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Minimal Quality Criteria for Acceptability of *in vivo* Bio-assays with Bt-proteins

Adinda De Schrijver, Yann Devos, Patrick De Clercq, Achim Gathmann, Jörg Romeis

Introduction

As part of the regulatory authorization process, the potential risks that the cultivation of genetically engineered (GE) plants may pose to non-target organisms (NTOs) and the ecosystem services they provide are assessed in many jurisdictions. A typical risk hypothesis addressed during the environmental risk assessment (ERA) of *Bt*-plants is that the newly expressed *Bt*-proteins are not toxic to valued NTOs at concentrations present in the field. Potential harmful effects on NTOs are evaluated within different tiers that progress from laboratory studies representing highly controlled, worst-case exposure conditions (Tier 1) to bio-assays with more realistic exposure to the toxin (Tier 2) and (semi-)field studies carried out under less controlled conditions (Tier 3)[1]. Moving to a higher tier is only considered relevant if adverse effects are detected at the lower tier, or if unacceptable scientific uncertainty remains. Because not all NTOs potentially at risk can be tested from a practical viewpoint, a representative subset of species is selected for assessment. These species are usually selected because they are either of conservation concern or represent taxonomic or functional groups that contribute to ecosystem services (e.g., natural enemies for pest regulation).

We reviewed the early tier studies testing the hypothesis whether exposure to plant-produced Cry34/35Ab1 proteins as a result of cultivation of maize 59122 for controlling corn rootworms (*Diabrotica* sp.) is harmful to valued NTOs. Twenty-five laboratory studies with Arthropoda and Annelida were reviewed (Table 1) and assessed for their scientific quality against a set of eight criteria[2] determining their relevance and reliability[3].

Scientific quality criteria for laboratory studies

Equivalence of the test substance with the proteins produced in the GE plant

In the assessment of Bt-crops, large quantities of toxins are needed to conduct tests, and these are therefore often produced in microbes. The microbial produced proteins can then be used in safety tests as a surrogate for the plant-produced proteins provided that they are biochemically and functionally equivalent. The biochemical equivalence of the *Pseudomonas*-produced Cry34/35Ab1 proteins with the Cry toxins isolated from maize event 59122 was demonstrated by comparing the molecular size, immuno-recognition, and *N*-terminal amino acid sequence, and by confirming the lack of glycosylation of the plant-produced protein. Further, the bioactivity of maize event 59122 against a range of pest species was shown to be similar with the profile reported for the

bacterial-produced Cry34/35Ab1 proteins[4]. Further, in two early tier studies, plant material from different Cry34/35Ab1-expressing maize candidate events (i.e., TC5638, TC5639), containing a transformation cassette other than that of maize 59122, was used as a toxin source. The biochemical and functional equivalence of the *Bt*-proteins produced by the candidate events with the maize 59122 Cry34/35Ab1 proteins was also demonstrated. Therefore, the outcomes of the laboratory studies with NTOs fed a diet containing microbe-produced Cry34/35Ab1 proteins or plant material from the two different candidate events were considered informative to the ERA of maize 59122[4-5].

Exposure of the test organisms to high concentrations of the test substance

As part of the ERA, an exposure characterization is performed to determine how much of the plant-produced Bt-proteins a particular organism is likely to be exposed to under field conditions. This expected environmental concentration (EEC) is predicted from protein expression data in various plant tissues[1]. As the EECs reported in the various laboratory studies differ (e.g., because they are based on preliminary empirical expression data instead of data obtained from field trials), the highest concentration reported in literature was used to calculate the EECs for all the different groups of NTOs tested. A worst-case EEC was calculated for herbivores and pollinators; a realistic EEC for natural enemies, decomposers, and aquatic species. This approach of using a base EEC allowed inter-comparability of the study outcomes at the level of exposure. In all the studies, except three (number 10, 19, 21 in Table 1), the test organisms were initially exposed to at least the base EEC. These three studies were therefore considered of limited value for risk assessment[6-8].

Confirmation of intake of the test substance by the test organisms

Plant-produced *Bt*-proteins have no contact toxicity and must be ingested by a susceptible organism to be effective. Thus, direct dietary intake is required to evaluate the toxicity of *Bt*-proteins[1]. For seven laboratory studies it was uncertain whether the test organisms had actually ingested sufficient amounts of the test substance. These include: (i) studies where the toxic reference had not the same composition as the test diet; (ii) tri-trophic studies where *Bt*-maize fed aphids were used to expose predatory Coccinellidae larvae to the *Bt*-proteins, since there is strong evidence that aphids do not (or at very low levels) ingest Cry proteins when feeding on *Bt*-transgenic plants[9]; (iii) studies where the predatory insects were fed a mixture of a preferred prey that did not contain the Cry toxins (i.e., aphids) with an alternative food containing the Cry proteins (i.e., *Bt*-maize pollen); (iv) studies in which adult test specimens were simultaneously exposed to the test treatment and an alternative water source on which they can survive; or (v) studies using insects with piercing-sucking mouthparts such as *Chrysoperla carnea* and *Orius insidiosus* where the test compounds were provided coated to moth eggs or pollen. Predatory insects with piercing-sucking mouthparts do not consume the external surface of insect eggs[1,4]. These seven studies (6, 9, 11, 12, 14, 17, 21 in Table 1) were therefore not considered relevant to draw conclusions on risk.

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Nr	Test organism (Order: Family)	Reference	Endpoints tested	Results	Test substance
Non-target terrestrial (plant- and ground-dwelling) arthropods: herbivores					
1	*Gastrophysa viridula* (Coleoptera: Chrysomelidae)	Székács & Kong (2011)[a]	Survival	No effect	59122 pollen
			Adult weight	<10% decline at 600-800 grains/cm^2	
2	*Danaus plexippus* (Lepidoptera: Nymphalidae)	Sears & Rempel (2003)[a]	Survival, weight gain, consumption	No effect	59122 pollen
3	*Vanessa cardui* (Lepidoptera: Nymphalidae)	EFSA (2013a)	Survival, weight	No effect	59122 pollen
4	*Pieris rapae* (Lepidoptera: Pieridae)	EFSA (2013a)	Survival	No effect	59122 pollen
			Weight	32% lower	
Non-target terrestrial (plant- and ground-dwelling) arthropods: natural enemies					
5	*Poecilus cupreus* (Coleoptera: Carabidae)	Vinall (2005)[a,b]	Survival, development time, adult weight	No effect	Pure protein injected into blowfly pupae
6	*Hippodamia convergens* (Coleoptera: Coccinellidae)	Bryan et al. (2000a)[a]	Survival	No effect	Pure protein
7	*Coleomegilla maculata* (Coleoptera: Coccinellidae)	Higgins (2003)[a,b]	Survival	No effect	Pure protein
			Weight	< 80% decline	
8			Survival, adult weight, development	No effect	59122 pollen and moth eggs
9		Higgins (2000)[b]	Development time, adult mortality, weight	No effect	Aphids reared on TC5638 maize
10	*Coccinella septempunctata* (Coleoptera: Coccinellidae)	Califf & Ostrem (2009)[a]	Survival, development time, adult weight	No effect	59122 pollen and moth eggs
11		Takács et al. (2010)[c]	Survival, development	No effect	Aphids reared on 59122 maize supplied with or without 59122 pollen
12		Takács et al. (2012)[d]	Development time, fecundity, fertility	No effect	59122 pollen and aphids reared on 59122 maize
			Adult weight	10-15% lower male weight	
13		Vinall (2011b)[e]	Survival, adult weight	No effect	59122 pollen and moth eggs
14	*Orius insidiosus* (Hemiptera: Anthocoridae)	Patnaude (2008)[b]	Survival	No effect	Pure protein
15	*Orius laevigatus* (Hemiptera: Anthocoridae)	Vinall (2011a)[a]	Survival	No effect	Pure protein
			Development time	Longer (15-19h) at highest dose	
16	*Nasonia vitripennis* (Hymenoptera: Pteromalidae)	Porch & Krueger (2001)[a,b]	Survival	No effect	Pure protein
17	*Chrysoperla carnea* (Neuroptera: Chrysopidae)	Sindermann et al. (2001)[a,b]	Survival	No effect	Pure protein mixed with moth eggs
Non-target terrestrial (plant- and ground-dwelling) arthropods: pollinators					
18	*Apis mellifera* (Hymenoptera: Apidae)	Maggi (2001)[a,b]	Survival	No effect	Pure protein
19			Survival, development rate	No effect	TC5639 pollen

Non-target soil arthropods and annelids: decomposers					
20	*Folsomia candida* (Collembola: Isotomidae)	Teixeira (2001) [a,b]	Survival, reproduction	No effect	Pure protein
21		Teixeira (2006b) [a]	Survival, reproduction	No effect	Plant tissue
22	*Eisenia fetida* (Annelida: Lumbricidae)	Bryan et al. (2000b) [a,b]	Survival, weight	No effect	Pure protein
23		Teixeira (2006a) [a]	Survival, weight	No effect	Plant tissue
Non-target aquatic arthropods					
24	*Daphnia magna* (Cladocera: Daphniidae)	Marino & Yaroch (2001) [a,b]	Mobility	No effect	Pure protein
25	*Culex quinquefasciatus* (Diptera: Culicidae)	Fisher et al. (2012) [a]	Survival	No effect	Pure protein

[a] as referred to in EFSA, 2013a; [b] as referred to in US EPA, 2010; [c] published in Növénytermesztés 59S:625-628; [d] published in IOBC/wprs Bulletin 73:121-134; [e] as referred to in EFSA, 2013b

Inclusion of negative control treatments

All studies included an appropriate negative control treatment consisting of pure diet (either artificial diet, pollen, or untreated prey), allowing us to assess the suitability of the test system. The absence of unacceptable high mortality levels in all studies except one (number 14) in the negative control treatment indicated a good study design.

Confirmation of the stability and bioactivity of the test substance during the testing period

Over the course of the laboratory study, consistent exposure to the test substance is preferable. The stability of the Bt-proteins (total protein concentration) over the test duration was ensured in twelve laboratory studies by recording the test substance concentration, or replacing it at regular intervals. For one study with earthworms (number 22 in Table 1), the Cry34/35Ab1 protein concentrations were reported not to be monitored throughout the test period[7]; for the other studies, it is unclear whether the test organisms were constantly exposed to the Cry proteins.

Information on whether the test substance remained bioactive during the study period was not provided in the majority of studies. If optimal storage conditions are used for the test substance, one can presume that it remains stable and active. When suboptimal conditions are used, it is advisable to confirm bioactivity. For one study (number 1 in Table 1), suboptimal storage conditions were used and bioactivity was not confirmed. For this particular study, it is thus uncertain whether the test species were exposed to fully bio-active Cry proteins[7].

Measurement endpoints

In the laboratory studies, typical measurement endpoints to detect lethal and sublethal effects were considered, such as survival, development, or weight gain, the percentage of individuals that reach a certain life stage, and to a lesser extent reproduction and mobility. Appropriate measurement endpoints are those that are easy to evaluate and likely to indicate the possibility of adverse effects. For all studies, the endpoints measured were in accordance with international (e.g., OECD) standards and were considered appropriate.

Statistical power

Each experiment should be sufficiently replicated to detect a defined effect size (i.e., 20% is suggested by EFSA[10]; 50% is used by US EPA[1]) with an acceptable statistical power. A level of 80% power at an alpha level of 0.05 is usually considered acceptable[2]. For several laboratory studies, either a prospective or retrospective power analysis was performed to demonstrate that the studies had acceptable statistical power (> 80%) to detect an effect size that ranges from 20% to 40%. As effect sizes vary from jurisdiction to jurisdiction, the studies may be judged differently at

the same level of statistical power. Due to the lack of information, the statistical analysis criterion could often not be judged. In order to allow appraisal of the overall study design, we presumed that the power of those studies that lacked pertinent information was sufficient to detect a predefined effect size.

Representativeness of the tested species

The Cry proteins were tested against a range of NTOs. These species are usually selected because they are either of conservation concern or represent taxonomic or functional groups that contribute to ecosystem services. Given the activity of the Cry34/35Ab1 proteins towards corn rootworm beetles, several *in vivo* laboratory studies considered non-target beetle species. The green dock leaf beetle, a herbivorous chrysomelid, was tested as well as predators from the families of Carabidae (ground beetles, *Poecilus cupreus*) and Coccinellidae (ladybirds, *Coleomegilla maculata*, *Coccinella septempunctata*, *Hippodamia convergens*). Other species studied that provided ecosystem services were *Nasonia vitripennis* (parasitic wasps), *Orius* spp. (predatory flower bugs) and *C. carnea* (predatory lacewings). Further, pollinators (i.e., the honeybee *Apis mellifera*) and decomposers including soil-dwelling ones (i.e., the springtail, *Folsomia candida*, and the compost worm, *Eisenia fetida*) were examined, as well as some species of conservation concern (i.e., different species of Lepidoptera), and two non-target aquatic organism, *Daphnia magna* and *Culex quinquefasciatus*, both of which are a food source of fish that may occurlive in water bodies near maize fields.

Most of the selected non-pest species were considered representatives of important arthropod and annelid species in maize ecosystems. Exceptions are *N. vitripennis* which parasitizes fly pupae in bird's nests[4,6,7] and the southern house mosquito *C. quinquefasciatus*[6]. It was therefore suggested to use a more ecologically relevant parasitoid like *Macrocentrus grandii* (*Hymenoptera*: *Braconidae*), a parasitoid of the European corn borer (*Ostrinia nubilalis*; Lepidoptera: Crambidae)[4] or other Diptera, such as hoverflies or saprophytic dipteran larvae[6]. Nonetheless, studies as the one with *N. vitripennis* and *C. quinquefasciatus* add confidence to the risk assessment that the Cry34/35Ab1 proteins are unlikely to affect Hymenoptera and Diptera.

Conclusion

As a case-study, this exercise revealed that when not all eight quality criteria are met, weighing the robustness of a study and its relevance for risk assessment is challenging. Clearly some minimum criteria should be met to consider a study sufficiently robust to have relevance for ERA. The equivalence of the test substance with the one produced by the GE crop is considered a prerequisite for the study to be informative for ERA. If the test substance is not equivalent at the biochemical and functional level, deviating results might be obtained. Further, if the test substance provided is not bioactive or if the test organism is not exposed to the Cry protein(s) through oral ingestion, then a study is not suitable for ERA, as the results might be false negatives.

Another criterion considered of major importance to judge the robustness of the experiments is the use of an appropriate negative control. Without such a control, it is impossible to evaluate the appropriateness of the test system (including the diet) used and thus to interpret the effects observed. Rearing species on a sub-optimal medium may in itself cause unforeseen side-effects on the measurement endpoints[2]. Studies that do not fulfil these minimum criteria (eight studies in the case of maize event 59122, namely 1, 6, 9, 11, 12, 14, 17, 21 in Table 1) ought to be discarded. Studies fulfilling the minimum criteria can provide supportive information to the risk assessment. Their value in risk assessment will need to be judged depending on the additional quality criteria met. This exercise confirmed the importance of conducting studies meeting certain quality standards as this minimizes the probability of erroneous or inconclusive results and increases confidence in the results and adds certainty to the conclusions drawn.

References

1. Rose RI (2007) White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target invertebrates for regulatory risk assessment. USDA-APHIS and US Environmental Protection Agency, Washington, DC. Available from http://www.epa.gov/pesticides/biopesticides/pips/non-target-arthropods.pdf
2. Romeis J, Hellmich RL, Candolfi MP, Carstens K, De Schrijver A, Gatehouse AMR, Herman RA, Huesing JE, McLean MA, Raybould A, Shelton AM, Waggon er A (2011) Recommendations for the design of laboratory studies on non-target arthropods for risk assessment of genetically engineered plants. Transgenic Res 20:1-22.
3. De Schrijver A, Devos Y, De Clercq P, Gathmann A, Romeis J (2016) Quality of laboratory studies assessing effects of Bt-proteins on non-target organisms: minimal criteria for acceptability. Transgenic Research DOI 10.1007/s11248-016-9950-8.
4. US EPA (2010) Biopesticides registration action document: Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (PHP17662 T-DNA) in event DAS-59122–7 corn (OECD Unique Identifier: DAS-59122–7), PC code: 006490. http://www.epa.gov/oppbppd1/biopesticides/pips/cry3435ab1-brad.pdf
5. EFSA (2007) Opinion of the Scientific Panel on genetically modified organisms [GMO] on an application (Reference EFSA-GMO-NL-2005–12) for the placing on the market of insect-resistant genetically modified maize 59122, for food and feed uses, import and processing under Regulation (EC) No 1829/2003, from Pioneer Hi-Bred International, Inc. and Mycogen Seeds, c/o Dow Agrosciences LLC. EFSA J 470:1-25.
6. COGEM (2008) Cultivation of genetically modified maize line 59122. COGEM advice CGM/080207-02. http://www.cogem.net/index.cfm/nl/publicaties/publicatie/cultivation-of-genetically-modified-maize-line-1507x59122
7. EFSA (2013a) Scientific Opinion on an application from Pioneer Hi-Bred International and Dow AgroSciences LLC (EFSA-GMO-NL-2005-23) for placing on the market of genetically modified maize 59122 for food and feed uses, import, processing and cultivation under Regulation (EC) No 1829/2003. EFSA J 11:1-103.
8. EFSA (2013b) Statement supplementing the environmental risk assessment conclusions and risk management recommendations on genetically modified insect-resistant maize 59122 for cultivation in the light of new scientific information on non-target organisms and regionally sensitive areas. EFSA J 11:3443.
9. Romeis J, Meissle M (2011) Non-target risk assessment of Bt crops – Cry protein uptake by aphids. J Appl Entomol 135:1-6.

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Genetic Engineering Microalgae to Control Biological Contaminants in Open and Closed Culture Systems

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Microalgae are a diverse group of microscopic organisms present in almost all ecosystems on Earth, and together with macroalgae play a major role as primary producers. Similar to plants, they are able to manufacture their own food using sunlight as an energy source to convert carbon dioxide (CO_2) into carbohydrates and oxygen through photosynthesis. Microalgae are the most efficient organisms in converting solar energy into biomass and are able to produce much more biomass per unit area than land plants[1, 2, 3, 4]. Therefore, in addition to its ecological importance, microalgae deserve special attention due to economic factors.

Microalgal biomass holds great promise as energy-rich feed stocks, biofuels, and non-energy products. Microalgae produce numerous non-energy compounds that have important commercial applications for industrial use, such as pharmaceuticals, biodegradable polymers, natural pigments, cosmetics, polysaccharides, and bioflocculants, as well as for human and animal nutrition such as poly-unsaturated fatty acids and anti-oxidants[3, 5, 6]. The use of microalgae to produce biofuels such as biodiesel and bioethanol has invoked controversy because of the extremely large area of land needed to produce sufficient quantities of these products. Nevertheless, interest in designing efficient systems for high density and large-scale cultivation of microalgae has increased tremendously in the last fifteen years. To date, efforts mainly from private industry have allowed the design of both closed bioreactors and open pond systems that can be operated with certain success. In closed bioreactors the algal culture is isolated from the outside environment and grown under controlled conditions, including light (cycle and intensity), temperature, water quality, minerals, and CO_2 which are necessary to achieve higher productivity, as well as under sterile or semi-sterile conditions to avoid contamination by external biological organisms. Closed photobioreactors are specialized devices of high cost and high energetic consumption for which the maintenance of sterility during the production process is one of the most expensive components in their cost of operation.

Open-pond systems are shallow ponds in which the algae are exposed to natural environmental conditions of temperature, CO_2, and sunlight, while nutrient levels can be monitored and supplemented to provide adequate nutrition for algal growth. In this system water and nutrients are kept in motion by paddle wheels and algae are suspended in the water. Open ponds are easy to operate, their construction is low cost, and they are more environmentally sustainable, although they present limitations for controlling culture conditions and the contamination by biological pollutants. According to many experts, open ponds are the best systems for biomass production; in fact, over 90% of microalgal biomass production worldwide is currently achieved in large raceway ponds. However, the commercial production of microalgae in open culture systems is currently restricted only to organisms that can grow in extreme environmental conditions, such as high alkalinity (*Spirulina*), high salinity (*Dunaliella*), and high nutrient concentrations (*Chlorella*). Extreme environments limit contamination by "weedy" microalgae and bacteria that generally lead to unwanted competition for nutrients and sunlight, and result in an undesirable shift in overall biomass composition[4, 6, 7, 8]. Several strategies have been proposed to cultivate microalgal species unable to grow in extreme culture conditions in open ponds; however, it has been difficult to reliably cultivate microalgal monocultures outdoors, as they are unavoidably infested by non-photosynthetic microbes or out-competed by unwanted, faster-growing exogenous algae[8, 9].

To date, different genetic and metabolic engineering tools have been used to maximize the yield of several products such as lipids and pigments. However no attempts have been made for managing contamination through metabolic engineering to generate microalgae capable of outcompeting undesirable microalgae or

other microorganisms for restricted resources. We postulate that an effective way to create a selective environment for favouring monocultures of selected algal lineages or consortiums of microalgae species with desirable properties, without compromising growth and productivity, is the use of metabolic engineering to design microalgae strains capable of converting a non-metabolizable source of an essential nutrient into a chemical form that can be easily incorporated into their metabolism. These metabolically engineered organisms will have a competitive advantage over competitors when grown on media supplemented with the non-metabolizable form of the selected essential nutrient, allowing better control of contaminations in open systems and reducing the need for sterile conditions in closed reactors[10].

Phosphorus (P) is an essential nutrient that can only be used in the chemical form of inorganic phosphate (Pi, $^{=}PO_4$) by the vast majority of the organisms. However, some bacterial isolates have the capacity of using a different chemical form of P as nutrient source, namely the reduced chemical form of P named phosphite (Phi, $^{=}PO_3$). The best characterized bacterial isolate capable of using Phi as a sole P source is *Pseudomonas stutzeri* WM88. This bacterial strain has a set of genes integrated in an operon called *ptxABCDE,* where *ptxABC* genes encode a Phi transport protein, *ptxE* gene encodes a putative regulatory protein, and *ptxD* gene encodes a highly Phi-specific oxidoreductase that oxidizes Phi using NAD^+ as a cofactor, yielding Pi and NADH[11]. Thus, this bacterium absorbs P in the form of Phi and oxidizes it into Pi to use it in all its biochemical functions, including the synthesis of DNA, RNA, and proteins, as well as many other energy requiring metabolic processes. In contrast to bacteria, all the evidence suggest that eukaryotic organisms lack the required genes for the metabolism of Phi[12, 13]. In fact, it has been extensively documented that plants cannot metabolize Phi, and more recently, we showed that a number of microalgae species, including *Chlamydomonas reinhardtii*, *Ettlia oleoabundans*, and *Botryococcus braunii,* are unable to use Phi as a sole P source[14].

To test our theory that metabolic engineering could be used to produce microalgae capable of outcompeting other organisms in open and closed reactors, we introduced the *ptxD* gene from *P. stutzeri* WM88 in microalgae. We found that in contrast to the parental cells, the engineered *C. reinhardtii* lines are capable of using Phi as the sole P source (Fig. 1a). In fact, we found that the growth rate of the *C. reinhardtii* engineered lines in media containing Phi as sole P source was quite similar to that of the parental cells or its own in media containing Pi as a P source. Therefore, we demonstrated that it is possible to generate microalgae strains capable of converting a non-metabolizable source (Phi) of an essential nutrient (P) into a chemical form (Pi) that can be easily incorporated into their metabolism, without compromising growth and productivity[10].

As mentioned above, maintaining microalgae monocultures in open pound systems has been difficult, due to contamination by "weedy" microalgae and non-photosynthetic microbes. We hypothesize that an effective and selective environment for favouring monocultures is the use of culture medium containing Phi as sole source of P (Phi media) and in which the growth of *ptxD*-engineered microalgae lines would be dominant because non-engineered microalgae, including *C. reinhardtii* and most bacteria, are unable to metabolize Phi. To test this concept, we designed growth competition experiments using a multi-cultivator photobioreactor to determine whether an engineered *ptxD-C. reinhardtii* line has a competitive advantage to outgrow undesirable contaminants when cultivated in media supplemented with Phi as P source. We observed that when the *C. reinhardtii,* engineered or not to express the *ptxD* gene*,* was cultured in media containing Pi as P source under non-sterile conditions, it had limited growth as a consequence of the growth of contaminating bacteria present in the water or salts used to prepare the growth media. In contrast, when the engineered *C. reinhardtii lines* were grown in media containing Phi as a sole source of P, it grew vigorously and overcame contamination, showing that Phi media, in conjunction with an engineered microalgae strain, can be used to effectively control the growth of

biological contaminants naturally present in the water and reagents used to prepare media. The engineered *C. reinhardtii* was also co-cultured in direct competition with *Scenedesmus obliquus* and *Haematococcus pluvialis,* two microalgae species unable to metabolize Phi. When these mixtures of two microalgae were cultivated in media containing Pi, the two non-engineered species, which naturally grow faster than *C. reinhardtii*, were better competitors and outgrew the engineered *C. reinhardtii* strain. By contrast, under Phi treatments, we observed that engineered *C. reinhardtii* proliferated faster and dominated the culture system, quickly outgrowing the non-engineered *S. obliquus* and *H. pluvialis* microalga species. We also tested the capacity of the Phi system to control environmental contaminants in open, non-sterile, wall-shaped bioreactors. In media containing Pi as P source using this non-sterile system, *C. reinhardtii* was unable to grow because of biological contamination that produced a brownish coloured media, whereas in media containing Phi as a sole P source, the engineered *C. reinhardtii* line dominated the system producing a saturated green coloured culture, which continued to grow normally until 16 days after inoculation (Fig. 1b). These experiments clearly show that the capacity of metabolizing Phi can provide a competitive advantage to engineered strains, which is crucial to limit the invasion of open or closed culture systems by unwanted microalgae and many other microorganisms[10].

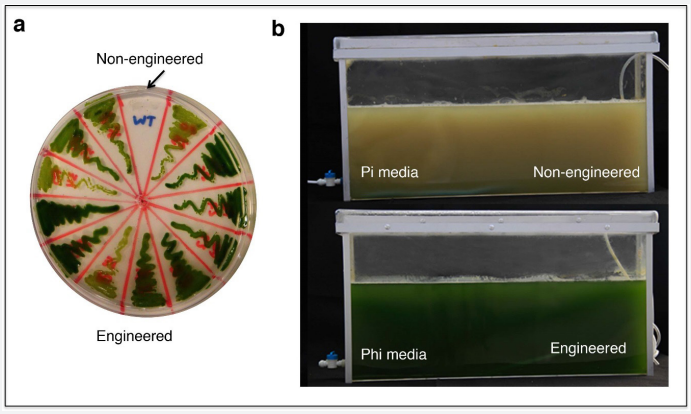

Figure 1. The non-engineered strain (WT) and twelve independent engineered Chlamydomonas reinhardtii clones harbouring the ptxD gene were grown in artificial media containing an antibiotic as the selective agent (a). One of these engineered clones was then selected to grow in wall-shaped bioreactors (b) using phosphite (Phi) containing media as the phosphorus source (bottom panel), in comparison with the WT grown in phosphate (Pi) containing media (top panel), under non-sterile conditions.

As mentioned previously, one of the major constraints for the production of microalgal biomass is the control of microalgae and bacterial contaminants. Some strategies have been adopted with some success such as monitoring the growth of potential contaminants in order to apply antibiotics and fungicides in the appropriate time, or using very high starting inoculum to attempt to decrease competition from contaminant organisms, together with meticulous cleaning of the reactors[4, 15, 16, 17]. The Phi approach is potentially a very effective alternative for generating an effective system to control biological contaminants for the cultivation of microalgae in open and closed culture systems and which eliminates the need for antibiotics, herbicides, and fungicides. A similar strategy was successfully shown to be effective for producing engineered plant varieties capable of using Phi as a sole source of P, which can outcompete weeds when fertilized with Phi instead of Pi[18].

The Phi system to control biological contamination in microalgae culture systems still requires testing in larger scale reactors. However, it potentially would significantly reduce the costs of operation of large photobioreactors, because by culturing Phi-metabolizing strains, the need for media and reactor sterilization would no longer be required. Moreover, the Phi-based system also has the potential to reduce cost of biomass production or industrially relevant compounds in closed bioreactors and raceway ponds. The Phi system would have an important impact on the microalgae industry by permitting different microalgae to be metabolically engineered, enabling the use of raceway ponds for the production of numerous industrially interesting microalgae species that are not currently exploited for comercial purposes. Additionally, the Phi system would facilitate the cultivation of microalgae consortia to produce more complex mixes of compounds or even the use of microalgae and algae-growth-promoting bacteria, engineering them both to

metabolize Phi. We are currently validating the Phi technology for massive algal culturing in large-scale photoreactors and race-way ponds for the generation of added value products.

References

1. Al-Qasmi, N. et al. A Review of Effect of Light on Microalgae Growth. *Proceedings of the World Congress on Engineering* 1, 1-3 (2012).
2. Williams, P.J. & Laurensa, L.M. Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. *Energy and Environmental Science* 3, 554-590 (2010).
3. Ghayal, M.S. and Pandya, M.T. Microalgae biomass: a renewable source of energy. *Energy Procedia* 32, 242–250 (2013).
4. Ramanana, R. et al. Algae–bacteria interactions: Evolution, ecology and emerging. *Biotechnology Advances* 4, 14–29 (2016).
5. Simas-Rodrigues, C. et al. Microalgae for economic applications: advantages and perspectives for bioethanol. *Journal of Experimental Botany* 66, 4093-4095 (2015).
6. Raja1, R. et al. Biomass from Microalgae: An Overview. *Oceanography* 1, 1-7 (2014).
7. Milledge, J. Commercial application of microalgae other than as biofuels: a brief review. *Environmental Science and Bio/Technology* 10, 31-41 (2010).
8. Zhang, X., et al. Current status and outlook in the application of microalgae in biodiesel production and environmental protection. *Front Energy Research* 2, 1–15 (2014).
9. White R.L. and Rebecca R.A. Long-Term Cultivation of Algae in Open-Raceway Ponds: Lessons from the Field. *Industrial Biotechnology* 11, 213-220 (2015).\
10. Loera-Quezada, M. et al. A novel genetic engineering platform for the effective management of biological contaminants for the production of microalgae. *Journal of plant Biotechnology*, Accepted Article; doi: 10.1111/pbi.12564 (2016).
11. White, A.K. and Metcalf, W.W. Microbial Metabolism of Reduced Phosphorus Compounds. *Annual Reviw of Microbiology* 61, 379-400 (2007).
12. Ávila, F.W., Faquin, V., Klynger, A., et al. Effect of phosphite supply in nutrient solution on yield, phosphorus nutrition and enzymatic behavior in common bean (Phaseolus vulgaris L.) plants. *AJCS* 7:713–722 (2013).
13. Berkowitz, O., Jost, R., Kollehn, D.O., et al. Acclimation responses of Arabidopsis thaliana to sustained phosphite treatments. *J Exp Bot* 64:1731– 1743. doi: 10.1093/jxb/ert037 (2013).
14. Loera-Quezada, M.M., Leyva-González, M.A., López-Arredondo, D.L., Herrera- Estrella, L. Phosphite cannot be used as a phosphorus source but is non-toxic for microalgae. *Plant. Sci.* 231:124–130. doi: 10.1016/j.plantsci.2014.11.015 (2015).
15. McBride, R.C. et al. Contamination Management in Low Cost Open Algae Ponds for Biofuels Production. *Industrial Biotechnol* 10, 221–227 (2014).
16. Kazamia, E. et al. An Engineered Community Approach for Industrial Cultivation of Microalgae. *Industrial Biotechnol* 10, 184-190 (2014).
17. Shurin, J.B. et al. Industrial-strength ecology: Trade-offs and opportunities in algal biofuel production. *Ecology Letters* 16:1393–1404 (2013).
18. López-Arredondo, D.L. and Herrera-Estrella, L. Engineering phosphorus metabolism in plants to produce a dual fertilization and weed control system. *Nat Biotechnol* 30:889–893. doi: 10.1038/nbt.2346 (2012).

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GE Salmon, GE Mushrooms, and Other Ingredients in a Stew of Controversy

Phill Jones

As highlighted in the February 2016 issue of the *Information Systems for Biotechnology News Report*, the US Food and Drug Administration has completed its two-decade review of genetically engineered (GE) salmon and approved it as fit for human consumption. The product of AquaBounty Technologies (Maynard, Massachusetts), the GE salmon mature to market size about twice as fast compared with conventional farm-raised Atlantic salmon. The engineered salmon have a recombinant DNA construct that includes a Chinook salmon growth hormone gene under the control of an ocean pout gene promoter that keeps the growth hormone gene active. The normal growth hormone gene of Atlantic salmon is only active during the summer. The GE salmon will be the first GE animal sold as food for humans in the United States unless protestors get their way.

During March, a group of environmental and food-safety organizations filed a lawsuit in a California federal district court against the US Department of Health and Human Services, the FDA, and the US Fish and Wildlife Service. The plaintiffs alleged that the FDA's approval of the GE salmon was unlawful, because the agency failed to adequately asses the full range of potentially significant environmental and ecological effects. Specifically, they claimed that the FDA completed "an extremely limited environmental assessment," rather than a full Environmental Impact Statement. Among the supposed environmental threats is "the risk that GE salmon will escape from the facilities where they are manufactured or grown and interbreed with wild endangered salmon." This may be asking too much of the GE salmon, which AquaBounty engineered to be reproductively sterile.

The plaintiffs further alleged that the FDA approval violated the Endangered Species Act. They said that the FDA had "failed to consult with the federal fish and wildlife agencies to insure that its approval of AquaBounty's application was not likely to jeopardize endangered and threatened species or adversely modify critical habitat."

One allegation about the limitations of the Federal Food, Drug, and Cosmetics Act could hobble the FDA's future regulation of GE animals. "FDA's decision to approve AquaBounty's GE salmon application should be vacated and set aside," the plaintiffs urged, "because FDA lacks the statutory authority to regulate GE animals as a 'new animal drug' under the FFDCA. The FFDCA does not explicitly grant FDA authority to regulate GE animals." The FDA regulates the GE salmon under the new animal drug provisions of the FFDCA on the basis that the recombinant DNA introduced into the fish meets the definition of a drug.

In their complaint, the plaintiffs also mentioned the FDA's decision that the agency will not require food from GE salmon to be labeled as such. The FDA concluded that food from GE salmon is as nutritious as food from conventional Atlantic salmon, and that there are no biologically relevant differences in the nutritional profile of GE salmon compared to that of other farm-raised Atlantic salmon. This aspect of the FDA's approval inspired several efforts in Congress to require labeling of GE salmon – so far, without effect.

Race to Block State-based GE Food Label Laws

In March, the US Senate Agriculture Committee approved a bill to preempt state labeling laws for "genetically modified organisms." The Safe and Accurate Food Labeling Act is similar to legislation passed by the House during June 2015. The bill faces an uncertain fate in the Senate.

Meanwhile, Senator Pat Roberts, Chairman of the Senate Agriculture Committee, introduced the Biotechnology Labeling Solutions bill, compromise legislation aimed to prevent the development of a patchwork of state-by-state labeling laws. The bill would establish a national voluntary labeling standard that would become mandatory if the marketplace fails to provide consumers with sufficient information

after several years. The legislation includes a federal preemption clause that would forbid a state or a subdivision of a state from establishing a requirement to label food produced by genetic engineering or food that contains an ingredient produced with genetic engineering.

Roberts emphasized that disparate state-based labeling laws would wreak havoc on the flow of interstate commerce of agriculture and food products. "That's exactly what this is about," he said, "the marketplace. Let me repeat that – this is about the marketplace. It's not about safety, or health, or nutrition…it's about marketing. Science has proven again and again that the use of agriculture biotechnology is 100 percent safe."

The compromise legislation, submitted as an amendment to the Defund Planned Parenthood Act of 2015, was the subject of a procedural vote on March 16. The bill failed to attract the necessary number of votes to advance in the Senate.

Earlier this year, Claire Parker, spokesperson for the Coalition for Safe Affordable Food, wrote about the urgent need to preempt state GE labeling laws. "Congress must act quickly to pass a national food labeling solution that offers farmers, families and food producers the certainty and access to the affordable and sustainable food supply they deserve," she said in a press release. "Time is running out, and consumers will ultimately pay the price of delay and inaction."

Vermont created this doomsday ticking clock. July 1 marks the date that the state's law mandating the labeling of food containing "GMO ingredients" will go into effect. Vermont will become the first state to require such labeling.

New Technology That Skirts APHIS Regulation Mushrooms

In recent years, the US Department of Agriculture's Animal and Plant Health Inspection Service has decided that about 30 GE plants fall outside the scope the agency's regulatory authority. Some of the plants were modified using zinc-finger nuclease and transcription activator-like effector nuclease systems. During April, APHIS decided that the agency will not regulate a GE white button mushroom (*Agaricus bisporus*). The mushroom had been engineered using CRISPR–Cas9.

Yinong Yang, plant pathologist at Pennsylvania State University, modified the mushroom to resist browning by introducing small deletions in a polyphenol oxidase gene. The anti-browning trait improves the mushroom shelf life and facilitates automated mechanical harvesting. The genetically engineered mushroom does not contain foreign DNA in its genome.

Last October, Yang asked Michael J. Firko, APHIS Deputy Administrator, for confirmation that the GE mushroom is not a regulated article. Firko provided that assurance, noting that the Plant Protection Act of 2000 gives APHIS the authority to regulate the importation, interstate movement, and environmental release of GE organisms that have the potential to be plant pests. APHIS scientists could see no reason to believe that the CRISPR-Cas9-edited mushrooms are plant pests.

Within a week, Firko provided similar news to DuPont Pioneer's Daria H. Schmidt, Director of Registration and Regulatory Affairs - North America. The company used CRISPR-Cas to develop waxy corn that has altered starch composition.

In a Penn State press release, Yang said that CRISPR-Cas9 technology could change the way that many people perceive genetic engineering. "There's too much divisiveness and hostility between pro- and anti-GMO camps," Yang said. "I hope development of the new technology will facilitate rational and productive dialogue among diverse groups of people, with a common goal to achieve food safety, food security, and agricultural and environmental sustainability."

Time to Deconstruct the Philosophy of US Biotech Regulation?

Despite Yang's hope for productive dialogue, new genetic engineering techniques may have the

opposite effect according to Jennifer Kuzma, co-director of the Genetic Engineering and Society Center at North Carolina State University. "With the wealth of possibilities now offered by newly developed gene-editing tools—particularly CRISPR–Cas9—debates about the safe and appropriate uses of GE are becoming more heated," Kuzma said in her *Nature* article. "In fact, in the 20 years that I have been involved in discussions about it, oversight of GE has never seemed so much like a powder keg waiting to explode."

Kuzma says that one issue that has dominated the debate is whether the focus of regulation of GE organisms should be on the GE organisms and the products made from the GE organisms, or on the process by which GE organisms were made. To a certain extent, the current product-based regulation system is an artifact of expediency. During the mid-1908s, the developers of the Coordinated Framework for Regulation of Biotechnology decided that the final product of genetic engineering could pose a potential risk to human health and the environment, but the process by which the product was made does not inherently pose a risk. This approach enabled agencies to shoehorn new products of biotechnology into established regulations. The tactic has fostered some odd stepchildren, such as the FDA's rationale for regulating certain GE animals on the basis that the animals harbor recombinant DNA molecules, which the FDA considers to be "new drugs."

Kuzma suggests that the time has come to reboot the debate about regulation. "Product-versus-process arguments reflect world views about the desired level of regulation for GE organisms," she says. "These underlying viewpoints should be made explicit, and the idea that product-based regulation is the only science-based approach rejected." Stakeholders could "help to establish a governance system that is both informed by the science and guided by the concerns and values of citizens."

Selected Sources

Brasher, Philip, "GMO Labeling Bill Stalls in Senate," Agri-Pulse Website (March 16, 2016). Available at: http://www.agri-pulse.com.

Cline, Meghan, "Chairman Roberts on Biotechnology Labeling: 'It's Time to Act'," U.S. Senate Committee on Agriculture, Nutrition, & Forestry Website (March 15, 2016). Available at: http://www.agriculture.senate.gov/newsroom.

Gill, Chuck, "Gene-edited Mushroom Created by Penn State Researcher Is Changing GMO Dialogue," Penn State News Website (April 19, 2016). Available at: http://news.psu.edu.

Kuzma, Jennifer, "Policy: Reboot the Debate on Genetic Engineering," Nature 531(7593):165-167 (March 10, 2016).

Parker, Claire, "New Economic Study Highlights Urgent Need for Congressional Action to Prevent Food Price Hikes on Families from Vermont GMO Labeling Law," Coalition for Safe Affordable Food Website (February 22, 2016). Available at: http://coalitionforsafeaffordablefood.org.

Salvatore, Cara, "Food-Safety Groups Sue FDA Over Approval of GE Salmon," Law360 Website (March 31, 2016). Available at: http://www.law360.com.

Waltz, Emily, "Gene-edited CRISPR Mushroom Escapes US regulation," Nature 532(7599):239 (April 21, 2016).

Wheeler, Lydia and Tim Devaney, "Voluntary GMO Labeling Bill Approved by Senate Agriculture Committee," Genetic Literacy Project Website (March 1, 2016). Available at: https://www.geneticliteracyproject.org.

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