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Ecological risk assessment for DvSnf7 RNA: A plant-incorporated protectant with targeted activity against western corn rootworm

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ABSTRACT

MON 87411 maize, which expresses DvSnf7 RNA, was developed to provide an additional mode of action to confer protection against corn rootworm (*Diabrotica* spp.). A critical step in the registration of a genetically engineered crop with an insecticidal trait is performing an ecological risk assessment to evaluate the potential for adverse ecological effects. For MON 87411, an assessment plan was developed that met specific protection goals by characterizing the routes and levels of exposure, and testing representative functional taxa that would be directly or indirectly exposed in the environment. The potential for toxicity of DvSnf7 RNA was evaluated with a harmonized battery of non-target organisms (NTOs) that included invertebrate predators, parasitoids, pollinators, soil biota as well as aquatic and terrestrial vertebrate species. Laboratory tests evaluated ecologically relevant endpoints such as survival, growth, development, and reproduction and were of sufficient duration to assess the potential for adverse effects. No adverse effects were observed with any species tested at, or above, the maximum expected environmental concentration (MEEC). All margins of exposure for NTOs were >10-fold the MEEC. Therefore, it is reasonable to conclude that exposure to DvSnf7 RNA, both directly and indirectly, is safe for NTOs at the expected field exposure levels.

1. Introduction

Over the past decade, a number of food crops utilizing RNA interference (RNAi), have received regulatory approvals from United States agencies such as the Environmental Protection Agency (U.S. EPA) and Department of Agriculture (USDA), as well as approval in other countries such as Canada, Mexico, Australia, New Zealand, Japan, Korea, and Brazil (CERA, 2012). The RNA-based products approved thus far have conferred resistance to specific viruses (e.g. plum-pox virus), extended produce quality (e.g. Arctic Apple) or nutritional enhancement (e.g. alfalfa, soy) (Auer and Frederick, 2009; CERA, 2012). Recently, genetically engineered (GE) insect-protected plants that confer resistance via RNA-based gene regulation have been developed and reported in the scientific literature (Bachman et al., 2013; Baum et al., 2007; Bolognesi et al., 2012; Mao et al., 2007). These plants express double-stranded RNAs (dsRNAs) targeted to suppress mRNA levels in a specific species or a small group of closely related species by utilizing the RNAi pathway. The sequence specific nature of RNAi allows these products to target pest species with a high level of specificity, while mitigating risk to non-target organisms (NTOs) (Bachman et al., 2013; Burand and Hunter, 2013; Whyard et al., 2009). Monsanto Company has developed a GE maize, MON 87411, that confers protection against corn rootworm (CRW) (*Diabrotica* spp.) utilizing RNAi as the mechanism of insecticidal action (Bolognesi et al., 2012). The DvSnf7 RNA expressed in MON 87411 is composed of a 968 nucleotide sequence containing 240 base pair dsRNA component plus the addition of a poly A tail (Urquhart et al., 2015) designed to target the western corn rootworm (*Diabrotica virgifera virgifera;* WCR) *Snf7* gene (DvSnf7). Upon consumption, the plant-produced RNA in MON 87411 is recognized by the CRW's RNAi machinery, which results in a rapid decrease of DvSnf7 mRNA and protein levels leading to growth inhibition followed by mortality (Bolognesi et al., 2012; Levine et al., 2015). It has been established that after ingestion of DvSnf7 by WCR, suppression of the DvSnf7 mRNA occurs within 24 h, followed by suppression of DvSNF7 protein and onset of mortality by day 5 (Bolognesi et al.,

Abbreviations: dsRNA, double stranded RNA; RNA, ribonucleic acid; RNAi, RNA interference; NTO, non-target organism; ERA, ecological risk assessment.

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2012). MON 87411 also contains a *cry3Bb1* gene that produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. The spectrum of activity of the Cry3Bb1 protein has previously been reviewed by the U.S. EPA and, at the levels expressed in GE maize, activity was only evident in the family Chrysomelidae within the order Coleoptera (U.S. EPA, 2010a). Corn rootworm active *Bt*-technologies, such as the Cry3Bb1 and Cry34Ab1/Cry35Ab1 proteins, have been safely marketed for over a decade, and have provided significant value to farmers (Prasifka et al., 2013). In addition, incorporation of multiple modes of action against CRW by pyramiding *Bt* and RNA-based traits will offer increased efficacy and durability of a product while maintaining a high degree of specificity for the target pest and environmental safety (Baum and Roberts, 2014).

A critical step in the deregulation and/or registration of a GE plant incorporated protectant (PIP) is performing an ecological risk assessment (ERA) to evaluate the potential for adverse ecological effects from cultivation. Assessment of potential ecological impacts, associated with the introduction of a PIP, is based on the characteristics of the crop and the introduced trait. The approach for evaluating ecological risks from pesticides is a multi-step iterative process (Romeis et al., 2013; U.S. EPA, 1998). Key steps include problem formulation, analysis of exposure and potential effects, and risk characterization. During problem formulation, the assessor defines protection goals, prepares a conceptual model to aid in identification of the relevant assessment and measurement endpoints, and then develops an analysis plan that serves as the basis for a risk characterization. Important information that was used to inform the problem formulation step for MON 87411 included the biology and familiarity with the crop and the trait, the mode of action (MOA), the spectrum of activity, the tissue specific expression profile, routes of exposure for ecological receptors and an assessment of potential persistence in the environment. In general, the scope of the ecological safety assessment for a PIP can be reduced when the MOA is well characterized, there is a narrow spectrum of activity, and expression levels of the trait are well characterized (Romeis et al., 2013). The MOA of DvSnf7 RNA has been well characterized (Bolognesi et al., 2012; Ramaseshadri et al., 2013) and has been shown to have a narrow spectrum of activity with activity only evident within a narrow subset of beetles, the Galerucinae subfamily in the order Coleoptera (Bachman et al., 2013). This limited range of activity reduces the potential for non-target effects and can narrow the scope of ecological testing. Additionally, the DvSnf7 RNA and Cry3Bb1 protein have been shown to act independently which allowed for Cry3Bb1 and DvDnf7 RNA to be tested and assessed independently (Levine et al., 2015). Taken together, information on the MOA, spectrum of activity, expression profile, lack of interaction, and routes of potential exposure were used to help inform and define the scope of NTO testing used for this ERA.

For the MON 87411 assessment, the protection goals were identified as the maintenance of ecological functions of NTOs 'in-field' and biodiversity of species 'off-field' that contribute to the structure and function of the environment. Ecological functions to be protected include pollination, predation and parasitism (i.e., biological pest control, referred to herein as biocontrol), decomposition of soil organic material, and soil nutrient cycling. Additional confirmatory data was collected to address regulatory requirements and to provide empirical data for a broad range of taxa for this first in class insecticidal RNAi product. This included a broader range of avian and other non-target vertebrate populations where a plausible risk hypothesis would typically not require such data given barriers to exposure in these taxa (see section 4.1 in Discussion). An important assessment endpoint for PIPs is the abundance of taxa within functional groups of NTOs. Primary

Table 1
The relationships between protection goals, assessment endpoints, indicators of effect, and measurement endpoints utilized in the DvSnf7 RNA/MON 87411 ecological risk assessment.

Non-target organism		Protection goals	Assessment endpoints	Indicators of effect	Measurement endpoints
Honey bee adult and larvae	*Apis mellifera*	Pollinationservices and pollinator biodiversity	Population size and function, biodiversity	Laboratory larval & adult bee toxicity studies	Adults (worker) survival Larval survival and development to adult
Ladybird beetle	*Coleomegilla maculata*	Biocontrol by non-target arthropods	Population size and function, biodiversity	Laboratory toxicity studies initiated neonates or adults	*C. maculata, P. chalcites, and O. insidiosus* survival, growth and development to adult
Rove Beetle	*Aleochara bilineata*				*A. bilineata and C. carnea* adult survival and reproduction
Ground Beetle	*Poecilus chalcites*				*P. foveolatus* adult survival
Green Lacewing	*Chrysoperla carnea*				*E. andrei* survival and body weight
Insidious Flower Bug	*Orius insidiosus*				*Collembola* survival and reproduction
Parasitic Wasp	*Pediobius foveolatus*				Carbon and nitrogen transformation
Earthworm	*Eisenia andrei*	Nutrient cycling by soil biota	Population size and function, biodiversity of soil macro-organisms	Laboratory toxicity studies initiated with neonates or adults	
Collembola	*Folsomia candida*		Functionality of microbially-mediated soil processes	Laboratory toxicity studies with soil microorganisms	
Beneficial soil microorganisms					
Bobwhite quail	*Colinus virginianus*	Avian and wild mammal populations	Population size, biodiversity	Toxicity studies with bobwhite quail, chickens, and catfish	Survival and growth
Chicken	*Gallus domesticus*				
Catfish	*Ictalurus punctatus*				

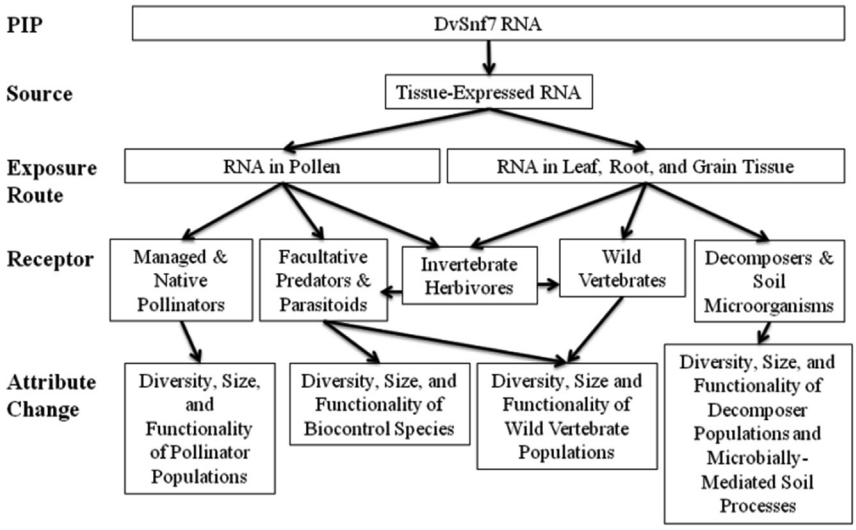

Fig. 1. Exposure-based conceptual model for MON 87411.

indicators of effect include impacts on survival, growth, development and reproduction. The relationship between protection goals, assessment endpoints, and indicators of effect (measurement endpoints) for DvSnf7 RNA are outlined in Table 1. Using an exposure-based conceptual model (Fig. 1), ecologically relevant routes of exposure for NTOs were identified and used to develop risk hypotheses. The over-arching risk hypothesis that was tested was that cultivation of MON 87411 will have no unacceptable adverse effects on NTOs resulting from environmental exposure to the DvSnf7 RNA. Testing this hypothesis required performing laboratory toxicity tests on individual species and placing the results into the context of an ERA. An *in silico* analysis, using publically available sequences for relevant NTOs associated with maize agriculture and/or key ecological functions, was conducted to assess potential effects to additional species.

2. Materials/methods

2.1. Conceptual model

An exposure-based conceptual model was developed for MON 87411 to illustrate routes of exposure to DvSnf7 RNA for ecological receptors (e.g. NTOs) that represent functional roles (Fig. 1). Key functional and measureable attribute changes were identified for the ecological receptors that were linked to the identified environmental protection goals (Table 1). Pollen was included as the route of exposure for pollinators, facultative predators and parasitoids, and invertebrate herbivores that could use pollen as a supplementary or life-stage specific food source. Leaf, root, and grain tissue were included as an exposure route for herbivorous invertebrates and wild vertebrates and senescent tissue was considered as the route of exposure for soil biota. The invertebrate herbivores feeding on leaf or root tissue were considered an indirect exposure route for biocontrol species (e.g. insect predators or parasitoids) and wild vertebrates. Aquatic exposures were considered but not included in the conceptual model because exposure of aquatic organisms to maize tissue after harvest is limited temporally and spatially; therefore potential exposure of aquatic organisms is low to negligible (U.S. EPA, 2010a). In addition, DvSnf7 RNA has been shown to rapidly degrade in aquatic systems (Fischer et al., 2016a,b). Measureable attribute changes (assessment endpoints) were identified for each ecological receptor including biodiversity, population size, and/or ecological functionality.

2.2. Effects testing

2.2.1. Test species selection and study design

Selection of test organisms was informed by the protection goals and conceptual model, and to meet the U.S. EPA's testing framework for PIPs (U.S. EPA, 2001). NTO testing included laboratory toxicity testing against a representative pollinator [honey bee (*Apis mellifera*)], six beneficial insect species that represent biocontrol species [parasitic wasp (*Pediobius foveolatus*), ladybird beetle (*Coleomegilla maculata*), carabid beetle (*Poecilus chalcites*), rove beetle (*Aleochara bilineata*), green lacewing (*Chrysoperla carnea*), and insidious flower bugs (*Orius insidiosus*)], representative soil biota [earthworm (*Eisenia andrei*), Collembola (*Folsomia candida*), and microbially-mediated soil processes], and representative wild vertebrates [bobwhite quail (*Colinus virginianus);* channel catfish *(Ictalurus punctatus);*and broiler chicken *(Gallus domesticus)*]. Survival, growth and/or developmental observations were examined in the ladybird beetle, carabid beetle, insidious flower bug, honey bee, and vertebrate studies; survival and reproduction with Collembola, rove beetle and green lacewing, and survival and biomass with earthworm. Carbon and nitrogen (C: N) transformation in soil mixed with root and shoot tissue derived from MON 87411 was measured to evaluate the functionality of soil nutrient cycling by microorganisms. In addition to the avian and catfish studies, the results of a 28-day (*Mus musculus*) repeat dose oral gavage study with the DvSnf7 RNA at doses up to 100 mg/kg/day (U.S. EPA, 2015; Petrick et al., 2016) was included as part of the ERA.

All NTO studies were conducted with diet-incorporation methodology and the organisms were fed *ad libitum*. Studies followed established regulatory guidelines or published methods from the authors' laboratory. Details for each test method are provided in Tables 2 and 3 and Supplementary Appendix A. Dietary exposures were initiated with the earliest life stages amenable to laboratory testing and consideration was given to selecting the life stage(s) with direct exposure to the PIP where applicable. The duration of each study was selected to exceed the known time to kill for DvSnf7 RNA to CRW (~5 days) as well as allow for the evaluation of ecologically relevant endpoints beyond mortality to adequately assess the potential for off-target effects. Where appropriate, positive control treatments were included to

Table 2
Non-target arthropod study design for DvSnf7_968 RNA laboratory studies.

Non-target organism	Guideline or method	Concentration of DvSnf7_968 RNA	Duration (days)	Environmental conditions	Sample size	Life stage at initiation
A. mellifera larvae	Tan et al., 2015	1000 ng/g diet; 11.3 ng/larvae	17[a]	Dosing: 24 °C; RH[b] 66%; Larval development: Ambient hive conditions; Adult emergence: 28 ± 2 °C; RH 60 ± 13%; 0L:24D	20 larvae × 4 replicates	2-3 day old larvae
A. mellifera adult	Tan et al., 2015	1000 ng/g diet	14	29 ± 1 °C; RH 50 ± 6%; 0L:24D	20 bees × 4 replicates	≤2-day old adults
C. maculata	Bachman et al., 2013	1000 ng/g diet	16–18	27 °C; RH 70%; 14L:10D	20 larvae × 3 replicates	≤32-h old larvae
P. chalcites	Bachman et al., 2013 and Duan et al., 2005	1000 ng/g diet	35	27 °C; RH 70%; 14L:10D	20 larvae × 3 replicates	≤24-h old larvae
A. bilineata	Grimm et al., 2000	1000 ng/g diet	70[c]	20±1 °C; RH 65 ± 10%; 16L:8D; 800–900 lux	20 (10 ♀ and 10 ♂) × 4 replicates	3-7 day old adults
C. carnea	Vogt et al., 2000	1001 ng/g diet	16–18	25±2 °C; RH 65 ± 16%; 16L:8D; 3600–4800 lux	20 (10 ♀ and 10 ♂) × 4 replicates	≤24-h old adults
P. foveolatus	Bachman et al., 2013	1000 ng/g diet	20	25 ± 5 °C; RH 70 ± 10%; 16L:8D	10 wasps × 4 replicates	24-hr old adults
O. insidiosus	Tan et al., 2011	1000 ng/g diet	10	25 ± 5 °C; RH 70 ± 10%; 16L:8D	40 nymphs/treatment	5-day old nymphs
E. andrei	OECD 207	5000 μg/kg soil	14	20 ± 1 °C; 24L:0D; 525–750 lux	10 worms × 4 replicates	~5-months old
F. candida	OECD 232	1000 ng/g diet	28	20 ± 2 °C; RH 71± 5%; 16L:8D; 470–540 lux	10 springtails × 4 replicates	9-10 day old juveniles

[a] Single exposure on Day 0.
[b] RH = relative humidity.
[c] 28day continuous dietary exposure followed by 42 day observation for emergence of F_1 generation.

Table 3
Beneficial soil microbe and non-target vertebrate study design or DvSnf7_968 RNA and/or tissue derived from MON 87411.

Non-target organism	Guideline or method	Concentration of DvSnf7_968 RNA	Duration (days)	Environmental conditions	Sample size	Life stage at initiation
Carbon Transformation	OECD 217	MON 87411 root & shoot tissue	28	22 ± 3 °C	5 replicate samples	
Nitrogen Transformation	OECD 216	MON 87411 root & shoot tissue	28	22 ± 3 °C	3 replicate samples	
C. virginianus	OPPTS 850.2200 U.S.EPA, 1996	1000 μg/kg diet	14	Days 0–6: 37.3 ± 4.0 °C; Days 7–14: 30.1 ± 1.2 °C; RH[a] 32± 12%; 16L:8D; 400 lux	5 quail × 6 replicates	14 day old
G. domesticus	Taylor et al., 2005	~57% MON 87411 grain	~42	Days 0–4: 24L:0D; 1.0–1.3 fc[b] Days 5–10: 10L:14D; 1.0–1.3 fc Days 11–18: 12L:12:D; 0.2–0.3 fc Days 19+: 16L:8D; 0.2–0.3 fc	100 birds/treatment (10 birds per pen × 5 pens ♀ and 5 pens ♂)	Approximately 1 day old chicks
I. punctatus	OECD 215; Hammond et al., 1996	30% MON 87411 grain	8 weeks	30 ± 2 °C; DO[c] 5.00–6.41 mg/L; 14L:10D; Flow 750–1667 mL/min.	20 catfish × 5 replicates	11 months old; mean wt 5.1–5.5 g

[a] RH = relative humidity.
[b] fc = footcandles.
[c] DO = Dissolved Oxygen.

Table 4
DvSnf7 RNA levels in selected maize tissues used to determine maximum expected environmental concentrations (MEEC) from MON 87411. The highest values in the range were used to determine MEECs.

Tissue type[a]	Developmental stage[b]	Mean (SD) Range μg/g
Pollen (fwt[c])	VT-R1	0.103×10^{-3} (0.069×10^{-3}) 0.056×10^{-3} - 0.224×10^{-3}
Leaf (fwt[c])	V14-R1	14.4×10^{-3} (6.71×10^{-3}) 5.40×10^{-3} - 33.8×10^{-3}
Root (fwt[c])	V3-V4	3.15×10^{-3} (1.79×10^{-3}) 1.74×10^{-3} – 8.00×10^{-3}
Whole Plant (dwt[d])	V6-V8	55.1×10^{-3} (23.1×10^{-3}) 33.0×10^{-3} – 106×10^{-3}
Grain (dwt[d])	R6	0..104×10^{-3} (0.033×10^{-3}) 0.056×10^{-3} - 0.175×10^{-3}

[a] For multiple over season tissue types (e.g. leaf) the tissue stage with the highest maximum expression is reported.
[b] The crop development stages at which each tissue was collected. The growth stages were described by Ritchie et al. (1997).
[c] The DvSnf7 RNA levels are calculated as microgram (μg) of DvSnf7 RNA per gram (g) of tissue on a fresh weight (fwt) basis. The sample means, SDs, and ranges (minimum and maximum values) were calculated for each tissue type across all 5 sites (n = 20), except for pollen n = 5 due to expressions from two pollen samples < LOD and from the rest of the samples for < LOQ).
[d] The DvSnf7 RNA levels are calculated as μg of DvSnf7 RNA per gram of tissue on a dry weight (dwt) basis. The sample means, SDs, and ranges (minimum and maximum values) were calculated for each tissue type across all 5 sites (n = 19).

demonstrate the effectiveness of the test system to detect an adverse effect as recommended by Romeis et al. (2011).

2.2.2. Test material

All terrestrial invertebrate NTO studies and the quail study were conducted using *in vitro* produced DvSnf7 RNA, referred to as DvSnf7_968 RNA that was prepared as described in Urquhart et al. (2015). *In vitro* synthesized DvSnf7_968 RNA was shown to be functionally equivalent to the DvSnf7 RNA produced *in planta* (Urquhart et al., 2015). This is critical information to support the risk assessment because it demonstrates that the DvSnf7 material used in testing was equipotent to DvSnf7 that non-target taxa would potentially be exposed to in the field. Soil microorganism testing was conducted using MON 87411 root and shoot tissue incorporated into a sandy loam soil and the catfish and broiler chicken studies were conducted using MON 87411 grain. With the exception of the earthworm study, all studies utilizing the *in vitro* produced test substance included a diet analysis. Diet analyses were performed using a sensitive insect (*Diabrotica undecimpunctata howardi*; Southern corn rootworm, SCR) to measure biological activity and/or concentration or a DvSnf7-specific Quantigene® assay to measure DvSnf7_968 RNA levels along with an insect bioassay to assess biological activity. Additionally, where appropriate based upon the diet matrix, the homogeneity of the test material and stability over the period of storage was also evaluated. A dose confirmation was not appropriate for the earthworm study due to the rapid degradation of RNA in the soil matrix (Dubelman et al., 2014; Fischer et al., 2016a,b).

2.2.3. Estimation of maximum expected environmental concentration

DvSnf7 RNA expression values from MON 87411 across a range of tissue types were used to determine the maximum expected environmental concentration (MEEC) for dietary or soil concentrations. Diet concentrations for specific studies were based on the highest expressing tissue type that the NTO would most likely be directly or indirectly exposed to in the maize agroecosystem and included pollen, leaf, senescent root and grain (Table 4) and concentrations were selected that represented a worst-case scenario exposure of greater than 10-times the MEEC (U.S. EPA, 2010a). DvSnf7 RNA expression levels were quantified using a validated QuantiGene® Plex 2.0 (Affymetrix Inc.) assay (Armstrong et al., 2013). Tissue samples were collected from MON 87411 plants produced at five sites during 2011–2012. The DvSnf7 RNA level in each tissue type was calculated on a microgram (μg) per gram (g) of fresh weight tissue (fwt) or dry weight tissue (dwt) basis.

Many of the invertebrate NTOs that were tested primarily feed upon pollen in the agroecosystem; therefore the maximum DvSnf7 RNA expression in pollen was used for the MEEC with honey bees, wasps, the ladybird beetle and the insidious flower bug. For predatory insects and insectivorous birds that consume herbivorous prey and have an indirect exposure to maize expressed DvSnf7 RNA, the maximum expression value from the leaf development stage with the highest expression (V14-R1) was used to represent worst-case scenario to calculate the margin of exposure (MOE). For other wild vertebrates, the most likely route of exposure to the DvSnf7 RNA is from grain produced by MON 87411 within the agroecosystem. The most ecologically relevant route of exposure for soil-dwelling organisms, such earthworms and Collembola, was considered primarily to be from root tissue with some addition of late season plant tissue that enters the soil environment. Of these tissue types the highest expressing tissue (root V3-V4) was used as a worst-case exposure scenario for these taxa. For the C: N transformation studies, lyophilized MON 87411 shoot and root tissues (V7) were incorporated into soil at 20 mg dwt tissue/g dwt soil. This concentration was used as a worst-case scenario and assumed the biomass of 1-acre of maize containing 25,000 plants at 650 g dry wt/plant (Sims and Holden, 1996) was incorporated into the top 6 inches of soil. Additionally, the use of lyophilized tissues provided a higher concentration of DvSnf7 for the respective tissue used in the ERA, therefore the maximum dry weight expression in V7 plants was used as the MEEC for soil microorganisms. Based upon knowledge of agronomics of maize, and that the amount of root or shoot tissue would be less than that for total plant tissue, it was concluded that this soil concentration would be in excess of the root and shoot tissue concentration occurring under normal cultivation of MON 87411.

2.3. In silico analysis

To provide additional data to evaluate the laboratory studies, bioinformatics analyses was conducted to evaluate whether non-target species have sufficient genomic match to the DvSnf7 sequence that would render them potentially susceptible to MON 87411 maize (Supplementary Appendix B). Twenty-three NTOs were selected based upon the following criteria: plausible exposure to MON 87411 maize, availability of public transcriptomes, and potential susceptibility based on current knowledge from laboratory bioassays (Supplementary Appendix B). The evaluation was conducted using STELLAR software (version 1.3, July 2012) and compared the DvSnf7 sequence with transcript (22 organisms) or EST (1 organism) sequences from the 23 organisms. The STELLAR searches were conducted to identify exact 21 or greater nucleotide (nt) matches between the DvSnf7 query and sequences contained in transcript or EST collections. The species selected included vertebrate (birds, fish and mammals) and invertebrate species (arthropods, insects, worms and crustaceans). Although bioinformatics were evaluated for several vertebrate species, direct feeding of dsRNA to induce RNAi has not been successful in vertebrates without the use of encapsulation to prevent degradation, or addition of chemical stabilization and penetration enhancers such as transfection agents (Petrick et al., 2013; Sifuentes-Romero et al., 2011; Ubuka et al., 2012). These species were included as part of the

Table 5
No significant ($p > 0.05$) adverse effects of DvSnf7_968 RNA in diet bioassays against a battery of non target arthropods demonstrates negligible risk to these taxa from exposure to MON 87411 maize.

Non-target organism	Endpoint	DvSnf7_968 RNA treatment	Assay control	Positive control	Statistical test	Analytical confimation[b]
A. mellifera adult	Mean Survival (%)	92.5	91.3	0[a]	T-test	SCR bioassay
A. mellifera larvae	Mean Survival (%)	100	100	0[a]	N/A	SCR bioassay
	Mean Capped Brood (%)	100	100	0[a]	N/A	
	Mean Time to 50% Adult Emergence (Days ± SE)	15.5 ± 0.3	15.6 ± 0.4	N/A	T-test	
C. maculata	Mean Survival (%)	91.7	90.0	16.7[a]	T-test	SCR bioassay
	Mean Development Time to Adult (Days ± SE)	14.9 ± 0.23	15.1 ± 0.32	N/A	T-test	
	Mean Adult Biomass (mg)	10.2 ± 0.19	10.2 ± 0.08	N/A	T-test	
P. chalcites	Mean Survival (%)	93.3	91.7	65[a]	T-test	SCR bioassay
	Mean Adult Emergence (%)	70.0	75.0	N/A	T-test	
	Mean Development Time to Adult (Days ± SE)	32.9 ± 0.38	32.9 ± 0.11	N/A	T-test	
	Mean Adult Biomass (mg)	31.9 ± 1.02	32.3 ± 0.99	N/A	T-test	
A. bilineata	Mean Survival (%)	88.7	92.5	95.0	Fischer's Exact Test	SCR bioassay and Quantigene
	Mean Number of F_1 Progeny per replicate	1028.0	991.8	39.0[a]	Dunnett's *t*-test	
C. carnea	Mean Survival (%)	93.3	81.7	70.0	Fisher's Exact test	SCR bioassay and Quantigene
	Mean Number of Viable eggs/female/day	20.3	18.2	1.0[a]	Dunnett's *t*-test	
P. foveolatus	Mean Survival (%)	100.0	100.0	0[a]	N/A	SCR bioassay
O. insidiosus	Mean Survival (%)	93.0	93.0	0.0[a]	T-test	SCR bioassay
	Mean Adult Emergence (%)	98.0	95.0	13.0[a]	T-test	
	Mean Development Time to Adult (Days ± SE)	10.9 ± 0.13	11.1 ± 0.15	10.6 ± 0.40	T-test	

[a] Significant difference from assay control at $\alpha = 0.05$.
[b] Confirmation of biological activity, concentration, stability and/or homogeneity of DvSnf7_968 in Diet.

assessment to provide a comprehensive approach to expand the range of NTOs that were evaluated.

3. Results

3.1. Effects testing

For all species tested, no statistically significant adverse effects from ingestion of or exposure to DvSnf7_968 RNA were detected when compared to the control for any of the measured endpoints (Table 5, Table 6, Table 7, and Supplementary Appendix C). It is important to recognize that all of the NTO studies, with one exception (wasp), conducted for MON 87411 assessed sub-lethal endpoints in addition to survival. Additionally, all studies met the prescribed validity or performance criteria for control survival, reproductive performance, and positive control response, and where applicable the stability, homogeneity and nominal concentration of DvSnf7 RNA was confirmed.

For the NTOs, MOEs were calculated based on the ratio of the no observed effect concentrations (NOECs) from the laboratory studies to the MEECs. The NOECs and MOEs determined for each of the species under a worst case exposure scenario are summarized in Table 8. Included in Table 8 is the no observed adverse effects level of 100 mg/kg as described in U.S. EPA (2015) and the calculated MOE for the 28-day repeat dose oral toxicity study with *M. musculus*. As no long-term adverse effects were observed in the C: N transformation studies with MON 87411 tissue, as well as the chicken and catfish feeding studies with MON 87411 grain at maximum incorporation rates, the MOEs for these organisms were considered to be ≥ 1.

3.2. In silico assessment

A comprehensive *in silico* evaluation with available genomes

Table 6
No significant ($p > 0.05$) adverse effects of DvSnf7_968 RNA or MON 87411 on non-target soil biota demonstrates negligible risk to these taxa from exposure to MON 87411 maize.

Non-target organism	Endpoint	DvSnf7_968 or MON 87411 treatment	Assay control	Positive control	Statistical test	Analytical confirmation [b]
F. candida	Mean Survival (%)	100.0	97.0	7.0[a]	Fisher's Exact test	SCR bioassay[c]
	Mean Number of Progeny	167	169	0.3[a]	Dunnett's T-test	
E. andrei	Mean Survival (%)	100.0	100.0	LC_{50} within reference range	N/A	No
	Mean Change in Biomass (% fwt)	8.4 ± 1.4 decrease	9.4 ± 2.4 decrease	N/A	T-test	
Carbon Transformation	CO_2 Production (% dev from control)	≤25%			≤25% dev from control	N/A
Nitrogen Transformation	NO_3–N Production (% dev from control)	≤25%			≤25% dev from control	N/A

[a] Significant difference from assay control at $\alpha = 0.05$.
[b] Confirmation of biological activity, concentration, stability and/or Homogeneity of DvSnf7_968 in Diet.
[c] Conducted as method development external to the definitive study.

Table 7
No significant ($p > 0.05$) adverse effects of DvSnf7_968 RNA or MON 87411 on non-target vertebrates demonstrates negligible risk to these taxa from exposure to MON 87411 maize.

Non-target organism	Endpoint	DvSnf7_968 or MON 87411 treatment	Assay control	Positive control	Statistical test	Analytical confirmation [a]
Colinus virginianus	Mean Survival (%)	100	100	N/A	N/A	SCR bioassay and Quantigene
	Mean Weight (g)	74.0 ± 9.0	75.0 ± 7.0	N/A	T-test	
	Mean Weight change (g)	43.0 ± 7.0	43.0 ± 6.0	N/A	T-Test	
G. domesticus	Mean Survival (%)	97.0	96.0	N/A	Fischer's Exact Test	Event specific PCR to verify identity of test substance and absence of test substance in control
	Mean Weight (g/bird ± SEM)	3004 ± 36.8	3011± 15.0[b]	N/A	ANOVA	
	Mean Weight Gain (g/bird ±SEM)	2963 ± 36.9	2970± 15.1[b]	N/A	ANOVA	
I. punctatus	Mean Survival (%)	100	100	N/A	N/A	N/A
	Mean Diet consumed (g/fish± SD)	30.6 ± 1.4	29.0 ± 1.8	N/A	ANOVA	
	Mean Weight Gain (g/fish± SD)	14.0 ± 2.2	14.1 ± 1.3	N/A	ANOVA	
	Diet conversion ratio (±SD)	2.3 ± 0.4	2.1 ± 0.1	N/A	ANOVA	

[a] Confirmation of biological activity, concentration, stability and/or homogeneity of DvSnf7_968 in Diet.
[b] Control and reference diets pooled.

and transcriptomes did not identify any ≥21 nt contiguous matches for the 23 species (Supplementary Appendix B). Therefore, no adverse effects of DvSnf7 RNA against these species are predicted. As mentioned above, honey bee adult and larvae were evaluated in dietary bioassays with DvSnf7_968 RNA and no adverse effects were observed (Tan et al., 2015). The results of that bioinformatics analysis confirm the results of the toxicity testing and provide an additional line of evidence to explain why no adverse effects were detected with larval and adult honey bees. Likewise, this bioinformatics analysis provides additional evidence for the lack of adverse effects to other NTOs (jewel wasp, *Nasonia vitripennis*) that also were evaluated in previous laboratory studies (Bachman et al., 2013).

4. Discussion

The ERA for MON 87411 has taken into consideration the MOA, the spectrum of insecticidal activity, routes and levels of exposure

Table 8
Maximum expected environmental concentrations (MEECs), no observed effect concentrations (NOECs) from non-target organism (NTO) studies and estimated margins of exposure (MOEs).

NTO	MEEC [a]	NOEC [b]	MOE [c]
A. mellifera larvae	0.000448 ng[d]	≥11.3 ng/larvae [e]	≥25,223
A. mellifera adult	0.224 ng/g fwt pollen	≥1000 ng/g	≥4464
C. maculata	0.224 ng/g fwt pollen	≥1000 ng/g	≥4464
P. chalcites	33.8 ng/g fwt leaf [f]	≥1000 ng/g	≥29
A. bilineata	33.8 ng/g fwt leaf [f]	≥1000 ng/g	≥29
C. carnea	33.8 ng/g fwt leaf [f]	≥1001 ng/g	≥29
P. foveolatus	0.224 ng/g fwt pollen	≥1000 ng/g	≥4464
O. insidiosus	0.224 ng/g fwt pollen	≥1000 ng/g	≥4464
E. andrei	8.0 ng/g fwt root [g]	≥5000 μg/kg dry soil	≥625
F. candida	8.0 ng/g fwt root [g]	≥1000 ng/g dry soil	≥125
Soil microorganisms (C:N Transformation)	106 ng/g dwt plant[h]	≥106 ng/g dwt plant	≥1
C. virginianus	33.8 ng/g fwt leaf [f]	≥1000 μg/kg [i]	≥29
G. domesticus	0.175 ng/g dwt grain	≥0.175 ng/g dwt grain	≥1
M. musculus	0.045 mg/kg/day[j]	≥100 mg/kg/day[k]	>2958
I. punctatus	0.175 ng/g dwt grain	≥0.175 ng/g dwt grain	≥1

[a] Maximum expression levels determined from MON 87411.
[b] NOECs reflect nominal test concentrations.
[c] MOE values were calculated based on the ratio of the NOEC to MEEC. The MOE was determined based on the maximum expression level of the DvSnf7 RNA in the tissue from MON 87411deemed most relevant to the NTO exposure.
[d] MEEC based upon mean quantity of DvSnf7 RNA expressed in 2 mg of MON 87411pollen (fwt). The average consumption of pollen by honey bee larvae is 2 mg during development (Babendreier et al., 2004). The MEEC was calculated as follows: (2 mg pollen × (0.224 ng DvSnf7 RNA/1000 mg pollen)).
[e] The NOEC represents a single dose of 10 μl of 1000 ng/g solution added to each larval cell. The total mass added and consumed in each larval cell was 11.3 ng DvSnf7/cell. The concentration of 1000 ng/g DvSnf7_968 RNA in the diet solution is calculated based on the density of the 30% sucrose/water (w/v) solution of 1.127 g/ml.
[f] The maximum expression value from the leaf development stage with the highest expression (V14-R1) was used to represent worst-case-scenario for a predator consuming a herbivorous prey.
[g] The maximum expression value from the root development stage with the highest expression (V3-V4) was used to represent worst-case-scenario for a soil dwelling invertebrates.
[h] For the C:N transformation studies, lyophilized MON 87411 80% shoot and 20% root tissues (V7) were incorporated into soil at 20 mg dwt tissue/g dwt soil. The highest expressing whole plant tissue dwt was used for the MEEC as this value exceeded all root expression values.
[i] The NOEC of ≥1000 μg/kg diet is equivalent to 190 μg DvSnf7 RNA/kg/day.
[j] The MEEC for *M. musculus* is based on a daily dietary dose (DDD). The DDD = Food Intake Rate (FIR)/body weight × dietary concentration, and was calculated for the grass eating herbivorous mammal with the highest FIR (1.33), the common vole that consumes 100% maize shoots. The highest leaf expression highest expression (V14-R1) was used to represent worst-case-scenario. Therefore, (1.33 × 0.0338 mg/kg fwt = 0.045 mg DvSnf7 RNA g body weight or mg/kg/body weight) following EFSA, 2009 and Crocker et al., 1998).
[k] U.S. EPA, 2015 and Petrick et al., 2016.

levels to DvSnf7 RNA produced by MON 87411 and the results from a taxonomically and functionally diverse group of NTO studies. NTO studies followed established methods and the tiered testing framework developed by the U.S. EPA to assess the environmental safety of PIPs. These studies evaluated ecologically relevant apical endpoints (survival, growth, development, and reproduction) to assess potential impacts to NTO populations and communities. Tier 1 NTO studies for PIPs are generally initiated with neonates, because they are typically thought to be the most sensitive life-stage, and the assays were run for a sufficient duration to evaluate developmental milestones (i.e. development to adult and/or reproduction). By evaluating a significant portion of the life cycle under conservative high dose exposure scenarios, it can be concluded with reasonable certainty that there is low likelihood of potential chronic adverse off-target effects at realistic field exposure levels. The Tier 1 studies for this ERA were conducted with concentrations (single limit dose) that far exceeded anticipated exposure of DvSnf7 RNA to maximize the potential for observing and documenting off-target effects. A limit dose is a treatment level that provides a high "worst-case" exposure level (i.e., 10 × anticipated field exposure level) and a large margin of exposure. Importantly, a lack of adverse effects in high dose testing has traditionally provided EPA with sufficient confidence to address uncertainties, conclude that there is no unacceptable risk to the environment, and conclude that no further data are required.

In an ecological assessment for PIPs, MOEs that are ≥10 are indicative of minimal risk in worst-case sub-chronic and chronic laboratory assays (U.S. EPA, 2010a). All of the MOEs calculated for the NTO species in this ERA were >10-times a high end predicted exposure level (Table 8). Of particular importance is the lack of adverse effects from exposure to DvSnf7 RNA in both adult and larval honey bees (*A. mellifera*). These results are consistent with (Velez et al., 2015), which found no adverse effects of adult or larvae honey bees fed high concentrations of a dsRNA with 100% sequence match to the honey bee. Additionally, no long-term adverse effects were observed on microbially-mediated soil nutrient cycling with MON 87411 tissues incorporated into soil at levels that exceed expected environmental concentrations. In vertebrate feeding studies at concentrations that approximate realistic field concentrations and at worst-case exposures, no adverse effects of MON 87411 or the DvSnf7 RNA were observed. In addition to the data reported herein, a 28-day mouse (*Mus musculus*) repeat dose oral gavage study with the DvSnf7 RNA at 100 mg/kg/day was performed and no adverse effects attributable to the DvSnf7 were observed (U.S. EPA, 2015; Petrick et al., 2016). An MOE for the mouse as a representative wild mammalian species can be calculated assuming a worst−case scenario for a herbivorous mammal consuming maize shoots (e.g. the common vole, *Microtus arvalis)* at a level of 133% of its body weight each day (Table 8) (Crocker et al., 1998; EFSA, 2009). This food intake rate of 1.33 exceeds a worst-case food intake rate corrected for body weight for an insectivorous mammal. In addition, insects would likely not accumulate DvSnf7 RNA to higher levels than what is expressed *in planta* because it is known that nucleic acids do not bioaccumulate. There is presently no evidence that the DvSnf7 RNA will persist or accumulate to levels higher than *in planta* expression in insects that feed on MON 87411 (Ivashuta et al., 2015). Therefore a worst-case assumption is that the concentration of DvSnf7 RNA in insects will equal that of the maximum expression in fresh weight MON 87411 plant tissue. Under these assumptions, given the NOEC for mice of 100 mg/kg/day and a maximum expression in leaf tissue of 33.8 ng/g fwt, the MOE for a herbivorous mammal is ≥ 2958 (Table 8).

Therefore, as with the previously assessed Cry3Bb1 protein, DvSnf7 RNA is not likely to produce adverse effects on terrestrial beneficial invertebrate and vertebrate species at field exposure levels. This conclusion is in agreement with prior published literature which reported that DvSnf7 activity is restricted to the Galerucinae subfamily within the Chrysomelidae family in the Order Coleoptera (Bachman et al., 2013). Further confirmation of results from laboratory studies were provided in a field study by Ahmad et al. (2015), where no adverse effects from MON 87411 maize were observed to non-target arthropod communities.

Recently, consideration has been given to whether the existing ERA framework is applicable to GE crops expressing RNA-based traits, especially insecticidal traits (Auer and Frederick, 2009; Lundgren and Duan, 2013; Scott et al., 2013). In their recent review of the risk assessment approach for GE plants containing RNA-based traits, Lundgren and Duan (2013) postulated that unintended off-target effects of insecticidal RNAs against NTOs could be widespread. This assertion was largely based upon data from pharmaceutical-specific publications that examined the effects of high concentrations of dsRNA in *in vitro* cell monolayers (Jackson and Linsley, 2010) and is not directly applicable to levels for the ecological assessment of MON 87411. Although off-target effects have been reported in *in vitro* systems in the pharmaceutical literature at high concentrations, these studies are not relevant to exposure scenarios for NTOs in agroecosystems. Only realistic routes and levels of exposure for NTOs, to a trait such as DvSnf7 RNA in MON 87411 maize, should be considered in the risk assessment (Fig. 1). Therefore, *in vitro* studies with RNA are not predictive of potential impacts to NTOs following dietary exposures due to much lower exposures in the environment and the absence of significant uptake afforded by use of transfection reagents in cultured cells. Additionally, pools of small RNAs, as would arise from dicing of a long dsRNA tend to eliminate off-target effects due to a dilution effect of a complex siRNA pool (Hannus et al., 2014). When off-target effects have been observed, gene suppression has been shown to be orders of magnitude less potent than that observed with small RNAs having full complementarity (Vaishnaw et al., 2010).

Lundgren and Duan (2013) also identified other reputed risks to NTOs based on the pharmaceutical literature such as immune stimulation and over-saturation of the RNAi machinery. The off-target effects observed in *in vivo* studies from the pharmaceutical literature result from exposure to large amounts of chemically stabilized dsRNA delivered specialized formulations via injection into the organism (Petrick et al., 2013). Therefore, these papers need to be interpreted with caution particularly in the context of low exposure scenarios to DvSnf7 RNA expressed by MON 87411. Under *in vitro* conditions, RNAi machinery saturation was shown to occur in a dose-dependent manner after transfection of relatively high doses of small RNAs into cells (Khan et al., 2009). This exposure condition in cell lines has limited or no relevance to risk an ERA for a PIP (Table 1). There are no reports to date suggesting that interferon or inflammatory responses occur following oral exposure (Petrick et al., 2013). Similar to humans and livestock, the diets of NTOs consist of plant or animal sources which naturally contain dsRNAs and there exists a long history of safe consumption of these endogenous dsRNA across eukaryotes. This has been illustrated specifically for grain from food and feed crops such as soybean, corn, and rice (Heisel et al., 2008; Ivashuta et al., 2009; Jensen et al., 2013), and as the result of viral infection in crops such as kidney bean, pepper, and barley (Fukuhara et al., 2006). With constant oral exposure to environmental dsRNA endogenously present in natural food sources, unintended effects in non-target organisms from immune stimulation and RNA machinery saturation are extremely unlikely to result from relatively low exposures to dsRNA resulting from cultivation of MON 87411.

Contrary to concerns regarding non-specific off-target effects, numerous studies have demonstrated that RNAi technology can

achieve sequence-specific gene silencing in some insects by feeding dsRNAs (Bachman et al., 2013; Baum et al., 2007; Whyard et al., 2009). Therefore, RNAi PIPs have the potential to selectively target economically important pest species and greatly reduce the likelihood of adverse effects on non-target organisms, including those beneficial to agriculture. The DvSnf7 RNA sequence in MON 87411 was carefully selected for its high degree of divergence between species to mitigate potential adverse effects on organism not closely related to the target pest species, WCR. This sequence has been shown to diverge rapidly within the subfamily level Galerucinae (Bachman et al., 2013), therefore, activity outside this subfamily is not predicted. The purposeful selection of the DvSnf7 sequence to reduce non-target effects is in alignment with recommendations from the 2014 Scientific Advisory Panel (SAP) on RNAi that recognized that targeting genes with a high degree of divergence will help "hone the specificity of RNAi to the target pest" (U.S. EPA, 2014). The SAP recommended that dsRNA sequences should be chosen that target a region of gene with no shared 21 nt sequences with other species (U.S. EPA, 2014). These recommendations are in alignment with previous studies by Baum et al. (2007), Whyard et al. (2009), and Bachman et al. (2013) that demonstrate how the insecticidal activity of ingested dsRNAs is directly related to the degree of sequence match to the target gene between species. Whyard et al. (2009) demonstrated that species-specific activity can be achieved in insects with dsRNAs that diverge at the species level. Bachman et al. (2013) demonstrated that for ingested dsRNAs, contiguous sequence matches of $\geq$21 nt to the target gene are necessary for biological activity to occur in insects, and that when no significant sequence match existed to the target gene then no adverse effects were observed in NTO testing. Finally, while a potential adverse effect from a dsRNA can be likely excluded when a 21 nt alignment is not present, it should be noted that NTO diets are continuously exposed to RNA that have 21 or greater bioinformatic alignments with the ingesting organism without evidence of a potential for hazard (Frizzi et al., 2014; Ivashuta et al., 2009).

The application of bioinformatics can have an important role in the selection and design of the dsRNAs and in informing the assessment process for NTOs. When bioinformatics data for non-target arthropods are available and indicate that the minimum sequence requirements for RNAi activity are not met, then the need for toxicity testing is diminished and the likelihood of detecting adverse effects is low. However, when the minimum sequence requirements are met, the converse is not true; these data cannot be reliably used to predict the presence of RNAi activity. Nevertheless, bioinformatics can assist with the developing a hypothesis-based taxonomic approach for characterizing the spectrum of activity for pest control, understanding the relationship between taxonomic relatedness and activity, and aid in the selection of test species for NTO testing (Bachman et al., 2013). This approach is in alignment with recommendations from RNAi –focused Scientific Advisory Panel (SAP) held in 2014 (U.S. EPA, 2014), which recommended that while a bioinformatics analysis is not an absolute predictor of effects, *in silico* searches of published genomes could be used to perform a screening level assessment to identify potential NTOs for further evaluation based upon the presence of sequence matches. While there are currently only a limited number of published complete and partial arthropod genomes publically available, additional genomes are being published at a rapid rate and are likely to become increasingly useful as a screening level tool for ERAs.

The confirmatory *in silico* analysis of NTO transcriptomes performed as part of this ERA support the findings of the laboratory bioassays, widen the scope of the NTO assessment, and provide further support to the conclusions of no adverse effects to NTOs from exposure to DvSnf7 RNA and cultivation of MON 87411 maize.

4.1. Further considerations for the ERA; exposure limitations of insecticidal RNA PIPs

In addition to sequence specificity, physical and biochemical barriers to the oral toxicity of dsRNAs exist in arthropods and other non-target taxa. As identified by the recent SAP on RNAi (U.S. EPA, 2014) these barriers vary across taxa and for insects include feeding behavior and diet, potential degradation of the dsRNA prior to ingestion, and the inherent sensitivity of the insect to ingested dsRNA based upon conservation and function of components of the RNAi machinery (Whyard et al., 2009).

4.1.1. Exposure/uptake

For a transgenic plant expressing an insecticidal trait, ingestion of the RNA via plant material is the most likely route of exposure. Induction of RNAi-mediated gene suppression in insects via an oral route of exposure requires efficient uptake of dsRNAs by midgut cells followed by suppression of the target mRNA leading to significant effects on growth, development and survival. In plants, nematodes and some basal arthropods (e.g. Acari), exogenous dsRNAs that enter the cell can be amplified via RNA-dependent RNA polymerases (RdRPs) to produce endogenous dsRNAs that supplement the RNAi pathway and prolong the RNAi effect (Grbic et al., 2011; Miller et al., 2012). However, in it important to note that insects, crustaceans and mammals have been shown to lack RdRPs (Grbic et al., 2011; Miller et al., 2012) and the ability of WCR and *Tribolium castaneum* to produce dose-dependent responses with RNAi is consistent with the absence of an endogenous amplification mechanism (Bolognesi et al., 2012; Miller et al., 2012). The lack of an endogenous amplification mechanism in insects suggests that exposure to dsRNA in higher trophic levels, via ingested prey species, will be limited because a mechanism for bioamplification is not evident. Other factors can also influence the efficiency of RNAi in insects, including concentration, potency and efficacy against the target, sequence and length, persistence of gene silencing and the insect life-stage (Baum et al., 2007; Huvenne and Smagghe, 2010; Whyard et al., 2009). In general, long dsRNAs that incorporate a high degree of sequence match to mRNAs in the target insect have greater potential for efficacy as a result of the number of siRNAs that can be produced from the sequence of each long dsRNA (Baum et al., 2007; Miller et al., 2012). Another mechanism that can affect RNAi efficiency in insects, and potentially limit environmental exposure, is the length of the dsRNA. Bolognesi et al. (2012) and Miller et al. (2012) demonstrated that a dsRNA must be of sufficient length (e.g. $\geq$ 60 bp) to result in efficacy against WCR and *T. castaneum*, respectively. Additionally, Bolognesi et al. (2012) demonstrated that a single 21 nt contiguous sequence match in a large carrier molecule was sufficient to induce biological activity in the southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi*). Further, as demonstrated in Miller et al. (2012), the potency of a dsRNAs is positively related to the number of potential 21 nt matches contained in the sequence and therefore the number of 21 nt matches should be considered as part of the relevant environmental exposure necessary for biological activity under realistic exposure scenarios for NTOs in the agroecosystem.

4.1.2. Barriers

Physical and biochemical barriers to the oral toxicity of dsRNAs also exist in many arthropod taxa. These include potential degradation of the dsRNA prior to ingestion as well as the inherent sensitivity of the organism to ingested dsRNA (Whyard et al., 2009). For example, recent studies on the tarnished plant bug (*Lygus*

lineolaris, Hemiptera) demonstrated that endonucleases present in saliva rapidly degrade dsRNA creating a barrier to an RNAi effect in this species by oral delivery of dsRNA (Allen and Walker, 2012). In addition, as summarized in recent reviews (Baum and Roberts, 2014; Huvenne and Smagghe, 2010), insects display a wide range of sensitivities to ingested dsRNA, with the order Coleoptera demonstrating significantly greater sensitivity than other insect orders. For example, the order Lepidoptera has demonstrated variable sensitivity to ingested dsRNA and high concentrations are required to elicit a response in this order relative to coleopterans (Huvenne and Smagghe, 2010; Terenius et al., 2011). Additionally, rapid degradation of dsRNA in the hemolymph of *Manduca sexta* has been reported and attributed to nuclease activity, indicating that sensitivity to RNAi may be influenced by the instability of dsRNA within the insect (Garbutt et al., 2013). Successful induction of RNAi in aquatic invertebrates (shrimp, e.g. *Penaeus monodon*) via ingestion has been achieved, however all reported successful cases involved stabilization of the dsRNA in the diet either via of nanoparticle encapsulation or feed coated with bacteria expressing the dsRNA (Sarathi et al., 2008). Therefore, RNAi in aquatic invertebrates from ingestion of RNA-based PIPs or other unformulated dsRNAs is not expected.

Similar to the above barriers described for arthropods, all vertebrate digestive systems display commonalities in regards to structure and function such as enzymes that aid in digestion. The digestive systems of mammals and other vertebrates such as fish, reptiles and birds contain physical barriers such as the cellular membranes of the gut epithelium in addition to salivary endonucleases, harsh conditions in the stomach, and ribonucleases that hydrolyze nucleic acids in the gut lumen, and even nucleases in the blood (Houck, 1958; Park et al., 2006; Stevens and Hume, 1995). Therefore, the same digestive barriers that prevent oral activity of ingested RNA in insects are also applicable to other vertebrates.

To date, no successful feeding studies with naked (without transfection reagents) dsRNAs to induce an RNAi response have been achieved in vertebrate systems. Using mammal models (i.e. surrogate for non-target wild mammals), systemic delivery of RNA via the oral route has only been successful through the use of encapsulation to prevent degradation, or addition of chemical stabilization and penetration enhancers (Petrick et al., 2013). In avian species, successful RNAi has only been achieved with cell lines and/or embryos and has required the use of electroporation or other invasive techniques (Sifuentes-Romero et al., 2011; Ubuka et al., 2012). Likewise, successful RNAi with fish, amphibians and aquatic reptiles has only been achieved with cell lines and/or embryos and has required the use of transfection agents, direct injection, or other invasive techniques (Schyth, 2008; Sifuentes-Romero et al., 2011). In this ERA we evaluated a worst-case scenario exposure for an insectivorous avian species, *C. virginianus*. As would be predicted from the physiological barriers present in vertebrates and the selective activity of the DvSnf7 RNA, no adverse effects from 14-day of continuous exposure to DvSnf7 RNA were observed. As discussed previously, and consistent with these findings, no adverse effects were observed in a 28-day mouse repeat dose oral gavage study with the DvSnf7_968 RNA or a 42-day broiler chicken feeding study with MON 87411 grain containing the DvSnf7 RNA (U.S. EPA, 2015). Based on low exposure levels, physiological barriers to exposure, the likelihood of adverse effects to non-target terrestrial vertebrates from cultivation of MON 87411 is concluded to be extremely low.

Though aquatic habitats may be located near agricultural areas, the exposure of aquatic organisms to GE crops is limited temporally and spatially and the potential exposure of aquatic organisms is therefore low to negligible (U.S. EPA, 2010a). Additionally, DvSnf7 RNA has been shown to rapidly degrade in both terrestrial (Dubelman et al., 2014; Fischer et al., 2016b) and aquatic systems (Fischer et al., 2016a), further limiting the potential for exposure to aquatic taxa. Due to the aforementioned barriers, the lack of meaningful ecologically-relevant exposure to aquatic organisms from maize, other than through purposeful feeding of processed maize products, and the reported rapid degradation of DvSnf7 RNA in the environment, Tier 1 effects tests on aquatic species were not conducted for MON 87411. An 8-week channel catfish growth study has shown that no adverse effects are expected from feeding of processed maize products to with a diet consisting of 33% MON 87411 grain containing the DvSnf7 RNA (U.S. EPA, 2015).

In these studies, no adverse effects were observed in any NTO tested. Though barriers exist to systemic exposure in vertebrate species, the potential barriers to exposure in each invertebrate NTO was not characterized. Therefore, we cannot know which, if any, of these species (especially invertebrates) are recalcitrant to environmental/oral RNAi and hence cannot determine if the lack of adverse effects was related to the presence of barriers or lack of sequence match. In the absence of barriers, the bioinformatics assessment provided herein lends confidence to a conclusion that should exposure occur, significant sequence match does not exist between the DvSnf7 RNA and NTOs to elicit an adverse effect.

5. Conclusions

No adverse effects on NTOs were observed in a comprehensive battery of laboratory tests evaluating the potential adverse effects of DvSnf7 RNA/MON 87411 maize. These effects data, along with information on relevant exposure levels within the agroecosystem, were assessed with an approach that is consistent with EPA's current testing and assessment framework for genetically engineered plants (*e.g. Bt*-expressing plants). This ERA framework has enabled scientifically sound regulatory decisions with adequate certainty of acceptable risk and within the standards established by FIFRA (i.e., no unreasonable effects to the environment) (U.S. EPA, 2010b). Additionally, a tripartite group (government, industry and academia) evaluated this ERA approach and concluded that the current ERA framework and effects testing requirements for NTOs are applicable to plants engineered to express insecticidal RNA (ILSI-CERA, 2011).

As discussed, a key component of problem formulation is the identification of plausible risk hypotheses and evaluation of relevant routes of exposure through the conceptual model. In the case of MON 87411, based on the expected environmental exposure routes in the maize agroecosystem, the known environmental exposure concentrations, and the natural digestive barriers and physiological differences between NTOs, there is little probability of NTOs encountering DvSnf7 RNA in high enough concentrations to induce off-target effects as observed in the pharmaceutical literature and cautioned by Lundgren and Duan (2013). It is well established that RNAi is a sequence-specific mechanism, and activity is only possible when sufficient uptake and sequence complementarity to the target mRNA exists that leads to mRNA cleavage followed by gene silencing. There must be sufficient exposure to and uptake of the DvSnf7 RNA, sequence match, and sensitivity to RNAi in a given taxa for there to be a potential adverse effect.

Combining the lines of evidence from i) bioassays designed with appropriate duration and relevant endpoints to detect adverse and off-target effects specific to the known MOA of the DvSnf7 dsRNA in the target pest, ii) a spectrum of activity limited to within the Galerucinae, and iii) no adverse effects to NTOs from oral exposure to environmental dsRNA at MOE factors >10, and iv) rapid degradation in the environment, it can be concluded with reasonable certainty that there is low likelihood of MON 87411 maize adversely affecting NTOs at field exposure levels.

MON 87411 is the first commercial RNAi insecticidal PIP. As such, the studies incorporated in this ERA were not only designed to address specific risk hypotheses, but also intended to the lay the foundation for regulatory approvals of a new class of insecticides and provide data that will aid in communicating the environmental safety for an insecticidal RNA. For future RNAi products, consideration should be given to whether representatives of wild birds and mammals that have barriers to systemic exposure to RNA should be tested for an RNA-based product with low environmental exposures. Additionally, as a sequence based mechanism with a high potential for specificity, the selection of dsRNAs to have a narrow spectrum of activity can limit the potential for adverse effects beyond a select and closely related group of insects, thus building a case for the reduction of the number and types of invertebrate NTOs required for testing to make a sound and science-based conclusion on potential ecological risks. This opinion was expressed in the consensus points on the 2011 ILSI-CERA conference on "Problem Formulation for the Environmental Risk Assessment of RNAi Plants" where it was recognized that bioinformatic data coupled with activity spectra evaluations can be used to reduce the scope of NTO testing (ILSI-CERA, 2011).

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.08.001.

Appendix A. Supplementary data

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