http://www.arb-silva.de/ (Pruesse, Quast et al. 2007).

3.2 Life-cycle of algae

Since algae are such a diverse group it is not surprising that they exhibit a wide range of reproductive strategies, from simple, asexual cell division to complex forms of sexual reproduction. Due to the diversity and to the lack of information on many algal strains only a few strains have been studied in detail.

In this report the life cycle of a number of algae are presented[25].

- Dinophyta: A dinoflagellate strains life cycle comprises four main phases: growth (mitotic and asexual), sexuality (meiotic), quiescence (a sexual or asexual immobile stage with a low metabolic rate also known as cyst) and senescence (population decline and death). Most dinoflagellates have haplontic life cycles, meaning that the vegetative stage is haploid. (http://tolweb.org/notes/?note_id=5512)
- *Chlorachniophyta:* The basic life cycle of the chlorarachniophytes comprises amoeboid, coccoid and flagellated cell stages. However, the patterns of the life cycle vary among strains, and some strains lack one or two of those stages. Asexual reproduction is carried out by either normal mitotic cell division or zoospore formation. Sexual reproduction has been reported from two strains: *Chlorarachnion reptans* and *Cryptochlora perforans*. In *C. reptans*, two different types of cells, amoeboid and coccoid, fuse to form a zygote (anisogamy), while in *C. perforans*, the fusion occurs between two amoeboid cells (isogamy) (http://tolweb.org/20515).
- *Cryptophyta*: Reproduction asexual, sexual doubtful (Peter Robert Bell 2000)
- *Euglenophyta*: Sexual reproduction is unknown in euglenids. Asexual reproduction occurs by mitosis followed by cytokinesis (http://tolweb.org/Euglenida/97461).
- *Glaucophyta*: Glaucophytes are reproduced by binary fission, zoospores or endospores. Sexual reproduction is unknown (http://www.shigen.nig.ac.jp/algae_tree/GlaucophytaE.html).

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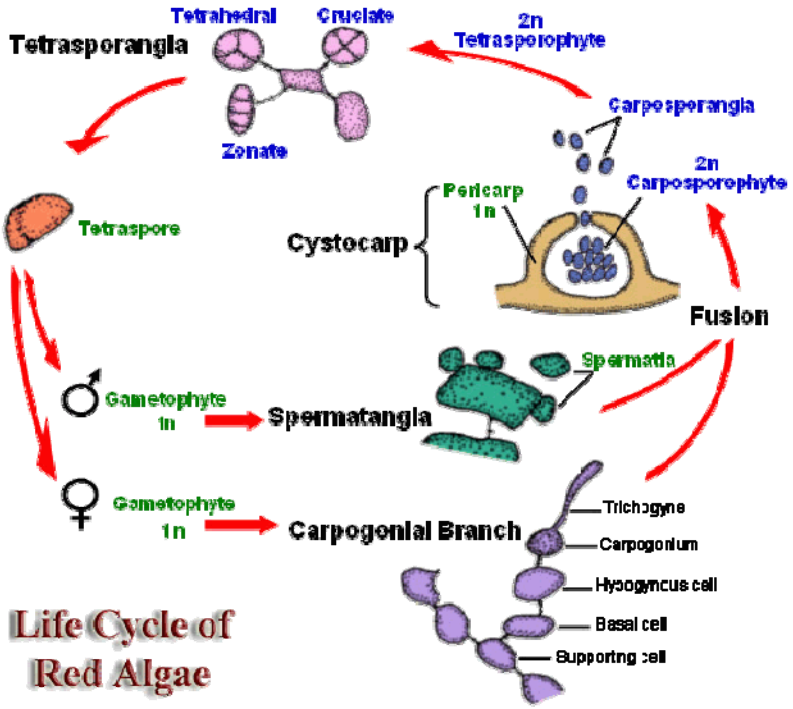
[24] An approved list of generic cyanobacterial names can be found at www.cyanodb.cz/valid_general

[25] Information on the life cycles of several algae groups can be found on http://academic.kellogg.edu/herbrandsonc/bio111/algae.htm#ygalgae.

http://www.rbgsyd.nsw.gov.au/science/Plant_Diversity_Research/marine_algae/life_cycle

- *Haptophyta*: Most haptophytes exhibit a haploid-diploid life cycle in which both stages are capable of independent asexual reproduction.
- *Rhodophyta:* The standard life cycle of rhodophytes includes three distinct stages, each a separate organism in the loop of reproduction. The three stages are the tetrasporophyte, the gametophyte and the cystophyte carpospores (see also Figure 13). Each of these forms of algae will produce offsprings which may take the form of another stage. The algae produce gametes which are 1n When these gametes are fertilised, they grow into a carposporophyte (carposporangia), a separate generation (2n), that is often housed within a cystocarp. These spores are eventually released, germinate and grow into another generation known as the tetrasporophyte (2n). Sometimes this tetrasporophyte generation looks identical to the male and female gametophytes that originally produced it. This is known as an isomorphic alternation of generations. But sometimes they look completely different and form a thin felt-like crust on the seabed. This is known as a heteromorphic alternation of generations. These tetrasporophytes then produce tetrasporangia (by meiosis resulting in four 1n spores) that are released, germinate and grow into more male and female gametophytes and thus the cycle is complete.

Figure 13 Life cycle of red algae



- Heterokontophyta (includes brown algae and diatoms):
  - Reproduction in brown algae can be either sexual or asexual and some form of alternation between free-living haploid and diploid generations (iso- or heteromorphic) is nearly universal. Asexual reproduction can occur in the diploid sporophyte phase of the life history by way of either mitotic divisions within plurilocular sporangia (2n) that result in the recapitulation of the sporophyte, or meiotic divisions in unilocular sporangia, resulting in haploid (1n) spores that germinate into the gametophyte phase. The gametophyte produces gametes through mitotic divisions in plurilocular gametangia. In

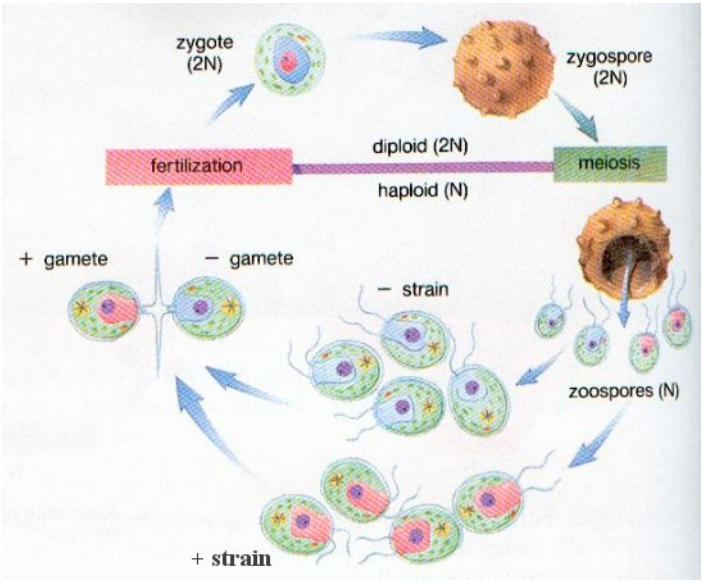
many cases the haploid gametes (spores) can settle and develop directly back into a gametophyte if they do not fuse with another gamete[26].

- In the 'centric' diatoms (a paraphyletic group of basal lineages), sexual reproduction is oogamous, i.e. fertilization occurs between small motile sperm and larger immobile eggs. Pennate diatoms, on the other hand, are usually isogamous, with similar large, non-flagellate, amoeboid gametes. In this case, there is often no differentiation into 'male' and 'female'. The vegetative cells of diatoms are diploid (2N) and so meiosis can take place, producing 1N gametes, which then fuse to form the zygote[27].

- Chlorophyta: In green algae there is in general an asexual phase where the cells are diploid, a sexual phase where the cells are haploid followed by fusion of the male and female gametes. Asexual reproduction is advantageous in that it permits efficient population increases, but less variation is possible.

  Vegetative cells of *C. reinhardtii* are haploid. Under stress conditions e.g. nitrogen starvation, haploid gametes develop. There are two mating types, identical in appearance and known as mt(+) and mt(-), which can fuse to form a diploid zygote.

Figure 14 The life cycle of *Chlamydomonas reinhhardtii*



Source: http://tolweb.org/notes/?note_id=52

The zygote is not flagellated, and it serves as a dormant form of the strains in the soil. In the light the zygote undergoes meiosis and releases four flagellated haploid cells that resume the vegetative life cycle.

- Cyanobacteria: The only means of reproduction in cyanobacteria is asexual. Filamentous forms reproduce by trichome fragmentation, or by formation of special hormogonia. Hormogonia are distinct reproductive segments of the

[26] http://tolweb.org/Phaeophytes/129402

[27] http://rbg-web2.rbge.org.uk/algae/auxospores/LifeCycle_vegetative.html

trichomes. They exhibit active gliding motion upon their liberation and gradually develop into new trichomes (Bartram 1999).

3.3 Pathogenic and toxin producing algae

3.3.1 Pathogenic algae

There are only a few species of algae that are known pathogens to humans or animals:

- Protothecosis is a disease caused by a strain of green algae called *Prototheca wickerhami*. It is a rare infection that usually only affects humans and other mammals living in tropical climates. The source of infection is often unknown but can be related to a penetrating injury in some cases. This strain of algae lacks chlorophyll. Recently a related *Prototheca cutis* was identified from a biopsy of a human chronic skin ulcer (Kazuo Satoh 2010). Prototheca infection is known to cause bovine mastitis in cattle.
- *Chaetoceros*, another alga, has spines which can physically clog and damage fish gills, leading to the death of cage-reared salmon and other strains. According to Tomas (1997) *Chaetoceros* is one of the largest, if not the largest genus of marine planktonic diatoms, with ca. 400 strains described. High concentrations of Chaetoceros spp. may clog the gills of farmed fish and the spiny *Chaetoceros setae* can penetrate the gill tissue. Fish mortality is therefore caused by induced hypoxia (mucus produced by the gill tissue) and hypercapnia (excessive amount of carbon dioxide in the blood).

The IOC-Unesco has created a Taxonomic Reference List of Harmful Micro Algae[28] with the following aims:

- to provide a catalogue of the world's harmful micro-algal strains;
- to promote stability in harmful micro-algal nomenclature;
- to act as a tool for higher taxonomic revisions and regional monographs;
- to provide a base link for other online databases that use harmful micro-algal nomenclature.

The list contains both species producing toxins and species that cause harm due to biomass, mucus, morphology (spines etc).

Harmful algae mentioned on the IOC-Unesco list, with exception of the toxin producing dinoflagellates and diatoms (described in the next section), are shown in Figure 15.

Figure 15 Harmful algae according to the IOC-Unesco list.

Haptophyta	**Heterokonthophyta**
Chrysochromulina leadbeateri	*Chattonella globosa*
Chrysochromulina polylepis	*Chattonella japonica*
Phaeocystis globosa	*Chattonella marina*
Phaeocystis pouchetii	*Chattonella subsalsa*
Prymnesium calathiferum	*Fibrocapsa japonica*
Prymnesium faveolatum	*Heterosigma akashiwo*
Prymnesium parvum	*Heterosigma carterae*
Prymnesium patelliferum	*Pseudochattonella farcimen*
Prymnesium zebrinum	*Pseudochattonella verruculosa*

[28] http://www.marinespecies.org/hab/

3.3.2 Toxin producing algae

Certain marine algae produce potent toxins that impact human health through consumption of contaminated shellfish and finfish and through water or aerosol exposure. Over the past three decades, the frequency and global distribution of toxic algal incidents appear to have increased, and human intoxications from novel algal sources have occurred (Van Dolah 2000). Also animals are affected, obviously mammals (sea lions, dolphins, whales, dogs swimming, cattle drinking) but also birds and fish.

Toxic algae can be filtered from the water by shellfish, such as clams, mussels, oysters, or scallops, which then accumulate the algal toxins to levels which can be lethal to consumers, including humans (Shumway 1990, Ahmed 1991). Typically, the shellfish are only marginally affected, even though a single clam can sometimes contain sufficient toxin to kill a human. Fish and shellfish can also be subject to sub-lethal effects, including increased susceptibility to disease and reduced growth.

Algal toxins can give rise to a number of different poisoning syndromes:

- NSP - neurotoxic shellfish poisoning;
- PSP - paralytic shellfish poisoning;
- ASP - amnesic shellfish poisoning;
- DSP - diarrhoeic shellfish poisoning;
- Ciguatera fish poisoning.

Strains from two algal groups, the dinoflagellates and diatoms are best known to produce toxins that impact humans but there are a few other strains also producing toxins.

In certain conditions estuarine, marine, or fresh water algae accumulate rapidly in the water column and results in discoloration of the surface water. These so called algal blooms are associated with the production of natural toxins, depletion of dissolved oxygen or other harmful effects, and are generally described as harmful algal blooms (HABs). The occurrence of HABs in some locations appear to be entirely natural (they are a seasonal occurrence resulting from coastal upwelling, a natural result of the movement of certain ocean currents) while in others they appear to be a result of increased nutrient loading from human activities (Dolah 2000). Certain dinoflagellates colour the water red when blooming and cause the toxic red tides. Algal blooms can also occur in fresh water lakes and water reservoirs and are becoming a growing concern. In the US for instance Congress initiated the scientific assessment of freshwater algal blooms[29].

The Department of Botany of the Smithsonian National Museum of Natural History has developed an Internet site with an overview of harmful *Dinoflagellates* and diatoms[30]. See Figure 16 for this overview.

Figure 16 Toxin producing *Dinoflagellates* and diatoms

Dinoflagellates			
Alexandrium	***Cochlodinium***	***Dinophysis***	***Gambierdiscus***
A. acatenella	*C. polykrikoides*	*D. acuminata*	*G. toxicus*
A. catanella		*D. acuta*	

[29] http://lakes.solarbee.com/system/files/Lopez,Jewett,dortch,walton,hudnellFreshwaterReport_final_2008.pdf

[30] http://botany.si.edu/references/dinoflag/index.htm

A. minutum		*D. caudata*	
A. monilatum	***Coolia***	*D. fortii*	***Gonyaulax***
A. ostenfeldii	*C. monotis*	*D. mitra*	*G. polygramma*
A. pseudogonyaulax		*D. norvegica*	
A. tamarense		*D. rotundata*	
A. tamiyavanichi		*D. sacculus*	
		D. tripos	
Gymnodinium	***Gyrodinium***	***Noctiluca***	***Pfiesteria***
G. breve	*G. galatheanum*	*N. scintillans*	*P. piscicida*
G. catenatum			
G. mikimotoi			
G. pulchellum	***Lingulodinium***	***Ostreopsis***	***Prorocentrum***
G. sanguineum	*L. polyedrum*	*O. heptagona*	*P. arenarium*
G. veneficum		*O. lenticularis*	*P. balticum*
		O. mascarenensis	*P. belizeanum*
		O. ovata	*P. concavum*
		O. siamensis	*P. faustiae*
			P. hoffmannianum
			P. lima
			P. maculosum
			P. mexicanum
			P. micans
			P. minimum
			P. ruetzlerianum
Diatoms			
Amphora coffeaeformis	*Nitzschia navis-varingica*	*Pseudo-nitzschia australis*	*Pseudo-nitzschia calliantha*
Pseudo-nitzschia cuspidata	*Pseudo-nitzschia delicatissima*	*Pseudo-nitzschia fraudulenta*	*Pseudo-nitzschia galaxiae*
Pseudo-nitzschia multiseries	*Pseudo-nitzschia multistriata*	*Pseudo-nitzschia pseudodelicatissima*	*Pseudo-nitzschia pungens*
Pseudo-nitzschia seriata	*Pseudo-nitzschia turgidula*		

Source: http://botany.si.edu/references/dinoflag/index.htm

Also certain strains of cyanobacteria produce toxins. Cyanobacteria reproduce explosively under certain conditions also resulting in so-called blooms, which can become harmful to other strains if the cyanobacteria involved produce toxins. These toxins can be neurotoxins, hepatotoxins, cytotoxins, and endotoxins, and can be toxic and dangerous to humans as well as other animals and marine life in general (Bartram 1999). See Figure 17 for an overview on toxin producing cyanobacteria.

Figure 17 Toxin producing cyanobacteria

Genus*	**Toxins produced**
Anabaena	Anatoxins, Microcystins, Saxitoxins
Anabaenopsis	Microcystins
Aphanizomenon	Saxitoxins, Cylindrospermopsins
Cylindrospermopsis	Cylindrospermopsins, Saxitoxins
Hapalosiphon	Microcystins
Lyngbya	Aplysiatoxins, Lyngbyatoxin a
Microcystis	Microcystins
Nodularia	Nodularin
Nostoc	Microcystins
Phormidium (Oscillatoria)	Anatoxin
Planktothrix (Oscillatoria)	Anatoxins, Aplysiatoxins, Microcystins, Saxitoxins
Schizothrix	Aplysiatoxins
Trichodesmium	yet to be identified
Umezakia	Cylindrospermopsin

* Not all strains of the particular genus produce toxins
Source: http://www-cyanosite.bio.purdue.edu/cyanotox/toxiccyanos.html

3.3.3 Industrial relevant algae that are pathogenic or produce toxins

Screening the list of industrial relevant algae for pathogenic or toxin producing algae resulted in Figure 18 in which potential pathogenic and toxin producing algae have been indicated. As shown only a few strains on the list of industrial interesting algae are pathogens or produce toxin. Some algae have been given the GRAS (generally regarded as safe) status by the FDA.

Figure 18 Industrial relevant algae and their safety aspects (potential pathogenic, toxin producing algae, GRAS) for those algae where information is available.

Organism	**Species**	**Safety aspect**	**Organism**	**Species**	**Safety aspect**
Cyanobacteria	*Arthrospira sp.*		**Heterokon-tophyta**	*Alaria esculenta*	
	*Phormidium sp.**	Toxin		*Undaria pinnatifida*	GRAS
	*Anabaena sp.***	Toxin		*Ascophyllum nodosum*	
	Synechococcus sp.			*Fucus sp*	
Chlorophyta	*Caulerpa sp.*			*Himanthalia elongate*	
	Ulva sp.			*Cystoseira sp.*	
	Cladophora sp.			*Halidrys siliquosa*	
	Codium sp.			*Sargassum muticum*	
	Ostreococcus sp			*Laminaria sp*	GRAS
	Tetraselmis sp			*Saccharina latissima*	
	Botryococcus braunii			*Saccorhiza polyschides*	
	Chlamydomonas reinhardtii			*Amphora coffeaeformis*	Toxin
	Haematococcus pluvialis			*Amphiprora hyalina*	
	Dunaliella sp.			*Chaetoceros muelleri*	
	Chlorococcum sp.			*Cyclotella cryptica*	
	Neochloris oleoabundans			*Cylindrotheca sp*	
	Scenedesmus			*Navicula sp.*	
	Desmodesmus sp			*Nitzschia dissipata*	
	Chlorella sp	GRAS		*Phaeodactylum tricornutum*	
	Parietochloris incisa			*Thalassiosira pseudonana*	
	*Prototheca sp****	Pathogen		*Odontella aurita*	
Rhodophyta	*Chondrus crispus*			*Skeletonema sp.*	
	Mastocarpus stellatus			*Monodus subterraneus*	
	Grateloupia turuturu			*Nannochloropsis sp.*	
	Palmaria palmate		**Haptophyta**	*Isochrysis sp.*	
	Solieria chordalis			*Pavlova sp3*	
	Porphyridium cruentum	GRAS	**Dinophyta**	*Crypthecodinium cohnii*	GRAS

* Phormidium, not all strains produce toxins; ** *Anabaena circinalis, Anabaena flos-aquae* produce toxin; *** *Prototheca wickerhami, Prototheca cutis* are human and animal pathogens.

4. Genetic modification of algae

As a result of increased research on eukaryotic algae and cyanobacteria a large amount of data, protocols and publications on the molecular biology of algae has become available. Due to the rapid evolving DNA-sequencing methods and DNA-data analysis software, sequencing a genome is now within the reach of every medium-sized research program. The table in Appendix Dgives an overview of genome projects on algae (situation 2011). Also transcriptome analyses in the form of expressed sequence tags (EST) projects have been performed and several strains have been genetically modified.

This chapter describes the state of the art on transgenic research on algae. First of all an overview is presented of the genetically transformed algae strain (4.1), followed by an overview of the DNA delivery methods (4.2) and of the targets of genetic modification of algae (4.3).

4.1 Genetically modified algal strains and their stability

Transformation of the cyanobacterium *Synechocystis* was already reported in 1970 (Shestakov 1970). Successful transformation of the green alga *Chlamydomonas reinhardtii* was reported in 1989 (Harris 2009). *C. reinhardtii* has become the model species in molecular biology of (eukaryotic) algae and is therefore the best described one (Harris 2009). Since then successful genetic transformation of approximately 30 algal species has been demonstrated (Hallmann 2007; Radakovits, Jinkerson et al. 2010). See Figure 19 for an overview.

Figure 19 Overview of genetically transformed algal species

Species	**Stability of transformation***	**Species**	**Stability of transformation***
Chlorophyta		***Heterokontophyta***	
Chlamydomonas reinhardtii	stable	*Laminaria japonica*	stable
Chlamydomonas reinhardtii	stable (chloroplast)	*Undaria pinnatifida*	stable
Volvox carteri	stable	*Phaeodactylum tricornutum*	stable
Dunaliella salina	stable	*Navicula saprophila (Fistulifera saprophila)*	stable
Dunaliella viridis	stable	*Cylindrotheca fusiformis*	stable
Haematococcus pluvialis	stable	*Cyclotella cryptic*	stable
Chlorella sorokiniana;	stable	*Thalassiosira weissflogii*	transient
Chlorella kessleri (Parachlorella kessleri)	stable	*Nannochloropsis sp.*	stable
Chlorella ellipsoidea	stable	**Dinoflagellates**	
Chlorella vulgaris	transient	*Amphidinium sp.*	stable
Ulva lactuca	transient	*Symbiodinium microadriaticum*	stable
Ostreococcus tauri	stable		
Rhodophyta		**Cyanobacteria**	

Cyanidioschyzon merolae	stable	*Spirulina platensis (Arthrospira platensis)*	stable
Porphyra yezoensis	stable / transient	*Anabaena sp*	stable
Porphyra miniata	transient	*Synechocystis sp.*	stable
Kappaphycus alvarezii	transient	*Synechococcus*	stable
Gracilaria changii	transient	*Nosctoc muscorum*	stable
Porphyridium sp	stable (chloroplast)		
Porphyridium sp	stable	**Euglenids**	
Gracilaria	stable	*Euglena gracilis*	stable (chloroplast)

*nuclear transformation unless otherwise mentioned.

4.2 Methods for DNA delivery

Several methods for DNA delivery have successfully been applied. These methods are micro-particle bombardment (or biolistic), cell agitation with micro- or macro-particles (e.g. glass beads), protoplast transformation with polyethylene glycol or protoplast or whole cell transformation by means of electroporation and finally *Agrobacterium* mediated transformation (Coll 2006). Cells from the late logarithmic growth phase are commonly used for transformation.

As Cyanobacteria are bacteria they can be transformed by established techniques, e.g. by means of electroporation, by conjugative transfer of vectors from *E. coli* (Wolk 1984), and by a natural DNA uptake system which is present in *Synechocystis* sp. PCC 6803 and in *Thermosynechococcus elongatus* (Iwai, Katoh et al. 2004).

In the transformation experiments of algae a number of selectable markers have been shown to be successful in obtaining genetically modified strains. The table in Appendix Egives an overview of selectable markers that have been successfully used in algae. Most selection systems for these algae have been tested in *Chlamydomonas reinhardtii* because of its prominent position in the eukaryotic algae molecular biology. The number of selection markers for cyanobacteria exceeds the amount of markers for eukaryotic algae.

The promoters used to drive gene expression in transgenic algae are either homologous promoters e.g. the Rubisco small subunit (RbcS2) or the ubiquitin (Ubi1) promoter or the heterologous promoters CaMV35S and SV40. CaMV35S, the cauliflower mosaic virus promoter, a typical promoter for strong expression in higher plants, works well in several algal strains while the SV40, the simian virus 40 promoter a polyomavirus promoter, has been shown to work in *H. pluvialis* and in *C. reinhardtii* (Coll 2006).

Nuclear transformation of algae generally results in random integration of transgenes. In *C. reinhardtii* and *C. merolae* and *Ostreococcus* homologous recombination has been achieved but the frequency is low (Radakovits, Jinkerson et al. 2010). Recently one alga, the oil producing algae *Nannochloropsis sp*., was shown to have a high frequency of homologous recombination after transformation and selection (Kilian, Benemann et al. 2011). In contrast chloroplast transformation often results in homologous recombination (Miri Lapidot 2002; Purton, León et al. 2007).

Contrary to the eukaryotic algae, homologous recombination is easy to achieve in cyanobacteria (Xiaonan Zang 2007). Moreover also autonomously replicating vectors can be used in the cyanobacteria *Synechococcus* and *Synechocystis* (Mermet-Bouvier, Cassier-Chauvat et al. 1993).

RNA silencing by either antisense or RNAi technology has also been applied to algae. Several examples of RNA silencing and RNAi technology in *C. reinhartii* have been reviewed by Schroda (Schroda 2006) while RNAi has also been applied to *Euglena*

gracilis and *Phaeodactylum tricornutum* and is predicted to become a valuable tool in algae genetics (Cerutti, Ma et al. 2011).

4.3 Targets of algal genetic modification

Genetic modification as a tool to improve algal performance is more and more considered a necessity to achieve new and economical viable productions systems (Barbosa 2010; Greenwell, Laurens et al. 2010; Michael Hannon 2010; Scott, Davey et al. 2010; Gressel 2008; Holger Schuhmann 2012).

We can distinguish between three types of targets for genetic modification of algae:

1. Improvement of photosynthetic efficiency

Biofuel production efficiency with algae is directly dependent on the solar photon capture and conversion efficiency of the system. However daylight intensity is most of the time above the maximum photosynthetic efficiency of algae and therefore growth is reduced, a phenomenon known as photo inhibition. Research in this area focuses on the light harvesting antenna complex (LHC) (Mussgnug, Thomas-Hall et al. 2007; Anastasios 2009).

2. Improve productivity of selected products

The rising market demand for pigments from natural sources has promoted large-scale cultivation of microalgae for synthesis of such compounds. Genes encoding enzymes that are directly involved in specific carotenoid syntheses have been investigated and further development of transformation techniques will permit considerable increase of carotenoid cellular contents, and accordingly contribute to increase the volumetric productivities of the associated processes (Ana Catarina Guedes 2011). One example of such a gene (a phytoene desaturase) has already been published (Steinbrenner and Sandmann 2006). Figure 20 gives an overview of carotenoids produced by selected microalgae.

Figure 20 Carotenoids produced by selected microalgae

Microalga source	**Active compound**
Dunaliella salina	B-carotene
Haematococcus pluvialis	Astaxanthin, cantaxanthin, lutein
Chlorella vulgaris	Cantaxanthin, astaxanthin
Coelastrella striolata var. multistriata	Canthaxanthin, astaxanthin, β-carotene
Scenedesmus almeriensis	Lutein, β-carotene

As mentioned earlier in this report research on lipid production has increased in the past decades due to interest in developing algal biofuels. Genetic modification is part of the strategy to increase lipid production with algae. Target genes are lipid biosynthetic genes, lipid storage genes and lipid degradation genes. Obviously, the first two categories have to be enhanced while the third category of genes should be reduced (Radakovits, Jinkerson et al. 2010; Scott, Davey et al. 2010).

The figure in Appendix Fgives an overview of the lipid biosynthesis pathway in algae.

Another interesting aspect is the modification of the lipid characteristics. This could increase the quality of the lipids with regards to suitability as diesel fuel feedstock but could also make the lipids suitable for other applications like industrial applications, food or feed (Radakovits, Jinkerson et al. 2010). Genes for this purpose will originate from the group of fatty acid modifying enzymes, such as desaturases and thioesterases which have been studied in genetically modified plants in detail for a long time already (Napier 2007).

3. New products

An emerging field in the biotechnology of algae is the introduction of genes or metabolic pathways in order to produce components of economic interest and that are not yet present in the wild type. Figure 21 gives an overview of new products that have been made by algae through genetic modification. Two major groups of new products can be distinguished: energy products (like ethanol, hydrogen and fatty acids) and recombinant proteins.

Figure 21 New products that have been made by algae through genetic modification

Product	**Algae used**	**Reference**
Hydrogen	*Chlamydomonas reinhardtii*	(Happe 2001)
Hepatitis B antigen protein (HBsAg)	*Dunaliella salina*	(SUN 2003)
Human growth hormone (HGH)	*Chlorella vulgaris* *Chlorella sorokiniana*	(Hawkins and Nakamura 1999)
Poly-3-hydroxybutyrate (PHB)	*C. reinhardtii*	(Chaogang, Zhangli et al. 2010)
Erythropoietin; Human fibronectin 10FN3 and 14FN3; Interferon β; Proinsulin; Human vascular endothelial growth factor (VEGF); High mobility group protein B1 (HMGB1)	*C. reinhardtii*	(Rasala, Muto et al. 2010)
Bovine lactoferricin (LFB)	*C. reinhardtii*	(Li and Tsai 2009)
Avian and human metallothionein type II; Antigenic peptide P57; Antigenic proteins VP19,24,26,28; Foot and mouth disease virus VP1 protein; Anti-glycoprotein D of herpes simplex virus; Anti-rabbit IgG; Human tumour necrosis factor; Bovine mammary-associated serum amyloid; Classical swine fever virus E2 viral protein; Human glutamic acid decarboxylase 65; Human erythroprotein; Anti-anthrax protective antigen 83 antibody; D2 fibronectin—binding domain	*C. reinhardtii*	(Griesbeck, Kirchmayr et al. 2012)
Flounder growth hormone (FGH)	*Synechocystis*	(Liu, Zhang et al. 2008)
Ethylene	*Synechocystis*	(Sakai, Ogawa et al. 1997)
Ethanol	*Synechococcus*	(Coleman 1999)
Fatty acid	*Synechocystis*	(Xinyao Liu 2011)
Isobutyraldehyde	*Synechococcus elongatus*	(Atsumi, Higashide et al. 2009)
Isoprene	*Synechocystis*	(Lindberg P 2010)
Poly-3-hydroxybutyrate (PHB)	*Phaeodactylum tricornutum*	(Franziska Hempel 2011)

There are no commercialized products from the figure above already available. However, research on the application of algal systems for the production of these products is increasing (Angermayr 2009; Beer, Boyd et al. 2009; Specht 2010; Griesbeck, Kirchmayr et al. 2012).

Research on the use of algae for CO_2 capture and wastewater treatment is also performed but since this is not a priority in GM-algae research this application will not be discussed in this report.

A review on recent research involving engineering cyanobacteria for the production of valuable compounds has been published by Ducat, Way et al. (2011).

5. Risks of GM-algae: results of desk research

This chapter starts with a short description of the Netherlands legislation concerning GMOs (5.1). In the other two sections of the chapter an overview is given of what is already known about the risks related to production systems of (GM-)algae (5.2) and the potential risks of GM-algae for human health and the environment (5.3).

5.1 Regulations on working with GMOs

Working with genetically modified organisms (GMOs) in the Netherlands is governed in the Netherlands legislation by the "Besluit genetisch gemodificeerde organismen Milieubeheer" and the "Regeling genetisch gemodificeerde organismen". These regulations implement the EC directives 2009/41/EC and 2001/18/EC that deal with contained use of GMOs and with deliberate release into the environment of GMOs respectively.

A risk assessment is the key element in both directives. Guidance notes to the EC directives, laid down in annexes to the directives, describe in detail the different aspects of such a risk assessment. Both in Directive 2001/18/EC and 2009/41/EC it is stated that the performance of an environmental risk assessment (ERA) is mandatory. In 2001/18/EC an ERA is defined as 'the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose'. Under 2001/18/EC 'human health' is taken into consideration only as far as incidental exposure is concerned; food and feed safety are taken into consideration in the EU regulation 1829/2003.

The EC directives on GMOs make a clear distinction between contained use and deliberate release into the environment:

- Contained use is defined as 'any activity in which organisms are genetically modified or in which such organisms are cultured, stored, transported, destroyed, disposed of or used in any other way and for which specific containment and other protective measures are used to limit their contact with the general public and the environment';
- Deliberate release is defined as 'any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment'.

5.2 Risks related to production systems of (GM-)algae

In this report three different production systems for large-scale production of algae were distinguished: natural locations, open ponds (raceway ponds) and closed systems (PBRs).

Natural locations clearly are deliberate release in into the environment since there are no effective protective measurements to prevent the algae from entering the surrounding environment.

Open ponds can be regarded as deliberate release. Since the ponds are not covered there is contact with the environment through open air which could be considered intentional introduction into the environment.

Closed systems could be considered contained when placed inside a building. Cultivation of a GMO in a closed system which is placed outside may be considered under the regulation of contained use when it meets the following criteria: ' "contained use" means any activity in which micro-organisms are genetically modified (...) and for

which specific containment measures are used to limit their contact with the general population and the environment' (Directive 2009/41/EC, Article 2c).

In the Netherlands, a safety level of MI-I may be applied to the use of micro-organisms in industrial settings. The safety level MI-I is based on the concept of Good Industrial Large Scale Practice (GILSP). This concept, already developed in the OECD 'Blue Book'[31], implies that, if a host organism has a long history of safe use in an industrial setting, the same industrial setting offers adequate containment for the use of a GMO derived from this host organism.

The rules of GILSP can be applied to the use of a GMO if:

- the host organism is non-pathogenic and has a long history of safe use under industrial conditions;
- the GMO is derived from this host organism using a 'safe' vector (if applicable) and a 'safe' insert, and the resulting GMO has a reduced fitness in the environment compared to the host organism.

The concept of GILSP implies, *inter alia*, that living organisms of a culture grown under GILSP may be released in the environment in as much as that is usual also for the host organism.

Until this moment there is still limited practice of algae production systems. In the Netherlands local municipalities that have granted environmental approval for growth facilities for non-modified algae but have done so according to different regulations. For example the algae production systems of AlgaePARC needed to be contained, while for the production systems of Ingepro no risk assessment was required.

The case study in Appendix G shows that the biosafety assessment on the non-GM algal production facility fully focuses on the containment of the facility itself. The biological safety is not taken into account. This is in contrast with the EC regulations on GMOs in which the properties of the GMO are considered as the most important factor in the risk assessment.

5.3 Overview of potential risks of GM-algae for human health and the environment

The EC has developed guidance notes for risk assessment on the use of GMOs. The guidance note (2000/608/EC) to Annex III to directive 90/219/EEC deals with risk assessment on contained use of genetically modified micro-organisms and the guidance note (2002/623/EC) to Annex II to directive 2001/18/EC deals with the risk assessment on deliberate release into the environment of genetically modified organisms. In this paragraph elements of the risk analysis as mentioned in these aforementioned guidance notes will be discussed using the available information for these elements found through the desk study.

5.3.1 Safety of the algae, the insert, vector and the GM-algae

With respect to contained use, the risk assessment is aimed at identification of harmful properties of the algae due to characteristics of the recipient organism, the insert, the vector and the resulting GM-algae with respect to human health and the environment.

As described in Chapter 2 there are only a few species of algae that are classified as pathogens in humans or animals. These algae belong to the Prototheca or Chaetoceros or are mentioned on the IOC-Unesco list of harmful algae. However quite a number of algal species, especially belonging to the dinoflagellates and the diatoms produce toxins that impact humans, animals and birds. In addition some cyanobacteria also

[31] Recombinant DNA safety considerations, 1986, OECD, Paris.

produce toxins that are harmful to humans and animals. Also, we showed that a few species on the list of industrially interesting algae are pathogens or produce toxin.

In the examples of GM-algae mentioned in this report the DNA inserted in the recipient algae has been characterised. Although it is unlikely that GM-algae intended for use in outdoor cultivation systems contain inserts that have not been characterised, a differentiation between donor organisms in terms of toxin producer, pathogens or non-toxin producer non-pathogen will influence the risk assessment when uncharacterised genes have been used to produce the GM-algae, as uncharacterised genes may be involved in toxin production or pathogenicity.

When looking at the targets of genetic modification of algae the following groups of genes used as inserts, can be distinguished:

- Genes involved in photosynthesis;
- Genes involved in carotenoid biosynthesis;
- Genes involved in lipid biosynthesis;
- Genes encoding (pharmaceutical) proteins;
- Regulatory genes such as transcription factors or other metabolic regulators.

In general the genetic modification of algae aimed at modifying either photosynthesis, carotenoid biosynthesis or lipid biosynthesis is not expected to generate harmful strains with respect to human health. None of the genes used encode for toxins or are suspected to lead to toxin production through enhanced metabolic steps or metabolic pathways, especially when they are expressed in “safe” algae hosts.

However, introducing genes in the host may have phenotypic effects and for that reason it is argued that these effects should be analysed. When expressing pharmaceutical proteins (e.g. antibodies) potential effects of these proteins on humans have to be addressed in the risk assessment.

In eukaryotic algae the donor DNA is integrated in the genomic or chloroplast DNA. Only *Chlamydomonas reinhardtii* has a history of stable genetic modifications and subsequent cultivation of the GM-strains. Stability of other GM-algae (which is mainly an issue in the production using these algae) still has to be confirmed especially under non-selective conditions since stability will most likely be gene and integration dependent. As cyanobacteria are bacteria, vector DNA can be integrated into the genome but also vectors, which can replicate in the cytoplasm, are used. The methodology of risk assessment used for GMOs can be applied to cyanobacteria without major modifications.

5.3.2 Transfer of genetic material to other organisms

An important aspect to be addressed in the ERA is the transfer of inserted genetic material to other organisms. Therefore horizontal gene transfer (HGT) - the transfer of genetic material from one organism to another which is a natural mechanism and has played an important role in evolution - is a point of concern.

In cyanobacteria where ~50% of extended gene families putatively have a history of HGT (either between cyanobacteria and other phyla, or within cyanobacteria, or both) HGT has played an important role in evolution (Zhaxybayeva, Gogarten et al. 2006; Monier 2009). In these bacteria HGT is a mechanism in real time adaptation and for that reason it is part of the risk assessment of GM-bacteria.

In eukaryotic algae HGT has been part of the evolutionary development, however in these organisms this is not a real time event and poses no additional risk in GMOs[32].

Vertical gene transfer uses reproduction as a means of gene transfer through generations and may be a risk with GM-algae when the species used has a sexual reproduction cycle and wild type partners are present in the environment.

The transfer of antibiotic resistance or herbicide resistance is an issue in the debate on the safety of GMOs. Several governments in the European Union have recommended the phasing out of GM-crops containing any antibiotic resistance markers (EFB 2001). Therefore, the use of GM-algae, without antibiotic resistance genes, for outdoor cultivation will almost certainly be easier accepted by the public. However, as discussed above, in most of the genetic modification protocols for algae, antibiotic resistance is being used as the selection criterion. Some alternative selection systems have been used in algae (the nitrate reductase selection system, uracil selection) but more research on alternatives for antibiotic selection of algae GMOs is necessary. Genetic deletion of the antibiotic selection gene after generation of a stable transgenic line has also been achieved for some algae transgenic systems, so technology to avoid antibiotic genes in GM-algae is under development (Mayfield pers. communication).

[32] HGT from GM-plants to prokaryotes has been studied and was shown to pose negligible risks (Keese 2008). Horizontal gene transfer from bacteria has also been studied in relation to mechanisms and barriers (Thomas and Nielsen 2005) and to risk assessment of GMOs (Heuer and Smalla 2007).

6. Risks of GM-algae: results of the workshop

The questions that were central to the workshop (see Section 1.2) were very useful in structuring the discussion, but could not be answered easily and in a straightforward way. However, the workshop provided an overview of issues that are relevant when taking into consideration the risks of GM-algae for human and the environment, how they could be assessed and more important, contained and prohibited. The workshop started with presentations: Prof Alison Smith on algae-bacteria consortia and Prof Jonathan Gressel on mitigating possible risks from transgenic algae. Short summaries of both presentations can be found in the first two sections of this chapter: 6.1 and 6.2. The results of the workshop discussion are summarised in the sections on taxonomy (6.3), competition of GM-algae and wild types (6.4), fitness (6.5), horizontal gene transfer (6.6), ecological baseline (6.7) and mitigation (6.8).

6.1 Algae-bacteria consortia

Professor Alison Smith (Plant Sciences, University of Cambridge, UK) argued that understanding algal community biology in more detail is a key both to effective algal production systems and to containment thereby avoiding contamination. Prof. Smith's group studies vitamin metabolism in plants and algae, and one of the group's research subjects deals with algal-bacterial consortia and vitamin auxotrophy that is widespread in microalgae. Research surveying 306 species of algae showed that more than 50% required cobalamin (vitamin B12), 22% thiamine (vitamin B1) and about 5% biotin (vitamin H or Coenzyme R). No phylogenetic relationship was found between requirers and non-requirers of these vitamins, so it can be concluded that this was due to environmental pressures. As concentration of free vitamin B12 in environment is extremely low and only bacteria can make B12 (the synthesis requires more than 20 enzymatic steps) and bacteria are frequently found associated with algae[33] it was concluded that these bacteria could very well be the B12 source for the algae.

This finding can be used in algal production systems: as vitamin B12 is an expensive micronutrient, bacteria (introduced in the system) can provide the vitamin B12 (or other essential nutrients). This requires further research into controllable production processes of algae. At the same time, algal-bacterial consortia may be more robust to invading species, since if the bacterial niche is occupied, contamination might be more difficult.

For risk assessment of algae the fact that they often live in symbiotic relation with other (prokaryotic) organisms (such as bacteria) is important. For that reason working with clean cultures should be checked by DNA-analysis of the potential present endo-symbiotic organisms. On the other hand, rather than genetically modifying the algae, it might be possible to modify the symbiotic bacteria instead either accidental or deliberately. Accidental genetic modification of symbiotic bacteria should be avoided however targeted genetic modification of symbiotic bacteria might help in facilitating the other aspects of industrial algal cultivation (for example by expressing lytic enzymes that might facilitate release of the product from the algal cells). This could be extended to devise ways of containment (if a spill occurred, lysis of the algae could be induced).

[33] Bacteria are even found in culture collections of algae. They are hard to remove, and antibiotic treatment often leads to death of algae too.

6.2 Mitigating possible risks from transgenic algae

Professor Jonathan Gressel started with presenting the advantages of marine microalgae: they do not compete with crops for land and fresh water, they consume industrial carbon dioxide, are fertilizer efficient and are highly productive with possibilities for a number of different products. However, in order to use algae for large scale production they have to be domesticated, just as all crops have been. Genetic modification is one of the main tools to do this.

Firstly, one has to agree on what is an acceptable baseline for risks of GM-algae. The risks of the transgenes differ, depending on the transgene. However, he argues that many domestication traits reduce fitness, so the effects are minor should there be an inadvertent leak to natural ecosystems. There are exceptions as some traits may increase fitness. Although the potential negative effects of domesticated transgenes might be negligible in the long term (as ecosystems recover and are changing/evolving all the time), the potential negative effects on native populations might transiently be very large.

What will happen with the large open pond production systems or even closed systems in case of natural disasters such as hurricanes, earthquakes and tsunami's, or human failure? Millions of litres water with algae will spill to natural ecosystems; what will be the effect on the ecosystem? While many domesticated transgenes may reduce risk through lower fitness (but which fitness level is low enough?), some transgenes may increase risks through increased competitiveness.

Professor Gressel's answer to this potential problem is the use of mitigator technology. In this case risks may be mitigated transgenically to prevent ability to reproduce in natural ecosystems. Mitigator technologies couple a primary (e.g. for high oil-content) transgene in tandem with mitigator genes that are positive or neutral to the algae but deleterious to its offspring. In this case genes are needed that are carriers of incompetence in volunteers and offspring, but not in algae (such as for instance dwarfing genes that are used as a mitigator in oilseed rape).

Professors Gressel mentions suppressed carbon capture as a mitigator for algae grown in high CO_2. Other possible mitigators for algae include: decreased-Rubisco, (anti) nitrate/nitrite reductases (for algae cultivated with urea), (anti) cilia/flagella, reduced PS2 antennae and reduced metabolite storage. These mitigators allow growth of transgenic algae in culture, but are devastating for transgenic algae in nature. However, he argues that one also should ask the question about the environmental risks from massive spills of non-transgenic algae. He proposes that wild type algae be mitigated by mutagenic gene deletion of nitrate reductase (chlorate resistance; prove deletion), Zinc finger or TALEN gene deletion. He finished his presentation by concluding that biotechnology can prevent or mitigate accidents, and regulators should ensure it is done where needed.

6.3 Identity and taxonomy of algae

Taxonomy was one of the first issues discussed during the workshop. It is very important: knowing the specific identify of the algae strains is essential in the communication with other researchers and for using the results of their research on the strain. Establishing the strain identity is especially crucial for gathering information for risk assessment research. Taxonomists can be considered as important service providers in risk assessment also because the 'history of safe use' of algae (which is an important aspect in risk assessment) is only valid in case the identity of the algae strain is known.

Knowing the identity of the strain enables literature search on its toxicity and pathogenicity assuming the authors have correctly identified the described strain. In risk assessment not only a distinction has to be made between pathogenic and toxicogenic algae or between prokaryotic and eukaryotic algae, but also between the different strains of algae and their properties.

Currently for many of the commonly used algae information on their pathogenicity and toxicity is available as a result of research on fishery, water storage and algal blooming in general.

A history of safe use for a certain algae implies that production has proven to be safe over a longer period of time (this also implies some forms of environmental exposure). As mentioned above, when collecting knowledge on the - safe - use it has to be certain that the historical data refers to the same species as the one you are intending to use: here identity and taxonomy come in because in case the identity is not known, no history of safe use can be build. It was recommended during the workshop to develop the concept of GILSP (Good Industrial Large Scale Practice, see Section 5.2) to be applied to algae strains.

6.4 Competition of GM-algae and wild types

The ERA that is mandatory in case of deliberate release into the environment of GMOs has to take into account the properties of the GMO and its insert as discussed in the previous paragraph. However, in addition there should also be strong emphasis on potentially adverse effects of the GMO on the environment. Ecological implication of the accidental release of algae from production systems and interactions (other than pathogenic or toxicogenic effects which have been described in this report) of the released algae with other (micro) organisms should be part of a risk assessment.

Research should be done on the competition between GM-algae and wild types, persistence of GM-algae in the environment and spreading of GM-algae. Relevant information to back up such studies is already available from studies with GM-bacteria, GM-yeast (Orvos, Lacy et al. 1990; Grossmann, Kiessling et al. 2010) and with GM-plants.

Also a distinction has to be made between survival of the inserted gene and the survival of the algae. Although many transformation experiments on algae have been described to result in stable transformants one should bear in mind that except for *Chlamydomonas reinhardtii*, algal molecular biology has a very short history. During the workshop the stability of the modification was discussed (and it was recognised that for industrial purposes only stable GM-algae would be used), but at the same time it was concluded that the stability of strain itself (vulnerable to spontaneous occurrence of mutations) was perhaps even more important than that of the GM-variant. Strain stability has been studied for heterotropic organisms, but testing the stability of strain is very time consuming.

6.5 Fitness

Fitness is defined here as the ability to exist/survive in the surrounding environment. A number of experts (including those that have been interviewed) argued that it applies for almost all GM-algae that their modification theoretically will reduce their fitness i.e. their survival in the wild. The risks of GM-algae are considered low with regard to long-term effects on the environment. In the short term GM-algae can have an impact on the environment. Some inserted genes will give the algae a competitive advantage. Example of such genes is antimicrobial peptides, avermectins for controlling sea lice and others, which you do not want in the ocean.

In this respect there was a reference to domesticated algae in industrial production processes. It was expected that the more domesticated the strain is and the more adapted to its production environment, the harder it is for the strain to exist in the wild environment. However, this presumption is not very well documented. Based on this aspect of the discussion, one of the conclusions was that with respect to deliberate release, it should be investigated what type of domestication effects could be desirable in algae in relation to survival outside the production system.

More in general, it was concluded that research was needed on the fitness of the GM-algae to exist and survive in native environments (it would be desirable that it would express its functions in such a way that its effect can be measured). Molecular

approaches - such as metagenomics - can be used to genetically characterise the (changes in the) environment after release of the GM-algae.

6.6 Horizontal gene transfer

Although there was no real consensus between the workshop participants about the frequency or timescale of HGT (in case of cyanobacteria) and there is hardly any knowledge available on the characteristics of the system dynamics of cyanobacteria evolution, there was agreement on this as a point of concern. One of the experts argued that a distinction has to be made between GM-cyanobacteria that contain only genes from other cyanobacteria, and those that contain genes from animals, bacteria or fungi. As HGT between cyanobacteria is already well documented (and because they are not very closely related to each other there is less HGT), GM-cyanobacteria that have genes only from other cyanobacteria are likely to exist in the environment already, hence the "risk" of unforeseen consequences from this type of GM-cyanobacteria can be considered lower.

It was concluded that HGT between cyanobacteria and other organisms should be addressed in the risk assessment of GM-cyanobacteria. The focus must be on the likelihood of potential adverse effects of HGT to microorganisms such as toxicity, pathogenicity, antibiotic resistance competitive advantage, utilization of novel substrates, in the environment. In case of expected adverse effects a close examination of the potential for gene transfer is warranted.

6.7 Ecological baseline

In this respect it was mentioned that it was very important to know the ecological baseline: one has to define and specify in sufficient details the ecological baseline for criteria such as spreading, survival and development, specific niches, etc. These experiments should be done in different environments, including that of the controlled growth conditions (extreme salt etc.) under which algae production systems operate. As described above algae exhibit a wide range of reproductive strategies, from simple, asexual cell division to complex forms of sexual reproduction. Due to the diversity only a few algae have been studied in detail. A careful analysis of the life cycle of the GM-algae used and of the ecological niche in which the GM-algae might be released should give an indication of the risk of sexual interaction and thereby gene transfer from the GM-algae to compatible wild type algae.

6.8 Mitigation

Experiments with deliberate release of GM-algae also focus on the conditions under which the algae will survive or will disappear. One technical solution for making algae (and other micro-organisms) unfit to survive outside the defined environment that has received attention is the mitigation of GM-algae.

Mitigation of algae makes them less fit for the other environment than the one they are cultivated in. It can be considered as a kind of 'biological containment'. Suggestions for mitigation of algae include the growing of salt-water algae in the country, or algae that need vitamin B12 for survival. In case the production system falls out and the algae wash away on the land and in the (sweet water) rivers, they will not survive (see also Section 6.2 with the suggestions made by Prof Gressel).

The mitigation technology makes the impact of the potential harmful effects of GM-algae on human and the environment less harsh or hostile. The mitigation approach is considered a suitable approach that needs validation.

It was recommended that an overview should be made of all industrial algae that are now being used in research and production and to investigate which mitigation is most feasible. Second, experiments with these mitigated algae should be done (inside and outside) and closely monitor the survival of the algae and the specific niches in which they survive, cq die.

7. Conclusions

Research on algae and genetic modification of algae is rapidly expanding due to great expectations with respect to the production of biofuel, bio-chemicals and other bio-products by algae. Algal technology is a sustainable technology that may contribute to the solution of societal problems like climate change and fossil fuel depletion and genetically modified algae will be part of that technology. Large investments from governments and industries stimulate the research on GM-algae. In the Netherlands there are several on-going research projects on algae and this research is bound to increase. Currently the GM activities with algae in the Netherlands are limited to contained use.

The technology is still rather immature, and a lot needs to be done before commercial production of these products by GM-algae will take place. Nevertheless, the potential risks involved in the mass cultivation of these GM-algae should be addressed as soon as possible in order to be prepared for the future.

This study provides an overview of the developments in research and production of genetically modified algae, of the potential risks of GM-algae, of the knowledge already available and knowledge that is required. The conclusions of the study are presented in this chapter.

7.1 General conclusions

Risk assessment of GM-algae and GM-cyanobacteria fits well in current regulations on GMOs.

The rules of GILSP can possibly be applied to outdoor cultivation of specific GM-algae and GM-cyanobacteria that have a history of safe use and are genetically modified with a safe vector and a safe insert.

In those cases where GILSP is not applicable an ERA for deliberate release instead of contained use is applicable. Key issues are strain identity, strain fitness, and vertical or horizontal gene transfer.

It is recommended to build theoretical cases to test whether the GILSP approach can be applied to large-scale cultivation of selected GM-algae or GM-cyanobacteria.

7.2 Conclusion on the regulations concerning GM-algae

Directives 90/219/EEC and 2001/18/EC cover all issues related to a risk assessment on GM-algae and GM-cyanobacteria.

Closed alga production systems could be considered contained when placed inside a building, in this case an ERA according to the directive 2009/41/EC is applicable.

Cultivation of a GM-algae and GM-cyanobacteria in a closed system, which is placed outside may be considered under the regulation of contained use when it meets the following criteria:

- the system has a long history of safe use under conditions known as GILSP (good industrial large scale practice) for cultivation of the particular host organism;
- the particular GMO is composed of a non-pathogenic host organism, a ‘safe’ vector and insert, and the resulting GMO has a lower fitness in the environment than the host organism, in agreement with the criteria for organisms acceptable for use under GILSP (MI-I, in Netherlands regulation).

Cultivation of GM-algae and GM-cyanobacteria not meeting the criteria of GILSP in outdoor closed systems and open pond systems will be subject to an environmental risk assessment (ERA) in accordance with directive 2001/18/EC.

7.3 Conclusions on risk assessment issues of GM-algae.

7.3.1 Strain identity

Strain identity is an important parameter for determining the potential risk of mass cultivation of industrial GM-algae.

The 'history of safe use' of algae (which is an important aspect in risk assessment) is only valid in case the identity of the algae strain is known.

A few algae species are known pathogens in humans or animals; they belong to the Prototheca or Chaetoceros or are mentioned on the IOC-Unesco list of harmful algae. A number of algal species, especially belonging to the dinoflagellates and the diatoms, produce toxins that impact humans, animals and birds. Also some cyanobacteria produce harmful toxins.

However only few species on the list of industrial interesting algae are pathogens or produce toxin so if the identity of the strain is established potential pathogenicity or toxicity can be evaluated.

7.3.2 Strain fitness

The fitness of the GM-strain in relation to wild types in the environment should be an important aspect of the ERA. Insight in the fitness of the GM-algae to exist and survive in native environment is needed.

Effects of introducing genes encoding enzymes not found naturally in the host may have phenotypic effects. These effects should be analysed and monitored over time.

Using a mitigation technology could be an approach to reduce the survival of the GM-algae in the environment.

7.3.3 Vertical or horizontal gene transfer

Horizontal gene transfer is a point of concern with cyanobacteria. The focus must be on the likelihood of potential adverse effects of HGT to microorganisms such as toxicity, pathogenicity, antibiotic resistance competitive advantage, utilization of novel substrates, in the environment.

A careful analysis of the life cycle of the GM-algae used and of the ecological niche in which the GM-algae might be released will provide indications of the risk of sexual interaction and thereby the risk of gene transfer from the GM-algae to compatible wild type algae.

Appendix A References

A.1 References used in the text

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A.2 Databases and other internet sites

http://www.plant.wageningen-ur.nl/projects/angel/ An EU project on gene flow from GMO-crop to wild forms.

http://www.aquafuels.eu/attachments/079_D%204.3%20Report%20on%20ongoing%20RD%20Projects%20FINAL.pdf An inventory on algae research project in the EU

http://www.algaebase.org/ A database on algae

http://tolweb.org/tree/ A database on all living organisms

http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/ A taxonomy browser

http://www.arb-silva.de/ A ribosomal RNA database

http://academic.kellogg.edu/herbrandsonc/bio111/algae.htm#ygalgae Some examples of the reproduction of algae

http://botany.si.edu/references/dinoflag/index.htm An overview of harmful Dinoflaggelates

http://www.marinespecies.org/hab/ A Taxonomic Reference List of Harmful Micro Algae

http://www.aquafuels.eu/attachments/079_D%201.2%20Taxonomy.pdf An overview of industrial relevant algae

Appendix B Industrially relevant algae and cyanobacteria for biofuel production

Cyanobacteria		**Eukaryotic microalgae**	
	Arthrospira sp.	*Chlorophyta*	*Ostreococcus sp*
	Phormidium sp		*Tetraselmis sp*
	Anabaena sp		*Botryococcus braunii*
	Synechococcus sp		*Chlamydomonas reinhardtii*
			Haematococcus pluvialis
Eukaryotic macroalgae			*Dunaliella sp.*
Chlorophyta	*Caulerpa sp*		*Chlorococcum sp.*
	Ulva sp		*Neochloris oleoabundans*
	Cladophora sp		*Scenedesmus*
	Codium sp		*Desmodesmus sp*
Rhodophyta	*Chondrus crispus*		*Chlorella sp*
	Mastocarpus stellatus		*Parietochloris incisa*
	Grateloupia turuturu		*Prototheca sp*
	Palmaria palmate	*Rhodophyta*	*Porphyridium cruentum*
	Solieria chordalis	*Heterokontophyta*	*Amphora sp.*
Heterokontophyta	*Alaria esculenta*		*Amphiprora hyalina*
	Undaria pinnatifida		*Chaetoceros muelleri*
	Ascophyllum nodosum		*Cyclotella cryptica*
	Fucus sp		*Cylindrotheca sp*
	Himanthalia elongate		*Navicula sp.*
	Cystoseira sp.		*Nitzschia dissipata*
	Halidrys siliquosa		*Phaeodactylum tricornutum*
	Sargassum muticum		*Thalassiosira pseudonana*
	Laminaria sp		*Odontella aurita*
	Saccharina latissima		*Skeletonema sp.*
	Saccorhiza polyschides		*Monodus subterraneus*
			Nannochloropsis sp.
		Haptophyta	*Isochrysis sp.*
			Pavlova sp3
		Dinophyta	*Crypthecodinium cohnii*

Appendix C European algae-related projects

Figure 22 Overview of EU-funded algae projects

Project title	Acronym	Coordinating country	Status
Adolescence for Renewable Energies in Transport	ADORE IT	THE NETHERLANDS	Execution
Biofuel from Algae Technologies	BIOFAT	SPAIN	Negotiation
Align Biofuel GHG Emission Calculations in Europe	BioGrace	THE NETHERLANDS	Execution
Sustainable Fuels from Marine Biomass Project	BIOMARA	IRELAND	Execution
Biofuels and Electric Propulsion Creating Sustainable Transport in Tourism Resorts	BIOSIRE	SPAIN	Execution
Biowaste and Algae Knowledge for the Production of 2nd Generation Biofuels	BIOWALK4BIOFUELS	ITALY	Execution
Energetic Algae	ENALGAE	UNITED KINGDOM	Execution
Utilization of Microalgae for Wastewater Treatment with Energy Purposes	ENERBIOALGAE	SPAIN	Execution
Real-Time Non-Invasive Characterization and Selection of Oil-Producing Microalgae at the Single-Cell Level	FUEL MAKING ALGAE	CZECH REPUBLIC	Execution
Marine Algae as Biomass for Biofuels	MABFUEL	IRELAND	Execution
Renewable Hydrogen from Sun and Water	SOLAR-H2	SWEDEN	Execution
Biotechnological Exploitation of Marine Products and By-Products	BIOTECMAR	FRANCE	Execution
European Multilevel Integrated Biorefinery Design for Sustainable Biomass Processing	EUROBIOREF	FRANCE	Execution
Genetic Improvement of Algae for Value Added Products	GIAVAP	ISRAEL	Execution
Control of Light Use Efficiency in Plants and Algae - From Light to Harvest	HARVEST	NETHERLANDS	Execution
Towards a Better Sunlight to Biomass Conversion Efficiency in Microalgae	SUNBIOPATH	BELGIUM	Execution
Sustainable Production of Biologically Active Molecules of Marine Based Origin	BAMMBO	IRELAND	Execution
Enabling European SMEs to Remediate Wastes, Reduce GHG Emissions and Produce Biofuels via Microalgae Cultivation	BIOALGAESORB	Norway	Execution

Source:
http://www.aquafuels.eu/attachments/079_D%204.3%20Report%20on%20ongoing%20RD%20Projects%20FINAL.pdf.

Figure 23 An overview of national or regional funded algae projects in Europe (situation 2010)

Project title	**Acronym**	**Coordinating country**	**Status**
Biofuel Production from Algae	SHAMASH	FRANCE	Started in 2006
Algohub-Roquette	ALGOHUB	FRANCE	Execution
VICI:Photosynthetic Cell Factories		The Netherlands	Execution
Lipid-based, high value products and renewable energy from microalgae	Sunlight	Belgium/ The Netherlands	Execution
Biofuels from Microalgae		The Netherlands	Execution
Advanced Water Treatment		The Netherlands	Execution
Recycling of Nutrients from Wastewater with Microalgae		The Netherlands	Execution
Optimal Design for a Tubular PBR		The Netherlands	Execution
Maximization of Photosynthetic Efficiency of Microalgae Outdoor Sunlight Conditions		Spain/The Netherlands	Execution
Algicoat		The Netherlands	Execution
Algae for Chemicals Production and Emission Abatement	Alchemis	Belgium/ The Netherlands	Execution
Natural Food Colorants from Algae		The Netherlands	Execution
Algobioloop		The Netherlands	Execution
Emerald Oils		The Netherlands	Execution
Refining of Microalgae		The Netherlands	Execution
AlgaeParc		The Netherlands	Execution
Microalgae, Starting Material for Bio oil	MAMBO	Italy	Execution
NutraMara, The Marine Functional Foods Research Initiative	NutraMara	Ireland	Execution
Seaweed Biorefinery		The Netherlands / Ireland	Execution
SUPERGEN Bioenergy - Phase II		UK/Ireland	Execution
Sustainable and Cost-Efficient Production of Marine Micro-Algae for Aquaculture Use	Halosydne	Belgium	Execution
Integrated New Concept(s) for the Production of SCO on an Economic Scale	Bi-Cycle	Belgium/ Germany	Execution
Combined Algal and Bacterial Waste Water Treatment for High Environmental Quality Effluents	ALBAQUA	Germany	Execution
Capture and Valorization of CO2 from Power Station Using Microalgae	AlgaPlanE	Spain	Execution
Utilization of Industrial Effluents (Gases and Liquids) for the Production of Microalgae	MicroAqua	Spain	Execution

Biomass			
Production of Biofuels from Microalgae with High Content of Starch and Lipids Using Flue Gas CO2 as a Source of Carbon		Czech Republic / Portugal/ Switzerland /Germany	Execution
Adhesion of Microalgae onto Solid Surfaces		Czech Republic	Execution
Competence Centre for Bio-refining and Bio-energy (CCBB)	CCBB	Ireland	Execution
Vlaams Algen Platform (Flemish Algae Platform)	VAP	Belgium	Execution

Source: http://www.aquafuels.eu/attachments/079_D%204.3%20Report%20on%20ongoing%20RD%20Projects%20FINAL.pdf.

Appendix D Overview of algae genome sequencing projects

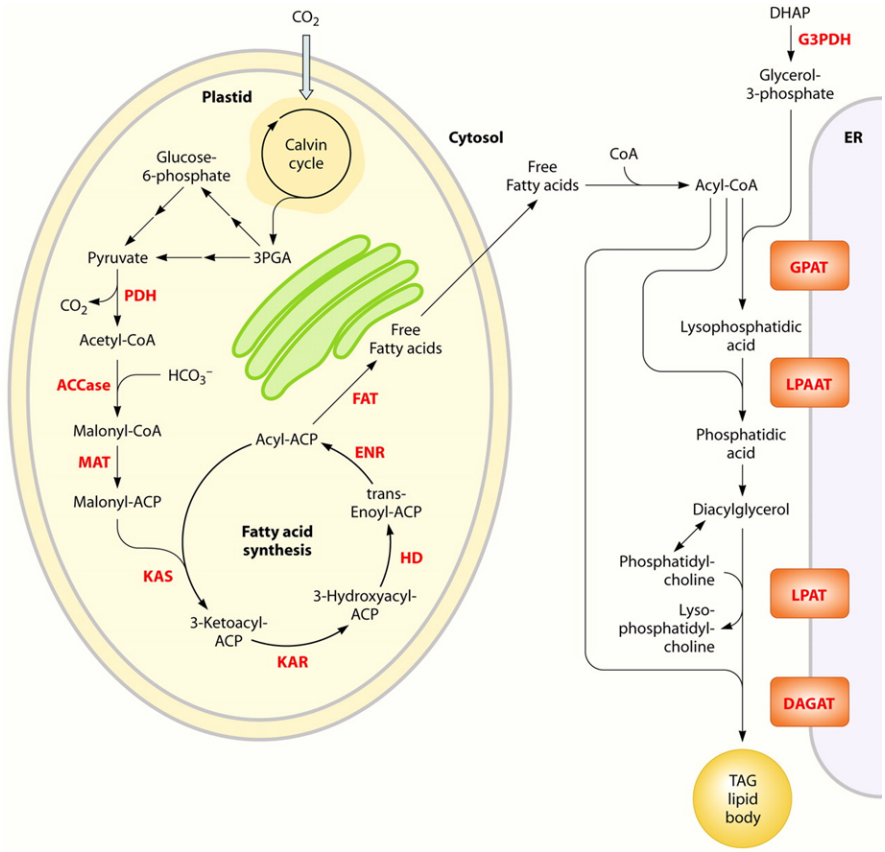
Organism	Genome Size (Mb)	Status	Data available at
Chlorophyta			
Chlamydomonas reinhardtii	100	Completed	http://genome.jgi-psf.org/Chlre4/Chlre4.home.html
Chlorella sp NC64A	46.2	Draft	http://genome.jgi-psf.org/cgi-bin/searchGM?db=ChlNC64A_1
Chlorella vulgaris C-169	49.1	Completed	http://genome.jgi-psf.org/Chlvu1/Chlvu1.info.html
Coccomyxa sp. C-169	49	Completed	http://www.jgi.doe.gov/genome-projects/ data not yet available
Dunaliella salina CCAP 19/18	130	In Progress	http://www.jgi.doe.gov/genome-projects/
Micromonas pusilla CCMP1545	15	Completed	http://genome.jgi-psf.org/MicpuN3/MicpuN3.home.html
Micromonas pusilla. RCC299	21.09	Completed	http://genome.jgi-psf.org/MicpuN3/MicpuN3.home.html
Ostreococcus lucimarinus CCE9901	13.25	Completed	http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
Ostreococcus tauri OTH95	12.5	Draft	http://genome.jgi-psf.org/Ostta4/Ostta4.info.html
Ostreococcus sp. RCC809	13.3	Completed	http://genome.jgi-psf.org/OstRCC809_2/OstRCC809_2.home.html
Volvox carteri f. nagariensis	120	In Progress	http://www.jgi.doe.gov/genome-projects/
Botryococcus braunii		In progress	http://www.jgi.doe.gov/sequencing/why/bbraunii.html
Asterochloris sp.	56	Draft	http://genome.jgi-psf.org/Astpho1/Astpho1.home.html
Rhodophyta			
Cyanidioschyzon merolae	16.5	Completed	http://www.biomedcentral.com/1741-7007/5/28/
Galdieria sulphuraria	12	In Progress	http://genomics.msu.edu/galdieria/
Heterokontophyta			
Thalassiosira pseudonana	34	Completed	http://genome.jgi-psf.org/thaps1/thaps1.home.html
Phaeodactylum tricornutum	30	Completed	http://genome.jgi-psf.org/Phatr2/Phatr2.info.html
Fraqilariopsis cylindrus	80.5	Completed	http://genome.jgi-psf.org/Fracy1/Fracy1.home.html
Haptophyta			
Emiliania huxleyi	220	Completed	http://genome.jgi-psf.org/Emihu1/Emihu1.home.html
Cryptophyta			
Guillardia theta	87.2	Draft	http://genome.jgi-psf.org/Guith1/Guith1.home.html
Chlorarachniophytes			
Bigelowiella natans	94.7	Draft	http://genome.jgi-psf.org/Bigna1/Bigna1.home.html
Cyanobacteria			
*Nostoc azolla**	5.5	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
Acaryochloris mmarina	8.4	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
*Synechococcus**	2.2-4.4	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
*Prochlorococcus marinus**	1.8	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
*Cyanothece**	6	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
Microcystis aeruginosa	5.8	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
Arthrospira platensis	6.8	Completed	http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

Appendix E Selection systems used in the transformation of algae and cyanobacteria

Gene	Gene product	Selection method
Chlamydomonas reinhardtii		
ARG7	Argininosuccinate lyase	Rescue of arg7 mutants to arginine prototrophy
NIA1 (=nit1)	Nitrate reductase	Rescue of nit1 mutants to growth on nitrate as the sole nitrogen source
NIC7	Putative quinolinate synthetase	Rescue of nic7 mutants to nicotinamide prototrophy
CRY1 (L153P)	Cytosolic ribosomal protein S14	Resistance to cryptopleurine and emetine
PPX1(V389 M)	Protoporphyrinogen oxidase	Resistance to S-23142
ALS (K257 T)	Acetolactate synthase	Resistance to sulfonylurea herbicides
ble	Zeomycin-binding protein	Resistance to zeomycin and phleomycin
aadA	Aminoglycoside adenyltransferase	Resistance to spectinomycin and streptomycin
aphVIII (aphH)	Aminoglycoside phosphotransferase	Resistance to paromomycin
aph7"	Aminoglycoside phosphotransferase	Resistance to hygromycin B
NIA1 (=NIT1)	Nitrate reductase	Sensitivity to chlorate
MAA7	Tryptophan synthase β-subunit	Sensitivity to 5-fluoroindole
Volvox carteri		
ble	Zeomycin-binding protein	Resistance to zeomycin and phleomycin
aphVIII (aphH)	Aminoglycoside phosphotransferase	Resistance to paromomycin
NIA1 (=nit1)	Nitrate reductase	Rescue of nit1 mutants to growth on nitrate as the sole nitrogen source
Haematococcus pluvialis		
pdsMod4.1	Phytoene desaturase	Resistance to norflurazon
Chlorella vulgaris		
hpt	Aminoglycoside phosphotransferase	Resistance to hygromycin B
Porphyridium		
AHAS	Acetohydroxyacid synthase	Resistance to sulfometuron methyl
Laminaria japonica		
hpt	Aminoglycoside phosphotransferase	Resistance to hygromycin B
Phaedactylum tricornutum		
ble	Zeomycin-binding protein	Resistance to zeomycin and phleomycin
nat, sat-1		Resistance to nourseothricin
CAT	Chloramphenicol acetyltransferase	Resistance to chloramphenicol
nptII	Neomycin phosphotransferase	Resistance to G418
Cylindrotheca fusiformis		
ble	Zeomycin-binding protein	Resistance to zeomycin and phleomycin
Navicula saprophila, Cyclotella cryptica		
nptII	Neomycin phosphotransferase	Resistance to G418
Amphidium sp., Symbiodinium microadriaticum		
hpt	Aminoglycoside phosphotransferase	Resistance to hygromycin B
Dunaliella viridis, Chlorella sorokiniana, Ulva lactuca		
NIA1 (=nit1)	Nitrate reductase	Rescue of nit1 mutants to growth on nitrate as the sole nitrogen source
Cyanidioschyzon merola		
ura	UMP-synthase	Selection for uracil phototrophy
Euglena gracilis		
aadA	Aminoglycoside adenyltransferase	Resistance to spectinomycin and streptomycin
Synechocystis, Synechococcus, Anabaena, Nostoc muscorum, Arthrospira platensis		
Resistance to Kanamycin, Streptomycin, Neomycin, Erythromycin, Chloramphenicol or Spectinomycin Glutamine auxotrophy (N. muscorum)		

Appendix F Lipid biosynthesis pathway in algae

The figure from Radakovits, Jinkerson et al. (2010) provides a simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes shown in red. Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER.



ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, gycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerols.

Appendix G Case study

G.1 Case study on the biosafety of the pilot algae (non GMO) production facility AlgaeParc

On the location Nergena near Bennekom Wageningen UR has built the research facility AlgaePARC (=Algae Production and Research Centre). For the purpose of a 5 years research program several small algae production systems have been build.

Goal of the research is:

- Comparison of different production systems under identical conditions;
- The development of new photo bioreactors;
- The design of productions systems on a larger scale.

In four medium-sized and three small systems algae will be cultivated. The feed will consist of water, CO2 and feed supplements. On a regular basis part of the cultures will be harvested. The harvest will be stored in tanks. After storage the water will be separated from the biomass by means of centrifugation. The concentrated biomass will be used for analysis in a laboratory. After analysis the biomass will be sterilized en discarded as waste.

On a yearly basis approx. 470 kg of biomass will be produced. The centrifugation procedure will harvest over 99% of the biomass after which the remaining water will be sterilized and discarded.

AlgaePARC will also research the possibility to reuse the water from the algae cultures.

The water that is used will be either freshwater or salt water.

Technical description

Outdoor systems.

- 25 m2 open pond made of plastic (4.8 m3).
- 25 m2 horizontal tubular photo bioreactor (0.4 m3). Transparant plastic tubes.
- 25 m2 vertical tubular photo bioreactor (1.1 m3). Transparant plastic tubes.
- 25 m2 plastic foil reactor (0.3 m3). In closed transparent plastic bag water flows along small lamella.
- 2.5 m2 horizontal tubular photo bioreactor (40 l).
- 2.5 m2 vertical tubular photo bioreactor (110 l).
- 2.5 m2 plastic foil reactor (30 l).
- The content of closed systems does not have any contact with the environment. The feeding water circulates to closed tanks with degassing equipment.
- Circulation pumps are inside a building or in in safety cupboards.
- Centrifuges for harvesting are inside a contained room (18 m2).
- Cooling equipment has been installed.
- CO2 supply comes from a rack with 16 CO2-cylinders.

- Containers for storage of seawater (15 m3), rainwater (6 m3), tap water (6 m3), a container for storage of harvest water and a container for wastewater (6 m3) are present.
- A harvest tank for the open pond (6 m3), a harvest tank for the horizontal tubular reactor (1 m3), a harvest tank for the vertical; tubular reactor (1 m3) and a harvest tank of the plastic foil reactor (0.5 m3) are present.
- A sterilisation tank (2 x 1 m3) in a safety tray, outside the concrete platform is also present.

Biological safety, discharges and soil protection

Process water: The wastewater obtained after the harvest process contains remainders of nutrients and remainder of biological material. This waste water is treated as described hereafter.

After centrifugation the harvest water is stored in the harvest tank. This water is pumped through an ultrafiltration (UF)-membrane which separates the algae from the water. The cleaned water is discharged. The retained biological material is stored in a sedimentation tank. After some time the sediment is tapped and sterilised (autoclave). The remaining water is pumped through the UF-membrane and discharged. The retained material is added to the sedimentation tank. This process is repeated with addition of new waste water. The whole process is validated on a regular basis.

The estimated water volume in this process is 670 m3/yr.

Protective measures:

- The closed cultivation systems are situated in a concrete bin with sufficient capacity to hold the content of the cultivation systems even during heavy rainfall. This concrete bin has a drain which automatically closes in case of leakage of a cultivating system.
- The open pond is secured against overflow by means of a level detector. At maximum water level a pump will automatically transport the overflow to a storage tank. If the level does not sink within 5-10 minutes the drain of the concrete bin will close automatically.
- Soil threatening activities will be organized in such a way that they comply with soil-risk level A (regulations G1).

Security against animals: A fence is installed surrounding the facility in order to prevent dogs, cats and rabbits from entering. The fence is buried 30 cm in the surface. A bird detector has been installed next to the open pond which produces ultrasonic sounds when necessary.

Appendix H Program of the workshop

Date: Monday March, 19, 2012

Venue: Forum-building (Gebouw/Building 102), Room 031, Droevendaalsesteeg 2, Wageningen, The Netherlands

Algae are a large and diverse group of simple, typically autotrophic (self-feeding) organisms, ranging from unicellular to multi-cellular forms, such as the giant kelps that grow to 65 meters in length. Research on algae for the production of biofuel, food, feed or chemicals is rapidly expanding. Since long time, algae have been used for producing food, food ingredients and for ingredients of cosmetics. However, their potential for bio-fuel production has accelerated the research and development of algae-based production systems. One of the developments in algae research is genetic modification often with the aim of increasing the productivity or the composition of the anticipated products.

Genetic modification of algae has already been researched outside Europe for some time but is currently also part of research projects in Europe and the Netherlands.

The Netherlands Commission on Genetic Modification (COGEM) wants to be prepared for future research on and use of genetically modified algae and has initiated a project with the aim of providing an inventory on the technical developments, the risks associated with GM-algae and the elements of a risk analysis necessary for a license application on the use of GM-algae. As part of the project Wageningen UR has made an inventory report (draft available for the workshop participants) and a workshop is organised. The project is a cooperation of Wageningen University and Research Centre (Wageningen UR) and Technopolis BV.

Aim of the workshop

The workshop's aim is to discuss and answer the following questions:

1. On which risk aspects of GM-algae is **sufficient** knowledge available in order to make the risk assessments that are necessary for applications made in the framework of environmental regulation of GM-algae in the Netherlands/Europe?

2a. On which risk aspects of GM-algae is **no or insufficient** knowledge available in order to make the environmental risk assessments that are necessary for judging applications in the framework of environmental regulation of GM-algae in the Netherlands/Europe?

2b. Given the above: to what new research questions in GM-algae and risk research (addressing the knowledge needs) does this lead?

Programme of the workshop

11:00 Welcome by the chair of the workshop Prof. Dr. Louise Vet (Director NIOO), presentation of the goal and the programme of the workshop

11:05 Tour de table with short introduction by each of the participants of the workshop

11:20 'Reasons for COGEM to initiate the project and the future implementation of results of the project', presentation by Leen van den Oever (Chair of the Steering Group of this project, COGEM, Director NIBI)

11:35 'Algae and genetic modification: state of the art on algae research with focus on genetic modification and risk assessment related to the cultivation of genetically modified algae' presentation of the Wageningen UR-report by author Jan Springer, Q&A and discussion

12:00 'Algal microbial consortia: possible ways in which microbial consortia could be used to mitigate any deleterious effects' Presentation by Prof. dr. Alison Smith (Plant Sciences, University of Cambridge), Q&A and discussion

12:30 'Mitigating the risks of transgenic algae', presentation by Prof. dr. Jonathan Gressel (Plant Science, Weizmann Institute of Science), Q&A and discussion

13:00 Lunch

14:00 Introduction to the discussion by a short summary of morning session, highlighting the risk-aspects of GM-algae for which sufficient data/information is already available (Q1), by Prof. dr. Rene Wijffels (WUR)

14:15 Discussion aimed at answering Q1

15:00 Introduction to the discussion aimed at answering Q2a and Q2b, by Dr. Hans Bergmans (GMO Office)

15:15 Discussion aimed at answering Q2a-b

16:15 Conclusions by the chair Prof. Dr. Louise Vet

16:30 Closing, drinks and bites etc.

Appendix I Participants of the workshop

Dr. Maria Barbosa	Wageningen UR
Dr. Hans Bergmans	Genetically Modified Organisms Office (Bureau GGO)
Dr. Willem Brandenburg	Wageningen UR
Dr. John Chapman	Unilever R&D
Dr. Piet van Dijck	DSM Nutritional Products
Prof. dr. Ellen van Donk	Netherlands Institute of Ecology (NIOO-KNAW)
Drs. Rene Draaisma	Unilever R&D
Prof. dr. Gerrit Eggink	Wageningen UR
Prof. dr. Dick van Elsas	University of Groningen
Dr. Christien Enzing	Technopolis Amsterdam
Bart Erkamp MSc	Commissie Genetische Modificatie (COGEM)
Dr. Tanja Fernandes	Netherlands Institute of Ecology (NIOO-KNAW)
Prof. dr. Jonathan Gressel	Weizmann Institute of Science, Israel
Prof. dr. Klaas Hellingwerf	University of Amsterdam
Drs. Lenny de Jaeger	Wageningen UR
Dr. Ingrid van der Meer	Wageningen UR
Drs. ir. Anke Nooijen	Technopolis Amsterdam
Drs. Leen van den Oever	Netherlands Institute for Biology (NIBI)
Dr. Leo van Overbeek	Wageningen UR
Prof. dr. Alison Smith	University of Cambridge, UK
Ing. Jan Springer	Wageningen UR
Prof. dr. Lucas Stal	Royal Netherlands Institute of Sea Research (NIOZ)
Dr. Anthony Verschoor	Ingrepro B.V.
Prof. dr. Louise Vet	Netherlands Institute of Ecology (NIOO-KNAW)
Dr. ir. Cecile van der Vlugt	Genetically Modified Organisms Office (Bureau GGO)
Prof. dr. Rene Wijffels	Wageningen UR
Dr. ir. Frank van der Wilk	Commissie Genetische Modificatie (COGEM)

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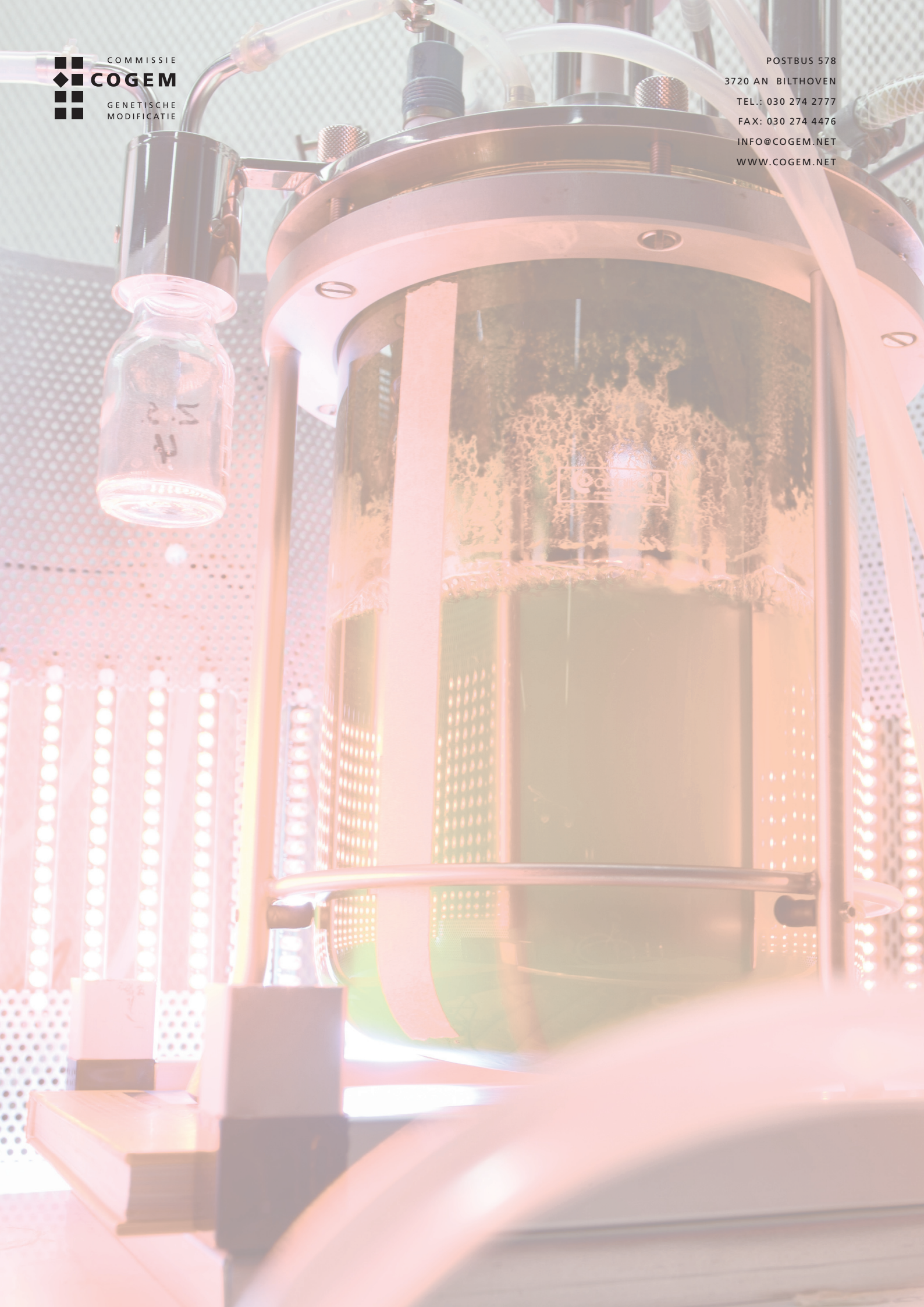

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