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Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides

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No. 104

Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides

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Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2020

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FOREWORD

The purpose of the OECD work leading to this paper was to develop a broad set of recommendations relating to risk assessment considerations for exogenously-applied double-stranded RNA (dsRNA)-based products, with a focus on issues relating to data requirements for determining the environmental fate of sprayable RNA molecules and for examining the potential risks to non-target organisms. This document is intended to provide an overview of available scientific information related to RNA interference (RNAi), and considerations on regulating this technology for pest control. The document does not provide guidance as further experience needs to be gained in this field. This document builds on current knowledge. Therefore, the OECD will consider revising the document to reflect new scientific insights when they are available.

This document has been developed in the framework of the OECD Ad Hoc Expert Group on RNAi-based pesticides, a sub-group of the OECD Working Group on Pesticides (WGP) that helps member countries to harmonise the methods and approaches used to assess exogenously-applied dsRNA-based products. The initial draft of this document was developed by Les Davies, former co-chair of the Ad Hoc Expert Group on RNAi-based pesticides, who served as a consultant to the Secretariat.

The development of this document was overseen by the Chair of the Expert Group, Mike Mendelsohn (US) with input from Expert Group members Emily Hopwood (Canada), Denis Rochon (Canada), Achim Gathmann (Germany), Ann-Kristin Diederich (Germany), Antje Dietz-Pfeilstetter (Germany), Dimitra Kardassi (EFSA), and John Kough (US).

The working document was approved by the Working Group on Pesticides on 26 June 2020.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology, which has agreed that it be declassified and made available to the public.

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1. Introduction

1. RNA interference (RNAi) is a biological process in which small ribonucleic acid (RNA) molecules inhibit gene expression, typically by causing the enzymatic destruction of specific messenger RNA (mRNA) molecules, which are the templates for the synthesis of proteins. This process is commonly referred to as post-transcriptional gene silencing (PTGS); that is, mRNA is transcribed from the DNA gene but before the message is translated into proteins by ribosomes, the mRNA is blocked or otherwise destroyed by an enzymatic process guided by a specific non-coding small interfering RNA (siRNA) (see 2.1.1) or microRNA (miRNA) (see 2.1.2).

2. Although observations of PTGS had been reported in the scientific literature prior to 1989, it was not until that year that the biological mechanism underlying PTGS was attributed to non-coding RNA molecules (Fire et al, 1989).

3. Since that time, research has demonstrated that gene silencing is part of a sophisticated network of interconnected pathways for cellular defence, RNA surveillance, and control of ontogenetic development. Furthermore, the mechanism is providing a powerful tool to manipulate gene expression experimentally.

4. In recent years, the application of RNAi-based technology has been investigated in human and animal therapies, and in the development of agricultural products. The potential utility of RNAi for insect pest control was suggested by two studies published in 2006 which demonstrated that PTGS can be elicited in insects by oral administration of double-stranded RNA (dsRNA) (Araujo et al, 2006; Turner et al, 2006). Subsequently, investigations in *Aedes aegypti* mosquitoes provided the first demonstration that RNAi could be induced in insects by topical application of dsRNA (Pridgeon et al, 2008).

5. The potential applications of dietary/exogenous RNAi, also termed environmental RNAi, were quickly recognised by researchers interested in human therapeutics (Lares et al, 2011; Witwer & Hirschi, 2014; Hirschi et al, 2015) and in plant protection (Baum et al, 2007; Mao et al, 2007; Mao et al, 2011; Burand & Hunter, 2013; Koch & Kogel, 2014). Progress in the use of RNAi in the human therapeutic space has been limited by the existence of multiple barriers, as discussed later in this working paper (see 5.1.1).

6. Using RNAi-based technology, plant protection against pests may be achieved by:

- genetic modification of the crop plant to express a long precursor dsRNA specifically directed against a pest species feeding on the crop (referred to as a plant-incorporated protectant (PIP) or *in planta* RNAi);
- topical application (spraying) of double-stranded RNA (dsRNA) molecules (with a nucleotide sequence specifically developed to target a pest species) onto the plant; the dsRNA on the leaf surface may be directly ingested by feeding pests or, if the uptake of the dsRNA is facilitated in some way to aid penetration of the cuticle[1], the absorbed dsRNA molecules could possibly be processed to small RNA molecules in plant cells and distributed systemically throughout the plant (if the

[1] Multiple publications have demonstrated that the plant cuticle and cell wall create significant challenges for the movement of topically-applied dsRNA into the plant RNAi pathway (e.g., Dalakouras et al, 2016); special application methods or formulations are needed to bypass plant structural and biochemical barriers (see Section 5.2.4).

sequence had significant similarity to the plant transcriptome and was amplified)[2]; dsRNA may also be applied to the plant via trunk injection or petiole absorption - the dsRNA then resides in the apoplast, is not subject to the plant RNAi machinery and is directly taken up by insects or pathogens;

- topical application (spraying) of a mixture of nonviable bacteria that had been engineered to produce double-stranded RNA (dsRNA) molecules with the appropriate sequence.

7. Other application methods have been utilised in laboratory or small-scale experiments (e.g., uptake by plant roots or stem injections; reviewed by Joga et al. 2016; Liu et al. 2020; Dalakouras et al. 2020), but it is unclear if these types of application methods will be commercially developed. To date, RNAi has been commercially applied in a number of genetically-engineered plant species, including the development of blue carnation (Tanaka et al, 2005) [3], apples that do not go brown when cut (Waltz, 2015), 'Flavr Savr' tomatoes (Redenbaugh et al, 1993) that remained firmer for longer, compositionally-modified soybeans and alfalfa (lucerne), virus-resistant papaya, squash, plums, potatoes and beans (Fuchs and Gonsalves, 2007), and of potatoes that are bruise-resistant (Halterman et al, 2016). The potential to use RNAi in the development of novel plant protection products and human and animal therapies has been recognised by researchers in industry and academia.

8. The use of dsRNA-based active ingredients could provide a valuable additional mode of action to mitigate pest pressures in a number of agricultural crops world-wide, in a manner that is specifically directed at pest targets, without adversely impacting non-target[4] species, including beneficial insects; in this regard, the deployment of dsRNA-based active ingredients holds great promise as compared with conventional chemical insecticides, acaricides, fungicides and herbicides (see e.g., Scott et al, 2013). Further, the long established view that dietary intake of nucleic acids, including dsRNAs from plant viruses, does not present a health risk to humans and other vertebrates means that the adoption of RNAi technology in agriculture is likely to present a lower human health risk than the use of conventional pesticides (see e.g. Joga et al, 2016; Kamthan et al, 2015).

9. Regulators need to consider human and environmental health and safety issues relating to technologies based on RNAi. In most, if not all OECD member countries, genetic modification of organisms to produce dsRNAs aimed at pest control will be regulated by those agencies, which currently regulate genetically-modified organisms (GMOs), using well-established procedures. dsRNA molecules, which are developed for topical application to crops for pest control will be assessed by relevant government agencies using a similar set of considerations to those applied to chemical and biological

[2] While miRNA-driven RNAi in plants has been shown to involve amplification and systemic movement, it would be unlikely for a topically-applied dsRNA to enter the plant RNAi pathway due to stoichiometric limitations (e.g., high copy levels of siRNAs per cell are needed), and the requirement for the applied dsRNA to have significant complementarity to the plant transcriptome.

[3] An Australian company, Florigene, and a Japanese company, Suntory, engineered a 'blue' rose in 2004. This involved adding a gene for the blue plant pigment delphinidin and the application of RNAi to depress all other color production by endogenous genes.

[4] In the context of an RNAi-based pesticide, **off-target** effects occur when an siRNA incorporated in the RNA-Induced Silencing Complex (RISC) down-regulates unintended targets; this could be in the target pest or in **non-target** species exposed to the pesticide.

pesticides. However, as pointed out in subsequent sections of this document, additional issues need to be taken into account, including, *inter alia*, (1) the potential to silence genes with significant sequence identity with the target gene in the intended pest; and (2) the fate and any possible effect of remnant small RNAs in the environment, treated crop or animal.

10. In cooperation with regulators in member countries and researchers in academia and industry, the OECD is coordinating this work to consider specific issues, which may need to be taken into account in considering applications for pesticides based on PTGS.

1.1. Purpose of this working paper

11. The pesticide industry and government regulators need to understand how best to collect and analyse data for informing ecological risk assessment of dsRNA-based pesticide products, with a particular focus on the effects on non-target organisms (NTOs) found in diverse habitats and representing diverse ecological functions (Roberts et al, 2015).

12. The purpose of the OECD work leading to this paper was to develop a broad set of recommendations relating to risk assessment considerations for exogenously-applied dsRNA-based products, with a focus on issues relating to data requirements for determining the environmental fate of sprayable RNA molecules and for examining the potential risks to non-target organisms.

13. Different sections of this document cover:

- an overview of RNAi mechanisms, noting that it is not intended to be a detailed review of the complex biology of these mechanisms but a guide to regulators, whose job it is to carry out human health and environmental safety assessments of new products based on RNAi technology.
- current and likely future applications of RNAi-based pesticides.
- environmental risk assessment, including:
  - problem formulation (i.e., identifying the issues specific to RNAi that are not already being considered by regulators in assessing the safety of using chemical and biological pesticides or introducing genetically-modified organisms into the environment)
  - the environmental stability of dsRNA in formulated products designed to control pests by an RNAi mechanism (including the impact of product formulation on dsRNA stability)
  - possible exposure of non-target organisms to dsRNA molecules applied in the environment to control a targeted pest
  - possible adverse effects on non-target organisms, including target and/or off-target effects.

14. This document is intended to provide an overview of available scientific information related to RNAi (basic biological mechanisms of RNAi, the environmental fate and behaviour of dsRNA molecules, and the possible impacts of small RNAs on non-target organisms), and considerations on regulating this technology for pest control.

15. It is important to note that the document does not address issues related to the risk assessment of genetically-modified crop plants, which incorporate the machinery to synthesise RNAi molecules specifically directed against a pest species feeding on the crop

(so-called ‘plant-incorporated protectants’, or PIPs); that is, the focus is solely on the risk assessment of the application of exogenous RNA.

16. Furthermore, it is not the intention of this document to consider in any detail the biological process referred to as RNA-induced transcriptional silencing (RITS) in which components of the RNA interference pathway are used in many eukaryotes in the maintenance of the organisation and structure of their genomes (described in Appendix 4).

17. Finally, it is considered that the product formulation may substantially impact the stability, efficacy, delivery and uptake of dsRNA. In addition, an important consideration might be the potential exposure of users and bystanders to dsRNA-based pesticides and the impact of the formulation type and formulation ingredients with regards to its environmental fate and persistence. As a general principle, the test requirements stipulated in the current nomenclature for conventional pesticides should be applied also to the dsRNA based products. However, the properties, behaviour and effects of the formulated products should not be automatically assumed to be similar to its naked form of dsRNA. This means that in addition to the data and information generally considered in risk assessment of the active ingredient certain additional specific aspects would need to be considered depending on the formulation type.

2. Overview of RNA interference

This section provides a summary overview of RNA interference. For a more detailed explanation, the reader should refer to the several reviews cited in this document.

18. Interference or suppression of gene expression by naturally-occurring dsRNA was largely unknown until the basic mechanism was elucidated in a scientific paper by Andrew Fire and Craig Mello (Fire et al, 1998); this work led to the award of the 2006 Nobel Prize in Physiology or Medicine[5].

19. Much research on modifying plants, insects, mammals and other organisms with transgenes or antisense constructs producing single-stranded RNA (ssRNA) had been published (see USEPA, 2013) prior to the discovery that dsRNA was at least 10-times more potent in its effect on gene expression than ssRNA (Fire et al, 1998). This finding unleashed a major research focus into the biochemical mechanism of what was termed 'RNA interference' (or RNAi).

2.1. Gene Silencing Pathways

20. The term 'gene silencing' refers to the interruption or suppression of the expression of a gene at either the transcriptional, post-transcriptional or translational level. Transcriptional gene silencing is a mechanism of epigenetically directed alterations in gene expression based on chemical modifications of nucleotides and histones without changes to DNA sequences, but with potential heritability.

21. As noted above, RNAi corresponds to a post-transcriptional gene silencing process, initiated by dsRNA molecules, that inhibits expression of specific genes by inactivation of specific mRNAs (Zamore et al, 2000).

22. The discovery of the RNAi mechanism did not occur with a single event or publication. The phenomenon had been observed in plants (called 'Post Transcriptional Gene Silencing' or 'co-suppression'; see USEPA, 2013 and references cited therein) and in fungi (called 'quelling'; Vance & Vaucheret, 2001; Mello & Conte, 2004). However, it was only after these apparently-unrelated processes were understood that it became clear that they all described the same phenomenon, called RNA interference (RNAi) by Andrew Fire and Craig C. Mello in their 1998 paper on blocking gene expression in the nematode worm *Caenorhabditis elegans* by the application of dsRNA (Fire et al, 1998). Their observation that specific and more robust gene silencing could be achieved using micro-injected dsRNA (rather than ssRNA) in this model organism led to the elucidation of the RNAi machinery as we now understand it. The initial discovery of the potency of dsRNA as an elicitor of gene silencing was quickly followed by the finding that, at least in *C. elegans*, dsRNA from the environment (in this case produced in *Escherichia coli* bacteria used as a food source for the worms) could also trigger gene specific silencing (Timmons & Fire, 1998).

23. RNAi has been demonstrated as an important endogenous pathway used in many different organisms to regulate gene expression post-transcriptionally, as for example not

[5] This was an even shorter period than that between the 1983 paper reporting the polymerase chain reaction (PCR) and Kary Mullis's award of the 1993 Nobel Prize in Chemistry (with Michael Smith).

only is RNAi a vital part of the immune response of plants to viruses and bacteria and fungi (Stram & Kuzntzova, 2006; Katiyar-Agarwal et al, 2006; Obbard et al, 2009; Zhao et al., 2018). In addition, in both juvenile and adult *Drosophila*, RNAi is important in antiviral immunity and is active against pathogens such as Drosophila X virus (Zambon et al, 2006; Wang et al, 2006a). This immune-type response, triggered by the application of exogenous long dsRNA molecules, probably resembles the response to double-stranded nucleic acid from RNA viruses. Some species, such as flies and plants, use RNAi as part of their immune system because RNA from infecting viruses triggers an RNAi response.

24. There are several variations in RNAi pathways which differ in the source of the RNA and the specific mechanism through which gene silencing is accomplished, but they all are triggered by the presence of a double-stranded RNA (dsRNA) molecule and all follow a similar order of events (see e.g., Roberts et al, 2015 and references cited therein). The dsRNA is processed into smaller RNAs (normally between 21–25 base pairs in length) by an enzyme called 'Dicer' or its homologs (part of the RNase III family of ribonucleases[6]) and incorporated into a protein complex known as RISC - the 'RNA-Induced Silencing Complex' (Figure 1) (Elbashir et al, 2001b; Tijsterman & Plasterk, 2004: Vance, 2011). RISC then uses one strand of the siRNA as a guide to find and bind to a complementary sequence on a specific mRNA, noting that some sequence mismatch may be allowed (Du, 2005); in plants, endogenously-produced forms of small RNA (micro RNA, or miRNA – see Section 2.1.2 below) usually bind with perfect or near-perfect complementary to their complementary mRNA sequences (and induce direct mRNA cleavage by RISC), while miRNAs in animals tend to be more divergent in their requirements for target complementarity with some mismatching (and induce translational repression) (Saumet and Lecellier, 2006; Bartel et al., 2009). The binding of RISC to mRNA leads either to its degradation or the interruption of its translation into protein. The following sections provide more detail about several RNAi pathways, which are distinct but significantly conserved in various functions. The two main types of small RNAs are siRNA and miRNA.

[6] Dicer is also known as 'endoribonuclease Dicer' or 'helicase with RNase motif', names which reflect its function. Being part of the RNase III family, it cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments called small interfering RNA and microRNA, respectively.

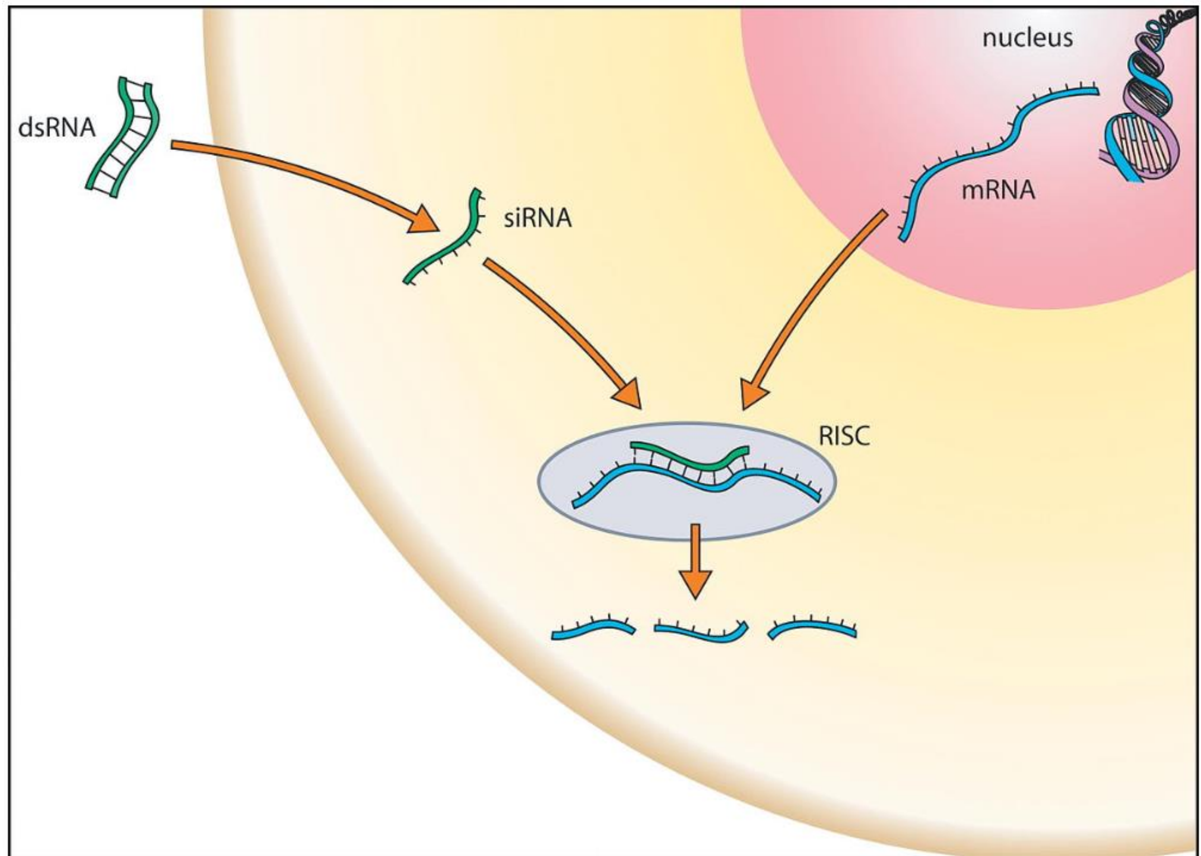

Figure 1: Simplified schematic of environmental RNAi: A double-stranded RNA is taken up by the cell and cleaved into small interfering RNAs. A guide strand from the siRNA incorporates in the RNA-Induced Silencing Complex (RISC), which undergoes binding to a complementary sequence on a messenger RNA (mRNA) and leads to its degradation. [Figure from Wikimedia Commons (https://commons.wikimedia.org/wiki/File:RNAi.jpg). Source: Robinson, 2004]

2.1.1. *Small interfering RNA (siRNA)*

25. An inducible RNAi pathway is triggered by the introduction of **exogenous** dsRNA. The dsRNA is cleaved by 'Dicer' into a pool of siRNA duplexes 21 to 25 nucleotides in length. The siRNAs comprise a population representing the entire length of the exogenous dsRNA; they are double stranded because they derive from the long dsRNA that triggers the process. One strand of the siRNA duplex (the so-called 'guide strand') incorporates into the RNA-induced silencing complex (RISC). The 'passenger' strand of the siRNA is degraded. Which strand becomes the guide or passenger strand is determined by the thermodynamic properties of the duplex. Once incorporated in RISC, the siRNA strand acts as a guide to find complementary sequences in mRNAs that it can bind to by Watson-Crick base-pairing. In post-transcriptional silencing, RISC contains a ribonuclease that cleaves

the target mRNA to which the siRNA guide has bound, triggering degradation of the target. In this way, targeting of mRNA degradation is sequence-specific and RNA complementary to the dsRNA trigger is silenced.

26. siRNAs generally show full complementarity to their target mRNA, and cleavage occurs 10–12 bases from the 5′ end of the guide strand binding site (Davidson & McCray, 2011 and references cited therein).

2.1.2. *Micro RNA (miRNA)*

27. The second pathway is used by **endogenous** micro RNAs (miRNAs). These small RNAs are formed from endogenously-expressed transcripts in the nucleus of the cell. miRNAs are derived from inverted repeat sequences that form one or more stem-loop structures, where the stem consists of dsRNA and the loop is unpaired, single-stranded RNA. In plants these primary miRNAs (pri-miRNAs) are processed by DICER-LIKE 2 (DCL2) in a two-step process in the nucleus which finally produces mature miRNA of 20- to 22 nucleotides (reviewed in Bologna and Voinnet, 2014 and in Axtell et al, 2011). In animals pri-miRNAs are processed in the nucleus into pre-miRNA by the ribonuclease III enzyme Drosha. After the pre-miRNAs are exported to the cytoplasm, Dicer processes these pre-miRNAs into mature miRNAs that are approximately 22 bp in length and function similar to siRNAs (see above). Because of the size and structure of the primary miRNA transcript and the specificity of the processing, the result is a single miRNA, which may target multiple mRNA transcripts (Siomi & Siomi, 2010). Either processed strand can mediate post-transcriptional gene silencing, but many miRNAs show asymmetry, primarily loading one strand into the RISC. The miRNA guides RISC to the mRNA target, where the miRNA typically binds to the 3′ UTR. In animals, Watson-Crick base pairing between miRNAs and their targets is usually partial, but with high complementarity from bases 2–8 of the miRNA, which is known as the ‘seed’ region. Base pairing can also occur between central miRNA nucleotides and target mRNAs. Data from several laboratories showed that miRNAs repress the initiation of translation, although more recent work indicates that miRNA–mRNA complexes can be transported to cytoplasmic processing bodies, after which de-adenylation, decapping and mRNA degradation occurs. It appears that some miRNA-mediated translational repression is reversible (see Davidson & McCray, 2011 and references cited therein).

28. Plant miRNAs usually have near-perfect pairing with their mRNA targets, which induces gene repression through cleavage of the target transcripts. In contrast, mammalian miRNAs can recognise their target mRNAs by using as little as 6–8 nucleotides (the seed region) at the 5' end of the miRNA, which is not enough pairing to induce cleavage of the target mRNAs but may result in translational pausing. As noted above, a given miRNA may have hundreds of different mRNA targets; conversely a given target might be regulated by multiple miRNAs.

29. More detail about the formation and processing of miRNAs can be found in Appendix 1 -‘Endogenous formation of miRNAs; pri- and pre-miRNAs’ (Section 10).

2.1.3. *siRNA vs miRNA*

30. In considering the related siRNA and miRNA pathways for RNA interference, the differences between them can be summarised as follows (see also Lam et al 2015):

- miRNAs are endogenously-derived from specific loci within the genome of the cell, while siRNA may be derived from transposons (Ghildiyal et al, 2008), viruses, heterochromatic DNA[7] or exogenous dsRNA.
- miRNAs are processed from longer precursor hairpin transcripts, whereas siRNAs are processed from long dsRNA (bimolecular) or small hairpin RNAs.
- Each miRNA hairpin precursor molecule produces a single miRNA duplex, whereas each long dsRNA molecule produces multiple siRNA duplexes.
- siRNA sequences are rarely conserved (e.g., different plant viruses don't generate the same siRNA sequences in a common host), while many miRNA sequences are well conserved across both plant and animal species (and are thought to be a vital and evolutionarily-ancient component of gene regulation) (Carrington & Ambros, 2003).
- All bases within an siRNA generally contribute to its target specificity, whereas only the 5' half of miRNA contributes to its target specificity.
- miRNA often binds to the 3' untranslated region of target transcripts, whereas siRNAs can form a complementary duplex anywhere along a target mRNA.

2.1.4. *PIWI-interacting RNA*

31. PIWI-interacting RNAs (piRNAs) are a class of longer-than-average miRNAs, about 26-31 nucleotides long as compared to the more typical miRNA or siRNA of about 21 nucleotides. Found in most metazoans (multicellular animals with differentiated tissues and organs), they bind to particular Argonaute proteins called PIWI proteins; the term 'PIWI' (sometimes also 'piwi') is derived from 'P-element induced wimpy testis' proteins described in research on *Drosophila*. piRNAs have specialised roles in the nuclei of some cells. For example, they are involved in silencing sequences of DNA called 'transposable elements' or transposons. Silencing stops these sequences moving around the genome, which can eliminate the potential for transposon-induced mutations (see Siomi et al, 2011 and references cited therein).

32. PIWI proteins and their bound piRNAs are key components of a regulatory pathway that is essential for germline establishment and maintenance. Loss of PIWI proteins in *Drosophila*, mice, and zebrafish (*Danio rerio*) leads to a loss of fertility, due to a disruption in germline stem-cell formation or maintenance, arrest in meiosis, and other gametogenic defects (Juliano et al, 2014).

33. PIWI proteins are also expressed outside the germline, primarily in various kinds of stem and progenitor cells. For example, PIWI genes are expressed in the pluripotent stem cells of planarians, sponges, and tunicates and are required for epimorphic regeneration in these organisms. PIWI expression is also found in somatic stem cells in cnidarians and ctenophores, mesenchymal stem cells in mice, and hematopoietic stem cells in humans (Juliano et al, 2014).

34. However, detailed investigations have largely been confined to the function of the PIWI-piRNA pathway in the germline and gonadal somatic cells in a few model bilaterians (animals with bilateral symmetry), with a focus on transposon silencing. The potential

[7] Non-coding RNAs transcribed from heterochromatic DNA repeats function in the assembly of heterochromatin and keep heterochromatic domains silent (Bühler & Moazed, 2007).

significance of the pathway in stem cells outside the gonad and on non-transposon sequences is yet to be investigated to any significant extent (see Juliano et al, 2014 and references cited therein).

35. It appears that piRNAs require less sequence complementarity than siRNAs and have been more frequently involved in translational pausing in mammals and higher organisms than direct mRNA cleavage (USEPA, 2013).

2.1.5. *Deadenylation and decapping pathways*

36. In plants, fungi and animals, mRNA can be degraded as part of eukaryotic RNA ‘quality control’. Decapping and deadenylation pathways result in removal of the 5´ cap or 3´ poly(A) tail, respectively, thereby destabilising the mRNA. Modulating the length of the poly(A) tail of an mRNA by deadenylation is a means of controlling protein production and mRNA stability (Humphreys et al, 2005); changes in the length of mRNA poly(A) tails are catalysed by a diverse range of deadenylase enzymes (Goldstrohm & Wickens, 2008). In plants, for example, as the tail is shortened, the mRNA typically undergoes decay by exoribonucleases present in the cytoplasm (Abassi et al, 2013).

37. The removal of the 5’ cap (7-methylguanylate) exposes a 5’ monophosphate cap, leading to the 5’-3’ degradation of mRNA sequences by 5’ exonucleases such as XRN1 in eukaryotes (Poole & Stevens, 1997); decapping may or may not be linked to the 3’-5’ degradation of mRNAs (USEPA, 2013). In mammals, miRNAs are known to bind to the 3’ untranslated region of the mRNA, which is often followed by deadenylation and mRNA decay (Beilharz et al, 2009). miRNAs are also known to effect translational repression of mRNAs in *Drosophila* and zebrafish (*Danio rerio*) through deadenylation and subsequent destabilisation of mRNA.

38. While RNA turnover and PTGS are functionally linked (see e.g. Martínez de Alba et al, 2015), note that deadenylation and decapping pathways are not RNAi gene silencing pathways *per se* but are part of RNA turnover. In this document, they are considered in relation to the interplay between RNAi and RNA decay pathways; ‘aberrant’ RNA without a polyA tail may be subject to RNA-dependent RNA polymerase 6 (RdRP6), thereby generating dsRNA which can then enter the RNAi pathway (Baeg et al, 2017). Meanwhile decapped and deadenylated mRNAs, and siRNAs and miRNA-cleaved mRNAs are substrates for exonucleolytic decay.

39. In prokaryotes, the initial mRNA transcripts naturally possess a 5'-triphosphate group after transcription; the bacterial enzyme 5' pyrophosphohydrolase (RppH) removes a pyrophosphate molecule from the 5' end, converting the 5'-triphosphate to a 5'-monophosphate and triggering mRNA degradation by ribonucleases (Deana et al, 2008; Hsieh et al, 2013).

2.2. Cell-autonomous, Non-cell-autonomous, Systemic, & Environmental RNAi

40. In responsive organisms, RNAi silencing can act in a cell-autonomous or non-cell-autonomous manner (see Figure 2).

2.2.1. *Cell-autonomous RNAi*

41. In the case of cell-autonomous RNAi, the silencing process is limited to the cells in which the dsRNA is introduced (or expressed) and encompasses the RNAi process within those exposed cells. The varied biology of dsRNA-induced silencing is exemplified

by the apparently cell-autonomous, non-heritable silencing in *Drosophila* and mammals compared with the systemic nature of silencing in *C. elegans* (see the following sub-sections). In *C. elegans* and in a number of arthropods RNAi was found to be not only systemic, but also heritable (parental RNAi) due to RNA signal transmission to progeny (Zotti et al, 2018; Bucher et al, 2002; Abdellatef et al., 2015).

2.2.2. *Non-cell-autonomous RNAi*

42. In the case of non-cell-autonomous RNAi, the interfering effect can propagate across cell boundaries and takes place in tissues or cells separate from the location of application of the dsRNA. Non-cell-autonomous RNAi was observed in the first RNAi experiments, conducted in *C. elegans* (Fire et al, 1998); when these nematodes are microinjected with dsRNA into head, tail, intestine or gonad arm, soaked in dsRNA solution, or fed with bacteria expressing dsRNA, RNAi is induced and the effect is transmitted across cellular boundaries (see also Tabara et al, 1998; Timmons & Fire, 1998). Non-cell autonomous RNAi has been described in parasitic nematodes[8] (Geldhof et al, 2007), hydra (Chera et al, 2006), planaria (Newmark et al, 2003; Orii et al, 2003), insects (Tomoyasu et al, 2008; Xu & Han, 2008) and plants (Himber et al, 2003).

43. There are two different kinds of non-cell-autonomous RNAi, ‘environmental RNAi’ and ‘systemic RNAi’ (Hunter, 2006; Whangbo & Hunter, 2008; Huvenne & Smagghe, 2010).

2.2.3. *Environmental RNAi*

44. Environmental RNAi refers to sequence-specific gene silencing in response to environmentally-encountered dsRNA. Certain organisms can take up dsRNA from their environment, with subsequent triggering of RNA silencing. At least two pathways for dsRNA uptake have been described: (1) a specific trans-membrane channel-mediated uptake and (2) an endocytosis-mediated uptake (reviewed in Whangbo & Hunter, 2008; Huvenne & Smagghe, 2010).

45. Environmental RNAi was first observed in *C. elegans*; RNAi was induced when the nematodes were soaked in a dsRNA solution or fed with bacteria expressing the dsRNA molecules (Meng et al, 2013). *C. elegans* has an impermeable cuticle covering nearly its entire surface and environmental uptake is thought to occur *via* the intestinal lumen while feeding (Whangbo & Hunter, 2008). Thus, environmental RNAi in *C. elegans* requires the following steps:

1. dsRNA uptake by the intestinal cells
2. export of dsRNA or dsRNA-derived silencing signals from the intestinal cells
3. import of the silencing signals into other tissues (e.g., muscle, epidermis, germline)
4. targeted gene silencing *via* cell-autonomous RNAi machinery.

46. Winston et al (2007) reported the identification and characterisation of SID-2 (systemic RNAi defective protein-2), an intestinal luminal transmembrane protein required for environmental RNAi in *C. elegans*. When the gene for SID-1 protein was expressed in the environmental RNAi-defective species *C. briggsae,* an environmental RNAi response was conferred.

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[8] *C. elegans* is not a parasitic nematode but a free-living one, feeding on dead and decaying organic matter viz. it is a saprobic nematode.

47. Environmental RNAi has been observed in a number of phyla including planaria (flatworms), hydra, ticks, honey bees and parasitic nematodes, but not in vertebrates (Whangbo & Hunter, 2008; Huvenne & Smagghe, 2010). Sections 6.2.2 and 6.4.1 (below) discuss environmental RNAi in plants.

2.2.4. *Systemic RNAi*

48. 'Systemic RNAi' is the term used when the silencing phenomenon is locally initiated in the organism but then spreads from cell to cell throughout the whole organism. Systemic RNAi was first observed in *C. elegans* when ingested or injected dsRNAs (microinjections into the head, tail, intestine or gonad arm) led to the systemic spread of RNAi throughout the organism and transmission to its progeny. It had been suggested that transporting channels for dsRNA, encoded by sid-1 and sid-2 genes, are responsible for the spread of the silencing signal among cells in *C. elegans* (Winston et al, 2002). However, while SID proteins are involved in the uptake of endogenous RNA (see Section 5.2.2), the evidence is less clear on their role in systemic spread from cell to cell since sid-like genes have not been found in insects that are highly responsive to environmental RNA. SID-1 (systemic RNAi deficient-1) protein is a trans-membrane protein that forms a dsRNA channel (Feinberg & Hunter, 2003; Shih et al, 2009; Shih & Hunter, 2011); it has orthologs in a wide range of animals, including mammals. SID-1 mutants have intact cell-autonomous RNAi, but do not display either systemic RNAi or environmental RNAi effects in response to feeding, soaking, or injection of dsRNA (Winston et al, 2002). SID-2 is a transmembrane protein localised to the apical membrane of intestinal cells; it is necessary for the initial import of dsRNA from gut lumen but not for the systemic spread of silencing signals among cells (McEwan et al, 2012). It appears that *C. elegans* can also transport endogenous and exogenous RNA silencing signals between many different tissues *via* at least two SID-1 independent export pathways (Jose et al, 2009).

49. In plants, movement of siRNAs can occur in a localised fashion *via* plasmodesmata and systemically throughout the whole plant *via* the phloem network.

50. In plants, nematodes and fungi, the systemic spread of an RNAi silencing signal can be aided by an amplification process; this occurs through the production of secondary siRNAs by RNA-dependent RNA polymerases (RdRPs) (e.g., Vazquez & Hohn, 2013). This mechanism allows very low copy numbers of imported dsRNA to generate a robust RNAi response. Insects and mammals do not appear to have RdRP-mediated RNAi amplification (Gordon & Waterhouse, 2007); for example, although the Western corn rootworm (*Diabrotica virgifera virgifera*) is highly responsive to orally-delivered dsRNA, systemic RNAi appears to be derived from the original dsRNA molecules taken up from the gut lumen rather than being caused by transitive RNAi amplification (Li et al, 2016).

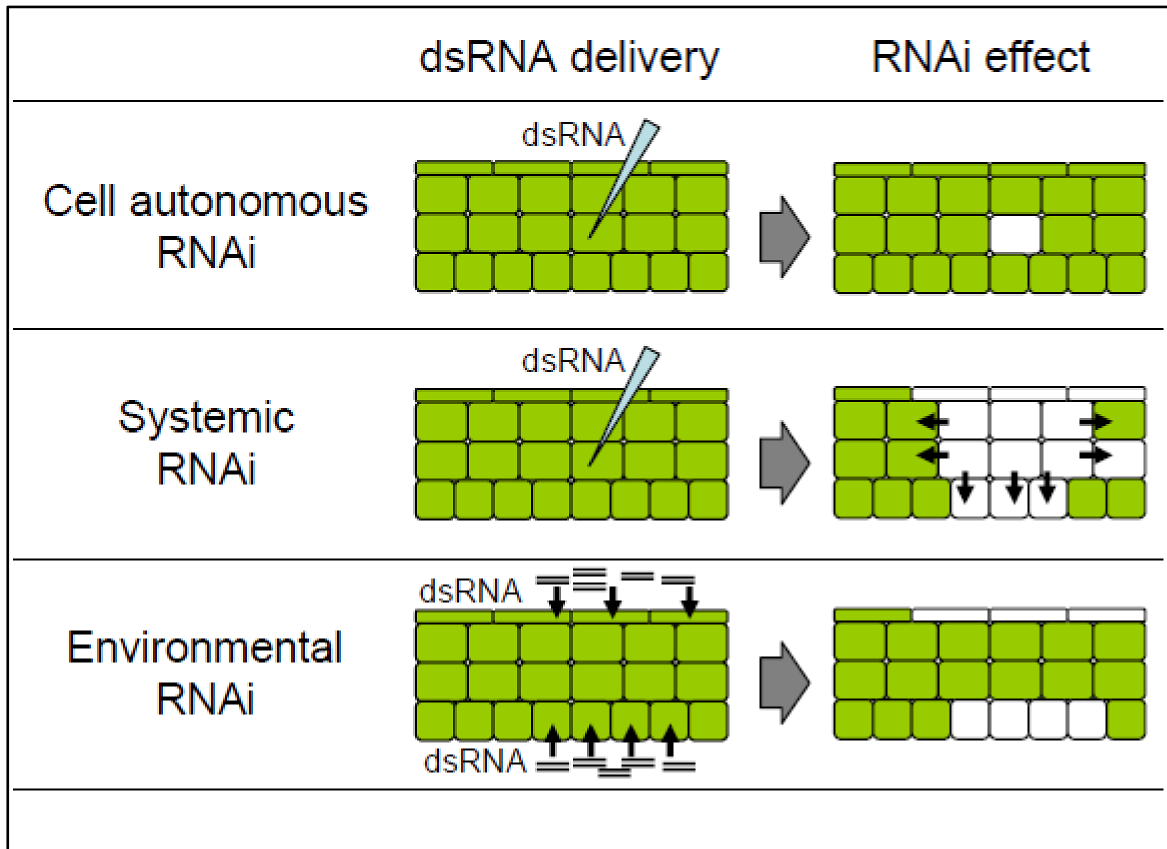

Figure 2: Cell autonomous, systemic and environmental RNAi: RNAi can act in a cell-autonomous manner, affecting only cells directly exposed to dsRNA e.g., by injection (top row of figure). Cell non-autonomous RNAi includes systemic RNAi, in which the RNAi effects propagate across cellular boundaries, mediated by transport of small RNAs (middle row). Environmental RNAi refers to the take-up of dsRNA from the environment (bottom row). [This figure is modified from Figure 5 in Paces et al, 2017. A similar illustrative diagram is found in Huvenne & Smagghe, 2010.]

Text Box: Brief summary of transcription, translation and RNA interference

1. RNA interference — or RNAi — is a cellular process that can silence the expression of specific genes: that is, it can stop proteins being produced from specific genes. RNAi can involve different types of small RNA molecules. The best-known types of RNAi act by targeting messenger RNA (mRNA) in the cytoplasm.
2. In eukaryotic cells, protein-coding genes are transcribed by RNA polymerase II (RNAPII). During transcription, an RNA molecule — the primary transcript — is synthesised using the DNA sequence as a template. The primary transcript is processed to form mRNA; regions of sequence that do not code for the protein (introns) are removed by splicing and a 'cap' is added to the 5′ end of the RNA.
3. The mRNA travels from the nucleus into the cytoplasm through the nuclear pore complex. The nuclear pore complex is one of the largest protein complexes in the cell. Filaments on the cytoplasmic side of the pore help to channel the mRNA towards the protein synthesis machinery.
4. In the cytoplasm, ribosomes carry out translation of the mRNA to form a polypeptide chain, which folds to form a protein. Ribosomes are made up of proteins and RNA molecules called ribosomal RNAs (rRNAs). Some protein folding happens during translation, but the endoplasmic reticulum is an important site of protein folding and many ribosomes are associated with it.
5. The RNAi mechanism targets the mRNAs to stop this synthesis of proteins. Because siRNAs can trigger degradation of specific mRNAs, they stop production of specific proteins.
6. RNAi is now widely used in the laboratory to explore the functions of genes by experimentally silencing them. Specific siRNAs (commonly 'hairpin' RNAs, molecules that fold back on themselves so that they become double-stranded) are now available to silence almost any gene in human cells or model organisms.
7. Researchers hope to use siRNAs to correct faulty gene expression in humans and to control economically- and environmentally-important pest species. Delivery mechanisms are a key consideration in the development of RNAi-based human therapies and RNAi-based pest-control products; the siRNA trigger needs to be delivered efficiently and in a targeted manner.

3. RNA – Molecular structures, chemical modifications, assay methods, commercial production & product formulation

Determination of the possible risks of exogenously-applied dsRNA to non-target species in the environment will require a good understanding of likely levels and persistence (or lack thereof) of dsRNA molecules in the environment. Therefore, an understanding of the structure of dsRNA molecules and any chemical modifications, their chemical stability, assay methodology, will be important in carrying out a risk assessment.
Sections 7.1.1 and 7.4.1 contain discussion and recommendations for regulatory data relating to the technical issues considered in this section.

3.1. Molecular structures of RNA

51. RNA is inherently less stable than DNA due to its chemical structure. The presence of 2'-OH group on the ribose sugar makes it susceptible to nucleophilic attack in the presence of the OH group on the 5'-phosphorus atom, thus causing breakage of the phosphodiester link and hence, the polynucleotide chain.

52. Steric factors influence the access of nuclease enzymes to nucleotide chains. Unlike double-stranded DNA, in many of its biological roles, RNA molecules are single-stranded or have single-stranded sections and are more accessible to nucleases.

53. Nucleic acid helices have two grooves; the major groove provides access to the bases while the minor groove is lined by the ribose (in RNA) or deoxyribose sugars (in DNA). The 2'-OH groups on the ribose sugars in the shallow groove of RNA are good hydrogen bond donors and acceptors, absent in DNA.

54. RNA nucleotide sequences can form various structures – A-form double helices – or fold into specific base-paired conformations that contain single-stranded regions, hairpin loops, internal loops, bulges, junctions, pseudoknots, kissing hairpins etc. Loops, bulges and mismatch base pairs open up the major groove of adjacent double helix sections of RNA molecules, facilitating the access of functional groups that are inaccessible in A-form helix geometry.

55. While these distinct RNA structures are crucial to their various biological functions, knowledge about the likely thermodynamic and biochemical stability of their secondary and tertiary structures is incomplete (see e.g., Nowakowski & Tinoco, 1997).

56. Compounding the fact that RNA is inherently less chemically stable than DNA, RNA-degrading enzymes (RNases) are more prevalent in organisms and the environment than DNases. RNA molecules play major roles in cellular signal processing as the intermediate message templates for the synthesis of proteins with amino acid sequences based on the genetic code contained in nuclear DNA. They also act as control molecules, regulating key cellular functions, including transcription and translation. Since so much RNA is being produced, but high levels are not measured, active RNA degradation systems (e.g. RNases) exist. RNA degradation is a necessary cell activity and there are substantial similarities in the RNA degradation processes between bacteria, archaea, and eukaryotes. The three major classes of RNA-degrading enzymes (ribonucleases or RNases) include: endonucleases that cut RNA chains internally; 5′ exonucleases that hydrolyse RNA from the 5′ end; and 3′ exonucleases that degrade RNA from the 3′ end (Housley & Tollervey, 2009 and references cited therein).

3.2. **Chemical modifications of dsRNA**

57. Various chemical modifications may be made to RNA in order to make it more suitable for various purposes. Generally, these modifications include covalent modifications, conjugation of dsRNA, and formation of dsRNA complexes.

3.2.1. ***Covalent modifications***

58. Chemical modifications of siRNAs which have been used to render them suitable for therapeutic purposes include:

- modifications to the phosphodiester backbone – to make siRNA more resistant to nucleases and also improve bio-distribution and cellular uptake.
- modification of the ribose 2'-OH group – to increase thermostability, potency and to reduce immune stimulation; artificially-introduced 2'-O-methyl groups in siRNAs can stabilise them in serum without affecting their RNA interference activities in mammalian cells (Yu et al, 2005).
- modifications to the ribose ring and nucleoside base – to increase stability and influence base-pairing.

3.2.2. ***Conjugation of dsRNA***

59. siRNA can be chemically bound to various biochemical components to increase cellular uptake. Examples include:-

- linking cholesterol to the 3'-OH of the siRNA – promotes uptake through receptor-mediated endocytosis (Kim & Rossi, 2007; Rettig & Behlke, 2012).
- binding of ligands such as cationic lipids (i.e., transfection reagent lipofectamine), polymers and dendrimers to the siRNA – promotes cell uptake *via* adsorptive endocytosis.
- conjugation to cell-penetrating peptides (CPPs) such as penetratin and transportin – promotes endocytosis of the siRNAs. Receptor-specific peptides, hormones, antibodies and even vitamins have been used.
- conjugation with bile acids or various long chain fatty acids – can promote cellular uptake.

3.2.3. ***dsRNA Complexes and nanoparticles***

60. Research on the therapeutic application of RNAi in treating human disease has shown that siRNAs can be packaged in larger complexes in order to protect them from degradation in, and clearance from, the human body. Liposomes have been a popular delivery system for use in RNAi therapy because they are simple to synthesise and do not activate the immune system. However, they might offer limited efficiency due to their neutral nature. Stable nucleic acid-lipid particles (SNALPs) are a similar concept to liposomes; it is reported that their positive charge allows for more effective delivery and lower toxicity than using liposomes.

61. The potential uses of nanomaterials in the development of plant protection products are under active investigation. Different forms of nanocapsules (sometimes called 'Functional Nano-Dispensers' or FNDs), based on polymeric encapsulation of active

ingredients offer promise as carriers for controlled delivery of dsRNAs. For example, chitosan nanoparticles have been used as a carrier for controlled delivery of dsRNAs because of their biocompatibility, biodegradability, absorption (Kashyap et al., 2015) and lack of oral toxicity (Baldrick, 2010). A potential delivery system for exogenously applied dsRNAs is nanoparticles. In clinical studies on human therapeutics, a variety of natural and synthetic nanocarriers, including liposomes, micelles, exosomes, synthetic organic polymers, and inorganic materials have been investigated for siRNA delivery (Shen et al, 2012). Das et al. (2015) found that carbon quantum dot (CQD) nanoparticles[9] were the most efficient carriers for dsRNA delivery compared to chitosan and silica complexes when used to target SNF7 and SRC in *Aedes aegypti* larvae.

62. However, unlike potential human therapies, the economic realities of agriculture are likely to limit the formulation technologies that will be developed and introduced for crop protection.

3.3. **RNA assay methods**

63. This section summarises the main assay methods that have been used for detecting and measuring specific dsRNAs/siRNAs in and on plants and in non-target organisms; it does not provide any guidance on the validation of the analytical methodology. As per existing regulations in OECD member countries, before an assay can be used as a detection and quantification method in regulatory studies, it must be shown to meet appropriate performance criteria, including relevance, reliability and reproducibility.

64. The expanding use of RNA interference in therapeutics and agricultural biotechnology necessitates tools for characterising and quantifying dsRNA. Identification and quantification of small RNAs (siRNAs) may be challenging in some instances because of their relatively short length but also due to low abundance in cases of siRNA pool generated by a specific dsRNA. In the case of miRNAs, characterisation may be challenging due to the high sequence similarities within miRNA family members, and the existence of miRNA isoforms and O-methyl 3′ modifications. , For siRNAs, there is a need for accurate determination of sequences targeting specific pest transcripts.

65. Methods for the detection and measurement of dsRNA in biological samples historically include various PCR and nucleic acid hybridisation type methods, including Northern Blots, reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (RT-qPCR), assays based on the ‘QuantiGene’ Plex 2.0 RNA assay platform, and Nuclease Protection Assays.

66. While Liquid Chromatography (HPLC or UHPLC/UPLC) is not particularly relevant for analysing dsRNA in environmental or other matrices because it is not a sequence-specific analytical method, it can be used in the analysis of test materials or manufactured products where a specific RNA is being produced.

67. These methods and several others are briefly summarised below; several references are provided as sources of much more detailed information on their application and utility.

[9] An aqueous mixture of polyethylene glycol and polyethylene-imine was heated in a microwave for several minutes, resulting in functionalised carbon quantum dots. These were then loaded with dsRNA (suspended in sodium sulfate) by mixing at 4°C.

68. In addition to analytical methods, in cases where an appropriate bioassay exists it might be utilised to augment the results from the other analytical methods (see further discussion in later sections).

3.3.1. *Northern Blots*

69. Northern blotting[10] involves the use of electrophoresis to separate RNA molecules by size, and detection with a hybridization probe complementary to part of, or the entire target sequence. Following the introduction of the technique in 1977, northern blotting has been used extensively for relative RNA quantification because it can resolve RNA species (including single- and double-stranded RNA) but the method is time-consuming, requires a relatively large quantity of RNA, is semi-quantitative at best, and is quantitatively inaccurate for low levels of RNA. In addition it may be difficult or not possible to distinguish and quantify specifically the mature small RNAs (si RNA or mi RNA) and their precursor (long ds RNA) molecules (EFSA GMO Panel, 2018a).

3.3.2. *Polymerase chain reaction (PCR) methods*

70. The discovery of reverse transcriptase during the study of viral replication of genetic material led to the development of reverse transcription PCR (RT-PCR); the method became the benchmark for the detection and quantification of RNA because of its relative simplicity, specificity and sensitivity. In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. By enabling sample amplification, the method eliminated the need for abundant starting material required for northern blot analyses. The method allows a very wide range of RNA abundance to be measured and it can be high-throughput.

71. In quantitative PCR (qPCR), the quantification of DNA in samples is made after each amplification cycle; this is the reason this method is often called 'real-time PCR' or RT-PCR and hence confused with reverse-transcription PCR. [Note: In this document, RT-PCR refers to reverse transcription PCR.] Yang et al (2009) describe a quantitative reverse transcriptase PCR (RT-qPCR) method capable of quantifying miRNA and siRNA in the femtomolar range, equivalent to ten molecular copies per cell or fewer[11]. This provides a linear readout of miRNA concentration spanning 7 orders of magnitude and allows the discrimination of small RNAs that differ by as little as one nucleotide.

72. Pitfalls of RT-PCR amplification of dsRNA include potential self-priming of RNA when it is expressed in both orientations which can interfere with template specificity, and the need for modifications to the standard RT-PCR method when strand-specific detection of dsRNA is desired. Furthermore, for all RT-PCR applications, RNA purification is required to eliminate hybridization to related sequences in genomic DNA. Self-annealing of the inverted repeats of the two RNA strands makes it challenging to reverse transcribe or amplify through PCR, therefore the method is quantitative for ssRNAs but not for dsRNAs (Haddad et al, 2007).

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[10] The term 'northern blot' refers to the step in the method involving capillary transfer or electroblotting of RNA from the electrophoresis gel to the blotting membrane.

[11] Note that the Yang paper calls this method a "high-performance real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay".

73. Designing primers to the correct RNA strand is critical to the success of this assay, as illustrated by the retraction of a 2016 article which had concluded that *Brassica oleracea* microRNAs could be detected in the blood of humans who ate broccoli. The retraction advised that the authors no longer had confidence in the data to support their central conclusion; in response to concerns raised about the incorrect design of the microRNA primers used in the study, primer sequences were checked and antisense design of all (bar one) of the forward primers for broccoli microRNA detection was found (Pastrello et al, 2017).

74. Droplet digital PCR (ddPCR) is a method used for absolute quantification of RNA for a variety of applications (e.g., Maheshwari et al, 2017; Zhong et al, 2018). The ddPCR method is based on partitioning of the reaction into nanolitre-sized droplets, such that either one or zero analyte molecules are present in each droplet, which can then be analysed by rapid microfluidics (Hindson et al, 2013). This technique circumvents the challenge of appropriate reference gene selection or standard curve preparation for RT-qPCR, which can be particularly advantageous for quantifying small RNAs).

3.3.3. *'QuantiGene' Plex 2.0 RNA assay*

75. The QuantiGene assay utilises cooperative binding of multiple oligonucleotide probes with specificity for the target sequence, resulting in exceptionally high assay specificity. This hybridization-based assay incorporates branched DNA (bDNA) technology, which uses signal amplification rather than target amplification for direct measurement of RNA or DNA. Direct signal amplification more precisely quantifies the RNA expression because variations or errors inherent to reverse transcription of RNA and subsequent cDNA amplification are avoided. Moreover, the QuantiGene assay can be strand-specific and is not affected by self annealing of dsRNA. Like the PCR and northern blot assays (see above), prior to hybridization the assay requires heat denaturation in the presence of the oligonucleotide probes, in order to dissociate the duplex dsRNA transcripts.

76. In the first report of a non-polymerase chain reaction-based quantitative assay for dsRNA-containing transcripts, Monsanto used this assay to characterise and quantify double-stranded RNA (dsRNA)-containing transcripts that are expressed in transgenic maize lines engineered to control western corn rootworm (Armstrong et al, 2013). Validation studies indicated that the assay was sensitive (to 10 pg of dsRNA/g fresh tissue), highly reproducible, linear over *ca* 2.5 logs, and quantitative in crude tissue lysates. This data was considered acceptable to the USEPA. However, EFSA (EFSA GMO Panel, 2018a) did not take into account these data because the dsRNA is an intermediate molecule which is processed by dicer to siRNA molecules and EFSA considers the levels of dsRNA are not a good proxy for the levels of the active siRNAs in the plant (Paces et al., 2017). Therefore, the levels of the DvSnf7 dsRNA were not considered relevant for the EFSA risk assessment of the GM maize.

77. Since then, the method has been further developed to provide a fast and high-throughput assay, allowing the simultaneous measurement of a large number of sequences of interest in a single well of a 96- or 384-well plate. The assay does not require RNA purification and is straightforward to use, with a simple ELISA-like workflow. It has been adapted to measuring dsRNA in soil (Dubelman et al, 2014; Fischer et al, 2016) and water-sediment systems (Albright et al, 2016; Fischer et al, 2017).

3.3.4. *Nuclease Protection Assays*

78. Nuclease protection assays are used to identify individual RNA molecules in a heterogeneous RNA sample extracted from cells. The extracted RNA is first mixed with radioactive antisense RNA or DNA probes complementary to the sequence(s) of interest and the complementary strands are hybridised to form double-stranded RNA (or DNA-RNA hybrids). The mixture is then exposed to ribonucleases that specifically cleave only single-stranded RNA but have no activity against double-stranded RNA; the surviving RNA fragments are those that were complementary to the added antisense strand and thus contain the sequence of interest. These products are separated on a denaturing polyacrylamide gel and visualised by autoradiography (Eyler, 2013). The high sensitivity of these assays makes them well-suited for detecting low-abundance targets. For dsRNA quantification, however, this method may not be well suited due to the double-stranded nature of the RNA which will lead to competition between labelled and non-labelled RNA strands.

3.3.5. **In Situ** *Hybridization (tissue sections)*

79. *In situ* hybridization (ISH) uses labelled complementary DNA, RNA or modified nucleic acid probes to localise specific DNA or RNA sequences in tissue sections, cells, and whole mounts. This *in situ* hybridization method ('RNA *in situ* hybridization' or RNA-ISH) localises RNAs (mRNAs, miRNAs, lncRNAs) with either radio-labelled or hapten-labelled probes, with analysis by light or electron microscopy; the method is semi-quantitative, at best (see e.g., Jin & Lloyd, 1997), This method is time and labor intensive.

3.3.6. *Liquid Chromatography (HPLC and UHPLC/UPLC)*

80. During the development and manufacture of agricultural end-use products, chromatographic analysis of active constituents and final product formulations is necessary to characterise and control the quality of the materials concerned. Liquid chromatography enables rapid and reliable determination of active ingredients and related compounds, as well as excipients and impurities in complex formulation matrices designed to stabilise dsRNA and help deliver it to the target plant or pest species.

81. Double-stranded RNA may be synthesised by *in vitro* transcription methods or by fermentation; both approaches may result in mixtures of RNA molecules as well as by-products or other impurities. Even for small dsRNA molecules containing, say, tens of base pairs, the complexity of the sequences and the similarity of the various RNA sequences that may be produced during synthesis means that standard high-performance liquid chromatography (HPLC) may not adequately separate closely-related compounds. Ultra-high-performance liquid chromatography (UHPLC, also known as ultra-performance liquid chromatography – UPLC) systems developed in recent years (e.g., Waters Corporation, 2004) can significantly increase the resolution of oligonucleotide profiles as compared to HPLC (e.g., Cramer et al, 2016). An informative overview of the application of UHPLC for the successful chemical characterisation of formulations of siRNA in liposome nanoparticles is given by Li et al (2010).

82. However, for larger dsRNA molecules containing, say, hundreds or thousands of base pairs, it is not generally possible to resolve closely-related sequences and UHPLC offers no particular advantages over HPLC. Furthermore, some stationary phases that are useful for analysing these larger nucleic acid molecules are not available in UHPLC format. Consequently, large dsRNA molecules are often analysed using ion-pair reverse-phase HPLC (e.g., Dickman et al, 2006; Waghmare et al, 2009; Nwokeoji et al, 2017).

3.3.7. *MicroRNA sequencing (miRNA-seq)*

83. miRNA-seq uses next-generation sequencing or massively parallel high-throughput sequencing to sequence miRNAs. Like other miRNA profiling technologies, miRNA-Seq has advantages (sequence-independence, coverage) and disadvantages (high cost, time and resource intensive, run length, and potential artifacts, including inadvertently favouring miRNAs that are highly expressed) (see e.g., Aldridge & Hadfield, 2012).

3.3.8. *Commercial measurement platforms for miRNA*

84. A number of commercial measurement platforms have been developed to determine relative miRNA abundance in biological samples using different technologies such as small RNA sequencing, reverse transcription-quantitative PCR (RT-qPCR) and (microarray) hybridization; a paper by Mestdagh et al (2014) described 20 platforms available at that time and reported on their relative performance.

85. Leshkowitz et al (2013) studied the detection performance of three high-throughput commercial platforms; the ability to detect miRNAs was shown to depend on the platform and on miRNA modifications and sequence. The analysis in this paper suggests that, as for conventional crop protection products, data should be provided on the performance and validity of the analytical method(s) used in regulatory studies on dsRNA-based pesticide products.

3.4. **Commercial dsRNA production**

86. One of the challenges in the commercial development of dsRNA for pest control is the availability of cost-effective methods for producing large quantities of dsRNA. dsRNA can be produced by *in vitro* transcription using RNA polymerases or by chemical synthesis, based on chemical polymerisation of nucleotides.

87. More cost-efficient methods for mass production are being developed, including production in bacteria (e.g., Palli, 2014; Timmons et al, 2001; Ongvarrasopone et al, 2007; Zhu et al, 2011; Niehl et al, 2018). The use of genetically-engineered bacteria to produce dsRNA is currently one of the most cost-effective methods, and biotechnology companies are investing heavily in this production method. cDNA complementary to the target gene is inserted into the bacterial genome using an appropriate plasmid vector and is subsequently expressed in the microbial host.

88. Hazards which may be associated with the microbial production of large quantities of dsRNA include:

- Potential contamination of the required product with microbial/viral contaminants, microbial toxins, allergens and/or other metabolic products that could pose a hazard to non-target organisms in the environment. Contaminants may arise from expression of residual virus vector, or of expression of genes of the bacterial host. (Note: This concern is not unique to production of dsRNA; the knowledge of microbial fermentation that exists can serve as guide for identifying appropriate endpoints for assessment.)
- Carry-over of viable production microorganisms in the end product. If this occurred in a product applied in the environment as a pest-control agent, it could potentially lead to increased persistence and levels of the dsRNA, with a consequent increase in the exposure of non-target organisms to the dsRNA. Furthermore, viable

microorganisms in the product could potentially transfer plasmid/genes to other organisms, thereby increasing the likelihood of non-target effects.

- Generation of unwanted infectious effects arising from the utilisation of an infectious virus vector; the likelihood of this is low with modern methods of genetic manipulation.
- Incorrect expression of the desired dsRNA or inconsistency in the product due to the instability of the inserted gene over time.

89. For these reasons, like all regulations relating to microbially-produced products, the microbial production of dsRNA will need to address strict quality-control criteria, including a possible prohibition on the presence of any live microorganisms in end-use products. Otherwise, a potential product registrant would need to apply for approval of the genetically-modified microorganism as well as for the dsRNA. Since the presence of viable GM microorganisms in a dsRNA preparation would create significant regulatory issues, it seems unlikely that such an approach would be taken by registrants.

90. OECD documents on the regulation of microbial biopesticides can be found at the OECD website on 'Biological Pesticides' (at http://www.oecd.org/chemicalsafety/pesticides-biocides/biological-pesticides.htm). Of particular relevance to the quality control of commercial dsRNA production is the Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products (Series on Pesticides No. 65, 2011).

91. Alternative methods have been developed more recently, e.g. a cell-free bioprocessing platform allowing cost-efficient RNA production without the use of genetically modified microorganisms (Taning et al. 2019). Other contaminants arising from the manufacturing process that could pose hazards to non-target organisms, if carried through in the technical-grade active constituent (TGAC[12]) and formulated product, may include chemical reaction by-products, sequence heterogeneity of the dsRNA product, fermentation residues, reaction starting materials, and mutant, or alternative forms of the mutant or alternative forms of dsRNA arising from re-arrangement of the plasmid during the fermentation process.

3.5. Formulation chemistry – effects on dsRNA stability and environmental persistence

92. A formulation ingredient is a substance other than the active ingredient that is intentionally added to a pest control product to improve its physical characteristics (e.g., the stability of the active ingredient, the solubility of the active ingredient, product sprayability, product spreadability).

93. In addition to considering the chemical and biological stability of dsRNA, any risk assessment of dsRNA-based products will need to take into account whether the dsRNA in a proposed commercial product has been stabilised in some way, in order to reduce the rate of abiotic and/or biotic degradation in the environment. The intent of this is to increase residence time in or on the treated crop or target pest in order to allow sufficient absorption or ingestion of the active constituent for it to have the desired pesticidal effect. However, the increased persistence of stabilised dsRNAs is likely to lead to increased non-target

[12] Also Technical-Grade Active Ingredient (TGAI) or Technical-Grade Active Substance (TGAS), depending on the jurisdiction.

exposure, potentially increasing the chances of unwanted off-target effects caused by the dsRNA.

94. A number of formulation strategies have been developed in the past decade to address issues related to the delivery, bioavailability, or potential toxicity of RNAi in human therapeutic products. It is likely that some of these will be adapted to exogenously-applied dsRNA products in agriculture. Howeverit is likely that the economic realities of agricultural and horticultural production will have an impact on the development of complex formulation technologies. In general, agricultural chemical companies need to balance multiple factors in the development of end-use product formulations, including: product stability during shipment and storage; product performance (i.e., loading, mixing, efficacy); the environmental load of formulation components; and the costs of development, formulation components and production (see e.g., http://news.agropages.com/News/NewsDetail---26237.htm). In formulating dsRNA-based products, companies will endeavour to find formulation technologies compatible with a biological active constituent in order to: ensure product stability in commercial channels; not negatively impact product efficacy in the field; find formulation types that do not require significant changes in handling or application as *cf.* conventional pesticide products.

3.5.1. *Non-active constituents in EPs*

95. Formulation ingredients[13] such as wetting agents, dispersing agents, stabilisers, preservatives, soil conditioners etc., that could be used in dsRNA-based formulations for pest-control may affect dsRNA stability, delivery and availability to the target organism or pest. However, in most cases it is unlikely that effects of non-active constituents in formulations will be theoretically predictable and thus applicants / companies will need to conduct empirical studies (similarly to what is done for other formulated pest-control products).

3.5.2. *Manufacturing impurities in EPs*

96. As discussed in some detail in Section 3.4 (‘Commercial dsRNA production’), genetically modified microorganisms (GMOs, GMMs or GEMs) can be used to produce large quantities of dsRNA for use in dsRNA-based pesticide products. Consideration may need to be given to prohibiting the presence of viable GMOs in dsRNA-based end-use products or requiring applicants to provide theoretical and empirical data, on a case-by-case basis, to show that their presence would not be a cause for concern. Moreover, the presence of viable GMOs would be subject to GMO legislation in OECD member countries

97. In addition to impurities introduced with a microbially-produced dsRNA TGAI, the potential for impurities of concern to be introduced with other formulation excipients will need to be considered (as is done for existing pesticide products). And therefore the nature and content of certain impurities should be specified.

[13] Termed ‘co-formulants’ in some jurisdictions. According to Regulation (EC) No. 1107/2009 co-formulants are substances or preparations which are used or intended to be used in a plant protection product or adjuvant, but are neither active substances nor safeners or synergists.

4. Types of dsRNA-based end-use products & application methods

An understanding of the type of product for which a registration is sought, and a knowledge of way(s) in which it is proposed to be applied is an essential part of the problem formulation step; this information will help determine the approach to estimating likely environmental exposures.

98. Exogenously-applied dsRNA products will be and are being designed and developed to be sprayed on crop and ornamental plants or directly applied in situations where pest control is needed e.g., in bee hives to control the varroa mite (*Varroa destructor*).

4.1. Types of dsRNA-based products

99. It is likely that dsRNA active ingredients that could be formulated in exogenously-applied or 'environmental' dsRNA-based end-use products[14] (EPs) will fall into one of the following activity categories: (1) Direct-control agents; (2) Resistance-factor suppressors; (3) Developmental disruptors; and (4) Growth enhancers (USEPA, 2013).

4.1.1. *Direct Control Agents*

100. A dsRNA direct-control agent is defined as a dsRNA active ingredient that has direct adverse effects upon the metabolism or other vital biological processes of the target pest species, resulting in mortality of that pest. The difference between dsRNA-based products and conventional chemical pesticides is that, whereas traditional pesticides exert their toxic physiological effects at higher levels of the organism's physiology and metabolism, the dsRNA operates at the level of gene expression.

101. dsRNA-based EPs falling into the category of dsRNA direct control agents are likely to include products with insecticidal, fungicidal and herbicidal activity. Because their mode of action is different from that of 'conventional' chemical pesticides, they offer the promise of being incorporated into integrated pest-management (IPM) systems, resulting in reduced chemical pesticide use and possible lower potential for resistance development by target pests. Zhang et al (2017) have noted that even a weak RNAi response may still be effective when it interacts with a plant's own chemical defence. As part of U.S. EPA insect resistance management requirements for a plant-incorporated protectant based RNAi product, and as reported in 2018 a laboratory selected colony of WCR was developed which was resistant to ingested DvSnf7 dsRNA. The WCR in this colony had impaired luminal uptake and resistance was not DvSnf7 dsRNA-specific, as indicated by cross resistance to all other dsRNAs tested (see Section 8.2.3), https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0197059.

4.1.2. *Resistance Factor Suppressors*

102. A dsRNA resistance-factor suppressor is defined as a dsRNA active ingredient that suppresses genetic resistance to a conventional chemical pesticide. Many pest species have developed resistance to chemical controls since the advent of widespread chemical pesticide use in the 1940s; repeated use of the same chemical or other chemicals with a similar mode of action has led to the selection of resistant weeds, fungi and insects. As the

[14] Referred to as 'Non-PIP dsRNA-EPs' in USEPA nomenclature.

number of pesticide-resistant weed, fungi and insect species increases, the interest in developing dsRNA products that specifically target the genes responsible for the resistance mechanisms to specific herbicides, fungicides and insecticides increases (Lundgren & Duan, 2013; Preuss & Pikaard, 2003). As an example, Bona et al (2016) have demonstrated the efficacy of RNAi in restoring the susceptibility of adult mosquitoes to a widely used pyrethroid insecticide. It is likely that the dsRNA and conventional chemical products will be used as a combination spray although it is feasible that the dsRNA-based product could be sprayed at a suitable interval before the application of a chemical pesticide.

103. Apart from resistance-factor suppressors that suppress resistance to the action of chemical pesticides, it is likely that dsRNA active ingredients will be developed that specifically target genes responsible for pathogen resistance in organisms. Such dsRNA-based products could be engineered to: (1) stimulate pathogen resistance in desirable food and ornamental plants[15]; and (2) suppress pathogen resistance in weed species[16]. In addition, it is conceivable that dsRNA products may be developed to suppress disease resistance in other pest taxa, in a targeted manner.

4.1.3. *Developmental Disruptors (Growth Regulators)*

104. A dsRNA developmental disruptor is defined as a dsRNA active ingredient that interferes with the normal development or growth of the target pest such that the target pest or its progeny either die (indirect mortality), are less fit to survive in the environment, or are sterile. Examples of developmental disruptors that are currently registered for use in agriculture include the insect growth regulators (IGRs); these chemicals fall into two main categories: (1) juvenile hormone mimics that disrupt hormonal control of larval development and inhibit metamorphosis (e.g., methoprene); and (2) chitin synthesis inhibitors (e.g., triflumuron) that prevent chitin formation and replacement of the old cuticle following ecdysis (moulting).

105. It is possible that dsRNA active ingredients will be developed that specifically target genes responsible for the ontogenetic development of important pest species.

4.1.4. *Plant Growth Regulators*

106. A dsRNA plant growth regulator is defined as a dsRNA active ingredient that stimulates, inhibits, or otherwise mimics the activity of a naturally-occurring plant hormone; in this case, the target is the crop plant, not the pest species (as considered in the previous section). Plant hormones and their synthetic analogues are typically applied to food crops and ornamental plants for a range of reasons including harvest control (delaying or advancing fruit ripening, inhibiting or promoting abscission), increasing plant resistance to environmental stress, and improving aesthetic qualities (colour, odour, taste, shape, uniformity).

4.1.5. *Application methods for dsRNA-based pesticides*

107. In developing guidance on conducting environmental risk assessments for dsRNA-based pesticide products, it is necessary to determine the most-likely application methods for these exogenously-applied products since the application method will have a direct

[15] There are differences in how different OECD countries regulate ‘plant strengtheners’.

[16] This use may fall outside the existing pesticide definition and /or regulations in some jurisdictions.

bearing on the potential routes of exposure of non-target organisms in the environment to the product being applied.

108. It is likely that dsRNA-containing end-use products could be applied using the same methods as traditional chemical pesticides, viz:

- Foliar sprays
- Foliar dusts and powders
- Soil sprays and drenches
- Seed treatments
- Granules/powder (topically applied to soil or soil incorporated)
- Baits and traps

109. As for conventional chemical pesticides, both outdoor and indoor uses are expected; outdoor use sites are likely to include both terrestrial and aquatic habitats while indoor use sites are likely to include crop storage facilities, greenhouses, and buildings (USEPA, 2013).

5. Effects on non-target organisms from exposure to RNAi-based pesticides

5.1. General considerations

110. The following Table (Figure 3) summarises the possible outcomes for gene silencing in target and non-target organisms and the likely consequence, following the application of an RNAi-based product.

111. Small interfering RNA, if it (or its precursor) is correctly designed:

- may block the expression of the target gene in the target organism, leading to a biologically-meaningful effect (e.g., reduced fitness or mortality) – the desired effect (Table: Top left-hand side (LHS)).
- may also block the expression of the same transcript in non-target organisms which might be exposed (Table: Top RHS). This may create the potential for a biologically meaningful effect or hazard.
- may also block an off-target gene (or genes) in the target organism (Table: Bottom LHS). If this occurs, it is unlikely to be of any consequence if the effect of silencingthe off-target gene compromises the fitness of the target organism (pest) as intended.
- may block the expression of a non-target gene (or genes) in non-target organisms, which may be exposed (Table: Bottom RHS). This may create the potential for a biologically- meaningful effect or hazard.

Off-target Gene Silencing

	Target Organism	Non-target Organism
Target Effect	✓	X or -
Off-target Effect	-	X or -

✓ desired effect
- effect of no consequence
X unintentional effect (e.g. loss of fitness, impact on reproduction, death)

Figure 3: Summary table for off-target gene silencing

112. Note that this table is intended to provide a general illustration of the potential for off-target effects; it should not be taken to imply that a 'target' effect in a non-target organism (Table: Top RHS) will necessarily be as potent or significant as the sought-after effect (loss of fitness or mortality) in the target organism. For example, a decrease in production of a particular protein in the target pest might have been shown to have an adverse impact (i.e., pesticidal efficacy has been demonstrated), but the level of production of that protein in the non-target organism may be more abundant than in the target pest, meaning that a downshift in its production may be of no biological consequence.

113. With respect to possible off-target effects in non-target organisms (Table: Bottom RHS), the unintentional effect may be detrimental or it may be of no consequence with respect to fitness or mortality; the kinetics and biochemical pathway of the putatively-impacted protein(s) may be different in the target pest and the non-target organism.

114. The potential for a biologically-meaningful interaction with a dsRNA should be influenced by the phylogenetic 'distance' between the non-target organism and the pest organism[17].

115. Three key factors must be considered in the environmental risk assessment for non-target organisms:

1. *Potential for exposure*: A pre-requisite for a biological effect (including an off-target effect) is exposure. This will be determined by a range of factors, including the requested use pattern of the dsRNA-based product.
2. *Responsiveness of non-target organisms*: This is a critically important factor and includes a consideration of barriers in organisms to environmental RNA.
3. *Sequence alignment*: This is one requirement for biologically meaningful interaction of a dsRNA active ingredient.

116. These and other factors are relevant to the consideration of the off-target effects; they are covered in more detail in the following sections of this document.

117. *In silico* comparisons of sequence similarities between siRNAs and sequences present in target organisms suggest that siRNAs could have off-target effects within a targeted cell or organism (Davidson & McCray 2011; Lundgren & Duan, 2013). However, it is relevant to note that the off-target effects reported in these review articles were in human cell lines, with the use of transfection agents, an exposure dynamic that is unlikely to be relevant to topical dsRNA applications in agriculture. Furthermore, it is important to note that a sequence similarity does not indicate an effect – it simply indicates that one of the requirements for effective RNAi is met.

5.1.1. *Lessons from research on medical applications of dsRNA*

118. A great deal of clinical research is being carried out in order to try and treat human diseases by silencing specific cellular functions with RNAi. This research has resulted in a range of information which is relevant to human health risk assessment of possible pest-control products using dsRNA.

119. A 2016 review (Sullenger & Nair, 2016) of the status of clinical research on the application of RNA-based therapeutics in treating a range of human diseases concluded

[17] Evolutionarily-conserved genes would not be appropriate targets for a selective RNAi-based pesticide.

that there are two major pharmacokinetic challenges in the development of synthetic oligonucleotide therapeutics:

1. their limited oral bioavailability; and
2. the rapid rate at which short RNAs are cleared from the circulation.

120. This rapid clearance from circulation was also observed with RNAs designed to be nuclease-resistant. All the five Phase-3 clinical trials of synthetic siRNAs underway up to the time of the review either used local delivery of the siRNA (e.g., intraocularly to treat age-related macular degeneration or retinal ganglion apoptosis) or involved modifications to the siRNA in order to target delivery to the liver or kidney after intravenous administration (e.g., formulating the siRNA with N-acetylgalactosamine to facilitate uptake by and release into the cytoplasm of hepatocytes).

121. Another 2016 review on the clinical application of therapeutic oligonucleotides concluded that "the effective delivery of oligonucleotides to their intracellular sites of action remains a major issue" (Juliano, 2016). This review did not discuss barriers to uptake following oral ingestion of oligonucleotides but considered those in place following intravenous administration of oligonucleotides, including those packaged in nanoparticle delivery systems. These barriers include:

122. *The vascular endothelial barrier*: the capillary lumen is surrounded by a tightly-abutted layer of endothelial cells, forming a barrier between blood and the parenchymal space. The vascular endothelium allows passage of molecules the size of individual oligonucleotides but limits the passage of those in nanoparticle packaging.

123. *The reticuloendothelial system (RES)*: The administration of oligonucleotides in nanoparticles will usually result in a large fraction of the material being taken up by the RES, particularly Kupffer cells.

124. *Renal excretion*: Renal clearance plays an important role in the pharmacokinetics of oligonucleotides. siRNA and uncharged oligonucleotides do not bind extensively to plasma proteins and are readily cleared by the kidney. Many oligonucleotides fall in the size range of 3–6 nm or less and are ultra- filtered by the kidneys (see review by Juliano, 2016). For siRNAs, the liver and kidneys are the key sites of siRNA uptake. Phosphorothioate-modified oligonucleotides bind to plasma proteins, slowing their renal clearance and permitting greater distribution to tissues, with most found in liver and kidneys; their renal excretion mainly involves nuclease degradation products. Uncharged morpholino oligonucleotides are rapidly cleared by the kidneys, largely as intact molecules, and display lower levels of tissue accumulation than phosphorothioates (Juliano, 2016). Molitoris et al (2009) showed that an siRNA targeted to p53, a pivotal protein in the apoptotic pathway, was cleared within minutes of intravenous administration to Wistar and Sprague-Dawley rats; the kidneys, in particular, proximal tubule cells, were overwhelmingly the primary site of tissue distribution. The authors noted that the rapid clearance of the oligonucleotide from the body minimised exposure of other organs/cells. Thus, renal clearance appears to play an important role in the pharmacokinetics and bio-distribution of all types of 'free' oligonucleotides.

125. In summary, while the effectiveness of a dsRNA-based pharmaceuticals might be shown in cell culture studies, *in vivo* delivery to target organs by the oral, dermal and inhalation routes has been problematic. Most dsRNA therapies based on native forms of dsRNA have not shown efficacy by the oral route of administration without some protection from exposure to stomach acids and RNases (Petrick et al, 2013). For dermal

and inhalation routes of drug delivery, some form of conjugation, encapsulation or chemical modification is necessary to facilitate trans-membrane movement and to reduce rapid renal clearance. A literature review of baseline information to support the food and feed risk assessment of RNAi-based GM plants (Davalos et. al., 2019, EFSA Supporting publications) also supports the facts that exogenous dsRNA first need to reach the intented organism in sufficient quantity levels by overcoming the biological barriers. Thus, available evidence suggests that the likelihood of systemic exposure of mammals to RNA molecules applied in the field as pesticides is very low assuming no modification or addition of other product ingredients to facilitate pesticidal action in the target organism. (see also Section 6.5.3).

126. While clinical research has provided significant amounts of information about the pharmacokinetics of dsRNA, it has also provided information on possible off-target effects. Off-target effects of RNAi therapy can be broadly classified as siRNA specific or nonspecific. The former can arise from limited siRNA complementarity to non-target mRNAs and has been characterised in highly-exposed *in vitro* systems and following systemic RNA administration, in experiments employing agents to facilitate delivery. The latter, resulting in immune- and toxicity-related responses, arise due to the construction of the siRNA sequence, its modification, or the delivery vehicle. Off-target effects associated with siRNA delivery can be divided into three broad categories: (1) miRNA-like off-target effects, referring to siRNA-induced sequence-dependent regulation of unintended mRNA transcripts through partial sequence complementarity; (2) inflammatory responses through the activation of Toll-like receptors (TLRs) triggered by siRNAs or delivery vehicles (e.g., cationic lipids and viruses); *and* (3) high-dose effects on miRNA processing and function through the saturation of the endogenous RNAi machinery by exogenous siRNAs (for a recent review, see Yang & Yang, 2016 This suppression of RNAi machinery has been observed following very high doeses in in vitro experimental systems (e.g., cultured cells using transfection agents or stable hairpin expression), not from routine therapeutic use of exogenous RNAs (and would be unlikely at levels of exposure expected from agricultural use of dsRNA-based products).

127. An example of siRNA activation of TLRs was reported by Cho et al (2009); 21-nt or longer non-targeted siRNAs were able to suppress hemangiogenesis and lymphangiogenesis in mouse models of neovascularisation, independently of RNA interference, by directly activating Toll-like Receptor 3 (TLR3), a double-stranded RNA immune receptor, on the surface of blood endothelial cells. This action was as efficient as a 21-nt siRNA specifically targeting vascular endothelial growth factor-A. In contrast, a 7-nt non-targeted siRNA, which was too short to activate TLR3, did not affect angiogenesis in these models.

128. Whitehead et al (2011) commented that "one of the significant challenges facing the field [viz. human siRNA therapeutics] today is the differentiation between therapeutic effects caused by target-specific, RNAi-mediated gene silencing and those caused by non-specific stimulation of the innate immune system". Their review highlighted ways in which siRNA could be engineered either to avoid or provoke an innate immune system response.

129. In its white paper on the use of RNAi technology as a pesticide, the USEPA commented that since no new protein is being expressed by RNAi, the use of RNA interference technology should not present any allergenicity issues (USEPA, 2013). Despite the lack of allergenicity concerns for RNA, allergenicity issues may still occur in RNAi-based exogenous pesticides if other biologically- derived molecules are present as contaminants (see also Sections 3.4 and 3.5).

130. The literature from pharmaceutical research on undesired effects arising from RNAi-based therapies (e.g., the potential interaction with the innate immune system of humans and other mammals) needs to be put in context. The route of exposure for most of this literature is not directly relevant for non-target organism assessment of topically-applied dsRNA-based products. While the majority of clinical studies utilised intravenous injection, the primary route of exposure for mammals to topical dsRNA-based pesticide products would most likely be *via* the diet. Thus, an innate immune response to a topically-applied dsRNA would be unlikely since dsRNA would not be absorbed in meaningful amounts from the gastrointestinal tract; these barriers are discussed in more detail in Section 6.5.3. Secondly, the oligonucleotides utilised in clinical studies are usually extensively formulated to improve persistence and bypass cellular barriers to RNA uptake. RNAi machinery in different organisms

5.1.2. *Potential for Off-Target Gene Silencing*

131. There is reasonably extensive literature on the potential for off-target gene suppression mediated by small RNAs through partial complementarity to a non-target mRNA or through complementarity between the 'seed' region (the 7 bases of nucleotides 2 to 8 of the guide strand) of a small RNA and the 3'-untranslated region (UTR) of a non-target mRNA[18] in mammalian cells (reviewed by Petrick et al, 2013). While RNA-mediated gene suppression is hybridization-dependent and thus occurs in a sequence-specific manner, suppression of genes with less-than-perfect complementarity has been documented in, for example, *in vitro* mammalian screening assays, albeit employing high doses and transfection agents (Jackson et al, 2003; Jackson et al, 2006a; Jackson et al, 2006b; Vaishnaw et al, 2010). Given the nature of the test system in these studies, it may be questioned whether they are relevant for consideration in a risk assessment.

132. Off-target gene suppression can occur through hybridization with mRNA from genes that have a high degree of sequence similarity to the intended target gene, especially between the siRNA seed region and the 3'-untranslated region of the mRNA transcribed from a mammalian off-target gene (Jackson et al, 2006b) (see also Section 5.4.2). The seed region is critical for mRNA recognition by RISC-incorporated animal small RNAs. Seed pairing has been shown to be both necessary and sufficient for target regulation by some miRNAs (Doench & Sharp, 2004; Krek et al, 2005; Lewis et al, 2005; Lewis et al, 2003), and a single base mismatch within the central region of the siRNA may eliminate detectable siRNA-mediated silencing of the target in mammalian cells (Amarzguioui et al, 2003; Du et al, 2005).

133. The importance of other criteria beyond sequence complementarity has been described by Liu and colleagues (Liu et al, 2013); they noted that G/C base content, thermodynamic stability between the siRNA and its target, sequence context outside the small RNA target region, target accessibility (e.g., is the target protein bound?) and target secondary structure are also significant determinants of the potential for a small RNA to suppress a target gene.

134. In animals, gene suppression through seed matches is significantly less potent than that observed with small RNAs having full complementarity (Vaishnaw et al, 2010),

[18] The three-prime untranslated region (3'-UTR) is the section of mRNA that immediately follows the translation termination codon; it often contains regulatory regions that post-transcriptionally influence gene expression.

perhaps owing to the need for approximately 5 to 6 seed-matching small RNAs binding cooperatively to the 3' UTR to achieve sufficient gene suppression of a given target (Broderick et al, 2011).

135. However, it appears that sequence complementarity outside the seed region is also required for efficient target suppression by siRNAs and some miRNAs (Didiano & Hobert, 2006; Ha et al, 1996; Tay et al, 2008; Vella et al, 2004); in some cases, single base mutations outside the seed region can eliminate target suppression (Du et al, 2005; Duxbury et al, 2004; Elbashir et al, 2001a). Investigations by Grimson et al (2007) indicated that factors such as site context and sequence context can contribute to the efficacy of target silencing; thus, a match between the seed region and a target gene is not necessarily sufficient for gene suppression.

136. An *in -silico* study examined the potential for off-target binding sites for potential initiation of RNAi, employing transcriptome sequence data from *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Homo sapiens* (Qiu et al, 2005). Considering sequence similarities alone, the probability of RNAi off-target binding ranged from 5 to 80% for each of the organisms, when using as a parameter the exact identity between any possible siRNA sequences (arbitrary length ranging from 17 to 28 nt) derived from a dsRNA (range 100–400 nt) representing the coding sequences of target genes and all other siRNAs within the genome. Interestingly, siRNA sequences of 21 nt, the length most commonly observed *in vivo*, optimally balanced target specificity and a lower probability of off-target RNAi; siRNA sequences of <21 nt increased the probability for off-target effects while longer sequences did not gain adequate target specificity. If biological and thermodynamic descriptors of effective siRNA were included in the analyses, the off-target error rate was reduced to *ca*. 10%. These computational predictions did not include the effects of siRNA concentration (i.e., the need for multiple siRNA copies per cell for effective RNAi), the potential for exposure, or responsiveness to environmental RNA, nor did they attempt to account for any synergistic or mutually interfering interactions of a pool of siRNAs (e.g., off-target effects tend to be eliminated by the dilution effect of a complex siRNA pool[19] (Hannus et al, 2014). Thus, while these results clearly overestimate likely off-target effects, they do indicate that, based on sequences only, off-target binding is possible and that, despite an apparently optimal length for specific sequence-based recognition, 21 nt siRNAs still have a chance of causing off-target effects when considering all coding domains within a transcriptome. Not surprisingly in this *in silico* analysis, the potential for off-target interactions increased when sequence mismatches between the siRNA and potential targets were allowed. It should also be noted that, in mammals, matching of the seed region sequence might be sufficient for an off-target effect as siRNAs can also enter the miRNA pathway (refer to Section 7.4.4).

137. Another study showed that 17% of dsRNAs in a *D. melanogaster* dsRNA library contained putative 21-mers with complete sequence homology with off-target binding sites in the *Drosophila melanogaster* transcriptome (Kulkarni et al, 2006). Given the small sizes of siRNAs, it is perhaps not surprising that predicted off-target binding sites are prevalent within the genomes of all organisms evaluated to date (Lundgren & Duan, 2013). Although predicted off-target binding would not appear to be a concern in target organisms, off-target binding in non-target organisms could be a hazard if (1) the non-target organisms are sufficiently exposed to the RNA; (2) the non-target organism is responsive to environmental RNA; and (3) the down-regulation of a protein (or proteins) leads to an impact on growth, reproduction, or mortality. Further limiting the potential for suppression

of mRNA, it is possible that potential binding sites may be protein bound or possess secondary structures that limit the access of the environmental RNA.

5.2. **RNAi machinery in different organisms**

138. Key to an understanding of possible off-target risks of applying dsRNAs in the environment is knowledge of how different species respond to exogenous RNAs. Available data indicate that organisms vary significantly in their ability to take up foreign dsRNA and use it in the RNAi pathway. Although RNAi follow a generally conserved pathway across eukaryotes, some components are significantly different depending on the taxonomic kingdom or group, with regard to the molecular mechanism behind cellular uptake and systemic spread of silencing (see Joga et al, 2016 and references cited therein). Some limited information about core RNA silencing components (such as Dicer, Argonaute proteins, and RdRP) in different taxa is provided below, but specialist research papers and reviews should be consulted for more detailed information on what is currently known about RNAi mechanisms in different species. In an overview paper on RNAi in insects, Zotti & Smagghe (2015) noted that, while differences in components of RNAi pathways are generally limited, they are sufficient to provide a high degree of variability in RNAi responsiveness amongst insect orders. This high variability has been largely ascribed to different mechanisms of dsRNA uptake and spread; however, while extensive research has been carried out in an attempt to correlate the diverse results with differences in RNAi efficiency, “no conclusions were reached” (Zotti & Smagghe, 2015). Since uptake of dsRNA and spread of the interference signal within organisms have a direct bearing on systemic exposure (and hence the risk assessment for non-target organisms), the focus of the following subsections is more on these mechanisms than on the core RNAi mechanisms.

5.2.1. ***Micro-organisms***

139. Micro-organisms include bacteria, archaea[20] and most protozoa, plus some fungi, algae, and some micro-animals such as rotifers.

140. RNAi is predominately a eukaryotic pathway. Bacteria do not have homologous RNAi machinery, but they can recognise invading DNAs and RNAs. Indeed, RNA molecules that act as regulators were known in bacteria for many years before the first microRNAs (miRNAs) and short interfering RNAs (siRNAs) were discovered in eukaryotes. For example, in *Escherichia coli* small non-coding RNAs can cause gene silencing by translational repression, in the absence of mRNA destruction (Morita et al, 2006). A unique class of regulatory RNAs in *ca*. 40% of bacteria and *ca*. 90% of archaea sequenced to date is part of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system (see e.g., Wiedenheft et al, 2013). Mature CRISPR RNAs (crRNAs), which provide resistance to bacteriophages and prevent plasmid conjugation, share limited similarities with eukaryotic siRNA-driven gene silencing, although CRISPR is phylogenetically and mechanistically different than eukaryotic RNAi as none of the protein components are orthologous, and the systems exhibit distinct features. Neither messenger RNAs (mRNAs) nor siRNAs or miRNAs contain the secondary structures

[20] Archea have morphological similarity to bacteria but possess genes and several metabolic pathways that are more closely related to those of eukaryotes.

required for interaction with CRISPR-associated (Cas) proteins and thus are not substrates for the CRISPR system (Marraffini, 2015). In summary, the only similarity between RNAi and CRISPR in bacteria is that they use sequence complementarity and they can act as a deterrent to viral infection. Waters & Storz (2009) have reviewed RNA regulatory systems in bacteria and archea.

141. In many fungi, the mechanism of quelling is generally considered to be equivalent to RNAi in animals because core RNA silencing components such as Dicer, Argonaute, and RdRP genes are used in their pathways (Quoc & Nakayashiki, 2015). Comparative phylogenetic analyses suggest that the number of Dicer, Argonaute, and RdRP genes vary significantly among fungal species, suggesting that RNA silencing pathways have diversified during the evolution of fungi. While the mechanisms of RNA silencing are conserved in most fungal species, there are some exceptions: most or all of the components are missing in certain fungi, most notably in the model organism *Saccharomyces cerevisiae*. However, introducing two RNAi-related proteins from the budding yeast *Saccharomyces castellii* facilitates RNAi in *S. cerevisiae* (Drinnenberg et al, 2009). In *Neurospora crassa* several additional genes in the quelling pathway have been identified (e.g., QDE-3, a DNA helicase). That certain ascomycetes and basidiomycetes are missing RNA interference pathways indicates that proteins required for RNA silencing have been lost independently from some fungal lineages, possibly due to the evolution of a novel pathway with similar function, or to the lack of selective advantage in certain niches (Nakayashiki et al, 2006).

142. Some eukaryotic protozoa such *as Leishmania major* and *Trypanosoma cruzi* lack the RNAi pathway entirely (DaRocha et al, 2004; Robinson & Beverley, 2003).

5.2.2. *Non-arthropod Invertebrates*

143. As noted above (Section 2; 'Overview of RNA Interference'), the discovery that feeding dsRNA to the nematode worm *Caenorhabditis elegans* could trigger gene specific silencing (Timmons & Fire, 1998) led to this species becoming a model organism in RNAi research. This section provides a summary overview of RNAi machinery in this species from the phylum Nematoda[21].

144. *Caenorhabditis elegans* is a free-living (not parasitic), transparent nematode[22] (roundworm), about 1 mm in length, which lives in temperate soil environments. In 1998, it was the first multicellular eukaryotic organism to have its whole genome sequenced (The *C. elegans* Sequencing Consortium, 1998) and in 2011, a near-complete 'connectome' (neuronal wiring diagram) was published (Varshney et al, 2011).

145. The function of specific genes can be blocked by environmental RNAi in *C. elegans* by soaking, injecting, or feeding the organism with dsRNA targeting the relevant mRNA. Environmental RNAi uptake is much less efficient in most other species of worms in the

[22] Because it is one of the simplest organisms with a nervous system, it has been used for many decades as a model organism for the investigation of neural development and neural mechanisms in animals. The experimental advantages of *C. elegans* include the following: bulk populations of the organism can be grown inexpensively and easily (a few hundred nematodes can be kept on a single agar plate and suitable growth medium); individuals can be frozen and remain viable when subsequently thawed, allowing long-term storage; its transparency facilitates the investigation of cellular differentiation and other developmental processes in the intact organism; males are clearly distinguishable from females; and it is a convenient organism for genetic analysis (Brenner, 1974).

Caenorhabditis genus; although injecting RNA into the body cavity induces gene silencing in most species, only *C. elegans* and several other distantly-related nematode species can take up orally-ingested dsRNA (Félix, 2008).

146. Another aspect of RNAi extensively studied in this nematode is intercellular or systemic spreading of gene suppression (see e.g., Jose & Hunter, 2007). The phenomenon of intercellular spreading of RNAi appears to be restricted to plants, fungi, and some invertebrate species, including *C. elegans* (Jose & Hunter, 2007; Voinnet, 2005); it does not appear to be present in *Drosophila* or vertebrates (Tomari & Zamore, 2005).

147. In *C. elegans*, the Sid-2 gene encodes a membrane protein which is situated in the intestinal cells. The protein SID-2 imports dsRNA from the intestinal lumen (Winston et al, 2007; McEwan et al, 2012) through endocytosis and exports the silencing RNAs to other neighbouring cells through SID-1 channels from the internalised vesicles by way of passive movement (Whangbo & Hunter, 2008; McEwan et al, 2012; Baum & Roberts, 2014). Thus, environmental RNAi in *C. elegans* involves both SID-1 and SID-2 proteins. When inserted as a transgene in the other species, sid-2 allows them to take up dsRNA for RNAi as *C. elegans* does (Winston et al, 2007).

148. *C. elegans* possesses an RNAi amplification system; secondary siRNAs are created *via* an RNA-dependent RNA polymerase (RdRP) mechanism which amplifies and prolongs the silencing effect.

149. While *C. elegans* is an important model species for elucidating the RNAi biochemical pathway, this species is not fully representative of the responsiveness to environmental RNA in the *Caenorhabditis* genus. Nuez and Felix (2012) demonstrated a wide variation in the responsiveness to environmental RNA within this genus and adduced evidence that the range of responsiveness evolved convergently several times. Similarly, it cannot be assumed that all free-living nematodes are responsive to environmental RNA based on the *C. elegans* data; Wheeler et al (2012) demonstrated a lack of responsiveness in several grassland nematode species. (The wide variation in responsiveness to environmental RNA even in well-studied genera needs to be borne in mind when considering problem formulation in the non-target risk assessment for dsRNA-based topically-applied crop protection products.)

5.2.3. *Arthropods*

150. The phylum 'Arthropoda' is the largest phylum in the animal kingdom; about 84% of all known animal species are members of this phylum. Arthropods show a huge variety of adaptations and are represented in every habitat (terrestrial, aquatic, atmospheric). From a pest control perspective, two important groups within the phylum are insects (class: Insecta) and arachnids (class: Arachnida). Insects form by far the largest group of hexapod invertebrates and are the most diverse group of animals on the planet, representing more than half of all known living animal species. Within the class Arachnida (which includes spiders, ticks, mites, and scorpions), spiders (order: Araneae) are the most important predators of insects because of their abundance.

151. Since arthropods are often targets for RNAi-based products under consideration in this document, an understanding of similarities and differences in RNAi systems across different orders within the phylum is important. However, our current knowledge is still incomplete; as noted by Joga et al (2016), "a reasonable understanding [of systemic RNAi] in insects remains elusive and still precludes several potential practical applications for insect pest control". Similarly, Zhang et al (2017) noted that "a better understanding of the

striking differences between insects in their responsiveness to exogenously-supplied dsRNAs could aid the design of more efficient RNAi-based pest control strategies”. For a systematic review of the current knowledge in this area, refer to Christiaens et al (2018).

152. From a pest management perspective, effective control requires a functional systemic RNAi system: a localised effect (e.g., dsRNA uptake in the midgut) may or may not cause mortality. Although RNAi acts *via* a mechanism which is generally conserved, some components are significantly different across the different taxa, especially with respect to the molecular mechanisms behind cellular uptake and the systemic spread of silencing (Joga et al, 2016 and references cited therein).

153. Thus, while the application of RNAi technology has demonstrated its potential to control certain insect pests, the efficiency of RNAi varies significantly between the different insect orders. In many RNAi-recalcitrant insect species, the gene silencing is around 60% or lower and is often only temporary (Huvenne & Smagghe, 2010; Li et al, 2013). Gene silencing in RNAi-responsive coleopterans can be 90% or higher, requires only very small doses and the effect can be long-lasting (Baum et al, 2007; Zhu et al, 2011; Bolognesi et al, 2012; Rangasamy & Siegfried, 2012).

Insects

154. Insects display a wide range of responsiveness to ingested dsRNA (Bellés, 2010), with coleopterans showing significantly greater sensitivity than other arthropod orders within this class. Lepidopteran species have variable responsiveness to ingested dsRNA and require high concentrations of dsRNA to elicit a response comparable to coleopterans (Terenius et al, 2011; Ivashuta et al, 2015). *Drosophila melanogaster* (order: Diptera) only shows a transient response to dsRNA at the site of delivery by microinjection (Price & Gatehouse, 2008); this species is not responsive to environmental dsRNA through soaking or feeding, although this can be overcome with the assistance of transfection agents (e.g., liposome encapsulation; Whyard et al, 2009). Other insects vary widely in their ability to respond to environmental dsRNA as well as in their ability to elicit systemic RNAi through microinjection or other means (Bellés, 2010; Huvenne & Smagghe, 2010; Terenius et al, 2011; Gu & Knipple, 2013), despite possessing the necessary cellular machinery and functional cell-autonomous RNAi. The correlation between the genes in the model organism *C. elegans*[23] identified as important for dsRNA uptake with homologs in responsive insects is low, as is the correlation between homologs for systemic RNAi (Huvenne & Smagghe, 2010). This suggests that even when insects are responsive to environmental RNAi, the mechanism may not be the same (Huvenne & Smagghe, 2010).

155. After arrival of dsRNA at the gut membrane surface, the epithelial cells must take up the dsRNAs and convey these to the intracellular RNAi machinery. Two separate pathways have been described for dsRNA uptake in insects. These are the trans-membrane SID-1 channel protein-mediated pathway and the endocytic pathway. In the nematode *C. elegans*, the *sid-2* gene encodes a membrane protein which is situated in the intestinal cells. The protein SID-2 imports dsRNA from the intestinal lumen (Winston et al, 2007; McEwan et al, 2012) through endocytosis and exports the silencing RNAs to other neighbouring cells through SID-1 channels from the internalised vesicles by way of passive movement (Whangbo & Hunter, 2008; McEwan et al, 2012; Baum & Roberts, 2014). Thus, environmental RNAi in the nematode *C. elegans* involves both SID-1 and SID-2 proteins.

[23] See section above – ‘Non-arthropod invertebrates’

156. Unlike the model nematode organism *C. elegans*, no Sid-2 genes have been found in the genomes of any insect species sequenced to date (e.g., Tomoyasu et al, 2008; Xu & Han, 2008; Zha et al, 2011; Cappelle et al, 2016). Analyses suggest that Sid-1 like genes in *Tribolium* species (red flour beetles) are not orthologous to Sid-1 genes in *C. elegans* but rather to the *C. elegans* Tag-130 gene which is not associated with systemic RNAi in nematodes (Tomoyasu et al, 2008); hence they are referred to as 'SID-1-like' channel proteins. These SID-1-like channel proteins have been shown to be involved in dsRNA uptake in some insect species, such as the brown plant hopper *Nilaparvata lugens* (Xu et al, 2013), the Colorado potato beetle (*Leptinotarsa decemlineata*) (Cappelle et al, 2016) and the red flour beetle (*Tribolium castaneum*) (Tomoyasu et al, 2008). The number of Sid-1-like genes varies between insects belonging to different orders (see Table 1 in Joga et al, 2016); while insects in most orders appear to have only one Sid-1-like gene, 2 or even 3 Sid-1-like genes have been identified in the genomes of several coleopteran insects (Tomoyasu et al, 2008; Miyata et al, 2014).

157. Dipteran insects such as *D. melanogaster* seem to lack Sid-1-like genes altogether. Studies in *Drosophila* S2 cells[24] indicate that two endocytic receptors together account for a major part (>90%) of dsRNA uptake into S2 cells. These structurally-unrelated type-I membrane proteins play a role in the phagocytosis of bacterial pathogens and display multi-ligand specificity reminiscent of mammalian scavenger receptors. The clathrin heavy chain (chc) gene, a known component of the endocytosis machinery, was identified as being necessary for RNAi (Ulvila et al, 2006). Further, chemical blocking of endocytosis resulted in a lack of RNAi effect (Ulvila et al, 2006). These results suggest that in *D. melanogaster*, the uptake of dsRNA relies on receptor-mediated endocytosis.

158. Saleh et al (2006) confirmed the role of endocytosis uptake in the *D. melanogaster* S2 cell line and identified that vacuolar H+ ATPase as well as several other genes involved in endocytosis were integral for dsRNA uptake and processing. Additionally, Saleh et al (2006) confirmed that orthologues of these genes were also critical for RNAi response in *C. elegans*, indicating the likely conservation of the endocytotic uptake pathway in animal cells. More recently, the role of endocytosis in dsRNA uptake in insects (from haemocoel injections) has been reported for the beetle *T. castaneum* (Xiao, 2015) and the locust *Schistocera gregaria* (Wynant et al, 2014).

159. Cappelle et al (2016) have shown that in the coleopteran Colorado potato beetle, both the transmembrane channel proteins as well as receptor-mediated endocytosis are involved in dsRNA uptake. In contrast to uptake of dsRNA, no information is currently available on transport systems for dsRNA within the bodies of insects.

160. As noted above, systemic RNAi as it exists in the nematode *C. elegans* is not present in insects since Sid-1 and Sid-2 homologs are not found in insect genomes. Another difference between insects and nematodes relates to amplification of the RNAi system. In insects, no clear homologs for the RNA-dependent RNA polymerase (RdRP) system in the nematode *C. elegans* (Section 5.2.2) have yet been described. This does not necessarily mean that insects do not have an amplification system based on another enzyme with a mechanism similar to RdRP or a distinct mechanism that remains to be elucidated. Indeed, in some coleopterans, the RNAi effect is so strong and can last so long that an amplification system may be present. In early 2018, an international research consortium published the

[24] S2 cells are a commonly used *Drosophila melanogaster* cell line. These cells were derived from a primary culture of late-stage (20–24 h old) *Drosophila* embryos, probably from a macrophage-like lineage.

genome of the Colorado potato beetle (*L. decemlineata*) (Schoville et al, 2018); RNA-dependent RNA polymerase (coded by the Elp-1 gene) was listed as one of the genes associated with RNA interference in this species[25] (although at the time of drafting this report, it was not clear whether this gene is part of the CPB transcriptome). However, research by Li et al (2016) provided no evidence of an RNA amplification system in Western corn rootworm, a species responsive to environmental RNA. It remains to be elucidated why coleopterans like the WCR are so responsive to environmental RNA while many other insect species require large amounts of dsRNA to elicit even a moderate effect, often short-lived.

161. Several publications have compared sensitivity to RNAi across different taxonomic ranks of insects (e.g., Terenius et al, 2011; Christiaens & Smagghe, 2014; Kolliopoulou & Swevers, 2014). Appendix 3 provides a table with examples of RNAi-mediated gene silencing in different insect orders. Many members of the superorder Dictyoptera (cockroaches, termites, mantids), the Order Hemiptera ('bugs' with sucking mouthparts including cicadas, aphids, planthoppers, leafhoppers and shield bugs), the order Orthoptera (various superfamilies of grasshoppers, crickets, katydids etc), the order Coleoptera (beetles, weevils, ladybirds etc) and the infraorder Isoptera (termites) seem to be responsive toward dsRNAs (Katoch et al, 2013), while species in the order Lepidoptera (butterflies and moths) and the order Diptera (flies) have demonstrated variable sensitivity to ingested dsRNA and high concentrations are required to elicit a response (Huvenne & Smagghe, 2010; Terenius et al, 2011; Katoch et al, 2013). Thus, in the lepidopteran species Squinting bush brown butterfly *Bicyclus anynana*, Soybean Looper *Chrysodeixis includens* and the African/Egyptian cotton leafworm *Spodoptera littoralis*, high doses of dsRNA (more than 1 mg/mg of tissue) did not result in any silencing effects while less than 10 ng per mg tissue was needed to induce silencing in the lepidopteran species cecropia moth *Hyalophora cecropia*, the Chinese (oak) tussar moth *Antheraea pernyi* and the moth *Manduca sexta* (Terenius et al, 2011).

162. As discussed in some detail for mammals (Sections 5.1.1 and 5.5.2), barriers exist in insects to the systemic uptake of exogenous dsRNA. For example, a comparative *ex vivo* and *in vivo* study examined the fate of a dsRNA and the activity of dsRNAse in the tobacco hornworm *Manduca sexta*[26] and the German cockroach *Blatella germanica*[27]. It was found that dsRNA was rapidly degraded by *M. sexta* haemolymph but persisted much longer in *B. germanica*. Since experimental RNAi responses are difficult to achieve in the hornworm while the cockroach is highly responsive, it appears that the persistence of dsRNA in insect haemolymph, mediated by the action of nucleases, is an important factor in determining the responsiveness of insect species to RNAi (Garbutt et al, 2013). Baum and Roberts (2014) presented data on the differing persistence of dsRNA in the midgut fluids of Western corn rootworm[28] and Fall armyworm[29]; while dsRNA was stable for 60 min at both pH 7.4 and pH 10.5 in WCR, dsRNA degradation was seen after 40 and less than 10 minutes at these difference pHs in Fall armyworm midgut fluid. While entrapment of internalised dsRNA in endosomes has been reported as a significant contributor to inefficient RNAi

[25] CPB is very adaptive - resistance has developed to many conventional pesticides.

[26] Order: Lepidoptera

[27] Order: Blattodea

[28] Order: Coleoptera

[29] Lepidoptera

response in lepidopterans including the Fall armyworm (Yoon et al, 2013), it appears that differences in the stability of dsRNA in midgut fluid might also be a contributing factor in the differing responsiveness of coleopterans and lepidopterans. Research published in early 2018 may be a significant contribution to helping explain why certain lepidopteran insects are not very responsive to RNAi (Guan et al, 2018). Using transcriptome analysis of the Asian corn borer *Ostrinia furnacalis*, the authors reported that they had previously identified a gene, termed *up56*, that is up-regulated in response to dsRNA. They have now shown that this Lepidopteran-specific gene encodes a nuclease that contributes to the RNAi insensitivity in this insect order. Sequence analysis indicated that *up56* encodes a previously-uncharacterised protein with homologous sequences in seven other lepidopteran species. Its computationally-predicted 3D structure revealed a high structural similarity to human exonuclease I. Exposure to dsRNA in *O. furnacalis* strongly up-regulated the expression of this gene, and the protein product can digest both single-stranded and double-stranded RNA and DNA, both *in vitro* and *in vivo*. The up-regulation of *up56* expression is faster than that of the gene encoding the key RNAi-associated nuclease, Dicer. Silencing of *up56* in *O. furnacalis* significantly enhanced RNAi efficiency and significantly increased the amount and diversity of small RNAs. Furthermore, *up56* over-expression in *Drosophila melanogaster* suppressed RNAi efficiency. In view of these findings, the *up56* gene product was named RNAi efficiency-related nuclease (REase). As noted above, not only do silencing effects vary between insect orders and between species, they also vary within species. For example, Chu et al (2014) observed that the silencing effects of a dsRNA targeting DvRS5 (a cysteine proteases gene) varied between three different populations of the coleopteran Western corn rootworm (*Diabrotica virgifera virgifera*).

163. Furthermore, silencing effects have also been reported to vary with life stage of the insect (see Katoch et al, 2013 and references cited therein). It appears that RNAi effects may be more prominent in early life stages as compared to late stages. Guo et al (2015) found that dsRNA targeting S-adenosyl-L-homocysteine hydrolase decreased the target gene expression in an instar-dependent manner. Silencing of the nitrophorin 2 gene was 42% in second instars of the bug *Rhodnius prolixus*[30] as compared to none in fourth instars treated with the same concentration of dsRNA (Araujo et al, 2006). In *Spodoptera frugiperda*[31], a higher gene silencing was observed in the fifth instar larvae as compared to adult moths (Griebler et al, 2008). When dsRNA is injected at the last larval stage of the red flour beetle (*Tribolium castaneum*), the RNAi effect can last for many months, perhaps even extending to the entire lifespan of the individual (Miller et al, 2012). Bucher et al (2002) also showed that, if the female pupae or adults of the same species are injected with dsRNA, the effect is seen in the offspring for several months. Miller et al (2012) suggested that this so-called ‘parental RNAi’ is less efficient when last instar larvae are injected with dsRNA, one possible reason being that the female reproductive organs do not complete formation until the pupal stage; for the oocytes to efficiently uptake dsRNA, they must be formed at the time of dsRNA introduction to the body.

164. In addition to the life stage of the insect, many other factors can affect RNAi efficiency in insects, including dsRNA concentrations, lengths of dsRNA fragments, the timing and duration of exposure, activation of RNAi machinery, and dsRNA uptake and degradation activities (Bolognesi et al, 2012; Chu et al, 2014; Coleman et al, 2014;

[30] The vector of Chagas disease (Order: Hemiptera, Subfamily: Triatominae)

[31] The Fall armyworm is the larval life stage of a Fall armyworm moth (Order: Lepidoptera)

Huvenne & Smagghe, 2010; Ivashuta et al, 2015; Terenius et al, 2011; Bachman et al, 2020).

165. Moreover, silencing effects may vary, depending on the target site. Terenius et al (2011) suggested that in Lepidoptera, the genes involved in immunity are the most likely to be responsive to dsRNA-induced RNAi, whereas in hemipteran species the genes expressed in the gut, salivary glands and gnathal appendages are the most likely to be responsive to RNAi.

166. Information on factors influencing the exposure of non-target insects, including the potential degradation of dsRNA prior to exposure, barriers to cellular uptake, stability of exogenous dsRNA within the organism following ingestion or exposure, and the inherent sensitivity of the organism to ingested or absorbed dsRNA will help refine exposure estimates and facilitate risk assessment predictions across non-target taxa. Currently, however, our limited understanding of such factors does not allow any broad generalisations to be made (Ramon et al, 2014; USEPA, 2014). A better understanding of how and why responsive insects take up dsRNA from the environment, and what insect orders possess the capacity for systemic RNAi, will help to identify those species that may require consideration during risk assessment, or those species that, because of their inability to respond to environmental RNAi, can be eliminated from risk-assessment considerations.

167. It is important to note that, while a given insect may be very responsive to environmental RNAi, sequence match is still a key determinant of RNAi efficiency. For example, as described in Bachman et al (2013), Colorado potato beetle, which lacks ≥21 nt matches to the DvSnf7 sequence for western corn rootworm, showed no adverse effects when fed this DvSnf7 dsRNA but did demonstrate high mortality when fed the Snf7 ortholog with 100% sequence match to the Colorado potato beetle.

Arachnids

168. An important pest species is the honeybee ectoparasite, *Varroa destructor*. An arachnid, not an insect[32], the Varroa mite is major contributor to pest and disease-related loss of bees in most countries. Varroa is responsive to environmental RNAi but the route of delivery is unusual; immersion of Varroa mites in a saline solution containing dsRNA can lead to gene silencing (Campbell et al, 2010), as can feeding dsRNA to the honey bees upon which the mites subsequently feed (Inberg et al, 2017). This presents a strategy for control of this economically-important agricultural pest. A bi-directional interspecies transfer of dsRNA from bee to mite and back to bee has been claimed (Garbian et al, 2012), but data in this publication are not conclusive.).

169. RNAi responses were investigated in the mite *Metaseiulus occidentalis*, an important biological control agent by feeding dsRNA of *RpL11*, *RpS2*, *RpL8*, or *Pros26.4* genes in 20% sucrose (Wu & Hoy, 2014). In order to elicit an RNAi effect (nearly complete loss of egg production), treated females needed to subsequently feed on two-spotted spider mites (*Tetranychus urticae*); dsRNA-mediated gene silencing was robust and long-term. No RNAi effect was induced if the predatory mites were only provided with the sucrose diet after ingesting dsRNAs. However, once the RNAi-mediated gene silencing commenced, the spider mite diet was not needed to sustain it.

170. Khila & Grbic (2007) studied RNAi in the two-spotted spider mite *Tetranychus urticae*, focusing on Distal-less (Dll), a conserved gene involved in appendage specification

[32] Order: Parasitiformes

in metazoans. Fluorescently labelled dsRNA and siRNA molecules injected into the abdomen of adult females were incorporated into the oviposited eggs, suggesting that RNA can be systemically distributed in spider mites. Injection of longer dsRNA as well as siRNA induced canonical limb truncation phenotypes as well as the fusion of leg segments.

171. Using a dsRNA-permeated leaf disc feeding assay, Kwon et al (2013) demonstrated systemic gene silencing and significant mortality in the two-spotted spider mite[33], *Tetranychus urticae*, at applied concentrations of 160 ppm dsRNA. Most of the efficacious targets surveyed, including genes encoding the COPI coatomer β0 subunit, *V-ATPAse* subunits A and B, and ribosomal protein S4, had been previously identified. Reduction of target gene transcripts following dsRNA treatment was confirmed by quantitative PCR, demonstrating the effectiveness of dsRNA feeding in killing *T. urticae*.

172. The above studies, carried out in a limited number of arachnid species, show that oral delivery of dsRNA is likely to be a valuable tool for (1) efficient genome-wide functional screens in this class of arthropods; and (2) control of arachnid pest species. As well as uptake after oral feeding of dsRNA, systemic distribution of RNAi signals appeared to take place in the species tested. For the gene targets studied in *Tetranychus urticae* at least, dsRNA-mediated gene silencing was robust and long-term.

173. Published data suggest that mites have RdRP; in fact, *Tetranychus urticae* has five duplicated copies of an RdRP (Van Leeuwen et al, 2013). However, there appears to be a paucity of published research to date examining the detail of RNAi mechanisms in arachnids.

5.2.4. *Plants*

174. An overview of RNAi in plants can be found in Lindbo (2012) and in Borges and Martienssen (2015).

175. The cuticle in plants represents a significant barrier (Yeats & Rose, 2013), making it difficult for unfacilitated dsRNA to penetrate plant surfaces. San Miguel & Scott (2015) demonstrated that dsRNA could be taken up by detached petioles placed in dsRNA:water solutions and was effective in producing RNAi effects. However, uptake took a number of hours and the application method is not particularly relevant to any foreseen practical application method for dsRNA-based plant-protection products, apart from e.g., stem injection for individual high-value plants. Most published research shows that formulation, leaf bombardment, or other techniques are required to bypass the physical and biochemical barriers in plants to the uptake of long dsRNA. For example, a high-pressure spray (from an air brush pistol) was required to achieve penetration of siRNAs (21 – 24 nt) and effective RNAi in *Nicotiana benthamiana* plants viz. systemic silencing of a GFP transgene (i.e., not a native target); low-pressure spraying, or wiping leaves with a solution, suspension or emulsion of siRNAs, with or without surfactants, was without effect (Dalakouras et al, 2016).

176. In growth chamber work with a fungal pathogen of barley (*Fusarium graminearum*), Koch et al (2016) demonstrated that a spray application of a long non-coding dsRNA (791 nt *CYP3*-dsRNA which targets the three fungal cytochrome P450 *lanosterol C-14α-demethylases* required for biosynthesis of ergosterol), inhibits symptom development and fungal growth in directly-sprayed (local) as well as non-sprayed (distal) parts of leaves; however, as for the experiments reported by San Miguel & Scott (2015)

[33] Also called the red spider mite.

(see previous paragraph), these studies by Koch et al were performed on detached leaves. It was noted that the spray-induced control of fungal infections in the distal tissue of the detached leaves involved passage of the dsRNA *via* the plant vascular system and processing into siRNAs by fungal DICER-LIKE 1 (FgDCL-1), after uptake by the pathogen. Wang et al (2016) showed that *Botrytis cinerea*, a fungal disease that causes severe crop losses, can take up external small RNAs (sRNAs) and dsRNAs; applying sRNAs or dsRNAs that target *Botrytis* DCL1 and DCL2 genes on the surface of fruits, vegetables and flowers significantly inhibited this grey mould disease. In this work, dsRNA was applied in droplets and then *Botrytis* spores were applied in droplets to the same application point; there was no evidence that the *Botrytis* uptake of the dsRNA occurred inside the plant rather than just on the plant surface. In recent research, Mitter el al (2017) reported that a single spray of dsRNA afforded virus protection for at least 20 days on sprayed and newly-emerged unsprayed leaves, provided that the dsRNA was loaded on layered double hydroxide (LDH) clay nanosheets. Evidence was provided for the following sequence: degradation of the clay nanosheet complex, dsRNA uptake in plant cells, and silencing of the complementary mRNA.

177. In plants, the effects of RNA interference can be systemic; this is also the case in *C. elegans*, although not in *Drosophila* or vertebrates (e.g., Tomari & Zamore, 2005). In plants, RNAi is thought to propagate by the transfer of siRNAs between cells through plasmodesmata[34] (Lodish et al, 2004; Molnar et al, 2011). A classic example of the intercellular spreading phenomenon in plants is the systemic transport of viral resistance from a local site of infection to distant sites (e.g., Jose & Hunter, 2007; Ding & Voinnet, 2007; Brosnan & Voinnet, 2011).

178. While it has been demonstrated that RNAi does not result in heritable effects, there are dsRNA-based mechanisms in plants that do lead to heritable effects. Heritability is based on the change of methylation patterns of promoters triggered by dsRNAs; the new methylation pattern may be maintained in subsequent generations of the cell (Jones et al, 2001). [dsRNAs act as key regulators that trigger either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS), depending on their origin/size class (Ghildiyal & Zamore, 2009) and on whether they are complementary to promoter or coding sequences (Waterhouse et al, 1998; Mette et al, 2000). As noted in the 'Introduction' section, this document is not intended to consider components of RNA pathways in eukaryotes involved in the maintenance of the organisation and structure of the genome. It is important to make a distinction between the process of PTGS initiated by siRNAs and the process of TGS initiated by another set of siRNAs derived from heterochromatic DNA. For example, while the first mechanism - what is called RNA interference in this paper - results in mRNA degradation and/or translational arrest and is not heritable (i.e., not necessarily correlated with epigenetic modifications like DNA methylation), the second mechanism (TGS) is often heritable due to promoter methylation[35] and chromatin modifications.]

179. A broad general distinction between plants and animals lies in the targeting of endogenously produced miRNAs; in plants, miRNAs are usually perfectly or nearly perfectly complementary to their target genes and induce direct mRNA cleavage of the target mRNA transcript by RISC (Jones-Rhoades et al, 2006). By contrast, in animals,

[34] Channels in plant cell walls that enable communication and transport.

[35] See Law & Jacobsen (2010) for a more in-depth description of the methylation pathways in plants and animals.

miRNAs tend to be more divergent in sequence and recognise their target mRNAs by using as little as 6–8 nucleotides (the seed region) at the 5' end of the miRNA (Lewis et al, 2003; Lewis et al, 2005; Ellwanger et al, 2011); this is not enough pairing to induce cleavage of the target mRNAs (Bartel, 2009) but can induce translational repression (Saumet & Lecellier, 2006; Doench & Sharp, 2004). This translational effect may be produced by inhibiting the interactions of translation initiation factors with the messenger RNA's polyadenine tail (Humphries et al, 2005). In animals, a given miRNA may have hundreds of different mRNA targets, and a given target might be regulated by multiple miRNAs (Friedman et al, 2009; Krek et al, 2005).

180. Notwithstanding the above, there is some evidence that miRNAs can act in a translationally-inhibitory mode in plants, separable from endonucleolytic cleavage of the mRNA strand (Brodersen et al, 2008) and that miRNAs in animals can target mRNA cleavage (Guo et al, 2010; Huntzinger & Izaurralde, 2011).

181. Unlike animal miRNAs, plant miRNAs possess a 2′-O-methyl group on the ribose of the 3′ terminal nucleotide, which helps to protect plant miRNAs from degradation in the plant (Yu et al, 2005).

182. In plants, miRNA-directed cleavage of target mRNA sequences can lead to the production of secondary siRNAs, which in turn can lead to the degradation of non-targeted mRNA through a process known as transitive RNAi (Himber et al, 2003; Manavella et al, 2012).

183. One class of secondary siRNAs are transacting siRNAs (tasiRNAs), endogenous siRNAs which down regulate genes at loci different from which they arose (Peragine et al, 2004; Vasquez et al, 2004). TAS genes, from which tasiRNAs are derived, have been found in the small flowering weed *Arabidopsis thaliana*, Asian rice *Oryza sativa*, other higher plants and mosses, but have not yet been identified in animals or fungi. While they post-transcriptionally silence gene expression like miRNAs and other siRNAs, the mechanism of their biogenesis is somewhat different. They are derived from longer TAS transcripts which are targeted by miRNAs, followed by processing to short siRNAs. TasiRNAs are incorporated into the RISC complex and direct the cleavage of target mRNAs, albeit with reduced fidelity relative to sequence alignments. This reduced sequence specificity in tasiRNAs is shared by some miRNAs.

184. While our understanding of extracellular transport of sRNAs in plants is incomplete, available evidence suggests that packaging strategies may be similar to those in mammals, in which extracellular sRNA is transported after incorporation in exosomes or microvesicles or inclusion in ribonucleoprotein complexes (reviewed in Fritz et al, 2016). Exosomes or microvesicles are a heterogeneous group of membrane-bound vesicles that can be released from cells as part of a process which delivers diverse macromolecules to other cells within an organism (Colombo et al, 2014). Plants may possess exosome-like particles known as nanoparticles (Zhang et al, 2016) which, when experimentally-generated from plant material in the laboratory, can contain sRNAs, lipids, and proteins, although, to date, they have not been shown to deliver bioactive sRNA to cells. (It should be noted that, since such nanoparticles can be produced during destructive mechanical processing of plant material, it is possible that they do not naturally occur in plants.) In addition to nanoparticles, miRNAs (i.e., from endogenous sources) complexed with

proteins have been found in the vascular systems of plants (Yoo et al, 2004) and animals (Fritz et al, 2016) and are likely to stabilise extracellular sRNAs[36].

185. With regard to packaging, Witwer (2016) suggested that any protein binding that might occur in packing like a vesicle would also potentially prevent an RNA from achieving protein translation i.e., 'protection limits accessibility'.

5.2.5. *Fish, Reptiles and Birds*

186. In 2011, Sifuentes-Romero et al noted that whether dsRNA can initiate a specific RNAi response, and whether all the factors required for RNAi are present in non-mammalian vertebrates, were questions which were yet to be answered.

187. Successful RNAi in vertebrates such as fish, reptiles, and birds has only been achieved with cell lines or embryos and has required the use of transfection agents, direct injection, electroporation, or other invasive techniques (Schyth, 2008; Sifuentes-Romero et al, 2011; Ubuka et al, 2012). Based on the existing scientific literature, the US EPA considered that the barriers to dietary dsRNA uptake discussed in Section 5.1.1 were operable in all vertebrates (USEPA 2016b, USEPA, 2017).

5.2.6. *Mammals*

188. Although there has been speculation that amplification of the RNAi signal and systemic transport and spreading might be present in mammals under certain environmental conditions (Jose & Hunter, 2007), there is no *in vivo* evidence for these functions to date (Petrick et al., 2015).

189. In 2012, the publication of a paper by Zhang et al suggested that ingested plant miRNAs could act to regulate animal metabolism; this report described the uptake of plant-derived miRNAs into the serum, liver and a few other tissues in mice following consumption of rice, as well as apparent gene regulatory activity in the liver. The paper was met with much scepticism but served to stimulate a significant amount of research since the notion of uptake of active diet-derived small RNAs in recipient organisms would have significant implications for the safe use of RNA interference technology in agriculture, both with respect to the environment and to human health and safety. Indeed, it would have very significant implications for our understanding of human nutrition and therapeutics.

190. Most research cannot replicate and validate such findings regarding the bioactive potential of dietary RNAs in mammals. It has been shown that the human diet contains small RNAs with 100% sequence complementarity to the human genome without any biologically meaningful impact (Ivashuta et al., 2009). A 2017 review confirms this conclusion and outlines the type of experimental evidence that would be needed to support the theory of biologically meaningful dietary uptake of dsRNA (Chan & Snow, 2017).

191. In studies designed to evaluate the reproducibility of the reported findings of the Zhang et al (2012) publication, Dickinson and co-workers found little evidence for the presence of miRNAs in blood prior to or following dietary intake of miRNA-rich plant material and observed that the reported physiological changes in mice ascribed to RNAi effects were due to dietary imbalance (100% raw rice) and variability in detection methods

[36] Most miRNAs in mammalian blood are in free Argonaute complexes, with a small percentage in vesicles (Witwer, 2016); small RNAs without an Agronaute protector have no regulatory function

(Dickinson et al, 2013). More recently, Chan and Snow (2017), whose own research has been unable to validate the bioavailability of dietary plant miRNAs, reviewed the literature and suggested that studies in this field have suffered from "technical artifact and a lack of reproducibility"; they note that data reported as supportive "typically reveals descriptive phenomenology where multiple interpretations, including technical artifact, could explain the results". Thus, in a 2016 paper by Pastrello and co-workers, it was reported that, in a large nutrigenomics study cohort and in a randomised dose-controlled trial, there was a significant positive correlation between the daily amount of broccoli (*Brassica oleracea*) consumed and the amount of miRNA in the blood. They also suggested that these Brassica miRNAs regulate expression of human genes and proteins *in vitro*, and that miRNAs cooperate with other Brassica-specific compounds in a possible cancer-preventive mechanism. However, this paper was retracted by the authors on 22 May 2017 as they "no longer [had] confidence in the data to support [their] central conclusion – the detection of *Brassica oleracea* microRNAs in the bloodstream of humans who consumed broccoli". This retraction was based on the incorrect design of the miRNA primers, with anti-sense design of all the forward primers for broccoli miRNA detection (Pastrello et al, 2017).

192. This lack of biologically-meaningful uptake of plant miRNA from the diet was also demonstrated in primates by Witwer et al (2013) and in mice by several independent groups using different dietary sources (Dickinson et al, 2013; Snow et al, 2013).

193. Chan and Snow (2017) concluded that, unless some unknown mechanisms are involved, insufficient levels of plant miRNAs are present in mammalian plasma or serum to be active *via* canonical RNA interference mechanisms. This conclusion is supported by a 2017 report (Kang et al, 2017), which is probably the most rigorous assessment of diet-derived miRNAs to date; examination of small RNA (sRNAs) in >800 datasets from human tissues and body fluids revealed that, although dietary sRNAs could be detected, they were present at *ca* 5 copies per cell or fewer, far below the levels shown for their endogenous counterparts, which may reach 50,000 copies per cell for some miRNA entities. Furthermore, feeding experiments using different plant diets in rats and different milk diets[37] in pigs did not find any evidence of substantial uptake of dietary sRNA.

194. The conservative conclusions of the reviews by Chan and Snow (2017) and Kang et al (2017) is that, based on the weight of evidence, the uptake and canonical RNA interference activity of dietary miRNAs is not a "prevalent" mechanism in mammals. Whether such transfer might occur in specialised contexts, including those which could occur under various conditions of dsRNA concentration, modification, formulation, and application, is still a subject of debate. However, the USEPA's 2016 Science Advisory Panel extensively reviewed a large number of studies and concluded that there was no reliable evidence that exogenous dsRNA could be taken up from the gut into mammalian circulation to exert its functions in the ingesting organism (USEPA, 2016a; see section 5.5.2 for references).

5.3. **Minimum threshold of dsRNA/siRNA for RNAi in different species**

195. The gene-silencing effects of dsRNA are understood to be dose-dependent, and for some organisms, exposure must be continuous to result in effective gene silencing (Katoch et al, 2013). Evidence also exists of the potential for upper and lower thresholds to the

[37] miRNAs in human and bovine milk have been reported to be resistant to RNases, based on their incorporation into extracellular vesicles (Admyre et al, 2007; Hata et al, 2010; Lasser et al, 2011; Zhou et al, 2012).

effects of gene silencing (USEPA, 2013). Cell culture studies indicate that at least 100 copies of siRNA molecules need to reach a targeted cell site to induce RNAi in mammalian cells (Brown et al, 2007; Snow et al, 2013). This number may be as high as 1,000 – 10,000 copies/cell (Title et al, 2015; Witwer, 2016). This contrasts with the findings in *Caenorhabditis elegans* suggesting that only a few RNA molecules are sufficient to initiate the RNAi pathway (Fire et al, 1998).

196. Huvenne and Smagghe (2010) suggested that in insects an optimum concentration exists for gene silencing for each target gene and organism, and that exceeding this optimum does not necessarily result in additional gene silencing. Based on this observation, there may be environmental exposure levels below which no effects, or above which no greater degree of effects, would necessarily occur. It is also just possible that lower limits of exposure may be essentially non-existent for some very responsive species, although the extensive amount of research carried out to date on RNAi across different phyla suggest that the number of extremely responsive species is likely to be very limited.

197. More clarity on the issue of minimal thresholds in different organisms is required before firm conclusions can be reached about their dose dependency and responsiveness to low levels of dsRNA in the environment. [This issue will be considered in the context of risk assessment in later parts of this document.)

5.4. Responsiveness of different insect taxa to dsRNA-mediated gene silencing

198. The information contained in Section 5.2.3 is also relevant to the discussion here; however, the following section focuses on dose-responsiveness rather than on mechanisms. An overview table of dsRNA uptake experiments in different insect orders including cellular uptake mechanisms can be found in the EFSA literature review by Christiaens et al. (2018).

199. A number of coleopteran species are exceedingly responsive to ingested dsRNAs, with LC50 values in the range of 1 to 10 ppb in the diet (cf. >400 ppm and 1000 ppm in dipteran and hemipteran species; see Table in Baum & Roberts, 2014); among the responsive species are the Western corn rootworm (*Diabrotica virgifera virgifera*), the Southern corn rootworm (*D. undecimpunctata howardii*) and the Canola flea beetle (*Phyllotreta striolata*), with both larval and adult stages being responsive. The response is dose-dependent, systemic, occurs in the absence of any amplification by RNA-dependent RNA polymerase, and depends on the size of the dsRNA, presumably due to less efficient uptake of smaller dsRNAs. (Note that at least 60 bp dsRNA length is required for dietary uptake of environmental dsRNA for effective RNAi in Western corn rootworm; Bolognesi et al, 2012.) However, not all coleopteran species tested to date are as responsive. Most studies in other insect orders have reported successful oral delivery of dsRNA at much higher doses, with LC50s greater than 10 ppm in the diet (Baum & Roberts, 2014).

200. Terenius et al. (2011) reviewed the status of RNAi studies in lepidopteran species and noted that, with respect to oral delivery, gene suppression has been reported in a wide range of lepidopteran species but only appeared to be successful when high concentrations of dsRNA were provided in the diet. Even a 'successful' RNAi response in this order was still three orders of magnitude lower than that observed among sensitive coleopteran species (Baum & Roberts, 2014).

201. Across all studies with hemipterans, the dietary concentrations of dsRNA required for gene silencing and/or causing lethal phenotypes vary widely (even between studies with

the same organism) but tend to be at least three orders of magnitude higher than effective doses in responsive coleopteran species (Baum & Roberts, 2014).

202. In the order Orthoptera, locust species tested displayed a highly responsive systemic RNAi effect when structural barriers were bypassed but were refractory to environmental RNAi. Injection of as little as 10 ng/insect affected target gene expression in the desert locust, *Schistocerca gregaria*. Likewise, the migratory locust, *Locusta migratoria* was highly responsive to injected dsRNAs in a dose-dependent manner but was not responsive to environmental RNAi. Notwithstanding the lack of response to environmental RNAi, the Locusta genome revealed putative orthologs for almost all of the genes implicated in dsRNA uptake in the dipteran *Drosophila melanogaster*, another species which is unresponsive to environmental RNAi (Baum & Roberts, 2014; and references cited therein).

5.5. **Unintended siRNA effects**

5.5.1. *Gene silencing in non-target species*

203. As described by Roberts et al (2015), there are two ways to approach the question of how likely it would be for off-target gene effects to be seen in non-target organisms, with examples of both approaches in the current literature (e.g., Whyard et al, 2009; Bolognesi et al, 2012; Bachman et al., 2013). The first is a bioinformatics-based approach (see Section 7.1.3 and 7.4.4 below for more detailed discussion). If reliable bioinformatic data indicate that minimum sequence requirements for RNAi are not met between non-target and target species, then further assessment may not be necessary, as the likelihood of adverse effects would be low. However, this approach is currently subject to substantial limitations (EFSA, 2014; Ramon et al, 2014; USEPA, 2014; Casacuberta et al, 2015, Christiaens et al, 2018) as knowledge of genome sequence information may not be available for all species of interest. Furthermore, it may also be subject to differences between organisms in terms of how the RNAi machinery functions in relation to base pair mismatches etc; and there are scientific uncertainties about the rules governing small RNA-mRNA matches/interactions. (Ongoing research on RNAi mechanisms, generation of genomic data libraries for relevant species, and design of algorithms to make reliable predictions are likely to increase the usability of bioinformatic data in support of environmental risk assessments of dsRNA-based products.)

204. The other approach is an empirical one i.e., to introduce dsRNA (that is perfectly complementary to the target gene in a target organism) to a range of other organisms, starting with close relatives and then moving outwards, to see how more phylogenetically-distant organisms respond (Bachman et al., 2013). This approach enables characterisation of the activity spectrum of dsRNAs and does not require sequence information from the tested species. Results published by Bachman et al (2013) indicated that, in tested insects, close phylogenetic relationships are required for off-target gene effects, provided the target gene selected is not highly conserved, and that at least one sequence match of greater than 19 bp to the target sequence is necessary to see significant activity.

5.5.2. *Unintended effects in mammals*

205. In considering organisms in the environment which could be exposed to dsRNA following the application of dsRNA-based pesticide products in cropping or other primary-production scenarios, it is possible that mammalian species may be exposed. While the focus of this document is on environmental risk assessment and not on human health risk

assessment, it is recognised that extensive ongoing research on the application of RNAi in human therapeutics can provide information useful for the risk assessment of environmental dsRNA on non-target mammalian species which may be exposed to it during the intentional application of RNA-based pesticides.

First human RNAi therapeutic approved in 2018 – stabilised in a lipid nanoparticle and delivered by IV injection

In August 2018, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) gave marketing authorisation to Onpattro™, an RNAi therapeutic, for the treatment of the polyneuropathy of hereditary transthyretin-mediated (hATTR) amyloidosis in adults (Alnylam Pharmaceuticals, 2018). The active substance of 'Onpattro' is patisiran, a double-stranded siRNA that specifically targets all mutant and wild-type transthyretin (TTR). The TTR gene is mutated in hereditary transthyretin amyloidosis, resulting in accumulation of TTR protein fragments as amyloid deposits in multiple organs. Patisiran causes the catalytic degradation of mRNA for TTR in the liver, resulting in a reduction of serum TTR protein and thus reducing amyloid deposition.

On 26 July 2018, the European Committee for Medicinal Products for Human Use (CHMP) recommended the granting of a marketing authorisation for 'Onpattro' (European Medicines Agency, 2018).

'Onpattro' has to be administered by IV infusion and the active constituent is formulated as a lipid nanoparticle to provide the required stability.

206. In considering human health risks from the application of RNAi technology in agriculture, the USEPA's Science Advisory Panel (SAP) concluded that "the combination of RNases and acids found in the human digestive system are likely to ensure that all forms of RNA structure are degraded throughout the digestive process" (USEPA, 2014). Furthermore, in considering the susceptibility of plant and animal RNAs to RNases, the panel concluded that the available evidence "supports the likelihood that PIP and non-PIP RNAs expressed in plant material consumed by humans are likely to be degraded, no matter the type of RNA or its structural status when entering the human digestive system".

207. Extensive physical and biochemical barriers present a significant challenge to oral delivery of nucleic acids (reviewed by Petrick et al, 2013). These biological barriers to absorption include nucleases in the saliva and gastrointestinal tract, acidic conditions in the stomach, and multiple membrane barriers, including in gastro-intestinal (GI) cells, from GI cell to endothelium, across endothelium to blood and tissues, across tissue membrane, etc. Together, they severely limit the delivery of ingested RNA into blood and tissues. While there may be some individuals in a diverse mammalian population (animal or human) with a higher intestinal pH or more permeable GI epithelium, the other barriers would preclude functional uptake[38] of ingested RNAs. : The assessment of ncRNA should be based on a weight of evidence approach, taking into account the various aspects described in this section, and, in summary, related to:

[38] 'Functional uptake' of dsRNA means the ability to reach a target tissue at a sufficient concentration to mediate the RNAi process.

EXPOSURE:

- very limited (local and systemic), exposure (particularly after oral intake, due to various physicochemical barriers at tissue and cellular level;

- this low exposure would limit achieving sufficient levels to trigger effects;

- the presence/absence of chemical modifications that would increase its stability and cellular uptake in the gastrointestinal tract following oral administration.

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- on a case by case basis, taking into account the type of ncRNA under assessment, its possibility to be processed intracellularly in mammalians/vertebrates as compared to target species and reach the target at a sufficient level, the presence of specific motifs considered relevant in activating immune responses (e.g. Robbins, 2009). The possibility to identify possible off target effects by in silico tools is debatable in mammalian species (Davalos et al. 2019; EFSA supporting publication).

208. Supporting these conclusions, pharmaceutical studies indicate a very short half-life for injected RNAs that have been chemically stabilised (Christensen et al, 2013), suggesting that absorption from the GI tract and digestive barriers are not the only barriers to potential activity of ingested RNAs. Similarly, intravenous injection of a stabilised RNA targeting a mouse gene did not demonstrate distribution to, or gene suppression in the liver or jejunum, despite a very high dose of 50 mg/kg bw (Soutschek et al, 2004); this also suggests that barriers outside the GI tract are sufficient to preclude activity of exogenous naked nucleic acids.

209. Furthermore, well-conducted studies examining the potential for cross-kingdom gene regulation in mammals via ingested RNAs have demonstrated a lack of biologically-meaningful uptake of plant miRNA in mice, non-human primates, and humans (see Sections 5.2.6 and 6.5.3).

210. A library of small RNAs from corn was compared with a library of 98,650 human transcript sequences. The analysis of sequences (21 nt or greater) revealed that there were 150 endogenous corn-grain small RNAs that had 100% sequence identity to a total of 500 protein-coding human transcripts (Petrick et al, 2016). Since there is no indicationthat ingestion of corn by humans has ever resulted in suppression of transcription of human genes, this bioinformatics analysis supports that even the consumption of RNA transcripts with 100% sequence identity to human transcripts is not of concern at the concentrations in which these small RNAs are found in plants. It is not currently known how the concentrations of these miRNAs would compare to the concentrations of exogenously-applied dsRNA. Barriers to exogenous RNA molecules (described elsewhere in this document) play a key functional role in higher organisms that regularly consume these dietary components in significant amounts.

211. A number of repeat-dose toxicity studies in mammalian test species did not indicate toxicity of large doses of dsRNAs:

- In a 28-day oral toxicity study, *DvSnf7_968* dsRNA was administered to CD-1 mice (10/sex per dose) by gavage at doses of 0 (vehicle control - nuclease free water), 1.06, 11.0, or 105 mg/kg bw per day. Negative controls received 104 mg/kg bw per day of yeast RNA. The animals were examined for clinical signs, mortality, body weight, food consumption, clinical pathology, organ weights, and gross and histopathology examination. There were no treatment-related effects on clinical

signs, mortality, body weight parameters, food consumption, organ weights, or gross and histopathological findings at any dose (Petrick et al, 2016). (The doses tested were many orders of magnitude higher than likely human dietary exposures arising from the expression of this dsRNA as a plant-incorporated protectant for the control of WCR; exposures were calculated from measurements of dsRNA residues in crop commodities and expected dietary consumption.) The USEPA found this study acceptable. This study was considered in the risk assessment of a GMO product, but the EFSA GMO Panel could not derive any conclusions due to several deviations from OECD TG 407 requirements observed (EFSA GMO Panel, 2018a). ntravenous injection of therapeutic siRNAs with 100% identity to the mouse ApoE gene did not produce gene silencing at the expected target site (mouse liver) at doses of 50 mg/kg bw, unless the apoB-1-siRNA was conjugated with a cholesterol tag[39] to facilitate distribution of the siRNA to organs (liver and jejunum); these results showed that presence of a specific siRNA in blood does not necessarily indicate that gene silencing or toxicity will result (Soutschek et al, 2004).

- At intravenous doses of up to 200 mg/kg bw in rats, injection of stabilised siRNA matching rat p53, a key transcript in many pathways including cell cycle regulation, was readily degraded and was not toxic (Thompson et al, 2012).
- A lack of toxicity in mice was noted for orally-administered siRNAs and a long dsRNA with 100% sequence identity to mouse vacuolar ATPase at doses of up to 48 mg/kg and 64 mg/kg, respectively (Petrick et al, 2015).

212. *Immune system stimulation*: Innate immune systems of higher organisms rely on pattern recognition proteins and other factors to identify potentially pathogenic invaders, including foreign dsRNAs. There is at least a theoretical potential for environmental dsRNAs to stimulate innate immune responses in mammals. siRNAs are able to trigger mammalian endosomal immune cascades or cytoplasmic pathways (Sioud, 2015). The injection of small RNA fragments (fewer than 30 nt) stimulated an immune reaction in mammals; some Toll-like receptors recognise and respond to the sequence, length, and structure of siRNAs (Robbins et al, 2009). It also appears that small changes in nucleotide sequence can mitigate immune-stimulatory effects in a given organism. In mice, the immune-stimulation by siRNA injection led to reductions in lymphocytes and platelets, largely correlated with a cytokine response (Judge et al, 2005). Systemic inflammation and damage to organs including the gut was reported when 5 µg/g weight of foreign RNA was injected into mice (Zhou et al, 2007). It has been suggested that Petrick et al (2015) observed inflammation in 1/8 male mice at an oral dose of 64 mg/kg bw per day with 218-bp dsRNA and 1/8 female mice at 45 mg/kg bw per day with siRNA pool. However, according to the study pathologist, no treatment-related gross lesions or microscopic findings were observed after dosing with a 21-mer siRNA pool or 218 bp dsRNA test materials. For females only, there was minimal inflammation in one (1) animal given siRNAs but not in the dsRNA-treatment group. This was cited in the paper as being not treatment related as it was not seen in both sexes or in both RNA treatment groups, nor in a separate oral RNA study. Further, this type of infiltration is a common background finding and does not provide evidence of inflammation following oral exposure. It should be noted that these effects seen in some studies in mammals are unlikely to be relevant to risk assessment of exogenously-applied dsRNA-based pesticide products since they were

[39] Chol-siRNAs were synthesised by linking cholesterol to the 3' end of the sense strand *via* a pyrrolidine linker.

seen only after a systemic routes of exposure (injection) or at oral doses many-fold in excess of exposures, which would follow application of such products. The pathologist in the gavage study reported in Petrick et al (2016) concluded that, at high doses of dsRNA (100 mg/kg), there was no inflammation in the stomach or the GI tract after direct exposure by gavage. Furthermore, the study pathologist and the reviewing pathologist considered that any gross or histological changes seen in individual animals were incidental findings and not related to the test substance, leading to the conclusion that there was no dsRNA-related inflammation in this oral dosing study.

213. Although there are some similarities in the innate immune response of insects and mammals (Lundgren & Jurat-Fuentes 2012), it is not clear how the immune systems of other organisms will react to an influx of small RNAs, nor whether any immune-stimulation, which could occur would affect the fitness of non-target organisms. It has been hypothesised that the risk of immune-stimulation by dsRNAs may be one reason why RNA-dependent RNA polymerase (RdRP) enzyme, responsible for amplifying the abundance of siRNAs in some organisms, had not been found in mammals and insects[40] (Agrawal et al, 2003; Dillin, 2003).

214. The conclusion reached above, viz. that it is unlikely that novel dsRNAs as PIPs would cause an immune response in non-target mammals, has been reached by regulatory authorities in Australia, Canada, and the USA (FSANZ, 2014; CFIA, 2015; USEPA, 2014).

215. *Saturation of RNAi machinery*: High levels of siRNAs occurring in a cell as a result of the introduction of exogenous dsRNA can saturate a cell's RNAi machinery and thereby alter endogenous gene expression (Agrawal et al, 2003; Dillin, 2003; Katoch et al, 2013). Essentially, there are a limited number of RISCs present within a cell, and excess exogenous siRNAs may saturate these, thereby preventing them from carrying out typical homeostatic functions in regulating endogenous gene expression (Kahn et al, 2009). Jackson and Linsley (2010) suggested that small RNAs could have "effects on the expression of genes predicted to be under the control of endogenous microRNAs". Saturation could also lead to reduced defenses against viral infection (Dillin, 2003). It should also be noted that saturation of RNAi machinery could disrupt other pathways in which RNAi machinery is involved, such as RITS (Appendix 4), which could result in heritable changes in gene expression due to histone modifications.

216. The process of saturation has been better documented with expression of small hairpin RNA, although it is also known from high-dose *in vitro* studies with transfected siRNAs (Jackson & Linsley, 2010). Grimm et al (2006) hypothesised that the toxicity and mortality observed in mice Infused with a high dose of short hairpin RNAs (shRNAs), was due to competition with miRNA components. Kahn et al (2009) found that siRNAs concentrations from 4 nM were able to saturate RISC components, while saturation of Exportin-5 and Argonaute proteins (especially AGO2) was observed in mice when 5×10^{11} to 2×10^{12} copies of exogenous siRNAs were introduced (Grimm, 2011). High copies of viral-associated RNA (10^8 copies/cell) were also able to saturate the RNAi pathway (Andersson et al, 2005); it is noted that these data come from experiments with transformed cells in culture and the relevance to *in vivo* systems is yet to be determined.

[40] In early 2018 Schoville et al published the Colorado potato beetle (*L. decemlineata*) genome; RNA-dependent RNA polymerase (coded by the Elp-1 gene) was listed as one of the genes associated with RNA interference in this species, although at the time of drafting this working paper, it was not clear whether this gene is part of the CPB transcriptome (see also Section 5.2.3 under 'Insects').

217. However, it seems unlikely that, following the application of a dsRNA-based pesticide, the extent of systemic exposure of mammals, and indeed, of other organisms in the environment, would be sufficient to saturate their RNAi machinery (EFSA, 2014; USEPA, 2014; Paces 2017).

218. The conclusion that saturation is an unlikely hazard is supported by the ubiquitous presence of RNA in the environment, leading to the need for eukaryotic organisms to develop barriers and other mechanisms in order to avoid saturation of the RNAi machinery that is critical to maintaining their cell processes. For example, long endogenous dsRNAs are prevalent in plants and sequence analysis of predicted long dsRNA transcriptomes of major crops reveals complementarity with human genes (Jensen et al, 2013); *in silico* evaluations also note the potential for sequence alignments. Species responsive to RNAi are being exposed to many thousands of endogenous and exogenous RNAs - see Ivashuta et al (2015) for a description of exogenous RNA sources in the diet of environmental RNAi-responsive species. Given this, it seems reasonable to assume that organisms, including mammals, possess mechanisms to limit the potential for saturation of their RNAi biochemical pathways (in the same way that they have developed mechanisms to limit amplification of sRNAs). Finally, given the stochiometric requirements for effective RNAi (100-1,000 copies per cell required) and most non-target organisms are made up of billions to trillions of cells, saturation of RNAi machinery appears unlikely to create a potential concern for non-target organisms.

219. *Effects on the gut microbiome*: Bacteria and archaea have RNA-based regulatory systems, but the machinery differs from those in eukaryotic systems (see Section 5.2.1). Dietary composition (e.g., fibre, fat content) has been reported to alter the microbiome, but there is no evidence that specific dietary RNA sequences have any such effects (USEPA, 2014); RNA uptake by microbes is generally followed by catabolism to provide nutrition. There is no evidence that dsRNA is amplified by bacteria.

5.5.3. *Other potential hazard considerations in non-target organisms exposed to dsRNA*

220. *Immune stimulation*: Findings of immune stimulation in mammals in response to exposure to dsRNA and siRNA are discussed in Section 5.5.2. It is not clear how the immune systems of non-mammalian organisms will react to an influx of small RNAs nor is it clear how any resulting immune system stimulation would affect the fitness of non-target organisms (USEPA, 2014). Substantial empirical research would be required to investigate the effects of RNAi inputs on the immune responses of the many members of biological communities associated with agroecosystems; however, on the basis of research to date, it is unlikely that dsRNAs applied as pesticides would cause an immune response in non-target organisms (EFSA, 2014; USEPA, 2014, Paces 2017). Standard mortality, growth, and reproduction endpoints utilised in current ecological testing protocols should be adequate to evaluate the potential for hazard; the mechanism leading to a hazard only merits investigation if empirical testing demonstrates that there is a hazard.

221. *Saturation of RNAi machinery*: Similar RNAi pathway components are present in mammals and insects (Appendix I); saturation of Dicer, AGO2, and Exportin5 is theoretically possible. However, it would seem unlikely that, following the application of a dsRNA-based pesticide, the extent of systemic exposure of non-target organisms in the surrounding environment would be sufficient to saturate their RNAi machinery (EFSA, 2014; USEPA, 2014). Saturation of the RNAi machinery and immune stimulation by RNAi-based GM derived products were considered as theoretically possible in EFSA,

2014. However, no supporting evidence to suggest that these effects occur in humans and animals after oral administration is currently present (Paces et al., 2017). Keeping in mind that potential RNAi-related effects, either in the plant or in consumers, should not be considered hazards *per se*, a list of RNAi-related effects, which could be considered hazards, may include non-specific silencing in non-target organisms (Christiaens et al, 2018) including plants and/or in humans and livestock animals and effects on the microbiome in the human and animal gut and the possible influence on their homeostasis (EFSA, 2014). However, it should be noted that limited regulatory testing of specific non-target organisms for pesticidal dsRNA-based active ingredients to date has not shown any potential for hazard *via* any potential underlying mechanism (USEPA 2016b; USEPA 2017; Bachman et al, 2016). As was noted above (in relation to immune stimulation), in the context of safety assessment of RNAi products, empirical testing for effects on defined end-points such as mortality, growth and reproduction should adequately address the hazards associated with RNAi in non-target organisms, including saturation of RNAi machinery.

5.6. **Effects on birds, fish, aquatic invertebrates, plants, bees, non-target arthropods, earthworms, non-target soil micro-organisms etc**

222. As noted in Section 5.2.5, successful RNAi in non-target vertebrates such as **fish**, **reptiles**, and **birds** has only been achieved with cell lines or embryos and has required the use of transfection agents, direct injection, electroporation, or other invasive techniques (Schyth, 2008; Sifuentes-Romero et al, 2011; Ubuka et al, 2012).

223. Possibly the most extensive studies to date on the effects of dsRNA on a number of organisms across a range of phyla have been carried out by Monsanto with a dsRNA directed against the Western Corn Rootworm (WCR) *Snf7* gene. The double-stranded RNA transcript containing a 240-bp fragment of the WCR *Snf7* gene is recognised by the WCR's RNA interference machinery resulting in down-regulation of the targeted WCR *Snf7* gene coding for an essential vacuolar sorting protein, and target insect mortality (USEPA, 2016).

224. Avian dietary toxicity studies included tests on broiler chickens (*Gallus domesticus*) and northern bobwhite quail (*Colinus virginianus*). There were no effects of dietary exposure to *Diabrotica virgifera* (*Dv*)*Snf7* dsRNA on either of these bird species, under the conditions of the assays.

225. A freshwater fish dietary toxicity study was carried out on channel catfish (*Ictalurus punctatus*). After 8 weeks of exposure, there were no effects on food consumption, weight gain, or diet conversion, and no apparent adverse effects.

226. Annelid **earthworms** (*Eisenia Andrei*) were exposed to DvSnf dsRNA (active against Western Corn Rootworm) in a 14-day contact study. However, the USEPA concluded that the study contained too many uncertainties to rely on it. (USEPA, 2016b). Springtails (*Folsomia candida*), another soil invertebrate[41], was also tested; there were no effects on survival or reproduction in a 28-day dietary study at 1,000 ng DvSnf7/g of diet. While degradation of the test material may have occurred in the earthworm study, no adverse effects were noted in the study and any degradation would have reflected what would be expected in the agroecosystem under actual use conditions.

[41] Phylum: Arthropoda

227. **Honeybee** larvae (2 – 3-days old) and adults (newly-emerged) were exposed to DvSnf7 dsRNA, adults to a nominal concentration of 1,000 ng/g diet in a 14-day continuous feeding study and larvae at a concentration of 1,000 ng/g delivered in a single 10 µl aliquot to each larval cell, with observations until adult emergence (USEPA, 2016b). In adults, no adverse effects on survival, behaviour or appearance were observed. No adverse morphological or behavioural effects were noted in treated larvae (NOEL *ca*. 11.3 ng per larva).

228. Toxicity testing with DvSnf7 dsRNA was carried out in a **range of non-target insects**, including larvae of Lady Beetles (*Coleomegilla maculata*) (21-day study; 1,000 ng/g diet), newly-emerged parasitic wasps (*Pediobius foveolatus*) (20-day study; 1,000 ng/g in 30% v/v honey in water solution), Insidious Flower Bug (*Orius insidiosus*) (10-day study, starting as 5-day-old nymphs; 1,000 ng/g diet), Carabid Beetle (*Poecilus chalcites*) (35-day study, starting at the first-instar larval stage; 1,000 ng/g diet), newly-emerged adult Green Lacewing (*Chrysoperla carnea*) (18-day study; 1,000 ng/g diet), and adult Rove Beetle (*Aleochara bilineata*) (28-day study; 1,000 ng/g diet). There were no effects on survival, development or growth of Lady Beetle larvae; parasitic wasp survival; Insidious Flower Bug larvae survival or development; Carabid Beetle larval survival, development, or growth; Green Lacewing survival or reproductive performance; or on Rove Beetle survival or reproductive success.

229. The above results on DvSnf7 are on only one dsRNA construct and hence there needs to be a degree of caution in generalising the results (i.e., lack of adverse effects) to other dsRNAs; however, the suite of tests conducted comprised the most extensive dataset on a dsRNA at the time this report was drafted, and will help serve as a guide to the development and testing of other RNAi-based pesticide products.

6. Fate and Distribution of dsRNA from RNAi-based Pesticides in the Environment

Determining the rate of biodegradation of double-stranded RNA (dsRNA) in the environment is an important element of a risk assessment of an RNAi-based agricultural product. This information is used to help define relevant routes and durations of exposure for organisms in the environment to the applied dsRNA.

230. In assessing the environmental risks from the application of dsRNA-based pesticides will depend on the distribution and fate of the dsRNA in the environment. The distribution and fate of dsRNA within the environment, and therefore possible routes of direct and indirect exposure of NTOs to the dsRNA, as well as their duration of exposure will depend on the following factors (USEPA, 2013):

- Product formulation
- Application rate of the exogenously-applied dsRNA active ingredient
- Application method
- Application timing
- Number of applications
- Stability and persistence of the dsRNA following application
- Off-site movement of the dsRNA

231. Key considerations are the stability and persistence of the dsRNA following product application and its movement from the target plant or site. The speed and extent of *in situ* dsRNA degradation *via* abiotic and/or biotic processes will affect the extent of off-site movement of applied dsRNA from the treatment area to other environments (terrestrial or aquatic); its stability and hence persistence, may be different in different environmental media. These issues are considered below.

6.1. Stability of dsRNA in the environment

232. A determination of the potential risks of exogenously-applied dsRNA to non-target species in the environment requires an understanding of the likely stability and persistence (or lack thereof) of dsRNA molecules in the environment.

233. As discussed in some detail in Section 3.1 (above), RNA is inherently less chemically stable than DNA due to its chemical structure. Furthermore, RNA-degrading enzymes (RNases) are more prevalent in organisms and the environment than DNases and there are substantial similarities in the RNA degradation processes between bacteria, archaea, and eukaryotes.

234. A complicating factor in any risk assessment of a dsRNA-based pesticide product is the need to consider whether the dsRNA TGAI has been stabilised in any way; it would be reasonable to expect that stabilised dsRNA would degrade at a slower rate in soil and water, and in or on biological matrices than unstabilised dsRNA, unless empirical evidence to the contrary was provided or the stabilisation was targeted only to increase in-container shelf life (e.g., inclusion of an antimicrobial agent). Section 3.2 discusses various approaches to dsRNA stabilisation.

6.2. Studies on dsRNA degradation in the environment

235. Methods and models have been developed by regulatory agencies in OECD member countries to estimate environmental concentrations of chemical pesticides, following application at label use rates; these data are then used to provide estimates of the likely exposure of non-target organisms. Such models can serve as a useful basis for estimating the environmental concentrations of exogenously-applied dsRNAs provided that information is available on degradation rates of dsRNA in soil and water matrices under aerobic and anaerobic conditions and variable conditions of UV light and pH; these data will need to be generated by registrants/applicants if they are not available in the literature. Soil degradation study protocols for biologically-derived materials can be adapted for generation of soil persistence data.

236. Once applied at the use site, dsRNA will be subject to the same abiotic and biotic degradation processes as traditional chemical and biological pesticides. However, data regarding the degradation of dsRNA in the environment are somewhat limited (see review by Lundgren & Duan, 2013). There are more data on the fate of DNA in soil and sediment (e.g., Levy-Booth et al, 2007; Pietramellara et al, 2009) but this information is not particularly applicable to understanding the fate of dsRNA because of the different chemical characteristics of the two molecules (see discussion above; Section 3.1). DNA in soil may degrade in as little as 7 days or persist for several years, with persistence enhanced by binding to soil humus, or reduced by rapid microbial degradation (Levy-Booth et. al, 2007 as cited in Lundgren & Duan, 2013). Available data indicate that RNA is likely to degrade more rapidly than DNA.

237. From a risk assessment perspective, a further consideration with dsRNA as *cf.* conventional chemical pesticides is the question as to whether exogenously-applied dsRNA might amplify within plants once it has been taken up and if so, the degree of amplification that would occur. If amplification within plant tissues occurred to any significant extent, this would complicate the estimation of environmental levels and the conduct of off-target risk assessments. However, there is no reported evidence as discussed elsewhere in this document. Amplification would require the long dsRNA to be able to bypass structural and cell wall barriers, have a >21 nt sequence complementarity to an endogenous transcript, and bypass the multi-layered self-protection safeguards from 'unwanted' silencing in plants (Baeg et al, 2017; Pak et al, 2012). That is, if amplification were to occur, the applied dsRNA would have to be formulated and applied in a manner that bypassed the cuticle and circumvented any other barriers; then it would have to enter the plant RNAi pathway, and have a sequence match to a transcribed gene (see Sections 6.4.2 and 8.2.2).

6.2.1. *dsRNA degradation in soil*

238. For exogenously-applied dsRNA active ingredients, soil degradation is likely to be the main compartment for their environmental dissipation since the soil is likely to receive the majority of the applied dsRNA applications, either directly or from leaf surfaces of sprayed crops (deflected from the leaves during application, dislodged by wind or rain after application, or from crop trash being incorporated into the soil post-harvest).

239. In soil, the fate of dsRNA is likely to be affected by abiotic (e.g., temperature, pH, soil structure/type, UV light) and biotic (microbial and nuclease degradation) factors. Nucleic acids present a nutrient source for all organisms, a factor that needs to be considered in the environmental fate assessment of dsRNA-based products. Binding of dsRNA to soil organic matter may decrease degradation, but such binding may also

decrease its availability to organisms (USEPA, 2014). Dubelman et al (2014) conducted a study to determine the biodegradation of a DvSnf7 dsRNA transcript derived from a Monsanto GM maize product and produced in vitro, respectively that confers resistance to corn rootworm. Degradation of dsRNA in soil was measured by two independent methods, molecular analysis ('QuantiGene') based on RNA hybridization to specific DNA probes (Armstrong et al, 2013), and an insect bioassay, which used a functional activity endpoint (viz. corn rootworm mortality)[42]. Within approximately 2 days after application to soil (7.5 µg of DvSnf7 RNA per gram of soil), it had degraded and biological activity was undetectable[43], regardless of pH, clay content and soil type (Figure 3). The application rate, which ranged over two orders of magnitude (0.3, 1.5, 7.5 and 37.5 µg RNA/g soil) did not impact the rate of degradation.

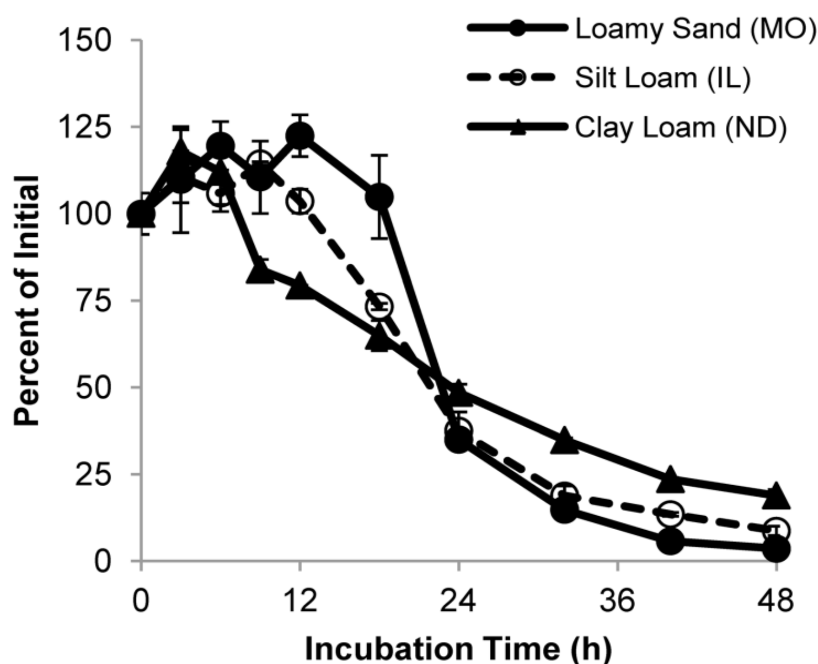

Figure 4: Degradation of DvSnf7 RNA degradation in three soil samples (Dubelman et al, 2014): DvSnf7 RNA was added (7.5 µg per gram of soil) in the absence of maize tissue, then its concentration determined after different incubation times with a QuantiGene assay. Error bars represent one standard error of the mean (n = 2). Soil samples were from Missouri (MO), Illinois (IL), and North Dakota (ND). [Equivalent results were obtained from soil samples amended with lyophilised insect-protected maize tissue and fortified with 7.5 mg DvSnf7 RNA per gram of soil.]

240. In research ostensibly carried out to investigate methods to accurately measure dsRNA in soil, Fischer et al (2016) incubated DvSnf7_968 (hairpin-structured dsRNA) and a linear 100-bp ds RNA (with no significant sequence similarity with DvSnf7) in vessels containing silt-loam soil. Both dsRNAs degraded rapidly with similar kinetics, being

[42] Both methods produced comparable DT50 and DT90 values, suggesting that either method could be used alone for analysis of dsRNA in soil. However, insect bioassays can only be used for measuring dsRNAs with an quantifiable insecticidal endpoint.

[43] Testing for biological activity is important since molecular methods can detect fragments indicating their presence, but these fragments may not be biologically active.

undetectable in the soil within 32 – 48 hours (see Figure 5). The soil degradation kinetics were independent of the dsRNA sequence, molecular weight, and structure.

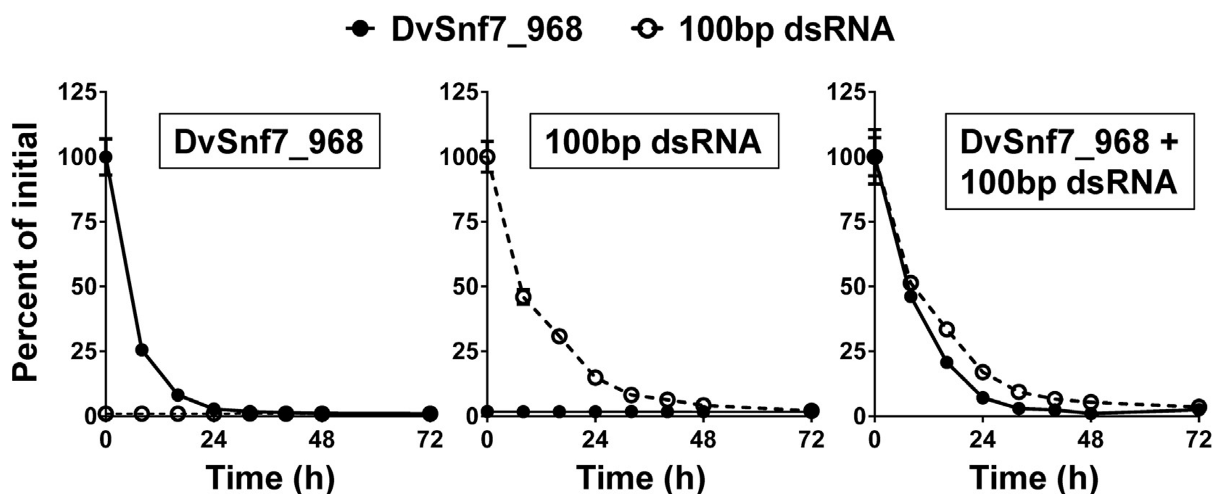

Figure 5: Sequence-specific QuantiGene results indicate that dsRNA degrades in soil, independent of size, sequence, and structure (Fischer et al, 2016). DvSnf7_968 (left panel), a surrogate 100 bp dsRNA (middle panel), or both RNA species (right panel) were applied to vessels containing silt loam soil. The 100 bp dsRNA (left panel) and DvSnf7 (middle panel) were not detected in the vessels to which they were not added (n = 3).

Note: In their 16 May 2017 'Proposed Registration Decision' for commercial-use corn products containing the DvSnf7 dsRNA plant-incorporated protectant, the USEPA requested the sponsor, Monsanto, to provide measurements of DvSnf7 dsRNA concentrations in soils collected during the growing season and after harvest from fields planted with the engineered corn; it was suggested that these data would provide *in situ* concentrations of DvSnf7 dsRNA and provide an additional degree of certainty to the field study reported above (USEPA, 2017). However, follow up discussions, submissions, and further EPA review may potentially alter the scope of this request (Monsanto Company, *pers comm*).

241. On the basis of data available to date, it appears that once dsRNA reaches soil, it is unlikely to persist or accumulate.

6.2.2. *dsRNA degradation on plants*

242. Applications of dsRNA will contact plant surfaces. The hydrophobic cuticle in plants represents a significant barrier to the unfacilitated entry of dsRNA (reviewed in Yeats & Rose, 2013). Additionally, plant surfaces are colonised by microbial communities (reviewed in Bulgarelli et al, 2013) that represent a source of nucleases that may contribute to the degradation of unprotected dsRNA on leaf surfaces.

243. However, San Miguel & Scott (2015) observed that foliar application of 5 µg Colorado potato beetle (CPB) actin-dsRNA per leaf protected potato plants for at least 28 days under greenhouse conditions. In laboratory feeding assays, second-instar CPBs placed on excised leaves (with petioles in water) treated with 5 µg of actin-dsRNA ceased feeding between 2 and 3 days. None of the larvae reached fourth instar, with low weight gain (~8% of the controls) and 98% mortality. As little as 1 µg of actin-dsRNA per leaf resulted in significant weight gain reduction compared to the control. However, no mortality or delay in development was observed at this concentration.

244. The study also tested the effects of simulated rain on the activity of the dsRNAs applied to excised leaves (petioles in water). It was found that the dsRNA did not significantly wash off with 3 swirls in 250 mL of water (3 seconds per swirl) when the dsRNAs was allowed to dry for 1 h on the leaves. The biological activity of the CPB actin-dsRNA was not significantly different between leaves that had been rinsed or not rinsed. It is possible that the swirling of leaves in water does not mimic the effect of rainfall or the effect of additional environmental degradation mechanisms in field situations.

245. The study further tested the likelihood of dsRNA degradation under UV exposure. After 2 hours exposure to 1500 µW/cm of 254 nm UV light, the 297 base-pair CPB actin dsRNA (applied thinly on a glass surface) lost its biological activity. However, as mentioned before, the biological activity of the dsRNAs on the plant surface was retained even after 28 days in the greenhouse, suggesting that the dsRNA is more stable on the leaf surface than on the glass surface used for the UV stability studies. It was hypothesised that the variation is due to the fact that the spray may be protected by shade from tiny hairs on the leaf or perhaps the spray soaks into the leaf (Ramanujan, 2015).

6.2.3. *dsRNA degradation in water*

246. Double-stranded RNA from agricultural products may reach water bodies from spray drift (when spraying near water), direct overspray, and from soil run-off following rainfall or an irrigation event, especially since dsRNA is hydrophilic. However, the amount of exogenous dsRNA required to be applied per unit area of crop in order to achieve acceptable effective pest control is likely to be very low, thus limiting the amount available for run-off or leaching. Furthermore, as indicated in the previous section, the rapid degradation of dsRNA in soil (< 2 days) will limit its presence in nearby water bodies *via* run-off or leaching.

247. Seitz et al (2011) noted that purified Norwalk virus RNA (extracted from Norwalk virus virions) persisted for 14 days in groundwater, tap water, and reagent-grade water. Tsai et al (1995) observed that viral RNA extracted from poliovirus could not be detected by RT-PCR after two days of incubation in unfiltered seawater but in filter-sterilised seawater, was detected after 28 days. Similarly, Limsawat & Ohgaki (1997) showed that bacteriophage Qß RNA in autoclaved wastewater and autoclaved Milli-Q water was detectable for up to 88 days but not after 30 and 60 minutes incubation in raw domestic wastewater and filtered wastewater. These results suggest that the activities of microorganisms present in wastewater are involved in RNA degradation.

248. It should be noted that the above RNAs are single-stranded. Double-stranded RNAs are known to be more resistant to common and ubiquitous endonucleases that cut single-stranded RNAs although double-stranded RNAs can be efficiently cleaved by the less abundant type III bacterial RNAses (Espinosa et al, 2008).

249. A study was conducted to measure the rate of double-stranded RNA degradation in aerobic water–sediment systems (Fischer et al, 2017). DvSnf7 dsRNA (60 µg, approximately equivalent to a final concentration of 300-330 ng RNA per mL of water) was directly applied to sediment-water microcosms containing clay loam (GR) and loamy sand (GL) sediments with overlays of river and lake water sources, obtained from the environment. Using molecular (QuantiGene) and bio-assays (insecticidal activity against Southern Corn Rootworm), it was found that DvSnf7 dsRNA dissipated rapidly from the water phase and was undetectable within 7 days. Degradation kinetics estimated a half-life (DT50) of less than 3 days and a time to 90% dissipation of approximately 4 days. Further analyses indicated that DvSnf7 dsRNA had DT50 values of less than 6 days in both

sediment-free natural water and in sediment only. Results indicate that dsRNA-based agricultural products are unlikely to persist in aquatic environments.

250. A non-bioactive 100-bp dsRNA was spiked into the water column of 3 different water and sediment microcosms, in order to mimic drift from a spray application and run-off of unbound dsRNA; the microcosms were: laboratory water over sterilised sediment; sterilised pond water over sterilised sediment; and active pond water over active sediment. dsRNA (measured by an Affymetrix QuantiGene 2.0 Singleplex assay kit) was detected at 48 hours in all 3 water columns but then dissipated and was below the limit of detection after 96 hours; DT50s were estimated as 63 h, 72 h and 56 h, respectively, in the 3 water columns. Interestingly, in this experiment there was little difference in the DT50 values in the presence or absence of biotic factors[44]. The levels detected in the sediment were not significant at any time and may indicate degradation in the water column prior to any partitioning to sediment (Albright et al, 2016).

251. It was noted that these results involved testing sediment and water from only two locations; using sediments and water samples collected across a broader range of locations would yield more robust results. However, the results were consistent with other studies examining degradation of RNA or DNA in water. For example, Zhu (2006) observed plasmid and plant DNA to be completely or mostly degraded in river and ground water by 96 h. In lake water, DNA- and RNA-hydrolysing bacteria degraded DNA and RNA[45] to undetectable levels by 10 and 8 days, respectively (Takata et al, 1993). Eichmiller et al (2016) observed that the degradation rate of environmental DNA was influenced by water quality and temperature; the slowest observed occurred with a DT90 of 6.6 days at 5°C.

6.3. **Off-site movement of applied dsRNA**

252. Offsite movement of dsRNA from the treatment site may occur via the following pathways (USEPA, 2013):

- Surface runoff from foliage and soil, following rain
- Infiltration into the soil and movement into groundwater
- Spray drift
- Physical movement of pollen from treated crops and dust from treated fields
- Physical movement on insect pollinators and other animals moving through treated crops
- Physical movement with plant debris.
- Uptake by plants through the roots.

253. Regardless of whether the dsRNA is unstabilised or stabilised, it may be distributed throughout the environment *via* some or all of these pathways. Volatilisation of dsRNA into the atmosphere is not likely. The first three pathways listed indicate how dsRNA could quite easily migrate from the application site to offsite terrestrial or aquatic environments.

[44] Although the authors noted that some RNases (e.g., RNase A) can slowly re-activate after autoclaving.

[45] RNA extracted from yeast (*Saccharomyces cerevisiae*) was used in these experiments, containing RNA molecules with single-stranded and double-stranded sections.

254. Pollen movement could raise exposure concerns if exogenously-applied dsRNA was applied during crop anthesis (flowers fully open and functional) or if the RNA was amplified in treated plants and transported into or expressed in pollen. Insect pollinators, if present on plants at the time of application, or collecting pollen and nectar after application, could possibly carry dsRNA residues back to their nests or hives and horizontally transfer residues to other insects. Although this route is very unlikely to be a significant pathway for moving dsRNA off-site from treated areas, dsRNA residues could accumulate within bee hives and potentially cause unanticipated off-target effects. Garbian et al. (2012) suggested that dsRNA ingested by honey bees could be transferred to Varroa mites feeding on the bees' haemolymph and reciprocally, back from dsRNA-carrying mites to other bees; however, as discussed above (see section 5.2.3 under 'Arachnids'), this conclusion about reciprocal transfer has been called into question. Furthermore, recent data (Ramsey, 2017) suggest that, contrary to a long-held belief, Varroa mites actually feed on the fat bodies of bees, not on the haemolymph.

255. Migration of dsRNA from the application site may occur *via* other animal vectors. Random movements of insects, as well as terrestrial and avian wildlife through treated areas at, or following dsRNA application, could carry unknown amounts of dsRNA to offsite areas. However, this is not likely to be a major pathway and would not be easy to model. As for other pesticides, regulatory consideration of edge-of-field exposure could be considered as a worst-case for offsite exposure.

6.4. Foliar uptake of dsRNA and possible amplification in plants

6.4.1. *Plant uptake and translocation*

256. It is highly likely that many dsRNA-based products will be developed to spray on crops. Thus, dsRNA will be deposited on the foliage of crops, weeds and other non-target plants. While evidence exists that exogenously-applied dsRNA can enter plant cells (reviewed in Cai et al., 2018, Cagliari et al., 2018, Dubrovina & Kiselev, 2019), the exact mechanism of foliar uptake is currently unknown. This section will describe the studies to date that have provided direct and indirect evidence for foliar uptake and translocation, and conclude with a summary of the outcomes and limitations of current knowledge.

257. For this section, databases of scientific literature were searched for studies which described *Foliar application of dsRNA* and either (A) *Testing for direct uptake of dsRNA* and/or (B) *Testing for systemic movement of dsRNA.* Any study where dsRNA foliar application was used for control of a pest but either (A) and/or (B) were not also examined was excluded from the literature search as not providing evidence for uptake and/or transport, since control of the pest achieved from activity of the dsRNA on the leaf surface could be a confounding factor to examination of uptake.

Mechanism of uptake

258. For dsRNA to enter plant cells, it first must escape degradation on the leaf surface by abiotic factors as well as plant-derived or microbial nucleases. Plant surfaces are colonised by microbial communities (reviewed in Bulgarelli et al., 2013) that are likely to represent a source of nucleases that contribute to the observed rapid degradation of unprotected or unmodified dsRNA in the environment (Monsanto, 2014); such degradation would limit the availability of dsRNA for uptake across the cuticle. As noted in Section 6.2.2, the hydrophobic cuticle also represents a significant barrier to unfacilitated entry of RNA (Koch & Ensikat, 2008; Yeats & Rose, 2013; Backman et al, 2020).

259. After escaping degradation and bypassing the cuticle, the dsRNA would need to cross the cell wall, apoplast, and cell membrane to enter the cytosol and be processed by the plant cell's RNAi machinery. In part due to these barriers, many of the available studies have tested foliar uptake of dsRNA using detached leaf assays, specialized formulations, or application after treatment with an abrasive agent.

Direct evidence for uptake and translocation

260. One of the initial studies suggesting foliar uptake of dsRNA was conducted by San Miguel and Scott (2015), where they demonstrated that Colorado potato beetle (CPB) actin-dsRNA in water could be taken up by excised leaves if their petioles were incubated in the solution. This method was effective in reducing the effects of the CPB on plant leaves. It has to be noted that uptake of dsRNA into leaves after foliar application on whole plants was not demonstrated, and that no systemic biological activity was observed in the nearby untreated leaves. Similar results were obtained testing dsRNA uptake after spraying on coleoptiles instead of leaves (Song et al., 2018). In this study, fluorescently-labeled dsRNA was internalized, translocated to tracheal elements and parenchyma, and conferred protection against fungus in distal cells, but only when the dsRNA was applied to cut coleoptiles (Song et al., 2018). Uptake and systemic movement of dsRNA was not observed if applied to intact coleoptiles.

261. In a study by Koch et al. (2016), detached barley leaves were sprayed locally with dsRNA targeting the expression of green fluorescent protein (GFP), and the distal (non-sprayed) parts of the leaves were inoculated with GFP-expressing fungus. Silencing of GFP expression in the fungus was observed by fluorescence microscopy and qPCR analysis of the target transcript levels. When CYP3 dsRNA (targeting three fungal genes involved in ergosterol biosynthesis in *Fusarium graminearum*) was similarly applied, a reduction in target gene expression and a reduction in fungal growth were observed in distal (non-sprayed) portions of the leaves. This indicated semi-systemic movement of dsRNA from the application site to a distal site within the same leaf.

262. The study by Koch et al. (2016) also examined the morphological distribution of fluorescently-labeled dsRNA within sprayed leaves, and found that the dsRNA was taken up by phloem cells after 24 hours. The dsRNA was also observed in other vascular tissue components, and stomata. Overall, the study gave evidence of dsRNA uptake at the cellular level, and showed systemic movement of dsRNA in the leaf, i.e. locally sprayed CYP3 dsRNA conferred gene silencing in and protection against *Fusarium graminearum* infecting non-sprayed segments of barley leaves (Koch et al., 2016).

263. Another approach (Dalakouras et al., 2016) applied high-pressure (7–8 bar) foliar spray of an aqueous solution of 21-24nt siRNAs targeting the GFP coding region to transgenic GFP-expressing *Nicotiana benthamiana*. In contrast to low-pressure spray, use of surfactants and leaf infiltration, this approach resulted in efficient induction of local GFP silencing 2 days post application.

264. Niehl et al. (2018) found that exogenously-applied tobacco mosaic virus (TMV)-derived dsRNA could inhibit the spread of TMV-GFP virions after mechanical virus inoculation in the presence of celite.

265. Enhanced stability and foliar uptake of dsRNA was achieved by Mitter et al. (2017) by loading fluorescently-labeled dsRNA on layered double hydroxide (LDH) nanoclay particles before spraying onto *Arabidopsis thaliana* seedlings, thereby protecting dsRNA from RNase degradation. After 48 hours, confocal microscopy revealed that fluorescently-

labeled dsRNA was present in the xylem of treated leaves, demonstrating uptake into the vascular tissue. The fluorescently-labeled dsRNA was also observed in apical untreated leaves, suggesting systemic translocation through the plant (Mitter et al., 2017). To examine dsRNA uptake further, another experiment in the same study tested RNAi effects of β-glucuronidase (GUS)-specific dsRNA applied to seedlings, but only a slight reduction in GUS activity was observed. Altogether, additional studies are needed for direct evidence of foliar uptake and biological activity of externally-applied dsRNA.

Indirect evidence for uptake

266. Indirect evidence for foliar uptake of dsRNA comes from studies investigating systemic movement of dsRNA. In the following studies, while the uptake of dsRNA was not directly visualized in a locally-treated leaf (e.g. by detection of dsRNA via fluorescence microscopy or PCR or by proof of RNAi effects), detection of dsRNA or RNAi effects in distal untreated leaves was assumed to be indicative of uptake and systemic movement.

267. Several studies (Konakalla et al., 2016; Kaldis et al., 2017; Gogoi et al., 2017) tested the ability of dsRNAs to control virus or insect pests in distal leaves, when introduced by mechanical application to a local leaf after dusting with an abrasive agent (carborundum or calcite) which disrupts some of the physical barriers to uptake. Konakalla et al. (2016) reported dsRNA detection in systemic (distal) leaves as early as 1 hour post-infection, reaching maximum strength 1 day post-infection (dpi), and then becoming undetectable at 6 dpi. Kaldis et al. (2017) detected the dsRNA itself as well as viral protection in distal leaves at 9 and 21 dpi; and Gogoi et al. (2017) demonstrated the presence of the exogenous dsRNA and corresponding siRNA fragments in insects feeding on distal leaves at 14 dpi. The study by Gogoi et al (2017) suggests that insects feeding on systemic leaves take up the dsRNA from within the plant; however, these results cannot rule out contamination of the distal leaf by exogenous dsRNA during initial application to the local leaf. In the study by Mitter et al. (2017) systemic virus protection in newly emerging, unsprayed leaves was observed after spray application of viral dsRNA covered LDH nanoclay particles on *Nicotiana tabacum*, however exogenous movement of stabilized dsRNA to the shoot apex could not be ruled out.

Conclusions on foliar uptake of dsRNA

268. Overall, the current evidence indicates that foliar uptake of exogenously-applied dsRNA followed by systemic translocation to a distal untreated portion is possible (Koch et al., 2016; Song et al., 2018; Konakalla et al., 2016; Kaldis et al., 2017; Gogoi et al., 2017; Mitter et al., 2017). However, these studies use either a detached leaf assay (Koch et al., 2016), abrasion of the leaf surface (Konakalla et al., 2016, Kaldis et al., 2017, Gogoi et al,. 2017, Niehl et al., 2018), wounding at the application site (Song et al., 2018), or stabilizing formulations (Mitter et al., 2017) to investigate uptake and/or systemic movement. There is no convincing evidence to date for uptake and systemic transport from application of unformulated dsRNA to intact tissue in the absence of these delivery approaches (San Miguel and Scott, 2015; Song et al., 2018). Therefore, to the best of our current understanding, the available studies indicate that dsRNA can be taken up by foliar application, but only if specialized formulations and/or delivery methods are used. However, given that limited data exist on this topic, knowledge of these mechanisms and possibilities may change as research in this area progresses.

6.4.2 Amplification in plants

269. Organisms with an amplification system can generate a robust RNAi response from very low copy numbers of imported dsRNAs. Like the model organism *Caenorhabditis elegans* and in contrast to insects and mammals, plants are able to amplify dsRNA signals *via* RNA-dependent RNA polymerases (RdRPs) (Zhang & Ruvkin, 2012; Vaistij et al., 2002; Frizzi & Huang, 2010).

270. In *C. elegans*, gene inactivation by RNA interference can be very potent due to amplification of initial silencing triggers by RNA-dependent RNA polymerases (RdRPs). RdRPs catalyse the synthesis of abundant secondary small interfering RNAs (siRNAs) using the target mRNA as template. It appears that the interaction between primary siRNAs derived from the exogenous dsRNA trigger and the target mRNA is required for the recruitment of RdRPs (Zhang & Ruvkun, 2012; Ghildiyal & Zamore, 2009). In plants RNA-dependent RNA polymerase 6 (RDR6) uses aberrant RNAs, i.e. transcripts lacking a 5′ cap or a 3′ polyA tail, as substrates to synthesise long dsRNA (Luo and Chen, 2007). Besides the origin of substantial amounts of aberrant RNAs from viral RNAs, from truncated transcripts or from highly expressed transgenes, aberrant RNAs may also be derived from RISC-generated target mRNA cleavage triggered by primary siRNAs (Frizzi and Huang, 2010). Target RNA cleavage products serve as template for secondary dsRNA and eventually secondary siRNA production (Rajeswaran et al, 2012; Voinnet, 2008), thereby amplifying RNA silencing.

271. Secondary siRNAs are not restricted to the initial target RNA region, but have been found to match to upstream and downstream sequences (transitivity). They also appear to be important for a systemic spread of the silencing signals (non-cell-autonomous RNA silencing) within the plant. In contrast to transgenes, with few exceptions (e.g. *TAS* genes) plant endogenes are generally not susceptible to transitivity and systemic silencing, which has been attributed to lower production of aberrant mRNAs and to the presence of introns, counteracting the generation of secondary siRNAs (Dadami et al, 2014; Christie et al, 2011). Voinnet (2008) noted that in view of the large quantity of endogenous siRNAs in plants there must be strong safeguards against undesired transitivity and that ‘plants appear to have evolved sophisticated measures to tolerate or exploit amplified silencing under specific biological circumstances’. Recent research also emphasises the existence of multi-layered self-protection safeguards against ‘self-attack’ of endogenous plant mRNA by RNA silencing (Baeg et al, 2017).

272. Therefore there are uncertainties as to whether exogenously-applied dsRNA, if it bypasses plant barriers, can be amplified within living plant tissue once it has been absorbed and if so, the degree of amplification (USEPA, 2013). As has been suggested based on experimental evidence, only highly-expressed mRNAs or viral RNAs would provide sufficient amounts of aberrant RNAs needed as substrates for RDR6 and as amplification trigger in plants (Frizzi & Huang, 2010; Dubrovina and Kiselev, 2019). Evidence against signal amplification comes also from reports on tissue-specific silencing of plant endogenes by the expression of dsRNA under control of tissue-specific promoters in transgenic plants (Sunilkumar et al, 2006; Frizzi & Huang, 2010). Nevertheless, some highly expressed endogenes may be prone to RNAi signal amplification.

273. With respect to the size of small dsRNAs functioning as primers for the induction of secondary (transitive) siRNAs, 22 bp siRNAs are hypothesized to have the greatest efficiency (Cuperus et al, 2010; Chen et al, 2010). 22 bp siRNAs, but not 21 bp siRNAs were also found to be able to trigger systemic silencing of the GFP (green fluorescent protein) transgene in *Nicotiana benthamiana* (Dalakouras et al 2016). However, the target

transcripts for production of secondary siRNAs in these reports were from *TAS* genes or from transgenes, both known to be susceptible to transitivity and systemic silencing.

274. Even for highly expressed plant genes and in the presence of 22 bp siRNAs an inevitable prerequisite for amplification of a silencing signal is sequence homology between plant mRNA and the delivered dsRNA. Therefore amplification is highly unlikely in the case of dsRNAs targeting plant pest sequences. In line with this, based on plant expression data from Monsanto's research on corn engineered to include the *DvSnf7* sequence for RNAi against the Western Corn Rootworm there is no evidence for amplification of this dsRNA as it does not have 21 nt sequence matches to any *Zea mays* sequences and hence does not target endogenous maize genes (Bachman et al, 2016; USEPA 2016b).

275. If the ability to amplify the RNAi signal was possible in plants and other organisms, this could affect the usefulness of exposure estimates involving those organisms, since environmental exposure determined to be 'low' may not translate to the same exposure within the organism. On the other hand, if efficient cellular uptake and systemic spread of RNAi signals does not occur, as has been found for some organisms (Terenius et al, 2011; Joga et al, 2016), then the environmental levels, regardless of whether amplification occurs within e.g. crop plants, would not be relevant to the risk assessment for these organisms.

7. Ecotoxicological risk assessment

7.1. Problem formulation

276. The focus of this section of the working paper is to highlight the specific issues that will need to be considered in environmental and ecological risk assessments of dsRNA-containing end-use products (dsRNA-EPs) that are not encountered in assessments for conventional chemical pesticides. The aim of regulators is to effectively manage the risk of unacceptable effects in non-target species in the environment, i.e., to ensure that protection goals for non-target organisms are met.

277. At the time this document was being drafted, the OECD was not aware that any regulatory agency had conducted a risk assessment of a dsRNA applied directly to the environment as a constituent of an end-use product intended for pest control.

278. The current approach used by regulatory agencies in OECD countries to assess the ecological risks of chemical pesticides provides the basic framework for environmental and ecological risk assessment of dsRNA-EPs. Due to the specific mode of action of dsRNA active ingredients (viz, Watson-Crick base pairing of complementary or near-complementary RNA sequences), the assessments currently used for traditional chemical pesticides will need to be adjusted to specifically investigate potentially harmful off-target gene effects of the dsRNA.

279. As with conventional pesticides, an understanding of the underlying mode of action of dsRNA-based active ingredients will help to inform the risk assessment but a complete understanding of all the steps of a biochemical pathway are not required to complete a risk assessment; key components of risk assessment - an estimation of exposure and identification of any hazards (effects on mortality, growth, and/or reproduction) – have been, and will continue to be carried out without necessarily having this information to hand.

280. Special factors to consider during problem formulation for risk assessment of dsRNA products fall under three main areas, exposure, relative sensitivity and sequence match; these are outlined below.

7.1.1. *Exposure – identification of plausible exposure scenarios*

281. An important step in initial problem formulation is the identification of plausible exposure scenarios for species that may be adversely affected by the dsRNA; this will depend on the product and its proposed use and application methodology. It is to be expected that the currently-used exposure scenarios will be applicable to dsRNA products.

282. Since the availability of data on the environmental fate (stability, persistence, mobility etc) of dsRNA molecules is limited at this stage of the development and application of such products, these aspects will need a particular focus. While the available evidence suggests limited stability and persistence of dsRNA molecules, empirical data will need to be provided by applicants, particularly on those that are chemically modified and/or packaged to improve stability and efficacy. Such information will allow better estimation of the extent and duration of exposure of species in the environment following product application.

283. Another element of exposure is a consideration of the barriers to systemic exposure in non-target organisms. For example, is the dsRNA active constituent (modified or

complexed or not) susceptible to endonucleases in the saliva, midgut fluids or haemolymph?

7.1.2. *Bioavailability to non-target species/limits for dsRNA exposure in different species*

284. There are several factors that may play a part in determining the bioavailability of dsRNA to an organism:

- Exposure route
- Formulation
- Natural host barriers
- Mechanisms for dsRNA uptake;
- Other interspecies variations in RNAi machinery.

7.1.3. *Exposure route*

285. Since exogenously-applied dsRNA products are likely to be applied using the same methods as traditional chemical pesticides (see Section 4.2), various routes of exposure of non-target species are possible. These routes include oral, aquatic, respiratory, ocular and dermal exposure *via* spray drift or from subsequent aerosolisation of applied product. These routes are relevant for the safety of workers who are processing the raw agricultural commodity[46], and to non-target organisms that may contact a dsRNA-based pesticide.

Oral exposure

286. Direct ingestion *via* consumption of treated plants, incidental ingestion *via* water, soil, pesticide spray, dust or granules, and indirect ingestion *via* predator/prey interactions are all possible oral exposure routes for non-target organisms. Several, physiological and biochemical barriers that limit dsRNA exposure after oral ingestion in most organisms have been reported (refer to Section 7.1.5. However, at present, it is considered that there is insufficient understanding on specific barriers to make any generalisations across non-target taxa. More reliable and robust data would be needed (EFSA, 2014, Davalos et al. 2019).

Aquatic exposure

287. If a dsRNA-based pesticide enters water systems, exposure could occur to aquatic species. For fish, uptake *via* the gills is theoretically possible; the extent of this will be influenced by a number of factors such as the physiochemical properties of the dsRNA, the degree of mixing of the water in the waterbody, the rate of waterflow through the gills, and the rate of gill blood flow. To the best of the authors' understanding however, as of the drafting of this document, no studies have tested dsRNA uptake *via* the gills. The existing literature of dsRNA effects in fish describe mainly injection studies into adult tissue or embryos which use specialized formulations such as exosome nanovesicles (Yang T et al 2017), PEG nanoparticle encapsulation (Diao et al 2015), or intramuscular injection

[46] Operator exposure to aerosols of sprayed dsRNA-based products is planned to be addressed in a subsequent human health working paper.

coupled with *in vivo* electroporation for dsRNA delivery (Terova et al 2013), which are of limited applicability to aquatic exposure routes that could be relevant to pesticide exposure assessments. One study (Bachman et al, 2016) investigated effects of dietary DvSnf7 dsRNA on channel catfish (*Ictalurus punctatus*); no mortality was observed and there were no treatment-related differences in food consumption or body weight gain relative to the assay control. However, since the study focused on maize-derived dsRNA, aquatic exposure was not considered. Therefore, in the absence of studies specifically testing dsRNA uptake via the gills in fish, no conclusions can be drawn about the impact of aquatic respiration as an exposure route for dsRNA. Further data, taking into account the environmental fate and behaviour of dsRNA in aqueous environments (Section 6.2.3), will be required.

Respiratory exposure

288. Respiratory exposure relating to RNAi in vertebrates has been studied mainly in a therapeutic context (reviewed in Lam et al, 2012), using siRNAs of usually 21–23 base pairs which are designed to bypass the interferon response elicited by longer dsRNA. Similar to the barriers to oral uptake of dsRNA discussed for vertebrates (Section 7.1.5), several barriers to uptake of dsRNA along the airways of mammalians exist. These include the presence of mucus, phagocytosis by macrophages, alveolar fluid, and the highly branched structure of the lungs limiting particle sizes which can be deposited in the lower airways (Lam et al, 2012). These barriers may limit respiratory exposure to dsRNA.

289. However, unlike oral exposure routes, respiratory exposure appears to induce RNA interference responses more readily under certain conditions. For example, in a study using unmodified siRNA targeting plasminogen activator inhibitor-1 (PAI-1), a gene involved in disease progression of pulmonary fibrosis, intranasal administration in mice reduced target protein levels in bronchoalveolar fluid after 14 days and improved survival rate (Senoo et al, 2010). Intranasal administration of slightly modified anti-viral siRNAs (containing deoxythymidine dinucleotide [dTdT]) reduced viral symptoms in both Rhesus macaques (Li BJ et al, 2005) and humans (DeVincenzo et al, 2010). However, while these studies suggest that respiratory exposure to siRNA can lead to induction of an RNAi response, the impact of chemical modification and formulation should also be considered. When intratracheal administration of naked unmodified siRNA in mice was compared to either locked nucleic acid (Moschos et al, 2011) or 2-O-methylated siRNA (Gutbier et al, 2010), rapid renal filtration was observed for unmodified siRNA within minutes, while the chemically-modified/formulated siRNA was still detected hours after administration in peripheral blood and pulmonary tissue (Gutbier et al, 2010).

290. Overall, the existing literature on respiratory exposure to RNA indicates that, under certain conditions, an RNAi response can be induced by both unmodified and formulated siRNA. Until respiratory toxicity studies are conducted with putative dsRNA-based pesticides, the majority of data informing our current understanding of respiratory exposure in vertebrates to dsRNA will be derived from the clinical literature. However, limitations of the current data should be kept in mind when assessing respiratory exposure to dsRNA-based pesticides. The existing studies mainly use short sequences of RNA designed to bypass potential immune responses such as binding to Toll-Like Receptors and activating the interferon response; dsRNA-based pesticides are likely to employ longer dsRNA, which could potentially activate these pathways, and clinical data on inhalation of longer dsRNAs are lacking. The majority of studies on respiratory exposure to RNA use mammalian models, so the impact of lung structures on inhalation effects of dsRNA across different types of non-target organisms is currently unknown. As well, respiratory exposure routes

such as intratracheal administration used in animal studies for RNAi-based therapeutics may not be wholly applicable to the exposure scenarios that may be expected with dsRNA-based pesticides.

291. Inhalation of pollen, if exogenously applied dsRNA happened to be taken up and or translocated to the pollen, could be a possible source of exposure. However, this route of exposure is unlikely to be of significance as pollens are limited in number and both pollen and agricultural dust/aerosols tend to be large particles that do not migrate to the small capillaries of the lungs (EFSA, 2014). Such particles will be deposited in and cleared from the upper respiratory tract, resulting in secondary oral exposure rather than pulmonary exposure[47]. Supporting this conclusion, Sherman et al (2015) noted that there was a history of safe inhalation exposure of dsRNA contained in plant pollen and dust; however, since the particle size of plant dust and pollen is generally larger than inhalable particles (generally considered to be <10 µm), inhaled pollen and plant dust particles would be cleared from the upper airways and result in secondary oral exposures, not inhalation exposures.

Dermal exposure

292. Organisms in the target area (including those inhabiting crops, in soil, sediment or aquatic zones) during product application, or movement of organisms through the treated area following product application, could also be dermally exposed. However, physical barriers (e.g., cuticle, exoskeleton or integument in insects; skin; fur) are likely to prevent or limit dermal absorption (see Section 7.1.5).

293. Information on dermal exposure of dsRNA in vertebrates is limited[48], and generally comes from studies developing delivery methods for siRNA therapeutics for treatment of skin diseases. siRNA has poor dermal permeability due to its size, charge, susceptibility to degradation; therapeutics using siRNA must employ strategies to cross the outer layer of epidermis including conjugation to cholesterol, cell-penetrating peptides, liposomes, lipid nanoparticles, and transfection reagents (Chen et al, 2014; Ibaraki et al, 2019; Colombo et al, 2019). In these studies, short siRNAs (21-23nt) are used to bypass the innate immune response, and specialized formulations and modifications are employed to facilitate cellular uptake. Therefore, the unmodified siRNA controls in these studies give preliminary data to understand possible effects from dermal exposure to RNA.

294. The general trend emerging from current studies on dermal exposure to siRNA is that, in its unmodified form, siRNA does not readily cross dermal barriers and is not capable of uptake on a level that causes silencing of the target gene. Both *in vitro* (e.g. Chen et al, 2014; Ibakari et al, 2019) and *in vivo* studies (e.g. Hsu & Mitragotri, 2011; Ibaraki et al, 2019; Kasiewicz & Whitehead, 2019) indicate that modification of the siRNA and/or specialized delivery methods are required for siRNA to cause significant effects upon dermal exposure.

[47] On this basis, oral toxicity studies would be sufficient to cover investigations of this exposure route.

[48] For this working paper, only studies using topical application of RNA were examined for information on dermal exposure. Studies in which RNA was administered through more invasive routes such as subcutaceous injection were disregarded, since they are of limited relevance to exposure routes expected for non-target organisms.

295. Cell culture studies give preliminary insight into results of exposure to siRNA when certain barriers to uptake such as the outer epidermal layer are not present. Human keratinocytes, when treated with 25 µM siRNA alone, displayed target gene silencing of ~18%; however, when the siRNA was conjugated to a cell-penetrating peptide and formulated within a phospholipid nanovesicle, target gene silencing increased to 80% (Chen et al, 2014). Likewise, Ibakari et al (2019) observed 10% uptake of siRNA alone in murine macrophage cell culture, but nearly 100% uptake with a liposome formulation and transfection agent.

296. In a study on mice by Hsu and Mitragotri (2011), 10µM of siRNA (2-O-methyl modified) complexed with a dermal penetrating peptide (or appropriate controls) were applied topically to a 3 cm^2 area of skin on the back of the animal for 24-72 hours. The siRNA or peptide administered alone did not result in silencing of target protein, but in complex with the dermal penetrating peptide, significant target silencing was observed (Hsu and Mitragotri, 2011). Similar results were obtained by Colombo et al (2019), where 40-50mg of a 21-bp siRNA formulation (containing up to 10 µM RNA) did not elicit a silencing effect in mice after application to undamaged skin, while siRNA possessing several modifications (such as including several dNTPs in the siRNA, and conjugating the siRNA to cholesterol) resulted in significant suppression of the target gene.

297. Evidence that dermal exposure to siRNA alone does not result in cellular uptake also comes from Ibaraki et al (2019), where 5µg of fluorescently-labelled siRNA were nearly undetectable by fluorescence microscopy on a skin biopsy after 10 hours of siRNA exposure, even when the stratum corneum (outer layer of epidermis) was removed before siRNA application. In contrast, siRNA encapsulated in liposomes visibly permeated up to 100µm into the skin and significantly reduced target protein production (Ibakari et al, 2019). In a diabetic mouse model, application of 100–500 nM therapeutic siRNA to wounded skin resulted in significant target silencing, localization within wound tissues, and accelerated wound healing when encapsulated in fluorescently-labelled lipid nanoparticles (Kasiewicz & Whitehead, 2019). Localization of nanoparticles was visualized in and around wound bed cells, suggesting that siRNA can enter tissue and elicit silencing effects when formulated (Kasiewicz & Whitehead, 2019).

298. Overall, unformulated siRNAs do not appear to result in significant dermal permeation, cellular uptake, or target silencing following dermal exposure. Given that dsRNA differs from siRNA only in length, it is tempting to speculate that dermal exposure to longer dsRNA would not result in adverse effects if the dsRNA is unformulated. However, further study of dermal exposure to dsRNA-based pesticides is required. The existing studies relating to dermal exposure are limited, focusing on mammals and using siRNAs in specialized formulations; effects of longer dsRNAs from dermal exposure in a wider range of vertebrates and other NTOs are currently unknown.

299. It should be noted that exposure alone is not enough to induce an RNAi response. High enough exposure concentrations as well as accessibility of the dsRNAs to intracellular target site, and at a sufficiently-high concentration, are required for a gene-silencing effect to be observed in responsive organisms.

7.1.4. *Formulation*

300. Product formulations may have a significant effect on absorption and the bioavailability of dsRNA (e.g., Brown & Ingianni, 2013; see also Section 3.5 and the sub-section headed ‘Test Material for Non-target Organism Testing’ in Section 7.2.3). For example different types of dsRNA carriers have been demonstrated to affect stability and

uptake of dsRNA into plant cells. Jiang et al. (2014) used water-soluble cationic fluorescent nanoparticles with peripheral cationic groups for electrostatic binding to dsRNA for efficient delivery of dsRNA into plant cells. Application of mixtures of 3.6 nm nanoparticles and specific dsRNAs to root tips of *Arabidopsis* seedlings resulted in uptake of dsRNA nanoparticle complexes into the root system and into plant cells, followed by systemic silencing of target genes after 3 days continuous treatment.

301. Not only cellular uptake, but also dsRNA stability can be affected by dsRNA carriers. Numata et al. (2014) complexed a fusion peptide of a polycation and a cell-penetrating peptide with siRNA via ionic interactions. dsRNA-peptide complexes which are in the size range of 100 to 300 nm were much more resistant to RNase treatment than naked dsRNA. Moreover, there was evidence for cellular uptake of siRNA-peptide complexes and transient local gene silencing in leaf epidermal cells after infiltration of *Arabidopsis thaliana* and poplar leaves. As a more sophisticated delivery vehicle Zhang et al. (2019) designed DNA nanostructures with attachment loci for conjugation to biological molecules. For DNA nanostructures of 2.4 to 16 nm conjugated to siRNA and applied on the abaxial side of *Nicotiana benthamiana* leaves via infiltration, a high degree of colocalization with the plant cytosol was observed. Loading on these DNA nanostructures protected siRNA from degradation inside the cell and enabled efficient transient siRNA mediated silencing of a marker transgene. The conformation and compactness of the DNA nanostructure did not only affect the degree of cellular uptake, but also seemed to affect the type of gene silencing mechanism (mRNA degradation vs. translational inhibition) triggered. Although these reports demonstrate the effects of carrier molecules on dsRNA stability and uptake into plant cells, it has to be noted that leaf infiltration is highly unlikely to be used in agricultural field applications.

302. In contrast to the above mentioned methods which relied on carrier uptake, Mitter et al. (2017a, 2017b) used positively charged layered double hydroxyde (LDH) clay nanosheets (size range 15 – 120 nm) as dsRNA carrier in order to protect dsRNA from RNase degradation and wash-off from the leaf surface and to facilitate steady release of the dsRNA through slow LDH breakdown under environmental conditions. By permitting prolonged dsRNA persistence on plant leaves as compared to naked dsRNA, LDH nanosheets have been demonstrated to enable sustained protection against infecting plant viruses.

7.1.5. *Natural barriers to uptake*

303. In the digestive tract, RNA is subject to both non-enzymatic and enzymatic degradation. In mammals, the breakdown begins with mastication and exposure to degradative RNases in saliva (Park et al, 2006), followed by further digestion in the stomach and gut (USEPA, 2014); the harsh acidic conditions of the stomach denature and depurinate nucleic acids (Loretz et al., 2006; O'Neill et al, 2011). Pancreatic and intestinal nucleases and degradative enzymes (and possibly bile salts) eventually catabolise RNA to nutritionally-available mono-nucleotides and subsequent nucleosides and bases (O'Neill et al., 2011; Carver & Walker, 1995; Hoerter et al, 2011; Rehman et al, 2011; Sorrentino et al, 2003). In addition to the RNases encoded within the genome, there are likely to be numerous others RNAses provided by the collection of micro-organisms that colonise the gastrointestinal tract (USEPA, 2014). If the RNA avoids all the degradation processes, uptake of short RNA sequences in humans is predicted to be limited to the upper small intestine (Carver & Walker, 1995). Due to its size and charge, diffusion across cell membranes is difficult for RNA.

Mammals

304. The mammalian gut also provides a physical barrier to uptake of hydrophilic compounds like siRNAs. In the absence of encapsulation to prevent degradation or the inclusion of chemical stabilisation and penetration enhancers, the absorption of RNA across the GI tract is unlikely(Akhtar, 2009; Jain, 2008). The low percentage of ingested nucleic acids that might be systemically absorbed from the GI tract encounter nucleases in the blood (Houck and Berman, 1958) and renal clearance (Molitoris et al, 2009). The effects of co-formulants and packaging or complexing of dsRNAs may affect the uptake and degradation of dsRNA molecules; these issues would need to be addressed by registrants in seeking to market a dsRNA-based pesticide product.

305. To affect gene expression in cells any remaining RNAs in circulation must: (1) cross cellular membranes; (2) escape from early endosomes to enter the cytoplasm; *and* (3) avoid degradation by nucleases found within lysosomes (Gilmore et al, 2004; Manjunath & Dykxhoorn, 2010; Sioud, 2005). The cumulative impact of these barriers would result in an insufficient amount of intact siRNA to impact cellular function[49].

306. Thus, when metabolism and barriers to RNA absorption are considered, it is extremely unlikely that oral ingestion of naked/unformulated dsRNAs will reach mammalian cells in sufficient quantities to mediate any RNAi effects.

Non-mammalian vertebrates

307. All vertebrate digestive systems display commonalities with regard to structure and function such as enzymes that aid in digestion (Stevens & Hume, 1995; Finegan & Stevens, 2012), therefore, the same digestive barriers that prevent oral activity of ingested RNA in mammals are likely to be applicable to non-mammalian vertebrates (Monsanto, 2014). The USEPA's risk assessment of DvSnf7 dsRNA plant-incorporated protectant in corn (MON 87411) noted that "birds, wild mammals, and other vertebrates would be expected to have similar barriers [to those in humans], and although there is likely some variation such that not all of them may be present, several of them would be" and that "these barriers are expected to significantly limit uptake and the potential for effects" (USEPA, 2016b).

Insects

308. In insects, the first barrier encountered by ingesta is the midgut peritrophic matrix (PM), a chitin and glycoprotein layer that prevents large molecules and toxins from entering midgut cells (Hegedus et al, 2009). Disruption of the PM structure improves midgut permeability and causes adverse effects (Barbehenn, 2001); this disruption can be brought upon by cysteine proteases (Pechan et al, 2002). In contrast to mammals, some insects (including coleopterans) have high levels of cysteine proteases in the gut (USEPA, 2014). Cysteine proteases can enhance oral RNAi in insects; 3rd instar cotton bollworms larvae were fed an artificial diet supplemented with plant cysteine proteases, GhCP1 and AtCP2, and *E. coli* cells for 2 days. The larvae were then transferred to leaves of transgenic *Arabidopsis* plants expressing the dsRNA against the bollworm P450 gene CYP6AE14. While the transcript level of CYP6AE14 was moderately decreased in controls, a stronger decrease of CYP6AE14 expression occurred in the larvae pre-treated with His-GhCP1 or His-AtCP2 (Mao et al, 2013).

[49] This is consistent with the clinical experience in endeavouring to deliver therapeutic RNAs.

309. In addition to physiological barriers, enzymic barriers appear to exist in insects. Studies on the tarnished plant bug[50], *Lygus lineolaris*, demonstrated that endonucleases present in saliva rapidly degrade RNA (Allen & Walker, 2012). Rapid degradation of dsRNA in the haemolymph of *Manduca sexta*[51] has been attributed to nuclease activity (Garbutt et al, 2013).

310. Dermal absorption of dsRNA *via* the integument in insects and mites has been reported, but in a number of cases, successful RNAi after soaking or droplet application may have been due to oral ingestion (e.g., Whyard et al, 2009). In studies by Campbell et al (2010), topically-applied dsRNA to immobilised *Varroa destructor* mites for several hours failed to induce gene silencing, presumably because the dsRNA was unable to cross the cuticle. Effective gene silencing required the total immersion of mites overnight in a saline solution of dsRNA; systemic entry was presumed to be either by ingestion or though the tracheal system *via* the spiracles. The penetration of topically-applied dsRNA bearing a fluorescent label was reported in larvae and eggs of the Asian corn borer[52], *Ostrinia furnalalis* (Wang et al, 2011); however very high concentrations of the dsRNA (0.5 µl drop of a 5 µg/µl solution) were used. When 50 ng/µl of 4 different dsRNAs (DS2, DS10, DS28 and DS35) were sprayed on corn borer larvae in the presence of their artificial diet, 5-day mortalities of 90-100% were seen. Since there were two possible pathways of dsRNA uptake (either by penetration of the body wall or by feeding), a second experiment was conducted in which 50 ng/ml two dsRNAs (DS10 and DS28) were sprayed on newly hatched larvae before they were placed on their artificial diet; 5-day mortalities were only 40% and 52%, suggesting significantly less dermal penetration than oral uptake (Wang et al, 2011). It is likely that, even in the latter experiment, there was some oral ingestion of the sprayed dsRNA; if oral ingestion could have been eliminated, it is likely that mortalities would have been lower. It remains to be clearly demonstrated that dsRNAs, without chemical enhancers and high doses, can induce a significant RNAi effect in arthropods through a contact/dermal route of exposure. In an overview paper, Yu et al (2013) discussed microinjection, ingestion and soaking to deliver dsRNA in insects, and noted that in "RNAi-based pest control strategies, the biggest challenge is the delivery of dsRNAs".

7.1.6. *Relative responsiveness*

311. As discussed above, not all taxa demonstrate measurable RNAi responses and there is a very large range of responsiveness to dsRNA across different taxa. When selecting NTO species for risk assessment for a particular product, those non-target species that are mosre likely to be exposed should be identified. In parallel the environmental stability, dispersion and levels of the dsRNA active constituent need to be determined so that responsiveness of the NTOs identified can be assessed as needed.

7.1.7. *Sequence matching information*

312. As discussed in more detail below (Section 7.4.4), sequence information should only be used to indicate whether a non-target organism is potentially responsive to Watson-

[50] Order: Hemiptera

[51] Commonly known as the Carolina sphinx moth or the tobacco hawk moth (as adults) and the tobacco hornworm or the goliath worm (as larvae). Order: Lepidoptera

[52] Order: Lepidoptera

Crick complementary binding to its mRNAs. Bioinformatics can be used to reduce uncertainties in the risk assessment, but its application in the risk assessment is of limited value because of the extent of environmental exposure, significant barriers to systemic exposure (limiting target-site access) and differences in RNAi machinery between organisms. Furthermore, chemical and thermodynamic factors will influence selection of the proper guide strand and the binding of siRNAs to complementary, or near-complementary mRNA strands. Thus, sequence information alone should not be used to predict the hazard of a dsRNA product to non-target organisms. Nevertheless, an examination of the use of sequence information by the product inventor/developer/applicant in designing the dsRNA to be as selective as possible for the target insect will be a crucial aspect of the risk assessment; the rationale for the design of the dsRNA should be an important guide to the likely selectivity of the resulting siRNA for the target pest and its specificity for the target gene. In addition, the support of bioinformatics could be valuable, but the limited availability of insect genomic sequences, the possible silencing in the presence of mismatches between the target and the siRNA sequences, and the possibility of sequence-unrelated off-target effects indicate the fundamental role of bioassays in assessing the actual activity spectrum of dsRNA (Christiaens et al., 2018, Annex II of the minutes of the 118th GMO plenary meeting (https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf)).

313. As available genomes of environmental species expands, it may be reasonable to exclude a non-target organism (or a group of non-target organisms) from consideration as possibly being susceptible to sequence-dependent dsRNA-mediated silencing, on the basis that no sequence match to the construct occurs within the genome of that non-target organism (or group of organisms) and the potential for sequence-independent off-target effects (e.g. RNAi machinery saturation) is determined not to be applicable (see Emprical Testing Decision Tree in Section 7.2.3).

7.2. **Exposure Assessment**

314. Section 4 of this document summarises the types of products that are likely to be developed (Section 4.1) and the likely application methods (Section 4.2). These are relevant to assessing exposure of organisms in the environment to dsRNA-based products. In terms of general factors to consider, the assessment approach is likely to be very similar to that for ‘conventional’ chemical pesticides because the application methods and proposed use patterns are likely to be the same or very similar. As noted elsewhere, one particular issue relevant to exposure assessment for dsRNA-based products is the potential for signal amplification (see Section 8.1.2).

315. Although estimates of degradation rates may be calculated using the limited available data on the environmental fate of similar nucleic acids, specific information is lacking on dsRNA, particularly if it has been stabilised for formulation into end-use products. The USEPA has stated that selected Tier-II biochemical pesticide environmental fate studies may be used to determine the degradation rate and environmental fate of dsRNA following application of dsRNA-based pesticide products to plant and soil surfaces (USEPA, 2013); data obtained from these studies will more completely inform exposure assessments for exogenously-applied dsRNA products (as well as for dsRNA-PIPs). These Tier-II data requirements may be found in the table at 40 CFR 158.2060(d) and are summarised as follows:

- Sediment and soil adsorption/desorption for parent and degradates

- Soil column leaching
- Hydrolysis
- Aerobic soil metabolism
- Photodegradation in water
- Photodegradation on soil
- Anaerobic soil metabolism
- Aerobic aquatic metabolism
- Anaerobic aquatic metabolism

316. These studies must be supported with robust and independently-validated analytical methods that are accurate and precise for the detection of dsRNA in the various environmental matrices.

317. It is generally recognised in the risk assessment of pesticides that soil is the primary environmental compartment for consideration. Similarly, a standard assumption is that some off-site movement from the application site *via* spray drift or soil runoff will occur, leading to exposure of non-target organisms in water bodies to the pesticide. The impact of the product formulation on the environmental persistence of constituents of chemical and biological pesticides is also considered in the existing risk assessment paradigm; for example, if a formulation utilises encapsulation technology, data may be required by regulatory authorities to show that the formulation has no significant impact on soil persistence of the active ingredient, or that the capsules break down in the spray tank so that the formulation has no impact on soil persistence or fate of the active ingredient.

- While the general framework for the exposure component of the risk assessment can be applied to dsRNA-based products, within OECD member states a range of requirements exist with respect to the data that are required to elucidate the environmental profile of active ingredients in microbial and biochemical pesticides. For example, the US EPA has created a specific set of data requirements for microbial and biochemical pesticides[53]. The European Union (EU) utilises one set of official data requirements for all chemical substances, and a second set of data requirements for microorganisms; EU Regulation 283/2013 Part B lists the data required for microorganisms that are proposed for use as plant protection products[54].

318. Given that there is currently no existing EU guidance of this type for dsRNA-based active ingredients, the complete list of environmental exposure data requirements is presented in this working paper in order to indicate the possible range of data requirements that may be needed to support the exposure component of risk assessments for dsRNA-based products within OECD member states (see Table 1).

[53] The division within US EPA (Biopesticides and Pollution Prevention Division; BPPD https://www.epa.gov/pesticide-contacts/contacts-office-pesticide-programs-biopesticides-and-pollution-prevention) that deals with existing biologically-based products has stated publicly their intention to regulate topically-applied dsRNA-based products within this division (see USEPA, 2013).

[54] See http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0001:0084:EN:PDF

Table 1: The possible range of environmental exposure data requirements for dsRNA-based products within OECD member states.

US EPA (From Part 158, Subpart U Biochemicals[55])	EU Member States SECTION 7. Fate and behaviour in the environment (From Regulation EU 283/2013)
No exposure data are required in Tier I. However, on a case-by-case basis, laboratory soil degradation studies utilising non-radiolabelled test material have been required in some circumstances. All environmental fate data requirements are in Tier II and are conditionally/may be required. 163-1 (835.1230) Sediment and soil adsorption/desorption for parent and degradates 163-1 (835.1240) Soil column leaching 163-2 (835.1410) Laboratory volatilization from soil 161-1 (835.2120) Hydrolysis 161-1 (835.4100) Aerobic soil metabolism 161-2 (835.2240) Photodegradation in water 161-3 (835.2410) Photodegradation on soil 162-2 (835.4200) Anaerobic soil metabolism 162-4 (835.4300) Aerobic aquatic metabolism 162-3 (835.4400) Anaerobic aquatic metabolism 880.4425 Dispenser - water leaching	7.1. Fate and behaviour in soil 7.1.1. Route of degradation in soil 7.1.1.1. Aerobic degradation 7.1.1.2. Anaerobic degradation 7.1.1.3. Soil photolysis 7.1.2. Rate of degradation in soil 7.1.2.1. Laboratory studies 7.1.2.1.1. Aerobic degradation of the active substance 7.1.2.1.2. Aerobic degradation of metabolites, breakdown and reaction products 7.1.2.1.3. Anaerobic degradation of the active substance 7.1.2.1.4. Anaerobic degradation of metabolites, breakdown and reaction products 7.1.2.2. Field studies 7.1.2.2.1. Soil dissipation studies 7.1.2.2.2. Soil accumulation studies 7.1.3. Adsorption and desorption in soil 7.1.3.1. Adsorption and desorption 7.1.3.1.1. Adsorption and desorption of the active substance 7.1.3.1.2. Adsorption and desorption of metabolites, breakdown and reaction products 7.1.3.2. Aged sorption 7.1.4. Mobility in soil 7.1.4.1. Column leaching studies 7.1.4.1.1. Column leaching of the active substance 7.1.4.1.2. Column leaching of metabolites, breakdown and reaction products 7.1.4.2. Lysimeter studies 7.1.4.3. Field leaching studies 7.2. Fate and behaviour in water and sediment 7.2.1. Route and rate of degradation in aquatic systems (chemical and photochemical degradation) 7.2.1.1. Hydrolytic degradation 7.2.1.2. Direct photochemical degradation 7.2.1.3. Indirect photochemical degradation 7.2.2. Route and rate of biological degradation in aquatic systems 7.2.2.1. 'Ready biodegradability' 7.2.2.2. Aerobic mineralisation in surface water 7.2.2.3. Water/sediment study 7.2.2.4. Irradiated water/sediment study

[55] https://www.ecfr.gov/cgi-bin/text-idx?SID=f1bd9313cf7ae85d48c8630c7d016adc&mc=true&node=sp40.26.158.u&rgn=div6

	7.2.3. Degradation in the saturated zone 7.3. Fate and behaviour in air 7.3.1. Route and rate of degradation in air 7.3.2. Transport via air 7.3.3. Local and global effects 7.4. Definition of the residue 7.4.1. Definition of the residue for risk assessment 7.4.2. Definition of the residue for monitoring 7.5. Monitoring data

Soil degradation

319. When considering what data are needed for conventional chemical pesticides within the context of the environmental exposure component of the risk assessment, key questions to be addressed are:

- How rapidly does the active ingredient degrade in soil (i.e., DT50 and/or DT90, the time needed for 50% or 90% of the active ingredient to degrade)?
- What is the degradation pathway - microbial and/or physicochemical?
- What metabolites are formed and how rapidly are they degraded?
- Are there any environmentally relevant metabolites that may be potentially hazardous, and if so, should they be included in the risk assessment?

320. When soil degradation data have been required for biological pesticides, the US EPA normally has accepted laboratory soil degradation data using protocols similar to those described in Dubelman et al (2014); only the soil half-life or degradation rate has been considered in these studies. However, studies utilising these protocols may also provide information on e.g., whether degradation is abiotic or biotic.

321. By comparison, in some OECD member countries all the different categories of data may be required encompassing rate and route of degradation and metabolite formation (e.g., EU 7.1.1 – 7.1.2.2.2).

322. Many tests can possibly be used. The need for extensive environmental fate data on dsRNA-based active ingredients may however be questioned. For instance, ubiquitous presence of nucleic acids in the environment[56], the existing scientific knowledge on the degradation of nucleic acids in various biological and environmental matrices, and the limited routes for potential hazard from exposure to dsRNA-based active ingredients, the need for extensive environmental fate data on dsRNA-based active ingredients may be questioned.

323. The following discussion points provide reasoned analysis to support the conclusion that laboratory soil degradation data should be sufficient for these active ingredients:

324. *Degradation rate (DT50 and/or DT90)*: Key data for the exposure assessment is the degradation rate. The potential period of biologically meaningful exposure is one of the most important components of risk assessments for any pesticide, and it is equally

[56] The ubiquitous detection of RNA in the environment can be attributed to its deposition from the earth's massive biosphere (see e.g., Hanna et al, 2015; Yinon et al, 2018).

important for dsRNA active ingredients. Since it is long dsRNAs that are taken up and processed by Dicer into 21-24 nt siRNA fragments, the enzymatic, microbial, or physiochemical degradation of a long dsRNA active ingredient into small fragments will prohibit entry of siRNAs into the RNAi complex. For example, studies have shown that for insects that are responsive to environmental RNA, a length of at least 60 bp is required for effective dsRNA. This was indicated in western corn rootworm (Bolognesi et al, 2012) but similar findings were also reported by Miller et al. (2012) with Tribolium castaneum. Further information are reported in Christiaens et al., 2018 (Section 5.3.3 Factors involved in oral RNAi efficiency). Furthermore, degradation of a long dsRNA will limit its ability to elicit a response in the innate immune system of non-target organisms.

325. Available literature has demonstrated that naked dsRNA has a very short half-life in the environment (see e.g., Section 6.2.1; Dubelman et al, 2014). Thus, data confirming the predicted rapid rate of degradation should be sufficient for the risk assessment, especially if no hazard potential has been demonstrated in available empirical tests on non-target organisms.

326. Even in cases in which the dsRNA active ingredient has been formulated to protect it from degradation, the dsRNA must be biologically available for processing by Dicer and loading into RISC complexes for it to be active in the RNAi pathway and efficacious against the target pest. Therefore, formulations that irreversibly bind dsRNA to protect it from degradation are unlikely to be utilised for crop protection purposes. That is, given that free dsRNA would be needed for pest control efficacy, formulations may be designed to slow degradation in the environment, but the same free form of dsRNA needed for pest control would also be subject to degradation in the soil. In the case of formulations designed to reduce degradation of the dsRNA active ingredient, parallel laboratory soil degradation studies can be performed to demonstrate the difference in degradation rates between the formulated and free dsRNA active ingredient – thus providing relevant data for the exposure assessment.

327. *Metabolites from unmodified dsRNA active ingredients are known*: For new conventional chemical pesticide active ingredients, data on the formation of soil metabolites are needed to elucidate the route of degradation as well as the metabolites formed. In many cases, there is insufficient information available to predict with any certainty the degradation pathway and the metabolites that are formed in soil. In contrast, an unmodified dsRNA-based active ingredient consists of polynucleotides, and there are no known biological or chemical transformation processes that could lead to the formation of unknown metabolites of toxicological concern.

328. Even in case in which the unmodified dsRNA active ingredient is formulated to protect it from degradation, it is highly unlikely that this would lead to unknown metabolites, for the same reason as described above (e.g., for a dsRNA to be active in the RNAi pathway, it must be biologically available for processing by Dicer and loading into RISC complexes).

329. *Route of degradation for dsRNA and other nucleic acids are known*: Nucleic acids like DNA and RNA are found throughout the living world and are ubiquitous in the environment; over the evolutionary time scale, this created a need for natural environments to degrade these moieties. For example, dsRNAs of 1-15 kb in length are ubiquitous in both the plant and fungal kingdoms (for reviews, see Dodds et al, 1984; Boccardo et al, 1987; Nuss & Koltin, 1990) and exposure to dsRNA in the environment can perhaps most effectively be demonstrated by the acknowledged history of the existence and ubiquitous presence of RNA-containing plant viruses. Plant viral RNA measured in the faeces of

humans (Rosario et al, 2009; Hamza et al, 2010) and many other vertebrates (two avian species; Rosario et al, 2009: multiple rodent species; Phan et al, 2011: bats; Li et al, 2010: domesticated pigs; Sachsenröder et al, 2012) indicate environmental exposure to plant viruses. RNA from plant viruses has been found in diverse aquatic environments including ditches and drainage canals, ponds, lakes, streams, creeks, rivers, and marine environments; RNA-containing viruses have been found in water particles suspended in air (i.e., clouds and fog) demonstrating ongoing aquatic as well as terrestrial exposures to RNA (Roossinck, 2012; Djikeng et al, 2009; Mehle & Ravnikar, 2012).

330. The inherent structure of RNAs makes these molecules markedly susceptible to degradation by nucleases found in the environment (Sambrook & Russell, 2001; Desai & Shankar, 2003). Ribonucleases (RNases) from microbial sources are likely to contribute to the environmental degradation of dsRNA, as has been shown to be the case for DNA degradation by DNases in the soil (e.g., Blum et al, 1997; Levy-Booth et al, 2007). In bacteria, RNAs are degraded by enzymic mechanisms that target RNA sites with different efficiencies, with accessibility of sites depending on several factors, including RNA secondary or tertiary structure and polyadenylation status. RNases can be divided into endoribonucleases that cleave the RNA internally and exoribonucleases that cleave the RNA from one of the ends. In *E. coli* alone, for example, there are >20 different RNases. RNase III is an endoribonuclease that is responsible for the cleavage of double-stranded RNA substrates (e.g., Arraiano et al, 2010). The conserved Eri1 family of RNases has been shown to degrade double-stranded silencing RNAs (Kennedy et al, 2004; Iida et al, 2006). Exogenously-applied dsRNAs are also likely to be degraded by nucleases in plants and by exposure to UV irradiation (Ramachandran & Chen, 2008; Simonet & Gantzer, 2006).

331. In considering existing testing guidelines, it is likely that some revisions of test guideline protocols will be needed for dsRNA-based products. For example:

- Laboratory studies and the lysimeter study for conventional chemical pesticides require the use of radiolabelled test material; this approach is not relevant for dsRNA-based products since it is likely that nucleotides would be incorporated by organisms present in soils, thus confounding the measurement of half-life etc. (Hungate et al, 2015; Lueders et al, 2006; Radajewski et al, 2006).
- Relatively harsh extraction methods normally utilised for conventional chemicals are not applicable for dsRNA-based products as they would be likely to destroy the test material prior to analysis; this is an important consideration since long segments of dsRNA are essentially negatively charged biopolymers that are likely to bind to soil particles (Draper, 2004; Pietramellara et al, 2009; Trevors, 1996); thus, attention would need to be paid to appropriate extraction methods prior to assay.

7.3. Hazard identification – identification of the characteristics of dsRNA pesticides which may cause adverse effects

332. With respect to toxicity testing, the current battery of non-target organism tests for assessment of potential hazard may be adequate[57] for the investigation of dsRNA-based products. However, not all of these tests may be required. As is the case in the existing environmental testing guidelines (see Section 7.2), it is likely that some revisions of test guideline protocols will be needed for dsRNA-based products. For example:

- *Study duration*: In general, when comparing dsRNA active ingredients with conventional chemical pesticides regarding the onset of efficacy (e.g., time from application to pest mortality), dsRNA active ingredients commonly take longer to demonstrate efficacy against the target pest (see Baum & Roberts, 2014; Zotti & Smagge, 2015; Zhang et al, 2015). For example, results of Zhang et al (2015) indicated that 120 hours were required to demonstrate mortality in the Colorado potato beetle after feeding on plants with targeted dsRNA constructs, whereas less than 24 hours were required for a conventional chemical pesticide, imidacloprid (Chen et al, 2014). Reasons for this difference could include the stoichiometric requirements of effective RNAi, time to access the cellular target, time for incorporation into the RNAi pathway (e.g., efficacy and effect relies on entry into the biochemical pathway of the exposed target and non-target organisms), and protein turnover; these factors will have an impact on the time of onset of the effect of the RNA. Given this difference, it is suggested that acute non-target study protocols be adapted by extending the evaluation period; this is discussed in Bachman et al (2016).
- *Oral/dietary exposure*: Given the existing literature demonstrating that effective RNAi in environmentally-responsive organism most commonly requires oral or dietary exposure, any empirical non-targeting organism testing should focus on this route of exposure and consider limiting the need for contact/non-dietary exposure testing. Section 6.5.1 of this document provides additional information on this aspect of the risk assessment.
- *Dose selection*: Given that adverse effects in the standard battery of surrogate organism testing may not be anticipated, consideration in initial non-target organism testing protocols should be given to utilising a single dose that allows for a safety factor (10x – 100x, depending on OECD member country regulations) over the estimated environmental exposure concentration. If needed, test protocols that employ multiple doses to elucidate a no-effect concentration could be conducted after the results of the single dose study are available (Bachman et al, 2016).
- *Dose quantification*: As some test protocols include specifications for the analytical method to be used for confirming dose levels achieved in the study, changes in the analytical methodology will be needed for the accurate measurement of dsRNA. Many of the methods described in Section 3.3 of the document may be appropriate for dose confirmation.

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[57] It is noted that discussion in Section 7.4.5 (‘Effects assessment’) and the assessment ‘schema’ (in Section 7.2.3) allow for flexibility in the need to conduct studies on the species in the standard test battery – and in the addition of species selected on the basis of their phylogenetic relationship to the target pest.

- *Degradation of dsRNA during the study*: Degradation of dsRNA in dosing material (e.g., diet) or in the testing matrix is likely to occur where nucleases or other environmental factors known to degrade RNA are present. For example, degradation of dsRNA would be expected in study protocols examining effects on earthworms and other soil-inhabiting organisms. The risk assessor should consider how to address this anticipated degradation; options include considering this degradation as an indication of a lack of any hazard potential due to the lack of exposure or, if possible, adapt the protocol to allow for multiple recharge dosing of the testing matrix to account for anticipated degradation. If this is done, it needs to be ascertained that doses do not reach exaggerated or unrealistic environmental levels (USEPA, 2016b).

333. To date, an ecological risk assessment of an *in vitro*-produced dsRNA, DvSnf7_968 RNA, designed to be active against Western Corn Rootworm, has been conducted; a battery of tests on non-target organisms included invertebrate predators, parasitoids, pollinators, soil biota, and aquatic and terrestrial vertebrate species (Bachman et al, 2016). Laboratory tests evaluated endpoints including survival, growth, development, and reproduction and were of a duration selected to adequately assess the potential for adverse effects. Taking into account the potential for RNA degradation in the different tests, all margins of exposure for NTOs were >10-fold the expected environmental concentrations of the dsRNA [as confirmed in the USEPA assessment and approval decision (USEPA, 2016b; USEPA, 2017; EFSA, 2018).

7.4. Effects assessment – identification of possible hazards arising from the use of exogenously-applied dsRNA pesticides

334. As for the exposure component of risk assessments, OECD member countries use different sets of data requirements to characterise the hazard profile of active ingredients in microbial and biochemical pesticides. The US EPA Biopesticides and Pollution Prevention Division has created a specific set of data requirements for microbial and biochemical pesticides. The European Union (EU) utilises one set of official data requirements for all chemical substances, and a second set of data requirements for microorganisms (see Section 7.2).

335. Ultimately, there may be some differences in regulatory requirements for hazard data for dsRNA-based products in different OECD member countries[58]. However, available hazard and risk assessment paradigms of toxicity testing for biopesticides[59] can be applied as a guide to the assessment of the environmental fate and potential adverse effects of dsRNA. Data requirements have been developed by regulatory agencies to determine the hazard to non-target organisms from exposure to active ingredients and formulated products.

336. For the purposes of this working paper the full list of likely regulatory data requirements for the US EPA and the EU is provided in order to illustrate the spectrum of

[58] Many jurisdictions are yet to finally determine how products based on environmental dsRNA are to be classified and regulated.

[59] The term 'biopesticides' covers a diverse group of compounds and is not defined in the same way between OECD members; testing and assessment may be 'tailored' to the nature of the organism or substance, not necessarily formally tiered.

non-target organism testing that may be needed to support risk assessments for dsRNA-based products in OECD member states (see Table 2).

Table 2: The range of non-target organism hazard testing data requirements that may be adopted for dsRNA-based products, based on current US and EU requirements.

US EPA (From Part 158, Subpart U Biochemicals[60])	EU Member States SECTION 8. Ecotoxicological studies (From Regulation EU 283/2013)
Note that these requirements may be fulfilled by use of a TGAI[61] or by a typical EP[62]. Tier I Avian Testing 850.2100 Avian acute oral toxicity 850.2200 Avian dietary toxicity Aquatic Organism Testing 850.1075 Fish acute toxicity, freshwater 850.1010 Aquatic invertebrate acute toxicity, freshwater Nontarget Plant Testing 850.4100 Terrestrial Plant Toxicity, Seedling emergence 850.4150 Terrestrial Plant Toxicity, Vegetative vigor Insect Testing 880.4350 Nontarget Insect Testing Note that EPA normally utilises the acute oral mammalian toxicology data to assess the potential hazard to wild mammals for biochemicals. Tier II Nontarget Plant 850.4225 Seedling emergence 850.4250 Vegetative vigor Tier III • Freshwater fish/invertebrate testing (TGAI) • Marine/Estuarine fish/invertebrate animal testing (TGAI) • Aquatic field fish/invertebrate testing (EP) • Terrestrial Wildlife o Avian Reproduction (TGAI)	Note that for many of these data requirements, the EU requires data from both the active ingredient and the end use product; end-use product data requirements are outlined in EU284/2013 – and reproduced in Appendix 5 (below). *SECTION 8. **Ecotoxicological studies*** Introduction 8.1. Effects on birds and other terrestrial vertebrates 8.1.1. Effects on birds 8.1.1.1. Acute oral toxicity to birds 8.1.1.2. Short-term dietary toxicity to birds 8.1.1.3. Sub-chronic and reproductive toxicity to birds 8.1.2. Effects on terrestrial vertebrates other than birds 8.1.2.1. Acute oral toxicity to mammals 8.1.2.2. Long-term and reproductive toxicity to mammals 8.1.3. Active substance bioconcentration in prey of birds and mammals 8.1.4. Effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians) 8.1.5. Endocrine disrupting properties 8.2. Effects on aquatic organisms 8.2.1. Acute toxicity to fish 8.2.2. Long-term and chronic toxicity to fish 8.2.2.1. Fish early life stage toxicity test 8.2.2.2. Fish full life cycle test 8.2.2.3. Bioconcentration in fish 8.2.3. Endocrine disrupting properties 8.2.4. Acute toxicity to aquatic invertebrates 8.2.4.1. Acute toxicity to *Daphnia magna* 8.2.4.2. Acute toxicity to an additional aquatic invertebrate species 8.2.5. Long-term and chronic toxicity to aquatic invertebrates 8.2.5.1. Reproductive and development toxicity to *Daphnia magna*

[60] See 40 CFR 158.2060(d); https://www.govregs.com/regulations/title40_chapterI_part158_subpartU_section158.2060 or https://www.ecfr.gov/cgi-bin/text-idx?SID=f87f49e73d82a2b1588981dc3b08dfac&mc=true&node=pt40.24.158&rgn=div5#se40.26.158_12060

[61] TGAI - Technical grade active ingredient; also TGAC (constituent) or TGAS (substance)

[62] EP– end-use product

◦ Wild mammal acute toxicity (TGAI) ◦ Terrestrial field testing (EP) • Beneficial Insects ◦ Field testing for Pollinators (TEP[63]) • Nontarget Plants ◦ Nontarget plant (TGAI)	8.2.5.2. Reproductive and development toxicity to an additional aquatic invertebrate species 8.2.5.3. Development and emergence in *Chironomus riparius* 8.2.5.4. Sediment dwelling organisms 8.2.6. Effects on algal growth 8.2.6.1. Effects on growth of green algae 8.2.6.2. Effects on growth of an additional algal species 8.2.7. Effects on aquatic macrophytes 8.2.8. Further testing on aquatic organisms 8.3. Effect on arthropods 8.3.1. Effects on bees 8.3.1.1. Acute toxicity to bees 8.3.1.1.1. Acute oral toxicity 8.3.1.1.2. Acute contact toxicity 8.3.1.2. Chronic toxicity to bees 8.3.1.3. Effects on honeybee development and other honeybee life stages 8.3.1.4. Sub-lethal effects 8.3.2. Effects on non-target arthropods other than bees 8.3.2.1. Effects on *Aphidius rhopalosiphi* 8.3.2.2. Effects on *Typhlodromus pyri* 8.4. Effects on non-target soil meso- and macrofauna 8.4.1. Earthworm — sub-lethal effects 8.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms) 8.4.2.1. Species level testing 8.5. Effects on soil nitrogen transformation 8.6. Effects on terrestrial non-target higher plants 8.6.1. Summary of screening data 8.6.2. Testing on non-target plants 8.7. Effects on other terrestrial organisms (flora and fauna) 8.8. Effects on biological methods for sewage treatment 8.9. Monitoring data

337. The left-hand side column of the table outlines the Tier-I and II non-target organism studies that must be addressed to support the registration of a new biochemical pesticide active ingredient in the USA; the test material is the TGAI but tests on the EP may be required if, for example, the formulation contains non-active ingredients that may be toxic to non-target organisms. These studies have generally been considered sufficient by the USEPA to inform non-target risk assessments for new biochemical pesticides to date. The studies primarilytest at the limit dose.

338. If required, more extensive toxicity testing of the TGAI or EP may be necessary. These tests (listed in Table 2) are included in Tier III of 40 CFR 158.2060(d); the grounds for extra testing are listed in the footnote 62.

[63] TEP = Typical end-use product

339. It should be noted that, depending on the proposed use of the product or from other considerations during the problem-formulation stage, it may be possible to waive individual data requirements or address issues by scientific argument.

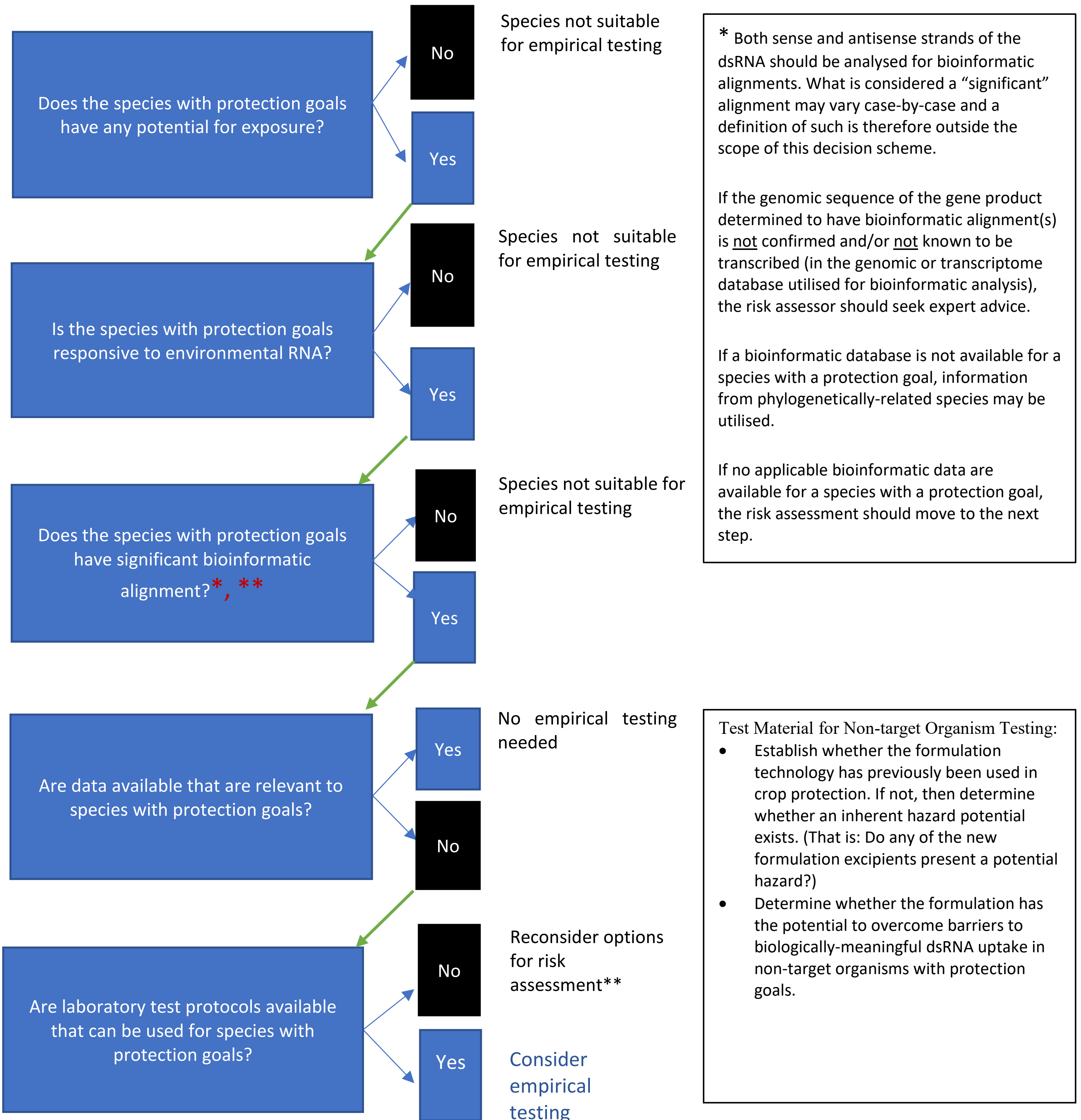
340. When considering what data or approaches from this testing spectrum are needed, there are hazard identification considerations that are particular to dsRNA-based products:

- *Potential for exposure*: A pre-requisite for a biological effect (including an off-target effect) is exposure. This will be determined by a range of factors, including the requested use pattern of the dsRNA-based product.
- *Responsiveness of non-target organisms*: Responsiveness to RNAi is a critically important factor and includes a consideration of barriers in organisms to environmental RNA
- *Bioinformatic analysis of sequence alignment*: This is a requirement for biologically meaningful interaction of a dsRNA active ingredient with its target.

341. As stated in preceding sections of this document, the key need from empirical hazard testing is to characterise adverse effects on the endpoints of survival, growth, development, and/or reproduction that are relevant to assessing the population-level impacts on surrogate species that are representative of non-target organisms. To date it has been demonstrated that laboratory tests of sufficient duration were acceptable for evaluating the potential unintended latent adverse effects of a dsRNA PIP product (USEPA, 2016b). Therefore, the goal of empirical testing for other dsRNA-based products would be to characterize the hazard for both acute and latent adverse effects and investigate/ quantify any adverse effects on survival, growth, development and/or reproduction in test systems in order to assess long-term biological consequences and characterise potential risk.

342. Working from these principles, the following schema is proposed for determining whether empirical testing of a specific test organism may be useful to inform the hazard identification phase of the risk assessment.

External dsRNA: Decision Tree for selecting NTOs for empirical testing



***Note: If the schema leads to the conclusion that no empirical testing is required for any non-target organism with protection goals, the risk assessor could still consider requiring a subset of normally-required terrestrial non-target organism and/or aquatic non-target organism data to confirm the lack of a hazard potential. If this approach is taken, the risk assessor should utilise information from the problem-formulation step (e.g. use pattern) and select a subset of representative non-target organisms that may potentially be exposed. For example, if registration of a foliar application of the dsRNA-based product is requested in the registration action and no empirical testing is required (according to this schema), the risk assessor may determine it is appropriate to request the following tests: non-target insect (e.g., honey bee acute oral), avian acute oral, freshwater aquatic invertebrate (e.g., Daphnia), and a fresh water fish acute (e.g., rainbow trout). If these studies do not demonstrate any potential for hazard, the regulatory review process can proceed to the next step.*

Test Material for Non-target Organism Testing

343. With respect to the impact of the formulation on the hazard potential of a dsRNA-based EP, existing regulatory approaches may be employed to consider whether testing should be done on the active ingredient, the EP, or on both. Iif a novel formulation technology or ingredient included in a ds-RNA based EP has no history of safe use in conventional chemical pesticides, consideration should be given to the following:

- Does the new technology or ingredient have any an inherent hazard potential? (That is, does the novel ingredient itself present a potential hazard?)
- Does the new technology or ingredient have the potential for overcoming barriers to biologically meaningful uptake of the dsRNA active ingredient in non-target organisms with protection goals?

344. If this analysis concludes there is a potential for hazard, then additional testing of the EP may be merited.

7.5. **Regulatory issues**

345. Toxicity testing generally follows internationally-harmonised test guidelines, thus enabling data transportability and mutual acceptance of data. However, for plants genetically-engineered to contain insecticidal properties (so-called plant incorporated protectants or PIPs), country- or region-specific ecological effects information is often required by regulatory agencies. That this has occurred is most likely primarily due to differences in national laws and approaches relating to the regulation of the genetic manipulation of plants.

346. International efforts, including by the OECD, are being focussed on the testing and regulation of novel pesticide products based on the mechanism of RNA interference, using dsRNA targeted against the pest species as the active constituent. Achieving harmonisation of test methods and regulatory approaches will help minimise differences in core data requirements, maximise efficiency of data development, and facilitate the interchange of data between countries/regions.

347. As noted in some detail in the foregoing sections, most of the existing test methods (environmental fate and toxicity) can be used for dsRNA-based products, modified as appropriate to take into account specific features of these products. It is only for specific RNAi-related effects that new tests and/or guidance may need to be developed or adopted from other areas of the life sciences (e.g., bioinformatic procedures for sequence matching and determining whether there is meaningful alignment of sequences in non-target organisms to the sequence(s) designed to act against the target organism).

7.5.1. *Data Considerations*

348. The diagram presented at Section 7.2.3 outlines a proposed scheme to help direct considerations of the need for empirical testing of non-target species in the environment.

349. Note: If the schema leads to the conclusion that no empirical testing is required for any non-target organism with protection goals, the risk assessor could still consider requesting a subset of normally required terrestrial and/or aquatic non-target organism data, in order to confirm the lack of any hazard potential. If this approach is taken, the risk assessor should utilise information from the problem-formulation step (e.g. use pattern) and select a subset of representative non-target organisms that may potentially be exposed. For example, if registration of a foliar application of the dsRNA-based product is requested and no empirical testing is required (according to the above schema), the risk assessor may determine it is appropriate to request the following tests: non-target insect (e.g., honey bee acute oral), avian acute oral, freshwater aquatic invertebrate (e.g., *Daphnia*), and a freshwater fish acute (e.g., rainbow trout). If these studies do not demonstrate any potential for hazard, the regulatory review process can proceed to the next step.

350. In considering what data are needed, there are two important points that merit repeating:

- The presence of barriers to RNAi: physical, enzymatic, and biochemical barriers exist in many non-target organisms that preclude biologically meaningful exposure to dsRNA.
- Lack of sequence complementarity: most of the potential impacts of dsRNA-based products require significant sequence similarity to elicit a hazardous effect (e.g., target and off-target gene silencing in non-target organisms). Although substantial sequence complementarity is needed to trigger off-target gene effects, data from mammalian cells transfected with high doses of siRNA containing some mismatches indicated the potential for some effective silencing (Jackson et al., 2006). Off-target gene silencing, if present, would most likely be observed in bioassays.

351. With these in mind, the following points address particular non-target organism testing guidelines:

- *Avian and mammalian testing* (US EPA 850.2100, 850.2200 and EU 8.1-8.1.5): As indicated in Section 5.2.5, there is to this date no evidence from the literature that birds or mammals are responsive to environmental dsRNA following *in vivo* dietary exposure, nor do nucleic acids bioaccumulate in these species. For example, the anticipated effect of barriers to oral exposure to exogenous dsRNA was confirmed in testing done for the Dvsnf7 dsRNA (see Sections 5.2.6 and 7.4.5). Based on the proposed steps in the schema, avian testing may not be warranted unless there is a significant sequence alignment with an avian species with a protection goal, as well as the potential for routine and repeated exposure of the species under consideration

to the dsRNA-based product. Furthermore, with respect to endocrine disrupting properties (EU 8.1.5.), the long history of ubiquitous exposure to exogenous nucleic acids, the existence of effective barriers to dsRNA uptake in organisms with endocrine systems, and the properties of nucleic acids, it is quite unlikely that dsRNA would have any direct impact on endocrine systems.

- *Aquatic testing* (USEPA 850.1075, 850.1010 and EU 8.2-8.2.8): As indicated in Section 5.2.5, there is no evidence from the literature that fish are responsive to environmental dsRNA following *in vivo* exposureSimilarly, effective RNAi in *Chironomus* species[64] has required soaking for long periods, injection, or special formulation (Klomp et al, 2015; Tang et al, 2017; Zhang et al, 2018).
- *Arthropod testing* (USEPA 880.4350 and EU 8.3 – 8.5): As summarised in Baum & Roberts (2014) and elsewhere in this document (see Section 5.6), different orders of insects and arachnids show a wide variation in their responsiveness to environmental RNA, with beetles being the most responsive arthropod order. Therefore, as indicated in the schema, if the problem formulation step indicates a potential for exposure of a beetle species that has a protection goal, bioinformatic analysis should be conducted to determine if there is significant alignment (e.g., > 21 bp with the transcriptome sequence, or if a transcriptome is not available, a 21 bp transcribed genomic sequence) with the dsRNA active ingredient. Also, considering the societal and regulatory importance given to pollinators in agriculture and the desire to apply a high degree of caution, empirical acute oral testing data in honey bees may be appropriate in order to confirm the lack of any hazard associated with a dsRNA active ingredient
- *Non-target plant testing* (US EPA 850.4100, 850.4150, 850.4225, 850.4250, and EU 8.6-8.6.2): As discussed in this document, plants have effective barriers to the biologically-meaningful uptake of unmodified exogenously applied dsRNA (see Section 5.6, 6.2.2 & 6.4) and empirical testing of non-target plants is unlikely to be warranted, except when weed control is the purpose of the product or if the dsRNA is formulated or modified in any way. RNAi-based weed control products must be designed to bypass barriers in the target weed and therefore non-target plant testing would be required.

352. Notwithstanding the foregoing comments about barriers to RNAi and their effect on the extent of testing to be undertaken, if an end-use product formulation being developed is demonstrated to bypass these barriers in the target pest, then there needs to be additional consideration of non-target organism testing e.g., the development of an herbicide product that bypasses target weed plant barriers should include non-target plant testing.

7.5.2. *Testing - test species selection; relevant measurement endpoints; test protocols*

353. *Test species selection*: testing to assess the toxicity of conventional chemical pesticides to non-target organisms (NTOs) is routinely carried out using representative surrogate species. Because not all NTOs present in the environment where a pesticide is applied (or a GM plant is grown) can feasibly be tested in an environmental risk assessment

[64] *Chironomus* is a genus of non-biting midges; the larvae of several species inhabit deep zones of lakes and ponds, below the level of effective light penetration.

(ERA), it has been the practice of regulatory agencies to select a representative subset of surrogate[65] species. For laboratory and semi-field studies, test organisms from representative taxa are typically chosen based on the likelihood of exposure of (an) organism(s) in that taxa to the applied pesticide (= relevance) as well as the ability to dependably test the organism in the laboratory (= assay reliability).

Considerations under EU framework

On the basis that the general risk assessment framework for GM crops incorporating genes for plant protectants such as Bacillus thuringiensis (Bt) toxins is a good starting point for assessing RNAi-based technologies (see e.g., Velez et al, 2016), it is appropriate to consider the approach to test species selection for assessing the safety of GMO crops in the EU. For the selection of surrogate species in the EU, for example, the European Food Safety Authority (EFSA) utilises a 4-step approach (EFSA, 2010). Functional groups of non-target organisms [e.g. herbivores, pollinators, predators of the target pest(s), decomposers] relevant to ERA are defined. Subsequently (step 2), non-target species occurring in the receiving environment of the pesticide/GM plant are categorised within the relevant functional groups. If necessary, endangered species are also listed. A first prioritisation of possible species (step 3) is based on ecological criteria e.g., exposure of the species to the pesticide/GM plant, abundance of the species, feeding habits, and information about likely sensitivity to the pesticide/GM plant. When finally choosing the most appropriate species for testing (step 4), practical criteria are considered e.g., availability of adequate numbers of the species for testing and the practicability of conducting the proposed test(s) on that species (viz, ecotoxicological criteria). This approach results in the selection of testable species belonging to relevant functional groups in the receiving environment. At least one surrogate species per relevant functional group is required for testing (De Schrijver, 2013).

However, in the EU, a set of test organisms is defined by Regulations EC 283/2013 (authorisation of active substances) and EC 284/2013 (plant-protection products); Part A of both sets of regulations deals with chemicals while part B deals with microorganisms. As outlined above, most of the described test systems are designed to test chemical substances. The EU is yet to determine which of these test systems are appropriate to assess dsRNA-based pesticides. While specific regulations are yet to be adopted for RNAi-based products, European authorities might apply an interim risk assessment approach for a transition period, while any new regulations are developed. Therefore, the approach described above (De Schrijver, 2013; EFSA, 2010) might be helpful. In selecting non-target species on which to conduct toxicity testing as part of the ERA for a dsRNA-based pesticide, similar consideration as outlined above may apply. However, as cf a conventional pesticide or many plant-incorporated protectants, dsRNA-based products rely, to a very significant degree, on sequence identity between siRNA (formed intracellularly from the dsRNA) and a target mRNA in the target pest organism; this means that, with the available (and ever-

[65] Called 'focal species' by EFSA. Focal species have been defined by EFSA as "real species that represent others in a crop resulting from their potential higher level of exposure to pesticides" (Dietzen et al, 2014). Dietzen C, Edwards PJ, Wolf C, Ludwigs J-D & Luttik R (2014) Focal species of birds in European crops for higher tier pesticide risk assessment. Integr Environ Assess Manag 10: 247–259. DOI: 10.1002/ieam.1487.

developing) database of genome sequences from non-target organisms, bioinformatics information will provide an additional and potentially quite powerful tool to better inform the selection of surrogate species for tiered toxicology testing to inform the ecological assessment (see subsection 'Bioinformatics in the selection and design of dsRNAs' in Section 7.4.4).

Uncertainties in ERAs associated with the use of a limited number of surrogate species can be addressed in part by requiring sufficient margins of safety between conservative estimates of environmental exposure and measurement endpoints [no observed adverse effect concentration/level (NOAEC/NOAEL) or LCx value]. Safety factors allow for extrapolation among related species, inter-assay variation, laboratory to field extrapolations, and uncertainties associated with routes and levels of exposure (Klaassen, 2008). However, considering the unique mode of action of dsRNA-based pesticide products (reliance on sequence similarity), a 'sufficient' margin of safety may not cover the uncertainties arising from testing on a limited number of surrogates. The focus should be on ensuring that closely-related species are included among the surrogates selected for testing, and that a sufficient range of endpoints are examined, to account for off-target effects.

A 2012 scientific review reported that, for most arable crops, the taxa identified in the major beneficial insect groupings were comparable across the geographies in the EU (Meissle et al, 2012; Riedel et al, 2015). A similar conclusion was reached in an assessment of maize crops; the major beneficial insects were similar across geographies. Thus, for most arable crops, groupings of beneficial non-target organisms identified in a particular geography are likely to be quite similar across geographies. This finding has helped to limit the range of appropriate and representative indicator organisms needing to be tested across different ERAs.

354. *Relevant measurement endpoints*: Established test protocols that assess mortality, growth, and reproduction endpoints should be adequate to inform ecological/environmental risk assessment for dsRNA-based products; whether an observed effect is due to silencing of gene expression, immune stimulation, or saturation of RNAi machinery is not directly relevant to hazard identification. The potential hazard arising from these and other potential interactions should be accounted for in the risk characterisation phase of the risk assessment. This information can be utilised to determine empirical non-target organism testing to identify biologically meaningful measures of hazard viz. mortality, growth, and reproduction endpoints.

355. *Test protocols*: In the same way that conventional testing on a representative range of non-target organisms was applied to the testing of plant incorporated pesticides (taking into account additional considerations, such as the presence of transgenic DNA), it seems reasonable to apply a similar approach to the testing of dsRNA-based pesticides. For this novel technology, there are no issues of concern with respect to any in-planta formation of transgenic RNAs. While the possibility of off-target effects - including gene silencing - of RNA sequences designed to target a specific mRNA in the target organism, is an additional and specific consideration as *cf.* conventional chemical pesticides, the potential down-regulation of a protein that does not result in mortality or impact growth and/or reproduction is not directly relevant to the role of the test protocols viz. identification of

potential hazards. That is, the key issue in testing is to identify potential hazards, not the route or biochemical mechanism underlying any hazards that might be identified.

356. Testing shall provide: (1) valuable information on taxonomic specificity, which can significantly reduce the extent of testing required if biological activity is shown to have high taxonomic specificity; *and* (2) a conservative screen to identify potential adverse effects on susceptible organisms or beneficials such as pest insect predators, plant pollinators, decomposers in the pesticide application area.

357. To evaluate the predictive and protective capability of a battery of tests on non-target organisms, a retrospective meta-analytical validation of the tiered approach for plant-incorporated protectants was carried out by Duan et al (2010); they concluded that Tier-1 laboratory studies were appropriate to evaluate the environmental safety of crops engineered to contain the *Bacillus thuringiensis* (Bt) toxin. This conclusion is consistent with the field work of Rauschen et al (2010), which suggested that potential effects of *Bt* maize varieties on key predators of pest insects (e.g., Coccinellids) would be most reliably assessed in low-tiered laboratory studies because of the high level of natural variability in insect densities observed in field situations. It is likely that a similar conclusion pertains to the application of a similar test battery when examining the responses of non-target organisms to dsRNA-based pesticides.

358. The test battery needed for hazard characterisation should consider:-

- existing molecular biological knowledge of RNAi mechanisms in the target organism(s) and relevant non-target organisms;
- bioinformatics; and
- estimates of systemic exposure (based on amount of product applied, environmental stability of the TGAI, and physiological and biochemical barriers in the surrogate species selected for testing, and any formulation of the end-use product that facilitates the bypassing of physiological and/or biochemical barriers to biologically-meaningful exposure to dsRNA).

7.5.3. *Possible tools/approaches for aiding selection of relevant species for investigating possible effects on NTOs*

359. To address concerns for effects on non-target species and to potentially reduce the requirements for *in vivo* toxicology testing, the following three areas need to be considered in some detail:

- the mode-of-action of the dsRNA, including its degree of specificity for the target gene;
- the specificity of the dsRNA for the target organism, adopting a hypothesis-based taxonomic approach and utilising bioinformatics tools and available genomic databases; and
- relevant routes and levels of exposure of non-target organisms.

360. With this information to hand, a relevant testing and assessment strategy can be prepared that should address potential risks of an RNAi-based product to non-target organisms.

361. Gebert & Rosenkranz (2015) commented that "though the principles of RNAi pathways are essentially the same in all eukaryotic organisms, remarkable differences can be observed even in closely-related species, reflecting the astonishing plasticity and diversity of these pathways".

362. It is not reasonable to expect that scientific risk assessors in regulatory agencies would have a detailed knowledge and understanding of RNAi systems across a diverse range of phyla. This diversity of RNAi mechanisms and the complex molecular biology of interference pathways means that regulators will need to work cooperatively with relevant experts (including academic and industry-based researchers, regulatory affairs professionals in industry, and government scientists involved in gene technology research and/or assessment) in developing appropriate approaches to the risk assessment of novel dsRNA-based products. In recognition of the fact that utilising RNA interference may add an extra layer of complexity as *cf* 'conventional' chemical pesticides, the expertise of the industry and/or academic researchers involved in the design and development of the dsRNA active constituent (and any modifying product excipient which modifies its activity/stability), as well as independent researchers in the RNAi field, must be drawn upon.

7.5.4. *Bioinformatics*

363. Bioinformatics can play a role in (1) the selection and design of dsRNAs; (2) informing the selection of non-target organism test species for toxicology testing in dsRNA product development; and (3) informing the subsequent risk assessment process for non-target organisms.

364. Bioinformatics has been proposed as a tool for predicting the potential for off-target binding from exposure to RNA derived from dsRNA-based products. In like manner, it may be used as a tool to exclude the possibility of off-target binding. Critical to the application of sequence analysis in assessing the likelihood of off-target effects of dsRNA products is an understanding of the degree of sequence similarity necessary for effective RNA-mediated gene suppression[66], as well as an understanding of a number of structural and biological factors which can affect binding of small RNAs to mRNA. Note that bioinformatic prediction of a potential binding site for a dsRNA does not necessarily mean that a hazard will result i.e., a bioinformatics assessment is not a hazard assessment.

365. As described in the literature review of baseline information on RNAi-based GM plants (Paces et al, EFSA supporting publication 2017), assuming that there is relevant exposure to the RNA target site, the degree and position of base-pairing between the small RNA and RNA transcript is the primary factor determining the efficiency of silencing (Liu et al, 2014). Therefore, in silico target prediction algorithms are designed based on the binding energy and base-pairing among other specific filtering parameters (Pasquinelli, 2012, Rhoades et al. 2002). In addition, other factors can influence the potential for this interaction to result in a silencing effect. Among them, the most important is the abundance of each small RNA produced (i.e. lower off target potential when (heterogeneous) siRNA pools are generated by a longer dsRNA, compared to a homogeneous miRNA- pool

[66] For example, as pointed out in Section 5.2.4, there is a broad general distinction between plants and animals in the targeting of endogenously-produced miRNAs; plant miRNAs are usually perfectly or nearly perfectly complementary to their target genes and induce direct mRNA cleavage of the target mRNA transcript by RISC, while animal miRNAs tend to be more divergent in their sequence recognition.

generated by miRNA hairpins. Off-target effects bioinformatics analysis to predict off-target effects is required in EFSA GMO applications for RNAi-based plants (Annex II of the minutes of the 118th GMO plenary meeting (https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf), EFSA GMO Panel, 2018).

366. Thus, sequence complementarity alone is not informative for hazard identification or risk assessment, since *in silico* identification of matches to a putative target is not sufficient to determine the potential for gene suppression or potential for downstream effects (even assuming that a given small RNA was able to reach a target in sufficient quantities to mediate an RNAi effect).

367. The analysis by Vaishnaw et al (2010) of the use of RNAi in human therapeutics generally indicated that, despite high degrees of off-target complementarity for a number of lead molecules, *in vitro* potency for on- and off-target effects was clearly distinguishable, with IC50s separated by several orders of magnitude. They concluded that hybridization-dependent off-target gene suppression was unlikely to be a significant concern for *in vivo* safety in preclinical studies; when this was considered together with low exposures to small RNAs, they concluded that the likelihood for any toxicologically relevant off-target gene suppression effects in humans or animals due to applications of RNAi was likely to be very low.

368. Vaishnaw and colleagues noted that unformulated siRNAs have generally been well tolerated in preclinical safety studies at intravenous doses >100 mg/kg, suggesting that potential changes in transcriptional profiles (e.g., target or off-target gene suppression) do not appear to occur with unformulated RNAs nor impact their safety *in vivo* (Vaishnaw et al, 2010). This was underscored in studies by Thompson et al (2012) in which intravenous doses of up to 200 mg/kg of a stabilised RNA[67] targeting an endogenous gene was administered to rats without adverse effects.

369. *Bioinformatics in the selection and design of dsRNAs*: The sequence-specific nature of RNAi allows genomics and transcriptomics data and target gene sequence conservation across taxa to help inform the design and potential taxonomic activity spectrum of the guide strand of a proposed dsRNA-based product (Swevers & Smagghe, 2012). In a 2010 'status report' on the use of RNAi in human therapeutics, Vaishnaw et al (2010) stated that bioinformatics plays a "crucial role" in minimising the potential for off-target binding by helping to avoid the selection of guide strand sequences that have significant off-target complementarity; they suggested that it in most cases it was possible to avoid sequences of interest that have high off-target potential.As previously discussed, there are additional barriers to achieving an RNAi-mediated effect in insects and consequently not all species are responsive to ingested dsRNA or may not be responsive at environmentally relevant exposure concentrations. The lack of any significant response of honey bees (*Apis mellifera*) to *vATPase subunit A* dsRNA from either *Diabrotica virgifera virgifera* or *A. mellifera* is a clear illustration that the activity spectrum of dsRNA not only depends on sequence identity to the target gene, but also on the inherent ability of the organism to respond to orally ingested dsRNA (Vélez et al, 2016). Similarly, the monarch butterfly (*Danaus plexippus* (L.)) was shown not to be responsive to dietary *vATPase subunit A* dsRNA from either *Diabrotica virgifera* or *D. plexippus* (L.) (Pan et al, 2017). Because of these barriers, bioinformatics data indicating that minimum sequence requirements for

[67] Partially protected from nuclease degradation using a methoxy modification on the 2' position of the ribose sugar; this modification occurs naturally in mammalian cells.

RNAi activity are met can provide supplemental information to the results from insect bioassays but cannot be reliably used as a stand-alone to predict the presence of RNAi activity.

370. *Bioinformatics in the selection of non-target organism test species:* Bioinformatics can assist in developing a taxonomic approach for characterising the spectrum of activity for an RNAi-based pest-control agent under development, and in helping to understand the relationship between taxonomic relatedness of target and non-target species and the activity of the RNAi product. Thus, bioinformatics can aid in the selection of relevant test species for non-target organism testing using appropriate empirical bioassays.

371. Conversely, if bioinformatic data for relevant[68] non-target species are available which indicate that the minimum sequence similarity requirements for RNAi activity are not met, the likelihood of adverse effects would be low. This conclusion does not take into account 'non-canonical' effects of ingested RNA, including innate immune system stimulation, or saturation of the native RNAi machinery; such effects are possible but, to the extent they might occur, would appear to require very high levels of exposure. A proposed empirical testing schema addresses the issue of these non-sequence specific effects – see Section 7.2.

372. With regard to investigating the possible effects of a proposed dsRNA-based product on non-target insects, it may be noted that, at the time this document was prepared, only a limited number of complete and partial insect genomes and transcriptomes had been published (see Bachman et al, 2013). However, that number is constantly increasing and web-based tools are being developed to search the database of arthropod transcriptomes so that pest-specific RNAi sequences can be identified. One such is 'OfftargetFinder' (Good et al, 2016); the output demarks sequences that should be avoided in RNAi designs to ensure taxonomically-limited RNAi pesticides.

373. Large-scale comparison of genomic and transcriptomic sequences requires reliable tools to search for local alignments. Several computer-based applications have been available for some time to find local alignments between sequences; examples of such programs are BLAST (Basic Local Alignment Search Tool) (Altschul et al, 1990) and STELLAR (SwifT Exact LocaL AligneR) (Kehr et al, 2011).

374. Tools such as these will help technology developers to design RNAi sequences with appropriate target selectivity, as well as aid in the selection of those non-target species which, on the basis of sequence overlap or gene similarity, should be assessed in the risk assessment process.

375. *The use of bioinformatics to help inform the risk assessment process*: Target gene sequence conservation across taxa and the sequence-specific nature of RNAi means that the use of genomics and transcriptomics data can help inform the design and potential taxonomic activity spectrum of a proposed dsRNA-based product (Swevers & Smagghe 2012). Therefore, uncertainty in the environmental risk assessment of a dsRNA product should be able to be reduced by using bioinformatics to develop a hypothesis-based taxonomic approach for characterising the spectrum of activity of the product, and to understand the relationship between taxonomic relatedness and the activity of the product's active constituent.

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[68] Species that are reasonably likely to be exposed to the dsRNA product being considered, based on its use and use-pattern.

376. In 2002 the USEPA issued an Interim Policy on Genomics which stated that genomics data alone were insufficient as a basis for risk assessment and management decisions (Dix et al, 2006). A subsequent USEPA white paper titled *Potential Implications of Genomics for Regulatory and Risk Assessment Applications at EPA* (EPA 2004) indicated that criteria needed to be developed to accept genomics data for use in a risk assessment. In various guidance documents for agency-wide use, the USEPA has identified these criteria, but they remain to be fully developed. To date, the USEPA had not published anything further on how to utilise genomics in the regulatory process and the 'interim' approach is still in place. It may be noted that the USEPA Biopesticides and Pollution Prevention Division (BPPD) considered and utilised the bioinformatics analysis that was submitted by Monsanto for approval of DvSnf7 dsRNA expressed in GM corn (USEPA, 2016a; 2016b; 2017). Canadian regulatory bodies also reviewed the same bioinformatics analysis submitted for DvSnf7.

7.5.5. *Tiered risk assessment*

377. Pesticide regulatory agencies use a tiered-testing framework for ecological assessments, in order to make scientifically-sound regulatory decisions in a time- and resource-efficient manner. The tiered approach organises testing and assessment in such a way that unnecessary testing, more comprehensive lines of investigation, and assessment/evaluation tasks are avoided or minimised. The tiered testing framework is structured to initially consider unrealistic 'worst-case' scenarios and if these high exposure levels (generally unrealistic or unlikely) are assessed as not presenting any environmental hazard, then further testing and refinement of the risk assessment is not necessary. If tier-1 (screening level) testing and assessment fail to indicate adequate certainty of acceptable risk (i.e., unacceptable effects are probable or possible), then further testing must be carried out. Higher-tier studies encompass definitive hazard level determinations, and longer-term testing under actual conditions of use (e.g., greenhouse or field testing).

378. The tiered approach to testing and assessment has helped to minimise data needs and requirements and helped avoid unnecessary testing of conventional (chemical) pesticides, biological pesticides, microbial pesticides, biochemical pesticides and plant-incorporated protectants (PIPs). The approach has enabled scientifically-sound regulatory decision-making, with the goal of adequate certainty of acceptable risk i.e., no unreasonable adverse effects on the environment.

379. For pesticide products based on the application of exogenously-applied dsRNAs, an approach based on the current framework for tiered testing and assessment, in practice, could be sufficient to address potential adverse effects on non-target organisms. However, in the risk characterisation phase of the risk assessment, attention will need to be paid to potential effects specific to dsRNA-based products, including those caused by both target and off-target gene silencing in non-target organisms, immune stimulation, and saturation of the RNAi machinery in cells. As previously noted, the identification of hazard potential (impacts on mortality, growth, and reproduction) is the goal of empirical testing and the focus of test protocols should be on measuring the dose:hazard response.

380. Tier I studies can generate statistically-reliable results by testing a sufficiently large number of organisms at a limit dose, which represents a 'worst-case' exposure (e.g., 10 – 100 times the anticipated field exposure depending on OECD member state regulations). A lack of adverse effects at the limit dose can allow regulators to conclude that there is no unacceptable risk to the organism being tested, and thus conclude that no further test data

are required. If similar negative results are obtained in empirical tests against all organisms in the environment reasonably assessed as likely to be exposed to the product when used as proposed, then the conclusion can be made that the product does not present an environmental hazard. However, if screening studies, generally conducted in a laboratory setting, indicate potentially unacceptable risk, then additional studies can be conducted under more realistic field conditions. This approach is just as applicable to dsRNA-based pesticide products as it is to other types of pest-control agents.

381. In designing and conducting toxicology tests on proposed dsRNA-based pesticide products, the route of exposure needs to be considered. There have been some reports of dermal absorption of dsRNA *via* the integument in insects and mites, however successful RNAi often requires transfection agents or other chemical enhancers to facilitate absorption of the dsRNA into the test organism (Whyard et al, 2009; Campbell et al, 2010, Wang et al, 2011). Because of the potential for rapid degradation of the dsRNA active constituent and the uncertainties regarding dermal or integument absorption in non-target organisms being tested, dietary bioassays, as employed in the existing tiered hazard testing approach, are likely to present worst-case exposure scenarios for evaluating the potential adverse off-target effects of dsRNA-based products.

382. Characterisation of the spectrum of activity (e.g. based on bioinformatics and activity bioassays) of a proposed dsRNA-based product can provide critical information to help shape a testing program for non-target organisms[69]. If a biopesticide has a narrow spectrum of activity the scope of hazard testing may be narrowed to focus on organisms that are taxonomically-related to the target organism, are closely associated with the target organism (e.g., natural enemies), or provide important environmental functions (e.g. pollinators) (Romeis et al, 2013, Levine & Brown, 2013).

383. A knowledge of the likely spectrum of pesticidal activity of a proposed dsRNA-based pesticide product will help regulators to better understand the potential hazards to non-target organisms of the exogenously-applied dsRNA by indicating which species, taxonomically-related to the target, need to be considered for testing. If species-specificity is adequately characterised, this information may be used to reasonably indicate a lack of toxicity to some non-target organisms and thus support a waiver of some empirical testing of some non-target organisms, despite the possibility of their being exposed following product application. Thus, an understanding of the data on the spectrum of activity of the proposed dsRNA-based product data can both alleviate concerns about some non-target organisms in the environment and indicate areas of focus for the risk assessment.

384. An example of the use of this taxonomic approach has been outlined by Bachman et al. (2013); to evaluate a dsRNA-based product for insect control, Bachman and her research colleagues followed a hypothesis-based taxonomic approach for activity spectrum testing to establish the relationship between taxonomic relatedness to the target pest and activity. In order to characterise the spectrum of insecticidal activity of a dsRNA with activity against Western Corn Rootworm (WCR; *Diabrotica virgifera* LeConte) non-target species for testing were selected on the basis of phylogenetic relatedness to WCR, relevance of the organism as a surrogate for beneficial insects (e.g., predator, parasitoid), the availability of published genome sequence data, and the ability to conduct laboratory tests on the organism with sub-chronic or chronic dietary exposure.

[69] For example, Syngenta has made their data on the spectrum of activity of their putative insecticidal dsRNAs available to the public at http://opendata.syngenta.agroknow.com/rna. Note that these data have not been peer-reviewed.

385. Since the WCR belongs to the order Coleoptera, 5 species from this order were assayed. In addition, 4 species from the order Lepidoptera, 2 species from the order Hymenoptera[70], and one species from the order Hemiptera were assayed; an insect from the order Diptera was not examined since a Dipteran species (*Drosophila* spp.) had previously been shown not to be responsive to dietary dsRNA, with insecticidal activity only achieved by soaking larvae in solutions of dsRNA encapsulated within cationic liposomes (Whyard et al, 2009).

386. To assess risk to the environment from PIPs, the USEPA requires toxicity data/information on non-target organisms, generally consisting of testing with birds; mammals; freshwater and marine/estuarine fish and invertebrates; non-target insects, including honey bees; non-target plants; and soil invertebrates. Other data are also considered, including the environmental persistence of the PIP (relevant to products based on environmental dsRNA), the potential for gene flow (not relevant) and the development of weediness (not relevant).

387. The USEPA published a proposed registration decision (later finalised) for the commercial use of a Monsanto-developed GM corn MON 87411 expressing a dsRNA targeting the mRNA of a key gene (*DvSnf7*) in the Western Corn Rootworm (USEPA, 2017), while EFSA has conducted a risk assessment for this GM event only for food and feed uses, import and processing (EFSA, 2018). DvSnf7 dsRNA is a plant-incorporated protectant (PIP), and the USEPA environmental risk assessment (the first for a PIP product incorporating an RNAi mechanism to protect against insect pests) presents a reasonable framework for future environmental assessments of pesticide products based on environmental dsRNA: this takes into account the fact that a significant amount of testing for approval of this GM corn was done on free *DvSnf7* dsRNA, not just on the GM plant or plant extracts. Thus, the key elements[71] of the data and arguments considered by the regulator are outlined as follows:

- Environmental persistence: The terrestrial and aquatic fate of the PIP was assessed, based on degradation studies with synthesised *Dvsnf7* dsRNA.
- Non-target organisms
    - Birds
        - 6-week dietary study with broiler chickens fed diet containing 57% grain from the GM corn (MON 87411). (Note: This study is required only for GM crops and would be unlikely to be applicable to the assessment of topically applied dsRNA-based products.)
        - 14-day dietary toxicity study with Northern bobwhite quail with 1.0 mg *DvSnf7* RNA/kg diet.
        - Bioinformatic analyses of the genomes of red junglefowl, rock pigeon and mallard duck.

[70] Tests on honey bees (*Apis mellifera*) are routinely required by regulatory agencies and thus were not included in the activity spectrum assessment.

[71] Note that this particular risk assessment did not rely solely on toxicology data, bioinformatics, and exposure arguments; where there were uncertainties, the risk conclusions drew upon multiple lines of evidence.

- Mammals
  - 28-day gavage toxicity study in mice with *DvSnf7* RNA (up to 105 mg/kg bw/day). (Note: For testing of topically applied dsRNA, the rat would also be an appropriate test species.)
  - 90-day toxicity study in rats fed a diet of 33% (w/w) of grain from MON 87411 corn. (Note: This study is required only for GM crops and would be unlikely to be applicable to the assessment of topically applied dsRNA-based products.)
  - Bioinformatic analyses of the genomes of beef cattle, domestic dog, horse, house mouse, Norway rat, and pig.

- Freshwater invertebrates
  - Argument: Expected aquatic concentrations of *DvSnf7* RNA are far below (2 to 3 orders of magnitude) the dietary LC50s for target insects which are presumably the most sensitive.

- Freshwater fish
  - Channel catfish fed a diet of 30% MON 87411 grain. (Note: This study is required only for GM crops and would be unlikely to be applicable to the assessment of topically applied dsRNA-based products.)
  - Bioinformatic analyses of the genomes of Zebra fish and Medaka
  - Argument: The argument presented for freshwater invertebrates (see above) also applied to the assessment for freshwater fish.
  - Argument: Barriers to uptake by vertebrate organisms was also a line of evidence used to draw risk conclusions.

- Marine and estuarine fish and invertebrates
  - Argument: As for freshwater invertebrates (above).
  - Argument: Barriers to uptake by vertebrate organisms was also a line of evidence used to draw risk conclusions.

- Non-target plants
  - Argument: *DvSnf7* RNA not expected to be present at high levels or to persist in soils (based on soil degradation studies with *DvSnf7* RNA), so that significant plant uptake would not be expected.

- Non-target insects
  - 5 species from the order Coleoptera, 4 species from the order Lepidoptera, 2 species from the order Hymenopter, and one species from the order Hemiptera were assayed by Monsanto
  - The USEPA also considered several other studies examining *DvSnf7* RNA activity on 8 other insect species across 6 different orders.

- Bees
  - Testing for effects in larvae and adult honey bees (using *DvSnf7* RNA)
  - Bioinformatic analyses of the genomes of honey bee and bumble bee

- Other invertebrates
  - Argument: Based on the specificity of the effect of *DvSnf7* RNA and a lack of adverse effects on a range of non-target arthropod species.
  - Argument: 'Other invertebrates' in this particular case were primarily soil invertebrates - the low expected levels of the *DvSnf7* RNA and rapid breakdown in soil were also factors in the risk determination. [Note: Studies were submitted but were not necessarily used in the assessment.]

388. Thus, technical arguments prepared by the applicant dealt with a range of issues and concerns specific for product acting *via* an RNAi mechanism; these arguments drew on a sound knowledge of the mechanism of action and specificity of the *DvSnf7* dsRNA, knowledge of likely environmental concentrations, and on bioinformatic information. The regulator noted that the empirical data on the effects of *DvSnf7* dsRNA on non-target species included testing across a wide range of taxa, with additional and more intensive focus on species most likely to be impacted (viz. insects and other arthropods). Testing had been performed at high concentrations, with continuous dietary exposure over many days and with study durations that were considered sufficient to allow observation of adverse effects. For dvSnf7 dsRNA in corn, known barriers to uptake in mammals were one of several lines of evidence used to support a conclusion of low risk to non-target vertebrates. Therefore, the EPA concluded that unintended effects, if they occurred, were unlikely to be of biological significance for *DvSnf7* dsRNA (as expressed in the GM corn).

8. Uncertainties

389. There are many uncertainties with respect to our current understanding of RNAi technology and its application in pest control. Nevertheless, ongoing basic research into the molecular biological mechanisms of RNAi, applied clinical research into the potential therapeutic applications of RNAi, and the experience of regulatory agencies in dealing with engineered plants incorporating RNAi constructs – e.g. for expression of so-called plant-incorporated protectants, or PIPs – provide a sound basis for developing a rational approach to the assessment of pest control products containing dsRNA as the active ingredient. As is the current practice for pesticide risk assessment, the application of safety factors to address uncertainties should be employed for dsRNA-based products.

390. Two particular areas of uncertainty relate to the estimation of environmental exposure and the determination of possible effects in non-target organisms, the two major components of an ecological risk assessment.

8.1. Uncertainties in exposure estimates

391. Uncertainty can be reduced by determining the most likely exposure routes and developing realistic estimates of expected environmental concentrations over time after application. The more accurate these estimates are, the more accurate will be the estimates of the likelihood and extent of exposure of non-target organisms in the environment.

8.1.1. *Stability of dsRNA active constituent*

392. While the stability of the 'technical grade' dsRNA active constituent can be determined under laboratory conditions, what is likely to be less certain is its stability in the environment in its formulated state. This is an issue which needs to be addressed during product development and adequately considered by regulatory risk assessors. Note that this is no different from the situation with conventional pesticides i.e., environmental fate data have to be provided.

8.1.2. *RNAi signal amplification*

393. If an organism has the ability to amplify an RNAi signal, this could affect the usefulness of an environmental exposure estimate, since an exposure determined to be 'low' may then not translate to the same exposure within the organism. However, if physiological and biochemical barriers exist to prevent systemic uptake of environmental dsRNA in a range of organisms, then whether amplification occurred in e.g., the crop plant or not, would not be relevant to the risk assessment for those organisms.

394. The effectiveness of RNAi in many organisms is potentiated through the signal-amplifying activity of an RNA-directed RNA polymerase (RdRP) system that can convert a small population of exogenously-encountered dsRNA fragments into an abundant internal pool of small interfering RNA (siRNA). Pak et al (2012) investigated this system since, as for other biological amplification systems, they expected there would be other mechanisms to limit the ability of a randomly-encountered trigger to produce an uncontrolled and self-escalating response. In *Caenorhabditis elegans*, they found that feed-forward amplification was limited by biosynthetic and structural distinctions at the RNA level between (1) triggers that can produce amplification and (2) siRNA products of the amplification reaction. By assuring that initial (primary) siRNAs can act as triggers but not templates for activation, and that the resulting (secondary) siRNAs can enforce gene

silencing on additional targets without unbridled trigger amplification, the system achieves substantial but fundamentally limited signal amplification.

395. A more extensive molecular biological understanding across different phyla of (1) amplification mechanisms; (2) mechanisms to limit uncontrolled feed-forward amplification; *and* (3) physiological and biochemical barriers to systemic uptake of dsRNAs in organisms would help reduce uncertainty in assessing likely systemic exposure in those organisms.

8.2. **Uncertainties in the determination of possible effects in non-target organisms**

8.2.1. *Mechanisms of off-target silencing*

396. Analysis of sequence data (bioinformatics) can indicate whether a non-target insect might potentially be a target; this is on the basis of Watson-Crick base pairing of sequences of the guide strand of the siRNA and the target mRNA. What is less certain, in the early stage of development of dsRNA-based pesticide products, is the impact of other possible silencing mechanisms viz. the imperfect binding of siRNAs to the 3' UTR of mRNAs and potentially binding to a number of mRNA targets (Deng et al, 2014; Bramsen & Kjems, 2012; also see above under 'Bioinformatics'); *and* the selection of the wrong strand by RISC, so that the passenger strand becomes the guide strand and genes complementary to this strand are silenced (Kanasty et al, 2012).

397. It should be noted that a transient reduction of mRNA quantity does not automatically mean that an adverse effect has occurred; manifestation of adverse effects or creation of a hazard will depend on function and turn-over rate of the target protein and other factors.

8.2.2. *Amplification*

398. Amplification is discussed in Section 8.1.2 with respect to uncertainty in exposure estimates. If it occurred in a non-target organism, a low systemic exposure could lead to sufficient copy numbers of siRNA molecules to have an effect in cells of the non-target organism. Uncertainties regarding amplification include 1) our knowledge of the existence of amplification mechanisms in the range of non-target organisms which may be exposed to the applied dsRNA product in the application zone and 2) the triggering mechanism in the target organism for the recognition of the dsRNA molecule for amplification .

8.2.3. *Development of resistance to environmental dsRNA*

399. While a consideration of the possible development of resistance by target insects to exogenously-applied dsRNAs may not appear to be immediately relevant to risk assessment, an awareness of possible resistance mechanisms in target organisms may help inform a better understanding of RNAi mechanisms. Arising from the use of dsRNA products, the possible selection of target organisms with reduced responsiveness to RNAi could create a level of uncertainty with respect to product efficacy, and hence, any risk-benefit analysis.

400. Multiple mutants impaired in environmental and/or systemic RNAi have been isolated in *C. elegans*, a species normally extremely responsive to RNAi. A potential risk is that target genes will be selected for single nucleotide polymorphism variants that evade RNAi processing – however, large dsRNAs could presumably mitigate this risk (Bachman et al, 2013; Bolognesi et al, 2012). The concern is with mutations that up-regulate nucleases

or alter dsRNA uptake, processing, or systemic spread; these could compromise the triggering and spread of the RNAi response in a pest organism. The observation that most species in the *Caenorhabditis* genus are unresponsive to environmental RNAi (Nuez & Félix, 2012; Whangbo & Hunter, 2008) and that several other nematode species are completely unresponsive to RNAi (Wheeler et al, 2012) suggests that mechanisms bestowing responsiveness to RNAi can change.

401. A review by Zhang et al (2017) on RNAi-mediated crop protection includes a reasonably-detailed discussion on whether insects can develop resistance to RNAi, similar to the widespread resistance to many chemical pesticides and the emerging resistance to *Bacillus thuringiensis* (Bt) toxins. They discussed three theoretically-possible sources of resistance:

- Mutations affecting the efficiency of the RNAi machinery of the insect (or inactivating it) could render exogenously applied dsRNAs less effective (or ineffective). However, they considered that, given the essential functions that RNAi machinery has in insect development and physiology (RNA metabolism and small RNA biogenesis), mutations inactivating the RNAi machinery would be likely to be incompatible with insect fitness and survival and thus, an unlikely source of resistance development to environmental RNAi by pest insects.
- Resistance could arise through the accumulation of mutations in the sequence of the target gene. A number of point mutations or small deletions could reduce the complementarity between the target gene and the dsRNA to a point where the siRNAs generated from the dsRNA no longer efficiently recognise the mRNA of the target gene. However, the authors considered it unlikely that enough mutations would accumulate within a reasonable timeframe to affect the responsiveness of the target mRNA to RNAi[72]. However, what is considered a "reasonable timeframe" will depend on exposure period and the generation time of the target organism and the evolutionary costs of accumulating so many point mutations to gene function.
- Insects could become resistant to RNAi through the acquisition of mutations that affect the stability of ingested dsRNAs in their digestive tract and/or the uptake of dsRNAs by intestinal cells. As detailed elsewhere in this working paper, there are substantial variations in the effectiveness of RNAi among different groups of insects, suggesting that the changes in mechanisms involved in dsRNA uptake and metabolism could be a source of resistance evolution (see also Chu et al, 2014). As part of U.S. EPA insect resistance management requirements for a plant-incorporated protectant based RNAi product, and as reported in 2018 by Khajuria et al, a laboratory selected colony of WCR was developed which was resistant to ingested DvSnf7 dsRNA. It had impaired luminal uptake and resistance was not DvSnf7 dsRNA-specific, as indicated by cross resistance to all other dsRNAs

[72] They further commented that, even if a particular target gene accumulated enough mutations to render it insensitive to the dsRNA, this type of resistance could be dealt with by switching the target to a different essential gene in the insect. Another strategy to prevent resistance development arising from the accumulation of target gene mutations would be to target more than one essential gene at the same time, either by producing long chimeric dsRNAs that are composed of two or more target sequences (or for a plant-incorporated protectant, co-expressing two dsRNAs from two distinct transgenes in the same plant).

tested. This dsRNA resistance was inherited recessively, located on a single locus, and autosomal.

8.3. **Other Uncertainties**

402. These include questions like:

- How rapidly would a dsRNA product be degraded?
- Do applications have to be repeated for acceptable efficacy?
- What about rain fastness on a leaf surface?
- What about the photostability of a dsRNA product on a plant surface?
- How long is the silencing-effect caused by a dsRNA, and is this affected by the dose applied?

403. Answers to these issues will depend on the dsRNA product under consideration and the target pest - just as it does for all other pesticide types. Thus, such uncertainties should be addressed in the data package developed in order to bring a new product to market.

9. OECD Conference "Regulation of Externally Applied dsRNA-based Products for Management of Pests 10-12 April 2019

404. The OECD's two and a half-day Conference summarised the current state of knowledge and on-going developments that are relevant for the regulation of externally applied dsRNA-based products that are proposed for use as pesticides. Invited speakers included academic, industry and government experts in varying aspects of RNAi and their presentations summarised product developments, environmental fate, exposure to externally applied dsRNA in non-target organisms, lessons gathered from human therapeutic use of dsRNA, and key points from previous regulatory reviews of dsRNA-based crop traits.

Purpose and Scope of the Conference

405. The main objectives of the OECD Conference were:

- to provide participants with a summary of the state of the art of dsRNA-based product used in agriculture;
- to exchange information on the current status and future possibilities for the regulation of externally applied dsRNA-based products that are proposed for use as pesticides;
- to facilitate exchanges on their implications in environment, health and regulation;
- to inform regulatory policy and facilitate harmonised approaches.

Participation – details of total number of participants, countries they came from, backgrounds (academia, industry, etc.)

406. Around 60 people from academia, government, industry and other stakeholders participated in this event. Countries represented included: Australia, Austria, Belgium, Canada, Czech Republic, Denmark, Estonia, France, Germany, Hungary, the Netherlands, Switzerland, the United Kingdom and the USA. Experts representing the European Commission were also present.

407. People attending the OECD conference included:

- members of the OECD Ad Hoc Expert Group on RNAi-based pesticides;
- invited experts from the pesticide industry (BIAC) and manufacturers of these products;
- invited experts from research institutes (academia), and
- regulators and evaluators from governmental or intergovernmental bodies.

Major highlights from the presentations

408. The first session provided a background on the molecular mechanism of RNAi and relevant related pathways, reviewed the current understanding of cross-kingdom RNAi, examined the species-specificity of these dsRNAs on non-target species and discussed challenges related to the RNAi-efficiency in insects.

409. The second session dealt with the research to date that investigated the factors that affect insects' responsiveness to environmental dsRNA, covered approaches to assess biodegradation of dsRNA in different matrices and explored the role of certain technology to predict genetic changes and the impact in terms of efficacy of the RNAi products in target pests. It also summarised the published literature related to dietary uptake of external dsRNA in humans and addressed the lessons learnt from human therapeutic use of dsRNA.

410. The third and final session focused on how problem formulation can guide risk assessments for spray applications of pesticides containing dsRNA, and considered potential pathways and testable risk hypotheses in the context of environmental risk assessment. It also covered the experience of experts involved in the risk assessment of genetically-modified crop plants, which incorporate the machinery to synthesise RNA molecules specifically directed against a pest species feeding on the crop (so-called 'plant-incorporated protectants', or PIPs).

Significant considerations in terms of policy relevance.

411. These considerations represent varied input from multiple participants during the conference and do not always reflect consensus views and include the following.

- The potential for exposure of non-target organisms as well as responsiveness to environmental RNAi were seen as the first parameters to consider in the risk assessment of external dsRNA applications before looking at sequence data.
- Sequence information alone should not and cannot be used as a stand-alone predictor of off-target effects.
- Protocols for addressing risk with dsRNA-based products require some revisions compared to how they are carried out for conventional pesticides because dsRNA-based pesticides often take longer to display efficacy. Any evaluation needs to account for this time lag by extending the study period. Additionally, any evaluation of a dsRNA-based pesticide should include monitoring for degradation of the dsRNA over time.
- Impact of product formulation on environmental persistence of dsRNA and uptake by non-target organisms requires consideration.
- The potential uptake of dsRNA by mammals is likely low due to substantial barriers in the oral and topical uptake pathways.
- Evidence from human clinical studies suggest that systemic exposure of mammals to dsRNA when dsRNA is applied in the field as a pesticide is likely quite low.
- The use of established laboratory protocols for studying the persistence of Bt proteins from transgenic plants was proposed for evaluating the environmental fate and persistence of dsRNA.

- While it may be possible to generalise the applicability of barriers to dsRNA uptake identified in mammals to other vertebrates, it is currently not possible time to predict responsiveness across invertebrate taxa to environmental dsRNA.

Website for further details

412. Information about the conference, including the programme, speakers, abstracts, presentation files and other related material is available online (see below) and in Appendix 6. The full proceedings of the conference on “Regulation of Externally Applied dsRNA-based Products for Management of Pests” with papers prepared subsequently by each of the speakers, has been published online at the Frontiers Research Topic RNAi Based Pesticides. The conference report summarises input from presenters and Conference participants during the panel discussions at the end of each session.

413. Website: http://www.oecd.org/chemicalsafety/pesticides-biocides/conference-on-rnai-based-pesticides.htm

10. Conclusions

414. The document summarises the current state of the art in scientific and technology knowledge regarding environmental risk assessment of the application of sprayed or externally applied ds-RNA-based pesticides. It is based on available literature, the results of an OECD Conference on this topic, and input from experts in OECD member countries. From that, the following conclusions can be drawn:

1. In general, exposure to dsRNA in dsRNA-based pesticide products is expected to be limited because of low application rates in comparison to many conventional chemical pesticides and the anticipated rapid degradation and dissipation of dsRNA in the environment.

2. To assess fate and degradation processes, the use of established laboratory protocols for studying the persistence of Bt proteins from transgenic plants could be a starting point for evaluating the environmental fate and persistence of dsRNA.

3. The potential for exposure of non-target organisms as well as responsiveness to environmental RNAi were seen as the first parameters to consider in the risk assessment of external dsRNA applications. Sequence information alone should not and cannot be used as a stand-alone predictor of off-target effects. Bioinformatics will inform the selection and prioritisation of non-target species for toxicity and effects testing.

4. Available evidence suggests that dsRNAs have a long record of safe consumption by humans and other vertebrates. These dsRNAs include both long and short dsRNAs with sequence identity to genes/exons in humans and other organisms consuming them. Nucleic acids are naturally-occurring components of plant- and animal-derived foods and feed and are routinely consumed by humans and animals.

5. Significant physiological and biochemical barriers exist in humans and other vertebrates to limit the uptake and distribution of exogenous RNAs. These barriers include nucleases in saliva, denaturation and depurination in the acid pH of the stomach, nucleases in the digestive tract, pancreatic secretions of bile salts and degradative enzymes, cellular membrane barriers, the polysaccharide coating of the intestinal epithelium, and intracellular degradation in endosomes and lysosomes. The widespread distribution of such barriers is likely to be a consequence of the widespread presence of RNAs in the environment, molecules which could be biologically active if they were easily able to access the cytoplasm or nucleus of living cells. While it may be possible to generalise the applicability of barriers to dsRNA uptake identified in mammals to other vertebrates, it is currently not possible to predict responsiveness across invertebrate taxa to environmental dsRNA.

6. As with all pesticide products, the impact of the dsRNA pesticide use pattern and application method (e.g. greenhouse, outdoor, bait, sprayable) require consideration.

7. In some cases, specific product formulations may be designed to overcome barriers to uptake for the target organism. Special attention should be paid to how barriers to uptake are proposed to be overcome for the target organism and the impact this could have on environmental persistence of dsRNA as well as to barriers to and uptake of the dsRNA by non-target organisms. Information and/or studies on the

impact to uptake and environmental persistence that the formulation presents are important to characterize exposure to the dsRNA.

8. Where specific product formulations impact barriers to and uptake of the dsRNA, product-specific formulation toxicology testing on organisms or test surrogates would help better characterize the potential for hazard. Thus, product-specific empirical testing may be required to characterize hazards and exposure to inform the risk assessments of dsRNA-based pesticides. The necessity of such testing depends on the legal requirements in the different OECD member countries as well as the characterization of the product.

9. Protocols for addressing risk with dsRNA-based products require some revisions compared to how they are carried out for conventional pesticides because dsRNA-based pesticides often take longer to display efficacy. Any evaluation needs to account for this time lag by extending the study observation period. Additionally, any evaluation of a dsRNA-based pesticide should include monitoring for degradation of the dsRNA over time. For organisms that have been demonstrated to be responsive to environmental RNA, consideration of life cycle studies (growth, development and reproduction) and studies on other non-lethal effects could be considered.

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12. Glossary and Abbreviations

Argonaute – proteins of the Argonaute family are essential components of the RNA-induced silencing complex (RISC). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). Small RNAs guide Argonaute proteins to their specific targets through sequence complementarity, leading to mRNA cleavage or translation inhibition. The repertoire of different Argonaute proteins varies among species. For example, there are more than 25 Argonautes in the nematode worm *Caenorhabditis elegans* compared with five in the fly *Drosophila melanogaster*.

cDNA - complementary DNA is DNA synthesised from a single-stranded RNA template (e.g., messenger RNA or microRNA) using reverse transcriptase. To express a specific protein in a cell that does not normally express that protein (so-called heterologous expression), cDNA that codes for the protein is inserted into the genome of the recipient cell. cDNA is often used to clone eukaryotic genes in prokaryotes. cDNA is produced by retroviruses and then integrated into the host's genome to create a provirus. The term is also used in a bioinformatics context to refer to the sequence of an mRNA transcript, expressed as DNA bases (GCAT) rather than RNA bases (GCAU).

Cell-autonomous RNAi - refers to the RNAi response that individual cells carry out when encountering dsRNA, a response that is executed by a broadly conserved or core RNAi machinery found in eukaryotic cells.

CpG oligonucleotides – Immunostimulatory dinucleotide motifs that interact with Toll-like receptor 9.

Dicer - A member of the RNase III family of ribonucleases that cleaves double-stranded RNAs into small interfering RNAs, and precursor microRNAs and mirtrons (see definition below) into microRNAs. In most species, cleavage of longer dsRNAs by Dicer produces double-stranded siRNAs that are ~21 nucleotides long and have a two-nucleotide overhang at their 3′ end, as well as a 5′ phosphate and a 3′ hydroxyl group.

Environmental RNAi - refers to the triggering of RNAi by environmental exposure to dsRNA by soaking or feeding. Environmental RNAi may or may not be followed by systemic movement of the silencing signal, perhaps a key step in determining the biological activity of a dsRNA.

Exportin-5 - a nuclear export receptor for certain classes of double-stranded RNA (dsRNA), including pre-micro-RNAs, viral hairpin RNAs, and some tRNAs.

GEM - genetically-engineered microorganism. GEM refers only to bacteria, fungi, yeast and other microorganisms.

GMO - genetically-modified organism. The acronym can apply to plants, animals or microorganisms, whereas the term genetically-engineered microorganism (GEM) refers only to bacteria, fungi, yeast or other microorganisms.

lncRNAs - long non-coding RNAs, i.e. RNA transcripts longer than 200 nucleotides which are not translated into proteins

Microprocessor Complex - a protein complex consisting of at least two proteins, ‘Drosha’ and DGCR8. Drosha is an RNase III type enzyme in animals that cleaves RNA molecules with double-stranded regions. The function of the Microprocessor Complex is to recognise and release the hairpin structure

precursor of microRNAs from the primary miRNA transcript. The complex is active in the nucleus and generates the pre-miRNA molecules.

miRNA* - The precursor microRNA (pre-miRNA) processed by Dicer generates a miRNA duplex containing the miRNA strand and the miRNA* strand, one of which is loaded into the RNA-induced silencing complex (RISC). The ratio of one strand to the other being loaded into RISC to mediate silencing activity can vary among species, tissues, and disease or developmental settings.

Mirtron - Mirtrons are a type of microRNA that are located in the introns of genes. Mirtrons arise from spliced-out introns. Mirtrons, first identified in *Drosophila melanogaster* and *Caenorhabditis elegans*, have also been described in mammals and plants. Plant miRNAs are derived from the sequential DCL1 cleavage from pri-miRNA to give pre-miRNA (precursor miRNA), but mirtrons bypass DCL1 cleavage and enter as pre-miRNA in the miRNA maturation pathway.

Non-cell autonomous RNAi – While cell-autonomous RNAi refers to the RNAi response that individual cells carry out when encountering dsRNA, a response that is executed by a broadly conserved or core RNAi machinery found in eukaryotic cells, non-cell autonomous RNAi includes the phenomenon of systemic RNA, the movement of a silencing signal, presumably siRNA and/or dsRNA, from cell to cell and from one part of an organism to another. Non-cell autonomous RNAi also includes the phenomenon of environmental RNAi, the triggering of RNAi by environmental exposure to dsRNA by soaking or feeding.

Non-target Effects – see 'Off-target' effects

Off-target effects - Any detectible phenotypic change that is triggered by the RNAi treatment, other than those that are derived directly or indirectly from silencing the targeted mRNA. In the context of an RNAi-based pesticide, **off-target** effects occur when an siRNA processed by the RNA-Induced Silencing Complex (RISC) down-regulates unintended targets; this could be in the target pest or in **non-target** species exposed to the pesticide.

Parental RNAi – used to describe cases in which female organisms are treated with dsRNA and the interference effect extends to the offspring.

Precursor miRNAs (pre-miRNAs). Hairpin precursors of microRNAs formed by the cleavage of primary microRNAs by DCGR8 and Drosha in animals or by DCL1 in plants.

Primary miRNAs (pri-miRNAs) - The initial transcriptional products of microRNA genes. They are generally >100 nucleotides long and may contain one or more microRNA stem loops that are processed by the microRNA biogenesis pathway.

Plasmodesmata (singular: plasmodesma) - Microscopic channels which traverse the cell walls of plant cells and some algal cells, enabling transport and communication between them.

RNA-induced silencing complex (RISC) - RISC is composed of a group of proteins, including one of the Argonaute proteins, that induces target mRNA cleavage, based on loaded small interfering RNA or microRNA guide strands.

Seed region - A sequence of seven bases in a microRNA that is complementary to the mRNA target. This sequence is essential for the initial binding of the microRNA to most targets. Seed regions can also exist arbitrarily in small interfering RNAs and processed short hairpin RNAs, causing microRNA-like silencing.

sRNA – small RNA.

Stable nucleic acid lipid particle (SNALP) - A lipid nanoparticle formulation for the systemic delivery of small interfering RNAs to tissues.

Toll-like receptors (TLRs) - A family of receptors that recognise pathogen-associated molecular patterns (PAMPs), including some DNA and RNA molecules.

Trans-acting RNAs – Small RNAs which act upon mRNA derived from largely unrelated endogenous loci. These siRNAs originate from genes to which the targeted mRNA bears little resemblance (Vazquez et al, 2004.

Transitive RNAi - the observation that RNAi silencing specificity can spread along a target mRNA to target sequences not in the original trigger dsRNA. Mechanistically, this occurs during RNA-directed RNA polymerase (RdRP) production of secondary siRNAs. Subsequently, these secondary siRNAs can then target other mRNA transcripts (Zhuang et al, 2013).

Transposon - A segment of DNA that is capable of independently replicating itself and inserting the copy into a new position within the same or another chromosome or plasmid. Transposons act somewhat similarly to viruses and, in humans, are an underlying factor in the development of haemophilia, certain cancers, and other diseases.

Three prime untranslated region (3' UTR) - The section of messenger RNA (mRNA) that immediately follows the translation termination codon. Several regions of the mRNA molecule are not translated into protein including the 5' cap, the 5' untranslated region, 3' untranslated region, and the poly(A) tail. The 3'-UTR region often contains regulatory regions that post-transcriptionally influence gene expression.

Type I interferon response - An innate immune response to dsRNA, ssRNA, CpG DNA and other stimuli that triggers a protective antiviral response in host cells. Signalling elicits α- and β-interferon release, which activate multiple components of innate and adaptive immunity.

Appendix 1: Endogenous formation of miRNAs; pri- and pre-miRNAs

MicroRNAs (miRNAs) are endogenously-synthesised small RNAs that bear some structural similarities to small interfering RNAs (siRNAs), but are unique in several aspects including biogenesis (the focus of this Appendix). MicroRNAs originate from distinct genomic loci that, when transcribed, fold into short hairpins through localized regions of self-complementarity. In contrast, siRNAs are typically derived from longer bimolecular duplex RNA (Bartel, 2004). MicroRNAs are abundant in many cell types and, based on the number of annotated miRNA sequences in the human genome, are estimated to have identity to 30% - 80% of human genes (Friedman et al, 2009; Lu & Clark, 2012).

MicroRNAs are non-coding RNA molecules of approximately 21 nucleotides, found in plants, animals and some viruses; they regulate gene expression through post-transcriptional gene silencing (reviewed in Gebert & MacRae, 2019). MicroRNAs are evolutionarily conserved in both plants and animals and, in addition to their functions in gene regulation, may represent an anti-viral defense mechanism in several types of organisms (see e.g., Obbard et al, 2009).

MicroRNAs are initially transcribed mainly by RNA polymerase II (Pol II) (Lee et al., 2004a) as part of a longer stem-loop structure termed a **primary** miRNA (or **pri-miRNA**) (Gebert & MacRae, 2019). A single pri-miRNA may contain several miRNA precursors. In animals, processing of the pri-miRNAs is carried out in the nucleus by the "Microprocessor Complex" comprising Drosha (an RNase III-type enzyme) in association with the protein DiGeorge Syndrome Critical Region 8 (DGCR8) (Denli et al, 2004; Gebert & MacRae, 2019). In some invertebrates, including the common model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, the protein which carries out this function is known as 'Pasha' (Denli et al, 2004; Gebert & MacRae, 2019). The Microprocessor Complex cleaves the pri-miRNA near the base of the stem-loop structure, yielding a shorter hairpin known as a **precursor** miRNA (**pre-miRNA**). In plants, both pri-miRNA and pre-miRNA are processed by DCL1 (Kurihara & Watanabe, 2004).

Precursor miRNAs can be heterogeneous in length, but in general are approximately 60 – 70 nucleotides with 5'-monophosphate and 3'-OH groups, and possess a 3' overhang of 2 nucleotides (Starega-Roslan et al., 2011). Sequence motifs downstream of the pre-miRNA such as CNNC (where N is any nucleotide) contribute to recognition and processing (Auyeung et al, 2013). Pre-miRNA-like structures can also arise directly from introns through splicing and debranching; these are known as 'mirtrons', and have been found in *D. melanogaster*, *C. elegans,* and mammals (Berezikov et al, 2007). Certain sequence characteristics of pre-miRNAs may be altered through editing, such as adenosine to inosine transitions catalysed by adenosine deaminases acting on RNA. These modifications can then affect the processing, degradation, or target specificity of miRNAs (Wulff & Nishikura, 2011).

In animals, pre-miRNA hairpins are exported from the nucleus to the cytoplasm in an energy-dependent process involving the nuclear export receptor, Exportin-5, which recognises the two-nucleotide overhang at the 3' end of the pre-miRNA resulting from Drosha or DCL1 cleavage (Gebert & MacRae, 2019). miRNAs are also found in the nucleus, where they may function in gene expression regulation at the transcriptional level (refer to Appendix 4).

In human cells, it has been shown that the 3' overhang and the 5'phosphorylated ends of the pre-miRNA hairpin are recognized in the cytoplasm by Dicer, an RNase III enzyme (Park et al, 2011). Dicer cleaves the pre-miRNA approximately 21 nucleotides from the 5' end, removing the loop region from the hairpin structure to yield a short duplex miRNA (Park et al, 2011). In plants, this step of miRNA processing is

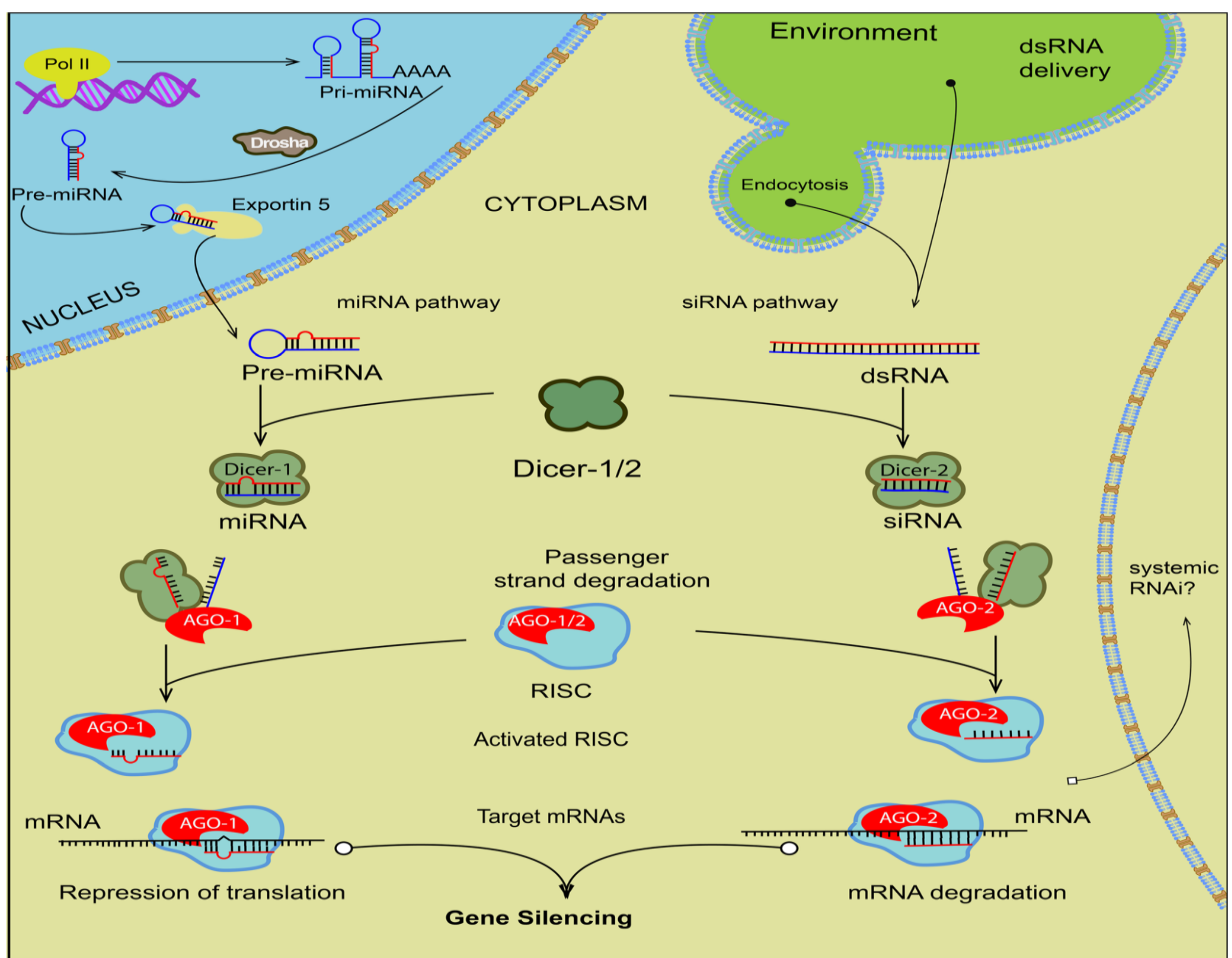
carried out in the nucleus by DCL1 (Kurihara & Watanabe, 2004), and by Dcr1 in *D. melanogaster* (Lee et al, 2004b). One strand of the miRNA duplex, known as the guide strand, is loaded onto the Argonaute (AGO) protein within the RNAi-induced silencing complex (RISC); the guide strand directs binding of RISC to the target mRNA (Park et al, 2011). Guide strand selection is influenced by thermodynamic characteristics of the dsRNA duplex (reviewed in Noland & Doudna, 2013).

Once associated with the target mRNA, RISC mediates silencing of the target. If the miRNA guide strand is perfectly complementary to the target mRNA, as is the case in plants, a catalytically-active AGO will cleave the mRNA (refer to Section 5.1.5 and references cited therein). However, in animals, binding between the seed region of the miRNA guide strand and the target mRNA is typically just partially-complementary. In this case, AGO proteins along with GW proteins recruit downstream effectors which initiate translational repression, de-adenylation, and de-capping leading to 5’-to-3’ decay of the mRNA (see e.g. Jonas & Izaurralde, 2015). This mechanism of seed-region complementarity potentially allows a single miRNA to regulate many mRNAs; conversely, the expression of a single mRNA may be regulated by multiple miRNAs (Gebert & MacRae, 2019).

Appendix 2: Mechanistic overview of RNA pathways involving miRNAs and siRNAs

The following diagram is taken from Zotti & Smagghe (2015) and provides a mechanistic overview of RNAi pathways involving miRNAs and siRNAs. Only the miRNA and siRNA pathways are shown, although a number of RNAi pathways use dsRNAs to generate sRNAs (e.g., Bernstein et al, 2001, Ketting, 2011); the 'canonical' miRNA pathway is the most understood. The source of sRNAs is different between pathways; for miRNAs the short RNA duplexes are derived from miRNA genes in plants and animal genomes or in invading viral genomes. siRNAs are derived from long dsRNAs that may be exogenous – including long 'environmental dsRNA' taken up from the environment - (exo-siRNA) or endogenous (endo-siRNA).

For a more detailed description of the RNAi mechanism, the reader is referred to the text of the Zotti & Smagghe (2015) overview paper.



Appendix 3: Examples of RNAi-mediated gene silencing in different insect orders

Insect (*Order*; Name)	Species	RNAi response	Notes
Diptera			
Savannah tsetse fly	*Glossina morsitans*	silencing of the immune-responsive midgut-expressed gene *TsetseEP*	dsRNA delivered by injection and in the bloodmeal. First demonstration in Diptera of gene silencing by feeding (Walshe et al, 2009).
African malaria mosquito	*Anopheles gambiae*	Reduced expression of chitin synthase gene AgCHS1	AgCHS1 repressed by chitosan/AgCHS dsRNA-based nanoparticles through larval feeding. AgCHS1 transcript level and chitin content were reduced by 62.8 and 33.8%, respectively (Zhang et al, 2010).
Yellow fever mosquito,	*Aedes aegypti*	Greatly-reduced fertility in males. Production of a male-biased population.	Mosquito larvae were fed dsRNAs that targeted testis genes and a female sex determination gene (Whyard et al, 2015)
Lepidoptera			
Light brown apple moth	*Epiphyas postvittana*	Transcript levels of a larval gut carboxylesterase gene (EposCXE1) reduced to <50% of controls within 2 days of EposCXE1 dsRNA feeding.	Droplet feeding of larvae (Turner et al, 2006).
Cotton bollworm; corn earworm	*Helicoverpa armigera*	Gene silencing of AChE, with mortality, larval growth inhibition, pupal weight reduction, mal-formation & reduced fecundity.	Larvae fed with siRNA (Kumar et al, 2009).
Asian corn borer	*Ostrinia furnacalis*	Larval mortalities were *ca.* 40-50%. qRT-PCR analysis verified the correlation between mortality and target gene down-regulation. dsRNA penetrated the body wall and circulated in the body cavity.	dsRNAs for the genes DS10 and DS28 were directly sprayed on newly-hatched larvae (Wang et al, 2011)
Fall armyworm	*Spodoptera frugiperda*	Silencing of neuropeptides allatostatin AS-C-type	Feeding with dsRNA. Juvenile Hormone titre in the hemolymph affected by the stimulatory (allatotropins) or inhibitory

		(Spofr/Manse-AS) or the allatotropin AT 2 (Spofr-AT 2) genes - transcript levels reduced in brain and gut of last instar larvae as well as of adults.	(allatostatins) neuropeptides (Griebler et al, 2008).
Oriental leafworm; cotton leafworm; tropical armyworm	*Spodoptera litura*	Reduced expression of Aminopeptidase N gene	Soaking and in artificial diet of larvae (Rajagopal et al, 2002).
	Spodoptera litura	vitellogenin receptor (VgR) gene	Injection of VgR dsRNA in 4th or 6th day pupae led to a phenotype with high vitellogenin (Vg) accumulation in the haemolymph, low Vg deposition in the ovary, and the failure of insect spawning (Shu et al, 2011).
Diamondback moth	*Plutella xylostella*	CYP6BG1 (a Cytochrome P450) gene	Oral delivery of dsRNA in larvae efficiently reduced the expression of CYP6BG1 (Bautista et al, 2009).
Coleoptera			
Western corn rootworm	*Diabrotica virgifera virgifera*	Vacuolar ATPase Subunit A gene	dsRNA in an artificial diet (Baum et al, 2007).
Western corn rootworm	*Diabrotica virgifera virgifera*	Target mRNA was the Snf7 ortholog (DvSnf7) which encodes an essential protein involved in intracellular trafficking.	After dsRNA feeding to WCR larvae, DvSnf7 suppression was time-dependent, with mRNA suppression preceding suppression of protein synthesis (Bolognesi et al, 2012).
Striped flea beetle	*Phyllotreta striolata*	An odorant receptor PsOr1 gene	Adult beetles unable to sense the attractant or repellent odour stimulus after microinjection of dsRNA against PsOr1 (Zhao et al, 2011).
	Phyllotreta striolata	Arginine kinase gene	Ingestion of dsRNA retarded the development and increased the mortality of adults, and greatly reduced fecundity and fertility (Zhao et al, 2008).
Red flour beetle	*Tribolium castaneum*	Enhanced Green Fluorescent Protein (EGFP) gene	When long dsRNA (520 bp) was injected into last instar larvae, silencing of EGFP was seen in both the larval and pupal stages. A siRNA (21 bp) did not affect EGFP expression (Miller et al, 2012).
Hymenoptera			
Western honey bee	*Apis mellifera*	Vitellogenin gene. Almost all (96%) showed the mutant phenotype.	Abdominal injection in newly emerged bees of a dsRNA derived from a 504bp stretch of the vitellogenin coding sequence. (Amdam et al, 2000).
Western honey bee	*Apis mellifera*	Vitellogenin gene	dsRNA mixed with natural diet of second-instar larvae (Nunes et al, 2009).
Western honey bee	*Apis mellifera*	Toll-related receptor 18W	dsRNA delivered by a feeding-soaking delivery method (Aronstein et al, 2006).
Hemiptera			

Brown planthopper	*Nilaparvata lugens*	Trehalose phosphate synthase (TPS) gene	Feeding dsRNA led to rapid reductions in levels of TPS mRNA and enzymatic activity. The development of larvae was disturbed, with reduced survival (Chen et al, 2010).
Triatomine bug	*Rhodnius prolixus*	Salivary nitrophorin 2 (NP2) gene (nitrophorins are salivary hemeproteins)	NP2 dsRNA was introduced by injection or ingestion. injections in fourth-instar nymphs and feeding of second-instar nymphs significantly reduced gene expression (Araujo et al, 2006).
Pea aphid	*Acyrthosiphon pisum*	A target salivary gland transcript, C002.	Injection of siRNA into parthenogenetic adult pea aphids led to dramatic depletion of a target salivary gland transcript over a 3-day period. The siRNA was generated from double stranded RNA that covered most of the open reading frame of the transcript (Mutti et al, 2006).
Pea aphid	*Acyrthosiphon pisum*	A putative aquaporin referred to as ApAQP1; its transcript was localised to the stomach and distal intestine.	RNAi-mediated silencing of its expression resulted in elevated osmotic pressure of the haemolymph (Shakesby et al, 2009).
Order: ***Blattodea*** Infra-order: ***Isoptera***			
Eastern subterranean termite	*Reticulitermes flavipes*	Genes for a cellulase enzyme and a caste-regulatory hexamerin storage protein	Silencing of either gene through high-dose dsRNA feeding led to significantly reduced group fitness and mortality (Zhou et al, 2008).
Orthoptera			
Desert locust	*Schistocerca gregaria*	peptide hormones, insulin-related peptide Scg-IRP and neuroparsin Scg-NP.	dsRNA injection in adult female desert locusts led to reduced mRNA transcript levels and peptide hormones (Badisco et al, 2011).
Desert locust	*Schistocerca gregaria*	alpha-tubulin 1a and gapdh (glyceraldehyde 3-phosphate dehydrogenase) genes	Intra-abdominal injection of dsRNAs led to a potent reduction of their corresponding mRNAs, an effect increasing with time and, with silencing of the alpha-tubulin 1a gene, resulting in high mortality (Wynant et al, 2012).

Notes

Developed from data tables in Katoch & Thakur (2013) and Katoch et al (2013). The information is as provided in the respective references but it should be noted that, on their own, observed changes in mRNA transcript levels do not necessarily confirm a direct regulatory interaction with an sRNA from a particular dsRNA (Chan & Snow, 2017); these authors summarise approaches to demonstrating that a given sRNA directly regulates a target transcript and conclude that *in vivo* genetic modification of putative target genes via traditional knock-in techniques or novel ones, such as CRISPR/Cas9, may be required to provide final definitive evidence of a regulatory relationship between a given sRNA and a specific transcript (see also Bassett et al, 2014).

Bellés (2010) has suggested that the reluctance of researchers to publish negative results has led to an over-estimation of the general responsiveness of insects to RNAi techniques.

Appendix 4: RNA-induced transcriptional silencing (RITS)

In addition to the modulation of gene expression via mRNA degradation or inhibition of translation (post-transcriptional gene silencing or PTGS), as directed by some miRNAs and siRNAs, heterochromatic siRNAs can modify histone proteins and DNA associated with heterochromatin in insects,plants, and fungi (Reinhart & Bartel, 2002; Martienssen & Moazed, 2015); methylation of histone proteins and cytosine bases in DNA can provide transgenerational alteration of phenotype by affecting gene expression in particular regions of the chromosome. Thus, by altering chromatin rather than mRNA, these siRNAs influence gene expression at the transcriptional rather than the post-transcriptional/translational level. The overarching term used for this process is RNA-induced transcriptional silencing (RITS). Importantly, heterochromatin formation is an epigenetic modification which modulates gene expression in a way that may be heritable even in the absence of RNAi stimulus (Grewal and Elgin, 2007).

RITS was discovered in the fission yeast Schizosaccharomyces pombe and has been shown to be involved in centromere organisation and the initiation and spreading of heterochromatin in the mating-type region (viz. the region regulating the mating type of the organism and determining key events in the life cycle, such as whether it will reproduce sexually or asexually).

In Drosophila, so-called endo-siRNAs, derived from transposable element (TE) transcripts, play a role in heterochromatin formation in somatic tissues during larval development and in adults. The proper nuclear localisation of these siRNAs is essential to the regulation of chromatin dynamics in Drosophila (Fagegaltier et al, 2009).

The role of RNAi in transcriptional gene silencing (TGS) in plants has been well characterised, and functions primarily through histone methylation leading to heterochromatin formation, as well as DNA methylation via the RdDM pathway. Recruitment of the RITS complex (containing an Ago protein) and histone methyltransferases to nascent transcripts results in degradation of the transcript and RNAi-dependent methylation of lysine 9 of histone H3, leading to heterochromatin formation and silencing of transcription (Holoch & Mozaed, 2015). RNA-directed DNA methylation is an epigenetic process first discovered in plants (Wassenegger et al, 1994). During RdDM, dsRNAs are processed to 21-24 nt siRNAs which guide methylation of homologous DNA loci. In this process, which is distinct from the process described in fission yeast, Argonaute-bound siRNA recognises nascent RNA transcripts or the target DNA to guide the methylation and silencing of the target genomic region (Matzke et al, 2009).

The relevance of observations in Schizosaccharomyces pombe to mammals is not clear, as there is evidence to suggest that heterochromatin maintenance in mammalian cells is independent of the components of the RNAi pathway (Wang et al, 2006b). However, plants and animals have analogous mechanism for small RNA-guided heterochromatin formation, and it is believed that the mechanisms described above for S. pombe are quite highly conserved and play some role in heterochromatin formation in mammals. In higher eukaryotes, RNAi-dependent heterochromatic silencing appears to play a larger role in germline cells than in primary cells or cell lines and is but one of many different forms of gene silencing used throughout the genome (Volpe & Martienssen, 2011).

Appendix 5: End-use product data requirements in the EU

These data requirements are detailed in Commission Regulation (EU) 284/2013 – see https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0085:0152:EN:PDF
SECTION 10: Ecotoxicological studies
Introduction
10.1. Effects on birds and other terrestrial vertebrates
10.1.1. Effects on birds
10.1.1.1. Acute oral toxicity to birds
10.1.1.2. Higher tier data on birds
10.1.2. Effects on terrestrial vertebrates other than birds
10.1.2.1. Acute oral toxicity to mammals
10.1.2.2. Higher tier data on mammals
10.1.3. Effects on other terrestrial vertebrate wildlife (reptiles and amphibians)
10.2. Effects on aquatic organisms
10.2.1. Acute toxicity to fish, aquatic invertebrates, or effects on aquatic algae and macrophytes
10.2.2. Additional long-term and chronic toxicity studies on fish, aquatic invertebrates and sediment dwelling organisms
10.2.3. Further testing on aquatic organisms
10.3. Effects on arthropods
10.3.1. Effects on bees
10.3.1.1. Acute toxicity to bees
10.3.1.1.1. Acute oral toxicity
10.3.1.1.2. Acute contact toxicity
10.3.1.2. Chronic toxicity to bees
10.3.1.3. Effects on honey bee development and other honey bee life stages
10.3.1.4. Sub-lethal effects
10.3.1.5. Cage and tunnel tests
10.3.1.6. Field tests with honeybees
10.3.2. Effects on non-target arthropods other than bees
10.3.2.1. Standard laboratory testing for non-target arthropods
EN 3.4.2013 Official Journal of the European Union L 93/93
10.3.2.2. Extended laboratory testing, aged residue studies with non-target arthropods
10.3.2.3. Semi-field studies with non-target arthropods
10.3.2.4. Field studies with non-target arthropods
10.3.2.5. Other routes of exposure for non-target arthropods
10.4. Effects on non-target soil meso- and macrofauna
10.4.1. Earthworms
10.4.1.1. Earthworms — sub-lethal effects
10.4.1.2. Earthworms — field studies
10.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)
10.4.2.1. Species level testing
10.4.2.2. Higher tier testing
10.5. Effects on soil nitrogen transformation
10.6. Effects on terrestrial non-target higher plants
10.6.1. Summary of screening data
10.6.2. Testing on non-target plants
10.6.3. Extended laboratory studies on non-target plants
10.6.4. Semi-field and field tests on non-target plants
10.7. Effects on other terrestrial organisms (flora and fauna)
10.8. Monitoring data

Appendix 6: Additional Information Regarding the OECD Conference "Regulation of Externally Applied dsRNA-based Products for Management of Pests 10-12 April 2019

Information below is also available online, http://www.oecd.org/chemicalsafety/pesticides-biocides/conference-on-rnai-based-pesticides.htm.

Programme

OECD Conference on Regulation of Externally Applied dsRNA-based Products for Management of Pests

Welcome by the OECD

Richard Sigman, OECD Environment Directorate

Welcome by the CRP and introduction to the Programme

András Székács, CPR Scientific Advisory Body

Session 1: Summary of the State of the Art: dsRNA-based Product Use in Agriculture

***Chair*: Achim Gathmann**, Federal Office of Consumer Protection and Food Safety (BVL), Germany

This part of the first session will give some background on the molecular mechanism of RNAi and relevant related pathways, review the current understanding of cross-kingdom RNAi, examine the species-specificity of these dsRNAs on non-target species and discuss challenges related to the RNAi-efficiency in insects.

An introduction to RNAi technology

Petr Svoboda, Institute of Molecular Genetics of the ASCR, Czech Republic

Potential for dsRNA-based management of plant diseases

Karl-Heinz Kogel, Justus Liebig University Giessen, Germany

RNA interference technologies to control pests and pathogens

Steve Whyard, University of Manitoba, Canada

RNAi as a novel technology in pest control: current status and challenges

Olivier Christiaens, Ghent University, Belgium

***Chair*: Dimitra Kardassi**, European Food Safety Authority, EU

The following series of presentations will further discuss the research to date investigating the factors that affect insects' responsiveness to environmental dsRNA, cover approaches to assess biodegradation of dsRNA in different matrices and explore the role of RNA-Seq to predict genetic changes and impact the efficacy of RNAi products in target pests. They will also summarise the published literature related to dietary uptake of external dsRNA in humans and address the lessons learnt from human therapeutic use of dsRNA.

Variation in responsiveness to environmental dsRNA in insects

Ana María Vélez Arango, University of Nebraska, United States

Potential for off-target effects in topically applied dsRNA-based products used for crop protection purposes

Gunter Meister, University of Regensburg, Germany

Environmental dissipation of dsRNA in soil, aquatic systems and plants

Pam Bachman, Bayer

Validation of RNA interference by RNA-Seq: How to see the big picture

Brenda Oppert, United States Department of Agriculture, United States

Dietary uptake of environmental dsRNA in humans and other vertebrates

Thais B Rodrigues, Greenlight Biosciences, United States

Regulatory experience with antisense oligonucleotides for human use

Frank Holtkamp, Medicine Evaluation Board, The Netherlands

Panel Discussion Session 1

Moderator: **Achim Gathmann**, Federal Office of Consumer Protection and Food Safety (BVL), Germany

Session 2: Summary of Regulatory & Risk Assessment Experience with dsRNA-based Products

***Chair*: Mike Mendelsohn**, US Environmental Protection Agency

The second session will describe how problem formulation can guide risk assessments for spray applications of pesticides containing dsRNA, and consider potential pathways and testable risk hypothesis in the context of environmental risk assessment. It will also cover the experience gathered from the risk assessment of genetically-modified crop plants, which incorporate the machinery to synthesise RNA molecules specifically directed against a pest species feeding on the crop (so-called 'plant-incorporated protectants', or PIPs).

Problem formulation considerations for externally applied dsRNA-based products

Alan Raybould, Syngenta

Ecological assessment of topically applied dsRNA-based products

Jörg Romeis, Agroscope, Switzerland

Review of EFSA's activities on the risk assessment of RNAi-based GM crops

Nikoletta Papadopoulou, European Food Safety Authority, EU

The European perspective on regulatory aspects and experiences with dsRNA-based products

Achim Gathmann, Federal Office of Consumer Protection and Food Safety (BVL), Germany

Ecological Risk Assessment Considerations for in planta Expressed and Exogenously Applied dsRNA at the U.S. EPA

Shannon Borges, US Environmental Protection Agency, United States

A perspective on risks associated with dsRNA-based products

Neena Mitter, Centre for Horticultural Science, Australia

Panel discussion Session 2

Moderator: **Mike Mendelsohn**, US Environmental Protection Agency

Session 3: Discussion themes

Background to the draft OECD Working Paper on 'Environmental Risks from the Application of dsRNA-Based Pesticides'

Les Davies, OECD consultant

Discussions themes

Environmental Fate of dsRNA

Facilitator 1: **Pam Bachman**

Non-target Organism Assessment (Part 1)

Facilitator 2: **Shannon Borges**

Facilitator 3: **Joerg Romeis**

Non-target Organism Assessment (Part 2)

Facilitator 2: **Shannon Borges**

Facilitator 3: **Joerg Romeis**

Summary of discussions

Facilitators 1-3

Human Health Assessment

Facilitator 4: **Les Davies**

Facilitator 5: **Alan Raybould**

Summary of discussions

Facilitators 2-5

Wrap-Up and Leaving Address by the CRP

András Székács, CPR Scientific Advisory Body

Conference Results and next Steps

Mike Mendelsohn, US EPA; Dimitra Kardassi, EFSA; Achim Gathmann, BVL; Magda Sachana, OECD

Conference closes

Presentation Abstracts

Introductory session

Welcome by the CRP and introduction of the Programme

András Székács – CPR Scientific Advisory Body; National Agricultural Research and Innovation Centre, Hungary

The OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems (CRP) funds cutting-edge research on food, agriculture, fisheries and forestry with a focus on global issues such as sustainability, food security and nutrition, climate change and the inter-connectedness of economies through trade and scientific co-operation. The CRP helps achieve globally agreed policy objectives by facilitating international co-operation among research scientists and institutions. In doing so, it strengthens scientific knowledge and innovation.

With this focus on global issues, CRP-funded research generates benefits for people around the world. To deliver on these overarching challenges, the CRP has three themes into which funding proposals need to fit: Theme 1. Managing natural capital for the future; Theme 2. Managing risks in a connected world; Theme 3. Transformational technologies and innovation.

It achieves its objectives through two distinct activities:

• The CRP funds short-term research projects for individual scientists in other CRP member countries by providing travel bursaries to strengthen the exchange of ideas and increase international co-operation.

• The CRP sponsors international conferences and workshops to keep policy makers, industry and the academic world informed of innovative research, scientific developments and opportunities.

The CRP places a policy emphasis on all the activities it funds, and the findings from the activities provide valuable evidence and information to support policy makers in promoting the sustainable use of natural resources in food, agriculture, fisheries and forestry.

Find out more by visiting the CRP website: www.oecd.org/agriculture/crp.

Session 1: Summary of the State of the Art: dsRNA-based Product Use in Agriculture

An introduction to RNAi technology

Petr Svoboda – Institute of Molecular Genetics of the ASCR, Czech Republic

RNA interference (RNAi) refers to the selective degradation of mRNA induced by double-stranded RNA (dsRNA), first discovered *in Caenorhabditis elegans*. Related silencing pathways are present in almost all eukaryotes. The common feature of all RNA silencing pathways is utilization of small RNA molecules (20-30 nucleotides long), which form ribonucleoprotein complexes with proteins from the Argonaute family. In addition to Argonaute proteins, biogenesis of small RNAs often (but not always) involves activity of RNase III Dicer. Small RNAs in RNA silencing come from many different sources and have diverse biological roles such as regulation of protein-coding genes (genome-encoded microRNAs), innate antiviral immunity (RNAi) or genome protection by repression of mobile elements (RNAi and piRNA pathway in animals). Consequently, the ability of small RNAs to induce a sequence-specific gene silencing has been adapted for specific gene silencing in cultured cells and *in vivo* and became a common experimental strategy, which is being explored also in therapeutic and biotech applications. Importantly, achieving efficient and highly specific silencing requires appropriate experimental design and controls because artificial induction of RNAi may be accompanied with undesirable effects, particularly off-targeting where repression of other genes occurs in addition to the targeted one. In my contribution, I will provide an overview of the molecular mechanism of RNAi and relevant related pathways, will describe basic strategies for silencing using RNAi, and discuss potential non-specific silencing issues and available remedies.

Potential for dsRNA-based management of plant diseases

Karl-Heinz Kogel, Justus Liebig University Giessen, Germany

In plants, small RNA (sRNA)-mediated RNA interference (RNAi) is essential for regulating host immunity against bacteria, fungi, oomycetes, viruses, and pests. Similarly, sRNAs from pathogens and pests also play an important role in modulating their virulence and in developing a disease. Most strikingly, recent evidence supports that some sRNAs can travel between interacting organisms and induce gene silencing in the counter party, a mechanism termed cross-kingdom RNAi.

Our research aims at exploiting the natural role of RNA in plant - microbe /pest interactions as a blue print for novel plant protection strategies based on RNA (for *rev.* Cai et al. 2018, doi 10.1016/j.mib.2018.02.003). In recent years, several reports showed that double-stranded (ds)RNA has the potential to become an important tool for disease-control. In general, RNA can be used in two different technology application strategies. One strategy uses the expression of dsRNA in crop plants, where the dsRNA sequence is partially identical to a devastating target microbial pathogen or pest. This technology is called host-induced gene silencing (HIGS) and has shown unprecedented effectiveness in reducing pest and diseases; traits based on the new technology (such as the dsRNA-producing DvSnf7 transgenic corn) have already been approved by the United States Environmental Protection Agency (EPA) and by the Canadian Food Inspection Agency (CFIA). Alternatively, dsRNAs have been used to control pest and diseases by direct spray applications. Direct application of dsRNAs or sRNAs onto host plants or post-harvest products leads to spray-induced gene silencing (SIGS) of the target microbe/pest by environmental RNAi and may confer efficient disease control. Yet, few examples have been reported in literature. My talk will review the current understanding of cross-kingdom RNAi, its implications for HIGS and SIGS and how these findings can be developed into novel effective strategies to fight diseases caused by microbial pathogens and pests.

RNA interference technologies to control pests and pathogens

Steve Whyard, University of Manitoba, Canada

Chemical pesticides are widely used to protect crops from pests and pathogens and to reduce the spread of insect-borne disease. Increasing incidences of pesticide resistance and growing concerns about the adverse effects of broad-spectrum chemicals on non-target species is driving the need to find safer and more effective alternatives for pest control. RNA interference (RNAi), with

its in-built sequence-specific capabilities, is viewed by many research groups as a potentially species-specific method of pest and pathogen control. My research team has been developing a variety of RNAi-based methods to control crop insects, fungal pathogens, and disease-vectoring mosquitoes. Using topically-delivered ingestible dsRNA-based insecticides, we have demonstrated effective control of flea beetles in greenhouse settings, and are now conducting field trials to assess the technology under natural environmental conditions. We are examining the species-specificity of these dsRNAs on non-target species, to ensure that the dsRNAs do not adversely affect beneficial species in the canola farming landscape. We have also been developing topically-applied dsRNA-based fungicides to protect canola against pathogenic fungi. Small-scale field trials will be initiated to assess the dsRNA's effectiveness and specificity to control the pathogens without impacting beneficial fungi within the cropping system.

In addition to developing insecticidal formulations of dsRNA, we are also developing RNAi-based methods of producing sterile male insects for Sterile Insect Technique (SIT) programs. To date, we have focused our efforts on the mosquito *Aedes aegypti*, and on the fruit fly, *Bactrocera tryoni*. Through some of our advances in dsRNA delivery formulations, we have observed improved efficacy of RNAi in these insects, and believe that there is potential for this method of sterilization to complement existing methods to produce sterile males for other SIT programs. The prospect of taking this technology to the field and the various challenges that it will face will be discussed.

RNAi as a novel technology in pest control: current status and challenges

Olivier Christiaens, Ghent University, Belgium

Over the past decade, RNA interference (RNAi), the sequence-specific suppression of gene expression triggered by specific double stranded RNA (dsRNA) molecules, has proven to be a very promising strategy in crop protection. Some of the most alluring aspects of this technology are its species-selectivity and the short persistence in the environment of the dsRNA. Here, we will give an overview of some of the promising results in insect control and the different application strategies, which could be used, in the field, including host-induced gene silencing (HIGS), spray-induced gene silencing (SIGS) and virus-induced gene silencing (VIGS).

Furthermore, we will also address associated sequence-dependent and –independent biosafety aspects and some of the important challenges and barriers that need to be addressed before RNAi could be implemented as a widely used pest control strategy. One of these challenges is a variable efficiency. While some insects show a very robust, efficient and systemic RNAi response, many others show a limited or variable RNAi response. Possible causes for this variability in sensitivity are degradation of the dsRNA in the insect body, insufficient uptake into the cells, an impaired endosomal release, viral interactions or problems with the RNAi machinery in the cells. In recent years, many efforts have been made to increase RNAi-efficiency in some insensitive insects, using different formulations, which could be vital in future sprayable dsRNA applications. An overview of these will be given, with special attention to the use of formulations improving cellular uptake and the dsRNA persistence in the insect body.

Finally, we will also briefly discuss a recent EFSA report which was co-produced by our group, which gave an overview of (cellular) uptake of dsRNA, potential adverse effects, exposure routes, RNAi efficiency in insects and the question whether or not bioinformatics could ever have a role to play in environmental risk assessment of RNAi-based products.

Variation in responsiveness to environmental dsRNA in insects

Ana María Vélez Arango, University of Nebraska, United States

RNA interference (RNAi) is a sequence-specific regulation of gene expression triggered by the presence of double-stranded RNA (dsRNA). RNAi has been explored as a pest management tool for the control of insect pest for the last 12 years. For RNAi to work as an insect management tool, the insect must uptake dsRNA from the environment to regulate endogenous gene expression. The responsiveness to environmental dsRNA in arthropods is highly variable. This variability affects the ability to target different insects orders and the potential effects on non-target species. Coleopterans have shown to be the most susceptible species to environmental dsRNA, while the susceptibility of insects within the orders Hemiptera, Hymenoptera, Diptera, and Lepidoptera is variable. Differences in the response among species have been attributed to the presence of RNases, variations in the processing of dsRNA within the cell, and environmental factors. This presentation will provide an overview of the research to date investigating the factors that affect the responsiveness to environmental dsRNA in different insect orders.

Potential for off-target effects in topically applied dsRNA-based products used for crop protection purposes

Gunter Meister, University of Regensburg, Germany

For efficient gene inactivation, siRNAs are either exogenously applied or expressed as double stranded RNA (dsRNA) precursors from endogenous sources. SiRNAs are 21 nucleotides in length and one strand is incorporated into the RNA-induced silencing complex (RISC) and becomes the guide strand (also referred to as the antisense strand). It directly binds to a member of the Argonaute protein family (Ago proteins) and guides it to fully complementary sequences on target RNAs. A catalytically active Ago protein (Ago2 in mammals, for example) cleaves the complementary target RNA, which is subsequently removed from the cell. This process is generally referred to as RNA interference or short RNAi.

SiRNAs, however, can have off-target effects and target unrelated genes directly or indirectly. Known causes of off-target effects in animals will be summarized and reviewed in the talk. In many animal species, siRNAs mimic endogenous microRNAs (miRNAs) and utilize the miRNA machinery. MiRNAs are incorporated into RISC as well but do not guide it to fully complementary target sequences. Instead, miRNAs bind to only partially complementary sequences and induce mRNA de-adenylation, decapping and decay of the mRNA. The so-called 'seed sequence' spanning nucleotides 2-8 of the miRNA is sufficient to achieve efficient gene knock down. Most siRNAs that are applied possess unwanted seed-matched targets in the cell. In fact, based on our own analysis but also on published results, siRNA screening datasets in human cells are highly dominated by 'miRNA-like' seed matches and thus off-target effects. These data will be presented in the talk. One possibility to overcome such off-target problems, are siRNA-pooling strategies. This allows for a dilution of effects that are specific to individual siRNA sequences. In nematodes and insects, for example, where long double stranded RNA is used as RNAi trigger, such pools naturally occur and seed sequence-based off-target effects are most likely not as problematic as in higher animals such as mammals.

Environmental dissipation of dsRNA in soil, aquatic systems and plants

Pam Bachman, Bayer Crop Science

Determining the rate of biodegradation of double-stranded RNA (dsRNA) in the environment is an essential element of a comprehensive environmental risk assessment (ERA) of an RNA-based agricultural product. This information is used during problem formulation to define relevant routes and durations of environmental exposure for *in planta*–expressed and topically applied dsRNA based on the use pattern of the product. This presentation will examine the technical approaches and results of quantification of dsRNA in terrestrial, aquatic, and plant matrices in context of an ERA. These evaluations include a QuantiGene® assay to quantify the amount of dsRNA, and insect bioassays to measure functional toxicity where appropriate. Although exposure to these dsRNA products in terrestrial and aquatic environment is predicted to be minimal, little is known regarding the fate of dsRNA in these environments. To assess exposure to terrestrial and aquatic environments, a series of studies were conducted to measure the rate of biodegradation of dsRNAs in terrestrial soils and aerobic water–sediment systems. Results support the conclusion that dsRNA-based agricultural products rapidly degrade and consequently are unlikely to persist in terrestrial and aquatic environments. Data are also available that demonstrate barriers to the meaningful uptake of foliarly applied dsRNA, effectively exposing the dsRNA to degradation factors in the environment. Finally, data from *in planta*-expressed dsRNA demonstrates a quantifiable low level of residue can be discerned to inform the regulatory risk assessment.

Validation of RNA interference by RNA-Seq: How to see the big picture

Brenda Oppert, United States Department of Agriculture, United States

Targeting genes via RNA interference (RNAi) has become a successful method to reduce some pest insect populations. Ideally, pest-specific gene expression is targeted with dsRNA to disrupt a gene critical for life function, via spray or oral delivery. Experts have developed working guidelines for the development and regulation of RNAi as a pesticide. In this summary, I argue that an important step in the validation of RNAi is understanding global gene expression in the target pest before and after dsRNA is introduced via RNA-Seq. To support this hypothesis, I provide data from our studies of RNAi in the coleopteran model Tribolium

castaneum. These studies have led to new discoveries of insect responses to injected dsRNA, including transcriptome compensation responses that are similar to what has been observed at the protein level. Gene expression compensation can mask RNAi responses and thus can be an obstacle in predicting efficacy of dsRNA treatment. We also have observed differential expression of 52 long noncoding RNAs following knockdown of a cuticle protein gene ($p<0.01$), providing insight into the mechanism of knockdown on overall gene expression. In some cases, we identified new gene interactions that were previously unassociated with the target gene. For example, knockdown of aspartate 1-decarboxylase, a cuticle sclerotization gene, caused increased expression of a dopamine receptor and consequently reduced mobility in RNAi-treated insects. These data emphasize the importance of using RNA-Seq as a tool to understand known and unknown effects of target gene knockdown. This information can be used to predict genetic changes that will impact the efficacy and long-term durability of RNAi products in target pests.

Dietary uptake of environmental dsRNA in humans and other vertebrates

Thais B Rodrigues, Greenlight Biosciences, United States

Products incorporating double-stranded RNA (dsRNA) as the active ingredient have emerged as environmentally friendly biological-based solutions for pest control. These products function by engaging the endogenous RNA interference (RNAi) pathway present in most eukaryotic organisms to silence expression of a specific gene in the target pest. This sequence-dependent mode-of-action contrasts sharply with the broad-spectrum activity of traditional synthetic insecticides and enables development of products designed to kill a specific target pest without impacting non-target organisms. This selective activity, coupled with the rapid degradation of dsRNA in the environment, support a favorable overall safety profile. As a non-transformative spray, dsRNA has the potential to be used in a variety of agricultural applications, ranging from control of specific pests and pathogens on diverse crops, to protection of bees from viral infection. As a new mode of action, dsRNA is also well positioned to add an additional tool to existing pesticide resistance management strategies. However, as with any emerging technology, the potential range of future products, potential future regulatory frameworks, and public acceptance of the technology are all evolving topics. The aim of this presentation is to summarize the published literature related to dietary uptake of external dsRNA in humans and other vertebrates, providing a solid foundation for focused discussion sessions and panel discussions that will help draw recommendations and inform further improvements to regulatory processes. Information gathered from studies examining the effect of dietary uptake of external RNAs by mammals and other vertebrates will be summarized to review our current understanding of the potential impacts and risks on these non-target organisms. Mammalian barriers to uptake and systemic distribution of dietary dsRNA, such as RNA degradation in the digestive system, will be discussed. We will also discuss the potential of ingested RNAs to regulate specific metabolism in mammals, as well as the influence of human health conditions on susceptibility to RNA uptake.

Regulatory experience with antisense oligonucleotides for human use

Frank Holtkamp, Medicine Evaluation Board, The Netherlands

The European Medicines Agency (EMA) is a decentralised agency of the European Union (EU) responsible for the scientific evaluation, supervision and safety monitoring of medicines in the EU. For new medicinal products submitted for marketing authorisation, the EMA reviews whether a positive benefit risk balance can be identified to recommend for possible approval within the EU. EMA has reviewed several antisense oligonucleotides (AONs) submitted for marketing authorisation. These AONs are currently primarily focused on treating rare occurring diseases caused by a genetic problem, therefore the available clinical safety data is relatively limited. Current knowledge on the safety aspects of these AONs will be presented and discussed.

DAY 2

Session 2: Summary of Regulatory & Risk Assessment Experience with dsRNA-based Products

Problem formulation considerations for externally applied dsRNA-based products

Alan Raybould, Syngenta

Risk comprises the probability and severity of harm that may result from undertaking an activity, such as applying a pesticide. Risk is high when severe effects are likely and low when harmful effects are predicted to be rare or trivial, or both. Estimates of risk contribute to decisions about whether to take certain actions; other relevant factors may be estimates of the likely benefits of those actions and the risks from not taking action. Efficient and effective risk assessment relies on problem formulation, which includes several vital steps: 1. agreement on what effects should be regarded as harmful; 2. formulation of hypotheses about how the proposed activity may lead to such harmful effects; 3. tests of those hypotheses with existing data; and 4. a plan to acquire new data for hypothesis testing should tests with existing data be insufficient for decision-making. By beginning with defining harm and how it may be caused by the proposed activity, problem formulation encourages the development of decision-making criteria early in the risk assessment; in effect, risk assessment becomes a test of a hypothesis that the proposed activity does not pose unacceptable risk. This approach may be particularly valuable when assessing uses of innovative products, such as those with new modes of action, where distinguishing between basic research hypotheses ("nice to know") and hypotheses about decision-making criteria ("need to know") may be more difficult than for more familiar products. We illustrate how problem formulation can guide risk assessments for spray applications of insecticides containing dsRNA active ingredients that induce RNAi in target insects.

Ecological assessment of topically applied dsRNA-based products

Jörg Romeis[1], Franco Widmer[2], [1] Research Division Agroecology and Environment, [2]Competence Division Method Development and Analytics, Agroscope, Zurich, Switzerland

RNA interference (RNAi) is an emerging and powerful technology that offers new opportunities for pest control through the silencing of target genes in arthropod pests. Because the RNAi effect is sequence-specific, dsRNA can be designed to have a very narrow spectrum of both activity and target organisms, thus allowing very targeted pest control. While successful RNAi has been reported from a number of arthropod species belonging to various orders, the impact of environmental RNAi (i.e., an RNAi response after dsRNA uptake) is more limited. However, RNAi in pest control may be achieved by applying dsRNA in foliar sprays. One of the main concerns related to the use of dsRNA in pest control is that it could cause adverse effects on the environment and in particular on valued non-target species. Arthropods form a major part of the biodiversity in agricultural landscapes and contribute to important ecosystem services. This includes, in particular, regulating services such as pollination and biological control of herbivores, supporting services such as nutrient cycling, and cultural services in the case of species of conservation concern. Consequently, environmental risk assessment (ERA) to assess the potential impacts that plant protection products may have on valued non-target arthropods is legally required prior to their placement on the market.
Early in the ERA, problem formulation is used to set the problem context and to develop plausible pathways on how the application of plant protection products containing dsRNA could harm valued non-target arthropod species. In this presentation, potential pathways will be presented and testable risk hypotheses will be identified. Furthermore, suggestions will be presented on how these hypotheses can be tested in a proportionate and tiered manner.

Review of EFSA's activities on the risk assessment of RNAi-based GM crops

Nikoletta Papadopoulou, Fernando Álvarez-Alfageme, Yann Devos, Anna Lanzoni, Claudia Paoletti, Elisabeth Waigmann, European Food Safety Authority, EU

Genetically modified (GM) plants intended for market release can be designed to induce silencing of specific genes *in planta* or in target pests through RNA interference (RNAi). As part of the pre-market risk assessment (RA), the European Food Safety Authority (EFSA) evaluates any risks that GM plants may pose to the animal and human health and the environment. Potential risks associated with the use of RNAi in GM plants were considered at an international scientific workshop organised by EFSA. Experts from academia, RA bodies and the private sector discussed the biology underlying the RNAi mechanisms, current and future applications of RNAi-based GM plants, and RA approaches. The outcome of the workshop helped determine in which areas

the existing approaches for RA are appropriate, and whether complementary or alternative RA strategies need to be developed for RA of RNAi-based GM plants (EFSA, 2014; Ramon et al., 2014, Casacuberta et al., 2015). In addition, limitations in methods to unequivocally identify potential off-target effects, and remaining scientific uncertainties on the likelihood of exposure of humans, animals and the environment to dsRNA and deriving small RNAs (miRNA, siRNA) were addressed at the workshop. To further address these issues, EFSA commissioned separate external scientific reports, in which relevant scientific literature has been reviewed to inform food and feed and environmental risk assessment of RNAi-based GM plants (Paces et al., 2017; Christiaens et al., 2018). Furthermore, the EFSA GMO Panel, considering the available scientific information and the technical limitations for small RNA off-target bioinformatics studies, developed a strategy on how to identify and assess plant off-target effects in RNAi-based GM plants[73], which was recently implemented on the safety assessment of DvSnf7 dsRNA-expressing GM maize (EFSA GMO Panel, 2018).

1) European Food Safety Authority, 2014. International scientific workshop 'Risk assessment considerations for RNAi-based GM plants' (4–5 June 2014, Brussels, Belgium). EFSA supporting publication 2014:EN-705, 38 pp.

2) Ramon M., Devos Y., Lanzoni A., Liu Y., Gomes A., Gennaro A. and Waigmann E., 2014. RNAi-based GM plants: food for thought for risk assessors. Plant Biotechnology Journal, 12 (9): 1271-1273.

3) Casacuberta J.M., Devos Y., du Jardin P., Ramon M., Vaucheret H., Nogué F. 2015. Biotechnological uses of RNAi in plants: risk assessment considerations. Trends Biotechnology, 33(3):145-147.

4) Paces J, Nic M, Novotny T and Svoboda P, 2017. Literature review of baseline information to support the risk assessment of RNAi-based GM plants. EFSA supporting publication 2017:EN-1246, 314 pp. https://doi.org/10.2903/sp.efsa.2017.en-1246.

5) Christiaens O, Dzhambazova T, Kostov K, Arpaia S, Joga MR, Urru I, Sweet J, Smagghe G, 2018. Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. EFSA supporting publication 2018:EN-1424. 173 pp. doi:10.2903/sp.efsa.2018.EN-1424

6) EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H,Birch AN, Casacuberta J, De Schrijver A, Gralak AM, Guerche P, Jones H, Manachini B, Messean A,Nielsen EE, Nogue F, Robaglia C, Rostoks N, Sweet J, Tebbe C, Visioli F, Wal J-M, Ardizzone M,De Sanctis G, Fernandez Dumont A, Gennaro A, Gomez Ruiz JA, Lanzoni A, Neri FM, Papadopoulou N, Paraskevopoulos K and Ramon M, 2018. Scientific Opinion on the assessment of genetically modified maize MON 87411 for food and feed uses, import and processing, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2015-124). EFSA Journal 2018;16(6):5310, 29 pp.

The European perspective on regulatory aspects and experiences with dsRNA-based products

Achim Gathmann, Federal Office of Consumer Protection and Food Safety (BVL), Germany

RNA interference (RNAi) is a means of reducing or switching-off the expression of individual genes, often described as 'gene silencing'. RNAi is a natural process with important defence and regulatory functions in animals, plants and fungi. RNAi technology is widely used in GM plants. Sprayable dsRNA-based plant protection products are in the pipeline aiming at different targets such as flea beetles in oil seed rape, fusarium diseases in barley, or weed control to overcome resistant weeds.

RNAi constitutes a new mode of action in "conventional plant protection products". In the past, the authorisation process of plant protection products focused on chemical substances. This new type of products places new demands on the authorisation process. While the characteristics of dsRNA as active ingredient might ease the assessment of some risk areas, for other risk areas there is a need for adaptions of existing or the development of new risk assessment tools. Additionally, new formulations to resist rapid degradation of dsRNA after product application such as liquid encapsulation, conjunction with polymers or nanoparticles might challenge the risk assessment.

The presentation will give a short introduction into the authorisation process in the EU. Some aspects regarding specific challenges for risk assessment and risk management of this new type of plant protection product will be highlighted. A short view on activities

[73] Annex II of the minutes of the 118th GMO plenary meeting https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf

and experiences gained in the assessment of GM plants using RNAi mechanisms in Europe will complement the presentation considering similarities and differences in risk assessment of biotechnical and classical plant protection products

Ecological Risk Assessment Considerations for in planta Expressed and Exogenously Applied dsRNA at the U.S. EPA

Shannon Borges, US Environmental Protection Agency, United States

Double-stranded RNA (dsRNA)-based pesticides require special considerations for ecological risk assessment (ERA) because of their unique nature and mode of action. The U.S. EPA has developed problem formulations for dsRNAs expressed in planta and applied exogenously. These problem formulations were presented to the FIFRA Science Advisory Panel (SAP) in 2014, and relevant recommendations by this SAP were applied to the ERA for the DvSnf7 dsRNA expressed in planta in corn (a plant incorporated protectant [PIP]). This ERA relied primarily on the results of bioassays, but also considered other lines of evidence, including environmental fate in soil and water, barriers to dsRNA uptake in vertebrates, bioinformatics analyses, and bioassays to determine specificity to the target pest. Additional bioassays beyond the standard data set for PIPs were required to assess the potential for unintended effects, such as off-target gene silencing. The EPA determined that this DvSnf7 dsRNA corn PIP did not present risks to non-target organisms or the environment. While the EPA has not yet conducted an ERA specifically for an exogenously applied dsRNA-based pesticide, many of the lessons learned from this experience are applicable to exogenously applied dsRNA-based pesticides.

A perspective on risks associated with dsRNA-based products

Neena Mitter, Centre for Horticultural Science, Australia

By 2050, it is estimated that up to 10 billion people will populate the planet and in order to meet their daily need of two trillion calories the global agriculture industry will have to sustainably increase production by 70%. The emerging concept of pre- and post-harvest topical application of dsRNA to trigger gene silencing in pest and pathogens offers a paradigm shift in agriculture that promises to contribute to this need. As with any new technology, there are potential risks, including to human health, the environment, and trade. The regulation of dsRNA-based topical products needs to be viewed differently from GM crops. Importantly, the Office of the Gene Technology Regulator (OGTR) in Australia has proposed that topically applied dsRNA be exempt from GMO regulations. The development of dsRNA-based crop protection products leveraging the naturally occurring RNAi pathway share the history of exposure to, and consumption of, small RNAs. The registration of dsRNA-expressing crops such as western corn rootworm-resistant maize provides some regulatory synergy in this area. The presence and persistence of dsRNA in environmental matrices as well as the exposure and uptake by non-target organisms (NTOs) need consideration. Some of these risks are addressed by available information on barriers to exposure and cellular uptake, potential degradation of dsRNA, instability of dsRNA in soil/environment and within the recipient organism, and the inherent sensitivity of the organism to ingested dsRNA. Bioinformatics-based design to avoid off-target impacts in NTOs mitigates risks further. Potential risks associated with formulation ingredients including carriers for delivering external dsRNA should be examined on a case-by-case basis. Finally, early engagement with all stakeholders will be paramount to acquire consumer acceptance and public support given the fundamental advantages of the new technology.

Session 3: Discussion themes

Background to the draft OECD Working Paper on 'Environmental Risks from the Application of dsRNA-Based Pesticides'

Les Davies,OECD Consultant, Canberra, Australia

The purpose of the working paper is to (1) provide an overview of available scientific information on RNAi relevant to externally-applied dsRNA-based pesticides (including basic biological mechanisms of RNAi, the environmental fate and behaviour of dsRNA molecules, and the possible impacts of small RNAs on non-target organisms); and 2) highlight issues for consideration by regulatory authorities in conducting environmental risk assessments of dsRNA-based pesticide products. The document is organised into sections, which provide an overview of available scientific information relating to RNAi, regulatory experience to date with pesticide products involving an RNAi mechanism, and considerations regarding environmental risk, including

environmental fate and non-target effects. While not directly focussing on the risk assessment of plants which have been genetically modified to incorporate the machinery to synthesise dsRNA directed against pest species, regulatory history and risk considerations for in-planta RNAi are discussed in the context of externally-applied dsRNA-based pesticides.

The initial draft was prepared by Les Davies, OECD consultant, and was based on publicly-available scientific and regulatory information.