

## Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants

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### Abstract

This report is the outcome of an EFSA procurement (OC/EFSA/GMO/2015/02) which aims at reviewing relevant scientific information on RNA interference (RNAi) that could serve as baseline information for the environmental risk assessment of RNAi-based genetically modified (GM) plants. The report is based on a systematic literature search on the use of RNAi molecules in arthropods, nematodes, annelids and molluscs with dsRNA, siRNA and miRNA applied primarily through feeding and soaking (oral ingestion). The numbers of retrieved publications covering these areas are reported, along with the species name, life stages tested, the target gene and its function, details of the test substances and their concentrations used, methods of delivery and effects. Separate sections discuss the available information on: (1) the uptake and systematic spread of RNAi activity, including a description of the various components involved in this process; (2) the mechanisms of dsRNA-, siRNA- and miRNA-elicited gene silencing and the different factors involved in RNAi efficiency; (3) routes of exposure of the biotic and abiotic environment to dsRNA, siRNA and miRNA from GM plants; (4) the environmental fate of dsRNA, siRNA and miRNA; and (5) the various factors that may limit non-target effects including exposure, factors influencing the silencing efficiency of dsRNA, siRNA and miRNA, possible unintended and off-target effects, and their mechanisms. Finally, an overview of the species of arthropods, nematodes, annelids and molluscs for which genomic data are available is also presented. The report identifies some of the challenges involved in developing plants with RNAi systems which affect invertebrate gene expression. The report also concludes that, currently, knowledge on issues such as exposure, specificity, offtarget effects, sequence similarities and bioinformatics is very limited, as only a few RNAi expressing plants which specifically target invertebrate species have been developed and comprehensively studied.

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**Key words:** Genetically modified (GM) plants, RNA interference (RNAi), systematic literature search, invertebrates, biosafety, specificity, bioinformatics

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## Summary

A systematic literature search was used for a review of the scientific information on RNA interference (RNAi) that supports the environmental risk assessment (ERA) of RNAi-based genetically modified (GM) plants. The process involved identifying and retrieving scientific evidence for the review considering the following major aspects: definition of the review questions; identification of the information sources which are likely to yield relevant studies; definition of the keywords and search strings for identifying relevant studies; strategy for management of the references and the documents retrieved; documentation and reporting of the searches; definition of the inclusion and exclusion criteria; selection procedure for relevant studies. Web of Science (WoS) and CAB were the main information sources used. The search strings were tested against a reference collection of relevant articles and refined to minimise the number of irrelevant studies. The literature searches in online databases WoS and CAB Abstracts resulted in a total number of 17,680 records, which were reduced to 13,867 unique studies after removing duplicates, books, book sections and patents. The initial screening of these 13,867 records/studies revealed eight major methods for delivery of interfering RNA molecules in around 300 invertebrate species belonging to annelids, nematodes, arthropods and molluscs.

By far the most information on the mechanisms and pathways for RNAi is available in nematodes, and more specifically, in the free-living *Caenorhabditis elegans*. This nematode possesses a high sensitivity for feeding RNAi, due to a well-developed system for cellular uptake, systemic spread and an amplification system which produces secondary small interfering RNAs (siRNAs). Furthermore, the silencing signal in *C. elegans* can be observed over multiple generations. However, as has been clearly shown in the literature, a considerable degree of variation exists within nematodes concerning these pathways. Most nematodes outside of the genus of *Caenorhabditis* are missing several genes which are considered important in *C. elegans*, which may explain observed differences in RNAi efficiency in different nematode species. While there are other factors involved in explaining variability in RNAi sensitivity in invertebrates, cellular uptake of the small RNA (sRNA) and systemic spread of the silencing signal have been shown to be critical mechanisms for environmental RNAi.

More differences and variability in terms of uptake and systemic spread pathways were found in arthropods, where it seems that cellular uptake is much less efficient in some arthropods, especially some insect orders such as Diptera and Lepidoptera. While arthropods contain some elements of the elaborate cellular uptake and systemic spread systems found in *C. elegans*, their requirement and involvement in successful environmental or systemic RNAi has not yet been clearly demonstrated. In insects, and possibly some other taxonomic groups, cellular uptake is regulated by two pathways which may or may not be linked. One of these pathways involves the *sid-1-like* gene found in insect genomes, the other is based on clathrin-mediated endocytosis. The occurrence and robustness of systemic activity of RNAi in arthropods is variable and no evidence for an amplification system of the silencing signal, as is present in *C. elegans* and many other nematodes, has been reported yet.

Of the 4,612 studies which included all oral intake and soaking exposure, only 122 studies were considered relevant for the review of environmental exposure and fate. In addition, a few studies that were published after the completion of the literature search were also considered relevant and five additional studies in which transgenerational transmission of interference was studied *in vitro* via injection of RNAi, were also selected as relevant for this task. The analysis of the studies reporting GM plants transformed with double-stranded RNA (dsRNA) indicates that, for the majority of the cases, information on dsRNA expression is insufficient to run a robust exposure analysis and in several of these studies, detection of dsRNA in plants was not conducted. When the results of dsRNA detection in plants are reported, relative values of expression compared to reference genes are indicated. Similarly, only occasionally were expression levels detailed in different plant tissues or different phenological stages of the plant life cycle. Studies of *in planta* relative expression of dsRNA compared to housekeeping genes do not indicate actual amounts of dsRNA detected in plants, therefore they only orientate qualitatively an exposure analysis. By contrast, in the studies of the

characterization of GM maize MON 87411, a quantitative determination of dsRNA expression in various plant parts was conducted using a commercial hybridization-based molecular kit, which allows sensitive detection of dsRNA in all plant tissues.

From the relatively small amount of data on expression of dsRNA in plants, it can be inferred that in different transformation events with dsRNA targeting different genes, expression in plant parts was variable and the highest expression was detected in different tissues (from leaves to flowers) in different studies. Therefore, it will be necessary to characterize the expression levels in each GM event in order to determine exposure levels of both target organisms (TO) and non-target organisms (NTO).

Environmental persistence of dsRNA is mostly limited, as laboratory studies found dsRNA to be mostly degraded within 48 hours in soil and aquatic environments. However, in a few cases low levels of dsRNA could be detected for several days longer. Movement of dsRNA along trophic chains and the persistence of its biological activity have been shown in a few multi-trophic systems. The likelihood of a biological effect is primarily linked to the uptake of dsRNA in taxonomically different organisms and the efficiency of RNAi in the exposed organism. Similarly, intergenerational effects have been studied in a handful of cases where exogenous dsRNA was observed in a number of generations after exposure.

Reports on studies of RNAi sensitivity and efficiency in invertebrates were reviewed, mainly focusing on feeding RNAi. Several influencing factors, for example stability of dsRNA in the invertebrate body, the cellular uptake of dsRNA from the gut, the RNAi core machinery and the possible effect of viral infections, have been identified by various authors. The reports show that sensitivity and efficiency is very variable, not only between species, but also sometimes between strains, (laboratory) cultures, life stages or due to experimental aspects such as the siRNA molecule that is used. While some of these factors, such as dsRNA stability in the insect body and cellular uptake, have attracted a lot of attention from researchers, a number of other factors, such as the influence of the life stage or the impact of viral infections have not been studied in depth.

The systematic search identified 42 studies of target specificity, off-target effects and/or effects on non-target invertebrates, linked to interference triggered by dsRNA. However, there were few in-depth studies of off-target and non-target activity of ingested dsRNA particularly from GM plant sources. There were some reports of silencing effects in the presence of mismatches between sequences and non-specific silencing which indicate that the accurate design of the dsRNA to induce interference does not exclude the possibility of off-target and/or non-target effects. The support of bioinformatics is identified as being valuable, but the limited availability of invertebrate genomic sequences, the possible silencing in presence of mismatches between the target and the siRNA sequences, and the possibility of sequence-unrelated off-target effects indicate the requirement for bioassays in assessing the actual activity spectrum of dsRNA.

An overview of the available genomic data (in February 2017) for invertebrates belonging to the phyla of nematodes, arthropods, molluscs and annelids in four major databases or platforms is presented. Although studies indicated that successful gene silencing requires a high degree of homology, there is no consensus yet on exact 'rules' for siRNA/RISC binding to the homologous messenger RNA. Therefore, relying only on the use of bioinformatics to predict silencing effects in targets, off-targets and non-target invertebrates is difficult at present. Furthermore, there is no clear evidence for the number of siRNAs, processed from long dsRNA for example, necessary to incite gene silencing. Therefore, genomic data alone is not guaranteed to predict absence of silencing effects. Additionally, one must consider that genome sequences never have a 100% coverage of the genome, nor are they always 100% accurate.

The report concludes by highlighting the gaps in our baseline knowledge that warrant further research in order to support environmental risk assessments.

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## 1. Introduction

### 1.1. Background and Terms of Reference as provided by EFSA

This report is an outcome of an EFSA procurement titled "*Literature review of baseline information to support the risk assessment of RNAi-based GM plants – lot 2*" (reference number OC/EFSA/GMO/2015/02), awarded by the European Food Safety Authority to Ghent University, in consortium with Agrobioinstitute (ABI), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), and JT Environmental Consultants Ltd.

### 1.2. Tasks as defined by EFSA

The following tasks were provided by EFSA in the tender specifications:

- **Task 1:** To perform a systematic literature search, so as to provide an overview on the use of host-delivered RNAi molecules in arthropods, nematodes, annelids and molluscs with double stranded, small interfering and micro RNAs (dsRNAs, siRNAs and miRNAs, respectively) applied through feeding (oral ingestion).

The following relevant factors should be reported per experimental study published in the peer-reviewed scientific literature: (1) Test organism: taxonomy (phylum, order, family, and species name), functional group, life stage tested, number of organisms tested, number of replications per treatment, number of generations tested (if appropriate); (2) target gene and its function as well as the gene location in the recipient organism; (3) test substance (purified dsRNA, siRNA and miRNA), small RNA expressing bacteria or genetically modified (GM) plant tissue) and method of delivery (e.g., artificial diet, GM plant tissue); (4) test concentrations (or expression levels); (5) length of dsRNA, siRNA and miRNA; (6) test duration/duration of exposure; (7) measurement endpoints followed (lethal and sublethal); (8) controls used (negative control, positive control); (9) observed effects in terms of messenger RNA (mRNA) silencing, including the amount of silencing (relative expression of the target gene compared to the control), affected measurement endpoints, the recovery of gene expression, and evaluation method; (10) any other additional information considered relevant (e.g., statistical power); (11) the reference of the scientific publication.

- o **Task 1.1:** To report on the approach followed and the outcomes of the systematic literature search performed to identify and retrieve relevant publications published in the peer-reviewed scientific literature (e.g., search terms/string, scientific literature databases, inclusion/exclusion criteria for the identification of relevant scientific publications).
- o **Task 1.2:** To report on the outcomes of the systematic literature search in terms of relevant scientific publications identified and retrieved.
- o **Task 1.3:** Based on the retrieved information, to summarise the activity spectrum of the tested dsRNAs, siRNAs and miRNAs.
- o **Task 1.4:** Based on the retrieved information, to assess whether, and if so under which conditions, siRNA and miRNA delivered to arthropods, nematodes, annelids and molluscs through feeding trigger (efficient) RNAi.
- **Task 2:** Based on available scientific literature, to review mechanisms of dsRNA (and siRNA and miRNA, if relevant) uptake and systematic spread in arthropods, nematodes, annelids and molluscs, including a description of the various components involved in this process (narrative review).
- **Task 3:** Based on available scientific literature, to review plausible routes of exposure of the biotic and abiotic environment to dsRNA (and siRNA and miRNA, if relevant) expressed in RNAi-based GM plants, the environmental fate of dsRNA (and siRNA and miRNA, if relevant), and the various factors that may limit non-target organism (NTO) exposure (barriers to exposure:

e.g., enzymatic barriers, pH in gut of recipient organisms, (in)stability of RNAi molecules in recipient organisms) (narrative review).

- **Task 4:** Based on available scientific literature and the information retrieved in the previous tasks, to summarise which factors largely influence silencing efficiency of dsRNA (and siRNA and miRNA, if relevant) across arthropods, nematodes, annelids and molluscs delivered through feeding (narrative review).
- **Task 5:** Based on available scientific literature, to assess the plausibility of unintended adverse effects on arthropods, nematodes, annelids and molluscs associated with the cultivation of RNAi-based GM plants (covering lethal, sublethal and chronic effects in the various life stage of NTOs), and involved mechanisms (e.g., unintended gene suppression, immunostimulation, saturation of RNAi machinery, potential for resistance evolution in target pests) (narrative review).
- **Task 6:** To provide an overview of species (including model species) belonging to the taxa of arthropods, nematodes, annelids and molluscs for which complete or partial genome data are available (narrative review).

### 1.3. Content of the report

- **Section 1** presents the tasks defined by EFSA (Section 1.2) and summarises the content of the report (Section 1.3). It also includes a general introductory section (Section 1.4), providing some background information on RNA silencing and its development for control of invertebrate pests. Detailed information on the mechanisms and functions of RNA silencing in a range of organisms including some invertebrates is provided in the report on the parallel lot "*Literature review of baseline information to support the risk assessment of RNAi-based GM plants – lot 1*" (reference number OC/EFSA/GMO/2015/01), awarded by EFSA to the Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic (IMG) in consortium with EcoMole Ltd. (Pačes et al., 2017)
- **Section 2** describes the methodology used to perform the systematic literature search (Task 1).
- **Section 3** describes the results of the systematic literature search for baseline information to support the environmental risk assessment (ERA) of RNAi-based GM plants (Tasks 1.1 and 1.2).
- **Section 4** analyses the primary data from the selected studies, and describes the activity spectrum of the tested dsRNAs, siRNAs and miRNAs (Task 1.3), based on the relevant studies retrieved from the systematic literature search. Scientific literature on invertebrate species (i.e., arthropods, nematodes, annelids and molluscs) studied and the range of genes targeted by RNAi systems is reported, and the activity spectrum of the target gene that is altered described, including the impact on the physiology and metabolism of the target (and non-target) organism and its ultimate demise (e.g., loss of fertility, mortality). The specificity of each dsRNA, siRNA and miRNA is described in terms of its activity spectra within the different tissues, and also the range activities in non-target species. This section also assesses whether, and if so under which conditions, siRNA and miRNA delivered to arthropods, nematodes, annelids and molluscs through feeding trigger (efficient) RNAi (Task 1.4). Studies of soaking and oral introduction of siRNA and miRNA into invertebrates are reviewed for the methods, dose and longevity of feeding exposure, results and outcomes of each study. This part discusses which systems provide sufficient siRNA and miRNA to trigger efficient RNAi and whether they also persist or stimulate multiplication/replication within these invertebrates. It also considers their activity spectra within the different tissues of an organism and also the range of off-target activities and non-target effects. The implications of the efficiency of oral



RNAi on resistance development, non-specific/off-target effects and non-target effects are discussed.

- **Section 5** contains the narrative literature reviews, as defined by EFSA (Tasks 2-5):
  - o *Section 5.1: Uptake and systemic spread of small RNAs in invertebrates (Task 2)*. This section includes a study of entry routes of sRNA into the body of invertebrates and cellular uptake and systemic transport and the influence of the small RNA (sRNA) molecule on cellular uptake and systemic RNAi efficiency. The literature on nematodes is discussed with a focus on cellular uptake, transport and systemic RNAi efficiency in *Caenorhabditis elegans*, as this is one of the most intensively studied species, and this is contrasted with information from studies with other nematode species, including plant parasitic species. The literature on other invertebrates is discussed with a focus on cellular uptake, transport and systemic RNAi efficiency in arthropods where insects such as *Drosophila* have been extensively studied, and in annelids and molluscs. Reports of amplification of the silencing signal and transgenerational activity of parental RNA are reviewed.
  - o *Section 5.2: Environmental exposure and fate of dsRNA, siRNA and miRNA (Task 3)*. In this section, the information on exposure routes and pathways and the fate of dsRNA and siRNA is discussed and considered in relation to possible exposure routes to invertebrates and the evaluation of exposure for risk assessment purposes. Data on persistence of dsRNA in plant and animal material, soil and soil water is examined. Exposure is also an important element in resistance development in target organisms so that the exposure information also informs the studies of resistance discussed in Section 5.4 (Task 5).
  - o *Section 5.3: Factors influencing silencing efficiency of dsRNA, siRNA and miRNA delivered orally in arthropods, nematodes, annelids and molluscs (Task 4)*. In this section studies related to the silencing efficiency and efficacy of dsRNA, siRNA and miRNA in this range of invertebrates are described and discussed. The factors involved in restricting and promoting the efficiency of RNAi in invertebrates are identified and examined, both at the molecular mechanism level and in relation to the uptake studies in Section 5.1 (Task 2) and the exposure studies in Section 5.2 (Task 3). This section considers the published information relating to these factors and discusses the evidence that feeding studies of the different RNAs are resulting in effective RNAi of target invertebrate species. The factors affecting the success or failure of systems are analysed, and influences of these factors on non-target and off target effects discussed.
  - o *Section 5.4: Off-target, non-target and unintended effects of RNAi-based GM plants (Task 5)*. This section reviews the studies on target specificity, off-target effects and/or effects on non-target invertebrates, linked to interference triggered by dsRNA. It focuses on studies of off-target and non-target activity of ingested dsRNA from GM plant sources. It considers reports of silencing effects in presence of mismatches between sequences and non-specific silencing. It examines whether the accurate design of the dsRNA to induce interference excludes the possibility of off-target and/or non-target effects. The possible silencing in presence of mismatches between the target and the siRNA sequences is considered, and the possibility of sequence-unrelated off-target effects are discussed. In addition, the mechanisms and potential for resistance evolution in target pests is considered.
  - o *Section 5.5: Genomic data on arthropods, nematodes, annelids and molluscs (Task 6)*. This section gives an overview of the available genomic data for arthropods, nematodes, annelids and molluscs in four major databases or platforms. The potential use of bioinformatics to predict silencing effects in targets, off-targets and non-target

invertebrates is considered in relation to the 'rules' for binding of siRNA, which is incorporated in the RNA induced silencing complex (RISC), to the homologous mRNA.

- **Section 6** summarises the main findings and conclusions from each section and identify gaps in data and knowledge, whereas in **Section 7** further studies are recommended to provide more information on areas where knowledge and experience is limited, in order to support the ERA of GM-based RNAi plants.

#### 1.4. Introduction to RNAi-based GM plants for invertebrate control

RNAi was discovered in 1998 in the nematode *C. elegans*, where injection of dsRNA caused a specific post-transcriptional gene silencing (Fire et al., 1998). Soon after, it was shown that this mechanism is conserved in most Eukaryota, including insects. When it became clear that oral delivery of dsRNA was also able to elicit RNAi, scientists soon realised the potential of this technique as a pest control strategy. This has encouraged research on developing plants that could be protected from herbivorous pests by engineering them to express dsRNAs targeting vital genes in the pest. In recent years, there have been several studies of this approach further demonstrating the potential of this mechanism for crop protection. The modes of action and mechanisms associated with sRNAs in a range of animal and plant species have been the subject of another review commissioned by EFSA (Pačes et al., 2017). However, the variable efficiency of RNAi in many arthropods is a challenge that must be addressed before RNAi can be widely used in pest control (Terenius et al., 2011; Christiaens and Smagghe, 2014). In this report, we use the term sRNAs to refer to miRNA, siRNA and longer dsRNA (25-1500 bp).

Mao et al. (2007), working with cotton bollworm (*Helicoverpa zea*), and Baum et al. (2007), working with a range of insects that includes western corn rootworm (WCR, *Diabrotica virgifera virgifera*), southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi*) and Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) have shown that transgene-encoded ingestible dsRNA can specifically target certain pest insect genes and prevent their function, resulting in mortality of the pest. Since then, a number of studies with other arthropod species have demonstrated similar effects with ingested dsRNAs, and there are studies also with other sRNAs such as siRNAs and miRNAs (Zotti and Smagghe, 2015). In 2013, the US Environmental Protection Agency (US EPA) approved an experimental use permit (No. 524-EUP-104) for field evaluation of a GM maize expressing a transgene construct which includes the *Snf7* gene which suppresses mRNA encoding vacuolar ATPase subunits A and E. This gene targets the WCR, and published data indicate the specificity of the *Snf7* gene to the target pest ATPase mRNAs (Bachman et al., 2013). This has raised a number of issues relating to the risk assessment of RNAi plants as indicated by Ramon et al. (2014), Casacuberta et al. (2015) and Zotti and Smagghe (2015), and the need to clearly identify all the available information that can underpin and support ERA of these GM-based RNAi plants.

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## 2. Data and Methodologies

### 2.1. Data

Data was obtained from available scientific literature and from relevant publications retrieved from the systematic literature search.

### 2.2. Methodologies – Systematic literature search

A systematic literature search was performed to obtain/retrieve relevant studies/information to support the ERA of RNAi-based GM plants. The process towards identifying and retrieving scientific evidence for the review involves the following major steps:

- Definition of the review questions;
- Identification of the information sources which are likely to yield relevant studies;
- Definition of the keywords and search strings for identifying relevant studies;
- Strategy for management the references and the documents retrieved;
- Documentation and reporting of the searches;
- Definition of the inclusion and exclusion criteria;
- Selection procedure for relevant studies.

#### 2.2.1. Definition of the review questions

The team defined five review questions which aim to address the topics described in the tender specifications, as follows:

- Activity spectrum and conditions for efficient RNAi through feeding (oral ingestion) of arthropods, nematodes, annelids and molluscs with dsRNAs, siRNAs and miRNAs;
- Mechanisms of dsRNA, siRNA and miRNA uptake and systematic spread in arthropods, nematodes, annelids and molluscs, including a description of the various components involved in this process;
- Plausible routes of exposure of the biotic and abiotic environment to dsRNA, siRNA and miRNA expressed in RNAi-based GM plants, the environmental fate of dsRNA, siRNA and miRNA and the various factors that may limit NTO exposure (barriers to exposure: e.g., enzymatic barriers, pH in gut of recipient organisms, (in)stability of RNAi molecules in recipient organisms);
- Factors which influence silencing efficiency of dsRNA, siRNA and miRNA across arthropods, nematodes, annelids and molluscs delivered through feeding;
- Plausibility of unintended adverse effects on arthropods, nematodes, annelids and molluscs associated with the cultivation of RNAi-based GM plants (covering lethal, sublethal and chronic effects in the various life stage of NTOs), and involved mechanisms (e.g., unintended gene suppression, immunostimulation, saturation of RNAi machinery, potential for resistance evolution in target pests).

#### Review question 1

*What is the activity spectrum and under which conditions dsRNAs, siRNAs and miRNAs delivered to arthropods, nematodes, annelids and molluscs through feeding (oral ingestion) trigger efficient RNAi?*

The aim of this question is to identify the range of genes targeted by RNAi systems applied through feeding of dsRNA, siRNA and miRNA to arthropods, nematodes, annelids and molluscs. This includes the activity spectrum of the gene that is altered, the impact on the physiology and metabolism of the target (and non-target) organism and its ultimate demise (e.g., loss of fertility, mortality).

## Review question 2

*What are the components and mechanisms of dsRNA, siRNA and miRNA uptake and systematic spread in arthropods, nematodes, annelids and molluscs?*

In the focus of this question are the articles studying components and mechanisms of dsRNA, siRNA and miRNA uptake and systematic spread in invertebrates. It aims to consider specific aspects of RNAi such as the important differences in uptake and systemic transport mechanisms, the genes involved and the implications on uptake capacity of ds-, si- or miRNA molecules by arthropods, nematodes, annelids and molluscs.

## Review question 3

*What are the exposure routes and the environmental fate of dsRNA, siRNA and miRNA?*

This question aims at retrieving information on the various aspects of environmental exposure including exposure of soil invertebrates through plant roots, bio-pesticides (containing dsRNA), plant and animal residues, as well as studies which investigate persistence and degradation of dsRNA in the gastrointestinal tract of the invertebrates and the biological and biophysical factors driving these processes. It also includes the collection of data on persistence of dsRNA in plant and animal material and soil and the discussion of possible exposure routes to invertebrates.

## Review question 4

*What are the factors influencing silencing efficiency of dsRNA, siRNA and miRNA delivered orally in arthropods, nematodes, annelids and molluscs?*

This question addresses one of the main topics in RNAi research at the moment related to the silencing efficiency and the observed variability between different experiments, species, delivery systems, uptake routes, etc. Getting a clear understanding of the factors that influence this efficiency and how they are linked to each other is of great importance for the development, ERA and management of this technology. This question considers the evidence that feeding studies using different RNAs are effective for RNAi of target invertebrate species. This question also examines the factors affecting the success or failure of systems and the influence of these factors on non-target and off target effects.

## Review question 5

*What are the off-target, non-target and unintended effects of RNAi-based GM plants to arthropods, nematodes, annelids and molluscs?*

This question aims at retrieving studies which examine the off-target and non-target effects of RNAi-based GM plants as well as studies of other unintended effects such as: gene suppression; immunostimulation; saturation of RNAi machinery; degradation of non-targeted mRNA by transitive RNA silencing and stability of the silenced phenotype, e.g., considering removal of toxins. The collected information is examined to determine whether RNAi systems developed in plants for silencing plant metabolic activities, or directed at other targets such as viruses, have off-target or non-target effects in invertebrates.

### 2.2.2. Elements of the review questions

The key elements of the review questions such as the population of interest, the intervention and the type of exposure were defined and used as an input for the definition of the keywords and the content of the search strings. In the next table, a description of these elements is given:

**Table 1:** Key elements of the review questions

Question	Population	Intervention	Outcomes
<i>What is the activity spectrum and under which conditions dsRNAs, siRNAs and miRNAs delivered to arthropods, nematodes, annelids and molluscs through feeding (oral ingestion) trigger efficient RNAi</i>	Arthropods, nematodes, annelids and molluscs	Oral delivery (including soaking) of dsRNA, siRNA and miRNA	Silencing or knockdown of gene expression in target and non-target populations
<i>What are the components and mechanisms of dsRNA, siRNA and miRNA uptake and systematic spread in arthropods, nematodes, annelids and molluscs?</i>	Arthropods, nematodes, annelids and molluscs	Any type of exposure in laboratory or field conditions (oral, injection, soaking, etc.) to dsRNA, siRNA and miRNA	Silencing or knockdown of gene expression in target and non-target populations
<i>What are the exposure routes and the environmental fate of dsRNA, siRNA and miRNA?</i>	Arthropods, nematodes, annelids, molluscs and different environments	Any type of exposure in laboratory or field conditions (oral, injection, soaking, etc.) to dsRNA, siRNA and miRNA	Silencing or knockdown of gene expression in target and non-target populations
<i>What are the factors influencing silencing efficiency of dsRNA, siRNA and miRNA delivered orally in arthropods, nematodes, annelids and molluscs?</i>	Arthropods, nematodes, annelids, molluscs	Oral delivery (including soaking) of dsRNA, siRNA and miRNA	Silencing or knockdown of gene expression in target and non-target populations
<i>What are the target, off-target, non-target and unintended effects of RNAi-based GM plants in arthropods, nematodes, annelids and molluscs?</i>	Arthropods, nematodes, annelids, molluscs	GM plants and other types of exposure in laboratory or field conditions (oral, injection, soaking, etc.) to dsRNA, siRNA and miRNA	Silencing or knockdown of gene expression and any unintended effects in target and non-target populations

### 2.2.3. Sources of information

The sources of information that may yield relevant scientific studies for the review topic were selected after a discussion within the team following the best practices for conducting systematic reviews described in methodological guidelines (EFSA, 2010, 2011) and systematic review protocols (Meissle et al., 2014; Sweet and Kostov, 2014).

#### 2.2.3.1. Journals, conference abstracts or proceedings in electronic bibliographic databases

The main sources to retrieve relevant information are journals and conference abstracts or proceedings available in electronic bibliographic databases. Bibliographic databases are the most efficient way to identify an initial set of relevant scientific studies. They are usually designed to facilitate effective information retrieval with information presented in structured ways and often with the addition of indexing. Multi-disciplinary and subject-specific databases were used to allow consideration of complementarity and redundancy. It also minimised the impact of publication bias, and helped to address limitations in research reporting and indexing. The selected electronic databases include the largest abstracting literature databases, full text engines that provide

comprehensive lists of information sources. These databases were selected because they cover studies in all the relevant areas of the biological sciences relating to RNAi in plants and invertebrates.

The search was carried out in the following electronic bibliographic databases:

- **Web of Science (WoS)** (ISI Web of Knowledge 1900 – 2016) (Thomson Reuters, New York, USA), a multi-disciplinary keyword database, contains peer-reviewed scientific studies. The WoS platform conducts searches in the following databases according to the subscription of ABI:
  - o Web of Science™ Core Collection
  - o BIOSIS Citation Index<sup>SM</sup>
  - o Current Contents Connect
  - o Data Citation Index<sup>SM</sup>
  - o Derwent Innovations Index<sup>SM</sup>
  - o KCI-Korean Journal Database
  - o MEDLINE®
  - o Russian Science Citation Index
  - o SciELO Citation Index
  - o Zoological Record®
- **CAB Abstracts** (1984 – 2016) (CABI, Wallingford, UK), comprehensive database for the applied life sciences – agriculture, environment, veterinary sciences, applied economics, food science and nutrition. This database also includes local and non-English studies.

### 2.2.3.2. Reference lists

A list of references was compiled, which consisted of 131 RNAi studies in invertebrates, to be used as a validation of the search string. The compilation was made based on the reference list of six recent reviews on relevant topics and methodological articles (Kumar et al., 2013; Nandety et al., 2015; Owens and Malham, 2015; Roberts et al., 2015; Schumpert et al., 2015; Zotti and Smagghe, 2015), or studies provided by the team members based on their experience in the subject.

### 2.2.4. Search terms and strings used in Web of Science

The search in WoS covered time period from 1998 until July 2016. The time span of the search started with 1998, the year when gene silencing, after introduction of dsRNA in invertebrate species (*C. elegans*), was described and the term RNAi was introduced (Fire et al., 1998). Relevant original studies written in English were retrieved through the search interfaces of WoS using specified search terms (key words) organised in strings. For the selection of the search terms, the following factors were taken into account:

- The key elements of the review questions;
- The terms and indexers used by the authors to describe their documents in the database records;
- The search tools and options given by the electronic databases such as search operators (e.g., AND, OR, NEAR) and filters;
- The number of hits received when using the search terms;
- Major groups of irrelevant studies among the hits;
- Language: only English search terms and taxonomic terms were used.

In the choice of the key elements, their number and syntaxes, a stepwise approach was taken. After the inclusion of each element, a search was performed in WoS to assess the influence of each element and their combinations to the number of hits. The list of studies relevant to the review (presented in Appendix A) was used to define common keywords and assess the relevance of the results in the pilot searches in the database.

When included in the search string, the terms were modified according to the differences of spelling and other types of variation in the representation (such as the use singular and plural nouns) using asterisks (\*) to represent any number of characters that may be attached before or after the term. Quotes (") were used to search exact phrases. The terms which are often used in studies (e.g., NTOs, invertebrates, arthropods, insects, diet, feeding, delivery, ingestion, inoculation) produced large numbers of irrelevant studies when used alone. In order to reduce the number of irrelevant hits, additional conditions were used in the search string, requiring these terms to be positioned within a specified number of words with RNAi terms. This was achieved by using the "NEAR" search operator.

#### 2.2.4.1. Search string element 1: Population terms

The population terms are the primary element of the searches when conducting systematic reviews (EFSA, 2010). The selected population terms describe different types of organisms in the focus of the review. In this review, these population terms include general terms such as: NTOs, invertebrates, model, insects and arthropods; specific population terms include common names and names of major taxonomic groups:

- Molluscs;
- Annelids;
- Flies;
- Bugs;
- Aphids;
- Beetles;
- Bees;
- Moths;
- Ants;
- Wasps;
- Spiders;
- Worms;
- Snails;
- Leeches;
- Shrimps;
- Acari;
- Arachnids;
- Nematodes.

Six major orders of insects containing a large number of target and non-target species (i.e., Coleoptera, Collembola, Diptera, Hemiptera, Hymenoptera and Lepidoptera) were also included among the population terms, as well as terms referring to the developmental stage (i.e., adult, larva, imago, pupae and nymph;) and specific species names (i.e., *Drosophila melanogaster* and *C. elegans*). The species names of the well-known model invertebrates *D. melanogaster* and *C. elegans* were included in the search terms since in some cases the authors do not use other definitions for these organisms (e.g., invertebrate, insect, fly or nematode) in the title, the abstract or the keywords of their studies. The need for inclusion of these terms in the search string was noted during the test of the search string when some of the reference studies used for sensitivity analysis were missing from the results due to the lack of the population term (e.g., Clemens et al. 2000; Marques et al. 2013, see list with reference studies in Appendix A).

The inclusion of *D. melanogaster* and *C. elegans* as terms in the search string resulted in a substantial increase in hits when tested in the WoS. The reason for this was found to be not just because of the large number of studies containing these terms in their title and/or abstract, but also due to the presence of these terms in the "Keywords Plus". The "Keywords Plus" is a special feature of the WoS, and adds additional keywords to the ones provided by the authors that are searchable and automatically assigned by the WoS system based on the references used in the study. While this



might be considered as a useful feature that increases the sensitivity of the search, it produced a large number of irrelevant hits. The analysis of the hits and the major groups of irrelevant studies revealed this effect when using *Drosophila* and *C. elegans* and general population terms such as NTOs, adult, arthropod, etc. To limit this effect, the "NEAR" search operator was used to find records where the terms joined by the operator are in the same field (e.g., title or abstract), and within a specified number of words of each other. In this way, the hits only contained studies which included the specified terms in combination (e.g., 20 words distance) with the most common intervention terms RNAi and RNA interference in their title and/or abstract. This also enabled exclusion of studies that have these terms only in the "Keywords Plus". The sensitivity of the search string was confirmed using the 131 reference studies.

When the species names of the model organisms were included among the population terms, the missing studies were present within the search results. These two model organisms are the most commonly used invertebrates in studies that include RNAi, as evident from the preliminary analysis of the search string results.

The following string of population terms was defined:

coleopter\*<sup>1</sup> or collembol\*<sup>1</sup> or dipter\*<sup>1</sup> or hemipter\*<sup>1</sup> or hymenopter\*<sup>1</sup> or lepidopter\*<sup>1</sup> OR mollus\*<sup>1</sup> OR annelid\*<sup>1</sup> OR acari\*<sup>1</sup> OR arachn\*<sup>1</sup> OR nematod\*<sup>1</sup> OR fly<sup>1</sup> OR flies<sup>1</sup> OR bug<sup>1</sup> OR bugs<sup>1</sup> OR aphid\*<sup>1</sup> OR beetle\*<sup>1</sup> OR butterfly\*<sup>1</sup> OR Apis<sup>1</sup> OR bee<sup>1</sup> OR moths<sup>1</sup> OR moth<sup>1</sup> OR "ant"<sup>1</sup> OR "ants"<sup>1</sup> OR wasp\*<sup>1</sup> OR spider\*<sup>1</sup> OR \*worm\*<sup>1</sup> OR snail\*<sup>1</sup> OR leech\*<sup>1</sup> OR shrimp\*<sup>1</sup> OR larva\*<sup>1</sup> OR imago<sup>1</sup> OR nymph\*<sup>1</sup> OR pupa\*<sup>1</sup> OR "nontarget organism\*"<sup>1</sup> NEAR/20 RNAi OR "non-target organism\*"<sup>1</sup> NEAR/20 RNAi OR invertebrate\*<sup>1</sup> NEAR/20 RNAi OR arthropod\*<sup>1</sup> NEAR/20 RNAi OR insect\*<sup>1</sup> NEAR/20 \*RNA\* OR model<sup>2</sup> NEAR/20 dsRNA OR adult\*<sup>2</sup> NEAR/20 RNAi OR invertebra\*<sup>1</sup> NEAR RNAi OR *Drosophila*<sup>2</sup> NEAR RNAi OR *Drosophila*<sup>2</sup> NEAR dsRNA OR "*Caenorhabditis elegans*"<sup>2</sup> NEAR RNAi OR "*Caenorhabditis elegans*"<sup>2</sup> NEAR dsRNA OR "*C. elegans*"<sup>2</sup> NEAR RNAi OR soil<sup>2</sup> NEAR \*RNA\* OR environment\*<sup>2</sup> NEAR RNA

<sup>1</sup> Keywords included after internal discussion among the review team members;

<sup>2</sup> Keywords included after initial test of the search string and content analysis of the missing reference studies from the retrieved studies.

The NEAR operator was used to limit the number of irrelevant studies and exclude the influence of the "Keywords Plus" terms to the search results in WoS. This enabled combining the keyword with the most common RNAi related terms appearing within a specified number of words from each other.

#### 2.2.4.2. Search string element 2: Intervention terms

The intervention terms include the various types and wordings of RNAi and RNAi molecules such as RNA interference, dsRNA, miRNA and siRNA. These terms limit the query to studies that include RNAi molecules.

The following string of intervention terms was defined:

"double stranded ribonucleic acid\*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA interference" OR "RNA-interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA"

In the definition of these terms, the team took into account the various RNA molecules that can be used for RNAi. All the terms were defined after internal discussion and minor adjustments were made during the test of the search string mainly due to the different ways of naming and spelling.

#### 2.2.4.3. Search string element 3: Exposure and outcome terms

As the third element, terms that describe different types of exposure to RNAi molecules and relevant outcomes were defined. This includes various types of exposure and administration terms such as

oral, topical, injection, spray, as well as relevant outcomes such as silencing, knock-out, gene inhibition, gene function, disruption. The inclusion of outcome terms was found necessary since some of the studies do not mention the routes of exposure to RNA molecules in their abstracts.

Most of the exposure terms are common words that are often used in various types of scientific studies. To limit the irrelevant results, the "NEAR" operator was used to make sure that the exposure term is positioned within 15 words distance from any type of RNA molecule in the title or the abstract of the study. For the outcome terms, which consist of two words in combination, the NEAR operator was used to capture differences in wording, e.g., gene NEAR/3 silenc\* which covers many forms of wording such as "gene was silenced", "gene is silenced", "gene silencing", "silencing of genes", "silenced gene", "silencing of (name of a gene) gene".

The following string of exposure/outcome terms was defined:

plant\* NEAR \*RNA OR diet\* NEAR \*RNA OR deliver\* NEAR \*RNA OR ingest\* NEAR \*RNA OR \*inject\* NEAR \*RNA OR inoculat\* NEAR \*RNA OR topical\* NEAR \*RNA OR target NEAR/5 gene\* OR spray\* NEAR \*RNA OR oral\* NEAR \*RNA OR feed\* NEAR \*RNA OR fed NEAR \*RNA OR field OR gene NEAR/3 silenc\* OR RNA\* NEAR/3 silenc\* OR gene NEAR/3 knockdown OR gene NEAR/3 knock-out OR gene NEAR/3 express\* OR \*RNA NEAR/3 pathway OR \*RNA NEAR/3 uptake OR environment\* NEAR/3 assessment OR inhibit\* NEAR/3 expression OR inhibit\* NEAR/3 function\* OR disrupt\* OR formation NEAR RNAi

#### 2.2.4.4. Additional search string elements in order to reduce the number of irrelevant studies in Web of Science

When the search string containing the three key elements alone was used in WoS, the number of hits was above 30,000. This number was too large for a manual selection for relevance, and imposed the need to reduce the number of hits without losing sensitivity of the search string. This was achieved by the inclusion of two additional elements in the search string and through the use of the advanced search options of WoS.

*Additional element 1:* excluding studies in rodents, mammals and humans by title, abstract and key words

The aim of this element of the search string was to exclude studies which involve RNAi with organisms that are not relevant to the review, such as rodents, mammals and humans. This includes terms often used in these types of studies, such as rats, rodents, clinical, patients. The Boolean operator "NOT" was used to exclude the studies that contain the terms in their title, abstract or key words. These terms were defined after carrying out a content analysis of the titles and abstracts of the major groups of irrelevant studies. The lack of these terms in the titles and the abstract of the reference studies was confirmed using text editing program.

The following string of terms that exclude studies in rodents, mammals and humans was defined:

drug\* OR rat OR rats OR rodent\* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor\* OR pig OR pigs OR cattle\* OR patient\*

*Additional element 2:* excluding studies in rodents, mammals and humans by title.

Major terms that occasionally appear in the abstracts in the relevant studies, but that are never present in the title are used to limit the search results.

The following string of terms that exclude studies in rodents, mammals and humans by the title was defined:

mammal\* OR mouse OR human\* OR cancer\*

In conclusion, the constructed search string consisted of five parts (linked with "AND" or "NOT"), where the terms in each part were linked with "OR". This provided the following full search string that was used in WoS:

TS=(coleopter\* or collembol\* or dipter\* or hemipter\* or hymenopter\* or lepidopter\* OR mollus\* OR annelid\* OR acari\* OR arachn\* OR nematod\* OR fly OR flies OR bug OR bugs OR aphid\* OR beetle\* OR butterfly\* OR Apis OR bee OR moths OR moth OR "ant" OR "ants" OR wasp\* OR spider\* OR \*worm\* OR snail\* OR leech\* OR shrimp\* OR larva\* OR imago OR nymph\* OR pupa\* OR "nontarget organism\*" NEAR/20 RNAi OR "non-target organism\*" NEAR/20 RNAi OR invertebrate\* NEAR/20 RNAi OR arthropod\* NEAR/20 RNAi OR insect\* NEAR/20 \*RNA\* OR model NEAR/20 dsRNA OR adult\* NEAR/20 RNAi OR invertebra\* NEAR RNAi OR Drosophila NEAR RNAi OR Drosophila NEAR dsRNA OR "Caenorhabditis elegans" NEAR RNAi OR "Caenorhabditis elegans" NEAR dsRNA OR "C. elegans" NEAR RNAi OR soil NEAR \*RNA\* OR environment\* NEAR RNA) AND TS=("double stranded ribonucleic acid\*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA interference" OR "RNA-interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA") AND TS=(plant\* NEAR \*RNA OR diet\* NEAR \*RNA OR deliver\* NEAR \*RNA OR ingest\* NEAR \*RNA OR \*inject\* NEAR \*RNA OR inoculat\* NEAR \*RNA OR topical\* NEAR \*RNA OR target NEAR/5 gene\* OR spray\* NEAR \*RNA OR oral\* NEAR \*RNA OR feed\* NEAR \*RNA OR fed NEAR \*RNA OR field OR gene NEAR/3 silenc\* OR RNA\* NEAR/3 silenc\* OR gene NEAR/3 knockdown OR gene NEAR/3 knock-out OR gene NEAR/3 express\* OR \*RNA NEAR/3 pathway OR \*RNA NEAR/3 uptake OR environment\* NEAR/3 assessment OR inhibit\* NEAR/3 expression OR inhibit\* NEAR/3 function\* OR disrupt\* OR formation NEAR RNAi) NOT TS=(drug\* OR rat OR rats OR rodent\* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor\* OR pig OR pigs OR cattle\* OR patient\*) NOT TI=(mammal\* OR mouse OR human\* OR cancer\*)

### 2.2.5. Testing the search strategy

The search strategy was tested to determine its suitability to retrieve studies relevant to the review topic. The aim of this exercise was to develop the exact content of the search string to be used in the systematic review and was conducted in one of the largest literature databases: WoS.

To test the relevance of the results from the search string in WoS, we used 131 studies (titles are provided in Appendix A), which comply with the inclusion criteria. The compilation was made based on the reference list of six recent reviews on relevant topics and methodological articles, or studies provided by the team members based on their experience in the subject (see Section 2.2.3.2). The presence of the reference studies among the results from the search string was confirmed manually.

When using the defined search string, on 23 July 2016, the number of hits retrieved in WoS was 22,824 (Table 2). However, this number was not the final number of hits since the WoS system did not remove the duplications automatically, but only when the user browsed through all the results. After the duplications were removed, there were 11,643 hits. The relevance check revealed that all the 131 reference studies were among the results.

**Table 2:** Scoping exercise results (search performed on 23 July 2016)

Platform	Search string	Number of hits	Presence of reference studies
Web of Science	TS=(coleopter* or collembol* or dipter* or hemipter* or hymenopter* or lepidopter* OR mollus* OR annelid* OR acari* OR arachn* OR nematod* OR fly OR flies OR bug OR bugs OR aphid* OR beetle* OR butterfly* OR Apis OR bee OR moths OR moth OR "ant" OR "ants" OR wasp* OR spider* OR *worm* OR snail* OR leech* OR shrimp* OR larva* OR imago OR nymph* OR pupa* OR "nontarget organism*" NEAR/20 RNAi OR "non-target organism*" NEAR/20 RNAi OR invertebrate* NEAR/20 RNAi OR arthropod* NEAR/20 RNAi OR insect* NEAR/20 *RNA* OR model NEAR/20 dsRNA OR adult* NEAR/20 RNAi OR invertebra* NEAR RNAi OR Drosophila NEAR RNAi OR Drosophila NEAR dsRNA OR "Caenorhabditis elegans" NEAR RNAi OR "Caenorhabditis elegans" NEAR dsRNA OR "C. elegans" NEAR RNAi OR soil NEAR *RNA* OR environment* NEAR RNA) AND TS=("double stranded ribonucleic acid*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA interference" OR "RNA-interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA") AND TS=(plant* NEAR *RNA OR diet* NEAR *RNA OR deliver* NEAR *RNA OR ingest* NEAR *RNA OR *inject* NEAR *RNA OR inoculat* NEAR *RNA OR topical* NEAR *RNA OR target NEAR/5 gene* OR spray* NEAR *RNA OR oral* NEAR *RNA OR feed* NEAR *RNA OR fed NEAR *RNA OR field OR gene NEAR/3 silenc* OR RNA* NEAR/3 silenc* OR gene NEAR/3 knockdown OR gene NEAR/3 knock-out OR gene NEAR/3 express* OR *RNA NEAR/3 pathway OR *RNA NEAR/3 uptake OR environment* NEAR/3 assessment OR inhibit* NEAR/3 expression OR inhibit* NEAR/3 function* OR disrupt* OR formation NEAR RNAi) NOT TS=(drug* OR rat OR rats OR rodent* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor* OR pig OR pigs OR cattle* OR patient*) NOT TI=(mammal* OR mouse OR human* OR cancer*)	22 824 <sup>a</sup> 11 643 <sup>b</sup>	131 from 131

(a): Total number of hits.

(b): Number of hits after the removal of duplications.

The presence of all reference studies within the results of the WoS search, and the fact that the search did not produce excessive numbers of hits, indicated the suitability of the search strategy to retrieve relevant studies.

### 2.2.6. Search terms and strings used in CAB abstracts

The search strategy developed for WoS was adapted and transferred in CAB Abstracts. For the reasons explained above, the time span of the search was the same as in WoS, covering from 1998 to July 2016. For the definition of the content and the syntax of the search string the same factors considered for the WoS search were taken into account. The content of the WoS string, all the keywords organised and connected in elements, was transferred to the string for CAB Abstracts. Additionally, the following specifics of CAB Abstracts were considered:

- The search terms should be present in the title, abstract and the keywords defined by the authors;
- The number of hits in CAB Abstracts while testing the string was much smaller than in WoS.

Since there were less searchable fields and less hits in comparison to WoS, and in order to increase the sensitivity of the search, the proximity operator "NEAR" was removed from the string. Thus, the following full search string was used in CAB Abstracts:

(coleopter\* or collembol\* or dipter\* or hemipter\* or hymenopter\* or lepidopter\* OR mollus\* OR annelid\* OR acari\* OR arachn\* OR nematod\* OR fly OR flies OR bug OR bugs OR aphid\* OR beetle\* OR butterfly\* OR Apis OR bee OR moths OR moth OR ant OR ants OR wasp\* OR spider\* OR \*worm\* OR snail\* OR leech\* OR shrimp\* OR larva\* OR imago OR nymph\* OR pupa\* OR "nontarget organism\*" OR "non-target organism\*" OR invertebrate\* OR arthropod\* OR insect\* OR model OR adult\* OR invertebra\* OR Drosophila OR "Caenorhabditis elegans" OR "C. elegans" OR soil OR environment\*) AND ("double stranded ribonucleic acid\*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA interference" OR "RNA-interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA") AND (plant\* OR diet\* OR deliver\* OR ingest\* OR \*inject\* OR inoculat\* OR topical\* OR "target gene\*" OR spray\* OR oral\* OR feed\* OR fed OR field OR "gene silenc\*" OR "RNA\* silenc\*" OR "gene knockdown" OR "gene knock-out" OR "gene express\*" OR "\*RNA pathway" OR "\*RNA uptake" OR "environment\* assessment" OR "inhibit\* expression" OR "inhibit\* function\*" OR disrupt\* OR formation) NOT (drug\* OR rat OR rats OR rodent\* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor\* OR pig OR pigs OR cattle\* OR patient\*) NOT title:(mammal\* OR mouse OR human\* OR cancer\*) AND yr:[1998 TO 2016])

Complete documentation of the performed searches was made in order to make the process transparent and reproducible. This includes:

- The name of the database;
- The dates of the search for each database and the period searched;
- Any restrictions or filters used (region, language or publication status);
- The full search strategy (all terms and set combinations) and the numbers of records retrieved from each database;
- The total number of records retrieved from the information sources before and after removing duplicates.

All the documented searches and results were recorded and provided as an Endnote file (.enl) (Annex 1).

### 2.2.7. Inclusion and exclusion criteria

In order to be considered relevant for the review questions, and hence to be included in the review, a study needed to comply with each of the following requirements:

- Relevant intervention(s): Any type of exposure (e.g., oral ingestion, injection, spraying, *in planta* expression, *in vitro*, *in vivo*, in laboratory or field conditions, cross-species exchange) to exogenous RNAi molecules (e.g., miRNA, dsRNA or siRNA) or systems;
- Relevant population(s): Any invertebrate species belonging to annelids, nematodes, arthropods and molluscs, or any receiving relevant environment (e.g., soil, above ground, aquatic environments);
- Relevant outcome(s): Knockdown, upregulation and downregulation of gene expression; changes in any enzymatic, metabolic or physiological processes and functions; any survival, developmental, reproduction, behavioural, immunity or longevity effect; any non-target or off target effects and consequences for species concerned; any process related to the fate of RNAi molecules (e.g., uptake, delivery, mobility, transport, stability, persistence, efficacy, functionality, degradation) in invertebrates and the environment.

A study was excluded during the selection process for the following reasons (Reasons for exclusion - REx):

- Relevant intervention is missing (REx1);
- Relevant population is missing (REx2);
- Relevant outcome is missing (REx3);
- No original data is reported (this includes records such as patents, books, book chapters and commentary and review publications) (REx4);
- Duplicate data - the same data are reported in another study (REx5); or
- The full text is not accessible and the attempts for acquiring the studies were not successful (REx6).

### 2.2.8. Selection of relevant studies

The selection of relevant studies was done following a two-step process. In the first step, studies complying with the inclusion criteria were selected by two reviewers, initially by title and abstract, and later by screening the full text. The level of agreement between the reviewers was assessed using Kappa statistics (<http://www.vassarstats.net/kappa.html>).

In the second step, the team separately examined each study selected during the first step, and subsequently indicated which task(s) it related to, and whether it contained original data or it was a review of other studies. The studies were classified as follow:

1. Fully relevant studies – outcomes provide valuable information to one or more of the tasks;
2. Supplemental studies – outcomes do not provide new or substantial evidence useful for any of the review tasks;
3. Studies with unclear relevance – outcomes could not be assigned to any of the review tasks.

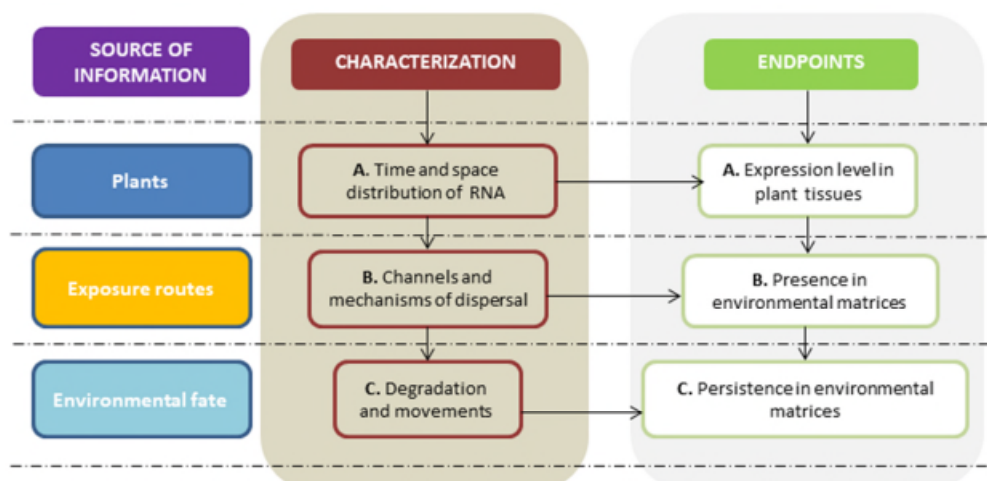
### 2.2.9. Relevance assessment criteria for studies on environmental exposure (Task 3) and off-target, non-target and unintended effects (Task 5)

For selecting studies relevant to Task 3 (Section 5.2), components of the environmental exposure routes and factors affecting each of these exposure pathways and fate of dsRNA, siRNA and miRNA were considered for target and NTOs in different environments, specifically referring to the literature concerning RNAi.

Studies from the database obtained with the systematic search described in this section were screened based on the conceptual framework, presented in Figure 1. Three categories of information were deemed relevant for the selection procedures: (1) reports dealing with the molecular characterization of RNA-expressing GM plants; (2) studies investigating possible exposure routes for RNA-expressing GM plants; and (3) reports about the environmental fate of RNA in environmental matrices.

Studies reporting on the production of RNA-expressing GM plants were reviewed by searching in particular for data relative to exogenous RNA expression levels in plant tissues. Studies presenting data on the presence of RNA (or DNA) in environmental matrices and studies presenting information on the possible movement and degradation of RNA (or DNA) in environmental matrices were also considered relevant.

Data were stored in MS Excel (Version 2007 SP3) format and can be easily used for reviewing the outcomes and results and for possibly updating when further studies will become available.



**Figure 1:** Conceptual framework for selecting studies relevant for the exposure characterization of RNA-expressing GM plants

The list of studies retrieved during the systematic search was screened for relevance to Task 5 (Section 5.4). Articles were deemed relevant if they were dealing with: target specificity of the dsRNA (i.e., bioinformatics analyses and/or gene silencing experiments were conducted comparatively on the target as well as on some non-target species); reports of unintended effects on the target species; bioassays with dsRNA on non-target species; evaluation of trophic chain effects.

In addition, information retrieved from studies published after the systematic search (August 2016 – November 2017) were also reviewed.

### 2.2.10. Management of the references and documents retrieved

When possible, the search results were exported from the databases in a format suitable for import in a reference manager program (e.g, "ris" or "ciw"). The EndNote program (EndNote X7) was used to create a library with the overall search results. Duplicate records were removed using the program's function. All the remaining duplications (due to differences in the reference representation) were removed manually. If not possible to be extracted in the reference manager format, the records were saved as plain text or as a table for screening separately from the EndNote library after removal of the duplications.

## 2.3. References

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### 3. Results of the systematic literature search (Task 1.2)

#### 3.1. Search results in Web of Science

The search in WoS was performed on 24 July 2016 and included all the databases in the subscription of ABI (Table 3). The search used the developed and tested for relevance search string (Table 2), with the time-span of the search from 1998 to July 2016. The search resulted in a total of 11,643 hits. All references were exported as "ciw" files into an EndNote library. The automatic function of the EndNote program "Find Duplicates" was able to find 199 duplications. From the overall results there were 354 references to books and book sections and 272 to patents, which were removed from the list. Thus 10,818 references from the WoS search were left to be screened for relevance.

#### 3.2. Search results in CAB abstracts

The search in CAB abstracts was performed on 12 August 2016 and details are summarised in Table 3.

A total of 6,037 hits were retrieved from CAB abstracts. All the results were exported as "ris" files and imported into the EndNote library.

An EndNote library was made uniting the results from the WoS and CAB abstracts searches. It contained all the 17,680 records (11,643 from WoS plus 6,037 from CAB abstracts). The automatic "Find Duplicates" function of EndNote was able to detect 1,645 records in CAB abstracts which duplicated records present in the WoS results. Together with the 199 duplicates detected within the WoS results, the total number of duplicates we detected automatically was 1,844. They were removed from the list, but kept in the "Trash" folder of the library file.

The remaining 10,818 records from WoS and 4,392 records from CAB abstracts were extracted in table format separately from the library and the files were used for an additional, semi-manual comparison of the references in order to identify duplications which could not be detected by the program due to the differences in spelling. This was done by using text comparing plug in MS Excel. In this way another 1,334 CAB abstracts records results duplicating WoS records were found. Finally, the total number of duplicates removed from the CAB abstracts records list was 2,979 (1,645 automatically detected, plus 1,334 semi-manually detected) or 49%, leaving 3,058 unique records for screening. Together with the 199 duplicates detected among in the WoS results, the final number of duplicates removed from the list was 3,178% or 18% of all the hits.

From the overall 17,680 records, the total number of records retrieved after removing duplicates (3,178), books (82), books sections (272) and patents (272) was 13,876 (10,818 records from WoS, plus 3,058 from CAB abstracts).

#### 3.3. Selection of relevant studies

All the records from the EndNote library created as a result of the search were extracted in a table format which was used for the selection of relevant studies.

The initial selection was performed by two reviewers at ABI independently. They assessed whether or not a study fulfilled the inclusion criteria, marking each of the reviewed studies in the MS Excel sheet with YES or NO. The screening was based on the content of the abstracts of the studies, however during the process it was noted that in many cases the amount of information was not sufficient to make a definitive decision. In these cases, the full text of the studies was examined as well.

**Table 3:** Details of the searches in Web of Science and CAB Abstracts

<b>Data requirement(s) captured in the search</b>	<b>Details of the searches</b>	
<p><b>Intervention(s):</b> Any type of exposure (e.g., oral ingestion, injection, spraying, in planta expression, in vitro, in vivo, in laboratory or field conditions, cross-species exchange) to exogenous RNAi molecules (e.g., miRNA, dsRNA or siRNA) or systems.</p>	<p><b>Database 1:</b> Web of Science including the following databases:</p> <ul style="list-style-type: none"> <li>• <i>Web of Science™ Core Collection</i></li> <li>• <i>BIOSIS Citation Index<sup>SM</sup></i></li> <li>• <i>Current Contents Connect</i></li> <li>• <i>Data Citation Index<sup>SM</sup></i></li> <li>• <i>Derwent Innovations Index<sup>SM</sup></i></li> <li>• <i>KCI-Korean Journal Database</i></li> <li>• <i>MEDLINE®</i></li> <li>• <i>Russian Science Citation Index</i></li> <li>• <i>SciELO Citation Index</i></li> <li>• <i>Zoological Record®</i></li> </ul>	<p><b>Database 2:</b> CAB Abstracts (1984-2016) (CABI, Wallingford, UK)</p>
<p><b>Population(s):</b> Any invertebrate species belonging to annelids, nematodes, arthropods and molluscs or any relevant receiving environment (e.g., soil, above ground, aquatic)</p>	<p><b>Justification for choosing the source:</b> WoS is a platform which allows simultaneous search across the major multi-disciplinary databases for scholarly literature.</p>	<p><b>Justification for choosing the source:</b> CABI is a comprehensive database for the applied life sciences – agriculture, environment, veterinary sciences, applied economics, food science and nutrition.</p>
<p><b>Outcome(s):</b> Knockdown, upregulation and downregulation of gene expression; changes in any enzymatic, metabolic or physiological processes and functions; any survival, developmental, reproduction, behavioural, immunity or longevity effect; any non-target or off target effects and consequences for species concerned; any process related to the fate of RNAi molecules (e.g., uptake, delivery, mobility, transport, stability, persistence, efficacy, functionality, degradation) in invertebrates and the environment.</p>	<p><b>Date of the search:</b> 24 July 2016  <b>Language limitation:</b> no limitation  <b>Date span of the search:</b> 1998 to 24 July 2016</p>	<p><b>Date of the search:</b> 12 August 2016  <b>Language limitation:</b> no limitation  <b>Date span of the search:</b> 1998 to 12 August 2016</p>
	<p><b>Search strategies used for this data requirement</b></p> <p>TS=(coleopter* or collembol* or dipter* or hemipter* or hymenopter* or lepidopter* OR mollus* OR annelid* OR acari* OR arachn* OR nematod* OR fly OR flies OR bug OR bugs OR aphid* OR beetle* OR butterfly* OR Apis OR bee OR moths OR moth OR "ant" OR "ants" OR wasp* OR spider* OR *worm* OR snail* OR leech* OR shrimp* OR larva* OR imago OR nymph* OR pupa* OR "nontarget organism*" NEAR/20 RNAi OR "non-target organism*" NEAR/20 RNAi OR invertebrate* NEAR/20 RNAi OR arthropod* NEAR/20 RNAi OR insect* NEAR/20 *RNA* OR model NEAR/20 dsRNA OR adult* NEAR/20 RNAi OR invertebra* NEAR RNAi OR</p>	<p><b>Search strategies used for this data requirement</b></p> <p>(coleopter* or collembol* or dipter* or hemipter* or hymenopter* or lepidopter* OR mollus* OR annelid* OR acari* OR arachn* OR nematod* OR fly OR flies OR bug OR bugs OR aphid* OR beetle* OR butterfly* OR Apis OR bee OR moths OR moth OR ant OR ants OR wasp* OR spider* OR *worm* OR snail* OR leech* OR shrimp* OR larva* OR imago OR nymph* OR pupa* OR "nontarget organism*" OR "non-target organism*" OR invertebrate* OR arthropod* OR insect* OR model OR adult* OR invertebra* OR Drosophila OR "Caenorhabditis elegans" OR "C. elegans" OR soil OR environment*) AND ("double stranded ribonucleic acid*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA</p>

	<p>Drosophila NEAR RNAi OR Drosophila NEAR dsRNA OR "Caenorhabditis elegans" NEAR RNAi OR "Caenorhabditis elegans" NEAR dsRNA OR "C. elegans" NEAR RNAi OR soil NEAR *RNA* OR environment* NEAR RNA) AND TS=("double stranded ribonucleic acid*" OR "double-stranded RNA" OR "double stranded RNA" OR RNAi OR "RNA interference" OR "RNA- interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA") AND TS=(plant* NEAR *RNA OR diet* NEAR *RNA OR deliver* NEAR *RNA OR ingest* NEAR *RNA OR *inject* NEAR *RNA OR inoculat* NEAR *RNA OR topical* NEAR *RNA OR target NEAR/5 gene* OR spray* NEAR *RNA OR oral* NEAR *RNA OR feed* NEAR *RNA OR fed NEAR *RNA OR field OR gene NEAR/3 silenc* OR RNA* NEAR/3 silenc* OR gene NEAR/3 knockdown OR gene NEAR/3 knock- out OR gene NEAR/3 express* OR *RNA NEAR/3 pathway OR *RNA NEAR/3 uptake OR environment* NEAR/3 assessment OR inhibit* NEAR/3 expression OR inhibit* NEAR/3 function* OR disrupt* OR formation NEAR RNAi) NOT TS=(drug* OR rat OR rats OR rodent* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor* OR pig OR pigs OR cattle* OR patient*) NOT TI=(mammal* OR mouse OR human* OR cancer*)</p>	<p>interference" OR "RNA-interference" OR dsRNA OR miRNA OR siRNA OR microRNA OR "small RNA") AND (plant* OR diet* OR deliver* OR ingest* OR *inject* OR inoculat* OR topical* OR "target gene*" OR spray* OR oral* OR feed* OR fed OR field OR "gene silenc*" OR "RNA* silenc*" OR "gene knockdown" OR "gene knock-out" OR "gene express*" OR "*RNA pathway" OR "*RNA uptake" OR "environment* assessment" OR "inhibit* expression" OR "inhibit* function*" OR disrupt* OR formation) NOT (drug* OR rat OR rats OR rodent* OR clinical OR "Stem Cells" OR carcinoma OR leukaemia OR tumor* OR pig OR pigs OR cattle* OR patient*) NOT title:(mammal* OR mouse OR human* OR cancer*) AND yr:[1998 TO 2016])</p>
	<p><b>Total number of records received: 11,643</b></p>	<p><b>Total number of records received: 6,037</b></p>
	<p><b>Total number of records retrieved after removing duplicates, books, books sections and patents: 13,876</b></p>	

In the beginning of the selection process, after the first 1,000 records were screened the level of agreement between the two reviewers was assessed using kappa statistics. The calculation based on

the reviewers' decisions resulted in kappa coefficient of 0.7, and according to the scale given by Landis and Koch (1977)<sup>1</sup> the reviewers agreement is "substantial" ( $k > 0,6$ ).

Studies in which the decision on relevance differed between the two reviewers, were subjected to additional reviewing by another two independent reviewers, i.e., Ghent University and JT Environmental Consultants. Based on their judgment the final decision on relevance was made.

The assignment of the selected studies to one or more of the review questions was made during the accomplishment of the respective tasks in the project proposal. Studies were excluded based on the defined inclusion and exclusion criteria defined in Section 2.2.7.

During the initial screening, the reviewers found that 8,801 records did not fulfil the inclusion criteria or were excluded and these studies were excluded from the list (Figure 2). The 5,075 eligible reviewed studies were those that mostly included exposure of different types of invertebrates to RNAi molecules and were marked as relevant by the reviewers. While this number is surprisingly high, to a great extent it is due to the large number of RNAi studies involving the two model organisms: free living nematode *C. elegans* and fruit fly *D. melanogaster*. Both organisms have been used for studying gene functions, metabolic pathways, and a variety of physiological functions.

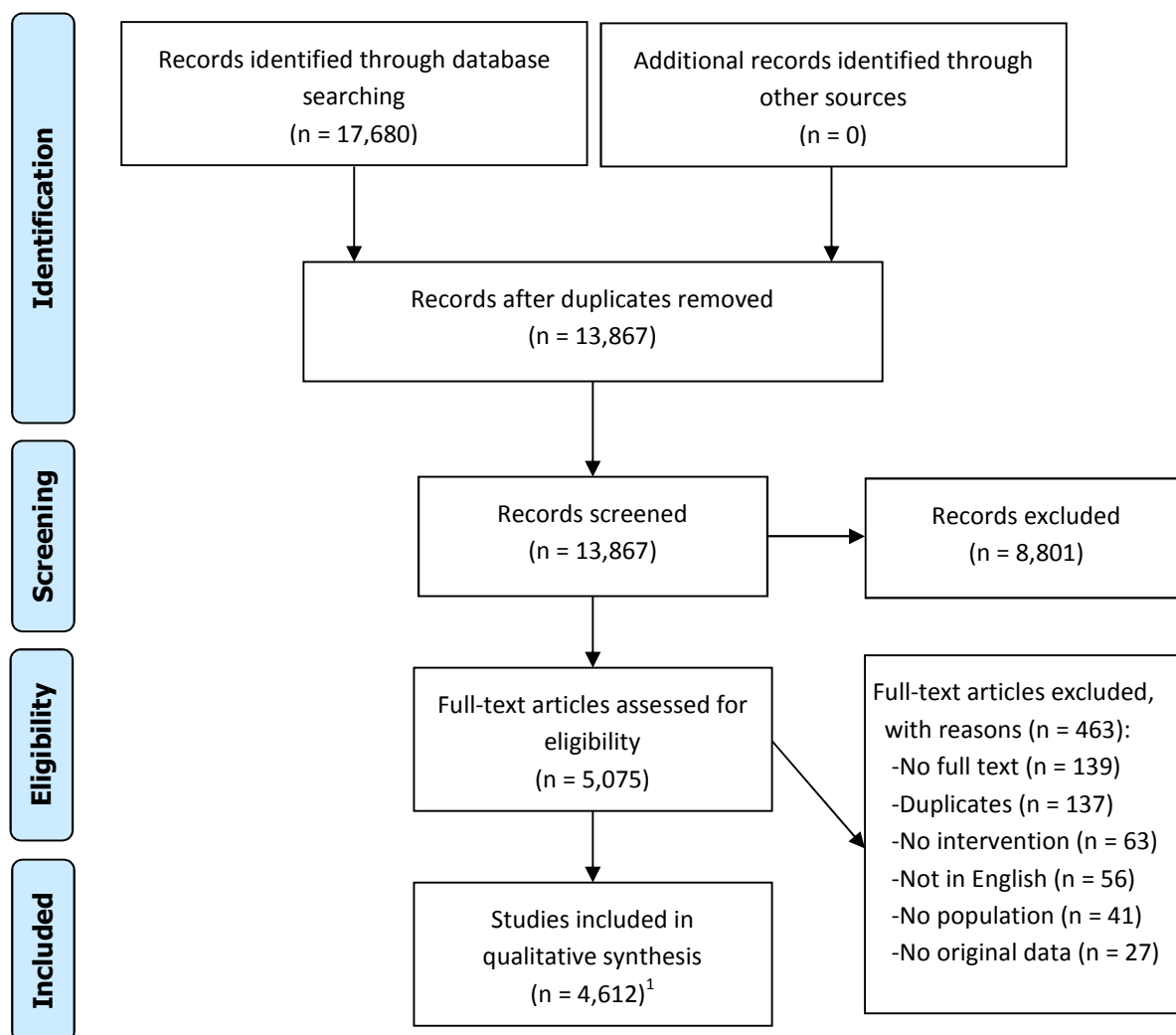
The selection of the studies continued with the retrieval of the full text of the selected studies from the databases. The full text was used for an additional screening to confirm the presence of the inclusion criteria. During this stage, primary data related to the used invertebrate species and the delivery method of the RNAi molecules was extracted from the relevant studies. As a result, 463 references were removed from the selected studies for the following reasons: no full text (e.g., poster references, meeting abstracts) - 139 references; duplications - 137 references; relevant intervention is missing - 63 references; full text is not in English - 56 references; relevant population is missing - 41 references; no original data is present (reviews and commentary publications) - 27 references. Thus, 4,612 full text studies were finally selected and primary data was extracted for all of them.

### 3.4. Conclusions on literature search

The literature searches in online databases WoS and CAB Abstracts resulted in a total number of 17,680 records, which were reduced to 13,867 unique studies after removing duplicates, books, book sections and patents. During the initial screening of these 13,867 records/studies, we have identified eight major methods for delivery of interfering RNA molecules in around 300 invertebrate species belonging to annelids, nematodes, arthropods and molluscs. To what extent the selected studies are relevant (fully relevant; supplemental studies or studies with unclear relevance) to the review questions are discussed in the following sections. After manual relevance assessment, a total of 4,612 studies were considered relevant.

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<sup>1</sup> Landis R and Koch GG, 1977. The measurement of observer agreement for categorical data. *Biometrics*, 33, 159–174.



**Figure 2:** Flow diagram of the selection process

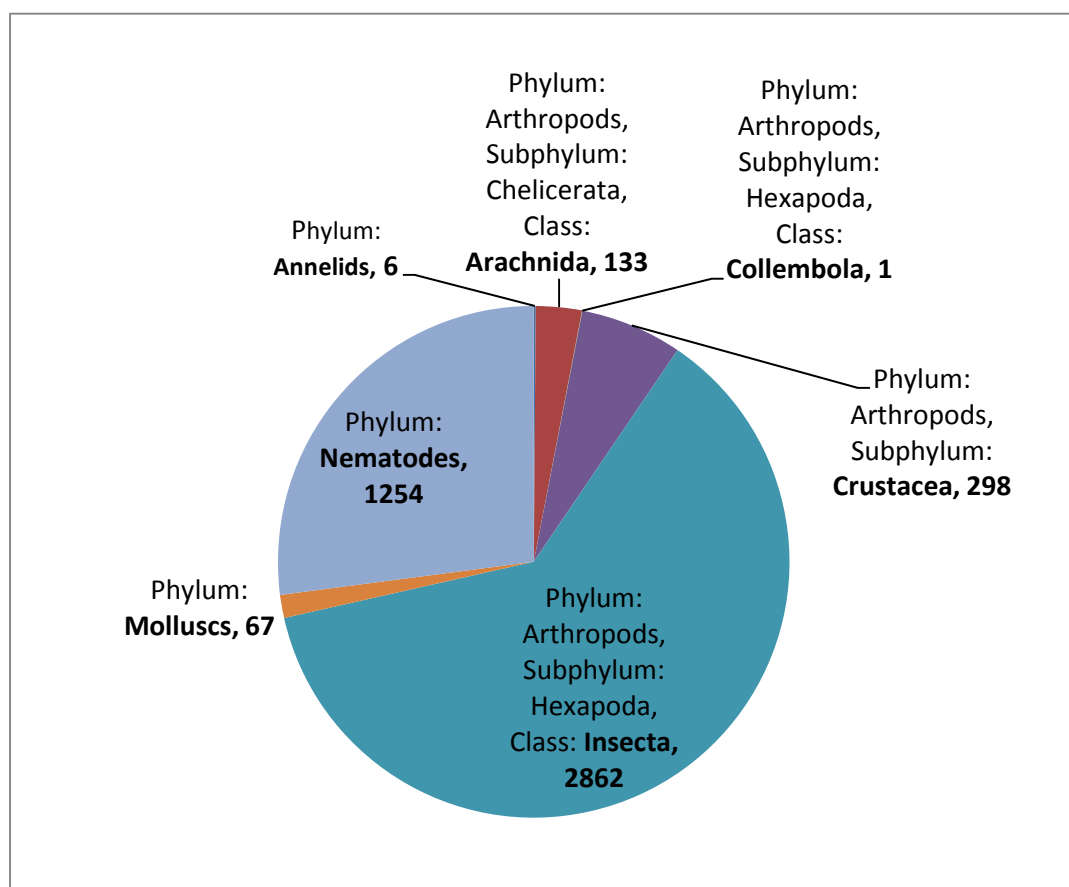
## 4. Analysis of the primary data from the selected studies

### 4.1. Activity spectrum of the tested dsRNAs, siRNAs and miRNAs in invertebrates (Task 1.3)

The extracted data was used to create a list of invertebrate species studied in the relevant RNAi publications. The list consists of 341 species (Appendix B) belonging to the four major groups of invertebrates in the focus of this report: annelids, nematodes, arthropods and molluscs. The number of studies in which each species appears is also included in Appendix B.

Arthropods are the most abundant group of species used in the RNAi experiments, representing approximately 62% of all the studies (Figure 3). The major subgroup is that of the subphylum Hexapoda (86%, or 2,862 studies), followed by Crustacea (10%, or 298 studies) and Arachnida (133 studies). Only one study involved the soil-inhabiting springtails (Collembola).

The second largest group of invertebrates are the nematodes with a total of 1,254 studies. The number of RNAi studies which involve molluscs and annelids is much smaller compared to the other two groups of organisms, with 67 and six studies, respectively.



**Figure 3:** Invertebrates used in RNAi studies retrieved in the systematic literature search. The numbers refer to the number of studies found for each taxonomic group

#### 4.1.1. Arthropods

In the conduct of RNAi experiments, the largest group of species from arthropods belong to the hexapods and, more specifically, the insect class. They are represented by more than 180 different species, which is around 60% of all the studied invertebrate species. Approximately 44% of all insect RNAi studies involved one species, namely the - the fruit fly *D. melanogaster* (1,243 studies). Following in numbers are the red flour beetle *Tribolium castaneum* (184 studies) and the silkworm *Bombyx mori* (176 studies) and, model insects belonging to the orders of Lepidoptera and Coleoptera, respectively (Table 4).

**Table 4:** Insect species which appear in more than ten RNAi studies

No.	Species	Number of studies
1	<i>Drosophila melanogaster</i>	1,243
2	<i>Tribolium castaneum</i>	184
3	<i>Bombyx mori</i>	176
4	<i>Aedes aegypti</i>	133
5	<i>Apis mellifera</i>	78
6	<i>Helicoverpa armigera</i>	67
7	<i>Anopheles gambiae</i>	66
8	<i>Nilaparvata lugens</i>	55
9	<i>Plutella xylostella</i>	42
10	<i>Blattella germanica</i>	39
11	<i>Locusta migratoria</i>	39
12	<i>Spodoptera exigua</i>	36
13	<i>Leptinotarsa decemlineata</i>	32
14	<i>Gryllus bimaculatus</i>	31
15	<i>Spodoptera frugiperda</i>	30
16	<i>Manduca sexta</i>	27
17	<i>Rhodnius prolixus</i>	25
18	<i>Spodoptera litura</i>	25
19	<i>Laodelphax striatellus</i>	24
20	<i>Bactrocera dorsalis</i>	23
21	<i>Oncopeltus fasciatus</i>	22
22	<i>Schistocerca gregaria</i>	21
23	<i>Culex pipiens</i>	19
24	<i>Diabrotica virgifera virgifera</i>	17
25	<i>Aedes albopictus</i>	16
26	<i>Glossina morsitans morsitans</i>	16
27	<i>Acyrtosiphon pisum</i>	15
28	<i>Bemisia tabaci</i>	15
29	<i>Nasonia vitripennis</i>	13
30	<i>Myzus persicae</i>	11

Mosquito species, vectors of human diseases, such as *Aedes aegypti* (133 studies) and *Anopheles gambiae* (66 studies) are also frequently studied. The honey bee (*Apis mellifera*) is also in the top list with 78 studies (Table 4).

The agricultural insect pests most frequently occurring in RNAi studies are four Lepidoptera species (*Helicoverpa armigera* (67 studies), *Plutella xylostella* (42 studies), *Spodoptera exigua* (36 studies), *Spodoptera litura* (25 studies), the Hemiptera *Nilaparvata lugens* (55 studies), and the Coleoptera – *L. decemlineata* (32 studies).

Shrimps are the most commonly used crustacean species in RNAi studies, including the ones used as sources of food: *Litopenaeus vannamei* (88 studies), *Penaeus monodon* (71 studies), *Marsupenaeus japonicus* (39 studies) and *Fenneropenaeus chinensis* (14 studies).

From the class Arachnida, the most commonly occurring species are the ticks *Haemaphysalis longicornis* (29 studies), *Ixodes scapularis* (20 studies), *Amblyomma americanum* (16 studies) and *Ixodes ricinus* (12 studies).

#### 4.1.2. Phylum: Nematodes

The free-living nematode *C. elegans* is involved in 1,109 studies which is nearly 90% of all the studies within the phylum of nematodes. More than 30 other nematode species are subjects of RNAi experiments, of which the most represented are the plant-parasitic species *Meloidogyne incognita* (44 studies), *Heterodera glycines* (16 studies), *Bursaphelenchus xylophilus* (15 studies), *Caenorhabditis briggsae* (14 studies), *Meloidogyne javanica* (nine studies), *Heterodera schachtii* (eight studies) and *Radopholus similis* (seven studies).

#### 4.1.3. Phylum: Molluscs

The great pond snail (*Lymnaea stagnalis* – 10 studies), which is a model for neurological studies, and economically important mollusc species, such as the pacific oyster (*Crassostrea gigas* – 10 studies), pearl oyster (*Pinctada fucata* – 10 studies) and Farrer's scallop (*Chlamys farreri* – nine studies), are the most often used species in RNAi studies from this group.

#### 4.1.4. Phylum: Annelids

One annelid species, *Hirudo medicinalis*, is present in six RNAi studies including ring worms. This species has been used as a model organism in cellular analyses of nervous system function.

#### 4.1.5. Methods used to trigger RNAi in invertebrates

According to the extracted primary data, the most commonly used method to deliver interfering RNA molecules in invertebrate species is by injection, found in approximately 40% of the studies. Injecting solutions of RNA molecules in the thorax of adults and larvae at different stages, or directly into eggs or embryos (microinjection), took place in half of the RNAi studies with arthropods. It is also the most used method in molluscs, but less common in nematodes (Table 5).

Oral delivery of dsRNA is the most often used method to trigger RNAi in nematodes. This is partially due to the existence of a routine experimental protocol for RNAi screening of *C. elegans* by feeding worms with dsRNA expressing bacteria. However, the delivery of dsRNA as a supplement to natural or artificial diets or feeding with dsRNA expressing transgenic plants is also being used in many arthropod species.

Incubation and transfection are being used for induction of RNAi in cell cultures and embryos of many arthropods. Soaking, spraying or topical application of dsRNA are other methods for delivery of different groups of invertebrates. In nearly half of the *Drosophila* studies RNAi was induced by



generating transgenic insects or so-called RNAi lines. We have noted that in 140 studies there is more than one method used for dsRNA delivery.

**Table 5:** Delivery of interfering RNA molecules in invertebrates

Delivery method	Annelids	Arthropods	Molluscs	Nematodes	Mixed Phyla	Total
<b>Single method studies</b>						
Injection (embryos, eggs, larvae, adults)	5	1,547	53	208	2	1,815
Oral		314		820		1,134
Transgenic insects		743			1	744
Cells (incubation or transfection)		554	10	10		574
Soaking	1	31	2	100		134
Transgenic nematodes				21		21
In silico		13		7	1	21
Other		10		1	1	12
Lysate (cells, embryos)		6		2		8
Topical		6				6
Spraying		3				3
<b>Multi-method studies</b>		57	2	77	4	140
<b>Total</b>	<b>6</b>	<b>3,284</b>	<b>67</b>	<b>1246</b>	<b>9</b>	<b>4,595</b>

#### 4.2. Oral introduction of siRNA and miRNA and the induction of efficient RNAi in arthropods, nematodes, annelids and molluscs

For the purpose of ERA baseline information, studies using a feeding or soaking delivery are especially important. Therefore, we have identified all such studies and have prepared an overview table (Annex 2) listing the relevant information for each study, including:

- Species;
- Phylum;
- Order;
- Life stage tested;
- Number of generations investigated;
- RNAi triggering molecule (dsRNA, miRNA, siRNA);
- Length of the RNAi triggering molecule;
- Exposure duration;
- Delivery method (e.g., soaking, feeding bacteria producing dsRNA, in planta, via artificial diet,);
- Target gene;
- The dose or concentration used in the experiment;
- The observed effects of RNAi silencing.

## 5. Narrative reviews

### 5.1. Uptake and systemic spread of small RNAs in invertebrates (Task 2)

For exogenous small RNAs (sRNAs) to have a silencing effect, they need to be internalized into the cell and released into the cytoplasm. Once there, they are then processed further into 20-25 nt long small interfering RNAs (siRNAs) by the RNase III Dicer enzymes, which constitutes the start of the cytoplasmatic RNAi pathway. In this part, we review the current evidence on the cellular uptake mechanisms of sRNAs in invertebrates and the pathways involved in this process. While these mechanisms are still being investigated and under debate, a sizable body of research is already available, mainly in nematodes and arthropods. Here, we explore the similarities and differences between these mechanisms in the different taxonomical groups and we also discuss the implications on the efficiency of RNAi in invertebrate species. Furthermore, we also discuss systemic RNAi, which involves the spread of the silencing signal from the cells or tissues that were exposed to environmental sRNAs to other tissues in the body.

#### 5.1.1. Introduction

Whangbo and Hunter (2008) described three concepts relating to RNAi and cellular uptake or transport: (1) cell-autonomous RNAi, which is the RNAi process that happens within a cell after the sRNA has been introduced into the cytoplasm; (2) environmental RNAi, in which the cell is able to take up sRNAs from its environment (e.g., gut lumen, cell medium), leading to silencing in those cells that are exposed to the environmental sRNAs; and (3) systemic RNAi, whereby the sRNAs, after uptake from the environment, are also transported further to neighbouring cells and tissues, eventually leading to RNAi-induced gene silencing there. Systemic RNAi has been shown to be functional in several nematode and arthropod species, including the nematodes *C. elegans* (Hunter et al., 2006; Winston et al., 2002b) and *Panagrolaimus superbus* (Shannon et al., 2008), the coleopterans *T. castaneum* (Miller et al., 2012; Tomoyasu et al., 2008a), *Di. virgifera* (Li et al., 2016) and *Aethina tumida* (Powell et al., 2017), the honeybee *A. mellifera* (Aronstein et al., 2006; Jarosch and Moritz, 2011), the cricket *Gryllus chico* (Dabour et al., 2011), the grasshopper *Schistocerca americana* (Dong and Friedrich, 2005), the locusts *Locusta migratoria* (Luo et al., 2013) and *Schistocerca gregaria* (Wynant et al., 2012), the mollusc *C. gigas* (Fabioux et al., 2009). However, this is not the case for all invertebrates. In both the nematode and arthropod clades, there are species in which efficient (systemic) RNAi has not been achieved yet, or RNAi success is variable at best. This is the case for example in several lepidopteran, dipteran and hemipteran insect species, as well as animal- and plant-parasitic nematodes and several species from the *Caenorhabditis* genus (Christiaens and Smagghe, 2014; Christiaens et al., 2014; Terenius et al., 2011). This insensitivity to environmental and systemic RNAi could be linked to several different phenomena, such as a slow or limited cellular uptake, a lack of dsRNA stability in the animal's gut or haemolymph, issues with the cellular core machinery, viral interactions, amplification of the silencing signal, etc. Most of these factors are discussed in Task 4, which deals with the efficiency of RNAi in invertebrates.

In this part, we focus on issues related to cellular uptake of sRNAs, systemic transport and amplification of the silencing signal. Understanding these mechanisms and uptake routes could be vital for assessing possible implications in terms of environmental exposure to sRNAs. We start by discussing the possible entry routes of (dietary) sRNA into the body of invertebrates. Next, an overview is given of what is known so far regarding the cellular uptake of sRNAs and the components that have been identified as being involved in this process. Furthermore, we discuss the evidence available for systemic transport and the mechanisms involved in this process. Finally, the amplification of the silencing signal, which has only really been identified in nematodes, is discussed. Since there is very little knowledge available on these processes in annelids and molluscs, this review mainly focuses on nematodes and arthropods.

### 5.1.2. Entry routes of sRNA into the body of invertebrates

Before going into details on cellular uptake, the first question, when considering exposure to environmental sRNAs, is how these molecules could naturally enter the body of an invertebrate and reach the internal tissues. Quite early on in RNAi research, scientists found that both nematodes and arthropods could be targeted for RNAi by oral ingestion of the sRNAs (Baum et al., 2007b; Soares et al., 2005; Timmons et al., 2001; Timmons and Fire, 1998; Turner et al., 2006; Whyard et al., 2009). Once inside the digestive tract, sRNAs can be taken up intracellularly, by epithelial cells lining the gut, and potentially also passed through to the body cavity by transcytosis, leading to a systemic response (Calixto et al., 2010; Chan and Snow, 2017; Jose and Hunter, 2007; Jose et al., 2009). sRNAs could potentially also pass the digestive tract barrier paracellularly, meaning they are transported by diffusion between the epithelial cells, via the septate junctions. This mode of action was suggested by Chan & Snow (Chan and Snow, 2017), as this type of transport has been found to occur for a number of other molecules and viruses (Bonning and Chougule, 2014; Casartelli et al., 2007; Fiandra et al., 2009; Hardy et al., 1983; Huang et al., 2015; Jeffers and Roe, 2008). However, to the best of our knowledge, no experimental evidence for paracellular transport of sRNAs in invertebrates is available.

Several studies in both nematodes and arthropods have shown that topical application, by spraying or soaking for example, could also trigger an efficient silencing response (Bakhetia et al., 2005; Fanelli et al., 2005; Gu and Knipple, 2013; Killiny et al., 2014; Pridgeon et al., 2008; Tabara et al., 1998; Wang et al., 2011; Whyard et al., 2009). At present, topical uptake routes have not been investigated thoroughly and the exact uptake mechanisms are not well understood. It is known that, for nematodes, the cuticle is permeable to some degree for water molecules, certain ions and nonelectrolytes including some organic nematicides (Bird and Bird, 2012). Whether this is also the case for nucleic acids is unclear. It has been hypothesized that differences in cuticle permeability could be a factor in explaining the variability in RNAi efficiency observed between different nematode species (Dalzell et al., 2011a). However, we were unable to find direct evidence for transport of sRNAs through the cuticle. An alternative explanation for successful RNAi using soaking as the delivery method could be the fact that the dsRNA is actually taken up orally from the soaking solution. In fact, fluorescence microscopy experiments performed by Urwin et al., (2002) showed that soaking *C. elegans* in a solution containing fluorescein isothiocyanate (FITC) led to oral uptake, rather than uptake through the integument. This hypothesis might also explain why the silencing of *sid-2* in *C. elegans*, a gene coding for a protein which is critical for sRNA-uptake in the gut, led to a strong decrease in RNAi efficiency, both in feeding and soaking experiments (Winston et al., 2007).

In insects, the chitin-based cuticle itself is thought to be impenetrable, protecting the insect from desiccation. Therefore, topical uptake of sRNAs has to happen in another way. One plausible theory is that the topically applied sRNAs enter the body and reach the internal tissues through the tracheal system (Gu and Knipple, 2013). The tracheal system is comprised of a network of branched tubes inside the insect, which are connected to the outside environment through valved spiracles on the cuticle of the insects. Through these spiracles, air can enter into the trachea, where gas exchange can happen and oxygen can be taken up. However, we found no direct evidence of tracheal uptake of sRNAs in arthropods. A number of insect dsDNA viruses, for example nuclear polyhedrosis viruses and baculoviruses, have been shown to infect tracheoblasts, but these are considered to be secondary infections coming from the midgut (Barrett et al., 1998; Clem and Passarelli, 2013; Engelhard et al., 1994).

Another possible entry way was proposed by Killiny et al., (2014), who managed to induce RNAi by topically applying a dsRNA solution on the thorax of adult citrus psyllids (*Diaphorina citri*). They hypothesized that the dsRNA could enter the body through the intersegmental membranes of the thorax. Direct proof for uptake through the integument was provided by Wang et al. (2011), using a topical application of fluorescently labelled dsRNA in eggs and larvae of the Asian corn borer *Ostrinia furnacalis*.

Whyard et al. (2009) published a study investigating several delivery strategies in insects and found that after soaking one-day old *D. melanogaster* larvae in a dsRNA solution targeting a GUS construct led to a small degree of transcript silencing (5-8%), but only in gut tissues. Addition of Lipofectamine to the dsRNA solution, a transfection agent which can increase cellular uptake efficiency, increased the silencing efficiency to 50% in isolated guts. No silencing in other tissues was reported. Adding a colouring dye to the dsRNA solution also confirmed that the liquid was merely ingested, and no evidence for transport through the integument or via the trachea could be found. These findings showed that in soaking experiments, a silencing effect due to oral uptake, rather than uptake through the cuticle cannot be excluded. Given the fact that entry through parts of the body integument has been shown in other insect species, as noted earlier, it also raises the question about variability in uptake routes and uptake mechanisms between different species and different orders within the Insecta.

### 5.1.3. Cellular uptake and systemic transport

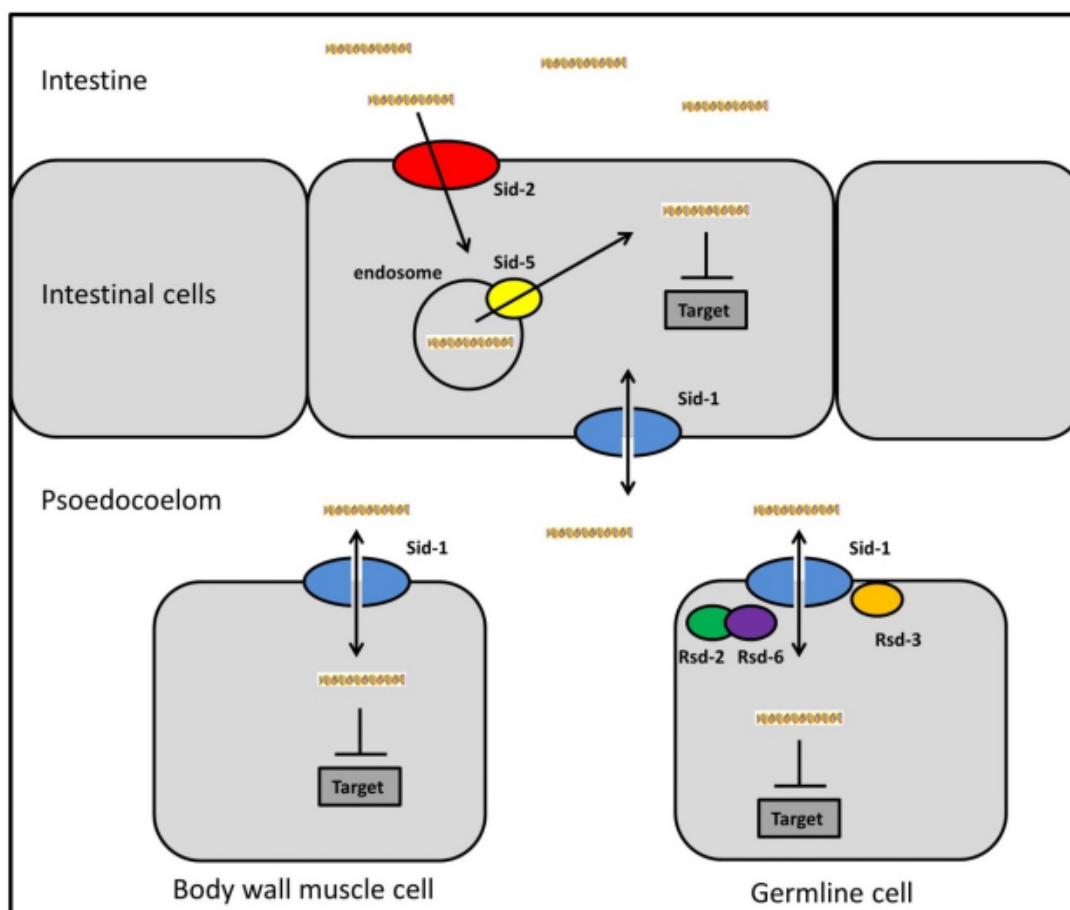
Much of what we know about RNAi, including sRNA cellular uptake mechanisms, was first reported in nematodes, more specifically in *C. elegans*. However, given the recent interest in the use of RNAi for pest control, more and more knowledge is being gathered on the molecular processes that drive RNAi in arthropods as well. In this section, we review the information available on cellular uptake and systemic transport in invertebrate species, since both are strongly linked. Given the considerable differences between different invertebrate subphyla in terms of sRNA cellular transport mechanisms and pathways, this part will be structured per subphylum.

#### 5.1.3.1. Nematodes

Since the discovery of RNAi in the free-living nematode *C. elegans* in 1998 (Fire et al., 1998), this species has become a model organism for RNAi research. Most of the molecular mechanisms involved in RNAi, including those of cellular uptake and systemic spread of sRNAs, were first described in this species. We review what is known on cellular uptake and transport of sRNAs in *C. elegans*, and discuss these systems in other nematodes, and the differences that exist.

##### *Cellular uptake and transport of sRNAs in Caenorhabditis elegans*

The complete picture of the cellular uptake of sRNAs in *C. elegans* has not yet been elucidated. However, several different types of proteins and mechanisms have been shown to be involved in this process, including certain elements from the endocytosis pathway and dsRNA-specific receptors or importers (Jose, 2015; Saleh et al., 2006b). Several Systemic RNA Interference Deficient (*sid*) genes have been discovered and characterized in nematodes and their involvement in uptake, export and systemic spread of sRNAs has been described (Feinberg and Hunter, 2003; Hinas et al., 2012; Jose et al., 2012; Krautz-Peterson et al., 2010; McEwan et al., 2012; Rocheleau, 2012; Shih et al., 2009; Shih and Hunter, 2011; Winston et al., 2002a). Despite the common name, these genes encode for proteins that belong to different protein families and have different working mechanisms and functions, besides all being involved in cellular uptake or transport of the silencing signal. Some of these *sid* genes are mainly active in the gut tissue, while some are active in other tissues and are involved in systemic transport. Besides the *sid* genes, several other components involved in cellular uptake have been identified as well, such as RNAi spreading-defective (*Rsd*) genes and components of the endocytosis pathway. A schematic overview of sRNA uptake pathways in *C. elegans* is given in Figure 4. Additionally, Table 6 lists all genes that have been implicated so far in feeding RNAi and systemic transport in *C. elegans*. The table is based on the table published by Saleh et al. (2006b) and expanded with additional known elements of the feeding/systemic RNAi pathway.



**Figure 4:** Overview of our current understanding of cellular uptake and transport of dsRNA in *Caenorhabditis elegans*. **A.** The situation in cells lining the intestinal lumen involves Sid-2, and has a dual role for Sid-1. Uptake from the intestinal lumen is facilitated by Sid-2. Sid-1 is involved in the release into the cytoplasm. Uptake from the body cavity is facilitated by Sid-1 directly, in association with Sid-3. **B.** In non-intestinal cells, Sid-2 is not involved and environmental uptake is mainly facilitated by Sid-1 and Sid-3. In both locations, the endosome-associated Sid-5 is involved in export out of the cell and transport of the silencing signal. In gonad cells, Rsd-2, Rsd-3 and Rsd-6 are thought to be involved in cellular uptake, although their exact roles are unknown. More recent research indicates that they might have an indirect effect through the biogenesis of secondary siRNAs (Zhang et al., 2012). Figure based on (Jose and Hunter, 2007); (Dalzell et al., 2011); (Rocheleau, 2012); (McEwan et al., 2012), (Meng et al., 2013) and (Sarkies and Miska, 2014)

### *Sid-1*

Using *C. elegans* mutant strains which were resistant to systemic RNAi, but not to autonomous RNAi, Sid-1 was first discovered in 2002 as a protein which is required for systemic RNAi. It is expressed in all non-neural cells and possesses predicted transmembrane domains (Winston et al., 2002). The same research group published a study in 2003, where the *C. elegans sid-1* gene (*CeSid-1*) was expressed in *Drosophila* Schneider 2 (S2) cells, which have a strong cell-autonomous RNAi response, but lack a *sid-1* homologue of their own and an environmental RNAi response. Upon expression of *CeSid-1*, these S2 cells showed a 25- to 130-fold increase of dsRNA uptake, indicating its role in the

cellular uptake of dsRNA. The study also showed that the transport via Sid-1 is passive (Feinberg and Hunter, 2003). Since *C. elegans sid-2* mutants were found to be resistant to environmental RNAi, it seemed clear that *sid-1* alone is not sufficient to fully explain environmental cellular uptake (Winston et al., 2007). McEwan et al. (2012) later showed that, in cells lining the intestinal lumen, it is actually part of a multi-step uptake mechanism involving endocytosis-mediated uptake. First, dsRNA in the intestinal lumen is specifically recognized by Sid-2 membrane proteins on the cell membrane and then taken up by endocytosis. Sid-1 is then responsible for the release of dsRNA from the endosomes into the cytoplasm (McEwan et al., 2012). This also explains the observation that in *sid-1* defective intestinal cells, environmental RNAi was not functional, but transport across the intestine was found to occur (Winston et al., 2007).

The involvement of endocytosis in sRNA uptake in *C. elegans* was unexpected, since earlier studies suggested no involvement in these import (or export) processes (Tijsterman et al., 2004). Further studies also showed that besides dsRNA, Sid-1 is capable of importing pre-miRNA and hairpin-RNA. However, this uptake did not occur as efficiently as 500 bp long dsRNA, possibly due to stretches of single-stranded RNA in those sRNAs (Shih and Hunter, 2011). Finally, a study from José et al. in 2009 also demonstrated that Sid-1 is not necessary for export of sRNAs out of the cell, transport across the cells lining the intestinal lumen or further spreading of the systemic silencing signal (Jose et al., 2009).

### *Sid-2*

The protein encoded by the *sid-2* gene is a membrane-bound protein which has been shown to be required for environmental RNAi in *C. elegans* (McEwan et al., 2012; Winston et al., 2007). Winston et al. (2007) first demonstrated that *Sid-2* mutants were resistant to RNAi by bacterial delivery, while transgene-mediated RNAi was successful in these mutants. The study also showed that Sid-2, contrary to Sid-1, was not necessary for export of sRNAs out of the cells and subsequent spread. It is expressed mainly in cells lining the intestinal lumen and was found not to be necessary for uptake of silencing information in muscle cells (Winston et al., 2007). McEwan et al. (2012) reported that the Sid-2 transmembrane receptor is pH-dependant and only active in acidic environments. Furthermore, it was shown to be selective for dsRNA of 50–1,500 bp long. The same study also discovered that the Sid-2 uptake system is linked to vesicle transport and endocytosis, whereby Sid-2 is the receptor selectively binding dsRNA from the intestinal lumen before endocytosis-mediated uptake occurs. McEwan et al. (2012) also suggested that similar systems, using tissue-specific and environment-specific proteins like Sid-2, could exist in other tissues as well to transport dsRNA to environments which are more suitable for broadly expressed proteins such as Sid-1 to function.

### *Sid-3*

Recently, a tyrosine kinase encoded by the gene *sid-3* was discovered and was shown to be implicated in cellular uptake of dsRNA in *C. elegans* (Jose et al., 2012). This kinase is a homolog of the mammalian activated cdc-42-associated kinase (ACK), which is known to be directly associated with endocytic vesicles (Jose et al., 2012; Teo et al., 2001; Yang et al., 2001). José et al. found the Sid-3 protein to be localized in the cytoplasm of many *C. elegans* tissues and, using *sid-3* mutants, found that this protein was required for efficient environmental RNAi, including uptake of dsRNA from the intestine. However, some silencing in the *sid-3* mutants was still observed, contrary to what was observed in for example *sid-1* mutants. Cell-autonomous RNAi was not affected by the absence of Sid-3, and the researchers could also prove Sid-3 is only involved in import, and not export, of dsRNA (Jose et al., 2012).

### *Sid-5*

In 2012, the systemic RNAi pathway expanded further with the discovery of Sid-5, a protein encoded by the *sid-5* gene and which is found to be associated with the endosomes of somatic cells (Hinas et al., 2012). Hinas et al. discovered that *C. elegans sid-5* mutants are refractory to systemic RNAi and are only partially sensitive to environmental RNAi (Hinas et al., 2012). While these effects are similar

to those observed in *sid-3* mutants, the protein encoded by *sid-5* functions entirely differently. Immunohistochemistry experiments revealed that *sid-5* is expressed in somatic cells and is associated with late-endosomes in the cells (Hinas et al., 2012). These late endosomes have been implicated as sites of RNAi activity within cells (Gibbings and Voinnet, 2010; Lee et al., 2009). Experiments performed with *sid-5* mutants showed that rescue of *sid-5* expression in intestinal cells only restored successful RNAi in body wall muscle (bwm) cells, while rescue of *sid-5* in bwm cells only, did not recover functional RNAi in these cells, demonstrating that Sid-5 is necessary for transport across the intestine and spreading of the silencing signal. This is contrary to what was discovered for Sid-1, where rescue in the intestine had no effect on bwm cell RNAi functionality, but rescue in bwm cells successfully recovered RNAi there (Hinas et al., 2012; Jose et al., 2009).

### *Rsd* genes

Similar to the *sid* genes, RNA Spreading Defective (*rsd*) genes comprise a group of structurally and functionally diverse proteins. What they have in common is that they are somehow involved in systemic RNAi and the spreading of the silencing signal in *C. elegans*. Tijsterman et al. (2004) discovered these genes through screens with mutant worms, which were defective for systemic RNAi. They identified 5 genes, which they divided into two groups based on the mutant phenotype; *rsd-4* (identified as *sid-2*) and *rsd-8* (found to be an allele of Sid-1 eventually (Winston et al., 2002)) (Class I) mutations resulted in worms which were defective for RNAi of both somatic and germline specific genes, while *rsd-2*, *rsd-3* and *rsd-6* (Class II) mutations led to worms which were only refractory to RNAi in the germline cells but did not impair initial uptake from the intestine and transport to somatic cells.

The exact role in the cellular uptake or transport mechanisms has not been elucidated yet for most of these genes. One of the best characterized RSD proteins is RSD-3, a homolog of epsinR in mammalian cells, which is implicated in clathrin-mediated vesicular transport (Legendre-Guillemain et al., 2004). Originally thought to be a germline cell-specific gene (Tijsterman et al., 2004), Imae et al. (2016) recently found that the gene is ubiquitously expressed, also in somatic cells, and is required for systemic RNAi in both tissue types. Co-localization studies showed that RSD-3 is associated with the Trans-Golgi-network (TGN) and endosomal vesicles. The *rsd-3* mutation caused only a partial resistance to RNAi in somatic cells, contrary to for example knockout of *sid-1*. The study also showed that RSD-3 is not involved in cellular uptake from, or export out of, intestinal cells, but is required for subsequent uptake from the pseudocoelom. Given these characteristics, the authors hypothesized that RSD-3 might be associated with the same transport pathway involving Sid-1, which is also implicated in uptake and internal cellular transport of sRNAs (Imae et al., 2016).

Tijsterman et al. (2004) further reported that RSD-2 exhibited no known motifs or close homologs in other organisms that could give us a clue about its function, while RSD-6 contains a Tudor domain, frequently found in RNA-binding proteins. Yeast two-hybrid experiments showed that RSD-2 and RSD-6 potentially act as a complex. Zhang et al. (2012) demonstrated that RSD-2 and RSD-6 are required for the accumulation of secondary siRNAs in *C. elegans*, which could explain these earlier observations, and would imply that they are not directly affecting sRNA uptake. Furthermore, both are also implicated in maintaining chromosome integrity (Han et al., 2008), and in antiviral RNAi through the production of secondary viRNAs (Guo et al., 2013).

### *fed* genes

Timmons et al. (2003) identified two more mutants, which were insensitive to RNAi by feeding, but did exhibit systemic RNAi when the dsRNA was injected. However, the genes involved in these mutants were not further investigated at that time. *fed-1* and *fed-2* are likely to be allelic with *sid-2/rsd-4* and *rsd-2*, respectively, based on phenotype and genetic map position (Jose and Hunter, 2007; Whangbo and Hunter, 2008).

### *Other genes or pathways involved in uptake and systemic spread*

At least twelve additional proteins which are likely to be involved in uptake and systemic spread of sRNAs were identified after a genetic screen for feeding RNAi in *Drosophila* S2 cells was performed by Saleh et al. (2006a). *C. elegans* homologs of the genes, which were found to be involved in *Drosophila* feeding RNAi, were knocked out in worms and the effect on feeding RNAi was observed. Most genes which were found in this screen were linked to cellular vesicular transport, such as vacuolar protein sorting-41 (*vps-41*), conserved oligomeric Golgi complex subunit 2 (*cgo-2*) and ADP-ribosylation factor-like protein 1 (*arl-1*). Furthermore, genes involved in lipid metabolism were also identified, together with a few genes of unknown function. These data further proved that endocytosis and vesicular transport is critical in dsRNA uptake and systemic spread. However, the exact role of these genes in the different uptake and transport pathways has not been further investigated yet. A full list of these genes is given in Table 6.

**Table 6:** Overview of all genes implicated in *Caenorhabditis elegans* feeding RNAi uptake and systemic spread (adapted from Saleh et al. (2006b))

Group	<i>C. elegans</i> gene ID	<i>C. elegans</i> gene name
Sid genes	C04F5.1	<i>Sid-1</i>
	ZK520.2	<i>Sid-2</i>
	B0302.1	<i>Sid-3</i>
	F14B8.2	<i>Sid-5</i>
Rsd genes	F52G2.2	<i>Rsd-2</i>
	C34E11.1	<i>Rsd-3</i>
	F16D3.2	<i>Rsd-6</i>
Vesicle mediated transport	F54C9.10	<i>arl-1</i>
Intracellular transport	F22G12.5	
	C06G3.10	<i>cgo-2</i>
	ZK1098.5	<i>trpp-3</i>
	F32A6.3	<i>vps-41</i>
Lipid metabolism	R01H2.5	<i>ger-1</i>
	B0025.1	<i>vps-34</i>
Other	B0464.4	<i>bre-3</i>
Unknown	W05H7.3	<i>sedl-1</i>
	Y45G12B.2	
	C54H2.1	<i>sym-3</i>

Finally, Sundaram et al. (2006) reported the involvement of at least one ATP binding cassette (ABC) transporter, encoded by *haf-6*, in environmental RNAi of *C. elegans*. In their study, RNAi by injection, soaking and feeding was applied on wild type and *haf-6* mutants. The experiments demonstrated that the *haf-6* mutants had become less sensitive to oral RNAi in germline cells, in a concentration-dependant manner. Interestingly, the assays also showed loss of RNAi phenotype in intestine cells, but not in other somatic cells. Subsequent localization experiments provided an explanation when it was discovered that *haf-6* is mainly expressed in intestinal and germline cells and thus the loss of RNAi sensitivity in these *haf-6* mutants mainly occurs in the cells where *haf-6* is normally expressed in wild type forms. However, exactly how *haf-6* influences RNAi and whether it is really related to uptake or transport is not known. ABC transporters in general are known to be able to transport substrates in or out of the cells, but also between intracellular compartments, so an involvement in transport of the silencing signal is likely.



A more recent study suggested involvement of another ABC transporter, *haf-2*, in the RNAi machinery upon exposure to a non-coding RNA (ncRNA) called OxyS from the *Escherichia coli* bacteria used in the feeding studies (Liu et al., 2012a). This OxyS ncRNA contains a 17nt homologous sequence to the *C. elegans* gene *che-2*, encoding a G-protein containing WD40 protein. The authors observed depletion in *che-2* mRNA upon feeding on *E. coli* and linked this to a silencing effect induced by OxyS uptake. In their investigation, knockout of *sid-1* and *sid-2* revealed no change in silencing efficiency, suggesting a different pathway being involved in uptake. Mutants in which *haf-2* was knocked out affected the observed *che-2* silencing, suggesting an involvement of this ABC transporter (Liu et al., 2012a). However, Akay et al. (2015) further investigated this phenomenon, using OxyS-overexpressing and –knockout strains small RNA mapping and came to the conclusion that *che-2* silencing was not caused by OxyS ncRNA and that the latter does not cause RNAi in *C. elegans*. In a recent review, Waqas and Shan pointed to the fact that in the small RNA screens performed by Akay et al., there was a shift from 21 nt to 22 nt RNAs between the wild type nematodes and RNAi deficient nematodes used in the study, which was not noticed or mentioned by the authors. Furthermore, the review also points out that different development stages were used in both studies, which can make comparisons difficult (Waqas and Shan, 2016).

#### Cellular uptake in other nematode species

In the past 15 years, it has become clear that the mechanisms in *C. elegans* are not always representative of the whole nematode subphylum. Indeed, several species such as the soil nematodes *Caenorhabditis briggsae* (Winston et al., 2007), *Caenorhabditis remanei* (Winston et al., 2007), *Caenorhabditis brenneri* (Winston et al., 2007), *Oscheius tipulae* (Louvet-Vallée et al., 2003; Wheeler et al., 2012), *Rhabditis* sp. (Wheeler et al., 2012), *Mesorhabditis* sp. (Wheeler et al., 2012), *Acrobeloides* sp. (Wheeler et al., 2012) and *Pristionchus pacificus* (Pires-daSilva and Sommer, 2004), as well as the animal-parasitic nematodes *Haemonchus contortus* (Geldhof et al., 2006), *Trichostrongylus colubriformis* (Issa et al., 2005) and *Ostertagia ostertagi* (Visser et al., 2006) exhibit a lower or variable sensitivity to (environmental) RNAi. Plant-parasitic nematodes generally seem to be susceptible to environmental RNAi, although variability in gene silencing efficiency has been observed during *in planta* experiments (Charlton et al., 2010; Huang et al., 2006; Ibrahim et al., 2011; Klink et al., 2009; Li et al., 2010; Lilley et al., 2012; Patel et al., 2010; Patel et al., 2008; Sindhu et al., 2009; Steeves et al., 2006; Urwin et al., 2002). Furthermore, researchers found that *Brugia malayi* is competent for environmental RNAi, but has no homologs of *sid-1* and *sid-2* in its genome (Aboobaker and Blaxter, 2004; Ghedin et al., 2009). In the parasitic nematode *Globodera pallida*, neuronal cells are susceptible to exogenously supplied dsRNA, while this is not the case for most neuronal cells in *C. elegans* (Kimber et al., 2007; Whangbo and Hunter, 2008).

This led researchers to investigate the differences between these so-called RNAi effectors in *C. elegans* and in other nematode species. An extensive study by Dalzell et al. (2011) looked into the available genomic and transcriptomic data of 13 nematode species and revealed that *C. elegans* possesses an expanded repertoire of RNAi-related genes compared to many other nematodes. Most species which were included in the investigation, except those belonging to the *Caenorhabditis* genus, contained less than half of the genes considered involved in RNAi in *C. elegans*. Furthermore, several components which are known to be involved in cellular uptake in *C. elegans* were found absent in parasitic nematodes (Dalzell et al., 2011). Most striking was the observation that *sid-1* and *sid-2* are absent in most investigated nematodes not belonging to the *Caenorhabditis* genus, including the free-living nematode *P. pacificus*, the plant-parasitic *Meloidogyne* species and the human parasite *B. malayi*. Since both genes are instrumental in environmental RNAi and systemic spread in *C. elegans*, their absence might, at least partially, explain the lower sensitivity to RNAi in *P. pacificus* (Dalzell et al., 2011; Viney and Thompson, 2008). Interestingly though, several studies have shown successful systemic RNAi in *Meloidogyne* and *Globodera* species (Antonino de Souza Junior et al., 2013; Bakhietia et al., 2005; Dalzell et al., 2010a; Dalzell et al., 2010b; Kimber et al., 2007; Rosso et al., 2005) and successful environmental RNAi in *B. malayi* (Aboobaker and Blaxter, 2004; Song et al., 2010). This raises the question whether the loss of *sid-2* and its role in cellular uptake from the gut

environment is compensated by other proteins or whether these genes are fast evolving and species have developed alternative pathways for sRNA uptake and systemic spread. Dalzell et al. (2011) did report that all nematodes used in the study displayed similar coverage of the functional protein groups in the entire RNAi machinery, meaning they possessed genes from each functional group. The main difference was a further expansion and diversification, possibly due to gene duplication events, within these functional groups in *Caenorhabditis* species, and notably in *C. elegans*.

Within the group of *Caenorhabditis* species, the differences were not as profound as compared to other nematode species, especially concerning genes that are involved in cellular uptake and transport processes (Dalzell et al., 2011). However, evidence for a fast evolution of some components can be found within this *Caenorhabditis* genus as well. *Caenorhabditis briggsae* was found to possess a highly divergent homolog of *sid-2* (*Cbsid-2*), causing this species to lose its oral RNAi sensitivity. Their study showed that expression of *Cesid-2* in *C. briggsae* caused *C. briggsae* to become sensitive again for oral RNAi. Conversely, expression of *Cbsid-2* in *sid-2* mutants of *C. elegans* could not rescue the response/sensitivity to oral RNAi sensitivity (Winston et al., 2007).

#### *Influence of the sRNA molecule on cellular uptake and systemic RNAi efficiency*

Most of the knowledge on cellular uptake mechanisms and systemic transport of sRNAs in *C. elegans* which is discussed above, was discovered in dsRNA feeding assays, often using bacterial-based delivery constructs. Data on cellular uptake of miRNA in nematodes is scarcer. Furthermore, the length of the dsRNA also proved to be a determining factor for environmental and systemic RNAi. In this section, we discuss what is known on uptake and transport of miRNA and on the influence of dsRNA/siRNA length on efficient environmental and systemic RNAi.

#### *Uptake and transport of miRNAs in nematodes*

miRNAs are small (20-25 nt) RNA molecules, first discovered in *C. elegans*, which are important in internal gene regulation in all animals (Lau et al., 2001). For the most part, they require an RNAi pathway which is molecularly distinct from the dsRNA/siRNA pathway (Hoogstrate et al., 2014; Parry et al., 2007). While most miRNAs seem to be intracellular, some have been found in extracellular biofluids inside extracellular vesicles, where they can potentially act as signalling molecules. Furthermore, some human parasitic nematodes have also been found to secrete these miRNA-containing vesicles in their host (Buck et al., 2014; Coakley et al., 2015; Grainger et al., 2010; Maizels et al., 2012; Quintana et al., 2016). How these vesicles are then taken up by the recipient cells is not yet known. In humans, several hypotheses have been proposed, including endocytosis and membrane fusion (Boon and Vickers, 2013). These findings show that nematode cells are at least capable of exporting miRNAs, through the formation of these extracellular vesicles.

Regarding potential uptake of exogenous miRNA, not much is known in nematodes. An RNAi screening study to identify genes involved in the miRNA pathway did not identify any genes that are clearly related to uptake or extracellular transport. The authors did identify genes that are involved in intracellular trafficking and export from the nucleus (Parry et al., 2007), but whether they also have a role in import or export of the cell is unclear. Finally, while Shih and Hunter (2011) discovered that Sid-1 in *C. elegans* is capable of importing pre-miRNAs when expressed in S2 cells, we have found no evidence for oral or environmental miRNA uptake by nematodes.

#### *Length of sRNA*

The influence of dsRNA length on RNAi efficiency in nematodes was reported early in RNAi research. In 2000, Parrish et al. observed that in green fluorescent protein (GFP) construct carrying transgenic *C. elegans*, 717 bp-long double-stranded GFP (dsGFP) caused a stronger silencing effect of the GFP construct expression than 50-200 bp-long dsGFP (Parrish et al., 2000). At that moment, the reason for this was still unclear. Furthermore, Feinberg and Hunter (2003) suggested that this factor might be linked to the cellular uptake of dsRNA, and more specifically to Sid-1, important in systemic dsRNA transport. They found that for a 100 bp-long dsRNAs to have the same silencing efficiency as 500 bp-

long dsRNA in *Drosophila* S2 cells expressing the *C. elegans sid-1* gene, a 100-1000-fold higher concentration was necessary. Similarly, 21 bp siRNA required a 10<sup>5</sup>-fold higher concentration to have the same silencing efficiency as 100 bp-long dsRNA (Feinberg and Hunter, 2003). However, it was later found that Sid-1 does not have selectivity for length (Shih et al., 2009). In contrast, Sid-2, which is required for environmental uptake in the gut of *C. elegans*, does exhibit selectivity for dsRNA length and that dsRNAs longer than 25 bp are required for efficient uptake (McEwan et al., 2012).

In other nematodes, siRNA/dsRNA molecules ranging from 21 bp to 1799 bp have been shown to be capable of inducing functional dsRNA (Bakhetia et al., 2007; Dalzell et al., 2010b; Huang et al., 2006; Hussein et al., 2002; Urwin et al., 2002). Interestingly, when dsRNA of 1799 bp was compared to dsRNA of 204 bp in an RNAi study on *Nippostrongylus brasiliensis*, researchers found that the silencing effect after feeding on the 1799 bp dsRNA only lasted 1-2 days, while the effect after exposure to 204 bp long dsRNA lasted up to at least 6 days (Hussein et al., 2002). However, to date, no comprehensive study on the correlation between length and RNAi efficiency has been performed in these non-*C. elegans* nematodes.

### 5.1.3.2. Arthropods

#### *Drosophila*

After *C. elegans* in 1998, *D. melanogaster* became the second animal species to have its genome sequenced in 2000. Both species henceforth became model species for many molecular studies, including RNAi. Experiments with S2 cells, derived from a primary *D. melanogaster* embryo cell line, had already shown that cell-autonomous RNAi and cellular uptake from medium is fully functional (Clemens et al., 2000; Worby et al., 2001). The first attempts to trigger RNAi in flies, using transgenic flies containing a transgenic RNAi construct, suggested that RNAi in *D. melanogaster* may not be systemic (Giordano et al., 2002; Kalidas and Smith, 2002). This was later confirmed by Roignant et al. (2003) who demonstrated, using a transgenic RNAi construct, that effective gene silencing was only achieved in cells in which RNAi was triggered. In contrast, research on transmission of viruses in *D. melanogaster* suggests that there must be a systemic RNAi pathway, at least for viral RNA (Saleh et al., 2009). These authors hypothesized that, while sRNA might not be exported from healthy, uninfected cells, virus-infected cells could release sRNA either after apoptosis or through a virus-induced shedding mechanism which can then be taken up by other cells in the organism (Saleh et al., 2009).

Additionally, various experimental designs attempting feeding RNAi in *Drosophila* species showed a lack of silencing response when naked dsRNA was administered (Taning et al., 2016; Whyard et al., 2009). However, when a transfection agent such as Lipofectamine was added to the dsRNA solution, RNAi silencing was observed (Taning et al., 2016; Whyard et al., 2009). These results indicated that besides a lack of systemic RNAi, *Drosophila* also seems to lack an efficient uptake of dsRNA from the midgut.

Interestingly, the *Drosophila* genome contains no homologs of the *C. elegans sid* genes, which, as discussed earlier, are instrumental in cellular uptake and systemic spread of dsRNA in *C. elegans*. However, *in vivo* experiments delivering dsRNA via microinjection in *Drosophila* had been shown to cause successful silencing in different tissues, suggesting there is dsRNA-uptake in various cell types in *D. melanogaster* (Dzitoyeva et al., 2001a; Dzitoyeva et al., 2001b; Dzitoyeva et al., 2003; Goto et al., 2003). In 2006, two independent studies which looked into the cellular uptake mechanism of dsRNA in S2 cells were published almost simultaneously. Both demonstrated that receptor-mediated endocytosis is involved in dsRNA uptake in S2 cells (Saleh et al., 2006b; Ulvila et al., 2006). The first, by Ulvila et al. (2006) used an RNAi-induced lethality screen to investigate components of the RNAi pathway in *Drosophila* and found that silencing clathrin heavy chain, a protein required for endocytosis, was able to rescue the cells and thus inhibit RNAi. This suggested that the cellular uptake of dsRNA in S2 cells might be facilitated by clathrin-mediated endocytosis. This hypothesis was confirmed by fluorescence microscopy experiments which showed that the fluorescently-labeled

dsRNA were internalized by S2 cells via cytoplasmic vesicles and by the fact that RNAi-mediated knockdown of the genes encoding the Scavenger receptor (SR-CI) and Eater inhibited endocytosis of dsRNA, proving that dsRNA uptake in S2 cells happens via scavenger receptor-mediated endocytosis (Ulvila et al., 2006). Scavenger receptor and eater are two structurally similar membrane-bound proteins which are known to be involved in phagocytosis of bacterial pathogens and are thus not dsRNA-specific (Kocks et al., 2005; Rämetsä et al., 2001; Ulvila et al., 2006). Interestingly though, expression of both *sr-ci* and *eater* in post-embryonic stages was found to be restricted mainly to haemolymph plasmatocytes of fruit flies, meaning that uptake in other cell types may use other pathways (Kocks et al., 2005; Kroeger et al., 2012; Pearson et al., 1995; Rämetsä et al., 2001).

The second 2006 study reporting on a dsRNA uptake mechanism in S2 cells was published by Saleh et al. (2006b). They also demonstrated the role of endocytosis in dsRNA uptake in S2 cells through fluorescence microscopy and the use of pharmacological endocytosis-inhibitors and identified an extended list of genes involved in this process using a genome-wide screen, as we have discussed earlier (See 4.1). Furthermore, adding inhibitors of Scavenger receptor to the medium also caused inhibition of the RNAi response, confirming that, at least in S2 cells, SR-CI is involved in uptake. Genes identified in the Saleh et al. (2006b) study are listed in Table 7 and include some elements which are also implicated in *C. elegans* cellular uptake of dsRNA.

**Table 7:** Overview of all genes implicated in cellular uptake of dsRNA in *D. melanogaster* S2 cells (adapted from Saleh et al., (2006b))

Functional group	<i>Drosophila</i> gene ID	<i>Drosophila</i> gene name
Proton transport	CG3161	<i>Vha16</i>
	CG17332	<i>VhaSFD</i>
Vesicle mediated transport	CG9012	<i>Clathrin hc</i>
	CG7057	<i>AP-50</i>
	CG5915	<i>Rab7</i>
	CG6025	<i>Arf72A</i>
Intracellular transport	CG54125	<i>ninaC</i>
	CG6177	<i>IdlCp</i>
	CG3248	
	CG3911	
	CG18028	<i>light</i>
Lipid metabolism	CG3495	<i>Gmer</i>
	CG5373	<i>Pi3K59F</i>
	CG12070	<i>Saposin-r</i>
Proteolysis and peptidolysis	CG4572	
	CG5053	
	CG8184	
	CG8773	
Other	CG9659	<i>egghead</i>

### Other arthropods

As was the case with *C. elegans*, *Drosophila* does not seem to be the most representative species for other arthropods, in relation to cellular uptake of sRNAs and systemic RNAi. Current evidence suggests that *Drosophila* is more of an exception, rather than the rule for systemic uptake of RNAi. For example, systemic RNAi has been observed in several insect species, including Orthoptera (Dabour et al., 2011; Dong and Friedrich, 2005; Luo et al., 2013; Luo et al., 2012; Mito et al., 2008; Ronco et al., 2008; Santos et al., 2014; Wynant et al., 2012), Coleoptera (Bucher et al., 2002; Li et al., 2016; Miller et al., 2012; Powell et al., 2017; Tomoyasu et al., 2008; Zhu et al., 2011), Hemiptera (Araujo et al., 2006; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Pitino et al., 2011) and Hymenoptera (Aronstein et al., 2006; Jarosch and Moritz, 2011; Lynch and Desplan, 2006). Further evolved lepidopteran and dipteran species are generally thought to be less efficient for systemic RNAi. However, feeding of RNAi leading to effects in other tissues than the gut have been reported for some Lepidoptera (Tian et al., 2009; Turner et al., 2006; Zhang et al., 2015) and Diptera (Li et al., 2011). The first indications for functional systemic RNAi systems have also been reported for several non-insect arthropod species, including several tick and mite species (Aung et al., 2011; Khila and Grbic, 2007; Kyaw Min et al., 2011; Wu and Hoy, 2014), as well as the crustacean *Daphnia* (Schumpert et al., 2015). Additionally, inducing RNAi by feeding, whether systemically or restricted to gut epithelial cells, has been achieved for many arthropod species, including insects, chelicerates and crustaceans (See Section 5.3).

### *Sil*-mediated uptake

In contrast to *Drosophila* and some other dipterans, *sid-1* homologues have been found in various other insect species, including *T. castaneum* (Tomoyasu et al., 2008; Xiao et al., 2015), *L. decemlineata* (Cappelle et al., 2016), *D. virgifera* (Miyata et al., 2014), *B. mori* (Tomoyasu et al., 2008), *S. gregaria* (Wynant et al., 2014a), *L. migratoria* (Luo et al., 2012), *A. mellifera* (Aronstein et al., 2006), *N. lugens* (Xu et al., 2013). Interestingly, beetle species possess multiple distinct *sid-1* homologues (Cappelle et al., 2016; Miyata et al., 2012; Tomoyasu et al., 2008; Xiao et al., 2015). Given the highly efficient systemic RNAi in beetles, compared to other insects, it is tempting to hypothesize that this efficiency might be linked to an augmented repertoire of *sid-1* genes. Several studies have been published in various species investigating the role of these *sid-1* homologues in insects. A study by Tomoyasu et al. in 2008, investigating systemic RNAi in the red flour beetle *T. castaneum*, found that knockdown of these *Tcsid-1* genes did not affect systemic RNAi in *T. castaneum*, suggesting they might not have the same involvement in sRNA cellular uptake as they have in *C. elegans*. Furthermore, a more detailed analysis of these gene sequences revealed that they share more similarity to *tag-130*, a *C. elegans sid-1*-like gene, than to *sid-1* itself. Hence, they decided to name these genes *sid-1*-like (*sil*) genes. Also, knockout of *tag-130* in *C. elegans* showed no involvement in RNAi (Tomoyasu et al., 2008). Another observation casting doubt over the link between *sil* gene repertoire and efficient environmental or systemic RNAi, was the fact that several lepidopteran species, which are much less sensitive to (systemic) RNAi (Terenius et al., 2011), also contain three *sil* genes, including *B. mori*, *Danaus plexipus* and *Spodoptera littoralis* (Cappelle et al., 2016; Tomoyasu et al., 2008). Of course, a lack of sensitivity to systemic or environmental RNAi could be due to several factors. One example is degradation of dsRNA in digestive systems and/or haemolymph of insect species (Allen and Walker, 2012; Arimatsu et al., 2007; Christiaens et al., 2014; Garbutt et al., 2013; Liu et al., 2012b; Wynant et al., 2014b) which seems especially important in Lepidoptera.

More recently, studies on sRNA cellular uptake and systemic RNAi mechanisms have been conducted in several other insect species. In the coleopteran species *D. virgifera* and *L. decemlineata*, two *sil* genes were identified in the genome and, in contrast to *T. castaneum*, these *sil* genes were found to be required for efficient environmental or systemic RNAi in both species (Cappelle et al., 2016; Miyata et al., 2014). In the *D. virgifera* study, a two-step in vivo assay was used to investigate the effect of knocking down *silA* and *silC* on RNAi efficiency. Miyata et al. (2014) observed only a modest decrease

in RNAi efficiency after either *silA* or *silC* were silenced. The limited effect could just be due to the fact that only a partial knockdown of the effector genes was achieved (50 – 60%), but it could also indicate that these *sils* are both involved in the uptake mechanism and could exhibit some redundancy towards each other. Another possible explanation is that multiple distinct pathways are involved in uptake of dsRNA in *D. virgifera*. In the *L. decemlineata* study, Cappelle et al. (2016) also found a small effect on RNAi feeding efficiency after silencing of *silA* (14.4% rescue of expression) or *silC* (8.1% rescue of expression), but they also looked at the effect of simultaneous silencing and found a 24.1% rescue of the reporter gene expression upon simultaneous silencing of both *sil* genes. In this study, knockdown of *silA* and *silC* only resulted in a 59% and 66% gene silencing, respectively, making it difficult to draw conclusions on the importance of these genes for environmental or systemic RNAi.

In non-beetle species, *sil* has been suggested to be involved in environmental RNAi in the honeybee *A. mellifera* and the brown planthopper *N. lugens*, both containing one *sil* gene. In the honeybee, Aronstein et al. (2006) observed that upon feeding of dsRNA, honeybee sil expression increased 3.4-fold compared to the control group which was not fed dsRNA. While the authors concluded that this is proof for involvement of *sil* in cellular uptake or transport of the silencing signal, the evidence is indirect at best. Further research in honeybee *sil* is needed to confirm this hypothesis. In *N. lugens*, the evidence for sil involvement is stronger. The authors injected dsRNA specific for *N. lugens sil*, together with dsRNA targeting distal-less (*dll*), a gene which is involved in development of the distal limb structure. Silencing of *dll* causes claw defects in this planthopper. Injection of dsSil led to strong silencing of *sil* throughout the body (>90%) on day 3 and at day 6, the authors observed normal claw structures for most treated insects, while only a few had minor defects (Xu et al., 2013). In contrast, even though *sil* genes were discovered in the genomes of the orthopteran locusts *S. gregaria* and *L. migratoria*, knockdown of these genes had no adverse effect on RNAi efficiency, suggesting that they might not play an important role for dsRNA uptake (Luo et al., 2012; Wynant et al., 2014a). A full overview of what is known so far on sil-involvement in insect RNAi is presented in Table 8.

#### *Endocytosis-mediated uptake*

As discussed above, even though *sil* genes are not present in *Drosophila*, S2 cells are still capable of internalizing dsRNA efficiently due to the scavenger receptor-mediated endocytosis of dsRNA. In recent years, a number of studies have investigated whether this pathway also plays a role in other insects and found proof for involvement of this pathway in feeding or systemic RNAi in every species in which it was investigated (Table 8), including *T. castaneum*, *L. decemlineata* and *S. gregaria* (Cappelle et al., 2016; Wynant et al., 2014a; Xiao et al., 2015). In all three studies, so-called RNAi of RNAi experiments were used to implicate key endocytosis components in RNAi efficiency. Interestingly, Cappelle et al. (Cappelle et al., 2016) showed for the first time that both endocytosis and *sil* genes play a role in uptake or transport of dsRNA in an insect species, namely *L. decemlineata*. They found that silencing *chc* and *vha16*, both important members of the endocytosis pathway, impaired the feeding RNAi efficiency significantly. The observed effect was also more profound than when *silA* and *silC* were silenced, indicating that endocytosis might be more important in dsRNA uptake. Using a pharmacological inhibitor of endocytosis, there was a near-complete rescue of reporter gene expression (Cappelle et al., 2016). Recently, endocytosis was also shown to be involved in RNAi in a non-insect arthropod, namely the tick *H. longicornis* (Aung et al., 2011). In addition, this study used an RNAi approach to investigate the impact of Scavenger Receptor B (*SR-B*) silencing on the silencing efficiency of the reporter genes, in this case vitellogenin-1 (*Vg-1*) and vitellogenin receptor (*VgR*). Knockdown of *SR-B* before *Vg-1* or *VgR* silencing led to rescue of the hatching rate to 83%, compared to 0% and 13.7% after just *Vg-1* or *VgR* silencing, respectively (Aung et al., 2011).

#### *Conclusions on arthropods*

This study of the literature indicates that dsRNA uptake in insects, and possibly in other arthropods, is distinct from uptake mechanisms in nematodes, or in *C. elegans*, more specifically. The available

evidence suggests that *Sid-1*-like transmembrane channels might play some role in dsRNA uptake and transport in insects, but the proof for this is not unequivocal. While *Sil* has been implicated in *N. lugens*, *D. virgifera* and *L. decemlineata*, it did not appear to be necessary for systemic RNAi in *T. castaneum* and the orthopteran *S. gregaria* and *L. migratoria*. Perhaps there is a link with environmental RNAi here, as the species in which *Sil* has not been considered necessary are also not sensitive to environmental RNAi, while most insects for which *Sil* was found to be required for efficient systemic RNAi also exhibit a robust environmental RNAi response. One exception is *N. lugens*, for which feeding RNAi is not successful. But, as suggested before, other factors such as dsRNA stability in the digestive system could also play a role here. The studies conducted so far seem to indicate that endocytosis might play a more prominent role than *sil*-mediated uptake or transport of dsRNA. However, it cannot be excluded that both pathways act together and could have a synergistic effect (Cappelle et al., 2016).

**Table 8:** Overview of reported dsRNA uptake experiments in arthropods. Table adapted from Cappelle et al. (2016)

Insect order	Species	Environmental RNAi	Systemic RNAi	No. of <i>sid-1</i> homologs present	Application method	SID-1 is involved	Endocytosis is involved	Reference
Diptera	<i>Drosophila melanogaster</i> (S2 cell line)	+	+	0	S	no	yes	(Saleh et al., 2006b; Ulvila et al., 2006)
Coleoptera	<i>Bactrocera dorsalis</i>	+	+	0	F	no	yes	(Li et al., 2015c)
	<i>Tribolium castaneum</i>	+	++	3	I	no	yes	(Tomoyasu et al., 2008; Xiao et al., 2015)
	<i>Diabrotica virgifera</i>	++	++	2	F	yes	n.d.	(Miyata et al., 2014)
	<i>Leptinotarsa decemlineata</i>	++	++	2	F	yes	yes	(Cappelle et al., 2016)
Lepidoptera	<i>Bombyx mori</i>	-	+	3	I	no	n.d.	(Tomoyasu et al., 2008)
Orthoptera	<i>Schistocerca gregaria</i>	-	++	1	I	no	yes	(Wynant et al., 2014a)
	<i>Locusta migratoria</i>	-	++	1	I	no	n.d.	(Luo et al., 2012)
Hymenoptera	<i>Apis mellifera</i>	+	+	1	F	yes	n.d.	(Aronstein et al., 2006)
Hemiptera	<i>Nilaparvata lugens</i>	-	+	1	I	yes	n.d.	(Xu et al., 2013)
Ixodida	<i>Haemaphysalis longicornis</i>	+*	+*	?	I	n.d.	yes	(Aung et al., 2011)

++: present and robust, +: present but not robust, -: not present, F: feeding, I: injection, S: soaking, n.d.: not determined.

\* Assessments were based on a single soaking experiment and only a small number of injection experiments available for this tick species



Furthermore, as shown in Table 8, several of the studies used injection as a delivery method, making it hard to draw conclusions as to the necessity of Sil proteins in feeding RNAi, or even systemic RNAi. Indeed, several of the studies reporting systemic RNAi were actually performed using an injection delivery into the body cavity or haemolymph, so that systemic RNAi has not been clearly demonstrated. They showed that dsRNA can spread through the haemolymph to different tissues, but not necessarily that the silencing signal can be exported from one cell and imported into another. Further research will be necessary to unravel the uptake mechanisms in insects and to investigate why there is such variability in feeding and systemic RNAi in insects.

### 5.1.3.3. Annelids and molluscs

None of the studies described in Annex 2 on use of RNAi in annelids and molluscs reported mechanisms of cellular uptake or systemic transport of dsRNA. In total, three studies were retrieved reporting successful oral or soaking RNAi in molluscs (Chen et al., 2014; Knight et al., 2011; Wang et al., 2016), suggesting that snails and clams might possess an environmental RNAi capacity. No feeding RNAi studies for any annelid species has been found in the systematic literature searches, nor in subsequent manual searches.

### 5.1.4. Amplification of the silencing signal

For a strong systemic RNAi response, a sufficient number of siRNAs is logically required inside the body of the target animal, in order to invoke a silencing response in a sufficiently high number of cells. While this could be achieved by a constant supply of sRNAs through feeding (e.g., *in planta*), strong systemic RNAi responses are also observed in some species upon a single delivery of a small amount of these sRNAs. In *C. elegans*, a pathway has been identified causing an amplification of the silencing signal inside the body of these nematodes, similar to the one described in plants earlier (Alder et al., 2003; Pak and Fire, 2007; Sijen et al., 2001; Sijen et al., 2007; Smardon et al., 2000; Tsai et al., 2015; Zhang et al., 2012). In short, this mechanism is based on RNA-dependant RNA polymerases (RdRPs) which are able to produce secondary siRNAs from the mRNA that is being targeted by the primary siRNAs. Sijen et al. (2007) described these secondary siRNAs as being distinct from primary siRNAs in the sense that they contain di- or triphosphates at the 5' end, while primary siRNAs have a monophosphate group at the 5' end. Furthermore, the secondary siRNAs were found to be of antisense polarity only. They also showed that these siRNAs were not produced directly from the miRNA or exogenous dsRNA, but from the targeted mRNA, since single nucleotide mismatches in the primary siRNAs are not present anymore in the secondary siRNAs, who regain the full homology with the target mRNA (Sijen et al., 2007).

Pak and Fire, (2007) reported that these secondary siRNAs constitute the vast majority of siRNAs present in *C. elegans*, indicating its importance in RNAi-mediated gene silencing efficiency in these nematodes. Furthermore, since these secondary siRNAs are synthesized from the targeted mRNA, an important consequence of this mechanism is the occurrence of so-called transitive RNAi, which entails silencing regions of a mRNA which were originally not covered by the original dsRNA (Alder et al., 2003; Sijen et al., 2001; Sijen et al., 2007). Alder et al. (2003) confirmed the existence of this transitive RNAi in *C. elegans* by injecting dsRNA specific to GFP in mutant worms which contained a homologous *gfp* sequence fused to the sequence of a number of essential genes. The dsGFP resulted in silencing of these essential genes. In *C. elegans*, secondary siRNAs were found to mainly cover the region of the mRNA that is upstream of the silencing trigger (Alder et al., 2003; Pak and Fire, 2007; Sijen et al., 2001). Interestingly, siRNAs also seem to induce a so-called Slicer activity causing them to cleave target mRNAs much more efficiently than primary siRNAs. So enhanced RNAi efficiency is not only caused by the increased amount of siRNAs present, but also by a more efficient target mRNA cleavage (Aoki et al., 2007).

The exact mechanism of secondary siRNA production has not been elucidated yet. The main actors in this pathway appear to be complexes containing RRF-1 or EGO-1, which are RdRPs able to produce

secondary siRNAs in a Dicer-independent manner (Aoki et al., 2007). RRF-1 seems to create siRNAs in an unprimed manner using as a template 3' uridylated mRNA fragments produced by an argonaute enzyme called RDE-8 (Tsai et al., 2015). One more RdRP that has been identified is RRF-3. However, this protein seems to exert an inhibitory role on secondary siRNA production (Simmer et al., 2002). Furthermore, Zhang et al. (2012) identified several other genes involved in this amplification mechanism, including *rde-10*, *rde-11*, *rsd-2*, *rsd-6* and *haf-6*. RDE-10 and RDE-11 proteins form a complex that appears to be essential for amplification of siRNAs. Indeed, mutation in these genes resulted in worms which remained sensitive to high doses of dsRNA, but lose their sensitivity upon delivery of small doses of RNAi (Zhang et al., 2012). Over the years, it also became clear that secondary siRNAs and their gene silencing require a distinct RNAi pathway than primary siRNAs, including different argonaute effectors, called secondary siRNA-specific argonautes (SAGOs) and worm-specific argonautes (WAGOs) (Tsai et al., 2015). While the RNAi pathways for exogenous dsRNA and endogenous miRNA are for the most part distinct in *C. elegans*, this RdRP pathway seems to largely overlap in both pathways. Indeed, RDE-10 and RDE-11 have been shown to be required for the production of secondary siRNAs of both endogenous and exogenous RNAi-triggering sRNAs (Zhang et al., 2012).

While RdRP activity seems to be a crucial factor in RNAi efficiency in *C. elegans*, little data is available on this mechanism in other nematodes. Dalzell et al. (Dalzell et al., 2011) found that the EGO-1 RdRP is present in most nematodes that they studied, including several non-*Caenorhabditis* species such as *Ascaris suum*, *B. malayi*, *Meloidogyne* sp., *H. contortus*, *Oesophagostomum dentatum* and *P. pacificus* (Dalzell et al., 2011). In contrast, the *rrf-1* gene was absent in all but one non-*Caenorhabditis* species (Dalzell et al., 2011). The high conservation is perhaps not surprising, given that this RdRP plays a major role in endogenous RNAi processes including genome surveillance and germline development (Smardon et al., 2000). Dalzell et al. (2011) concluded that the presence of EGO-1 in most nematodes indicates that most species in this phylum are capable of at least some form of secondary siRNA production.

In arthropods, the presence of homologs for these *C. elegans* RdRPs in the genome has only been reported for two tick species, namely *Rhipicephalus microplus* and *I. scapularis* (Kurscheid et al., 2009), which both contain an EGO-1 homolog in their genome and in the two-spotted spidermite *Tetranychus urticae*, which contains 5 RdRP gene copies (Grbić et al., 2011). However, the activity of these RdRPs and possible involvement in the chelicerate RNAi pathway is still to be confirmed. So far, no homologs have been reported in insect or crustacean species. Whether this means that insects completely lack any amplification system is not sure. Based on the sensitivity of some species for environmental, systemic and sometimes even parental RNAi, notably in a number of beetle species, it would not be surprising to find that some insects have a similar but distinct mechanism by which the RNA-silencing signal can be amplified. However, no evidence for such a pathway has been reported.

### 5.1.5. Parental RNAi

Parental RNAi (pRNAi) or heritable RNAi, meaning RNAi-mediated gene silencing in the offspring of dsRNA-exposed adult females, has been observed in several invertebrate species, including the nematodes *C. elegans* (Alcazar et al., 2008; Grishok et al., 2000; Vastenhouw et al., 2006) and *Meloidogyne chitwoodi* (Dinh et al., 2014a; Dinh et al., 2014b), and several insect species belonging to different orders including the coleopterans *T. castaneum* (Bucher et al., 2002), *D. virgifera* (Khajuria et al., 2015), *Orchesella cincta* (Konopova and Akam, 2014), the orthopteran *Gryllus bimaculatus* (Mito et al., 2006), the hemipterans *Oncopeltus fasciatus* (Liu and Kaufman, 2004a), *Rhodnius prolixus* (Paim et al., 2013), *Myzus persicae* (Coleman et al., 2015), *Acyrtosiphon pisum* (Mao et al., 2013), the blattodean *Blattella germanica* (Piulachs et al., 2010) and the hymenopterans *Athalia rosae* (Yoshiyama et al., 2013) and *Nasonia vitripennis* (Lynch and Desplan, 2006).

In *C. elegans*, transgenerational silencing effects have been observed over multiple generations (Alcazar et al., 2008; Grishok et al., 2000a; Vastenhouw et al., 2006). Upon injection of dsRNA in the

parent, most RNAi effects remain visible in the next (F1) generation. The silencing effects typically recede over the generations eventually leading to a return to a normal phenotype. Interestingly, it was found that both males and females were able to pass the silencing signal on to the next generation in *C. elegans* so that both sperm and oocytes were responsible for transmitting the silencing signal (Alcazar et al., 2008). Furthermore, these studies also observed that the silencing signal is passed on unevenly between the progeny, leading to siblings with very different silencing effects (Alcazar et al., 2008; Vastenhouw et al., 2006). The exact mechanisms of this heritable silencing effect are still not fully understood. Grishok et al. (2000) observed that chromatin remodelling factors were necessary for a heritable silencing effect and that *rde-2* and *rde-4* were not required for successful heritable silencing, which suggested that while the initial silencing in the first (exposed) generation is due to RNAi, the inheritance mechanism could involve silencing at the transcriptional level by chromatin changes at the locus, rather than the post-transcriptional level (Vastenhouw et al., 2006).

Most of the pRNAi studies in arthropods involved heritable silencing effects upon injection of pupae or of the female adult. However, pRNAi has also been observed after feeding on dsRNA by the female adult. Khajuria et al. showed that feeding dsRNA specific to *brahma* and *hunchback* genes to adult female WCRs resulted in eggs that did not hatch, showing the involvement of both genes in embryonic development (Khajuria et al., 2015). Vélez et al. (2016) further investigated several parameters for successful pRNA in WCR, and demonstrated that pRNAi exhibits a dsRNA exposure dose- and duration dependant effect.

### 5.1.6. Conclusions on uptake and systemic spread

In this review we have provided an extensive overview of the available literature regarding mechanisms of cellular uptake and systemic spread of sRNAs in invertebrates. By far the most information on these mechanisms and pathways is available in nematodes, and more specifically the free living *C. elegans*. This nematode possesses a high sensitivity for feeding RNAi, due to a well-developed system for cellular uptake, systemic spread and an amplification system which produces secondary siRNAs. Furthermore, the silencing signal in *C. elegans* can be observed over multiple generations. However, as has been clearly shown in the literature, a considerable degree of variation exists within nematodes concerning these pathways. Most nematodes outside of the genus of *Caenorhabditis* are missing several genes which are considered important in *C. elegans*, which may explain observed differences in RNAi efficiency in different nematode species. While there are other factors involved in explaining variability in RNAi sensitivity in invertebrates, which are discussed further in other sections, cellular uptake of the sRNA and systemic spread of the silencing signal have been shown to be critical mechanisms for environmental RNAi.

Even more differences and variability in terms of uptake and systemic spread pathways were found in arthropods, where it seems that cellular uptake is much less efficient, especially some insect orders such as Diptera and Lepidoptera. While arthropods do contain some elements of the elaborate cellular uptake and systemic spread systems found in *C. elegans*, their requirement and involvement in successful environmental or systemic RNAi has not yet been clearly demonstrated. What is clear is that, in insects and possibly some other taxonomic groups, cellular uptake is regulated by two pathways which may or may not be linked. The occurrence and robustness of systemic RNAi in arthropods is variable and no evidence for an amplification system of the silencing signal, as is present in *C. elegans* and many other nematodes, has been reported yet. However, given the efficiency of environmental and systemic RNAi in some insects, notably Coleoptera, and the fact that parental RNAi has been described for several arthropods, there may well be such a system in some arthropods.

Given the very small number of RNAi studies in molluscs and annelids, no information was found regarding the uptake mechanisms in these phyla. A number of feeding studies in clams and snails suggest that these molluscs possess some sort of environmental RNAi capacity, but future research is

required to investigate whether mechanisms similar to those in nematodes or arthropods are involved.

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## 5.2. Environmental exposure and fate of dsRNA, siRNA and miRNA (Task 3)

### 5.2.1. Introduction

In this section, we consider studies on environmental exposure to and fate of dsRNA, siRNA and miRNA and the factors affecting exposure levels. We first focus on environmental exposure routes from plants to invertebrates in general, drawing on what is known from other plant biotechnologies. In a second part, we present the literature specifically dealing with dsRNA molecules in this context, such as expression levels in GM plants and stability of dsRNA in the environment. Furthermore, potential barriers in invertebrates are also considered, but are discussed more in detail in Section 5.3 which deals with efficiency of RNAi in invertebrates.

### 5.2.2. Environmental exposure routes from plants to invertebrates

Plants are the primary producers in both grazing and detritus food chains linked to cropping systems. Plant tissue-expressed compounds can enter food chains through primary and secondary routes, and a number of invertebrate organisms at different trophic levels can be exposed to them. A schematic representation of the possible routes of exposure discussed above is given in Table 9.

#### 5.2.2.1. Direct exposure through plant consumption

The primary route of exposure from plants to invertebrates is through direct feeding on plant living material (herbivory) above or below ground, or through plant residues in the case of detritivores.

Many primary consumers are usually linked to plants as their food source. The variety of feeding habits is quite large and, basically, all plant parts can be ingested by herbivorous invertebrates, including leaves, seeds, pollen, phloem sap, guttation, nectar, roots, tubers/corms. Initial exposure of a herbivore, and possibly the higher trophic species feeding on the herbivore, will depend on expression levels in the plant tissues consumed by the herbivore. In the case of some GM plants, the newly expressed trait is sometimes not expressed or expressed at low levels in certain tissues such as pollen, nectar or phloem. Hence, species feeding exclusively on these tissues may not be exposed or affected. It is therefore important to determine which plant tissues are expressing RNAi in order to determine exposure levels in invertebrate herbivores with different feeding habits.

Cultivated plants represent the first point of exposure for herbivores. However, during or after the growing season, plant tissues may be dispersed in the form of pollen and seeds, or, occasionally, as plant residues on the soil surface. Dispersal occurs at variable distances, due to air, water and soil movement, and by the activity of animals. Therefore, exposure can occur remotely from source plants, and may be significant in relation to pollen collected by insects and harvested seeds, roots, tubers and corms transported to storage and processing facilities.

Seed dispersal of most cultivated species normally occurs over short distances, though there are some exceptions: for example, some fruit crop seeds can be dispersed in animal faeces (Cain et al., 2000), and seeds of some grasses can be widely dispersed by wind, e.g., GM creeping bent grass (Zapiola et al., 2008). Insects and wind can be efficient means of dispersing pollen and the extent of pollen dispersal depends on the crop species, flower and pollen morphology, the vectors and the local meteorological conditions. Several studies on crops such as maize indicate that the large majority of wind-dispersed pollen grains were deposited at relatively short distances from the source (Lavigne et al., 1998; Treu et al., 2000). Pollen grains of other crop species may move over significantly larger distances, with bees foraging over distances of several kilometers (McCartney and Lacey, 1991).

Pollen and nectar feeders include bees, wasps, pollen beetles, etc., but invaders of nests and hives (e.g., wax moth, ants) could also be exposed to it. Foraging adult insects (e.g., bees) will transport pollen and nectar to larvae so that they are also exposed. Exposure to pollen may also occur through

other routes, e.g., maize pollen transported by wind can be deposited on the leaves of food plants of non-target species in the vicinity of cultivation areas. If the pollen is derived from GM plants expressing insecticidal traits (e.g., Cry proteins), the non target species may become exposed to it when feeding on the leaves of their food plants (Felke et al., 2010; Gathmann et al., 2006).

The data collected on the extent of pollen deposition on wild plants are quite variable (e.g., Gathmann et al., 2006; Hofmann et al., 2016; Lang et al., 2004). The actual exposure to GM pollen for a given non-target species will ultimately depend on the presence of individuals of the species during pollen shed in the area, the availability of their host plants, their distance from pollen source and the pollen concentration at the emission source. The combination of all these factors in the specific receiving environment can be very different even for the same crop species. For instance, the estimate of the potential exposure of non-target butterfly larvae to maize pollen in different growing regions, may produce substantially different results (Arpaia et al., 2018).

The possible exposure of NTOs to plants is not limited to the growing season. After harvest, plant residues and most of the root system remain in the soil until degradation. Depending upon the harvesting practices and the cultivated species, part of the residues might also be incorporated into the soil for enriching the reservoir of organic matter. Plant residues during and after mechanical harvesting may be transported outside cropped fields and enter soil and aquatic systems where it could remain near the site of entry or be further dispersed by water currents (e.g., Rosi-Marshall et al., 2007; Poté et al., 2009; Tank et al., 2010).

The fate of GM plant residues in soil has been previously studied. For instance, Cry proteins are known to bind to clay, suggesting that there is potential for their long term persistence and consequently long exposure to NTOs (Icoz and Stotzky, 2008). Degradation of plant tissue will affect the potential for exposure to nucleic acids, since DNA is degraded within the plant during senescence (Pietramellara et al., 2009). However, a certain amount of dsRNA could be expected to be released in the soil.

Depending on the crop species considered, possible outcrossing to wild/weedy relatives may occur. Consequently, the above exposure routes may be applicable to the progeny of the outcrossed plants expressing traits passed from a transformed crop. The potential for outcrossing and introgression of traits from crops to wild relatives has been extensively reviewed, (e.g., Eastham and Sweet, 2002; Ellstrand et al., 2013).

#### **5.2.2.2. Indirect exposure through the food chain**

Secondary exposure can occur through indirect feeding (exposure through multi-trophic interactions) e.g., natural enemies feeding on herbivores. Food webs in agro-ecosystems are typically simplified compared to natural habitats due to the major impact of human activities, the short time span for which plants remain in the field and the usual uniformity of cultivated plants. Even so, rather complex multi-trophic relationships are established between primary producers and consumers, at least up to the fourth trophic level (Verkerk, 2004). A herbivore represents a possible host/prey of one or more species of natural enemies. Upon ingestion, a plant compound can be present in the body of a herbivore as such, or as one of its metabolites. When predators or parasitoids feed on herbivores, they are consequently exposed to the plant-derived product and/or its metabolites.



**Table 9:** Possible channels and routes of exposure of non-target organisms to plant-expressed compounds

Channel	Mechanism	Exposed invertebrates	Site of exposure
Air	Pollen flow, seed dispersal	Herbivores ingesting pollen, seeds	Plants, soils and water bodies inside and outside cultivated areas
Plant	Ingestion of plant parts	Primary consumers (herbivores, including pollinators); Higher order consumers ( <i>predators, parasitoids, etc.</i> )	Cultivated areas, plants and plant propagules/parts removed from cultivated areas and sites of wild/weedy relatives of the crop
Soil (including soil water)	Release of plant products (e.g., exudates), Plant residues in the soil and soil water	Meso- and macro-fauna (detritivores, higher order consumers), aquatic species	Cultivated field, drainage systems, water courses

Predators usually consume several individuals of their preys when feeding, and therefore accumulation of plant products may also occur at higher trophic levels. Svobodová et al. (2017) found that, among different predators, ladybeetle larvae showed higher concentrations than lacewing larvae and juvenile spiders. A particular case of tritrophic relationships occurs in nature with some higher order consumers, feeding on products made by herbivores (e.g., aphids' honeydew). For instance, ants are well known for obtaining a large portion of their carbohydrate needs by collecting the sugar-rich honeydew of aphids, which is secreted as a waste product of the aphids diet of phloem sap (Way, 1963). Production of honeydew is related to sap ingestion from plants so that plant compounds present in the sap, including some defensive compounds, are excreted through aphids' honeydew.

Additionally, plant products may survive the gastrointestinal tract and occur in animal faeces and then pass into degrading organisms in soil and soil water, constituting another possible route of exposure for some invertebrate species.

### 5.2.2.3. Factors affecting RNAs physiological uptake

Even if an organism is physically exposed to plant compounds through one of the routes described above, further steps may be required after the ingestion of plant parts in order to become physiologically exposed.

When RNA is present in the environmental matrices described above, invertebrate organisms are primarily physically exposed to nucleic acid through ingestion, although some other possible exposure routes have been proposed as well, such as passage through the integument (see Section 5.1). When successful ingestion occurs, cells of the gut surface may take up dsRNA and genes of these cells may become possible targets for interference. Several mechanisms (see Section 5.1) may allow further systemic transport of dsRNA and potentially target active genes in other tissues and organs. Therefore, as a consequence of these mechanisms, dsRNA or siRNA derived from its cleavage, can be found in cells and tissues remote from where they were introduced into the invertebrate.

A large body of research is available indicating that, at least in arthropod species, various barriers to physiological exposure exist. These include the potential degradation of dsRNA prior to ingestion, breakdown by nucleases in saliva and gastrointestinal tract (Allen and Walker, 2012; Christiaens et al., 2014; Garbutt et al., 2013; Liu et al., 2013; Prentice et al., 2017; Shukla et al., 2016; Wynant et al., 2014), degradation of dsRNA in the haemolymph (Christiaens et al., 2014), barriers to cellular uptake (Dalzell et al., 2011; Taning et al., 2016; Tomoyasu et al., 2008; Whyard et al., 2009) and the mechanisms of transport of dsRNA within the organism (Dalzell et al., 2011).

The presence of these barriers and their effectiveness in decreasing RNAi is not common to all invertebrates. In insects, it has become clear that in some orders, such as Lepidoptera (Garbutt et al., 2013; Liu et al., 2013; Shukla et al., 2016; Terenius et al., 2011), Hemiptera (Christiaens et al., 2014) and Homoptera (Wynant et al., 2014), nucleolytic degradation of dsRNA can negatively affect RNAi efficiency. Even in Coleoptera, which are generally considered highly sensitive to oral RNAi, examples of dsRNA degradation in the gut negatively impacting RNAi efficiency have been reported (Prentice et al., 2017). Also, oral intake studies have shown that uptake of RNA varies between different orders of arthropods. Many coleopteran species and nematodes will absorb RNA from plant and/or synthetic diets very efficiently, whereas for example in dipterans, oral RNAi can only be achieved when dsRNAs are formulated together with polymers or liposomes enhancing their cellular uptake and stability (Kumar et al., 2016; Taning et al., 2016; Whyard et al., 2009; Zhang et al., 2010). Furthermore, also in nematodes, research has revealed that differences in dsRNA uptake capacity and the ability to spread the silencing signal within the body can affect RNAi sensitivity by ingestion of dsRNA (Dalzell et al., 2011). A more detailed discussion on these topics, and other factors influencing RNAi efficiency in invertebrates, are presented in Section 5.1 and Section 5.3.

### 5.2.3. Relevant publications from the systematic literature search

From the initial list of 4,612 oral exposure studies, only 122 studies were considered relevant for Task 3 applying the criteria listed in Section 2.2.9. Among these, 117 studies concern exposure characterization (Appendix C) and environmental fate of RNA (Appendix D). Five additional studies in which transgenerational transmission of interference was studied *in vitro* via injection of RNAi, have also been selected as relevant for this task.

#### 5.2.3.1. Molecular characterisation in RNAi-based GM plants

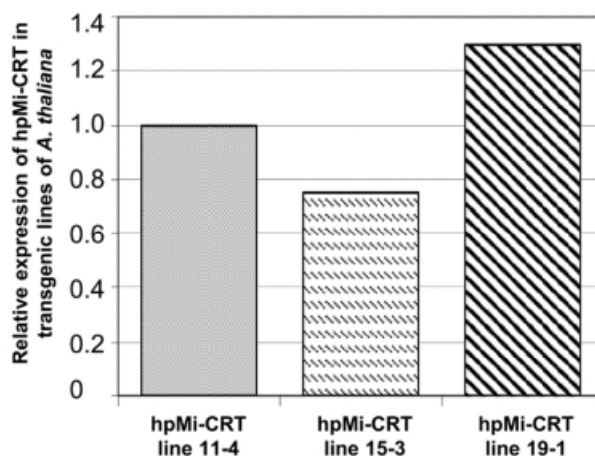
The primary exposure of organisms to environmental RNAs originating from transgenic plants is linked to their expression levels in the GM plant. Exposure studies on interfering RNAs have mostly focused on dsRNA, as little data was found on other interfering RNAs such as miRNA.

The plant-delivered expression level of an RNA-expressing construct can be measured by absolute or relative quantitative RT-PCR (qRT-PCR). The absolute quantification uses a calibration curve, while the latter technique, which is easier to perform, measures the relative change in mRNA expression levels of a target gene versus housekeeping genes (reference or control gene) (Livak and Schmittgen, 2001).

Analysis of *in planta* selected studies reveal that the expression of dsRNA is commonly assessed by qRT-PCR analysis and subsequently, transcript levels are determined with the comparative  $C_T$  method using the formula  $2^{-\Delta\Delta C_T}$  (Abdellatef et al., 2015; Agrawal et al., 2015; Clément et al., 2009).

In some other studies, quantification of gene expression is calculated using the Pfaff equation, that takes into account primers efficiency (Fairbairn et al., 2007). Data is normally presented as the fold change in gene expression normalized to one or preferably, multiple selected endogenous reference genes.

For example, in Jaouannet et al. (2012) the relative expression of the exogenous hpMi-CRT (a hairpin RNA for *M. incognita* targeting calreticulin, a calcium binding-protein) in three *Arabidopsis thaliana* transgenic lines, was normalized to the expression of the NADH dehydrogenase (AT1G02020) housekeeping gene (Figure 5).



**Figure 5:** Expression patterns of hpMi-CRT in three *Arabidopsis thaliana* genetically modified lines. From (Jaouannet et al., 2012)

An effective method to quantify the expression level of dsRNA-containing transcripts expressed in transgenic plants, was developed by Armstrong et al. (2013). In this study, the authors reported for the first time a non-polymerase chain reaction-based quantitative assay for dsRNA-containing transcripts, using the QuantiGene kit. The assay platform does not require RNA purification and has a simple workflow based on RNA hybridization to specific DNA probes containing a fluorescent marker so that the expression level is used to fit a regression with the amount of dsRNA present in the sample. They used RNA from multiple maize tissue types and tissue lysates of the transgenic maize engineered to control western corn rootworm (event MON 87411), to quantify the expression of the *DvSnf7* dsRNA transgene. Validation studies indicated that the assay was highly reproducible and sensitive to concentrations as low as 10 pg of dsRNA/g of fresh tissue.

Bachman et al. (2016) quantified the *DvSnf7* dsRNA expression levels in different tissue types collected from MON 87411 plants across different sites, using the validated QuantiGene assay described above (Armstrong et al., 2013). The authors also calculated the expression levels of *DvSnf7* dsRNA in micrograms of RNA per gram (g) of fresh or dry weight tissue (Table 10).

The *DvSnf7* dsRNA maximum expression levels found in these studies were then used to determine the maximum expected environmental concentration (MEEC) for testing some groups of exposed arthropods including representative pollinators, natural enemies and soil biota. This is further discussed in Section 5.4.

The same method was also used for efficiently measuring low concentrations of dsRNA in different soil and water samples (Albright et al., 2017; Dubelman et al., 2014; Fischer et al., 2016; Fischer et al., 2017).

**Table 10:** *DvSnf7* dsRNA expression levels in different tissue type from MON 87411. (Modified from (Bachman et al., 2016))

Tissue type	Developmental stage	Range µg/g
Pollen (fwt)	VT-R1	$0.056 \times 10^{-3} - 0.224 \times 10^{-3}$
Leaf (fwt)	V14-R1	$5.40 \times 10^{-3} - 33.8 \times 10^{-3}$
Root (fwt)	V3-V4	$1.74 \times 10^{-3} - 8.00 \times 10^{-3}$
Whole Plant (dwt)	V6-V8	$33.0 \times 10^{-3} - 106 \times 10^{-3}$
Grain (dwt)	R6	$0.056 \times 10^{-3} - 0.175 \times 10^{-3}$

fwt = fresh weight, dwt = dry weight

### 5.2.3.2. Environmental fate in different matrices

#### *Plant residues in soil*

Soils are chemically complex and spatially heterogeneous with surface-reactive particles (clay, sand, silt and humic substance) which can adsorb nucleic acids (Ranjard and Richaume, 2001). The presence of inorganic compounds and organic molecules on both soil particles and DNA molecules can influence the DNA adsorption and degradation and protect them against nuclease degradation (Pietramellara et al., 2009). The sorption of plant-derived insecticidal compounds may lead to irreversible adsorption to particle surfaces and a successive change of conformation upon adsorption. The adsorbed conformation of dsRNA depends on the adsorbent surface properties and solution chemistry (Parker and Sander, 2017). Existing knowledge on the fate of nucleic acids in soils indicate a low environmental persistence in most conditions (Levy-Booth et al., 2007; Poté et al., 2005). The fate of dsRNA will be affected by the biophysical characteristics of soil and sediments (e.g., hydrophobicity, salt concentration and pH), in addition to other abiotic and biotic factors. The biodegradation of dsRNA, as with DNA degradation in soil, is likely to be mediated by microbial-produced ribonucleases, possibly in combination with plant-derived nucleases (Blum et al., 1997; Dubelman et al., 2014; Green, 1994; Poté et al., 2005).

The presence of several active moieties in the molecules may lead to the formation of metabolites that are challenging to identify in the soil. Extraction protocols for dsRNA from soil systems are less established and extraction systems are considered of low efficiency (Parker and Sander, 2017). Studies of degradation in soil were conducted in laboratory conditions to determine the potential biodegradation of a *DvSnf7* dsRNA transcript derived from a GM maize product that confers resistance to *Diabrotica* spp. (Dubelman et al., 2014). Maize tissues (shoots and roots) were added to different types of soil to simulate post-harvest conditions. Three agricultural soils differing in their physicochemical characteristics were used (silt loam, loamy sand and clay loam). Purified (in vitro-transcribed) *DvSnf7* dsRNA was added to the soil (0.3, 1.5, 7.5, 37.5 µg RNA/g soil in four different treatments) to increase its initial concentration. The GM and control (non-GM maize) materials were added to each soil and incubated at 22°C for 48 hours. Samples were collected at set time intervals during the incubation period, extracted, and analyzed using QuantiGene molecular analysis and insect bioassay (Southern Corn rootworm, *D. undecimpunctata*). The DT<sub>50</sub> (half-life) values for *DvSnf7* dsRNA in the three soils were 19, 28, and 15 hours based on QuantiGene, and 18, 29, and 14 hours based on the insect bioassay, respectively. The DT<sub>90</sub> (time until 90% degradation) values for *DvSnf7* RNA in all three soils were 35 hours. *DvSnf7* dsRNA was degraded and biological activity was undetectable within approximately two days after application to soil, regardless of texture, pH, clay content and other soil differences. Furthermore, soil-incorporated *DvSnf7* dsRNA was non-detectable in soil after 48 hours, as measured by QuantiGene, independently from the initial concentration, at levels ranging more than two orders of magnitude (0.3, 1.5, 7.5 and 37.5 mg RNA/g soil).

A recent presentation given at the 14<sup>th</sup> International Symposium on the Biosafety of Genetically Modified Organisms (ISBGMO14)<sup>2</sup> described similar studies with two Brazilian soil samples, and reported similar levels of degradation of *DvSnf7* dsRNA with time.

#### *Plant residues in water*

Nucleic acids usually persist in water for a limited time. Gulden et al. (2005) found that DT<sub>50</sub> of plant DNA in leachate water ranged from 1.2 to 26.7 hours. Persistence in water of dsRNA was specifically investigated by Fischer et al. (2017) who conducted a study to measure the rate of biodegradation of *DvSnf7* dsRNA in aerobic water sediment systems. In laboratory conditions, water from natural aquatic systems and sediments that varied in physical and chemical characteristics were treated with dsRNA by applying 60 µg of *DvSnf7* dsRNA directly to the water column. The system consisted of about 90 g of sediment placed in the bottom of a 500 ml graduated cylinders and covered with about 200 ml of the corresponding water source (fresh water from a river or a lake). *DvSnf7* dsRNA dissipated rapidly from the water phase and was undetectable within seven days in the diverse systems, when measured by QuantiGene and a sensitive insect bioassay with *D. undecimpunctata*. Degradation kinetics estimated a DT<sub>50</sub> of less than three days and a time of about four days to reach DT<sub>90</sub>. When this laboratory setup was manipulated to obtain a “disturbed” system (i.e., the sediment was mixed into the water column following dsRNA application), DT<sub>50</sub> and DT<sub>90</sub> values for the water phases were less than one day regardless of the analytical method or the sediment-water origin.

Further analysis indicated that *DvSnf7* dsRNA had DT<sub>50</sub> values of less than six days in both sediment-free systems containing natural water as well as systems with only sediment.

In a recent study, Albright et al. (2017) spiked a non-bioactive dsRNA into a column of water and sediment microcosm to mimic a run off of unbound dsRNA or transport of plant tissues. Dissipation of dsRNA in the water column and partitioning into sediment was determined. The dsRNA rapidly dissipated in the water column and was below the limit of detection after 96 hours. The levels detected in the sediment were not significant and may indicate a rapid degradation in the water column prior to partitioning to sediment. In both studies, a QuantiGene assay kit (Affymetrix) was used to determine dsRNA concentrations in the samples. This method has been shown to be capable of detecting low concentrations of dsRNA in soil regardless of sequence, molecular weight, or structure.

dsRNA stability under different temperatures and outdoor conditions was also the subject of the study conducted by Li et al. (2015), who obtained contrasting results. During laboratory experiments conducted in a water system, the authors observed a relative stability of dsRNA-containing solutions over a period of 8 hours under conditions of continuous UV exposure and high temperature (40-60°C). When simulating outdoor conditions, independent of sunlight or shade, dsRNA began to gradually degrade after 1 month and the calculated half-life was 40 to 45 days. In this study, the dsRNA (fluorescent labelled) contents were detected using a NanoDrop ND-1000 spectrophotometer (NanoDrop ND-1000TM) and checked by agarose gel electrophoresis.

Considering the existing scientific literature on the subject, persistence of dsRNA in water is also expected to be short.

#### *Trophic chains from plants*

Transfer of RNA effectors between trophic levels is a mechanism which has been studied by several authors, in order to estimate possible impacts at higher trophic levels (i.e., consumers of different orders). However, no tritrophic studies demonstrating *in vivo* activity of plant derived RNA in prey on predator or parasitic species are available.

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<sup>2</sup> Available at <http://isbr.info/files/tiny/mce/uploaded/BOA%20-%2020062017%20VA.pdf>

Studies of dsRNA transfer from herbivores to natural enemies using non-plant derived RNA have been conducted. Garbian et al. (2012) discovered a bi-directional transfer of RNAi between honey bees and their parasitic mite *Varroa destructor*. The possible goal of the application of RNA interference mechanisms would be the control of this ubiquitous pest of apiaries. The experiments were originally conducted by using a “neutral” dsRNA carrying a segment of the gene for GFP. Adult bees were fed a sucrose solution containing dsRNA, which was also transferred to larvae via jelly produced by nurse bees and laid in cells where immatures develop. Subsequently, *Varroa* mites were allowed to feed on adult bees after sucrose feeding and examined after three days for direct dsRNA transfer. Exposure of mites to dsRNA from honeybee larvae was also induced by letting mites feed on larvae in cells (indirect transfer). Both direct and indirect successful transfer was confirmed via qRT-PCR. The authors also tested the bidirectional transfer of dsRNA from mites to bees by using *Varroa* individuals from previous experiments and allowing them to feed on new honeybee colonies which were not in contact with dsRNA. The dsRNA was detected in bees four days after infestation with dsRNA carrying mites, demonstrating that it was transferred to bees via parasitic activity.

After establishing the bidirectional transfer with marker genes, a number of dsRNAs were designed to target housekeeping genes of *Varroa* involved in cytoskeleton assembly, energy transfer, apoptosis inhibition, etc. The presence of phenotypic RNAi-mediated silencing effects was observed in experiments of direct and indirect transfer from bees. These results support the idea that dsRNA maintains its biological activity after across-species horizontal transfer. Diets containing different mixtures of dsRNA did not affect bee colony numbers, while a significant effect on the population of mites was detected.

Another demonstration of dsRNA transfer among different trophic levels is described by Itakura et al. (2009). In this study, the third trophic level was constituted by symbiotic protists of the termite, *Coptotermes formosanus*. The 21nt siRNAs designed for endoglucanases of the symbiotic protists *Pseudotrichonympha grassii* (PgEG), *Holomastigotoides mirabile* (HmEG), and *Spirotrichonympha leidy* (SIEG) were used to silence protist genes through their termite hosts. In the experiment disorganization of *P. grassii* and *H. mirabile* occurred within a few days in the hindgut of the termites that had ingested PgEG and HmEG siRNAs suggesting that this mechanism could be used to impair the normal development of termite colonies.

#### *Transgenerational effects from plant derived RNA in herbivores*

A particular type of exposure route is the transmission of the effects of gene silencing due to dsRNA from a directly exposed herbivore to its progeny. This may lead to the exposure of larger numbers of individuals of the given species to the stressor (indirect exposure), increasing the overall frequency of exposure.

The first evidence of this phenomenon was found in *C. elegans* by Grishok et al. (2000b) who first investigated inheritance properties associated with silencing through exogenous dsRNA. Transmission of the interference effect occurred through a dominant extragenic agent. The activities of the RNAi pathway genes *rde-1* and *rde-4* were required for the formation of this interfering agent but were not needed for interference thereafter. Different genes, *rde-2* and *mut-7* genes were required downstream for interference.

Burton et al. (2011) discovered that nuclear RNAi maintains heritable gene silencing and siRNA expression in the progeny of animals exposed to dsRNA. The epigenetic transmission pathway in the same species was further elucidated by Buckley et al. (2012) who discovered that the Argonaute protein HRDE-1 directs gene-silencing events in germ-cell nuclei that drive multigenerational RNAi inheritance.

The persistence of the epigenetic transmission was studied by Houri-Ze'evi et al. (2016) who showed that exposure to dsRNA activates a feedback loop, whereby gene-specific RNAi responses dictate the transgenerational duration of RNAi responses mounted against unrelated genes. Besides the induction of silencing, dsRNA-induced effects on the production of heritable endogenous small RNAs, which

regulate the expression of RNAi factors. Manipulating genes in this feedback pathway changed the duration of the heritable silencing.

However, the transgenerational transmission of silencing is not a general phenomenon and Ashe et al. (2015) found that antiviral RNA interference against natural Orsay virus infections is neither systemic nor transgenerational in *C. elegans*. While exogenous RNAi spreads throughout the organism and can be passed between generations, this phenomenon has not been observed for the endogenous RNA pathways.

Besides certain nematodes, evidence of transgenerational RNAi has also been found in certain insect species belonging to different orders including the coleopterans *T. castaneum* (Bucher et al., 2002), *D. virgifera* (Khajuria et al., 2015), *Orchesella cincta* (Konopova and Akam, 2014), the orthopteran *G. bimaculatus* (Mito et al., 2006), the hemipterans *O. fasciatus* (Liu and Kaufman, 2004), *R. prolixus* (Paim et al., 2013), *M. persicae* (Coleman et al., 2015), *A. pisum* (Mao et al., 2013), the blattodean *B. germanica* (Piulachs et al., 2010) and the hymenopterans *A. rosae* (Yoshiyama et al., 2013) and *N. vitripennis* (Lynch and Desplan, 2006).

Khajuria et al. (2015) described an RNAi mediated knockdown of two western corn rootworm developmental genes, (hunchback (*hb*) and brahma (*brm*)). After feeding adult females with dsRNA-containing artificial diet a significant decrease in *hb* and *brm* transcripts compared to negative control (treatment with GFP or water) was recorded in the tissues of eggs laid by the exposed females. Although total oviposition was not significantly affected, there was almost complete absence of hatching in the eggs collected from females exposed to dsRNA for either gene. This could be considered as a form of transgenerational effect, as the outcome of the silencing was expressed in embryos of the following generation, which was not directly exposed to the dsRNA.

Persistence and transgenerational effects of dsRNA were also studied on *M. persicae* feeding on GM plants (Coleman et al., 2015). The targeted gene was *Rack1*, a key component of various cellular processes. Maximal reduction of gene expression was ~70% and was achieved between four and eight days of exposure of the aphids to (dsRNA)-expressing *Arabidopsis thaliana* plants. Observations of the impact of RNAi was studied over three generations and the effects detected in the progenies lasted longer (12-14 days) than on the original individuals tested (approximately six days). Also, the effect on the population growth of aphids was more dramatic on the progeny (60% decline in reproduction) than in the parental aphids (40%).

#### 5.2.4. Conclusions

The exposure of invertebrates to transgene products expressed in GM plant material can follow several pathways, which can be direct and indirect, and will therefore not be limited to the in-field situation or the growing season of the GM plant. Therefore, case-specific and local conditions are crucial for determining the likelihood and the extent of exposure of invertebrate organisms to dsRNA-expressing plant material.

While extensive data from studies of "first generation" GM crops (i.e., expression of proteins conferring insect resistance or herbicide tolerance) have clarified many aspects of exposure of target and NTOs, the evidence base on exposure pathways and environmental fate of interfering RNAs is very limited. Most of the available information focuses on SmartStax Pro maize expressing, among others, *DvSnf7* dsRNA to control corn rootworms.

Scientific publications describing RNAi-expressing GM plants do not explicitly investigate potential exposure of invertebrate organisms. In most publications, information on dsRNA expression levels is limited to data on RNA silencing activity. Moreover, dsRNA expression levels in plants are rarely measured, probably because the primary objective of most of these studies is to investigate the effectiveness of RNA silencing. The evaluation of the effects of dsRNA was usually performed by qRT-PCR directly on the target gene expression of the invertebrate organism. In the studies reporting the results of dsRNA detection, relative values of expression compared to reference genes are usually

presented. Studies of *in planta* relative expression of dsRNA compared to housekeeping genes do not indicate actual amounts of dsRNA detected in plants. Therefore, these publications can only qualitatively orientate an exposure analysis. Similarly, only occasionally have detailed studies of expression in different plant tissues or different phenological stages of the plant life cycle been conducted. For example, Antonino de Souza Júnior et al. (2013) directly calculated the relative abundance of specific protease gene transcripts in *M. incognita* infesting dsRNA-expressing tobacco lines. The *M. incognita* 18S ribosomal subunit (mi18S) was used for normalization of qRT-PCR data. The analysis was performed at different stages of the nematode life cycle and the results were presented as fold change referenced to the stage that had the smaller relative expression value to which was arbitrarily assigned the value 1. Other examples where the efficiency of the silencing was directly evaluated on the target insect, include studies on the green peach aphid *M. persicae* (Pitino et al., 2011), lepidopteran pests *S. littoralis* (Apone et al., 2014) and *H. armigera* (Zhu et al., 2012) and the whitefly *Bemisia tabaci* (Raza et al., 2016).

The main exception pertains to the studies on characterization of GM maize MON 87411. In these publications, a quantitative determination of dsRNA expression in various plant parts was conducted using the molecular kit QuantiGene. This allows a sensitive detection of dsRNA in all plant tissues. Even with the availability of a relatively small amount of data, it can be inferred that in different transformation events with dsRNA, expression in plant parts was variable and the highest expression was detected in different tissues (from leaves to flowers) in the different studies.

Environmental persistence of dsRNA in decaying plant tissues and released from plants is expected to be limited. However, the use of different detection methods renders the available experimental results incomparable. The role of the different matrices in determining the environmental persistence of dsRNA (e.g., detailed information on the composition of soils, the presence of inorganic and organic compounds, as well as the microbial community of soil and sediments on site) might offer a better understanding of the possible persistence of dsRNA in soils.

No field studies were found in which the actual persistence of dsRNA derived from GM plants was evaluated on site. The published studies on the environmental degradation of dsRNA derived from GM plants are based on data obtained from soils studied under laboratory conditions.

The movement of dsRNA along trophic chains and the persistence of its biological activity have been shown in a few multitrophic systems. The likelihood of a biological effect is primarily linked to the uptake of dsRNA in taxonomically different organisms (see Section 5.1) and the efficiency of RNAi in the exposed organism (see. Section 5.3). Similarly, intergenerational effects have been studied in a few cases where exogenous dsRNA were observed in a number of generations after exposure. Due to the different mechanisms involved, predictions of similar effects in other biological systems are possible but yet to be demonstrated.

The case studies currently addressed by the scientific community are limited in terms of type of dsRNA used, plants species, NTOs selected, environmental conditions and agro-ecological landscape. Thus there is insufficient data to come to general conclusions concerning these factors. The availability of new dsRNA-expressing GM events will offer more opportunities for collecting specific relevant ecological data and filling the data gaps.

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### 5.3. Factors influencing silencing efficiency of dsRNA, siRNA and miRNA delivered orally in arthropods, nematodes, annelids and molluscs (Task 4)

#### 5.3.1. Introduction

While RNAi experiments have been performed since the 1980s, reported as antisense, quelling or co-suppression experiments in nematodes, fungi and plants, the true mechanism of RNAi was not known until Fire et al. (1998) demonstrated that this post-transcriptional gene silencing phenomenon in the nematode *C. elegans* is actually initiated by dsRNA, rather than sense or antisense RNA. After this discovery, researchers started using this technique in *C. elegans* on a large scale, mainly for research purposes. Initially, microinjection was used as a delivery method, but a feeding delivery was soon discovered to be functional and was adopted in many studies as an easy and efficient delivery method (Fraser et al., 2000; Timmons and Fire, 1998).

Soon after the discovery of RNAi in nematodes, the first RNAi-experiments in the model insect, the fruitfly *D. melanogaster* were reported, showing that the technique can be used in these flies for gene knockdown by microinjection (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Willert et al., 1999). This was confirmed in other arthropod species such as the coleopteran insect *T. castaneum*. Feeding experiments in *D. melanogaster*, using naked dsRNA, did not seem to work initially (Perrimon et al., 2010; Whyard et al., 2009). However, they were found to be successful in some other arthropods. One of the first reported successful oral RNAi studies was that in the tick *I. scapularis* (Soares et al., 2005) where researchers managed to silence the *isac* gene using capillary feeding of dsRNA. Not long after, Turner et al. (2006) showed that feeding dsRNA to the lepidopteran light brown apple moth *Epiphyas postvittana* could induce silencing, but only when feeding very high doses of dsRNA. Many more studies on arthropods, mainly insects, followed and researchers quickly recognised the potential of this technique for pest control. The first paper in this context was published by Baum et al. (2007), showing that feeding dsRNA to two beetle species, the Colorado potato beetle *L. decemlineata* and the Western corn rootworm *D. virgifera*, could be a very potent insecticidal pest control strategy as both species proved to be very sensitive to oral RNAi.

These early studies in nematodes and arthropods already gave an indication of the variability of RNAi efficiency in invertebrates. While *L. decemlineata* and *D. virgifera* seemed to exhibit a similar sensitivity to RNAi, as is the case for *C. elegans*, this was not the case for many dipteran and lepidopteran species for example, which were either insensitive or required to be exposed to very high doses of dsRNA in order to achieve any gene silencing. In this Section, we discuss the available data on RNAi sensitivity and efficiency in invertebrates, mainly focusing on feeding RNAi, and also discuss the factors that influence RNAi efficiency. Several factors, for example stability of dsRNA in the invertebrate body, the cellular uptake of dsRNA from the gut, the RNAi core machinery and the possible effect of viral infections have been identified by various authors and are discussed here.

#### 5.3.2. RNAi efficiency in invertebrates

In this section, we review oral RNAi experiments in different invertebrate taxonomic clades in order to give an indication on the sensitivity of RNAi in these groups and the doses and concentrations that were required to elicit efficient silencing responses. An overview is given in Table 11 and Table 12.

##### 5.3.2.1. Nematodes

###### *Caenorhabditis elegans*

The success of feeding RNAi in animals was first reported in *C. elegans* (Timmons and Fire, 1998). In this study, *E. coli* bacteria were transformed to express dsRNA specifically targeting three genes (*unc-22*, *fem-1* and *gfp*) and were fed to the *C. elegans* worms. This was a logical delivery method,

since *C. elegans* nematodes naturally feed on these bacteria. Feeding of these transformed *E. coli* led to a reversible gene silencing, similar to that induced by microinjected dsRNA. Furthermore, the effect was observed in somatic tissues, indicating transport of the RNAi silencing signal from the gut to other parts of the body. However, the authors reported that this feeding approach was less effective compared to microinjection, since only a part of the treated population exhibited the silencing phenotypes and the effects were found to be less severe. Indeed, while silencing *unc-22* led to a specific phenotype in 85% of the treated individuals, for *fem-1* and *gfp* this was only 43% and 12%, respectively (Timmons and Fire, 1998). Nevertheless, given the practical advantages of this delivery method compared to microinjection, feeding dsRNA-expressing bacteria as a delivery method became widely adopted for RNAi research in *C. elegans* and protocols were also improved, leading to higher efficiencies. Kamath et al. (2001) presented an optimized feeding method which leads to phenotypes which were at least as strong and frequent as those achieved by dsRNA microinjection. They used a specific strain of *E. coli*, called the HT115 strain, in which RNase III expression had been knocked out, to prevent dsRNA degradation inside the bacteria. Furthermore, they also optimized the expression induction conditions, finding that isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) induction at room temperature overnight leads to optimal RNAi results in the *C. elegans* worms (95-100% phenotype frequency).

One of the disadvantages of this bacterial feeding approach is the difficulty to actually assess the quantitative efficiency, since the amount of dsRNA eventually taken up via these bacteria is unknown. Only a minority of oral RNAi studies in *C. elegans* have been conducted using known concentrations of naked dsRNA in the form of a soaking solution (Annex 2). The soaking solution delivery was first reported by Maeda et al. (2001) who soaked L4 worms in dsRNA at concentrations of 1-5  $\mu\text{g}/\mu\text{L}$ , resulting in 100% RNAi phenotypes in the F<sub>1</sub> progeny. All soaking RNAi studies in *C. elegans* that were retrieved in the systematic literature search of this project used dsRNA concentrations in the  $\mu\text{g}/\mu\text{L}$  range. No experiments using lower concentrations were found. Many studies using soaking methods reported RNAi phenotypes in most treated worms. For example, silencing *hsp* genes in *C. elegans* using the protocol described by Maeda et al. (2001) caused developmental retardation in 73.8 % of the treated population after 72 hours (Al-Amin et al., 2016). When the *stip1* gene was silenced in L4 individuals, a 100% embryonic lethality of the progeny was observed (Ji et al., 2007). Silencing the *par-5* gene in L4 larvae caused sterility in 66% of the F<sub>1</sub> progeny (Morton et al., 2002). Unfortunately, most of these studies did not include qRT-PCR data, which would allow us to evaluate silencing efficiency at the transcript level. However, the widespread phenotypical effects do indicate that the soaking exposure to dsRNA can efficiently trigger RNAi, albeit at relatively high concentrations of 1-5  $\mu\text{g}/\mu\text{L}$ . In our systematic literature search, no studies were retrieved where miRNA was fed to nematodes. Interesting to note here is that several natural isolates of *C. elegans* (and other *Caenorhabditis* species) have been found which have defects in their RNAi pathways either in somatic cells or in the germline (Felix, 2008; Tijsterman et al., 2002). So, variability of RNAi efficiency can exist between different strains of *C. elegans*.

#### Other nematode species

As already mentioned in Sections 5.1 when discussing uptake mechanisms, some variability has been observed in RNAi sensitivity in nematode species (Felix, 2008). In the *Caenorhabditis* species *C. briggsae*, *C. remanei* and *C. brenneri*, exposure to oral intake of dsRNA does not elicit as effective silencing response as injected dsRNA. In the former two species, it has been reported that this is most likely due to the lack of *sid-2* genes in their genome, as the *C. elegans* Sid-2 protein is able to rescue functional feeding RNAi in these nematodes (Felix, 2008; Nuez and Felix, 2012; Winston et al., 2007). Also in other nematodes, RNAi sensitivity seems to be variable. Felix (2008) compared RNAi efficacy in 17 different nematode species and reported that oral RNAi is ineffective in six species (*C. briggsae*, *C. remanei*, *C. brenneri*, *O. tipulae*, *P. pacificus* and *Strongyloides stercoralis*) and is variably or moderately effective in three others (*H. contortus*, *O. ostertagi* and *B. malayi*) (Aboobaker and Blaxter, 2003; Aboobaker and Blaxter, 2004; Geldhof et al., 2006; Lok, 2007; Louvet-Vallée et al., 2003; Pires-daSilva and Sommer, 2004; Visser et al., 2006; Winston et al., 2007). These include

both animal and plant parasitic species. Furthermore, the free-living mycophagous nematode *Aphelenchus avenae* has also been found to be recalcitrant to oral RNAi (Reardon et al., 2010). In several grassland soil nematodes, insensitivity to RNAi was reported, including after dsRNA-injection (Wheeler et al., 2012). A meta study looking into the RNAi-pathway related genes showed that *Caenorhabditis* sp. have an expanded RNAi-related gene repertoire compared to parasitic nematodes and that especially the genes related to uptake and spread of the RNAi signal in *C. elegans* are poorly conserved in parasitic species (Dalzell et al., 2011). However, these authors indicated that, despite quantitative differences in terms of RNAi-related genes, there was no qualitative difference in terms of functional groups that were represented in these nematode species. Furthermore, they could also not identify a link between RNAi effector repertoire and RNAi functionality in parasitic nematodes.

Despite this observed RNAi variability and the differences with *C. elegans* in terms of RNAi effector repertoire, successful oral or soaking RNAi has been reported in many nematode species. Contrary to *C. elegans*, most RNAi studies in other nematode species used a non-bacterial feeding or soaking method. We found 34 studies using artificial diet or soaking and over 40 studies reporting successful RNAi using *in planta* delivery methods, either using GM plants or viral-/bacterial-mediated plant delivery. In most of the retrieved *in vitro* feeding or soaking studies, dsRNA concentrations in the 1-10 µg/µL range, similar to those in *C. elegans*, were used (Annex 2). In a study by Bakhietia et al. (2007), soaking of J2 stage *H. glycines* in a 2 µg/µL dsRNA solution targeting *eng-1* led to severe silencing at the transcript level for at least five days. Between day five and 10 post-soaking, the gene expression recovered. In another study, targeting *pat-10* and *unc-87* in *Pratylenchus coffeae* also led to a strong silencing response after 24 hours soaking in a 1 µg/µL dsRNA solution. However, recovery of expression happened quickly, in less than 24 hours. Phenotypically, silencing of both genes led to severe impairment of mobility of the nematodes (Joseph et al., 2012). In the pinewood nematode *B. xylophilus*, targeting *vap-1* by soaking in a 4 µg/µL solution led to a silencing efficiency of  $48 \pm 3\%$  24 hours after the start of the treatment, which resulted in a significantly reduced migration rate (Kang et al., 2012). Similar RNAi successes were also reported, for example in *Ditylenchus destructor*, *Panagrolaimus superbus*, *Globodera rostochiensis*, *R. similis* (Peng et al., 2014; Reardon et al., 2010; Rehman et al., 2009; Wang et al., 2016b). Interestingly, two studies reported successful RNAi silencing using 50 ng/µL and 25 ng/µL siRNA soaking solutions. The former observed a strong silencing effect lasting between 48-72 hours before transcript expression recovered upon soaking of *M. graminicola* in the siRNA solution (Nsengimana et al., 2013). Phenotypically, the RNAi-treated nematodes in this study were severely affected in their mobility after silencing of the *pat-10* and *unc-87* genes. In the latter study, *Heterodera avenae* were soaked in a 25 ng/µL siRNA solution. The researchers did not look at the gene's transcript levels in these soaking experiments, but did report significant mortality in the targeted nematodes (Zheng et al., 2015). Based on the few studies where nematodes were exposed to siRNA, rather than long dsRNA (Ma et al., 2011; Nsengimana et al., 2013; Zheng et al., 2015), there does not seem to be an obvious difference in efficiency between both molecules. Ma et al. (2011) noticed large differences in RNAi silencing efficiency between different siRNAs targeting a different region of the gene.

In the past decade, several studies have also shown that nematodes can be targeted for efficient RNAi through plants, either via a GM plant or transiently introduced dsRNA or siRNA in the plant (Annex 2). A study where hairpin dsRNAs targeting the *M. incognita* *splicing factor* and *integrase* genes were expressed in tobacco plants showed a dramatic decrease in the number of root knots, number of females per knot and number of egg masses on the plant, compared to the non-RNAi plant (Yadav et al., 2006). Furthermore, the authors also confirmed functional RNAi by semi-quantitative PCR, showing a complete absence of target mRNA. In another study, targeting the *16D10* gene in four different root-knot nematodes (*M. incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne hapla*) through *in vitro* soaking and through *in vivo Arabidopsis*, *in planta* RNAi caused significant silencing at the transcript and protein level (Huang et al., 2006). Another study investigated both soaking and *in planta* delivery in *Meloidogyne* species. Treatment of freshly hatched *M. incognita* J2s in a 1 µg/µL dsRNA solution caused 93-97% silencing at the transcript level and a



65–69% reduction at the protein level. In the *in vivo* experiments, dsRNA-expressing plants showed a 63–96% reduction in number of galls and a decrease in gall size for several *Meloidogyne* species. Furthermore, a 66–93% reduction in number of eggs per gram root was reported as well (Huang et al., 2006). These experiments indicate that *in planta* exposure to target-specific dsRNAs can cause a highly efficient silencing in these plant-parasitic nematodes.

**Table 11:** Overview of reported sRNA concentrations or single doses in genetically modified plants, *in vitro* feeding and soaking studies to elicit successful RNAi gene silencing in nematodes

Class	Order	<i>In planta</i>	<i>In vitro</i> feeding	<i>In vitro</i> soaking	Comments
Adenophorea	Trichocephalida	-	-	0.05 µg/µL	Only one soaking study available (Chen et al., 2012)
Secernentea	Rhabditida	-	1-5 µg/µL	1-10 µg/µL	Most studies using bacterial production systems (feeding or soaking)
	Tylenchida	Sni	1-2 µg/µL	0.05 - 10 µg/µL	Most studies using <i>in planta</i> delivery systems

Sni: Successful silencing but no information on concentration or expression level in the plant  
 - No studies found

### 5.3.2.2. Arthropods

In our systematic literature search, we retrieved nearly 350 studies in which dsRNA, siRNA or miRNA was delivered to arthropod species via feeding, soaking or through a topical application (Annex 2). In the majority of these studies it was achieved with dsRNA molecules, while siRNA or miRNA studies were less common. The majority of studies were done using insect species and the number of Crustacea or Chelicerata oral RNAi studies is limited. An overview of the doses or concentrations that elicited successful silencing in arthropods is given in Table 12.

### 5.3.2.3. Insecta

In insects, a diverse range of oral delivery methods have been used for sRNAs, including soaking, delivery through plants, topical application or mixing sRNAs with artificial diet. Not surprisingly, given the interest in RNAi for crop protection, most of the information available in this context of oral RNAi concerns pest organisms, as can be seen in Annex 2. In this section, we give an overview of the recorded RNAi sensitivity in insect species. While we mention possible factors explaining the differences in RNAi sensitivity in certain species, these factors are discussed in much more detail in Section 5.3.3 of this Technical Report .

#### *Diptera*

The first insect in which RNAi was attempted was the dipteran *D. melanogaster*. This was not surprising, given the fact that it has been considered a model insect for decades and that its genome was sequenced in the late 1990s (Adams et al., 2000). While RNAi by injection and by soaking of dechorionated embryos was functional in *D. melanogaster* (Baqri et al., 2006; Dzitoyeva et al., 2001; Dzitoyeva et al., 2003; Eaton et al., 2002; Misquitta et al., 2008; Schmid et al., 2002), this was not the case when flies were exposed to dsRNA orally (Perrimon et al., 2010; Whyard et al., 2009). However, Whyard et al. (2009) demonstrated that adding a liposomic transfection agent to the dsRNA significantly increased oral RNAi efficiency in these fruit flies, leading to the hypothesis that *Drosophila* lacks a functional dsRNA cellular uptake mechanism, possibly related to the lack of any *Sid-1*-like genes in its genome (Feinberg and Hunter, 2003; Miller et al., 2008; Tomoyasu et al., 2008; Ulvila et al., 2006; Whyard et al., 2009). In a close relative, *Drosophila suzukii*, similar findings were reported for the use of naked dsRNA and liposome-coated dsRNA (Taning et al., 2016b). A single naked dsRNA feeding event to *D. suzukii* larvae did not result in any observable silencing or

phenotype, while coating the dsRNA with liposomes caused up to  $42 \pm 2\%$  silencing on transcript level and an increase of up to  $42 \pm 7\%$  mortality compared to the control (Taning et al., 2016b). Furthermore, another study reported that continuous delivery to *D. suzukii* through dsRNA-expressing yeast symbionts also resulted in a significant RNAi silencing effect (up to 76% on transcript level) which led to decreased survival, mobility and fecundity of the fruit flies (Murphy et al., 2016). In total, only three studies were retrieved in the systematic literature search on oral RNAi in *Drosophila* and none of them reported successful RNAi using naked dsRNA.

In another genus of fruit flies, *Bactrocera*, we found eight studies investigating or using oral RNAi. Interestingly, all these studies targeted adult flies. No RNAi studies on *Bactrocera* larvae were found. Concentrations used in these studies were 0.5-2.0  $\mu\text{g}/\mu\text{L}$  in most cases. A study by Li et al. (2011) reported successful silencing of four genes in *B. dorsalis* after continuous feeding on artificial diet containing 2  $\mu\text{g}/\mu\text{L}$  dsRNA. Remarkably, the RNAi silencing effect only lasted for a short time (1-3 days) and in some cases, it was followed by a strong upregulation of the gene expression to a much higher level than the non-dsRNA fed control, while still being fed the target dsRNA. In another study, researchers fed dsRNA targeting the *rpl19* gene to *B. dorsalis* and *B. minax*, and also achieved significant silencing 24 hours after feeding. For *B. dorsalis*, a silencing efficiency of 54% and 69% was observed at 0.5  $\mu\text{g}/\mu\text{L}$  and 1  $\mu\text{g}/\mu\text{L}$  dsRNA concentrations, respectively. For *B. minax*, this was 90% and 91% (Chen et al., 2015). Yi et al. (2013) reported successful silencing of the *CSP2* gene in *B. dorsalis*, after continuous feeding on an artificial diet containing 1  $\mu\text{g}/\mu\text{L}$  dsRNA. Silencing on transcript level was detected at day 2 and expression further decreased as the flies spent longer on the dsRNA-containing diet, until gene expression was silenced by 79% at day 6 of the experiment. In all these studies, successful silencing at the transcript level was also linked to clear phenotypical changes in the insects, depending on the gene that was silenced. Despite these successful experiments, *Bactrocera*, like other dipterans, lacks the *sid-1* gene and therefore has to rely solely on the endocytotic pathway for cellular uptake of dsRNA, which might impact its sensitivity for oral RNAi. In fact, a study by Li et al. (2015b) seems to suggest that insects may develop an insensitivity to RNAi caused by feeding of RNAi, through the downregulation of clathrin-mediated endocytosis.

The 31 oral intake RNAi studies on non-fruitfly dipteran insects which we encountered in our literature search, targeted the housefly *Musca domestica* and important pathogen vectors such as the tsetse fly *Glossina morsitans* and mosquitoes of the *Aedes*, *Anopheles* and *Culex* genera. Successful RNAi was achieved using siRNA or dsRNA delivered either by soaking, artificial diet or bloodmeal. In *G. morsitans* adults, Walshe et al. (2009) demonstrated that RNAi could be achieved by mixing dsRNA to bloodmeal. Semi-quantitative PCR indicated a knockdown of around 50% could be achieved for several days when feeding a 10  $\mu\text{g}$  dose of dsRNA. In another study using the same feeding method, knockdown of serpins managed to cause a decrease of trypanosoma infection (Ooi et al., 2015). In *M. domestica*, an approach mixing dsRNA-producing bacteria was used to achieve up to 76% gene knockdown (Tang et al., 2012a; Tang et al., 2012b). In mosquitoes, several studies have shown successful RNAi, mostly in larvae. Interestingly, the dsRNA was encapsulated in several of these studies, indicating a less than optimal efficiency when feeding naked dsRNA. Zhang et al. (2010) demonstrated that soaking *A. gambiae* larvae in a chitosan-coated dsRNA solution could lead to efficient RNAi knockdown. In another study, dsRNA was encapsulated by a transfection agent and *A. aegypti* larvae were placed in a 20  $\text{ng}/\mu\text{L}$  solution of this encapsulated dsRNA for 16 hours. This delivery method led to very efficient knockdown (up to 98%, evaluated by semi-quantitative PCR) (Cancino-Rodezno et al., 2010; Cancino-Rodezno et al., 2012). Nonetheless, experiments with naked dsRNA have also been shown to be successful. The Bona et al. (2016) study of silencing the *VGSC* gene in larval mosquitoes showed that soaking *A. aegypti* larvae in a 0.5  $\mu\text{g}/\mu\text{L}$  concentration of dsRNA could lead to a significant silencing effect ( $\pm 75\%$ ), and also led to an increase of mortality of about 50%. Lower concentrations that were tested did not elicit a significant gene knockdown. The study also showed that the strong silencing effect was lost as larvae developed into adults. In another study, three different concentrations of dsRNA targeting the *NAT5* gene (0.05, 0.5 and 5  $\mu\text{g}/\mu\text{L}$ ) were tested in *A. aegypti* and, while the lowest concentration did lead to some silencing on day 4, the

other two concentrations led to much higher knockdown levels. Also in terms of phenotypic effect, there was a clear concentration-dependent effect (Meleshkevitch et al., 2013). These and other studies suggest that while soaking or feeding naked dsRNA is able to incite successful gene knockdown, the necessary concentrations are higher and the effect is weaker in comparison to experiments where encapsulated dsRNA was used.

### *Coleoptera*

Baum et al. (2007) investigated the use of RNAi for protection against coleopteran crop pest insects. In their study, they focused on the WCR, *D. virgifera*, and the CPB, *L. decemlineata*. These two species were known to be among the most sensitive insects to RNAi, with 50% lethal concentration (LC<sub>50</sub>) values in the 1-10 ppb (1-10 pg/μL) range (Baum and Roberts, 2014), which is lower than anything observed in other insect orders. Furthermore, some other coleopteran species, such as the canola flea beetle *Phyllotreta striolata* (Zhao et al., 2008) seem to show similar efficiency. However, even within Coleoptera there is variability in terms of RNAi sensitivity. In *T. castaneum* for example, dsRNA delivered through flour discs at a concentration of 100 ng/mg diet was necessary to achieve a significant silencing of the *Na<sub>v</sub>* gene (Abd El Halim et al., 2016) and Whyard et al. (2009) reported an LC<sub>50</sub> of 3.3 μg/mg of dsRNA targeting V-ATPase. Recently, two studies on the African sweet potato weevils *Cylas puncticollis* and *Cylas brunneus* demonstrated that even between two very closely related species, a significant amount of variability in RNAi sensitivity could be observed, with *C. brunneus* being much more sensitive to oral RNAi than *C. puncticollis*, which was rather recalcitrant to oral RNAi using naked dsRNA (Christiaens et al., 2016; Prentice et al., 2017). In *C. brunneus*, feeding on diet containing 1 μg/mL dsRNA led to 67%-73% gene knockdown, while in *C. puncticollis*, a concentration of 30 μg/μL was necessary to achieve similar knockdown levels. The authors also found the variable sensitivity to be related to differences in dsRNA stability in the gut of these two weevils. In most of the oral RNAi studies retrieved in our search, the dsRNA was mixed with artificial diet, coated on leaves or presented through GM plant material. However, a study of the banana root borer *Cosmopolites sordidus* demonstrated that a topical application of 200-2000 ng of dsRNA targeting the *ubiquitinE2* gene on the eggs also elicited successful gene silencing in hatching larvae, eventually causing 100% mortality 21 days after hatching (Ocimati et al., 2016).

### *Lepidoptera*

Lepidoptera comprise some of the most devastating agricultural pests and have therefore received a lot of attention from researchers investigating whether RNAi could be an effective pest control strategy. Experiments using microinjection in some of these lepidopteran species had already indicated that the RNAi pathway is present and functional, and that relatively small amounts of dsRNA were necessary for efficient gene knockdown (Bettencourt et al., 2002; Hirai et al., 2004; Terenius et al., 2007). However, when attempting feeding dsRNA to these insects, it became apparent that the sensitivity to oral RNAi in Lepidoptera was generally quite low, and very variable at best in comparison with Coleoptera. A meta-study by Terenius et al. (2011) gathered all data available on RNAi in Lepidoptera, both published and unpublished studies and concluded that while oral RNAi sometimes works in some species at very high doses of dsRNA, these species are generally refractory to feeding RNAi. They evaluated a total of 32 lepidopteran RNAi studies and reported low efficiency for 5 of those and a complete lack of successful silencing in 18 studies. An efficient silencing response was only observed in nine out of 32 studies.

*Manduca sexta* was one of the species generally showing moderate levels of oral RNAi efficiency. We retrieved eight studies in total on *M. sexta* RNAi, most of them delivering the dsRNA through feeding of a droplet of water containing 4-5 μg dsRNA to caterpillars (Cancino-Rodezno et al., 2010; Flores-Escobar et al., 2013; Gomez et al., 2015; Porta et al., 2011). Three studies also successfully targeted *M. sexta* larvae through *Nicotiana attenuata* plant-expression of dsRNA which elicited an efficient gene knockdown (Kumar et al., 2012; Kumar et al., 2014; Poreddy et al., 2015). The majority of the lepidopteran oral RNAi studies we retrieved were targeting *H. armigera*, even though the data from Terenius et al. (2011) suggested that oral RNAi in this species is rather unsuccessful. In most

*H. armigera* studies, concentrations in the 1-10 µg/µL range were used. However, one study also reported successful gene knockdown after feeding 35 ng dsRNA/µg artificial diet to second-instars, leading to near complete knockdown after 24 hours. Gene expression in this study started to recover after 48 hours (Zhang et al., 2015d). Interestingly, the potential for insecticidal miRNAs was also explored in *H. armigera*. Tomato plants were genetically modified to express an artificial miRNA-24 specifically silencing the *chitinase* gene in *H. armigera*, resulting in successful silencing and toxic effects similar to those seen when using dsRNA to silence *chitinase* (Agrawal et al., 2015). Other species for which successful RNAi was reported were *Heliothis virescens*, *Ostrinia* sp., *P. xylostella*, *Chilo* sp. and several *Spodoptera* species (Apone et al., 2014; Ayra-Pardo et al., 2015; Chen et al., 2014b; Khajuria et al., 2010; Qiu et al., 2015; Zhang et al., 2012; Zhu et al., 2016). Despite these successful experiments, often requiring high doses, there is a consensus that oral RNAi in Lepidoptera does not work very efficiently. Two possible explanations have been suggested, namely a very strong nucleolytic degradation of dsRNA in the digestive system of these caterpillars, and potential issues with cellular uptake or cytoplasmic release (Arimatsu et al., 2007; Garbutt et al., 2013; Liu et al., 2012; Shukla et al., 2016). These factors are discussed further in Section 5.3.3.

### Hymenoptera

Hymenopteran species are an interesting order, since they comprise economically important species such as pollinators and parasitic wasps. Most studies we retrieved on hymenopteran RNAi reported experiments on the honeybee, *A. mellifera*. Several studies explore the potential use of RNAi as a therapeutic, targeting honeybee-specific viruses and also the Varroa mite (*V. destructor*). Feeding a 1 µg dose of Israeli Acute Paralysis Virus (IAPV) specific siRNA mixed with sugar water was able to inhibit viral replication in the honeybees, demonstrating that the technology could indeed be used for therapeutic purposes (Chen et al., 2014c; Hunter et al., 2010; Maori et al., 2009). Similarly, feeding of 1 µg deformed wing virus (DWV)-specific dsRNA every day for six days led to significantly lower viral titres upon infection (Desai et al., 2012). Maori et al. (2012) also managed to decrease *V. destructor* incidence by 60% after feeding parasitized honeybees with a 40 µg/µL *V. destructor* specific dsRNA sugar water solution. Successful RNAi experiments targeting honeybee genes were also reported. Nunes and Simões (2009) reported around 90% silencing of the *vitellogenin* gene expression upon feeding of 0.5 µg dsRNA to honeybee larva.

Data in other Hymenoptera is scarce. One study showed that RNAi in the bumblebee *Bombus terrestris*, which is an important commercial pollinator, can be used efficiently to target IAPV virus infections in the bumblebee, using high doses of 2 µg dsRNA per day per individual (Piot et al., 2015). No studies targeting *Bombus*-specific genes by feeding dsRNA were found. Injection studies however reveal that even with that delivery method, high doses of 20 µg were necessary to elicit a silencing response of an endogenous *B. terrestris* gene (Niu et al., 2016). In another study, targeting the wasp *Polistes metricus*, very high doses of dsRNA (80 µg dsRNA over two days) resulted in only a modest silencing effect. It must be noted, however, that only one timepoint was chosen for sampling (Hunt et al., 2011). Finally, targeting three genes in the ant *Camponotus floridanus*, fed *ad libitum* on 2 µg dsRNA/µL diet, resulted in an observable silencing effect for only one of the two targeted genes in the body of minor workers, suggesting *C. floridanus* is not extremely sensitive to oral RNAi compared to other more sensitive insect species (Ratzka et al., 2013). Based on these data, RNAi efficiency in Hymenoptera in general does not seem to work highly efficiently. Of course, one has to take into account the size and weight of many of the hymenopteran species studied in these researches, such as honeybees and bumblebees, but, even taking this into account, the efficiency in certain Coleoptera and Hemiptera is considerably higher.

### Hemiptera

The order of Hemiptera also comprises many pest insects, including whiteflies, aphids, stinkbugs, planthoppers and thrips. In many of these species, RNAi by oral feeding has been shown to be moderately efficient, albeit variable in some species (Christiaens and Smagghe, 2014). In the pea aphid, *A. pisum*, several studies report successful RNAi at concentrations ranging from 750 ng/µL to

5 µg/µL (Mao and Zeng, 2012; Sapountzis et al., 2014; Shakesby et al., 2009; Whyard et al., 2009). However, the knockdown levels and phenotypical effects were not always that dramatic. For example, Mao and Zeng (2012) targeted hunchback in *A. pisum* by placing aphids on a 750 ng/µL dsRNA containing artificial diet. The highest transcript silencing was recorded at day seven, with 46% silencing. In terms of mortality, 45% of dsRNA-treated aphids were dead on day seven, while this was 20% in the control. In another study, Shakesby et al. (2009) targeted an aquaporin gene using a continuous feeding approach on a 1.8 µg/µL dsRNA containing artificial diet. After six days on this diet, the recorded mortality was 46%, compared to 19% in the GFP control. These authors observed significant silencing in at least one tissue for 12 aphids of the 15 aphids they analysed. In contrast, another study reported a failure to elicit efficient RNAi in *A. pisum*, both with feeding and injection and suggested possible strain-related variabilities in RNAi sensitivity (Christiaens and Smagghe, 2014; Christiaens et al., 2014). In *A. pisum*, degradation of dsRNA by saliva and in haemolymph was also reported, similar to observations in the tarnished plant bug *Lygus lineolaris* (Allen and Walker, 2012; Christiaens et al., 2014). Two other aphids, the green peach aphid *M. persicae* and the wheat aphid *Sitobion avenae* have been extensively studied and efficient oral RNAi was reported in both species. In *S. avenae*, very low doses of dsRNA, as low as 3-20 ng dsRNA/µL diet were sufficient to elicit successful gene knockdown (Deng and Zhao, 2014; Fan et al., 2015; Zhang et al., 2013; Zhang et al., 2016). In *M. persicae*, all studies which were retrieved via the systematic literature search used GM plants to elicit RNAi silencing. Target gene silencing was confirmed in aphids feeding on these transgenic plants both at the transcript level and based on phenotypical observations such as mortality, decrease in fecundity, etc. (Bhatia et al., 2012; Coleman et al., 2014; Elzinga et al., 2014; Pitino et al., 2011; Pitino and Hogenhout, 2013). Interestingly, one study also included a miRNA construct, targeting acetylcholine esterase in *M. persicae*. They observed similar results for the miRNA expressing plants compared to those expressing hpRNAs targeting the same gene. However gene knockdown levels did not exceed 30% (Guo et al., 2014). Successful oral RNAi has also been reported in *Bemisia tabaci* whiteflies. Most studies exposed the insects to concentrations around 200 ng/µL and 2 µg/µL to achieve successful RNAi. Two studies however used a range of concentrations, including some which were considerably lower. Shim et al. (2015) found that 2.5 ng/µL dsRNA, targeting hsp70, did not cause any observable silencing at the transcript level, while 25 ng/µL (66% silencing) and 250 ng/µL (near complete knockdown) did cause significant silencing. Phenotypically, feeding on 250 ng/µL dsRNA for 24 hours led to approximately 40% mortality at day six post treatment, while continuous feeding of dsRNA resulted in approximately 80% mortality after six days. Upadhyay et al. (2011) performed extensive research on the efficiency of RNAi in whiteflies, testing the difference between dsRNA and siRNA, testing different target genes and a range of concentrations. They found that dsRNA was only slightly more efficient than siRNA and reported an LC<sub>50</sub> value for dsRNA targeting V-ATPase of 3.08 ng/µL, indicating that oral RNAi in whiteflies is highly efficient. In the brown planthopper, *N. lugens*, most studies report dsRNA concentrations in artificial diet of 100-1000 ng/µL dsRNA. Hao et al. (2015) investigated different concentrations and found that while concentrations as low as 20 ng/µL could elicit significant knockdown after seven days, the effect was limited and concentrations of 100-500 ng/µL were necessary to elicit efficient knockdown leading to an observable phenotype. These results confirmed earlier observations by Chen et al. (2010) who reported that 500 ng/µL dsRNA targeting trehalose phosphate synthase was necessary to cause phenotypic effects, while 20 ng/µL and 100 ng/µL did not cause any observable effects. Thus, these studies indicate that higher concentrations seem to be necessary for successful RNAi silencing in brown planthopper.

### *Orthoptera*

Orthoptera are an interesting group of insects in terms of RNAi efficiency. Several publications have reported different locust species such as *S. gregaria* and *L. migratoria* to be highly sensitive to RNAi by microinjection, while feeding dsRNA, even very high doses, does not result in successful gene knockdown (Luo et al., 2013; Wynant et al., 2014b; Wynant et al., 2012). The possible mechanisms behind these observations will be discussed further on in this Technical Report.

## Blattodea

In Blattodea, no oral RNAi has been successfully achieved, to the best of our knowledge. However, RNAi by injection in the cockroach *B. germanica* works efficiently (Cruz et al., 2006; Garbutt et al., 2013).

## Conclusions on Insecta

Only a relatively small number of studies examine non-target insects, or even beneficial insects, exposed to dsRNA orally. Nevertheless, the insects for which data are available represent all major insect orders, giving us an idea of the range of RNAi efficiency and sensitivity in insects. Indeed, in general terms, the data seem to indicate that there are some inter-order differences in RNAi sensitivity. Generally, many Coleoptera seem to be very sensitive to oral RNAi while Lepidoptera are not. In Hemiptera and Diptera, the sensitivity seems rather variable between species while in orthoptera RNAi by injection seems to work highly efficiently, while locusts appear to be highly refractory to oral RNAi.

### 5.3.2.4. Crustacea

In our systematic literature search, we identified 21 studies reporting oral RNAi in Crustacea. The majority were studies on decapod *Penaeus* species. There is great interest in using RNAi to combat viral infections in these shrimps, which are economically important commodities in South-East Asia. Therefore, most *Penaeus* oral RNAi studies targeted viruses infecting these shrimps. In 2013, researchers used a bacterial approach to deliver dsRNA, specific for the Laem Singh virus (LSNV), to *P. monodon* shrimp. In these dsRNA treated shrimp, a reduction of 20-60 % LSNV was observed (Saksmerprome et al., 2013). Other studies also confirmed the functionality of orally delivered dsRNA expressing bacteria, as an antiviral therapeutic in *P. monodon*, *Penaeus merguensis* and *Penaeus vannamei* (Leigh et al., 2015; Saksmerprome et al., 2013; Sanitt et al., 2016; Sanitt et al., 2014; Sarathi et al., 2008; Sarathi et al., 2010). However, we found one study in which *Penaeus*-specific genes were targeted. Here, feeding of bacteria expressing dsRNA specific for the *Rab7* and *Stat* genes in *P. monodon* and *L. vannamei* caused successful silencing in both hepatopancreas and gill tissues (Attasart et al., 2013). In most of these studies, *E. coli* was used to deliver the dsRNA. However, two studies reported using nanoparticle-coated dsRNA. Sanitt et al. (2016) reported the use of cholesterol-based liposomic particles to increase RNAi efficiency against the yellow head virus (YHV) in *P. vannamei*. Another study reported the successful use of a chitosan-dextran sulfate nanocapsule to delivery dsRNA in *P. monodon* post-larvae (Ramesh Kumar et al., 2016).

Nanoparticle-coated dsRNA was also used in the copepod *Tigriopus californicus* (Barreto et al., 2015). However, the two carriers that were tested, chitosan polymer and a liposome transfection agent, did not result in successful RNAi silencing (Barreto et al., 2015). In the salmon louse, *Lepeophtheirus salmonis*, researchers managed to elicit RNAi knockdown by soaking larvae in a 10 ng/ $\mu$ L dsRNA solution. However, RNAi was only successful when animals were treated at the time they moulted from Nauplius I to Nauplius II stages (Eichner et al., 2015a; Eichner et al., 2014; Eichner et al., 2015b). In the waterfleas *Daphnia pulex*, *Daphnia melanica* and *Daphnia pulex*, successful RNAi was achieved by adding bacteria expressing *Daphnia*-specific dsRNA to the aquatic medium containing the animals. Semi-quantitative transcript analysis, as well as phenotypical observations, suggest an efficient knockdown using this method (Schumpert et al., 2016; Schumpert et al., 2015). Finally, one study also reported that soaking larvae and nauplii of the barnacle *Amphibalanus amphitrite* in 0.8 nL/ $\mu$ L siRNA concentration, using a liposomic transfection agent, was successful (Zhang et al., 2015a).

In conclusion, the amount of data on RNAi efficiency in Crustacea is rather limited, and most studies report successful RNAi in the decapod shrimps of the *Penaeus* genus using either bacteria or nanocarrier-coated dsRNA for virus control. The lack of studies using naked dsRNA may reflect a refractoriness in these shrimp species, or may be related to the short half-life of these dsRNA

molecules in the environment. In other Crustacea, the sensitivity to RNAi proved to be variable using naked dsRNA or siRNA.

### 5.3.2.5. Chelicerata

In our systematic literature study, we found reports that mites and ticks were susceptible to oral RNAi but no studies on species outside of the Acari subclass were found in the context of oral RNAi. One of the first demonstrations of successful feeding RNAi was delivered by Soares et al. (2005), who managed to elicit successful RNAi knockdown in *I. scapularis* tick nymphs. In this study, a capillary feeding approach was used, eventually delivering a dose of 2.4 µg dsRNA to each nymph. Northern blot analysis confirmed successful silencing of the *isac* gene and observed effects included a 40.6% reduction in weight. Sensitivity to oral RNAi was also demonstrated in another ixodid tick, namely *Haemaphysalis longicornis* (Galay et al., 2016). Immersion of *H. longicornis* ticks in a 1 µg/µL dsRNA containing solution led to efficient knockdown in all stages (Galay et al., 2016; Gong et al., 2009).

Also in mites, feeding RNAi seems to be functional. We identified four studies reporting successful gene knockdown in *Tetranychus* species. Two different delivery methods have been used in *T. urticae*. One entails a leaf-disk mediated delivery, whereby mites are placed on dsRNA-permeated leaf disks in a feeding apparatus. In one study, this delivery led to only 26% knockdown of the target gene *COPE* after 14 hours, and 14.6% knockdown after 72 hours. In another study, targeting other genes, the researchers managed to achieve 50% silencing for V-ATPase after 48 hours. For other genes, knockdown efficiency was lower (Kwon et al., 2016; Kwon et al., 2013). The same delivery method was used in *Tetranychus cinnabarinus*, leading to up to 64% knockdown efficiency (Shi et al., 2016). Another delivery method, which was used in *T. urticae*, was feeding of dsRNA through an impregnated piece of cotton, leading to a 42% transcript knockdown after three days feeding on the dsRNA (Ozawa et al., 2012). In another Acari species, feeding RNAi to the predatory mite *Metaseiulus occidentalis* also elicited a silencing response. In these studies, delivery of dsRNA was achieved by placing mites on a parafilm disc resting on a water-soaked piece of cotton containing a 350 ng/µL dsRNA (Pomerantz and Hoy, 2015; Wu and Hoy, 2014a; Wu and Hoy, 2014b; Wu and Hoy, 2015). Reported expression knockdown in these experiments was between 70% and 91%, confirming efficient silencing. Also in other mites such as the house dust mite, *Dermatophagoides pteronyssinus*, the citrus red spider mite, *Panonychus citri*, and the red poultry mite, *Dermanyssus gallinae*, RNAi by feeding dsRNA was shown to be functional (Kamau et al., 2013; Marr et al., 2015; Tian et al., 2015b).

**Table 12:** Overview of reported sRNA concentrations or single doses leading to successful RNAi-elicited gene silencing in *in planta*, *in vitro* feeding and soaking studies in Arthropoda

Subphylum	Order	<i>In planta</i> GM <sup>1</sup>	<i>In planta</i> non-GM <sup>2</sup>	<i>In vitro</i> feeding	<i>In vitro</i> soaking	Comments
Hexapoda	Diptera	-	-	0.02 - 6 µg/µL	0.05 - 0.5 µg/µL	Diptera are sensitive to RNAi by injection of naked dsRNA or siRNA, but require formulation with a polymer or liposome for feeding RNAi
	Lepidoptera	Sni	1-3 µg/cm <sup>2</sup>	0.015 - 10 µg/µL	-	Additional delivery methods, such as forcefeeding single doses (usually several µg) have been reported
	Coleoptera	50E-6 - 0.5 µg/µL 0.5 - 16 ng/cm <sup>2</sup>	Sni	1E-6 - 5 µg/µL	-	Very high sensitivity by feeding, compared to other insect orders, although some variability exists between different coleopteran species
	Hymenoptera	-	-	0.02 - 2.2 µg/µL 1-40 µg SD per day	-	
	Hemiptera	Sni	Sni	0.003 - 40 µg/µL	-	
	Orthoptera	-	-	3 µg/µL 6 µg SD per day	-	In <i>Schistocerca</i> , a dose of 1 mg per day for eight days could not trigger any RNAi knockdown
	Blattodea	-	-	0.15 µg/µL 2.2 - 5.1 mg/cm <sup>2</sup> 10µg SD	-	
Crustacea	-	-	6 - 12 mg/kg	0.01 - 1 µg/µL	<i>E. coli</i> - or <i>Artemia</i> -mediated delivery, or a polymer-formulation used in most Crustacea feeding studies	
Chelicerata	-	Sni	0.02 - 1.2 µg/µL	-		

Sni: Successful silencing but no information on concentration or expression level in the plant; SD: single dose

<sup>1</sup> Concentrations for *in planta* studies were most often not given; <sup>2</sup> These include leaf immersion, stem injection, root absorption delivery strategies

- : No studies found



### 5.3.2.6. Annelids and molluscs

Very little information is available concerning RNAi sensitivity for both annelids and molluscs. In both classes there are a number of studies demonstrating that injection of dsRNA or morpholinos can be used to elicit gene knockdown (e.g., Conzelmann et al., 2013; Song et al., 2002), but only three studies were retrieved demonstrating a soaking dsRNA approach in molluscs (Chen et al., 2014a; Knight et al., 2011; Wang et al., 2016a), and no studies of direct feeding were found. In one study, solutions ranging between 0.1 and 10 µg dsRNA/mL were used to target the Asiatic hard clam, *Meretrix meretrix*, and while knockdown efficiencies were not investigated at the transcript level, a near 100% mortality was observed in the RNAi-treated clam larvae, including at the 0.1 ng/µL concentration. This suggests that RNAi by soaking works efficiently in this species. Another study, investigating the role of *Prx* and *CathB* genes in schistosomiasis transmission by the freshwater snail *Biomphalaria glabrata*, demonstrated that soaking in a siRNA or dsRNA solution only resulted in silencing when the sRNAs were coated with the polyethyleneimine (PEI) polymer. Naked dsRNA or siRNA did not result in an observable knockdown (Knight et al., 2011), suggesting that the stability of sRNAs might be compromised in this animal, or in this experimental setup. Finally, in the abalone *Haliotis diversicolor*, efficient knockdown was reported using a 5 ng/µL soaking solution (Wang et al., 2016a). The concentrations used in some of these studies suggest that at least some molluscs are very sensitive to RNAi.

### 5.3.3. Factors involved in oral RNAi efficiency

In this section, we discuss different biotic, abiotic, biochemical and physiological factors that can influence RNAi efficacy in invertebrates. Many studies have been devoted to the variability in RNAi efficiency in invertebrates, notably in insects. While some factors, such as the stability of RNAs in the body and their cellular uptake, are thoroughly investigated in a range of species, others are still poorly understood, such as the effect of viral infections.

#### 5.3.3.1. RNAi machinery genes repertoire

RNAi efficiency is very much dependent on the presence of the RNAi (core) machinery genes in the genome. Despite the fact that RNAi is a conserved mechanism throughout Eukaryota, considerable differences in pathways and functioning proteins in these pathways exist between the different taxonomical clades. For example, while plants possess four Dicer-like (DCL) protein encoding genes in their genomes, insects only have two, and nematodes, annelids, molluscs and higher animals have only one (Mukherjee et al., 2013). Regarding Argonaute-related proteins, there is even more diversity to be found. For example, while most insects have five Ago-like proteins (Ago1, Ago2, Ago3, PIWI and Aubergine), eight Ago-like proteins have been found in humans, 10 in the plant *Arabidopsis thaliana* and 27 in nematodes (Höck and Meister, 2008). While not all of these Ago-like proteins have functions in the RNAi pathways, many do. And this diversity of core gene repertoire is also reflected in the diversity and compartmentalization of the different RNAi-related functions in eukaryotic cells. In insects for example, siRNAs, miRNAs and piRNAs are mainly processed by distinct pathways using different Dicer and Ago-like proteins. While in mammals, all sRNAs are processed by the one Dicer that is present. Since further comparisons of these RNAi pathways in non-invertebrates falls outside of the scope of this review, we can refer to some excellent recent research articles and reviews for further reading on the diversity of RNAi pathways in Eukaryota (Chapman and Carrington, 2007; Ding and Voinnet, 2007; Höck and Meister, 2008; Mukherjee et al., 2013; Obbard et al., 2009; Pačes et al., 2017).

So far, there is no report on cell-autonomous RNAi not being functional in any invertebrate, to the best of our knowledge. Given the important functions RNAi is involved in, this is no surprise. This also means that in terms of core genes involved in intracellular RNAi, such as Dicer and Ago, we should not expect important qualitative differences between species. Nonetheless, it has been reported that

RNAi-related genes belong to some of the most rapidly evolving genes, similar to other immunity pathway genes (Obbard et al., 2006), so differences do exist and will be discussed here, including whether they could have an impact on RNAi efficiency.

As discussed before, despite *C. elegans* being very sensitive to (oral) RNAi, other nematodes such as parasitic species are often more refractory. One of the possible reasons is a difference in RNAi-related machinery genes repertoire. A study looking into the RNAi-pathway related genes in a wide range of nematodes showed that *Caenorhabditis* species have an expanded RNAi-related gene repertoire compared to parasitic nematodes, especially concerning Ago genes and the genes related to uptake and spread of the RNAi signal (Dalzell et al., 2011). The variability in effector proteins involved in uptake and systemic spread was discussed in detail in Section 5.1. However, differences can also be found in other pathways, such as genes involved in small RNA biosynthesis, RISC elements and genes involved in amplification and secondary siRNA production. For example, proteins involved in the biosynthesis of small RNAs, such as RNase III enzymes (Drosha, Pasha, Dicer) were found to be broadly conserved, as were RNA helicases and exportins. However, orthologs of the dicer-cofactor RDE-4 were found to be absent in most parasitic nematodes. Another interesting find was that Ago genes involved in internal gene regulation were broadly conserved throughout nematodes, but this was not the case for Ago genes involved in RNAi induced by exogenous RNAi (Dalzell et al., 2011). *C. elegans* possesses at least 27 distinct Ago proteins. These Ago proteins, by being an important part of the RISC complex, are involved in different RNAi-related pathways. Different subsets could be identified such as those involved in miRNA processing (e.g., ALG-1), those that interact with endo-siRNA (e.g., ALG-4 and WAGO's) and the Ago that is responsible for RNAi triggered by exogenous dsRNA (RDE-1). While the former Ago's were found to be broadly conserved throughout nematodes, this was not the case for RDE-1 which was not found in plant-parasitic nematodes. However, the authors note that their identification strategy, which was based on BLAST analysis of known *C. elegans* RNAi effectors, could not account for the presence of other uncharacterized Ago's which could have assumed the role of those *C. elegans* Ago's that were not identified. Alternatively, other Ago's which were identified in these species could have assumed other roles than they have in *C. elegans* (Dalzell et al., 2011). In regard to the amplification of the RNAi signal through RDRP-dependent secondary siRNA production, most nematodes appear to have at least some of the RdRPs that are implicated in this mechanism, such as smg-2, smg-6 and ego-1. In contrast, smg-5 and rsd-2 seem to be only present in *Caenorhabditis* genomes. However, given the fact that in a number of plant parasitic nematodes efficient RNAi after exposure to very small doses has been observed, the absence of these RDRPs does not seem to affect RNAi efficiency in these species (Dalzell et al., 2010a; Dalzell et al., 2011). One important conclusion the authors made was that a clear link between the RNAi efficiency and the absence of certain effector genes in the investigated nematode species could not be made. In fact, while *Caenorhabditis* species, and especially *C. elegans*, do have an expanded gene repertoire, the difference is mainly quantitative rather than qualitative. All distinct pathways and essential proteins are represented throughout the nematode class (Dalzell et al., 2011).

For a long time, information on the RNAi core machinery in arthropods was limited to the model insect *D. melanogaster*. However, recently a number of in silico studies have been devoted to the RNAi-related gene repertoire in other arthropod species, including the Colorado potato beetle *L. decemlineata* (Swevers et al., 2013b), the red flour beetle *T. castaneum* (Tomoyasu et al., 2008), the common North Sea shrimp *Crangon crangon* (Christiaens et al., 2015), the Asian citrus psyllid *D. citri* (Taning et al., 2016a) and the soybean aphid *Aphis glycines* (Bansal and Michel, 2013). In 2016, a large scale study was published investigating 10 RNAi core genes in 100 insect species (Dowling et al., 2016). These included: the miRNA pathway elements Dcr-1, Ago-1, Loqs, Drosha and Pasha; the siRNA pathway components Dcr-2, Ago-2 and R2D2; the piRNA pathway elements Ago3 and Aub/Piwi; and *sid-1-like* genes. However, since transcriptomic data was used in the study, it is impossible to claim with any certainty that a certain gene is missing in a species. Nevertheless, the study did deliver a number of interesting results. In 2017, a similar study was published looking at the

RNAi core gene repertoire related to antiviral innate immunity in available Crustacea genomes (Lai and Aboobaker, 2017).

Looking at these arthropods, it is clear that the three RNAi pathways are present in all major lineages throughout both subphyla (Dowling et al., 2016), which is not surprising given their roles in antiviral defence, protection of the genome and internal gene regulation. However, some interesting differences could be found between different groups. For example, all insects possess two Dicer-like proteins, while this number varies in some Crustacea and Chelicerata. In the Crustacean *Daphnia magna*, three Dicer-like proteins were identified, for the first time in animal species (McTaggart et al., 2009). This extra Dicer is the result of a lineage-specific duplication of the Dicer that is involved in the antiviral immune pathway in arthropods, the siRNA pathway, suggesting that it might have an influence on the efficiency of siRNA RNAi as well. However, no further studies have been conducted to elucidate the role of this extra Dicer protein. Furthermore, in other crustacean species in which the RNAi pathways were investigated, such as the brown shrimp *C. crangon*, the black tiger shrimp *P. monodon* and the Pacific white shrimp *L. vannamei*, only two Dicers were found (Chen et al., 2011a; Christiaens et al., 2015; Li et al., 2013; Su et al., 2008; Yao et al., 2010). In ticks and mites (Acari), a variable number of Dicers was reported as well. The first Acari species to have its genome sequenced and annotated, the two-spotted spider mite *T. urticae* (Grbić et al., 2011), contains one gene copy for *Dicer-1* and *Dicer-2*, as in insects (Suzuki et al., 2017). However, in the ixodid tick *I. scapularis*, only one Dicer, homologous to *Dicer-1*, was identified (Kurscheid et al., 2009). Furthermore, five copies of the *Dicer-2* gene were identified in the genome of the predatory mite *M. occidentalis* (Hoy et al., 2016). While these differences in Dicer-encoding genes might influence the efficiency of the pathways involved, no actual proof of their effect on RNAi has been reported.

A number of these studies also shed more light on the issue surrounding R2D2. In *Drosophila*, R2D2 is an important cofactor of Dicer-2 and is required for successful RNAi and a successful antiviral response (Liu et al., 2003; Wang et al., 2006). In many Coleoptera, including *T. castaneum*, two distinct R2D2 genes were discovered (Dowling et al., 2016; Tomoyasu et al., 2008) while in several Lepidoptera, expression of R2D2 is either very low or the gene is absent (Dowling et al., 2016; Swevers et al., 2011). Furthermore, R2D2 has been reported absent in a number of other arthropod species, such as the insect *D. citri* and the crustacean *C. crangon* (Christiaens et al., 2015; Taning et al., 2016a). Dowling et al. (2016) confirmed that R2D2 was not present in the primary wingless insects they investigated and was also missing in their outgroup species, the crustacean *D. pulex* and the chelicerate *I. scapularis*. They also report that, to date, R2D2 has not been found outside of the class of Insecta. The authors speculate that Loquacious, a similar protein normally associated to the miRNA pathway, could take over the role of R2D2 in these basal insect species. This assumption, which was already suggested by Christiaens et al. (2015) and Taning et al. (2016a) is also supported by reports of Loquacious being functional as cofactor to Dicer-2 in the siRNA pathway in *D. melanogaster* (Czech et al., 2008; Marques et al., 2010; Okamura et al., 2008). In Crustacea, another protein has been identified as a cofactor for Dicer-2, namely an orthologue of the transactivating response (TAR) RNA binding protein (TRBP) (Lai and Aboobaker, 2017).

It is difficult to say whether these differences regarding R2D2 could be linked to RNAi efficiency in these species. Swevers et al. (2013b) have suggested that this could be the case, based on the observations that the RNAi sensitive Coleoptera possess two R2D2 copies (Swevers et al., 2013b), while less sensitive Lepidoptera have either no R2D2, or the gene is only expressed at very low levels as is the case in *B. mori* (Swevers et al., 2011). However, *Diaphorini citri* is sensitive to RNAi despite the absence of an R2D2 gene in the genome (Taning et al., 2016a). However, these authors did indicate that, despite quantitative differences in terms of RNAi-related genes, there was no qualitative difference in terms of functional groups that were represented in these nematode species. Furthermore, they could also not identify a link between RNAi effector repertoire and RNAi functionality in parasitic nematodes.

### 5.3.3.2. Persistence of dsRNA in the body

One of the major factors contributing to the variability in RNAi efficiency in invertebrates, especially insects, is the stability and persistence of dsRNA in the invertebrate body. Many studies have demonstrated that dsRNA can be rapidly degraded by nucleolytic activity in the saliva, the intestinal fluid or the haemolymph of a wide range of insect species (Allen and Walker, 2012; Arimatsu et al., 2007; Christiaens et al., 2014; Garbutt et al., 2013; Liu et al., 2012; Luo et al., 2013; Wynant et al., 2014a; Wynant et al., 2014b). In the pea aphid for example, researchers showed that the secreted saliva of an aphid into its artificial diet, was able to cause degradation of dsRNA in the diet. Additionally, the haemolymph of these plant suckers was also able to degrade dsRNA (Christiaens et al., 2014). The tarnished plant bug *L. lineolaris* and the Southern green stinkbug, saliva was also found to rapidly degrade dsRNA molecules (Allen and Walker, 2012; Lomate and Bonning, 2016). Even more evidence exists for degradation in the gut lumen. Studies in caterpillars of *M. sexta* (Garbutt et al., 2013), *B. mori* (Arimatsu et al., 2007; Liu et al., 2012), in African sweet potato weevils *C. puncticollis* and *C. brunneus* (Prentice et al., 2017), in the locusts *S. gregaria* and *L. migratoria* (Luo et al., 2013; Wynant et al., 2014b) and even in the Colorado potato beetle, which is considered very sensitive for oral RNAi, degradation of the dsRNA in the gut has been shown (Prentice et al., 2017; Spit et al., 2017). The fact that dsRNA degradation can be observed in the intestinal fluid of CPB, which is very sensitive to oral RNAi, shows that degrading nucleolytic activity in the gut does not necessarily mean that a species is insensitive to RNAi, but merely that RNAi efficiency can be compromised. Since these experiments were all carried out in vitro, often with extracted intestinal fluid from dissected midguts, it is difficult to say at what speed the dsRNA actually degrades in the gut in an in vivo situation. It is also difficult to compare stabilities between different studies, since gut juice is often collected with different methods, in different dilutions and the incubated dose of dsRNA differs as well. But it is clear that the persistence in some insects is higher than in others, and in some cases, this can be associated with a difference in RNAi efficiency. A recent publication by Prentice et al. (2017) provided data comparing the RNAi responses in different beetle species and comparing them with dsRNA persistence in intestinal fluid. They found a clear correlation between RNAi efficiency and the speed of degradation of the dsRNA. Notably the difference between the Colorado potato beetle, which is very sensitive to oral RNAi, and the sweet potato weevils *C. puncticollis* and *C. brunneus*, which are less sensitive, was striking. While dsRNA in sweet potato weevil extracts remained stable for 30 minutes to one hour, intact fragments could still be detected after 16 hours in CPB. Additionally, the difference in dsRNA stability in both weevil species corresponded to a difference in RNAi efficiency upon feeding with dsRNA (Prentice et al., 2017). Further evidence was also delivered when Spit et al. (2017) were able to improve RNAi sensitivity after silencing certain nucleases present in the gut.

A number of studies have also looked into the nucleases that could be implicated in this phenomenon. In the silkworm *B. mori*, a DNA/RNA non-specific nuclease was discovered that was highly expressed in the gut and was able to degrade dsRNA rapidly (Arimatsu et al., 2007; Liu et al., 2012). In fact, it was later shown that expression of this dsRNase is upregulated upon injection of dsRNA in *B. mori* (Liu et al., 2013). The authors hypothesized that this might be related to an antiviral mechanism. In *M. sexta*, Garbutt et al. (2013) concluded, based on their characterization studies, that the nucleolytic breakdown of dsRNA in the gut is caused by one or more metallo-enzymes, since addition of EDTA was able to stop degradation of the dsRNA. In *S. gregaria* and *L. decemlineata*, several nucleases were identified in the transcriptome that were able to degrade dsRNA (Spit et al., 2017; Wynant et al., 2014b). Interestingly, when these nuclease-encoding genes were silenced in both species, RNAi efficiency increased in CPB, but the *S. gregaria* locusts remained insensitive to RNAi. Analysis of the gut juice of these locusts showed that silencing the identified nucleases increased dsRNA persistence, but degradation could still be observed in the 50x diluted gut juice after 10 minutes incubation. This suggests that other nucleases might also be present in the locust which were not identified in these studies (Spit et al., 2017; Wynant et al., 2014b). In the Southern green stinkbug, *N. viridula*, researchers investigated the transcriptome, searching for potential dsRNA-degrading nucleases

(Lomate and Bonning, 2016). They found very strong dsRNA degradation activity in the saliva, while much lower activity was observed in the gut and salivary glands. An explanation for the difference between activity in the saliva and the salivary glands could be that the enzymes are released as zymogens, and are only activated once they are secreted in the saliva. Given the (extraoral) digestion behaviour of these plant sucking pest, by injecting saliva into the plant, the fact that the nuclease activity is higher in saliva than in the gut might not be surprising (Lomate and Bonning, 2016).

While a large body of evidence exists on dsRNA instability in insects and nucleolytic degradation in the digestive system, we were not able to find any studies investigating sRNA stability in nematodes, molluscs and annelids.

### 5.3.3.3. sRNA molecule

The characteristics of the sRNA molecule to which the invertebrate is exposed could also have a major impact on RNAi efficiency. For example, it is known that, in the nematode *C. elegans* and in some insects, cellular uptake efficiency is dependent on the dsRNA length. Length dependency in nematodes was already discussed in Section 5.1 in relation to the selectivity for dsRNA length exhibited by Sid-2. To briefly summarize, studies have shown that dsRNA longer than 25bp requires much lower concentrations than siRNA to have a similar RNAi effect and that this is due to Sid-2 being selective for length in uptake from the environment (Feinberg and Hunter, 2003; McEwan et al., 2012). However, nine studies were found reporting successful RNAi using siRNA soaking in nematodes, for example in *M. incognita* (Arguel et al., 2012; Dalzell et al., 2010a; Dalzell et al., 2010b), *B. xylophilus* (Ma et al., 2011) *M. graminicolis* (Haegeman et al., 2013), *G. pallida* (Dalzell et al., 2010a) and *C. elegans* (Sivamaruthi and Balamurugan, 2014).

In insects, similar observations have been reported. Saleh et al. (2006) found that long dsRNA were efficiently taken up in *Drosophila* S2 cells, while siRNAs required a transfection agent to be internalized. Bolognesi et al. (2012) compared cellular uptake of a Cy3-labeled long dsSnf7 fragment (240 bp) with that of a Cy3-labeled siRNA targeting Snf7 in *D. virgifera* midgut cells, and found that only the 240 bp fragment was taken up into the cells. These findings were also supported by biological activity data, since feeding of the long fragment led to mortality while feeding of the siRNA had no effect. In experiments with the Southern corn rootworm (SCR), Bolognesi et al. (2012) tested dsRNAs of different lengths and observed mortality from dsRNA ingestion increase from 16% for a 50 bp fragment to 68% mortality feeding a 60 bp fragment and eventually to 95% mortality using a 70 bp fragment. These results suggest that for SCR, the minimum length for efficient RNAi is around 60 bp. Important to note here is that the authors used only a single 27 nt sequence that was specific for the target insect in each of their dsRNAs, so these effects are not merely the result of having more specific siRNAs in the cells (Bolognesi et al., 2012). Miller et al. (2012) reported similar observations in *T. castaneum*, where 65 bp dsRNA fragments were successful in silencing a GFP construct, while 31 bp dsRNAs were not.

It is not yet clear whether this is a characteristic shared by all insects, let alone other invertebrates. For example, several publications have reported the use of 20-23 bp siRNA in RNAi experiments in a range of insect species, including the honeybee *A. mellifera* (Chen et al., 2014c), the diamondback moth *P. xylostella* (Gong et al., 2013), the cotton bollworm *H. armigera* (Zhang et al., 2015c), the pea aphid *A. pisum* (Mutti et al., 2006), the whitefly *B. tabaci* (Upadhyay et al., 2011), the potato/tomato psyllid *Bactericerca cockerelli* (Wuriyangan et al., 2011), termites (Zhou et al., 2006) and several others. Therefore, it appears to be probable that there are differences between different taxonomical clades concerning the influence of dsRNA length on RNAi efficiency. It is also important to note that the siRNA in some of these experiments was produced by *in vitro* dicing of dsRNA, meaning that a very small residual amount of long dsRNA might still have been present. However, most studies used chemically synthesized siRNA. In Table 13, we give an overview of invertebrate orders for which successful RNAi, using siRNA, has been reported. Remarkably, many of the insect species where

siRNA has proven to successfully elicit RNAi were Hemiptera, possibly suggesting that siRNA works more efficiently in Hemiptera than in other orders.

**Table 13:** Overview of type of sRNA used to successfully elicit gene silencing by oral feeding

Class	Order	siRNA	dsRNA	miRNA (mimic)
Hexapoda	Diptera	(*)	(*)	+
	Lepidoptera	+	+	+
	Coleoptera	-	+	N/A
	Hymenoptera	+	+	+
	Hemiptera	+	+	+
	Orthoptera	+	+	N/A
	Blattodea	+	+	N/A
Crustacea		-	+	N/A
Chelicerata		N/A	+	N/A
Adenophorea		N/A	+	N/A
Secernentea		+	+	N/A

\* Oral RNAi experiments in Diptera required polymer- or liposome-based formulations to elicit successful knockdown, both for siRNA and longer dsRNA

N/A: No studies were found exposing the invertebrate to this sRNA orally

Interesting to note in this context is that in GM plants expressing dsRNA, the plant RNAi machinery appears to process most expressed pest-specific dsRNA to siRNAs. Given the successes that have been reported using these insect-specific dsRNA expressing plants to induce gene silencing in the target insect, for example in Hemiptera (Abdellatef et al., 2015; Bhatia et al., 2012; Coleman et al., 2011; Coleman et al., 2014; Coleman et al., 2015; Khan et al., 2015; Khan et al., 2013; Raza et al., 2016; Zha et al., 2011), Lepidoptera (Apone et al., 2014; Kumar et al., 2012; Liu et al., 2015; Tian et al., 2015a; Zhu et al., 2012) and Coleoptera (Baum et al., 2007; Li et al., 2015a), it raises the question of whether siRNA is causing the gene silencing or residual dsRNA in the plant, not yet processed by the plant's RNAi machinery. In several studies, target RNA characterization of the transgenic plants showed that most dsRNA is processed by the plant into siRNAs. Li et al. (2015a) found a majority of 21 bp-24 bp fragments but also a clear band for the intact 275 nt dsRNA fragment that was used in this study. Similar observations of plant processing of the dsRNA were made in other studies (Pitino et al., 2011; Thakur et al., 2014; Wuriyangan and Falk, 2013; Zha et al., 2011). Interestingly, an *in planta* study using tobacco plants to express dsRNA specifically targeting a *M. sexta cytochrome P450 (CYP)* gene, found that gene silencing in the herbivorous insect was more efficient when the plant Dicer enzyme was silenced, providing further evidence of the importance of dsRNA length for efficient RNAi in insects (Kumar et al., 2012). Similarly, in another study using Dicer-mutant *Arabidopsis* lines, higher levels of dsRNA accumulation and less processing into siRNA in the mutant plants compared to wild type *Arabidopsis* plants, was accompanied by a higher RNAi efficiency in the cotton bollworm feeding on these plants (Mao et al., 2007).

The exact mechanism for this length dependency is unclear. In nematodes, Sid-2 has been shown to import dsRNA of greater length more efficiently than siRNAs. Possibly a similar mechanism is at work in arthropods through the sid-1-like uptake route. Uptake of siRNA through the endocytosis route could then explain why siRNAs can also still incite RNAi, but less efficiently than long dsRNA. But this is speculation at this moment. Further research at the cell level should be able to provide more information on the uptake capacity in invertebrates in the future. Recently, the production of dsRNA in the chloroplasts of the plant was reported as a way to increase RNAi efficiency against target

herbivores. The main advantage was that dsRNA accumulated in these chloroplasts and was not processed by the plant's own RNAi system (Jin et al., 2015; Zhang et al., 2015b). Another element to take into account here is that siRNAs are modified with 2'O-methylation when processed by the plant RNAi machinery, to protect them from nuclease activity. Whether this has any impact on the stability of these siRNAs in the invertebrate body when confronted with a nucleolytic environment is uncertain (Chan and Snow, 2017). If it does, this might compensate for the potentially less efficient uptake of these siRNAs by invertebrates.

Another characteristic of the dsRNA molecule that could influence RNAi efficiency is the location of the site/region in the gene that the dsRNA is homologous to. Several research studies have looked at the possibility of designing dsRNAs in the 3' untranslated region (UTR) or 5'UTR. The advantage of choosing these regions are that they are much less conserved than the part of the coding sequence (CDS) that is translated into protein, leading to a potentially much higher specificity of the pesticidal molecule. However, variable efficacies have been reported. For example, in a study targeting the inhibitor of apoptosis gene in *A. aegypti*, researchers compared the efficacies of different dsRNAs targeting different regions of the target gene and found that the 3' end targeting dsRNA had a greater effect on mosquito mortality than the dsRNA targeting the 5' end (Pridgeon et al., 2008). In contrast, Loy et al. (2012) found that the most effective dsRNA targeting the infectious myonecrosis virus in shrimp was the one targeting the 5' end. Finally, a study in the pea aphid *A. pisum* revealed no differences in efficiency between dsRNA targeting the 3' end and the 5' end of the hunchback gene (Mao and Zeng, 2012). These data demonstrate that target gene region can be an important factor affecting RNAi efficiency, and these will have to be determined empirically for every case.

#### 5.3.3.4. Cellular uptake of dsRNA

dsRNA cellular uptake efficiency varies between different species. For example, in nematodes, research has shown that *C. elegans* possesses an expanded gene repertoire encoding proteins which have different functions in cellular uptake and systemic spread, including several sid-like genes. However, many parasitic nematodes appear to have a more limited set of effectors in dsRNA uptake and systemic spread, possibly having an impact on RNAi efficiency (Dalzell et al., 2011). In insects, two different cellular uptake pathways have been identified (Cappelle et al., 2016; Saleh et al., 2006; Tomoyasu et al., 2008; Ulvila et al., 2006). One is based on clathrin-mediated endocytosis, while another involves a SID-1-like protein. Whether both pathways are completely independent is still unsure, but research in several species that have both pathways functional has shown that inhibition of one of these negatively impacts RNAi efficiency. Diptera, comprising flies and mosquitoes, do not possess a sid-1-like homologue and appear to rely solely on their endocytosis mechanism for cellular uptake of dsRNA. Research in S2 cells has demonstrated that expression of *sid-1* could significantly enhance the uptake of dsRNA. Additionally, feeding RNAi studies in *D. melanogaster* and *D. sukuzii* suggest that cellular uptake is a major reason why these fruit flies are refractory to oral RNAi (Taning et al., 2016b; Whyard et al., 2009). Recently, researchers found that in two lepidopteran cell lines, *S. frugiperda* Sf9 and *H. virescens* HvE6, RNAi was taken up from the medium, but never processed into siRNA, unlike in coleopteran cells. These results suggest that intracellular release of dsRNA is an additional problem in Lepidoptera possibly explaining their insensitivity for RNAi (Shukla et al., 2016).

There is a more detailed discussion on cellular uptake mechanisms and systemic spread of sRNAs, in Section 5.1.

#### 5.3.3.5. Amplification of the RNAi signal

One of the important factors influencing the efficiency of RNAi in the nematode *C. elegans* is the presence of an amplification system for the RNAi silencing signal, like that in plants. In *C. elegans*, an RdRP is at the center of this process, eventually leading to the production of secondary siRNAs. The mechanism itself has been discussed in depth in Section 5.1. Thus far, no homologue of this RdRP has been found in any insect or crustacean genome. There is however evidence for the presence of

this RdRP in certain tick species (Kurscheid et al., 2009). Whether or not this means that these ticks can amplify silencing RNAs, as in *C. elegans*, is not yet clear. Additionally, the absence of this RdRP does not necessarily mean that some species of insects or Crustacea do not possess a different mechanism to amplify the silencing signal. An alternative amplification mechanism could explain why in some species long lasting gene silencing or even a strong parental RNAi response can be observed. In fact, recent research in *D. melanogaster* has identified a siRNA amplification system, where secondary siRNAs are synthesized from viral dsRNA upon infection, via viral cDNAs. However, the study reported that this amplification mechanism was only activated in the presence of viruses and might therefore not be involved in an amplification of siRNA derived from delivery of exogenous dsRNA (Tassetto et al., 2017).

### 5.3.3.6. Viral infections

In invertebrates and plants, RNAi is a major component of the innate antiviral immunity response (Ding, 2010; Keene et al., 2004; Lu et al., 2005; Robalino et al., 2005; Schott et al., 2005; Sidahmed et al., 2014; van Rij et al., 2006; Wilkins et al., 2005). Several studies have shown that injection of non-specific dsRNA is able to activate this RNAi response, for example by upregulation of RNAi machinery core genes Dicer-2 and Ago-2 (Garbutt and Reynolds, 2012; Liu et al., 2013; Lozano et al., 2012). In *B. mori*, injection of dsRNA also led to the upregulation of a dsRNase capable of degrading dsRNA in the silkworm. A similar effect was seen when *B. mori* larvae were infected with *B. mori* cytoplasmic polyhedrosis virus (BmCPV), which is characterized by a genome of linear dsRNA elements (Wu et al., 2009). Given the close relationship between viruses and RNAi, it would not be surprising that viruses which have co-evolved with this line of defense, have developed mechanisms to inhibit the RNAi machinery. As hypothesized by Swevers et al. (2013a), such mechanisms could entail the production of viral suppressors of RNAi (VSRs) and saturation of the RNAi machinery due to accumulation of large quantities of viRNAs.

The first discovery of a VSR in invertebrate viruses was in Flock House Virus, which encodes the so-called B2 protein, capable of interfering with the RNAi machinery in different ways, including binding to dsRNA and siRNA, making it unavailable to be processed by the RNAi machinery, and directly interacting with Dicer-2 (Aliyari et al., 2008; Chao et al., 2005; Li et al., 2002; Singh et al., 2009). Another example was found with Drosophila C virus, which encodes an 1A protein capable of binding to dsRNA and, to a lesser extent, siRNA (van Rij et al., 2006). In Cricket Paralysis virus (CrPV) infections, the A1 protein was found not to bind to dsRNA/siRNA but was able to directly interact with Ago-2 and interfere with the function of RISC (Nayak et al., 2010). Interestingly, a study on bumblebees showed that infection with CrPV had no apparent effect on RNAi efficiency, while Israeli Acute Paralysis Virus infection actually enhanced RNAi efficiency (Cappelle et al., 2017). Also in human virus diseases, such as Dengue and West Nile virus, VSRs have been reported (Kakumani et al., 2013; Schnettler et al., 2012). Finally, VSRs in the form of nucleases have also been found to be expressed by invertebrate viruses. For example in *Heliothis virescens* ascovirus (HvAV) infections, an RNase III enzyme was found to be expressed capable of degrading dsRNA and siRNA and negatively affecting RNAi efficiency in the host (Hussain et al., 2010).

In vertebrates, a different mechanism based on the expression of a non-coding RNA by adenoviruses has been discovered allowing these viruses to suppress the RNAi response (by inhibiting siRNA and miRNA production) in adenovirus-infected vertebrate cells. This happens by interfering with nuclear export of short hairpin (shRNA) or pre-microRNA precursors, by competition for the Exportin 5 nuclear export factor or by interaction with Dicer function by direct binding to Dicer (Lu and Cullen, 2004). In contrast, production of so-called defective interfering RNAs (diRNAs) by a plant tombivirus actually enhanced RNAi efficiency due to hindering of the activity of VSRs produced by the virus (Havelda et al., 2005; Pathak and Nagy, 2009). Recently, suppression of RNAi in tick and insect cells by viRNAs produced by flaviviruses was also discovered, although the exact mechanism is not known yet (Schnettler et al., 2012; Schnettler et al., 2014).



Whether viral suppression of the RNAi machinery is a major problem in field populations of invertebrates is unclear. It could be one of the factors explaining variation sometimes observed between different strains or even different laboratory cultures of the same strain, such as the pea aphid which is a vector for several plant viruses (Christiaens and Smagghe, 2014). Interestingly, Swevers et al. (2013a) report that the vast majority of insect viruses are found in Lepidoptera, which are less sensitive to RNAi, while the RNAi sensitive Coleoptera are characterized by very low occurrences of viral infections. However, whether this observation indicates any causality is still to be determined. A study on lepidopteran cell lines indicated no apparent effect on RNAi efficiency upon persistent infection by viruses known to encode VSRs (Swevers et al., 2016).

#### 5.3.3.7. Invertebrate life stage

A few studies of the influence of developmental stage of invertebrates on RNAi efficiency were found. These include Terenius et al. (2011) who reported that in some experiments on lepidopteran insects, adults were more sensitive to RNAi when injected than juvenile stages. However, these observations should be confirmed by experiments using identical experimental conditions and setup. In contrast, some studies suggested that RNAi worked more efficiently in earlier juvenile stages than in later stages. For example, in the kissing bug *R. prolixus* no knockdown was observed when treating 4<sup>th</sup> instars with  $2 \times 80 \mu\text{g}$  *nitrospin2* dsRNA orally while 42% silencing was achieved in second instar individuals which had ingested approximately  $13 \mu\text{g}$  dsRNA on average (Araujo et al., 2006). Additionally, Griebler et al. (2008) also reported higher RNAi efficiency in larvae compared to adults of the fall armyworm *S. frugiperda*. Obviously, juvenile stages feed more than adult stages, which means that in most experiments with an *ad libitum* dsRNA supply, they will be exposed to a much higher dose of dsRNA. However, taking into account the dose of dsRNA/gram body weight, it seems that younger juvenile instars were more susceptible than older juvenile instars in some of these studies. Nevertheless, very little actual data is available and more research into this phenomenon is necessary before a more complete picture can be drawn.

#### 5.3.3.8. Protein stability or protein half life

Protein half-life varies enormously. For example in mammals, the half-life of certain collagen proteins have been reported to be longer than 100 years, while ornithine carboxylase has a reported half-life of 22 minutes (Iwami et al., 1990; Verzijl et al., 2000). Moreover, protein half-lives for the same protein can also vary between different species (Kuhar and Joyce, 2001). While this factor does not influence silencing at the transcript level *per se*, the stability or half-life of the protein encoded by the gene targeted for knockdown determines the degree in which phenotypical effects could be observed and is therefore something that has to be taken account when choosing target genes in a certain invertebrate species. Unfortunately, we found no reports on half-lives of invertebrate proteins associated with targets for RNAi and information on half-lives for most proteins are still unknown.

### 5.3.4. Conclusions

In this section, we provided an overview of the literature related to RNAi efficiency in invertebrates, which shows that it is very variable, not only between species, but also sometimes between strains, (laboratory) cultures, life stage or due to experimental aspects such as the sRNA molecule that is used. We have considered and discussed a number of factors and mechanisms that are known to affect the ability and hence the efficiency to induce RNAi-based gene silencing. While some of these factors, such as dsRNA stability in the insect body and cellular uptake, have attracted a lot of attention from researchers, a number of other factors such as the influence of the life stage or the impact of viral infections have not been studied in depth.

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## 5.4. Off-target, non-target and unintended effects of RNAi-based GM plants (Task 5)

### 5.4.1. Introduction

Food and energy turnover is one of the pillars of the functioning of every ecosystem, the way organic matter flows through an ecosystem is influenced by the organisms within the system. In agroecosystems, as in all other terrestrial ecosystems, plants constitute the primary source of energy supporting food webs on which other functional groups are sustained. Different orders of consumers in grazing or detritus food webs constitute the animal diversity which allows the provision of ecological services in agriculture (Table 14).

**Table 14:** Examples of functional groups of invertebrate species and ecosystem services provided to agriculture

Functional group	Examples	Examples of ecosystem functions and services
Herbivores	<u>Sap/cell feeders:</u> e.g., Acarina, Homoptera: Aphidoidea, Aleyrodidae, Cicadellidae; Thysanoptera: Thripidae. <u>Leaf feeders:</u> e.g., Coleoptera: Chrysomelidae, Curculionidae, Lepidoptera: Noctuidae, Pieridae, Tortricidae; Gasteropoda: snails, slugs <u>Leaf miners:</u> e.g., Diptera: Agromyzidae, Lepidoptera: Gelechiidae <u>Root feeders:</u> e.g., juveniles Hemiptera: Heteroptera, Diptera, Symphyla <u>Seed feeders:</u> e.g., Coleoptera: Carabidae, Hymenoptera: Formicidae	Nutrient cycling (as prey/hosts for natural enemies)
Predators	Thysanoptera (e.g., Aeolothripidae); Heteroptera (e.g., Anthocoridae, Miridae, Nabidae); Neuroptera (e.g., Chrysopidae, Hemerobidae); Coleoptera (e.g., Coccinellidae, Carabidae, Staphilinidae); Diptera (e.g., Syrphidae, Cecidomyiidae); Araneae; Acarina (e.g., Phytoseiidae) Nematoda	Population regulation (natural control of arthropod pests)
Parasitoids	Diptera (e.g., Tachinidae); Hymenoptera Parasitica	Population regulation (natural control of arthropod pests)
Pollinators, pollen feeders	Diptera: Syrphidae; Hymenoptera: Apidae; Coleoptera: Coccinellidae	Pollination, honey production
Decomposers	Nematoda, Collembola, Acarina, Isopoda, Haplotaxida: Lumbricidae	Decomposing litter, soil structure and fertility, Nutrient cycling (as prey/hosts for natural enemies)

GM plants expressing invertebrate resistance traits could introduce potentially harmful novel metabolites into existing food webs (e.g., Cry toxins, proteinase inhibitors, lectins). Therefore, higher order consumers might possibly be adversely affected (Lövei and Arpaia, 2005). All living organisms that are not meant to be affected by newly expressed compounds in GM plants, and that can be potentially exposed, directly or indirectly, to the GM plant and/or its products are considered as “non-target” organisms (Arpaia, 2010). NTOs could be affected either directly, by feeding on plant parts or preys containing RNAi-triggering molecules, or indirectly, by the reliance on a suboptimal diet due to the changed quality of the plants and/or the herbivore preys (Andow et al., 2006).

Another possible concern is that unintentional changes in expression of some metabolites in GM plants or changes in plant composition and structure may affect ecological interactions. Arpaia et al. (2017) showed that genetic modification can also induce a range of unintended effects on plant metabolism and overall phenotype, though their biological relevance needs to be evaluated case by case.

In this section, we will present the literature specifically dealing with off target and non-target effects of GM plants expressing sRNAs primarily targeted at invertebrate genes. Similar as in Section 5.3, we have first performed an assessment of relevant papers, dealing specifically with RNAi off-target effects and effects on NTOs, in the initial list of 4,612 oral exposure studies. In this search, we found a total of 41 studies which dealt specifically with these topics (Appendix E).

#### 5.4.2. Food chain effects

Traits of individual plants influence interactions between plants, herbivores and natural enemies (Price et al., 1980). These traits may be molecular (e.g., toxins, antifeedants, sRNAs) or physical (e.g., pubescence, toughness). Effects are often mediated between different trophic levels, i.e., sublethal effects such as delayed development, induced by plants may have consequences on the activity of natural enemies of the herbivores (Johnson and Gould, 1992). Therefore, any change in plant composition due to genetic modification may induce effects at different trophic levels.

Herbivores may acquire plant constitutive compounds and herbivores possess a number of mechanisms to deal with these compounds (e.g., degradation, sequestration, excretion). As a result, some products derived from plants, or their metabolites, are present in the body of herbivores.

Some invertebrate species show specialization with regard to the feeding sites they occupy on their host plants. Since plant compounds, including RNA molecules, are expressed at different levels in plant tissues, the feeding habits of the herbivores determine their exposure to plant compounds.

However, the occurrence of natural defensive compounds is usually concentrated in the regions where their presence may increase the fitness of the plant when attacked. Thus, knowledge of the expression of sRNA as well as endogenous compounds in different plant parts is important for determining exposure both in herbivores and in species at higher trophic levels exposed to these herbivores.

When a significant change in plant composition occurs in a host plant, a “counter-adaptation” is needed for the herbivore to continue feeding on this plant. Changes in host plants can consequently affect organisms at the second trophic level and may also produce changes in their quality as hosts/preys for natural enemies due to the additional presence of novel compounds in their body. In turn, natural enemies which normally feed on their hosts/preys with a “familiar” chemical composition will have to cope with new metabolites (intact or partly digested) which were acquired during herbivory. Finally, compounds originated from the host plant may interact with the herbivore’s constitutive compounds representing a new challenge to the natural enemies’ metabolism.

The possible outcomes of the changes in trophic interactions along food webs are therefore to be determined on a case by case basis.

#### 5.4.3. RNA-expressing genetically modified plants

The silencing effect of the siRNA is highly dependent on the active metabolism of the target organisms. Furthermore, since basically any gene of interest could be targeted with RNAi, some completely new modes of action are involved in the process. For instance, GM maize MON 87411 expresses, among other traits, a dsRNA targeting the *DvSnf7* gene, a gene involved in vacuolar sorting at cellular level, a mechanism which has not been previously targeted with any insecticidal compound or GM plant.

Several mechanisms triggered by dsRNA exposure were suggested to possibly produce unintended effects. Here, we divide them into two main categories, namely (1) sequence-dependent mechanisms such as off-target gene silencing, silencing genes in NTOs and (2) sequence-independent mechanisms such as immune stimulation and saturation of the RNAi machinery (Lundgren and Duan, 2013). Off-target effects in the target species may not necessarily be a safety issue, but we discuss the literature on both off-target effects and unintended effects on NTOs, since both rely on the same mechanisms, which is related to the specificity of RNAi, more specifically the minimum homology between an siRNA and the target mRNA sequence leading to successful gene silencing.

#### 5.4.4. Sequence-dependent mechanisms

Within a eukaryotic cell, long dsRNA is cleaved by the enzyme Dicer into siRNAs, which are about 21 nucleotides in length. The siRNAs, in conjunction with the RISC, scan the cell's mRNA molecules until a complementary match is found. Once recognized, the target mRNA is cleaved by the Argonaute in the RISC with consequent silencing of the corresponding gene. Thus, a highly sequence-specific gene silencing can be achieved in some invertebrates by feeding dsRNAs (Baum et al., 2007; Swevers and Smaghe, 2012; Whyard et al., 2009). Due to this mechanism of action, dsRNA-expressing plants targeting invertebrate herbivores are expected to be a highly selective means of pest control, since a high level of specificity can be achieved with the appropriate selection of target genes and target sequences within the gene of interest. Depending on the selected sequence, either a single species can be efficiently targeted or, in cases of more conserved sequences, a broader specificity can be achieved (Runo et al., 2011). Unfortunately, little fundamental biochemical information in invertebrates is available on the necessary homology between siRNA and mRNA to cause effective RNAi gene silencing. However, a number of studies conducted in invertebrates give some indication on the necessary level of homology. In this section, we review the available data on the necessary sequence homology to incite RNAi silencing, the potential for off-target effects and studies investigating the potential for unintended effects on NTOs.

##### 5.4.4.1. Target specificity of dsRNA

There is no consensus among scientists on the number of nucleotides that must match the target sequence identically or on the allowed number and type of mismatches in invertebrates. Although dsRNA expressed in crop plants as means of pest control has been claimed to have a high degree of specificity (Dillin, 2003; Petrick et al., 2013; Whyard et al., 2009) other studies have shown that siRNAs can silence unintended genes (Davidson and McCray, 2011). However, fundamental and direct evidence of the siRNA homology necessary to cause gene silencing in invertebrates not found in our literature search. In mammals, several studies have shown that mismatches between siRNA and target mRNA can be allowed while still leading to successful gene silencing (Huang et al., 2009; Jackson et al., 2003; Jackson et al., 2006; Schwarz et al., 2006). Further research is required to determine whether the same issues apply to invertebrates. It is also important to note that, when using long dsRNA, the effector molecules after Dicer processing of the dsRNAs are very diverse and the concentration of any single siRNA is much lower than the original dsRNA concentration. Therefore, a single siRNA able to cause gene silencing does not necessarily lead to a strong gene silencing or any phenotypical effects.

Whyard et al. (2009) obtained *V-ATPase* specific dsRNA for four different insect species from different orders: the fruit fly *D. melanogaster*, the flour beetle *T. castaneum*, the pea aphid *A. pisum*, and the tobacco hornworm *M. sexta*. When individuals of these species were fed with dsRNA-containing diets in laboratory experiments, high levels of mortality were achieved only when species-specific dsRNA was provided in the diets. In contrast, dsRNA targeting the homologous gene in other species did not produce adverse effects. Moreover, this study also investigated whether specificity between species belonging to the same genus (*Drosophila*) could be achieved. To this purpose, dsRNAs specific for the *gamma-tubulin* gene in *D. melanogaster*, *D. sechellia*, *D. yakubu* and *D. pseudoobscura* were

designed and fed to these fruitflies. The results confirmed that selectivity between these species was possible (Whyard et al., 2009).

Likewise, Baum et al. (2007) tested the potential for species selectivity based on nucleotide-sequence identity with the WCR, *D. virgifera virgifera* and the CPB, *L. decemlineata* using dsRNA that targets the gene encoding the V-ATPase subunit A and E for each species. The V-ATPase subunit A target sequences from CPB and WCR share 83% nucleotide-sequence identity whereas the V-ATPase subunit E target sequences from these organisms share 79% nucleotide-sequence identity. Feeding both WCR and CPB with the heterospecific dsRNA that targeted V-ATPase subunits A and E, caused mortality in both species. However, such response was expected given the multiple 21 nt shared sequence over the targeted portion of the gene for these two species.

Bachman et al. (2013) characterized the spectrum of insecticidal activity of a 240 nt dsRNA targeting the *Snf7* ortholog in the WCR. They first performed a similar study to the one of Baum et al. (2007), feeding heterospecific dsRNA to CPB and WCR. In contrast to the dsRNA targeting the v-ATPase subunits, the ds*Snf7* was species-specific in causing mortality. Analysis of sequence homology showed that the longest match between both homologous sequences was only 14 nt. The authors then selected and tested insects of 10 different families and four different orders (i.e., Hemiptera, Hymenoptera, Lepidoptera and Coleoptera) based upon their phylogenetic relatedness to WCR and measured potential lethal and sublethal effects in continuous feeding diet bioassays with *DvSnf7* dsRNA. Bioassay results demonstrated that the spectrum of activity for *DvSnf7* was narrow and activity was only evident in a group of beetles within the Galerucinae subfamily of Chrysomelidae (>90% identity with WCR *Snf7* 240 nt). A shared sequence length of  $\geq 20$  nt seemed required for efficacy against WCR (containing 221 potential 21-nt matches) and all orthologs susceptible for gene silencing by *DvSnf7* contained at least three 21 nt matches with the *DvSnf7* sequence.

Zhang et al. (2015) used a lepidopteran pest, the Asian corn borer *O. furnacalis*, as a target species. In order to investigate whether the dsRNA of a gene has species-specific or broad-spectrum activity on Lepidoptera, the authors identified a methionine-rich storage protein gene (*OfSP*) in the species' genome. dsRNA for the three functional domains of the gene were synthesized and were tested on the larvae of the target species and another lepidopteran, *H. armigera*. The dsRNA targeting the C-terminal domain, which was sprayed on the larvae, caused high mortality rates in both insects, whereas those targeting the M-segment and N-terminal domains only caused high mortality in the Asian corn borer. Sequence analysis revealed one perfect 21 nt match and four 20 nt matches between the dsRNA and the homologous gene in *H. armigera*. Furthermore, several 19 nt matches were found between this dsRNA and several other genes in *H. armigera* belonging to the hexameric storage protein family (Zhang et al., 2015).

#### 5.4.4.2. Off-target effects

The phenomenon of RNAi off-target effects (Birmingham et al., 2006) was described for the first time in eggs of *M. incognita* harvested from infected tobacco lines expressing dsRNA targeting proteases. Non-target proteases genes were up-regulated when the target gene was knocked-down, suggesting the presence of an innate response to compensate for the lack of some proteases, though their up-regulation gives no significant effects on nematode development (Antonino de Souza Júnior et al., 2013).

Off-target effects are commonly related to the siRNA sequence itself and most often arise from partial complementarity of the sense or antisense strands to an unintended target within an organism (Jackson et al., 2006). In particular, these effects appear to be related to partial sequence homology between the "seed region" (positions 2-7 or 2-8) of the siRNA and the 3' untranslated region of messenger RNA transcribed from a non-target gene, though different conclusions were reached by (Chen et al., 2015).

Given the small sizes of siRNAs, it is not surprising that off-target binding sites are quite common in different organisms (Qiu et al., 2005). Substantial sequence complementarity is needed to trigger off-target gene effects, but siRNAs containing some mismatches may still effectively trigger silencing (Jackson et al., 2006). The allowance for some mismatches could therefore increase the potential for off-target gene silencing effects.

Sequence dependent off-target effects were found in insects by Kulkarni et al. (2006). Based on genome-wide screenings they confirmed experimentally that dsRNAs containing  $\geq 19$  nt perfect matches lead to efficient knockdown of a cross-hybridizing transcript. Even though the use of long dsRNAs in *D. melanogaster*, where Dicer-mediated processing produces small RNAs inside cells, has been thought to reduce the probability of off-target effects, similar results were obtained by Ma et al. (2006). They conducted genome-wide RNAi screen for novel components of wingless signal transduction and showed that, even in the absence of candidate genes for positively acting wingless pathway components, off-target effects mediated by short homology stretches within long dsRNAs are prevalent in *D. melanogaster*.

Jarosch and Moritz (2012) worked on honeybees as a model system. Bees were tested by injecting three different dsRNAs: two obtained from the honeybee transcriptome (dsGPDH and dsVG) and one targeting a marker gene not present in the honeybee genome (*dsGFP*). The authors analyzed the gene expression of four non-target genes coding for proteins that are involved in different physiological processes. The genes selected lacked similarity with the dsRNA injected and were: *AmSID-I* coding for the production of a transmembrane channel protein, *amATF-2* a gene regulating transcription factors and stress proteins, *amDHAP-AT* involved in lipid metabolism and *amCPR* a cytochrome P450 reductase. All these proteins are commonly used as stress biomarkers. After treatment with three dsRNAs in two abdominal tissues (fat bodies and ovaries), the authors evaluated the tissue-specific responses of the dsRNA. The different dsRNA–off-target gene combinations showed altered transcript abundances after the treatment. All three dsRNAs, specifically designed to have no sequence homology longer than 20 bp with any gene in the honeybee genome, showed at least one unspecific off-target knockdown. However, the authors, considered the observed effects as non- sequence specific off-target effects and indicated the similarity of the metabolic function of the genes as a possible explanation for their results. The authors did not find similar effects when larvae of the studied non-target were injected with the dsRNA solvent, so they concluded that the observations were not the result of a wounding or septic reaction in response to injections. Other possible stressing factors, including injections, have been indicated as possible causes of stimulation of the immune system (see Section 5.4.5.2).

Off-target effects were shown in experiments with honeybees using dsRNA targeting *gfp* (Nunes et al., 2013). The use of dsRNA as an exogenous control for RNAi testing in arthropods is a well-established technique (Mutti et al., 2006; Sim et al., 2012; Tang et al., 2010). The GFP gene does not exist in the honeybee genome and it has therefore also been used as negative control in experiments with this species (Jarosch and Moritz, 2011; Kamakura, 2011; Nunes et al., 2013). Although dsGFP is not expected to trigger an RNAi response in bees, Nunes et al. (2013) report that phenotypical effects in pupal pigmentation and larval development have been observed in RNAi screens using dsGFP as a control. The authors fed honeybee larvae with diets containing dsGFP and checked the individuals at different time points through their development as worker bees. Three different experiments were conducted using different concentrations of dsGFP. Gene expression of the individuals under treatment and their respective negative control bees was determined through microarray analysis. A large number of genes (almost 10% of the whole genome) appeared to be upregulated or downregulated in bees treated in the larval phase, while a significantly lower number of activated genes appeared after treatment of adults. However, only five genes appeared in the list of differentially expressed genes in each of the different experiments, suggesting that the expression of most genes is changed due to a sequence-unspecific effect. Expression changes appear to be the result of both direct off-target effects and indirect downstream secondary effects; indeed, there were

several instances of sequence similarity between putative siRNAs generated from the dsGFP construct and genes whose expression levels were altered. Thus siRNA-induced silencing can generate specific and non-specific effects on an organism (Davidson and McCray, 2011).

Zhou et al. (2014) conducted a study in *C. elegans* and showed that nuclear Ago NRDE-3 protein associates with off target siRNAs in *C. elegans* following administration of exogenous RNAi. These findings supported the idea that the nuclear RNAi pathway is a primary source of exogenous and endogenous off-targeting effects in this species and suggest that off-target silencing is more than a mere biochemical limitation of the RNAi machinery but rather a genetically programmed aspect of the RNAi.

#### 5.4.4.3. Non-target effects

The possible exposure of NTOs to dsRNA expressed in plants is discussed in Section 5.2. In this section, we review the available information on the unintended and off-target effects of RNAi in invertebrate species other than the primary intended target organisms.

Phylogenetic relatedness to the target species and a high degree of homologous overlap between at least one siRNA and the gene sequence are considered to be two important requisites for RNAi effects (Bachman et al., 2013). Baum et al. (2007) reported that V-ATPase dsRNA targeted against the WCR, *D. virgifera virgifera*, proved to be also effective on the CPB, *L. decemlineata*, and noticed a sequence similarity of 83% of the *V-ATPase A* between the two species. More specifically, the CPB V-ATPase A dsRNA yielded an LC<sub>50</sub> value of 5.2 ng/cm<sup>2</sup> in the CPB bioassay, whereas the orthologous WCR dsRNA yielded a LC<sub>50</sub> value 452 ng/cm<sup>2</sup>, a greater than ten-fold difference in activity that could be attributed to divergence in target sequence.

Zhu et al. (2012) found that *H. armigera EcR* dsRNA, targeting a receptor complex of the steroid hormone 20-hydroxyecdysone, expressed in transgenic tobacco plants was also effective against another lepidopteran pest, *S. exigua*, due to the high similarity in the nucleotide sequences (89%) of the two *EcR* genes. In both species mortality levels of 40-50% were detected during bioassays with GM tobacco plants.

Pan et al. (2017) developed an *in vivo* toxicity assay to examine the impacts of ingested dsRNAs targeting the *V-ATPase A* gene on the larvae of the monarch butterfly *D. plexippus*. The full-length *v-ATPase A* cDNAs from the target pest, the WCR, *D. virgifera virgifera*, (Coleoptera) and the non-target *D. plexippus* were respectively cloned. To start from a 'worst case scenario', they opted for a 400 bp dsRNA located in a highly conserved region of a highly conserved gene (*v-ATPase A*). The *V-ATPase A* gene is highly conserved among holometabolous insects and, when analysing DNA sequences from data sets, the authors argued that Coleoptera were sister groups to Lepidoptera and Diptera and more phylogenetically distant from Hymenoptera and other insect orders. The sequence alignment of the *V-ATPase A* gene from *D. plexippus* and *D. v. virgifera* enabled identification of a highly conserved region of 400 bp which showed 77% identity between the two species. The bioinformatic analysis showed that 19–25 nt contiguous sequence matches between the two insect species were present. However, no significant differences in the survival of *D. plexippus* larvae across treatments were observed. The development time from the 1<sup>st</sup> instar to the adult was not affected by the treatments, though some differences appeared during each larval stage.

Although *D. plexippus* and *D. virgifera virgifera* shared nearly 80% sequence similarity within the selected 400 nt region of *V-ATPase A*, the lack of 21-mer matches between target and non-target insect species might explain the results of the bioassays. In addition, it must be remembered that silencing activity of dsRNA in Lepidoptera is very variable among families due to several factors such as non-target species, targeted tissue, delivery methods, dsRNA uptake, dsRNA degradation and gene function (Terenius et al., 2011) (reviewed in Section 5.3). The two above mentioned studies were based on molecular analyses, and there are other cases where adverse effects on NTOs have been identified.

Chen et al. (2015) investigated the effects of dsRNA targeting *rpl19* gene from *Bactrocera dorsalis* on non-target insects which are normally found in the same environment (*Citrus* cultivations) of the target species, by feeding the dsRNA to the congeneric species *Bactrocera minax*, the honeybee *A. mellifera* and the parasitoid wasp *Diachasmimorpha longicaudata*. Two different dsRNA were produced targeting two different regions of the cloned gene. Both dsRNA extracts were fed to the chosen NTOs in a series of laboratory bioassays, and subsequent gene silencing was measured with RT-PCR. The authors performed a homology search to reveal shared sequence in *rpl19* gene between target and non-target species. The expression levels of *rpl19* gene in the non-target insects were down-regulated by the *B. dorsalis rpl19* dsRNA, with the exception of honeybee. The maximum effect (approximately 90% down-regulation) was obtained on *B. minax*, but significant effects were also obtained on the hymenopteran *D. longicaudata* (approximately 40%) which shared 72% sequence homology with *B. dorsalis*. A sequence identity of 69% is shared between *B. dorsalis* and *A. mellifera*, however there was no apparent down-regulation of the *rpl19* gene on the latter. By contrast, the dsRNA targeting the 3' region did not induce effects on gene expression on any of the non-target species studied.

Pan et al. (2016) chose the collembolan *Sinella curviseta* as a non-target species to study possible effects of dsRNA targeting the *V-ATPase A*. The dsRNA was constructed in order to simulate a worst-case scenario, since the region with the highest homology (85% in a region 400 bp-long) between the gene of *D. virgifera virgifera* and *S. curviseta* was selected to prepare dsRNA. Several 19- through 23-mer matches were present in the targeted region of the *V-ATPase A* gene of the two species. Dietary RNAi toxicity assays were conducted and several measurement endpoints were used to determine effects on the collembolan, both at the molecular and phenotypical level. No significant degradation of *V-ATPase A* mRNA was observed. Similarly, there were no significant differences in adult survival rate, adult body length, fecundity and hatching rate. Larvae fed dsRNA developed faster compared to the control, however the biological significance of this difference is not clear. Vélez et al. (2016) evaluated the insecticidal activity of a known lethal dsRNA target for *D. virgifera*, the *V-ATPase A* in larvae and adult honeybees. A 400 nt *V-ATPase-A* dsRNA was designed based on the region of highest sequence similarity between the target and the non-target species. The dsRNA was tested via oral ingestion for specificity against both *D. virgifera virgifera* and *A. mellifera*. Larval mortality occurred only when species-specific dsRNA was provided to insects during bioassays. In bioassays with adults the relative expression of the *V-ATPase-A* in *A. mellifera* was visible up to 48 hours while it was not visible in adults collected at 96 h, suggesting that the reduced gene expression observed at 48 hours for *Am V-ATPase-A* dsRNA was transient. Overall the results indicate that honey bee larval development, adult eclosion and adult survival were unaffected by both *D. virgifera virgifera* as well as *A. mellifera* dsRNA, suggesting that honey bees are insensitive to *V-ATPase-A* dsRNA. The lack of response suggests that the activity spectrum does not only depend on the sequence specificity, but also on the ability of the organism to respond to RNAi. An overview of studies providing information on the relationship between sequence homology and RNAi silencing is given in Table 15.



**Table 15:** Overview of studies providing information on RNAi silencing effects in non-target species and sequence homology between the dsRNA and the homologous sequence in the non-target species

Non-target species	Target species	Target gene	Measurement endpoint	Sequence homology	Sequence overlap	Effect on nto	Reference
<i>Leptinotarsa decemlineata</i> (Coleoptera: Chrysomelidae)	<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	V-ATPase A	Mortality	83%	-	Mortality (10-fold less toxic)	Baum et al. 2007
		V-ATPase E		79%			
<i>Spodoptera exigua</i> (Lepidoptera: Noctuidae)	<i>Helicoverpa armigera</i> (Lepidoptera: Noctuidae)	EcR	Mortality	89%	-	Mortality	Zhu et al. 2012
<i>Danaus plexippus</i> (Lepidoptera: Nymphalidae)	<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	V-ATPase A	Mortality, development	77%	Twelve 19-, seven 20-, three 21-, two 22-, one 23-contiguous matches	No effects	Pan et al. 2017
<i>Bactrocera minax</i> (Diptera: Tephritidae)	<i>Bactrocera dorsalis</i> (Diptera: Tephritidae)	rpl19	Gene silencing	93%	Two 19-21 bp overlap	50-70% reduction of gene expression	Chen et al. 2015
<i>Apis mellifera</i> (Hymenoptera: Apidae)				69%	-	No effects	
<i>Diachasmimorpha longicaudata</i> (Hymenoptera: Braconidae)				72%	One 19-21 bp	40% reduction	
<i>Sinella curviseta</i> (Collembola: Entomobryidae)	<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	V-ATPase A	Gene silencing, survival rate, body length, fecundity, hatching rate	85%	Twelve 19-, seven 20-, three 21-, two 22-, and one 23-nt contiguous matches	No effects	Pan et al. 2016
<i>Apis mellifera</i> (Hymenoptera: Apidae)	<i>Diabrotica virgifera virgifera</i> (Coleoptera: Chrysomelidae)	V-ATPase A	Development, survival	High similarity	20-, 24-nt	No effects	Vélez et al. 2016

### Non-target studies on maize MON 87411

The most extensively studied case of GM plant-expressing dsRNA so far, relates to the characterization of the GM maize MON 87411, for which a non-regulated status has been recently declared by the US Department of Agriculture<sup>3</sup> ([https://www.aphis.usda.gov/brs/aphisdocs/13\\_29001p\\_det.pdf](https://www.aphis.usda.gov/brs/aphisdocs/13_29001p_det.pdf)). The applicant conducted an ERA of this maize which included an evaluation of impacts on NTOs (Ahmad et al., 2016; Bachman et al., 2016).

The potential for non-target effects of *DvSnf7* dsRNA was tested under worst-case laboratory conditions in several of NTOs, including the predators (*Coleomegilla maculata*, *Poecilus chalcites*, *Aleochara bilineata*, *C. carnea*, *Orius insidiosus*), parasitoids (*Pediobius foveolatus*), pollinators (*A. mellifera*), soil biota (*Eisenia andrei*, *Folsomia candida*) as well as aquatic and terrestrial vertebrate species (Bachman et al., 2016). A number of measurement endpoints were used to assess the potential for non-target effects, including mortality and possible indicators of sub-lethal effects (e.g., development time, adult biomass, adult emergence, fertility, fecundity).

The authors quantified the *DvSnf7* dsRNA expression levels in different tissue types collected from maize MON 87411 plants across different sites, using the validated QuantiGene assay (Armstrong et al., 2013) and calculated the expression levels in micrograms of RNA per gram of fresh or dry weight tissue (Table 16).

**Table 16:** *DvSnf7* RNA expression levels in different tissue type from MON 87411. Modified from Bachman et al. (2016)

Tissue type	Developmental stage	Range µg/g
Pollen (fwt)	VT-R1	0.056 x 10 <sup>-3</sup> - 0.224 x 10 <sup>-3</sup>
Leaf (fwt)	V14-R1	5.40 x 10 <sup>-3</sup> – 33.8 x 10 <sup>-3</sup>
Root (fwt)	V3-V4	1.74 x 10 <sup>-3</sup> – 8.00 x 10 <sup>-3</sup>
Whole Plant (dwt)	V6-V8	33.0 x 10 <sup>-3</sup> - 106 x 10 <sup>-3</sup>
Grain (dwt)	R6	0.056 x 10 <sup>-3</sup> - 0.175 x 10 <sup>-3</sup>

fwt = fresh weight; dwt = dry weight

The *DvSnf7* dsRNA maximum expression levels found in these studies were then used to determine the MEEC for testing the surrogate species of NTOs chosen. For all species tested, no statistically significant effects from ingestion of or exposure to *DvSnf7* RNA were detected when compared to the control. A synthesis of the experiment conducted is shown in Table 17.

<sup>3</sup> Available at: [https://www.aphis.usda.gov/brs/aphisdocs/13\\_29001p\\_det.pdf](https://www.aphis.usda.gov/brs/aphisdocs/13_29001p_det.pdf)

**Table 17:** List of laboratory bioassays conducted on invertebrate non-target organisms for the characterization of maize MON 87411 (Ahmad et al., 2016; Bachman et al., 2016).

Organism	Functional role	Measurement endpoint
<i>Apis mellifera</i>	Pollinator	Larval survival and development, worker survival
<i>Coleomegilla maculata</i>	Biocontrol agent	Survival, growth and development
<i>Aleochara bilineata</i>	Herbivore	Adult survival and reproduction
<i>Poecilus chalcites</i>	Biocontrol agent	Survival, growth and development
<i>Chrysoperla carnea</i>	Biocontrol agent	Adult survival and reproduction
<i>Orius insidiosus</i>	Biocontrol agent	Survival, growth and development
<i>Pediobius foveolatus</i>	Biocontrol agent	Adult survival
<i>Eisenia andrei</i>	Nutrient cycling	Survival and body weight
<i>Folsomia candida</i>	Nutrient cycling	Survival and reproduction

Bioinformatic analyses were conducted to evaluate whether the NTO species tested have sufficient genomic match to the *DvSnf7* sequence. *In silico* evaluation with available genomes and transcriptomes did not identify any 21-nucleotide contiguous matches for any of the species.

Ahmad et al. (2016) evaluated the potential impact of maize MON 87411 on non-target arthropods in field experiments. Field trials were conducted at 14 sites in the U.S.A., Argentina and Brazil providing geographic and environmental diversity within maize production areas. Abundance, measured with visual observations and the use of sticky traps, and damage assessment (for pest species) were chosen as endpoints. Twenty arthropod taxa were abundant enough to allow statistical analyses, and nine of them occurred in at least two of the three regions. Across the 20 taxa analyzed, no statistically significant differences in abundance were detected between MON 87411 and the conventional control for 123 of the 128 individual-site comparisons (96.1%). For the nine widely distributed taxa, no statistically significant differences in abundance were detected between MON 87411 and the conventional control.

#### 5.4.4.4. Trophic chain effects

While several non-target species have been assayed for possible nonspecific RNA interference, only a handful studies have performed trophic experiments which actually demonstrated movement of dsRNA along trophic chains.

Garbian et al. (2012) provided adult honeybees with a diet containing a dsRNA targeting *gfp*, used as a marker gene. Successively, individuals of the parasitic mite *V. destructor* were introduced into the bee colonies and allowed to feed on them. To test for bidirectional horizontal transfer, some of the mites were then removed after three days from the dsRNA-carrying bees and introduced into a container with untreated bees. DsGFP was detected in RNA extracts of parasitized bees that had not consumed dsRNA. After demonstrating the cross-species, reciprocal exchange of dsRNA between honeybees and mites, the same experiments were repeated with dsRNA aimed at silencing *Varroa* genes. The authors designed a number of dsRNA targeting fundamental housekeeping genes whose silencing was expected to harm the *Varroa* mites. The authors achieved a decrease in the mite population of over 60% when a mixture of dsRNA was fed to bees, demonstrating that dsRNA maintained its biological activity after transferring across species. Cedeño et al. (2015) similarly used this system to target *V. destructor* and also the deformed wing virus infecting bees.

## 5.4.5. Sequence-independent mechanisms

### 5.4.5.1. Saturation of the RNAi machinery

In the RNAi pathway, high levels of siRNAs could saturate the protein RISC complex (Lundgren and Duan, 2013), as there is a limited number of RNAi effectors, such as Dicer enzymes and RISCs, present within a cell. The saturation process can consequently temporarily inhibit cellular use of RNAi (Jackson and Linsley, 2010; Khan et al., 2009) and compromise some of its natural functions. For example, it could lead to lowering the efficiency of endogenous gene regulation (Agrawal et al., 2003; Dillin, 2003) and it could lead to reduced defenses against viral infection (Dillin, 2003). On the other hand, under realistic exposure conditions, it is not clear whether the oral dose would be sufficient to affect the RNAi machinery of exposed NTOs.

This process of saturation is better documented with a type of siRNA that targets a specific place on the mRNA named small hairpin RNA (shRNA) although it is also known to occur with siRNA (Jackson and Linsley, 2010). *In vitro* and *in vivo* shRNA transfection studies implied that one such factor, shared by the shRNA/miRNA pathways and readily saturated, is the nuclear karyopherin exportin-5 (Grimm et al., 2006). So far however, this phenomenon has not been reported in invertebrate species.

### 5.4.5.2. Immune stimulation

In invertebrates such as insects and nematodes, RNAi developed evolutionarily as a natural defense mechanism against viruses (Gammon and Mello, 2015; Karlikow et al., 2014). Recently it was found that the injection in the blood stream of small fragments (fewer than 30 nt) of nanocarrier-formulated siRNA (to facilitate cellular uptake) could activate the mammalian innate immune system via a toll-like receptor (TLR) pathway which recognized and responded to the sequence, length, and structure of siRNAs (Robbins et al., 2009). It is unclear how the immune system of other organisms responds to exogenous administered small RNA (Lundgren and Duan, 2013) and, despite similarities in the innate immune response of insects and mammals (Lundgren and Jurat-Fuentes, 2012), dsRNA-induced innate immune response has been rarely reported in invertebrates (Dong et al., 2009). Furthermore, it is also unclear whether such stimulation of the immune response could lead to adverse effects on the fitness of invertebrate species.

Recent studies described a non-sequence-specific immune response to dsRNA in honeybees, and a reduction of virus titer in virus-infected adult bees (Brutscher et al., 2017; Flenniken and Andino, 2013). Non-sequence-specific dsRNA has also been shown to reduce virus abundance and affect gene expression in bumble bees (Niu et al., 2016; Piot et al., 2015). This immune stimulation effect is not restricted to bees. Non-specific dsRNA-triggered antiviral response has also been reported in the Chinese oak silk moth *Antheraea pernyi* (Hirai et al., 2004) and in some crustacean species (Dong et al., 2009; Labreuche et al., 2010; Robalino et al., 2005; Robalino et al., 2007; Robalino et al., 2004). Overall, these findings indicate that an invertebrate immune system can recognize dsRNA as a virus-associated molecular pattern, and therefore activate an antiviral response (Robalino et al., 2004).

In several studies, it has been reported that dsRNA causes upregulation of the RNAi machinery components. For example, dsRNA-injection experiments in the tobacco hornworm *M. sexta*, revealed that dicer-2 mRNA levels and, to a lesser extent, argonaute-2 mRNA levels were elevated following injection in a specific and dose-dependent manner (Garbutt and Reynolds, 2012). An increase of Dicer-2 mRNA level was also observed in *B. germanica* after treatment with a nucleopolyhedrovirus dsRNA (Lozano et al., 2012).

Studies on other taxa, e.g., shrimps and fishes, reported increased levels of dicer-2 mRNA in response to challenge with dsRNA (Chen et al., 2011b; Su et al., 2009).

Some components of the innate immune response might also react to virus-specific dsRNA (Kingsolver et al., 2013). For example Paradkar et al. (2012) reported the activation of different (non-RNAi) innate immune pathways through Dicer-2.

In the fruit fly *D. melanogaster* Deddouche et al. (2008) reported the upregulation of the *Vago* gene after infection by the alphavirus Sindbis virus (SINV) and by a Drosophila C virus (DCV), while a third RNA virus, the nodavirus flock house virus (FHV) did not induce *Vago* expression. In a similar study by Paradkar et al. (2012) it has been shown that the West Nile virus (WNV) dsRNA was able to stimulate *Vago* production in *Culex quinquefasciatus* cells, while the bluetongue virus dsRNA did not cause *Vago* expression to increase. The induction of this gene was dependent on Dicer-2 but not by other RNAi pathway components (Deddouche et al., 2008). These outcomes suggest an RNAi-independent signaling mechanism for Dicer-2 and a cross-talk between the RNAi and Jak-STAT (Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathways (Kingsolver et al., 2013).

Although the exact immune response pathway is still unclear, it seems that non-specific dsRNA is recognized as a PAMP (Pathogen-Associated Molecular Pattern) triggering the anti-viral defense (Liu et al., 2013; Piot et al., 2015).

Dicer proteins, particularly Dicer-2 in *Drosophila* and mosquitoes, are recognized as the main PRR (Pattern Recognition Receptor) involved in recognition of virus-derived dsRNA and initiating not only the RNAi response, but also the Jak-STAT response (Deddouche et al., 2008; Ding, 2010; Paradkar et al., 2012).

#### 5.4.5.3. Resistance mechanisms

As for most means of pest control, the genetic plasticity of pests, their short life cycle and multiple number of generations favour the insurgence of new pathogenic strains. Possible resistance development mechanisms to RNAi have been proposed. For example, nuclease upregulation or dsRNA uptake malfunction, processing and systemic spread could stop the initiation and spread of RNAi response. RNAi resistance in mammalian cell cultures is known to occur and many RNAi viruses escape RNAi-mediated suppression through mutation of the target region, by encoding viral suppressors, or by cellular factors such as ADAR1 (adenosine deaminases acting on RNA), which is responsible for editing-mediated RNA resistance (Zheng et al., 2005).

While development of resistance to RNAi has not been documented in invertebrates so far, reports of RNAi (phenotypic) different effects and efficacy among insects strains (Kitzmann et al., 2013) give indications that such mechanism may arise. A recent study looking into the geographic variation in RNAi sensitivity in the migratory locust *Locusta migratoria*, showed that most individuals from two of the analyzed strains were sensitive to injection of different dsRNA, whereas those from two other strains were resistant (Sugahara et al., 2017). Furthermore, selection of dsRNA sensitive-individuals resulted in an increase in RNAi sensitivity in the following generation and the reciprocal crosses between a sensitive and a resistant strain resulted in F1 generations whose response to RNAi was comparable to that of the resistant strain, suggesting that the resistant phenotype might be dominant (Sugahara et al., 2017).

#### 5.4.6. Conclusions

The availability of GM plants-incorporating dsRNA has triggered discussions about their risk assessment and the possible adaptations needed for the current ERA frameworks (U.S. Environmental Protection Agency 2014; EFSA 2014). Particularly, the issue of the possible effects on NTOs is confronted with new challenges due to the lack of familiarity with the new modes of action of the RNA interfering mechanisms. At first sight, RNAi technology looks promising due to its potential to be highly species-specific. However, potential unintended off-target and non-target effects and knowledge gaps discussed in this report should be taken into account. The systematic literature

search confirmed that, to date, only a few systems have been specifically investigated for these impacts.

Several publications reported studies of off-target effects and effects on NTOs linked to interference triggered by dsRNA against *DvSnf7* or *V-ATPase*.

The impacts of *DvSnf7* expressed by GM maize event MON 87411 on NTOs and certain surrogate species representative of the main ecological groups in agro-ecosystems, have revealed no unintended effects in both *in vitro* procedures and field studies.

Several studies have also used dsRNA targeting different subunits of the *V-ATPase* gene. In a recent symposium on "Modern Biotechnology in Integrated Crop Management", Haller et al.<sup>4</sup> described experiments conducted with dsRNA targeting the *V-ATPase A* of *D. virgifera virgifera* and two ladybird species, *Coccinella septempunctata* and *Adalia bipunctata*. Both ladybirds were sensitive to dietary RNAi when ingesting their specific dsRNA, with *C. septempunctata* being much more sensitive than *A. bipunctata*. When assays were conducted with the dsRNA targeting *D. virgifera virgifera V-ATPase A*, adverse effects were detected in the two ladybird species (prolonged developmental time for *A. bipunctata* and significantly reduced survival rate for *C. septempunctata*). The results were supported by bioinformatic analyses that revealed a higher number of possible 21 nt matches of the targeted dsRNA sequence with the *V-ATPase A* of *C. septempunctata* (34 matches) compared to that of *A. bipunctata* (six matches).

This and other studies have identified an active silencing on NTOs in species taxonomically related to the target organism (i.e., Chrysomelidae and Coccinellidae) in some cases and, in one case, effects were also observed on a species belonging to a different insect order than the target (i.e., Coleoptera and Hymenoptera).

The likelihood of off-target effects has been suggested to be linked to the sequence similarity of the siRNA to any sequence in the genome of the NTO. In addition, some authors indicated that a certain number of mismatches in the siRNA sequence is allowed and silencing effects can still occur in such cases. Other sequence-independent mechanisms might also cause off-target effects such as similarity of functions of some genes, whose RNA might be similarly affected. In some cases, the use of dsRNA targeting genes absent from the genome of the tested species, caused expressional changes in a range of genes. Thus, the accurate design of the dsRNA to induce interference does not exclude the possibility of off-target and/or non-target effects. On the other hand, the question arises whether the presence of one 21 nt match siRNA in a pool of many different siRNAs after Dicer-processing of the long dsRNA would result in a silencing response strong enough to cause any significant effects.

The support of bioinformatics in this respect could be very valuable, but the limited availability of insect genomic sequences, the possible silencing in the presence of mismatches between the target and the siRNA sequences, and the possibility of sequence-unrelated off-target effects indicate the fundamental role of bioassays in assessing the actual activity spectrum of dsRNA.

Possible non-sequence dependent mechanisms leading to unintended effects on non-target invertebrates orally exposed to dsRNA have been hypothesized (e.g., immune-stimulation, saturation of the dsRNA machinery), however supporting experimental data are not available to date. Similarly, potential mechanisms which could lead to the onset of resistance in target organisms exist and will need to be duly considered.

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<sup>4</sup> Available at: [http://www.eigmo.info/sites/default/files/Book%20of%20abstracts\\_final.pdf](http://www.eigmo.info/sites/default/files/Book%20of%20abstracts_final.pdf)

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## 5.5. Overview of invertebrate species for which a sequenced genome is available (Task 6)

In this Section, we present an overview of the available genomic data for invertebrates belonging to the phyla of nematodes, arthropods, molluscs and annelids.

### 5.5.1. Introduction to genome sequencing in relation to RNAi

RNAi gene silencing is based on sequence homology between the effector molecule (dsRNA, siRNA, miRNA) and the target mRNA. Therefore, the availability of sequence data can be of great importance. It can be of importance for researchers and crop protectors when designing the sRNA and for avoiding off-target effects or gene silencing effects on NTOs. Furthermore, the availability of genomic sequence data on species in a given agroecosystem might also be of interest for risk assessors in determining potential effects on NTOs in that ecosystem. However, there has been some debate on the potential use of bioinformatics to predict silencing effects in target and NTOs. One of the main issues is that for invertebrates, there is no real consensus yet on the 'rules' for siRNA/RISC binding to the homologous mRNA (see Section 5.4).

Additionally, one must also consider that most genome sequences do not have a 100% coverage of the genome, nor are they always a 100% accurate. For example sequencing mistakes may occur or inclusion of contaminant sequences (e.g., bacterial symbionts) that sometimes remain in the genome after assembly and deposition in the public database can also cause errors (Lam et al., 2012; Merchant et al., 2014). Furthermore, automatic gene-predicting algorithms are also prone to mistakes, such as the failure to detect ORFs or the failure to correctly identify exon/intron structures. An additional issue is the fact that for most species, only one genome from one strain is publicly available, the so-called reference genome. Mutations in the genomes of other strains would be unknown.

Nevertheless, having an overview of the collection of genomic data can be of use. While using bioinformatics might not fully exclude potential off-target effects, it is helpful in identifying high-risk sRNAs for some NTOs, for example when multiple fully matching 21 nt siRNA sequence matches are present between the sRNA and a gene in the genome. Finally, the phenomenon of transitive RNAi, which entails the production of secondary siRNAs which can match regions of the target gene upstream or downstream the dsRNA triggering gene silencing, must be considered as well. This mechanism, which has been identified in the nematode *C. elegans*, has been discussed earlier in Section 5.1.4. So far, this mechanism has not been identified in arthropods, molluscs or annelids.

### 5.5.2. Genomic data

There are several databases which are receiving information from on-going international projects sequencing the genomes of a range of animals, microbes and plants. We present an overview of the available genomic data for invertebrates belonging to the phyla of nematodes, arthropods, molluscs and annelids extracted from four major databases or platforms:

- NCBI (Genbank): <https://www.ncbi.nlm.nih.gov/>
- JGI Genomes Online Databases (GOLD): <https://gold.jgi.doe.gov/>
- National human genome research institute (NIH): <https://www.genome.gov/>
- I5K (5,000 insect/arthropod genomes initiative): <http://i5k.github.io/>

Our search was performed on 17 February 2017, and it resulted in a list of 494 finished or ongoing invertebrate genome sequencing projects in total; 330 in arthropod species, 113 in nematodes, 40 in molluscs and 11 in annelids. Of these 494 genome sequencing projects, 333 (237 arthropods, 76 nematodes, 15 molluscs and 5 annelids) had been completed and have their genomic data publicly available on Genbank. For the other 161 genomes, which were only identified in the JGI database and

not in Genbank, projects are labelled 'incomplete' and are most likely still ongoing; for example, with assembly or annotation processes. Furthermore, an additional four genome sequencing projects, three *Caenorhabditis* strains and the chilopod *Strigamia maritima* were found on the NIH platform only. The I5K platform did not reveal any additional genomes that were not already found in Genbank.

The complete list of genome projects for which the data were available on 17 February 2017 is given in Table 18. The table also includes the genome Genbank ID and information on the assembly level for NCBI datasets (Contig, Scaffold or Chromosome). In genome sequencing, a contig refers to a collection of overlapping sequenced fragments. In other words, it forms a continuous stretch of sequence data created by read overlaps. These contigs can then be assembled into larger scaffolds, which still contain gaps of known length between the contigs. Several strategies are available to fill these gaps afterwards. Assembly at contig level means that nothing in the genome has been assembled beyond the level of sequenced contigs. Assembly at scaffold level means that at least some contigs have been assembled into larger scaffolds. At this level, scaffolds are still unplaced and unlocalized. Assembly at chromosome level refers to an available complete sequence for one or more chromosomes where scaffolds have been localized and placed. However, there may still be unlocalized and unplaced scaffolds. These levels give an indication of the coverage and assembly of the genome and the ability to annotate most genes in the genome. For some species there are genome projects and datasets for multiple strains. In those cases, only the so-called representative genome was referred to in the table.

**Table 18:** Overview of invertebrate species for which the genome sequence data was publicly available as of 17 February 2017

Phylum	Organism	Genbank ID	Assembly level
Arthropoda	<i>Achipteria coleoptrata</i>	37199	Scaffold
	<i>Acromyrmex echinator</i>	34551	Scaffold
	<i>Acyrtosiphon pisum</i>	29162	Scaffold
	<i>Aedes aegypti</i>	307340	Scaffold
	<i>Aedes albopictus</i>	304150	Scaffold
	<i>Agrilus planipennis</i>	202629	Scaffold
	<i>Amyelois transitella</i>	38909	Scaffold
	<i>Anopheles albimanus</i>	11556	Scaffold
	<i>Anopheles arabiensis</i>	11544	Scaffold
	<i>Anopheles atroparvus</i>	11555	Scaffold
	<i>Anopheles christyi</i>	11547	Scaffold
	<i>Anopheles culicifacies</i>	11552	Scaffold
	<i>Anopheles darlingi</i>	2624	Contig
	<i>Anopheles dirus</i>	11554	Scaffold
	<i>Anopheles epiroticus</i>	11546	Scaffold
	<i>Anopheles farauti</i>	11553	Contig
	<i>Anopheles funestus</i>	2697	Scaffold
	<i>Anopheles gambiae</i>	46	Scaffold
	<i>Anopheles koliensis</i>	36653	Contig
	<i>Anopheles maculatus</i>	11550	Scaffold
<i>Anopheles melas</i>	11549	Scaffold	

<i>Anopheles merus</i>	11548	Scaffold
<i>Anopheles minimus</i>	11551	Scaffold
<i>Anopheles nili</i>	17525	Scaffold
<i>Anopheles punctulatus</i>	36652	Contig
<i>Anopheles quadriannulatus</i>	11545	Scaffold
<i>Anopheles sinensis</i>	12170	Scaffold
<i>Anopheles stephensi</i>	2653	Scaffold
<i>Anoplophora glabripennis</i>	14033	Scaffold
<i>Apis cerana</i>	12051	Scaffold
<i>Apis dorsata</i>	15019	Scaffold
<i>Apis florea</i>	2740	Scaffold
<i>Apis mellifera</i>	937	Chromosome
<i>Athalia rosae</i>	14027	Scaffold
<i>Atta cephalotes</i>	2844	Scaffold
<i>Atta colombica</i>	44113	Scaffold
<i>Bactrocera cucurbitae</i>	11807	Scaffold
<i>Bactrocera dorsalis</i>	10754	Scaffold
<i>Bactrocera oleae</i>	17298	Scaffold
<i>Bactrocera tryoni</i>	15403	Scaffold
<i>Belgica antarctica</i>	14659	Scaffold
<i>Blattella germanica</i>	13223	Scaffold
<i>Bombus impatiens</i>	3415	Scaffold
<i>Bombus terrestris</i>	2739	Chromosome
<i>Bombyx mori</i>	76	Scaffold
<i>Caligus rogercresseyi</i>	38161	Contig
<i>Calliphora vicina</i>	15677	Scaffold
<i>Calycopis cecrops</i>	44594	Scaffold
<i>Camponotus floridanus</i>	2966	Scaffold
<i>Catajapyx aquilonaris</i>	17772	Scaffold
<i>Centruroides sculpturatus</i>	14105	Scaffold
<i>Cephus cinctus</i>	14134	Scaffold
<i>Cerapachys biroi</i>	55526	Scaffold
<i>Ceratina calcarata</i>	45025	Scaffold
<i>Ceratitis capitata</i>	2738	Scaffold
<i>Ceratosolen solmsi</i>	23331	Scaffold
<i>Chaoborus trivitattus</i>	38285	Scaffold
<i>Chilo suppressalis</i>	13084	Contig
<i>Chironomus riparius</i>	2978	Scaffold
<i>Chironomus tentans</i>	35154	Scaffold
<i>Cimex lectularius</i>	11279	Scaffold
<i>Clogmia albipunctata</i>	38294	Scaffold

<i>Coboldia fuscipes</i>	38275	Scaffold
<i>Condylostylus patibulatus</i>	38287	Scaffold
<i>Copidosoma floridanum</i>	12734	Scaffold
<i>Cotesia vestalis</i>	12200	Contig
<i>Culex quinquefasciatus</i>	393	Scaffold
<i>Cyphomyrmex costatus</i>	44114	Scaffold
<i>Dactylopius coccus</i>	36092	Contig
<i>Danaus plexippus</i>	11702	Scaffold
<i>Daphnia magna</i>	10953	Scaffold
<i>Daphnia pulex</i>	288	Scaffold
<i>Dendroctonus ponderosae</i>	11242	Scaffold
<i>Dermatophagoides farinae</i>	9138	Scaffold
<i>Diachasma alloeum</i>	40839	Scaffold
<i>Diaphorina citri</i>	867	Scaffold
<i>Dinoponera quadriceps</i>	40599	Scaffold
<i>Diuraphis noxia</i>	24062	Scaffold
<i>Drosophila albomicans</i>	2712	Scaffold
<i>Drosophila americana</i>	10917	Scaffold
<i>Drosophila ananassae</i>	244	Scaffold
<i>Drosophila arizonae</i>	45090	Scaffold
<i>Drosophila biarmipes</i>	3499	Scaffold
<i>Drosophila bipectinata</i>	3489	Scaffold
<i>Drosophila busckii</i>	38276	Chromosome
<i>Drosophila elegans</i>	3490	Scaffold
<i>Drosophila erecta</i>	250	Scaffold
<i>Drosophila eugracilis</i>	6863	Scaffold
<i>Drosophila ficusphila</i>	3491	Scaffold
<i>Drosophila grimshawi</i>	257	Scaffold
<i>Drosophila kikkawai</i>	3492	Scaffold
<i>Drosophila melanogaster</i>	47	Chromosome
<i>Drosophila miranda</i>	10915	Scaffold
<i>Drosophila mojavensis</i>	259	Scaffold
<i>Drosophila navojoa</i>	45091	Scaffold
<i>Drosophila persimilis</i>	265	Scaffold
<i>Drosophila pseudoobscura</i>	219	Scaffold
<i>Drosophila rhopaloea</i>	6853	Scaffold
<i>Drosophila sechellia</i>	271	Scaffold
<i>Drosophila simulans</i>	200	Chromosome
<i>Drosophila suzukii</i>	18317	Scaffold
<i>Drosophila takahashii</i>	3493	Scaffold
<i>Drosophila virilis</i>	300047	Scaffold

<i>Drosophila willistoni</i>	251	Scaffold
<i>Drosophila yakuba</i>	148	Chromosome
<i>Dufourea novaeangliae</i>	39971	Scaffold
<i>Ephemera danica</i>	14601	Scaffold
<i>Ephydra gracilis</i>	38282	Scaffold
<i>Ephydra hians</i>	38282	Scaffold
<i>Eristalis dimidiata</i>	38298	Scaffold
<i>Eufriesea mexicana</i>	44840	Scaffold
<i>Eurytemora affinis</i>	17731	Scaffold
<i>Eutreta diana</i>	38297	Scaffold
<i>Ferrisia virgata</i>	44261	Scaffold
<i>Fopius arisanus</i>	35518	Scaffold
<i>Frankliniella occidentalis</i>	14741	Scaffold
<i>Gerris buenoi</i>	17730	Scaffold
<i>Glossina austeni</i>	16689	Scaffold
<i>Glossina brevipalpis</i>	15576	Scaffold
<i>Glossina fuscipes</i>	15034	Scaffold
<i>Glossina morsitans</i>	55	Scaffold
<i>Glossina pallidipes</i>	15956	Scaffold
<i>Glossina palpalis</i>	15033	Scaffold
<i>Habropoda laboriosa</i>	39899	Scaffold
<i>Halyomorpha halys</i>	14101	Scaffold
<i>Harpegnathos saltator</i>	2965	Scaffold
<i>Heliconius cydno</i>	10843	Scaffold
<i>Heliconius elevatus</i>	42294	Scaffold
<i>Heliconius ethilla</i>	42293	Scaffold
<i>Heliconius hecale</i>	33769	Scaffold
<i>Heliconius ismenius</i>	36108	Scaffold
<i>Heliconius melpomene</i>	10844	Scaffold
<i>Heliconius numata</i>	12823	Scaffold
<i>Heliconius pardalinus</i>	42296	Scaffold
<i>Heliconius timareta</i>	15536	Scaffold
<i>Hermetia illucens</i>	38288	Scaffold
<i>Holcocephala fusca</i>	38300	Scaffold
<i>Homalodisca vitripennis</i>	13454	Scaffold
<i>Hyalella azteca</i>	16496	Scaffold
<i>Hypochthonius rufulus</i>	37200	Scaffold
<i>Hypothenemus hampei</i>	38265	Scaffold
<i>Ixodes ricinus</i>	16267	Scaffold
<i>Ixodes scapularis</i>	523	Scaffold
<i>Ladona fulva</i>	17240	Scaffold



<i>Lasioglossum albipes</i>	15038	Scaffold
<i>Lasius niger</i>	14569	Scaffold
<i>Latrodectus hesperus</i>	14107	Scaffold
<i>Lepeophtheirus salmonis</i>	2713	Contig
<i>Leptinotarsa decemlineata</i>	12832	Scaffold
<i>Lerema accius</i>	40057	Scaffold
<i>Limnephilus lunatus</i>	17773	Scaffold
<i>Limulus polyphemus</i>	787	Scaffold
<i>Linepithema humile</i>	2733	Scaffold
<i>Liriomyza trifolii</i>	16235	Scaffold
<i>Locusta migratoria</i>	10772	Contig
<i>Loxosceles reclusa</i>	14028	Scaffold
<i>Lucilia cuprina</i>	12732	Scaffold
<i>Lucilia sericata</i>	8289	Scaffold
<i>Lutzomyia longipalpis</i>	30816	Scaffold
<i>Maconellicoccus hirsutus</i>	44264	Scaffold
<i>Manduca sexta</i>	12037	Scaffold
<i>Mayetiola destructor</i>	2619	Scaffold
<i>Megachile rotundata</i>	6563	Scaffold
<i>Megaselia abdita</i>	38299	Scaffold
<i>Megaselia scalaris</i>	15767	Scaffold
<i>Melipona quadrifasciata</i>	12726	Scaffold
<i>Melitaea cinxia</i>	814	Scaffold
<i>Mengenilla moldrzyki</i>	14989	Contig
<i>Mesobuthus martensii</i>	14571	Contig
<i>Metaseiulus occidentalis</i>	3487	Scaffold
<i>Microplitis demolitor</i>	12766	Scaffold
<i>Mochlonyx cinctipes</i>	38286	Scaffold
<i>Monomorium pharaonis</i>	37124	Scaffold
<i>Musca domestica</i>	14461	Scaffold
<i>Nasonia giraulti</i>	759	Scaffold
<i>Nasonia longicornis</i>	760	Scaffold
<i>Nasonia vitripennis</i>	449	Chromosome
<i>Nasutitermes exitiosus</i>	40812	Contig
<i>Neobellieria bullata</i>	13341	Scaffold
<i>Neodiprion lecontei</i>	39861	Scaffold
<i>Nicrophorus vespilloides</i>	40824	Scaffold
<i>Nilaparvata lugens</i>	2941	Scaffold
<i>Oncopeltus fasciatus</i>	11434	Scaffold
<i>Onthophagus taurus</i>	12827	Scaffold
<i>Operophtera brumata</i>	39883	Scaffold

<i>Orussus abietinus</i>	14602	Scaffold
<i>Oryctes borbonicus</i>	41561	Contig
<i>Pachypsylla venusta</i>	14032	Scaffold
<i>Papilio glaucus</i>	36240	Scaffold
<i>Papilio machaon</i>	14159	Scaffold
<i>Papilio polytes</i>	33756	Scaffold
<i>Papilio xuthus</i>	13942	Scaffold
<i>Paracoccus marginatus</i>	44262	Scaffold
<i>Parasteatoda tepidariorum</i>	13270	Scaffold
<i>Parhyale hawaiiensis</i>	15533	Scaffold
<i>Pediculus humanus</i>	522	Scaffold
<i>Phlebotomus papatasi</i>	10999	Scaffold
<i>Phoebis sennae</i>	44038	Scaffold
<i>Phortica variegata</i>	38277	Scaffold
<i>Piezodorus guildinii</i>	34914	Scaffold
<i>Platynothis peltifer</i>	37201	Scaffold
<i>Plutella xylostella</i>	11570	Scaffold
<i>Pogonomyrmex barbatus</i>	2732	Scaffold
<i>Polistes canadensis</i>	16494	Scaffold
<i>Polistes dominula</i>	42066	Scaffold
<i>Priacma serrata</i>	11831	Contig
<i>Pseudococcus longispinus</i>	44263	Scaffold
<i>Rhipicephalus microplus</i>	2797	Scaffold
<i>Rhodnius prolixus</i>	447	Scaffold
<i>Sarcoptes scabiei</i>	36095	Scaffold
<i>Scaptodrosophila lebanonensis</i>	38279	Scaffold
<i>Solenopsis invicta</i>	2938	Scaffold
<i>Sphyracephala brevicornis</i>	38301	Scaffold
<i>Spodoptera frugiperda</i>	10985	Scaffold
<i>Steganacarus magnus</i>	8750	Scaffold
<i>Stegodyphus mimosarum</i>	12925	Scaffold
<i>Stomoxys calcitrans</i>	11278	Scaffold
<i>Strigamia maritima</i>	790	Scaffold
<i>Teleopsis dalmanni</i>	13232	Scaffold
<i>Tephritis californica</i>	38315	Scaffold
<i>Tetranychus urticae</i>	2710	Scaffold
<i>Themira minor</i>	38280	Scaffold
<i>Tipula oleracea</i>	38316	Scaffold
<i>Trachymyrmex cornetzi</i>	44115	Scaffold
<i>Trachymyrmex septentrionalis</i>	44116	Scaffold
<i>Trachymyrmex zeteki</i>	44394	Scaffold

	<i>Tribolium castaneum</i>	216	Chromosome
	<i>Trichogramma pretiosum</i>	14106	Scaffold
	<i>Trionymus perrisii</i>	44268	Scaffold
	<i>Triops cancriformis</i>	7259	contig
	<i>Trupanea jonesi</i>	38283	Scaffold
	<i>unclassified Sarcophagidae</i>	40370	Scaffold
	<i>Unclassified Trichoceridae</i>	38278	Scaffold
	<i>Varroa destructor</i>	937	Contig
	<i>Vollenhovia emeryi</i>	36511	Scaffold
	<i>Wasmannia auropunctata</i>	36651	Scaffold
	<i>Zootermopsis nevadensis</i>	17755	Scaffold
Nematoda	<i>Ancylostoma ceylanicum</i>	10936	Scaffold
	<i>Ancylostoma duodenale</i>	348	Scaffold
	<i>Angiostrongylus cantonensis</i>	30176	Scaffold
	<i>Angiostrongylus costaricensis</i>	9101	Scaffold
	<i>Anisakis simplex</i>	7896	Scaffold
	<i>Ascaris lumbricoides</i>	11969	Scaffold
	<i>Ascaris suum</i>	350	Scaffold
	<i>Brugia malayi</i>	42	Scaffold
	<i>Brugia pahangi</i>	13249	Scaffold
	<i>Brugia timori</i>	36524	Scaffold
	<i>Bursaphelenchus xylophilus</i>	11822	Contig
	<i>Caenorhabditis angaria</i>	3127	Scaffold
	<i>Caenorhabditis brenneri</i>	254	Scaffold
	<i>Caenorhabditis briggsae</i>	40	Chromosome
	<i>Caenorhabditis elegans</i>	41	Chromosome
	<i>Caenorhabditis japonica</i>	252	Scaffold
	<i>Caenorhabditis nigoni</i>	44874	Scaffold
	<i>Caenorhabditis remanei</i>	253	scaffold
	<i>Caenorhabditis tropicalis</i>	35261	Scaffold
	<i>Cylicostephanus goldi</i>	36526	Scaffold
	<i>Dictyocaulus viviparus</i>	779	Scaffold
	<i>Dirofilaria immitis</i>	10757	Contig
	<i>Ditylenchus destructor</i>	43946	Scaffold
	<i>Dracunculus medinensis</i>	10838	Scaffold
	<i>Elaeophora elaphi</i>	24454	Scaffold
	<i>Enterobius vermicularis</i>	8694	Scaffold
	<i>Globodera pallida</i>	32442	Scaffold
<i>Globodera rostochiensis</i>	44826	Scaffold	
<i>Gongylonema pulchrum</i>	36527	Scaffold	
<i>Haemonchus contortus</i>	16936	Scaffold	

<i>Haemonchus placei</i>	36529	Scaffold
<i>Heligmosomoides polygyrus</i>	36519	Scaffold
<i>Heterodera glycines</i>	852	Scaffold
<i>Heterorhabditis bacteriophora</i>	481	Scaffold
<i>Loa loa</i>	2686	Contig
<i>Meloidogyne floridensis</i>	33826	Scaffold
<i>Meloidogyne hapla</i>	260	Contig
<i>Meloidogyne incognita</i>	281	Contig
<i>Necator americanus</i>	770	Scaffold
<i>Nippostrongylus brasiliensis</i>	11002	Scaffold
<i>Oesophagostomum dentatum</i>	782	Scaffold
<i>Onchocerca flexuosa</i>	11948	Scaffold
<i>Onchocerca ochengi</i>	18268	Scaffold
<i>Onchocerca volvulus</i>	2687	Scaffold
<i>Oscheius sp. MCB</i>	36317	Contig
<i>Panagrellus redivivus</i>	16242	Scaffold
<i>Parascaris equorum</i>	36531	Scaffold
<i>Parastrongyloides trichosuri</i>	36401	Scaffold
<i>Pristionchus pacificus</i>	246	Contig
<i>Rhabditophanes sp.</i>	36407	Scaffold
<i>Romanomermis culicivorax</i>	23995	Scaffold
<i>Rotylenchulus reniformis</i>	11277	Scaffold
<i>Soboliphyme baturini</i>	36532	Scaffold
<i>Steinernema carpocapsae</i>	2699	Scaffold
<i>Steinernema feltiae</i>	17805	Scaffold
<i>Steinernema glaseri</i>	17808	Scaffold
<i>Steinernema monticolum</i>	17810	Scaffold
<i>Steinernema scapterisci</i>	17807	Scaffold
<i>Strongyloides papillosus</i>	36387	Scaffold
<i>Strongyloides ratti</i>	3496	Chromosome
<i>Strongyloides stercoralis</i>	13317	Scaffold
<i>Strongyloides venezuelensis</i>	12700	Scaffold
<i>Strongylus vulgaris</i>	9335	Scaffold
<i>Subanguina moxae</i>	37144	Scaffold
<i>Syphacia muris</i>	36393	Scaffold
<i>Thelazia callipaeda</i>	14733	Scaffold
<i>Toxocara canis</i>	8517	Scaffold
<i>Trichinella</i>	41654	Scaffold
<i>Trichinella britovi</i>	35242	Scaffold
<i>Trichinella murrelli</i>	35264	Scaffold
<i>Trichinella nativa</i>	35238	Scaffold

	<i>Trichinella nelsoni</i>	35237	Scaffold
	<i>Trichinella papuae</i>	35252	Scaffold
	<i>Trichinella patagoniensis</i>	41656	Scaffold
	<i>Trichinella pseudospiralis</i>	11844	Scaffold
	<i>Trichinella spiralis</i>	238	Scaffold
	<i>Trichinella zimbabwensis</i>	35251	Scaffold
	<i>Trichuris muris</i>	31518	Scaffold
	<i>Trichuris suis</i>	13427	Scaffold
	<i>Trichuris trichiura</i>	13417	Scaffold
	<i>Wuchereria bancrofti</i>	2616	Scaffold
<b>Mollusca</b>	<i>Aplysia californica</i>	443	Scaffold
	<i>Bankia setacea</i>	10465	Contig
	<i>Biomphalaria glabrata</i>	357	Scaffold
	<i>Conus tribblei</i>	40372	Scaffold
	<i>Corbicula fluminea</i>	15808	Contig
	<i>Crassostrea gigas</i>	10758	Scaffold
	<i>Crassostrea virginica</i>	398	Chromosome
	<i>Dreissena polymorpha</i>	13187	Contig
	<i>Lottia gigantea</i>	15113	Scaffold
	<i>Lymnaea stagnalis</i>	14639	Contig
	<i>Mizuhopecten yessoensis</i>	12193	Scaffold
	<i>Mytilus galloprovincialis</i>	12190	Contig
	<i>Octopus bimaculoides</i>	41501	Scaffold
	<i>Pinctada martensi</i>	55961	Chromosome
	<i>Radix auricularia</i>	55350	Scaffold
<b>Annelida</b>	<i>Amyntas corticis</i>	40146	Scaffold
	<i>Capitella teleta</i>	15118	Scaffold
	<i>Eisenia fetida</i>	12810	Scaffold
	<i>Helobdella robusta</i>	15112	Scaffold
	<i>Hydroides elegans</i>	45775	Scaffold

### 5.5.3. References

Lam HY, Clark MJ, Chen R, et al., 2012. Performance comparison of whole-genome sequencing platforms. *Nature Biotechnology*, 30, 78-82.

Merchant S, Wood DE and Salzberg SL, 2014. Unexpected cross-species contamination in genome sequencing projects. *PeerJ*, 2, e675.

## 6. Conclusions

In this Technical Report, an overview is given on the state of the art regarding RNAi in invertebrates. First, a systematic literature search was performed to identify all peer-reviewed studies conducted on RNAi in nematodes, arthropods, molluscs and annelids, leading to the collection of 13,867 studies. After this initial search, 4,612 studies which dealt with oral exposure to small RNAs were identified, analysed and listed in Annex 2. When we examined these studies, we found that most research on RNAi was conducted in the phyla of nematodes (27%) and arthropods (71%). Only a very limited number of studies have investigated RNAi in molluscs and annelids. In both nematodes and arthropods, there is a clear level of variability in sensitivity to RNAi. For example, nematodes belonging to the *Caenorhabditis* genus appear to be more sensitive than many other nematode species, due to an expanded RNAi-related gene repertoire in the genome. Similarly, in arthropods, a high degree of variability also exists, especially in the sensitivity of species to oral RNAi. Generally Coleoptera, which is comprised of beetles and weevils, appear to be more sensitive to RNAi than species in other orders and require smaller doses to elicit successful gene silencing. This indicates that the dose of sRNA to which target and NTOs are exposed is important when designing RNAi systems in plants.

In Section 5.1 and 5.3, several factors that influence this variability have been identified, including sRNA stability in the invertebrate body, the efficiency of cellular uptake of sRNAs, the presence of siRNA amplification systems, viral infections which could interfere with the RNAi machinery and RNAi-related gene repertoire. These factors have implications regarding the potential for silencing effects in non-target species and also the development of resistance to (oral) RNAi. Furthermore, we also found that while the cellular uptake machinery in nematodes (notably *C. elegans*) has been well-studied and described, this is not the case for species belonging to arthropods, molluscs and annelids. This cellular uptake has been studied in a limited number of insect species, but it seems clear that arthropods do not possess most of the genes involved in *C. elegans* sRNA specific uptake. While similar systems might exist in both phyla, they have not been entirely elucidated in arthropods yet.

In Section 5.2, we consider possible exposure routes and the fate of sRNAs produced in GM plants. Several direct or indirect exposure routes were identified. While methods exist to identify the sRNA expression levels in plants, we found very few studies actually reporting these. Interestingly, dsRNA seems to be very short-lived in the environment. Laboratory studies investigating the persistence of dsRNA in soil and aquatic environments indicated that most dsRNA is degraded within 48 hours. However, no field studies investigating the fate of dsRNA derived from GM plants are available yet.

We also reported on the potential for adverse effects on NTOs caused by sRNA exposure (Section 5.4). Several mechanisms have been identified in the literature which could cause unintended effects, including sequence-dependent silencing effects caused by sequence homology between sRNA and a (homologous) gene in the genome and sequence-independent effects such as immune system stimulation and RNAi machinery saturation. Only a limited number of studies have investigated unintended silencing effects in NTOs and all of these were conducted in insects using dsRNA targeting either the DvSnf7 or the DvV-ATPase in the Western Corn Rootworm. These studies indicated that successful gene silencing requires a high degree of homology and at least a full 20nt match between the dsRNA sequence and the mRNA. However, we could not find any study really fundamentally investigating siRNA specificity in invertebrates, making it difficult to identify a general rule on the necessary homology between dsRNA and mRNA to cause gene silencing. Phylogenetic relatedness between target and non-target species might favor the possible occurrence of non-target effects, though in some cases silencing has occurred after ingestion of dsRNA in test organisms belonging to different insect orders. Immune stimulation and RNAi machinery saturation have been proposed as potential adverse effects in some commentaries, but hard evidence that this might occur, let alone be a cause for a fitness cost in NTOs, is lacking. Furthermore, the studies conducted in the context of the ERA of the dsSnf7-expressing GM maize seemed to indicate the very high specificity of this product, especially when compared to other plant produced pesticides used to date.

An interesting question in the context of RNAi ERA is whether bioinformatics could play a useful role. In Section 5.5, we have compiled a list of invertebrate genomes that have been sequenced and are publically available and we discuss the potential usefulness of bioinformatics in predicting adverse effects in NTOs. In total, we identified 494 species for which the genomic data is available. This number represents only a very small part of the diversity of potential non-target invertebrates which could be exposed to sRNA in agroecosystems. Furthermore, the incomplete coverage of most genomes and the uncertainty regarding the necessary homology between sRNA and mRNA to cause gene silencing means that, at this time, bioinformatics alone cannot be relied on to predict the absence of any possible silencing effects. However, bioinformatics could still be useful at an early stage to identify highly conserved potential high risk sRNA for some NTOs, for example when multiple fully matching 21 nt siRNA sequence matches are present between the sRNA and a gene in the genome.

## 7. Recommendations

The evidence collected here indicates that RNAi is a very promising pest control method, compared to many of the currently available pest control strategies, due to the possibility of designing highly species-specific dsRNA and their short-lived persistence in the environment. However, there are several gaps in our knowledge of this mechanism, for example on the necessary homology between siRNA and mRNA and the potential adverse effects on immune systems of invertebrate NTOs. In order to support environmental risk assessments of RNAi GM plants, this report has indicated several areas which warrant further study and more bioinformatics information is required on a wider diversity of the invertebrate species occurring in agro-ecosystems. Bioinformatics could be a useful tool in ERA to screen and predict some sRNAs that have a 21 nt match against any of the available genomes but until we have more research information we cannot use it to determine the absence of silencing effects in NTOs with any certainty.

## Abbreviations

ABC	ATP-binding cassette
Bt	Bacillus thuringiensis
CDS	coding sequence
CPB	Colorado potato beetle
diRNA	defective interfering RNA
dsRNA	double-stranded RNA
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ERA	environmental risk assessment
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM	genetically modified
MEEC	maximum expected environmental concentration
miRNA	micro RNA
mRNA	messenger RNA
ncRNA	non-coding RNA
NTO	non-target organism
qRT-PCR	quantitative reversed transcription polymerase chain reaction
RdRP	RNA dependant RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
rsd	RNAi spreading defective
SAGO	siRNA-specific Argonaute
SCR	Southern corn rootworm
shRNA	short hairpin RNA
sid	spreading RNA interference defective
siRNA	small interfering RNA
sRNA	short RNA
TGN	Trans-Golgi network
TO	target organism
UTR	untranslated region
viRNA	viral RNA
VSR	viral suppressor of RNAi
WAGO	Worm-specific argonaute
WCR	Western corn rootworm
WoS	Web of Science



## Appendix A – List of reference studies used to assess the relevance of the results from the defined search string in Web of Science

No.	Authors	Year	Journal, volume, pages	Title
1	Abdellatef et al.	2015	Plant Biotechnology Journal 13, 849-857	Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid <i>Sitobion avenae</i>
2	Abdel-Latif and Hoffmann	2014	Peptides 53, 172-184	Functional activity of allatotropin and allatostatin in the pupal stage of a holometabolous insect, <i>Tribolium castaneum</i> (Coleoptera, Tenebrionidae)
3	Aditi et al.	2015	Transgenic Research 24, 791-801	Transgenic plants over-expressing insect-specific microRNA acquire insecticidal activity against <i>Helicoverpa armigera</i> : an alternative to Bt-toxin technology
4	Ahmad et al.	2016	Transgenic Research 25, 1-17	Transportable data from non-target arthropod field studies for the environmental risk assessment of genetically modified maize expressing an insecticidal double-stranded RNA
5	Allen and Walker	2012	Journal of Insect Physiology 58, 391-396	Saliva of <i>Lygus lineolaris</i> digests double stranded ribonucleic acids
6	Araujo et al	2006	Insect Biochemistry and Molecular Biology 36, 683-693	RNA interference of the salivary gland nitrophorin 2 in the triatomine bug <i>Rhodnius prolixus</i> (Hemiptera: Reduviidae) by dsRNA ingestion or injection
7	Aronstein et al	2006	Journal of Apicultural Research 45, 20-24	SID-1 is implicated in systemic gene silencing in the honey bee
8	Bachman et al	2013	Transgenic Research 22, 1207-1222	Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against western cornrootworm ( <i>Diabrotica virgifera virgifera</i> Le Conte)
9	Baum, et al	2007	Nature Biotechnology 25, 1322-1326	Control of coleopteran insect pests through RNA interference
10	Bautista et al	2009	Insect Biochemistry and Molecular Biology 39, 38-46	RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, <i>Plutella xylostella</i> , reduces larval resistance to permethrin
11	Belles	2010	Annual Review of Entomology 55, 111-128	Beyond <i>Drosophila</i> : RNAi in vivo and functional genomics in insects
12	Blandin et al	2002	EMBO Reports 3, 852-856	Reverse genetics in the mosquito <i>Anopheles gambiae</i> : targeted disruption of the Defensin gene
13	Boisson et al	2006	FEBS Letters 580, 1988-1992	Gene silencing in mosquito salivary glands by RNAi
14	Bolognesi et al	2012	PLoS ONE 7, e47534	Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm ( <i>Diabrotica virgifera virgifera</i> LeConte)
15	Burand and Hunter	2013	Journal of Invertebrate Pathology 112, S68-S74	RNAi: future in insect management
16	Chen et al	2013	Insect Biochemistry and	Conserved microRNAs miR-8-5p and miR-2a-

			Molecular Biology 43, 839-848	3p modulate chitin biosynthesis in response to 20- hydroxyecdysone signaling in the brown planthopper, <i>Nilaparvata lugens</i>
17	Chen et al	2014	PLoS Pathogens 10, e1004261	Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health
18	Christiaens et al	2014	Peptides 53, 307-314	DsRNA degradation in the pea aphid ( <i>Acyrtosiphon pisum</i> ) associated with lack of response in RNAi feeding and injection assay
19	Chu et al	2014	Pesticide Biochemistry and Physiology 110, 1-6	Differential effects of RNAi treatments on field populations of the western corn rootworm
20	Clemens et al	2000	Proceedings of the National Academy of Sciences of the United States of America 97, 6499-6503	Use of double-stranded RNA interference in <i>Drosophila</i> cell lines to dissect signal transduction pathways
21	Coleman et al	2014	Journal of Experimental Botany 66, 541-548	Persistence and transgenerational effect of plant-mediated RNAi in aphids
22	Coy et al	2012	Journal of Applied Entomology 136, 741-748	Gene silencing in adult <i>Aedes aegypti</i> mosquitoes through oral delivery of double-stranded RNA
23	Drake et al	2012	Journal of Visualized Experiments, e3479	RNAi-mediated gene knockdown and in vivo diuresis assay in adult female <i>Aedes aegypti</i> mosquitoes
24	Dubelman et al	2014	PLoS ONE 9, e93155	Environmental fate of double-stranded RNA in agricultural soils
25	Dutta et al	2014	Frontiers in Microbiology 5, nr. 760	The status of RNAi-based transgenic research in plant nematology
26	El-Shesheny et al	2013	PLoS ONE 8, e65392	Silencing abnormal wing disc gene of the Asian citrus psyllid, <i>Diaphorina citri</i> disrupts adult wing development and increases nymph mortality
27	Fabioux et al	2009	FEBS Journal 276, 2566-2573	In vivo RNA interference in oyster-vasa silencing inhibits germ cell development
28	Fang et al	2011	PLoS ONE 6, e21860	Identification of genes directly involved in shell formation and their functions in pearl oyster; <i>Pinctada fucata</i>
29	Feinberg and Hunter	2003	Science 301, 1545-1547	Transport of dsRNA into cells by the transmembrane protein SID-1
30	Fire et al	1998	Nature 391, 806-811	Potent and specific genetic interference by double-stranded RNA in <i>Caenorhabditis elegans</i>
31	Garbian et al	2012	PLoS Pathogens 8, e1003035	Bidirectional transfer of RNAi between honey bee and <i>Varroa destructor</i> : Varroa gene silencing reduces Varroa population
32	Garbutt and Reynolds	2012	Insect Biochemistry and Molecular Biology 42, 621-628	Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference
33	Ghanim et al	2007	Insect Biochemistry and Molecular Biology 37, 732-738	Tissue-specific gene silencing by RNA interference in the whitefly <i>Bemisia tabaci</i>
34	Gong et al	2013	PLoS ONE 8, e62990	Testing insecticidal activity of novel chemically synthesized siRNA against <i>Plutella xylostella</i> under laboratory and field conditions
35	Grimaldi et al	2004	Development Growth & Differentiation 46, 83-95	Muscle differentiation in tentacles of <i>Sepia officinalis</i> Mollusc is regulated by muscle regulatory factors MRF related proteins

36	Gu and Knipple	2013	Crop Protection 45, 36-40	Recent advances in RNA interference research in insects: implications for future insect pest management strategies
37	Guo et al	2014	PLoS ONE 9, e97410	Plant-Generated Artificial Small RNAs Mediated Aphid Resistance
38	Hajeri et al	2014	Journal of Biotechnology 176, 42-49	Citrus tristeza virus-based RNAi in citrus plants induces gene silencing in <i>Diaphorina citri</i> , a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing)
39	Hiruta et al	2013	BMC Biotechnology 13, nr. 96	Development of a microinjection system for RNA interference in the water flea <i>Daphnia pulex</i>
40	Hossain et al	2008	Insect Biochemistry and Molecular Biology 38, 1001-1007	Expression of 20-hydroxyecdysone- induced genes in the silkworm brain and their functional analysis in post-embryonic development
41	Huang et al	2006	Proceedings of the National Academy of Sciences of the United States of America 103, 14302-14306	Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene
42	Huvenne and Smagghe	2010	Journal of Insect Physiology 56, 227-235	Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review
43	Ivashuta et al	2015	RNA 21, 840-850	Environmental RNAi in herbivorous insects
44	Jaubert-Possamai et al	2007	BMC Biotechnology 7, nr. 63	Gene knockdown by RNAi in the pea aphid <i>Acyrtosiphon pisum</i>
45	Jiang et al	2006	Developmental and Comparative Immunology 30, 855-866	In vivo and in vitro knockdown of FREP2 gene expression in the snail <i>Biomphalaria glabrata</i> using RNA interference
46	Jiang et al	2013	Insect Biochemistry and Molecular Biology 53, 692-700	MicroRNA-281 regulates the expression of ecdysone receptor (EcR) isoform B in the silkworm, <i>Bombyx mori</i>
47	Kamath and Ahringer	2003	Methods 30, 313-321	Genome-wide RNAi screening in <i>Caenorhabditis elegans</i>
48	Kamath et al	2001	Genome Biology 2, nr. 0002	Effectiveness of specific RNA-mediated interference through ingested double stranded RNA in <i>Caenorhabditis elegans</i>
49	Kamath et al	2003	Nature 421, 231-237	Systematic functional analysis of the <i>Caenorhabditis elegans</i> genome using RNAi
50	Karim et al	2010	BMC Biotechnology 10, nr. 1	Functional genomics tool: gene silencing in <i>Ixodes scapularis</i> eggs and nymphs by electroporated dsRNA
51	Kato et al	2011	Development Genes and Evolution 220, 337-345	Development of an RNA interference method in the cladoceran crustacean <i>Daphnia magna</i>
52	Kennerdell and Carthew	1998	Cell 95, 1017-1026	Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway
53	Khajuria, et al	2015	Insect Biochemistry and Molecular Biology 63, 54-62	Parental RNA interference of genes involved in embryonic development of the western corn rootworm, <i>Diabrotica virgifera virgifera</i> LeConte
54	Killiny et al	2014	PLoS ONE 9, e110536	Double-stranded RNA uptake through topical application, mediates silencing of five CYP4 genes and suppresses insecticide resistance in <i>Diaphorina citri</i>
55	Korneev et al	2011	Journal of Neuroscience 22, RC227	Suppression of nitric oxide NO-dependent behaviour by double-stranded RNA-mediated

56	Kumar et al	2012	PLoS ONE 7, e31347	silencing of a neuronal NO synthase gene Tobacco rattle virus vector: a rapid and transient means of silencing <i>Manduca sexta</i> genes by plant mediated RNA interference
57	La Fauce and Owens	2013	Journal of Invertebrate Pathology 112, 162-165	Suppression of <i>Penaeus merguensis</i> densovirus following oral delivery of live bacteria expressing dsRNA in the house cricket ( <i>Acheta domesticus</i> ) model
58	La Fauce and Owens	2009	Journal of Invertebrate Pathology 100, 111-115	RNA interference reduces PmergDNV expression and replication in an in vivo cricket model
59	Labreuche et al	2010	Developmental and Comparative Immunology 34, 1209-1218	Non-specific activation of antiviral immunity and induction of RNA interference may engage the same pathway in the Pacific white leg shrimp <i>Litopenaeus vannamei</i>
60	Lee et al	2001	Learning and Memory 8, 220-226	Overexpression of and RNA interference with the CCAAT enhancer-binding protein on long term facilitation of <i>Aplysia</i> sensory to motor synapses
61	Lee et al	2004	Cell 117, 69-81	Distinct roles for <i>Drosophila</i> Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways
62	Lee et al	2011	Insect biochemistry and Molecular Biology 41, 236-243	RNA interference of pheromone biosynthesis-activating neuropeptide receptor suppresses mating behavior by inhibiting sex pheromone production in <i>Plutella xylostella</i> (L)
63	Levine et al	2015	PLoS ONE 10, e0118622	Independent action between DvSnf7 RNA and Cry3Bb1 protein in southern corn rootworm, <i>Diabrotica undecimpunctata howardi</i> and Colorado potato beetle, <i>Leptinotarsa decemlineata</i>
64	Li et al	2015	Journal of Applied Entomology 139, 432-445	Long dsRNA but not siRNA initiates RNAi in western corn rootworm larvae and adults
65	Li-Byarlay et al	2013	Proceedings of the National Academy of Sciences of the United States of America 110, 12750-12755	RNA interference knockdown of DNA methyltransferase 3 affects gene alternative splicing in the honey bee
66	Liu et al	2010	Insect Biochemistry and Molecular Biology 40, 666-671	Gene knockdown by intro-thoracic injection of double-stranded RNA in the brown planthopper, <i>Nilaparvata lugens</i>
67	Liu et al	2013	Journal of Insect Physiology 59, 646-654	Transcriptional response of BmToll9-1 and RNAi machinery genes to exogenous dsRNA in the midgut of <i>Bombyx mori</i>
68	Luan et al	2013	Insect Biochemistry and Molecular Biology 43, 740-746	Silencing the ecdysone synthesis and signaling pathway genes disrupts nymphal development in the whitefly
69	Luo et al	2012	RNA Biology 9, 663-671	The SID-1 double-stranded RNA transporter is not required for systemic RNAi in the migratory locust
70	Maeda et al	2001	Current Biology 11, 171-176	Large-scale analysis of gene function in <i>Caenorhabditis elegans</i> by high-throughput RNAi
71	Mao et al	2007	Nature Biotechnology 25, 1307-1313	Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol
72	Mao et al	2011	Transgenic Research 20, 665-673	Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to

				bollworms
73	Mao et al	2013	Plant Molecular Biology 83, 119-129	Cysteine protease enhances plant-mediated bollworm RNA interference
74	Mao and Zeng	2012	PLoS ONE 7, e48718	Feeding-based RNA interference of a gap gene is lethal to the pea aphid, <i>Acyrtosiphon pisum</i>
75	Mao and Zeng	2014	Transgenic Research 23, 145-152	Plant-mediated RNAi of a gap gene enhanced tobacco tolerance against the <i>Myzus persicae</i>
76	Maori et al	2009	Insect Molecular Biology 18, 55-60	IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion
77	Marques et al	2013	PLoS Pathogens 9, e1003579	Functional specialization of the small interfering RNA pathway in response to virus infection
78	Martin et al	2006	Journal of Insect Physiology 52, 410-416	RNAi studies reveal a conserved role for RXR in molting in the cockroach <i>Blattella germanica</i>
79	Miller et al	2008	Development Genes and Evolution 218, 505-510	Larval RNAi in <i>Drosophila</i> ?
80	Miller, et al	2012	PLoS ONE 7, e47431	Dissecting systemic RNA interference in the red flour beetle <i>Tribolium castaneum</i> : parameters affecting the efficiency of RNAi
81	Minakuchi et al	2008	FEBS Journal 275, 2919-2931	RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle <i>Tribolium castaneum</i>
82	Miyata et al	2014	PLoS ONE 9, e101661	Establishing an in vivo assay system to identify components involved in environmental RNA interference in the western corn rootworm
83	Miyoshi et al	2010	Nature Structural and Molecular Biology 17, 1024-1026	A direct role for Hsp90 in pre-RISC formation in <i>Drosophila</i>
84	Mussig et al	2010	Journal of Neuroscience 30, 7817-7825	Acute disruption of the NMDA receptor subunit NR1 in the honeybee brain selectively impairs memory formation
85	Mutti et al	2006	Journal of Insect Science 6, 1-7	RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, <i>Acyrtosiphon pisum</i>
86	Leigh et al	2015	Aquaculture 437, 360-365	Diet-delivery of therapeutic RNA interference in live E coli against pre-existing <i>Penaeus merguensis</i> hepadensovirus in <i>Penaeus merguensis</i>
87	Paldi et al	2010	Applied and Environmental Microbiology 76, 5960-5964	Effective gene silencing of a microsporidian parasite associated with honey bee ( <i>Apis mellifera</i> ) colony declines
88	Pitino et al	2011	PLoS ONE 6, e25709	Silencing of aphid genes by dsRNA feeding from plants
89	Pridgeon et al	2008	Journal of Medical Entomology 45, 414-420	Topically applied AaeIAP1 double-stranded RNA kills female adults of <i>Aedes aegypti</i>
90	Ramaseshadri et al	2013	PLoS ONE 8, e54270	Physiological and cellular responses caused by RNAi-mediated suppression of Snf7 orthologue in western corn rootworm ( <i>Diabrotica virgifera</i> ) larvae
91	Rangasamy and Siegfried	2012	Pest Management Science 68, 587-591	Validation of RNA interference in western corn rootworm <i>diabrotica virgifera virgifera</i> Le Conte (Coleoptera: Chrysomelidae) adults
92	Roberts et al	2015	Frontiers in Plant Science 6, nr. 953	Biosafety research for non-target organism risk assessment of RNAi-based GE plants

93	Rong et al	2013	Insect Science 20, 109-119	RNA interference to reveal roles of beta-N-acetylglucosaminidase gene during molting process in <i>Locusta migratoria</i>
94	Rosa et al	2010	Journal of RNAi and gene silencing 6, 361-366	RNAi effects on actin mRNAs in <i>Homalodisca vitripennis</i> cells
95	Rosa et al	2012	Pest Management Science 68, 995-1002	RNA interference is induced in the glassy winged sharpshooter <i>Homalodisca vitripennis</i> by actin dsRNA
96	Saleh et al	2009	Nature 458, 346-350	Antiviral immunity in <i>Drosophila</i> requires systemic RNA interference spread
97	Scott, et al	2013	Journal of Insect Physiology 59, 1212-1221	Towards the elements of successful insect RNAi
98	Seitz et al	2011	Silence 2, nr. 4	A 5'-uridine amplifies miRNA/miRNA* asymmetry in <i>Drosophila</i> by promoting RNA-induced silencing complex formation
99	Shakesby et al	2009	Insect Biochemistry and Molecular Biology 39, 1-10	A water-specific aquaporin involved in aphid osmoregulation
100	Shih and Hunter	2011	RNA 17, 1057-1065	SID-1 is a dsRNA-selective dsRNA-gated channel RNA
101	Sijen et al	2001	Cell 107, 465-476	On the role of RNA amplification in dsRNA-triggered gene silencing
102	Snow et al	2013	RNA Biology 10, 1107-1116	Ineffective delivery of diet-derived microRNAs to recipient animal organisms
103	Steeves et al	2006	Functional Plant Biology 33, 991-999	Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress <i>Heterodera glycines</i> reproduction
104	Surakasi et al	2011	Journal of Insect Physiology 57, 1537-1544	RNA interference of beta1 integrin subunit impairs development and immune responses of the beet armyworm, <i>Spodoptera exigua</i>
105	Terenius et al	2011	Journal of Insect Physiology 57, 231-245	RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design
106	Thakur et al	2014	PLoS ONE 9, e87235	Enhanced Whitefly Resistance in Transgenic Tobacco Plants Expressing Double Stranded RNA of V-ATPase A Gene
107	Tian et al	2009	PLoS ONE 4, e6225	Developmental control of a lepidopteran pest <i>Spodoptera exigua</i> by ingestion of bacteria expressing dsRNA of a non-midgut gene
108	Timmons et al	2001	Gene 263, 103-112	Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in <i>Caenorhabditis elegans</i>
109	Tomoyasu et al	2008	Genome Biology 9, R10	Exploring systemic RNA interference in insects: a genomewide survey for RNAi genes in <i>Tribolium</i>
110	Turner et al	2006	Insect Molecular Biology 15, 383-391	RNA interference in the light brown apple moth, <i>Epiphyas postvittana</i> (Walker) induced by doublestranded RNA feeding
111	Upadhyay et al	2011	Journal of Biosciences 36, 153-161	RNA interference for the control of whiteflies ( <i>Bemisia tabaci</i> ) by oral route
112	van Rij et al	2006	Genes and Development 20, 2985-2995	The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in <i>Drosophila melanogaster</i>
113	Walshe et al	2009	Insect Molecular Biology 18, 11-19	Prolonged gene knockdown in the tsetse fly <i>Glossina</i> by feeding double stranded RNA
114	Wang et al	2011	Developmental and Comparative Immunology	A primitive Toll-like receptor signalling pathway in mollusk Zhikong scallop <i>Chlamys</i>

115	Whitten et al	2016	35, 511-520 Proceedings of the Royal Society B 283, 20160042	<i>farreri</i> Symbiont-mediated RNA interference in insects
116	Whyard et al	2009	Insect Biochemistry and Molecular Biology 39, 824-832	Ingested double-stranded RNAs can act as species-specific insecticides
117	Winston et al	2002	Science 295, 2456-2459	Systemic RNAi in <i>C. elegans</i> requires the putative transmembrane protein sid-1
118	Winston et al	2007	Proceedings of the National Academy of Sciences of the United States of America 104, 10565-10570	<i>Caenorhabditis elegans</i> SID-2 is required for environmental RNA interference
119	Wuriyangan et al	2011	PLoS ONE 6, e27736	Oral delivery of double-stranded RNAs and siRNAs induces RNAi effects in the potato/tomato psyllid, <i>Bactericera cockerelli</i>
120	Wuriyangan and Falk	2013	PLoS ONE 8, e66050	RNA Interference towards the Potato Psyllid, Is Induced in Plants Infected with Recombinant Tobacco mosaic virus (TMV)
121	Xu et al	2013	PLoS Genetics 9, e1003535	Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, <i>Tribolium castaneum</i>
122	Yadav et al	2006	Molecular and Biochemical Parasitology 148, 219-222	Hostgenerated double stranded RNA induces RNAi in plantparasitic nematodes and protects the host from infection
123	Yigit et al	2006	Cell 127, 747-757	Analysis of the <i>C. elegans</i> Argonaute family reveals that distinct Argonautes act sequentially during RNAi
124	Zha et al	2011	PLoS ONE 6, e20504	Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect <i>Nilaparvata lugens</i>
125	Zhang et al	2010	Insect Molecular Biology 19, 683-693	Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito ( <i>Anopheles gambiae</i> )
126	Zhang et al	2013	BMC Genomics 14, 1-15	Identifying potential RNAi targets in grain aphid ( <i>Sitobion avenae</i> F.) based on transcriptome profiling of its alimentary canal after feeding on wheat plants
127	Zhang et al	2015	Science 347, 991-994	Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids
128	Zhang et al	2015	Entomologia Experimentalis Et Applicata 155, 218-228	Lepidopteran insect species-specific, broad-spectrum, and systemic RNA interference by spraying dsRNA on larvae
129	Zhou et al	2008	Insect Biochemistry and Molecular Biology 38, 805-815	RNA interference in the termite <i>Reticulitermes flavipes</i> through ingestion of double-stranded RNA
130	Zhu et al	2011	Pest Management Science 67, 175-182	Ingested RNA interference for managing the populations of the Colorado potato beetle, <i>Leptinotarsa decemlineata</i>
131	Zhu et al	2012	PLoS ONE 7, e38572	Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR

## Appendix B – List of invertebrate species or genera used in RNAi studies

Phylum	Subphylum/Class	Species	No. of studies
<b>Single species studies</b>			
Annelida			7
	Clitellata	<i>Hirudo medicinalis</i>	6
		<i>Hirudo verbana</i>	1
Arthropoda			3,420
	Chelicerata		149
		<i>Achaearanea tepidariorum</i>	8
		<i>Amblyomma americanum</i>	16
		<i>Amblyomma hebraeum</i>	2
		<i>Amblyomma maculatum</i>	7
		<i>Amblyomma variegatum</i>	1
		<i>Aquarius paludum</i>	1
		<i>Boophilus microplus</i>	1
		<i>Cupiennius salei</i>	6
		<i>Dermacentor marginatus</i>	1
		<i>Dermacentor variabilis</i>	7
		<i>Dermanyssus gallinae</i>	1
		<i>Dermatophagoides pteronyssinus</i>	1
		<i>Haemaphysalis longicornis</i>	29
		<i>Hyalomma anatolicum</i>	1
		<i>Ixodes ricinus</i>	12
		<i>Ixodes scapularis</i>	20
		<i>Limnaporus dissortis</i>	2
		<i>Metaseiulus occidentalis</i>	5
		<i>Ornithodoros erraticus</i>	1
		<i>Ornithodoros moubata</i>	3
		<i>Panonychus citri</i>	1
		<i>Parasteatoda tepidariorum</i>	1
		<i>Phytoseiulus persimilis</i>	1
		<i>Rhipicephalus decoloratus</i>	1
		<i>Rhipicephalus evertsi evertsi</i>	1
		<i>Rhipicephalus haemaphysaloides</i>	4
		<i>Rhipicephalus microplus</i>	5
		<i>Rhipicephalus sanguineus</i>	3
		<i>Tetranychus cinnabarinus</i>	1
		<i>Tetranychus urticae</i>	3
		<i>Varroa destructor</i>	3
	Collembola		1



	<i>Orchesella cincta</i>	1
Crustacea		300
	<i>Amphibalanus amphitrite</i>	1
	<i>Artemia diapause</i>	1
	<i>Artemia franciscana</i>	5
	<i>Artemia parthenogenetica</i>	7
	<i>Artemia salina</i>	2
	<i>Artemia</i> sp	1
	<i>Caligus rogercresseyi</i>	1
	<i>Callinectes sapidus</i>	1
	<i>Cherax quadricarinatus</i>	2
	<i>Daphnia magna</i>	2
	<i>Daphnia melanica</i>	1
	<i>Daphnia pulex</i>	1
	<i>Daphnia pulicaria</i>	1
	<i>Eriocheir sinensis</i>	7
	<i>Exopalaemon carinicauda</i>	1
	<i>Fenneropenaeus chinensis</i>	14
	<i>Fenneropenaeus merguensis</i>	1
	<i>Lepeophtheirus salmonis</i>	6
	<i>Litopenaeus schmitti</i>	1
	<i>Litopenaeus stylirostris</i>	1
	<i>Litopenaeus vannamei</i>	88
	<i>Macrobrachium nipponense</i>	6
	<i>Macrobrachium rosenbergii</i>	8
	<i>Mallotus japonicus</i>	2
	<i>Marsupenaeus japonicus</i>	39
	<i>Metapenaeus ensis</i>	3
	<i>Pacifastacus leniusculus</i>	5
	<i>Penaeus chinensis</i>	1
	<i>Penaeus japonicus</i>	2
	<i>Penaeus merguensis</i>	1
	<i>Penaeus monodon</i>	71
	<i>Penaeus vannamei</i>	4
	<i>Portunus trituberculatus</i>	1
	<i>Procambarus clarkii</i>	9
	<i>Scylla paramamosain</i>	1
	<i>Tigriopus californicus</i>	1
	<i>Uca pugilator</i>	1
Insecta		2,970
	<i>Achaea janata</i>	1
	<i>Acheta domesticus</i>	6

	<i>Acyrtosiphon pisum</i>	15
	<i>Aedes aegypti</i>	133
	<i>Aedes albopictus</i>	16
	<i>Aedes fluviatilis</i>	1
	<i>Aedes taeniorhynchus</i>	1
	<i>Agrilus planipennis</i>	1
	<i>Agrotis ipsilon</i>	1
	<i>Allonemobius socius</i>	1
	<i>Alphitobius diaperinus</i>	1
	<i>Anastrepha obliqua</i>	1
	<i>Anastrepha suspensa</i>	1
	<i>Annigeres subalbatus</i>	1
	<i>Anopheles aquasalis</i>	2
	<i>Anopheles coluzzii</i>	2
	<i>Anopheles dirus</i>	3
	<i>Anopheles gambiae</i>	66
	<i>Anopheles quadrimaculatus</i>	1
	<i>Anopheles stephensi</i>	4
	<i>Antheraea assama</i>	1
	<i>Antheraea pernyi</i>	7
	<i>Anthonomus grandis</i>	1
	<i>Aperiona germari</i>	1
	<i>Aphis gossypii</i>	7
	<i>Apis cerana</i>	4
	<i>Apis mellifera</i>	78
	<i>Apis sp</i>	1
	<i>Apolygus lucorum</i>	2
	<i>Apteranemobius asahinai</i>	3
	<i>Araneae spp</i>	1
	<i>Armigeres subalbatus</i>	10
	<i>Arthropoda spp</i>	1
	<i>Astylus atromaculatus</i>	1
	<i>Athalia rosae</i>	1
	<i>Autographa californica</i>	4
	<i>Bactericera cockerelli</i>	5
	<i>Bactrocera dorsalis</i>	23
	<i>Bactrocera minax</i>	1
	<i>Bactrocera oleae</i>	1
	<i>Bemisia tabaci</i>	15
	<i>Bicyclus anynana</i>	1
	<i>Blattella germanica</i>	39
	<i>Bombus ignitus</i>	3

	<i>Bombus terrestris</i>	3
	<i>Bombyx mandarina</i>	1
	<i>Bombyx mori</i>	176
	<i>Camponotus floridanus</i>	2
	<i>Carabidae</i> spp.	1
	<i>Ceratitis capitata</i>	7
	<i>Chaetocnema pulicaria</i>	1
	<i>Chilo infuscatellus</i>	1
	<i>Chilo suppressalis</i>	4
	<i>Choristoneura fumiferana</i>	2
	<i>Chrysomela populi</i>	2
	<i>Chrysopa perla</i>	1
	<i>Chrysopa septempunctata</i>	1
	<i>Chrysoperla</i> spp.	1
	<i>Chymomyza costata</i>	1
	<i>Cimex lectularius</i>	4
	<i>Circulifer haematoceps</i>	1
	<i>Coccinellidae</i> spp.	1
	<i>Cochliomyia hominivorax</i>	1
	<i>Cochliomyia macellaria</i>	1
	<i>Colaphellus bowringi</i>	1
	<i>Coleomegilla maculata</i>	1
	<i>Coptotermes formosanus</i>	1
	<i>Corcyra cephalonica</i>	1
	<i>Cosmopolites sordidus</i>	1
	<i>Cotesia plutellae</i>	1
	<i>Cryptotermes secundus</i>	2
	<i>Culex pipiens</i>	19
	<i>Culex quinquefasciatus</i>	9
	<i>Culicoides sonorensis</i>	1
	<i>Cyclommatus metallifer</i>	2
	<i>Cydia pomonella</i>	1
	<i>Cylas puncticollis</i>	1
	<i>Dalbulus maidis</i>	1
	<i>Danaus plexippus</i>	1
	<i>Delphacidae</i> spp.	1
	<i>Dendroctonus armandi</i>	1
	<i>Dendroctonus ponderosae</i>	1
	<i>Dermestes maculatus</i>	1
	<i>Diabrotica undecimpunctata howardi</i>	3
	<i>Diabrotica virgifera virgifera</i>	17
	<i>Diacamma</i> sp.	1

	<i>Diachasmimorpha longicaudata</i>	1
	<i>Diaphorina citri</i>	4
	<i>Diatraea saccharalis</i>	2
	<i>Diploptera punctata</i>	5
	<i>Diuraphis noxia</i>	1
	<i>Dolichopus spp</i>	1
	<i>Drosophila brahma</i>	1
	<i>Drosophila melanogaster</i>	1,243
	<i>Drosophila pseudoobscura</i>	1
	<i>Drosophila suzukii</i>	2
	<i>Epicauta chinensis</i>	1
	<i>Epilachna varivestis</i>	1
	<i>Epiphyas postvittana</i>	1
	<i>Episyrphus balteatus</i>	2
	<i>Eurygaster integriceps</i>	1
	<i>Euschistus heros</i>	1
	<i>Euxesta stigmatias</i>	1
	<i>Eyprepocnemis plorans</i>	2
	<i>Forficulidae spp</i>	1
	<i>Frankliniella occidentalis</i>	2
	<i>Gastrophysa atrocyanea</i>	3
	<i>Geocoris spp</i>	1
	<i>Georgecraigius atropalpus</i>	1
	<i>Gerris buenoi</i>	1
	<i>Glossina morsitans morsitans</i>	16
	<i>Glossina palpalis palpalis</i>	1
	<i>Graminella nigrifrons</i>	1
	<i>Grapholita molesta</i>	1
	<i>Gryllus bimaculatus</i>	31
	<i>Gryllus caudal</i>	1
	<i>Halyomorpha halys</i>	2
	<i>Harmonia axyridis</i>	3
	<i>Helicoverpa armigera</i>	67
	<i>Helicoverpa assulta</i>	1
	<i>Helicoverpa zea</i>	3
	<i>Heliothis virescens</i>	2
	<i>Henosepilachna vigintioctopunctata</i>	1
	<i>Hippodamia convergens</i>	1
	<i>Hodotermopsis sjostedti</i>	1
	<i>Homalodisca vitripennis</i>	3
	<i>Homoptera spp</i>	1
	<i>Hyalophora cecropia</i>	2

	<i>Hymenoptera spp</i>	1
	<i>Hyphantria cunea</i>	1
	<i>Laodelphax striatellus</i>	24
	<i>Leptinotarsa decemlineata</i>	32
	<i>Leptopilina boulandi</i>	1
	<i>Locusta migratoria</i>	39
	<i>Lucilia cuprina</i>	1
	<i>Lucilia sericata</i>	3
	<i>Lutzomyia longipalpis</i>	4
	<i>Lygus lineolaris</i>	3
	<i>Lymantria dispar</i>	5
	<i>Maecolapsis sp</i>	1
	<i>Mamestra brassicae</i>	2
	<i>Mamestra configurata</i>	1
	<i>Manduca sexta</i>	27
	<i>Mayetiola destructor</i>	3
	<i>Megacopta punctatissima</i>	1
	<i>Megaselia abdita</i>	4
	<i>Microplitis demolitor</i>	1
	<i>Microplitis mediator</i>	1
	<i>Modicogryllus siamensis</i>	1
	<i>Monochamus alternatus</i>	2
	<i>Musca domestica</i>	6
	<i>Mythimna separata</i>	2
	<i>Myzus persicae</i>	11
	<i>Nasonia vitripennis</i>	13
	<i>Nasutitermes takasagoensis</i>	1
	<i>Nephotettix cincticeps</i>	5
	<i>Nilaparvata lugens</i>	55
	<i>Nitidulidae spp</i>	1
	<i>Notoxus monodon</i>	1
	<i>Nysius plebeius</i>	2
	<i>Oncopeltus fasciatus</i>	22
	<i>Onthophagus binodis</i>	3
	<i>Onthophagus nigriventris</i>	1
	<i>Onthophagus sagittarius</i>	3
	<i>Onthophagus sp</i>	2
	<i>Onthophagus taurus</i>	2
	<i>Orius insidiosus</i>	2
	<i>Ostrinia furnacalis</i>	8
	<i>Ostrinia nubilalis</i>	3
	<i>Papilio xuthus</i>	2

	<i>Pardosa pseudoannulata</i>	1
	<i>Pectinophora gossypiella</i>	1
	<i>Pediobius foveolatus</i>	1
	<i>Peregrinus maidis</i>	1
	<i>Periplaneta americana</i>	8
	<i>Phaedon cochleariae</i>	5
	<i>Phalacrus politus</i>	1
	<i>Phenacoccus solenopsis</i>	2
	<i>Phlebotomus papatasi</i>	1
	<i>Phyllotreta striolata</i>	3
	<i>Pieris rapae</i>	4
	<i>Planococcus citri</i>	2
	<i>Plautia stali</i>	1
	<i>Plodia interpunctella</i>	4
	<i>Plutella xylostella</i>	42
	<i>Poecilus chalcites</i>	1
	<i>Polistes metricus</i>	1
	<i>Protaetia brevitarsis</i>	1
	<i>Pseudaletia separata</i>	1
	<i>Pseudoplusia includens</i>	1
	<i>Pteromalus puparum</i>	1
	<i>Pyrrhocoris apterus</i>	3
	<i>Recilia dorsalis</i>	3
	<i>Reticulitermes flavipes</i>	6
	<i>Reticulitermes speratus</i>	2
	<i>Rhodnius prolixus</i>	25
	<i>Rhopalosiphum pad</i>	1
	<i>Rhynchosciara americana</i>	1
	<i>Riptortus pedestris</i>	5
	<i>Romalea microptera</i>	1
	<i>Sarcophaga crassipalpis</i>	2
	<i>Sarcophaga peregrina</i>	2
	<i>Schistocerca americana</i>	2
	<i>Schistocerca gregaria</i>	21
	<i>Schizaphis graminum</i>	1
	<i>Scirpophaga incertulas</i>	1
	<i>Sesamia nonagrioides</i>	1
	<i>Sitobion avenae</i>	9
	<i>Sitophilus zeamais</i>	2
	<i>Sogatella furcifera</i>	8
	<i>Solenopsis invicta</i>	4
	<i>Spodoptera exigua</i>	36

	<i>Spodoptera frugiperda</i>	30
	<i>Spodoptera littoralis</i>	8
	<i>Spodoptera litura</i>	25
	<i>Tenebrio molitor</i>	10
	<i>Thermobia domestica</i>	6
	<i>Toxomerus</i> spp	1
	<i>Toxoptera citricida</i>	1
	<i>Triatoma brasiliensis</i>	2
	<i>Triatoma infestans</i>	1
	<i>Tribolium castaneum</i>	184
	<i>Tribolium orthodenticle</i>	1
	<i>Tribolium zer knullt</i>	1
	<i>Trichoplusia ni</i>	5
	<i>Trilocha varians</i>	1
	<i>Trypoxylus dichotomus</i>	1
	<i>Tuta absoluta</i>	1
	<i>Xanthine dehydrogenase</i>	1
	<i>Zootermopsis nevadensis</i>	2
Mollusca		67
	<i>Aplysia californica</i>	3
	<i>Aplysia</i> sp	3
	<i>Aplysia synapsin</i>	1
	<i>Argopecten purpuratus</i>	1
	<i>Biomphalaria glabrata</i>	7
	<i>Chlamys farreri</i>	9
	<i>Crassostrea angulata</i>	1
	<i>Crassostrea gigas</i>	10
	<i>Cyclina sinensis</i>	2
	<i>Haliotis diversicolor</i>	1
	<i>Hyriopsis cumingii</i>	1
	<i>Lymnaea stagnalis</i>	10
	<i>Meretrix meretrix</i>	2
	<i>Nipponacmea fuscoviridis</i>	1
	<i>Pinctada fucata</i>	10
	<i>Pinctada martensii</i>	3
	<i>Saccostrea glomerata</i>	1
	<i>Sepia officinali</i>	1
Nematoda		1292
	<i>Acrobeloides</i> sp	1
	<i>Aphelenchoides besseyi</i>	2
	<i>Aphelenchus avenae</i>	1
	<i>Ascaris suum</i>	2

	<i>Bursaphelenchus xylophilus</i>	15
	<i>Caenorhabditis briggsae</i>	14
	<i>Caenorhabditis elegans</i>	1109
	<i>Caenorhabditis nigoni</i>	1
	<i>Caenorhabditis remanei</i>	1
	<i>Ditylenchus destructor</i>	2
	<i>Globodera pallida</i>	6
	<i>Globodera rostochiensis</i>	4
	<i>Haemonchus contortus</i>	6
	<i>Heligmosomoides polygyrus</i>	1
	<i>Heterodera avenae</i>	5
	<i>Heterodera glycines</i>	16
	<i>Heterodera schachtii</i>	8
	<i>Heterorhabditis bacteriophora</i>	2
	<i>Meloidogyne paranaensis</i>	2
	<i>Meloidogyne arenaria</i>	3
	<i>Meloidogyne artiellia</i>	1
	<i>Meloidogyne chitwoodi</i>	5
	<i>Meloidogyne graminicola</i>	2
	<i>Meloidogyne hapla</i>	3
	<i>Meloidogyne incognita</i>	44
	<i>Meloidogyne javanica</i>	9
	<i>Mesorhabditis sp</i>	1
	<i>Nippostrongylus brasiliensis</i>	1
	<i>Oesophagostomum dentatum</i>	1
	<i>Onchocerca volvulus</i>	1
	<i>Oscheius sp</i>	1
	<i>Panagrellus redivivus</i>	1
	<i>Panagrolaimus superbus</i>	1
	<i>Pratylenchus coffeae</i>	1
	<i>Pratylenchus penetrans</i>	1
	<i>Pratylenchus thornei</i>	1
	<i>Pratylenchus vulnus</i>	2
	<i>Pratylenchus zaeae</i>	1
	<i>Pristionchus pacificus</i>	2
	<i>Radopholus similis</i>	7
	<i>Rhabditis sp</i>	1
	<i>Teladorsagia circumcincta</i>	1
	<i>Trichinella spiralis</i>	2
	<i>Trichostrongylus colubriformis</i>	1
	Environments - Soil	1
		4,786



## Appendix C – List of publications considered relevant for Task 3 “Environmental exposure and fate of dsRNA siRNA and miRNA” – Plant expression and intergenerational transmission

No.	Authors	Year	Journal, volume, pages	Title
1	Abdellatef et al.	2015	Plant Biotechnology Journal 13, 849-57	Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid <i>Sitobion avenae</i>
2	Agrawal et al.	2015	Transgenic Research 24, 791-801	Transgenic plants over-expressing insect-specific microRNA acquire insecticidal activity against <i>Helicoverpa armigera</i> : an alternative to Bt-toxin technology
3	Alcazar et al.	2008	Genetics 180, 1275-1288	Transmission dynamics of heritable silencing induced by double-stranded RNA in <i>Caenorhabditis elegans</i>
4	J. Antonino de Souza Junior et al.	2013	PLoS ONE 8, e85364	Knocking-down <i>Meloidogyne incognita</i> proteases by plant-delivered dsRNA has negative pleiotropic effect on nematode vigor
5	Apone et al.	2014	Journal of Insect Science 14	Targeting the diuretic hormone receptor to control the cotton leafworm, <i>Spodoptera littoralis</i>
6	Baum et al.	2007	Nat Biotech 25, 1322-1326	Control of coleopteran insect pests through RNA interference
7	Bhatia et al.	2012	PLoS ONE 7, e46343	Host generated siRNAs attenuate expression of serine protease gene in <i>Myzus persicae</i>
8	Calderon-Urrea et al.	2012	Plant Biotechnology Reports 6, 263-274	Overexpression of sense and antisense ced-9 in tobacco plants confers resistance to <i>Meloidogyne incognita</i>
9	Charlton et al.	2010	International Journal for Parasitology 40, 855-864	Additive effects of plant expressed double-stranded RNAs on root-knot nematode development
10	Chen et al.	2006	Journal of Integrative Plant Biology 48, 1458-1465	Functional characterization of Mi, a root-knot nematode resistance gene from tomato ( <i>Lycopersicon esculentum</i> L.)
11	Chi et al.	2016	International Journal for Parasitology 46, 105-113	Exposure to double-stranded RNA mediated by tobacco rattle virus leads to transcription up-regulation of effector gene Mi-vap-2 from <i>Meloidogyne incognita</i> and promotion of pathogenicity in progeny
12	Chronis et al.	2013	The Plant Journal 74, 185-196	A ubiquitin carboxyl extension protein secreted from a plant-parasitic nematode <i>Globodera rostochiensis</i> is cleaved in planta to promote plant parasitism
13	Yang et al.	2013	PLoS ONE 8, e69463	Molecular characteristics and efficacy of 16D10 siRNAs in inhibiting root-knot nematode infection in transgenic grape hairy roots

14	Clement et al.	2009	The Plant Cell 21, 2963-2979	Actin-depolymerizing Factor2-mediated actin dynamics are essential for root-knot nematode infection of <i>Arabidopsis</i>
15	Pitino et al.	2011	PLoS ONE 6, e25709	Silencing of aphid genes by dsRNA feeding from plants
16	Coleman et al.	2015	Journal of Experimental Botany 66, 541-548	Persistence and transgenerational effect of plant-mediated RNAi in aphids
17	Collins et al.	2010	Journal of Biotechnology 150, S116-S116	RNAi-mediated crop improvement for sustainable resistance to <i>Globodera pallida</i>
18	Dinh et al.	2014	Phytopathology 104, 1098-1106	RNA interference of effector gene Mc16D10L confers resistance against <i>Meloidogyne chitwoodi</i> in <i>Arabidopsis</i> and potato
19	Dinh et al.	2014	Nematology 16, 669-682	Plant-mediated RNA interference of effector gene Mc16D10L confers resistance against <i>Meloidogyne chitwoodi</i> in diverse genetic backgrounds of potato and reduces pathogenicity of nematode offspring
20	Dinh et al.	2015	Journal of Nematology 47, 71-78	Broad <i>Meloidogyne</i> resistance in potato based on RNA interference of effector gene 16D10
21	Dutta et al.	2015	Frontiers in Microbiology 6, 260	Tomato transgenic plants expressing hairpin construct of a nematode protease gene conferred enhanced resistance to root-knot nematodes
22	Elzinga et al.	2014	Molecular Plant-Microbe Interactions 27, 747-756	Suppression of plant defenses by a <i>Myzus persicae</i> (Green Peach Aphid) salivary effector protein
23	Fairbairn et al.	2007	Planta 226, 1525-1533	Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes
24	Guo et al.	2014	PLoS ONE 9, e97410	Plant-Generated artificial small RNAs mediated aphid resistance
25	Fuchs et al.	2007	Journal of Plant Pathology 89, 5-12	Safety assessment of transgenic plums and grapevines expressing viral coat protein genes: New insights into real environmental impact of perennial plants engineered for virus resistance
26	Hajeri et al.	2014	Journal of Biotechnology 176, 42-49	<i>Citrus tristeza virus</i> -based RNAi in citrus plants induces gene silencing in <i>Diaphorina citri</i> , a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing)
27	Hamamouch et al.	2012	Journal of Experimental Botany 63, 3683-3695	The interaction of the novel 30C02 cyst nematode effector protein with a plant beta-1,3-endoglucanase may suppress host defence to promote parasitism
28	Hu et al.	2013	Experimental Parasitology 135, 15-23	Molecular and biochemical characterization of the beta-1,4-endoglucanase gene Mj-eng-3 in the root-knot nematode <i>Meloidogyne javanica</i>
29	Huang et al.	2006	National Academy of Sciences 103,	Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a

			14302-14306	conserved and essential root-knot nematode parasitism gene
30	Huang et al.	2014	European Journal of Plant Pathology 138, 181-193	Molecular cloning and virus-induced gene silencing of MiASB in the southern root-knot nematode, <i>Meloidogyne incognita</i>
31	Iberkleid et al.	2013	PLoS ONE 8, e64586	Fatty acid- and retinol-binding protein, Mj-FAR-1 induces tomato host susceptibility to root-knot nematodes
32	Ibrahim et al.	2011	Experimental Parasitology 127, 90-99	Post-transcriptional gene silencing of root-knot nematode in transformed soybean roots
33	Jaouannet et al.	2013	Molecular Plant-Microbe Interactions 26, 97-105	The root-knot nematode calreticulin Mi-CRT is a key effector in plant defense suppression
34	Jin et al.	2015	Plant Biotechnology Journal 13, 435-446	Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts <i>Helicoverpa armigera</i> larval development and pupation
35	Khajuria et al.	2015	Insect Biochemistry and Molecular Biology 63, 54-62	Parental RNA interference of genes involved in embryonic development of the western corn rootworm, <i>Diabrotica virgifera virgifera</i> LeConte
36	Khan et al.	2015	Biotechnology Letters 37, 2083-2090	Inoculation of <i>Nicotiana tabacum</i> with recombinant potato virus X induces RNA interference in the solenopsis mealybug, <i>Phenacoccus solenopsis</i> Tinsley (Hemiptera: Pseudococcidae)
37	Khan et al.	2013	PLoS ONE 8, e73657	Use of recombinant tobacco mosaic virus to achieve RNA interference in plants against the citrus mealybug, <i>Planococcus citri</i> (Hemiptera: Pseudococcidae)
38	Klink et al.	2009	Planta 230, 53-71	A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female <i>Heterodera glycines</i> cyst formation during infection of <i>Glycine max</i>
39	Konopova and Akam	2014	EvoDevo 5	The Hox genes Ultrabithorax and abdominal-A specify three different types of abdominal appendage in the springtail <i>Orchesella cincta</i> (Collembola)
40	Kumar et al.	2012	PLoS ONE 7, e31347	Tobacco Rattle Virus Vector: a rapid and transient means of silencing <i>Manduca sexta</i> genes by plant mediated RNA interference
41	Kyndt et al.	2013	Nematology 15, 519-528	Transcriptional silencing of RNAi constructs against nematode genes in <i>Arabidopsis</i>
42	Li et al.	2015	Journal of Applied Entomology 139, 432-445	Long dsRNA but not siRNA initiates RNAi in western corn rootworm larvae and adults
43	Li et al.	2010	Planta 232, 775-785	Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of <i>Heterodera glycines</i> Ichinohe

44	Li et al.	2010	Plant Cell Reports 29, 113-123	Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants
45	Lin et al.	2013	Molecular Plant-Microbe Interactions 26, 55-66	A novel effector protein, MJ-NULG1a, targeted to giant cell nuclei plays a role in <i>Meloidogyne javanica</i> parasitism
46	Li et al.	2015	International Journal of Biological Sciences 11, 1073-1087	Cathepsin B cysteine proteinase is essential for the development and pathogenesis of the plant parasitic nematode <i>Radopholus similis</i>
47	Li et al.	2015	PLoS ONE 10, e0129351	A nematode calreticulin, Rs-CRT, is a key effector in reproduction and pathogenicity of <i>Radopholus similis</i>
48	Lourenco-Tessutti et al.	2015	Phytopathology 105, 628-637	Knock-down of heat-shock protein 90 and isocitrate lyase gene expression reduced root-knot nematode reproduction
49	Liu et al.	2015	International Journal of Biological Sciences 11, 67-74	Silencing the HaAK gene by transgenic plant-mediated RNAi impairs larval growth of <i>Helicoverpa armigera</i>
50	Mamta et al.	2016	Plant Molecular Biology 90, 281-292	Targeting chitinase gene of <i>Helicoverpa armigera</i> by host-induced RNA interference confers insect resistance in tobacco and tomato
51	Mao & Zeng	2014	Transgenic Research 23, 145-152	Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the <i>Myzus persicae</i>
52	Mao et al.	2007	Nature Biotechnology 25, 1307–1313	Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol
53	Mao et al.	2011	Transgenic Research 20, 665-673	Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms
54	Mao et al.	2013	Plant Molecular Biology 83, 119-129	Cysteine protease enhances plant-mediated bollworm RNA interference
55	Paim et al.	2013	Insect Biochemistry and Molecular Biology 43, 1015-1020	Long-term effects and parental RNAi in the blood feeder <i>Rhodnius prolixus</i> (Hemiptera; Reduviidae).
56	Pandey et al.	2008	Plant Physiology 147, 1212-1224	RNA-directed RNA Polymerase3 from <i>Nicotiana attenuata</i> is required for competitive growth in natural environments
57	Pant et al.	2015	Plant Signaling & Behavior 10, e977737	The syntaxin 31-induced gene, lesion simulating disease1 (LSD1), functions in Glycine max defense to the root parasite <i>Heterodera glycines</i>
58	Pant et al.	2014	Plant Molecular Biology 85, 107-121	Syntaxin 31 function in glycine max resistance to the plant-parasitic nematode <i>Heterodera glycines</i>
59	Niu et al.	2016	Scientific Reports 6, 19443	Msp40 effector of root-knot nematode manipulates plant immunity to facilitate parasitism

60	Papolu et al.	2013	PLoS ONE 8, e80603	Utility of host delivered RNAi of two FMRF amide like peptides, flp-14 and flp-18, for the management of root knot nematode, <i>Meloidogyne incognita</i>
63	Patel et al.	2010	Journal of Experimental Botany 61, 235-248	A nematode effector protein similar to annexins in host plants
64	Patel et al.	2008	Journal of Nematology 40, 299-310	Similarity and functional analyses of expressed parasitism genes in <i>Heterodera schachtii</i> and <i>Heterodera glycines</i>
65	Peng et al.	2016	Plos One 11, e0149959	Novel pectate lyase genes of <i>Heterodera glycines</i> play key roles in the early stage of parasitism
66	Pitino et al.	2011	Plos One 6, e25709	Silencing of aphid genes by dsRNA feeding from plants
67	Pitino and Hogenhout	2013	Molecular Plant-Microbe Interactions 26, 130-139	Aphid protein effectors promote aphid colonization in a plant-specific manner
68	Pogorelko et al.	2016	Molecular plant pathology 17, 832-44	A cyst nematode effector binds to diverse plant proteins, increases nematode susceptibility and affects root morphology
69	Rambo-Martin et al.	2009	In Vitro Cellular & Developmental Biology-Animal 45, S35-S35	Assessing RNAi gene targets of root-knot nematodes in composite transgenic soybean
70	Raza et al.	2016	Plos One 11, e0153883	RNA Interference based approach to downregulate osmoregulators of whitefly ( <i>Bemisia tabaci</i> ): potential technology for the control of whitefly
71	Porta et al.	2011	Insect Biochemistry and Molecular Biology 41, 513-519	Tobacco plants expressing the Cry1AbMod toxin suppress tolerance to Cry1Ab toxin of <i>Manduca sexta</i> cadherin-silenced larvae
72	Shabab et al.	2014	Febs Journal 281, 2769-2783	OPDA isomerase GST16 is involved in phytohormone detoxification and insect development
73	Shaheen et al.	2014	Pakistan Entomologist 36, 13-20	Screening and evaluation of insecticidal RNAi partial gene constructs in non-target insect species
74	Shang et al.	2016	Insect molecular biology 25, 422-30	Identification, characterization and functional analysis of a chitin synthase gene in the brown citrus aphid, <i>Toxoptera citricida</i> (Hemiptera, Aphididae)
75	Sindhu et al.	2009	Journal of Experimental Botany 60, 315-324	Effective and specific in planta RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success
76	Steeves et al.	2006	Functional Plant Biology 33, 991-999	Transgenic soybeans expressing siRNAs specific to a major sperm protein gene

				suppress <i>Heterodera glycines</i> reproduction
77	Tao et al.	2012	Molecular Ecology 21, 4371-4385	Gossypol-enhanced P450 gene pool contributes to cotton bollworm tolerance to a pyrethroid insecticide
78	Thakur et al.	2014	Plos One 9, e87235	Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of V-ATPase A gene
79	Tian et al.	2015	International Journal of Biological Sciences 11, 1296-1305	Transgenic cotton plants expressing double-stranded RNAs target HMG-CoA reductase (HMGR) gene inhibits the growth, development and survival of cotton bollworms
80	Tsygankova et al.	2013	Cytology and Genetics 47, 222-230	Increasing the resistance of rape plants to the parasitic nematode <i>Heterodera schachtii</i> using RNAi technology
81	Tzin et al.	2015	Journal of Insect Physiology 79, 105-112	RNA interference against gut osmoregulatory genes in phloem-feeding insects
82	Urquhart et al.	2015	Regulatory Toxicology and Pharmacology 73, 607-612	A novel method of demonstrating the molecular and functional equivalence between in vitro and plant-produced double-stranded RNA
83	Valentine et al.	2007	Plant Biotechnology Journal 5, 827-834	Delivery of macromolecules to plant parasitic nematodes using a tobacco rattle virus vector
84	Vandenborre et al.	2010	Journal of Experimental Botany 61, 1003-1014	<i>Nicotiana tabacum</i> agglutinin is active against Lepidopteran pest insects
85	Vastenhouw et al.	2006	Nature (London) 442, 882	Gene expression: long-term gene silencing by RNAi
86	Vargas et al.	2008	Virology Journal 5	Transient expression of homologous hairpin RNA interferes with PVY transmission by aphids
87	Van Eck et al.	2010	Plant Biotechnology Journal 8, 1023-1032	Virus-induced gene silencing of WRKY53 and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance
88	Vieira et al.	2015	Plos One 10, e0144674	The <i>Pratylenchus penetrans</i> transcriptome as a source for the development of alternative control strategies: mining for putative genes involved in parasitism and evaluation of <i>in planta</i> RNAi
89	Walawage et al.	2013	Bmc Genomics 14	Stacking resistance to crown gall and nematodes in walnut rootstocks
90	Wan et al.	2014	Journal of Insect Physiology 71, 105-113	A putative Delta(1)-pyrroline-5-carboxylate synthetase involved in the biosynthesis of proline and arginine in <i>Leptinotarsa decemlineata</i>
91	Wan et al.	2015	Pest Management Science 71, 1387-1396	Knocking down a putative Delta(1)-pyrroline-5-carboxylate dehydrogenase gene by RNA interference inhibits flight and causes adult lethality in the Colorado potato beetle

				<i>Leptinotarsa decemlineata</i> (Say)
92	Wang	2009	Plant Science 176, 279-285	Monogalactosyldiacylglycerol deficiency affects jasmonic acid biosynthesis and defense responses to insect herbivores in <i>Nicotiana tabacum</i>
93	Woldemariam et al.	2012	Plant Journal 72, 758-767	Jasmonoyl-l-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-l-isoleucine levels and attenuates plant defenses against herbivores
94	Wuriyanghan and Falk	2013	Plos One 8, e66050	RNA interference towards the potato psyllid, <i>Bactericera cockerelli</i> , is induced in plants infected with recombinant Tobacco mosaic virus (TMV)
95	Xie et al.	2016	Frontiers in Plant Science 7	A novel <i>Meloidogyne incognita</i> effector Misp12 suppresses plant defense response at latter stages of nematode parasitism
96	Xiong et al.	2013	International Journal of Biological Sciences 9, 370-381	Silencing the HaHR3 gene by transgenic plant-mediated RNAi to disrupt <i>Helicoverpa armigera</i> development
97	Xu et al.	2014	Transgenic Research 23, 389-396	Silencing of an aphid carboxylesterase gene by use of plant-mediated RNAi impairs <i>Sitobion avenae</i> tolerance of Phoxim insecticides
98	Xue et al.	2013	Phytopathology 103, 175-181	The 8D05 parasitism gene of <i>Meloidogyne incognita</i> is required for successful infection of host roots
99	Yadav et al.	2006	Molecular and Biochemical Parasitology 148, 219-222	Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection
100	Yang et al.	2013	Plos One 8, e69463	Molecular characteristics and efficacy of 16D10 siRNAs in inhibiting root-knot nematode infection in transgenic grape hairy roots
101	Youssef et al.	2013	Experimental Parasitology 134, 266-274	Post-transcriptional gene silencing of the gene encoding aldolase from soybean cyst nematode by transformed soybean roots
102	Yu et al.	2014	International Journal of Biological Sciences 10, 1171-1180	The insect ecdysone receptor is a good potential tTarget for RNAi-based pest control
103	Zha et al.	2011	Plos One 6, e20504	Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the Hemipteran Insect <i>Nilaparvata lugens</i>
104	Zhang et al.	2015	Science 347, 991-994	Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids
105	Zhu et al.	2012	PLoS ONE 7, e38572	Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR

## Appendix D – List of publications considered relevant for Task 3 “Environmental exposure and fate of dsRNA siRNA and miRNA” – Environmental fate

No.	Authors	Year	Journal, volume, pages	Title	Relevance
1	Ashe et al.	2012	Cell (Cambridge) 150, 88-99	piRNAs can trigger a multigenerational epigenetic memory in the germline of <i>Caenorhabditis elegans</i>	Multigenerational epigenetic effects
2	Ashe et al.	2015	Journal of Virology 89, 12035-12046	Antiviral RNA interference against Orsay virus ss neither systemic nor transgenerational in <i>Caenorhabditis elegans</i>	Lack of transgenerational effects
3	Bosch et al.	2016	Genetics 203, 109–118	Persistence of RNAi-Mediated knockdown in <i>Drosophila</i> complicates mosaic analysis yet enables highly sensitive lineage tracing	Transgenerational effects/Epigenetics
4	Buckley et al.	2012	Nature (London) 489, 447-451	A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality	Transgenerational effects/Epigenetics
5	Burton et al.	2011	Proceedings of the National Academy of Sciences of the United States of America 108, 19683-19688	Nuclear RNAi maintains heritable gene silencing in <i>Caenorhabditis elegans</i>	Transgenerational effects/Epigenetics
6	Dubelman et al.	2014	Plos One 9, e93155	Environmental fate of double-stranded RNA in agricultural soils	Environmental fate
7	Gamboa Cedeno et al.	2015	Journal of Apicultural Research 54, 99-100	Double-stranded RNA synthesized in bacteria can be transferred to bee and <i>Varroa</i> tissues	Trophic chains
8	Garbian et al.	2012	Plos Pathogens 8, e1003035	Bidirectional transfer of RNAi between honey bee and <i>Varroa destructor</i> : <i>Varroa</i> gene silencing reduces <i>Varroa</i> population	Trophic chains
9	Grishok et al.	2000	Science 287, 2494-2497	Genetic requirements for inheritance of RNAi in <i>Caenorhabditis elegans</i>	Transmission of effects to offsprings
10	Houri-Ze'evi et al.	2016	Cell 165, 88-99	A tunable mechanism determines the duration of the transgenerational small RNA inheritance in <i>Caenorhabditis elegans</i>	Transgenerational effects
11	Itakura et al.	2009	Sociobiology 54, 77-87	RNA Interference in symbiotic protists of the termite <i>Coptotermes formosanus</i> (Isoptera: Rhinotermitidae) through ingestion of siRNA by the host termite	Tritrophic
12	Jia et al.	2015	Scientific Reports 5, 12290	Nonfunctional ingestion of plant miRNAs in silkworm revealed by digital droplet PCR and transcriptome analysis	Cross-kingdom transfer of endogenous RNA



13	Khajuria et al.	2015	Insect Biochemistry and Molecular Biology 63, 54-62	Parental RNA interference of genes involved in embryonic development of the western corn rootworm, <i>Diabrotica virgifera virgifera</i> LeConte	Transgeneration
14	Li et al.	2015	Plant Cell and Environment 38, 2277-2285	New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests	dsRNA stability under different conditions
15	San Miguel and Scott	2016	Pest Management Science 72, 801-809	The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide	Persistence after plant spray or absorption

## Appendix E – List of publications deemed relevant for Task 5 “Off target, non-target and unintended effects of RNAi-based GM plants”

No.	Authors	Year	Journal, volume, pages	Title	Relevance
1	Ahmad et al.	2016	Transgenic research 25, 1-17	Transportable data from non-target arthropod field studies for the environmental risk assessment of genetically modified maize expressing an insecticidal double-stranded RNA	Non-target
2	Antonino de Souza Junior et al.	2013	PLoS ONE 8, e85364	Knocking-Down <i>Meloidogyne incognita</i> Proteases by Plant-Delivered dsRNA Has Negative Pleiotropic Effect on Nematode Vigor	Off-target
3	Asokan et al.	2012	Current Science 102, 1692-1699	Common siRNAs for various target genes of the fruit borer, <i>Helicoverpa armigera</i> Hubner (Lepidoptera: Noctuidae)	Remedies/minimizing off-target
4	Bachman et al.	2013	Transgenic Research 22, 1207-1222	Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm ( <i>Diabrotica virgifera virgifera</i> LeConte)	Specificity
5	Chen et al.	2015	Ecotoxicology 24, 595-603	The effects of RNA interference targeting <i>Bactrocera dorsalis</i> ds-Bdrpl19 on the gene expression of rpl19 in non-target insects	Non-target
6	Chen et al.	2011	Developmental and Comparative Immunology 35, 661-671	Identification and functional characterization of Dicer2 and five single VWC domain proteins of <i>Litopenaeus vannamei</i>	Immune stimulation
7	Danchin et al.	2013	PLoS Pathogens 9, e1003745	Identification of Novel Target Genes for Safer and More Specific Control of Root-Knot Nematodes from a Pan-Genome Mining	Non-target Bioinformatics
8	DasGupta et al.	2007	Genome Biology 8, R203	A case study of the reproducibility of transcriptional reporter cell-based RNAi screens in <i>Drosophila</i>	Non-target Bioinformatics
9	Deddouche et al.	2008	Nature Immunology 9, 1425-1432	The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in <i>Drosophila</i>	Immune stimulation
10	Dong et al.	2009	Fish & Shellfish Immunology 26, 438-442	The immune responses in Chinese mitten crab <i>Eriocheir sinensis</i> challenged with double-stranded RNA	Immune stimulation
11	Fisher et al.	2012	BMC Genomics 13, nr. 506	Advances in genome-wide RNAi cellular screens: a case study using the <i>Drosophila</i> JAK/STAT pathway	Off-target Bioinformatics
12	Flenniken et al.	2013	PLoS One 8, e77263	Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee	Immune stimulation

13	Gamboa Cedeno et al.	2015	Journal of Apicultural Research 54, 99-100	Double-stranded RNA synthesized in bacteria can be transferred to bee and <i>Varroa</i> tissues	Trophic chain
14	Garbian et al.	2012	Plos Pathogens 8, e1003035	Bidirectional Transfer of RNAi between Honey Bee and <i>Varroa destructor</i> : <i>Varroa</i> Gene Silencing Reduces <i>Varroa</i> Population	Trophic chain
15	Garbutt et al.	2012	Insect Biochemistry and Molecular Biology 42, 621-628	Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference	Immune stimulation
16	Hirai et al.	2004	Insect Molecular Biology 13, 399-405	Baculovirus and dsRNA induce Hemolin, but no antibacterial activity, in <i>Antheraea pernyi</i>	Immune stimulation
17	Jarosch et al.	2012	Apidologie 43, 128-138	RNA interference in honeybees: off-target effects caused by dsRNA	Off-target
18	Kingsolver et al.	2013	Journal of Molecular Biology 425, 4921-4936	Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections	Immune stimulation
19	Kitzman et al.	2013	BMC Genomics 14, nr. 5	RNAi phenotypes are influenced by the genetic background of the injected strain	Resistance mechanism
20	Kola et al.	2016	Frontiers in Physiology 7, nr. 20	Silencing of CYP6 and APN Genes Affects the Growth and Development of Rice Yellow Stem Borer, <i>Scirpophaga incertulas</i>	Non-target
21	Labreuche et al.	2010	Developmental and Comparative Immunology 34, 1209-1218	Non-specific activation of antiviral immunity and induction of RNA interference may engage the same pathway in the Pacific white leg shrimp <i>Litopenaeus vannamei</i>	Immune stimulation
22	Levine et al.	2015	PLoS ONE 10, e0118622	Independent Action between DvSnf7 RNA and Cry3Bb1 Protein in Southern Corn Rootworm, <i>Diabrotica undecimpunctata howardi</i> and Colorado Potato Beetle, <i>Leptinotarsa decemlineata</i>	Non-target
23	Liu et al.	2013	Journal of Insect Physiology 59, 646-654	Transcriptional response of BmTo119-1 and RNAi machinery genes to exogenous dsRNA in the midgut of <i>Bombyx mori</i>	Immune stimulation
24	Luan et al.	2012	PLoS ONE 7, e45163	The Unique GGA Clathrin Adaptor of <i>Drosophila melanogaster</i> Is Not Essential	Off-target
25	Lozano et al.	2012	Development Genes and Evolution 222, 229-235	Super-induction of Dicer-2 expression by alien double-stranded RNAs: an evolutionary ancient response to viral infection?	Immune stimulation
26	Moffat et al.	2007	Trends in Pharmacological Sciences 28, 149-151	Off-target effects associated with long dsRNAs in <i>Drosophila</i> RNAi screens	Off-target

27	Nunes et al.	2013	Insects 4, 90-103	Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays	Non-target/off-target
28	Piot et al.	2015	Viruses 7, 3172-3185	The Effect of Oral Administration of dsRNA on Viral Replication and Mortality in <i>Bombus terrestris</i>	Immune stimulation
29	Qiu et al.	2005	Nucleic Acids Research 33, 1834-1847	A computational study of off-target effects of RNA interference	Off-target
30	Robalino et al.	2007	Physiological Genomics 29, 44-56	Insights into the immune transcriptome of the shrimp <i>Litopenaeus vannamei</i> : tissue-specific expression profiles and transcriptomic responses to immune challenge	Immune stimulation
31	Robalino et al.	2005	Journal of Virology 79, 13561-13571	Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: Convergence of RNA interference and innate immunity in the invertebrate antiviral response?	Immune stimulation
32	Rual et al.	2007	BMC Genomics 8, nr. 106	Novel insights into RNAi off-target effects using <i>C-elegans</i> paralogs	Off-target Bionformatics
33	Runo et al.	2011	Pest Management Science 67, 129-136	RNA interference as a resistance mechanism against crop parasites in Africa: a 'Trojan horse' approach	Specificity
34	Seinen et al.	2011	Briefings in Functional Genomics 10, 206-214	RNAi-induced off-target effects in <i>Drosophila melanogaster</i> : frequencies and solutions	Remedies/minimizing off-target
35	Seinen et al.	2010	PLoS ONE 5, e13119	RNAi Experiments in <i>D. melanogaster</i> : Solutions to the Overlooked Problem of Off-Targets Shared by Independent dsRNAs	Remedies/minimizing off-target
36	Su et al.	2009	Aquaculture 289, 1-5	Grass carp reovirus activates RNAi pathway in rare minnow, <i>Gobiocypris rarus</i>	Immune stimulation
37	Velez et al.	2016	PLoS ONE 11, e0157520	Knockdown of RNA Interference Pathway Genes in Western Corn Rootworms ( <i>Diabrotica virgifera virgifera</i> Le Conte) Demonstrates a Possible Mechanism of Resistance to Lethal dsRNA	Non-target
38	Whyard et al.	2009	Insect Biochemistry and Molecular Biology 39, 824-832	Ingested double-stranded RNAs can act as species-specific insecticides	Specificity
39	Zhang et al.	2015	Entomologia Experimentalis Et Applicata 155, 218-228	Lepidopteran insect species-specific, broad-spectrum, and systemic RNA interference by spraying dsRNA on larvae	Specificity
40	Zhou et al.	2014	Genetics 197, 121-132	Nuclear RNAi Contributes to the Silencing of Off-Target	Off-target

41	Zhu et al.	2012	PLoS ONE 7, e38572	Genes and Repetitive Sequences in <i>Caenorhabditis elegans</i> Improvement of Pest Resistance in Transgenic Tobacco Plants Expressing dsRNA of an Insect-Associated Gene EcR	Non-target
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