Large scale cultivation of genetically modified microalgae: a new era for Environmental Risk Assessment

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Abstract

The genetic modification of microalgal strains for enhanced or modified metabolic activity shows great promise for biotechnological exploitation. However, of key concern for many is the safety of genetic modification technology and genetically modified organisms with regard to both the environment and human health, and how these concerns are met will play a key role in ensuring how successful commercialisation of genetically modified (GM) algae is achieved. Commercialisation opportunities for GM microalgae will inevitably require translation from laboratory to industrial settings, on scales beyond those typically associated with the current biotechnology sector. Here we provide an overview of the current situation with regards to genetic modification techniques and legislation, and the implications of large-scale cultivation with regards to developing a safe and effective risk assessment system for contained and uncontained activities. We discuss the rationale and options for modification and the implications for risks associated with scale up to human health and the environment, current grey areas in political/technical legislation, the use of contained/uncontained production systems, deliberate release and monitoring strategies. We conclude that while existing procedures are not entirely sufficient for accurate and exhaustive risk assessment, there exists a substantial knowledge base and expertise within the existing aquaculture, fermentation and (algal) biotechnology industries that can be combined and applied to ensure safe use in the future.

Key words

Genetic modification; microalgae; biotechnology, environmental exposure; hazard assessment, containment; risk management;

1. Introduction

Microalgae represent a highly diverse assemblage of photosynthetic microorganisms found over a wide range of environmental habitats, from fresh water through to hyper saline, and spanning a wide range of both temperature and pH tolerances [1, 2]. Containing both eukaryotic and prokaryotic (cyanobacteria) members, the general term 'microalgae' is used here to encapsulate this broad grouping of photosynthetic microorganisms with their diverse metabolic potential and function.

Production of microalgal biomass does not require high quality land resources, as is the case of plant crops, and in comparison to large scale fermentation vessel grown yeast or bacteria, these photosynthetic microorganisms have low input requirements (light and micronutrients) whilst producing large amounts of biomass over short periods of time [3]. Microalgae culturing has a significant requirement for water resources which are often scarce. However many species can be grown in saline or brackish waters, reducing impact on increasingly valuable fresh water supplies, or on nutrient rich waste waters that are not suitable for agriculture or human consumption [4]. Combining photosynthetic/heterotrophic growth with waste water treatment/remediation and/or CO_2 capture could not only reduce production costs but has the potential to offer "added value services" to the process of algal biomass generation.

Commercial viability of algal derived products will most likely be achieved by combining commercialisation of high-value, low-volume products such as β-carotene, docosahexaenoic acid and eicosahexaenoic acid with the production of low-value, high-volume products like feeds, fertilisers and biofuels [5].

GM microalgae and current legislation

Many algal species have become successfully established as suitable for mass culture [6, 7], predominantly aquaculture related, but including production for food and feeds, waste water treatment, fertiliser, biofuels, fine chemicals, and pharmaceuticals [8, 9] .The advent of the genomic era has heralded a new dawn in microalgal exploitation potential by allowing the combination and selection of key physiological characteristics with modified metabolic activities, enhancing production of native compounds relative to wild type strains or introducing genes for the production of additional non-native compounds or added functionality.

Microalgae have been commercially cultured for well over 40 years and the systems currently utilised at scale tend to be unsophisticated shallow open ponds with no artificial mixing or, alternatively, paddle wheel mixed raceway ponds, both of which can cover hundreds of hectares in size [10]. Commercialisation of genetically modified (GM) microalgae for industrial purposes will inevitably require the culturing of GM microalgae at this kind of large-scale, but this will require more stringent risk assessment and environmental management strategies than those utilised for the unmodified wild type algae currently being grown. Much can be learnt from existing ‘large-scale’ enclosed culture practices exploiting GM bacterial and yeast strains which are typically grown in fermenter-style reactors. Even at smaller scales (e.g. for the production of the highest value products), the utilisation of ‘closed’ photobioreactor (PBR) systems still requires the effective exposure of the algae to light, the agitation of liquid media to enhance nutrient mixing, and for the removal of toxic oxygen build up; creating multiple opportunities for environmental exposure and, therefore, potentially a significant barrier to commercialisation when these organisms are genetically modified.

The industrial biotechnology sector has so far been slow to respond to GM algae with most projects never leaving the research laboratory setting. Only a few collaborative ventures such as

a recent project carried out by Plymouth Marine Laboratory and Rothamsted Research utilising a genetically modified *P. tricornutum* strain expressing heterologous Δ5- elongase for the accumulation of high value omega 3 long chain fatty acids [11], and a commercial venture between Sapphire Energy and UC San Diego ever reach pilot scale . This is in part due to a fundamental lack of information and assessment tools available to researchers, industrial developers or regulators on the risks associated with the large scale propagation of GM microalgae, as well as a lack of suitable facilities to undertake essential pilot scale trials. Yet, even these relatively small trials (<2000 litres) have highlighted the pressing need for the development of tools and mechanisms to aid the technical aspects of GM microalgal cultivation, containment and risk assessment, and crucially to consider the legislative and political aspects of such activities.

To begin with, it is important to define exactly what is meant by the term 'Genetic Modification'. The term *genetically modified organism* (GMO) is used to refer to any microorganism, plant, or animal in which genetic engineering techniques have been used to introduce, remove, or modify specific parts of its genome. It should be noted however that techniques that replicate naturally occurring phenomenon such as random mutagenesis are not generally considered to result in GMOs under European guidelines and are therefore not subject to GM control measures or legislation[12]. Indeed, it is worthy of note that more than 2,500 plant varieties in 175 plant species, both crop and decorative, have been created by random mutagenesis and released without fanfare into the environment over the past 75 years [13].

There are many strategies for enhancing algal phenotypes, including random mutagenesis, traditional recombinant nucleic acid technologies, and genome editing tools including transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and

RNA-guided engineered nucleases (RGENs) derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)–Cas9 system [14].

Whether any of these new technologies produce a 'GMO' depends largely on the country involved: e.g. in European countries the definition of GMO is mostly associated with the synthetic introduction of genetic material into an organism to create a novel organism via the use of recombinant nucleic acid technologies, though there are ongoing debates about the definition of what constitutes a GMO and the genetic technologies involved. It is unclear how existing legislations around the world will address the new developments and capabilities around genome editing techniques such as CRISPR/Cas9. Direct delivery of guide RNA alongside purified Cas 9 protein into microalgal cells, as opposed to plasmid-mediated delivery for example, is likely to bypass the GMO legislation in the USA, since the genome editing complex is degraded in the recipient cell leaving no trace of foreign DNA [15]. Indeed, it is worthy of note that the US Department of Agriculture (USDA) has decided that it will not regulate a mushroom which has been genetically modified using the CRISPR/Cas9 gene editing tool [16], thus setting a precedent of CRISPR/Cas9 derived plants being considered non-GMO in the USA. Whether this technique will fall under GMO legislation in the European Union will depend on the interpretation of the 2001 Directive on the Deliberate Release of GM Organisms into the Environment [12] which stipulates that techniques of genetic modification include "recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but which they are capable of continued propagation". This legislation was formulated before the advent of gene editing techniques such as the

CRISPR/Cas9 technology and whether this technique is considered "targeted mutagenesis" (not GM) or the formation of new genetic material (GM) is likely to create significant debate in the future as more R&D projects are commercialised that incorporate this versatile and powerful technology. This failure of regulation to keep up to date with the GM technology advances has created an element of unease; while the European Commission debates this conundrum and repeatedly delays the decision, the legal limbo of gene editing is having a big impact on research [17] which will inevitably impact any commercialisation of genetically edited microalgae. Currently, within Europe there is legislation covering aspects of GMOs from deliberate release [12], environmental protection and remedying of environmental damage [18], GMOs in food and feed [19], and labelling [20], to list but a few. However, within the scope of these directives each member state is able to take further measures of regulation, management and control of GMOs. Other countries around the world follow their own sets of legislative rules. Despite the potential for wide disparity globally, fortunately most legislation is built on the requirements of the Cartagena Protocol on Biosafety to the Convention on Biological Diversity [21] which provides international guidelines on the regulation and management of living modified organisms (LMOs) .

Public concern

A major factor holding back industry uptake of GMOs is public concern resulting from intensive campaigns by both media and NGOs. Sensationalised press coverage and lack of appropriate communication from the scientific community to the general public has left many fearful and suspicious of GM technologies and, as a result, resistant to buying products containing them. Several reports commissioned by the UK Government and Research Councils have indicated that

communication between those involved in science and the general public must be improved and that engagement at an early stage is important for improving understanding [22]. It was also found that through free-flowing dialog, many issues surrounding the use of industrial biotechnology could be addressed and no longer present significant concerns to the general public [23]. Of key concern for many is the safety of GM technology and GMOs with regard to both the environment and human health, and how these concerns are met will play a key role in ensuring how successful commercialisation of GM algae is achieved. Thus, it is important that the potential of microalgae to contribute to future energy and food security, as well as human and environmental health, is not undermined before the platforms can become established. In a new era of increasingly ready access to genetically modified microalgae, there is a crucial requirement for an environmental risk assessment (ERA) system which can uphold and withstand the rigours of safety legislation, as well as be able to cope with a rapidly changing research and development backdrop.

Environmental and health risks

Release of microalgae into the environment could have potential negative ecological effects such as altering food webs, displacing native phytoplankton, causing local extinctions, hazardous algal bloom (HAB) formation, and having serious societal effects where harmful/toxic strains are involved [24]. Many of the risks to human health and the environment associated with production of a given GM microalgae will be specific to the types of traits and genes selected and the type of modifications performed. These GMO specific risks should be considered alongside the risks of general large scale algae production and potential release into the environment. In addition to the specific traits associated with the GM element of the microalgae

other considerations will need to be made such as choice of algae (HAB formers or known invasive strains will have a higher associated risk), type and location of growth and containment facility, and the risk of horizontal gene transfer from the GM algae to other organisms in the environment.

Many of the algae currently being modified are not native to the geographic areas in which they are generally cultivated and are often chosen for their rapid growth rate and overall hardiness which maximises biomass productivity. Whilst there is currently very little regulatory control over the importation and release of non-native algal strains into the environment, such as in the use of microalgae in aquaculture [24], the risks associated with non-native invasion should also be considered. The actual environmental risk associated with large algae spills therefore will not be limited to the GM aspect of these organisms but rather a combination of factors including the fitness of the invading algae, the fitness of the indigenous alga populations, modes of competition for the resident and invading species, and intricacies and population stability characteristics of the disrupted ecological system [25]. Indeed, since some transgenes reduce the fitness of recipient algae below the fitness of respective wild types, an important aspect of the risk analysis can therefore be based on the environmental risks associated with cultivating the wildtype [26].

That said, successful environmental invasion and establishment does not necessary require rapid growth rate of the invader or even population dominance, just a low level persistence or a potential for gene flow, which will be determined by the difference in relative resource limitation between the ‘alien’ and native species [27].

2. GM Microalgae: Initial Considerations

It is generally accepted that the deliberate release of GMOs into the environment is, in most cases, a necessary step in the development of new products derived from or containing GM algae, and that these organisms, whether released into the environment in large or small amounts, may survive, reproduce and spread, and that the effects of such releases on the environment may be irreversible [18]. Accordingly, before GM algae production can start, an application must be made to the relevant authorities for regulatory approval to release or market the algae and/or its derived products. These applications focus on a risk assessment covering human health, environmental protection, labelling and product use [28]. In addition, since public concerns could be a major barrier to commercialisation of GM algae (depending on the product type), information handling and release should be engaging and transparent, and be considered as part of, or in addition to, the risk assessment, to mitigate possibility of commercial failure due to product rejection by consumers in response to concerns raised by activist groups. Figure 1 describes a decision support system outlining the interacting components involved in industrial scale production of GM algae. Rather than a linear start at step 1 and end at step 11, each level interplays and is often dependent on the levels above and below, which can make the decision process complex. For example starting with any fixed parameters such as the type of algae to be produced and the end product marketed, Figure 1 can give the operator an indication of types of other decisions that would need to be considered and from there the risks involved can be assessed. The consideration of the risks associated with each aspect of the product and process, both independently and as a part of the whole, is a critical part of the risk assessment and failure to do so could result in rejection of an application and subsequent avoidable commercial failure. Further to the processes outlined below and in Figure 1, environmental monitoring (ideally prior to, during and post cultivation activity) must also be included as part of the environmental risk

assessment, however the financial implications of such activities can potentially be onerous and, in theory, *ad infinitum*. Lessons should be learned for example from the mining industry, to avoid tax payers shouldering the burden of any clean up, remediation and/or monitoring activities, long after industry has ceased production. Additionally, whilst a major aspect of the risk assessment should be focused on the GM component, other more general factors (traits of the non GM parent microalgae) should also be taken into consideration at this early stage– for example, is the algae of choice a native or wild-type to the area in which it will be cultivated, or is it considered a biosecurity hazard in certain environments or conditions? Non-GM algae discharged in to a non-native area could be just as much of a risk to the environment in the event of a release as any GM traits, and possibly more so if the GM algae are designed to be less competitive in natural ecosystems.

Choice of Microalgae

Since most GM modifications are built on the back of the natural algal metabolic potential, choice of species will be largely dependent on these base algae traits (e.g. oleaginous, high carotenoid production, rapid growth rate). The choices of algae and the nature of the modification will ultimately have a major impact on the risk assessment, since there are multiple factors to consider including local environmental conditions, existing infrastructure, budget, the growth medium, the scale of operation, as well as the final product. From cyanobacteria to dinoflagellates, as many as 300 diverse species of microalgae are reported to form blooms in the natural environment and nearly a quarter of these species are known to produce toxins. These species are known as 'Harmful Algal Bloom' (HAB) formers and fall into 2 categories [29]; The high-biomass producers, which can cause large regions of hypoxia resulting in indiscriminate kills of marine life after reaching dense concentrations [25], and the toxin producers such as

Gymnodinium mikimotoi [30] and *Karenia brevis* [31] that contaminate food supplies causing massive fish kills and the death of animals and birds [32]. Toxins are often present in the water where wave action can create aerosols containing toxins and cellular debris. Animals, including humans, are exposed to toxins when consuming contaminated seafood, have contact with contaminated water or inhale contaminated aerosols [33]. Some of these species such as *Alexandrium fundyense* [34] have toxic effects at low cell densities and do not need to form high density "blooms" to cause problems; the large scale, albeit controlled, cultivation of any such strains (and their GM derivatives) can therefore pose a serious risk to human health. Use of HAB forming algae should be avoided if possible (unless the toxin itself is the desired product), or strains should be additionally modified to reduce toxin production potential. Furthermore, assessment should assess the likelihood of genetic modification unintentionally causing a normally non-harmful alga (or any other organism capable of uptake of the genetic material), to start producing a toxin. Safety of human operators and any nearby populace is crucial and must be considered if a toxin producing strain is used in any situation. GM algal species used in an area not native to the non-GM wild-type parent must be considered as potentially invasive and risk assessed as such, since the release of such a species could pose a serious ecological threat regardless of the presence or absence of genetic modification.

Crop protection

Even without the GMO component, the sustainability of large-scale microalgae growth is a major challenge since, much like terrestrial crops, large algal monocultures will inevitably be invaded by pathogens and pests [35]. Microalgae growth facilities are an excellent habitat for a wide variety of unwanted microorganisms which are usually detrimental to productivity.

Parasites and predators such as fungi, protozoans, viruses or aquatic invertebrates [36, 37] will reduce productivity by consuming or killing the microalgae crop, and invasion by other algae could affect productivity by outcompeting the GM strain.

Approaches to mitigate crop losses could include identifying strains resistant to pathogens, or even using GM technologies to engineer specific pest resistance into production species. Given how rapidly pathogens evolve, new strains would need to be continually developed. GM algal strains prepared in this way would have a clear competitive advantage over their wild type counterparts and this would need to be taken into consideration when preparing the risk assessment concerning potential environmental impact in the event of a release.

The use of extremophile algae, tolerant to high or low temperature, pH or salinity gives a boost to productivity by enabling growth under conditions too extreme for most potential contaminants. A practical downside is that extremophiles often grow very slowly and so a balance needs to be sought between growth rate and the need to keep contaminants to a minimum. Whilst the majority of currently commercially produced (wild-type) algal strains are not extremophiles there are some significant exceptions such as carotenoid and astaxanthin rich halotolerant species *Dunaliella salina* and *Haematococcus pluvialis* [38]. The incorporation of novel genes into extremophiles not currently being exploited could open up new markets. Additionally, use of species such as thermophilic and acidophilic alga *Cyanidium caldarium,* which is cultivated at below pH 5 and temperatures up to 56°C [39], could allow for direct carbon capture from industrial flue gas, thus adding value while increasing crop protection. From an environmental protection stance, the use of genetically modified extremophiles offers a unique advantage in that the majority of these organisms if released into the local environment

would quickly die out due to inability to adapt to the altered conditions, or would be out-competed by the plethora of microorganisms already adapted to thrive under ambient environmental conditions.

Traits of Genetically Modified Microalgae

Targeted genetic modification is undertaken to enhance, redirect or reduce the production of enzymes or metabolites. Table 1 provides a brief overview of some of the ways in which researchers have already genetically modified algae with commercial exploitation in mind. However, the act of altering the function of one metabolic pathway often has implications for other non-targeted pathways, thereby potentially affecting their competitive fitness under natural conditions and possibly their role in the food web should escape/release occur. For example, increasing the cellular production of a given metabolite by changing the flux of material down a given pathway, could cause an unintended reduction in cell growth by disrupting natural intracellular resource allocation. In assessing the risk of a given GM algae to the environment, any advantages conferred by the new/modified genes/pathways and any corresponding disadvantages compared to the wild-type, and additionally how the transgenes may affect other environmental microorganisms should they be transferred via HGT will need to be considered. The potential adverse environmental consequences of GM algae will be intrinsically linked to how the organism has been modified [25]. In addition, many GM techniques use the transfer of selective or marker genes in addition to the main transgene, and as such the risks and impact posed by these peripheral heterologous genes will also need to be considered (see below). Information on the safety of the GM algae should also be sought, partially regarding any toxic,

allergenic or other harmful effects arising from the genetic modification, especially where the algae or algae product would be destined for the food feed or pharmaceutical sectors.

Selective genes and markers

Antibiotic, herbicide and fungicide resistance

There are two types of 'marker' genes used during genetic modification of algae: genes which confer resistance to a selective agent; and reporter genes which produce products that can be detected visually or by biochemical assay. The use of selective (antibiotic, pesticide and herbicide) and reporter (fluorescent protein) marker genes are initially required for efficient screening for successfully modified algal cells and are often inserted into the genome alongside the gene of interest. Although these marker genes often play no further role in the desired phenotypes of the GM algae at the production stage, they usually remain in the genomes. Additionally, selective genes can be used as an active trait in the final production strain – for example a strain engineered with a herbicide resistance gene can be treated with this compound to ensure monoculture growth of the GM strain and prevent invasion of the culture by faster growing competitor species [40]. In the context of use for both initial selection and as an active production trait these genes pose two potential risks. Firstly, their protein products may directly or indirectly have a negative effect on people and/or animals that consume or come into contact with the algae and secondly, algae possessing these genes may cause environmental harm by promoting gene transfer to other organisms or by providing the GM algae with a selective advantage in a normally inhospitable environment. Antibiotic, herbicide and pesticide resistance genes may provide GM algae with a significant advantage if inadvertently released into a watercourse fed with agricultural land run-off rich in such selective agents, and could therefore

cause substantial disruption of natural communities. Additionally, the horizontal gene transfer of antibiotic or pesticide resistance genes to other microorganisms in the environment has the potential not only to put humans at risk via the creation of so called "superbugs", but also to cause ecological imbalances by allowing previously innocuous microorganisms to grow unchecked [41]. Indeed, given the potential impact to human health surrounding the prevalence of antibiotic resistance and the paucity of new antibiotics on to the market, this aspect should be taken into particular consideration when conducting the risk assessment of GM algae containing such genes [18]. Safety concerns have led to the development of several strategies to eliminate these genes from the genome after they have fulfilled their purpose (transposition, site-specific recombination, homologous recombination, co-transformation and gene editing) [42, 43]. Removal of such selective genes prior to commercialisation would aid considerably in associated risk reduction. Indeed, in April 2004 The European Food Safety Authority's (EFSA's) scientific panel on genetically modified organisms issued a detailed opinion on the wide-scale use of antibiotic resistance genes in genetically modified plants, including considerations of the environmental risks [44]. Whilst this report was specifically with reference to GM plants, it is also directly applicable to the use of resistance genes in GM algae. EFSA concluded that each antibiotic resistance gene should be assigned to one of three groups (see Table 2).

Group 1 contains antibiotic resistance genes which are already widely distributed among micro-organisms in the environment (soil, plant, water and the mammalian gut) and confer resistance to antibiotics which have no or only minor therapeutic relevance in human medicine and restricted use in defined areas of veterinary medicine. Regardless as to whether the genes are left over from the transformation process or being actively used for maintaining a unialgal culture condition, the presence of these antibiotics resistance genes in the genome of transgenic algae is extremely

unlikely to change the existing spread of these genes in the environment or significantly impact human and animal health. Group 2 contains genes which confer resistance to antibiotics which are used for therapy in defined areas of human and veterinary medicine. These genes are already widely distributed among microorganisms in the environment and as such their presence in GM algae will have only a minimal effect on the spread of these genes and therefore have minimal impact on human and animal health. Group 3 contains antibiotic resistance genes which confer resistance to antibiotics highly relevant for human therapy and should therefore be avoided in the genome of transgenic algae [44], so as not to expedite the widespread proliferation of resistance to these "last resort" drugs, which currently have only low level of resistance but to which resistance is already growing in clinical settings [45, 46].

The choice of antibiotic selection for genetically modified microalgae is not straight forward and can be influenced by a plethora of factors including, photo, pH and temperature stability, salt compatibility and solubility of the antibiotic, liquid/solid media selection, as well as natural alga resistance and the impact of the antibiotic on associated microbiota. In the early stages of strain development at laboratory scale, such factors will likely take precedence over the downstream implications of scale up (i.e. resistance genes are chosen irrespective of their grouping). However, it is crucial to retain an awareness of the implications that marker selection can impose should the strain move forward to industrial production. At this later stage, the grouping of the antibiotic resistance gene could then be of fundamental importance and will influence risk assessment and whether additional modification for its removal is essential, advised or unnecessary.

Zeocin is a formulation of phleomycin D1, a glycopeptide isolated from *Streptomyces verticillus*. Although not considered in the April 2004 European Food Safety Authority's

(EFSA's) antibiotic resistance gene assessment, Zeocin has gained significant levels of popularity with algal genetic researchers over the past few years, so is worthy of note here. Resistance to Zeocin is conferred by the product of the *ble* gene from *Streptoalloteichus hindustanus* [47]. Belonging to the bleomycin family of antibiotics, it is effective against most bacteria, filamentous fungi, yeast, plant, and animal cells, and causes cell death by intercalating into DNA and inducing double-strand breaks [48]. Bleomycin is used to treat a range of cancers and is on the World Health Organization's List of Essential Medicines. It is therefore likely that the use of the *ble* resistance gene would be classified into group 3 and therefore if used in the creation of GM algae would need to be removed prior to commercialisation.

Use of a Group 1 resistance gene does not automatically ensure that its presence in genetically modified algae can be considered as entirely low-risk during the commercialisation process. For example, the *hph* and *hpt* genes encode a hygromycin phosphotransferase (HPH) enzyme which inactivates and therefore confers resistance to the antibiotic hygromycin B [49] which, like other aminoglycosides, kills bacteria, fungi and higher eukaryote cells by inhibiting polypeptide synthesis. As an example of a Group 1 resistance gene, *hph* has been isolated from *E. coli* and *Streptomyces hygroscopicus* [50, 51], and is one of the most common antibiotic resistance markers used in the transformation of plants and algae. Hygromycin B is not in human clinical use, but is licensed in the USA for veterinary use with swine and poultry. Even with a Group 1 resistance gene, a GM microalgae resistant to a veterinary medicine is likely to cause particular concern in areas of intense agriculture where run off may contain high levels of this antibiotic either permanently, sporadically or during particular times of the year. In such cases, interaction

with local agricultural, veterinary and water treatment stakeholders should form an essential part of the risk assessment process.

Similarly, herbicide based selection markers may also result in risk assessment issues. The *bar* gene confers resistance to the herbicide glufosinate which inhibits glutamine synthetase and as a result, leads to accumulation of toxic levels of ammonia. The *bar* gene was originally cloned from *Streptomyces hygroscopicus*, the gene product of which encodes a phosphinothricin acetyl transferase (PAT) enzyme. Interspecific transfer of this *Streptomyces* gene into *Escherichia coli* showed that it could be used as a selectable marker in other bacteria [52]. GM algae carrying this marker would have a significant selective advantage in media containing the glufosinate herbicide, which is potentially beneficial if the GM microalgae are prone to culture contamination and poor long term stability. Conversely in the event of a release, this advantage would also be translated to the natural environment in regions in which glufosinate is used and subsequently runs off into water courses through other agricultural uses. In addition to being used as an herbicide for GM crops, glufosinate is also used as a desiccant to facilitate harvesting of non-GM crops.

Visual and biochemical markers

A range of visual and biochemical markers are frequently used in algal genetic modification to allow researchers to determine which microalgae among a large population are modified and/or to determine the gene product localisation within the cell. This is in contrast to antibiotic selection, where all living microalgae can be considered to be genetically modified. The GUS gene product β-glucuronidase provides a reporter gene assay, the colour of which depends on the substrate provided [53]. The product of the Luciferase gene originally isolated from the firefly

Photinus pyralis is an oxidative enzyme that produces a bioluminescence [54]. A range of genes encoding a selection of fluorescent proteins are commonly used in selection or recombinant protein tagging, the most common of which is eGFP. Such markers are likely to be selectively neutral in the natural environment and should not confer any advantage or disadvantage on the GM strain. Indeed many marine organisms, including algae, produce fluorescent or chemiluminescent proteins naturally, although the actual function of such activity is poorly understood.

Nutritional Selection

Genetic modification can be used to create knock-out strains where one or more genes encoding for amino acid (AA) production is lost. These strains are then only able to grow in the presence of supplemented media and can then be used as a platform for further modifications where the gene is added back in as a selective gene (thereby returning them to the wild-type state) and the transformants selected in minimal media lacking the specific amino acid. Such strains would have no competitive advantage over their wild type counterparts. Additional pathways can also be engineered into algae to aid production efficiency: for example, a phototroph could be grown heterotrophically with the addition of a suitable sugar transporter. Such a modification may not have a direct impact on the actual target product itself, but would indirectly benefit the production process economics. The introduction of a new biochemical capacity in such a manner could confer lower, neutral or higher fitness depending on the modification and thus the fitness of the GM algae relative to the wild-type and would need to be considered in the environmental risk assessment. For example, it could have the potential to occupy new environments not

normally suited to the species where the sugar or other compound is present at biologically relevant concentrations, and thus cause a shift in community population dynamics.

Reproduction and gene transfer

Many microalgal species persist in a haploid state and reproduce asexually and there are many genera in which sexual reproduction has yet to be observed. In many species however, given specific environmental cues, asexual reproduction often switches to a sexual state enabling populations to increase the level of genetic recombination. Maintaining a production strain in an asexual state minimises opportunity to transfer genes to other compatible strains and also the frequency of horizontal gene transfer from contaminant strains. The risks from both gene introgression and contamination of cultures are therefore reduced. The use of sexually reproducing algae is likely to increase the potential for gene transfer unless there are specific incompatibilities between species. That being said, even species exhibiting complex sexual life cycles such as *Phaeodactylum tricornutum* [55] can be maintained in a non-sexual state by strict management of growth conditions [56], a state easily achievable in a highly controlled closed photobioreactor system, but much less so in an open system or in the event of an escape to surrounding surface waters.

Horizontal Gene Transfer

Horizontal gene transfer (HGT) refers to one of several natural processes for the acquisition of genetic information via the stable transfer of genetic material from one distantly related organism to another outside of reproduction and without human intervention.

The genome of almost every organism shows the result of many ancient HGT events [57] either as a result of direct DNA uptake or the result of virally or endosymbiosis-mediated DNA transfer. For example, analysis of ancient phylogenetic relationships and the non-lineal evolutionary origin of genetic material has demonstrated that both Prokaryotic and Eukaryotic genes have been transferred across diverse groupings such as chromalveolates via endosymbiotic gene transfer [58]. These kinds of events in Eukaryotes however are rare, but have led to the diversification of chromalveolata from a single ancestral cell to the major clade we see today. More common is the widespread occurrence of HGT involving bacteria and viruses, the most prominent example of which is the rapid spread of antibiotic resistance genes amongst pathogenic bacteria.

In order for viral genes and proteins to function correctly inside their hosts they must be suitably adapted to and be compatible with the genetic background of the host. This closely integrated host–virus compatibility creates the opportunity for genes to move between lineages via HGT [59]. The use of high throughput sequencing has enabled researchers to document the occurrence of historical HGT in eukaryote algae /virus systems including coccolithoviruses, chloroviruses and prasinoviruses (all of which infect microalgae). Significant HGT has occurred between the marine microalgae *Emiliania huxleyi* and the coccolithoviruses in both the virus to host direction and the host to virus direction, including the viral acquisition of a near complete pathway for sphingolipid biosynthesis [60]. A major concern for GM microalgae use therefore, is that the modifications created may be transferred from the GMO via HGT into natural algae, bacteria or virus species in the environment, and thereby cause damage to ecosystems via selective advantage conferred by the transferred genes. If the GM algae is to be released into the environment (deliberate or accidental), then determination of the likelihood of gene transfer from

the algae to an unintended recipient should be considered as part of any risk assessment, if data is available, as well as the impact that transfer of the transgene may have on unintended recipient populations.

Significant efforts have been made to ascertain the risk of HGT from GM Crops to soil bacteria, though HGT from plants to bacteria has not been conclusively demonstrated and, in most cases, cannot be simulated in an optimized laboratory environment. However, HGT may occur when transgenic plant material decomposes due to bacterial activity releasing plant DNA [61]. This has implications for directly using "waste" algal biomass as, for example, a crop fertiliser.

The chance of HGT depends on multiple factors: The frequency of HGT is strongly influenced by whether the organism is multicellular; eukaryotes, such as plants for example, have a much lower relative frequency HGT than single celled prokaryote/eukaryote such as microalgae, which in turn have a lower frequency than, for example, viruses [57]. The genetic relationship between the donor and the recipient will also affect the likelihood of HGT occurring, with the frequency between distantly related species being much lower compared to HGT between the same species or closely related strains.

The ecological relationship between the donor and the recipient is a particularly important consideration; microalgae often grow as a consortium of microorganisms in a symbiotic relationship and indeed many algae do not thrive when grown axenically. This is due to the fact that the majority of microalgae species lack the ability to synthesise their own B vitamins. Instead B vitamins produced by the associated bacterial consortia are used by the alga, and in a symbiotic relationship the bacteria appear to be able to use the carbon products of algal photosynthesis for their own growth [62, 63]. On an industrial scale it is unlikely that any algae could be grown truly axenically. The presence of other microorganisms and their close

association in the growth matrix, will therefore increase the chances of HGT, but it will not be possible to determine the relative increase when compared to an axenic culture and so the focus of the ERA should be on potential impacts.

The occurrence of HGT events will result in a secondary "GMO" which may give rise to adverse effects not controlled for by the management control measures imposed by the original licence or permit [57] and as such the initial risk assessment should try cover all possible outcomes. Whilst the emphasis tends to be on transfer of GM traits to wild organisms, perhaps an equally large risk is having GM algae acquiring wild type traits which could negate novel genetic traits in the GM algae designed to minimize its ability to survive in nature. Despite the theoretically low chances of HGT occurring from a GMO into the wild, HGT cannot be dismissed by the research community, and many have recognized that methods of monitoring HGT are often too insensitive [64]. Accordingly, the risk management (which would normally include a monitoring program) must make room for advances in monitoring methodology to ensure not only the greatest environmental security possible but also to provide robust reassurance to the public.

Choice of Growth facility

GM algae production will most likely make use of both open and closed systems. These options have significantly different challenges in terms of environmental exposure and risks to human health and the environment. Closed systems, such as PBRs, have the potential to minimize contamination and environmental exposure, but this comes at a high capital expense. Outdoor pond systems have lower initial capital costs, but rely on outcompeting potential contaminating organisms by using densely grown monoculture starter cultures (which are usually generated in closed systems) [35]. In addition, since there are few economically viable physical protective

measures for an open pond setting, the potential for GMO release is much higher due to aerosol dispersal, spillage, leakage, and vectors such as birds, insects and other animals (including humans).

The types of growth facility available are many and varied and the choice of which is utilised will depend on available infrastructure and resources, and the type of GM algae to be grown. In addition to the type of growth facility used, the materials used in the facility construction will also play a role, not only in economic productivity/losses, but also in the overall biosecurity and will need to be factored into the risk assessment process. For example in a large scale pond facility the pond wall structure is one of the most costly elements of the set up but is also important in determining the levels of environmental exposure through leakage. As such assessing the available materials (such as clay, concrete, asphalt, fiberglass, rubber, high-density polyethylene) early on will enable an informed choice of material which achieves an appropriate balance between initial costs, facility longevity, and overall suitability for algal growth and containment.

Large-scale cultivation of GM algae and extraction of derived products will require operations in accordance with good manufacturing practice. This can lead to a conflict between the measures designed to protect the operator and the environment and those designed to protect the product [65] and as such a balance must be struck to ensure protection of the environment and human health are not compromised.

Where high-value low-volume products such as nutraceuticals or pharmaceutical grade products are to be produced, high levels of production control will be required to ensure consistency, minimise levels of impurity’s and maintain maximal productivity. In such instances the use of

closed photobioreactors would be most appropriate. These units also carry the lowest risk of unintended release of the GM algae.

The majority of large scale manufacturing facilities involving GMOs in the UK operate in contained bioreactors under containment level 1 with a few at containment level 2 which are principally for virus based vaccine manufacturing processes [65]. The majority of the commercially interesting wild-type strains fall into hazard category 1 (unlikely to cause human disease) with the exception of *Chlorella spp.* [66, 67] which has been known to cause chlorellosis in humans and animals via ingress though open wounds. Whilst these events are very rare they would result in *Chlorella* potentially falling under hazard category 2 (can cause animal and in very rare instances human disease but is unlikely to spread to the community and effective treatments are available) [68]. As such, so long as the GM modification does not create, for example, enhanced pathogenicity or virulence in humans or animals [57] it is likely that GM microalgae production in closed PBR type facilities will also operate at containment level 2 or below.

For low-value, high-volume production of biomass for aquaculture, biofuel or chemical commodities, outdoor raceway ponds are likely to be the only cost effective set up. However growing GM algae in this kind of system offers no protection to the environment and therefore these kinds of commercial facilities for GM algae would be considered as deliberate release, which would require the full EU Part C application for commercialisation and release which involves an environmental risk assessment and post market environmental monitoring [12]. The use of industrial scale glass houses and polythene tunnels would offer a reasonable level of containment under most circumstances. These could provide not only a level of protection to the environment but simultaneously protecting the algae crop from predation and weather effects

such as storms and large temperature fluctuations across the year that could cause production inefficiencies [69]. However the cost of enclosing ponds is likely to be prohibitive for the majority of larger-scale production systems.

Environmental Exposure

There are a variety of mechanisms by which GM algae may become released into the environment during their production, processing and disposal, as well as their growth media. Release of GMOs into the environment can fall into two basic categories: deliberate and accidental, and measures should be taken to minimise unwanted releases and to manage their environmental impact if an event takes place.

Unintended Releases: Containment failure, system leaks, release during transport and sterilisation failure prior to disposal would all be considered accidental or unintended releases. Leaks from a bioreactor could lead to a significant algal release and containment measures should be considered to contain any such leaks so escaped algae do not disperse into the surrounding environment. This often involves forms of bunding, with bunded areas treated periodically to destroy residual algae.

Harvesting will involve the processing of large volumes of liquid including the transfer from the growth reactor to dewatering systems and then on to the product extraction system. At this stage leakage and spillage are almost inevitable. The water recovered during dewatering will need to be fed directly back into the growth reactor with additional nutrients, or processed to ensure any surviving algae and pathogens are rendered non -viable prior to disposal of the water. Failure of waste water treatment could lead to significant algal release directly into habitable environments.

606 Consideration should also be given as to how and where the GM algal biomass will be
607 processed. For example, will it need to be transported off site to a processing plant and if so will
608 the material need to be transported wet or dried, and will it be rendered non-viable before
609 transport? Dried algae, depending on the strain, may still be viable and therefore can still pose a
610 significant dissemination risk, despite the ease and preference for transporting a reduced biomass
611 volume. Live algal suspensions (either concentrated or not) are bulkier and could be
612 prohibitively expensive to transport, but may require less pre-processing to create and could be
613 considered under many circumstances to be easier to generate and control. A large, unplanned
614 release into a water course could however result in a high level of local exposure and a potential
615 for environmental harm.
616 Due to the risk of horizontal gene transfer, disposal methods for GMOs and their associated
617 waste streams need to address the destruction of both the organism and the genetic material [61].
618 There are various sterilisation methods employed which can be roughly classified into four
619 categories: heat, electromagnetic wave (UV, Gamma wave and microwave), filtration, and
620 chemical sterilisation [70].
621 For very low level contamination of waste water, the use of filtration and UV light treatment can
622 be very effective. However, microalgae are incredibly diverse and the resistance of some algae to
623 UV radiation and other treatment technologies can be significantly higher than that of others. In
624 addition high population loadings can cause significant reductions in efficacy, e.g. for UV
625 irradiation, as partial shading reduces effectiveness.
626 As with UV, not all organisms can be killed effectively with chemicals such as chlorine and if
627 chemical sterilisation is to be used the efficacy will need to be validated and monitored.
628 Chemical use can induce flocculation that reduces chemical exposure to shielded internal cells in

a similar manner to antibiotic resistance in biofilms. Furthermore, the ecological impact of the chemical utilised will also need to be assessed, heat and pressure (autoclaving) is the preferred method of sterilising solid waste but could be impractical and cost prohibitive for water treatment on an industrial scale. Inline heat treatment (like the systems used in milk pasteurisation) could be effective, however the temperature and exposure time required for effective sterilisation would need to be assessed (and monitored) for each individual GM algae strain.

Large volumes of biomass are unlikely to be disposed of directly since the algal biomass is in most cases the end product, and where the algae has been modified to produce a defined metabolite, the residual (waste) biomass can be used for added value in alternative applications such as biofuel, aquaculture or agricultural feedstocks [71]. If however, a large scale biomass disposal was required (presumably when the GM algae is employed in a bioremediation or similar application), composting could offer a cost effective method. The relatively high temperatures (greater than 55°C) over a prolonged period (15-21 days) combined with ammonia, sulphur and other toxic metabolite production can combine to destroy the GMOs and degrade cellular contents [61].

Deliberate release includes the use of open pond growth systems since they provide no protection against natural dispersion by weather and animal vectors of the GMO into the environment. Although not directly intended, release is inevitable. Escape may also occur through aerosol formation related to the turbulence and aeration necessary for cultivation. Additional consideration should also be given to accidental discharge, sabotage of systems, or natural disasters leading to a release. Such disaster scenarios are often envisaged as ‘worst case

scenarios' but in reality, the long term, low level release from a fully operational industrial activity is likely to have greater ecological impact than any one single unplanned release event.

Other factors associated with GM Microalgae

Enhanced lipid content

Several studies in recent years have focused on increasing the level of total lipid accumulation within algal cells, primarily by deregulating triacaylglyceride (TAG) storage [72, 73] such that the biomass can be used for the generation of biofuels. Additional studies have looked at elevating the accumulation of specific oil components such as polyunsaturated fatty acids (PUFAs) for use in the nutraceutical and aquaculture markets [11, 74, 75]. In the majority of studies, redirecting carbon metabolism to favour accumulation of lipids causes a reduction in growth rate, compared to the wild type though this is not always the case. It is therefore unlikely, given the suboptimal environmental growth conditions (compared to those of the mass culture conditions), that these released GM algae would persist in the environment at a significant or damaging level.

Since the biochemical and, therefore nutritional, content of these GM strains is altered, the impact of release on food webs should be considered. Dietary lipid content and composition is a critical factor for a range of organisms throughout the food web. Larval development and growth during early life stages in the Blue mussel *Mytilus galloprovincialis* and clam *venerupis pullastra*, for example, have a critical requirement for a specific composition of lipids, especially long chain polyunsaturated fatty acids (omega 3 and 6) [76, 77]. Exposure to (and consumption of) GM strains designed for biofuel applications, where short chain saturated fatty acid production predominates, could therefore have significant negative health impacts, whereas

675 omega 3 production platforms may actually have a positive impact on health at various trophic
676 levels.
677
678 **Enhanced Biomass productivity (shade effects and photosynthetic ability)**
679 The density of algae that can be grown in PBRs is invariably affected by the levels of light
680 received and ultimately self-shading by the growing culture, which limits the overall density that
681 can be achieved [78]. Improving biomass production can be achieved via a reduction in cellular
682 pigmentation (especially chlorophyll content), which results in a reduction in the shade effect
683 [79] and which can be achieved by altering the activity of genes involved in the chlorophyll
684 biosynthesis pathway [80]. Pigment binding complexes are required not just for light harvesting
685 but also required for photo-protection and as such strains with modified pigmentation are often
686 more susceptible to photosensitivity under high light conditions, which can have a negative
687 impact on production in a growth system with uncontrolled lighting (i.e. outdoor). A second
688 approach to improving biomass productivity is to modify strains to improve the overall
689 photosynthetic efficiency via a reduction in antenna size, defined as TLH (truncated light-
690 harvesting) mutant strains [81], by altering genes that encode light harvesting complex (LHC)
691 proteins, their import into the chloroplast, or translational regulation. In the event of escape,
692 increased photosynthetic ability or a reduction in pigmentation may confer an advantage since
693 these modified strains would be able to occupy a modified environmental niche location in
694 comparison to their wild type counterparts. Colonization of a deeper position in the water
695 column for example could impact on native strains with whom they are not normally in
696 competition the effects of which would be unknown.
697

Production of human therapeutic proteins

Recombinant therapeutic proteins are used widely in the biopharmaceuticals industry and whilst the majority of these are produced in bacteria, yeast or mammalian cell culture, interest in producing human therapeutic proteins from algae based platforms has grown in recent years [82]. It is unlikely that any of the therapeutic proteins such as antibodies and hormones [82-84] that are of primary interest for expression would confer any selective advantage on the GM algae in the natural environment, though as with all modifications this would have to be confirmed on a strain by strain basis comparison to the parental wild type strain. It is likely that the overall fitness of such GM algae would be considerably lower due to the metabolic pressure of over expressing "unnecessary" (as far as the algae are concerned) proteins.

Monitoring

A survey, both molecular and observational, of information on the environment surrounding production site such as local climate conditions, native flora and fauna, and details of any compatible (sexually or HGT) wild relatives to the GM algae should be made prior to production. This base level data can then be used in assessment programs, and will enable effective monitoring of long term cumulative effects in the event of a release [18]. Natural communities are usually in flux and can vary enormously over many spatial and temporal scales. The monitoring program should include keynote species representing the diversity and ecosystem functions of the natural fauna and flora, the GMO itself and species directly related to it within an area appropriate to the site and scale of activity. The strength and depth of the baseline survey will determine how easily GMO induced perturbations can be identified, and allow unexpected deviations to be investigated and acted upon if required. The establishment of

721 standard molecular based surveys to monitor not only for the transgene/s but also for community
722 alterations will be critical to the success of the ERA.
723
724 **Conclusions and recommendations**
725 In preparing a risk assessment and process design for large scale production of GM algae we
726 advocate that a common sense and precautionary approach should be used e.g. the use of
727 contained PBR facilities in preference to open ponds. Where this is not feasible, the ponds
728 should be contained within secondary containment such as glass houses or polythene tunnels if
729 appropriate. This would serve to restrict the release of the GM algae into the environment and
730 would benefit the grower through reduced productivity losses from predation, contamination and
731 weather events, and would provide a level of reassurance and security from those organisations
732 that may otherwise look to cause damage to the facility/ crop. Whilst the majority of GM algae
733 will display reduced fitness in comparison with wild type strains, the sheer abundance of GM
734 algae associated with an industrial monoculture process, could cause the displacement and
735 disruption of local species, creating unintended and unforeseen ecosystem damage in the event of
736 a large scale release.
737 Much can be learnt from existing industrial practises involving microalgae: the piecemeal
738 feeding of GM microalgae into the natural environment through normal operational conditions is
739 likely to have a similar effect as to the equivalent wild type species. Indeed, industrial activity
740 with GM microalgae is likely, in the first instances, to take place at existing production facilities
741 using modified versions of established strains, therefore a wealth of information on, and
742 experience of dealing with, the local biotic environment should already be available for these
743 ventures. The release of or transfer of modified genetic material to other organisms, and the

744 nature and impact of that material outside of controlled facilities is less well understood, and this
745 is where risk assessment will need to be as broad and forward thinking as is possible to ensure no
746 detrimental consequences are created. The removal of 'accessory' unused primary selection
747 associated material, such as antibiotic resistance, may prove to be an essential part of the R&D
748 pipeline to avoid unnecessary risk to both human and environmental health downstream. The
749 future is bright for algal biotechnology, the potential for microalgae to offer solutions relating to
750 energy, food & water security and health in the 21st century and beyond is without doubt, as is
751 the necessity that this will involve genetic modification. With this potential comes a
752 responsibility to the health and wellbeing of both the natural environment and the anthropogenic
753 environment (which can no longer be regarded as distinct), which will require careful thought,
754 deliberation, assessment and action as appropriate. The new era of environmental risk assessment
755 for GM microalgae has begun, whilst we do not yet have all the answers, we are at least
756 beginning to identify the right questions to ask.
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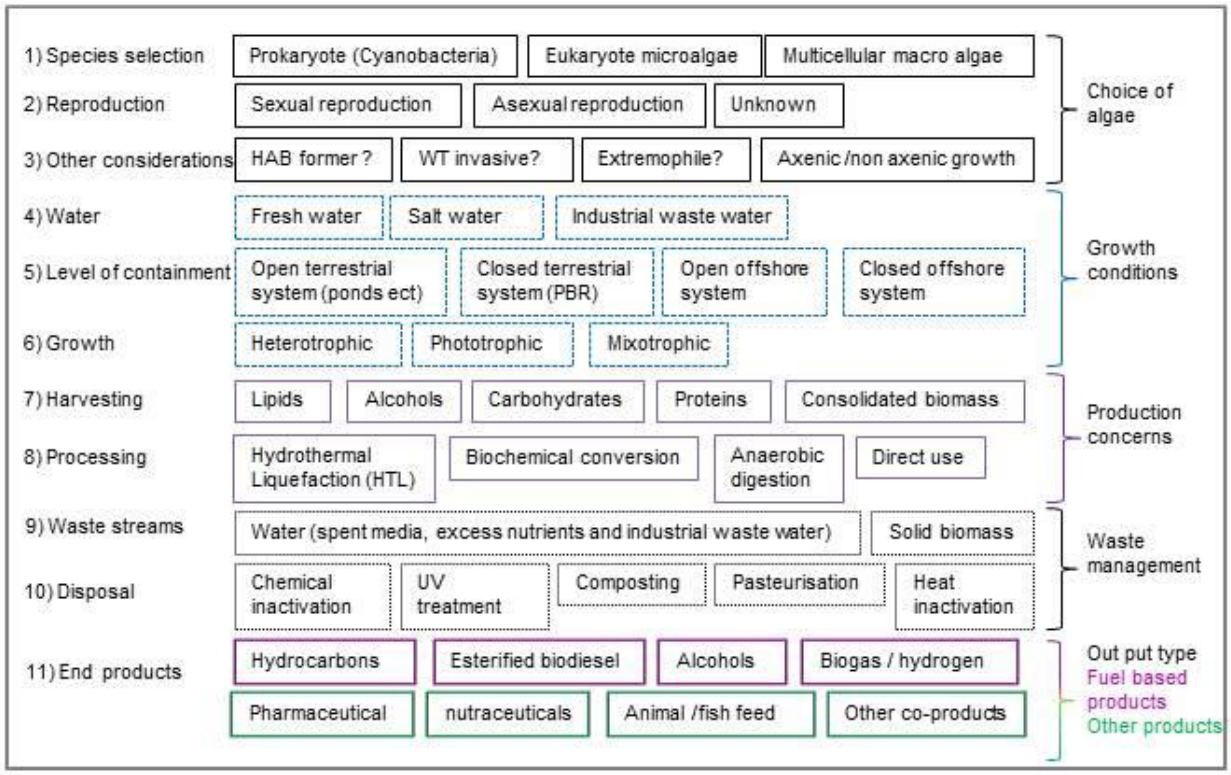

Figure 1. Risk Analysis Decision Support System: Factors to consider in relation to the "parent" wild type, the GM algae and the production life cycle.

Table 1. Examples of algae that have been modified to produce industrially relevant products

Genus and species	gene	Gene function	Purpose of modification	method of modification
Nannochloropsis salina (1,2)	Random	Lipid biosynthesis and regulatory pathways	Enhance lipid accumulation for biofuel production	EMS random mutagenesis
	DGA1 (Diglyceride acyltransferase)	Production of storage lipids (TAG)		Agrobacteria
Thalassiosira pseudonana (3)	Thaps3_264297	Multifunctional lipase/ phospholipase/ acyltransferase		Antisense and RNAi
Nannochloropsis gaditana (4)	Random	Light harvesting complex protein biosynthesis and regulation	Reduced cell pigmentation and or improved photosynthetic efficiency for increased biomass production	EMS and insertional mutagenesis
Chlamydomonas reinhardtii (5)	Tla1	Truncated light-harvesting chlorophyll antenna size		Insertional mutagenesis
Nannochloropsis Oceanica (6)	NoD12 (Δ12-Desaturase)	Long chain polyunsaturated fatty acid biosynthesis	Enhance production of essential fatty acids (EPA and DHA) - Human nutrition and aquiculture	Electroporation
Phaeodactylum tricornutum (7)	Δ5-elongase Δ6-desaturase			Biolistic
Chlamydomonas reinhardtii (8, 9)	Erythropoietin	Hormone that controls rate of production of red blood cells	Production of Human therapeutic proteins	Biolistic
	10fM3	Domains 10 and 14 of fibronectin		
	14Fn3			
	Interferon β	Signalling protein -maintains blood brain barrier -used to treat multiple sclerosis		
	Proinsulin	Hormone that regulates blood sugar levels		
	VEGF	Vascular endothelial Growth factor -treats pulmonary edema, erectile dysfunction and depression		
	HMGB1	High mobility group protein b1 - functions in wound healing		
	Large single chain antibody	Acts against glycoprotein D of the herpes simplex virus		
Chlorella vulgaris(10)	hGH	Human growth hormone (with an added extracellular secretion signal)		Chemical treatment of Protoplasts
Haematococcus pluvialis(11)	pds	Phytoene desaturase (with point mutation)	Enhanced carotenoid biosynthesis	Biolistic

Examples given refer to the following research: (1)[85], (2) [73], (3) [72], (4) [81] (5) [86], (6) [74], (7) [11, 75], (8) [82], (9) [83], (10) [84], (11) [87].

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Table 2 Antibiotic resistance (selective) marker genes

Resistance Gene	Substrates	Grouping
nptII	Kanamycin, Neomycin, Paromycin, Butirosin, Gentamicin B, Geneticin(G418)	Group 1 ; safe for use in field experiments and placing on the market
hph	Hygromycin B	
Cm^R	Chloramphenicol	Group 2; use should be restricted to field trial purposes only
amp^r	Ampicillin	
aadA	Streptomycin Spectinomycin	
ntpIII	Amikacin	Group 3; antibiotics highly relevant for human therapy and resistance genes should not be present in any GM algae
tetA	Tetracyclines	