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

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

Memorandum

DATE: DEC 2 7 2016

SUBJECT: Transmission of Meeting Minutes of the September 27-28, 2016 FIFRA SAP Meeting Held to Consider and Review Scientific Issues Associated with "RNAi Technology: Human Health and Ecological Risk Assessments for SmartStax PRO"

TO: Jack Housenger
Director
Office Pesticides Programs

FROM: Tamue Gibson, M.S.
Designated Federal Official FIFRA SAP
Office of Science Coordination and Policy

THRU: Laura E. Bailey, M.S.
Supervisory Physical Scientist/Executive Secretary FIFRA SAP
Office of Science Coordination and Policy

Stanley Barone, Ph.D.
Acting Director
Office of Science Coordination and Policy

Please find attached the meeting minutes of the September 27-28, 2016 Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) open public meeting held in Arlington, Virginia. This report addresses a set of scientific issues associated with RNAi Technology: Human Health and Ecological Risk Assessments for SmartStax PRO.

Attachment

cc:
Jim Jones
Louise Wise
Laura Bailey
Stan Barone
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Anwar Dunbar
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FIFRA Scientific Advisory Panel Minutes No. 2016-02

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

RNAi Technology: Human Health and Ecological Risk Assessments for SmartStax PRO

September 27-28, 2016
FIFRA Scientific Advisory Panel Meeting
Held at
EPA Conference Center
One Potomac Yard
Arlington, Virginia

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NOTICE

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (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. The meeting minutes have been written as part of the activities of the FIFRA SAP, and represent the views and recommendations of the FIFRA SAP and do not necessarily represent the views and policies of the EPA, or of other agencies in the Executive Branch of the Federal government. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use. The meeting minutes do not create or confer legal rights or impose any legally binding requirements on the EPA or any party. In preparing the meeting minutes, the FIFRA SAP carefully considered all information provided and presented by the EPA, as well as information presented in public comment.

These meeting minutes of the September 27-28, 2016 FIFRA SAP meeting held to consider and review scientific issues associated with "RNAi Technology: Human Health and Ecological Risk Assessments For SmartStax PRO" were certified by James McManaman, Ph.D., FIFRA SAP Session Chair and Jim Downing, Designated Federal Official. The minutes were prepared by Tamue L. Gibson, M.S., FIFRA SAP Designated Federal Official (Acting) and reviewed by Laura E. Bailey, M.S., FIFRA SAP Executive Secretary. The minutes are publicly available on the SAP website (https://www.epa.gov/sap) under the heading of "Meetings" and in the public e-docket, Docket Identification Number: EPA-HQ-OPP-2016-0349, accessible through the docket portal: http://www.regulations.gov. Further information about FIFRA SAP reports and activities can be obtained from its website at https://www.epa.gov/sap. Interested persons are invited to contact Jim Downing, Designated Federal Official, via email at downing.jim@epa.gov or Tamue Gibson, FIFRA SAP Designated Federal Official at gibson.tamue@epa.gov.

SAP Minutes No. 2016-02

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

RNAi Technology: Human Health and Ecological Risk Assessments For SmartStax PRO

September 27-28, 2016
FIFRA Scientific Advisory Panel Meeting
Held at
EPA Conference Center
One Potomac Yard
Arlington, Virginia

James McManaman, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel

Date: DEC 2 7 2016

Jim Downing
Designated Federal Official
Office of the Science Advisor/EPA

Date: DEC 2 7 2016

PANEL ROSTER

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COMMONLY USED ACRONYMS

ACRONYMS	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
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
exRNA	Extracellular RNA
mRNA	Messenger RNA
miRNA	microRNA
Non-PIP	Non plant-incorporated protectant
Not	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 product in a plant.
qPCR	Quantitative polymerase chain reaction
RDRPs	RNA-dependent RNA polymerases
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA interference	Gene silencing process initiated by dsRNA
RNAi	RNAi interference
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

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: Human Health and Ecological Risk Assessments For SmartStax PRO"** Advance notice of the SAP meeting was published in the *Federal Register* on June 30, 2016 (81 FR 42706).

The use of RNA interference (RNAi) gene silencing technology, particularly RNAi for pesticidal purposes to control macroorganism pests is a relatively recent innovation. Post-transcriptional silencing of gene function is a very rapid process where double-stranded RNA (dsRNA) directs sequence-specific degradation of single-stranded RNA (ssRNA). The discovery that dsRNA was 10 times or more potent in its effect on gene expression unleashed a wealth of research into the biochemical basis for RNAi technology. Thus, 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 RNAi have also been developed for pest control.

Development of new RNAi based pesticidal products led the EPA to convene its first SAP meeting in January 2014 to consider aspects of the biopesticide risk assessment framework. Biopesticide products are likely to be developed utilizing RNAi technology as both dsRNA of plant-incorporated protectants (PIPs) expressed in plants and non-PIP dsRNA end-use products may be externally applied for pest control.

In October 2015, EPA registered the Monsanto product MON 87411, a corn plant-PIP for seed increase with a time-limited registration of two years. The 2-year registration allowed for an increase in the amount of MON 87411 seed, however not for production of grain intended for human or animal consumption. Under the registration the company was allowed to grow seed on 15,000 acres (0.02% of corn acreage in the U.S.) per year and also produce *Bacillus thuringiensis (Bt)* Cry3Bb1 protein, for which MON 87411 expresses *DvSNf7* dsRNA. Upon consumption by the corn rootworm (CRW), the insect's RNAi machinery recognizes *DvSnf7* dsRNA, resulting in down-regulation of the targeted DvSnf7 gene leading to CRW mortality. Early in 2016, EPA received applications from Monsanto Company and Dow AgroSciences, LLC requesting permanent registration of RNAi PIPs expressing DvSnf7 dsRNA, also known by the name SmartStax PRO.

EPA relied upon the risk assessment process it had developed for RNAi-based PIPs, taking into account specific recommendations offered by the 2014 SAP. Because a seed increase registration is time and acreage limited, EPA only needed information to satisfy a subset of the SAP recommendations to support the seed increase registration. EPA indicated that it would need additional information, as recommended by the SAP, to support the permanent commercial registration.

The potential for increasing use of RNAi to control pests has lead EPA to conclude the Agency should convene additional SAP meetings to consider aspects of the human health and ecological

risk assessments and to determine whether an RNA interference based PIP can meet the human health and environmental safety standards of the FIFRA.

Therefore, the focus of this SAP meeting was to seek guidance on the natural processes in the environment and within non-target organisms that serve to reduce or eliminate exposure; the importance of the potential for unexpected effects of dsRNA PIPs and RNAi in non-target organisms; and additional testing that EPA may require to reduce uncertainties in risk estimates and risk conclusions.

US EPA presentations were provided during the FIFRA SAP meeting by the following (listed in order of presentation):

Welcome – Rick Keigwin, Deputy Office Director For Programs, Office of Pesticide Programs/EPA

Overview – Robert McNally Director, Biopesticides and Pollution Prevention Division (BPPD)/Office of Pesticide Programs/EPA

Ecological Risk Assessment for *DvSnf7* dsRNA Expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 – Shannon Borges, M.S., BPPD/OPP/EPA

Human Health Risk Assessment for DvSnf7 dsRNA Expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 – Judy Facey, Ph.D., BPPD/OPP/EPA

PUBLIC COMMENTS

Oral statements were presented by:

1) Jay Petrick, Ph.D., Associate Science Fellow, Macromolecule Toxicology Lead, Monsanto Company

2) Pamela Bachman, Ph.D.; Associate Science Fellow, Insect Environmental Assessment Team, Monsanto Company

3) Stephanie Burton, Biotechnology Regulatory Manager, Dow AgroSciences

Written statements were provided by:

1) On behalf of Center for Food Safety:
   a. Martha Crouch, Ph.D., Science Consultant
   b. Margaret Mellon, J.D., Ph.D., Science Policy Consultant
   c. Sylvia Wu, Staff Attorney
   d. Bill Freese, Science Policy Analyst

2) On behalf of the Agricultural Biotechnology Stewardship Technical Committee

a. Pamela Bachman, Ph.D., Monsanto Company and Chair, ABSTC NTO Technical Subcommittee
b. Jeffrey T. Bookout, Monsanto Company and Chair, ABSTC Steering Committee
c. Miles D. Lepping, Ph.D., Dow AgroSciences, LLC and Chair, ABSTC-IRM Technical Subcommittee

3) On behalf of The National Corn Growers Association:
    a. Chip Bowling, President

4) On behalf of themselves:
    a. Steve Whyard, Department of Biological Sciences, University of Manitoba

5) On behalf of the Multistate Research Project NC246 (Ecology and Management of Arthropods in Corn)
    a. Joseph Spencer, 2016 Chair NC246, University of Illinois
    b. Chris DiFonzo, Michigan State University
    c. Aaron J. Gassmann, Iowa State University
    d. Bruce E. Hibbard, USDA-Agricultural Research Service
    e. Thomas E. Hunt, University of Nebraska
    f. Bryan Jensen, University of Wisconsin
    g. Christian Krupke, Purdue University
    h. Charles Mason, University of Delaware
    i. Lance J. Meinke, University of Nebraska
    j. Patrick Porter, Texas A&M AgriLife Extension, Texas A&M University
    k. Susan Ratcliff, University of Illinois
    l. Thomas W. Sappinton, USDA-Agricultural Research Service
    m. Jeff Whitworth, Kansas State University

EXECUTIVE SUMMARY

The focus of this FIFRA SAP meeting was to provide advice to the Agency in considering the fate of ingested dsRNA and the potential for impacts on gene expression and the immune system; discuss the role that bioinformatics analysis may play in understanding and predicting possible off-target effects within the host genome, including predicting non-target effects as part of the ecological risk assessment; and recommend additional testing that the Agency may require to reduce uncertainties in risk estimates and risk conclusions.

The SAP addressed six charge questions divided into product characterization and human health risk assessment considerations (questions 1-2); environmental fate and exposure considerations (question 3); non-target organism hazard considerations (question 4); synergism considerations (question 5) and insect resistance considerations (question 6). The Panel provided the following overall summary of the major conclusions and recommendations detailed in the report.

Product Characterization and Human Health Risk Assessment Considerations – Major Conclusions and Recommendations

The Panel concurred with the Agency's human health risk assessment and considered it as robust and complete. However, for product characterization, there were no experiments addressed by the registrant to study unintended molecules over the generation of PIP-RNAi. The Panel suggested some "omics" studies in order to address unknown sequence signatures or secondary dsRNA as a result of introducing intended RNAi. In laboratory, the synthesis of in silico dsRNA frequently produces unintended structures that can be observed in agarose gel. The etiology of minute differences at individual gene, protein and metabolite levels may better characterize the PIP-RNAi characterization and any possible unknown changes, as well as increase the accuracy of risk assessment.

The Panel generally agreed with the Agency's conclusions of the DvSnf7 structure and concurred with the 2014 SAP on RNAi (Pesticides) by stating that the varying degree of enzyme-resistant forms of RNA can be engineered with no direct bearing on the dsRNA molecule. The basic RNA structure has no similarities to viroid-like structures and the stability tests conducted by Monsanto demonstrates its normal degradation by RNases, thus supporting the presence of its predictive and normal stem-loop structure.

Recommendations

- The Panel recommended the use of the following bioinformatics techniques in order to address unknown sequence signatures or secondary dsRNA as a result of introducing intended RNAi:
    - Transcriptome - microarray, RNA sequencing.
    - Proteomic - two-dimensional gel electrophoresis for proteome profiling.
    - Metabolomics - H-NMR fingerprinting and capillary gas chromatographic/mass spectrometric (GC/MS) - based metabolite profiling.
    - Genomics - addressing the chromatin methylation effects.

- The Panel recommended a comparison among the following three different plants with specific technical requirements to detect the overall effect caused by dsRNA insertion in PIP-RNAi.:
    - PIP-RNAi (SmartStax PRO).
    - PIP without RNAi (SmartStax).
    - No transgenic isoline over different generations.
      The technical requirements include:
        - The use of harmoization in experimental quality and data handling.
        - The use of several biological replicates to avoid other sources of variation besides the genetic modification.
        - The use of statistical analyses to simplify the interpretation of the large amount of data generated by the aforementioned comparative procedures among the three different plants.
- The Panel recommended the use of a combination of different "omics" techniques as well as the combination of "omics" and more sensitive techniques, such as Real Time PCR, to allow a comprehensive approach to the potential unintended effects that might be produced by genetic modification.
- The Panel recommended the use of in vivo studies and experimental evidence to be performed at all times in the overall assessment in order to validate the "omics" derived in silico results since in silico studies are not singularly conclusive.

Environmental Fate and Exposure Considerations – Major Conclusions and Recommendations

The 2014 SAP concluded that additional hazard and exposure tiered data were needed to reduce uncertainty in the environmental fate and ecological risk assessments. The 2014 SAP recommended a six step process for performing the risk assessment for DvSnf7. The 2016 SAP determined that the six step process were not followed by the registrant. One critical omission from the 2014 recommendation is that the organisms potentially at risk from exposure were not assessed in the data provided to the Panel. Consequently, the utility of all data were diminished for addressing previous Panel recommendations regarding subsequent steps for performing the risk assessment for DVSnf7. With incomplete data sets from the Tiered Framework Guidance and from the 2014 SAP recommendations, the 2016 SAP had a difficult time evaluating the biological importance of DvSnf7 fate data. The Panel agreed that the occurrence, transformation, persistence and bioavailability in terrestrial and aquatic environments were pertinent to assessing potential adverse impacts of DvSnf7 on the environment.

Recommendations

- The Panel recommended that the Agency identify higher detectable results of DvSnf7 in forage, root, and grain. These data are needed because the 90% upper confidence limit (CL90) or CL95 of concentrations in these materials should be used for exposure estimates in a Tier 1 assessment. Within the bioavailability data for DvSnf7 concentration within corn, data were presented which compared **c**oncentrations of DvSnf7 dsRNA in EPA'MON87144 alone with that of the hybrid combination.

DvSnf7 concentration in forage, root, and grain suggested non-normal distributions that were skewed toward accumulation of higher levels of DvSnf7 RNA products (refer to Table 1, page 6 of the EPA's Environmental Risk Assessment for FIFRA Section 3 Registration on MON89034 x TC1507 x MON 87411 x DAS-59122-7 dated August 16, 2016).

- The Panel recommended the collection of conclusive information regarding concentrations of DvSnf7 in plant constituent to complete the data set since the concentration of pollen was not tested for the combined plant construct.
- The Panel recommended that the concentrations in the hybrid construct for leaf, root and whole plant should be determined over the entire growing season. In evaluating Argentinian DvSnf7 concentrations in corn data, the Argentinian data showed concentrations for various parts of the plant over the growing season. Concentrations may have decreased over the growing season in some cases. However, it was unclear from these data if decreases were statistically significant for the majority of the time intervals.
- The Panel recommended the use of data from disturbed sediments in a refined or probabilistic risk assessments only (inappropriate for screening level (Tier 1) assessments). Transformation data showed that DvSnf7mean degradation kinetics depend upon conditions in various aquatic environments. Data published by Fischer *et al*. (2016) for sediments provided useful data indicating longer persistence of the dsRNA in sediments than was observed in soils. It is likely that more data will reduce this uncertainty (the longest half-life [90% dissipation time (DT90)] was 144± 9.6 hours (hrs.)) for undisturbed sediment. With only 2 replicates the 95% confidence interval of this DT90 estimates was ± 5.1 days (122 hrs.) which places the upper bound of these estimates at 11.1 days (166 hrs.). In the absence of this data, this conservative DTR90 estimate should be 11.1 days. The disturbance of sediments reduced the DT90. The Panel however, noted that the upper 90% confidence limit of the DT90 for distributed sediments was 8.6 days.
- The Panel recommended toxicity testing for sediment dwelling organisms (i.e. freshwater and estuarine) as part of the screening level (Tier 1) assessment. Although buildup in water with continual movement of post-harvest plant debris may be negligible, chronic or repeated exposure of macrofauna that dominate the biomass of surface sediment is possible where concentrations of DvSNF7 were detected past 28 days.
- The Panel recommended testing sediment dwelling organisms with dsRNA at concentrations of 10-100x the concentrations found in transgenic plants. This would be completed at values of 2130 nanogram/gram (ng/g) to 21300 ng/g (2.1 to 21.3 micrograms/gram (µg/g). The longer half-life of DvSnf7 in sediment compared to soil supports the need for toxicity testing involving sediment dwelling organisms.
- The Panel recommended addressing and fulfilling the 2014 SAP recommendation in determining the intersection of species and crop distributions (dsRNA stability in sediments) in corn growing regions and revising the endangered species statement to reflect empirical or modeled data derived from this study.
- The Panel recommended assigning a no-observed-adverse-effect-level (NOAEL) of 1 and the lowest-observed-adverse-effect-level (LOAEL) as 10 for the 28-day rodent study or classifying the study as deficient. Citing errors within the study procedure

cannot be the basis for allowing a study to be acceptable and to be utilized to establish the NOAEL.

- The Panel recommended addressing and fulfilling the 2014 SAP recommendation to increase the number of non-target organisms (> 25) in *in* silico testing to include species known to occupy corn growing areas, to include aquatic organisms as appropriate and to address the need for an alternative evaluation for assessing risks from DvSnf7 concentrations in plants.

Non-target Organism Hazard – Major Conclusions and Recommendations

The 2016 Panel determined that the non-target hazard data including the additional testing recommended by the 2014 SAP were adequate. However there were areas of uncertainty that addressed 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 challenged 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 accomplished without a better understanding of exposure to dsRNA PIPs or exogenously applied dsRNA products, and the RNAi modes of action.

Synergism – Major Conclusions and Recommendations

The Panel found the Agency's analysis of data on potential synergism among DvSnf7 and the Cry proteins appropriate, however there were concerns raised by the Panel. The Panel believes there is high scientific value in the data on potential interaction among PIPs because this could negatively impact the safety and efficacy of the final product. Additionally, the data would allow for the bridging of data on single PIPs. The concerns raised by the Panel include: the uncertainty of a robust approach to the Fixed Lethal or Concentration Addition Models for determining synergism; all relevant possible combinations of doses may not have been examined to ensure the effectiveness when determining the contribution of individual components within mixtures; and endpoints used in the studies may have been limited in mortality or growth. Other endpoints such as those associated with reproduction may be impacted at lower doses of the pesticide.

Insect Resistance – Major Conclusions and Recommendations

The Panel agreed that the corn rootworm could become resistant to DvSnf7 dsRNA treatment based upon their overall assessment of data, specifically Chu *et al*. (2014). The Panel attributed biologically relevant mutations in enzymes involved in RNAi machinery, dsRNA uptake, delivery, and processing as contributors to the development of resistance to transgenic RNAi crops. The Panel was inclusive of the following mechanistic results which could attribute to the development of resistance to RNAi crops: Changes in dsRNA target sequence; Changes in

characteristics of natural barriers to dsRNA uptake, transport, effect; Changes in the RNAi machinery enzymes or components; Behavioral avoidance.

Recommendations

- The Panel highly recommended the process of releasing and planting of SmartStax pro corn follow integrated pest management (IPM) principles which would mitigate the onset of corn rootworm resistance.
- The Panel recommended implementing the use of a resistance strategy (i.e., refuge areas) to better understand the target sequence/mode of action of dsRNA on the corn rootworm.

DETAILED PANEL DELIBERATIONS AND RESPONSE TO CHARGE

Charge Question 1. – Product Characterization and Human Health Risk Assessment

The 2014 SAP in general agreed that there were few issues with dietary exposure to dsRNA molecules in mammalian species but recommended several issues be examined to confirm these assumptions. These recommendations include: (1) confirm that special dsRNA forms (e.g., hairpins, super coils) may not degrade as quickly as simple dsRNA; (2) the blood of animals consuming food containing an RNAi-PIP should be examined for the presence of the dsRNA or pieces of dsRNA: (3) although bioinformatics would not give definitive answers, it can be predictive, and as such a useful tool, depending on the search methods and completeness of the database; and (4) consider the possibility that special subpopulations such as the elderly, children or people with gastrointestinal tract illnesses (e.g., Crohn's disease, colitis, or irritable bowel syndrome) may present a different pattern of uptake of dsRNA from the diet.

> **Question 1a.** Please comment on the feasibility of creating and successfully deploying in plants, dsRNA structures likely to present greater stability (e.g., supercoil, viroid-like structures). Please comment on EPA's conclusion that the single hairpin structure of the dsRNA for DvSnf7 is one of the simpler structures expected for RNAi inducing molecules, and that this structure is unlikely to present any unique stability to RNA degrading enzymatic attack.

Panel Response

The Panel stated that it was possible to engineer more and less enzyme-resistant forms of RNA through the introduction of complex structures and packaging strategies. However, they suggested that the basic science questions of RNA engineering and formulation did not have a direct bearing on the end-use product in question, since its structure was known and unremarkable.

The Panel agreed with the Agency's conclusions on the DvSnf7 structure that the dsRNA molecule formed a stem loop structure with a 240 nucleotide (nt) stem and a 150 nt loop. The Panel articulated that the basic RNA structure had no apparent purpose or ability to package similar to a viroid, and by all experimental accounts was not modified, sequestered, or packed in any proteinaceous or viroid-like particle. The stability results from Monsanto both in soil and in human blood supported its normal susceptibility to RNases and normal RNA breakdown (RNA is already an unstable molecule overall because of the 2'-hydroxyl), further supporting the presence of the predicted and normal stem-loop structure.

The Panel referred to the 2014 FIFRA SAP on RNAi (also referred to as the 2014 Panel) as a pesticide in their conclusion that "The combination of RNases and acids found in the human digestive system are likely to ensure that all forms of RNA structure are degraded throughout the digestive process." The 2016 Panel noted that evidence had not emerged since the 2014 Panel to suggest that this statement required amendment.

Question 1b. Recent evidence suggests that RNAs, including miRNAs, are normally present in blood and are transported to various target tissues and organs as part of normal homeostasis Freedman *et al*. (2016). Please comment on this evidence. How might this evidence relate to the subchronic and 28-day studies data supplied in support of this registration that show no effects on whole animals of the DvSnf7 dsRNA. What additional support might confirmatory testing in mammalian blood provide to a risk assessment?

Panel Response

The Panel stated that evidence strongly established presence of endogenous RNAs, including miRNAs, in blood, predominantly in blood cells including highly abundant red blood cells and platelets. Furthermore, there was also ample evidence to determine that extracellular endogenous RNAs (exRNAs) are present in the blood, mostly as complexes released from dead and dying cells. The Panel referenced the article by Freedman *et al*. (2016) which confirmed a diversity of circulating cell-free RNAs in blood that had previously been shown by other groups in the context of both unfractionated and fractionated fluids (Nolte-'t Hoen *et al*., 2012; Tosar *et al*., 2015). The Panel also noted that in the case of miRNAs, (to date the most studied classes of RNAs in this context), 95% of more of cell-free miRNA copies were reportedly found in free Argonaute (protein) complexes in blood (Turchinovich *et al*., 2011; Turchinovich *et al*., 2012; Arroyo *et al*., 2011). A single digit percentage of miRNA was thought to be contained within extracellular vesicles (EVs, also called exosomes, microvesicles, or microparticles), which may be specifically released by cells and may traffic to non-neighboring cells. The so-called extracellular RNA communication hypothesis suggested that these EVs or other carriers may transfer RNAs to recipient cells.

The Panel however asserted it had not been generally established, nor specifically shown by Freedman, *et al*. (2016) that endogenous RNAs in cell free blood "are transported to various target tissues and organs as part of normal homeostasis," implying function. Further, the Panel highlighted that Freedman *et al*. (2016) did not provide data to support this hypothesis and stated in their conclusion that "Whether they (RNAs) contribute to cellular homeostasis or are associated with disease pathobiology is not yet known." The Panel were in unison in principle that ongoing research sought to determine whether and to what extent extracellular particles such as EVs might transport RNA between non-neighboring cells (not just in non-physiologic in vitro experiments, but also in vivo experimentation). The Panel noted the strongest evidence to date that EV-transported RNAs may function in recipient cells in vivo involved messenger RNAs in mouse models containing reporter genes (Ridder *et al*., 2015; Zomer *et al*., 2015), though it was not always completely clear that RNA, not protein, mediated these effects. The evidence for extracellular miRNA function was mixed Turchinovich *et al*. (2016). Conclusions of positive effects were often based on a two-part approach in which first, a change in extracellular RNA was discovered in a disease or treatment condition, in vivo. Secondly, non-physiologic concentrations of RNA or of EVs enriched in it are introduced into or onto cells in vitro, and significant effects were reported (Witwer, K.W. and Halushka, M.K., 2016). It was emphasized that whether these effects were due to actual introduction of RNA into recipient cells, or how such introductions would occur in vivo, remained an open question.

The existence of cell-specific miRNAs somewhat challenged the theory of extracellular RNA communication in vivo. miR-451a is specific to and abundant in red blood cells (RBCs) Kent, *et al*. (2014), by far the most abundant cell type in the human body (Bianconi *et al*., 2013; Sender *et al*., 2016). Additionally, it was also one of the most abundant miRNAs in cell-free serum and would thus be a top candidate for involvement in intercellular communication. In continuation, the Panel noted although miR-451 was located throughout the body (a consequence of the presence of vasculature) it was not located in cells apart from RBCs. Reports of miR-451a in non-red blood cells are thought to be the result of RBC contamination, hemolysis, or contributions of exogenous serum in cell culture experiments Wei *et al*. (2016). The Panel recommended that similar conclusions must be drawn from examination of other cell-specific miRNAs including miR-126 (mostly endothelial), miR-122 (hepatocytes), and miR-486 (blood cells).

The Panel discussed how the evidence for endogenous extracellular RNA communication must also be weighed against the arguments of stoichiometry. Cellular concentrations of miRNAs necessary for target inhibition depended upon miRNA and targeted abundance and strength of miRNA-target interaction (Sergeeva *et al*., 2013; Seitz, H., 2009). The Panel introduced findings from a recent study by Phil Sharp's group which reported functional concentrations of specific miRNAs as being in the low-to-high nanomolar range in embryonic stem cells and mesenchymal stromal cells Bosson *et al*. (2014). Copy number calculations have suggested that hundreds to thousands of copies of a specific miRNA are necessary but not necessarily sufficient in a single cell to exert an effect Mullokandov *et al*. (2012). In contrast, total extracellular miRNA concentration in blood was reported by Thomas Tuschl's group to be in the femtomolar range Akat *et al*. (2014), and are thus, even as a class, at sub-hormonal concentrations in the blood. It is unclear how individual miRNAs would affect cellular concentrations or exert canonical regulatory functions at these levels, much less at the zepto- to attomolar concentrations which were reported in circulation for some less abundant miRNAs. It was noted that using data estimates for extracellular vesicle RNA content Sverdlov *et al*. (2012) should provide enough RNA-containing EVs in the blood at any given time to influence only a very small number of cells, even if uptake is 100%.

The Panel recommended that whatever the outcome of the ongoing research into the extracellular RNA communication hypothesis, studies of endogenous RNA have little relation to "the subchronic and 28-day study data supplied in support of this registration." This is because the dvSnf7 studies involved an exogenously supplied dsRNA. The Panel continued its discussions stating that there are no reliable evidence that exogenous dsRNAs are taken up from the gut into mammalian circulation to exert its functions in the ingesting organism. A small number of studies suggested that single-stranded small RNAs may enter the mammalian bloodstream from the gut. However, alternative explanations of these and other apparently positive “cross-organism” or “cross-kingdom” results have been advanced, suggesting that contamination or other artifacts were responsible (Tosar *et al*., 2014; Lusk, R.W., 2014; Bağcı, C. and Allmer, J. 2013). The Panel articulated that there does not appear to be any consistent and reliable indication that such RNAs exerted their function.

The Panel highlighted that other studies have been published with negative results (Dickinson *et al*., 2013; Petrick *et al*., 2015; Zhang *et al*., 2012; Snow *et al*., 2013; Witwer *et al*., 2013; Micó *et*

al., 2016; Laubier *et al*., 2015; Title *et al*., 2015; Baier *et al*., 2014) and that these negative data included several rigorous mouse feeding studies with miRNA knockouts to eliminate background (Laubier *et al*., 2015; Title *et al*., 2015). The Panel characterized another study as reported at an ISEV Workshop in Utrecht, the Netherlands, in 2015 with (Snow *et al.*, 2013; Witwer *et al.*, 2013; and Dickinson *et al.*, 2013) finding no or negligible uptake of plant and/or mammalian RNAs (Dickerson *et al*., 2013; Snow *et al*., 2013; Witwer *et al*., 2013). Likewise, Micó *et al* found no uptake of RNA from olive oil or beer in a human feeding study. Wang *et al*, often cited as a positive study, purported single-digit copies of an abundant plant miRNA, MIR168a, in human blood. Yet no other plant miRNAs were detected, and its mapping disappeared when a more stringent analysis strategy was utilized. Auerbach *et al* tested samples that had previously been reported by Zempleni's lab to show evidence of uptake of two milk miRNAs Baier *et al*. (2013), however finding no significant differences in the miRNAs or any other types Auerbach *et al.* (2016). Furthermore, sequencing data from Zempleni's lab were analyzed Auerbach *et al.* (2016) resulting in very similar conclusions. The Panel reviewed some of the available studies including the negative studies and provided general comments to them in the subsequent discussions below.

Because of the high-profile nature of some of the reports on dietary RNA uptake and function, as well as a continuing interest in this topic by funding agencies, the Panel sensed it was important to review the cases of several additional prominent putative positive studies.

Case 1. In a study in Cell Research, MIR168a, a plant miRNA, was predicted by Chen-Yu Zhang and colleagues to target a mouse transcript involved in cholesterol homeostasis Zhang *et al*. (2012). A mouse feeding study indicated, strikingly, that a plant-based diet resulted in increased circulating cholesterol. When carefully optimized in vitro transfection procedures were used to introduce millions of copies of exogenous miRNA "mimics" (short duplexes) of MIR168a into cells, these mimics were able to recognize a sequence from the putative target transcript. However, the in vitro protein results were not as clear, indicating that even under these non-physiologic conditions, knockdown was not strong. A subsequent replication study found no change in the target transcript in vivo, along with no evidence for substantial uptake of MIR168a from the diet. Importantly, the reported cholesterol upregulation was replicated, but more carefully prescribed control conditions showed that this was not due to knockdown of a cholesterol-related gene, but to a starvation response in which the nutritional insufficiency of the original plant diet led to a mobilization of cholesterol stores.

Case 2. Shizhen Wang and colleagues, also published in Cell Research, reported that blood of breast cancer patients contained a specific plant miRNA, MIR159a, with levels positively correlated with a good outcome. In high-throughput nucleic acid sequencing, it is important to establish cutoffs for background or experimental noise, since low-level sequences can represent trace contaminants of samples, reagents, or instruments. For small RNA sequencing, results may be expressed as "reads per million" miRNA reads (rpm). An appropriate cutoff might be 50 or 100 rpm. Notably, the authors of this study had, in a previous publication, used a cutoff of 50 rpm for analysis. In the MIR159a study, all MIR159a counts fell below this threshold. The study thus analyzed a sequence that was below a reasonable technical threshold and represented at most an attomolar level of miRNA in the blood. It is instructive to consider the EC50 of known substances in this context. To have any chance at effect, all copies in the blood of a human at any

given time would likely have to be transferred with 100% efficiency into regulatory complexes inside a single cell. The next part of the study was to introduce non-physiologic numbers of exogenous, easily processed miRNA "mimics" (short duplexes) into cancer cells, where they would form the overwhelming majority of small RNAs. On its own, the numbers would likely disrupt the stoichiometry of any ongoing RNA regulation in those cells. The two parts of this study thus had no informative relationship with each other. Conceivably, molecules from the gut, including RNAs, make their way into human circulation at very low levels that are at the edge of accurate quantitation. Cancer patients who were not eating well may have worse outcomes and ever so slightly lower levels of these molecules. But there were no expected function of any biological molecule at this level. Similarly, scientist are able to create artificial situations in the laboratory in which two sequences that would never encounter each other in an organism are instead placed in high-abundance juxtaposition. The results of such an experiment have nothing to do with dietary exposure. Thus the many orders of magnitude between actual exposures and known safe exposure resemble the difference between actual levels in vivo and experimental manipulation.

Case 3. Mlotshwa and colleagues gavaged approximately 10^15 copies of miRNA into a mouse GI cancer model each day for 30 days Mlotshwa *et al.* (2015). An apparent decline in tumor formation was reported in miRNA-exposed animals. This study was regularly cited as support for the exogenous RNA uptake hypothesis, and the authors also made this claim. However, uptake into blood was not tested, nor was uptake into gut epithelial cells clearly demonstrated. Quantitative PCR signal for only one of three chemically modified synthetic miRNAs was found in gut material, but a lack of rigorous washing (single PBS wash of gut) casts doubt that this signal, if specific, came from intracellular vs extracellular material. Unclear methods and statistics rendered this study largely uninterpretable, and should not be viewed as evidence for dietary RNA uptake.

Case 4. Zhou and colleagues reported that a putatively honeysuckle-specific miRNA delivered through the diet was an effective antiviral in a mouse model, providing a possible mechanism of action for a Traditional Chinese Medicine remedy Zhou *et al*. (2015). However, processing of honeysuckle to an aqueous decoction efficiently destroyed small RNAs with the exception of the MIR2911. This small RNA was in fact not a microRNA, but a highly GC-rich breakdown fragment of a ribosomal RNA (among the most abundant RNAs in the cell). It was also not specific to honeysuckle. Finally, the quantitative polymerase chain reaction (qPCR) assay used to detect the putative miRNA was for a sequence differing by one nucleotide from the honeysuckle rRNA fragment, highlighting potential specificity issues when highly GC-rich RNAs are amplified.

Case 5. The Hirschi laboratory investigated uptake of the same rRNA fragment (MIR2911), finding that gut and kidney damage facilitated approximately four-fold higher detection of MIR2911 in blood of mice Yang *et al*. (2015).

Case 6. The C-Y Zhang lab reported low-level uptake of microRNAs from 3 liters of watermelon juice in a human feeding study Liang *et al*. (2015). However, the apparently most abundant and efficiently absorbed miRNA was not a microRNA of dicots such as watermelon, raising the questions about the specificity of the assays used and the possibility of environmental

contamination Witwer *et al*. (2015). Furthermore, the negative control data consisted of abnormally strong positive signals.

Various technical explanations for the divergent findings have been hypothesized, and these were often repeated uncritically (to explain why so many orders of magnitude separate the observed in vivo levels and any reasonable level at which function might occur). However, a technical issue that might render the NGS library preparation fractionally less efficient for plant miRNAs, for example, would not affect ligation-independent forms of quantitative PCR or droplet digital PCR.

> **Question 1c.** Assuming that the SAP continues to believe that bioinformatics can be a useful tool, can the Panel offer advice on what level of similarity might trigger a biologically significant effect? Please comment on what RNA properties, in addition to sequence identity match, (for example, sequence length, context, or biophysical properties) are relevant in assessing the potential for a dsRNA molecule to mediate gene suppression.

Panel Response

According to the supporting materials, homology searches returned no 21-nt complementary matches of DvSnf7 sequences to human sequences. siRNA-like effects would not be predicted for the DvSnf7 sequence even if it were introduced into a human cell. Whereas DvSnf7 sequences do not consist of predicted matches, numerous endogenous RNA sequences of maize consist of predicted matches. Thus, the introduced RNA is less likely to have off-target effects than some sequences already present in maize, with no known adverse consequences for the consumer. This type of bioinformatics analysis can be useful in that it can discount the likelihood of off-target effects.

The Panel denoted the analysis in the preceding paragraph signifying the presence of a sequence match, however cautioning that this indication does not necessarily mean that a deleterious off-target effect would occur. The Panel provided an example of plants as being a traditional staple of the human diet and the lack of evidence that RNA in plants interfered with normal physiologic processes in humans despite perfect sequence matches. A bioinformatics "hit" does not signify function unless the RNA of interest is inserted into the appropriate molecular machinery and at the proper stoichiometry to its putative target.

The Panel intently described how bioinformatics become increasingly limited in predicting off-target regulation as the degree of complementarity between RNA and its putative target declined. The Panel went on to discuss how miRNAs, i.e., bind with imperfect complementarity to their target RNA molecules and assert only very low levels of regulation compared with siRNAs. The miRNA interactions were based on 6-8 nucleotide "seed" sequences near the 5' end of the miRNA. For short sequences, many spurious matches are predicted, or, viewed from a different angle, a given small RNA "sees" many matches that do not result in regulation. For example, only a small minority of perfect 8-nucleotide seed matches exert any measurable effect on targets. The differences between real and spurious matches are the subject of ongoing basic science research, and recent publications support an increasingly complex picture. The Panel made it a priority to emphasize that the preponderance of experimentally verified miRNA: target

binding, when it does occur, resulted in very small fold changes that were well within the range of the normal haplosufficiency of most of the human transcriptome (i.e. two-fold variation) (Seitz, H., 2009). Functional effects of such subtle fine-tuning might be noticeable only when applied to gene networks in which binding sites and microRNAs co-evolve. In contrast with the case of endogenous miRNAs and genes, or even those of viral, bacterial, or parasitic RNAs that are in intimate and reciprocal interactions with host RNAs, it is difficult to propose an evolutionary mechanism whereby plant small RNAs, or in this case a fragment of insect RNA, would co-evolve to achieve regulation of human gene target networks.

The Panel concluded by explaining how bioinformatics analyses that demonstrate 100% matches of product with human sequences do not establish risk, as widely known from studies of dietary plants which show no functionally relevant exposure. Moreover, bioinformatics analyses that demonstrated an absence of matches of product to humans may have some value in that even in the unlikely event of an unanticipated setting or condition or exposure (along with correct processing and incorporation into human RNA-induced silencing complex (RISC)), the sequence would be unlikely to have a biologically significant effect. Conversely, in the absence of demonstrated high-abundance and functionally relevant exposure, analysis of sequence context, length, biophysical properties, and other variables (product or target), is of limited or no value for risk assessment.

> **Question 1d.** Please comment on EPA's analysis regarding stability and potential for uptake of DvSnf7 dsRNA by special subpopulations with altered absorption or digestion (e.g., Crohn's disease, colitis, IBS).

Panel Response

The Panel agreed with the Agency's analysis that "There are extensive and redundant systems that degrade free nucleic acids from an oral exposure to DvSnf7 RNA."

The Panel discussed in detail that prior to oral exposure there must be the presence (of RNA) in the food material. Supporting materials related to DvSnf7 RNA reported that RNA in grain were several orders of magnitude less abundant than in non-food parts of the plant. Since few humans eat raw grain, the Panel stated that they were subject to consider the effects of storage and processing, which have been universally reported to diminish RNA in food materials. The Panel also discussed that conservative calculations assumed 100% retention of RNA from the plant. Yet, the Panel noted that dependent on the type and degree of processing, most RNAs may be degraded before dietary exposure.

Most notably, the Panel characterized physiologic barriers that commence with physical, chemical, and enzymatic transformation of food materials in the mammalian digestive tract (from mastication to stomach acids to pancreatic RNases, etc.). The Panel discussed how irrespective of the existence or non-existence of free nucleic acids, the chemical and enzymatic environment of the different parts of the digestive tract will be hostile to RNA and that free RNAs are exposed to an aqueous and in places highly acidic environment. Furthermore, a diverse array of powerful RNases that are present throughout the tract and in the gut are able to digest even dsRNAs. Moreover, the RNAs that are protected by lipids or proteins are first exposed by lipases and proteases. The Panel summarized the process by which some RNAs in

the gut contents are un-degraded, and protected in larger, undigested bits of food that are destined for excretion and that these RNAs are unavailable for uptake into or through the gut and should not be mistaken for free RNA. It was underscored, that in the gut, the microbiome assists with degrading food material and transforming larger molecules into nutrition.

The cells of the gut epithelium and the vascular endothelium form physical barriers to uptake macromolecules. In the intact gut, it is not clear how larger nucleic acid molecules and their carriers would cross into the bloodstream. Transporters for neither dsRNA nor small single stranded RNAs are well established in mammals. Studies reporting uptake have relied on apparent transport of fluorescent tags or lipid dyes, or have not eliminated the possibility of association with the outside of cells in the gut lumen. A study of extracellular vesicles artificially produced from plant material reported that a therapeutic substance in ginger was delivered to the liver; however, there were no appropriate controls to rule out that the EV preparations simply delivered the small molecule to the gut in a form (i.e. highly processed plant material) that was relatively digestible. In vitro studies purporting uptake into cells or across a barrier model have used non-physiologic quantities of nucleic acid carriers such as extracellular vesicles.

The Panel emphasized that the next barrier were the conditions within the blood. While generally less harsh than the digestive tract, blood is replete with nucleases that degrade free RNAs within seconds to minutes.

Clearance: In the discussions related to clearance, the Panel provided an example of how excess free nucleic acids, specifically those introduced by intravenous injection, were cleared within minutes by the kidneys. Complex nucleic acids, such as those located in EVs or artificial nanoparticles, are cleared by the liver and that the half-life of exogenous EVs in circulation have been estimated in the minutes to tens of minutes range.

Tissue and vasculature: In the realms of tissue and vasculature discussions, the Panel discussed how for the exception of cells in direct contact with blood, nucleic acid carriers would need to penetrate into tissue parenchyma to reach target cells.

Cellular barriers: During the cellular barrier process discussions, the Panel noted that the cells of the gut consisting of transporters of long dsRNA or small RNAs or of RNAs in complex with proteins are not known in other mammalian cells. For this reason, in vitro experiments involving introduction of foreign RNAs into cells must rely on cell membrane disruption by electrical pulsing (electroporation), infection with viruses or virus-like particles (transduction) or carefully optimized liposome formulations (transfection). Extracellular vesicles have been proposed to fuse with recipient cells, but EVs are in general not easily fusogenic and are instead taken up into the endosomal system, where they appeared to remain until degradation or uptake involving incompletely understood mechanisms.

Endosomal system: Several studies of apparent nucleic acid or nucleic acid carrier uptake by cells of the endosomal system do not distinguish between delivery into the cytoplasm and sequestration and eventual degradation in the endosomal system. Foreign material entering the endosome is degraded as the endosome acidifies and becomes a lysosome.

Molecular barriers. True cytoplasmic uptake of dsRNA or derivatives would result in function only if three conditions were satisfied. First, processing of dsRNA into effector molecules (short RNAs) or delivery of pre-processed effectors. Second, incorporation of the effectors into mammalian Argonautes. Normally, the Argonaute is loaded with one strand of the mammalian precursor miRNA during processing. Argonautes can be loaded with pre-processed single stranded RNAs only at much higher concentrations. Third, the RNA: Argonaute complex must associate with other RISC components as well as a target RNA. In most cells of the adult organism, there appears to be only limited RISC: target association LaRocca *et al*. (2015). Work by Craig Thompson's lab at Memorial Sloan Kettering Cancer Center suggests that, in most organs of the adult mammal, regulatory RNAs are found in low molecular weight complexes, i.e., not associated with target RNAs LaRocca *et al*. (2015). Only in a small number of organs harboring activated, differentiating, or high-metabolism cell types, and in cancers, did there appear to be high molecular weight Argonaute-containing complexes. Thus, even if exogenous RNA were present in the blood and could be delivered reliably to Argonautes in the cell cytoplasm, these complexes would have no regulatory effect in most cells.

Immune responses. Whether in the blood or in the cell, sufficient levels of dsRNA provoke non-specific, innate immune responses in mammals. The fact that humans eat foods replete with RNA including dsRNA viruses suggests that this response does not occur because dietary RNA is digested, not transported intact across the gut and throughout the body.

Stoichiometric barriers. The minute quantities of foreign nucleic acids that have been reported in mammalian blood would be insufficient to influence gene expression in the mammalian body. Even if one assumes that all foreign nucleic acids accumulate in the liver and are taken up into the cytoplasm of hepatocytes, and one further credits the highest reported concentration of foreign miRNA in the Zhang's Cell Research paper Zhang *et al*. (2012), there would be less than one copy of miRNA per liver cell in circulation at any given time. The many barriers described above suggest that exogenous extracellular RNA would be required to reach a molar excess of many orders of magnitude compared with the desired functional concentration in the cell. Instead, the Panel noted that they observed concentrations many orders of magnitude below a functional concentration.

Charge Question 2. – Product Characterization and Human Health Risk Assessment

Please comment on EPA's risk assessment of the combination PIP product including the evaluation of the DvSnf7 gene with regard to product characterization and human health. Does the SAP have any suggestions to improve it?

Panel Response

As it relates to human health, the Panel deliberated on all comments submitted to Charge Question 2, and considered the Agency's human health risk assessment robust and complete. However, for product characterization, the Panel suggested some "omics" studies in order to address unknown sequence signatures or secondary dsRNA as a result of introducing intended RNAi Abdurakhmonov, I. (2016). Thus far, there are no experiments addressed to study those unintended molecules over the generation of PIP-RNAi. In the laboratory setting, the synthesis of in silico dsRNA frequently produces unintended structures that can be observed in agarose

gel. In an effort to provide an additional example, the Panel provided a quote from Thermo Fisher's Scientific manufacturer instructions' manual by stating "It probably represents persistent secondary structure or re-initiation of transcription without transcript release". Those unknown molecules should be carefully identified to predict and prevent the silencing of unintended genes in off-target organisms, as well as the development of insect resistance. The Panel mentioned that this concern had already been pointed out by the scientific community (Abdurakhmonov, I. 2016; Heinemann *et al*., 2013) showing the importance to clarify this specific issue.

The Panel further supported the use of bioinformatics technology, specifically "omics" techniques such as transcriptome (microarray, RNAseq), proteomic (two-dimensional gel electrophoresis for proteome profiling), metabolomic (H-NMR fingerprinting and capillary gas chromatographic/mass spectrometric (GC/MS)-based metabolite profiling), and genomics (addressing the chromatin methylation effects) as efficient and precise tools that can detect and identify the main sources of even small differences at individual gene, protein and metabolite levels. These technologies can be effective in better characterizing the PIP-RNAi and any possible unknown changes, as well as increase the accuracy of risk assessments (Yang *et al*., 2015; Ricroch *et al*., 2011; Barros *et al*., 2010; Heinemann *et al*., 2011). The Panel suggested executing a comparison among three different plants: PIP-RNAi (SmartStax PRO), PIP without RNAi (SmartStax) and no transgenic isoline over different generations. This could detect the overall effect caused by dsRNA insertion in PIP-RNAi. However, the Panel agreed that the use of those techniques demands specific requirements, such as homogenization in experimental quality and data handling Lay *et al*. (2006); many biological replicates to avoid other sources of variation besides the genetic modification and also to allow statistical analyses to simplify the interpretation of the large amount of data generated by any of these technologies Barros et al. (2010). Also, the combination of different "omics" as well as the combination of "omics" and more sensitive techniques, such as Real Time PCR, allows a holistic approach to the potential unintended effects that might have been caused by genetic modification (Barros *et al*., 2010; Gong, C.Y., and Wang, T. 2013). The Panel also agreed that in an overall assessment, the unintended and non-specific effects with PIP-RNAi are probably lower than the current standard agricultural practices (Bachman, P. EPA-HQ-OPP-2016-0349-0062). Therefore, it was imperative to reinforce that "omics" related to in silico studies cannot be self-conclusive and that in vivo studies and experimental evidences should always be performed in order to validate the in silico results, and contextualize them.

One panel member disagreed with the suggestion in considering "omics" studies for risk assessment purposes in this effort. Ultimately, the Panel did not consider the use of "omics" techniques as a basic study approach for RNAi. As an alternative, the Panel recommended that the studies to be utilized to minimize the potential for off-target effects, ensuring the safeguard of the lifespan of bioinformatics technology, as well as providing an overall improved understanding of product characterization, in order to develop clarity in the safety of PIP-RNAi to the more conservative research colleagues.

Charge Question 3. – Environmental Fate and Exposure

The 2014 SAP recommended an exposure based model for testing related to dsRNA based pesticides, which places emphasis on the environmental fate and exposure data and analyses. To inform the environmental fate and exposure analysis for the DvSnf7 dsRNA, EPA has reviewed

data submitted by Monsanto Company that describes degradation in soil, water, sediment, and DvSnf7 concentration levels, and has also examined information from public literature. Based on these data, EPA has concluded that exposure in the terrestrial environment primarily is limited to organisms that consume plant tissue directly and to those that may be exposed secondarily through consumption of herbivorous arthropods. In the aquatic environment, EPA has determined that exposure to DvSnf7 in corn detritus is minimal, and while some exposure may occur in the water column, the exposure will be short lived and not significant.

Question 3a. Please comment on the completeness of the data set considered for determining exposure and environmental fate of DvSnf7 in both terrestrial and aquatic environments, taking into consideration the scope of EPA's needs for environmental risk assessment and the recommendations of the 2014 SAP.

Panel Response

The Panel acknowledges the Agency experienced many challenges in formulating and implementing the tiered framework of risk assessment for DvSnf7 within FIFRA while including the 2014 FIFRA SAP recommendations (also referred to as 2014 SAP). The 2014 SAP concluded that additional hazard and exposure tiered data were needed to reduce uncertainty in the environmental fate and ecological risk assessments. The Panel suggested improvements in implementing the tiered framework risk assessment process. The Panel was optimistic in addressing these in the context of existing data and possible paths forward.

The FIFRA Risk Assessment guidelines requires studies that proceed in a Tiered Fashion. It was noted during the 2014 SAP regarding this PIP that the extant guideline studies were unlikely to adequately or accurately assess risk from the RNAi mode of action. The 2014 SAP recommended a six step process for performing the risk assessment for DvSnf7:

1) Characterize dsRNA chemical and biological behavior
2) Identify species likely to be exposed and then to perform in-silico evaluations to determine which species are likely to have some response to dsRNA
3) Identify a "curtailed community" that is likely to experience exposure and that may exhibit effects so that feeding studies can be selected and tested
4) Conduct cellular and molecular studies
5) Determine population level effects in situ, and
6) Determine any mitigation needs.

These six steps have not been followed in preparing the assessments of DvSnf7 that were reviewed by the 2016 SAP. The organisms potentially at risk from exposure were never determined although many such data sets exist for vertebrate, invertebrate and plant species distribution in agroecosystems. Development of a repository of such information within the regulated community was recommended by previous FIFRA SAP's over several previous years during early assessments of probabilistic risk assessment frameworks. Omissions of the 2014 SAP recommendations diminishes the utility of all data that addresses recommendations in the remainder of step two as well as subsequent steps. Couple these facts with the lack of data fulfilling requirements of the standard tiered approach and the Agency and the Panel are left with

an array of information that while useful leaves significant uncertainty regarding risks that are posed to non-target organisms.

The 2016 Panel is being asked to evaluate new and possibly paradigm shifting pest control technology and to assess the adequacy of current data and possible data needs. With incomplete data sets from the Tiered Framework Guidance and from the 2014 SAP recommendations, the 2016 SAP had difficulty determining the adequacy of provided data to assess exposure and hazard. Furthermore, in the public meeting, the registrant asserted that follow-up evaluations were not done to refine or replace flawed data because such follow-up studies were not guideline studies. The Panel discussed with caution how the registrant utilized the guideline framework when benefiting themselves, and also when performing other less labor intensive evaluations. The Panel noted that this approach left the Agency at a disadvantage in discharging their duties to protect the environment from undue risk.

The Panel reiterated that DvSnf7 occurrence, transformation, persistence and bioavailability in terrestrial and aquatic environments are pertinent to assessing potential adverse impacts of DvSnf7 on the environment and that the data provided to the Panel regarding these parameters were well done and very beneficial to the understanding of DvSnf7's persistence in the environment.

DvSnf7 Expression in Corn: With respect to bioavailability, data was presented which compared concentrations of DvSnf7 dsRNA in MON87144 alone with that of the hybrid combination. Expression values for the leaf, root whole plant, forage, forage root and grain were compared between the two. The Panel discussed that the data for DvSnf7 concentrations in forage, root, and grain (refer to Table 1, page 6 of the Environmental Risk Assessment for FIFRA Section 3 Registration on MON89034 x TC1507 x MON 87411 x DAS-59122-7 dated August 16, 2016) suggested that non-normal distributions were skewed toward higher concentrations. The Panel expressed the difficulty in assessing this information without the underlying data, and recommended the variance within the data will need to be expressed as CL 90 or CL 95. The Panel also recommended that one of these upper exposure bounds is more appropriate for a Tier 1 assessment, and that the skewed distribution should be represented in any higher tier evaluations. Regardless of the manner of the variance computations, exposure estimates in a Tier 1 assessment should use the upper 90^{th} or 95^{th} percentile of occurrence as the exposure.

The Panel noted that the concentration of DvSnf7 in pollen were not measured for the combined construct. The Panel further described that although the concentrations in pollen were low, and the distribution by its component were considered limited, the results produced from this expression within this component of the plant is necessary to complete the data set.

Data are now available for concentrations of DvSnf7 RNA in various parts of the corn plant in the US and Argentina. Results of the concentrations in several parts of the plant measured at similar growth stages are comparable between the two growing regions. However, the Argentinian study does not provide information on concentrations within the hybrid product.

Unlike the US data set, the Argentinian data showed concentrations for the various parts of the plant over the growing season. A decrease in concentrations over the growing season was suggested some cases, but it was unclear from these data if decreases were statistically significant for the majority of the time intervals. It appeared that DvSnf7 concentrations in leaf tissue (where they are the highest) remain elevated over the growing season. Concentrations in the hybrid construct for leaf, root, and whole plant should be determined over the entire growing season. Both issues may alter projected exposures of non-target organisms.

The Panel acknowledged that excellent information was provided concerning in-field and off-sight movement of various sections of the corn plant during the growing season. Post-harvest data also provided additional useful information about availability.

Degradation of plant leaf material at 3x the maximum amount measured by molecular analysis and bioassay in soil from 3 different states in the US was provided. Average times required to reduce DvSnf7 by 50-90% was location dependent and ranged from 15-35 hrs. The average decline in insecticidal activity by 50-90% was 22-55 hrs. suggesting that there was residual insecticidal activity after appreciable degradation.

Persistence in Terrestrial Environments: The Panel determined that many aspects of the environmental fate studies were well done and provided data that allowed reasonable estimates of exposure duration. Document MRID 493151-22 and the associated publication by Monsanto scientists in Plos One provide a good description of the persistence of dsRNA related toxicity in soils. Data in Table 2 of that article provide precise and accurate estimates for 50% dissipation time (DT50) and DT90. The upper confidence limit for the longest DT90 was 36.6 hrs. as determined from insect toxicity data (which would be a conservative estimate of persistence of toxicity). While these are not technically chemical persistence, the measures demonstrated reduction in toxicity, which would be the basis of risk or hazard assessments, and actual persistence data are available in the Plos One document. Data in Fisher *et al.* (2016) in Chemosphere showed that the upper limit of DvSnf7 persistence was shorter than the upper limit of toxicity persistence, which support comments from the Panel that: 1) there may be residual although diminished activity of degraded dsRNA and 2) the nature and extent of microbial degradation of dsRNA is likely to be variable.

Pesticidal substances will be produced continually in a PIP crop plant. It is expected that DvSnf7 will be deposited in the soil throughout the growing season, and afterwards for as long leaf tissue expressing or containing reside are present. Moreover release of DvSNF7 into soil is expected to decrease throughout the growing season. Data in general, support these assumptions (although as noted above) there is little change in concentration in the leaf of the plant over the growing season. The Panel noted that there were no in situ measurements of DvSnf7 in soils. This presents an unanswered question of how much DvSnf7 is present in root zone soils.

Aquatic Environments [AEs]:Information was presented for the fate of DvSnf7 in aquatic environments. Movement of corn plant foliage beyond the planted field and into nearby aquatic habitats is limited prior to harvest, with the exception of pollen and precipitation-induced run-off. Concentrations in AEs will largely reflect the movement of plant debris. It is unclear

whether or not modeling estimates considered the movement of plant debris and run-off at multiple time points post-harvest.

Transformation data showed that DvSnf7mean degradation kinetics depend upon conditions in various AEs. Degradation kinetics for DvSnt7 in sediment and water collected from a lentic (with loamy sand) and a lotic (with clay loam) system were provided. DT_{50} and DT_{90} values were calculated for water and sediment from disturbed and undisturbed sediment-water microcosms and in filtered water or sediment alone. Data for sediments were available in a very useful study by Fischer *et al.* (2016) (recently released in Environmental Toxicology and Chemistry). This study showed longer persistence of the dsRNA in sediments than was observed in soils. The longest DT 90 was 6± 0.4 days (144± 9.6 hrs.) for undisturbed sediment. With only 2 replicates the CL95 of this DT90 was ± 5.1 days (122 hrs.) which places the upper confidence limit at 11.1 days (166 hrs.) in undisturbed sediment. This was identified as a non-trivial persistence and equivalent to many currently used insecticides. It is likely that more data will reduce the uncertainty and may reduce the upper bound of DT. But in the absence of said data, the conservative DT90 estimate at this time is 11.1 days. The disturbance of sediments reduced the DT90. The Panel noted that the upper 90% confidence limit of the DT90 for disturbed sediments is 8.6 days. Data for disturbed sediments could be used in refined or probabilistic risk assessments, but would be inappropriate for a screening level (Tier 1) assessment which must be conservative in their assumptions.

Across all treatments, the mean half-life of DvSnf7 in water from undisturbed sediments were < 3 days, with DT90 < 5 days; in water from disturbed sediment treatments the mean half-life were < l day. In disturbed sediments in the clay loam sediment, the mean DT_{50} was >2 days, and in all samples DvSnf7 was undetectable within 4 to 6 days In sediments with no overlaying water half-lives were similar to those observed in other sediment samples. DT90s from undisturbed systems should be used when incorporating these dissipation data to risk assessments.

The same publication describes DvSnf7 degradation in water. Among the 4 mean DT90's reported, the maximum was 4.2 ± 0.5 days (mean ± SE). Again with two replicates the CL95 for this DT90 is ±6.35 days, which establishes the upper confidence bound at 10.55 days. The Panel noted that DvSnf7 was detectable for longer periods, ranging between 14 and > 28 days. The upper limit for DT90 from water from Goose Bay test media is 8.38 days. The percent recovery of DvSnf7 were low (57%) in the Goose river sediment. This called into question the accuracy of any absolute concentration determination, but does not influence the computation of degradation kinetics, as such computations/processes are concentration independent, unless the biotransformation systems of organisms are depleted. Reporting of 150% of maximum concentrations was somewhat challenging, but the Panel interpreted that as 150% of nominal.

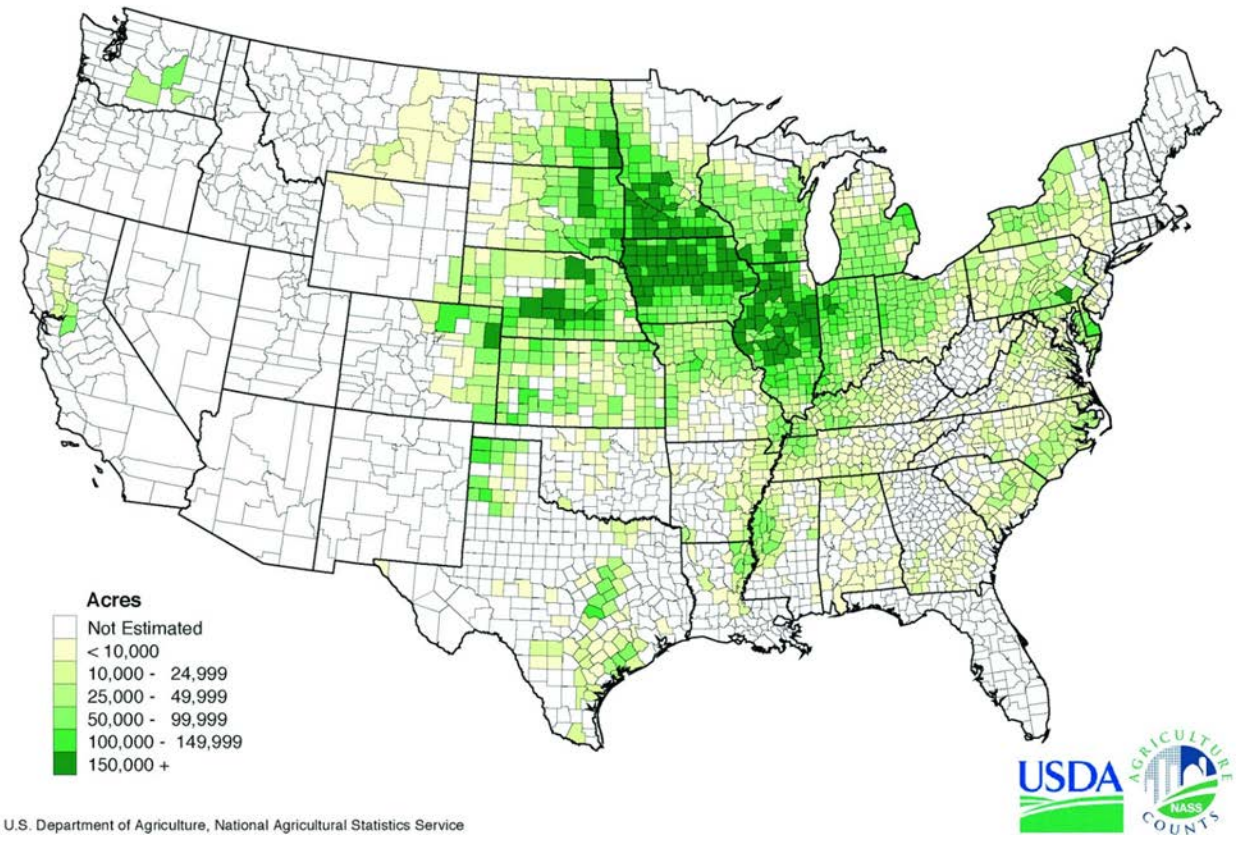
Sediment and water persistence data discussed in the preceding paragraphs above raised the concern that sediment dwelling organisms should have been tested as part of the screening level (Tier 1) assessment. Although buildup in water with continual movement of post-harvest plant debris may be negligible, chronic or repeat exposure of macrofauna that dominate the biomass of surface sediment is possible where concentrations of DvSNF7 were detected past 28 days. This also suggested that exemptions allowed with Tier 1 testing should not have been granted, especially for aquatic organisms in order to be deemed sufficient for the risk assessment for

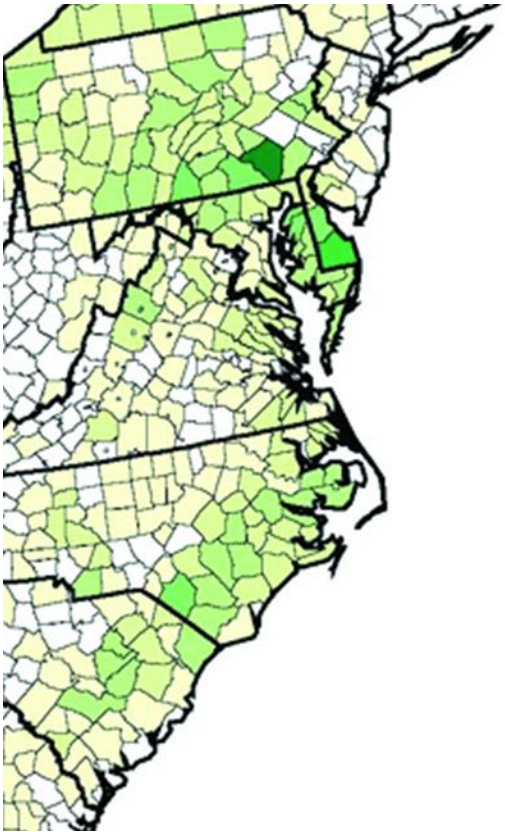
DvSnf7. Specifically, omission of sediment dwelling organisms from toxicity testing is inadequate and leaves many important aquatic species with indeterminate risks. This was identified as a significant limitation of the aquatic component of the DvSnf7 risk assessment. This limitation includes both freshwater and estuarine organisms, the latter having been discounted as not having an opportunity to receive runoff or plant debris from corn fields.

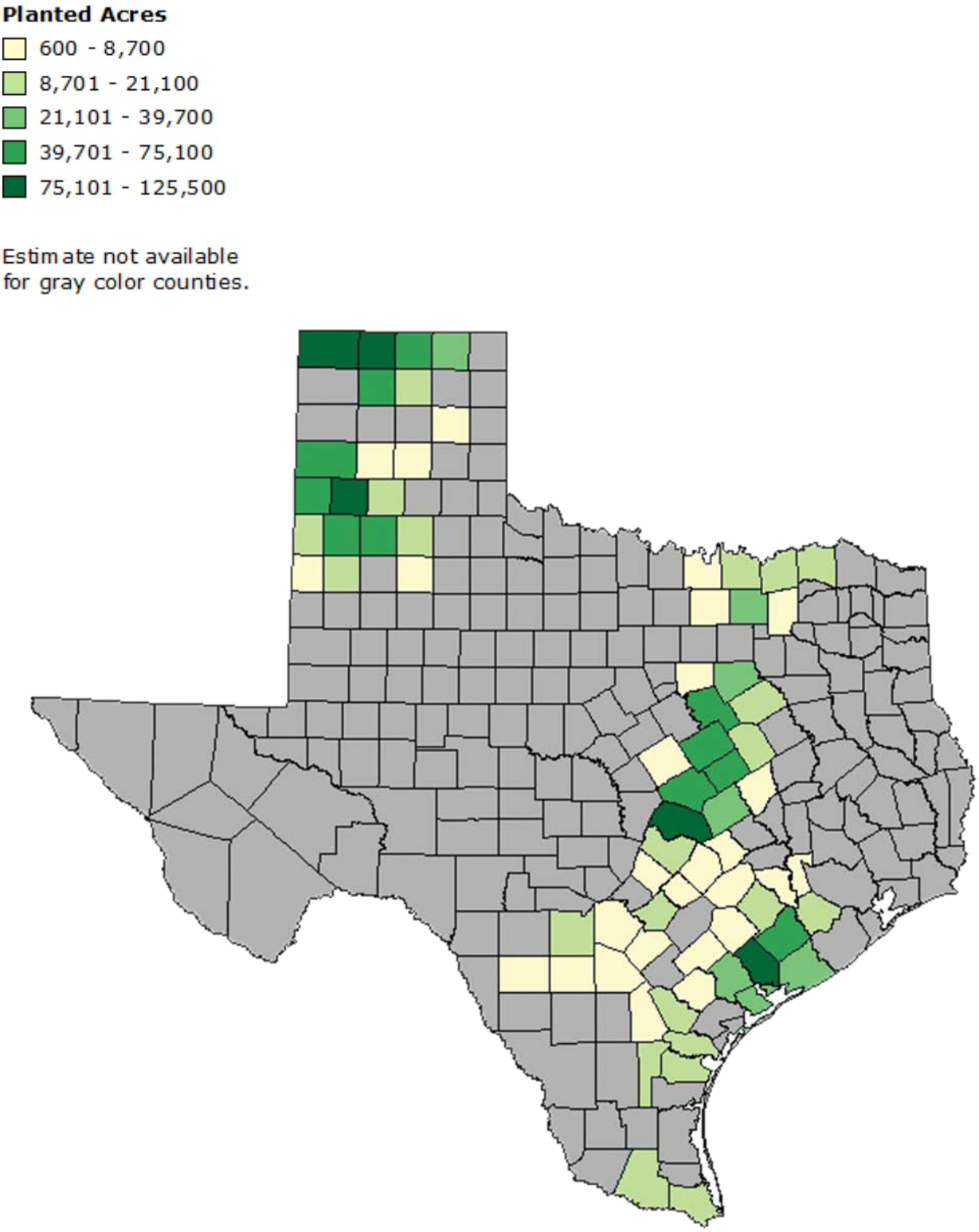
Coastal Areas: Corn use in coastal areas (Refer to Figure 1: Corn Use in Coastal Regions in the United States) do not support the Agency's conclusion that estuarine organisms will not be exposed. Numerous counties in important coastal areas in the United Sates included the Chesapeake Bay, the Outer Banks, and embayment's influencing the South Padre Island National Seashore. It should be noted that many areas of the country have requirements for buffer zones between agricultural fields and water bodies, including estuaries. The problem with these requirements lies in enforcement. Many jurisdictions have insufficient resources or even authority to observe/inspect the appropriateness of such buffer strips/zones or to enforce placement of buffer zones. The longer half-life of DvSvf7 in sediment compared to soil supports the need for toxicity tests involving sediment dwelling organisms including estuarine organisms.

Sediment dwelling organisms could be tested with dsRNA at concentrations of 10-100x the concentrations found in transgenic plants. This would be 2130 ng/g to 21300 ng/g (2.1 to 21.3 µg/g).

Figure 1. Corn Use in Coastal Regions in the United States







In the absence of dsRNA stability in sediments and biological species distributions in corn growing regions, the endangered species statement provided by the Agency is not supported by empirical or modeled data. This data gap should have been filled in addressing the

recommendation of the 2014 SAP that intersection of species and crop distributions be determined.

The deficiencies in the 28-day rodent study have been discussed in previous questions. Thyroid effects (smaller size) were noted in two treatments and possible ovarian effects (smaller size) were noted Petrick *et al*. (2016). Ovarian size was similar to negative control. In the absence of other data to indicate no adverse thyroid effect, citing an error in procedure cannot be used to allow a study to be used to establish a NOAEL. Either the study cannot be allowed or if the study is allowed, the NOAEL is 1 and the LOAEL is 10. With a flawed study, the conservative approach would be to make no determination of the NOAEL or LOAEL

The Panel discussed the importance to ensure that short half-life is accompanied by reduction in toxicity. Transformation can increase, decrease or leave toxicity relatively unchanged. Previous data that was submitted showed that transformation reduced the presence and biological activity of dsRNA. Small RNA molecules are far more stable, however small molecules also are far less active possibly to the point of being inactive.

The in-silico evaluation included less than 25 species that did not necessarily target potentially sensitive species that occupy corn growing regions and as such represent a small number of species given the scope of the proposed use and the number of animal species that could be exposed. The target species for in silico testing should have been assessed as suggested by the 2014 SAP to address the need for alternative paradigms for assessing risks from DvSnf7 concentrations in plants.

> **Question 3b.** EPA has based its determination of the aquatic environmental fate of DvSnf7 on assumptions developed for Bacillus thuringiensis derived Cry proteins, which are largely based on information from the literature. Please comment on the applicability of these assumptions to DvSnf7 and describe any additional or alternative information and/or analyses that EPA should consider.

Panel Response

While the current approach using assumption for Bt-derived PIPs may be sufficient, the general uncertainties with the current state of knowledge concerning dsRNAs indicate there are insufficient data to support or refute the applicability of the assumption developed for Bt-derived Cry proteins for DvSNf7. Cry is a protein and RNAi is a nucleic acid; RNAi mode of action is fundamentally different from Bt proteins. The fundamental difference in their chemical structures and mode of action suggests that the ecological risk assessment should be modified to address unique fate and exposure scenarios posed by dsRNA PIPs.

The Panel is unaware of data supporting the comparability of Cry protein leaching from plant material or persistence in the environment with similar parameters for DvSnf7 or other dsRNAs from plants. It is not apparent that a protein would represent the behavior of a double stranded RNA. Strain and Lydy, 2015 showed the presence of Cry I AB in runoff. However, given the amount of debris that can enter waters adjacent to corn fields, leaching from debris in the water may be a bigger contributor to the presence of Cry proteins in the water column. Thus the

statement in the supporting documents contain no empirical or defensible rational for assuming that Cry proteins leaching would be a "bigger contributor" than would DvSnf7.

The best approach would be to measure the dissipation of DvSnf7 in controlled laboratory and field studies. Sediment and soil data are provided although not for actual plant materials. So persistence after release from plants is relatively well characterized. The remaining question is the magnitude and duration of DvSnf7 from corn plants or runoff. If the Agency decides this is of minimal concern these data could be required in post registration studies.

The data by Gulden *et al*. (2005) demonstrating aquatic fate of dsRNA was similar to DNA which reduced its uncertainty. This type of comparison may prove more useful than comparisons to CRY proteins. The Panel noted that when the 2014 SAP considered comparison approaches for stability assessments, the available data indicated that RNA degradation/transformation was much faster than that of DNA. The newly released data by Gulden *et al*. (2005) highlights the error of using available data and not requiring that critical data gaps be filled.

"Rationales justifying why guideline testing is not required were also submitted to address certain data requirements." These rationales are often unacceptable and several decisions to reduce data requirements were inappropriate in the case of DvSnf7. No data were provided that document minimal exposures in estuarine areas. Corn production in the USA does in fact occur in reasonable measure near estuarine areas and cannot be excluded. The non-target plant exposures were similarly expected (given that the mode of action was targeted for animals), thus an omission is likely to be acceptable. Finally, the omission of endangered species assessments cannot be supported based on previously reported endangered species, including aquatic insects, in corn growing areas of the USA.

> **Question 3c.** Due to the nature of the data provided, EPA has based estimates of non-target organism exposure on environmental concentrations. However, as indicated in several publications, a certain threshold of molecules is required to induce RNAi and subsequent gene silencing. An analysis considering these thresholds was included in Monsanto Company's white paper on human safety of DvSnf7. EPA recognizes that a similar analysis for non-target organisms might provide more refined exposure estimates for DvSnf7 and for future risk assessments of dsRNA pesticides; however, it is unclear whether sufficient data are available to develop a similar analysis for non-target organisms. Please comment on the risk assessment for DvSnf7 by such a threshold of exposure based approach, considering the scope of EPA's ecological risk assessments, and any additional information needed to implement such a threshold based analysis.

Panel Response

The majority of the Panel's recommendations to this question is included into the Panel's response to Question 1 and the majority of the Panel's response was deferred to those answers. Uptake of plant miRNA is limited to less than 1 copy per cell and is deemed insufficient for mediating RNA in ingested organisms which is believed to require at least 100 copies/cell. Moreover, existing barriers in terrestrial vertebrates provide significant protection and RNAs are rapidly degraded.

The Panel identified no evidence of bioaccumulation Witner *et al*., (2013), so that this aspect of classical chemical assessment is unimportant for dsRNA. Current data suggests a low probability that exposures would exceed any toxic threshold for terrestrial organisms and for aquatic vertebrates. Aquatic invertebrates have not been well enough characterized to adequately determine minimal hazard or risk. The Panel noted that microbes were not considered and may become important considerations in future PIPs.

Information provided in the agency's human health risk assessment demonstrated that barriers exist to dsRNA uptake by humans that provide significant protection from native-unprotected dsRNA reaching organ tissues. This information may be sufficient to support protection of mammals. The agency's DEEM-FCID exposure model estimate of no concern for human exceedance of Chronic Population adjusted Dose (cPAD).

The Panel explicitly noted that the Agency has taken the position that "cessation of exposure is expected to result in reduction and eventual cessation of effects." The Panel concluded that one time exposures can have durable effects. With this in mind, additional testing in non-target organisms representing aquatic biota and experiments designed to address off-target and other unintended effects related to dsRNA exposure are warranted to conclude this question.

Charge Question 4. –Non-target Organism Hazard

EPA has reviewed non-target hazard data developed for DvSnf7 on two species of birds, two mammal species (from human health testing), a freshwater fish, seven species of non-target arthropods, an earthworm, and honey bees, and has included these studies in its consideration of non-target organism risk. This approach for dsRNA is consistent with EPA's testing framework for Bt derived PIPs, with additional data required on non-target insect reproduction. EPA has also included assumptions about common barriers to dsRNA uptake in vertebrates and bioinformatics analysis as additional lines of evidence in this consideration. Based on the whole of these data, EPA has concluded that adverse effects to non-target organisms are not anticipated to result from cultivation of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 corn.

Panel Response:

Additional testing in non-target organisms representing soil biota and experiments designed to address off-target and other unintended effects related to dsRNA exposure were warranted to conclude this question.

> **Question 4a.** Please comment on the completeness of the non-target organism hazard data reviewed forDvSnf7 as it pertains to the needs of the environmental risk assessment and the recommendations for testing made by the 2014 SAP.

Panel Response:

Non-target hazard data, including the additional testing recommended by the Agency's 2014 SAP, for the most part, were adequate where conducted. However, the Panel listed several of their concerns:

1) The Panel concurred with the Agency's assessment that the broiler study defined does have limited suitability, and the studies used 50% of DvSnf7 seed. Conservative designs would have used 100% of DvSnf7. If there was a desire to increase the dose, seeds could be treated with *in vitro* derived dsRNAs (similarity this has been asserted by the registrant and the agency). Also the registrant can assess exposures and toxicities to avian species *in situ* by conducting limited field or field-pen trials where approved seed production is occurring.

The bobwhite study (MRID 49886501) was deemed acceptable and the 1,000 µg/kg rather than the 5000 µg/kg maximum hazard dose (or limit dose) was reasonable as the expression *in planta* would not produce dsRNA at concentrations any higher than those tested. It appeared unnecessary to attempt studies at the 5,000 µg/kg for constituents that are expressed *in planta* at concentrations 6 orders of magnitude lower.

The catfish test for nutrition was not designed to assess hazard from DvSnf7. As such the supplemental nature is appropriate, and the need for added testing is largely dependent on the extent of the transcriptome data for individual fish species and the number of species in the database.

The endangered species statement is not supported by empirical or modeled data. This data gap would have been filled had the 2014 SAP Panel recommendation regarding determining intersection of species and crop distributions been followed.

The Panel explicitly noted that the Agency had taken a position that "cessation of exposure is expected to result in reduction and eventual cessation of effects." One-time exposure can have durable effects.

2) The Panel recommended that additional surrogate species representing the soil biota should be included in the non-target organism hazard analysis. This group of organisms is ecologically most relevant to the receiving environment where RNAi transgenic maize is used. The DvSnf7 dsRNA is intended for the control of CRW, the soil-dwelling corn pest. Soil biota is extremely diverse, and has enormous influences on soil characteristics and plant growth. Based on their size, soil biota includes megafauna (>20 millimeters (mm)), macrofauna (2-20 mm), mesofauna (0.1-2 mm), microfauna and microflora (1-100 micrometer (µm)). The interaction between transgenic plants and soil biota is dynamic and complex. The potential non-target impacts not only involved the direct effects to individual groups, but only include other members within the maize rhizosphere through trophic interactions. Therefore, the Panel recommends to test whether the RNAi transgenic maize can affect structural (i.e., individual species) or functional (i.e., biodiversity and ecosystem services provided by the soil biota) endpoints.

The Panel indicated they had access to only two of the soil detritivores (a springtail species representing mesofauna and an earthworm species covering macrofauna) risk assessment studies. However, the design of the earthworm study (MRID No. 493151-16) consisted of major flaws and the results were deemed questionable. In a 14-day toxicity assay, the registrant placed the earthworm *Eisenia andrei* in an artificial soil substrate spiked with naked DvSnf7 dsRNA. As a detritivore, the earthworm feeds on decomposing plant and animal parts as well as feces. The logical delivery method should be through oral ingestion (dietary RNAi) rather than contact exposure (environmental RNAi). Also, the stability of the test substance appeared to be compromised. In MRID No. 49315122 entitled "Soil Degradation of a dsRNA Transcript Derived from the DvSnf7 Suppression Cassette and purified DvSnf_968 RNA" stated "Results from the soil degradation study indicate that DvSnf7_968 degraded within approximately 2 days after application to soil, regardless of texture, pH, clay content and other soil differences, as measured by molecular analysis (QuantiGene) and by insecticidal activity (bioassay)". Based on the soil stability study, naked DvSnf7 dsRNA can, at most, persist in artificial soil substrates for 2 days in a 14-day contact study. Even for the first two days, registrant did not provide the bioactivity data of the test substances. Nevertheless, the registrant concluded that "Based on the results of assessments for mortality, behavior and change in biomass, the NOEC (no-observed-effect concentration) for DvSnf7_968 RNA was found to be 5,000 nanogram (ng) RNA/g soil dry weight." The Panel recommended to avoid future confusion, the registrant should remove the earthworm study (MRID No. 493151-16) from the supplementary materials.

The Panel further discussed that the rationale for excluding freshwater invertebrate and other insect testing may not be protective of non-target organisms. These organisms should be included in the data provided by the registrant.

The majority of invertebrate toxicity testing provided good information regarding the potential toxicity of DvSnf7. However, the Panel recommended that the registrant should have included the reproduction as an endpoint measurement for the phylogenetically and its most relevant non-target insects, specifically the coleopteran species, carabid beetle, *Poecilus chalcites*, insidious flower bug, *Orius insidiosus*, and pink-spotted lady beetle, *Coleomegilla maculata*.

In an effort to provide clarity, the Panel stated that it would have been more appropriate to assess the non-target effects in one of the various root nematodes closely associated with maize such as the sting nematode**,** *Belonolaimus gracilis*, ring nematodes, *Criconemoides* spp., corn cyst nematode, *Heterodera zeae*, root-knot nematodes, *Meloidogyne* spp., stubby-root nematodes, *Paratrichodorus* and *Trichodorus* spp. or one of the five lesion nematodes, *Pratylenchus* species. Additionally, the studies should have included species within the rhizosphere microbial community of maize such as, *Fusarium* which are the major soil-borne fungal pathogens and the bacterial flora *Trichoderma viride* or *Fusarium moniliforme*.

One of the associate panel members assigned to charge question four was not in agreement on several points raised by the lead respondent panel member.

Regarding the need for extensive additional testing, the associate respondent panel member cautioned that, in general, the regulatory burden proposed was unprecedented, particularly considering the ephemeral and apparently specific nature of the product. The associate respondent's sentiments regarding the new request for data were discovery-driven in nature and irrelevant in the risk assessment setting (to include data from the rhizosphere microbial community of maize). The associate respondent also expressed that the characterization of the trophic interactions appeared to be rooted in speculation despite the previously provided results on stability and toxicity and the RNA-mediated mode of action which included whether or not the relative efficacies of the exact sequence(s) within the hairpin have been defined.

The lead respondent emphasized that, in contrast with what the associate respondent considered to be speculation on disruption to communities, microbes were not known to be adversely affected by the presence of RNA, even assuming that the product was efficiently secreted from the plant material. The lead respondent had previously stated that data from Dubelman *et al* reported changes to the microbial community in a soil treatment study involving many orders of magnitude higher to DvSnf7 exposures than would ever occur in the field. However, these changes were quantitative, i.e., the communities had responded to the presence of new nutrients (large quantities of RNA) as expected. Another panel member (referred to as "PMC") was in agreement with this assessment, noting that microbes up take RNA components to avoid having to make their own. PMC concluded that the results of Dubelman *et al*. (2014) were consistent with the current understanding of foreign RNA as bacterial nutrition.

PMC was notably in opposition to the recommendation of omics-based or additional microbiome studies for several reasons. First, bulk data had already been generated indicating that the soil microbiome had functional equivalence in the presence and absence of the agent. Second, exposure and hazard were unclear, as previously discussed within this report. Third, the infancy of the microbiome field ensured that even scientifically interesting data were difficult to interpret. This was as a direct result from the fourth reason, the ever-present influence of contamination in sampling and sequencing, which had been reported in many microbiome studies (refer to a previously referenced study from Charlson *et al*., 2011). Fifth, although previous Panel discussions suggested that standardized approaches were being developed, soil sampling locations (taken at locations next to the root, one centimeter and forty centimeters away from the root) were certain to provide different results (this had been the consensus since the 1980s). Moreover minor differences in sampling would reveal apparent differences that had already existed in the field.

The associate panel member conceded and eventually agreed to the recommendation that testing of a nematode species might be scientifically sound given the response of several nematode species to dsRNA.

Regarding the earthworm study, the associate panel member noted the registrant had clearly marked the study as a contact exposure study, and provided a rationale for not classifying the data as a feeding study. The associate panel member cited some confusion as to why the study had been described as "erroneous" and that it should be expunged from the record versus being classified as "supplemental" data. The lead panel member concurred and for the record also noted—the study represented additional data.

Question 4b. EPA has concluded that DvSnf7 is unlikely to cause adverse effects to vertebrate non-target organisms. This conclusion relies in part on an assumption that biological barriers that limit uptake in mammals (see the human health risk assessment for DvSnf7) would also apply to other vertebrates. Please comment on the biological barrier assumption as a line of evidence supporting EPA's conclusion of minimal risk to vertebrate non-target organisms.

Panel Response:

The Panel found the Agency's human health risk assessment to be appropriate and to refer to their response to Charge Question 1 within this report.

Question 4c. EPA concluded that off-target and other unintended effects related to dsRNA exposure are unlikely in non-target organisms, based on the lack of effects observed in non-target testing. Please comment on EPA's conclusions regarding these effects.

Panel Response:

There is a level of concern for horizontal transfer of the transgenic gene cassettes from the MON 87411 corn strains. The stability of *Agrobacterium tumefaciens* Ti plasmid insertions has been well documented but the presence of new DNA structural forms to produce double stranded RNA should result in a reaffirmation of the considered insertion stability. If the potential exists for transgene mobilization in the host species, then an assessment must be made for potential integration into a symbiotic, infectious, or predatory system that could mediate transmission to another host. Such systems can be prokaryotic bacteria or viruses, or eukaryotic predators such as mites or aphids. In the event of lateral transfer of the DvSnf7 concentration cassette it can potentially lead to high concentrations of DvSnf7 dsRNA within the new host leads to non-target impacts where it either acts directly or indirectly to suppress non-target transcript, immune response or saturate the RISC system (Kyndt *et al.*, 2015; Soucy *et al.*, 2015; Stegemann *et al*., 2012).

Another associate panel member emphasized that these concerns about cassette stability would apply to all transgenic plants made in this manner and do not affect this product specifically.

According to the Panel, the registrant's assertion that "DvSnf7 is not toxic to mammals after 28 consecutive days of repeat oral exposure" is inaccurate. The deficiencies in the 28-day rodent study have been discussed in previous questions. Thyroid effects (smaller size) were noted in two treatments and possible ovarian effects (smaller size) were noted. Ovarian size was similar to negative control. In the absence of other data to indicate no adverse thyroid effect, the Panel in consensus stated that citing an error in procedure cannot be utilized to allow a study to establish a NOAEL. Either the study cannot be allowed, or the NOAEL is 1 milligram per kilogram per day (mg/kg/d) and the LOAEL is 10 mg/kg/d. Test organisms should be exposed to at least 5 concentrations to determine if doses are behaving monotonically. If not more doses

should be considered, and the question remaining about thyroid increases the likelihood of non-monotonic dose response.

Another panel member pointed to the Agency's response to questions about the assay, indicating that no dose response was observed and that the Agency had carefully evaluated these assays and found that trimming differences, not a biological effect, was responsible. Referring back to the Panel's position on dietary delivery of RNA, the Panel member emphasized that the dietary uptake of this RNA, to the extent it occurred, is extremely low. Any action of this dsRNA would be at sub-hormonal levels and unsupported by any known biological mechanism. Thus, the suggestions of non-monotonic dose responses appear to be hypothetical and speculative.

Charge Question 5. – Synergism

EPA requires data to demonstrate that PIPs expressed in combination within the same plant are not synergistic. The purpose of these studies is to allow bridging of data developed on individual PIPs to the combined trait product; otherwise, new data generation would be required on the combination to determine non-target risk. EPA reviewed five studies examining synergism among DvSnf7 and the Cry proteins expressed in MON 89034 x TC1507 x MON 87411 x DAS-59122-7 and determined that no synergism is expected. Please comment on EPA's analyses of these data and the scientific value of these data in the risk assessment.

Panel Response:

Overall the Panel concurred with the Agency's analysis of data on potential synergism among DvSnf7 and the Cry proteins. The Panel believed there was high scientific value in the data on potential interaction among PIPs because this could negatively impact the safety and efficacy of the final product. Thus, the data would allow for the bridging of data on single PIPs.

Several studies were reviewed and none demonstrated potential interactions among PIPs and DvSnf7. The Agency's assessment of the 5 studies that concluded synergism did not exist was considered valid. However, some concerns were raised by the Panel.

One study, 49505803, used lethal concentration, 50% (LC50) values of the Cry protein in the presence or absence of DvSnf7, at fixed sublethal concentrations. Although several concentrations were used in this study the Agency classified this study as supplemental, and stated that additional concentrations were needed. The Panel agreed with this assessment.

The Levine *et al*. (2015) paper submitted to the Agency utilized 3 concentration combinations using the response addition model and found no significant evidence other than additivity occurred.

It is not certain to the Panel if the Fixed Lethal or Concentration Addition models represented the most rigorous approaches for determining synergism. The effect of dose is critical when determining the contribution of individual components within mixtures, and all relevant possible combinations of doses may not have been examined. In pharmacology for example, complete isobolgrams are generated when studying drug-drug interactions. However, given the Agency's assurance that the current methodological approaches have been previously utilized successfully

with chemical mixtures and other PIPs, it was determined that the models used in the 5 studies under review were adequate.

Given that the methodology and models were adequate, one panel member noted that the endpoints used in the studies may have been limited. Mostly in mortality or growth. Other endpoints such as those associated with reproduction may be impacted at lower doses of the pesticide.

Charge Question 6. – Insect Resistance

Although the Agency has not requested comment from previous Scientific Advisory Panels on the potential for resistance in corn rootworm to RNAi-based pesticides, the Agency believes this to be an important consideration in its regulation of such pesticides, and uses this opportunity to collect advice and information.

Corn rootworm has demonstrated an ability to adapt to a wide range of chemical and cultural controls, including Bt Plant-Incorporated Protectants. Please discuss the likelihood and potential mechanisms by which corn rootworm could develop resistance to an RNAi-based pesticide such as DvSnf7 dsRNA.

Panel Response:

The Panel members indicated that there were no known reports of insects that had developed natural resistance to an RNAi-based pesticide. The Panel agreed that the corn rootworm could become resistant to DvSnf7 dsRNA treatment. Potential mechanisms by which corn rootworm could develop resistance to DvSnf7 dsRNA were indicated to luckily be the same as conventional Resistance Mechanisms to synthetic insecticides: A) target site insensitivity in the RNAi machinery (e.g., insensitivity or saturation of RNAi machinery) B) metabolic resistance in the dsRNA degradation enzymes (e.g., overexpression or upregulation of dsRNA degradation enzymes, such as dsRNases), and C) reduced permeability and reduced uptake (e.g., reduced expression or down regulation of SID-1 or endocytosis, and behavioral resistance).

During the discussion, it was noted that since insects have great genetic and phenotypic plasticity, individuals in a corn rootworm populations could be already more or less resistant to doses of DvSnf7 dsRNA. Chu *et al*. (2014) determined three field populations of the western corn rootworm, two collected in fields with crop rotation-resistance populations and one in an area without crop rotation-resistance, exhibited differential response to two different dsRNA treatments. This study showed that phenotypic responses to RNAi-based pesticides vary across corn rootworm populations, and that there is the potential for individuals in a corn rootworm population or for populations to naturally be more or less resistance to silencing. The RNAi resistant corn rootworm individuals could be selected to become predominant in a population subjected to strong evolutionary constrains, thus the Panel urged that release and planting of SmartStax PRO corn should follow IPM principles that would mitigate this selection and retard the onset of corn rootworm resistance.

Members of the Panel noted that the development of corn rootworm resistance in RNAi transgenic traits, including DvSnf7 dsRNA, seemed inevitable. The innate differences exhibited in the sensitivity to ingested dsRNAs among corn rootworm individuals within the same species Chu *et al.*, (2014) or differential responses to dietary RNAi among species within phylogenetically closely related taxon of insects were taken as a proof of a pre-adaptive phenomenon that would enable the corn rootworm to become resistance to SmartStax PRO corn. Biologically relevant mutations in enzymes involved in RNAi machinery, dsRNA uptake, delivery, and processing were indicated as contributors to the development of resistance to transgenic RNAi crops.

Additional mechanisms reported by the Panel were listed:

1) Changes in dsRNA target sequence. The Panel recognized that this scenario would be unluckily with a 240 bp target such as in the case of DvSnf7 dsRNA, however the Panel stated that changes in dsRNA target sequences could occur with shorter targets where presence of SNPs could compromise the complementarity of the dsRNA to its target.

2) Changes in characteristics of natural barriers to dsRNA uptake, transport, effect. Some examples could be an increase in RNAses levels in the gut lumen, changes in SID-1 permeability, etc.

3) Changes in the RNAi machinery enzymes or components. Genes involved in the RNAi pathways had been artificially downregulated in insects (for instance, in the brown planthopper by Zhang *et al.*, 2013) without adverse effects to the longevity of the insects. Velez *et al*. (2016) showed that suppression of *Dicer 2* and *Argonaute 2* expression in the western corn rootworm reduced their mortality after exposure of lethal doses of *vATPase A* dsRNA, demonstrating that under laboratory conditions the corn rootworm can survive without a functional RNAi machinery. In another case, the oriental fruit fly Li *et al*. (2015) was fed with dsRNA to target the endocytic pathway blocking dsRNA uptake in cells. In Drosophila, *Dcr2*, *R2D2*, and *Ago2* were reported to be among the fastest evolving 3% of all genes, more than components of innate immunity and housekeeping genes Obbard *et al*. (2006), thus this team hypothesized that natural mutants in genes with antiviral functions could evolve in the corn rootworm.

The Panel did not have an indication on how insects without functional antiviral defenses would perform in the field (for instance these insects could be more susceptible to viral infections), but during evolution some eukaryotes lost parts of their RNAi machinery that uses dsRNA/siRNAs to start silencing (the budding yeast *Saccharomyces cerevisiae* was reported as an example; vertebrates were also reported to have lost the antiviral function of the RNAi pathway).

4) Behavioral avoidance, in the case of plants modified to present different olfactory or gustative cues. At this point the members of the team noted that there were no indications if transgenic SmartStax PRO corn showed any difference with its non-transgenic isoline.

The Panel agreed that concerns pertinent to the development of resistance of the corn rootworm to SmartStax PRO corn were not part of a risk assessment evaluation, but the Panel cautioned that development of western corn rootworms resistance could occur in the near future. A report from Dow AgroSciences presented during the Panel meeting showed that about 134 adults of corn rootworm emerged in corn plots treated with SmartStax PRO. The Panel members recognized that there was no evidence that those individuals were resistant to DvSnf7 dsRNA, but suggested that similar trials could provide a window of opportunity to monitor insurgence of resistance. Members of the Panel suggested also that a better understanding of the mode of action of dsRNA on the corn rootworm would be a prerequisite to implement a resistance strategy, such as the use of refuge areas. The work of Shakula *et al*. (2016) that indicates differences between silencing mechanisms in beetles vs. lepidopteran was cited in support of this suggestion.

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