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Abstract

Considering the current rapid rate of fossil fuel depletion, many countries are heavily investing in biofuel generation from algae and including the use of genetic modification (GM) research to enhance their baseline biofuel qualities. The latter has become especially important as there is still no robust, commercially-viable strain available from the last decade of non-GM algae research. Hence, GM algal strains are being investigated to make algal biofuel commercially competitive to fossil fuels. However, the commercial mass cultivation of GM algae in open ponds with the potential impact of escape into the environment is an important concern. In addition, concerns exist over the potential that GM algae can form new GMO combinations via sexual reproduction with their wild-type counterparts or via horizontal gene transfer to cyanobacteria. Cyanobacteria have been more popular candidates for genetic transformation than green algae due to their greater receptiveness to the incorporation of foreign genes, though however this also means that they are more amenable to horizontal gene transfer than green algae. The genetic engineering of green algae is still at a primitive stage and the stability of the transgene in the modified organism genome is still a source of uncertainty, which makes them currently unsuitable for immediate commercial release. However, green algae are haploid in nature and have a tendency to reproduce asexually, both attributes helping to minimise the potential for transgene escape. Moreover, the current transgenes being considered for GM green algae are unlikely to confer a selective advantage in nature and may pose minimum ecological risk. For example, GM algae are generally less fit than their wild-type counterparts due to their special nutrient requirements and excess transgene-load. In addition, countermeasures can be incorporated into the design of GM algae in order to reduce their survival potential should they escape into the natural environment. The present review addresses the available information related

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to the mass production of GM algae with the focus restricted to green algae (chlorophytes) and blue green algae (cyanophytes), which are the most popular candidates currently being researched for biofuel production.

Keywords: algae breeding, biofuel, biosafety, GM algae, risk, transgene containment.

Riassunto

Alla luce dell'attuale rapido esaurimento dei combustibili fossili, al fine di migliorarne la qualità, molti paesi stanno investendo fortemente nella produzione di biocarburanti da alghe, comprese quelle geneticamente modificate (GM). Queste ultime sono particolarmente importanti in quanto negli ultimi dieci anni di ricerca con le alghe non-GM non è stato trovato alcun ceppo valido da un punto di vista commerciale. Ceppi algali GM sono quindi in fase di studio per ottenere biocarburanti competitivi ai combustibili fossili. Tuttavia, c'è una forte preoccupazione per il potenziale impatto dovuto ad un rilascio accidentale nell'ambiente durante la loro coltivazione commerciale di massa in vasche aperte. Inoltre, si aggiungono le preoccupazioni legate alla potenzialità delle alghe geneticamente modificate a formare nuove combinazioni OGM attraverso la riproduzione sessuale con le controparti wild-type o tramite il trasferimento genico orizzontale con i cianobatteri. I cianobatteri, per la loro maggiore recettività ad incorporare geni estranei, sarebbero in realtà candidati migliori delle alghe verdi per la trasformazione genetica, questo però significa anche una loro maggiore suscettibilità al trasferimento genico orizzontale. L'ingegneria genetica delle alghe verdi è ancora in una fase primitiva e la stabilità del transgene nel genoma dell'organismo modificato resta ancora fonte di incertezza, quindi allo stato attuale sono inadatte per un immediato rilascio commerciale. Comunque, il rischio di rilascio accidentale del transgene è minimizzato dal fatto che le alghe verdi sono in natura aploidi e hanno la tendenza a riprodursi asessualmente. Inoltre, i transgeni attualmente presi in considerazione per le alghe verdi GM difficilmente conferiscono un vantaggio selettivo, rappresentando un rischio ecologico minimo. Ad esempio, le alghe GM sono in genere meno idonee rispetto alle controparti wild-type per le loro particolari esigenze nutrizionali e per il carico eccessivo di transgene. Inoltre, nella realizzazione di alghe GM si possono introdurre contromisure in modo

da ridurre il potenziale di sopravvivenza in caso di fuoriuscita nell'ambiente naturale. Il presente lavoro riguarda le informazioni disponibili relative alla coltivazione massiccia di alghe GM, con una attenzione particolare per le alghe verdi (Chlorophytes) e le alghe verdi-azzurre (Cyanophytes), che sono attualmente le più utilizzate nella ricerca per la produzione di biocarburanti.

1. INTRODUCTION

Microalgae are currently the most promising sources of clean and renewable energy (Chisti, 2008). Microalgae are particularly attractive as biofuel producers as they possess short life cycles, can perform photosynthesis, and can utilise saline or wastewater for growth, as well as non-arable land. Moreover, valuable by-products such as animal feed and chemical precursors can be obtained from microalgae-based biofuel generation (Larkum *et al.*, 2012). The main reasons therefore that algae are being explored with respect to biofuel production are that they do not compete with food crops for agricultural land use and that they have the potential to be used in the current energy and transportation systems without altering the existing combustion engine system. This alternative source of energy is under investigation with a focus on economic feasibility and environmental safety considering the challenges associated with coal and petroleum.

Researchers have been studying algal fuels extensively since the 1970s, but no commercially-viable strain has yet been isolated (Vuttipongchaikij, 2012). In the past, some programmes had to be shut down due to the higher cost of algal biofuels as compared to fossil fuels. The use of recombinant DNA techniques offer the greatest range of options to improve upon the performance of wild strains. Therefore, the transgenic approach has been investigated as a potential method of increasing the productivity of algae and to compete with relatively cheaper fossil fuels. Cyanobacteria and green algae are amongst the most common algal groups being explored for biofuel production. However, public perception regarding GM algae is relatively untested. In addition, many countries are facing a deadlock over the approval of field trials of GM crops due to the indecisiveness of the GMO regulatory authorities (Kumar *et al.*, 2014).

The main concern with GM algae appears to be with regard to the potential escape of either viable GM algae or the relevant transgene(s) into natural ecosystems, the latter by sexual reproduction or horizontal gene transfer (HGT). HGT is the transfer of genetic material between organisms in a manner other than traditional reproduction and plays an important role in evolution. It is distinguished from the process of vertical gene transfer, which is the hereditary transfer of genetic material from parents to offspring during reproduction. HGT is particularly common in cyanobacteria, which are more

popular candidates than green algae for genetic manipulation. This is due to some cyanobacteria being easier to transform (Robertson *et al.*, 2011) than other species. HGT has been observed in members of the same species of cyanobacteria, between different species in the phylum, and even between cyanobacteria and eukaryotic algae (Waller *et al.*, 2006). For some eukaryotes, functionally significant HGT from bacteria has occurred and is due to the latter's greater metabolic diversity (Keeling, 2009). HGT in green algal species and in eukaryotic species has not been well studied (Ochman, 2000). However, HGT of the nuclear gene *psbO* from *Vaucheria litorea* (yellow green alga) to the photosynthetic sea slug *Elysia chlorotica* has been recorded; the organelles survived inside the mollusc's digestive system to carry out photosynthesis for months in the absence of algal nucleocytoplasm (Rumpho *et al.*, 2008). However, it is not clear yet whether the role of the sequestered chloroplasts is to provide camouflage or solar-based energy in the sea slugs (Christa *et al.*, 2014). As green algae are haploid and primarily reproduce asexually, the risk of sexual reproduction-based transgene transfer to wild-type cells is minimal with this group of algae.

GM algae-based biofuel projects are underway in many countries and are being supported by their respective governments and/or private agencies. A key aspect of making algal biofuel commercially viable is the requisite large-scale cultivation in open ponds, near oceans and in marine water, as opposed to sophisticated photobioreactors. Therefore, the introduction of GM algae into the environment and the associated biosafety issues should be addressed carefully and meticulously, especially the potential of unintended gene transfer of transgenic traits to compatible wild-type cells or others (Wolt *et al.*, 2010; Snow *et al.*, 2012). Therefore, GMO regulatory agencies should provide guidelines to the laboratories that are currently involved in the genetic manipulation of algal systems for enhanced biofuel values.

2. STATUS OF GM ALGAE

Several research groups have reported the genetic modification of green and blue green algae as model species. However, this review is limited to those which have shown promise as commercially-viable candidates for biofuel-related applications.

2.1. Green algae

The green algae *Chlamydomonas reinhardtii* has been genetically modified to express several important traits of biofuels (Ahmad et al., 2014), but low biomass production rates and lipid content in most of the model strains have prevented them from becoming industrially-relevant. Major green algal species, such as *Chlorella*, *Parachlorella*, *Nannochloropsis*, *Scenedesmus*, *Botryococcus*, and *Neochloris* have been identified as being rich in lipid content and thus as potential candidates for biofuel production. To date, genetic modification has largely been limited to formative studies transferring reporter genes and/or selectable marker genes, only two species, *Chlorella* and *Dunaliella*, transformed with biofuel-related traits (see Table 1).

Table 1. Important green algal species which have the potential for biofuel production, and the status of their genetic modification with biofuel-related traits.

Algal Species	Genetic transformation with biofuel related traits	Reference
<i>Chlorella</i>	Hydrogenase (<i>HydA</i>)	Chien et al., 2012
<i>Dunaliella</i>	Xylanase, α -galactosidase, phytase, phosphate anhydrolase, and β -mannanase	Georgianna et al., 2013
<i>Nannochloropsis</i>	NIL	-
<i>Scenedesmus</i>	NIL	-
<i>Botryococcus</i>	NIL	-
<i>Neochloris</i>	NIL	-
<i>Parachlorella</i>	NIL	-

2.2. Blue-green algae

Blue-green algae (cyanobacteria) accumulate relatively low lipid content as compared to the green algae, so they are not important candidates for biofuel production. However, despite this, cyanobacteria may be engineered to produce other important biofuel-related molecules due to their ability

to grow in extreme environments and the relative ease of their genetic transformation. In cyanobacteria, most genetic engineering has been carried out in model organisms, *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7492, *Synechococcus* sp. PCC 7002 and *Anabaena* sp. PCC 7120. However, the ideal production host remains difficult to predict (Savakis & Hellingwerf, 2015). Cyanobacteria have also been engineered to produce a range of different biofuel-related molecules (see Table 2).

Table 2. Blue-green algae genetically modified with biofuel related traits.

Algal Species*	Genetic transformation with biofuel-related traits	Reference
<i>Synechocystis</i> sp. PCC6803	Acyl-acyl carrier protein reductase and aldehyde-deformylating oxygenase (alkanes)	Wang <i>et al.</i> , 2013
	Acyl-acyl carrier protein thioesterase gene	Liu <i>et al.</i> , 2011
	Kivd and adhA from <i>Lactococcus lactis</i> (for isobutanol)	Varman <i>et al.</i> , 2013
<i>Synechococcus elongatus</i> PCC 7942	Knockout of the FFA-recycling acyl-ACP synthetase and expression of a thioesterase (fatty acids)	Ruffing & Jones, 2012
	Acyl-ACP thioesterase and acetyl-CoA carboxylase (fatty acids)	Ruffing, 2013
	2-methyl-1-butanol (alcohol)	Shen & Liao, 2012
	Acetyl-CoA acetyl transferase (encoded by <i>thl</i>), acetoacetyl-CoA transferase (encoded by <i>atoAD</i>), acetoacetate decarboxylase (encoded by <i>adc</i>) and secondary alcohol dehydrogenase (encoded by <i>adh</i>) (for isopropanol)	Kusakabe <i>et al.</i> , 2013
	Acyl-ACP reductase (<i>Aar</i>) overexpression (fatty aldehydes)	Kaiser <i>et al.</i> , 2013

* Model cyanobacterial strains. The ideal cyanophyte host for biofuel production has yet to be identified (Savakis & Hellingwerf, 2015).

Cyanobacteria fix CO₂ more efficiently than plants, and can be engineered to produce carbon feedstocks useful for biofuel. However, extension of this technology to commercial levels is disappointing due to the current low yields (Oliver *et al.*, 2014).

3. GENETIC INSTABILITY IN GM ALGAE

Generally, GM algae should show a high degree of stability (Savakis & Hellingwerf, 2015). However, achieving transgene stability in GM algae is an enormous challenge, as shown in studies of green and blue-green algal model systems.

3.1. Green algae

The storage of *Chlamydomonas* cultures in solid medium generally results in the loss of interested traits or spontaneous mutations (Hannon *et al.*, 2010; Ahmad *et al.*, 2014). This may be attributed to the inherent genetic instability and high mutation rate of naturally-occurring green algal cells. It has been estimated that there are >24,000 changes (including single nucleotide variations and insertions/deletions) between two common laboratory strains of green algae (Dutcher *et al.*, 2012). This high rate of mutation is largely due to the haploid genome in *Chlamydomonas* (Siaut *et al.*, 2011). It is a major obstacle for generating useful transgenic algal strains due to rapid gene silencing (Cerutti *et al.*, 1997; Fuhrmann *et al.*, 1999; Neupert *et al.*, 2009). In the unicellular green alga *C. reinhardtii*, epigenetic silencing of transgenes occurs at both the transcriptional and post-transcriptional levels. In the case of single-copy transgenes, transcriptional silencing takes place without detectable cytosine methylation of the introduced DNA (Cerutti *et al.*, 1997; Wu-Scharf *et al.*, 2000; Jeong *et al.*, 2002). The introduced genes are often unstably expressed, leading to the loss or reduced manifestation of newly-acquired traits. *Chlamydomonas* cultures are therefore most commonly maintained as vegetative cells on agar-containing medium for short periods of time. To maintain their quality over the long-term, *Chlamydomonas* cultures can be cryopreserved under liquid nitrogen (González-Ballester *et al.*, 2005; González-Ballester *et al.*, 2011).

3.2. Blue-green algae

Cyanobacteria are believed to be the most primitive organisms on Earth and were one of the first organisms to be genetic transformed - *Synechocystis*

was successfully transformed in the early 1970s (Shestakov, 1970). Banack *et al.* (2012) reported that cyanobacteria naturally produce N-(2-aminoethyl) glycine (AEG), a small molecule, which may play an important role in gene silencing. After the successful creation of the GM *Synechocystis* strain, it was sequentially transformed with a plasmid bearing *pdh/adh* genes. Several isolates containing the complete ethanol production cassette did not however demonstrate stable ethanol production (Dexter & Fu, 2009), indicating the instability of transgenes in cyanobacteria. Concerns have also been raised about the segregation of transgenes in polyploid strains (Berla *et al.*, 2013).

4. MODE OF TRANSGENE ESCAPE AND INTEGRATION

During large-scale cultivation of GM algae in open ponds, individual cells have the potential to escape into the environment by various means, such as being carried by the wind, water, birds, other animals, natural disasters or accidents. The transgenes may integrate into wild-type species via sexual hybridisation with the GM green algae and by HGT to cyanobacteria. These two methods are discussed below.

4.1. Sexual reproduction

Only haploid (haplotypes) GM green algae have the potential to transfer the transgene to sexually-compatible wild-type species. In sexual reproduction, genetic material from two individuals is combined to produce new individuals. In *C. reinhardtii*, the two genetically-determined mating types (+ and -) are morphologically similar, but express mating-related genes that allow fusion with a partner of the opposite mating type (Goodenough *et al.*, 2007; Geng *et al.*, 2014). Gametic differentiation in *C. reinhardtii* is triggered by the absence of nitrogen or unfavourable environmental conditions (e.g. the lack of nutrients or moisture). Thus, haploid daughter cells undergo sexual reproduction instead of forming spores for asexual reproduction. In asexual reproduction, green algae simply divide and produce genetically identical offspring. Thus, asexual reproduction cannot result in transgene transfer to wild-type species.

Cyanobacteria do not reproduce sexually; they only reproduce asexually by binary fission, spore production, or fragmentation.

4.2. Horizontal Gene Transfer (HGT)

HGT occurs when an organism transfers genetic material to other non-related via non-sexual methods. In eukaryotic algae, HGT has been an integral part of evolutionary development, however details of HGT are poorly studied in green algae (Ochman, 2000). In cyanobacteria, about 50 % of gene families have a history of HGT either between different cyanobacteria or within cyanobacteria (Waller *et al.*, 2006). Further, some cyanobacteria can release and take up “naked” DNA from their surroundings (Thomas & Nielson, 2005) and can both import and export their DNA through viral vectors (Lindell *et al.*, 2004). Considering that HGT can also occur between different species, in theory there is a very remote possibility of transgenes from GM algae transferring into edible aquatic species and, thus, into the human food chain.

5. CONTAINMENT OF GM ALGAE

There are many industrial, economic and societal interests in cultivating algae on a large scale. In this context therefore, for the production of biofuels from GM algae to become feasible, the next step is to scale-up production by moving from contained indoor facilities to outdoor open ponds.

5.1. Enclosed system and photobioreactors

For small-scale algae production, algae can be cultivated in photobioreactors or closed systems. Such closed systems prevent contact between the enclosed algae and the environment. GM cyanobacteria can be grown in photobioreactors, which for decades have been observed to be safe for growing bacterial cultures. Closed systems i.e. photobioreactors can also be placed outdoors to utilise natural sunlight or in greenhouses (Figure 1).

5.2. Outdoor open ponds

Outdoor systems represent a low-cost option for the generation of large quantities of green algae biomass. Care should be taken however to minimise the possibility of any spillage, especially when cultivating GM algae. One spillage mitigation option is to use a moat of extra source water around the cultivation tanks, as shown in Figure 2. The buffer zone of water can be also raised to 4-6 feet above the ground to reduce the prevailing wind intensity.

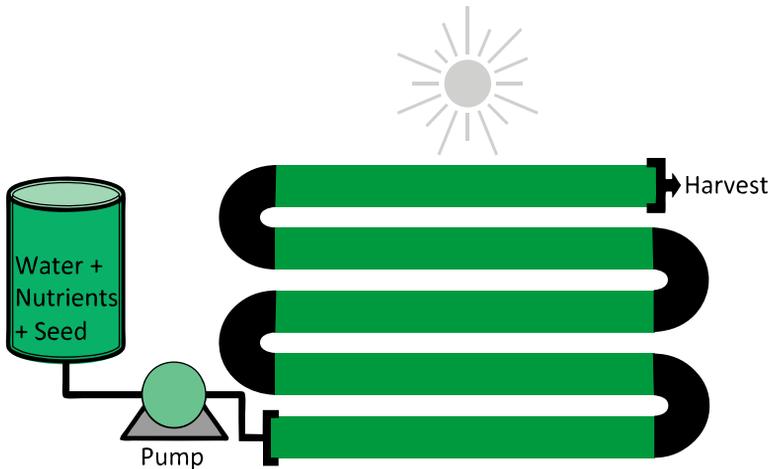


Figure 1. Outdoor cultivation of GM algae in an enclosed system to tap solar energy for growth. Such a system may be suitable for temperate climates to scale-up GM algae culture.

5.3. Genetic approach

Should the GM algae be hypothetically more robust than nearby wild-type species, there is a potential risk that any escaped GM algae could establish in the surrounding ecosystem to the extent that it may out-compete and eventually replace the wild-type species. A genetic approach to greatly reduce this potential risk is to couple the gene of interest with antisense or RNAi of genes that may increase the fitness of GM algae in the natural ecosystem (Gressel *et al.*, 2013). However, this may be unnecessary or considered excessive due to the general laboratory-based observations that most of the traits used for higher biofuel values generally reduce the ability of GM algae to compete with the wild-type host, due to transgene load and the requirement of higher and specialised nutrition to produce the desirable product.

Any antibiotic resistance marker genes used in the genetic transformation may themselves be a source of risk as there is the potential that they can be transferred to wild-type relatives and thus introduce novel antibiotic resistance. If necessary, they should be removed from the GM organism prior to commercial cultivation (Shao *et al.*, 2014), or instead either non-

antibiotic selection markers used e.g. as demonstrated in *Nannochloropsis oculata* using red fluorescent marker (Li and Tsai, 2009) or homologous recombination approaches exploited to avoid the use of selectable marker genes altogether (Kilian *et al.*, 2011).

In addition to the genetic material in the nucleus, green algae also have genetic material in their chloroplast, a singular organelle which occupies about two thirds of the cell. Due to its prokaryotic nature, this provides a unique opportunity for genetic modification to exploit site-specific recombination between homologous DNA sequences (Mayfield & Franklin, 2005). Chloroplast genes in green alga (*C. reinhardtii*) are known to transfer via maternal inheritance, and this is due to chloroplast DNA of mt^- gametes being preferentially lost in subsequent zygotes following mating with mt^+ gametes. This uniparental inheritance of chloroplast traits in green algae (Kuroiwa *et al.*, 1982; Nishimura *et al.*, 2002) can therefore be exploited to help reduce the potential of transgene integration into wild-type sexually-compatible species.

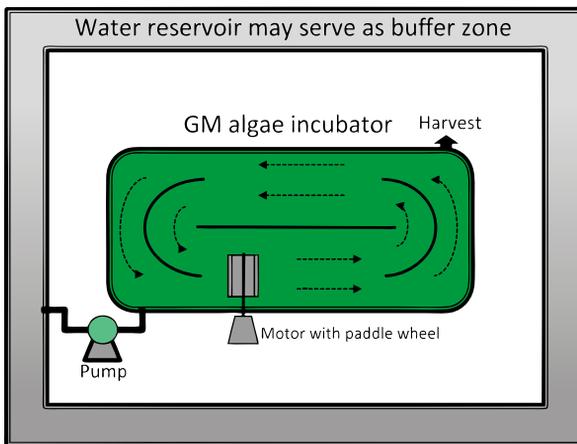


Figure 2. Outdoor cultivation in an open pond surrounded by a water reservoir buffer zone (~6-10 ft wide) to isolate the GM algae from any surrounding natural ecosystems. In addition to acting as a physical barrier, the water reservoir may also be used to store the nutrient-supplemented water or medium for cultivating GM algae.

6. BIOSAFETY AND REGULATORY ASPECTS OF GM ALGAE

GM algae represent a relatively new research frontier, and so far, no internationally-agreed regulations, policies or standards are currently in use. Examples of national regulations, policies and risks related to GM algae are discussed in this section.

6.1. Regulatory Polices and Risks

The USA Environment Protection Agency (EPA) has defined microorganisms (cyanobacteria and green algae) according to Whittacker's (1967) 5 kingdom system (Box 1). Similarly in the USA, the Department of Agriculture (USDA) regulates transgenic crops used for agricultural or other commercial uses under the Plant Protection Act. Green algal species and cyanobacteria are photosynthetic organisms, but it may be ambiguous for algal species to be regulated under existing biotechnology regulations related to the Plant Protection Act. Genetic modification research of biofuel-related algal strains is lagging way behind yeast or plants with only a few companies or research laboratories thus far able to generate GM alga to be explored for commercial use. Such genetic transformation research of algae may also draw the attention of different regulatory agencies. For example, the USDA's Animal and Plant Health Inspection Service (APHIS) consider *Agrobacterium* as a potential plant pest. If algae is transformed using *Agrobacterium* as a vector, or a selectable marker from so-called "plant pest" organisms is used, then additional clearance from the relevant regulatory agency will be required, making the entire process more complicated and lengthy. Due to the lack of experience with engineered algae, USA regulatory agencies (i.e. FDA, USDA, EPA) had difficulty in determining how best to evaluate the application from Mera Inc. to grow GM *Chlamydomonas* in Hawaii for the purpose of pharmaceutical production in 2005. Predicted risks included unintentional HGT of transgenic DNA to unrelated organisms in the surrounding environment from any GM algae present in untreated waste. In Europe, directives 90/219/EEC and 2001/18/EC cover all concerns pertaining to the risk of GM algae (EC, 2014). Accordingly, when GM algae are cultivated in closed systems, they should be considered as contained and thereby fall under Directive 90/219/EEC. Any cultivation of GM algae which does not fit the criteria of Good Industrial Large Scale Practice (GILSP) in outdoor closed systems and open ponds will instead be regulated according to Directive 2001/18/EC. Scientists in Australia are also extensively involved in GM algae research related to biofuel production,

and are regulated by the Gene Technology Regulator (OGTR, 2014). In China, the Agriculture Ministry has primary responsibility of managing agricultural GMOs (Ministry of Agriculture, China, 2002). The government of India recently introduced a new Biotechnology Regulatory Authority of India (BRAI) Bill in Parliament that will allow Indian scientists to conduct GM research without any political interference, in addition to single-window clearance of GMOs (Kumar *et al.*, 2014).

BOX 1. THE CLASSIFICATION OF MICROORGANISM USING THE 5 KINGDOM CLASSIFICATION OF THE LIVING WORLD

According to this classification, unicellular bacteria and photosynthetic cyanobacteria (blue green algae) are grouped together in the Kingdom **Monera**, while unicellular eukaryotes and photosynthetic organisms like green algae, which are autotrophic in nature, have flagella/cilia and reproduce both by sexual and asexual methods, are grouped together in the Kingdom **Protista** (Whittaker, 1969). In the proposed rule (59 FR 45550-51), the EPA has defined “microorganisms” in Sec. 725.3 according to this 5 kingdom system.

6.2. GMO regulatory bodies and decision-making

GMO regulatory bodies play important roles in mitigating any risks arising from the use of GM algae, following the steps in the proposed decision-making tree (Figure 3).

GM algae generated for commercial purposes at research institutes or university laboratories may first come under the oversight of an Institutional Biosafety Committee (IBSC). Should research prove successful, the GM strain can be transferred to a commercial body for further product development. Part of this process requires an application to be filed with the State GMO Regulatory Body (SGRB). The SGRB may consult with different scientific experts to evaluate the potential risks associated with any spillage of the GM algae into the wider environment. Any relevant scientific reports may be reviewed by the apex GMO regulatory body, the National GMO Biosafety Review Committee (NGBRC) for an approval or rejection of the application. If the GM alga and its open cultivation do not present an unacceptable risk, the GM strain may be approved for a confined open trial during which

further data related to any identified risks may be required to be generated. Finally, after reviewing all of the biosafety parameters, the GM strain may be approved for commercial release by the NGBRC (see Figure 3).

Ideally, should a decision to approve a GM algae be given by the regulatory authorities, its use should be free from political interference. Unfortunately, this has not been the case for a number of previous approvals of GM crops. For example, an herbicide-tolerant GM sugar beet approved in 2005 by the USDA for outdoor cultivation was banned in 2010 by a Californian district court due to lawsuits from various NGOs. Subsequently in June 2012, the USDA again deregulated it for commercial production (Waltz, 2012). In a developing country like India, legal battles over field trials of several GM crops are pending in the Supreme Court for regulatory clearance (Kumar *et al.*, 2014), which is a setback to commercial and scientific communities.

6.3. Risk characterisation

Key characteristics of the GM algal strain, for example, its overall fitness, the potential for HGT and any antibiotic selection markers used together combine to help identify and evaluate any risk to the natural ecosystem. The non-modified host strain should first be thoroughly investigated with respect to pre-existing risk from its mass cultivation, for example information on known harmful algae is available in a database hosted by the Intergovernmental Oceanographic Commission (IOC-UNESCO, 2014). Some cyanobacteria are well known for producing harmful toxins and algal blooms that may kill natural flora and fauna. The fitness study of GM algae in comparison with the wild-type strain may further help understand any risks involved. The characteristics of the transgene may/may not provide the subsequent GM algae with a competitiveness advantage over the wild-type, and thus may/may not establish in the natural environment. Sexual reproduction or HGT are also mechanisms by which the transgenes can spread beyond the GM algae. Depending upon the transgenic traits conferred, the results of such transfers may possibly cause toxicity, antibiotic resistance, and competitive advantage in the recipient organisms. Therefore, a careful study of the reproduction method and introduced characteristics should be carried out in advance to mitigate any harmful impact of GM algae upon the natural ecosystem. Thus, advanced characterisation of strain and risk assessment may help in the safe cultivation of GM algae and will protect the surrounding environment, and human and animal health.

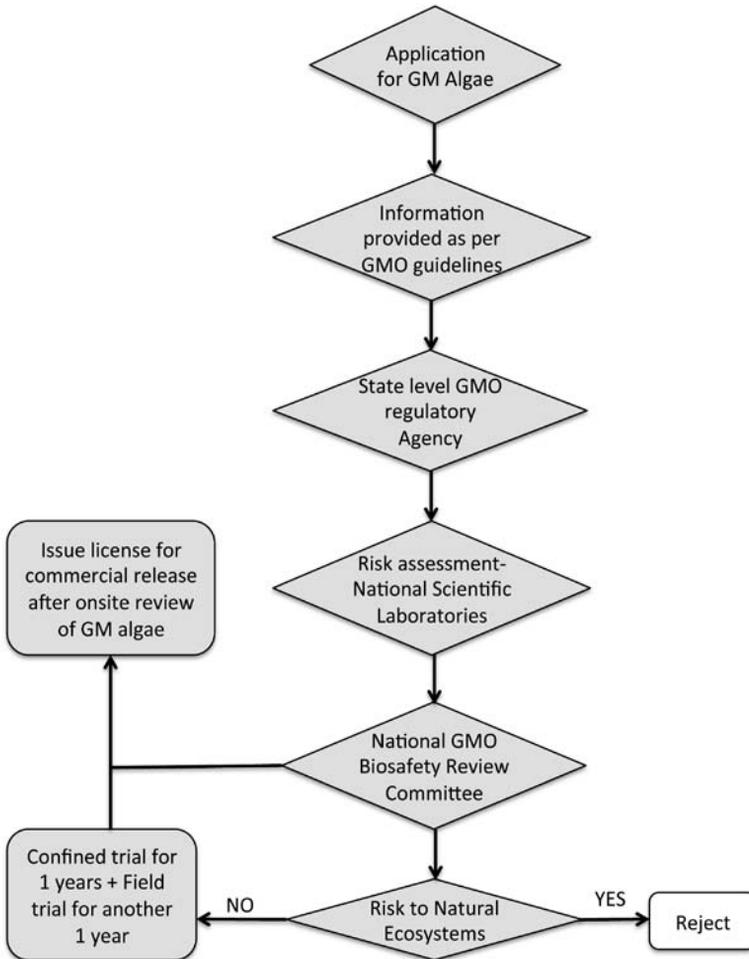


Figure 3. Theoretical decision-making tree for GM algae authorisation. After thorough testing and regulations related to GM algae by expert scientific laboratories, the overseeing GMO regulatory bodies should accept or reject the GM algae application for commercial release.

6.4. Hazard assessments

The first step of hazard assessment is to identify any detrimental impacts posed by the GM algae itself or the transferred trait. In general, green algae are harmless to the environment except a few like *Prototheca wickerhami*, which causes Protothecosis disease in dogs, cats, cattle and humans. However, it is not an important candidate for biofuel production. Some cyanobacteria produce toxins and may grow profusely under favourable conditions resulting into blooms and causing major ecological and human health problems worldwide. Blooming cyanobacteria can produce cyanotoxins, which are harmful to fishes, animals and humans. They can accumulate in fish and shellfish, and enter the human food-chain. Common toxins reported in some cyanobacteria are neurotoxins, hepatotoxins, cytotoxins, and endotoxins, which are health hazards to humans, animals and marine life. However, there is as yet no specific cyanobacterial candidate for biofuel production on the commercial scale. Most of the strains that have been genetically modified up until now are only model species.

6.5. Exposure assessment

Cultivating GM algae on a commercial scale may lead to spills into the natural ecosystems. A definitive exposure assessment may require an in-depth analysis of routes by which an organism may interact with the surrounding natural environment. In addition, sophisticated molecular tools like Digital PCR can be developed to specifically detect the presence of GM algal strains in the surrounding environment. However, as there is as yet no GM algae in commercial scale open cultivation, no definitive exposure assessment can be made, with only preliminary generalisations and likely mitigation measures speculated at this time.

7. CONCLUSIONS

There is currently plenty of hype and speculation concerning the use of GM algae as a source of biofuels. Algae-related research, including genetic modification, is in progress in the hopes of developing a lucrative level of biofuel production, to circumvent the problem of fossil fuel depletion. The production of biofuels using lipid-/carbohydrate-rich microalgae is a very promising alternative to conventional biofuel production, however, most of the genetic engineering processes are not economically viable yet. This technology is still in its preliminary phase due to practical difficulties in genetic

manipulation of algae and the present problem concerning transgene stability. Suitable solutions may take another 5-10 years to devise and develop, therefore much research is needed before commercial production using GM algae can become a reality. Potential risks involved in the mass cultivation of GM algae need to be addressed in advance in order to smooth the road for overall acceptance. Based on experience with current GM algal strains, any commercial strain may not be able to compete with existing non-GM strains in the wider environment. If considered necessary, through the co-transfer of "lethal genes", the GM algae can be developed to be unable to survive if accidentally released into natural ecosystems. Genetic and mechanical containment of GM algal strains in energy-efficient photobioreactors are alternative options for mass production systems. In general, the genetic modification of algae aimed at modifying either photosynthesis, biomass or lipid biosynthesis is not expected to generate harmful strains with respect to human health or for perturbing the surrounding natural ecosystem.

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