Chapter 4

The need and risks of using transgenic microalgae for the production of food, feed, chemicals and fuels

René H. Wijffels
Wageningen University, Bioprocess Engineering, Netherlands

This chapter provides a brief overview of the targets of algal genetic modification followed by a short description of the Netherlands legislation concerning genetically modified organisms, an overview of what is already known about the risks related to production systems of (GM-) algae, and the potential risks of GM-algae for human health and the environment.[1]

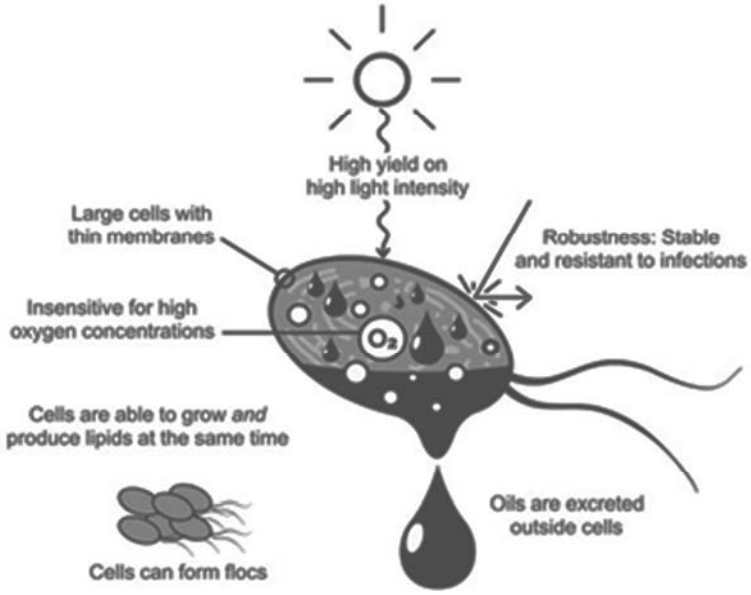
[1] This chapter is based on a study commissioned by the Netherlands Advisory Commission for Genetis Modification (COGEM), performed by Technopolis (2012).

Importance of transgenic microalgae

Microalgae may be used for the sustainable production of various commodities and products, such as feedstock and biofuels. Microalgae can be cultivated on seawater, using residual nutrients (carbon dioxide [CO_2], nitrogen [N], phosphorus [P]), and produce valuable co-products, e.g. lipids and proteins. Microalgae can be grown very efficiently. As an example, the total need for all transport fuels in Europe can be covered by microalgae cultivated on the surface area of Portugal. Four hundred million tons of protein would be produced as by-product, which is about 40 times the amount of soy protein imported into Europe. The EU FP7 programme[1] has funded a large number of research programmes aimed at further development of the use of (micro-) algae for various sustainable purposes.

There is a clear need for genetic improvement of the strains of microalgae that are currently being used, to create the "ideal micro-alga" (Figure 4.1). Features that could be improved include high biomass productivity, in particular of required molecules, such as proteins, saturated neutral lipids and unsaturated fatty acids, possibilities to grow under selective conditions, ease of harvesting and possibilities to use mild extraction conditions.

Figure 4.1. **Ideal microalga**



Genetic modification of algae

This section provides a short overview of the state of the art on transgenic research on algae, the algal strains that have been used as hosts for genetic modification and the DNA delivery methods. It then presents the targets of genetic modification of algae.

Genetically modified algal strains and their stability: DNA delivery methods

A first prerequisite transformation of the cyanobacterium *Synechocystis* was already reported in 1970 (Shestakov and Khyen, 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 et al., 2010). Table 4.1 provides an overview of genetically transformed algal species.

Table 4.1. **Overview of genetically transformed algal species**

Species	Stability of transformation[1]	Species	Stability of transformation[1]
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)*	
Porphyra yezoensis	Stable/transient	*Anabaena sp*	
Porphyra miniata	Transient	*Synechocystis sp.*	
Kappaphycus alvarezii	Transient	*Synechococcus*	
Gracilaria changii	Transient	*Nosctoc muscorum*	
Porphyridium sp	Stable (chloroplast)		
Porphyridium sp	Stable	**Euglenids**	
Gracilaria	Stable	*Euglena gracilis*	Stable (chloroplast)

Note: 1. Nuclear transformation unless indicated otherwise.

Methods used for DNA delivery into eukaryotic algae are micro-particle bombardment (or biolistic), cell agitation with micro- or macroparticles (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), i.e. methods that are also used for DNA delivery into plants.

Selectable traits used include resistance against antibiotics, chemical agents such as herbicides and genes that rescue mutations such as auxotrophies; marker genes allowing election of transformants include Gus and GFP genes (León-Bañares, 2004; Technopolis, 2012).

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 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 et al., 2011). In contrast, chloroplast transformation often results in homologous recombination (Lapidot, 2002; Purton, 2007).

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 (Wijffels and Barbosa, 2010; Greenwell et al., 2010; Hannon et al., 2010; Scott et al., 2010; Schuhmann et al., 2012).

Three types of targets can be distinguished for genetic modification of algae: improvement of photosynthetic efficiency, improvement of productivity of selected products and new products.

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 et al., 2007; Anastasios, 2009).

Improvement of 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 (Guedes et al., 2011). One example of such a gene (a phytoene desaturase) has already been published (Steinbrenner and Sandmann, 2006). Table 4.2 gives an overview of carotenoids produced by selected microalgae.

Table 4.2. **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

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 et al., 2010; Scott et al., 2010).

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 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 (Napier, 2007).

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 which are not yet present in the wild type. Table 4.3 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.

Table 4.3. **New products that have been made by algae through genetic modification**

Product	Algae used	Reference
Hydrogen	*Chlamydomonas reinhardtii*	Melis and Happe (2001)
Hepatitis B antigen protein (HBsAg)	*Dunaliella salina*	Sun et al. (2003)
Human growth hormone (HGH)	*Chlorella vulgaris* *Chlorella sorokiniana*	Hawkins and Nakamura, (1999)
Poly-3-hydroxybutyrate (PHB)	*C. reinhardtii*	Chaogang 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 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; Antianthrax protective antigen 83 antibody; D2 fibronectin-binding domain	*C. reinhardtii*	Griesbeck and Kirchmayr (2012)
Flounder growth hormone (FGH)	*Synechocystis*	Liu et al. (2008)
Ethylene	*Synechocystis*	Sakai et al. (1997)
Ethanol	*Synechocystis*	Deng and Coleman (1999)
Fatty acid	*Synechocystis*	Xinyao et al. (2011)
Isobutyraldehyde	*Synechococcus elongatus*	Athumi et al. (2009)
Isoprene	*Synechocystis*	Lindberg and Milis (2010)
Poly-3-hydroxybutyrate (PHB)	*Phaeodactylum tricornutum*	Hempel et al. (2011)

None of the products from Table 4.3 are commercially available at the time. However, research on the application of algal systems for the production of these products is increasing (Angermayr et al., 2009; Beer et al., 2009; Specht et al., 2010; Griesbeck and Kirchmayr, 2012).

Examples of other research are the use of algae for CO_2 capture and wastewater treatment.

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

European regulations for working with genetically modified organisms

Working with genetically modified organisms (GMOs) in the Netherlands is governed by national regulations that implement the EC Directives 2009/41/EC (European Union, 2009) and 2001/18/EC (European Union, 2001) 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 Directive 2001/18/EC and 2009/41/EC state that the performance of an environmental risk assessment (ERA) is mandatory. In Directive 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 Directive 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 (European Union, 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".

This chapter considers the environmental risks and the risk assessment of engineered algae in the context of these regulations.

Risks related to production systems of (GM-)algae

Three different production systems for large-scale production of algae can be distinguished: natural locations, open ponds (raceway ponds) and closed systems (photo bioreactors [PBRs]).

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

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

may also occur due to spillage which may occur due to, for example, large winds or floods, especially in very large-scale ponds.

Closed systems could be considered contained when placed inside a building. Cultivation of a GMO in a closed system which is placed in open air 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" (European Union, 2009: Article 2c).

In the Netherlands, a safety level of Good Industrial Large Scale Practice may be applied to the use of micro-organisms in industrial settings. This safety level is based on the concept of Good Industrial Large Scale Practice (GILSP) that was originally developed in the OECD "Blue Book" (OECD, 1986). It 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 inasmuch as that is usual also for the host organism.

Until now, there is still limited practice of algae production systems in Europe. In the Netherlands, local municipalities 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[2] needed to be contained, while for the production systems of Ingepro, no risk assessment was required.

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

The European Commission has developed guidance notes for risk assessment of the use of GMOs. Guidance Note 2000/608/EC (European Union, 2000) deals with risk assessment of contained use of genetically modified micro-organisms while Guidance Note 2002/623/EC (European Union, 2002) deals with the risk assessment of deliberate release into the environment of genetically modified organisms. This section discusses elements of the risk analysis methodology as developed in these guidance notes.

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

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

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 produce toxins that are harmful to humans and animals. For example, some genera that are industrially relevant contain species that are known to produce toxins, e.g. *Phormidium* (some strains do not produce toxins), *Anabaena circinalis*, *A. flos-aquae*, while *Synechococcus wickerhami* and *Prototheca cutis* are human or animal pathogens.

In the examples of GM-algae mentioned above, 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), the 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 vectors, which can replicate in the cytoplasm, are also used. The methodology of risk assessment used for GMOs can be applied to cyanobacteria without major modifications.

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 et al., 2006; Monier et al., 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.[3]

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 (European Federation of Biotechnology, 2001). Therefore, the use of GM-algae, without antibiotic resistance genes, for outdoor cultivation will almost certainly be more easily 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, personal communication).

Table 4.4. **Important data for environmental risk assessments of algae**

Data	Effect
Strain identity	Pathogenicity, toxin production
Growth conditions	Spreading into the environment
Algae production system	Open pond, closed tubes
Specific GMO properties	Enhanced or reduced growth, antibiotic resistance
Stability of the GMO	Horizontal gene transfer
Harvesting method	Chance of escape

Notes

1. http://ec.europa.eu/research/fp7/index_en.cfm.
2. www.algaeparc.com.
3. 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).

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