Food Webs 9 (2016) 46–54

Contents lists available at ScienceDirect

Food Webs

journal homepage: www.journals.elsevier.com/food-webs

An exposure-based, ecology-driven framework for selection of indicator species for insecticide risk assessment

Kelton D. Welch *, Jonathan G. Lundgren

United States Department of Agriculture — Agricultural Research Service, North Central Agricultural Laboratory, 2923 Medary Avenue, Brookings, SD 57006, USA

ARTICLE INFO

Article history:
Received 8 September 2015
Accepted 28 February 2016
Available online 2 March 2016

Keywords:
Trophic transfer
Food webs
Biodiversity
Natural enemies
Detritivores
Lasius neoniger

ABSTRACT

In the current "tiered" paradigm for evaluating risks of insecticidal products, one of the first decisions that must be made is the selection of indicator species to be used in toxicity assays. However, as yet, no formal system has been developed to determine whether proposed indicator species are relevant to the ecology of the crop system where the product will be released. Here, we propose a protocol that provides information on the ecology and trophic linkages of organisms within agro-ecosystems, and demonstrate its implementation within maize agro-ecosystems, which have been a major focus of recent insecticidal developments. We use molecular gut-content assays and network analysis to identify species that are likely to be exposed to plant-incorporated products, and that likely have important functional roles in interaction webs in the maize ecosystem. The vast majority of arthropod abundance was found in the soil (97% of specimens per m^2 were found in the soil column). Only nine of the 382 morphotaxa met all three of the ecological criteria (high abundance, corn consumption, degree of connectedness within the network) for inclusion as indicator species, only one of which, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), has routinely been considered in risk assessment. Ecological data collected in studies such as this one can be used to ensure that insecticide risk assessments are ecologically relevant. We advocate the use of large-scale field bio-inventories, combined with molecular gut-content assays and ecological network analysis as regular components of the preparation and design phases of all future risk-assessment programs.

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1. Introduction

Ecological risk-assessment programs aim to ensure that biodiversity and ecological dynamics in agricultural landscapes are preserved and protected from unsustainable disturbances by agronomic practices. However, developing and implementing programs that meet this ostensible goal are hampered by the inherent challenges in identifying and measuring important ecological processes, and in identifying which organisms can serve as appropriate indicators for those ecological processes. In most risk-assessment programs, risk is regarded as comprising two elements: hazard and exposure (National Research Council Committee on the Institutional Means for Assessment of Risks to Public Health, 1983). Hazard refers to a defined adverse effect of a product on an organism. Exposure refers to a measure of the amount of contact with the product in the field. Risk is considered to exist where both hazard and exposure can be demonstrated through a series of prescribed tests. Current protocols arrange these tests in a series of "tiers," in which the hazards of a product for a group of indicator species are evaluated in a succession of tests with increasing levels of ecological complexity (García-Alonso et al., 2006). For example, a risk assessment may begin with isolated organisms in laboratory assays, proceeding to tri-trophic assays in microcosms, before finally concluding with diversity assays in field cages or open field plots. However, later-tier testing is typically only considered necessary if early-tier testing reveals a physiological hazard to non-target organisms (García-Alonso et al., 2006). In effect, ecological dynamics are evaluated mainly for their potential to mitigate observed physiological hazards to indicator species, rather than for the susceptibility of these dynamics to potential disturbances from the product or practice. As a consequence of this, many authors have raised concerns with the ecological relevance of risk-assessment practices (Obrycki et al., 2001; Andow and Hilbeck, 2004; Andow et al., 2013; Lundgren and Duan, 2013). All phases of the risk-assessment process would benefit from being driven by ecological principles, and ensuring that measured indices are ecologically relevant.

Current risk-assessment practices can be enhanced by incorporating ecological principles earlier in the risk-assessment process, even before the first tier of risk-assessment tests are conducted. The first decision point in any risk-assessment protocol is the selection of indicator species to be used for testing. This step is crucial, as hazards are often species-specific, and the likelihood of discovering a hazard is thus contingent on which species are tested. Typical risk-assessment practices use an informal process of species selection based on informal heuristics

* Corresponding author at: Department of Plant Science, South Dakota State University, Brookings, SD 57007, USA.
E-mail address: keltondouglaswelch@gmail.com (K.D. Welch).

http://dx.doi.org/10.1016/j.fooweb.2016.02.004
2352-2496/Published by Elsevier Inc.

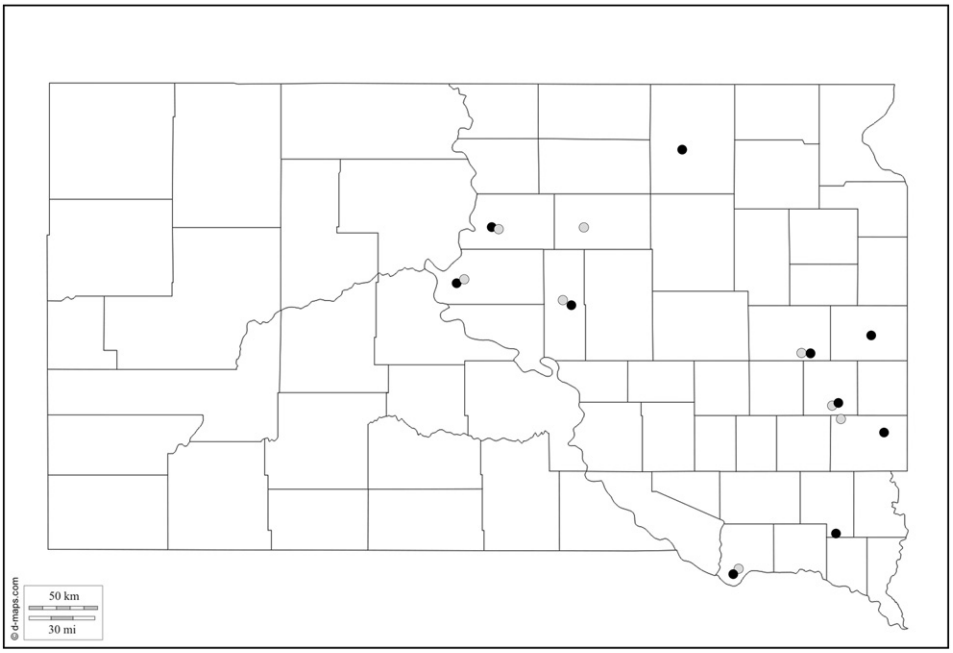

Fig. 1. A map of the U.S. state of South Dakota, with all collection sites for this study marked. Dark circles = sites sampled in 2013; light circles = sites sampled in 2014. The map is borrowed from http://d-maps.com/carte.php?num_car=19977&lang=en (used with permission).

and general information about a species' ecology, phylogeny and interest to society (García-Alonso et al., 2006) (Bachman et al., 2013). This usually involves qualitatively selecting organisms that represent different functional groups (e.g., pollinators, predators, and detritivores) or that have special relevance to producers or society (e.g., honey bees and aquatic insects). Additionally, we know of at least one attempt that has been made to institute a more rigorous system of risk assessment based on large-scale ecological data (Andow et al., 2013). Here, we propose a straightforward, data-driven method for quantifying various ecological aspects of organisms within an agro-ecosystem where transgenic or other plant-incorporated insecticidal products will be used. Our proposal builds on these previous systems by (1) formalizing a simple, practical process that can be integrated into the existing risk-assessment apparatus with minimal alteration, and (2) incorporating modern, high-throughput analytical techniques to generate multi-faceted matrices of ecological attributes that can aid in the identification of organisms of primary relevance to the function of ecosystems and the movement of insecticidal products within them. This method will allow scientists to increase the transparency with which indicator species are selected for risk-assessment studies based on their importance within the target ecosystem, and improve the ecological relevance of the tests conducted.

Current risk-assessment protocols have resulted in somewhat uneven coverage of different components of agro-ecosystems. While pollinators and natural enemies have featured prominently in risk-assessment studies, some groups of organisms, such as detritivores and other soil-dwelling organisms, have been less thoroughly investigated from an ecological perspective (Wagg et al., 2014), and are consequently under-represented in the risk-assessment literature. Recent trends in agriculture and ecology are increasingly recognizing the importance of soil health for crop production (Bender and van der Heijden, 2015) and ecosystem functioning (Gessner et al., 2010). The major role that soil food webs play in cycling nutrients and maintaining soil health, combined with their high sensitivity to land-management practices (de Vries et al., 2013), make it essential that soil dynamics be investigated more thoroughly, and their interactions with insecticidal products be evaluated. However, soil communities are inherently difficult to study, and researchers must rely on novel approaches to collect and analyze data from these communities.

The emergence of modern research methodologies and analytical tools makes possible an evaluation of ecosystems with detail and efficiency. Molecular gut-content methodologies, such as polymerase chain reaction (PCR) and enzyme-linked immuno-sorbent assay (ELISA), provide access to previously inaccessible data on the diets of organisms in the field, thereby accommodating large-scale evaluation of trophic webs (Sheppard and Harwood, 2005; Juen and Traugott, 2007; Weber and Lundgren, 2009), which are important components of organisms' ecology and a primary vehicle for transmission of insecticides through ecological communities. Additionally, techniques for network analysis that were pioneered for use in sociological and economic studies are increasingly being used to characterize associations among organisms in ecological networks (Proulx et al., 2005; Fath and Grant, 2007; Blüthgen, 2010; Lundgren and Fausti, in press). This facilitates visualization of the large-scale properties of entire ecosystems, and identification of species that play significant ecological roles via the frequency and strength of network interactions with other organisms. These two modern ecological techniques in combination with traditional approaches for creating bio-inventories can provide the foundation for a robust framework to guide risk-assessment for plant-incorporated insecticidal agents.

Here, we demonstrate the fundamentals of ecology-based indicator-species selection, using a common agroecosystem from the Great Plains region of the United States. Maize, *Zea mays* L. (Poales: Poaceae), is one of the most widely-grown crops in the world, and its primary pest, the Western corn rootworm, *Diabrotica virgifera* LeConte (Coleoptera: Chrysomelidae) (hereafter "rootworm"), has been a major focus of commercial insecticidal transgenic products (e.g., Rice, 2003; Baum et al., 2007; Bolognesi et al., 2012). We collect field data on the distributions and trophic links of arthropod taxa associated with maize fields over a 95,000 km^2 area in one U.S. state, South Dakota. Although we focus on one example system, the principles outlined here can be readily adapted to other crop systems, regions, and other types of insecticidal agents.

Table 1
A list of the 49 morphotaxa from maize fields in South Dakota, USA that met at least one of three ecological criteria indicating relevance to insecticide toxicity assays ("maize feeder", "highly connected (number of networks)" and "widespread"). Morphotaxa are listed by the number of criteria met. Dark shading = morphotaxa that met all three criteria; light shading = morphotaxa that met two of the three criteria; no shading = morphotaxa that met only one criterion.

Morphotaxon	Functional group	Primary habitat domain(s)	Criteria met
Isotomidae sp. 01 (Collembola)	detritivore	epigeal, soil	· maize feeder · highly connected (4) · widespread
Lasius neoniger Emery (Hymenoptera: Formicidae)	predator	foliar, epigeal, soil	· maize feeder · highly connected (3) · widespread
Mesostigmata sp. 02 (Acari)	predator	soil	· maize feeder · highly connected (3) · widespread
Tetragnatha laboriosa Hentz (Araneae: Tetragnathidae)	predator	foliar	· maize feeder · highly connected (3) · widespread
Oripodoidea (Acari: Oribatida)	detritivore	soil	· maize feeder · highly connected (2) · widespread
Dictyna sp. 01 (Araneae: Dictynidae)	predator	foliar	· maize feeder · highly connected (1) · widespread
Japygidae (Diplura)	predator	soil	· maize feeder · highly connected (1) · widespread
Orius insidiosus (Say) (Hemiptera: Anthocoridae)	predator	foliar	· maize feeder · highly connected (1) · widespread
Rhopalosiphum padi (L.) (Hemiptera: Aphididae)	herbivore	foliar	· maize feeder · highly connected (1) · widespread
Entomobryidae sp. 01 (Collembola)	detritivore	epigeal, soil	· maize feeder · highly connected (4)
Lygus sp. (Hemiptera: Miridae)	herbivore	foliar	· maize feeder · highly connected (2)
Coleomegilla maculata De Geer (adults) (Coleoptera: Coccinellidae)	predator	foliar	· maize feeder · highly connected (1)
Hippodamia convergens Guérin-Méneville (Coleoptera: Coccinellidae)	predator	foliar	· maize feeder · highly connected (1)
Lepidocyrtus sp. (Collembola: Entomobryidae)	detritivore	epigeal	· maize feeder · highly connected (1)
Elaphropus sp. (adults) (Coleoptera: Carabidae)	predator	epigeal, soil	· highly connected (4) · widespread
Phthiracaridae (Acari: Oribatida)	detritivore	soil	· highly connected (3) · widespread
Staphylinidae sp. 20 (adults) (Coleoptera)	predator	foliar, epigeal	· highly connected (3) · widespread
Frankliniella sp. 02 (Thysanoptera: Thripidae)	herbivore	foliar	· highly connected (2) · widespread
Anthicidae (larvae) (Coleoptera)	predator	soil	· highly connected (1) · widespread
Frankliniella sp. 01 (Thysanoptera: Thripidae)	herbivore	foliar	· highly connected (1) · widespread
Galumnidae (Acari: Oribatida)	detritivore	soil	· highly connected (1) · widespread
Mesostigmata sp. 01 (Acari)	predator	soil	· highly connected (1) · widespread
Chrysoperla carnea Stephens (adults) (Neuroptera: Chrysopidae)	herbivore	foliar	· maize feeder
**Diabrotica barberi* Smith & Lawrence (adults) (Coleoptera: Chrysomelidae)	herbivore	foliar	· maize feeder
**Diabrotica virgifera* LeConte (adults) (Coleoptera: Chrysomelidae)	herbivore	foliar	· maize feeder
Hemerobiidae (adults) (Neuroptera)	predator	foliar	· maize feeder
Scarabeidae sp. 01 (larvae) (Coleoptera)	herbivore	soil	· maize feeder
Staphylinidae sp. 01 (adults) (Coleoptera)	predator	soil	· maize feeder
Diptera (larvae)	detritivore	soil	· highly connected (3)
Empididae sp. 01 (adults) (Diptera: Brachycera)	predator	foliar, epigeal	· highly connected (3)
Elateridae (larvae) (Coleoptera)	herbivore	soil	· highly connected (2)
Hypoponera sp. (Hymeoptera: Formicidae)	predator	epigeal, soil	· highly connected (2)
Mermessus sp. (Araneae: Linyphiidae)	predator	foliar, epigeal	· highly connected (2)
Pauropoda (Myriapoda)	detritivore	soil	· highly connected (2)
Tennesseellum formica (Emerton) (Araneae: Linyphiidae)	predator	epigeal	· highly connected (2)
Aeolothripidae (Thysanoptera)	predator	foliar	· highly connected (1)
Aeolus sp. (adults) (Coleoptera: Elateridae)	predator	epigeal	· highly connected (1)
Cecidomyiidae (Diptera: Nematocera)	herbivore	foliar	· highly connected (1)
Coccinellidae (larvae) (Coleoptera)	predator	foliar	· highly connected (1)
Delphacidae sp. 01 (Hemiptera)	herbivore	foliar, epigeal	· highly connected (1)
Dolichopodidae sp. 01 (adults) (Diptera: Brachycera)	predator	foliar, soil	· highly connected (1)

(*continued*)

Morphotaxon	Functional group	Primary habitat domain(s)	Criteria met
Hypogastruridae sp. 01	detritivore	soil	· highly connected (1)
Malloewia sp. (adults) (Diptera: Chloropidae)	herbivore	foliar, epigeal	· highly connected (1)
Mycetophilidae sp. 01 (adults) (Diptera: Nematocera)	detritivore	foliar, epigeal, soil	· highly connected (1)
Psocoptera	detritivore	foliar, soil	· highly connected (1)
Tettigoniidae (Orthoptera)	herbivore	foliar	· highly connected (1)
Thripidae sp. 04 (Thysanoptera)	herbivore	foliar	· highly connected (1)
Anthicidae sp. 01 (Coleoptera)	predator	foliar	· widespread

*Denotes the focal pest species.

2. Materials and methods

We present the results of a field bio-inventory taken in a maize ecosystem in South Dakota, USA, combined with molecular gut-content assays and network analyses based on patterns of rank-abundance, to identify relevant indicator species. We follow four steps: (1) perform field collections across habitat domains (foliage, soil, ground surface) using standardized sampling techniques and intensities to characterize the composition of ecological communities in the target crop and identify abundant taxa; (2) perform molecular gut-content analysis on abundant taxa to identify trophic links to the target crop; (3) perform network analysis to identify species that show ecological connections with other species, with particular emphasis on the ecological connections of species that are trophically linked to the target crop; and (4) compile a list of species that display both high potential for exposure to plant-incorporated insecticidal agents (via molecular gut-content analysis) and high connectedness within rank-abundance networks (via network analysis).

2.1. Bio-inventory of maize arthropods

As a first step toward understanding the ecological dynamics of insecticide risk, a bio-inventory was conducted in untreated fields of maize (*Z. mays* Linnaeus, Poaceae: Andropogoneae) in the major maize-growing region of South Dakota, USA, during the growing seasons of 2013 and 2014. Each year, arthropods were collected from private farms spanning the eastern half of South Dakota (Fig. 1; approximately 95,000 km^2), in which non-Bt maize was grown without insecticidal seed treatments or sprays. Collections were conducted at each farm during two sample periods: once early in the season (when the majority of maize plants had reached the V4 stage), and once during anthesis. These two sample periods were chosen as pivotal time periods for ecosystem services in annual field crops: the early season as a time of crop colonization and establishment by pests and natural enemies (Settle et al., 1996; Landis and Van der Werf, 1997; Harwood et al., 2009), and anthesis as a time of high potential for exposure to plant-incorporated products due to the abundance of maize pollen as a food resource (Lundgren et al., 2004; Peterson et al., 2009, 2010). Sampled fields were at least 4 ha in size. One field from each of 10 farms was sampled in 2013, and one field from each of eight farms was sampled in 2014, yielding 18 site-years as replicates.

At each sample date, we sampled arthropods in three broadly-defined habitat domains — (1) the foliar domain, including organisms in and on the aboveground parts of the maize plants; (2) the soil-surface, or epigeal, domain; and (3) the subsurface soil domain — using sampling protocols modified from Lundgren and Fergen (2010). All samples were taken at least 10 m from the edge of the field, to avoid spillover effects from surrounding habitats.

The foliar domain was sampled using whole-plant searches. At each collection date, 50 maize plants were randomly selected and searched exhaustively for arthropods on leaves, stems, ears and tassels. Thereafter, each plant was cut at the ground level and transported carefully to a white sheet, where additional arthropods were dislodged by shaking and collected by hand. The plant was then carefully dissected to locate arthropods hidden within leaf whorls and inside stem tissue. All arthropods located were hand collected and stored in ethanol for identification and molecular analysis.

The epigeal domain was sampled using quadrats. At each site, arthropods were collected within five quadrat samples, which consisted of 5-min visual searches within 0.25-m^2 quadrat frames by teams of two observers. All arthropods on the soil surface or buried within the upper layers of debris were collected via aspirator and preserved in ethanol for identification and molecular analysis. Quadrats were delimited by metal frames pressed firmly into the soil to prevent any arthropods from entering or exiting the quadrat area during the search period.

The subsurface soil domain was sampled using soil cores. At each site, seven soil cores were collected with golf cup cutters (10 cm diam) to a depth of 10 cm and stored individually in plastic bags. Due to differences in transport times from each of the farms, all soil samples were chilled on ice for 24 h after collection, before being placed in a Berlese funnel apparatus for extraction of arthropods. Arthropods extracted during the first 24 h in the Berlese funnels (i.e. up to 48 h after field collection) were stored in 70% ethanol and stored at −20 °C for molecular gut-content analysis. Arthropods extracted in Berlese funnels over the following 6 d were collected separately and incorporated into faunistic analyses.

All specimens collected during bio-inventories were sorted and identified to the lowest possible taxon (usually family or morphospecies) using a variety of keys and other resources available in the lab, and voucher specimens are deposited in the laboratory's reference collection. Each morphotaxon was assigned to a broad functional group (predator, parasitoid, herbivore, pollinator, detritivore or other/unknown), and a habitat domain based on its pattern of occurrence across all three sampling techniques. All specimens were kept chilled during sorting and identification to preserve molecular remains of gut contents for later molecular analysis.

2.2. Molecular gut-content analysis

In order to determine which arthropods are potentially trophically exposed to maize-incorporated insecticidal products, specimens from representative morphotaxa were assayed for the presence of maize DNA within their gut contents using quantitative PCR (qPCR). Morphotaxa for these analyses were selected based on abundance in bio-inventories of South Dakota maize fields conducted over several previous years (Lundgren et al., 2015; Lundgren and Fausti, in press). DNA was extracted from crushed whole-body specimens using DNeasy tissue extraction kits (QIAGEN, Valencia, California, USA) according to product instructions. All extracted DNA samples were stored at −20 °C, and surface-sterilized in bleach prior to qPCR assay to eliminate false positives due to *Z. mays* DNA from the environment contaminating the outer body of the specimens. A pair of *Z. mays*-specific PCR primers developed previously (Lundgren and Weber, 2010) was selected for use in these assays at 150 nM concentration. These primers amplify a 141-bp region of the COI mitochondrial gene of *Z. mays*.

The presence of maize DNA in arthropod gut contents was assessed via 25-μL PCR reactions containing 12.5 μL 2× Brilliant SYBR Green qPCR Master Mix (Qiagen), 1 μL of each 150-nM primer solution, 1 μL

template DNA and 8.5 μL molecular-grade water (Sigma-Aldritch, St, Louis, Missouri, USA). Well-to-well variation in detection was normalized using the ROX dye. Extractions were amplified using an MX3000P qPCR system (Stratagene, La Jolla, California, USA), using the following temperature-cycle protocols: 95 °C for 10 min, followed by 50 cycles of 94 °C for 15 s, 54 °C for 30 s, and 72 °C for 30 s. Fluorescence was recorded at 492 nm (for SYBR Green) and 582 nm (for ROX) during the annealing step of each cycle, and fluorescence thresholds were adjusted manually to account for sample-specific background fluorescence. To generate a dissociation curve for each specimen, all reactions were subject to an additional temperature-cycle protocol: 95 °C for 1 min, followed by a gradual increase from 55 °C to 95 °C at a rate of 0.2 °C/s, monitoring fluorescence continuously.

To supplement previously-published evaluations of the target-specificity of the primers (Lundgren et al., 2009), samples of maize leaf tissue were collected from study fields and from maize plants grown in the greenhouse, along with tissue samples from 39 common weed species and other plants found in and around study fields. Plants were identified to genus or species by Dr. Randall Anderson (USDA-NCARL, Brookings, SD). Additionally, DNA was extracted from the legs of 130 arthropod species (including 80 voucher specimens from this study) and tested for cross-reactivity.

2.3. Network analysis of abundance data

In order to evaluate the structure of ecological interactions within maize arthropod communities, network analysis was used to identify patterns in rank-abundance of arthropod taxa across all site-years in the maize bio-inventory. In network analysis, an important metric is the *degree* of a given taxon, which in this case is the number of significant rank-abundance correlations (as determined by Spearman rank-correlation) between the focal taxon and other taxa in the network (De Nooy et al., 2011). A rank-abundance correlation between two morphotaxa indicates similar patterns of distribution across samples, suggesting either similarity in underlying ecological variables or a mutualistic or antagonistic interaction. Thus, morphotaxa with high degree share their patterns of regional distribution and abundance with many other species, making them potentially relevant as ecological indicators. Confidence intervals for each pairwise correlation test were generated via resampling (1000 bootstrap samples), and the degree for each morphotaxon was calculated by counting all correlations that were found to differ significantly from zero. We were particularly interested in the network connections of maize-feeding morphotaxa, as these connections represent potential routes of transfer for plant-incorporated insecticidal products. To evaluate these maize-feeder connections, we considered only the subset of significant correlations that included at least one maize-feeding morphotaxon. A species is listed in Table 1 as "highly connected" within a network if either its degree or its number of connections with maize-feeding taxa is in the upper quartile for that network.

The data were analyzed in three ways for comparative purposes: an overall analysis, a phenological analysis, and a habitat-domain analysis. For the overall analysis, all samples were pooled by site-year (n = 18), with specimen counts converted to densities (e.g., arthropods per m^2) to facilitate combination across habitat domains. Whole-plant samples were converted to density using seeding-rate data provided by the farmers, and quadrat and soil-core counts were converted to density using the surface area of the sampling unit. Because rank-abundance analysis is influenced by both presence and absence data, uncommon taxa sometimes display high mutual rank-abundance correlations due to frequent absences (lowest abundance rank), thereby inflating the degree and apparent ecological connectedness of taxa with repeatedly low rank. To guard against this overvaluing of low-ranking taxa, only taxa that met or exceeded a threshold incidence of 50% of site-years (9 of 18 site-years) were included in this analysis.

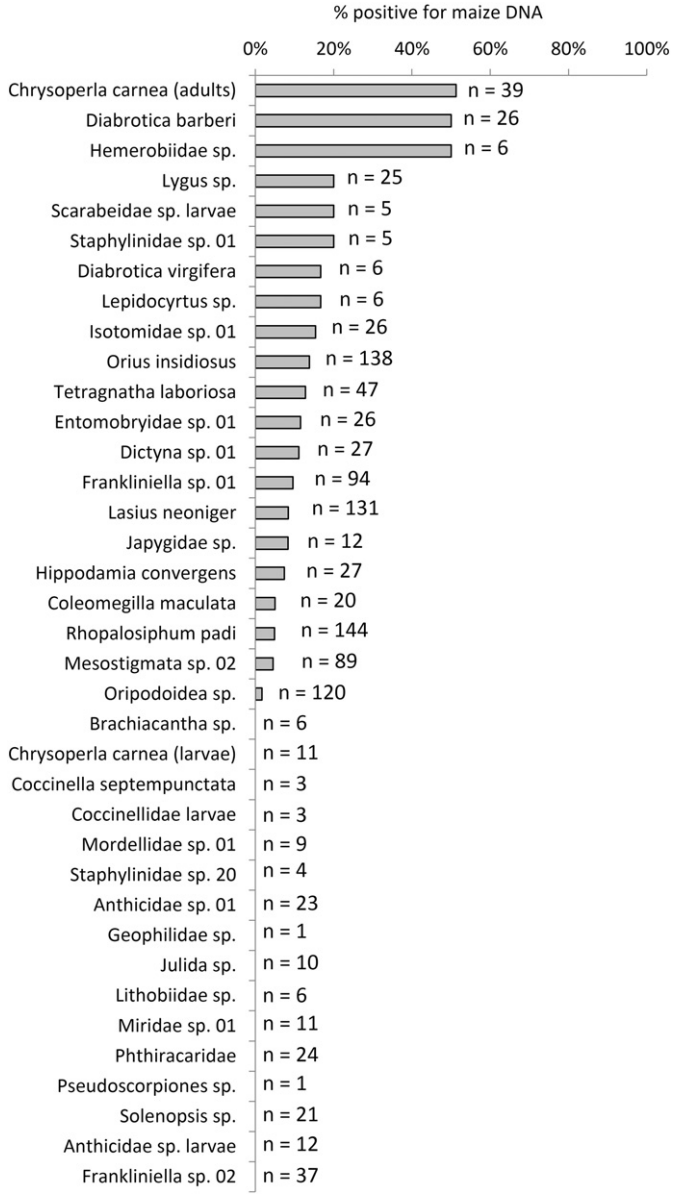

Fig. 2. Proportion of molecular assays in which the DNA of maize was detected in the gut contents of field-collected specimens. Numbers printed beside bars refer to the numbers of specimens tested.

For the phenological analysis, separate networks were constructed for early-season and anthesis collections, using the same analysis techniques as described above. However, partitioning the data into smaller networks yielded lower sample sizes, and fewer morphotaxa were able to meet the 50% incidence threshold. Consequently, the threshold was lowered to presence in 33% of fields (6 of 18 site-years).

For the habitat-domain analyses, separate networks were constructed for each habitat domain (foliar, epigeal and soil), using the same analysis techniques as described above. In these analyses, the threshold incidence for inclusion was lowered to presence in 25% of fields (5 of 18 site-years), to yield networks with at least 30 morphotaxa.

2.4. Final selection of indicator species

We compiled a list of species that met the main ecological criteria for suitability as indicator species, which were (1) widespread in maize fields across the region, (2) trophically linked to maize, and (3) highly connected within ecological networks (either in terms of overall degree, or in terms of specific connections with maize-feeding morphotaxa).

For criteria 1 and 3, we used an upper-quartile heuristic: that is, all species in the upper quartile for proportional incidence across site-years, or for network degree or maize-feeder connections are listed as meeting the corresponding criterion. For criterion 2, any species for which at least 1 specimen tested positive for maize DNA is considered trophically linked to maize.

3. Results

3.1. Bio-inventory of maize arthropods

In bio-inventories in South Dakota maize fields, a total of 12,560 specimens representing 382 morphotaxa were collected across all site-years. Of these morphotaxa, 131 (4636 specimens) were classified as "predators," 71 (3119 specimens) were classified as "herbivores," 30 (73 specimens) were classified as "parasitoids," 26 (3551 specimens) were classified as "detritivores," 6 (15 specimens) were classified as "pollinators," 13 (82 specimens) were classified as "other," and 105 (1085 specimens) were classified as "unknown."

The three habitat domains showed differences in abundance and diversity of morphotaxa. The epigeal domain was inhabited by 191 morphotaxa (4.24 morphotaxa/m^2), comprising 2530 specimens (56 specimens/m^2). The soil domain was inhabited by 158 morphotaxa (79.8 morphotaxa/m^2), comprising 5813 specimens (2936 specimens/m^2). The foliar domain was inhabited by 249 morphotaxa (0.84 morphotaxa/m^2), comprising 4217 specimens (14 specimens/m^2).

Four morphotaxa were collected in all 18 site-years of the analysis: Oripodoidea sp. (Acari: Oribatida), *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae), and *Frankliniella* sp. 02 (Thysanoptera: Thripidae). These morphotaxa were considered "widespread," and were included in the list of candidate taxa (Table 1). Morphotaxa that were collected in at least 14 (80%) site-years were included the upper quartile for incidence, and included the following morphotaxa: Anthicidae larvae (Coleoptera), Anthicidae sp. 01 (adults) (Coleoptera), unidentified coccinellid larvae (Coleoptera), *Dictyna* sp. 01 (Araneae: Dictynidae), *Elaphropus* sp. (Coleoptera: Carabidae), *Frankliniella* sp. 01 (Thysanoptera: Thripidae), Galumnidae (Acari: Oribatida), Isotomidae sp. 01 (Collembola), Japygidae (Diplura), *Lasius neoniger* Emery (Hymenoptera: Formicidae), 2 morphotaxa of predatory mites (Acari: Mesostigmata), Phthiracaridae (Acari: Oribatida), Staphylinidae sp. 20 (Coleoptera), and *Tetragnatha laboriosa* Hentz (Araneae: Tetragnathidae).

3.2. Molecular gut-content analysis

The DNA primers used in this study amplified products from 17 of 39 weed and plant species tested. The *Z. mays* amplicon produced by the primers used in this study can be distinguished from other plant amplicons by melting temperature (T_M = 77.8–78.1 °C for most specimens of *Z. mays*), with the exception of crabgrass (*Digitaria* sp.), kochia (*Bassia scoparia*) and some specimens of giant foxtail (*Setaria faberi*). However, these species were either rarely observed at sample locations, or did not penetrate into maize fields, so false positive errors generated by these species were likely infrequent. No false positives were observed for any of the 130 arthropod specimens tested. We are thus confident that an amplicon with T_M = 77.8–78.1 °C is representative of maize DNA from the gut contents of a specimen, and indicates recent ingestion of maize tissue or ingestion of a maize-feeding herbivore. Taxa that yield positive gut-content assay results are thus likely to be exposed to any transgenic or plant-incorporated insecticidal agents in a maize field, and are thus potentially useful indicator species for risk assessment.

In total, 1201 specimens from 37 morphotaxa were assayed for the presence of maize DNA in gut contents (mean ± se = 32.5 ± 6.8 specimens/morphotaxon). These morphotaxa represent the predator, herbivore and detritivore functional groups. Of these, 21 morphotaxa yielded at least one individual testing positive for maize DNA, including all four morphotaxa that were collected in all site-years. Percent of specimens testing positive within these morphotaxa ranged from 2% up to 51% (overall specimen-wise percent positive was 9.7% across 1201 total specimens tested). Chi-squared analysis revealed significant differences in specimen-wise percent-positive results across functional groups (χ^2 = 13.76, df = 2, p = 0.001), primarily driven by the low percent-positive result for detritivores (χ^2 contribution = 9.37). Nevertheless, the majority of species in each functional group included at least one individual testing positive (78% of herbivore species, 53% of predator species, and 67% of detritivore species), indicating that all functional groups can readily be exposed to plant-incorporated products.

3.3. Network analysis of abundance data

To analyze ecological similarities and interactions within the maize agro-ecosystem, network analyses were conducted to compare patterns of rank-abundance across taxa. In the overall network analysis, 55 of the original 382 morphotaxa met the threshold for inclusion in the network analysis (i.e., incidence ≥50% of site-years). The taxon with the highest degree was *L. neoniger* Emery (Hymenoptera: Formicidae), which was correlated with 13 other morphotaxa. Eleven additional morphotaxa were found to be "highly connected" in terms of overall degree, and five others were also found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Fig. 3).

When only early-season collections were considered, 44 of 283 morphotaxa met the 33% incidence threshold for inclusion in the network analysis. In this analysis, the morphotaxa with the highest degree were Isotomidae sp. 01 and *Tennesseellum formica* (Araneae: Linyphiidae), each with nine significant correlations. Three additional morphotaxa were also found to be "highly connected" on the basis of overall degree, and eight others were also found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Supplementary Figure 1).

When only anthesis collections were considered, 63 of 339 morphotaxa met the 33% incidence threshold for inclusion in the network analysis. In this analysis, the morphotaxa with the highest mean degree were Entomobryidae sp. 01 and Mesostigmata sp. 02, with 11 significant correlations. Ten additional morphotaxa were also found to be "highly connected" on the basis of overall degree, and five others were also found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Supplementary Fig. S2).

When only foliar collections were considered, 41 of 249 morphotaxa met the 25% incidence threshold for inclusion in the network analysis. In this analysis, the morphotaxon with the highest degree was *R. padi*, with six significant correlations. Seven additional morphotaxa were also found to be "highly connected" on the basis of overall degree, and three others were also found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Supplementary Fig. S3).

When only epigeal collections were considered, 31 of 191 morphotaxa met the 25% incidence threshold for inclusion in the network analysis. In this analysis, the morphotaxon with the highest degree was Entomobryidae sp. 01, with 7 significant correlations. Five additional morphotaxa were also found to be "highly connected" on the basis of overall degree, and one other was also found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Supplementary Fig. S4).

When only soil collections were considered, 39 of 158 morphotaxa met the 25% incidence threshold for inclusion in the network analysis. In this analysis, the morphotaxon with the highest degree was Staphylinidae sp. 20, with 10 significant correlations. Four additional morphotaxa were found to be "highly connected" on the basis of overall degree, and four others were found to be "highly connected" on the basis of correlations with maize-feeding taxa (Table 1, Supplementary Fig. S5).

Altogether, 90 morphotaxa met the incidence threshold for inclusion in at least one network analysis, of which 71 morphotaxa met the incidence threshold for inclusion in at least two analyses. However, only one taxon, the ant *L. neoniger*, met the incidence thresholds for inclusion in all six analyses, and it was found to be highly connected in three of those analyses.

The average degree across all morphotaxa in a given habitat domain was significantly higher for the soil network than for either the foliar (Mann–Whitney $U = 385.5$, $p < 0.001$) or the epigeal ($U = 367$, $p < 0.005$) network. No significant difference in average maize-feeder connections was observed across habitat domains.

3.4. Final selection of indicator species

When all of these data are combined, we found 49 morphotaxa that met at least one of the three criteria for consideration in tier-1 insecticide risk-assessment trials (Table 1). These include all morphotaxa that were listed in previous sections as maize-feeding, widespread, and/or highly-connected morphotaxa. Altogether, 9 morphotaxa met all three ecological risk criteria (maize-feeding, highly connected and widespread), 13 additional morphotaxa met two of the three criteria, and 27 morphotaxa met only one of the three criteria.

4. Discussion

Properly assessing the ecological risks of insecticidal products first requires a robust understanding of the underlying ecological dynamics that exist in an agroecosystem. Here we have demonstrated a simple method for incorporating specific ecological information about organisms in an agro-ecological community into the planning and designing stages of a risk-assessment program. We have applied this method using molecular gut-content assays and ecological network analysis to identify a number of taxa that are of potential interest for risk assessment in one important study system, maize fields in the central United States. This data supplements existing criteria used in indicator-species selection, such as phylogenetic relatedness and special economic interests. In some cases, the addition of ecological data confirms the usefulness of taxa already being used as indicator species. For example, two commonly-tested natural enemies, *O. insidiosus* and *Coleomegilla maculata* (Lundgren and Wiedenmann, 2002, 2005; Duan et al., 2007), met at least two of the three criteria used in our study, and *O. insidiosus* met all three. Our study thus confirms the ecological relevance of these common indicator species, and advocates their continued use as indicators.

Our analysis also suggests some ways in which the current indicator-species selection process must be advanced. For example, four of the nine morphotaxa that met all criteria for ecological relevance were arachnids (two spiders, *T. laboriosa* and *Dictyna* sp. 01; and two mites, Oripodoidea and Mesostigmatida sp. 02). Spiders have been the focus of at least one meta-analysis of the effects of Bt crops on spiders (Peterson et al., 2011). However, tier-1 tests for an upcoming commercial product based on insecticidal RNAi were only conducted on insect taxa (Bachman et al., 2013); and to our knowledge, no arachnid taxa have yet been evaluated in conjunction with this product. Given their high potential for exposure, their prominence in the maize ecosystem, their important biocontrol and decomposition services, and their potential to amplify and modify the RNAi machinery, it would make sense to consider arachnid taxa in risk-assessment programs.

The current results also confirm that organisms from all major functional groups can be regularly exposed to plant-incorporated products. Of particular note is that the DNA of maize was detected in the gut contents of soil-dwelling detritivores, including multiple species of springtails and mites, 48 h after being removed from the field. This long waiting period, though unavoidable in our study, is not ideal for the detection of residual DNA within gut contents; thus it is likely that the relatively low rates of maize-DNA detection in detritivores are an underestimate of actual maize-feeding frequency (Fig. 2). In general, interspecies variation in the inherent detectability of gut-content DNA limits the ability to draw comparisons between species or functional groups, although mathematical techniques and laboratory experiments can provide some estimates or comparisons of feeding rates in at least

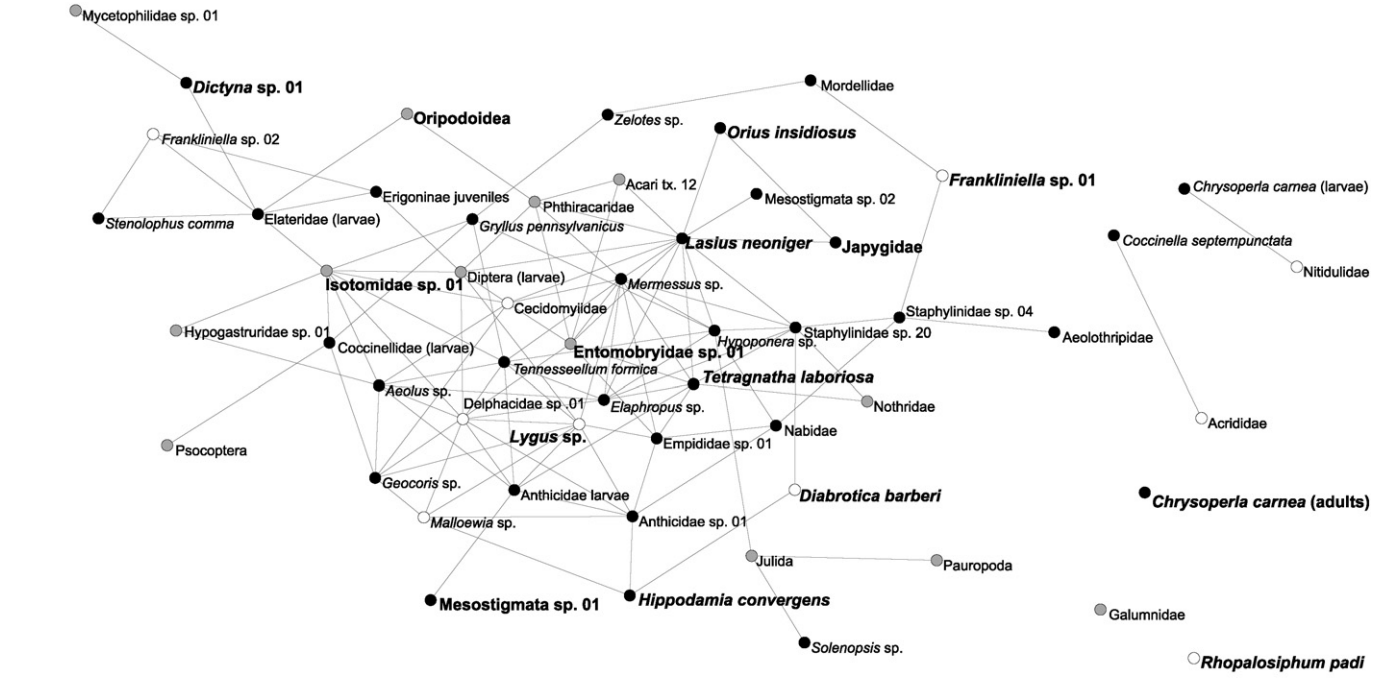

Fig. 3. Network diagram displaying rank-abundance correlations among all morphotaxa that were collected in at least 50% of all site-years. An edge (line) connecting two morphotaxa indicates a positive linear correlation in rank abundance across all site-years. More network diagrams are presented in the online supplementary files. Network diagrams were produced in the freeware program Pajek (version 4.04).

some cases (Naranjo and Hagler, 2001; Greenstone et al., 2014; Welch et al., 2014). However, such comparison is not strictly necessary for the purposes of insecticide risk assessment: rather, simply uncovering potential trophic routes and elucidating their ecological context may be sufficient to identify and prioritize indicator species for tier-1 insecticide tests. For example, there was only one taxon, the ant *L. neoniger*, which met the minimum incidence criterion for inclusion in all network analyses, making it unique as a common factor across all three habitat domains and both phenological periods. This ant was also found to feed on maize (8% of specimens tested positive for maize DNA), and it showed high network connectivity with other maize-feeding taxa in both the early-season and anthesis collection periods. Finally, as abundant ecosystem engineers, *Lasius* ants have previously been shown to have wide-reaching effects on the behavior and populations of other organisms, including aphids (Tegelaar et al., 2013), lady beetles (Oliver et al., 2008), spiders (Mestre et al., 2014) and even corn rootworms (Kirk, 1981). In combination, these three factors suggest that *L. neoniger* may fill a critical role in the structure of arthropod communities in maize, and in the transmission of plant-incorporated products through those communities.

Another area in which risk-assessment practices can be advanced is by greater focus on the soil-dwelling arthropod community. The detritivore food web is often neglected in risk-assessment studies. The results of the present study clearly demonstrate the ecological importance of soil fauna, and the likelihood for plant-incorporated products to reach the gut contents of soil-dwelling arthropods. Based on the present study, the top candidate for risk-assessment is a soil-dwelling springtail of the family Isotomidae. Indeed, the soil habitat domain was 50–200 times more densely populated and displayed 20–100 times higher species richness than either the foliar or epigeal habitat domains. Furthermore, soil networks showed a higher level of connectivity than epigeal or foliar networks.

In this experiment, we used three ecological criteria to identify potential indicator species: (1) abundance and distribution across a geographical area, (2) trophic linkage to the target crop, and (3) connectedness within ecological networks. Using ecological data such as this allowed us to identify taxa that likely have important roles in the structure and function of maize agro-ecosystems. In Table 1, a number of morphotaxa are ranked based on the number of criteria met (out of three). These data can serve as a guide for efficient selection of species for laboratory risk-assessment assays. Our study made use of standard field-sampling methods in order to facilitate replication of the study across a larger geographic region, which will be essential to maximizing the relevance of study findings for an insecticidal product's consumer base. In addition, this work demonstrates the utility of modern molecular techniques for assessing and monitoring risks of modern insecticidal products. Molecular gut-content analysis facilitates evaluation of trophic links in the field, enabling scientists and industry to construct a database of ecological information that can easily be referred to for future risk assessments. The current study is the first step in constructing such a database, which will help ensure that ecological risk assessment is being soundly guided by ecological principles.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fooweb.2016.02.004.

Acknowledgments

We are grateful to the following people, who aided extensively in field collections and associated laboratory support: Janet Fergen, Greta Schen, Dr. Kristine Nemec, Dr. Christina Mogren, Michael Bredeson, Cally Strobel, Jacob Pecenka, Marissa Layman, Claire Bestul, Nathan Koens, Kae Januschka, Nicole Berg, Ryan Bell, Ryan Schmid, and Mark Longfellow. The following farmers kindly allowed us access to their farms for insect collections: Ron Kaufman, Charlie Johnson, Mark Weinheimer, Melvin Gimble, Dan Forgey, Alan Ward, Ernie Namminga, Tom Wolles, Kevin Koester, Ted Fiegen and Lannie Mielke. Mention of a commercial product in this manuscript does not constitute endorsement by the USDA.

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