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Review article

Should dsRNA treatments applied in outdoor environments be regulated?

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ABSTRACT

The New Zealand Environmental Protection Authority (EPA) issued a Decision that makes the use of externally applied double-stranded (ds)RNA molecules on eukaryotic cells or organisms technically out of scope of legislation on new organisms, making risk assessments of such treatments in the open environment unnecessary. The Decision was based on its view that the treatment does not create new or genetically modified organisms and rests on the EPA's conclusions that dsRNA is not heritable and is not a mutagen. For these reasons EPA decided that treatments using dsRNA do not modify genes or other genetic material. I found from an independent review of the literature on the topic indicated, however, that each of the major scientific justifications relied upon by the EPA was based on either an inaccurate interpretation of evidence or failure to consult the research literature pertaining to additional types of eukaryotes. The Decision also did not take into account the unknown and unique eukaryotic biodiversity of New Zealand. The safe use of RNA-based technology holds promise for addressing complex and persistent challenges in public health, agriculture and conservation. However, by failing to restrict the source or means of modifying the dsRNA, the EPA removed regulatory oversight that could prevent unintended consequences of this new technology such as suppression of genes other than those selected for suppression or the release of viral genes or genomes by failing to restrict the source or means of modifying the dsRNA.

1. Introduction

In May 2018 the Decision-Making Committee of the New Zealand Environmental Protection Authority (EPA) announced that eukaryotic cells or organisms treated with double-stranded (ds)RNA are not new organisms (EPA, 2018a). The Committee's Decision in a biotechnology context concerns the application of exogenous (*exo*)-dsRNA for the purpose of causing RNA-interference (RNAi), the name given to a number of related pathways that regulate gene expression (Box 1).

Exo-dsRNA is dsRNA introduced into the cells of organisms to alter gene expression using techniques that cause it to penetrate cell walls and/or membranes. Both because the nature and source of the dsRNA applied as *exo*-dsRNA is undefined by the EPA in its Decision, and because dsRNA may have effects in addition to the intended RNAi, herein and like others (Parker et al., 2019) I will often use the term *exo-ds*RNA as a more generic description than exo-*si*RNA, the term used by EPA.

As explained in more detail in Box 1, RNAi-instigating dsRNA reagents are known as, among other names, miRNA (micro-RNA), siRNA (short-interfering RNA) and piRNA (piwi-interacting RNA). These reagents interact with mRNA (messenger RNA) molecules that contain similar nucleotide sequences to either prevent their translation, or cause *de novo* methylation of chromosomal DNA and histones and thereby inhibit transcription of genes with a shared nucleotide sequence (Fig. 1).

Research on dsRNA-mediated gene regulation has advanced rapidly, but there is much still unknown about its environmental fate (Parker et al., 2019) and biochemistry, even in the relatively few model organisms in which it has been studied (Djupedal and Ekwall, 2009; Ghildiyal and Zamore, 2009). Already it is clear that the biochemistry of dsRNA-mediated gene regulation is different between plants, animals, and fungi (Ghildiyal and Zamore, 2009). Perhaps even more importantly, almost nothing is known about RNAi pathways in species unique to New Zealand, where there remains much biological diversity to be explored. According to the Encyclopedia of New Zealand, Te Ara, “over 80% of the 2500 species of native conifers, flowering plants and ferns are found nowhere else.” “The best guess of the numbers of land-based native plants and animals is around 70,000 species. Insects and fungi dominate, each having an estimated 20,000 species – many are not yet described” (Manatū Taonga Ministry for Culture and Heritage).

Herein I analyze the major arguments and information sources on which the Decision-Making Committee and EPA staff based their finding that dsRNA treatments do not create genetically modified organisms and point out flaws in their decision-making (Fig. 2). The potential hazards (FIFRA, 2014; Heinemann et al., 2013; Parker et al.,

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Box 1
The science of RNAi

RNA interference (RNAi) is a form of gene regulation in eukaryotes that has many potential biotechnological applications which are being discussed by regulators worldwide (FIFRA, 2014; Heinemann et al., 2013). RNAi pathways are found in nearly all eukaryotes (Agrawal et al., 2003). RNAi is often referred to as gene silencing, but it also is known to sometimes cause an increase in the expression of genes (Carthew and Sontheimer, 2009; Kim et al., 2009).

RNAi results in what is called post-transcriptional gene silencing and transcriptional gene silencing (Kalinava et al., 2018). Post-transcriptional gene silencing occurs through dsRNA-mediated endonucleolytic cleavage or exonucleolytic destruction of the transcript or inhibition of translation of the transcript (Carthew and Sontheimer, 2009; Rechavi, 2014). In some organisms, dsRNA-mediated transcriptional gene silencing is caused by the modification of histones and DNA, while in others it may only be modification of histones, resulting in formation of heterochromatin and a decrease in transcription (Matzke and Birchler, 2005).

The nomenclature for dsRNAs is expansive, but the main classes include siRNA (short-inhibitory RNA), miRNA (microRNA) and piwi-interacting RNAs (piRNA) (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009). These types are foundation substrates in biochemical pathways involving Argonaute proteins that cause RNAi (Fig. 1).

The nomenclature should be used as an indicative guide to biogenesis of the dsRNA, but not the activity of the active form. This is because regardless of their source, dsRNAs share the same pathways in the cell (Ghildiyal and Zamore, 2009). “For example, siRNA is able to mimic microRNA (miRNA) to inhibit translation or elicit the degradation of [messenger RNAs] with partial sequence complementarity” (Zhou et al., 2014).

All three active forms derive from longer dsRNAs. Cytoplasmic Dicer converts the longer form of siRNA and miRNA into the active form of about 21–23 nucleotides. Argonaute proteins bind to the RNA and carry out the regulatory functions (Carthew and Sontheimer, 2009). Drosha (or Dcl1) acts in the nucleus to process pri-miRNA into pre-miRNA, which after transport to the cytoplasm is further processed to miRNA by Dicer (Kim et al., 2009).

Once associated with the Argonaute proteins, one strand of the dsRNA molecule is degraded and the other serves to guide the protein complex to its target. Some eukaryotic species have Argonaute proteins that can bind either miRNA or siRNA, and some that specialize in one or the other, while other species have Argonaute proteins that distinguish between miRNA and siRNA based on the structural features of the dsRNA.

It is the Argonaute proteins that determine the mechanism of silencing (Rankin, 2015; Rechavi, 2014). Some Argonaute proteins, such as AGO2 in humans, have an endonuclease activity called slicer. These complexes cleave the target messenger RNA molecule. Argonaute proteins AGO1–4 from humans and AGO1 from *Drosophila melanogaster* are examples that cause translational inhibition or degradation of the target transcript through exonucleolytic decay. Ago1 of *Schizosaccharomyces pombe* and AGO4 and AGO6 of *Arabidopsis thaliana* are examples that cause transcriptional gene silencing through heterochromatin formation (Kim et al., 2009).

The dsRNA is sorted among competing Argonaute proteins according to the number of mismatches and structural distortions, not because particular dsRNAs are genetically determined to exclusively follow pathways dedicated to miRNA or siRNA (Ghildiyal and Zamore, 2009). In *Drosophila*, AGO1 tends to favor duplexes with more bulges and mismatches and results in translation inhibition while AGO2 prefers duplexes with near perfect complementarity and results more often in messenger RNA cleavage (Ghildiyal and Zamore, 2009). However, even these rules are different between animals such as *Drosophila* and plants (Ghildiyal and Zamore, 2009) making it difficult to generalize for all eukaryotes. In short, intending a particular dsRNA to be an siRNA or an miRNA does not mean that it will be.

The binding strength of the guide strand and target influences the outcome of the interaction. The combination of near perfect antisense pairing between guide strand and target involving an Argonaute with slicer activity results in strand cleavage by an endonuclease activity (Massirer and Pasquinelli, 2013). The larger the number of mismatches between the guide and target RNA, the more likely the silencing will be caused by exonucleolytic decay or translational inhibition (Massirer and Pasquinelli, 2013).

While *endo*-siRNA, miRNA and piRNA may be born differently, they are not reliably distinguished by the silencing biochemistry. Both miRNA and piRNA arise from transcription of genomic DNA. Although this can also be true for siRNA, such as from transgenes or transposons (endo-siRNAs), the term is also often reserved for *exo*-siRNAs even if they have a hairpin structure. In general, miRNAs are not transcribed from the protein coding region of a gene and may have more mismatches with their targets. The converse is true for siRNAs. Thus, miRNA, piRNA and endo-siRNA all first appear in the nucleus and exo-siRNA does not (Carthew and Sontheimer, 2009).

It is not possible to confidently extrapolate the outcome of exposure to exo-siRNA based on similarity of nomenclature to endo-siRNA, as EPA has tried to do. Because of differences between organisms and differentiated cell types, generalizations based even on the structure of the dsRNA molecule often fail.

2019) that may arise from the use of dsRNA in the open air also will be briefly addressed.

2. The decision

Environmental biotechnologies that could cause adverse effects to New Zealand's biodiversity are regulated by the EPA under the Hazardous Substances and New Organisms (HSNO) Act http://www.legislation.govt.nz/act/public/1996/0030/93.0/DLM381222.html (1996). Under the Act, EPA makes two kinds of decisions (Fig. 3). The first kind, *which is the kind relevant to this article*, is under section 26: whether or not an organism or substance will be regulated. If it is not regulated, then a risk assessment by the Authority will not be done regardless of whether or not a risk assessment would be useful. The second kind, under section 25 of the HSNO Act, is whether or not a regulated organism or substance could be used in a safe manner. This determination follows from a mandatory risk assessment.

EPA could have determined that dsRNA used to modify an organism's genes or genetic material could be a hazardous substance and therefore should be regulated. However, RNA is not listed as a hazardous substance according to a search of the EPA's databases: “Approved hazardous substances with controls”, “Chemical Classification and Information Database”, or “New Zealand Inventory of Chemicals.” Moreover, the Ministry of Primary Industries includes RNA in the “Negligible Risk Register” (MPI, 2018).

The EPA could instead regulate the use of dsRNA if its use resulted in a genetically modified organism as defined by the HSNO Act, because genetically modified organisms are defined as new organisms. The HSNO Act says that a “genetically modified organism means…any organism in which any of the genes or other genetic material—(a) have been modified by *in vitro* techniques; or (b) are inherited or otherwise derived, through any number of replications, from any genes or other genetic material which has been modified by *in vitro* techniques” (1996). Some mutagens that modify genes or other genetic material are exempted from the regulations (Fig. 2). The exemptions are limited to

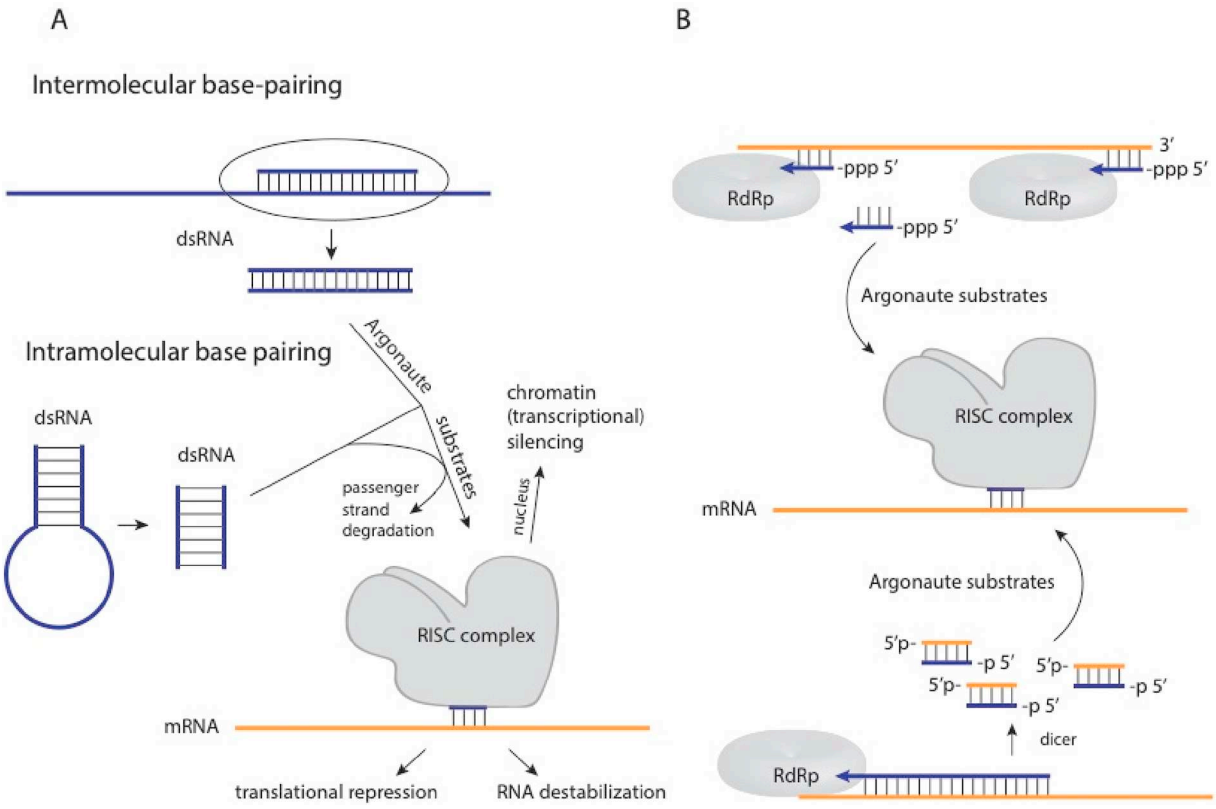

Fig. 1. Source of dsRNA substrates used in RNA interference.
(A) Dicer/drosha processed ~21 nucleotide precursors of linear double-stranded (dsRNA) or stem-loop structures are bound to Argonaute proteins. One strand (passenger) is degraded and the other strand is used to identify a target RNA molecule by base-pairing. The Argonaute-RNA becomes part of a larger RNA-induced silencing complex (RISC) or directed to the nucleus where it results in transcriptional inhibition. (B) Secondary and tertiary dsRNA molecules with either triphosphate or monophosphate (p) ends can be generated as part of RNA interference, either by primed or unprimed RNA-dependent RNA polymerase (RdRP) synthesis (Maida and Masutomi, 2011).

only "organisms that result from mutagenesis that uses chemical or radiation treatments that were in use on or before 29 July 1998" (2016). Other mutagens are regulated as confirmed by a 2015 High Court Decision (Kershen, 2015).

EPA received a request by an applicant to determine whether: "Eukaryotic cells that have been transiently transfected with synthetic molecules of double stranded RNA to inhibit (temporarily) the activity of the complementary RNA" (Trought, 2018) were new organisms. Staff rephrased the purpose of the application to "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" (emphasis added to EPA, 2018b), largely preserving the narrow scope set by the applicant and further clarifying that the dsRNA must be used to suppress the expression of a particular gene. The Decision-Making Committee significantly broadened the purpose to all "eukaryotes treated with double-stranded RNA molecules" (EPA, 2018a).

The Decision-Making Committee concluded that *exo*-dsRNA treatments do not create genetically modified organisms. The main reason given for coming to this conclusion was that externally applied (exo-) dsRNA is not inherited by the organism (Fig. 3). The Committee identified several factors that prevented inheritance. These factors were that exo-dsRNA molecules could not enter the nucleus and genes were not in the cytoplasm and exo-dsRNA molecules are not reverse transcribed into DNA, and for all of these reasons exo-dsRNA molecules therefore could not integrate into the DNA of the genome of the organism to which they are applied and modify it (paragraph 4.6 of Ref EPA, 2018a).

Although the Decision-Making Committee did not refer to any publications in their documentation, they did rely on the relevant EPA Staff Assessment Report (EPA, 2018b). The Staff Report had 16 items in its bibliography (Table 1). Of these, only 5 references dominated the sources of information relevant to the criteria used by the Decision-Making Committee to reach its Decision. As described below, these sources were sometimes misinterpreted or contained information contradictory to that described in the Staff Report. As describe in more detail below, this misinterpretation of bibliographic references lead to serious errors in understanding the current state of the science of dsRNA and to extrapolation well beyond the available data.

This EPA determination is important because there is growing interest in the use of dsRNA in medicine (Lam, 2012; Sardh et al., 2019) and agriculture, such as for pest control (Parker et al., 2019; Sammons et al., 2011; Van et al., 2011; Whyard et al., 2011). To the best of my knowledge it is now possible for anyone in New Zealand to treat eukaryotes (that are not already listed as biosecurity threats) in the open environment with any dsRNA material without any previous approval because EPA has concluded that all RNA is neither listed as a hazardous substance nor can it create a new organism.

2.1. *Exo-dsRNA is not confined to the cytoplasm*

The Committee understood that exo-dsRNA remains "solely as RNA molecules in the cell cytoplasm outside the nucleus" (EPA, 2018a), consistent with advice received from staff (paragraph 2.9 of Ref EPA, 2018b). The Committee's members concluded that physical isolation of the genes and other genetic material in the nucleus would be a biological barrier to inheritance of *exo*-dsRNA because the exo-dsRNA was confined to the cytoplasm (Fig. 3).

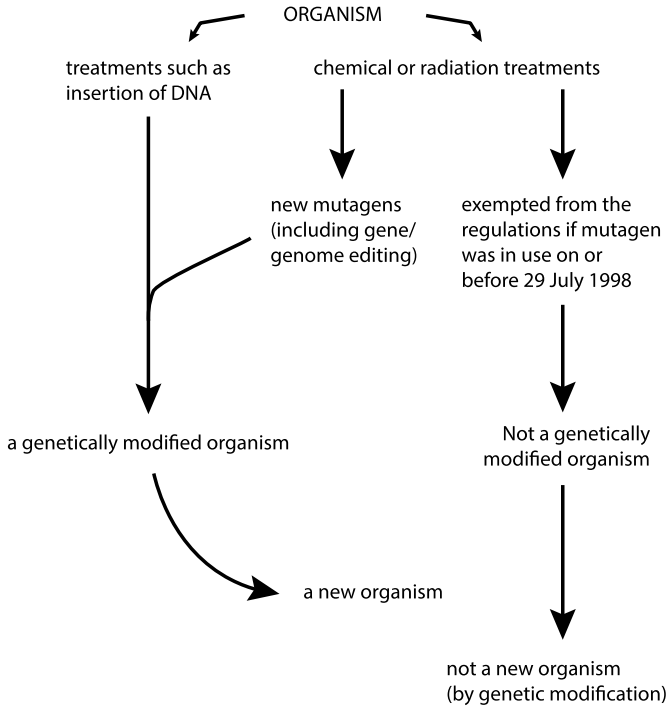

Fig. 2. What is a new or genetically modified organism?
The HSNO Act and Orders in Council describe a new organism that is a genetically modified organism as having had genes or other genetic material modified through the use of *in vitro* techniques. Modifications through chemical or radiation treatments in use prior to 1998 are excluded from scope, but those whose use is more recent are included in scope along with the most common form of modification, the insertion of DNA.

However, the Committee incorrectly concluded that exo-dsRNA always would be confined to the cytoplasm of exposed cells of organisms. This error undermines the conclusion that dsRNA is not heritable or cannot act as a modifier of genes or other genetic material (Fig. 2).

Firstly, processed exo-dsRNAs may be conducted to the nucleus in association with a variety of proteins including Dicer and NRDE-3 (Mao et al., 2015; Various, n.d.). Already a decade ago researchers reported that "NRDE-3 binds siRNAs generated by RNA-dependent RNA polymerases [RdRP] acting on messenger RNA templates in the cytoplasm and redistributes to the nucleus" (Guang et al., 2008).

Further evidence of transport is provided by Djupedal and Ekwall (2009) writing about heterochromatin formation. They said: "Exogenous siRNAs are thus capable of stable and specific epigenetic regulation of target genes." Those genes are located in the nucleus and the epigenetic regulation comes from chemical modifications made to DNA nucleotides and/or histones. Djupedal and Ekwall were cited in the underlying research provided by staff to the Committee (*e.g.*, paragraph 2.9 of Ref EPA, 2018b).

Carthew and Sontheimer (2009), also cited by EPA staff (*e.g.*, paragraph 2.2 of Ref EPA, 2018b), said that miRNA and exogenous siRNA are biochemically interchangeable once in the cytoplasm (Box 1). The biochemistries of these types of dsRNA overlap, and no clear distinction can be made in the kinds of silencing that they cause, further undermining certainty that *exo*-dsRNA could be relied upon to stay out of the nucleus.

Secondly, in many eukaryotes cytoplasmic and nuclear compartments regularly mix. The nuclear envelope breaks down every cell cycle in eukaryotes with open mitosis, resulting in mixing with the cytoplasm (Gorlich and Kutay, 1999; Smoyer and Jaspersen, 2014). This cyclic breakdown provides, for example, the Argonaute protein-associated RNA access to the chromosomes (Li, 2008). In animals at least, the nuclear envelope can also rupture, resulting in mixing of content (Hatch and Hetzer, 2014).

2.2. Genes are not confined to the nucleus

Even if it were the case that exo-dsRNA was confined to the cytoplasm, eukaryotes have genes there too. Cytoplasmic organelles called mitochondria and chloroplasts have DNA genomes. Separate from them, some eukaryotes have self-replicating DNA and RNA elements in the cytoplasm.

The yeast and filamentous fungi are host to self-replicating dsRNA agents located in the cytoplasm (Frank and Wolfe, 2009; Wickner, 1986). These RNA elements range in size from 1.5 kilobase-pairs to over 76 kbp. Parts of these dsRNA elements have transported to the nucleus and converted to DNA, where they were identified in the chromosomes (Frank and Wolfe, 2009). Moreover, these elements have acquired genes from other organisms and other dsRNA elements through RNA-RNA recombination, making it possible for them to acquire sequences directly from exo-dsRNAs (Ramírez et al., 2017).

Presumptive exclusion of dsRNAs from the nucleus does not prevent interaction with these cytoplasmic genes and therefore possible ongoing replication of the exo-dsRNA through linkage. Neither the EPA staff nor the Decision-Making Committee addressed the broader diversity of genes or other genetic materials that exist outside of those found in nuclei of eukaryotes.

2.3. Replication by reverse transcription

Another potential barrier to inheritance would exist if *exo*-dsRNAs could not be reverse transcribed (Fig. 3). Reverse transcriptase has the ability to synthesize a DNA molecule using an RNA molecule as a co-factor (template), similar to how DNA itself replicates using a DNA strand as a co-factor in DNA replication. Once a DNA strand has been synthesized by reverse transcriptase, that strand can serve as a co-factor in the synthesis of a complementary strand to produce a double-stranded DNA molecule. Unfortunately, neither the Decision (EPA, 2018a) nor the Staff Report (EPA, 2018b) provided references or analysis related to the existence of such a barrier. Meanwhile, there is substantial evidence indicating that RNAs can be, and have been, reverse transcribed and incorporated into eukaryotic genomes.

A variety of enzymes commonly found in eukaryotes have reverse transcriptase activity (Goic et al., 2013). By some estimates, as much as 30% of the mammalian genome, and 10% of the human, was created by the action of reverse transcriptase activity originating from retroviruses (de Parseval et al., 2003). Reverse transcriptases are also routinely used in transcriptomics experiments, in the first step of amplification of the transcriptome, demonstrating that amplification of RNAs even as small as siRNAs is possible (Dard-Dascot et al., 2018).

Reverse transcriptase requires a primer, *i.e.* another nucleic acid such as a dsRNA molecule called a tRNA, to initiate synthesis. The primer provides a 3′OH group for strand extension. The primer may come from the secondary structure (*e.g.*, a hairpin structure), as is common in precursors of siRNA (Fig. 1). Alternatively, the primer can be a separate "anti-sense" molecule that binds to the template RNA strand. The primer gives the reverse transcriptase reaction specificity because it binds by complementarity to a target sequence. At least in the laboratory, it is possible for a reverse transcriptase reaction to proceed without the addition of any particular primer molecule because there are sufficient numbers of small RNA molecules naturally present in the cytoplasm to serve this purpose (Frech and Peterhans, 1994).

We do not know whether all *exo*-dsRNA molecules could serve as substrates for reverse transcriptase, but it is unlikely that none could. RNA from viruses can be captured by reverse transcriptase for conversion into DNA molecules and integration into chromosomal DNA, as well as by Dicer for production of siRNA (Goic et al., 2013). RNA elements also could be converted into DNA by the action of reverse transcriptase in eukaryotes. For example, a DNA virus that infects animals is known to have evolved *via* recombination between a DNA virus that infects plants and an RNA virus that infects animals (Gibbs and Weiller,

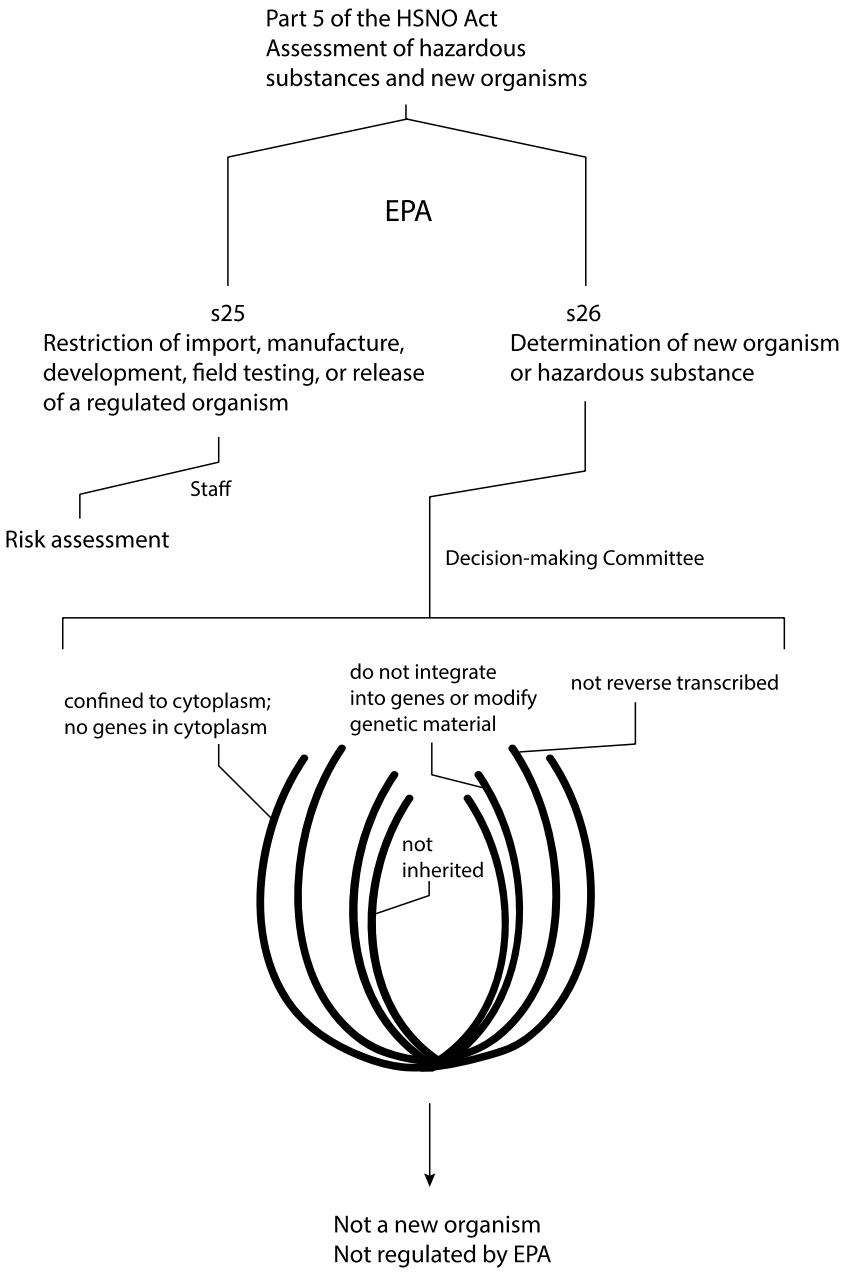

Fig. 3. Context of the EPA decision.
The EPA has two different activities described by sections 25 and 26 of the HSNO Act. (Left) If EPA decided that treatment of eukaryotic cells or organisms with dsRNA modified genes or genetic material by *in vitro* techniques, then s25 would apply. Such processes and products would be regulated as the normal work of EPA staff. (Right) Under s26, EPA decided that eukaryotes treated with *exo*-dsRNA were not new or genetically modified organisms because exo-dsRNA did not result in changes that EPA recognized as heritable. That conclusion is pictured as the center of an onion (center bottom), further protected by several additional conclusions, that form layers of the "onion" depicted in this figure, that contribute to increasing certainty in the conclusion. The layers are, from outermost, that exo-dsRNA: is confined to the cytoplasm and that genes are confined to the nucleus; cannot be reverse transcribed into DNA and only that way could it recombine with genes.

1999). The process involved reverse transcriptase from a third virus acting on the animal RNA virus to convert an RNA genome into DNA.

Significantly, an enzyme from bacteria was found to reverse transcribe RNA templates and create short DNA fragments that were subsequently recovered in the chromosome (Silas et al., 2016). The possibility that DNA molecules could be generated *in vivo* from *exo*-dsRNA constructs is made even more plausible by this discovery because the bacterial enzyme is most closely related to the reverse transcriptase of retrotransposons found in eukaryotes.

Thus, under the right conditions reverse transcriptase is able to use exo-dsRNA as a substrate. The smaller the RNA molecule the less likely it would be a substrate. However, this does not matter for the Decision because the EPA placed no size or structural constraints on the *exo*-dsRNA that can be used.

2.4. Means of modifying genes or other genetic material

dsRNA also can cause at least three other kinds of changes to DNA already comprising chromosomes in the nucleus of a cell: DNA deletions (Matzke and Birchler, 2005); changes in chromosome copy numbers (Khurana et al., 2018); and modification of nucleotides (Matzke and Birchler, 2005). In short, dsRNA can cause the same range of modifications that can be caused by mutagens. Had this been considered by the EPA it would have constituted evidence to support classifying eukaryotes treated with dsRNA molecules as new organisms.

2.4.1. Deletion

The eukaryote *Tetrahymenia thermophila* has an "RNAi-mediated process that directly alters DNA sequence organization" (Mochizuki and Gorovsky, 2004). Approximately 12,000 DNA fragments, comprising 46 M-bases, are deleted (Noto and Mochizuki, 2017). DNA fragments removed from *Paramecium tetraurelia* chromosomes by a dsRNA-guided mechanism are ligated together to form an extra-chromosomal element that is transcribed and processed into more dsRNAs (Rechavi and Lev, 2017). While this process has been described for endogenous dsRNAs, the example further cautions against making generalizations about dsRNA effects on DNA.

Table 1
Critical resources accessed by EPA.

Item[a]	Evidence in support[b]	Number of times cited
McGraw Hill Encyclopedia of Science and Technology	1	1
Arteaga-Vazquez & Chandler, 2010	2	1
Carthew and Sontheimer, 2009	2,3,4,5,6,**7**	11
Bartlett & Davis, 2006	**8**	3
Borel B, 2017	9	3
Djupedal and Ekwall, 2009	6,**7**,10,**11**	5
Gong et al., 2013	9	1
Götz et al. 2016	6	1
Ledwith et al., 2000	12	1
Mitter et al, 2017a	**8**,9	4
Mitter et al, 2017b	**8**	2
Nowrouzi et al., 2012	12	1
OriGene 2018	13	1
Sato & Siomi 2013	3,4	2
Shabalina & Koonin 2008	3,4	8
ThermoFisher 2018	13	1

1. Size of a genome.
2. Supports statement that dsRNA can be produced by transcription.
3. Describing RNAi and *e.g.* Argonaute proteins or evolutionary context of RNAi.
4. Describing exo-dsRNA, *endo*-dsRNA, piRNA and other kinds of dsRNAs.
5. Figure reproduced.
6. Link between endo-dsRNA and heterochromatin formation.
7. Staff assertion that exo-dsRNA cannot induce heterochromatin formation.
8. Biochemistry/estimates of stability of dsRNA in a cell.
9. Examples of uses of dsRNA.
10. Chromosome structure.
11. Staff interpretation of the secondary dsRNA structure and roles.
12. Evidence of DNA integration into chromosomes.
13. Sources of kits for research using dsRNA.

[a] Items in bold are considered to have been very important based on frequency of citation and specialty of information for criteria used by the Decision-making Committee and have special call-outs in the text. For further details on these items, see EPA, 2018b.

[b] Description of evidence; bold indicates of particular relevance to conclusions.

dsRNA also causes heritable DNA rearrangements in the eukaryote *Oxytricha trifallax*. Organisms of this species have two nuclei in each cell. The somatic macronucleus contains the genes being actively transcribed in somatic cells. During development of the macronucleus, 95% of the germline genome is destroyed resulting in extensive fragmentation followed by permutations and inversions (Nowacki et al., 2008). RNA guides the rearrangement process. Exo-dsRNA that targeted these guides prevented reassembly of DNA fragments in the macronucleus (Nowacki et al., 2008).

2.4.2. Copy number

The number of copies of chromosomes in the macronucleus in the cells of *O. trifallax* is regulated by dsRNA. The number of duplicates of chromosomes in the macronucleus was shown to increase from exposure to *exo*-dsRNA (Khurana et al., 2018). The exposure did not noticeably alter gene expression, but the effects on chromosome number were dependent on Dicer and RdRP activity. Using antibodies that recognize DNA:RNA hybrid molecules, small dsRNAs were shown to directly associate with chromatin. Moreover, the exo-dsRNA effect on the copy number of the DNA chromosomes was heritable (Nowacki et al., 2010).

2.4.3. Modification

In paragraph 4.9 of their Decision the Committee said that it required evidence of dsRNA integrating into the genome (*i.e.*, according to Decision paragraph 4.6, to be chemically attached to the DNA of chromosomes in the nucleus), or the dsRNA itself had to in some other way become heritable, for the conclusion to be reevaluated. Implicit in the Decision text was that the modification had to be the continued propagation of the dsRNA, rather than dsRNA modifying genes or other genetic material as through mutation.

Certainly if the dsRNA were propagated that would satisfy international definitions of modification, which also can include the change to the primary order of nucleotides in a DNA molecule as would result from linkage to a dsRNA molecule, if that could occur. However, the terms used in international instruments are also consistent with what agencies such as the UN Food and Agriculture Organization include, such as the "*chemical* modifications of DNA and chromatin, for instance, affecting the degree of chromatin compaction or the accessibility of regulatory sequences to transcription factors" (emphasis added to Ref CGRFA, 2015). As discussed above, that is a kind of modification that can result from a treatment with *exo*-dsRNAs without reliance on continued transcription (Rechavi, 2014). Furthermore, as discussed below, RNA-directed DNA methylation caused by dsRNA can result in heritable effects, such as through transition mutations, without needing to propagate along with the modifications that it makes.

Transcriptional gene silencing is caused by *chemical modifications* in the form of methyl groups added to nucleotides and histones by RNA-directed DNA methylation, promoting heterochromatin formation (Djupedal and Ekwall, 2009; Woodhouse et al., 2018). Methylation of DNA also influences RNA splicing patterns in insects, altering protein structure and diversity (Brevik et al., 2018).

The modification of histones and DNA nucleotides can pass through mitosis and meiosis (CGRFA, 2015). Once methylation has occurred, it can be propagated independently of further stimulation by exo-dsRNA. As Djupedal and Ekwall (2009), who also were cited by EPA staff, say: "It is easy to visualize how DNA methylation is inherited from mother cell to daughter cell considering that DNA replication is semi-conservative and the newly synthesized strand may be methylated with the 'old' strand as template. Likewise, half of the histones are partitioned to each DNA helix during S-phase, and may thereby guide histone modifications to newly incorporated histones. This would provide means for maintenance of the chromatin setting over cell divisions." This mechanism has been shown for both sexual and asexual reproduction of eukaryotes (Chong and Whitelaw, 2004).

Moreover, methylation can also change the frequency of DNA base mutations because methylated cytosines deaminate to thymine, causing transition mutations. T:G mismatches are 10 times less likely to be repaired than other mismatches (Holliday and Grigg, 1993). In both humans and plants methylation is more frequent in genes with naturally lower numbers of C residues, presumably because of historical deleterious transition mutations at these loci (Zilberman, 2017). The use of *exo*-dsRNA could result in targeted mutations in the eukaryotes that have RNA-directed DNA methylation pathways, making it a class of mutagen covered by the HSNO Act (Fig. 2).

The examples above would fall well within the parameters of evidence that dsRNA causes modification of genes or other genetic material that is "capable of being inherited by the progeny of the organism, or…capable of causing a characteristic or trait that can be inherited" (EPA, 2018b). Such modification, and not just transmission during reproduction, should also be considered in cases of dsRNA treatments of long-lived species of conservation or agricultural value, such as trees.

2.5. dsRNAs can be heritable

dsRNA molecules themselves can be amplified by RdRP acting on the target messenger RNA (Fig. 1). The EPA Staff Report (EPA, 2018b) made two significant errors related to this point. The first is that contrary to the claims made in the Staff Report, secondary small RNAs generated through RNAi (Fig. 1) can prime tertiary small RNAs in the germline cells of the nematode *Caenorhabditis elegans* "and therefore set in motion a feed-forward process that could theoretically preserve transgenerational inheritance ad infinitum" (Rechavi and Lev, 2017).

The second is that the Staff Report errs in generalizing an observation from nematodes to all eukaryotes in paragraph 2.14 (EPA, 2018b). The Staff Report says that "primary siRNAs have 5′-monophosphate groups at their 5′ ends, while secondary siRNAs have triphosphate groups at their 5′ ends (Djupedal and Ekwall, 2009)." The reference the staff rely upon actually says: "*In nematodes*, *many* of the secondary siRNAs arise as single-stranded, unprimed transcripts with 5´-triphosphates and do not require Dicer processing" (emphasis added to Djupedal and Ekwall, 2009). Secondary dsRNAs with 5´ monophosphate ends are the norm in plants (Baulcombe, 2007).

Possibly as a result of these or other errors, EPA staff viewed RdRP amplification of regulatory dsRNA molecules as a self-limiting reaction (paragraph 2.14 of Ref EPA, 2018b), but while RNAi can be self-limiting (Houri-Zeevi and Rechavi, 2017), it does not in all cases self-extinguish. It has been shown to transmit usually for around 3–5 generations, but has been observed to transmit for up to 80 generations (Houri-Zeevi and Rechavi, 2017).

Critically, where transgenerational effects of *exo*-dsRNA have been studied at all, there is evidence that the self-limiting behavior of RNAi can be an active process (Houri-Ze'evi et al. 2016), not the outcome of dilution as hypothesized in the evidence relied upon by the EPA staff (paragraph 2.6 of Ref EPA, 2018b). This could mean that there are other eukaryotic organisms in the vast repository native to New Zealand that lack this second tier of biochemistry modulating the response, or natural mutants that lack it. Interestingly, mutations in these limiting pathways in *C. elegans* cause hypersensitivity to exo-dsRNA stimulation (Houri-Zeevi and Rechavi, 2017).

The limiting mechanisms are also not assurances that the transience of the effect is shorter than necessary to prevent a harmful effect of the treatment, should there be one. Additionally, the limiting response can be reduced by repeat exposures to the exo-dsRNA (Houri-Zeevi and Rechavi, 2017). Repeat exposures are possible under the EPA Decision, even expected for applications such as pest control (EPA, 2018b).

2.5.1. Unintended heritable changes

Exo-dsRNA and *endo*-dsRNA compete for biochemical components of the RNAi pathway (Waldron, 2016). Traits made stable and heritable by endo-dsRNA may be destabilized through competition with exo-dsRNA. If the outcome of the competition for Argonaute or other proteins is an alternative heritable pattern of gene expression, then this too is a heritable effect of treatment with exo-dsRNA.

Exposing the eukaryote *C. elegans* to exo-dsRNA downregulated the production of endo-dsRNAs that are necessary for the inheritance of endo-dsRNA effects (Houri-Ze'evi et al. 2016). This effect was not specific to the sequence of the genes controlled by particular endo-dsRNA, but to production of proteins necessary for intergenerational transmission of RNAi caused by endo-dsRNAs.

A critical feature of this observation is that any attempt to determine the longevity of *exo*-dsRNA-mediated RNAi must define how often an organism will be exposed to exo-dsRNA. This is because the "'transgenerational timer' is being reset by initiation of new RNAi responses, and therefore 'second triggers' extend the inheritance of ancestral silencing" (Houri-Ze'evi et al., 2016). Exposure frequencies will determine the duration of the effect both in time and number of generations.

2.5.2. Commercial applications demonstrate heritability

EPA assertions that *exo*-dsRNA treatments are not heritable through modification of genes or other genetic material directly contradicts industry intellectual property rights claims (Crawford et al., 2014; Deikman et al., 2017; Fillatti et al., 2012). In the patent "Methods and compositions for introducing nucleic acids into plants" which includes use of dsRNA, the claim is for both treated organisms and their progeny:

"Several embodiments include *progeny seed or propagatable plant part* of such plants, and commodity products produced from such plants… wherein the modification of the target gene is non-heritable silencing of the target gene, *or heritable or epigenetic silencing of the target gene*, or a change in the nucleotide sequence of the target gene; embodiments include *the directly regenerated plant* exhibiting modification of the target gene and *plants of subsequent generations grown from the directly regenerated plant* and exhibiting modification of the target gene" (emphasis added to Ref Huang et al., 2018). The type of patent used in this case is a utility rather than plant variety patent and extends to the ownership of organisms and future generations of organisms treated with exogenous dsRNA similarly to how utility patents claim the use of genetically modified organisms.

"Several embodiments include a plant or a field of plants treated by a method, composition, or apparatus described herein, wherein the plant exhibits a desirable phenotype (such as improved yield, improved tolerance of biotic or abiotic stress, improved resistance to disease, improved herbicide susceptibility, improved herbicide resistance, and modified nutrient content) resulting from the treatment and when compared to an untreated plant. Several embodiments include progeny seed or propagatable plant part of such plants, and commodity products produced from such plants" (Huang et al., 2018). The maker of the dsRNA would apparently own an organism because it was exposed to the dsRNA, potentially including entire fields of conventional crops or long-lived trees and their seeds that have never been modified by insertion of DNA.

3. Risk scenarios

3.1. Unintended human exposures

The EPA Decision removes any need to notify the public of their potential exposures. The various kinds of exposures are through spray drift of dsRNA-based pesticides or brushing against treated plants, and ingestion of treated food items. The different exposure pathways – ingestion, inhalation or contact – have not been studied to the same extent. While the most research involves ingestion exposure and so far suggests that unmodified dsRNAs are unlikely to cause an effect in humans, this is still not fully certain and reports of dietary dsRNA exposure continue to appear (FIFRA, 2014; Luo et al., 2017). The other exposure pathways have received very little attention (FIFRA, 2014; Heinemann et al., 2013).

Hypothetical uses of *exo*-dsRNA on post-harvest or retail foods include for the purposes of delaying ripening or spoilage. For example, genetically modified tomatoes were engineered to produce dsRNA to silence the expression of 1-aminopropane-1-carboxylate synthase, the rate-limiting enzyme in the production of the ripening hormone ethylene. The expression of dsRNA was controlled by a promoter that was mainly active late in development so as to not interfere with the production of ethylene at other stages of fruit development (Gupta et al., 2013). The effects of silencing at the wrong time could alternatively be avoided by spraying exo-dsRNA on harvested but unripe tomatoes. Other approaches are to use topically applied exo-dsRNA to silence genes that encode receptors of ethylene (Deikman et al., 2017).

3.2. Exposures of non-target organisms

The EPA Decision did not preclude the use of dsRNA that might result in other kinds of effects either inadvertently or on purpose. dsRNA (and RNA in general) can have effects on organisms (*e.g.*, Refs. Kalluri and Kanasaki, 2008; Kleinman et al., 2008) other than RNAi, including heritable effects that are not associated with RNAi.

Potential unavoidable exposures of non-eukaryotic organisms, such as bacteria, to RNA that could result in effects other than RNAi also were not evaluated in the documents released by EPA. Applications of dsRNA in the open environment would result in exposures to non-target organisms, such as bacteria, including on the surface of target organisms.

Small RNA molecules are gene regulatory agents in bacteria, but do

not use the biochemistry of RNAi (Mars et al., 2016; Papenfort and Vanderpool, 2015). The intercellular trafficking of regulatory RNA molecules indicates that exo-RNA is relevant to their biology too (Sjöström et al., 2015).

Regulatory RNA in bacteria influences the transition from planktonic to biofilm growth (Ashley et al., 2017) and colonization of the intestine by pathogens (Han et al., 2017). RNA molecules serve as guides for the action of the nuclease Cas9 in the CRISPR/Cas9 system (Marraffini and Sontheimer, 2010). Exo-dsRNA secreted by intestinal cells has been implicated in adjusting the growth rate of different species of bacteria in the human gut (Liu et al., 2016). Nowhere in nature, and even rarely in the laboratory, would eukaryotic organisms (as opposed to tissue culture cells) be free of prokaryotes. RNA contaminants of any structure or size were ignored in the EPA Decision because it specifically excluded any *in vitro* modification technique from consideration.

3.3. Exposures to contaminants of RNA isolation procedures and RNA modifications

The EPA Decision specifically avoided consideration of how dsRNA was isolated or modified for use as exo-dsRNA. The Decision-Making Committee rejected the need to evaluate *in vitro* techniques of any kind because it decided that dsRNA treatments were out of legislative scope because it concluded that dsRNA is not heritable. I consider this to be a procedural error that could compound the potential for the Decision to lead to harm.

dsRNA may be directly isolated from organisms, or single-stranded RNA isolated from organisms may be converted to dsRNA *in vitro* using RdRP. Contaminants of RNA preparations from whole cells or tissues could include mRNA that might, upon entry to cells, be used to produce proteins that could be a source of allergens or toxins, and some RNA molecules could comprise substantial if not complete viral genomes (Ngo et al., 2017).

After all, RNA is itself the material of genes, such as in RNA viruses and retroviruses. These viruses can have either dsRNA or single-stranded RNA genomes. They replicate independently of human intervention once inside a eukaryotic cell and can have DNA intermediates that integrate into chromosomes (Ali et al., 2016).

The Committee did not address the physical description of the dsRNA in the approved treatments. The applicant sought permission to use “synthetic” dsRNA, restricted as well to those that would cause a temporary effect on the “activity of the complementary RNA” (Trought, 2018). Although siRNAs tend to get processed down to < 30 nucleotides, the EPA Decision is not restricted to externally applied dsRNA molecules of < 30 nucleotides. The dsRNA molecules possibly could be further chemically modified to mimic other classes of RNAs such as piRNAs (Ghildiyal and Zamore, 2009) or to affect their longevity and stability (Table 2). At least 128 different modifications have been reported so far in the literature (Dar et al., 2016; siRNAmod, 2018) and many synthesized dsRNAs can be routinely ordered with modifications (Bioland, 2018; Sigma, 2018).

Moreover, dsRNA or single-stranded RNA may be expressed in bacteria and packaged *in vivo* into virus-like particles (Arhancet et al., 2016; Killmer et al., 2016). These techniques can increase stability of the RNA, allow selective release of the RNA cargo, and also allow for significant increases in quantities of RNA that may be produced and purified.

Beyond modifications to the dsRNA molecules are the formulations or materials, such as cell penetrating proteins (Numata et al., 2014), that might be used to improve penetrance. The Decision imposed no restriction on method or material for causing the dsRNA to be taken up by organisms.

Responsible use of dsRNA for treating eukaryotes would unlikely include the purposeful amplification or modification of RNA viruses. However, the Decision specifically removes EPA from responsibility for protecting against inadvertent amplification of RNA viruses by saying “it was not necessary to consider whether *in vitro* techniques were involved.” This is surprising given the accessibility of both genetic databases and recent revelations that a poxvirus was assembled by purchasing the component DNA fragments through “the mail” and the expectation that portable synthesizing equipment will be more common in the future (Sharples, 2017). Even well-intentioned molecular biologists, not to mention citizen scientists, could use molecules of unknown potential to replicate in some eukaryotes.

Therefore, a significant concern is that the Committee did not consider the *in vitro* techniques that could be used either to create, isolate or amplify the dsRNA. The Committee:

- put no constraints on the size of the dsRNA molecules.
- constrained treatment to organisms that are not excluded by the Biosecurity Act, *but did not constrain the source of the dsRNA to be used*.
- removed any obligation to notify the use of *in vitro* conversion or synthesis of RNA genomes into dsRNA molecules.
- did not describe what it meant by external treatments, leaving chemical and biological vectors (Kolliopoulou et al., 2017) of any description possible.

4. Discussion

The EPA Decision defines the use of dsRNA applied externally to eukaryotes as out of scope of New Zealand biosafety legislation. The Decision has important implications because all native and endogenous eukaryotes, even those yet to be discovered, as well as those described as exotics, with the exception of organisms banned by biosecurity laws, come under the jurisdiction of the HSNO Act.

The EPA was convinced that *exo*-dsRNA molecules could not be inherited by eukaryotes and this was the primary rationale for the determination that eukaryotes treated with them were not new or genetically modified organisms for the purposes of the HSNO Act. In summary, the “facts” EPA relied upon (Fig. 3) and the uncertainties presented above, are:

- exo-dsRNA does not mix with material in the nucleus of the cell. This, however, was shown to be false. Moreover, the EPA failed to account for replicating RNA elements in the cytoplasm of some eukaryotes, and the literature on RNA-RNA recombination.
- exo-dsRNA is not reverse transcribed. This was shown to be plausible for some dsRNA molecules but demonstrably false for others.
- exo-dsRNA is not heritable because it does not modify the DNA genome. This was shown to be false. First, exo-dsRNA may replicate

Table 2
Common dsRNA *in vitro* chemical modifications.

Modification	Description	Effect
2′-O-Methyl phosphoramidites	2′-O-Me-rA, 2′-O-Me-rC, 2′-O-Me-rG, 2′-O-Me-rU	Increase stability, longer lasting RNAi effects
2′-Fluoro phosphoramidites	2′-FluoC, 2′-FluoU	
5′ modifications	5′-Amino, 5′-Biotin, 5′-Cholesterol, 5′-Phophorylation and 5′-Thio	Various reasons, *e.g.*, cholesterol for improved penetration through membranes.
3′ modification	3′-amino	

Table content amalgamated from several sources (Refs Bioland 2018; Dar et al., 2016; Sigma 2018).

independently of the DNA genome using RdRP-based amplification, as can other RNA-based elements in eukaryotes that are clearly genetic material. Second, exo-dsRNAs can modify DNA in chromosomes in some cell types or species. Modifications include heritable methylation of nucleotides and histones, DNA deletions and rearrangements, and changes in chromosome copy number.

These errors were compounded by a narrow survey of the literature and over-reach of conclusions. For example, the two 2017 papers by Mitter et al. along with the paper by Bartlett and Davis (2006) form the basis for asserting that exo-dsRNA is short lived inside eukaryotic cells, and therefore cannot be inherited (Table 1). This extrapolation to all eukaryotic organisms was based on measurements of the dsRNA molecules made in just two studies, one using mice and one using tobacco plants, combined with observations of the longevity of the silencing effect in only a few more (Table 1). Meanwhile, prolonged inheritance in nematodes was not considered as contrasting evidence.

The EPA Decision was based on hypothetical barriers to inheritance that are not present in all eukaryotes. Furthermore, it makes possible the use of *in vitro* techniques that until recently were confined to the laboratory, wherein it was possible to evaluate the resulting genetically modified organisms created by *in vitro* techniques before their release into the environment.

In contrast to the EPA, the industry developing dsRNA treatments for broad scale environmental applications is convinced that the treatments result in heritable changes. For example, an exo-dsRNA treatment was used to effect a color change in flowers of petunia plants that produced progeny that retained the modified trait. Those progeny were used to illustrate the multi-generational claim of ownership made by the patent holder (see paragraph 0173 of Ref. Huang et al., 2018).

It is important to emphasize that RNA effects are still rapidly being described even in model research organisms. “Among some animal groups [in New Zealand], new species are being discovered faster than scientists can cope with them” (Manatū Taonga Ministry for Culture and Heritage) much less test them for dsRNA responses. The clear statements made about there likely being much more to discover about dsRNA effects as more species are studied, statements made in the references used to develop advice from staff, were not mentioned in the advice provided to the Committee (Table 1 and EPA, 2018b). The narrow treatment by EPA of how dsRNA could modify genes or genetic material is surprising given the nation's pride in its native biodiversity.

5. Conclusion

In the future, it might be determined that some or all uses of externally applied dsRNA create no unmanageable risks to human health, the environment, or to society. This would be a welcome finding because there is potential for dsRNA-based products to be at least short-term remedies for some societal or environmental problems. However, coming to this determination should be an evidence-based and precautionary process. Only that kind of process can build trust in responsible providers of biotechnology and agencies that serve to protect the public's interest in the environment. The New Zealand EPA should revisit this Decision after accessing the full range of scientific evidence available, and use more precaution when it does so.

Declaration of Competing Interest

None.

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