RISK PARAMETERS AND ASSESSMENT OF DIETARY dsRNA EXPOSURE IN FOLSOMIA CANDIDA

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RISK PARAMETERS AND ASSESSMENT OF DIETARY dsRNA EXPOSURE IN

*FOLSOMIA CANDIDA*

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THESIS

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

Jeffrey Edward Noland

Lexington, Kentucky

Director: Dr. Xuguo Zhou, Associate Professor of Entomology

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2017

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ABSTRACT OF THESIS

RISK PARAMETERS AND ASSESSMENT OF DIETARY dsRNA EXPOSURE IN

FOLSOMIA CANDIDA

Assessing the risk of transgenic crop products is essential when determining the safety of a crop for deregulation and commercialization. The Organization of Economic and Cooperative Development (OECD), International Standards Organization (ISO), and governmental regulatory agencies require a battery of tests to demonstrate the safety of a GM product against several surrogate species of organisms that perform various ecosystem services. Assays are performed using toxicology methods established for pesticides. These methods have been applied to testing the safety, specificity and fate of Bacillus thuringiensis (Bt) Cry protein toxins engineered into crop plants and information exists on the effects on non-target organisms (NTOs). Toxicology assays are typically evaluated through a tier-based approach, where, if no or negligible risk via oral toxicity or phenotypic changes then a risk decision can be made. Long term exposure studies are often performed after commercial release of the crop occurs and provide a more in depth understanding of environmental impacts. Risk analyses are currently being performed on the product of the next generation of GM crops that express dsRNAs against Western Corn Rootworm. This thesis provides another such study, primarily focused on Folsomia
candida, a microarthropod that is the subject of numerous toxicological studies. I describe the development of dsRNA stability assays, which indicate stability of dsRNA across assay time, both with and without F. candida. When exposed to dsRNA levels several orders of magnitude higher that what would be encountered in the environment, F. candida is not negatively impacted as seen through gene expression and life history trait analysis.

KEYWORDS: Risk Assessment, dsRNA, Folsomia candida, Dietary, Exposure
RISK PARAMETERS AND ASSESSMENT OF DIETARY dsRNA EXPOSURE IN

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This thesis is dedicated to my wife, Holly, my sons, Collin and Connor, my parents, James and Jean, and the many other family members and countless friends who have helped me along the way. If it was not for their constant interaction, encouragement, and positivity, this work could not have been completed.
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Table of Contents

Acknowledgments ........................................................................................................ iii

List of Tables .................................................................................................................. vi

List of Figures ............................................................................................................... vii

Chapter One: Literature Review

Abstract ....................................................................................................................... 1
Introduction
Ecological Risk Assessment ..................................................................................... 2
RNAi-based gene silencing ......................................................................................... 6
Applications
RNA-based gene silencing technology ..................................................................... 8
Technical Challenges
dsRNA design ........................................................................................................ 11
dsRNA uptake ........................................................................................................... 13
dsRNA stability and delivery ................................................................................... 13
in planta RNAi ........................................................................................................... 15
Regulatory
Overview ................................................................................................................... 17
General parameters for assessing risk of GM crops ............................................... 18
Risk assessment of GM crops ................................................................................... 18
Regulatory concerns for RNAi based genetically modified crops ......................... 21
Summary and Perspectives
RNAi-based genetic manipulations ....................................................................... 23
Assessment of RNA-based controls ....................................................................... 25

Chapter Two: Development of dietary RNAi toxicity assay and assessment of oral
toxicity of dsRNAs in Folsomia candida Willem (Collembola: Isotomidae)

Abstract ..................................................................................................................... 31
Introduction ................................................................................................................ 33
Results
dsRNA stability ......................................................................................................... 40
Artificial diet consumption and in vivo dsRNA detection ....................................... 40
Temporal profile of RNAi effects in F. candida ........................................................... 41
Dietary RNAi toxicity assay ..................................................................................... 41
Discussion ..................................................................................................................... 42
Materials and Methods
Insect culture ............................................................................................................. 47
Dietary RNAi toxicity assay ..................................................................................... 47
Diet preparation ......................................................................................................... 47
Potassium arsenate toxicity assay ............................................................................. 48
Chapter Three: Future research: environmental fate and risk of plant incorporated dsRNAs

Abstract ..................................................................................................................65
Introduction .............................................................................................................66
Future Work
   Mode of Action ..................................................................................................68
   Environmental fate of dsRNAs and exposure hazards in the soil community ......68
Possible Studies
   Environmental fate and modeling ....................................................................71
   Non-target soil microarthropod and microbial community interactions ...........71
Significance and Conclusion ...............................................................................74

Literature Cited ......................................................................................................75
Vita ..........................................................................................................................95
List of Tables

Table 1, Risk assessment and regulatory considerations for RNAi-based crops.............29

Table 2, General regulatory concerns, listed as questions from the transgenic event to plant composition when seeking regulatory approval of a GM crop.............................30

Table 3, Primers used for dsRNA synthesis, gene expression and detection ................64
List of Figures

Chapter One

Figure 1, Risk assessment framework in coordinated stages, from initial planning between risk managers and assessors, to forecasting risk associated with all determine general ecological assessment endpoints (GEAEs). Adapted from US EPA 1992, 1998, and 2004.................................................................27
Figure 2, RNAi-based gene silencing .................................................................28

Chapter Two

Figure 3, Diet consumption assay .........................................................................55
Figure 4, dsRNA stability assay in the absence of F. candida ....................................56
Figure 5, dsRNA stability assay in the presence of F. candida ....................................57
Figure 6, dsRNA detection after feeding .................................................................58
Figure 7, v-ATPase A expression in F. candida following dsRNA consumption .......59
Figure 8, Egg production (Fecundity) of F. candida following dsRNA ingestion ......60
Figure 9, Body weight of F. candida following dsRNA ingestion .............................61
Figure 10, Survival rate following dsRNA ingestion .................................................62
Figure 11, Egg viability following dsRNA ingestion .................................................63
Chapter One: Literature Review

RNA interference (RNAi) is a cellular mechanism that processes double-stranded RNA (dsRNA), binds, cuts, unwinds and uses the processed RNA as a guide to target cellular messenger RNA (mRNA) transcripts in a sequence specific manner. This targeting process is responsible for down regulating gene expression through the interaction with small interfering RNAs (siRNAs). mRNAs can be silenced through two canonical pathways, specifically through endogenous nucleolytic degradation (e.g. RNAi pathway), or, via translation inhibition (e.g. MicroRNAs—miRNA). In the last decade, RNAi has broadly been applied in entomological research to decipher the roles of genes and gene networks in insects. Due to this successful application in functional genomics, a shift towards RNAi as a pest control option in agriculture has been given much attention and has shown extensive potential. The manipulation of plant genomes to produce heritable traits by knocking-in genes that increase tolerance or many alter plants more hardy when challenged with biotic stressors, or knocking-out genes in pest insects rendering them sterile, thus limiting population growth, are at the nexus of genetic-based pest management. In this chapter, I briefly summarize current knowledge and protocols for assessing RNAi-based gene silencing, the technical and regulatory hurdles for this next generation of RNA-based pest controls, and I also share insights to altering the current environmental risk assessment framework to better measure RNA-based pest strategies. Given the current discussion and concern of genetically modified organisms (GMOs), I Review the past and current standards of assessing the risk of GM crops and where RNA crops fit into this framework. Also, I, respectfully, weigh the pros and cons of RNAi and
culminate this chapter by discussing regulatory issues that should be addressed before this biotechnology moves from farm to table.
Introduction

Ecological Risk Assessment

Ecological Risk Assessment (ERA) is defined by the United States Environmental Protection Agency (US EPA) as an evaluation process that determines the likelihood of detrimental effects, ecologically, that might occur or do occur in response to one or multiple biotic or abiotic stressors (US EPA, 1992; 1998; 2004). This determination sets forth an assumption that a risk cannot occur unless two general parameters about the stressor occur, which are 1) the stressor having the innate property/ability to incur one or multiple adverse effects, and 2) interacts with a component in an ecological setting, specifically organisms, populations, communities, or the entire ecosystem for a long enough time and intensity that a determined detrimental effect is elicited (US EPA, 1992). The framework set forth by the US EPA will be focus of this section, as this body of work follows the ERA guidance.

Defining ERA can be useful in determining problems to the environment, establish priority to assess risks, and contribute a base, scientific understanding for possible or needed regulatory actions. ERAs can also be predictive, in that, scientists and appropriate regulatory bodies can forecast risks of stressors not currently in a given ecosystem, as well as establish risks of existing stressors (in minute amounts, deemed to not be a risk) and the rate at which existing stressors might become detrimental (US EPA 1992, 1998). The US EPA has conceptualized a framework that was adapted from Human Health Risk Assessment guidelines that were originally developed by the Natural Resource Council (NRC), and have been adapted throughout the years. While comprehensive descriptions of these guidelines exist through the Risk Assessment Forum
of the US EPA, a general diagram is provided, here, with brief descriptions to help organize the framework for ERA. The focus, for the majority of the following sections regarding the framework, has been generated by the US EPA. Guidances from other regulatory agencies from other countries, continents, or agencies are not discussed here.

**ERA Framework**

The general framework for ERA outlines the interactions between various groups as well as various points along the process where data is collected and analyzed. In Figure 1, the top trapezoid represents the planning stage of ERA framework. In this stage, interactions between two parties, the risk managers and risk assessors—those to monitor risks and perform analyses, respectively—will be communicating to determine parameters to assess of a known or possible ecological stressor. From this point, the ERA framework funnels in to the first phase, which focuses on problem formulation. This is a matter of characterizing preliminary effects of exposure. This data will be collected, analyzed, and will be compared to existing data to determine if sufficient information exists, or if any gaps exist, as well as determine regulatory and policy concerns, the applicability and overall direction of the ERA (US EPA, 1992). During planning and problem formulation stages, prescribed risk assessments will address stressor effects on a range of species that are likely to encounter the stressor. This list defines the surrogate species, or non-target organisms (NTOs), the major representatives that provide unique ecosystem services or are relevant to the crop of interest (US EPA, 1992). This list is not exhaustive, as this undertaking would not be possible (Dutton et al., 2003; US EPA 2007; Carstens et al., 2013).
In phase two, the risk is quantified by characterizing results of exposure and potential ecological impacts. During this phase, the stressor will be modeled to forecast risk in the ecosystem, as well as defining the distribution of effects in time and space in reference to such NTOs (US EPA, 2007). Ecological characterization in phase two allows, where possible, for the definition of causal relationships with respect to the effects caused by the given stressor. Phase three is the culmination of the data collected in phase two, in that, phase three characterizes the risk associated with the stressor through ecological effects and exposure. The purpose here is to define the parameters of risk, from effects of the stressor of interest, temporospatial distribution, and recovery potential (US EPA 1992, 1998, 2004, 2007). This summarizes all assumptions tested, gaps in scientific knowledge, as well as the strengths and pitfalls of the given analyses. Once the ERA evidence is compiled, interactions and discussions with risk managers and assessors determine the avenues to take to mitigate concerns through management of risks of the stressor of interest (US EPA 2007).

The work presented in this thesis uses this ERA framework to guide the set of studies that assesses the exposure risk to a surrogate NTO, *Folsomia candida* Willem (Collembola: Isotomidae) when exposed to purified dsRNA that is used to control the Western Corn Rootworm, *Diabrotica virgifera virgifera*. From here, I briefly discuss the discovery and understanding of RNA interference (RNAi), the mechanism of action (MOA) of dsRNA-directed RNAi in gene silencing, the uses of RNAi in functional genomics, and transition of RNAi into agriculture focusing on applied uses and regulatory concerns for this new biotechnology.
RNAi-based gene silencing

Gene silencing was discovered as far back as the early studies of viral infections in plants (Wingard, 1928). Wingard (1928) demonstrated that new leaf growth in several plants were resistant to ring-spot virus infections after older leaves were inoculated with the virus. The induction of anti-viral resistance to repeated infection was later shown to be associated with RNA-based gene silencing of viral RNAs, or virus-induced gene silencing (VIGS) (Waterhouse et al., 2001). Insertion of the chalcone synthase (CHS) transgene into petunia to increase flavonoid biosynthesis led to down regulation of another endogenous gene responsible for producing anthocyanin (Napoli et al., 1990). This simultaneous reduction in gene expression of a homologous gene in tandem with a transgene, known as co-suppression, was later discovered to be posttranscriptional gene silencing (PTGS) (Napoli et al., 1990). Major advances in our understanding of the RNA interference (RNAi) mechanism and the core machinery involved started with the discovery of reduced gene expression after ingestion of dsRNA by Andrew Fire and Craig Mello (Fire et al., 1998; Dykxhoorn et al., 2003; Agrawal et al., 2003). RNAi is a highly conserved mechanism for posttranscriptional regulation triggered by small interfering RNAs (siRNAs), which results in the knockdown of genes at the mRNA level (Xu et al., 2015). Deeming RNAi as an evolutionarily conserved process does not fully shed light on the range of taxa that execute RNAi. This process is documented in metazoans (RNAi), fungi (quelling), plants (PTGS), virus-induced gene silencing (VIGS), and transcriptional gene silencing (TGS), which serves as a biological protection system against transposons and viruses (Catalanotto et al., 2002; Agrawal et al., 2003; Reardon et al., 2010). A recent survey using the UniProt website (uniprot.org) shows the
diversity of taxa with the core RNAi machinery, specifically Argonaute, documented and described in viruses (n=35), bacteria (n=17), archaea (n=8), as well as eukaryotes (n=7,720). The diversity of taxa with these proteins speaks to the conservation of this pathway.

The RNAi-based gene silencing mechanism uses an endogenous sequence-specific target bound by non-coding RNAs that serve as a guide strand to trigger mRNA degradation. Immediately following input from this trigger, the RNAi mechanism carries out two steps that reduce gene expression. First, processing of dsRNAs by Dicer endonucleases, which takes longer dsRNA segments and cuts the segments in to shorter siRNAs ranging from 21-to-25 nucleotides (nt) in length (Hamilton and Baulcombe, 1999; Li et al., 2013; Xu et al., 2015). These newly generated siRNAs serve as guide sequences for the RNA-induced silencing complex (RISC), an RNase complex with an Argonaute domain that binds the target (homologous mRNAs) with the siRNA guides to degrade mRNA (Figure 1) (Xu et al., 2015).

RNAi-based gene silencing has been documented in several insect orders, including Coleoptera, Hemiptera, Diptera, Lepidoptera, Isoptera, and Hymenoptera (Galiana-Arnoux et al., 2006; Wang et al., 2006; Zhou et al., 2006; Baum et al., 2007; Zhou et al., 2008; Zha et al., 2011; Bansal and Michel, 2013; Xue et al., 2015; Xu et al., 2015). Lepidopterans are generally associated with difficulties in inducing the RNAi response, but few examples are documented. RNAi was used to determine the role of the molecular clock gene, period (per), in Spodoptera littoralis. The upper vas deferens (UVD) complex was treated with per dsRNA, indicating the presence of an UVD oscillator involving per, which regulates the expression of V-ATPase A and pH (Kotwina-
Rolinska et al., 2013). Transgenic RNAi cotton was capable of efficiently knocking down gene expression in the cotton bollworm, *Helicoverpa armigera*, after consuming tissue that expressed CYP6AE14 dsRNA (targeting a cytochrome P450 monooxygenase). Increased gossypol-susceptibility was observed in larvae following dsRNA consumption (Mao et al., 2007). In other studies, insect intestinal mucins 1 and 4, as well as PM protein 1 were silenced through plant expressed dsRNAs in *Mamestra configurata* larvae (Toprak et al. 2013). Interestingly, other, more ancestral, lineages of insects and closely related non-insect arthropods (Hexapoda), are not sensitive to dietary dsRNA-based gene silencing include the Protura, Diplura, springtails (Collembola), silverfish (Zygentoma), mayflies (Ephemeroptera) and the damsel- and dragonflies (Odonata) (Cullen, 2012).

**Applications**

*RNAi-based gene silencing technology*

In the last decade, a dramatic increase in RNAi research has documented successful gene knockdown, which coincides with the application of this biotechnology from functional genomics to applied areas, namely medical and agricultural sciences. RNAi has become a vital tool that recast functional genomics research, which allowed for the decryption of gene function. From here, prodigious investments in RNAi have been devoted to diagnostics, therapeutics and agriculture. Ocular disease was shown to be effectively treated with the application of siRNAs targeting vascular endothelial growth factors (VEGFs) (Fattal and Bochot, 2006; Guzman-Aranguez et al., 2013). In tandem with the potential of a new class of pharmaceutical drugs, RNAi has successfully improved crop traits, expanding resistance to dynamic environmental and biological
stresses, and facilitate RNA-based pest control strategies (Whyard et al., 2009; Zhu, 2013; Zotti and Smagge, 2015; Xu et al., 2015).

As it stands in agriculture, RNAi has three likely applications in pest management, specifically, 1) *in planta* RNAi (Mao et al., 2007; Whyard, 2015; Zhang et al., 2015), 2) baiting (Zhou et al., 2006; 2008) and 3) formulation (Zhu et al., 2010; Miguel and Scott, 2015; Xu et al., 2015). A high-throughput dietary RNAi system was developed to screen for candidate targets in *D. v. virgifera* to be used to develop transgenic RNAi maize (Baum et al., 2007). Out of the initial 290 possible targets, 14 candidates genes were selected that exhibited the highest potential to control pest larvae, of which the most effective dsRNA targets V-type ATPase subunit-A. Ingestion of this dsRNA resulted in rapid knockdown of VATPase A mRNA by 24 hours post feeding (h.p.f.) with minimal concentrations of dsRNA. Two alternate dsRNAs targeting V-ATPase E and β-tubulin can induce an RNAi response that resulted in high larval mortality. Mao and colleagues proposed the use of RNAi crops can inhibit target pest’s ability to detoxify plant defense allelochemicals by reducing the expression of critical xenobiotic metabolism genes (Mao et al., 2007). Here, suppression of CYP6AE14, a cytochrome P450 gene increased larval sensitivity in *Helicoverpa armigera* (Hübner). Zhou et al. (2008) used a similar approach (dietary RNAi) to silence the expression of two critical termite genes responsible for expression of a digestive cellulose enzyme and a caste-regulatory hexamerin storage protein, respectively (Xu et al., 2015). Inhibition of either gene or a combination of both reduced the overall fitness of the colony, which led to significant mortality. The dietary delivery of RNAi approach synchronizes with current baiting strategies employed for urban and structural pests, which provides a platform to
integrate RNAi urban pest managements. Bacterial expressed dsRNA was capable of significantly reducing gene expression in Colorado potato beetles (CPB) (Zhu et al., 2010). RNAi has even been applied to sterile male release programs, which was successfully demonstrated in *D. melanogaster* (Lin and Wang, 2015).

Financial losses due to insect pest damage and management cost the agricultural sector billions of dollars annually. These costs are correlative to pest resistance to control methods, including synthetic chemicals and biorational (*Bacillus thuringiensis* (Bt) toxins) controls (Huvenne and Smagghe, 2010). The ultimate goal in pest management is to find a control platform that combines environmental sustainability, efficiency, target specificity, while maintaining pest populations under the respective economic injury thresholds. One of the many reasons RNAi-based pest controls are a desirable option is due to their target specificity. To test the activity of DvSnf7 dsRNA—the dsRNA found in the currently deregulated maize variety MON 87411, phylogenetically related surrogate insect species representing 10 families from four orders were exposed to lethal and sub-lethal doses of dsRNA (Bachman et al., 2013). DvSnf7, is a housekeeping gene from *D. v. virgifera* encoding a key ESCRT (endosomal sorting complex required for transport)-III protein responsible for endocytic trafficking in eukaryotes (Weiss et al., 2009). Given the sequence-specific manner and narrow activity range of the endogenous RNAi pathway, a sequence homology of ≥21nt is required to induce the RNAi effect in *D. v. virgifera.*
Technical Challenges of RNAi-based gene silencing

dsRNA Design

Factors that can affect the efficacy of RNAi in the target insect include design and construction of dsRNA, concentration, delivery method, target gene of interest, half-life of dsRNA and target insect life stage (Pan et al., 2016). Detection methodology and the above mentioned factors can together explain the variable results of RNAi in insects (Noland et al., in prep). Comparisons of existing data sets, however, are difficult due to the variation in susceptibility among different model species (i.e. greater efficiency in Coleoptera compared to Lepidoptera) (Kennedy et al., 2004; Price and Gatehouse, 2008).

The first and most fundamental step in developing an RNAi-based pest control option is the overall design and construction of the dsRNA. Here, designing the dsRNA is in silico, which based work that selects the target gene, region that the dsRNA will target, as well as the length of the dsRNA segment required. Target gene selection can only be improved if reliable genome information exists for that particular target species. Target gene selection typically involves a housekeeping gene, responsible for maintaining the metabolic homeostasis where silencing of such genes would disrupt biological functions and may lead to death of the organism (Baum et al., 2007; Xu et al., 2015). Understanding this key feature of regulatory genes allows for an understanding in target gene selection for other pathways. As with the case of detoxification/metabolic resistance to xenobiotics, stimulation of insecticide resistance genes, such as cytochrome P450s, can confer resistance, thus targeting these genes would inhibit detoxification and reduce metabolism of plant secondary metabolites and/or synthetic chemicals. Mao and colleagues demonstrated this by silencing CYP6AE14, a cytochrome P450
monooxygenase, in *H. armigera* larvae by *in planta* RNAi. Larvae were significantly more sensitive to gossypol after consumption of RNAi cotton. Despite the success of targeting housekeeping genes under high dose strategies, broad taxonomic conservation has led researchers to investigate other targets (Bolognesi et al., 2012). Numerous examples exist concerning these differing targets, which include, but not limited to targeting virus-derived suppressor proteins in transgenic crop development, neuron related genes that are targeted to disrupt insect mating and host finding behaviors, as well as targeting of several neuronal genes that display complete suppression (Ramesh, 2013; Kennedy et al., 2004; Price and Gatehouse et al., 2008; Xu et al., 2015). The length and region within target genes should be analyzed meticulously when designing dsRNA to limit, if not eliminate the incidence of non-target (between species) and off-target (amongst genes within and between species) as these may arise due to sequence homology between target and non-target organisms. Although synthetic siRNAs have been demonstrated to produce similar effects as long as dsRNAs in *C. elegans* and *D. melanogaster* (Yang et al., 2000; Elbashir et al., 2001), Bolognesi et al. (2012) suggested that a minimum of 60bp length is required for dsRNAs to be biologically active against target insects (Xu et al., 2015). This is consistent with results reported by Li et al. (2015), which demonstrated high levels of mortality in *D. v. virgifera* with 60bp length dsRNA targeting *V*-ATPase subunit *C*, but demonstrated a minimum length of 184bp dsRNA segment induced 100% mortality. A critical difference between this report and that reported in *C. elegans* and *D. melanogaster* is that siRNAs (15, 25, 27, or pooled 21bp siRNA fragments) did not induce an RNAi effect, whereas long dsRNAs did (Li et al., 2015).
dsRNA uptake

Two separate, but complementary processes for dsRNA uptake have been characterized in insects following the study of *C. elegans* (Fire et al., 1998), specifically, cell autonomous RNAi, the sum total of RNAi effects within cells, and non-cell autonomous RNAi, or cell-to-cell spread of the RNAi signal (Fire et al., 1998; Calixton et al., 2010; Zhuang and Hunter, 2012). The potential for pest control utilizing RNAi strategies is enormous, and for this reason, insects should be both autonomously receptive to dsRNA (Huvenne and Smagghe, 2010), while capable of producing a systemic RNAi response, where both requirements must be fulfilled for efficient protection (Price and Gatehouse, 2008). Systemic RNAi has been observed in nematodes, specifically *C. elegans* (Hinas et al., 2012), as well as in insects including the red flour beetle, *Tribolium castaneum* (Tomoyasu et al., 2008), the soybean aphid *Aphis glycines* (Bansal and Michel, 2013), *Drosophila spp.* (Karlikow et al., 2014) termite *Reticulitermes flavipes* (Zhour et al, 2006; 2008), *Apis mellifera* (Jarosch and Mortiz, 2011) and the African sweet potato weevil *Cylas puncticollis* (Prentice et al., 2015; Xu et al., 2015). Spread of the RNAi silencing signal from tissue-to-tissue was reported by Zhou et al. (2006; 2008), where ingestion of dsRNAs induced an RNAi signaling response that translocated from the gut to the salivary gland and fat body by observing a reduction of gene expression of *Cell-1* and *Hex-2*, respectively.

dsRNA stability and delivery

dsRNAs can be produced in a few ways, *in vitro, in vivo*, and *in planta* using commercially available kits, bacterial expression systems or by transgenic plants. A major technical hurdle in control insects is the inherent lack of an RNAi amplification
mechanism. RNA-dependent RNA polymerase (RdRp), a polymerase used to amplify siRNAs, has not been identified in the current set of available insect genomes (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008). As this amplification mechanism is not present, the current challenge is to determine a mechanism that can deliver sufficient and continuous doses of dsRNA to achieve optimal suppression. This challenge is also confounded by the stability of RNAi in multiple organisms across a trophic scale, from the producer (dsRNA plant) to primary consumers (insect herbivores). Hunter et al. (2012) showed that dsRNAs can still be effective 57 days post treatment, demonstrating the persistence of dsRNA in plants. The dsRNA stability assays and dietary RNAi bioassays reported by Zhou et al. (2008) and Pan et al. (2016) showed that Cell-1 dsRNAs were active during a 24-day assay period, whereas dsRNA was stable in laboratory microcosms with and without collembola present during dsRNA bioassays, respectively (Xu et al., 2015; Noland et al. in prep).

Mode of delivery can also determine the rate of successfully inducing an RNAi effect in insects. While some insects are receptive to injection and ingestion of dsRNAs, like coleopterans, others are receptive to injection only, like orthopterans (Smagghe and Swevers, 2014). Once a delivery method is selected, the form of RNAi-inducing agent, a long dsRNA or as siRNAs or miRNAs, to the target organism presents another subset of major technical challenges (Zotti and Smagghe, 2015). Current delivery methods include reagent-mediated transfection or electroporation (cell culture), soaking/drenching (as done with grapevines), through the cuticle, transgenic plants, injection, and feeding, either with “naked” dsRNAs or dsRNAs produced by bacteria (Agrawal et al., 2003; Zhu et al., 2010; Pitino et al., 2011; Xu et al., 2015). RNAi has been achieved in C. elegans by
injection, soaking, and by feeding dsRNA expressing bacteria (Fire et al., 1998; Tabara et al., 1998; Joseph et al., 2012; Timmons and Fire, 1998). Additionally, depending on the field of work, delivery method can change as seen by the difference for functional genomics studies, where the preferred delivery method is via microinjection (Price and Gatehouse, 2008).

Alternatively, in field applications, the only practical delivery method of dsRNAs is via autonomous uptake, e.g. ingestion (Huvene and Smagghe, 2010; Zha et al., 2011). Insects have chitin-containing exoskeletons that readily protect the insect from direct exposure to various environmental conditions, which in turn limits the probability of direct interaction with externally applied dsRNAs. While physical limitations exist external to the insect, internally, some insects can readily degrade dsRNAs prior to reaching the intended target genes, as seen with Acrythosiphon pisum (Christiaens et al., 2014). A continual/repetitive dosing strategy could potentially solve this issue, which is best provided by transgenic plants, where constitutive expression of dsRNAs redoses the insect feeds (Baum et al., 2007; Price and Gatehouse, 2008; Li et al., 2015).

in planta RNAi

Production of insect-resistant, dsRNA expressing transgenic plants, a dsRNA sequence is designed against an insect-specific gene, which is then inserted into a plant via recombinant viral vectors, typically the Tobacco mosaic virus (TMV). This virus induces the RNAi system in plants during active infection, where the induction of the RNAi response leads to the production of processed dsRNAs and siRNAs. The dsRNAs expressed in the plant, can be constitutively active (i.e. always present/active), or inducible (i.e. production after a stimulus triggers a signaling event). This platform relies
on the natural feeding behavior of the insect herbivores where dsRNA in ingested following consumption of plant tissue or phloem sap. This *in planta* approach has been demonstrated previously, which was shown to protect crops against parasitic nematodes, resist phytopathogenic infections, in addition to resisting pestiferous invertebrates (Mao et al., 2007; Price and Gatehouse, 2008; Kurth et al., 2012). This method is successful at targeting coleopterans, lepidopterans, and hemipterans (Baum et al., 2007; Baum et al., 2007; Mao et al., 2007; Zha et al, 2011). Baum et al. (2007) demonstrated that transgenic maize expressing western corn rootworm *VATPase* dsRNA caused nearly 100% larval mortality and significantly reduced root damage (Xu et al., 2015).

The RNAi pathway can also be used to regulate endogenous gene expression through virus-induced gene-silencing, as demonstrated by Kurth et al. (2012), who introduced desirable traits in several varieties of grapevine. This system provides unique advantages over other, traditional transgene-triggered systems, like that of RNAi inducing *Agrobacterium tumefaciens*. This system uses a self-replicating RNA virus expressing target genes in plant tissues without requiring genomic integration (Mansoor et al., 2006; Kurth et al., 2012). The modification of plant viruses in this manner is similar to a “plant-based vaccination”, where the modified virus can be used to protect the host plant against a variety of plant pathogens and insect herbivores by delivering single or multigene cassettes designed against the target pest (Kurth et al., 2012).

Transient RNAi can also be achieved by spraying siRNAs. Virus-specific siRNAs produced by bacteria can by sprayed on to plants five days prior to challenge, inducing sufficient resistance to infection (Masoor et al., 2006). Making this process even simpler, crude lysate of dsRNA expressing bacteria were demonstrated to sufficiently protect
plants against RNA viruses (Tenllado et al., 2003). Bacterial expression systems are a cost-effective alternative to in planta RNAi, as bacteria can produce large amounts of dsRNA.

Transgenic plant-based RNAi has been shown to be effective in controlling pests that do not have effective control programs, specifically phloem-sucking hemipterans including planthoppers, aphids and whiteflies. *Nilaparvata lugens* (Stål), the brown planthopper, was shown to have three midguts genes silenced following dsRNA treatment, which leads to the possibility of applying this control method to the field (Zha et al., 2011). Interestingly, two psyllid species were controlled by root drenching with exogenous dsRNAs. This method relies on the plant vasculature to incorporate the dsRNA having the insect ingest the dsRNA in the phloem inducing the desired RNAi effect. This was demonstrated by Hunter et al. (2012), where gene expression was knocked down in the glassy-winged sharpshooter, *Homalodisca vitripennis*.

Overall, the three major technical hurdles for dsRNA in agriculture is properly designing the dsRNA, maintaining constitutive expression and determining the most appropriate delivery method. These three factors should be considered as well as developmental stages of the target pest (particularly younger stages), as there is a correlation between age and sensitivity to dsRNA (Araujo et al., 2006; Xu et al., 2015).

**Regulatory**

*Overview*

The case-by-case basis to identify and examine possible detrimental effects of a genetically modified organism is dictated by a formal framework of science-based risk analysis and management determines the likelihood of release. The European Parliament
(2001) states that this effect is either immediate, delayed, direct, or indirect on human, animal, or environmental health. Competent authorities (federal regulatory agencies of a given country; see Table 2) use risk analyses to determine level of management needed and/or if approval should be given for commercialization (Craig et al., 2008). While general parameters exist that are somewhat agreed upon internationally, variation exists among data required for depth of research needed by each regulatory authority. In this section, I will provide a brief and general overview of what is required for an accurate risk assessment.

General parameters for assessing risk of GM crops

Risk assessment is defined as a stepwise process, which consists of several steps before culminating in a decision with an acceptable level of ambiguity. The level of ambiguity produced during this type of work is always present, even if minimal during risk assessment and management protocols (NRC, 1993; OECD, 1993; Hill and Sendashonga, 2003). Factors that can influence this ambiguity can be altered to varying degrees by political, economic and social pressures (Johnson, 2007; Wilkinson and Ford, 2007).

The risk assessment process typically follows a previously determined set of analyses that increase in intensity (USEPA 1998a; Hill and Sendashonga, 2003; EFSA, 2004). Here, when considering a possible adverse effect of a given compound, possible outcomes through causal events of which a particular effect might occur need to be described scientifically with a summary of the current knowledge regarding each possible step. If it is determined, herein, that the consequences, or risks are acceptable, a determination can be made. These determinations can be supported through a tiered
system approach, weight of evidence, or hierarchical decision tree (Hill and Sendashonga, 2003; Wilkinson et al. 2003a; EFSA 2004; Andow and Zwahlen, 2006; Raybould and Cooper, 2006; CAC, 2003a; USEPA, 2005b; Prescott and Hogan, 2006; FAO/WHO, 2001). Each of these approaches rely on different features to generate decision power. For a tiered system approach, each tier is progressively less stringent, allowing for the addition of more variables at each level of assessment. The first tier, typically the most stringent is usually defined as the worst-case scenario. At each tier, the outcome can proceed in one of two ways, either 1.) make a risk decision based on the current evidence, or 2.) request addition evidence to address identified issues (USEPA, 1998a; Craig et al., 2008). Each higher tier following the worst-case scenario incorporates more realistic conditions to assay the given risk. In weight of evidence based approach, a determination is based off metadata analyses incorporating all of the current studies that detail the safety and nutritional assays of a GM crop to determine the level of associated risk. This approach also utilizes scientific data to draw appropriate conclusions in lieu of knowledge gaps. The last approach, the hierarchical decision tree (HDTs), is more of a predictive measure as it comprises a flow chart from a plan-to-goal (Craig et al., 2008). This framework uses the results of one test to determine the subsequent tests needed until the goal is reached. The HDT model incorporates cost and risks in relation to the goal, which is useful for both internal (corporate) and federal regulatory groups.

Risk assessment of GM crops

The primary step when assessing the risk of a GM crop is to identify the possible detrimental environmental effect, also known as the hazard. Hazards from GM crops typically are associated with the release of the product, which are profiled for potential
consequences and a decision is made about the overall likelihood of the hazards occurring. These decisions are made based off two types of hazards, 1.) unintended effect in the target population and 2.) unintended effects in a non-target population (Craig et al., 2008). For example, unintended effects in the target population can be associated with the evolution of resistance if the hazard is a GM crop with an insect resistance trait. Conversely, an example of an unintended effect in a non-target population can be the reduction in overall biodiversity where a GM crop is released, or possibly the integration of the GM trait from the crop to a weed species. To ensure these hazards are fully assessed, a common, overarching approach has been recommended by various regulatory agencies, including the USDA APHIS (1996), EFSA (2004), ASEAN (2003), CFIA (2005) and König et al. (2004). Once these hazards are identified and the relevant information concerning them has been collected, a regulatory dossier will need to be generated and submitted to a given regulatory agency that follows a typical outline that has internationally agreed upon standards (UNEP 1995; OECD, 2000a; CAC, 2003a; Craig et al., 2008). Briefly, regulatory dossiers take the organisms of transgene origin and destination into account, and includes information on how the transgene(s) were incorporated into the new host, how this/these transgene(s) are expressed in the new host and the transgene(s) end product(s). An overall description of the nutrition, allergen, toxicity, agronomic performance, environmental profiles are documented and how these affect the crop conversion in to food and feed ingredients, if and how dietary uptake changes, and if any potential long term nutritional impacts exist. Following these steps, a Molecular and Biological description of the GM crop determines the impacts on how the plant is engineered and how it performs in the field, which is reviewed extensively by
Craig et al. (2008). Table 3, adapted from Craig et al. (2008), covers specific concerns of these molecular and biological descriptions. As these prominent features of risk profiles are needed for the approval of any new variety or platform of plant incorporated protectants, as well as other incorporated transgenes, RNAi-based GM crops are at the subject of this scrutiny.

Regulatory concerns for RNAi-based genetically modified crops

Despite overwhelming scientific evidence noting the safety of GM crops, debate over the safety of GM crops, the public and some in the scientific community do not accept the current data. As one pursues research and development with genetically modified organisms, specifically crops, regulatory concerns are always at the nexus of product development and commercialization. Prior to the commercial release of RNAi GM crops, issues related to safety should be analyzed through a series of risk assessment-based studies. These steps should be taken, in general, for the sustainability of the future product and to limit the impact this new product has on the environment, while garnering the supportive view of the public, which has not always been the case. In 1999, following the publication by Losey et al., the anti-GMO community and the media erupted with the study’s data that monarch butterfly larvae are susceptible to Bt-toxins found in the pollen of GM maize. Rosi-Marshall et al. (2007) was criticized by the scientific community to prevent anti-GMO groups from improperly using the published research and extrapolating beyond the study’s scope (Waltz, 2009; Xu et al., 2015). Statements like these damage not only the reputation of the GM crop that can benefit countless people, but also can damage the public perception of agricultural companies and researchers who work in developing these products.
Risk assessments for RNAi GM crops and RNAi associated products are currently at an early stage, with some laboratory risk analyses complete (Pan et al., 2016; Noland et al., in prep), debates are ongoing concerning the improvements needed for building new risk assessment guidelines. Currently, the ecological risk assessment (ERA) framework for RNAi crops used was originally designed for first generation of GM crops (e.g. Bt crops). However, the predictive capability of ERA for RNAi crops and the unique role of non-coding RNA (ncRNA)-based GM plants requires adaptations and newly formed considerations (Auer and Frederick, 2009; Lundgren and Duan, 2013; Ramesh, 2013; Xu et al., 2015). Auer and Frederick (2009) discussed the predictive ERA for RNAi-based crops using a transgenic crop expression Bt endotoxin and a host-delivered (HD)-RNAi crop producing a small RNA with toxicity to insect pests as inferences (Xu et al., 2015). Modifications incurred by dsRNA expression involve redesigning the way plants express and regulate their genes, which is an unknown area as far as unintended environmental consequences.

Several possible risks associated with RNAi GM crops should be considered due to the unique nature of ncRNA-mediated gene manipulation, incorporating adapted Bt-framework. These risks include increased invasiveness, intra- and inter-specific hybridization resulting in gene transfer (e.g. crops-to-weeds), and potential adverse effects on human health and other non-target organisms (Heinemann et al., 2013; Lundgren and Duan, 2013; Casacuberta et al., 2015; Xu et al., 2015). As the RNAi effect depends on the sequence identity, in silico bioinformatics analyses are crucial for predicting the potential effects associated with the RNAi-expressing GM crops (Ramesh, 2013, Xu et al., 2015).
RNAi-based gene silencing technologies demonstrate immense potential as a genetic based pest control option. When applying RNAi-based technology in the development of next generation GM crops, technical challenges will need to be addressed, however, much more attention will need to be directed to risk analyses of biosafety concerns. This entails more direct communication between the scientific community and the general public on how assessments for potential risks are studied, what risks are involved with the new GM crop and how the scientific community can reassure the general public that these transgenic plants are safe for human consumption. Adapting an environmental risk assessment framework designed specifically for RNAi-mediated pest control is crucial.

**Summary and Perspectives**

**RNAi-based genetic manipulations**

As the global population continues to grow, with the greatest need for sustainable crops in developing countries, the need for GM crops is crucial for several reasons including, but not limited to: 1. Increasingly productive agricultural practices; 2. Improved nutritional yields of crops; 3. Increased benefits for farmers; reducing use of remaining arable land. An ultimate goal for agriculture is to produce crops with high yields, while limiting resource allocations and reducing/eliminating adverse impacts on the environment. RNAi-based genetic controls could serve as an alternative strategy to solve a variety of agriculturally relevant problems. RNAi is a conserved mechanism that can be employed to control pest insects to a high degree of specificity, reducing non-target interactions. This genetic technology boasts the stable inheritance of the suppressed phenotype produced by RNAi transgenes through the fifth generation of offspring (Singh
et al., 2011). RNAi can also be controlled by tittering dsRNA expression using tissue specific promoters to restrict dsRNA expression in highly specific regions of the GM plant (Singh et al., 2011).

The development of pesticide resistance decreases productivity and sustainability as more resources will be allocated in to management or mitigation (Christou et al., 2006). Following the initial commercial releases of Bt crops, some pest insects have modified natural behaviors and evolved resistance to an extensive array of Bt Cry-proteins (Bates et al., 2005). With the success of gene knock down by dietary and in planta RNAi, researchers have speculated that RNAi crops are sufficiently protected (Gordon and Waterhouse, 2007). One would expect that RNAi GM crops should control pests to a similar or better degree than Bt crops, however, development of resistance to RNAi is an ever-looming possibility. Even though Bachman et al. (2013) demonstrated that insect resistance to DvSnf7 dsRNA is highly unlikely due to the theoretical presence of as many as 221 potential 21nt matches (Xu et al., 2015). This means that, even with the occurrence of single nucleotide polymorphisms (SNPs) in a target sequence of 240nt would not likely alter the biological effect of dsRNA on the target species (Bachman et al., 2013).

For a plant to develop resistance to viral infections, pathogen-derived resistance (PDR) can be achieved by transforming plants with virus-based gene sequences, where the newly gained resistance is mediated by PTGS of RNAs (Mansoor et al., 2006). Once initiated, the gene silencing signal can transduce throughout the entire plant leading to plant-wide pathogen resistance (Mansoor et al., 2006). This same systemic spread of gene silencing in the plant is only theoretical when concerning pest insects. As plant viruses
have co-evolved with their respective hosts, these viruses have evolved counter-silencing mechanisms allowing the virus to disrupt the host plant’s anti-viral defenses, specifically, VIGS-based RNAi disruption (Ramesh, 2013). As this gene silencing pathway (siRNA-mediated gene silencing) is a primitive and conserved defense mechanism, an even more complex interaction exists between plants and their respective herbivores. As RNAi-based pest controls are soon to be out on the market (recently deregulated as of October 2015 in the United States and seeking approval), management of RNAi resistance should be monitored extensively to ensure the long-term sustainability.

Assessment of RNA-based pest controls

Derived from cellular processes, RNAi-based gene silencing is responsible for regulating gene expression and immunity against foreign DNAs. After several studies, optimization, RNAi-based gene silencing has become a powerful tool capable of manipulating genetic elements in reverse genetics, which has been applied to many fields. This includes the development of RNAi in pest management in agricultural applications through transgenic crops (Mao et al., 2007; Wang et al., 2011).

Despite this broad application in the many fields, there are fundamental regulatory concerns and risk analysis approaches for dsRNA-based gene silencing (Table 1). By degrading cognate mRNA in the cell, dsRNA-based RNAi regulates gene expression without eliciting changes to the genome, which is the target of the CRISPR/Cas9 system. Another inherent feature of this method is that RNAi manipulations are reversible. Due to partial gene disruption following RNAi application, few genes are lethal, whereas more genes could be lethal following different RNA-based platforms, such as the burgeoning genome editing-based gene disruption. The length of RNA for dsRNA applications is
typically 300-500bp, with the minimum length at 60bp. The generation of short siRNA fragments has the potential to produce guides that can increase the frequency of off-target effects, thus requiring more preliminary work, *in silico*, to reduce this occurrence. The impact this technology has in agriculture, the environment, and human health is tremendous and the benefits herein outweigh the possible risks associated with RNA-based pest management platforms.
Figure 1. Risk assessment framework in coordinated stages, from initial planning between risk managers and assessors, to forecasting risk associated with all determine general ecological assessment endpoints (GEAEs). Note, the stages represented as trapezoids funnel in to each box until risk can be forecasted for each determined endpoint.
Figure 2. RNAi-based gene silencing. The schematic drawing depicts dsRNA-based RNAi: 1: Exogenous dsRNA is taken into the cell either through receptor mediated endocytosis or SID-channel transfer across the membrane, to the cytoplasm. Upon uptake into the cell, 2: the dsRNA is processed into short, 21-25nt double-stranded fragments with 3′- and 5′-overhangs (siRNAs). The processed siRNAs, 3: are transferred either to neighboring cells (systemic RNAi), or are bound in the cytoplasm by the RNA-induced silencing complex (RISC-argonaute, a major component in the RISC multiprotein complex), 4: the RISC-bound siRNAs are then processed further leaving the anti-sense strand exposed, which is used to, 5: target homologous sequences on the mRNA target, 6: targeted mRNA sequences are degraded and the target gene is silenced.
Table 1. Risk assessment and regulatory considerations for RNAI-based crops

<table>
<thead>
<tr>
<th>Risk Parameters</th>
<th>Gene Silencing</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genetic/Bioinformatic</strong></td>
<td>Define target site specificity and how many base pairing matches exist between target and potential non-target species. Numerate possible cross interactions.</td>
<td>Determine nt matches (19, 20, 21-mer sequence overlaps).</td>
</tr>
<tr>
<td><strong>Laboratory</strong></td>
<td>Test dsRNAs via standard protocols using feeding, injection, and drenching/soaking methods in target and non-target species. Quantify possible interactions using RT-qPCR and life history traits.</td>
<td>Assess risk in lab and semi-field settings prior to field approval; design new regulatory framework off existing protocols.</td>
</tr>
<tr>
<td><strong>Ecological</strong></td>
<td>Stability of expressed dsRNAs in a given vector (<em>in planta</em>, bacteria, viruses, etc.). Determine half-life of dsRNAs, siRNAs, or miRNAs in field-level substrate.</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Release Facilities</strong></td>
<td>Controlled field plot releases on small, large, and full field scales.</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Profiling and Genetic Mixing</strong></td>
<td>Quantify effects and document loss (if seen, or observable) on target and non-target species.</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Reversibility</strong></td>
<td>Ensure half-life of dsRNA effect in target can be lost in 1-2 generations of defined target.</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Federal and/or Regulatory Agencies</strong></td>
<td>Strict regulation of dsRNA crops; release crops in cycles and measure quantifiable risks according to ERA framework to determine suitability of other crops.</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 2. General regulatory concerns, listed as questions, from the transgenic even to plant composition when seeking regulatory approval of a GM crop.

<table>
<thead>
<tr>
<th>Regulatory Concern</th>
<th>Inserted DNA</th>
<th>GMO Expressed Proteins</th>
<th>Biology of GM Crop</th>
<th>Composition of GM Crop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the insert in question the actual insert? No introduced indel mutations?</td>
<td>Direct transgene encoded protein concerns: Is transgene an allergen? If YES, does transgene accumulated and how does it behave in GI tract of consumers? Is protein anti-nutritive? Is the protein stably expressed of the lifetime of the crop and across generations?</td>
<td>Possibility of horizontal gene transfer? Does GM variety have alter agronomic traits compared to isolate variety? Increase propensity to become an agricultural weed? Possibility of compatible crop hybridization? Non-target organisms: Will this GM crop alter the abundance and/or diversity of NTOs, such as soil microarthropods, pollinators, predators, biological control and/or infectious agents? Environmental effects as determined by ecology and/or agronomy?Delayed/indirect negative effects on the environment? Analyses of nutritional and intended use of GM crop adequate? (Un)Intended changes to quantity and variety of toxins, allergens, or anti-nutritive compound profile? If YES, a specific RA will be needed for any/all listed changes.</td>
<td>Analyses of nutritional and intended use of GM crop adequate?</td>
<td></td>
</tr>
<tr>
<td>Is the amino acid sequence altered? If YES, perform associated RNA. Tests for inserted DNA must reveal site of integration and if proteins are developed from this insert. Has any component of the integration vector been incorporated (antibiotic marker, ori, promoter from vector, etc.)? Expression of inserted gene(s): Inducible or constitutive? Does the final product have selection markers? If YES, specific RA for safety will be performed. Do insert(s) segregate properly?</td>
<td>Indirect transgene encoded proteins: Has a fusion or chimeric protein been generated from insertion? Are there notable changes to gene expression? Does the transgene have high sequence similarity to a homologous gene? If YES, total plant composition is needed.</td>
<td>Dietary changes associated with end product? If YES, a specific RA will be needed. Post Market Analyses: Is end product performing according to predictions? Predicted or unexpected side effects documented.</td>
<td></td>
<td></td>
</tr>
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</table>
Chapter Two: Development of dietary RNAi toxicity assay and assessment of oral toxicity of dsRNAs in *Folsomia candida* Willem (Collembola: Isotomidae)

Abstract

Assessing the risk of transgenic crop products is essential when determining the safety of a crop for deregulation and commercialization. United States and Foreign-based regulatory agencies and international standards institutions require a battery of tests to be performed on organisms that perform various ecosystem services. These assays are performed using ecotoxicology testing methods established for pesticides (Candolfi et al., 2001; Romeis et al., 2008; Rombke et al., 2009; Hilbeck et al., 2011). This methodology have been applied to testing *Bacillus thuringiensis* (Bt) Cry protein toxins engineered into crop plants and a wealth of information exists on non-target organism (NTO) interaction. Toxicology assays are typically evaluated through a tier-based approach, where, if no or negligible risk is identified in lower tiers (oral toxicity or phenotypic changes), then a risk decision can be made. Long-term exposure studies are often performed after commercial release of the crop occurs and provide a more in depth understanding of impacts on the environment, such as the persistence of soil deposited Cry protein (Douville et al., 2007). Risk analyses have been recently performed on the next generation of GM-crops (expressing dsRNAs—focus on dsRNAs against *Diabrotica virgifera virgifera* LeConte). These analyses have demonstrated the safety of ingested dsRNAs that were *in vitro* transcribed and have shown negligible negative impacts in several surrogate species. Here, we report the establishment and standardization of laboratory protocols for testing dsRNA produced *in vitro* through stability assays in the presence and absence of the non-target surrogate detritivore, *Folsomia candida*. We show that *in vitro* transcribed dsRNA
targeting \( vATPase \) \( A \) is stable across the duration of the feeding assay time (\( F_{2,15} = 0.574, P = 0.575 \)), whereas dsRNA degraded by 50% by 48 hours in the presence of \( F. \) \textit{candida} (\( H(3) = 14.201, P = 0.007 \)), which denotes the time to consumption/replacement of the artificial diet for all bioassays. Uptake (ingestion) of \( vATPase \) \( A \) dsRNA was quantified and profiled across five assay days (\( F_{3,28} = 2.911, P = 0.005 \)). When exposed to an acute dose of \( vATPase \) \( A \) dsRNA, gene expression is not negatively impacted (\( F_{6,12} = 2.11, P = .1315 \)) and life history traits (survival (\( F_{3,12} = 5.647, P = .130 \)), egg production (\( F_{3,12} = 3.904, P = .272 \)), and egg viability (\( F_{3,12} = .284, P = .997 \)) are not altered significantly in response to chronic doses of dsRNA. Interestingly, growth was the only life history trait that was significantly altered, specifically those \( F. \) \textit{candida} individuals that fed dsRNA treated diet grew significantly more (\( F_{3,12} = 33.690, P = <0.001 \)). The combined results of \( vATPase \) \( A \) dsRNA stability, lack of negative impacts on gene expression and life history traits lead to the conclusion that dsRNA has a negligible impact on this surrogate microarthropod.
Introduction

Some of the most serious pests in agriculture are beetles, because of the direct (feeding) and indirect (e.g., vectoring plant pathogens) damage they do to plants. Management of pest beetles has historically been through the application of chemical insecticides and cultural changes (e.g. crop rotation, planting refugia); however, environmental toxicity and the development of resistance have made management difficult. One of the many goals of sustainable agriculture is to reduce the use of pesticides, which has partially been achieved by implementing transgenic crops in corn, cotton, potato, rice and tobacco (Phillips, 2008, Xu et al., 2015). The introduction of GM crops has reduced the broad application of insecticides, thus limiting contamination of synthetic pesticides to the environment (Phillips, 2008). Reduction of applied pesticides was partially achieved through the development of crops expressing Bacillus thuringiensis crystalline toxin. Bacillus thuringiensis (Bt) is a soil dwelling bacterium that is found in numerous soil types, both in agricultural and non-agricultural settings. Due to their potency and specificity, Bt toxins have been engineered into many crops. Despite their usefulness, there are limits to Bt toxin success, specifically the kinds of insects Bt-toxins are capable of targeting (e.g. highest efficiency with Lepidoptera, moderate targeting in Coleoptera and low-to-moderate efficiency in Diptera) (Flores, Saxena and Stotzky, 2005). Quick adaptation to planting GM-crops expressing Bt-toxins has led to the evolution of resistant populations of beetles, particularly seen with the western corn rootworm, Diabrotica virgifera virgifera LeConte (Gray et al., 2009).

The western corn rootworm has been a serious pest of maize in the United States since the 1940s following initial expansion from isolated regions of the western plain
states, Kansas and Colorado (Gray et al., 2009). Spread from these localized populations were likely due to continual planting of maize without rotation, which allowed for subsequent invasion into Midwestern states from the mid-1950s to 1970 and as far as Virginia by the 1980s (Chiang, 1973; Youngman and Day, 1993). This problem, however, is not isolated to the United States. It was documented in 1992 that a small population of *D. v. virgifera* was found in Serbia (Yugoslavia) at the Belgrade Airport that was likely due to international flights between airports close to infested maize in the United States that traversed to Europe (Gray et al., 2009). Since their discovery in Europe, western corn rootworm has been found in 20 European countries (Miller et al., 2005; Gray et al., 2009). Control of western corn rootworm has been a marred by the insect’s ability to adapt to both cultural practices (crop rotation), pesticide controls (chemical pesticides) and commercial GM maize (*Bacillus thuringiensis* Cry3Bb1 toxin).

While physiological adaptations to crop rotation was primarily thought to be the route of success for the western corn rootworm, researchers found that corn phenology induced changes in adult behavior. O’Neal et al. (2002) had discovered that aging, less suitable maize plants facilitated increased consumption of non-host plants, particularly soybeans. Feeding on alternate plant hosts allowed for adaptations to crop rotation, while simultaneously developing resistance to overused pesticides. Chemical control of western corn rootworm is a case study in itself. Amassing numerous founder populations with large amounts of genetic variation combined with partial tolerance led to selection of beetles that were resistant to cyclodiene application (Gray et al. 2009). As founding populations that had genes responsible for pesticide resistance and host plant expansion, numbers of beetles increased overtime and generated a highly adaptive and resistant
population of western corn rootworm (Metcalf, 1983; Miller et al., 2005; Onstad et al., 2003; Gray et al., 2009).

In 2003, the first line of genetically modified maize plants was commercialized to control western corn rootworm by *in planta* expression of Cry3Bb1, a Coleopteran-specific Bt toxin (James, 2010). By 2009, this Bt-maize constituted nearly half of all maize planted in the United States (James, 2009). With the rapid adoption of this GM-maize variety, in tandem with the lack of compliance by farmers (limited or no planting of refuges—unmodified maize that has no resistance), the beetles rapidly evolved resistance to Cry3Bb1 (Gassman et al., 2011). This resistant population shares cross-resistance to a modified Bt toxin variety, mCry3A, and is associated with severe injury to GM-maize in the field (Gassman, et al., 2014). Due to this resistance, novel methods in pest management were developed that used an entirely different ingested control from GM-maize that was unlike Bt toxin. *In planta* expression of dsRNA was a controls pest insects via gene expression knockdown was shown to be highly effective (Baum et al., 2007; have been developed and the GM-maize trait DSnf7 (MON 87411; a 240bp dsRNA segment targeting the Snf7 gene of *D. v. virgifera*) is currently deregulated by the United States Department of Agriculture. This maize variety is corn rootworm protected using RNA Interference (RNAi).

RNA Interference is a widely conserved cellular mechanism that produces short interfering RNAs (siRNAs) that are 20-30 nucleotides in size that promotes the degradation of homologous messenger RNAs (mRNAs). Delivery of long, double-stranded RNAs (dsRNAs) expressed in plant tissue (highest in the roots) is through ingestion, which then allows for dsRNAs to be internalized by cells, processed into
shorter fragments, transferred to neighboring cells and further processed to target specific
genes that are critical for survival of western corn rootworm. These so-called ‘genetic
pest control’ alternatives promise to be highly specific to a given pest, have low rates of
toxicity to non-target species, and dramatically decrease economical losses on a broad
scale (Price and Gatehouse, 2008; Kos, van Loon, Dicke, and Vet, 2009). Engineering
plants to express dsRNAs proved to be a highly potent method, reducing crop damage by
95% and increasing larval stunting and mortality, particularly with western corn
rootworm larvae (Baum et al., 2007; Mao et al., 2007; Mao et al., 2011; Mao et al.,
2013).

Despite the proposed safety of this new GM-maize, some concerns still remain
regarding the overall safety of this new biotechnology on non-target organisms (NTOs).
Due to the conserved nature of the RNAi pathway and the fundamental targeting of
housekeeping genes, microarthropods could be at risk of off-target gene knockdown as
many microarthropods exist in the same habitat that western corn rootworm larvae. This
risk is exacerbated by length of exposure to exuded dsRNAs from plant tissues that exist
both during (pre-harvest roots) and after the growing season (post-harvest roots, stalks,
and some leaf tissue). Several reports offer observations of increased survival of GM-
plant product survival, specifically Cry-toxin exudation from Bt-maize plants, lowered
rates of decomposition of Bt-maize types, as well as transgene survival in plant tissue
post-harvest (Douville, et al. 2007; Saxenaa, 2002; Clark et al., 2005; Flores, Saxena and
Stotzky, 2005), dsRNAs exuded into soil have an increased likelihood of longer half-life
that is influenced by soil type and protection by plant residuals.
Soil microarthropods play an incredibly diverse role in soil ecosystems and provide diverse ecosystem services. One major group of soil microarthropods, collembola (known colloquially as spring tails), are found globally and are responsible for nutrient cycling across many habitats (Fountain and Hopkin, 2005). Most species of collembola feed on leaf litter and fungi in soils (Hopkin, 1997). As many species have adapted to soil ecosystems, their integration is critical in evaluating the overall “health” of given conditions, where health refers to stabilized pH, growing conditions (if cultivatable or agricultural soils), as well as level of contamination by pollutants (Fountain and Hopkin, 2005). Collembola are highly sensitive to soil contamination, which has led to the use of abundance and diversity studies as a method to test the impact of many environmental pollutants (de Boer et al., 2010; Frampton et al., 2006; Domene, Alcaniz and Andres, 2006; Bakonyi et al., 2011). One such collembola species that has been implemented in many ecotoxicology studies is *Folsomia candida* Willem (Collembola: Isotomidae), a cosmopolitan species that has been shown to demonstrate changes in life history, behavior and effects of bioaccumulation in response to soil contaminants (Fountain and Hopkin, 2005).

The International Standards Organization (ISO) and Organization for Cooperative and Economic Development (OECD) have used *F. candida* as a model or “standard” species for soil toxicology assays and have developed a series of protocols to determine the ecotoxicology of a given toxicant (Crouau, Gisclard, and Perotti, 2002; Diez et al., 2001; Fava and Bertin, 1999; Fava, Digioia, and Marchetti, 2000; Fava and Piccolo, 2002; Frische, 2003; Gejlsbierg et al., 2001; Juvonen et al, 2000; Kratz and Riesbeck, 1998; Krogh, 2009; Schafer and Achazi, 1999; Van Gestel et al, 2001; OECD Chemicals...
Testing Guidelines, 2009). *Folsomia candida* has been widely used in numerous laboratory experiments due to ease of rearing (parthenogenetic, limited food requirements—specifically potato; small laboratory rearing space needed) and thus was used as a candidate surrogate species for our work. Genetically engineered maize expressing *Bacillus thuringiensis* toxins have been assessed for risk using *F. candida* and such framework was developed for assaying the toxicity of GE-plant products under protocols set forth by OECD and the US EPA FIFRA Act through Code of Federal Regulations, Title 40, Part 158 (publications through OECD biotrack website; Marvier, 2002). These regulations have set forth timed bioassays and specific species that can be surrogates to verify the safety of each GE-plant produced insecticidal compound. A set of criteria for experimental design was set forth and for *F. candida* when exposed to varying levels of Bt toxin from different plant sources: potato (99-173-01p and 94-257-01p), cotton (97-287-01p) and corn (96-317-01p and 94-319-01p). In each, four to five replicates per treatment and 10 individuals per replicate are used for laboratory experiments over the course of 28 days (Marvier, 2002). Studies with *F. candida* using Bt toxin exposure have focused on life history traits (reproductive output, survival, hatching rate) over a 28 day period have shown little or negligible negative impacts on life history parameters (Marvier, 2002; Romeis et al., 2008; Bakonyi et al., 2011). These types of studies were incorporated to assessing the effects of dsRNA on *F. candida*.

For our study, we adapted existing Bt risk assessment framework and altered it to examine dsRNA stability in laboratory microcosms and the potential effects dsRNA incurred to *F. candida*. Following the established guidelines set forth by ISO, OECD, USDA and US EPA, we developed a worst case scenario (high level dosing) of dietary
consumption of dsRNAs, much further beyond what would typically be encountered in the ambient environment. To answer the question of whether or not *F. candida* was susceptible to the effects of dietary RNAi, three *in vitro* transcribed (IVT) dsRNAs, β-glucoruronidase (plant control gene; 540bp segment; dsGUS) and two, 400bp dsRNAs targeting a conserved region of *F. candida* (dsFc) and *D. v. virgifera* (dsDvv) v-ATPase subunit A were fed to 10 replicates of 17 *F. candida* (10 days old) individuals over the course of 28 days. The stability of IVT dsRNA was measured through the duration of feeding time needed for *F. candida* individuals to consume whole pieces of diet and was quantified using densitometry. Diet consumption assays were performed to mimic feeding conditions in each microcosm with 15-20 individuals by staining artificial diet and visualizing collembola guts using digital photography. Both gene expression and life history trait analyses were used to determine if any negative impacts occurred from feeding on dsRNA laced diet. Information from the above experiments indicate negligible negative impacts on *F. candida* when fed dsRNAs from multiple sources.
Results

dsRNA stability assay

Percent (%) pixel intensity of each gel band of expected size was recorded every 12 hours over the course of 2.5 assay days that was normalized to the 0h time point. This was performed in the presence and absence of *F. candida* to demonstrate the impact of external degradation on dsRNA laced artificial diet. For *F. candida* absent diet, no significant degradation of *F. candida* v-ATPase A dsRNA was observed \([F_{2,15} = 0.574, P = 0.575]\) (Figure 4). For artificial diet that was exposed to *F. candida*, v-ATPase A dsRNAs were similarly stable across the first 36h of the assay, which started to decrease at 48h (~ 53% was detected of the total) and approximately 32% remained after 60h \([H(3) = 14.201, P = 0.007]\) (Figure 5). These results should not impact the dietary RNAi study as diet was replenished every 48h, prior to the significant degradation of dsRNAs.

Artificial diet consumption and in vivo dsRNA detection

As the artificial diet system is a critical component to properly assaying the potential impact of dsRNAs on the organismal and suborganismal level, two diet specific assays, a diet consumption assay and dsRNA detection assay, were developed to determine if this delivery system is sufficient. Following two days of feeding on diet that was stained with a 0.1% Nile Blue dye solution, the gut tract of *F. candida* larvae was a light-blue to gray tint, compared to those from the *en masse* culture, which guts were tinted brown (Figure 3). Following consumption of artificial diet laced with v-ATPase A dsRNA, dsRNA was detected using a dsRNA specific primer (Table 1). High concentrations were measured 12h post feeding, and significantly lower levels of dsRNA were detected at each time
point through 72h \([F_{3,28} = 2.911, P = 0.005]\). Two additional time points, 96h and 120h, were profiled to indicate the duration of dsRNA in \(F. candida\) (Figure 6).

**Temporal profile of RNAi effects in \(F. candida\)**

Gene expression of \(\nu\text{-ATPase A}\) was not affected by the treatment of dsRNAs \([F_{3,12} = 2.426, P = 0.116]\), time \([F_{2,12} = 2.44, P = 0.805]\), or interactions between these factors \([F_{6,12} = 2.11, P = .1315]\) (Figure X).

**Dietary RNAi toxicity assay**

Compared to the control treated larvae (100% survival), all treated larvae died from consumption of two different doses of potassium arsenate. Those larvae that fed on either concentration (36 µg/µl and 100 µg/µl) died within 7 assay days. No significant changes in adult survival was detected when \(F. candida\) was exposed to dsRNAs \([F_{3,12} = 5.647, P = .130]\) (Figure 10). Similarly, fecundity (egg production) \([F_{3,12} = 3.904, P = .272]\) (Figure 8) and egg viability \([F_{3,12} = .284, P = .997]\) (Figure 11) were not statistically significantly different. Conversely, \(F. candida\) grew significantly when feeding on diet laced with dsRNAs compared to the control \([F_{3,12} = 33.690, P = <0.001]\) (Figure 9).
Discussion

Assessing the risk of new transgenic technology by hypothesis driven research is driven by assessing initial low tier, laboratory based studies exclusively, or prior to analyses at higher tiers, given the possibility of making risk decisions. These studies focus on to facets of risk analyses: hazard and exposure. Typically, hazard focused studies utilize short-term, direct interactions (toxicology analyses, phenotypic, and/or phylogenetic studies) against non-target species, which refers to the worst case scenario. Exposure studies are longer term and primarily attempt to reproduce more realistic conditions of which pose most risk to non-target species.

As the risk assessment platform for this new transgenic technology is adapted directly from established Bt risk assessment and synthetic pesticides, it is imperative to incorporate work pertaining to modes of action (MOAs) of dsRNAs. Comparatively speaking, the MOA of Bt crystalline toxin is well documented and generally understood. No previous study has demonstrated negative impacts of Bt-toxins on collembola, particularly F. candida (Yu et al., 1997; Al-Deeb et al., 2003; Clark and Coats, 2006; Yuan et al., 2013; Römbke et al., 2010; Bai et al., 2011, 2013; Bakonyi et al., 2011; Yang et al., 2015). Conversely, the MOA of plant expressed dsRNAs is not clearly understood, thus studies such as this provide critical information that support its use in the field. This support is validated by producing endpoint measurements that demonstrate potential chonic, sublethal and acute, high dose effects to target and non-target species (Bolognesi et al., 2012; Lundgren and Duan, 2013; Xu et al., 2015; Roberts et al., 2015). While no negative impacts were observed, F. candida gained significantly more weight during the 28 assay day life history analysis when challenged with three dsRNAs.
To ensure laboratory conditions are suitable for measuring the toxicity of dsRNAs in non-target arthropods (NTAs), we developed a set of assays that examined the consumption of the artificial diet by *F. candida* (stained diet assay), used a potassium arsenate toxicity assay, and the dsRNA detection assay. By taking advantage of the translucent nature of the body of *F. candida*, individuals transferred from the *en masse* culture showed a brown tinted gut tract, compared to the individuals that fed on stained diet, which stained the gut tract a light-blue/gray color, demonstrating the collembola are capable of eating the artificial diet. The potassium arsenate assay, used as “proof of concept” to demonstrate lethality of ingested compounds, yield results similar to those of Yang et al. (2015) and Pan et al. (2016), where either *F. candida* or *Sinella curviseta*, a closely related collembola species, fed on potassium arsenate laced diet at varying concentration, lowering the overall fitness or killing the larvae exposed to the toxicant. Here, we used concentrations that closely represent the µg doses used for the dsRNA, and a concentration that was more than two fold higher (0—the control, 36, and 100 ng/µl) from both studies and incurred similar results. At these concentrations, *F. candida* died in approximately 5 assay days at 100 ng/µl and those exposed to the 36 ng/µl showed a rapid decline by 5 assay days (~40% survival) and were completely dead (all individuals) at 7 assays days. Detecting ingested dsRNAs over the course of five assay days and the tracking the overall concentration demonstrated *F. candida* ingested the dsRNA that individuals were challenged with under laboratory conditions.

Suitable assessments of worst case scenario as set forth by the USEPA (10-fold greater exposure) use purified active ingredient in artificial/control diet (USEPA 1998; 2014). In this study, 3.30 µg artificial diet was supplemented to 17 individuals every 48
44 hours for 28 assay days, resulting in each \textit{F. candida} individual consuming 3.63 µg/µl dsRNA \[(1.875 \times 3.30 \times 10)/17\]. This high of a concentration is 363 times higher than the \textit{LC}_{50} found to be lethal to \textit{D. v. virgifera} larvae (Baum, 2007). As \textit{F. candida} is a major soil decomposer, the likely route of exposure of dsRNAs is through the remaining plant residuals during the post-harvest season, or during the growing season through the dislodging of various plant tissues and pollen. The recently deregulated RNAi maize trait demonstrates fresh weight environmental concentrations of \textit{DvSnf7} expressing maize as 0.224 ng/g in pollen, 33.8 ng/g in leaf tissue, 3.68 ng/g for root remnants, 4.61 ng/g in forage root, 1.04 ng/g in the stover, and 9.02 ng/g in the silk. By comparison, the concentrations used in this study were 16025, 107, 373, 787, 3490, and 402-fold higher, respectively.

Not only did we use concentrations that surpass those documented in these deregulated maize plants, we also selected for a region of the \textit{\nu-ATPase A} gene that indicated the highest degree of similarity between \textit{D. v. virgifera} and \textit{F. candida} to synthesize potentially active dsRNAs in the NTA. As previously described, a single 21bp sequence match is capable of inducing the RNAi effect, which has been described and demonstrated in \textit{D. v. virgifera} (Bachman et al., 2013; Vélez et al., 2016). Here, an 86% sequence similarity exists between \textit{D. v. virgifera} and \textit{F. candida}, as well as having several possible 19-23bp matches between the target and non-target species. These factors, together with the high concentrations should have hypothetically incurred an effect in \textit{F. candida}, considering 1 µg of dsRNA fed to \textit{D. v. virgifera} leads to 100% mortality within 9 assay days, post feeding (Bachman et al., 2013; Vélez et al., 2016).
Pan et al. (2016) designed a similar study using a closely related species of collembola, *Sinella curviseta*. This group assayed the laboratory stability of dsRNAs in the microcosms used for the critical bioassays of life history and gene expression analyses in this collembola. In this study, Pan et al. demonstrated that *S. curviseta* feeding on an artificial diet laced with dsRNA designed against *D. v. virgifera*, β-glucuronidase, and against *S. curviseta*, were not only able to survive at rates equally between dsRNA treatments, but fecundity and hatching rate were also not significantly different. It was documented that development time from neonates to sexual maturity was significantly quicker when feeding on dsRNA treated diet, compared to the water (vehicle) control. Gene expression in *S. curviseta* was not statistically altered over the course of several assay days, which corroborates the work that we have found in *F. candida*. The results, taken together with those described above and the study by Pan et al., demonstrate negligible impact of RNAi toxicity and temporal RNAi treatment effect of ingested dsRNAs on *F. candida*.

While this new transgenic technology proves to be highly specific with negligible non-target impacts in previously studied surrogate species, challenges remain for the efficacy of RNAi in insects. Not only does phylogenetic relatedness play a major role in the efficacy of RNAi, but length, concentration and structure of expressed dsRNAs can affect RNAi efficiency (Price and Gatehouse, 2008; Terneius et al., 2011). Barriers to success not only exist in the biochemical profile of the dsRNA, but the proteins responsible for systemic transfer of dsRNAs, channels capable of facilitating dsRNA internalization, induction of RNAi machinery (let alone having RNAi machinery), or expressing RNases in the gut or hemolymph greatly impede the broad application of
dsRNAs as a control (Baum et al., 2007; Whyard et al., 2009; Bachman et al., 2013; Scott et al., 2013; Chistiaens and Smagghe, 2014). Bioinformatics research into the sequence of *F. candida* reveal a high rate of nucleic acid metabolism (158) genes (Timmermans et al., 2007). This could leave to another set of analyses that delve into specific genes that can confer resistance to ingested dsRNAs. Although the sequences used to produce dsRNAs against *D. v. virgifera* and *F. candida* showed numerous matches, no observable negative effects occurred in both gene expression or phenotypic assays, suggesting this type of study can support data obtained from *in silico* analyses.

In conclusion, ingestion of dsRNAs does not induce changes in gene expression or negatively impact life history traits and survival of *F. candida*. This suggests that RNAi crops have negligible impact on this species of collembola. This study developed confirmatory assays for both diet consumption, toxicity of the feeding system, internal detection of dsRNAs under laboratory conditions and standardized a dietary dsRNA toxicity assay and provides a subset of guidelines for testing the risk of newly produced transgenic crops expressing nucleic acid controls. While this report demonstrates negative results, this sets a precedence for our understanding of non-target interactions between biorational control agents and non-target species, while broadening our guidelines for assessing risk of transgenic crops.
Materials and Methods

Insect culture

*Folsomia candida* Willem (Collembola: Entomobryidae) was reared in-house at the University of Kentucky. *Folsomia candida* cultures were provided with ruby red potato *ad libidum* and maintained in plastic microcosms with a plaster of paris-charcoal substrate (80 g Plaster of Paris + 10 g Charcoal +70 ml dH2O; 5 cm D x 4 cm H). Microcosms were covered with paper towel 23 ± 0.5 °C that were seated on saturated paper towels to maintain 100% relative humidity/

Dietary RNAi toxicity assay

To quantify the efficacy of arthropod-active compounds in surrogate non-target species, an *in vivo* dietary RNAi toxicity assay was developed using dsRNA-laced artificial diet. Potassium arsenate (KH2AsO4), a derivative of arsenic, was laced into the diet and used as a positive dietary exposure control. Temporal stability and *in vivo* detection of arthropod-active dsRNAs was measured using stability assays to ensure dsRNAs were ingested by *F. candida*.

Diet preparation

Artificial diet was prepared following Giordano et al. (2010) with slight modifications. Specifically, 0.1 g agar and 1.0 g yeast were dissolved in 5 ml distilled water, respectively and heated to 80 °C, maintained for 15min, and combined. Prior to solidification, 25 µl dsRNA solutions (15 µg/µl) were incorporated into a 200 µl yeast-agar mixture. The final concentration of dsRNA in the artificial diet was 1.875 µg/µl. When cooled, diet was poured into a 48-well plate, allowed to solidify, and stored at 4°C. In the next phase of the
research, 17 individuals, in average, consumed approximately 3.63 μg of artificial diet in 3 days. Diet was cut into small pieces to ensure complete consumption and avoid fungal contamination. Approximately 3.3 μg artificial diet was supplemented to each microcosm on wax paper. Diet was provided ad libidum every 2.5-to-3 days.

**Potassium arsenate toxicity assay**

Potassium arsenate (Sigma-Aldrich, St. Louis, MO, USA), a known inorganic stomach toxin, was used to test whether the diet assay system is sufficient in determining dietary RNAi toxicity. Two doses, one similar to the doses of dsRNA used in this study (36 μg) and one dose that is nearly 2 and a half fold higher (100 μg) of potassium arsenate were laced into the artificial diet as described above, and the final concentration were 1.8 μg/μl, and 5 μg/μl, respectively. The potassium arsenate toxicity assay was initially designed to run the same length (28 days) with dietary RNAi toxicity assay, using three replicates with ten 10-day old neonate larvae for each replicate.

**dsRNA synthesis**

Gene specific primers containing a T7 promoter sequence were used to generate the dsRNAs, including dsFc, dsDVV, and dsGUS, which are provided in Table 1. The β-glucuronidase (GUS) gene was cloned as performed by Pan et al., 2016. Briefly, GUS was cloned into the pBTA2 plasmid and PCR amplified using GUS specific primers, yielding a 560bp fragment with a T7 polymerase promoter region at the 5’ end of the amplicon (Table 1). All PCR reactions were performed according to Pan et al., 2016; specifically, 50 μl reaction volumes containing 10 μl 5×PCR Buffer (Mg²⁺ Plus), 1.0 μl dNTP mix (10 mM of each nucleotide), 5.0 μl of each primer (10 μM each), and 0.25 μl
of GoTaq (5 u/μl) (Promega, Madison, WI, USA) were run under the following conditions: one cycle of 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 59 °C for 45 s and 72 °C for 1 min; a final cycle of 72 °C for 10 min. PCR products served as the template for dsRNA synthesis using the T7 MEGAscript kit (Ambion, Austin, TX, USA) following manufacturer’s protocol. Purified dsRNAs were resuspended in nuclease-free H2O, quantified with a NanoDrop 2000c UV-VIS spectrophotometer and then stored at -20 °C.

**dsRNA stability assay**

The stability of dsRNAs incorporated into artificial was determined in the presence and absence of *F. candida*. Pieces of diet (3 mm³) were placed into plaster of paris-coal microcosms on small pieces of waxed paper, using four replicates per 12-hour time point. A proportion of diet pieces was harvested every 12 hours across two and a half assay days (0, 12, 24, 36, 48 and 60 h). The resulting diet pieces were transferred to appropriately labelled 1.5mL microcentrifuge tubes containing 100 μl nuclease free H2O. Each diet piece was homogenized using a micropestel to liberate and resuspend the incorporated dsRNAs. Following brief vortexing and centrifugation at 700 g for 5 min, approximately 50-75 μl supernatant was collected, and subsequently analyzed on a 1% agarose gel. Double-stranded RNA stability was quantified by the intensity of the gel band, and the pixel density for each band at each time point was measured using a Bio-Rad Gel Imager (BIO-RAD Inc., Hercules, CA).
Artificial diet consumption and in vivo dsRNA detection

To ensure that *F. candida* consumed the prepared artificial diet, yeast-agar diet was produced as described previously. Two microliters of a 0.1% Nile Blue dye were applied to a 3mm³ yeast-agar diet piece, which was then supplemented to a microcosm containing 20 individuals. Digital photographs of *F. candida* individuals were taken to image the diet filled gut tract of *F. candida* to demonstrate the difference between *en masse* reared individuals and those that fed on stained artificial diet. Positive results indicate a change in gut tract color from brown-to-light blue.

Following positive identification of consumption of diet, dsRNA consumption needed to be positively confirmed from a dietary source. Using two internal dsRNA specific primers for dsFc, RT-qPCR was performed, as described previously, to determine if individuals consumed dsRNA across five total assay days. Internal dsRNA primers are listed in Table 1.

Dietary RNAi toxicity assay

10-day old larval *F. candida* were provided with artificial diet containing either dsGUS, dsFc, or dsDVV, or H₂O as a vehicle control. In order to obtain the amount needed to assess the effects of dsRNAs on the life history traits of *F. candida*, adults were transferred to individual plaster of paris-charcoal microcosms and provisioned with artificial diet and allowed to lay eggs for one day. Once eggs were laid, adults were transferred to a new container, and the diet was removed to reduce fungal contamination. Upon first documentation of neonate presence, fresh artificial diet was supplemented and replaced every three days to further avoid fungal contamination. At day 10, these larvae were selected and transferred to a labelled container representing a replicate of a given
treatment. A total of 17 to 18 individuals were used in one replicate, and 10 replications were used for each treatment. Assays were carried out in the plaster of paris-charcoal (70 g plast of paris:10 g charcoal in 80 mL ddH₂O) containers (D = 5cm, H = 4cm). Each microcosm was saturated prior to use and placed in to large plastic boxes on top of saturated paper towel to ensure 100% humidity. Assays were conducted in darkness at 23 ± 2°C.

Life history traits, specifically fecundity (number of eggs laid), egg viability (egg production), weight, as well as survival were measured. Larvae fed on artificial diet laced with dsRNAs for a total of 28 consecutive days. For each replicate, 3.30 µg diet was used to feed 17 individuals every three days, thus the average consumption of dsRNA by each individual for 28 consecutive days was 3.634 µg (calculated by \[1.875 \mu g / \mu g \times 3.30 \times 10)/17\]). The total number of adults survived at the end of the 28-day feeding test was recorded and the survival rate per replicate was calculated and averaged among treatments. Weight was calculated at the end of each week (seven-day cycle, performed four times) by transferring all individuals from the microcosm to a 1.5mL microcentrifuge tube and weighed. An empty tube was served as a blank to calculate the difference (tube + \textit{F. candida} – tube = total weight gain). It is critical to note the exact same blank tube was used for every weight measurement. Fecundity was measured by transferring adults to a fresh, saturated microcosm to lay eggs for seven days. At the end of seven days, the adults were transferred to another fresh, saturated microcosm and allowed to repeat for the duration of the 28-day study. Eggs were counted every week in each replicate and averaged for each week. Egg viability (hatching rate) was calculated
by the total number of neonates emerged divided by the total number of eggs counted and reported as a percentage.

**Dietary RNAi temporal profile in *F. candida***

*Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)*

RT-qPCR primers for 28S ribosomal RNA (28S rRNA) and v-ATPase A were designed based on the sequences obtained from GenBank (EF192441) and this study, respectively, using a web-based tool, https://www.idtdna.com/Primerquest/Home/Index. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer’s instruction. First-strand cDNA was synthesized from 1.0 μg of total RNA using the M-MLV reverse transcription kit (Invitrogen, Carlsbad, CA) and a random N primer according the manufacturer’s recommendations.

Gene-specific primers (Table 1) were used in PCR reactions (15 μl) containing 5.25 μl of ddH₂O, 7.5 μl of 2×SYBR Green MasterMix (BioRad, Hercules, CA, USA), 4 μM of each specific primer, and 1.0 μl of first-strand cDNA template. The RT-qPCR program included an initial denaturation for 3 min at 95 °C, followed by 40 cycles of denaturation at 90 °C for 10 s, annealing for 30 s at 55 °C, and extension for 30 s at 70 °C. For melting curve analysis, a dissociation step cycle (5 °C for 10 s, and then 0.5 °C for 10 s until 95 °C) was added. Relative expression of v-ATPase A was normalized to a reference gene, 28s rRNA using the \(2^{-\Delta\Delta C_t}\) method (Livak and Schmittgen, 2001). The reactions were set up in 96-well format Microseal PCR plates (BioRad, Hercules, CA, USA) in triplicate. Three biological replicates were conducted for each experiment.
**Temporal profile of RNAi effects in F. candida**

While the toxicity assay focuses on the effect of dietary RNAi at the organismal level, including life history traits and survival rate, this study focuses on the impact at the suborganismal level. Using the same experimental design as the dietary RNAi toxicity assay, v-ATPase A expression was quantified across all treatments and controls. *Folsomia candida* samples were collected every other day for eight total assay days (day 2, 4, 6, and 8) and normalized to day 0 to determine if there are changes in v-ATPase A expression over time when 10-day old larvae were subjected to dsRNAs in artificial diet. Samples were flash frozen in liquid nitrogen at each time point and stored in 1.5 mL microcentrifuge tubes at -80 °C until processed further.

**Statistical analysis**

A one-way ANOVA was used to compare the survival rate, development time, fecundity, hatching rate and the adult body length across different treatments. A two-way ANOVA was used to compare the gene expression dynamics of v-ATPase A under different treatments and time. Due to a non-normal distribution of datasets, the non-parametric Kruskal-Wallis test was adopted to analyze the average percent pixel intensity of the gel band for the dsRNA stability assay. Means were compared with LSD tests at $P < 0.05$. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.
Figures and Tables

Figure 3. Diet consumption assay. Immediately following selection from \textit{en masse} rearing container, \textit{F. candida} individuals were imaged (A). The black triangle indicates the gut tract brown coloration through the translucent body. The blue-gray gut tract in (B) shows the color difference in the gut of individuals following consumption of Nile Blue stained diet.
Figure 4. dsRNA stability in the absence of *F. candida*. dsRNA was laced on to diet and allowed to sit, unperturbed, for three assay days, or 72 hours. Recovered dsRNA was excised from diet and run on a 1% agarose gel. As indicated by gel imaging and gel band desitometry, dsRNA was stable (e.g. no statistical difference in degradation [$F_{2,15} = 0.574$, $P = 0.575$]) through the duration of three assay days.
Figure 5. dsRNA stability assay in the presence of *F. candida*. Similar to the dsRNA stability assay above, this stability assay included *F. candida* feeding on the diet, which was removed every 12 hours over three assay days. dsRNA was recovered as done previously, but recovered dsRNA indicated degradation post 48 hours \([H(3) = 14.201, P = 0.007]\). It is likely that saliva is partially responsible for this degradation.
dsRNA levels were quantified via qRT-PCR following consumption by *F. candida* individuals every 12 hours over three assay days and once daily on the fourth and fifth assay day. This assay indicates that the consumed dsRNA is either degraded or expelled (e.g. excreted) from the collembola over the first-three assay days \([F_{3,28} = 2.911, P = 0.005]\). Days four and five, levels remain equivalent, which is likely masked by the expression of *F. candida* v-ATPase A.
Figure 7. v-ATPase A Expression in F. candida Following dsRNA Consumption. Relative expression levels of v-ATPase A did not significantly change \([F_{3, 12} = 2.426, P = 0.116]\), in F. candida following consumption of 30μg doses (~2.5 μg/individual). Expression was stable over an eight assay day period \([F_{2,12} = 2.44, P = 0.805]\), and the interaction of dsRNA treatment and time did not impact expression \([F_{6, 12} = 2.11, P = .1315]\).
Figure 8. Egg production (Fecundity) of *F. candida* following dsRNA ingestion. Average egg produced over a 28 day assay period. The number of eggs did not differ significantly between dsRNA treatments and the water control \( F_{3, 12} = 3.904, P = .272 \).
Figure 9. Body weight of *F. candida* after dsRNA ingestion. Average weights were calculated at the end of each week over a 28 day assay period. Weights were significantly higher when fed dsRNAs (dsGUS—gray, dsFc—dark gray, dsDvv—light gray) compared to the water control (black) \([F_{3,12} = 33.690, P = <0.001]\).
Figure 10. Survival rate following dsRNA ingestion. *Folsomia candida* survived equally across all treatments over the 28-day assay period [F$_3$, 12 = 5.647, P = .130].
Figure 11. Egg viability following dsRNA ingestion. Eggs laid during the 28-day assay period had an average hatching rate between 95% and 99%, which were not significantly different between treatments and time [$F_{3, 12} = .284$, $P = .997$].
Table 3. Primers used to synthesize dsRNAs, for gene expression analyses, and *in vivo* detection.

<table>
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<tr>
<th>Primer Name</th>
<th>Sequence (5’-to-3’)</th>
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</thead>
<tbody>
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<td><em>dsRNA Synthesis</em></td>
<td></td>
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<tr>
<td><em>Folsomia candida vATPase A</em></td>
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<td><em>Diabrotica virgifera virgifera</em></td>
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<td><em>Diabrotica virgifera virgifera</em></td>
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Chapter Three: Future research: environmental fate and risk of plant protected dsRNAs

Abstract

Recent advances in our understanding of gene silencing using RNA Interference (RNAi) has led to the development of the next generation of genetically modified (GM) crops expressing double-stranded RNAs targeting a specific pest. The information presented in this chapter respectfully describes the areas of study/interest that environmental risk assessment needs to pursue for a more comprehensive understanding of RNAi crops and how they interact with the biotic components in the field. The main areas of focus deal with concerns of the mode of action (MoA) of insecticidal dsRNAs, the environmental fate of dsRNA released from GM crops, how the RNAs could be profiled, specifically with regards to retained stability and insecticidal activity of dsRNA. I also discuss the adaptation of latter tier risk assessment for short- and long term studies that are necessary to show negligible negative impact on the rhizosphere. This chapter ends with a description of possible technical challenges associated with the described future work, but provide some recommendations to alleviate these pitfalls.
Introduction

As insecticide-resistant pest insect populations adapt to current control methods, novel biorational control agents have been developed to circumvent this evolving conundrum. Coleoptera (beetles and weevils), an order of insects that are the focus of much research, are some of the most serious pests in agriculture. Coleopterans have been managed, historically, through the application of chemical insecticides and physical changes to crop fields (e.g. crop rotation), however, have proven difficult as increasing awareness of environmental toxicity and the evolution of resistance to pesticides is more evident. Implementation of transgenic crops, such as corn, cotton, potato, rice and tobacco have partially reduced the application of chemical insecticides (Phillips, 2008, Xu et al., 2015). The transgene used to genetically modify these crops are primarily from *Bacillus thuringiensis* (Bt) crystalline toxin. *Bacillus thuringiensis* is a soil dwelling bacterium, typically found in agricultural and non-agricultural soil types. The specificity and efficacy of this toxin group made it a desirable element to use in genetically engineering crop plants. Despite their usefulness, resistance has been documented and is prevalent in some insect populations, specifically Bt resistant western corn rootworm (WCR), *Diabrotica virgifera virgifera* (LeConte). Rapid adaptation to planting WCR-resistant crops, in tandem with a lack of crop rotation, resulted in a selective pressure for those beetles that were resistant, allowing for expansion of resistant *D. v. virgifera* populations (Gray et al., 2009). The WCR has a history with expansion, seen in the late 1940s associated with minimal crop rotation, which lead to invasions of corn fields in the Midwest by the 1970s, Virginia by the 1980s (Chiang, 1973; Youngman and Day, 1993; Gray et al., 2009), eventually founding a population in Serbia and expanding to 20 other
European countries (Miller et al., 2005; Gray et al., 2009). Methods to control the WCR have been impaired by the physiological adaptation to crops rotated with corn, traditional chemical controls, and more recently, resistance to commercial GM maize (expressing B. thuringiensis Cry3Bb1 toxin). Due to this resistance, novel methods in pest management needed to be developed. As a result, the GM-maize trait DSnf7 (MON 87411; a 240bp dsRNA segment targeting the Snf7 gene of D. v. virgifera) is currently deregulated by the United States Department of Agriculture (USDA) and seeking approval from the US Environmental Protection Agency. It is not yet understood if dsRNA negatively impacts the soil community of invertebrates (particularly microathropods) or microorganisms. This final chapter will briefly cover where the research for RNAi crops should pursue and what are the next logical studies.
Future work

Mode of Action

RNAi has been the focus of numerous publications that have defined the mechanism of action (MOA) of the RNAi pathway, namely the introduction of exogenous, long dsRNAs to a cell, the uptake of the long dsRNA, its processing by Dicer into duplexed siRNAs, spread or further processing of these siRNAs into single stranded segments that are used as guides by RISC to target homologous mRNAs (Yang et al., 2011, Xu et al., 2015, Noland et al., in prep). As the MOA has been defined, the rapid adaptation of this technology from functional genomics research tool to use as a plant incorporated protectant, the mode of action (MoA) is unclear. The difference, here, is that a mechanism of action is a description of events at the molecular level, whereas the mode of action is the impact of a substance that causes cellular level changes (Boobis et al., 2006). Despite the knowledge that gene expression is reduced, the scientific community has not defined the impact of RNAi at an anatomical level. It is possible that the effects are often too difficult to visualize without having the insect succumb to the effects before denoting broad physical changes to the cell. For this product to be fully approved, it is imperative to understand, even if rudimentary, what is occurring anatomically to the intended target species after challenge with dsRNA.

Environmental fate of dsRNAs and exposure hazards in the soil community

RNAi products (purified, in vitro transcribed dsRNAs) have been tested in several major representative insect species, which helps guide risk decisions (Xu et al., 2015; Pan et al., 2016; Noland et al., in prep). These studies, while greatly helpful, do not include all possible interactions in each surrogate species’ habitat. This type of study can only be
done under realistic field conditions using dsRNA expressing corn. Due to the conserved nature of the RNAi pathway and the fundamental targeting of housekeeping genes, soil microarthropods could be at risk of non-target gene knockdown as these microarthropods exist in the same habitat as the WCR larvae. As these organisms occupy similar space, they likely interact directly with the plant during growth and postharvest during plant degradation. Dubleman et al. (2014) explain that bacteria likely breakdown dsRNAs in soil, yet the study conducted offers this explanation without confirming microbial involvement at any point. There are, in fact, no published studies on bacterial interactions with dsRNAs. Several reports offer observations of increased numbers of bacteria during exposure of genomic DNA of crop plants in soil and bacterial nucleases aid in DNA degradation, not fungal nucleases (Blum, Lorenz and Wackernagel, 1997; Levy-Booth et al., 2007; Gulden et al., 2005; Arriano et al., 2010; Iida, Kawaguchi, and Nakayama, 2006). Other microbes might be negatively impacted by dsRNAs. It is known that dsRNAs can induce an RNAi response in fungi, known as quelling. Therefore, it is possible that dsRNAs could inhibit growth or performance of fungi when bacteria increase in number when metabolizing nucleic acids in soil (Blum, Lorenz and Wackenagel, 1997; Dubleman et al., 2014). This leaves a sizeable knowledge gap for environmental risk assessment for genetic-based pesticides in regards to agricultural systems. Thus, the goal of future studies should examine the interactions of dsRNA, both from purified (in vitro transcribed and exogenously applied) and plant-derived (leachate) sources with microarthropods and microbes. As with Cry-toxin found in exudate from Bt-maize plants, as well as transgene survival in post-harvest plant tissue (Douville, et al. 2007; Saxenaa, 2002; Clark et al., 2005), dsRNAs leached into soil have an increased
possibility of prolonged half-life, which could possibly lead to an increased possibility of non-target interaction. Extended half-life could lead to partially degraded forms of dsRNAs that give rise to guide sequences that potentially expand the targeting possibility to a wider range of non-target hosts, ultimately leading to knock down of other species. Unfortunately, it is not yet known if plant protected dsRNAs in soil change the dynamics of soil communities of microarthropods, let alone indicate potential effects of plant tissue increasing dsRNA half-life. Dubleman et al. (2014) showed that naked dsRNAs (not exuded by plant tissue or envelopment of any kind) became undetectable in agricultural soils within 48 hours. These results provide no description of dsRNA protection and do not replicate real world conditions. One observation was an increase in the observable counts of bacteria, but do not incorporate microarthropods into the study, which could potentially encounter plant-protected dsRNAs prior to microorganisms. Microarthropods could also alter the total half-life and concentration of dsRNA at a different rate compared to a system without microarthropod presence. To circumvent this knowledge gap, studies that need to be performed are those that seek to further understand the route of dsRNA from the plant (at any stage of growth and through plant residuals post-harvest), how much dsRNA is released from the plant, and if there are changes to microbial and invertebrate communities. These kinds of knowledge gaps are critical, as this information is not only unique to this chapter, but is also a source of scrutiny by the US EPA. In the minutes released from the 2014 Scientific Advisory Panel (SAP), Questions 4-through-7 ask for specific information regarding environmental fate, routes of exposure to NTOs, unintended effects and NTO testing, as well as the SAP meeting in 2016, where questions regarding NTO exposure to dsRNA from the plant or exogenously
applied dsRNA were still not answered fully. While some of these questions have been answered, I will briefly describe the possible studies that can be performed to satisfy these questions.

**Possible studies**

*Environmental Fate and modeling*

These studies, while difficult to conceptualize due to the vast nature of samples that could be taken and the time necessary during the year, or over one or more harvest cycles, can be performed after the product is commercialized. Using certain technologies that have been used in past experiments (Armstrong et al., 2013; Dubleman et al., 2014), QuantiGene analysis can be used to demonstrate the persistence of plant protected dsRNAs in soil. This could be performed using latter tiers (II and III) of environmental risk assessment framework by assessing dsRNA half-life in both greenhouse and agricultural soils. Information here could be used to produce a model to determine rate at which dsRNAs are exuded during growth, post-harvest (from residuals) and during decay (plant rotting/turnover). At all key intervals, dsRNA can be isolated and purified to be fed to *D. v. virgifera* larvae to assay for retained insecticidal activity. If, at any point, the insecticidal activity of the dsRNA decreases, or if the amount of dsRNA is not in high enough concentrations to detect, the model could predict, with a higher level of certainty, that a range of activity and detection is only viable for a given time frame. This information could be used to construct a mathematical model of environmental fate.

*Non-target soil microarthropod and microbial community interactions*

Plant incorporated dsRNA leakage into the soil will likely be the source of the highest rate of exposure during the post-harvest and plant degradation stages. This set of
studies should utilize buried plant parts/residuals (stalks, roots, leaves) that express dsRNAs and exogenously applied dsRNAs. Impacts on microarthropod communities (changes in mite and collembola numbers) should be assessed using techniques that target the soil and quantify by counts taken via Tullgren Funnel isolation. This process uses a wire mesh screen that is placed in a narrow aluminum funnel seated in a well under a heating element (light source). The heat released from the light source drives the invertebrates in the soil downwards through the screen, allowing them to be collected in a container under the funnel that is usually filled with ethanol. Sampling soil invertebrates in this manner allows for both short- and long term analyses of dsRNA exposure. Here, if an impact is to be detected, dsRNA treated samples would be compared to samples that were from an isogenic variety of RNAi crop. Impacts seen would be in broad categorical representations of various soil taxa, such as counts of family level representatives of collembola, mites, spiders, insects, annelids, nematodes, etc. Information from these studies will provide evidence for which soil fauna members are potentially sensitive to dsRNA, or impacted by the changes associated with the presence of dsRNA in soil.

The soil microbial community can be analyzed by a few different methods. Few microbes from soil can be cultured, a limiting factor in this study. While a small subset can be cultured (1%), soil samples can be taken to isolate cultureable bacteria and fungi. Pure isolates of these microbes should be sequenced to reveal their identity. Each pure isolate can be subjected to dsRNA in two ways, by dsRNA incorporated into the agar substrate, or like an antibiotic resistance test, having dsRNA applied to punch out rings in the culture substrate. Microbes would either grow well/normally in the presence of dsRNA, or be mild, moderately, or highly inhibited in the presence of dsRNA if
incorporated into the agar. Zones of inhibition would be visualized (if present) around the punched-out regions of substrate if the microbes would be sensitive to the presence of dsRNA. While this study can be performed over a short period of time (dependent upon culturing time of each microbe), metagenomics and PLFA-analyses are powerful methods for determining the impact of dsRNAs on the remaining 99% of unculturable microbes in soil samples (Berstein et al., 2017). PLFA (phospholipid fatty acid)-analyses take advantage of the phospholipid fatty acids that are a main constituent of cellular membranes in microbes. This technique utilizes certain markers, or fatty acid derivatives, that are only found in certain groups of microbes, allowing for a broader identification of the microbes present in the soil. Changes in the relative frequency of certain fatty acid markers can lead to the conclusion that certain categories of microbes are possibly affected by the presence of dsRNAs. While PLFA analyses give rise to broad changes, metagenomics can be utilized to further determine the microbes affected by dsRNAs. It has been proposed that bacteria counts will likely increase in the presence of extra nucleic acids, which is like that described by Dubleman et al. (2014). However, this change is likely short-lived, as the consumption or degradation of dsRNA would be linked to the short generation time between bacteria generations. While this change in bacterial titer might increase, it is possible to see a decrease in microbial diversity overall due to certain microbes outcompeting others. Those bacteria types that are readily able, or more specifically, capable of utilizing the exogenous source of nucleic acids as carbon and nitrogen sources would be more likely to succeed. This growth in bacterial density can modulate microbe-microbe interactions through direct competition for resources and the production of anti-microbial agents during competition. Fungi, specifically yeast—the
typical food source for collembola, could be impacted by dsRNAs directly, as quelling in fungi can impact gene expression through non-target knock down. The interactions between the changing microbial community in a short window of time could have longer term impacts on the microarthropod community. This could lead to poor performance, and even death, in neonatal microarthropods through the first instars due to the lack of typical food sources.

**Significance and conclusion**

As ‘genetic pest controls’ will be on the market in the coming years, it is important to develop framework to assess risk beyond direct effects between the target and the plant. When implemented, this framework will apply the existing biotechnology risk assessment framework to make predictions on risks of *in planta* RNAi on key soil microarthropods and major microbial constituents in the soil. Current perceptions of risk assessment do not focus on realistic scenarios with dsRNA degradation under environmental conditions or soil invertebrate interactions. Additional risk testing will provide critical information for assessing exposure risks of plant exuded dsRNAs on important soil dwelling organisms. The microbial community, while critical in the interactions in the soil environment for the health of plants, is often left out of these analyses, thus this provides a platform for incorporating microbes into risk analyses and decisions. The future work presented in this chapter describes the studies that need to be performed as the knowledge gap still exists regarding the impact on the soil community.
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131. Park, W., J. Li, R. Song, J. Messing and X. Chen. 2002. CARPEL FACTORY, a dicer homology, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Current Biology 12, 1484-1495


Vita

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M.Sc.—Biological Sciences; Molecular Biology, Western Illinois University, GPA: 3.88
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Professional Employment


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2014 Graduate Teaching Assistantship. Horticultural Entomology. University of Kentucky, Lexington, KY.
2012 Plant-Insect & Insect Genomics Laboratory Manager, Department of Biological Sciences, Western Illinois University, Macomb, IL
2011-2012 Graduate Teaching Assistant, Department of Biological Sciences, Western Illinois University, Macomb, IL
2011-2012 Plant-Insect & Insect Genomics Laboratory Manager, Department of Biological Sciences, Western Illinois University, Macomb, IL
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Professional Affiliations

*Beta Beta Beta*—Biological Honors Society, Western Illinois University, Gamma Iota Chapter
*American Physiological Society*—Kentucky Branch, University of Kentucky
*Entomological Society of America*—Biochemistry, Physiology and Toxicology Group
*Ohio Valley Entomological Association*—University of Kentucky Chapter
**Gamma Sigma Delta**—International Agricultural Honors Society, University of Kentucky University of Kentucky Chapter

**Omicron Delta Kappa**—National Honors Society Nomination, University of Kentucky

**Society of Industrial Microbiology and Biotechnology**—Illinois Chapter for Professionals

**American Society for Microbiology**—Illinois Chapter for Professionals

### Grants and Scholarships

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<td>the pinworm <em>Leidynema appendiculata</em>.</td>
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### Manuscripts, Publications and Protocols

#### Manuscripts Submitted or In Preparation


**demonstrates negligible impacts on *Folsomia candida* (Collembola: Isotomidae) gene expression and life history traits. In preparation for Scientific Reports**

#### Published Manuscripts

