

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

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14 **Abstract**

15

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

33

34 **Key words**

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

37 **1. Introduction**

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

44 Production of microalgal biomass does not require high quality land resources, as is the case of
45 plant crops, and in comparison to large scale fermentation vessel grown yeast or bacteria, these
46 photosynthetic microorganisms have low input requirements (light and micronutrients) whilst
47 producing large amounts of biomass over short periods of time [3]. Microalgae culturing has a
48 significant requirement for water resources which are often scarce. However many species can
49 be grown in saline or brackish waters, reducing impact on increasingly valuable fresh water
50 supplies, or on nutrient rich waste waters that are not suitable for agriculture or human
51 consumption [4]. Combining photosynthetic/heterotrophic growth with waste water
52 treatment/remediation and/or CO₂ capture could not only reduce production costs but has the
53 potential to offer “added value services” to the process of algal biomass generation.

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

58

59 **GM microalgae and current legislation**

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

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

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

83 a recent project carried out by Plymouth Marine Laboratory and Rothamsted Research utilising a
84 genetically modified *P. tricornutum* strain expressing heterologous $\Delta 5$ - elongase for the
85 accumulation of high value omega 3 long chain fatty acids [11], and a commercial venture
86 between Sapphire Energy and UC San Diego ever reach pilot scale . This is in part due to a
87 fundamental lack of information and assessment tools available to researchers, industrial
88 developers or regulators on the risks associated with the large scale propagation of GM
89 microalgae, as well as a lack of suitable facilities to undertake essential pilot scale trials. Yet,
90 even these relatively small trials (<2000 litres) have highlighted the pressing need for the
91 development of tools and mechanisms to aid the technical aspects of GM microalgal cultivation,
92 containment and risk assessment, and crucially to consider the legislative and political aspects of
93 such activities.

94 To begin with, it is important to define exactly what is meant by the term ‘Genetic Modification’.

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

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

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

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

129 CRISPR/Cas9 technology and whether this technique is considered “targeted mutagenesis” (not
130 GM) or the formation of new genetic material (GM) is likely to create significant debate in the
131 future as more R&D projects are commercialised that incorporate this versatile and powerful
132 technology. This failure of regulation to keep up to date with the GM technology advances has
133 created an element of unease; while the European Commission debates this conundrum and
134 repeatedly delays the decision, the legal limbo of gene editing is having a big impact on research
135 [17] which will inevitably impact any commercialisation of genetically edited microalgae.
136 Currently, within Europe there is legislation covering aspects of GMOs from deliberate release
137 [12], environmental protection and remedying of environmental damage [18], GMOs in food
138 and feed [19], and labelling [20], to list but a few. However, within the scope of these directives
139 each member state is able to take further measures of regulation, management and control of
140 GMOs. Other countries around the world follow their own sets of legislative rules. Despite the
141 potential for wide disparity globally, fortunately most legislation is built on the requirements of
142 the Cartagena Protocol on Biosafety to the Convention on Biological Diversity [21] which
143 provides international guidelines on the regulation and management of living modified
144 organisms (LMOs) .

145

146 **Public concern**

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

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

165

166 **Environmental and health risks**

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

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

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

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

196

197 **2. GM Microalgae: Initial Considerations**

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

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

232 **Choice of Microalgae**

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

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

261

262 **Crop protection**

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

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

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

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

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

292

293 **Traits of Genetically Modified Microalgae**

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

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

313

314 **Selective genes and markers**

315 **Antibiotic, herbicide and fungicide resistance**

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

334 cause substantial disruption of natural communities. Additionally, the horizontal gene transfer of
335 antibiotic or pesticide resistance genes to other microorganisms in the environment has the
336 potential not only to put humans at risk via the creation of so called “superbugs”, but also to
337 cause ecological imbalances by allowing previously innocuous microorganisms to grow
338 unchecked [41]. Indeed, given the potential impact to human health surrounding the prevalence
339 of antibiotic resistance and the paucity of new antibiotics on to the market, this aspect should be
340 taken into particular consideration when conducting the risk assessment of GM algae containing
341 such genes [18]. Safety concerns have led to the development of several strategies to eliminate
342 these genes from the genome after they have fulfilled their purpose (transposition, site-specific
343 recombination, homologous recombination, co-transformation and gene editing) [42, 43].
344 Removal of such selective genes prior to commercialisation would aid considerably in associated
345 risk reduction. Indeed, in April 2004 The European Food Safety Authority’s (EFSA’s) scientific
346 panel on genetically modified organisms issued a detailed opinion on the wide-scale use of
347 antibiotic resistance genes in genetically modified plants, including considerations of the
348 environmental risks [44]. Whilst this report was specifically with reference to GM plants, it is
349 also directly applicable to the use of resistance genes in GM algae. EFSA concluded that each
350 antibiotic resistance gene should be assigned to one of three groups (see Table 2).
351 Group 1 contains antibiotic resistance genes which are already widely distributed among micro-
352 organisms in the environment (soil, plant, water and the mammalian gut) and confer resistance to
353 antibiotics which have no or only minor therapeutic relevance in human medicine and restricted
354 use in defined areas of veterinary medicine. Regardless as to whether the genes are left over from
355 the transformation process or being actively used for maintaining a unialgal culture condition,
356 the presence of these antibiotics resistance genes in the genome of transgenic algae is extremely

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

367 The choice of antibiotic selection for genetically modified microalgae is not straight forward and
368 can be influenced by a plethora of factors including, photo, pH and temperature stability, salt
369 compatibility and solubility of the antibiotic, liquid/solid media selection, as well as natural alga
370 resistance and the impact of the antibiotic on associated microbiota. In the early stages of strain
371 development at laboratory scale, such factors will likely take precedence over the downstream
372 implications of scale up (i.e. resistance genes are chosen irrespective of their grouping).

373 However, it is crucial to retain an awareness of the implications that marker selection can impose
374 should the strain move forward to industrial production. At this later stage, the grouping of the
375 antibiotic resistance gene could then be of fundamental importance and will influence risk
376 assessment and whether additional modification for its removal is essential, advised or
377 unnecessary.

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

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

389

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

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

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

417

418 **Visual and biochemical markers**

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

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

432

433 **Nutritional Selection**

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

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

449

450 **Reproduction and gene transfer**

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

464

465 **Horizontal Gene Transfer**

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

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

479 In order for viral genes and proteins to function correctly inside their hosts they must be suitably
480 adapted to and be compatible with the genetic background of the host. This closely integrated
481 host–virus compatibility creates the opportunity for genes to move between lineages via HGT
482 [59]. The use of high throughput sequencing has enabled researchers to document the occurrence
483 of historical HGT in eukaryote algae /virus systems including coccolithoviruses, chloroviruses
484 and prasinoviruses (all of which infect microalgae). Significant HGT has occurred between the
485 marine microalgae *Emiliana huxleyi* and the coccolithoviruses in both the virus to host direction
486 and the host to virus direction, including the viral acquisition of a near complete pathway for
487 sphingolipid biosynthesis [60]. A major concern for GM microalgae use therefore, is that the
488 modifications created may be transferred from the GMO via HGT into natural algae, bacteria or
489 virus species in the environment, and thereby cause damage to ecosystems via selective
490 advantage conferred by the transferred genes. If the GM algae is to be released into the
491 environment (deliberate or accidental), then determination of the likelihood of gene transfer from

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

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

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

507 The ecological relationship between the donor and the recipient is a particularly important
508 consideration; microalgae often grow as a consortium of microorganisms in a symbiotic
509 relationship and indeed many algae do not thrive when grown axenically. This is due to the fact
510 that the majority of microalgae species lack the ability to synthesise their own B vitamins.

511 Instead B vitamins produced by the associated bacterial consortia are used by the alga, and in a
512 symbiotic relationship the bacteria appear to be able to use the carbon products of algal
513 photosynthesis for their own growth [62, 63]. On an industrial scale it is unlikely that any algae
514 could be grown truly axenically. The presence of other microorganisms and their close

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

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

529

530 **Choice of Growth facility**

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

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

541 The types of growth facility available are many and varied and the choice of which is utilised
542 will depend on available infrastructure and resources, and the type of GM algae to be grown.

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

552 Large-scale cultivation of GM algae and extraction of derived products will require operations in
553 accordance with good manufacturing practice. This can lead to a conflict between the measures
554 designed to protect the operator and the environment and those designed to protect the product
555 [65] and as such a balance must be struck to ensure protection of the environment and human
556 health are not compromised.

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

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

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

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

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

586

587 **Environmental Exposure**

588 There are a variety of mechanisms by which GM algae may become released into the
589 environment during their production, processing and disposal, as well as their growth media.

590 Release of GMOs into the environment can fall into two basic categories: deliberate and
591 accidental, and measures should be taken to minimise unwanted releases and to manage their
592 environmental impact if an event takes place.

593

594 **Unintended Releases:** Containment failure, system leaks, release during transport and
595 sterilisation failure prior to disposal would all be considered accidental or unintended releases.

596 Leaks from a bioreactor could lead to a significant algal release and containment measures
597 should be considered to contain any such leaks so escaped algae do not disperse into the
598 surrounding environment. This often involves forms of bunding, with bunded areas treated
599 periodically to destroy residual algae.

600 Harvesting will involve the processing of large volumes of liquid including the transfer from the
601 growth reactor to dewatering systems and then on to the product extraction system. At this stage
602 leakage and spillage are almost inevitable. The water recovered during dewatering will need to
603 be fed directly back into the growth reactor with additional nutrients, or processed to ensure any
604 surviving algae and pathogens are rendered non-viable prior to disposal of the water. Failure of
605 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

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

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

645

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

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

654

655 **Other factors associated with GM Microalgae**

656 **Enhanced lipid content**

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

667 Since the biochemical and, therefore nutritional, content of these GM strains is altered, the
668 impact of release on food webs should be considered. Dietary lipid content and composition is a
669 critical factor for a range of organisms throughout the food web. Larval development and growth
670 during early life stages in the Blue mussel *Mytilus galloprovincialis* and clam *venerupis*
671 *pullastra*, for example, have a critical requirement for a specific composition of lipids, especially
672 long chain polyunsaturated fatty acids (omega 3 and 6) [76, 77]. Exposure to (and consumption
673 of) GM strains designed for biofuel applications, where short chain saturated fatty acid
674 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

698 **Production of human therapeutic proteins**

699 Recombinant therapeutic proteins are used widely in the biopharmaceuticals industry and whilst
700 the majority of these are produced in bacteria, yeast or mammalian cell culture, interest in
701 producing human therapeutic proteins from algae based platforms has grown in recent years
702 [82]. It is unlikely that any of the therapeutic proteins such as antibodies and hormones [82-84]
703 that are of primary interest for expression would confer any selective advantage on the GM algae
704 in the natural environment, though as with all modifications this would have to be confirmed on
705 a strain by strain basis comparison to the parental wild type strain. It is likely that the overall
706 fitness of such GM algae would be considerably lower due to the metabolic pressure of over
707 expressing “unnecessary” (as far as the algae are concerned) proteins.

708

709 **Monitoring**

710 A survey, both molecular and observational, of information on the environment surrounding
711 production site such as local climate conditions, native flora and fauna, and details of any
712 compatible (sexually or HGT) wild relatives to the GM algae should be made prior to
713 production. This base level data can then be used in assessment programs, and will enable
714 effective monitoring of long term cumulative effects in the event of a release [18]. Natural
715 communities are usually in flux and can vary enormously over many spatial and temporal scales.
716 The monitoring program should include keynote species representing the diversity and
717 ecosystem functions of the natural fauna and flora, the GMO itself and species directly related to
718 it within an area appropriate to the site and scale of activity. The strength and depth of the
719 baseline survey will determine how easily GMO induced perturbations can be identified, and
720 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.

757

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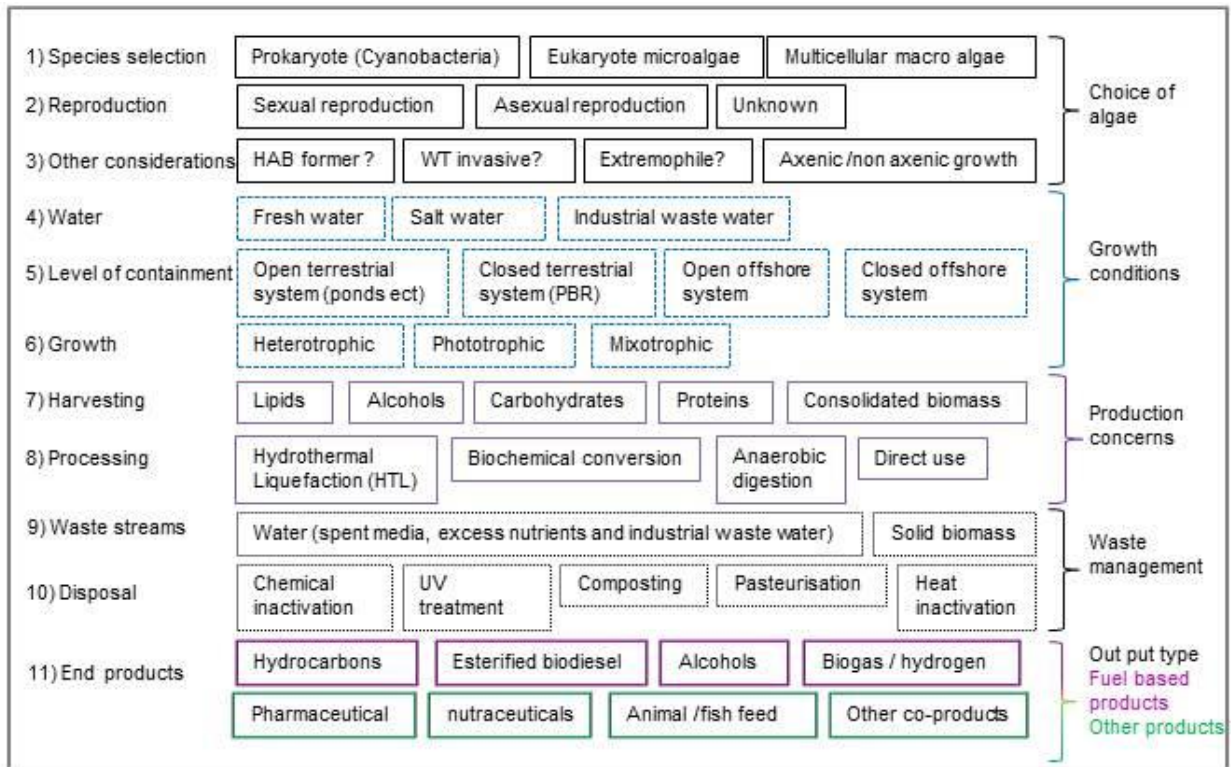
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1002 **Figure 1. Risk Analysis Decision Support System: Factors to consider in relation to the**
1003 **“parent” wild type, the GM algae and the production life cycle.**

1004

1005 **Table 1. Examples of algae that have been modified to produce industrially relevant**
 1006 **products**

Genus and species	gene	Gene function	Purpose of modification	method of modification
<i>Nannochloropsis salina</i> (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
<i>Thalassiosira pseudonana</i> (3)	Thaps3_264297	Multifunctional lipase/ phospholipase/ acyltransferase		Antisense and RNAi
<i>Nannochloropsis gaditana</i> (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
<i>Chlamydomonas reinhardtii</i> (5)	Tla1	Truncated light-harvesting chlorophyll antenna size		Insertional mutagenesis
<i>Nannochloropsis Oceanica</i> (6)	NoD12 (Δ 12-Desaturase)	Long chain polyunsaturated fatty acid biosynthesis	Enhance production of essential fatty acids (EPA and DHA) - Human nutrition and aquiculture	Electroporation
<i>Phaeodactylum tricornutum</i> (7)	Δ 5-elongase Δ 6-desaturase			Biolistic
<i>Chlamydomonas reinhardtii</i> (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		
<i>Chlamydomonas reinhardtii</i> (8, 9)	Proinsulin	Hormone that regulates blood sugar levels	Production of Human therapeutic proteins	Biolistic
	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		
<i>Chlorella vulgaris</i> (10)	hGH	Human growth hormone (with an added extracellular secretion signal)		Chemical treatment of Protoplasts
<i>Haematococcus pluvialis</i> (11)	pds	Phytoene desaturase (with point mutation)	Enhanced carotenoid biosynthesis	Biolistic

1007
 1008 Examples given refer to the following research: (1)[85], (2) [73], (3) [72], (4) [81] (5) [86], (6)
 1009 [74], (7) [11, 75], (8) [82], (9) [83], (10) [84], (11) [87].

1011 **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	

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