**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY**
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

May 1, 2014

**MEMORANDUM**

**SUBJECT:** Transmittal of the Meeting Minutes of the FIFRA SAP Meeting Held January 28, 2014 on the Scientific Issues Associated with the use of "RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment"

**TO:** Jack Housenger
Director
Office of Pesticide Programs

**FROM:** Sharlene Matten, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel Staff
Office of Science Coordination and Policy

**THRU:** Laura Bailey, M.S.
Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

David Dix, Ph.D.
Director
Office of Science and Coordination Policy

Attached, please find the meeting minutes of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) open meeting held in Arlington, Virginia on January 28, 2014. This report addresses a set of scientific issues associated with the use of "RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment." An electronic copy of this report is available on the FIFRA SAP website:
http://www.epa.gov/scipoly/sap/meetings/2014/012814meeting.html

Attachment

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# SAP Minutes No. 2014-02

# A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

# RNAi Technology: Program Formulation for Human Health and Ecological Risk Assessment

**January 28, 2014**
**FIFRA Scientific Advisory Panel Meeting**
**Held at**
**One Potomac Yard**
**Arlington, Virginia**

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## NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (EPA or the Agency). The content of the meeting minutes does not represent information approved by the Agency. The meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and established under the provisions of FIFRA as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the Environmental Protection Agency (EPA), Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. FQPA Science Review Board members serve the FIFRA SAP on an *ad hoc* basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Sharlene R. Matten, Ph.D., SAP Designated Federal Official, via e-mail at matten.sharlene@epa.gov. This report was reviewed by Laura E. Bailey, M.S., FIFRA SAP Executive Secretary.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by EPA, as well as information presented in public comment. This document addresses the information provided and presented by EPA within the structure of the charge.

# SAP Minutes No. 2014-02

# A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

# RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment

## January 28, 2014
## FIFRA Scientific Advisory Panel Meeting
## Held at
## One Potomac Yard
## Arlington, Virginia


**Daniel Schlenk, Ph.D.**
**FIFRA SAP Chair**
**FIFRA Scientific Advisory Panel**

**Date:** 4/28/14

**Sharlene R. Matten, Ph.D.**
**Designated Federal Official**
**FIFRA Scientific AdvisoryPanel Staff**

**Date:** 4/28/14

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## COMMONLY USED TERMS AND ACRONYMS

| Term/Acronym | Description |
|---|---|
| Argonaute | Members of the Argonaute protein family are the part of RNA-induced silencing complex responsible for cleavage of the mRNA as directed by small RNAs. |
| *Bt* | *Bacillus thuringiensis*, a soil microorganism |
| DNases | Deoxyribonucleases |
| Dicer | Dicer is a ribonuclease RNase III-like enzyme that processes long double-stranded RNA (dsRNA) or pre-micro RNA hairpin precursors into small interfering RNAs (siRNAs) or micro RNAs (miRNAs). |
| dsRNA | Double-stranded RNA |
| GI tract | Gastrointestinal tract |
| mRNA | Messenger RNA |
| miRNA | Micro RNA |
| Non-PIP | Non plant-incorporated protectant |
| nt | Nucleotide |
| PIP | Plant-incorporated protectant is a term used by the EPA to describe the pesticidal active ingredient and the genetic material necessary for its production in a plant. |
| qPCR | Quantitative reverse transcription PCR assays |
| RDRPs | RNA-dependent RNA polymerases |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNA interference | Gene silencing process initiated by dsRNA |
| RNAi | RNAi interference |

| Term/Acronym | Description |
|---|---|
| RNA-Seq | An approach to transcriptome profiling that uses deep-sequencing technologies |
| RNases | Ribonucleases |
| RNPs | ribonucleoprotein complexes |
| rRNA | Ribosomal RNA |
| siRNA | Small interfering RNA |
| ssRNA | Single-stranded RNA |
| SID-1 | Transport protein that mediates cellular uptake of dsRNA |
| *Sid1* | Gene encoding SID-1 |
| SID-2 | Transport protein that mediates cellular uptake of dsRNA |
| *Sid-2* | Gene encoding SID-2 |
| SNP | Single nucleotide polymorphism |

## INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) has completed its report of the SAP meeting regarding scientific issues associated with **"RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment."** Advance notice of the SAP meeting was originally announced in the *Federal Register* on August 15, 2013 (78 FR 49750)**.** Following the extended government-wide shutdown, the Agency issued a notice of cancellation in the *Federal Register* on October 28, 2013 (78 FR 64211). The meeting was rescheduled for January 28, 2014 and announced in the *Federal Register* on November 15, 2013 (78 FR 68836).

The SAP reviewed the Agency's charge in an open Panel meeting on **January 28, 2014** at One Potomac Yard, Arlington, Virginia. Materials for this meeting are available in the Office of Pesticide Programs (OPP) public docket or via www.regulations.gov, **Docket No. EPA-HQ-OPP-2013-0485**.

Daniel Schlenk, Ph.D., chaired the meeting. Sharlene Matten, Ph.D., served as the Designated Federal Official. Steven Bradbury, Ph.D., Director, Office of Pesticide Programs (OPP), and Robert McNally, Director, Biopesticides and Pollution Prevention Division, OPP, provided introductory remarks at the meeting. Technical presentations were provided by the following individuals: Chris Wozniak, Ph.D., John Kough, Ph.D., Shannon Borges, and Russell Jones, Ph.D, all from the Biopesticides and Pollution Prevention Division, OPP.

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. Working with *Caenorhabditis elegans*, a common saprobic nematode, the laboratories of Andrew Fire and Craig Mello ascertained that injected double-stranded RNA (dsRNA) sequences corresponding to known gene sequences in the nematode genome were quite effective at gene silencing (Fire et al., 1998; Grishok and Mello, 2002). 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. Long dsRNA molecules are thought to be critical to triggering RNA interference (RNAi). The long strands of dsRNA that trigger RNA interference can originate outside the cell or be internally produced. Small interfering RNAs (siRNAs) are the product of Dicer (a ribonuclease III-like enzyme) activity on longer molecules of dsRNA. The siRNA molecules produced by Dicer are also double-stranded from 21-24 nucleotides (nt) in size with a 2 nt overhang at the 3'end.

In the past several years, a number of pharmaceutical and agricultural products based on RNAi 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. Development of new RNAi based pesticidal products led the EPA to convene this SAP meeting to consider aspects of the current biopesticide risk assessment framework.

The current framework for the risk assessment of biopesticides is found in the EPA's RNAi issue paper (EPA, 2013). The approach for mammalian safety assessment of plant-incorporated protectants (PIPs) and biochemical pesticides can be found at the start of section III. The framework for ecological safety assessment for PIPS and biochemical pesticides can be found in section IV A and B. Biopesticide products are likely to be developed utilizing RNAi technology as both dsRNA PIPs expressed in plants and non-PIP dsRNA end-products that may be externally applied for pest control.

The focus of this SAP meeting was on the problem formulation phase of risk assessment of pesticidal products based on RNA interference (RNAi). Problem formulation is the first step in the risk assessment process and guides the subsequent questions about hazard and exposure (EPA 1998). In problem formulation, available information is used to define assessment endpoints and to develop a preliminary understanding of potential risks (i.e., develop a risk hypothesis and conceptual model) associated with the use of a pesticide. Problem formulation also serves as an opportunity to identify missing information and uncertainties that may limit the assessment and any assumptions that may be made in the absence of such data.

EPA consulted with the SAP on scientific issues that might be unique to RNAi and how they could fit under the existing risk assessment framework. The SAP addressed seven charge questions divided into human health considerations (questions 1-3) and ecological risk considerations (questions 4-7) as shown below.

**Human Health Considerations:**

1. Nature and extent of uncertainty in the specificity of long sequences of dsRNA targeted at pest species and relatedness to human sequences;
2. Factors that may limit exposure to dsRNA: extent of digestion of dsRNA and effect on absorption;
3. Effect of the structure of dsRNA on degradation in the gut and exposure to dsRNA.

**Ecological Risk Considerations:**

4. The environmental fate of the dsRNA;
5. Factors that may limit non-target organism exposure to dsRNA;
6. Potential unintended effects in non-target organisms exposed to dsRNA;
7. Framework of testing for non-target effects.

## PUBLIC COMMENTERS

**Oral statements were presented by:**

1. Taiwo Koyejo, Ph.D., Manager, Regulatory Affairs, Plant Biotechnology, CropLife International
2. Raymond Layton, Ph.D., on behalf of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) - Non-Target Organism Subcommittee
3. Jay Petrick, Ph.D., David Carson, Ph.D., and Steven Levine, Ph.D. on behalf of Monsanto Company
4. Craig Mello, Ph.D., Howard Hughes Medical Institute and Department of Molecular Medicine, University of Massachusetts Medical School (The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA."*)
5. Alaina Sauvé, M.S., Syngenta
6. James White, Ph.D. Regulatory Affairs Manager, Citrus Research Development Foundation
7. Michael Barrett, Ph.D., Weed Science Society of America
8. Scott Slaughter, Center for Regulatory Effectiveness
9. Stephen McFadden, M.S., Independent Scientific Research Advocates (ISRA)

**Written statements were provided by:**

1. Jack A. Heinemann, Ph.D. University of Canterbury, Christchurch, New Zealand
2. Doug Kornbrust, Ph.D., D.A.B.T. President, Preclinsight
3. Brett Adee and Dave Hackenburg, Co-Chairs, National Honey Bee Advisory Board
4. Belinda Martineau, Ph.D., University of California-Davis
5. Stephen Whyard, Ph.D., Department of Biological Sciences, University of Mannitoba
6. James D. Thompson, Ph.D., Vice President, Development, Quark Pharmaceuticals
7. Andrew F. Roberts, Ph.D., Deputy Director, Center for Environmental Risk Assessment, International Life Sciences Institute (ILSI) Research Foundation
8. Subba Palli, Ph.D., Department of Entomology, University of Kentucky
9. Robert Sears, President, Eastern Missouri Beekeepers Association
10. private citizen anonymous
11. Dan Jenkins on behalf of Monsanto Company
12. Stephen Chan, M.D., Ph.D., Harvard Medical School
13. E. Keith Menchey, Manager, Science & Environmental Issues, National Cotton Council (NCC)
14. Diana L. Cox-Foster, Ph.D., Department of Entomology, The Pennsylvania State University
15. Nancy B. Beck, Ph.D., D.A.B.T., Senior Director, Regulatory and Technical Affairs, American Chemistry Council (ACC)
16. James White, Ph.D. Regulatory Affairs Manager, Citrus Research Development Foundation
17. Craig Mello, Ph.D., Howard Hughes Medical Institute and Department of Molecular Medicine, University of Massachusetts Medical School
18. James Carrington, Ph.D., President, Donald Danforth Plant Sciences Center

19. Raymond Layton, Ph.D. on behalf of the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) - Non-Target Organism Subcommittee
20. Randy Oliver, contributor to the American Bee Journal and site manager of Scientific Beekeeping.com
21. Taiwo Koyejo, Ph.D., Manager, Regulatory Affairs, Plant Biotechnology, CropLife International
22. Ray Gaesser, President, American Soybean Association
23. Ian MacLachlan, Ph.D., Executive Vice-President and Chief Technology Officer, Tekmira Pharmaceuticals Corp.
24. Lee Van Wychen, Ph.D., Director of Science Policy, Weed Science Society of America
25. Barbara Glenn, Ph.D., Senior Vice President, Science & Regulatory Affairs, CropLife America
26. Cyndi Heintz, Executive Director, Project Apis m.
27. Wenonah Hauter, Executive Director, Food & Water Watch
28. Martin Barbre, President, National Corn Growers Association

## OVERALL SUMMARY

The focus of this SAP meeting was on the problem formulation phase of the human health and environmental risk assessment of pesticidal products using RNA interference technology (RNAi). EPA consulted with the SAP on scientific issues that might be unique to RNAi and how they could fit under the existing risk assessment framework. The SAP addressed seven charge questions divided into human health considerations (questions 1-3) and ecological risk considerations (questions 4-7). The Panel provided the following overall summary of the major conclusions and recommendations detailed in the report.

### Human Health Considerations – Major Conclusions and Recommendations

The Panel agreed that bioinformatic analysis can be used to identify specific nucleotide identity in long sequences of dsRNA or processed shorter products that could bind in a siRNA-like fashion, provided the sequences are in the database and the appropriate algorithm is used. Sequence identity does not mean that the dsRNA would provoke an RNAi response, but should caution the potential for unintended interactions and further review.

The Panel agreed with EPA's assessment that the primary route of exposure is through the oral ingestion of dsRNA PIPs in food plants or food plants treated with exogenously applied dsRNAs end-products. Dietary RNA is extensively degraded in the mammalian digestive system by a combination of ribonucleases (RNases) and acids that are likely to ensure that all structural forms of RNA are degraded throughout the digestive process. There is no convincing evidence that ingested dsRNA is absorbed from the mammalian gut in a form that causes physiologically relevant adverse effects. Evidence of any dietary uptake plant micro RNAs (miRNAs) in mammals is nominal and non-specific.

### Recommendations

- The Panel recommended collection of data on dsRNAs PIP abundance and tissue distribution to investigate factors that may affect absorption and effects of dietary dsRNAs.
- The Panel recommended experimental testing of the mammalian blood and exposed tissues be done to ensure that the siRNAs processed from the PIP dsRNAs are not present, since these could have off-target effects after human consumption.
- The Panel recommended that stability of different structural forms of dsRNAs be addressed for dermal and inhalation routes of exposure.
- The Panel recommended that the stability of dsRNA in individuals that manifest diseases, immune compromised, elderly, or children be investigated.

### Ecological Risk Considerations – Major Conclusions and Recommendations

Overall, the Panel agreed with the concerns raised by the EPA regarding the inadequacies of the current environmental fate and non-target effects testing frameworks for dsRNA PIPs and exogenously applied dsRNA products. Uncertainties in the potential modes of action in non-target species, potential

for chronic and sublethal effects, and potential unintended consequences in the various life stages of non-target organisms are sufficient justification to question whether the current Agency framework for ecological effects testing is applicable to dsRNA PIPs or exogenously applied non-PIP end-use products. Due to the modes of action of RNAi, no one set of test species will serve as an adequate representation of non-target species for all pesticidal products using RNAi technology. The classic approach of developing and assembling effects data for a standard set of test species will likely not work well for this technology.

Current requirements for soil degradation studies will provide some insight for non-target testing, but are inadequate to address the environmental fate of dsRNA PIPs or exogenously applied dsRNA products and potential exposures to non-target organisms. The inability to predict dsRNA exposure to non-target organisms using the current non-target testing framework challenges the evaluation of potential non-target effects.

The Panel concluded that additional data are needed to reduce uncertainty in the environmental fate and ecological risk assessments; however, the task of prescribing additional tests or additional test species cannot be done without a better understanding of exposure to dsRNA PIPs or exogenously applied dsRNA products, and the RNAi modes of action.

**Recommendations**

- The Panel recommended an exposure-based conceptual model to reduce the uncertainties in the ecological risk assessment of dsRNA PIPs and dsRNA non-PIP end-products. This approach would identify non-target organisms that might be exposed and narrow the spectrum of non-target organisms that would need to be tested for effects. Using the exposure-based conceptual model for ecological risk assessment will further the understanding of the likelihood of adverse outcomes in non-target species, facilitate the selection of endpoints, and identify potential data gaps that would require further study.
- The Panel recommended the following information be collected to address uncertainties in the environmental fate and ecological risk assessments:
  - Determine soil degradation kinetics for both dsRNA PIPs and exogenously applied dsRNA end-products. Collect data representing different soil types, soil biology, and abiotic factors such as weather, soil temperature and moisture under which dsRNA PIP crops are grown or where dsRNAs products are applied.
  - Determine plant degradation kinetics for dsRNA PIPs and consider plant materials (above and below ground), off-site movement of plant materials, and *in planta* exposure for organisms that directly ingest plant materials.
  - Determine environmentally-relevant dosages, the importance of physical barriers in non-target organisms concerning degradation and cellular uptake of dsRNA, and off-target effects to non-target organisms.
  - Evaluate the potential for chronic and sublethal effects and unintended consequences in the various life stages of non-target organisms.
  - Consider possible immunostimulatory effects and impacts on food webs.
  - Evaluate the potential for pest resistance.

## EXECUTIVE SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

### Human Health Considerations

**Question 1. Please discuss the nature and extent of uncertainty in the specificity of long sequences of dsRNA targeted at pest species, if bioinformatic analysis shows no significant similarity to mammalian genes.**

***Summary***

The Panel agreed that bioinformatic analysis can be used to identify specific nucleotide identity in long sequences of dsRNA or processed shorter products that could bind in a siRNA-like fashion, provided the sequences are in the database and the appropriate algorithm is used. The certainty in the matches is dependent on the reliability and completeness of the sequence information in existing databases, the algorithm used, and the presence of single nucleotide polymorphisms (SNPs). It is important to emphasize that this is a predictive tool, but not absolute. Sequence identity does not mean that the dsRNA would provoke an RNAi response, but should caution the potential for unintended interactions and further review. The Panel suggested that dsRNA sequence-specific factors may increase target specificity and reduce the likelihood of unintended effects. If the charge question is interpreted more broadly to include miRNA-like effectors and modes of binding, sequence predictions are much less reliable.

**Question 2. Based on data indicating degradation of the majority of dsRNA in the digestive system, please discuss the strengths and limitations in concluding there will not be significant absorption of dsRNA with possible mammalian effects on oral exposure.**

***Summary***

Dietary RNA is extensively degraded in the mammalian digestive system. In contrast with some other organisms, mechanisms for RNA uptake from the environment, RNAi amplification by RNA-dependent RNA polymerases (RdRPs), and systemic RNAi are not known or are not robust in mammals. The Panel concluded that available evidence supports the conclusion of no significant absorption of dsRNA in mammals and minimal likelihood of adverse effects. This evidence consists of:

- Observations that ingested dsRNAs undergo rapid degradation in the mammalian digestive tract,
- The history of safe consumption of plants containing RNA by humans and other mammals,
- The history of challenges developing effective oral RNAi therapeutics which can withstand the hostile environment of the human gut,
- Findings from three published studies of no significant mammalian uptake of dietary miRNA resulting in functional consequences. These findings call into question the study of L. Zhang et al. (2012), which reported mammalian uptake of dietary miRNA resulting in an effect on cholesterol metabolism.

However, the Panel stressed that data are lacking in this area and there are no published PIP-specific studies. The Panel recommended that additional data on dsRNA PIP abundance and tissue distribution is needed, and that factors that affect absorption and effects of dietary RNA should be investigated further. The Panel recommended experimental testing of the mammalian blood and exposed tissues be done to ensure that the siRNAs processed from the PIP dsRNAs are not present, since these could have off-target effects after human consumption.

**Question 3. To what extent does the specific structure of dsRNA, if it is super coiled or in a hairpin structure, make it more likely to survive degradation in the gut and lead to possible mammalian effects with oral exposure?**

***Summary***

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. RNA levels are below the limit of detection even when using very sensitive PCR amplification methods following treatments of total RNA samples from both plants and animals with a mixture of both ss- and ds-RNases (Li et al., 2012 a, b; Silverman and Gregory, 2013; Silverman et al., 2014; Zheng et al., 2010). Thus, this 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. However, the Panel recognized that other issues concerning the stability of dsRNA should be addressed and made the following recommendations:

- The question of the ability of different structural forms of dsRNAs to survive degradation in the gut should also be addressed for dermal and inhalation routes of exposure.
- The stability of dsRNA should be tested in individuals that manifest specific diseases (e.g., Crohn's, colitis, irritable bowel syndrome, etc.), the immune compromised, elderly, as well as children. These individuals may have compromised digestion or increased sensitivity to dsRNA exposure.
- Modifications may be used to stabilize dsRNAs as exogenously-applied end-products. Possible effects on the uptake of dsRNA should be evaluated.

## Environmental Considerations: Questions directed toward dsRNA expressed as part of a PIP and applied to the plant as a biochemical

**Question 4. EPA needs a clear understanding of the environmental fate of dsRNAs in terrestrial and aquatic environments. In sections IV.A.1. and IV.B.3, respectively, EPA has presented potential scenarios for dsRNA movement within the environment that may result from their pesticidal uses as PIPs or as exogenously applied dsRNAs. Environmental fate of dsRNAs is not well understood; however, EPA has frameworks in place for PIPs and Biochemicals to obtain data related to degradation and movement of pesticides in the environment. These data can be used to refine environmental exposure estimates to dsRNAs.**

*a. While nontarget exposure to dsRNA PIPs may result primarily from consumption of PIP crop plant tissue, EPA must also assess the exposure of nontarget organisms to dsRNA that may enter the environment through plant exudates and plant tissue breakdown. In section IV.A.2.a, EPA presented the current approach for testing to assess environmental fate for PIPs, which focuses on degradation within soil.*

*i) To what extent will data on degradation in soil inform EPA on nontarget exposure to dsRNA PIPs?*

***Summary***

The Panel commended the Agency for initiating this critical dialogue and for the meticulous background research done to provide the Panel with useful information. Panel members agreed that additional information is needed to understand the persistence and stability of dsRNA PIPs in soil. There are few studies in the published literature that describe the stability and effects of dsRNA and degraded products (if present) in the environment. The Panel emphasized the importance of understanding how degradation of plant materials proceeds as part of efforts to determine the extent to which *in planta* dsRNA and degraded products (if present) are released over time. Duration of dsRNA persistence in soil will define the season(s) during which dsRNA or any active transformation products might be present in soils and soil associated organisms. Fields with summer crops that produce or are treated with dsRNA may have enhanced microbial or abiotic processes to degrade dsRNA compared to fields in the winter.

The Panel made the following recommendations:

- Depending on the degradation profiles *in planta* and cropping practices, tests of multiple soil temperatures and soil moistures may be needed to evaluate degradation occurring as plant material overwinters (above or below soil surfaces).
- Degradation of root material and any above ground parts that may remain in the field should be evaluated.
- In addition, both fresh and dry plant materials should be evaluated.

The Panel added that it is important to determine if products of degradation in environmental and biological samples are bioactive in target and non-target organisms, e.g., siRNA.

*ii) What additional information, if any, would EPA need to assess environmental fate of dsRNA PIPs?*

***Summary***

The Panel agreed that the proposed testing scheme used for *Bacillus thuringiensis* (*Bt*) PIPs presented covers a portion of the information needed to understand environmental fate, but is insufficient to address all potential risks associated with the use of dsRNA. The Panel stated that there will be significant differences in evaluations of PIP dsRNAs, dsRNA in plant tissue and in the rhizosphere, and exogenously applied dsRNA end-products, e.g., sprays. Plant tissue may be desiccated and remain in fields during quite cold intervals. Thus, dsRNA may remain in plant material for long

periods of time allowing slow release into the environment. Double-stranded RNA used as a PIP may be exuded into the rhizosphere, where biotic and abiotic processes can vary quite significantly between growing and dormant seasons. The Panel noted that roots of annual plants will decompose under soil chemistry conditions (nutrients, oxygen, temperature, moisture, microbial activity) that may be dissimilar to growing season conditions. The Panel emphasized that additional data are critical to understanding the stability of dsRNA in soil and aquatic environments. While there is a reasonable body of literature describing the environmental persistence of nucleic acids in soil, there is little information on the persistence of dsRNA and small RNA (siRNAs and miRNAs). The Panel recommended collection of environmental fate data representing different soil types, water bodies, or weather conditions under which crops expressing dsRNA PIPs may be cultivated. The Panel also recommended significant changes to the overall risk assessment paradigm as discussed in the response to question 7.

*b. In section IV.B.4, EPA proposed environmental fate data needs for exogenously applied dsRNAs. To what extent is the proposed testing sufficiently robust to inform nontarget organism exposure for these dsRNAs?*

***Summary***

The Panel agreed that EPA's standard testing paradigm represents a good beginning for data sets needed to understand soil degradation and environmental fate of dsRNAs in the environment; however, there are deficiencies in the testing regime. Non-target species are more likely to be exposed to exogenously applied dsRNA end-products than to dsRNA PIPs. Non-PIP dsRNA exposures are likely multi-route while dsRNA PIP exposure is more likely via diet.

The Panel stressed the importance of assessing the availability of the dsRNA to non-target organisms as one of the earliest steps in the environmental fate evaluation process (a first tier evaluation). Data are lacking on cellular uptake of dsRNA at various taxonomic levels. In the absence of data, the Panel recommended that the present focus should be on evaluating uptake in non-target organisms closely related to the target (e.g., species in the same genus and related genera in the same family). The Panel indicated that the life stage exposed to dsRNAs should be considered in the design of non-target tests and recommended that multiple species be evaluated for some of the required tests. Test species might include organisms not currently listed in the testing paradigm. Special consideration should be given to non-target amphibian testing as part of a tier 1 testing regime. See the Panel's response to Question 7.

*c. For both dsRNA PIPs that may be free in the environment and exogenously applied dsRNAs, knowledge of the initial residue burden, and the dissipation rate of residues, is essential for developing environmental exposure estimates, particularly if models of environmental fate are used to arrive at these estimates. What analytical methods are available to accurately and precisely measure dsRNAs in diverse plant, soil, and water matrices?*

***Summary***

The Panel indicated that existing RNA extraction methods and subsequent use of quantitative PCR, high throughput sequencing, or hybridization analysis are the most current tools to assess the presence of a particular small RNA within a sample. The Panel emphasized the critical nature of using a reliable method for extracting and measuring the dsRNA to track the environmental fate of the dsRNA in soil.

**Question 5. The primary route of exposure for nontarget organisms to dsRNA PIPs is assumed to be ingestion, either of dsRNAs contained in plant tissue or free in the environment. However, some evidence also indicates the potential for exposure by direct contact in nematodes and some arthropods. For dsRNAs that are applied to plants and/or the environment, the primary routes of exposure are expected to be both ingestion and direct contact.**

*a. In section III.C. and IV.A.1.c. of the issue paper, EPA discusses potential barriers to uptake of dsRNA in the gut of mammals and arthropods; however, little information exists for other taxa.*

*i) In addition to the conditions of the gut environment and enzymes influencing digestion, what other factors may play a role in uptake within the gut and potentially limit exposure to dsRNA?*

***Summary***

The Panel stated that additional information is needed to understand what factors may play a role in uptake within the insect gut (or other organisms) and potentially limit non-target insect exposure to dsRNA and small RNAs. The published literature indicates there is a wide range of cellular uptake mechanisms that vary from species to species and across taxa. For example, cellular uptake of dsRNA is mediated by transmembrane proteins, SID-1 and SID-2, or through endocytosis (Hannon, 2002; Huvenne and Smagghe, 2010). Factors that influence cellular uptake and activity include, but are not limited to: tissue- or cell-specific expression, transport proteins, dose, dsRNA sequence length, and degradation.

*ii) Please comment on how these barriers can be generalized across all nontarget taxa that are considered in EPA's risk assessments (e.g., birds, plants, fish, etc.) for both dsRNA PIPs and exogenously applied dsRNA.*

***Summary***

The published literature indicates there is a wide range of environmental uptake mechanisms that vary from species to species and across taxa. The Panel stated that there is insufficient understanding of the uptake mechanisms of dsRNAs (PIPs or non-PIPs) and potential barriers to make any generalizations across non-target taxa. The Panel also commented that the ability to cross physiological barriers has to be combined with binding in the gut (where there is an actual target to be affected). The Panel

recommended collection of additional information to evaluate the types of cellular uptake mechanisms that exist and whether there are any patterns among different species, genera, or families, for example. For now, the Panel recommended a case-by-case evaluation of cellular uptake mechanisms for non-target taxa, as needed, based on exposure.

*b. The degree of exposure by direct contact is likely to differ between nontarget risk assessments for dsRNA PIPs and nontarget risk assessments for exogenously applied dsRNAs.*

*i) Please comment on the importance of the contact route of exposure for nontarget risk assessments for each of these types of dsRNAs*

***Summary***

There are insufficient data on the contact route of exposure to comment on the importance of this route in non-target risk assessment. The Panel found only two studies that examined direct contact as route of exposure to dsRNA. The limited information available indicates that the dietary route of exposure was probably the primary route of entry leading to activity and not the contact route of exposure. The Panel recommended collection of additional information to evaluate the importance of this route of exposure. For now, the Panel recommended a case-by-case evaluation to determine whether this is a likely route of exposure before pursuit of an in-depth study.

*ii) What barriers are likely to exist for this route of exposure for both terrestrial and aquatic organisms, and how can these be generalized across nontarget taxa?*

***Summary***

The Panel indicated that there are insufficient data on the contact route of exposure for both terrestrial and aquatic organisms to make any comparisons or generalizations across non-target taxa. See response to question 5.b.*i*. For now, the Panel recommended that each dsRNA PIP or exogenously applied dsRNA end-product should be evaluated on a case-by-case basis, as needed, based on exposure.

**Question 6. In the issue paper, EPA discussed possible effects other than silencing of the target gene in the target organism that may occur as a result of exposure to dsRNA. These unintended effects of dsRNA include off-target effects, silencing the target gene in nontarget organisms, degradation of non-targeted mRNA by transitive RNA, and effects resulting from immune stimulation and saturation of the RNAi machinery. EPA has little information to estimate the range of unintended effects that may occur and their probability of occurrence as a result of exposure to dsRNA in the environment.**

*a. Please comment on the unintended effects that EPA might reasonably anticipate in nontarget organisms exposed to dsRNAs, the likelihood of such unintended effects, and the biological significance of these effects in nontarget organisms, should they occur.*

***Summary***

The Panel stated that there is uncertainty in defining the spectrum of insecticidal activity. One concern expressed is that the potential scale for use of pesticidal RNAi in the agroecosystem (in particular) will increase the potential of unintended effects in non-target organisms. The Panel discussed several possible unintended effects that may result from exposure to dsRNA. For example, dsRNA used to silence the target gene may also silence the same conserved gene in non-target organisms. But one of the advantages of RNAi technology is that the RNAi effect is sequence and target gene dependent. Therefore, it is possible to design the dsRNA sequence to induce highly species specific gene suppression which would reduce the potential for off-target effects. Saturation of RNAi machinery and immunostimulation have been observed in animals (in mice or in cell cultures), but both require a fairly high dose to cause deleterious effects. The dose required to affect the RNAi machinery and induce immunostimulation in insects is unknown. There is also a lack of information on the risk of insect resistance to RNAi-mediated gene suppression. The knowledge gaps make it difficult to predict with any certainty whether unintended effects will occur in non-target species as a result of exposure to dsRNA. More data to close these gaps will increase the certainty in predicting the likelihood of unintended effects.

*b. To the extent that additional information would reduce uncertainty in addressing these issues, please describe specifically the nature of additional information that EPA may need and the degree to which this information would reduce uncertainty in the ecological risk assessment.*

***Summary***

As discussed in the response to Question 6.a., there is uncertainty in how to define the spectrum of insecticidal activity. The spectrum of activity depends on the target gene, dsRNA chosen, where and how much the dsRNA PIP is expressed in the plant, and the persistence and stability of the effect. What is a realistic level of non-target exposure based on the stability and persistence of the dsRNA? While the Panel noted that the mode of action of RNAi (by siRNAs) is, by definition, very specific (see discussion in response to question 1), there was some concern that unintended effects could occur across multiple insect orders. There could also be unique effects for each non-target organism. The question is how do you decide what non-target should be tested? For example, insect species representing 10 families and four insect orders were tested for DvSnf7 dsRNA activity (Bachman et al., 2013). Uncertainty in the spectrum of activity makes it more challenging to understand the environmental exposure levels and potential uptake pathways, important information that can narrow the scope of non-target organism testing for an ecological risk assessment.

The Panel concluded that the proposed ecological risk paradigm should be modified to reduce uncertainty in the risk assessment. In addition, data are needed in several areas. The Panel recommended an exposure-based decision framework for ecological risk assessment of dsRNA PIPs and dsRNA non-PIP end-products (see response to question 7).

**Question 7. In sections IV.A.2.a. and IV.B.4. of the issue paper, EPA presents the current framework of testing for determining nontarget effects resulting from exposure to PIPs and biochemicals, respectively. In section IV.A.2.c, EPA also raised potential issues related to nontarget testing with dsRNAs that may arise given their unique mode of action, which included 1) the potential influence of latent effects on results of nontarget testing, 2) the appropriate life stage for testing, and 3) the possibility for chronic effects.**

a. *Please comment on how each of EPA's current PIP and biochemical frameworks for nontarget effects testing will inform risk assessment for dsRNA PIPs and exogenously applied dsRNAs. In providing a response, please address the potential for unintended effects as described in Question 7, as well as the three issues outlined as concerns for nontarget testing listed above.*

***Summary***

The Panel agreed with the concerns the Agency expressed regarding application of current protocols for identifying non-target effects and characterizing environmental fate. Uncertainties in the potential modes of action in non-target species, potential for chronic and sublethal effects, and potential unintended consequences in the various life stages of non-target organisms are sufficient justification to question whether the current Agency framework for environmental fate and ecological effects testing is applicable to dsRNA PIPs or exogenously applied non-PIP end-use products. Due to the modes of action of this technology, no one set of test species will serve as an adequate representation of non-target species for all pesticidal products using RNAi technology. Hence, the Panel concluded that the classic approach of developing and assembling effects data for a standard set of test species will likely not work well for this technology.

The Panel discussed the following limitations in the current ecological risk assessment framework:

- Spectrum of activity - determination of non-target organisms that might be exposed.
- Maximum hazard dose assays alone may not be sufficient for assessing off-target effects associated with the use of RNAi technology.
- Lack of consideration for different life stages of a non-target organism and under different environmental conditions.
- The current Tier 1 testing scheme does not provide insight into potential chronic effects and limited insight to the potential for sublethal effects.
- Inability to determine possible synergistic effects (of co-exposures to other PIPs) with respect to target and non-target organisms
- Lack of consideration of other endpoints, e.g., immune response, potential for saturation of the RNAi machinery in a non-target organism.

b. *What additional nontarget effects testing, if any, should EPA consider to gain a full understanding of the potential for dsRNAs to cause effects to nontarget organisms?*

c. *What other approaches, such as bioinformatics analysis, may be used to address concerns for effects on nontarget species and reduce the set of data requirements?*

d. *In providing answers to the above subquestions, please be specific in discussing the extent to which additional information would reduce the nature and magnitude of these specific areas of uncertainty.*

***Summary***

The Panel provided a combined response to questions 7b.-d.

The Panel found it difficult to prescribe additional tests or additional test species without a better understanding of RNA-based modes of action, spectrum of activity, degradation kinetics of dsRNA in soil, and what non-target organisms are likely to be exposed to dsRNA PIPs or dsRNA non-PIPs. To address the limitations of the proposed ecological risk assessment framework, the Panel developed an exposure-based decision framework or conceptual network model (figure 7-1). Data needs are linked to various steps in the framework.

One important outcome of the proposed framework is identification of non-target organisms that might be realistically exposed to dsRNA PIPs or dsRNA non-PIPs. This result will eliminate the need to test a battery of standard species (current tier 1) many of which will either not be exposed or be insensitive. The Panel suggested that using this framework would further the understanding of the likelihood of adverse outcomes in non-target species, facilitate the selection of endpoints, and identify potential data gaps that would require further study.

The Panel provided the following general recommendations for data needed in the ecological risk assessment:

- Before any testing, identify what non-target organisms will be exposed to the dsRNA PIP or exogenously-applied dsRNA end-products. See exposure-based conceptual model for ecological risk assessment (figure 7-1).

- Bioassays should include doses that mimic the *in planta* expression levels of the PIP and where possible, use plant tissue.

- Mortality is one of many possible endpoints that should be considered. The potential for chronic effects and for sublethal effects should be considered.

- The Panel recommended the evaluation of entire life cycles, while simultaneously examining multiple life history parameters (intrinsic population growth rate is a robust metric) rather than simply relying on toxicity testing. The Panel recommended that

strategic research is need to determine under what conditions and what life stages should be assayed for RNAi activity.

- The Panel recommended inclusion of an immunological endpoint in the non-target organism bioassays.

- The Panel also recommended an endpoint to measure the potential for saturation of the RNAi machinery in a non-target organism.

- The Panel recommended that EPA consider possible synergistic effects (of co-exposures to other dsRNA PIPs) with respect to target and non-target organisms.

- The Panel also recommended that EPA consider the evolution of pest resistance.

- One panelist identified potential scenarios that could minimize the concern for non-target exposure and effects:

  - When the dsRNA PIP is very specific without homology to any of the sequence databases;
  - When the dsRNA PIP is not modified and therefore likely to degrade rapidly;
  - When the dsRNA is expressed at extremely low levels and is tissue specific (e.g., in the roots).

## OVERVIEW OF THE RNA INTERFERENCE PATHWAY

RNA interference (RNAi) is nearly ubiquitous in plants, animals, fungi, and microorganisms. Using the model nematode, *Caenorhabditis elegans*, Fire et al. (1998) discovered that injected dsRNA sequences matching known gene sequences in the nematode genome were highly effective at silencing these loci. RNAi processes are known in most eukaryotes. RNAi describes a group of related processes whereby small RNA molecules and their associated protein partners interfere with the production of specific genetic transcripts by binding in a partly or completely sequence-specific manner to RNA transcripts (see figure I-1 for a simple illustration of this process). This process depends on the degree of sequence homology, protein partners, and cellular context. Specific binding may lead to translational suppression, mRNA degradation, or target sequestration (Wilson et al., 2013; Bartel 2009). The unifying feature of the RNAi mechanisms is the use of entirely or partially double-stranded precursors to produce single stranded effector molecules that recognize sequences in target RNAs. Particular attention has been given to RNAi triggered by introduced long dsRNA that is cleaved by an RNase III enzyme called Dicer into 20-25 nucleotide (nt) siRNAs with a two base overlap overhang at the 3' end. The siRNAs are incorporated into the multi-protein RNA-induced silencing complex (RISC) which removes the non-guide strand and retains the guide strand. The Argonaute protein of the RISC complex cleaves a single-stranded messenger RNA (mRNA) with sequence complementary to the guide strand and leads to destruction of the mRNA. Therefore, the mRNA can no longer be translated into a protein product, i.e., interference in translation. Additional discussion on RNAi in eurkaryotic organisms is found in the response to question 6.a.

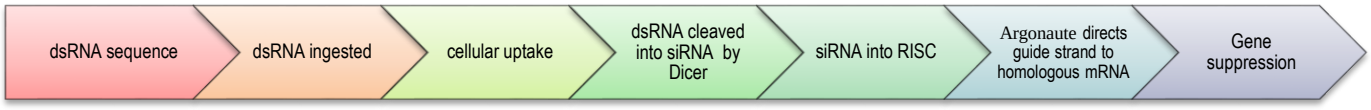


**Figure I-1.** Simple illustration of the RNAi process. Abbreviations: RISC = RNA-induced silencing complex; dsRNA = double-stranded RNA; siRNA = small interfering RNA; Dicer = a family of RNA endonuclease III enzymes; mRNA = messenger RNA; Agronaute = family of proteins that cleave mRNA

## DETAILED RESPONSES TO CHARGE QUESTIONS

### Human Health Considerations

**Question 1. Please discuss the nature and extent of uncertainty in the specificity of long sequences of dsRNA targeted at pest species, if bioinformatic analysis shows no significant similarity to mammalian genes.**

**Panel Response**

The Panel agreed that bioinformatic analysis can be used to identify specific nucleotide identity in a long sequence of dsRNA or processed shorter products that could bind in a siRNA-like fashion, provided the sequences are in the database and the appropriate algorithm is used. The certainty in the matches is dependent on the reliability and completeness of the sequence information in existing databases, the algorithm used, and the presence of single nucleotide polymorphisms (SNPs). The Panel emphasized that bioinformatics analysis is a useful, but not absolute, predictive tool. Sequence identity does not mean that the dsRNA would provoke an RNAi response, but should caution the potential for unintended interactions and further review. The Panel suggested that dsRNA sequence-specific factors may increase target specificity and reduce the likelihood of unintended effects. If the charge question is interpreted more broadly to include miRNA-like effectors and modes of binding, sequence predictions are much less reliable.

The Panel discussed the following major points concerning certainty of bioinformatic analysis to identify sequences of dsRNA targeted at pest species that are homologous to mammalian genes. Specific discussion with respect to non-mammalian organisms is found in question 6.

**1) Bioinformatic analysis can reduce uncertainty in sequence similarity**

The Panel stated that coverage of nearly all of the human transcriptome is now sufficient to allow accurate identification of nucleotide (nt) sequences with identity to "long sequences of dsRNA" or even short processed products, such as ~20-25-mers, that could bind in a siRNA-like fashion. Algorithms can be used to determine if there is overlap to mammalian sequences with the goal of minimizing unintended effects. While “long” dsRNA may have no similarity to mammalian genes, processing of dsRNA into shorter siRNAs may present additional issues if these siRNAs have a high degree of similarity to sequences in non-target species including mammals. Algorithms such as Blastn (Altschul et al., 1990), ClustalW (Thompson et al., 1994), and MegAlign (DNAStar, Madison, WI) can be used to identify shared sequence identity and scan for 21 nt matches among databases, but certainty in the matches will depend on the reliability and completeness of the sequence information in existing databases, the algorithm used, and the presence of single nucleotide polymorphisms (SNPs).

**2) To what degree does the siRNA have to match the target genetic sequence to trigger RNAi?**

The Panel indicated that current evidence suggests that a high degree of homology between the siRNA and target mRNA is necessary for induction of RNAi effects (Sabariegos et al., 2006). However, other studies question this dogma (see discussion in Question 6). Sequence identity in the end of the guide strand of siRNA, known as the seed region (located 2-8 nts from the 5' end) of the siRNA sequence and loaded into the RISC is critical for target gene silencing, but may also be responsible for silencing off-target genes (Jackson et al., 2006, Jackson and Linsley, 2010). For example, substituting the second base of the seed region with *O*-methyl ribosyl can reduce off-target siRNA binding within the target organism (Jackson and Linsley, 2010), which may reduce the potential for non-target effects, but there are no data to confirm this conclusion.

**3) Choice of dsRNA sequence makes a difference to target specificity**

The Panel discussed how the choice of dsRNA sequences can limit target specificity and minimize the likelihood of unintended effects in non-target organisms. See also related discussion in response to Question 6.

- Choose dsRNA sequences with perfect or near perfect sequence homology to regions of the target gene. Some regions may work better than others and have to be determined experimentally. The more specific the dsRNA sequence is to the target sequence, the greater the insurance will be against unintended effects. For example, select dsRNA sequences that target regions of genes with no shared 21 nt sequences with other species.

- Choose target genes that are unique to the genome and physiology of the target pest, if possible.

- An ideal target gene encodes a protein with a short half-life (see discussion in Scott et al. 2013); this is because the duration of the effect of RNAi will be shorter to achieve the desired phenotypic suppression. Phenotypic analysis of gene function at any life stage becomes more difficult if the protein product of the target gene has a long half-life.

- Careful selection of siRNA sequences that favor incorporation of the antisense strand into the RISC may improve efficacy and specificity of RNAi (Aronstein et al., 2011).

- Reducing the size of the dsRNA to the minimal effective length will likely result in fewer siRNA fragments in the mammalian gut and lower the subsequent "dose" (Scott et al., 2013). For the purpose of target specificity, the shorter the sequence, the better; however, this has to also be balanced with efficacy (in which case, the longer the sequence, the better). At least 100 copies of siRNA are needed to induce RNAi in mammalian cells (Brown et al., 2007).

- Screen for sequences that have been demonstrated to induce innate immune response (Judge

et al., 2005) and eliminate them from further consideration. See also discussion in response to questions 2 and 6.

**4) miRNA-like molecules: less certainty in sequence predictions**

The Panel noted that if the database for bioinformatics screening is increased more broadly to include miRNA-like effector sequences, predictions of non-target effects against mammals may be much less reliable. In contrast to siRNAs that require high sequence complementarity to the target mRNA with few off-target exceptions, miRNAs have less exact complementarity to the target mRNA. A single miRNA could be specific to a single sequence or could target hundreds of mRNAs (off-target possibilities). Depending on the *in silico* target prediction algorithm used, up to thousands of binding sites may be predicted for miRNA in the 3' untranslated regions of known transcripts alone. This number increases if 5' untranslated regions (UTRs) and coding sequences are included in the search. MicroRNAs are also known to bind to each other, ribosomal RNAs, long non-coding RNAs, transfer RNAs, and other forms of RNA in the cell (Helwak et al., 2013). The factors governing miRNA binding are not well defined.

Various algorithm targeting rules invoke perfect or near-perfect "seed" sequences at the 5' end of the miRNA, but these are imperfect. For example, there are predicted high-scoring target interactions that cannot be validated in the laboratory (even under non-physiologic conditions), and conversely, there are laboratory validated interactions that are not predicted by the algorithms (e.g., Ha et al., 1996; Lal et al., 2009). A recent technical advance apparently provides direct evidence of small RNA-target interaction, which suggests that a large proportion of miRNA-target pairings are non-canonical (Helwak et al., 2013). Thus, the Panel indicated that it would be a difficult task to predict all possible miRNA-like interactions. This means that the effect of a miRNA on a particular transcript, even when direct binding is confirmed by *in vitro* assays may be meager and, hundreds or thousands of copies of miRNA must be present in the cell to exert an observed effect (Brown et al., 2007; Mullokandov et al., 2012).

**Question 2. Based on data indicating degradation of the majority of dsRNA in the digestive system, please discuss the strengths and limitations in concluding there will not be significant absorption of dsRNA with possible mammalian effects on oral exposure**.

**Panel Response**

The focus of this charge question is on the potential dietary exposure to RNAi PIPs through oral ingestion. The Panel addressed the following areas in its discussion.

- Safe consumption of RNA
- RNA degradation in the mammalian digestive system
- Substantial systemic distribution of dietary RNA as input into RNAi pathways is not predicted for mammals by comparative biology
- Inflammatory responses to plant RNAi molecules
- Evidence for uptake and distribution of dsRNA or processing products in mammals
- Human disease and RNA uptake/clearance.

- Dose, stoichiometry and delivery: diet-derived miRNAs and gene regulation
- Other routes of exposure

**1) Safe consumption of RNA**

The Panel stated that to date there appears to be no convincing evidence in the literature that ingested dsRNA is absorbed from the mammalian gut in a form that produces biological effects. Although, some panel members argued that there is also no convincing evidence to the contrary. As noted in the public comments and in the literature, the history of safe mammalian consumption of RNA in plants suggests that nominal uptake of dietary RNA by mammals does not lead to adverse effects, despite the fact that siRNAs and miRNAs are abundant in soybean, corn, and rice, some with exact matches to human gene sequences (Ivashuta et al., 2008, 2009). The Panel pointed out that a perfect sequence match does not always correlate with gene silencing, one of the false positive issues with algorithms. The Panel added that the FDA considers RNA as GRAS ("Generally Regarded As Safe"), with the only concern that ingestion of high levels of purines resulting from nucleic acid degradation (e.g., through consumption of high levels of RNA-rich foods like brewer's yeast supplements) could aggravate gout. The Panel pointed out that this technology was developed because of the promise of improved efficacy and minimal unintended effects compared to traditional pesticides.

**2) RNA degradation in the mammalian digestive system**

The mammalian digestive system functions to break apart first tissues, then cells, then subcellular components including ribonucleoprotein complexes (RNPs). Breakdown of food into constituent nutrients begins with mastication and exposure to degradative enzymes in saliva, followed by further digestion in the acidic environment of the stomach. Passage into the gut is marked by increased pH, continued digestion (including by resident microorganisms), and absorption of liberated nutrients. Digestive strategies vary with diet: mammals that consume chiefly plant materials with tough cell walls have evolved more complex digestive processes than others.

In the digestive tract and elsewhere, RNA is subject to both non-enzymatic and enzymatic degradation. On the non-enzymatic side, in contrast with DNA, the 2′ hydroxyl group on the ribose moiety of RNA renders RNA sensitive to non-enzymatic hydrolysis. RNAs of all structural forms are relatively labile compared with DNA in the sense that RNA is more susceptible to non-enzymatic hydrolysis. However, all forms of RNA are not equally susceptible to certain nucleases. Even RNA that has been purified in the laboratory to exclude RNase enzymes, stored in properly buffered solutions, and maintained at extremely low temperatures will degrade over time.

The physiological conditions of the mammalian gut are barriers to the uptake of foreign dsRNA. The digestive system of mammalian organisms is replete with enzymes such as nucleases (Fort et al., 1969). As presented in detail in the response to question 3, these include RNases. Of course, RNA is often found in complex with proteins and lipids and this greatly affects stability. Extracellular RNA may also be carried within vesicles that are mobile *in planta* or are the product of tissue destruction during feeding. RNA contained within such protect packaging can be liberated by lipases and proteases.

Pancreatic and intestinal nucleases and enzymes eventually metabolize RNA to mono-nucleotides and subsequent nucleosides and bases, which are primarily absorbed in the upper small intestine (Carver and Walker, 1995; Hoerter et al., 2011; Rehman et al., 2011; Sorrentino et al., 2003).

In fact, the RNA-hostile nature of the various microenvironments of the mammalian digestive system, extreme acidic pH and RNA nucleases in the stomach, has hampered the development of dsRNA for oral therapies in humans (Petrick et al., 2013). Members of the Panel, public commenters, as well as evidence in the published literature referred to the difficulties in the development of orally active RNAi therapeutics as compelling arguments for the safety of oral exposure to dsRNA and derivatives. For example, limited success has been reported for oral siRNA delivery using encapsulation into engineered nanoparticles (Aouadi et al., 2009; Ballarin-Gonzalez et al., 2013). However, these approaches may be generally more effective for distribution to cells of the gut rather than across the gut and into the general circulation (Kriegel et al., 2013). Indeed, for candidate RNAi therapeutics to survive the extra- and intracellular biological environments, special modifications and/or formulations are needed even when molecules are injected into mammals at high concentrations. However, miRNAs that are resistant to low pH and RNases have been reported in milk. These miRNAs appear to be resistant based on their incorporation into extracellular vesicles (Admyre et al., 2007; Hata et al., 2010; Lasser et al., 2011; Zhou et al., 2012). Although there has been speculation as to the biological effects of these milk miRNAs, direct demonstration of such effects or transfer of RNA to the infant, to our knowledge, has not been done. The extent to which dsRNA PIP products could be similarly protected within "plant-specific packaging" is lacking and should be evaluated.

One panel member noted that some insects have high levels of cysteine proteases in the gut (e.g., some coleopterans), but there is no consistent response following oral ingestion of dsRNA. In contrast to insects, mammals lack cysteine proteases in the gut.

The Panel also discussed possible effects of ingested RNA on the gut microbiome. Dietary composition, e.g. fiber or fat content, have been reported to alter the microbiome, but there is no evidence that specific dietary RNA sequences have any such effects. As noted above, RNA import by microbes is generally followed by breakdown to provide nutrition. While bacteria and archaea have RNA-based regulatory systems, the machinery, RNA sequences, and binding behavior for these systems differ from those in eukaryotic systems. In addition, there is no current evidence that eukaryotic dsRNA is amplified by bacteria.

**3) Barriers to uptake and systemic distribution of dietary RNA in mammals vs. other organisms**

The Panel discussed barriers to the exogenous uptake and systemic distribution of dietary RNA into RNAi pathways in humans. Mechanisms for exogenous dsRNA import into RNAi pathways are not known in mammals, and reports of carrier-unmediated small RNA uptake await confirmation. In contrast, *C. elegans* and some plants have evolved mechanisms for uptake and use of exogenous dsRNA in sequence-specific RNA silencing processes that function as a type of defense against parasites and pathogens. Cellular uptake and distribution of dsRNA have been most extensively studied in *C. elegans* (e.g., Fire et al., 1998). These features render these species attractive targets for PIPs or non-PIP topical dsRNA pesticides, while limiting the impact of RNAi control measures on non-target organisms in

which these mechanisms are lacking or are not as robust, including mammals. Features include efficient uptake of dsRNA from the environment, amplification of RNAi by generation of secondary effector molecules, and RNAi spread throughout the organism. Ancillary processes that make RNAi particularly sensitive and effective in *C. elegans* do not appear to be shared by mammals or even some closely related nematodes (Felix, 2008).

***a. Uptake of dsRNA from the environment for input into the RNAi pathway.*** *C. elegans* assimilates dsRNA contained in dietary materials (Timmons and Fire, 1998) and when soaked in dsRNA solution (Tabara et al., 1998). Dietary uptake occurs when dsRNA of 60 nt or more in length (McEwan et al., 2012) is recognized by a single-pass transmembrane protein, SID-2 (McEwan et al., 2012; Winston et al., 2007) in the acidic environment of the gut. Binding leads to endocytosis, followed by import across the cell membrane via a multipass channel protein known as SID-1 (Hunter et al., 2006; Winston et al., 2002).

Gene homologs of *sid-1* can be found in many organisms, but with varying comparative functionality. In contrast, the *sid-2* gene is less conserved across organisms. Experiments in *C. elegans*, demonstrated that dsRNA uptake in refractory nematode species may become permissive when the *C. elegans* SID-2 gene is introduced (Nuez and Felix, 2012; Winston et al., 2007). While the *sid-2* gene is poorly conserved across organisms, there is some evidence for alternative uptake mechanisms. *C. elegans*, for example, does not require SID-2 (Liu et al., 2012). Such pathways may also exist in other organisms (or cell culture systems) that lack functional SID-2 but appear to import dsRNA (Saleh et al., 2006; Ulvila et al., 2006), e.g., through endocytosis-initiated processes (Price and Gatehouse, 2008).

***b. RNAi amplification.*** A second ancillary process that provides sensitivity to RNAi in some plants and animals is the production of "secondary" RNAi effectors (Axtell et al., 2006; Pak and Fire, 2007; Ruby et al., 2006; Sijen et al., 2001; Sijen et al., 2007). In *C. elegans*, RNA-dependent RNA polymerases are primed by binding of siRNA to a target mRNA.

The small RNAs formed through this process are multiple secondary siRNA molecules, each of which recognizes the target (Parrish and Fire, 2001). Additional amplification of dsRNA is also known to occur in some plants (Axtell et al., 2006; Sarkies and Miska, 2013). Even very low copy numbers of imported dsRNA may lead to a robust RNAi response in any organism with RDRPs that function in RNAi (e.g., *C. elegans*, some plants). However, many organisms, e.g., mammals, do not appear to have RDRP-mediated RNAi amplification (Duan et al., 2013; Lipardi et al., 2001). For mammals, each copy of a hypothetical PIP or non-PIP RNAi effector would need to come from the diet since RDRP-mediated RNAi amplification is absent.

***c. Conservation of cellular transport proteins*.** As mentioned above, the SID-1 transmembrane channel transfers long dsRNA across the cell membrane (Feinberg and Hunter, 2003; Hunter et al., 2006; Shih and Hunter, 2011; Winston et al., 2002). In *C. elegans*, this allows long dsRNA to silence target genes throughout the organism (systemic RNAi). Systemic distribution of dsRNA is also a key feature of plant RNAi (Brosnan and Voinnet, 2011; Voinnet, 2009). However, despite conservation of SID-1 sequences across many organisms (Hunter et al., 2006), effective systemic distribution seems to be the exception rather than the rule in animal species. Systemic RNAi of the type observed in *C. elegans* has not been reported in mammals. Cell-to-cell communication by extracellular vesicles has

been observed in mammalian cell culture systems, in some cases reportedly mediated by RNA cargo (Pegtel et al., 2010; Raposo and Stoorvogel, 2013; Skog et al., 2008; Valadi et al., 2007). However, its extent *in vivo* is not yet well known (Sverdlov, 2012; Turchinovich et al., 2012). Ties to dietary input, i.e., findings of vesicles that arrive from the diet, have not yet been reported.

***d. Barriers in mammals vs. insects.*** One panelist stated that the depth and breadth of human barriers to foreign RNA uptake and ultimate incorporation into cellular regulatory processes was previously advanced as an argument against off-target effects in insects. Yet, RNAi works in some insects. Why would there be variable effects of RNAi in insects versus humans, or observed effects in some insects and not others? In response, panel members remarked that amongst insects, there is a wide range of gut pH; diet composition and feeding practices; conservation and function of RNA receptors and transmembrane channels including, but not limited to, the SID proteins; and activity of RNases in digestive fluids and hemolymph. These factors may contribute to species differences in foreign RNAi effectiveness. A complete discussion of potential barriers to foreign uptake in insects in discussed in the Panel's responses to questions 5 and 6.

**4) Evidence for uptake and distribution of dsRNA or processing products in mammals**

Mechanisms for exogenous dsRNA import into RNAi pathways are not known in mammals, and reports of carrier-unmediated small RNA uptake await confirmation. The Panel stated that there is currently a lack of substantiated evidence that exogenous dsRNA and/or its processing products traverse the gut, are distributed throughout the organism in a manner analogous to plant or *C. elegans* RNAi processes, or are processed by native RNAi machinery to yield functional RNAi effector molecules. Of the various forms of RNA in dietary materials, Argonaute-bound and vesicle-protected small RNAs may well be among the most stable and thus the strongest candidates for survival and uptake. Small RNAs would be the ultimate effectors in tissue of hypothetical ingested and subsequently processed longer RNAs.

The question of potential dietary uptake of miRNAs arose after the publication of a recent study by L. Zhang et al. (2012). L. Zhang et al. (2012) reported that dietary plant miRNAs enter the mammalian bloodstream and regulate cholesterol metabolism in the ingesting animal through LDLRAP1 (a low-density lipoprotein). Four plant miRNAs were found in all samples, two of them occasionally reaching levels similar to those of abundant endogenous miRNAs. It was also reported that one plant miRNA, MiR168a, targeted an endogenous transcript involved in cholesterol metabolism, LDLRAP1, and raised circulating cholesterol counts. The Panel questioned the findings of L. Zhang and colleagues (L. Zhang et al., 2013) and identified the following issues:

- Sequencing data are currently unavailable for this study and thus the data cannot be independently analyzed.

- Despite the varied and abundant plant small RNAs in the human diet, only four plant miRNAs were detected in all pools of human sera and only two were abundant (Witwer, 2012).

- There was striking variability of read numbers despite a sample pooling strategy.

- At least one of the detected miRNAs is routinely used as a spike-in control in high-throughput sequencing library preparation, raising questions of possible cross-contamination. The presence of miRNA in pollen (Wei et al., 2011) also supports the notion of inadvertent contamination. Analysis of public sequence reads from numerous animal studies turned up no consistent evidence of high levels of plant miRNA in animal blood, cells, or tissues, except for what was described as an apparent over-representation of MiR168, suggesting contamination or other artifact(s) (Y. Zhang et al., 2012).

- The timing and magnitude of changes of both RNA and target in mouse feeding studies have been questioned (Witwer et al., 2014). No murine biological effects have been detected in subsequent feeding studies (Dickinson et al., 2013). If murine biological effects were to be confirmed in future studies, translation to human risk would need to account for gastric pH differences from mice to humans.

Three subsequent studies (Snow et al., 2013; Witwer et al., 2013, and Dickinson et al., 2013) failed to replicate the finding of high plant miRNA concentrations in mammals. Each study found negligible uptake of plant dietary miRNAs.

Snow et al. (2013) found that transfer of plant miRNAs from miRNA-replete diets in human and murine subjects, as well as adult bees was negligible, below the limit of detection of sensitive, amplification-based quantitative reverse transcription PCR assays (qPCR). This same study also reported that animal miR-21 in a lard diet was undetected in a miR-21 knockout mouse that completely lack this animal encoded miRNA, which implies that barriers to dietary uptake are not specific to plant RNAi effectors.

In a time-course study of non-human primates, Witwer et al. (2013) concluded that plant miRNAs were undetectable or nearly so in plasma before and after feeding, and that any low-level positive findings may be due to non-specific amplification. The miRNAs examined in both studies included highly abundant and less abundant miRNAs in the food sources (Witwer et al., 2013; Snow et al., 2013).

Miragen replicated the original mouse feeding study of L. Zhang et al. (2012) and found no consistently detectable plant miRNA from rice (Dickinson, et al., 2013). By administering several rice-containing diets to separate groups of animals, the investigators also uncovered evidence that the cholesterol results reported in the initial study (L. Zhang et al., 2012) may have been precipitated by dietary insufficiency, not MiR 168-mediated inhibition of LDLRAP1 (Dickinson et al., 2013).

In addition to these feeding studies, high-throughput sequencing was used to measure plant miRNA in human blood (Wang, et al., 2012) and in a mouse model of liver damage (Wang et al., 2013). Only one plant miRNA, MiR168 (see comments above regarding this miRNA), was detected, but at very low levels, <10 ppm in blood in the human study and <1 ppm in blood in the mouse study. Such low levels of apparent reads are difficult to distinguish from background. Public commenters stated that

additional, unpublished negative studies exist, and the Panel suggested that publication of these data would be helpful.

The Panel concluded that available evidence supports the conclusion of no significant absorption of dsRNA in mammals and minimal likelihood of adverse effects. Evidence of any dietary uptake plant miRNAs in mammals is nominal and non-specific. However, the Panel stressed that data are lacking in this area and there are no published PIP-specific studies. The Panel recommended collection of data on dsRNAs PIP abundance and tissue distribution to investigate factors that may affect absorption and effects of dietary dsRNAs. The Panel also recommended experimental testing of the mammalian blood and exposed tissues should be done to ensure that the siRNAs processed from the PIP dsRNAs are not present, since these could have off-target effects after human consumption. One panelist added that these evaluations should consider dietary exposure over an extended timeframe.

**5) Inflammatory and immune responses to plant RNAi molecules**

Mammals respond to the detection of foreign RNA in a strong general fashion. Mammalian cells sense foreign RNA by means of pattern recognition receptors including several Toll-like receptors (TLRs) and cytoplasmic sensors such as MDA5 and RIG-I (Dixit et al. 2013, Loo et al. 2011, Kawai et al., 2008). Evidence also exists for differences in innate immune responses to dsRNA from commensal and pathogenic bacteria (Kawashima, et al., 2013).

The Panel commented that recognition of foreign RNA triggers innate immune responses: degradation of RNA transcripts, shutdown of protein expression, and secretion of inflammatory cytokines. During acute viral infections, these responses might present as "flu-like symptoms." While such reactions may protect against infection, depending on their strength and longevity, tissue damage may occur. The Panel concluded that factors governing inflammatory responses to some foreign RNA, but not others, are incompletely understood.

***a. Could miRNA in plant extracellular vesicles or lipid-bound tissue breakdown products be relatively resistant to degradation, allowing delivery to gut cells and detection by pattern recognition receptors?***

Mammals regularly ingest plant material replete with all species of RNA without reported induction of innate immune responses. Additionally, although limited experimental evidence has been reported, oral administration of purified plant-derived nanovesicles in a murine-induced colitis model did not cause inflammation, but instead protected against inflammation (B. Wang, et al., 2013) resulting in significantly decreased production of pro-inflammatory cytokines such as IL-6. If active dsRNA PIPs are at relative abundance as low as indicated by industry representatives during the public comment session, the Panel thought it would be unlikely that PIPs would be a special case, differing from other plant RNA in their ability to trigger inflammatory responses. However, the absence of PIP-specific data suggests to some panel members that the potential of orally delivered plant-derived RNA to activate host innate immune mechanisms should be evaluated.

***b. Exosomes***. The Panel indicated that exosomes might be a method to encapsulate dsRNA and then it could be released inside an organism, but this is speculation. For example, An et al. (2007)

provided evidence that exosomes could be released upon fungal penetration (An et al., 2007). Regente et al. (2012) provided indirect evidence of exosomes in sunflower seeds.

***c. Possibility of plant RNA-stimulated immune responses in mammals.*** The Panel noted that there were a few published studies that raise questions about the possibility of plant RNA-stimulated innate responses in mammals.

- Experimental injection of animals with large quantities of foreign RNA may lead to systemic inflammation and damage to organs including the gut (Zhou et al., 2007).

- A report that fluorescent label from parasite extracellular vesicles was internalized by cultured intestinal epithelial cells (Marcilla et al., 2012) provides support for uptake and potential concentration of biologically active substances at the gut surface, potentially contributing to immune activation.

- An orally administered adenoviral vector engineered to overexpress dsRNA induced an inflammatory response in mice and ferrets (Scallan et al., 2013). The inclusion of the dsRNA enhanced systemic antibody responses, likely through interaction with Toll-like receptor 3 on dendritic or intestinal epithelial cells, protected the animals from lethal challenge with antigen. The Panel commented that presentation of antigen and adjuvant would not be expected to occur in the RNAi pesticide applications. However, Scallan et al. (2013) illustrated the potential issue of species differences in gastric pH. Specifically, while the mice were vaccinated by gavage because of their higher gastric pH, the ferrets (more like humans) required the use of an endoscope to administer the vaccine to the duodenum because of the lower gastric pH.

**6) Stoichiometry, delivery, and estimated dietary exposure**

***a. Stoichiometry.*** Measurable silencing of a particular target gene does not occur unless the stoichiometry of targeting molecules and target or targets is favorable. Hundreds to thousands of targeting RNAs per cell appear to be necessary for observable RNAi to occur (Brown et al., 2007). Many predicted binding sites for plant RNAs have been found in mammalian genes (Ivashuta et al., 2009), but lack of regulation of these genes by dietary intake suggests that insufficient copy numbers of foreign RNA are transported through the gut and then into cells to silence these targets. The Panel stated that the history of safe mammalian consumption of plants indicates that nominal uptake of dietary RNA by mammals is insufficient to lead to regulatory effects (see related discussion later in this response to this question). This conclusion is in agreement with statements by public commenters and findings of published studies.

As noted in the response to question 1, miRNAs have less exact complementarity to the target mRNA than siRNAs. However, a large number of targets will also dilute out the effects of any miRNA-like binding. Regardless of the type of off-target binding, measurable off-target effects are not necessarily expected even when large copy numbers of the effector are present. Studies that have reported regulation of mammalian transcripts by foreign sequences have usually involved *in vitro*

transfections of non-physiologic levels of nucleic acid. For example, transfection of several endogenous bacterial RNA sequences into mammalian cells altered cellular gene expression directly or indirectly (Wang et al., 2012). These researchers astutely noted, however, that RNA was transfected at a concentration many orders of magnitude higher than those at which any exogenous RNA species would have been present in the blood (Wang et al., 2012).

***b. Delivery.*** The form in which diet-derived RNA would exist in blood is not known, and it is unclear if these RNAs could be imported into cells in a manner consistent with regulatory function. Despite the growing evidence for endogenous RNA transfer by mammalian extracellular vesicles, it is unclear whether or how natively packaged mature miRNAs or other short ssRNA effectors shuttle between cells and influence gene regulatory programs *in vivo*. In *C. elegans*, long dsRNA and shorter dsRNA processing intermediates move from cell to cell and undergo ultimate processing in the destination cell (Jose et al., 2011). Single-stranded RNAi effector molecules (derived from processed short dsRNA) and single-stranded secondary molecules from the RNA-dependent RNA polymerase-mediated RNAi amplification pathway do not appear to spread with the same efficiency (Jose et al., 2011).

***c. Estimated human exposure to diet-derived RNAi.*** The Panel indicated that more detailed information is needed (more than that provided in the EPA background documents) to estimate human dietary exposure. This would include abundance data for different tissues. Where is the PIP expressed in the plant? What is the level of expression? Does expression change over time? Is the dsRNA expressed constitutively or transiently? Is the dsRNA stable or degraded *in planta*? The Panel recommended the collection of data on the abundance of PIP dsRNA relative to other dsRNAs in PIP plants.

Some panel members expressed concern that PIPs could be engineered to greatly over express RNAi effectors in comparison with other forms of RNA. Drastic over expression of specific dsRNAs would likely overwhelm the small RNA processing machinery in plant cells. In mammals, this can lead to loss of viability (Grimm et al., 2006).

Responding to direct questions from the Panel during the public comment period, Monsanto representatives indicated that levels of a dsRNA PIP under development for corn rootworm control were in the parts per billion of total RNA *in planta*, and that estimated human exposure to dsRNA PIPs in food materials would be in the nanograms per kilogram level. Panel members noted that published theoretical estimates of the human dietary intake necessary to achieve minimally relevant levels of foreign RNAi effectors would be physiologically impossible to achieve in mammals, e.g., 33 kg per day of rice (Petrick et al., 2013) or several thousand pounds of raw fruit (Snow et al., 2013).

The Panel encouraged publication of industry data on abundance and recommended independent verification of these data.

**Question 3. To what extent does the specific structure of dsRNA, if it is super coiled or in a hairpin structure, make it more likely to survive degradation in the gut and lead to possible mammalian effects with oral exposure?**

**Panel Response**

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. RNA levels are below the limit of detection even when using very sensitive PCR amplification methods following treatments of total RNA samples from both plants and animals with a mixture of both ss- and ds-RNases (Li et al., 2012 a, b; Silverman and Gregory, 2013; Silverman et al., 2014; Zheng et al., 2010). Thus, this 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.

**1) RNA structure**

All RNA molecules have an intrinsic structure. This structure is the collection of intricate folding patterns that an RNA molecule forms through specific base pairing interactions encoded within its primary sequence (Buratti et al., 2004; Cooper et al., 2009; Cruz and Westhof, 2009; Sharp, 2009). Many RNA molecules cannot properly function without the formation of an extremely precise secondary structure (Buratti et al., 2004; Cooper et al., 2009; Cruz and Westhof, 2009; Sharp, 2009). For instance, ribosomal RNAs (rRNAs) must form structural folds that enable interactions with the correct ribosomal subunits at specific locations along their length, thereby allowing the formation of functional ribosomes (Trappl and Polacek, 2011). Additionally, the structure of long non-coding RNAs (lncRNAs), not their primary sequence, drives their function in regulating gene expression (Hindorff et al., 2009; Khalil and Rinn, 2011). Structural elements also affect the overall steady state abundance and stability of many eukaryotic mRNAs (Goodarzi et al, 2012; Li et al., 2012 a, b). Finally, other post-transcriptional processes such as protein translation (Kozak, 1980; Kozak, 1986) and RNA-mediated silencing (Baulcombe, 2004; Jones-Rhoades and Bartel, 2004) are also tightly controlled by structural features within the RNA transcript. Thus, the structure of RNAs is often required for their functionality and regulation in diverse cellular and regulatory processes.

**2) RNA stability**

RNA is an intrinsically unstable molecule even in normal aqueous conditions no matter what structural confirmation (single-stranded or double-stranded) it assumes. This is due to its chemical nature, where the additional hydroxyl group at the 2' position on the ribose sugar ring provides the destabilizing moiety through intra-hydrolytic degradation. Both acidic and basic conditions can drive intra-strand hydrolysis of RNA chains irrespective of the structural confirmation of that molecule (Lilley, 2011). Additionally, numerous ribonuclease enzymes (RNases) are encoded by both prokaryotic and eukaryotic organisms (Sorrentino, 2010). RNases are a type of nuclease that catalyzes the degradation of RNA into single nucleotides. They can be divided into endoribonucleases and

exoribonucleases, and comprise several sub-classes of enzymes. In fact, RNases are classified based on their structural preference. Specific RNases prefer single-stranded RNAs (single-stranded RNases (ssRNases)), and others target double-stranded RNAs (double-stranded RNases (dsRNases)). Thus, there are RNases that can destroy all types of RNA molecules regardless of their structural confirmation expressed in both prokaryotes and eukaryotes (Sorrentino, 2010).

**3) RNases in humans (with a focus on the gastrointestinal (GI) tract)**

All organisms studied to date contain many RNases of many different classes, demonstrating that RNA degradation is a very ancient and important process. Humans specifically contain a large repertory of RNase enzymes, many of which are expressed and function throughout the GI tract. In fact, RNases are expressed and functional in saliva in the human mouth, throughout the esophageal tract, as well as in the stomach and small intestine. The RNases expressed and active in the human GI tract include both ss- and dsRNases (e.g., RNase A, T1s, T2s, Us, U-likes, etc.) (Sorrentino, 2010; Goo and Cho, 2013; Economopoulou et al., 2007; Uchida et al., 1970) and are present to degrade all types of RNAs (with their varied structures) present in foods consumed. The degradation of RNAs by RNases allow the breakdown of all types and forms of RNAs to be broken down to single nucleotides, which can be further degraded into their components or directly scavenged and used in cells throughout the body. This collection of RNAs can break down all structural conformations of both PIP and non-PIP RNAs that are ingested by humans. Overall, RNAs present in foods consumed by human beings are subjected to RNase exposure from the time that they enter the human body through the mouth during consumption (Sorrentino, 2010; Goo and Cho, 2013; Economopoulou et al., 2007; Uchida et al., 1970).

In addition to the RNases encoded within the human genome, there are likely numerous others present and functional (especially in the GI tract) provided by the collection of microorganisms that colonize the body (microbiome). Given the huge diversity of bacteria that constitute the microbiomes of the human GI tract, the RNases present in the human GI tract are likely to encompass a collection of enzymes that can degrade all RNA types no matter the structural confirmation (Hattori and Taylor, 2009). Thus, the collection of RNases in the human GI tract is incredibly varied and is present to function in breaking down RNAs from all consumed food products into their primary components without regard to their structure.

**4) Naked RNA in the human gut**

RNA is a fundamental constituent of all living cells. Therefore, humans unavoidably ingest natural dsRNA every day (with every meal) as a significant component of their diet. As mentioned previously, RNA is readily hydrolyzed under both acidic and basic conditions (Lilley, 2011). In the human GI tract, RNA encounters hydrochloric acid (HCl) within the human stomach, which is the acid released for digestion of consumed foods. The HCl of the stomach lowers the pH of the stomach to acidic conditions to aid in food digestion. This lower pH also favors hydrolysis of both PIP and non-PIP RNA molecules. The pH is then raised to ~8 when food moves into the small intestine for the completion of its digestion. These alkaline conditions strongly favor hydrolytic destruction of RNA molecules. Thus, no matter the structural confirmation in which an RNA molecule arrives into the human GI tract the non-specific

hydrolysis of RNA molecules is likely to result in significant degradation of both ss- and dsRNAs. These include all ss- and dsRNAs from PIP and non-PIP materials.

Additionally, collections of RNases that result in the degradation of all forms of RNA structure (both secondary and tertiary) are present throughout the human digestive tract (Sorrentino, 2010; Goo and Cho, 2013; Economopoulou et al., 2007; Uchida et al., 1970). Both PIP and non-PIP dsRNAs will first be confronted with a broad collection of RNases in the mouth, where they are found in human saliva. In both the stomach and small intestine a large battery of RNases that will degrade all types and structural confirmations of RNAs are also released during the digestion of consumed food products [16-19]. The combination of RNases and acids found in the human digestive system are likely to ensure the all forms of RNA structure are degraded throughout the digestive process. This hypothesis is also supported by other work that has demonstrated that after treatment of total RNA samples from both plants and animals with a cocktail of both ss- and dsRNases for more than 2 hours, RNA levels are below the limit of detection even when using very sensitive PCR (amplification-based) methods (Li et al., 2012a, b; Zheng et al., 2010; Silverman et al., 2013; Silver et al., 2014, and Gregory, B.D., unpublished results). Thus, this 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.

**5) Studies on potential dietary uptake of miRNAs**

The question of potential dietary uptake of miRNAs arose after the publication of a recent study by L. Zhang et al. (2012). The Panel discussed this study and subsequent studies in detail in response to question 2. A brief summary is included here. A recent study by L. Zhang et al. (2012) reported that dietary plant miRNAs enter the mammalian bloodstream and regulate cholesterol metabolism in the ingesting animal through LDLRAP1 (a low-density lipoprotein). Three subsequent feeding studies (Snow et al., 2013, Witwer et al., 2013, and Dickinson et al., 2013) reported that uptake of foreign miRNAs from the diet is negligible at best. Given the available evidence, the Panel strongly suggested that non-modified dietary RNAs (PIP or non-PIP) regardless of structural status are not transmitted at regulation-relevant levels out of the GI tract. Although this is likely true, experimental testing of the mammalian blood and exposed tissues should be done to ensure that the siRNAs processed from the PIP dsRNAs are not present, since these could have off-target effects after human consumption.

**6) RNase resistant RNAs**

There is evidence that microRNAs and other RNAs incorporated into microvesicles, for example as in milk, have been reported to be stabilized to degradation by acid conditions and ribonucleases (Hata et al., 2010; Izumi et al., 2012). Furthermore, the addition of specific proteins, including viral packaging or certain phage RNA-binding proteins, as well as other chemical modifications has been demonstrated to stabilize RNAs against digestion by RNases and from degradation in general (Pasloske et al., 1998; Ghidini et al., 2013; Sczepanski and Joyce, 2013; Li et al., 2014). The Panel indicated that it is conceivable that some exogenously applied dsRNA pesticide end-products will be formulated to increase the stability and protection of these products. Therefore, the Panel recommended further testing

of stabilizers used in exogenously applied dsRNA end-products applied to food plants that will be consumed by humans.

**7) Influence of human health conditions on susceptibility to RNA uptake/clearance**

The Panel commented on the potential influence of human health conditions (that, e.g., influence digestion conditions, intestinal permeability, or glomerular filtration) on uptake, distribution, and persistence of exogenous RNA in the body. As noted by one of the public commenters and further discussed by the Panel, reaction to ingested dsRNA may not be uniform among the population, and certain medical conditions may result in atypical responses. Susceptible subpopulations could include some individuals with (1) limited ability to degrade dsRNA in the digestive system, such as individuals with achlorhydria (producing lower than normal levels of stomach acid), as a result of genetics, disease or infections (e.g., *Helicobacter pylori*), or medication (e.g., proton pump inhibitors such as omeprazole), (2) increased intestinal permeability, facilitating uptake of dsRNA from the intestine, such as individuals with celiac disease and Crohn's disease or (3) individuals with reduced levels of RNase activity.

Although few studies are available, one panel member indicated that in one study comparing detection of foreign RNA in serum (human) of healthy controls, colitis patients, and individuals with colon cancer, there were no apparent differences in detection of the only plant miRNA (Wang et al., 2012). This panel member noted that MiR168 was previously reported to be a potential artifact or contaminant of sensitive high-throughput sequencing studies, and that it was detected in human blood at less than 10 reads per million. Studies on the effects of dietary PIP and individuals with special dietary conditions are not currently available. Given this situation, there are uncertainties in the assumptions of the degradation, uptake, and possible effects of dsRNAs in the absence of species- and susceptible population-specific data.

The Panel recognized that other issues concerning the stability of dsRNA should be addressed and made the following recommendations:

- This charge question focused on the stability of various structural forms of dsRNAs when ingested into the human gut. The Panel agreed that the dietary route of exposure is the most likely route of exposure to dsRNA PIPs, but other routes of exposure, such as dermal or intranasal (pollen and dermal) should also be considered as part of the human health risk assessment.

- The Panel recommended that the stability of dsRNA should be tested in individuals that manifest specific diseases (e.g. colitis, irritable bowel syndrome, etc.), the immune compromised, elderly, as well as children. These individuals may have compromised digestion or increased sensitivity to dsRNA exposure**.**

- Exogenously applied dsRNA end-products may use stabilizers to stabilize dsRNA. The Panel recommended that possible effects of stabilizers on uptake of dsRNA should be evaluated.

## Environmental Considerations: Questions directed toward dsRNA expressed as part of a PIP and applied to the plant as a biochemical

### Question 4. Environmental Fate

**EPA needs a clear understanding of the environmental fate of dsRNAs in terrestrial and aquatic environments. In sections IV.A.1. and IV.B.3, respectively, EPA has presented potential scenarios for dsRNA movement within the environment that may result from their pesticidal uses as PIPs or as exogenously applied dsRNAs. Environmental fate of dsRNAs is not well understood; however, EPA has frameworks in place for PIPs and Biochemicals to obtain data related to degradation and movement of pesticides in the environment. These data can be used to refine environmental exposure estimates to dsRNAs.**

**While nontarget exposure to dsRNA PIPs may result primarily from consumption of PIP crop plant tissue, EPA must also assess the exposure of nontarget organisms to dsRNA that may enter the environment through plant exudates and plant tissue breakdown. In section IV.A.2.a, EPA presented the current approach for testing to assess environmental fate for PIPs, which focuses on degradation within soil.**

***4.a.i) To what extent will data on degradation in soil inform EPA on nontarget exposure to dsRNA PIPs?***

**Panel Response**

The Panel noted that few studies in the published literature describe the degradation, persistence, and bioavailability of dsRNA and siRNA in the environment. The Panel recommended that environmental persistence of dsRNA and any active RNAi effectors (e.g., siRNA capable of binding Argonaute and forming RISC) should be documented as part of the environmental fate assessment. This documentation should include soil sampling representing all soil types, temperatures, and moisture content across the targeted pest-crop agroecosystem that will be affected by the proposed dsRNA pest management strategies. Binding of dsRNA to soil organic matter may decrease degradation, but such binding may also decrease availability to organisms. Both the persistence in soil and bioavailability should be evaluated in soil degradation studies and pesticidal activity studies. Measurement of the degradation of plant materials should be included to determine the extent to which *in planta* dsRNA and activated RNAi effectors are released over time. Results of these tests could inform and direct non-target effect testing (see response to question 7).

The Panel recognized that there will be significant differences in evaluations of dsRNA PIPs and exogenously applied dsRNA, e.g., dsRNA in plant tissue and in sprays. Plant tissue may be desiccated and remain in fields during quite cold intervals. Thus, dsRNA may remain in plant material for long periods of time allowing slow release into the environment. Double-stranded RNA used as a PIP may be exuded into the rhizosphere, where biotic and abiotic processes can vary quite significantly between

growing and dormant seasons. The Panel noted that roots of annual plants will experience different soil chemistry conditions (nutrients, oxygen, temperature, moisture, microbial activity) that may be dissimilar to growing season conditions.

The Panel provided the following comments and recommendations on the proposed environmental fate testing scheme. Many of these recommendations apply to both PIP and exogenously applied dsRNA.

**1) Bioavailability**

The Panel stressed the importance of assessing the bioavailability of the dsRNA to non-target organisms as one of the earliest steps in the environmental fate evaluation process (part of a tier 1 evaluation). Panel members agreed that persistence of dsRNA PIPs must be better understood to evaluate exposure potential. Persistence of dsRNA in the soil will be affected by a number of factors, e.g., temperature, soil structure and type, abiotic and biotic factors. For example, fields with summer crops that express dsRNA PIPs or are sprayed with dsRNA may have enhanced microbial or abiotic processes to degrade dsRNA as compared to fields in the winter. When conducting studies with plant tissue, care should be taken to ensure that studies of dsRNA degradation in above ground or root portions of plants are done in such a way that degradation/release from plant tissue is being measured and not simple degradation in soil. The Panel recommended studies be conducted to evaluate dsRNA degradation in the environment and bioavailability for uptake by unintended organisms. An example of the type of soil degradation study to be conducted was provided by one of the public commenters.

Soil dwelling invertebrates comprise a significant portion of the diet in adults and nestlings of selected avian species. For example, the primary route of avian exposure to pesticides may be through ingestion of earthworms. Thus, data on the soil degradation of dsRNA and degraded products (if present) would be useful to inform the bioavailability of environmental dsRNAs for uptake by soil dwelling invertebrates.

Some panel members commented that nanotechnology might be a potential delivery system for exogenously applied dsRNAs applied in or sorbed to nanoparticles.

**2) dsRNA stability in soils**

Little is known about the dsRNA stability and degradation kinetics of dsRNA PIPs and derivatives in soil. The Panel stated that specific data on dsRNA kinetics in soil and in plant material are needed to minimize uncertainties in exposure assessments. The importance of abiotic and biotic factors in the soil on the rate of degradation is discussed later in the response to this question.

In the absence of RNA stability data in dried tissues, the Panel looked at the relative stability data for RNA versus DNA in dried forensic samples. Results in Harteveld et al. (2013) indicated that saliva RNA can be more stable than saliva DNA in periods of up to 7 days at temperatures up to 20$^{o}$C and for 1 day at temperatures of 37$^{o}$C. At colder temperatures, approximately 50% of RNA remained after 1 day at 2$^{o}$C and 20% remained after 7 days. In the same study, drying blood samples stabilized RNA. The

Panel suggested that this information provides some insight on RNA stability in desiccated and cold plant materials above and below ground during fall and winter months.

Using *in vitro* cell cultures, Hoerter et al. (2011) found that dsRNA may be more stable in epithelial cells than serum. Furthermore, this study found that dsRNA is more stable than RNA/DNA hybrids in epithelial cells. The Panel suggested that this study addresses stability in animal tissues (potential non-target organisms) or in food items of carnivorous/omnivorous non-target species.

In contrast to these two studies indicating the relative stability of RNA vs. DNA, studies in aphids demonstrated that dsRNA is rapidly degraded in a manner of hours (Yu et al., 2013).

**3) Bioactive degradates**

The Panel added that it is important to determine if products of degradation in the environmental and biological samples are bioactive in target and non-target organisms. Recognizing that testing all RNAi processes for all degradation products is improbable, investigations should strive to identify dsRNA transformation products that have moderate to high likelihood of exhibiting RNAi activities in target and non-target organisms.

**4) Uptake by non-target organisms**

If the dsRNA is bioavailable to target and non-target organisms (either as the parent dsRNA or siRNA), the next step is to determine the potential exposure to non-target organisms. The EPA background document (EPA, 2013) discussed the possibility that some organisms have the ability to uptake environmental siRNA and amplify these siRNAs within the organism that may lead to additional exposure. For example, *C. elegans* can accomplish this through RdRP amplification (Sijen et al., 2001). Environmental uptake and RdRP amplification has also been noted for some plants and fungi (Dillin, 2003). If cellular uptake is not possible then environmental exposure would be irrelevant. RdRP amplification pathways are not present in mammals, insects, and bacteria. The presence of other amplification pathways over a wider range of organisms should be investigated if there is a potential for transfer. The Panel discussed amplification mechanisms in response to questions 2 and 6 and cellular uptake mechanisms in response to question 5.

**5) Trophic connections**

The Panel discussed exposures due to possible trophic connections (e.g., predation, herbivory, mycophagy, etc.). The focus was on the connection between soil and above ground organisms, e.g., rhizosphere microbes and invertebrates that can be consumed by above ground vertebrates and invertebrates. Given these trophic linkages, the Panel suggested that the potential of dsRNA transfer between soil and above ground organisms be investigated if the potential for transfer of dsRNA is determined in the previous studies. In these cases, the Panel also recommended that the uptake of dsRNA in herbivorous insects and non-target insects should be evaluated because of the possible impacts on predatory insects and arachnids. The Panel discussed amplification mechanisms in response to questions 2 and 6 and cellular uptake mechanisms in response to question 5.

**6) Non-target toxicity testing**

The current non-target testing paradigm for PIPs, most of which express proteins derived from the bacterium, *Bacillus thuringiensis* (*Bt),* is based on modes of action that are fundamentally different from RNAi. The Panel indicated that that EPA's proposed ecological risk assessment framework should be modified to address the unique environmental fate and exposure scenarios posed by dsRNA PIPs.

In any non-target testing paradigm**,** multiple non-target species should be tested; however, a standard suite of species may not be appropriate for all RNAi products. The life stage of potentially exposed non-target species should be considered in toxicity testing paradigms. For example, if toxicity is observed for vertebrates, juveniles should be considered as a sensitive alternative to the standard test design. Juveniles are most likely to be present at the time when insecticidal activity is needed and as such they are likely to be exposed at a susceptible life stage. Fossorial or other seasonally burrowing organisms (e.g., amphibians) may be exposed dermally. This could be particularly important for amphibians in the playa or prairie pothole regions. The Panel suggested having amphibian testing earlier as part of a tier 1 testing scheme. Much more descriptive information on the suitability of the current ecological risk assessment is found in the response to question 7.

*4.a.ii) What additional information, if any, would EPA need to assess environmental fate of dsRNA PIPs?*

**Panel Response**

The Panel agreed with EPA that dsRNA PIPs pose unique challenges not seen for other PIPs (see discussion in EPA's issue paper, pp. 13-19). There will be significant differences in evaluations of dsRNA PIPs, e.g., dsRNA in plant tissue and in the rhizosphere, and exogenously applied dsRNA end-products, e.g., sprays.

The Panel stated that current requirements for soil degradation studies will provide some insight for non-target testing, but additional data are needed to assess the environmental fate of dsRNA PIPs and potential exposures to non-target organisms, i.e., plant degradation (above and below ground), off-site movement of plant materials, *in planta* exposure for organisms that directly ingest plant materials. Plant tissue may be desiccated and remain in fields during quite cold periods. As a result, dsRNA may remain in plant material for long periods of time allowing slow release into the environment. Double-stranded RNA PIPs may be exuded into the rhizosphere, where biotic and abiotic processes can vary quite significantly between growing and dormant seasons. The Panel noted that roots of annual plants will decompose under soil chemistry conditions (nutrients, oxygen, temperature, moisture, microbial activity) that may be dissimilar to growing season conditions. The Panel emphasized that additional data are critical to understand the stability of dsRNA in soil and aquatic environments. While there is a reasonable body of literature describing the environmental persistence of nucleic acids in soil, there is little information on the persistence of dsRNA and small RNA (siRNAs and miRNAs). The Panel recommended the collection of environmental fate data for different soil types, water bodies, and weather conditions under which crops with dsRNA PIP may be cultivated.

**1) Soil type**

Persistence in the soil is affected by a range of soil qualities and environmental conditions. The Panel suggested that persistence be measured for different structural forms of the RNAi molecules (dsRNA, siRNAs and hairpin RNAs) across a range of temperatures, moistures, and soil type. Whether the dsRNA PIP bound to organic matter renders them unavailable for uptake or degraded is not known. Thus, in studies of RNA degradation, it will be important to indicate when and where synthetic RNA molecules are used, and what differences, if any, there may be between these substrates and the native plant materials. These might include chemical modifications, sequence differences, protein packaging, etc. The contribution of soil type on degradation kinetics of dsRNA PIPs expressed in crops should be further evaluated for multiple soil types and at various temperatures, etc.

During the public comment period, representatives from Monsanto presented unpublished data from preliminary soil degradation studies of a dsRNA PIP for corn rootworm control under development. One panelist was concerned that low PIP concentrations *in planta* in Monsanto's studies were insufficient to allow detection in degradation studies. In response, representatives from Monsanto stated that the active dsRNA PIPs are at such low concentration that in their soil studies, vast quantities of synthetic dsRNAs were added to biological samples in order to be able to measure the RNA. The Panel stated that these data should be made publicly available[1].

**2) Soil biology**

An important and overlooked component in EPA's list of required tests is the contribution of abiotic and biotic factors on the rate of soil degradation. For example, biodegradation of DNA is mediated by microbial-produced deoxyribonucleases (DNases) (Levy-Booth et al., 2007). The degree to which biota contribute to the degradation rate of dsRNAs in the soil is poorly understood, and as such, represents an important data gap that should be addressed. The Panel recommended that the contribution of soil biota on degradation kinetics of dsRNA PIPs expressed in crops (e.g., degrading plant biomass in the presence or absence of other organic matter) should be evaluated using multiple soil types, temperatures, etc. conducted to refine exposure assessments.

**3) Viability of dsRNA, siRNA transfer to other organisms**

The viability of dsRNA (siRNA) of uptake by other organisms is an issue that needs to be addressed. If the dsRNA (siRNA) is bioavailable then transfer to other organisms might be possible if there is cellular uptake. If uptake is not possible then the issue of viability is irrelevant. See also the discussion of cellular uptake in response to question 4.a.*i.2* and 5.

**4) Predictive value of models**

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[1] Note: These data were published subsequent to the SAP meeting as Dubelman et al. (2014). The Panel used only the information presented by Monsanto during the meeting.

Models are good tools if the model has sufficiently precise and accurate empirical data defining input parameters. However, this is not the case for dsRNA PIPs. The Panel stated that uncertainties involved in input parameters of the EPA and other stability/persistence models may be sufficiently large to provide outputs that are of little or no predictive use. For this reason, the Panel urges caution in using EPA models (discussed on p. 15 of EPA's issue paper) until more empirical data are available. This shortcoming can only be overcome with sufficient empirical data that describe PIP environmental fate and transport, persistence, bioavailability and biological effects.

*4.b. In section IV.B.4., EPA proposed environmental fate data needs for exogenously applied dsRNAs. To what extent is the proposed testing sufficiently robust to inform nontarget organism exposure for these dsRNAs?*

**Panel Response**

As noted for dsRNA PIPs, there are also unique challenges to the assessment of environmental fate of and exposure to exogenously applied dsRNA products. The response to question 4.a.*i.* regarding environmental fate data needs for dsRNA PIPs (soil type, soil biology, viability of dsRNA (siRNA) transfer to other organisms, and applicability of models) is also generally applicable to answer questions concerning environmental fate data needs for exogenously applied dsRNAs; however, there are some differences. Non-target species are more likely to be exposed to exogenously applied dsRNA end-products than to dsRNA PIPs. Future end-products might fit into several different pest control categories (p. 24, EPA issue paper). Non-PIP dsRNA exposures are likely multi-route while dsRNA PIP exposure is more likely via diet. Exposure may be through airborne particles, diet, or dermal/cuticle penetration depending on the end-product. However, the dsRNA in an applied product (e.g., spray application) may be more exposed to environmental factors, e.g., UV light. As such, more species may be exposed to exogenously applied dsRNA products, although this concern may be balanced by the likely increased instability of the sprayed product (duration of the RNAi).

The Panel agreed that EPA's standard testing paradigm (shown below) represents a good beginning for data sets needed to understand soil degradation and environmental fate of dsRNAs in the environment; however, additional data are needed to address exposure scenarios unique to exogenously applied dsRNA products (see also EPA's discussion of exposure scenarios on pp. 26-28 of the issue paper).

- 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

The Panel stressed the importance of assessing the bioavailability of the dsRNA to non-target organisms as one of the earliest steps in the environmental fate evaluation process (a first tier evaluation). Data are lacking on cellular uptake of dsRNA at various taxonomic levels. In the absence of data, the Panel recommended that the present focus should be on evaluating uptake in non-target organisms closely related to the target (e.g., species in the same genus and related genera in the same family). See additional discussion in response to question 5. Special consideration should be given to non-target amphibian testing as part of a tier 1 testing regime. The Panel indicated that the life stage exposed to dsRNAs should also be considered in the design of non-target tests. Early life stages often do not have protective enzymes or other detoxifying mechanisms. The Panel recommended that multiple species be evaluated for some of the required tests. Test species might include organisms not currently listed in the non-target testing paradigm. See the Panel's response to question 7.

For both dsRNA PIPs that may be free in the environment and exogenously applied dsRNAs, knowledge of the initial residue burden, and the dissipation rate of residues, is essential for developing environmental exposure estimates, particularly if models of environmental fate are used to arrive at these estimates.

*4.c. What analytical methods are available to accurately and precisely measure dsRNAs in diverse plant, soil, and water matrices?*

**Panel Response**

The Panel indicated that existing RNA extraction methods and subsequent quantitative PCR, high throughput sequencing, or hybridization analysis are the most current tools to assess the presence of a particular small RNA within a sample. The Panel stressed the importance of standardizing the extraction and isolation protocols and having accurate and precise analytical methods to quantify if active degradation products, dsRNA and associated small RNAs, are present in the biotic or abiotic samples.

The Panel emphasized the critical nature of using a reliable method for extracting the dsRNA to track the environmental fate of the dsRNA in soil. Each PCR is run on a VERY small quantity of soil or tissue, and thus it becomes very important to conduct power analyses to assess how many samples will be necessary in order to reliably detect the substance within environmental samples. Recovery of material from an environmental sample may be limited. The use of small sample sizes (mass/volume) may introduce significant intra sample variance due to sample heterogeneity. For example, metal distribution in soils can be extremely variable. With these considerations, extremely large numbers of samples may be required for a reliable analysis of agricultural systems under different field conditions.

## Question 5. Primary Route of Exposure for Nontarget Organisms to dsRNA.

**The primary route of exposure for nontarget organisms to dsRNA PIPs is assumed to be ingestion, either of dsRNAs contained in plant tissue or free in the environment. However, some evidence also indicates the potential for exposure by direct contact in nematodes and some arthropods. For**

**dsRNAs that are applied to plants and/or the environment, the primary routes of exposure are expected to be both ingestion and direct contact.**

*5.a.i) In addition to the conditions of the gut environment and enzymes influencing digestion, what other factors may play a role in uptake within the gut and potentially limit exposure to dsRNA?*

**Panel Response**

The published literature indicates there is a wide range of cellular uptake mechanisms that vary from species to species and across taxa. For example, cellular uptake of dsRNA is mediated by transmembrane proteins, SID-1 and SID-2, or through endocytosis (Hannon, 2002; Huvenne and Smagghe, 2010). It seems likely that other pathways for dsRNAs to enter cells will be discovered in the future. Factors that influence cellular uptake and activity include: tissue- or cell-specific expression, transport proteins, dose, dsRNA sequence length, and degradation. The Panel recommended additional research to study this issue.

The Panel commented on the following factors that influence cellular uptake and activity:

- ***Tissues.*** Gene expression may be tissue or cell specific. It remains unclear what processes make tissues amenable or not to RNAi. Indeed different tissues, even in target organisms, lack the ability to uptake dsRNA from the surrounding environment. The basis of this difference is still unknown, but may be due to fundamental developmental differences between tissue types, such as cell ploidy, or due to differences in gene expression required for the uptake and transport of dsRNA. The gut consists of different cell types, columnar cells with typical microvillar pole, goblet cells, endocrine cells, and stem cells. Data is lacking on cellular uptake in different cell types or in cellular uptake in different organisms (Hakim et al., 2010).

- ***Transport proteins.*** The impact of different transport proteins is not clear, e.g., SID-1, SID-1 like/SID-2, clathrin-mediated and/or caveolae-mediated endocytosis, and lipophorins can all bind dsRNA. For example, lipophorins can adhere to dsRNA, bacteria and fungi present in the insect hemolymph (Wynant et al., 2014) and assist in the cellular uptake of dsRNA in some insects (Terenius et al., 2011; Scott et al., 2013; Swevers and Smagghe, 2012). The Panel stressed that a generalization of physical barriers for environmental uptake of dsRNAs (from PIPs or exogenously applied) across non-target taxa (e.g., targeted versus non-targeted taxa or humans versus arthropods) is not possible at the present time due to the lack of information across species. The published literature indicates there is a wide range of environmental uptake mechanisms that vary from species to species and across taxa. No definitive conclusions can be made concerning specific physiological barriers at this time. The Panel also commented that ability to cross physiological barriers also has to be combined with the binding in the gut; i.e., whether there is an actual target to be affected. There is no predictable conserved pattern of transport mechanisms among all insects.

- ***dsRNA length.*** There is some information that dsRNA length impacts cellular uptake, but there is no generalized pattern among insect taxa.

- ***Dose.*** Dose information is needed for the assessment of exposure and effects of exogenous dsRNA. What dose of dsRNA will trigger effective gene silencing?

- ***Formulation of applied dsRNA end-products.*** Polymers and agents used in an end-product formulation to increase uptake/penetration kinetics to overcome physical barriers should be investigated for potential effects on non-target organisms.

Other factors suggested by some panel members:

- ***Metabolic degradation of dsRNA in insects.*** One panel member suggested that rapid degradation of dsRNA in some insects would minimize exposure to exogenous dsRNA. There are only two papers so far that confirm enzymatic degradation of dsRNA in insects (Lygus and aphids) in the saliva and in the gut (Allen and Walker, 2012; Christiaens et al., 2014). Additional information is needed for other insects before any generalization can be made regarding the role of degradation in reducing non-target exposure to exogenous dsRNAs (PIPs or non-PIPs)

- ***Peritrophic matrix.*** One panel member mentioned that the peritrophic matrix lining the midgut in many insects might act as a physical barrier, but no information exists to verify this role. Another panel member responded that exogenous dsRNA will readily pass through the peritrophic matrix because of its size.

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*5.a.ii) Please comment on how these barriers can be generalized across all nontarget taxa that are considered in EPA's risk assessments (e.g., birds, plants, fish, etc.) for both dsRNA PIPs and exogenously applied dsRNA.*

**Panel Response**

The published literature indicates there is a wide range of environmental uptake mechanisms that vary from species to species and across taxa. The strategy of using dsRNA as a species-specific pesticide against insects depends at least in part on dietary uptake that varies from species to species (see references including, but not limited to: Whyard et al., 2009; Zhou et al., 2008; Terenius et al., 2011; Bachman et al., 2013; Zhang et al., 2013). For example, amongst insects, there is a wide range of gut pH; diet composition and feeding practices; conservation and function of RNA receptors and transmembrane channels including, but not limited to, the SID proteins; and activity of RNAses in digestive fluids and hemolymph. These factors may contribute to species differences in foreign RNAi effectiveness. Gut conditions among organisms are variable. A low pH of 1-2 occurs in the human stomach, while the stomach pH is higher in mice. In Lepidoptera the midgut pH can reach 12. There is

no indication of interaction of insect dsRNAs with the gut microbiome communities (e.g., degradation, accumulation, amplification).

The Panel stated that there is insufficient understanding of the uptake mechanisms of dsRNAs (PIPs or non-PIPs) and potential barriers to make any generalizations across non-target taxa. The Panel also commented that the ability to cross physiological barriers has to be combined with binding in the gut (where there is an actual target to be affected). The Panel recommended the collection of additional information to evaluate the types of cellular uptake mechanisms that exist and whether there are any patterns among different species, genera, or families, for example. For now, the Panel recommended a case-by-case evaluation of cellular uptake mechanisms for non-target taxa, as needed (based on exposure).

*5.b. The degree of exposure by direct contact is likely to differ between nontarget risk assessments for dsRNA PIPs and nontarget risk assessments for exogenously applied dsRNAs.*

*5.b.i) Please comment on the importance of the contact route of exposure for nontarget risk assessments for each of these types of dsRNAs.*

**Panel Response**

There are insufficient data on the contact route of exposure to comment on the importance of this route in non-target risk assessments. The Panel found only two studies that examined direct contact as a route of exposure to dsRNA. The limited information available indicates that the dietary route of exposure was probably the primary route of entry leading to activity and not the contact route of exposure. The Panel recommended the collection of additional information to evaluate the importance of this route of exposure. For now, the Panel recommended a case-by-case evaluation to determine whether this is a likely route of exposure before pursuit of an in-depth study.

In both studies, insects were soaked or treated topically with a solution of dsRNA. In the first study (Singh et al., 2012), mosquito larvae (*Aedes aegypti*) were soaked in 0.5 µg/µL dsRNA solution for 2 hours. The mRNA transcripts for *β-tubulin*, *chitin synthase-1* and *-2*, and *heat shock protein 83* were reduced between 30 and 50% at three days post-dsRNA treatment. Based on the Panel's review of the data, and as reported by the authors, the gut was probably the primary route of entry for the dsRNAs leading to activity. In the second case (Wang et al. 2011), newly hatched *Ostrinia fumalalis* larvae (a lepidopteran) sprayed with 50 ng/ml dsRNAs of the genes *DS10* and *DS28* resulted in around 40-50% mortality. In this situation, uptake happened via the gut as caterpillars crawled over each other. The Panel remarked that in *C. elegans*, soaking is a common practice (Tabara et al., 1998). Here, as in the other described cases, the probable route of dsRNA uptake is through the digestive system and not through the integument itself. The Panel recommended that this route of exposure should be evaluated on a case by case basis, as indicated (based on exposure).

*5.b.ii) What barriers are likely to exist for this route of exposure for both terrestrial and aquatic organisms, and how can these be generalized across nontarget taxa?*

The Panel indicated that there are not enough experimental data available on the contact route of exposure and uptake for both terrestrial and aquatic organisms to make any comparisons or generalizations across non-target taxa. For now, the Panel recommended that each dsRNA PIP or exogenously applied dsRNA end-product should be evaluated on a case-by-case basis (based on exposure). See response to question 5.b.i.

## Question 6. Unintended Effects of dsRNA

**In the issue paper, EPA discussed possible effects other than silencing of the target gene in the target organism that may occur as a result of exposure to dsRNA. These unintended effects of dsRNA include off-target effects, silencing the target gene in nontarget organisms, degradation of non-targeted mRNA by transitive RNA, and effects resulting from immune stimulation and saturation of the RNAi machinery. EPA has little information to estimate the range of unintended effects that may occur and their probability of occurrence as a result of exposure to dsRNA in the environment.**

*6.a. Please comment on the unintended effects that EPA might reasonably anticipate in nontarget organisms exposed to dsRNAs, the likelihood of such unintended effects, and the biological significance of these effects in nontarget organisms, should they occur.*

### Panel Response

Note: The Panel focused the discussion on unintended effects of dsRNA on non-target insects.

The Panel discussed several possible unintended effects that may result from exposure to dsRNA. One concern is that the potential scale for use of pesticidal RNAi in the agroecosystem (in particular) will increase the potential of unintended effects. For example, dsRNA used to silence the target gene may also silence the same conserved gene in non-target organisms. But one of the advantages of RNAi technology is that the RNAi effect is sequence and target gene dependent. Therefore, it is possible that dsRNA sequence used to trigger RNAi can be designed to be highly species specific which should help reduce possible non-target effects. Saturation of RNAi machinery and immunostimulation has been observed in animals (in mice or in cell cultures), but both require a fairly high dose to cause deleterious effects. The dose required to affect the RNAi machinery and induce immunostimulation in insects is unknown. There is also a lack of information on the risk of insect resistance to RNAi-mediated gene suppression. The knowledge gaps make it difficult to predict with any certainty whether unintended effects will occur in non-target species as a result of exposure to dsRNA. More data to close these gaps will increase the certainty in predicting the likelihood of unintended effects.

**1) A standard suite of molecular machinery is present in RNAi-active eukaryotes**

The introduction to the report provided an overview of the elements involved in dsRNA-mediated gene interference. This section focuses on eukaryotic RNAi. There are four essential steps that potentially dictate which species will be affected by this technology (Agrawal et al., 2003). First, a dsRNA must be produced (either endogenously or exogenously) that has some minimal level of

specificity for a targeted mRNA. Next, this relatively long dsRNA (<1,000 bp) must be cleaved into small (20-25 nt) siRNAs using an enzyme in the Dicer family (Elbashir et al., 2001; Siomi and Siomi 2009). These siRNAs dissociate into single strands after integration into RISC. Finally, the target mRNA molecule is either cleaved (by an enzyme in the Argonaute family) or removed from translation through binding with the siRNA. Silencing in the absence of cleavage may result if the RISC unit simply binds to mRNA, thereby restricting its translation (Alemán et al., 2007). The amplified siRNAs are sometimes then transported to other cells of eukaryotic hosts via the SID-2 transport protein; this process is dubbed systemic RNAi. Although these steps appear to be universally present in eukaryotes that use RNAi, there are slight differences in the machinery, which potentially restrict the activity spectrum of a given dsRNA for non-target organisms (Agrawal et al., 2003; Reynolds, 2004; Leake et al., 2004; Alemán et al., 2007; Doench et al. 2007).

In plants, the amplification process is regulated by RNA-directed RNA polymerases (RdRP). In contrast, an RdRP amplification process or an equivalent has not been identified in insects (a primary target of RNAi-based genetically-modified crops) or in mammals (see discussion in response to question 2). An exception is embryonic *Drosophila melanogaster* (Agrawal et al., 2003; Dillin, 2003; Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). Although RNAi functions at low molar quantities in these organisms and many cell types and tissues remain to be examined. As such, some mechanism that facilitates the proliferation of siRNA within a target insect or mammal may be discovered upon further exploration.

**2) Unintended gene suppression by pesticidal RNAs (non- and off-target effects)**

Most of the work on off-target silencing is related to functional genomics within a cell culture or a single organism, and so the question of how the choice of dsRNA sequence affect target and off-target genes in non-target organisms that are potentially phylogenetically distant from the target pest has received very little attention. Nevertheless, substantial research has been conducted in this area from the perspective of functional genomics and gene therapy that can be used to inform the likelihood of this effect. Most of the experience with this technology is in cell cultures or sick individuals.

The potential scale for use of dsRNA technology as pesticides in agroecosystems (in particular) warrants exploration of the potential for unintended ecological effects. The Panel discussed several possible unintended effects that might occur if non-target organisms are exposed to dsRNA PIPs or exogenously applied dsRNA end-products. The knowledge gaps make it difficult to predict with any certainty whether unintended effects will occur in non-target species, and their severity should they occur, as a result of exposure to dsRNA. Additional information is needed to close these gaps and reduce uncertainty in predicting the likelihood of unintended effects.

***a. Silencing the target gene in a non-target organism using pesticidal RNA can be partially mitigated through design of the pesticidal RNAs.*** Carefully targeting genes that have species-level divergence in sequence will help to hone the specificity of RNAi to the target pest. Through the use of dsRNAs that produce multiple, distinct functional siRNAs, the genes of non-target organisms that are the focus of RNAi can have substantial sequence divergence from the target species' gene and still experience gene suppression. For example, in developing genetically-modified maize plants resistant to *Diabrotica virgifera virgifera* (Western corn rootworm), Baum et al. (2007) also examined the effects of

a few of the dsRNAs identified for plant transformation on several other beetle species. They found that the dsRNAs that targeted *D. virgifera* v-*ATPase A* and *E* also reduced survival of *Diabrotica undecimpunctata* (spotted cucumber beetle) and *Leptinotarsa decemlineata* (Colorado potato beetle) significantly, even though these pests shared only 79 and 83% sequence homologies in these genes with *D. virgifera*. Whyard et al. (2009) did not find increased mortality when *Drosophila* spp. ingested dsRNAs designed to suppress a congener's *tubulin* gene in laboratory feeding assays. These results were echoed when other, more phylogenetically distant, insect taxa ingested dsRNAs aimed at repressing other species' *γ-tubulin* or *v-ATPase* expression, although mRNA knockdown for the latter gene was minimal even for the targeted insect species.

Each organism's genome presents a unique set of potential off-target gene sequences (Qiu et al., 2005). The likelihood of off-target binding increases with the size of the genome, and so reduced hazard is posed to non-target species with relatively small genomes (Qiu et al., 2005). The length of the dsRNA is also positively correlated with off-target binding (Lew-Tabor et al., 2011), and using strictly siRNAs can substantially reduce the likelihood of off-target effects (Qiu et al., 2005). Structural characteristics of the siRNA, especially in the seed region (2' location of the guide strand) is particularly important in reducing off-target binding, and pesticidal RNAs can be engineered rationally (examples would be methylating the 2' position of the small RNA (Caffrey et al., 2011), or producing "bulge" RNAs) (Dua et al., 2011). Chances of off-target binding increase as the siRNA becomes shorter and if sequences mismatches between target and off-target sites occur (Qiu et al., 2005; Seinen et al., 2011). Small inducing RNAs are more likely to produce off-target effects when a higher dose than is needed is administered (Caffrey et al., 2011).

***b. Sequence homology and off-target gene silencing.*** *In silico* searches commonly reveal that 10-20% (but potentially up to 80%) of randomly generated siRNA sequences will bind to unintended mRNAs within a single organism (Qiu et al., 2005; Kulkarni et al., 2006). There is also experimental evidence of off-target binding (Jackson et al., 2003; Lin et al., 2005, Ma et al., 2006, Davidson and McCray, 2011; Dua et al., 2011; Lew-Tabor et al., 2011; Seinen et al., 2011; Jarosch and Moritz, 2012). Rates of mRNA and protein suppression may be correlated with rates of off-target binding predicted by *in silico* searches for sequence homologies with siRNAs (Lew-Tabor et al., 2011, Seinen et al., 2011), especially when considering the sequences of the seed region rather than complete sequence of the siRNA (Birmingham et al., 2006). Given the diversity of organisms present in and around agroecosystems and uncertainty in defining the spectrum of activity and non-target organisms exposed to potential dsRNA PIPs or exogenously applied dsRNA end-products, the Panel suggested that *in silico* searches may help identify potential non-target organisms that could be further evaluated (see related discussion in response to question 7). As stated in the response to question 1, *in silico* searches may be useful screening tools to identify potential non-target organisms, but should not be used as an absolute predictor of effects.

Evidence suggests that off-target binding can change the phenotypes of affected organisms, sometimes unpredictably. Suppression of mRNA by off-target binding reduces some phenotypes (Saxena et al., 2003; Lin et al.,2005), although RNAi effects on off-target protein levels tend to be less studied than mRNA regulation. Federov et al. (2006) found that 29% of off-target suppression of mRNAs resulted in the death of transfected cells. Off-target binding of siRNAs resulted in reduced

protein production in 7 of 30 cases involving culture cells; surprisingly this off-target suppression of genes was not accompanied by mRNA cleavage, but by binding of the siRNA and RISC unit with the targeted mRNA (Alemán et al., 2007). Thus, looking only at mRNA levels may overlook some off-target gene silencing (Saxena et al., 2003; Alemán et al., 2007). These off-target effects become particularly important when proteins within metabolic cascades or biochemical pathways; numerous phenotypes could be affected by disruptions of individual mRNA (Wheeler et al., 2013).

The Panel noted that off-target gene silencing will only affect phenotypes of the transcribed genes, and so it may be more effective to predict off-target effects by examining comprehensive transcriptomes rather than full genomes. Transcriptome changes would need to be related to apical effects (growth, development, survival, etc.) to confirm the prediction. This issue is also discussed below and in the response to question 7.

**3) Saturation of RNAi machinery can have severe consequences**

Saturation of RNAi machinery could have severe consequences for cellular functions, although it is unclear what dose is necessary to harm non-target organisms. High levels of exogenous siRNAs can saturate a cell's RNAi machinery, and thereby reduce the efficiency at which a cell regulates endogenous gene expression with miRNA (Agrawal i et al., 2003; Dillin, 2003; Grimm et al., 2010). Dicer was one of the first components of RNAi machinery that was demonstrated to be saturated by high levels of siRNA. Essentially, there are limited number of RISCs present within a cell, and if the augmented siRNAs saturate these complexes, then health and performance of the cell may be compromised (Kahn et al., 2009). In particular, Exportin-5 and Argonaute proteins (especially Ago-2) seem to be particularly limiting components of the RNAi machinery that have been demonstrated to be saturated by exogenous siRNAs (Grimm, 2011). Saturation occurred when hundreds of thousands of siRNA sequences were being produced per cell (Grimm et al., 2010), and dose is clearly an important component of this risk factor. Jackson and Lindley (Jackson and Linsley, 2010) found evidence that small RNAs could have "global effects on the expression of genes predicted to be under the control of endogenous microRNAs." This process of saturation is better documented with shRNA (small hairpin RNAs), although it is known from siRNA as well. One question that needs to be answered first is whether it is possible to deliver enough pesticidal dsRNA to saturate an organism's RNAi machinery. One panel member commented that saturation will not matter if the amount will be so minimal that it is inconsequential.

**4) Immune stimulation**

Innate immune systems of higher organisms rely on pattern recognition proteins and other factors to identify potentially pathogenic invaders, and these defenses recognize and eliminate dsRNAs that are potential pathogens (Elbashir et al., 2001; Kulkarni et al., 2006). Immunostimulation is more likely to occur with molecules smaller than 28 nt in length. Generally speaking, siRNAs are able to trigger mammalian endosomal immune cascades (e.g. Toll receptors) or cytoplasmic pathways (e.g., RIG-1, Mda-5, PKR) (Robbins et al., 2009; Forsbach et al., 2011; Menget al., 2013). Immune stimulation appears to be sequence dependent, and the length of the siRNA does not apparently affect immunostimulation (Forsbach et al., 2011). In mice the immunostimulation of RNAi led to reduced lymphocytes and platelet cells, largely correlated with cytokine response to the siRNA (Judge et al.,

2005). Although there are some similarities in the innate immune response of insects and mammals (Saleh et al., 2009; Lundgren and Jurat-Fuentes, 2012), it is unclear how the immune systems of other organisms will react to an influx of small RNAs. Nor is it known how this immunostimulation will affect the fitness of non-target organisms. Slight changes in nucleotide sequence, particularly at the 2' position of the guide strand, can reduce the immunostimulatory effects in a given organism (Jackson and Linsley, 2010; Meng et al., 2013).

The Panel concluded that there is no clear evidence that small RNAs could trigger the immune systems of non-mammalian non-target species or what dose is necessary to trigger a response. See also the Panel's discussion in response to question 2.

**4) Concern for insect resistance**

While the general consensus in the scientific community is that resistance to PIPs is unlikely, this has not been fully investigated. The idea of resistance (the selection of a genetically different population of insects from the target population that have a survival advantage when exposed to the biopesticide) is unlikely because it is assumed that the PIP would be designed to a highly conserved region of a gene that has low/no sequence polymorphisms. However, this information may not be available for different populations of the target pest, and failures could occur if SNPs were found in the gene for the critical target region of the dsRNA/siRNA (Scott et al., 2013). Therefore, caution is warranted in evaluating the potential for resistance in multiple field populations prior to release of the event, and for continued monitoring once the event is released for commercialization.

*6.b. To the extent that additional information would reduce uncertainty in addressing these issues, please describe specifically the nature of additional information that EPA may need and the degree to which this information would reduce uncertainty in the ecological risk assessment.*

**Panel Response**

As discussed in the response to Question 6.a., there is uncertainty in how to define the spectrum of insecticidal activity. The spectrum of activity depends on the target gene, dsRNA chosen, where and how much the dsRNA PIP is expressed in the plant, and the persistence and stability of the effect. What is a realistic level of non-target exposure based on the stability and persistence of the dsRNA? The Panel noted that the mode of action of RNAi (by siRNAs) is, by definition, very specific (see discussion in response to question 1). However, there is some concern that unintended effects could occur across multiple insect orders. There could be unique effects for each non-target organism. The question is how do you decide what non-target should be tested? For example, insect species representing 10 families and four insect orders were tested for DvSnf7 dsRNA activity (Bachman et al., 2013). Uncertainty in the spectrum of activity makes it more challenging to understand the environmental exposure levels and potential uptake pathways, important information that can narrow the scope of non-target organism testing for an ecological risk assessment.

The Panel suggested an exposure-based conceptual model to reduce the uncertainty in the ecological risk assessment of dsRNA PIPs and dsRNA non-PIP end-products. This approach would

identify non-target organisms that might be exposed and narrow the spectrum of non-target organisms that would need to be tested for effects. The conceptual model is explained in the Panel's response to question 7.

One panel member suggested a screening process to identify a diversity of responses at a "network level." Cell lines from different tissues could be used to screen for differential gene expression. Test endpoints could be better defined.

**Question 7. Framework of Testing for Nontarget Effects**

**In sections IV.A.2.a. and IV.B.4. of the issue paper, EPA presents the current framework of testing for determining nontarget effects resulting from exposure to PIPs and biochemicals, respectively. In section IV.A.2.c., EPA also raised potential issues related to nontarget testing with dsRNAs that may arise given their unique mode of action, which included 1) the potential influence of latent effects on results of nontarget testing, 2) the appropriate life stage for testing, and 3) the possibility for chronic effects.**

*7. a. Please comment on how each of EPA's current PIP and biochemical frameworks for nontarget effects testing will inform risk assessment for dsRNA PIPs and exogenously applied dsRNAs. In providing a response, please address the potential for unintended effects as described in Question 7, as well as the three issues outlined as concerns for nontarget testing listed above.*

**Panel Response**

The Panel appreciates all the time and effort spent by both the Agency and the public commenters in preparation and presentation of the materials for this meeting. Use of RNAi technology in pesticidal products poses some unique challenges to environment fate and ecological risk assessment.

The Panel agreed with concerns the Agency expressed regarding inadequacies of the current environmental fate and non-target effects testing frameworks for ecological risk assessment dsRNA PIPs and exogenously applied dsRNA products. As stated in Section IV of the EPA issue paper (p. 23, EPA, 2013):

- *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.*
- *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.*
- *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.*

Current requirements for soil degradation studies will provide some insight for non-target testing, but additional data are needed to address the environmental fate of dsRNA PIPs and potential exposures

to non-target organisms, i.e., plant degradation (above and below ground), off-site movement of plant materials, *in planta* exposure for organisms that directly ingest plant materials. Additional data are also needed to address the environmental fate of exogenously applied dsRNA products. Non-target species are more likely to be exposed to exogenously applied dsRNA end-products than to dsRNA PIPs. Future end-products might fit into several different pest control categories. Non-PIP dsRNA exposures are likely multi-route while dsRNA PIP exposure is more likely via diet. Exposure may be through airborne particles, diet, or dermal/cuticle penetration depending on the end-product. However, the dsRNA in an applied product (e.g., spray) may be more exposed to environmental factors, e.g., UV light. As such, more species may be exposed to exogenously applied dsRNA products, although this concern may be balanced by the likely increased instability of the sprayed product (duration of the RNAi). The Panel discussed these issues in detail in response to question 4.

The Panel indicated that the classic approach of developing and assembling effects data will not work well for this technology due to the modes of action of RNAi. No one set of test species will serve as an adequate representation of non-target species at risk for every dsRNA PIP or exogenously applied dsRNA products.

The Panel discussed the following limitations in the current ecological risk assessment framework.

- ***dsRNA PIP and dsRNA non-PIP characterization including spectrum of activity.*** There is limited environmental fate information for dsRNA PIPs or dsRNA non-PIPs to determine exposure and what non-target organisms might need to be tested. The specificity of control is dependent on the target gene selected (gene specificity), dsRNA selected and resulting siRNAs (sequence specificity), mechanisms of action, plant tissue expressed (dsRNA PIPs), dose needed to trigger RNAi, amplification, and duration of the effect. This is why the approach to defining the spectrum of activity for RNAi PIPs or RNAi end-products is critical for the assessment of non-target effects.

- ***Dose considerations in bioassays.*** Maximum hazard dose assays alone may not be sufficient for assessing off-target effects associated with the use of RNAi technology. There is limited information on the dose and duration of exposure needed to trigger a nontarget effect. Given these limitations, the Panel observed that it will be difficult to determine the range of doses to test and the duration of the bioassays. The Panel recommended that bioassays for dsRNA PIPs include doses that mimic the *in planta* expression levels of the PIP and where possible, use plant tissue.

- ***Consideration of chronic and sublethal effects.*** Mortality is one of several endpoints. Maximum hazard dose and measuring mortality offers limited information needed for risk assessment dsRNA PIPs and non-PIP dsRNAs. The current Tier 1 testing scheme does not provide insight into potential chronic effects and limited insight to the potential for sublethal effects.

- ***Prediction of non-target effects and exposure.*** In the Panel's response to Question 6.a., the following potential unintended effects were identified: silencing the target gene in non-target

species, silencing off-target genes in non-target organisms, saturating the RNAi machinery in non-target organisms, and stimulating immune responses in non-target organisms. The proposed testing scheme should include a process assessing the potential and severity of unintended effects if a non-target organism was exposed to the dsRNA PIP. Thus, an early step in the non-target testing scheme is determining what organisms are exposed to the dsRNA.

The Panel remarked that the most difficult effect to predict will be off-target binding of pesticidal RNAs to mRNAs that share a minimal level of sequence homology with the target gene. A complicating factor is whether potential effects of RNAi can only be observed if an off-target gene is expressed during the time and conditions of the observations. For example, different genes are up-regulated during different life stages of a non-target organism (this would also be true for the target organism), and under different environmental conditions. Therefore, the Panel indicated that strategic research will be important to determine under what conditions and what life stage should be assayed for RNAi activity.

- ***Genomics and sequence specificity.*** The Panel encouraged sequencing full genomes to expand existing databases of known genomes, but also recognized that associated costs may limit this application. The Panel encouraged sequencing full genomes to expand existing databases of known genomes. *In silico* searches could be performed using existing (and potentially expanded) databases of genomes to curtail the list of potential non-target organisms to be tested. The Panel also re-emphasized that bioinformatics analysis is a useful, but not an absolute, predictive tool (see related discussion in response to question 1). Sequence identity does not mean that the dsRNA would provoke an RNAi response, but should caution the potential for unintended interactions and further review. Results from *in silico* searches could be used to help identify potential non-target organisms that might be affected by off-target binding.

- ***Potential synergism.*** The Panel observed that there might be synergism between dsRNA PIP sand other PIPs expressed in a crop plant. The Panel recommended that EPA consider possible synergistic effects (of co-exposures) with respect to target and non-target organisms.

- ***Potential for evolution of resistance.*** The Panel stressed the importance of assessing the potential for the evolution of pest resistance to RNAi, as well as cross-resistance (or multiple resistance) to different modes of action (e.g., RNAi and *Bt* PIPs).

- ***Potential scenarios that could minimize the concern for non-target exposure and effects.*** One panelist suggested three potential scenarios that could minimize the concern for non-target exposure and effects:
  - When the dsRNA PIP is very specific without homology to any of the sequence databases;
  - When the dsRNA PIP is not modified and therefore likely to degrade rapidly in the environment; and
  - When the dsRNA is expressed at extremely low levels and is tissue specific (i.e., in the roots for corn rootworm).

In conclusion, the uncertainties in our understanding of the potential modes of action in non-target species, potential for chronic and sublethal effects, and potential unintended consequences in the various life stages of non-target organisms are sufficient justification to question the applicability of the current Agency framework for assessing the environmental fate and ecological effects of dsRNA PIPs or exogenously applied non-PIP end-use products. As a consequence, use of RNAi technology as dsRNA PIPs or exogenously applied dsRNA products poses some new (not insurmountable) challenges for risk assessment.

*7.b. What additional nontarget effects testing, if any, should EPA consider to gain a full understanding of the potential for dsRNAs to cause effects to nontarget organisms?*

*7.c. What other approaches, such as bioinformatics analysis, may be used to address concerns for effects on nontarget species and reduce the set of data requirements?*

*7.d. In providing answers to the above subquestions, please be specific in discussing the extent to which additional information would reduce the nature and magnitude of these specific areas of uncertainty.*

**Panel Response**

The Panel provided a combined response to questions 7b-d.

**1) Data needs**

Based on the responses to questions 4-6, the Panel concluded that additional data are needed to reduce uncertainty in the environmental fate and ecological risk assessments; however, the task of prescribing additional tests or additional test species cannot be done without a better understanding of the RNAi modes of action and what non-target organisms are likely to be exposed to dsRNA PIPs or exogenously applied dsRNA products. The inability to easily predict what non-target organisms might be exposed to the specific dsRNAs using the current framework challenges the evaluation of potential non-target effects.

The Panel provided the following general recommendations for data needed in the ecological risk assessment:

- Bioassays should include doses that mimic the *in planta* expression levels of the PIP and where possible, use plant tissue.

- Mortality is one of many possible endpoints. The potential for chronic effects and for sublethal effects should be considered.

- The Panel recommended evaluating entire life cycles, while simultaneously examining multiple life history parameters (intrinsic population growth rate is a robust metric) rather than simply relying on toxicity testing. These tests should be conducted under realistic conditions (as much as possible) to ensure that genes in operation in normal developmental conditions are

not suffering from off-target binding. Therefore, the Panel indicated that strategic research will be important to determine under what conditions and what life stage should be assayed for RNAi activity.

- In light of the discussion on potential immune system interactions in the EPA issue paper (EPA 2013) and during the meeting, the Panel recommended inclusion of an immunological endpoint in the non-target organism bioassays.

- The Panel also recommended an endpoint to measure the potential for saturation of the RNAi machinery in a non-target organism. Saturation of RNAi machinery could reduce the efficiency at which a cell regulates endogenous gene expression.

- The Panel recommended that EPA consider possible synergistic effects (of ds RNA PIP co-exposures to other PIPs) with respect to target and non-target organisms.

- The Panel also recommended that EPA consider the evolution of pest resistance.

**2) Proposed exposure-based conceptual model for assessment of ecological risk posed by dsRNA PIPs or exogenously applied dsRNA end-products (figure 7-1)**

To address the limitations in the EPA's proposed ecological risk assessment framework, the Panel designed an exposure-based conceptual model for use in ecological risk assessment of RNAi PIPs and non-RNAi PIPs (see figure 7-1). The Panel proposed a network approach that incorporates mode of action, exposure, species sensitivity, various endpoints, and adverse outcome pathway components (see Ankley et al., 2010) at the organism and population levels. Only non-target organisms that might be realistically exposed to RNAi PIPs or non-RNAi PIPs will be tested. This result will eliminate the need to test a battery of standard species (current tier 1) many of which will either not be exposed or be insensitive. Integration of the exposure-based conceptual model to non-target organism testing will further the understanding of the likelihood of adverse outcomes in non-target species, facilitate the selection of endpoints, and identify potential data gaps that would require further study. The Panel provided a detailed discussion of the framework for the proposed conceptual model.

**Box 1: Agent**

1. Characterize the dsRNA ("Agent")
    a. What is the dsRNA sequence?
    b. What is the gene targeted and what portion?
    c. What is the expected phenotypic effect? How was it measured? How long does it last?
    d. What are the target organisms?
    e. What is the dose to trigger an effect in the target organism?
    f. For PIPs, what is the expression level – systemic or tissue-specific?
    g. For sprays, what is the concentration?

2. What is the dsRNA degradation kinetics in decaying plant tissue, soil?

**Box 2 - Identify non-target species**

1. Agent studies define which species are likely exposed. Use this information to define the community that overlaps in space and time with the siRNAs (e.g., those organisms that are most likely to be exposed).

2. Based on the route of exposure (e.g., topical, ingestion), curtail the number of species of interest. For example, establish trophic linkages to the dsRNA PIP plant and determine the species likely to ingest plant.

3. Of this curtailed community define a set of species that will be tested *in vitro* based on relative abundance, importance in food web structure (e.g., trophic interactions), phylogenetic placement, and functional significance.

4. Perform *in silico* searches on the narrowed list of non-target species to help focus specific *in vitro* studies. Is there sequence complementarity with gene sequences in other organisms? As noted previously, *in silico* searches are not a predictive of effects. The Panel discussed different options to focus the *in silico* searches.

    a. Perform *in silico* searches using a database of known genomes.
    b. If full genomes do not exist, sequence full genomes of this curtailed non-target list and perform *in silico* searches for gene sequences with high complementarity to the dsRNA.
    c. Perform RNA-seq of organisms fed on modified and unmodified plants so that only organisms with significantly changed gene expression would need to be considered further in downstream analysis.
    d. Use primers specific for dsRNA to attempt to amplify sequences from non-target species; no amplification would be evidence of lack of sequence conservation in non-target.

**Box 3: Model feeding studies**

1. Conduct *in vitro* feeding bioassays on non-target organisms based on the *in silico* searches. Measure activity at various endpoints, e.g., mortality, immune response. Measure protein production. Examine different life stages and tissues rather than simply standard toxicity testing. What is the dose to achieve an effect? Can the dose be saturated? What is the consequence? Conduct these tests under realistic exposure conditions. Results will help elucidate potential mechanisms of action and inform selection of sublethal endpoints.

2. For those species that have reduced protein production of off-target genes, determine whether predicted phenotypic effects of the off-target gene suppression are measureable. When off target gene function is unknown, or multiple off target sites are identified, a comprehensive laboratory examination that examines lethality and a battery of sublethal effects should be

undertaken. These should form a composite fitness assessment, rather than each component being analyzed individually.

3. Determine possible synergistic effects (of ds RNA PIP co-exposures) with respect to target and non-target organisms.

4. Determine the potential for insect resistance and recommend resistance management strategies.

**Box 4: Cellular and molecular studies**

1. Is mortality, toxicity, sublethal effect the result of differential gene regulation?
2. Which gene(s) and what dose?
3. How persistent is the effect?
4. Is the response due to RNAi?
5. Is the response related to immunostimulation?
6. If RNAi is confirmed, what is the mechanism?
7. How is RNA taken up by cells, e.g., transporters? Other?

**Box 5: Population effects**

1. If necessary, conduct field tests over multiple years to determine how RNAi-based pesticides may affect biological networks and food web functioning under realistic conditions.
2. Field reports of pest resistance
3. Are there disturbances in the food web?

**Box 6: Mitigation**

1. Could non-target uptake be inhibited?
2. Could dsRNA be re-designed to minimize off-target effects? Resistance?

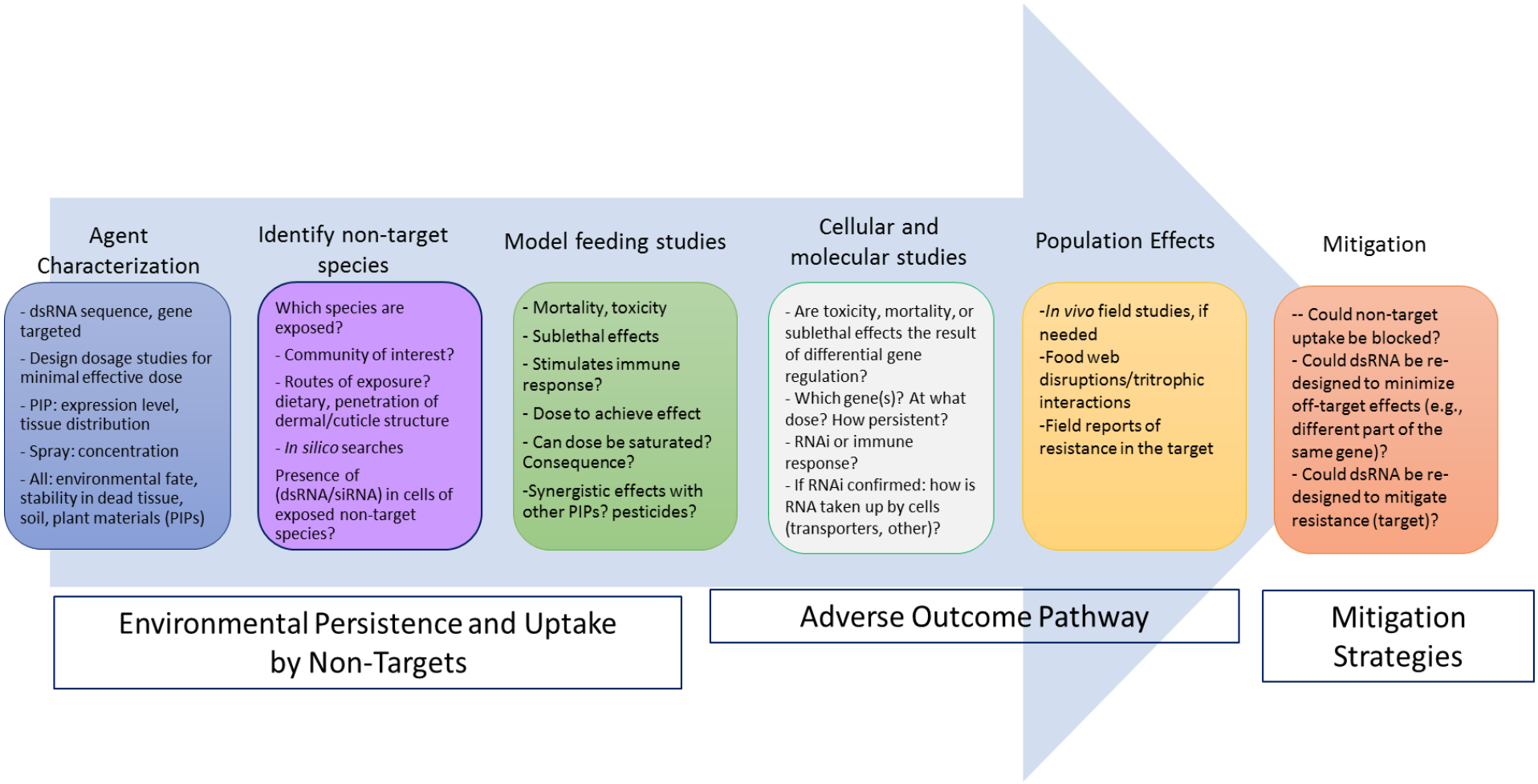


**Figure 7.1. Proposed exposure-based conceptual model for assessment of ecological risk posed by dsRNA PIPs or exogenously applied dsRNA end-products**

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