White Paper
on
RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment

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RNA-Interference FIFRA Scientific Advisory Panel White Paper

In the past several years, a number of pharmaceutical and agricultural products based on RNA interference have been developed. Most of these products are pharmaceuticals aimed at turning off aberrant protein expression (e.g., cancer genes or macular degeneration), but a few agricultural products utilizing RNA interference have also been developed for pest control. The potential for increasing use of RNA interference to control pests has lead EPA to conclude the Agency should convene a Scientific Advisory Panel (SAP) to consider aspects of risk assessment the Agency uses to determine whether an RNA interference based PIP can meet the environmental safety standard of the Federal Insecticide Fungicide and Rodenticide Act (FIFRA).

EPA is not requesting of the SAP a review of any of the Agency's past or current decisions related to RNA interference based products, but would appreciate a consultative discussion on issues that might arise as the technology develops and finds wider application in the future. Given the likelihood for development of both plant expressed RNA interference products for pest control (i.e., RNAi in Plant-Incorporated Protectants or PIPs) and other types of dsRNA pest control products (i.e., non-PIP dsRNA end use products, e.g., the dsRNA the Agency views as a biochemical), EPA addresses both types of products in this White Paper, and requests discussion on both types from the SAP.

I. Background: Agricultural Products Based on RNA Interference with an Emphasis on Those Reviewed by EPA

The FlavrSavr™ tomato, one of the first plant products of modern biotechnology to be approved for human consumption in 1992 by the Food and Drug Administration, , was developed using what was then termed "anti-sense" technology. The FlavrSavr™ tomato was engineered using the antisense technology to turn off expression of a polygalacturonase enzyme involved in the softening of the fruit during ripening. In 1998, a potato engineered to express the gene for potato leaf roll virus replicase was registered as a PIP by EPA (EPA, 1998a). This product differed from the products previously examined by EPA in that the company could not detect any replicase protein being expressed, yet its stable phenotype[1] was resistant to viral infection. The resistance traits of these plant transformations have subsequently been theorized as being due to the process of RNA interference.

More recently, EPA registered another PIP expressing a transgene which delivers viral resistance in European plum to the plum pox virus via an RNAi pathway; this PIP expresses a coat protein

[1] EPA was able to make a dietary safety finding for this virus resistant PIP based on the fact that virus infected produce is known to long be part of the food supply without any untoward effect on human and domestic animal health.

gene from the plum pox virus (EPA, 2010a). This PIP does not produce detectable quantities of coat protein as the coat protein transgene functions to suppress infection by the plum pox virus. In 2013, the EPA approved an experimental use permit (No. 524-EUP-104) for field evaluation of corn engineered to express a transgene construct which includes the *Snf7* gene which suppresses mRNA encoding vacuolar ATPase subunits A and E (Bachman et al., 2013). This PIP targets the Western corn rootworm, and published data indicate the specificity of the *Snf7* gene to the target pest ATPase mRNAs with no activity noted toward the other insect species evaluated.

II. RNA Interference – Overview

Interference or suppression of gene expression by naturally occurring double stranded ribonucleic acid-based mechanisms was largely unknown or misunderstood until the work of Andrew Fire and Craig Mello (Fire et al., 1998) which earned them the Nobel Prize in Physiology or Medicine in 2006. Previous efforts to modify plants, insects, mammals and other organisms with transgenes or antisense constructs producing single stranded RNA (ssRNA) were well documented (Izant and Weintraub, 1984; Fire et al., 1991; Lee et al., 1993; Guo and Kemphues, 1995; Matzke and Matzke, 1995; Goodwin et al., 1996; Akhmanova et al. 1997; Taylor, 1997), however, the discovery that double stranded RNA (dsRNA) was 10 times or more potent in its effect on gene expression than ssRNA unleashed a wealth of research into the biochemical basis for this mechanism known as RNA interference (RNAi) (Fire et al, 1998).

Post-Translational Gene Silencing (PTGS) or co-suppression was noted in many instances in the late 1980s and 1990s wherein transgenic sequences introduced into plant genomes often resulted in transient expression followed by silencing and return to the untransformed phenotype (de Carvalho et al., 1992; Finnegan et al., 1994; Flavell, 1994). Plant resistance to RNA viruses had been demonstrated to function well when DNA sequences matching the sequence of coat protein or other viral genes were introduced into plants as transgenes; however the mechanisms for both observations remained nebulous, although many theories were proposed.

Working with *Caenorhabditis elegans*, a common saprobic nematode, the laboratories of Andrew Fire and Craig Mello ascertained that injected dsRNA sequences matching known gene sequences in the nematode genome was quite effective at gene silencing (Fire et al., 1998; Grishok and Mello, 2002). Injection of dsRNA sequence matching a *mex-3* gene of *C. elegans* resulted in an absence of *mex-3* mRNA and an alteration in phenotype *(i.e.,* embryonic arrest) in contrast to ssRNA injections which yielded little change in corresponding mRNA populations and > 90% embryo production.

Evidence for naturally occurring gene silencing, and that resulting from introduction of foreign DNA or RNA sequences, has been noted in numerous prokaryotic and eukaryotic organisms to date (Waterhouse et al., 1998; Makarova et al., 2006; Pridgeon et al., 2008; Auer and Frederick, 2009; Huvenne and Smagghe, 2010; Younger and Corey, 2011; Adamo et al., 2012). As

research continues utilizing RNA interference (RNAi) for elucidation of biochemical pathways, genomic structure analysis and for practical applications like pest or disease resistance in plants, insects and humans, it is clear that differences exist at the molecular level regarding the details of the RNAi pathway to gene silencing between groups of organisms and sometimes even within species of a single genus.

A. General Features of the RNA Interference Pathway

While differences exist among organisms in the biochemical details of the RNAi pathway, some general features of the mechanism appear to be conserved. The various forms of dsRNA clearly show differences in structure and in initial production / processing stages; however, they also share a common mode of action. All of these dsRNAs are processed by Dicer or a Dicer-like nuclease, to yield small-interfering RNAs (siRNAs) which then attach to an Argonaute (AGO) family protein preferentially binding to one (guide strand) of the two strands of RNA in the 20 to 30 nucleotide (nt) molecule; the other RNA strand or passenger strand is typically degraded following release from AGO in the RNA Induced Silencing Complex (RISC). AGO is an RNAse H type enzyme, an endonuclease, which degrades mRNAs with complementarity to the bound miRNA or siRNA sequence. This activity, which utilizes the guide strand to target and bind complementary mRNA sequences for subsequent degradation is referred to as Slicer activity.

Long dsRNA molecules are thought to be critical to triggering RNA interference. The long strands of dsRNA that trigger RNA interference can originate outside the cell or be internally produced. Small interfering RNA is the product of Dicer activity on longer molecules of dsRNA. The siRNA molecules are also double stranded from 21-24 nucleotides (nt) in size with a 2 nt overhang at the 3'end. These were first described in studies of transgene-induced and viral-induced silencing in plants (Hamilton and Baulcombe, 1999; De Paoli et al. 2009). siRNAs may be processed from long dsRNAs and short hairpin RNAs, as well as being exogenously supplied by injection into a cell or via transformation or transfection. In addition to their role in RNAi through PTGS, siRNAs are known to effect chromatin modifications (e.g., cytosine or histone methylation, histone acetylation).

There are also other forms of RNA molecules triggering RNAi that can be generated internally from the host chromosome such as the micro RNA (miRNA), PIWI-interacting RNA (piRNA) and trans-acting RNA (tasiRNA). While all these RNA types are stated as being separate entities, they are similar in structure when involved in the RNA interference pathway. All these RNA forms are processed by cellular systems that result in short 21-31 nt single stranded RNA guide molecules loaded into the RNA induced silencing complex (RISC) (e.g., Argonaute or its homologues with perhaps other associated enzymatic activities; Pratt and MacRae 2009). The RISC enzymatic complex is guided by the 21-31 nt guide sequence loaded into Argonaute for specific recognition, binding and activity toward a target mRNA molecule. In the instances involving siRNA and miRNA, AGO is a part of RISC, which achieves gene silencing by degradation of the corresponding mRNA or by transcriptional repression (Wilson and Doudna,

2013). The outcome of this binding can be either direct cleavage of the target mRNA by RISC or repression of the targeted mRNA translation. The piRNA and tasiRNA have been associated with chromatin restructuring. The overall effect of RNAi is suppression of the targeted gene's phenotype.

siRNAs rely on a high degree of specificity or complementarity based upon sequence alignment with the target mRNA. miRNAs, in contrast, do not always require the same degree of base sequence complementarity in order to effect gene silencing through either mRNA degradation or chromatin modification.

dsRNA appears to be a key element in RNAi but there are several sources of the dsRNA. dsRNA molecules may be produced within the nucleus of a cell in the form of pri-miRNA (primary micro RNA) which are produced by RNA Polymerase II activity on a host gene and are approximately 1000 nt long. These long dsRNAs are processed by a ribonuclease III (RNAse) enzyme known as Drosha and a dsRNA binding protein known as Pasha (in invertebrates) or DGRC8 (DiGeorge Syndrome Critical Region 8; in mammals), collectively referred to as the microprocessor complex, to form pre-miRNA molecules. These pre-miRNA (precursor miRNA) molecules, approximately 70 nt in length and containing stem and loop structures, are exported to the cytoplasm via the nuclear pore complex and bind with a RNAse III enzyme called Dicer (or Dicer-Like 1 (DCL1) in plants; Dcr1 and Dcr2 in insects). This enzyme cleaves the RNA sequence into short sequences, miRNAs, typically 21-25 nt in length. This binding to Dicer also initiates association with the larger RISC complex where binding of corresponding mRNAs will ultimately take place. Interestingly in plants, the DCL1 activity occurs in the nucleus and not the cytoplasm. For animal miRNAs, (vertebrates and some invertebrates) the two reactions are split spatially, one in the nucleus and one in the cytoplasm. To aid in accurate processing of plant miRNAs and RNA strand selection, an RNA binding protein HYPONASTIC LEAVES 1 (HYL1) and the zinc finger protein SERRATE (SE) function coordinately with DCL enzymes (Manavella et al., 2012a).

piRNAs (PIWI-interacting RNAs) are short ssRNAs (26-31 nt) present in vertebrates and some invertebrates, found in both the nucleus and cytoplasm whose biogenesis is unclear as they do not require Dicer to produce an effect. They are known to cause gene silencing, often targeting retrotransposon sequences, and also have a role in epigenetic phenomena which may be maternally heritable. piRNA are especially present in germ-line cells and are known to influence spermatogenesis.

Within the group of small dsRNAs known as siRNAs, transacting siRNAs (tasiRNAs) are endogenous RNAs which down regulate genes at loci different from which they arose (Peragine et al., 2004; Vasquez et al., 2004). TAS genes, loci from which tasiRNAs are derived, are known from work in *Arabidopsis thaliana*, *Oryza sativa*, other higher plants and moss, but have not yet been identified in animals or fungi. While they have some similarities with miRNAs and other siRNAs in that they post-transcriptionally silence gene expression, the biogenesis of tasiRNAs

differs in the requirement for RNA-dependent RNA polymerases (RDRP) and SGS3 (suppressor of gene silencing), known to be present in plants in multiple forms. One or more forms of DCL enzymes are also required for proper processing to the final 21 nt length, as are components typical of the miRNA pathway (e.g., AGO1, HEN1, HYL1). tasiRNAs are derived from longer dsRNAs as compared to miRNAs.

TasiRNAs are incorporated into the RISC complex and thereby direct the cleavage of target mRNAs, albeit with less fidelity relative to sequence alignments, to degrade mRNA. This reduced sequence specificity in tasiRNAs is shared by some miRNAs as well. It has been demonstrated in plants that miRNA directed cleavage of target mRNA sequences can lead to the production of secondary siRNAs which can lead to the degradation of non-targeted mRNA through the formation of transitive RNAs (Himber et al., 2003; Manavella et al., 2012b). Transitivity can be triggered by the specific AGO involved in the RISC complex as well as the 22 nt length of the guide strand retained in the effector complex. Additionally, asymmetrical bulges in the miRNA:miRNA* duplex resulting from a lack of complete complementarity can trigger transitivity. Why some miRNAs trigger transitivity and some do not is not well understood at this time. A similar transitive effect has been seen in *C. elegans* following amplification of new dsRNA and a resulting non-autonomous effect (Alder et. al., 2003; Roignant et al., 2003). That is, the silencing of a gene targeted in one cell can lead to the silencing of a second gene in a distinct cell type. This phenomenon has not been noted in *Drosophila* and its absence attributed to the lack of RdRP activity in this insect (Roignant et al., 2003).

Small RNAs which act upon highly related sequences (mRNAs) are referred to as auto-silencing RNAs whereas small RNAs which act upon sequences derived from largely unrelated loci with minimal sequence similarity are referred to as hetero-silencing RNAs (Vasquez et al., 2004). The latter are termed trans-acting in that they target mRNAs from genes to which they bear little resemblance and the siRNAs which target closely related and highly homologous sequences are termed cis-acting RNAs.

In addition to the modulation of gene expression via mRNA degradation (e.g., PTGS), as directed by some miRNAs and siRNAs, heterochromatic siRNAs modify histone proteins and DNA (cytosine) associated with heterochromatin in insects and fungi (Reinhart and Bartel, 2002). By altering chromatin rather than mRNA, these siRNAs influence gene expression at the transcriptional level. These siRNAs derive from endogenous sequences through dsRNA precursors and are processed by Dicer, then bind to a RNA-Induced Transcriptional Silencing (RITS) complex, at least in *Drosophila* and *Schizosaccharomyces pombe* (Fagegaltier et al., 2009). RITS complexes contain Argonaute, a chromodomain protein Chp1, and Tas3, and they interact with a RNA-directed RNA polymerase, a polyA polymerase (Cid12) and a helicase (Hrr1), termed RDRC, necessary for the formation of dsRNA. Both RITS and RDRC interact with noncoding RNA transcripts derived from pericentromeric areas of the chromosome to trigger the RNA interference pathway and assembly of heterochromatin. A methyltransferase

(Clr4) aids in the association of RITS and RDRC with chromatin and is in part responsible for the formation of siRNA. Methylation of histone protein 3 is mediated by this methyltransferase in *Schizosaccharomyces pombe*, the fission yeast; methylation of histone proteins and cytosine bases can provide transgenerational alteration of phenotype through their impacts on gene expression of select chromosomal regions.

Ago1, RDRC and Dicer are all required in the formation of siRNAs from specific noncoding RNA transcripts (Halic and Moazed, 2010). In the absence of Dicer, transcriptomal degradation products termed primal RNAs interact with Ago1 to drive the initial formation and amplification of siRNA in the absence of heterochromatin (Conte and Mello 2010). Interestingly, it appears that heterochromatin formation in humans and other mammals is independent of the RNAi pathway.

B. Post-Transcriptional Repression Through Deadenylation

The RNAi pathway in plants, fungi and animals also includes post-transcriptional repression of mRNA translation through decapping and deadenylation pathways which serve to decrease the stability of mRNAs and to trigger their ultimate degradation. Three different types of deadenylase complexes (i.e., PARNS, CCR4-Not, PAN) are known to deadenylate mRNA following shortening of the 3' poly-A tail. As the tail is shortened, the mRNA typically undergoes decay by resident exoribonucleases, present in the cell cytoplasm in plants (Abassi et al., 2013). The removal of the 5' 7-methylguanylate cap (decapping) may or may not be linked to the 3'-5' degradation of mRNAs, however, it does preceed the 5'-3' degradation of mRNA sequences by exonucleases such as XRN1. In mammals, miRNAs are known to bind to the 5' untranslated region (UTR) 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 destabilization of mRNA.

C. RNAi and Cellular Maintenance

RNAi pathways can be seen as a normal part of cell maintenance and as a response to stress events (e.g., viral infection, abiotic stress) which seek to maintain homeostasis. Some have characterized RNAi pathways as a surveillance system to prevent translation of aberrant mRNAs and other nucleic acid sequences (e.g., viruses, transposons) (Zamore et al., 2000). Although the details of the RNAi pathways and their outcomes may differ among organisms, what is clear is that the influence of small RNAs on growth, development, defense and even transient heritability of traits is substantial. With that in mind, the potential for modification or manipulation of these pathways for pest management, agronomic production and essentially any other desired phenotypic alteration is great and will lead to a variety of approaches to delivery of small RNAs.

siRNAs are often delivered to plant or animal cells from exogenous sources, although, as described above, they can also arise from the host genome (i.e., miRNA, tasiRNA,

heterochromatic RNA). While most miRNAs arise from endogenous DNA sequences, efforts are underway to manipulate these sequences to effect the targeting of select mRNAs. Early experiments which helped divulge the existence of the RNAi pathway relied upon soaking in dsRNAs or ingestion of these dsRNAs by *C. elegans* (Fire et al., 1998). Injection of small RNAs into the gonads of *C. elegans* or tissues of other organisms has also been a well used delivery procedure for research purposes, however, not as a practical approach for agricultural uses.

Plant transformation, that is the introduction of novel nucleic acid sequences into the plant genome, is an obvious delivery system for expression of novel traits in plants, including dsRNAs (Bachman et al., 2013). However, delivery of dsRNAs to plants, insects or other organisms via spraying with dsRNA (Wang et al., 2011) or being carried in a microbe (Zhu et al., 2011) are also feasible approaches depending on the target organism and ability of that organisms to take up the RNA directly. Oral ingestion through feeding is a common approach to test the RNAi response in insects (Turner et al., 2006). In *C. elegans*, Sid-1 (Systemic RNAi Defective) has been shown to be critical to dsRNA uptake in some instances and capable of effecting systemic spread of the RNAi signal (Gu and Knipple, 2013). Receptor mediated endocytosis has also been demonstrated in these two organisms for uptake of dsRNA from the gut lumen.

The fate and impact of small dsRNAs on organisms has been divided into three principle groupings: cell autonomous, environmental and systemic (Gu and Knipple, 2013). Cell autonomous RNAi refers to instances wherein the effect of the dsRNA is in the cells where the dsRNA was produced or exogenously supplied and is therefore limited spatially. Environmental RNAi impacts result from exogenous uptake from the immediate environment, such as an insect gut or hemocoel, or even from incidental contact in the soil. If the silencing effect of the dsRNA continues to spread among neighboring cells, even cells in which the target RNA does not exist, this is referred to as systemic RNAi. Environmental and systemic RNAi are termed non-cell autonomous since the dsRNA does not have to be produced at the site (cell) of action.

RDRP is present and active in *C. elegans*, notably amplifying the level of siRNAs and aiding in the systemic RNAi response (Sijen at al., 2001). It is known, however, that siRNA amplification and even core RNAi components are not necessary for systemic RNAi induction (Tomoyasu et al., 2008). Among insect species, much of the RNAi machinery is largely conserved, although responses to dsRNA, including systemic spread are highly variable (Aronstein et al., 2011; Terenius et al., 2011). RDRP is not known from insects although one cannot rule out the presence of alternative enzyme activities with overall little direct homology to RDRP as known from other organisms.

As discussed further in the sections to follow, the EPA is formulating a risk assessment approach for products using RNAi-based pathways to perform its duties under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA), the Food Quality Protection Act (FQPA) and the Federal Food Drug and Cosmetic Act (FFDCA) relative to the safe use of a pesticide and any

impacts upon man and the environment. Uncertainties clearly exist with respect to a complete understanding of all current and future applications of this technology, however, the Agency's previous experience with RNAi-based PIPs and attention to ongoing developments in research on small RNAs provides a basis for such assessment. This FIFRA Scientific Advisory Panel will examine potential impacts upon humans from consumption of RNAi-based PIP and non-PIP products, interactions of dsRNA with non-target organisms, and the environmental fate of dsRNA when introduced as part of a PIP or as a direct spray application of dsRNA for pest management. The questions proposed to this panel will help to focus the discussion on the Agency's needs relative to data gaps which may exist.

III. Mammalian Safety Assessment

The current risk assessment for mammalian safety for PIP and biochemical products utilizes data and information determined on a case by case basis. The product characterization information accurately describes the pesticidal product, its original source, its mode of action, how it was introduced and how it can be detected. For biochemical pesticides, there is an additional discussion of the manufacturing method, including consideration of any materials used during manufacture or added to formulate the final product.

The hazard analysis of PIPs depends on information about the specific amino acid sequence and comparative analyses using computer algorithms to discover any significant sequence similarities to known toxins or allergens. There is also basic chemical information about the stability of the protein to heat and digestive enzyme activity. Finally, there is toxicity information from studies performed with a purified protein or from information in the peer-reviewed scientific literature. The studies for mammalian safety determinations of PIP proteins utilize the microbial guidelines (40 CFR 158.2140) and typically consist of a single acute toxicity study and the *in vitro* and bioinformatic analyses. For biochemical pesticides, the hazard analysis is based on the tiered testing scheme described in the guidelines (40CFR 158.2050) which considers the results of toxicity studies along with information from the peer-reviewed scientific literature to make a safety finding.

Safety determinations for non-PIP dsRNA products may need to rely on selected guideline studies under 40CFR 158.2050 due to likely stability differences between PIP and non-PIP dsRNAs. It is anticipated that non-PIP dsRNAs that are exogenously applied to target pests or targeted use sites, will be formulated into end-use products (EPs) in a manner that will increase their stability to abiotic and biotic degradation in the environment (see a more detailed discussion in Section IV.B. of this document), to provide sufficient residence time in/on the treated articles for the dsRNA to accomplish the desired pesticidal activity. The presence of "stabilized" dsRNA in/on treated articles increases the potential opportunities for topical, inhalation, and

dietary exposure that can be assessed with the traditional Tier I hazard testing for biochemical pesticides. These studies are listed below:

- Acute oral toxicity
- Acute dermal toxicity
- Acute inhalation toxicity
- Acute eye irritation
- Acute dermal irritation

Depending upon the residence time of "stabilized" dsRNA following application, additional longer term studies may be needed, such as:

- 90-oral toxicity
- 90-day dermal toxicity
- 90-day inhalation toxicity

Finally, an exposure assessment is performed on the product based on its intended use, if the results of the hazard analysis warrant that consideration.

A general approach to the risk assessment of mammalian health effects for dsRNA molecules used for pest control resembles the process described above for protein PIPs and biochemicals but can be further developed based on two considerations. These considerations are directed towards factors that might mitigate hazards and limit possible exposure. The first is the design of dsRNA sequences to control pests. Appropriate sequence selection to trigger pest control may lessen the potential for affecting non-targeted species. A thorough bioinformatics search and comparison should be performed to insure specificity of the pest control sequence; i.e., an appropriate sequence should display no significant similarity to other non-targeted gene sequences, especially those found in mammalian species. The second consideration is the low likelihood that any significant mammalian exposure could result from dietary or other anticipated exposures (e.g., dermal or inhalation) once the digestive degradation and environmental fate of dsRNA is examined. These suppositions need to be made explicit and examined for supporting data. Information supporting these suppositions include: (1) an understanding of the molecular basis of the RNA interference phenomenon as it relates to the safety assessment; (2) results of human therapeutic research on the use of RNAi technology to treat human disease, and (3) research indicating the level of sequence specificity and/or amount of dsRNA necessary to trigger successful RNA interference.

A. A Brief Overview of the Molecular Basis of the RNA Interference (RNAi) Phenomena as it Informs the Mammalian Safety Assessment.

As noted in the previous section of this White Paper, there are three forms of dsRNA molecules that are generally acknowledged to be involved in RNA interference in mammals: micro RNA (miRNA), PIWI interacting RNA (piRNA) and small interfering RNA (siRNA). miRNA is transcribed from the genome as a pri-miRNA then processed and exported from the nucleus as pre-miRNA with a distinctive small hairpin or stem-loop structure. The piRNA is involved in silencing transposons during germ cell formation. The siRNA is the product of Dicer activity on longer molecules of dsRNA. These longer strands of dsRNA can originate outside the cell or be internally produced. The siRNA molecules are also double stranded from 21-24 nucleotides (nt) in size with a 2 nt overhang at the 3'end. While all three of these RNA types are stated as being separate entities, they are similar in structure when involved in the RNA interference pathway. All these RNA forms are processed by cellular systems that result in short 21-31 nt single stranded RNA guide molecules loaded into the RNA induced silencing complex (RISC) (e.g., Argonaute or its homologues with perhaps other associated enzymatic activities) . The RISC enzymatic complex is guided by the 21-31nt sequence loaded into Argonaute for specific recognition, binding and activity toward a target mRNA molecule. The outcome of this binding can be either direct cleavage of the target mRNA by a slicer activity in the RISC complex or repression of the targeted mRNA translation. The overall effect of RNAi is suppression of the targeted gene's phenotype.

Differences between the miRNA, piRNA and siRNA are critical for their expected function. miRNAs originate in the nucleus and have the secondary structure of a hairpin or stem loop with the dsRNA sections that can be processed into 21-24 nt siRNAs. These smaller siRNAs can then be loaded into the RISC complex and have mostly host developmental functions of up or down regulation of the host genome. piRNA also originates in the nucleus, does not have such a complex secondary structure, may not be double stranded prior to loading into the RISC complex as 26-31 nt species and functions mostly to inactivate transposons in germ line cells, especially those associated with mammalian spermatogenesis. siRNA is thought to be the product of Dicer activity on longer dsRNA molecules, is generally 21-24 nt when functional in the RISC complex and is believed to have higher sequence complementarity to trigger mRNA cleavage. Both miRNA and piRNA require less sequence complementarity than siRNA and have been more frequently involved in translational pausing in mammals and higher organisms rather than direct mRNA cleavage.

The RNA interference phenomenon is found in most forms of life but there are significant differences in some of the components depending on the taxonomic kingdom or group. One significant difference has been the presence of an RNA dependent RNA polyermase (RdRP) in plants and nematodes but not in vertebrates. RdRP is responsible for increasing the number of dsRNA molecules present and for the systemic spread of RNAi in plants. Systemic spread has been associated with viral disease resistance and other systemic signaling effects in plants. The lack of systemic spread in vertebrates has made delivery of therapeutic agents for RNA interference more challenging.

B. Therapeutic Research in Humans

In mammals, and humans in particular, there has been a great deal of research to describe the RNAi system which has resulted in information that is cogent to dietary risk assessment. There has been extensive research using RNAi for reverse genetics to examine the effects of trait silencing. The most relevant use of RNAi applicable for the mammalian health risk assessment has been the development of pharmaceutical drugs to treat diseases by silencing specific cellular functions. The dsRNA based pharmaceuticals have generally been shown to be effective in cell culture studies but delivery to the target organ by the oral, dermal or inhalation route has been difficult (Castanotto and Rossi, 2009). 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 or encapsulation is necessary to facilitate transmembrane movement (Castanotto and Rossi, 2009). Some RNAi interventions have also shown off-target effects on the treated animal where other genome sequences were silenced along with the target sequence and immunostimulatory effects, particularly on the innate immune system have been noted (Whitehead et al., 2011; Meng et al., 2013). It is important to note that since there is no new protein being expressed, the use of RNA interference technology, should not present any allergenicity issues. The use of RNAi as PIPs presents a lower exposure scenario by all routes for mammals compared to the stabilized and sprayable formulations of dsRNA (see section V. on non-PIP dsRNA end-products)

C. Open Questions on Sequence Specificity

The implication that sequence specificity of the dsRNA molecule drives the recognition and subsequent degradation of the target mRNA should allow a robust bioinformatic analysis of the proposed target gene and the dsRNA product. EPA has not yet established what form this bioinformatic analysis should take and what level of similarity would trigger concern for off-target effects. There are already reports that rice, corn and other crop plant species have siRNA species present that have high homology to human gene sequences (Ivashuta et al., 2009). These represent similarities in the 21-24 nt range which would not be unexpected given simple probability analysis for such short sequences. The significance of the random short homologies versus longer contiguous homologies has not been examined. However, if significant homologies of the overall dsRNA product are found with mammalian genes or regulatory regions, it would be prudent to examine the dsRNA molecules potential effect more closely. The specificity of the Dicer enzyme in the generation of siRNA species is not clear so that the need for a 21nt stepwise or other modified bioinformatic analysis to predict off-target effects is still unsubstantiated. The presence of a 3’ overhang of one to three nucleotides is a dominant force for the specificity of Dicer activity but longer overhangs or blunt ends will also generate the siRNA species (Vermulean, 2005).

Indications are that the majority, if not all, orally administered dsRNA is degraded by the acid environment of the stomach and the action of pancreatic RNases (Rehman et al., 2011; Sorrentino et al., 2003; Hoerter et al., 2011). This degradation should lessen the probability that any dsRNA PIP could survive gut passage and be taken up at a concentration that would trigger RNAi. 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). If the need for high copy number delivery to target cells is accurate, there may also be levels of RNA molecules with potential similarity to mammalian genes that could be safely consumed without triggering RNA interference.

However, there are still questions of the stability of individual dsRNA molecules and if chemically modified forms or RNA molecules with significant secondary structure such as hairpins or super coils, could survive acid and enzymatic degradation leading to greater exposure. There are reports in the literature that miRNA molecules from plants consumed in the diet have been absorbed and shown to induce RNAi in humans (Zhang et al., 2012). There have also been subsequent reports that tend to refute the findings of significant uptake of miRNA from the diet (Witwer, 2013; Snow, 2013). There are also several reports on the lack of successful development of RNA based therapeutic drugs for oral delivery which also question the dietary exposure and uptake of RNA molecules (Petrick et al., 2013; Castanotto and Rossi, 2009).

IV. Environmental Risk Assessment

Double-stranded RNA may be utilized as a pesticide that is expressed as a plant incorporated protectant (PIP), or it may be applied to the plant or the environment in the form of a foliar spray, post-directed spray or drench, seed treatment, or granule/powder. While exposure scenarios may differ between dsRNA PIPs and exogenously applied dsRNAs, EPA would have similar considerations for environmental fate of dsRNAs and nontarget effects under the different exposure scenarios. The sections below discuss the unique issues relevant to exposure of nontarget organisms to dsRNA PIPs and exogenously applied dsRNAs, as well as the uncertainties related to the current approaches to risk assessment.

A. Environmental Considerations for PIP-Expressed dsRNA

Over the past two decades, ecological risk assessments have been performed for numerous PIPs, most of which express proteins derived from the bacterium *Bacillus thuringiensis* (*Bt*, e.g., see EPA, 2005; 2010b; 2010c). The current approach to ecological risk assessment for *Bt* PIPs provides a framework for risk assessment of dsRNA PIPs. However, as this section will describe, not all aspects of the fate of dsRNA in the environment and potential effects on nontarget organisms are necessarily understood. As a result, dsRNA PIPs may present unique challenges for ecological risk assessment that have not yet been encountered for other PIPs.

This section discusses issues and uncertainties related to the two major components of ecological risk assessment as these relate to dsRNA PIPs, which are the estimation of environmental exposure and determination of effects resulting from exposure. Details of the approach generally used by EPA to assess ecological risk for other types of PIPs (e.g., see EPA, 2005; 2010b; 2010c) are presented herein only where potential differences of approach may be required for the risk assessment for dsRNA PIPs. Some aspects of ecological risk assessment commonly considered for PIPs, such as potential for gene flow through outcrossing, potential for development of invasiveness, and horizontal gene transfer, are not discussed because EPA assumes that the current approach that has been used for *Bt* PIPs would also apply to dsRNA. The discussion in this section is focused on to assessing risks dsRNA PIPs may present to nontarget organisms.

1. Nontarget Exposure

Important considerations in any environmental risk assessment are the stability of the pesticidal substance, in this case the dsRNA, and the potential routes through which the pesticidal substance may be distributed to the environment. As this section of this issue paper examines dsRNA that is tightly integrated into the plant, i.e., is a PIP, the analysis will focus on the expression and stability of dsRNA in the plant and how dsRNA integrated into plant tissue may persist or move through the environment, thus creating the conditions of potential exposure to nontarget organisms.

The degree of nontarget exposure depends on the distribution and fate of the dsRNA PIP within the environment, as well as the potential routes of exposure, exposure duration, and potential for uptake. These factors are discussed here, as well as qualities unique to dsRNA PIPs that may introduce additional uncertainty into exposure estimates.

a. dsRNA Distribution and Fate in the Environment

Factors that affect the distribution of dsRNA in the environment and the potential for exposure to nontarget organisms include physical movement of the PIP crop plant tissue, persistence and physical movement of dsRNA released from the plant, either while the plant is living or following breakdown of plant tissue. EPA assumes that the dsRNA expressed in a PIP crop plant would be present wherever any of that crop plant tissue exists or is moved unless sufficient data are presented to show otherwise. On the crop field, living plant tissue will be present both above and below the soil surface where the crop plants are grown. During the growing season, plant tissue may be moved within the field through pollen release or the breakage and physical movement of other plant tissues (e.g., leaves) from the plants. Ultimately, these plant tissues are expected to reach the soil surface, though pollen may fall onto plant surfaces first. Once the plant is harvested, crop debris could be deposited on the ground surface or tilled into the soil. Root

tissue is expected to remain in the soil until it breaks down or is harvested, except in the case of perennial plant for which living root tissue would persist.

It is unclear at this point whether a dsRNA PIP also would be incidentally present in root exudates, guttation droplets, or nectar, providing additional on-field sources of nontarget exposure. This may be affected by the characteristics of the dsRNA or the plant. Most PIPs target insect pests that chew plant tissue, and exposure considerations thus have been focused on nontarget organisms that eat solid plant tissues. However, some current work is focused on using dsRNA expressed *in planta* to control plant hoppers and other sucking insects (Pitino et al., 2011; Zha et al., 2011), and Mlotshwa et al. (2002) indicate that systemic gene silencing may occur as a result of movement of the silencing signal in phloem, although the exact mechanism is not confirmed. These studies suggest that these sources of exposure may also need to be considered in a risk assessment.

Off-site movement of plant tissue is also possible. Depending on whether the dsRNA is expressed in the pollen, significant consideration may be given to the role pollen may play in off-field nontarget exposure. Some pollen is expected to move off site, and the amount and the distance moved will depend on the characteristics of the pollen (e.g., morphology, weight) and the mechanism relied upon for pollination (e.g., wind, pollinators, self-pollination). Animal-pollinated crops would be expected to play a smaller role in off-field nontarget exposure, and concerns in these cases would be more focused on the exposure of the pollinators themselves. Other plant tissues are also expected to move off site, and an assessment would consider whether these tissues could contribute significantly to nontarget exposure, as well. In some crops, post-harvest plant debris may be left on the field and in the immediate field margins. Debris may be moved further away from the field in terrestrial environments by wind, water, or another mechanism (e.g., physical movement by man), and may also enter aquatic systems where it could remain near the site of entry or be moved by water currents. Additionally, harvest of some crops can create dust out of dried plant material, which may move offsite. For determining the extent and degree of off-field nontarget exposure related to plant tissue movement, EPA would consider the expression and stability of the dsRNA PIP within plant tissue, and may also rely on data on pollen movement, which are often available. Where data are not available, EPA may make case-specific assumptions based on cultural practices and physical characteristics of the crop plants.

Plant material containing dsRNA would act as a source of exposure for organisms that directly ingest plant tissue; however, once plant tissue breaks down and cells lyse, the dsRNA may also be released into the environment. Degradation of the plant tissue will affect the potential for exposure, since RNA is degraded within the plant during senescence (Pietramellara et al., 2009). However, not all dsRNA is necessarily expected to be degraded by the time plant material reaches the ground. Once released from plant cells, dsRNA is expected to ultimately end up in the soil in terrestrial environments, where it may persist or be transported to other terrestrial or to

aquatic areas. Plant material that is moved into aquatic areas would provide a source of dsRNA in aquatic environments, where it may move into sediment or be present in the water column.

Studies show that DNA can persist in soil by binding to humic substances or minerals, or it may be degraded by microbes and nucleases (Levy-Booth et al., 2007; Pietramellara et al., 2009). DNA can persist for a few days in soil or for several years (Levy-Booth et al. 2007). Little is known about the fate of dsRNA in the environment, and the chemical and structural characteristics of RNA and DNA may be sufficiently different to prevent use of data developed on the environmental fate of DNA as a surrogate for RNA. In the absence of any environmental fate data, EPA can estimate conservative environmental exposure concentrations based on expression data and assumptions about persistence; however, EPA typically receives soil degradation data on *Bt*-derived PIPs, which provide information about the potential for their persistence in the soil. Thus far, these studies, in addition to longer term (i.e. multi-year) soil degradation studies, have been considered to be sufficient in developing an understanding of the persistence of *Bt*-derived PIPs in the environment. While focus only on soil degradation may also be adequate for dsRNA PIPs, additional information on the potential for dsRNA PIPs to move vertically and laterally with water in soil, bind to soil and sediment particles, and the effects of binding to soil particles on persistence and bioavailability (e.g., by protection from nucleases, see Pietramellara et al. 2009) could aid in determining the spatial extent of exposure and the on- and off-field levels of exposure to nontarget organisms.

b. Nontarget Exposure Opportunities

In risk assessments for PIPs, EPA typically considers exposure to birds, wild mammals, freshwater and marine/estuarine fish, aquatic invertebrates, nontarget insects, including honey bees, nontarget plants, and soil invertebrates. These taxa are considered sufficient representatives of the potential for risk to all nontarget organisms for purposes of screening level risk assessments, though refined risk assessments may consider exposure at lower taxonomic levels.

For most PIPs, since they are expressed in the crop plant, nontarget organism exposure is expected to occur primarily through ingestion of the living or dead plant material, which can occur on or off the field in which the crop is growing. Nontarget organisms, particularly insects, may consume pollen produced by the crop plant, either while it is on the plant or once it has fallen within the crop field or outside it. Pollen may be consumed directly as food (or, in the case of bees, may be carried back to the hive or nest to feed to larvae), or may be consumed incidentally on food plants upon which the pollen falls. While pollen consumption may be more likely to be a means of exposure for insects, it is also possible for other animals (e.g., grazing vertebrates) to consume plants upon which pollen has fallen. Plant material that becomes dust, as described above, could also incidentally expose nontarget organisms to dsRNAs.

Secondary exposure may also occur, where organisms that have ingested dsRNA in plant tissue (e.g., nonsusceptible pests) are consumed by nontarget predators or parasites. The factors influencing the possibility of exposure by this pathway (e.g., longevity of dsRNA once consumed, concentration resulting within the herbivorous insect) are not known. However, Whyard et al. (2009) observed susceptible insects feeding on dsRNA for several days before deleterious effects were observed, which would present an opportunity for this type of exposure. Garbian et al. (2012) also showed that dsRNA could be transferred from bees to *Varroa* mites, indicating that this type of exposure is possible.

Ingestion of pesticidal dsRNA that is free in the environment (following plant tissue breakdown) may also occur if it is present in soil, sediment or water. Incidental ingestion of dsRNA present in soil and sediments by nontarget organisms is a possibility. This is expected particularly of invertebrates that live in soil or sediment; however, incidental soil ingestion by vertebrates foraging along these substrates could also occur (e.g., birds probing soil for invertebrates). Any free dsRNA in the water column may be inadvertently consumed by both vertebrates and invertebrates, both by organisms living in such environments and those organisms that use them as a source for water.

Exposure to free dsRNA via the integument (in animals) is not a route that has been considered in nontarget risk assessments for *Bt*-derived PIP proteins; however, evidence exists that this may be a possibility for dsRNA. Whyard and colleagues cite examples in which RNAi was induced in nematodes and flatworms by soaking these animals in a solution of dsRNA. They also cite research in which gene silencing was observed in dechorionated *Drosophila* embryos (see Tabara et al., 1998; Orii et al., 2003; and Eaton et al., 2002 as cited in Whyard et al., 2009). Campbell et al. (2010) also observed significant gene knockdown in *Varroa destructor* with soaking in a solution of NaCl and dsRNA. More information is needed, particularly since the method of soaking in these experiments could have actually led to ingestion of the dsRNA, but these data may indicate the possibility of exposure through this route. Organisms that might be exposed in this way could include animals in aquatic environments as well as those inhabiting soil and sediment, though EPA recognizes that the exposure concentration in these environments may be low. Uptake by plants in both terrestrial and aquatic environments may also be possible, but little information also exists on the potential for plant exposure to dsRNAs in the environment.

The above is meant to provide a comprehensive listing, in light of the limited information available on dsRNA in the environment, of potential routes through which nontarget organisms might be exposed to dsRNAs expressed in crop plants. EPA recognizes that not all of the routes discussed above may be significant to the risk assessment, since some may have low likelihood of occurring and other factors (e.g., environmental concentration, barriers to uptake) may also limit exposure. Nonetheless, at this point in time, recognition of the potential of these scenarios

to affect nontarget organisms might be advisable in assessing the risk of dsRNA PIPs, even if only to show that these scenarios are not a consideration for the particular PIP.

c. Other Factors Affecting Exposure Levels

The level and duration of exposure can be influenced by additional factors, even once dsRNA exposure has occurred. Exposure by ingestion to dsRNA may be reduced by barriers that exist in the gut. Potential barriers for uptake in mammals were discussed previously for human health exposure and effects. Some evidence for similar barriers also exists for arthropods. For example, Allen and Walker (2012), observed successful gene knockdown in the tarnished plantbug (*Lygus lineolaris*) when dsRNA was injected into the hemocoel; however, when dsRNA was fed to the insect, RNAi was not successful. The lack of effects was attributed to dsRNA digestion by nucleases in the insect gut. This study also showed that the saliva of *L. lineolaris* degraded double-stranded RNA. On the other hand, exposure by both injection and feeding successfully resulted in reduced expression of targeted genes in the triatomine bug, *Rhodnius prolixus* (Araujo et al., 2006). Other conditions of the gut environment, such as pH, may also influence breakdown of dsRNA, and mechanisms of uptake may also vary (Terenius et al., 2011; Katoch et al., 2013). However, much of what we understand about RNAi in living organisms is limited to mammals and arthropods, and little work has focused on the barriers to uptake that exist in other organisms. Better understanding of the mechanisms influencing uptake, particularly if they can be extrapolated to other organisms, would reduce uncertainty in exposure assumptions and help to focus risk assessments on the most appropriate organisms.

For purposes of assessing risk, exposure levels are typically assumed to be equivalent to the highest amount expressed among various plant tissues, except where the focus may be exposure to a particular type of plant tissue (e.g., exposure via pollen collected by bees). The 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 effects of gene silencing. As discussed previously, mammalian cell culture studies indicate that a minimum number of copies of siRNA may be needed at a targeted site to induce RNAi, whereas Fire et al. (1998) suggest that only a few RNA molecules are sufficient to initiate the RNAi pathway. Huvenne and Smagghe (2009) also suggest that 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 evidence, there may be environmental exposure levels below which no effects, or above which no greater degree of effects, would necessarily occur. It is also possible that lower limits of exposure may be essentially non-existent for some species. More clarity on this issue and its potential influence on exposure estimates are needed.

Some organisms have the ability to amplify the RNAi signal by utilizing environmental siRNA as a template to produce additional siRNA, leading to exposure within the organism that is more prolonged and greater than that taken up. siRNA amplification has been observed in *C. elegans*, which accomplish this through RNA-dependent RNA polymerase (RdRP)(Sijen et al., 2001). This phenomenon has also been observed in plants and fungi (Dillin, 2003). While amplification by RdRP has been characterized in these organisms, whether a similar mechanism exists in other organisms has not been determined. If the ability to amplify the RNAi signal is possible in other organisms, this may 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 uptake at the cellular level is not possible in some organisms, then the environmental exposure would be irrelevant. Further understanding of these mechanisms would aid in deriving exposure estimates and would reduce uncertainty.

2. Ecological Effects to Nontarget Organisms

a. EPA's Current Testing Framework

Data requirements for PIPs are determined on a case-by-case basis, and are based primarily on the tiered microbial pesticide data requirements for environmental fate and nontarget effects in 40 CFR 158.2150. The list below describes what are considered Tier I nontarget effects and environmental fate studies that are typically received for new PIP active ingredients. These studies generally have been considered sufficient to inform nontarget risk assessments for *Bt*-derived PIPs to date. The preferred test material for these studies is the *Bt* protein, extracted from a microbial source (e.g., *Escherichia coli*) and purified; however, in some cases, plant material (e.g., grain, pollen, lyophilized leaf tissue) has also been used. In these studies, test subjects are typically fed a maximum hazard or limit dose, and mortality is the endpoint measured. Some studies with insects (historically the focus of *Bt*-derived protein PIP nontarget risk assessments) have also included observations of effects on growth and reproduction of test subjects. Studies that are considered higher tier studies, such as field studies on the effects of beneficial insects or multi-year soil persistence studies, have also been required, though they are currently not relied upon as heavily for new *Bt*-derived PIP active ingredients.

<u>Nontarget effects</u>

Wild mammal toxicity
Avian oral toxicity
Avian dietary toxicity
Freshwater fish toxicity
Freshwater invertebrate toxicity
Estuarine/marine animal toxicity
Honey bee toxicity (adult and larvae)
Nontarget plant toxicity
Arthropod toxicity
Non-arthropod invertebrate toxicity
Monarch butterfly (in specific cases)
Synergistic effects (for combination PIPs)

<u>Environmental Fate</u>

Soil degradation

b. Unique Challenges Presented by dsRNA PIPs

Risks of deleterious effects of *Bt*-derived PIPs on nontarget organisms assessed by EPA thus far have been determined to be minimal, and this is due in large part to the well-characterized specificity of *Bt* proteins. The current testing framework for PIPs, relying primarily upon first tier tests with focus on acute endpoints, is considered to be adequate for informing nontarget risk assessment for these types of PIPs. While dsRNA produced in crop plants as a means of pest control has been claimed to have a high degree of specificity (Petrick et al. 2013; Whyard et al. 2009), evidence suggests that it may have other unexpected effects. Therefore, dsRNA PIPs may present new challenges for ecological risk assessment.

In general, specificity does not appear to be guaranteed for dsRNAs when fed to insects. For example, Whyard et al. (2009) observed slight cross-species silencing among four insect species fed dsRNAs that were meant to target genes in other species. Terenius et al. (2011) also were not able to confirm specificity of RNAi following examination of data from numerous studies with RNAi in insects, and also proposed that variation in sensitivity of the target gene to RNAi and in the sensitivity of the tissue targeted may influence effects of dsRNA. Baum et al. (2007) observed target gene silencing and reduced survival in *Leptinotarsa decemlineata,* but silencing also occurred in an ortholog to the target gene in *Diabrotica virgifera virgifera*, although silencing in the latter species required exposure to greater dsRNA concentration. Therefore, even when intended to target a specific gene, effects among related organisms have been observed.

"Off-target" effects may also result from binding of siRNAs to genes other than the target. These effects appear to be related to partial sequence homology between the "seed region" (positions 2-7 or 2-8) of the siRNA and the 3' untranslated region of messenger RNA transcribed from a nontarget gene (Birmingham et al. 2006, Jackson et al. 2003), though Birmingham et al. (2006) note that such binding is likely one factor in a complex process that is not yet fully understood. Off-target effects are siRNA specific, instead of target-gene specific, and may influence other signaling and transcription pathways (Jackson et al. 2003). They have also been shown to reduce cell viability in controlled studies (Fedorov et al. 2006). Since RNAi reduces or eliminates translation of a mRNA gene product into protein, but does not alter gene expression directly (Huvenne and Smagghe 2010), off-target effects may not be severe or prolonged if they were to occur. This will, of course, depend on the sensitivity of the nontarget organism, the gene(s) targeted, and the potential for amplification of siRNAs. Nonetheless, little information is available with which to estimate the likelihood of these effects resulting from unpredicted interactions of dsRNA with genes not intended for silencing and they may be significant factors affecting nontarget risk (Auer and Frederick 2009, Lundgren and Duan 2013).

Systemic RNAi, discussed in more depth in section II.C., could be an additional contributing factor in the observation of unexpected effects. For example, Terenius et al. (2011) describe work with *Hyalophora cecropia* in which RNAi was observed in the offspring of insects injected as pupae, indicating uptake of the dsRNA by oocytes in the pupae. Such effects also indicate a potential influence of life stage on RNAi, which is discussed further below. On the other hand, Whyard et al. (2009) failed to observe systemic RNAi in *D. melanogaster*; therefore, systemic RNAi may be possible in some species but not others. The mechanisms driving the systemic RNAi among species are not known, and studies to date do not cover the possibility of this effect, or lack thereof, in all taxa that EPA considers in its risk assessments.

RNAi has necessary functions within cells that are important to growth, development and tissue homeostasis. Oversaturation of RNAi machinery as a result of introduction of environmental dsRNA could disrupt regulation of gene expression and normal cell function (Dillin 2003, Katoch et al. 2013, Lundgren and Duan 2013). Saturation could also lead to reduced defenses against viral infection (Dillin 2003). Exposure to RNAi may also stimulate immune response. This has been observed in mammals (see Lundgren and Duan 2013), and could be a factor for observed gene silencing in some insect studies (Terenius et al. 2011); however, how immune stimulation by RNAi may affect nontarget organisms is not known.

c. Possible Limitations of the Current PIP Testing Approach

Considering the unique mode of action of dsRNAs that may serve as PIPs and the unintended effects of RNAi gene silencing described above, it would seem that risk assessors should remain cognizant of the possibility of unexpected effects that may not be readily predicted based on the known specificity for the target gene. The current approach for testing/assessing PIPs for potential risk will serve to provide much information for risk assessment; however, owing to the nature of dsRNA and RNAi, some questions may remain as to how well the current suite of required studies can detect potential effects of dsRNA PIPs on nontarget organisms.

Better information with which to estimate exposure could be valuable to ecological risk assessments for dsRNA PIPs. Some data are available with which to determine the potential for dsRNA uptake in mammals, which may be extrapolated to other vertebrates with similar physiological characteristics (e.g., acid stomachs); however, less information is available for vertebrates with other characteristics. Additionally, some research has focused on barriers to uptake in arthropods, but clear trends are not available with which to make predictions. More information on barriers to dsRNA uptake or dsRNA degradation after uptake, particularly data that would facilitate predictions across nontarget taxa, could refine exposure estimates or allow assumptions of minimal exposure in certain organisms.

More information on specificity and the realistic possibility and impacts of the unintended effects described above on nontarget organisms may also help to focus testing needs. At this point, unintended effects primarily have been described from laboratory screening tests (e.g., microarray analyses) or testing in which the exposure was artificial (e.g., microinjection in insects). Without further information, it is not unreasonable to assume that some kind of related effect could occur in nontarget organisms; however, at this point the actual biological impacts are not known.

EPA would rely on environmental fate and nontarget toxicity testing to inform the ecological risk assessment; however, the unique nature of dsRNA and RNAi raise several issues of concern with respect to the typical data set submitted for nontarget effects:

1) The potential influence of latent effects on results of nontarget testing. As discussed above, arthropods in laboratory studies have been observed feeding for several days on dsRNA before the effects of gene silencing were apparent. Several of the toxicity studies submitted for PIPs are short term dosing studies, and latency of effects may impact observations of both sublethal effects and mortality. Some studies, such as nontarget insect studies, are carried out for sufficient time to observe effects on reproduction, and latent effects would more likely be observed. Therefore, the importance of this issue could vary between different types of studies.

2) The appropriate life stage for testing. Toxicity testing is often targeted toward young test organisms, as it is assumed that younger organisms are more sensitive to toxic substances than adults. Much of the work that has been done with arthropods suggests that younger life stages are more sensitive to RNAi (Huvenne and Smagghe, 2009). However, sensitivity may not follow a specific trend over time, but may be the result of the importance of a target gene, or a gene affected by off-target silencing, in the organism at a specific life stage. It is likely that dsRNA designed for delivery to pest insects in plants would target young life stages, so testing on those life stages would be appropriate. However, given the range of possible unexpected effects, it is conceivable that an effect could occur in the field that would not be observed in the lab.

3) The possibility of chronic effects. Current testing focuses on mortality as the endpoint. Off-target effects, or binding to target genes in a less sensitive organism, could cause effects that do not lead to lethality. This has been observed in studies with insects. For example, Zha et al. (2011) observed suppression of target genes encoding a hexose transporter, a carboxypeptidase, and a serine protease in the brown planthopper (*Nilaparvata lugens* Stål), but did not observe significant lethality. Suppression of genes without overt signs of toxicity may be considered insignificant following a single exposure; however, long-term exposure and continuous or repeated gene knockdown could result in chronic effects. Since pesticidal substances are produced continuously in a PIP crop plant, chronic exposure to some organisms is possible.

Approaches other than nontarget testing may provide additional information and reduce data requirements. For example, while there are currently limitations to the use of bioinformatics analysis, particularly for nontarget organisms for which there are limited genomic data, this type of approach may be useful in identifying groups of nontarget organisms that are more or less susceptible to the effects of dsRNA PIPs. Further development of predictive tools also has potential to reduce uncertainty as well as the scope of testing required to support a risk assessment.

3. Conclusions – dsRNAs Expressed As PIPs

RNAi gene silencing is a relatively recent discovery, and there are general uncertainties with the current state of knowledge about dsRNAs and their potential environmental fate, as well as the importance of effects observed in laboratory studies to actual nontarget hazard. The current approach as used for *Bt*-derived PIPs may be sufficient; however, information in the literature suggests the need to consider additional effects beyond those typically investigated for *Bt*-derived PIPs. At present, EPA could move forward with a risk assessment based on the current approach; however, the resulting risk predictions could have uncertainty or require conservative assumptions. Therefore, EPA seeks guidance on the natural processes in the environment and within nontarget organisms that serve to reduce or eliminate exposure, the importance of the potential for unexpected effects of dsRNA PIPs and RNAi in nontarget organisms, and additional testing that EPA may require to reduce uncertainties in risk estimates.

B. Non-PIP dsRNA End Use Products

The current approach used by the Agency to assess the ecological risks of traditional chemical pesticides (conventional chemicals and biochemicals) may also be used as a basis for ecological risk assessment of non-PIP dsRNA-containing end-use products (dsRNA-EPs). As stated above in Section IV of this paper (Environmental Considerations for PIP-expressed dsRNA), environmental fate and effects of dsRNA are poorly understood and present unique challenges for ecological risk assessment that have not yet been encountered in assessments for traditional chemical pesticides

The Agency has many years of ecological risk assessment experience with PIPs and the previous section of this document has explored the various pathways whereby dsRNA expressed in PIPs may move into the environment and result in nontarget exposure. Unlike PIPs, however, the Agency has not, to date, assessed the hazards or risks of dsRNA applied directly to the environment as components of end-use products intended for pest control under Section 3 of FIFRA. Numerous ecological risk assessments have been conducted on traditional chemical pesticides (EPA, 2012a). However, the screening level assessments currently used for traditional

chemical pesticides may not be applicable due to the unique modes of action of dsRNA active ingredients. As stated above in Section IV.A., EPA's goals are to ensure that unreasonable effects do not occur to nontarget populations. Details of the approaches used by Office of Pesticide Programs to conduct ecological risk assessments on traditional chemical pesticides may be found at EPA's Ecological Risk Assessments webpage (EPA, 2012b).

This section of the paper will focus on descriptions of the various exposure pathways that may result following the application of dsRNA-EPs. The potential human health and ecotoxicological effects of dsRNA have been discussed in detail in previous sections of this paper, and will be discussed here only in the context of uncertainties and likely data needs. In order to delineate the possible pathways of exposure, there first will be a discussion of the likely categories of dsRNA active ingredients intended for use in pest control products, as well as the likely end-use products and application methods. This section of the paper will conclude with a brief discussion of issues and uncertainties related to estimation of environmental exposure and determination of effects, the two major components of an ecological risk assessment, for non-PIP dsRNA-EPs.

At the time of this writing, no entity has approached the Agency with an application to register a non-PIP dsRNA-EP. While based on publically-available information, the following discussion regarding the different categories of dsRNA active ingredients and application methods, is speculative.

1. Likely Categories of Active Ingredients

At his time, the Agency anticipates four likely categories of dsRNA active ingredients that could be present in non-PIP dsRNA-EPs: (1) Direct control agents; (2) Resistance factor repressors; (3) Developmental disruptors; and (4) Growth enhancers.

a. Direct Control Agents

A dsRNA direct control agent is defined here as a dsRNA active ingredient that has direct toxic effects upon the metabolism of the pest resulting in mortality. The difference between dsRNA activity and traditional chemical pesticides is that dsRNA operates at the level of the gene, whereas traditional pesticides have toxic physiological effects at higher levels of organismal metabolism. The family of dsRNA direct control agents likely would include, but are not limited to herbicides, insecticides, and fungicides. This dsRNA active ingredient category does not depend upon chemical pesticide control, and could be rotated into integrated pest management (IPM) systems to reduce chemical pesticide use and lessen the possibility of resistance development by the target pests.

b. Resistance Factor Suppressors

A dsRNA resistance factor suppressor is defined here as a dsRNA active ingredient that suppresses genetic resistance to a traditional chemical control. Many pest species have become resistant to chemical control since the advent of widespread chemical pesticide use in the 1940s and there is a long history of the development of resistance to chemical controls by insects (IRAC, 2013). More recently, herbicide resistant weeds have becoming a major worldwide pest control problem (Mortensen et. al., 2012). As the list of pesticide-resistant insect and weed species grows longer, there is a proportional interest in developing dsRNA products that specifically target the genes for resistance to specific herbicides and insecticides (Lundgren and Duan, 2013; Preuss and Pikaard, 2003). This dsRNA active ingredient category does not reduce dependence upon chemical pesticide control, but does permit the continued use of existing chemistry by rendering formerly resistant pests susceptible.

c. Developmental Disruptors (Growth Regulators)

A dsRNA developmental disruptor is defined here 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 competitive, or are sterile. Developmental disruptors that are currently registered by the Agency are insect growth regulators (IGRs), and they fall into two main categories: (1) juvenile hormone mimics (juvenoids) 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 (molting). It is conceivable that dsRNA active ingredients could be developed that specifically target genes responsible for the proper growth and development of any taxa of pest species, not just that of insects.

d. Growth Enhancers

A dsRNA growth enhancer is defined here as a dsRNA active ingredient that stimulates, inhibits, or otherwise mimics the activity of a naturally-occurring plant hormone. Plant hormones and their analogues are considered pesticides under FIFRA (1996) and are regulated as such. Plant hormones are typically applied to food and ornamental crops for aesthetic purposes (color, odor, taste, shape, uniformity), as harvest controls (delayed or advanced maturity, inhibition or promotion of fruit ripening, inhibition or promotion of abscission), and for resistance to environmental stress. Another category of plant regulators not associated with the traditional plant hormones, the induced resistance promoters, are those substances that stimulate the internal defense mechanisms of plants such that they will have an enhanced capacity to resist infection by plant pathogens. The two most well-known mechanisms of induced resistance are known as Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) and they are

distinguished from one another primarily by how the respective resistance mechanisms are elicited, the subsequent pathways of resistance induction, and the specificity of the induced response (see review by Jones, 2012). It is conceivable that dsRNA active ingredients could be developed that specifically target genes responsible for pathogen resistance. This type of dsRNA product could be used in two ways: (1) stimulate pathogen resistance in desirable food and ornamental plants; and (2) suppress pathogen resistance in weed species. In addition, it is conceivable that a family of dsRNA products could be developed for the purpose of suppressing disease resistance in other pest taxa (e.g. birds, fish, insects, mammals etc.)

2. Potential Application Methods

It is anticipated that dsRNA-containing end-use products could be applied using the same methods as traditional chemical pesticides:

- Foliar sprays
- Post-emergence directed soil spray/drench
- Seed treatments (as a protectant)
- Granular/powder (soil applied topical/incorporated & baits/traps)

Depending upon the target pest, application method, and product type, both outdoor and indoor use sites also are anticipated. Outdoor use sites may include, but are not limited to field crops, nurseries, forests and other natural areas; outdoor use sites also may include both terrestrial and aquatic habitats. Indoor use sites may include, but are not limited to food storage facilities, greenhouses, and residential/structural treatments.

3. Nontarget Exposure

As stated above in Section IV.A., the degree of nontarget exposure depends on the distribution and fate of dsRNA within the environment, as well as the potential routes of direct and indirect exposure, exposure duration, and the potential for uptake by nontarget species. In regard to non-PIP dsRNA EPs, the distribution and fate of dsRNA within the environment, potential routes of direct and indirect exposure, and duration of exposure to nontarget organisms will be dependent upon the following:

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

Once the product has been applied, other factors will affect the distribution of dsRNA in the environment and the potential for direct and indirect exposure to nontarget organisms. The most important of these factors are: (1) *In situ* dissipation of dsRNA via abiotic (e.g. UV-light) and biotic (microbial degradation) processes; (2) Offsite (physical) movement of applied dsRNA from the treatment area; and (3) persistence of dsRNA in the environment.
Stabilized vs. Unstabilized dsRNA. The following discussion regarding the factors influencing the distribution and persistence of dsRNA in the environment presumes that exogenously applied dsRNA will be stabilized in some manner to inhibit abiotic and biotic degradation in the environment such that there will be sufficient residence time in/on the treated use site to permit the maximum desired pesticidal activity. Similarly, for products requiring ingestion by the pest (e.g. treated foliar surfaces, granules, etc.) the active ingredient likely will be stabilized for a sufficient period of time for the target pest to locate the treated article and consume a sufficient amount of dsRNA to have the desired pesticidal effect. It is further presumed that the degradation rate of "stabilized" dsRNA will likely be slower than "unstabilized" dsRNA.

a. In situ *Dissipation of dsRNA*

It is presumed that once applied at the use site, dsRNA will be subject to the same abiotic and biotic degradation processes as traditional chemical pesticides. However, as described above in Section IV.A., few data are available regarding the degradation of dsRNA in the environment (see review by Lundgren and Duan, 2013). Data regarding the fate of DNA in soil and sediment (Levy-Booth et. al, 2007; Pietramellara et al. 2009) may not be applicable to understanding the fate of dsRNA due to the different chemical characteristics of the two molecules. Available data indicate that RNA may degrade more rapidly than DNA, although DNA in soil may degrade in a little as 7 days or persist for several years, with persistence enhanced by binding to soil humic substances, or degraded rapidly by microbial activity (Levy-Booth et. al, 2007 as cited in Lundgren and Duan, 2013). The relative degradation rates of DNA vs. RNA in the environment remains nebulous.

There also is uncertainty regarding whether exogenously applied dsRNA will amplify within living plant tissue once it has been absorbed and if so, the degree of amplification that will occur prior to loss of the tissue by the plant. While dsRNA present on treated plant surface may begin to degrade, it is possible that amplification within plant tissues, if it occurs to any substantial degree, may result in tissue residue levels that are greater than at the time of application, further complicating EEC determinations.

Another complicating factor is the degradation rate of "stabilized" dsRNA vs. "unstabilized" dsRNA. The Agency presumes that stabilized dsRNA will degrade at a slower rate in soil and water, and in/on biological matrices than unstabilized dsRNA-EP.

b. Off-site Movement of Applied dsRNA

Offsite movement of dsRNA from the treatment site may occur via the following pathways:

- Surface runoff from foliage and soil following a precipitation event
- Infiltration into the soil and movement into groundwater
- Spray drift
- Physical movement of treated plant tissue and pollen
- Pollinators and other animal vectors.

Regardless of whether the dsRNA is unstabilized or stabilized it will be distributed throughout the environment via the pathways described above. Volatilization into the atmosphere is not expected. The first three pathways listed above are self-explanatory and indicate how dsRNA may migrate from the application site to offsite terrestrial and aquatic environments. Plant tissue and pollen movement were discussed above in Section IV and would raise the same exposure concerns if exogenously applied dsRNA is amplified within treated plant tissues and transported into, or expressed in pollen, or if dsRNA was applied during anthesis.

Pollinators such as honey bees, if present on plants at the time of application, may carry dsRNA residues back to their hive and distribute the residues throughout the hive via bee-to-bee contact. Similarly, dsRNA residues could be carried back to the hive in pollen if exogenously applied dsRNA is amplified in treated plant and expressed in the pollen. Although this may not be a major offsite pathway from the treated area, dsRNA residues could bioaccumulate within the hive and have unanticipated effects resulting from off target effects. Bees can also transfer dsRNA to other organisms. Garbian et. al. (2012) demonstrated that dsRNA ingested by bees could be transferred to Varroa mites (a pest of bees) and then from the mite to a Varroa-infested bee. Other horizontal transfers may be possible.

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 the time of dsRNA application, could carry unknown amounts of dsRNA to offsite areas although this is not likely to be a major offsite pathway and would be difficult to model.

c. Persistence of dsRNA in the Environment

Accurate determination of exposure, hazards, and risk of exogenously applied dsRNA requires a complete understanding of dsRNA persistence, or lack thereof, in the environment. At this time, there are no quantitative measures of dsRNA degradation in soil, water or biological matrices, under aerobic and anaerobic conditions, or under conditions of variable pH and UV light.

Modeling of Environmental Fate. There are a number of models developed by the EPA to estimate the environmental concentrations of chemical pesticides, following applications at maximum label use rates, for use in estimating pesticide exposure to non-target organisms (EPA, 2013). Unfortunately, these models are of limited utility for determining the estimated environmental concentrations (EECs) of exogenously applied dsRNA, due to the paucity of information regarding degradation rates of dsRNA in soil and water matrices and under aerobic and anaerobic conditions. Furthermore, it is unknown whether exogenously applied dsRNA will amplify within living plant tissue once it has been absorbed and if so, the degree of amplification that will occur prior to loss of the tissue by the plant. *In situ* amplification of dsRNA in plant tissue would complicate environmental exposure estimations calculated by the existing models.

4. Hazards to Nontarget Organisms and Environmental Fate

At this time, the Agency does not possess empirical data regarding the acute or dietary hazards of dsRNA to non-target birds, fish, aquatic invertebrates, insects, and plants. However, the Agency believes that the existing hazard and risk assessment paradigm of tiered toxicity testing for biopesticides may be applied to assess the environmental fate and effects of dsRNA. Specific data requirements have been developed by the Agency to assess the hazard to nontarget organisms from exposure to new active ingredients. The list below describes the Tier I nontarget organism and environmental studies that must be addressed to support the registration of a new biochemical pesticide active ingredients and are similar to those used to determine the acute hazards of microbial pesticides. These studies have been generally been considered sufficient to inform nontarget risk assessments for all new biochemical pesticides to date. The preferred test material is the technical grade active ingredient (TGAI), the product that is the purest and highest concentration of the active ingredient that is typically used to formulate one or more end-use products. The studies focus primarily on maximum hazard dose testing and use mortality as a toxicological endpoint. The Tier I biochemical pesticide nontarget organism and environmental fate data requirements may be found in the table at 40 CFR 158.2060(d) and are summarized below:

- Avian acute oral toxicity
- Avian dietary toxicity
- Fish acute toxicity, freshwater
- Aquatic invertebrate acute toxicity, freshwater
- Terrestrial plant toxicity, seedling emergence
- Terrestrial plant toxicity, vegetative vigor
- Non-target insect testing

As discussed in Section IV.B.3. above, the persistence of dsRNA in the environment is relatively unknown. Although rough estimations of degradation rates may be calculated using the limited data available for the environmental fate of similar nucleic acids, specific information is lacking on dsRNA, regardless of whether it is stabilized when formulated into end-use products. The Agency believes that selected Tier II biochemical pesticide environmental fate studies can be used to determine the degradation rate and environmental fate of dsRNA following application of dsRNA-EPs to plant and soil surfaces. Data obtained from these studies will more completely inform exposure assessments for exogenously applied dsRNA products, as well as for dsRNA-PIPs. The Tier II biochemical pesticide nontarget organism and environmental fate data requirements may be found in the table at 40 CFR 158.2060(d) and are summarized below:

- 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

All of the studies listed above must be supported with robust and independently validated analytical methods that are accurate and precise for the detection of dsRNA residues in various environmental matrices.

5. Possible Limitations of the Current Testing Paradigm for Biochemical Pesticides

Biochemical pesticides are naturally-occurring substances that have non-toxic, well-characterized modes of action against their respective target pests. Acute and subchronic hazard data, if not readily available from other sources, are typically easy to generate using the Tier I studies listed above in Section IV.B.3. Acute and subchronic hazards of dsRNA to nontargets may likewise be determined using the same battery of testing. The new categories of dsRNA products, however, will present additional hazard and risk assessment challenges due to their unique modes of action and other toxicological endpoints that cannot be measured using the traditional testing paradigm. In a recent review, Lundgren and Duan (2013) observed that the current tiered hazard assessment approach used by the Agency, is inappropriate to address the following unique hazards potentially posed by dsRNA products:

- Off-target gene silencing
- Silencing the target gene in unintended organisms
- Immune stimulation
- Saturation of the RNAi machinery in cells

All of the above potential dsRNA hazards have been discussed in detail in previous sections of this document and will not be revisited in this section of the document; other unanticipated dsRNA hazards also may occur. The Agency assumes that the hazards and risks to nontargets identified in Section IV.A., will be identical for non-PIP dsRNA-EPs, with the exception that the exposure suite will be much greater for exogenously applied products. We note that accurate, standardized methods for measuring and assessing the aforementioned hazards will be necessary to conduct robust nontarget species risk assessments on dsRNA products.

6. Conclusions

Non-PIP dsRNA end-use products (dsRNA-EPs) present new challenges in regard to ecological risk assessment for nontarget taxa. These challenges arise as a result of the unique modes of action of dsRNA active ingredients,and the paucity of reliable data regarding the persistence and environmental fate of exogenously applied dsRNA. Persistence and environmental fate will strongly influence the degree of exposure to nontarget organisms, nontarget effects, and final determination of risk. In regard to hazard effect, the current testing paradigm for nontarget species characterizations, which emphasizes limit dose testing and use of mortality as an endpoint, likely will not be adequate to assess adverse effects resulting from off-target gene silencing, silencing of the target gene in unintended organisms, immune stimulation, and saturation of the RNAi machinery in cells.

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