Centre for Integrated Research in Biosafety
School of Biological Sciences
Tel: +64 3 336 95597
Email: jack.heinemann@canterbury.ac.nz

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

Dr. Stephen Cobb
New Organisms Manager
Hazardous Substances and New Organisms (HSNO)
Environmental Protection Authority

Re: Requested submission for reconsideration of APP203395

16 September 2019

Kia ora Dr. Cobb

On 31 July 2019 you requested from me a formal submission to a Decision-Making Committee (DMC) reconsidering the 2018 determination (EPA 2018a) to not class cells or organisms treated externally with double-stranded (ds)RNA molecules as new or genetically modified organisms. I welcome EPA's decision to reconsider its determination on application APP203395. In response to your request I provide the following four documents as my complete submission to the DMC:

1. **This letter.**
2. **A submission on scientific and other matters.**
3. **My comprehensive and peer-reviewed paper examining the scientific basis of the New Zealand Environmental Protection Authority HSNO Decision-Making Committee's May 2018 determination, which I sent to EPA on 4 June 2019 (Heinemann 2019).[1] This paper provided the EPA with new information that demonstrated that the DMC of the day was mistaken in relying upon the stated biological barriers to dsRNA causing heritable effects in eukaryotic organisms and it overlooked known heritable effects, some in pre-commercial stages of exploitation. Moreover, it failed to account for effects on exposed inseparable prokaryotic organisms.**

   If a gene technology meets the test of heritability it is sufficient to conclude that external treatments of organisms with dsRNA creates genetically modified organisms. The barriers that the DMC relied upon to disqualify external treatments from causing heritable effects are stated in paragraph 4.6 of its decision. They are that the dsRNA remains "solely as RNA molecules in the cell cytoplasm outside the nucleus, and therefore they do not integrate into the DNA of the eukaryote genome", is "not reverse-transcribed into DNA", and is "therefore not inheritable by the organism." The DMC was wrong to rely on these barriers.

- The membrane surrounding the nucleus is not maintained in all eukaryotic organisms. In animals, it is degraded once per cell cycle leading to mixing of nuclear and cytoplasmic content. Moreover, in many eukaryotes dsRNA is recruited to the nucleus in association with nucleus-localising proteins.
- There was no evidence to assert that dsRNA cannot be reverse transcribed, and no literature foundation that I could find provided certainty for this assertion for all eukaryotic species.
- In fungi at least there are replicating RNA molecules in the cytoplasm that express their genes and

[1] https://www.sciencedirect.com/science/article/pii/S0160412019306038

recombine with new RNA molecules taken into the cytoplasm. Moreover, in some eukaryotes dsRNA modifies chromosomes by causing DNA rearrangements and can increase the transition mutation rate of G:C pairs to A:T pairs. Finally, the EPA and the DMC did not account for the heritable effects externally applied dsRNA has by competing with endogenously produced dsRNA.

Potential adverse effects to human health or the environment that might result from treatments using dsRNA would not be considered if the May 2018 determination stands. Implications of the determination include but are not limited to—

- heritable changes in both targeted and non-target but exposed organisms. Neither the EPA nor the DMC appeared to consider the potentially harmful effects to human health, companion animals, livestock and beneficial plants, insects, protists, fungi and bacteria from exposures to unregulated open-air dispersals of dsRNA, which are not fully predictable from sequence information alone (Heinemann et al. 2013; NASEM 2016).
- the release of partial or whole viruses with RNA genomes. Penetration of RNA into plant, animal and fungal cells can be accomplished using simple and otherwise non-hazardous chemicals as delivery vehicles. Because these same formulations also cause penetration of cells by DNA, unintended release of viruses with DNA genomes is also possible. These viruses may cause disease in organism exposed on purpose or by accident.
- use of this method anywhere, anytime and on any kind of organism (not controlled under the Biosecurity Act or already genetically modified), including potential disease-causing microorganisms.
- effects on still to be described species. Neither the EPA nor the DMC were hesitant to extrapolate from the literature on RNA interference which is informed by a relatively small number of research organisms to all eukaryotes, even the thousands that are yet to even be described in New Zealand. As the US National Academies concluded: “More research is required to address the sustainability of, and off-target effects arising from, RNAi approaches” (NASEM 2016). The paper most frequently cited by EPA staff in their report says in bold type at the very beginning: “Recent advances have revealed unexpected diversity in their biogenesis pathways and the regulatory mechanisms that they access.” Later the same review concludes: “We are rethinking our views as to what constitutes an siRNA or miRNA. The rules about biogenesis and action are much more fluid than we thought” (Carthew and Sontheimer 2009).

  Without knowledge of the genomes of undescribed species there would be no ability to design dsRNAs that had no targets in these organisms. Risk mitigation would require controls to limit exposures. Such controls could only be achieved if the use of external treatments was regulated.

4. **A comprehensive and peer-reviewed paper examining the kinds of chemical and physical vectors, mentioned briefly above, that are in development for use as delivery vehicles for external dsRNA treatments (Heinemann and Walker). It further evaluates risks that should be considered on a case-by-case basis by a precautionary regulator.**

- It is obvious that external application of dsRNA to cells or organisms can have adverse effects because most of the first such products are intended to have adverse effects (usually lethal, but sometimes intergenerational) because they are pesticides.
- Even lawful uses may cause harm through off-target effects, and these off-target effects cannot be predicted with accuracy. Exposures are designed to be at combination of concentrations, pathways of exposure and/or using active molecules that are not found in nature.

- The use of nucleic acids that do not occur in nature as well as stabilizing forms that otherwise would not be suitable for external treatments, including single-stranded DNA, single-stranded RNA (e.g. guide RNAs) and chimeric mixtures of DNA and RNA are described in patents. Over 100 known chemical modifications can be added to *in vitro* synthesized RNA molecules.
- Exposures to externally applied dsRNAs besides by ingestion have received far less attention, especially in humans and other large animals. Importantly, even less is known about the effects of co-formulants on the stability and effectiveness of the dsRNA. This cannot be known until tested and only will be tested if they are subject to regulatory oversight. Outside of pharmaceuticals, we did not find much discussion on purposeful inhalation exposure but we did find many references to application technologies that create the potential for such exposure, for example by aerosolization through spray applicators. At present, there is insufficient knowledge of these exposure pathways to justify deregulation of external dsRNA treatments.
- Nothing is known of the potential to tamper with products that cause uptake of dsRNA. Without post-market risk mitigation strategies that could come from a pre-market risk assessment, we may not know until after harm has been caused.

Biodiversity of potentially exposed environments is both high and largely undescribed (USDA ; Womack et al. 2010) making it both difficult to limit exposures to intended species and predict the effects of all exposures. Airborne microorganisms including eukaryotes can number in the hundreds of thousands per m^3 and "can be as diverse as those in terrestrial environments, including soils" (Womack et al. 2010).
Metagenomics surveys indicate from 10,000-830,000 species per gram of soil (Dance 2008). According to the United Nations Food and Agriculture Organization: "Over 1000 species of invertebrates may be found in a single m^2 of forest soils. Many of the world's terrestrial insect species are soil dwellers for at least some stage of their life-cycle. A single gram of soil may contain millions of individuals and several thousand species of bacteria. A typical, healthy soil might contain several species of vertebrate animals, several species of earthworms, 20-30 species of mites, 50-100 species of insects, tens of species of nematodes, hundreds of species of fungi and perhaps thousands of species of bacteria and actinomycetes. Soil contains the organism with the largest area. A single colony of the honey fungus, Armillaria ostoyae*, covers about 9 km^2" (FAO 2015).*

The Environmental Protection Authority will always be challenged in the fast-paced arena of biotechnology. Fortunately, the HSNO Act provides a solid foundation for guidance and direction on the kinds of biotechnologies that cause concern if or when they are used in the environment. In essence, if either an *in vitro* technique modifies a gene or genetic material of an organism, or an organism is descended from one with modified genes or genetic material, EPA has been tasked with evaluating it for safe use or release.

I believe that the use of dsRNA for external treatments of cells or organisms could provide benefits. However, it is a gene technology and requires oversight.

I wish to draw attention to use of confusing, ambiguous, sometimes conflicting, descriptions of terms, purposes and scope in APP203395. One clear example is the important distinction between how you described the determination in your 31 July 2019 letter to me, which is consistent with the May 2018 decision paragraph 1.1, but which is not consistent with decision paragraph 6.1. The decision as stated in paragraph 6.1 is not limited by the intention[2] of the treatment (EPA 2018a). Moreover, neither paragraph 1.1 nor 6.1 provides guidance on how the treatment would be restricted to causing only the intended response, range of sizes and other characteristics of molecules that might be described as siRNA or dsRNA, the temporal and biochemical range of potential responses, and what is meant by the undefined term "siRNA response".[3]

[2] Intention of treatment is limited in paragraph 1.1 of DMC decision: "treated…to induce a small interfering RNA (siRNA) response."
[3] I could find no mention of the term "siRNA response" searching within the key references in the EPA staff report.

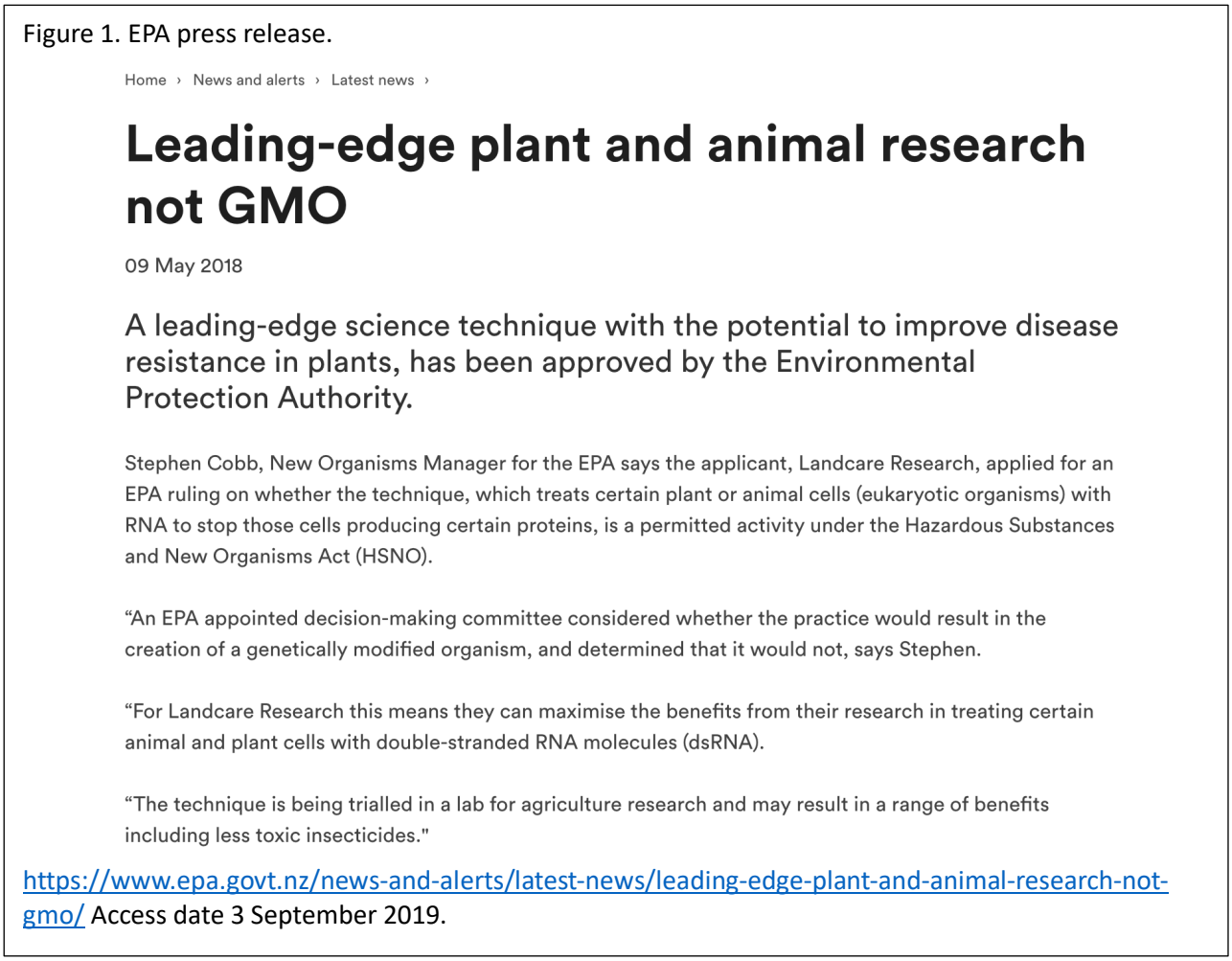
Table 1. Purpose of the application

Source	Description‡	Notes
Application "To obtain a determination of whether an organism is a new organism" APP203395 (Trought, 2018).	**Eukaryotic cells** that have been **transiently** transfected with *synthetic molecules of double stranded RNA* to inhibit (temporarily) the activity of the complementary RNA.	Application for eukaryotic cells (which may be tissue culture) becomes a determination for all eukaryotic organisms. Application for use of synthetic/artificial dsRNA molecules contrasts with determination for all dsRNA molecules of undisclosed source, size or other characteristics. Application originally limited to an activity on the mRNA target that is temporary changed to a decision allowing any form of expression suppression and then to any dsRNA treatment outcome.
EPA Staff Report "Determining whether eukaryotic cell lines treated with double-stranded RNA are genetically modified organisms" (EPA, 2018b).	[the applicant] seeks a determination…on whether **eukaryotic cells** treated with *artificially synthesised* dsRNA to **transiently** suppress the expression of user-selected genes are new organisms for the purpose of the Act.	
EPA Decision "Purpose of the Application" page 1 (EPA, 2018a).	"**eukaryotic cell lines** that have been *treated with externally applied double-stranded RNA* molecules for the purpose of inducing a transient small interfering RNA (siRNA) response are new organisms."	
EPA Decision section 2 (EPA, 2018a).	"**eukaryotes** *treated with double-stranded RNA* molecules were considered genetically modified organisms."	
‡Highlighted terms are inferred as homologous[4] in the different passages.		

[4] Homologous means related by descent.

The former DMC described the application in various, and significantly different, ways (Table 1) from the applicant (Trought 2018) or EPA staff (EPA 2018b). The applicant sought permission to use "synthetic" dsRNA, restricted as well to those that would cause a temporary effect on and only on the "activity of the complementary RNA" and in eukaryotic cells (Trought 2018). The purpose of the application was restated more or less similarly by EPA staff but not by the DMC (Table 1). The DMC introduced the phrase "eukaryotic cell lines" to the stated purpose, which could imply a narrowing of the scope to tissue culture or other "certain cells" as mentioned in a press release (Figure 1).[5] Simultaneously the DMC introduced the terminology "siRNA response". Whereas the applicant also conflates the active molecule siRNA with a process ("the siRNA process"), it never uses the term siRNA response, instead referring to RNA interference.

Figure 1. EPA press release.



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Leading-edge plant and animal research not GMO

09 May 2018

A leading-edge science technique with the potential to improve disease resistance in plants, has been approved by the Environmental Protection Authority.

Stephen Cobb, New Organisms Manager for the EPA says the applicant, Landcare Research, applied for an EPA ruling on whether the technique, which treats certain plant or animal cells (eukaryotic organisms) with RNA to stop those cells producing certain proteins, is a permitted activity under the Hazardous Substances and New Organisms Act (HSNO).

"An EPA appointed decision-making committee considered whether the practice would result in the creation of a genetically modified organism, and determined that it would not, says Stephen.

"For Landcare Research this means they can maximise the benefits from their research in treating certain animal and plant cells with double-stranded RNA molecules (dsRNA).

"The technique is being trialled in a lab for agriculture research and may result in a range of benefits including less toxic insecticides."

https://www.epa.govt.nz/news-and-alerts/latest-news/leading-edge-plant-and-animal-research-not-gmo/ Access date 3 September 2019.

Did the DMC mean RNA interference by the phrase "siRNA response", or something less comprehensive than RNA interference? If so, why and how would any treatment be able to avoid RNA interference? What about exposures to siRNA molecules that cause effects other than RNA interference, but would certainly then be siRNA responses and not necessarily RNA interference, such as occurs in environmentally inseparable prokaryotes[6] (Heinemann 2019; Shabalina and Koonin 2008)? An EPA press release implied that many kinds of eukaryotes that are not plants or animals also had not been considered (Figure 1). Only

[5] I consider the press release had the potential to mislead the public through its suggestion that the decision applies only to plant and animal exposures, only certain cells, and would be limited to effects on protein production. None of these kinds of restrictions are inherent in the decision.

[6] This quote is from the Shabalina and Koonin (2008) paper frequently cited by EPA staff in their report. "RNAi-like mechanisms do exist in prokaryotes and seem to show functional analogies both to the miRNA and the siRNA pathways of eukaryotes, even though the proteins involved in these processes are nonhomologous."

plants and animals were mentioned, and only “certain cells” of them. The announcement gave no indication of how treatments would be so finely controlled that only selected cells of only plants and animals would be exposed.

The scope changes later in the DMC decision and conclusion text. At this stage there is no longer a specific reference to cells. Now it becomes clear that the determination is not restricted to cell lines but applies to organisms and cells (and some eukaryotes are single-celled organisms). The rephrasing releases the treatment from effects only on mRNA molecules, making possible the many other kinds of effects dsRNA, including siRNA, can have on DNA or other genetic material (reviewed in Heinemann 2019; Heinemann et al. 2013). The final text also removed any need to confine the treatments to those that had only temporary effects (which in any case were never defined). While section 26 of the HSNO Act gives considerable power to the DMC, it surely cannot use any application as a pretext to make any kind of determination on anything. Therefore, how are these significant variations introduced after the application and staff report justified by the DMC?

The DMC removed the terms “synthetic” (used by the applicant) and “artificially synthesised” (used by EPA staff) for delimiting the kind of dsRNA molecules used in treatments. Those terms emphasise that treatments involve *in vitro* techniques. As I explain in my submission document on further scientific and other matters, use of *in vitro* techniques is an unavoidable test of whether an organism is a new organism. Accepting the use of these techniques as affecting genes or other genetic material, as they do, would have been sufficient evidence to determine that eukaryotic cells or organisms treated externally with dsRNA were new and genetically modified organisms.

However, even if the determination could be restricted somehow to avoid *in vitro* techniques in the construction of the dsRNA molecules - which I do not believe is possible to do - the determination would not change. This is because the methods used to penetrate cells or organisms with dsRNA molecules is also a kind of *in vitro* technique, just as it is when DNA molecules are used to create genetically modified organisms.

Finally, I would like to say that whereas dsRNA-based gene technologies may prove to be beneficial, only a proper risk assessment can determine when their use might be inappropriate. For example, the effects of using dsRNA on protists has different potential harm pathways (not extreme or unusual or unrepresentative, just different) than on plants. In some environments, dsRNA is far more stable than previously assumed because soil absorption has been mistaken for degradation, and it is also bio-available to microorganisms (Parker et al. 2019). The EPA decision as it stands rules out making case-by-case assessments because it does not require any future risk assessment to be made before use.

Some of the most interesting and unexpected effects of dsRNA on eukaryotes are observed in the protists. These organisms are ubiquitous in outside environments where dsRNA products may be used and yet many of these organisms are still to be described. “Toward the end of the last millennium, there was an estimated 4,300 described free-living ciliate species. In the new millennium, species of free-living ciliates are being described at an average rate of ~50 per year” (Warren et al. 2017).
They are far too numerous to be considered either as extreme or special cases. Populations range from 1000s of protozoans per gram of agricultural soil, to 10s of thousands in prairie soils up to 100s of thousands in forest soils (Fortuna 2012). They provide critically important ecosystem services. “Heterotrophic microeukaryotes such as ciliates are thought to be of considerable importance in aquatic ecosystems, as they are major predators of bacteria and constitute a nutritional resource for other protozoa, invertebrates, and probably fish larvae. In addition, protozoan bacterivory contributes to enhanced decomposition of leaf detritus—a vital nutrient resource in streams—by increasing turnover of bacterial populations through predation” (Dopheide et al. 2009). The effects of dsRNA on the DNA components of protozoan genomes demonstrates that treatments with dsRNA are within scope of the HSNO Act and effects should be assessed for risk on a case-by-case basis.

Recommendations

In view of the information I have presented in the four documents that compose this submission, I recommend to the DMC that it—

1. retract the 1 May 2018 decision on application APP203395.
2. issue a new decision that in accordance with section 26 of the Act, and having regard to the relevant information, the EPA has determined that cells or organisms that have been treated with externally applied double-stranded RNA are new organisms for the purpose of the Act.

Once it has been decided that external treatments of eukaryotes create genetically modified organisms, it may be appropriate to simplify risk assessment procedures in some cases. Tissue culture experiments in contained laboratories may have negligible potential to cause harm. Risk could similarly likely be managed for treatments occurring in contained glasshouses where plants are the subject of experimentation and so long as other potentially exposed organisms cannot escape. As forecast by the EPA, however, this technology is intended to be used out-of-doors at least by some and may be used by anyone using easily accessed chemicals or abrasives (see my submission document four). The outdoor applications will use chemical or physical vehicles that alter the efficiency of uptake of dsRNA to levels that do not occur in nature, and by exposure pathways that have been little studied. Therefore, regulation of external treatments of organisms with dsRNA is the option consistent with the purpose and principles of the new organism provisions of the HSNO Act.

Nāku iti noa, nā,

Dr Jack A Heinemann
Professor of Molecular Biology and Genetics[7]

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[7] This letter and all accompanying documents are provided in accordance with the University of Canterbury Critic and Conscience of Society and Academic Freedom Policy (2018) as the author's expert opinion and not as statements of the opinion of the University of Canterbury.

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