National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Risk assessment of plant protection products based on **dsRNA/RNAi**

RIVM report 2023-0456
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DOI 10.21945/RIVM-2023-0456

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This investigation was performed by order, and for the account, of the Ministry of Agriculture, Nature and Food Quality, within the framework of the programme 10B.4.3

Published by:
National Institute for Public Health and the Environment, RIVM
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

Synopsis

Risk assessment of plant protection products based on dsRNA/RNAi

Plant protection products are used to protect crops against diseases and pests. The EU Member States are looking to make agriculture more sustainable, among other things by using fewer chemicals. To that end, new plant protection products are being developed that are less harmful to the environment, growers, and consumers.

One of these developments concerns plant protection products on the basis of double-stranded RNA (dsRNA). These are natural alternatives to chemical pesticides that work specifically against certain diseases and pests. Plant protection products are assessed for their safety for humans and the environment before they receive market authorisation. However, it turns out that the existing risk assessment is unsuitable for assessing the potentially harmful effects of this latest type of plant protection products, as it focuses primarily on the effects of chemicals.

RIVM has made a number of recommendations to facilitate the assessment of dsRNA-based plant protection products. Among other things, it advises risk assessors about which data they need from the existing assessment methods. RIVM has also recommended assessing the entire product in order to evaluate its effects on the environment. Normally, dsRNA degrades rapidly in the environment, causing little exposure. When used in a plant protection product, however, it may be more stable.

For the purpose of this study, RIVM investigated which applications on the basis of dsRNA are currently in development. It also looked at the risks that dsRNA might pose for human health and the environment.

Keywords: plant protection products, dsRNA, RNAi, risk assessment

Publiekssamenvatting

Risicobeoordeling van gewasbeschermingsmiddelen op basis van dsRNA/RNAi

Gewasbeschermingsmiddelen beschermen landbouwgewassen tegen ziekten en plagen. Binnen Europa willen lidstaten de landbouw verduurzamen, onder andere door minder chemische middelen te gebruiken. Daarom worden nieuwe gewasbeschermingsmiddelen ontwikkeld die minder schadelijk zijn voor het milieu, de gebruikers van de middelen en voor consumenten.

Zo zijn gewasbeschermingsmiddelen op basis van dubbelstrengs RNA (dsRNA) in ontwikkeling. Dit zijn natuurlijke alternatieven voor chemische bestrijdingsmiddelen die specifiek tegen bepaalde ziekten en plagen werken. Deze producten worden beoordeeld op hun veiligheid voor mens en milieu, voordat ze op de markt worden toegelaten. Alleen blijkt dat de bestaande risicobeoordeling niet geschikt is om mogelijke schadelijke effecten van dit type gewasbeschermingsmiddelen te beoordelen. Deze is nu vooral gericht op effecten van chemische stoffen.

Het RIVM doet aanbevelingen om de beoordeling van dsRNA-middelen mogelijk te maken. Het adviseert onder andere aan risicobeoordelaars welke data uit de bestaande testen voor de beoordeling nodig zijn. Ook raadt het RIVM aan het hele product te beoordelen om de effecten op het milieu te kunnen onderzoeken. dsRNA breekt namelijk snel af in het milieu, waardoor het milieu er weinig aan wordt blootgesteld. Maar in een product kan het stabieler zijn.

Voor dit onderzoek heeft het RIVM in kaart gebracht welke toepassingen op basis van dsRNA in ontwikkeling zijn. Ook is gekeken welke risico’s van dsRNA er voor mens en milieu zouden kunnen zijn.

Kernwoorden: gewasbeschermingsmiddelen, dsRNA, RNAi, risicobeoordeling

Contents

Summary

Due to advancing developments in biotechnology, new crop protection applications are becoming available. For example, plant protection products based on dsRNA/RNAi are regarded as one of the possibilities to drive towards more sustainable farming, while at the same time allowing farmers to combat pests. In cells, dsRNA is broken down to short pieces of single stranded RNA which can interfere very specifically with the production of proteins in the cells. By targeting protein expression essential for the growth, development or reproduction of pests, plant protection products based on dsRNA can very selectively target plant pests.

To facilitate the approval of dsRNA-based plant protection products it is important that an assessment framework is in place, in order to assess the potential risks of these products. The current regulatory framework under Regulation (EC) 1107/2009 contains data requirements for either chemical substances or for living microorganisms such as bacteria, fungi and viruses. The aim of this study was to assess whether the current data requirements for plant protection products are suitable for assessing the potential risks of new biological techniques such as dsRNA/RNAi, and to make recommendations on how the requirements might be improved.

To this end, RIVM investigated which types of biotechnological application of dsRNA-based plant protection products are available or will be available soon. This overview was used to assess if specific risks of these products for human health and the environment need to be taken into account, and how these risks can be investigated in a regulatory context. This information was used to determine if the current assessment methods for plant protection products are suitable for dsRNA.

Research on the use of RNA interference in plant protection has shown promising results, for example in applications against the Colorado potato beetle. To increase the efficacy of topical applications of dsRNA, many developments on the formulation of dsRNA-based products are also taking place. An example is increasing the stability of dsRNA-based products in the field, which has been a limiting factor for spray application of these products.

Based on the available information on the potential risks of dsRNA and considering the low stability and bioavailability of dsRNA in the environment, it is clear that the risk assessment requires a different approach than that of conventional chemicals. Potential elements to consider in the risk assessment of dsRNA are sequence similarity and immune stimulation. The potential risks are expected to be lower for unformulated/unmodified dsRNA, as this rapidly degrades in the environment and shows low systemic availability. However, formulation or modification methods may increase the stability and increase the need for information on the potential risk. The production method of the dsRNA can also lead to certain elements that need to be considered in

the risk assessment. For example, if genetically engineered microorganisms are used in the production, the presence of toxins, microbial contaminants and viable microorganisms should be considered, similar to the risk assessment of microbial plant protection products.

Specific recommendations are made in this report on how these potential risks of dsRNA-based plant protection products used as a spray application may be addressed. Overall, the recommendations made provide guidance to risk assessors on how the environmental and human health risk assessment of dsRNA-based plant protection products should be conducted.

1 Introduction

Background

The EU Farm to Fork strategy[1], which is at the heart of the European Green Deal, aims to reduce the use of hazardous pesticides and to facilitate the placing of low-risk plant protection products and biopesticides on the market. At a national level, the Dutch "Uitvoeringsprogramma Toekomstvisie gewasbescherming 2023[2]" also identified the need to focus on improving the approval procedure for active substances with a low-risk profile. To be able to promote the availability of low-risk and biological plant protection products, it is important to ensure that the regulatory frameworks are ready for these products.

Due to advancing developments in biotechnology, new crop protection applications which can selectively suppress plant diseases and pests are becoming available. Plant protection products based on double stranded RNA (dsRNA) are seen as one of the opportunities to drive towards more sustainable farming, using fewer chemical plant protection products while at the same time allowing farmers to combat pests. These products are expected to play a bigger part in protecting crops in the future. To facilitate the approval of dsRNA-based plant protection products in the EU, an assessment framework needs to be in place to determine if these products do not pose a risk to humans and to the environment.

The current regulatory framework under Regulation (EC) 1107/2009 contains data requirements for either chemical substances or living microorganisms, such as bacteria, fungi and viruses. The aim of this study was to assess whether the current data requirements for plant protection products are suitable for assessing the potential risks of new biological techniques, based on dsRNA/RNAi. To achieve this aim the following research questions were studied:

1. What new biotechnological applications to protect plants from diseases and insects, based on dsRNA, are available or will be available in the short term?
2. What information is available on the potential risks of dsRNA to humans and the environment, e.g. based on available risk assessment methods for dsRNA applied by the OECD or the EU or on public literature information?
3. To what extent do the current EU data requirements for the evaluation of plant protection products cover the risks of dsRNA-based products?
4. What changes in the EU data requirements for plant protection products are needed to cover the potential risk of dsRNA-based products?

[1] https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en
[2] https://www.rijksoverheid.nl/documenten/kamerstukken/2020/09/28/uitvoeringsprogramma-toekomstvisie-gewasbescherming-2030

In view of the current developments in biotechnological applications based on dsRNA, the focus for the environmental risk part of this report is on the use of dsRNA against insect pests on crops and on the application of dsRNA as a spray. The recommendations in the human health risk assessment can be applied in a broader context, as these are less related to the target organism or application method.

Setup of the report

Chapter 2 describes the methodology used to create this report. The results section starts with an overview of available or soon to be available biotechnological applications of dsRNA-based plant protection products (chapter 3). This overview served to get a sense of the type of products for which an approval may be requested in the near future, and to assess if specific risks of these products need to be taken into account. The results section also gives an overview of the available information on potential environmental and human health risks of dsRNA products, and how these can be investigated in a regulatory context (chapter 4 and 5). After that it addresses the question if the current EU data requirements for plant protection products sufficiently cover the potential risk of dsRNA based products, or if changes are needed (chapter 6). The report ends with a discussion summarising the results (chapter 7) and the conclusions and recommendations (chapter 8).

2 Method

Two OECD working documents were used as a starting point in developing this report: the OECD working document on the considerations for the environmental risk assessment of the application of spray or externally applied dsRNA-based pesticides (ENV/JM/MONO(2020)26), and the OECD working document on the considerations for the human health risk assessment of externally applied dsRNA-based pesticides (ENV/JM/MONO(2023)26). Additionally, the summary document on the discussions from the 2019 OECD conference on RNAi based pesticides was used (Mendelsohn et al., 2020). Based on these publications, a first assessment on the potential environmental and human health risks of dsRNA-based plant protection products was made. This information was expanded with information derived from public literature. To this end the SCOPUS database was searched, using the search terms provided in the table below.

The literature research did not just focus on environmental and human health risks, but also included search terms relating to the biotechnological application of dsRNA-based plant protection products (keywords for chapter 3).

Table 1 Search terms used in the literature search.

Chapter	**Keywords**
Chapter 3	(dsRNA OR RNA interference OR RNAi OR spray induced gene silencing) AND (plant protection product OR pesticide OR crop protection)
	dsRNA AND commercial use OR prospects OR formulation technology
Chapter 4	dsRNA AND (Non-target effects) OR (ecological impact) OR (lethal dose) OR (saturation RNAi machinery)
	Environmental Risk Assessment AND dsRNA OR problem formulation AND dsRNA
	dsRNA stability AND transfer OR foliar uptake
	dsRNA AND ((Non-target organisms) OR developmental stage OR introduction environment OR immune response OR allergenicity)
	Bioinformatics AND dsRNA AND NON-target organism OR dsRNA fragment length
Chapter 5	dsRNA AND (human or mammals) AND (adverse OR human health OR toxicity OR allergenicity)
	dsRNA AND exposure AND human AND (plant protection)
Chapter 4 and 5	dsRNA AND dose quantification OR dose selection OR oral/dietary exposure OR study duration

The retrieved hits were compiled in separate endnote files. All hits were screened for their relevance, based on the title of the publication. Based on that screening, publications which were clearly not relevant to address the research questions of this study were rejected. Relevant publications and publications of undetermined relevance were further screened on the basis of the abstract. The full list of publications used in this study can be found in the list of references in chapter 9.

Based on the available information on the potential hazards of dsRNA, an assessment was made of the current data requirements for active substances (Regulation (EU) 283/2013) and products (Regulation (EU) 284/2013) to determine if they are sufficient to cover potential environmental and human health risks.

3 Spray Induced Gene Silencing

3.1 The mechanisms of RNA interference

RNA interference (RNAi) is an evolutionary conserved mechanism found in most eukaryotic organisms including animals, plants and fungi to defend themselves against pathogens and to regulate expression levels of their own genes (Guo et al., 2016, Koeppe, Kawchuk and Kalischuk 2023). Taking plants as an example, RNAi is initiated by the presence of free double-stranded RNA molecules (dsRNA) in a plant cell (Fletcher et al., 2020). The origin of this dsRNA is important for the response in the plant. dsRNA originating from the plant's own genome can be processed by the RNAi machinery to regulate its own gene expression levels. This is a common process in the growth and development of the plant (Guo et al., 2016). Alternatively, if the dsRNA originates from a different biological entity, for example a virus, the plant will recognise it as such and use the RNAi machinery to prevent the expression of potentially harmful messenger RNA (mRNA) sequences (Fletcher et al., 2020). This is the concept behind AbioProtect, a plant protection product based on mild, wild type strains of the *Pepino Mosaic Virus,* that prepares the plant for a subsequent systemic infection with more virulent strains of this virus (EFSA et al., 2021). Although effective, plants do not need to be exposed to an entire virus or microorganism to recognise it as a potential threat. A small RNA fragment can be enough for an effective RNAi response.

The RNAi machinery

The first step in RNAi, is for Dicer or Dicer-like proteins to cut dsRNA into smaller pieces of 20 to 24 base pairs called small interfering RNA (siRNA). After this, siRNA attaches to a multi-protein complex called the RNA-induced silencing complex (RISC). This protein complex binds to mRNA with the same sequence as the incorporated siRNA molecule and degrades the mRNA upon hybridisation, thereby preventing the translation of mRNA into protein (Fletcher et al., 2020).

Plants are vulnerable to a wide range of pests and pathogens, resulting in significant yield losses for the agricultural sector. Conventional crop management with chemicals is challenged by resistance incidence and environmental and human health concerns, stimulating research on alternative, more sustainable methods (Ghag 2017). RNAi is an adaptive system that can be used to target predetermined RNA sequences based on homology with the dsRNA sequence that initiates the RNAi response. RNAi is an interesting mechanism for researchers to investigate as an alternative to chemical products, because of its potential to ward of pests and pathogens.

HIGS and SIGS, two different RNAi approaches
The initial experimental approach to use RNAi for plant protection was based on genetically modified plants expressing dsRNA-constructs which - through the mechanism of RNAi - prevented the expression of essential genes in pests and pathogens or the expression of plant genes

that make the plant vulnerable to infections, so called susceptibility genes (Ghag 2017). RNAi based on genetic modification of plants is called 'host induced gene silencing' (HIGS). The genetically modified plant contains - or 'hosts' - a DNA-construct that results in the production of a dsRNA construct from within the plant cells. This dsRNA is readily available to the plant's RNAi machinery to process into siRNA and can be used to silence the expression of a specific mRNA target sequence (Ghag 2017). The process of developing a genetically modified plant is a long and costly process and requires efficient plant transformation protocols (Ray et al., 2022). A non-GMO based RNAi method with proven potential as a crop protection product, is 'spray induced gene silencing' (SIGS). For SIGS, the dsRNA is produced separate from the plants, using an *in vitro* production system. The produced dsRNA is then externally applied on the plant's surface (De Schutter et al., 2022). For example, the dsRNA may then be ingested by herbivorous insects feeding on treated plant tissue. This is lethal to the insect, if the dsRNA is designed to silence the expression of a gene that is essential for the life cycle of the insect (Kunte et al., 2020, Ray et al., 2022). It is also possible for topically applied dsRNA to enter plant cells and be processed into siRNA by the plant's RNAi machinery. However, efficient delivery of dsRNA into plant cells is challenging, due to the presence of a cuticle, cell wall and plasma membrane (Rêgo-Machado, Inoue-Nagata and Nakasu 2022).

3.2 Current use of RNAi in crop protection management

HIGS and SIGS are two approaches to RNAi, but only HIGS is currently commercially used. HIGS plants are already authorised in Europe for direct use or for processing in food or feed (but not for cultivation). For example, Vistive gold soybean (MON 87705) is a commercially available plant for food and feed uses in Europe which uses RNAi to alter the fatty acid biosynthesis pathway in soybeans (EFSA 2012). HIGS is also used to protect commercially available crops from pests and pathogens. In 2017, SmartStax Pro maize (MON 87411) was the first European authorised HIGS plant for food and feed purposes using RNAi against a pest organism (EFSA 2018a). SmartStax Pro maize expresses a dsRNA construct that, through the plants RNAi machinery, is processed into active siRNAs targeting the *Snf7* gene (required for transport of transmembrane proteins) in *Diabrotica virgifera virgifera*, also known as Western corn rootworm. When the worm feeds on the SmartStax Pro maize, it ingests the siRNA, resulting in the silencing of the *Snf7* gene, causing mortality (Head et al., 2017, EFSA 2018a). SIGS on the other hand is not yet commercially available for crop protection management, but commercial parties have been showing increasing interest in this method. The development of dsRNA sprays offers a way to use RNAi in crop protection at reduced costs and production time, compared to developing genetically modified HIGS plants (Ray et al., 2022). The first dsRNA sprays are currently in development and requests for market approval are expected in the near future. SIGS does not require any genetic modification of the plants. Authorisation is therefore not regulated via GMO legislations (Taning et al., 2020). This leads to the need for a regulatory framework to assess the environmental and human health risks of dsRNA sprays.

3.3 Enhancing stability and delivery efficiency for SIGS

The external application of dsRNA on plants comes with specific challenges, mainly associated with the delivery efficiency of the dsRNA to the target organism and the overall stability of the dsRNA in the environment (Ray et al., 2022). Rapid degradation of dsRNA under environmental conditions such as UV radiation, humidity and exposure to ribonucleases creates a bottleneck for dsRNA sprays in crop protection management (Bachman et al., 2020, Ray et al., 2022). One of the main focuses of SIGS research is to enhance the stability and delivery efficiency of dsRNA. There is a variety of methods to solve this problem. One of the potential solutions is to attach dsRNA to small carrier particles with properties that enhance the stability or delivery efficiency of the dsRNA. These carriers are often referred to as nanoparticles and come in different shapes and sizes. Many nanoparticles find their origin in medical studies and have recently been evaluated to determine if they can also be used for the delivery of dsRNA to crops (Ray et al., 2022).

- **Layered double hydroxide (LDH) clay nanosheets**
  LDH clay nanosheets (LDHs) are biodegradable ionic particles consisting of layers of positively and negatively charged ions. These sheet-like clay nanoparticles have an average particle size of 80 to 300 nm (Mitter et al., 2017a). The negatively charged ions are weakly bound and can be replaced by other negatively charged ions, such as dsRNA. As the LDHs gradually break down over time, they release dsRNA which has been shown to be successful at initiating the RNAi response to protect plants from viral infections for up to 20 days after application (Mitter et al., 2017a). Recent work with dsRNA loaded onto LDHs used SIGS to protect plants in greenhouses against a fungal infection for up to 60 days (Mosa and Youssef 2021).
- **Carbon dots and nanotubes**
  Carbon dots are a particularly small type of nanocarriers with a size of 1 to 10 nanometers. Carbon dots are formed by hydrothermal reactions or pyrolysis of carbon precursors, such as organics sugars, acids or amino acids (Schwartz et al., 2019). Unlike LDHs, carbon dots are not inherently able to bind dsRNA. Carbon dots need to be functionalised by coating them with a substance that is capable of binding dsRNA. Due to their small size, carbon dots are able to diffuse through the cell wall of plants, something LDHs can’t do. On the other hand, because of their small size carbon dots can only carry dsRNA constructs which are shorter compared to the ones on LDHs (Schwartz et al., 2019). A variation on the carbon dots are the nanotubes. Nanotubes have different dimensions compared to carbon dots, with a diameter of 0.8-1.2 nanometers and a length of 500 to 1,000 nm. These particles have a larger surface area for dsRNA to attach to, while still having dimensions that enable them to passively traverse into plant cells (Demirer et al., 2019).

Nanoparticles may be an essential component in the formulation of future dsRNA sprays. Prior to submitting their application for authorisation, commercial parties are generally secretive about the composition of their product formulations. Since there are currently no authorisations in Europe for dsRNA sprays, it is unclear whether or not nanoparticles will be commonly used for SIGS products and if there is a clear trend towards their use.

Besides the use nanoparticles there are other methods to protect dsRNA from degradation and to enhance delivery efficiency. One method is to alter the structure of the RNA itself. Paperclip RNA (pcRNA) is an example of how a different RNA structure can alter cellular uptake. This is a dsRNA construct with two partially closed ends, that has been shown to have a different - but as yet unknown - uptake route in mosquitos, compared to conventional dsRNA constructs with open ends (Abbasi et al., 2020). Some commercial parties are working on alternative RNA structures to improve translocation across cellular membranes and to improve stability (Trillium Ag and NanoSUR) (Taning et al., 2020).

Another method to enhance RNA stability is to encapsulate it like a package. Minicells are small bacteria-like cells formed as the result of asymmetrical cell division in bacteria, and can be used to encapsulate dsRNA. Minicells contain ribosomes, RNA and proteins but no chromosomal DNA. The absence of this DNA removes their ability to divide and grow (Islam et al., 2021). Like other carriers, minicells have previously been used in studies into animal cells, and recent studies are investigating the potential of minicells as a delivery system for dsRNA (Islam et al., 2021, Necira et al., 2021). AgroSpheres is a company specialised in the production of biodegradable capsules, which are expected to be based on bacterial minicells. Recent work has shown the effectivity of minicell-encapsulated dsRNA to protect plants against viral and fungal infections (Islam et al., 2021, Necira et al., 2021). A detailed description of the composition and content of the minicells was not provided for both studies.
In nature, encapsulation of RNA happens in plants and animals via extracellular vesicles (EVs) allowing transport of RNA between cells. Pharmaceuticals already make use of recent advances in synthetic liposome synthesis to make vesicles for drug delivery. Artificial nanovesicles (AVs) are synthetic liposomes used to encapsulate dsRNA, and have recently been used for gene-targeting pathogenic fungi (Qiao et al., 2023). The AVs were shown to be effective at preventing early RNA degradation.

3.4 The first dsRNA sprays

Experimental studies on SIGS for crop protection were aimed to target essential genes in a wide variety of plant pathogenic viruses, fungi and pest insects (Huvenne and Smagghe 2010, Gebremichael et al., 2021, Rêgo-Machado, Inoue-Nagata and Nakasu 2022). Successful reduction of infection severity or of damage to the plants as a result of SIGS have been reported for each of these groups. Despite the successful experimental studies on SIGS to combat viruses and fungi, there are currently only notifications for the commercialisation of dsRNA sprays related to insects. The susceptibility to RNAi varies greatly among

different orders of insects. The order of the Coleoptera (beetles) is considered to be highly susceptible to RNAi (Kunte et al., 2020). The potential of RNAi to control the Colorado potato beetle (CPB) was shown in multiple studies (Rodrigues et al., 2021, Pallis et al., 2022). A company by the name of GreenLight Biosciences is currently developing a dsRNA spray targeting the CPB under the product name Ledprona.

GreenLight Biosciences dsRNA products

- Ledprona is a 490 base pair long dsRNA construct targeting mRNA coding for the production of a proteasome in the CPB. Without this proteasome, the CPB will die due to protein aggregation in its cells. The efficiency of Ledprona has been tested on CPB larvae in bioassays and in a greenhouse trial. High CPB mortality rates up to 90% were accomplished within nine days, due to the lower mRNA levels of the target gene. In the greenhouse trial, Ledprona was compared to the chemical insecticide Spinosad. Both Ledprona and Spinosad treatment reached 100% mortality of the CPB, but the Ledprona treatment needed 14 days for this while Spinosad needed only 3 days (Rodrigues et al., 2021). Exposure of the CPB to low concentrations of Ledprona is not lethal, but can still contribute to overall pest management because it reduces mobility and fertility of the insects, resulting in reduced population sizes (Pallis et al., 2023).
- Besides Ledprona, GreenLight Biosciences has been working on another dsRNA product that is not meant for crop protection but targets the *Varrao destructor,* an ectoparasite infecting honeybees (*Apis mellifera*). Recent work showed how dsRNA taken up by the honey bees could reduce gene expression levels of the *V. destructor* mites infecting those honeybees, resulting in a 50% reduction of the *V. destructor* survival rate (Muntaabski et al., 2022).

In 2021, the Environmental Protection Agency (EPA) in the United States received an application from GreenLight Biosciences for an experimental use permit. This would allow GreenLight Biosciences to test Ledprona in different concentrations on multiple potato production fields in the US (Federal Register / Vol. 87, No. 83 / Friday, April 29, 2022 / Notices). The EPA gave GreenLight the experimental use permit on April 4, 2023 (Federal Register / Vol. 88, No. 86 / Thursday, May 4, 2023 / Rules and Regulations), bringing the authorisation of Ledprona in the US a step closer.

4 Environmental risk assessment of dsRNA sprays

Based on the OECD-report and the proceedings of the OECD-conference, a summary was made of the main points discussed regarding dsRNA sprays and data requirements of chemical plant protection products. Solutions suggested in this report and the proceedings are briefly summarised and discussed. Literature was also consulted, as was an internal policy note on the assessment of RNA sprays as plant protection products (2020). The focus is on the environmental risk assessment of dsRNA applications as a spray (SIGS). In 4.1 we will discuss the effects of dsRNA on the environment. In 4.2 the effects of dsRNA on target and non-target organisms will be discussed.

4.1 Distribution and persistence of dsRNA in the environment

Degradation and distribution
The degradation rate is an important element in the risk assessment of plant protection products, because it can be used to define relevant routes and time of exposure of an organism to a plant protection product (OECD 2023a). When the product degrades more rapidly, there will be less exposure to the environment, resulting in a lower possibility of distribution in the environment.
Unlike most chemical substances, dsRNA is unstable and will be degraded rapidly by enzymes that are naturally present in the environment. The rate of degradation depends on the environment where the dsRNA is introduced (Dubelman et al., 2014, Albright et al., 2017, Mitter et al., 2017b, Bachman et al., 2020). The biodegradation of insecticidal DvSnf7 dsRNA expressed in genetically modified MON87411 maize was determined for different types of soil, and this demonstrated that the estimated DT_{50} (time to 50% degradation) was <30 hours and the DT_{90} (time to 90% degradation) was <35 hours (Dubelman et al., 2014).

Stability and persistence
To maintain the efficacy of the dsRNA product, the dsRNA in sprays needs to be protected and/or stabilised, because of the rapid degradability of dsRNA in the environment. This stabilization means that the dsRNA product will have an increased persistence in the environment, leading to an increased chance of exposure of non-target organisms to the dsRNA. The effects of the non-active constituents in the formulation that contribute to the stabilization of the product can be assessed in a similar manner as for other formulated plant protection products.
Product formulations can be used to stabilise the dsRNA or to increase uptake of dsRNA into plant cells (Jiang et al., 2014). Some of these applications derive from clinical research, where more experience with the use of carriers to deliver RNA in cells is present. A variety of natural and synthetic carriers that can be used as a formulation is described in chapter 3, but more techniques and/or formulations are being developed.

Effects for environmental risk assessment
Due to the fast degradation of non-stabilised dsRNA, the exposure of the environment of the active ingredient will be low (Bachman et al., 2020). The effects of this low exposure for the environmental risk assessment and tests for the admission as a plant protection product will be discussed in chapter 6. Stabilised dsRNA will probably lead to an increased persistence in the environment and therefore to an increased exposure of the environment, including non-target organisms. This could potentially increase the chance of undesired off-target effects caused by dsRNA (OECD 2023a).

4.2 Effects of dsRNA on non-target organisms

Specificity of a dsRNA product
The specificity of a dsRNA product is based on a sequence similarity between the dsRNA and the target gene that needs to be silenced in the pest insect. In principle, the larger the sequence similarity to the target gene is, the more specific the dsRNA product will be (OECD 2023a). The dsRNA product is designed to act specifically against the pest organism (the target organism) and not against other (non-target) organisms. This may have consequences for the test protocols that are used for non-target organisms (Romeis and Widmer 2020). This point will be addressed later on in this report (see 4.2.5).

Effects on non-target organisms can occur for instance when the sequence of the dsRNA is not specific enough, and therefore is also active in the non-target organism (resulting in a non-target effect), or when the dsRNA blocks the expression of a different gene (off-target effect).

Sensitivity of organisms towards RNAi
The target gene that is chosen for a dsRNA product, should be essential for the target organism, but preferably share no sequence similarity with any genes in non-target organisms. However, despite sequence similarity of the dsRNA, it has been demonstrated that not all target organisms show a similar RNAi effect after oral uptake of the dsRNA (Cooper et al., 2019). Insects from the Coleoptera order are more sensitive to dsRNA than lepidopteran insects (Romeis and Widmer 2020, Nitnavare et al., 2021).

Other possible adverse effects in non-target organisms mentioned in the OECD-report (OECD 2023a) are the possibility of immune stimulation (see 4.2.2) (Brutscher, Daughenbaugh and Flenniken 2017, Dávalos et al., 2019) and saturation of the RNAi machinery (see 4.2.3) (Rodrigues and Petrick 2020). These effects are often related to mammals and are discussed further in chapter 5.

Whether these effects actually occur depends on a number of main factors, which are visualised in the OECD-report (2023a) in a so called Empirical Testing Decision Tree, or in literature in plausible pathways to harm (Romeis and Widmer 2020). This pathway to harm shows the steps in how a dsRNA spray insecticide could cause harm to natural enemies of the target arthropod. Possible adverse effects on non-target organisms are only expected if the organism is actually exposed to the

dsRNA, is capable of uptake of the dsRNA and possesses a RNAi machinery that contributes to the formation of siRNA (Romeis and Widmer 2020). The siRNA causes a decrease in expression of an essential gene in the organism, leading to death of the organism.

4.2.1 *Non-target and off-target effects*

RNAi efficiency

Besides the sequence similarity, the RNAi efficiency of a dsRNA product in a non-target organism depends on the life stage of the insect, the dsRNA concentrations, the length of the dsRNA fragment and the timing and duration of the exposure (Huvenne and Smagghe 2010, Bachman et al., 2020, OECD 2023a). Therefore, it is recommended to make several adjustments to the regular test protocols for non-target organisms for the assessment of dsRNA products (see 4.2.5) (Romeis and Widmer 2020, OECD 2023a).

Bioinformatics

Sequence data (bioinformatics) can give an indication whether a non-target effect will take place in a non-target organism (Bachman et al., 2016). This is because the dsRNA targets a specific target sequence in the pest insect. If this target sequence is not present in the non-target organism, no effect is expected on this non-target organism. Selection of a non-conserved region of other species or isoforms of the target gene is important to avoid non-target effects (Singewar and Fladung 2023). Thus, using appropriate empirical bioassays, bioinformatics can help in the selection of relevant non-target test species (OECD 2023a). Conversely, the absence of sequence similarities can be an argument to not perform standard tests. However, the genome sequences of potential non-target organisms are not available for all species (Romeis and Widmer 2020). In addition, the selection of non-target organisms based on sequence similarity may not be sufficient to rule out non-target effects (OECD 2023a). Reasons mentioned are possible differences between organisms with regard to the functioning of their RNAi machinery and effects that can occur independently of the RNA sequence, such as stimulation of the immune system and saturation of the RNAi machinery (OECD 2023a). This is discussed further in 4.2.2 and 4.2.3.

Product formulation

It is possible that the product formulation is aimed at promoting uptake of the dsRNA in the pest insect. In that case it is also important to research whether uptake is promoted in non-target organisms as well. The formulation could therefore lead to a wider effect of the dsRNA product.

4.2.2 *Effects on the immune system*

The discussion on immune stimulation (effects on the immune system) focusses on mammals in particular. The immune system of mammals can be stimulated after injection with a high dose of dsRNA (Judge et al., 2005). It is considered unlikely that the doses of unformulated dsRNA used as a plant protection product will lead to effects on the immune system, because the exposure of mammals to such a dsRNA spray will most likely take place via oral uptake (OECD 2023a) (see also chapter 5.1.2). However, it is unclear how the immune system of other

organisms will react to the uptake of dsRNAs and if immune stimulation will lead to fitness effects in non-target organisms (EFSA 2014, USEPA 2014). Therefore, more research would be required to investigate the effect of RNAi inputs on the immune response of non-target organisms. However, several reports (EFSA 2014, USEPA 2014, Paces et al., 2017) stated that - based on the current literature - it is considered highly unlikely that unformulated dsRNA pesticides would cause an immune response in non-target organisms (USEPA 2014, OECD 2023a), and that using standard mortality, growth and reproduction endpoints should be sufficient to determine the potential for hazard (OECD 2023a).

4.2.3 Saturation of RNAi machinery

Saturation of the RNAi machinery was mentioned as a putative risk of RNAi-based, genetically modified derived products (EFSA 2014). It was described as a result of a limited number of RNA-induced silencing complexes (RISCs) present within a cell and an excess of exogeneous siRNAs that may saturate the RISCs, resulting in a lack of performance of homeostatic functions in the cell (Khan et al., 2009). Saturation of the RNAi machinery may occur in cell cultures and mice, but is only demonstrated in experimental system setups with high doses of dsRNA (Rodrigues and Petrick 2020). The overall opinion is that it is very unlikely that a dsRNA-based pesticide used in agriculture would be sufficient to cause RNAi machinery saturation (EFSA 2014, USEPA 2014, Paces et al., 2017).

4.2.4 Which organisms to test

When chemical pesticides are tested, their effects on a standard series of non-target organisms are investigated, according to OECD-defined test protocols. Unlike chemical pesticides, dsRNA-based products are designed to act specifically against a single pest insect. Therefore, testing on a standard group of non-target organisms may not be the most useful method. Taking the specificity of the product into account, testing on non-target organisms should be focused more on taxonomically related species of the pest species, because the strongest effect can be expected in those species (Romeis and Widmer 2020). Bioinformatics can play an important role in the choice of non-target species (OECD 2023a). We agree with this statement, and are of the opinion that using a standard set of non-target species in the assessment of the active ingredient of RNA sprays used as crop protection product has no added value in principle.
At the OECD conference (Mendelsohn et al., 2020) there was a warning for the emergence of a random selection of tests, which cannot be validated by means a ring test, thus making the results variable. It was mentioned that it might be helpful to use the experience obtained in the environmental risk assessment of genetically modified plants with a specific effect against the pest insect, in which the choice of the tested non-target organism is based on a case-by-case assessment (Schrijver 2013). There is also experience with designing and performing laboratory feeding studies with insecticidal proteins active in the gut, such as Cry and VIP proteins from *B. thuringiensis* (Romeis et al., 2011, De Schrijver et al., 2016).
It may be possible to use specific tests from this framework. We agree with this point of view when it comes to testing the active ingredient. However, as long as there is unfamiliarity with possible side effects of

the formulated product (Zhang et al., 2022), like for instance the penetration of existing barriers in organisms, testing a set of standard organisms can provide additional certainty about the safety of the product.

4.2.5 Test protocols

Test protocols for chemical substances aim to demonstrate effects on a number of acute and chronical endpoints (mortality, growth, reproduction). Since dsRNA-based pesticides are not comparable to chemical pesticides, the OECD-report indicates that these protocols need some revision and adjustment for dsRNA products on the following points:

- Study duration: Effects of dsRNA active ingredients appear to show later than those of chemical products. This has consequences for the study duration (OECD 2023a). For instance, a chemical pesticide like imidacloprid demonstrated mortality in the Colorado potato beetle in less than 24 hours (Chen et al., 2014). Transplastomic potato plants producing dsRNA targeted against the Colorado potato beetle demonstrated mortality within five days after feeding from the plant (Zhang et al., 2015).
- Mode of exposure: Only oral or dietary exposure are relevant routes of exposure for dsRNA active ingredient (Romeis and Widmer 2020, OECD 2023a).
- Dose selection: Starting with an initial dose of ten times the estimated environmental exposure is considered, as there is no frame of reference for dsRNA products (OECD 2023a). This proposal requires an estimation of this concentration, or a methodology to determine the concentration in the section Fate and Behaviour in the environment (Section 7 in 283/2013). High and unrealistic doses of dsRNA should be avoided (USEPA, 2016).
- Analytical method: To measure the dose of dsRNA, the analytical method to determine the dose must be adjusted, as some test protocols include specifications on the analytical method (OECD 2023a).
- Repeated exposure as a worst-case scenario: It is most likely that dsRNA degrades during the test protocols. If possible, the protocol should be adjusted by including repeated exposure of the dsRNA, anticipating on the expected degradation (OECD 2023a). As mentioned above, high and unrealistic doses of dsRNA should be avoided (USEPA, 2016).
- The life stage of the insect: Insects sometimes only consume a plant at the larval stage and are therefore only exposed to the dsRNA as larvae (Cooper et al., 2019, Romeis and Widmer 2020).
- Length of the dsRNA fragment: The length of the dsRNA fragment is important in the effectivity of the dsRNA. Studies have demonstrated that a length of at least 60 base pairs in the dsRNA fragment is required to achieve an effect in insects (Wang et al., 2019, Christiaens et al., 2020, He et al., 2020). A length of 140-500 base pairs is considered to be the most effective (Huvenne and Smagghe 2010, Joga et al., 2016, Hofle et al., 2020).

5 Human health risk assessment

At EU level, there is currently no risk assessment methodology available that than can be applied directly to determine the risks to human health of externally applied dsRNA based plant protection products. The EFSA report (EFSA 2014) on the "International scientific workshop 'Risk assessment considerations for RNAi-based GM plants" provides some recommendations on how the risks to humans should be addressed, but these relate to oral exposure to unformulated/non-stabilised dsRNA and not to other exposure routes relevant for sprayed plant protection products, such as inhalation and dermal exposure, or to exposure to formulated dsRNA. The OECD document on the "Considerations for the Human Health Risk Assessment of the Application of Externally Applied ds-RNA-Based Pesticides" (OECD 2023b) lists a few potential human health risks, namely unintended gene suppression due to homology (see chapter 5.1.1) and immune responses (see chapter 5.1.2). These are the same potential risks that were identified in the literature search.

As indicated in chapter 4.2.3, saturation of RNAi machinery is not considered to be a concern for dsRNA-based plant protection products. It is therefore not addressed any further here. The conclusion that the saturation of the RNAi machinery is not a potential concern for non-target organisms is in line with the OECD-report (OECD 2023b).

Chapter 5.1 describes the potential risks of pure (unformulated/unmodified) dsRNA. In chapter 5.2 we discuss the impact of the production method of the dsRNA on the human health risks, while the potential risks associated with formulated or modified dsRNA are addressed in chapter 5.3. In chapter 5.4 we describe which exposure routes to dsRNA during and after application should be addressed in the human health risk assessment.

5.1 Potential adverse effects of dsRNA to humans

5.1.1 Sequence specific effects – Unintended gene suppression

The likelihood of sequence specific effects occurring in humans is low, due to expected low homology with the dsRNA used as plant protection product.

Even if homology would be evident, the risk to humans is likely negligible (Fletcher et al., 2020). For dsRNA to induce an adverse effect in a sequence specific manner, it would require systemic uptake and translocation into cells, rather than to bind to receptors on the surface of cells. dsRNA is unstable in biologic fluids. In the digestive tract, RNA is subject to both non-enzymatic and enzymatic degradation (OECD 2023b). Upon entering the blood it is easily degraded by nucleases (Chen et al., 2018). The degraded dsRNA is then filtered by the kidneys and taken up by phagocytes or the reticuloendothelial system. Cellular uptake is challenging as dsRNA, which is negatively charged, cannot diffuse passively into the cells (Chen et al., 2018). The lack of potential for a sequence specific effect of dsRNA via the oral route was illustrated by a 28-day toxicity study in mice, with dsRNA sequences matching the

mouse vATPase gene. Despite the dsRNA targeting a gene in the test species, there were no treatment-related effects on body weight, food consumption, clinical observations, clinical chemistry, haematology, gross pathology or histopathological endpoints (Petrick et al., 2015). This lack of effects was explained by the limited systemic bioavailability. Moreover, if dsRNA would reach the interior of a cell, significant barriers exist that prevent a response. For example, endosomes sequester a majority of RNA molecules that enter a cell (98-99%) (Rodrigues and Petrick 2020).

5.1.2 Immune response

A potential concern has been identified in literature regarding the possibility of an immune response for long dsRNA, in a non-sequence specific manner (Mendelsohn et al., 2020). Longer dsRNA (>30 nucleotides) can rapidly induce both interferon responses by binding to double-stranded-RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase-RNase L system or several Toll-like receptors (TLRs), depending on the length (Kim et al., 2019). These are evolutionarily conserved mechanisms aimed at combating invading viral pathogens (Aagaard and Rossi 2007). Shorter dsRNA (≤30 nucleotides) does not appear to have this problem.

In a study with adeno-associated virus (AAV)-mediated RNA interference as a therapy for chronic hepatitis B, significant liver toxicity with severe hepatocellular damage was observed, accompanied by increases in serum ALT levels and decreases in albumin (Sun et al., 2013). These liver effects were associated with hepatic leukocytes and inflammatory cytokines and chemokines, suggesting an inflammatory response as the cause of liver injury. In immunodeficient mice an hepatoxic response was completely absent, supporting this theory. It should be noted that the exposure in this study occurred via injection, which is not a realistic exposure route for plant protection products.

If such an immunostimulatory effect could occur after the use of dsRNA as plant protection depends on the systemic availability of the dsRNA. For consumers, the risk of a potential immune response seems low. Humans have a long history of ingesting numerous amounts of dsRNA through the consumption of virus-infected plants without any adverse effects. This is likely due to rapid degradation of dsRNA by nucleases in the gastrointestinal tract (Jensen et al., 2013). Other biological barriers include rapid degradation by nucleases in blood, degradation in the kidneys and limited cellular uptake as indicated in chapter 5.1.1. Regulatory bodies have previously concluded that the human health risk for consumers after oral uptake of unformulated dsRNA in plants is limited. For example, the US EPA concluded in 2016 that "*there are no reliable evidence that exogenous dsRNAs are taken up from the gut into mammalian circulation to exert its functions in the ingesting organism*" (USEPA 2016). Similarly, the food safety authority Food Standards Australia New Zealand (FSANZ) concluded that "*A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of these RNAs from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect."*

(FSANZ 2015). These conclusions are related to the evaluation of dsRNA as PIPs (plant-incorporated protectants) and are also relevant for unformulated/unmodified dsRNA.

Similarly, dermal exposure is not expected to lead to a risk of a potential immune response as dermal uptake is limited. For nucleic acids to pass the stratum corneum would require either diffusion via lipid channels and/or transcellular passage through corneocytes, or entry through sweat ducts or hair follicles. Large hydrophilic molecules like dsRNA undergo negligible transport across the skin without transport enhancers or cellular membrane disruption techniques such as microporation or electroporation (Rodrigues and Petrick 2020). The OECD-report also concluded that, based on the available information, unformulated/unmodified dsRNA does not appear to result in dermal permeation and cellular uptake (OECD 2023b).

It seems unlikely that exposure to unformulated/unmodified dsRNA through inhalation leads to a risk of a potential immune response, but the available data is limited. Inhalation exposure has been explored as a possible administration method for RNA therapeutics. Delivery of the RNA molecules for therapeutic purposes via this route has proven to be challenging, because it requires specifically engineered formulations to effectively deliver the RNAi based drugs (Man et al., 2016). The likelihood of an immunostimulatory response due to inhalation exposure seems low, due to the poor cellular uptake of unformulated dsRNA . Nevertheless, most studies addressing the safety of inhalation exposure to RNAi are related to clinical studies using shorter dsRNA which is designed to bypass the immune response, while longer sequence dsRNA tends to be applied for plant protection purposes (OECD 2023b). Overall, the available information on the potential immunostimulatory effects of dsRNA used as plant protection product after inhalation exposure is too limited to entirely exclude a potential risk. To address this concern, additional data may need to be generated, either on the hazard properties or on the potential inhalation exposure (see chapter 6.3).

The OECD-report highlights one additional exposure route which should be paid particular attention to, which is ocular exposure (OECD 2023b). Clinical studies indicate that even unmodified dsRNA applied via eye drops is more stable in ocular fluids than in plasma, and activation of the innate immune system via Toll-like Receptor 3 was observed. So even for unmodified dsRNA potential immunostimulatory effects after ocular exposure should be taken into account.

5.2 Impact of production methods on the human health risk assessment

Production of dsRNA can either be done via *in vitro* methods through enzymatic synthesis or chemical synthesis, or through the use of genetically engineered microorganisms such as *E. coli, Pseudomonas syringae* and *Yarrowia lipolytica*.

Choosing a microbial production system could impact human health (and the environment) through residual microorganisms in the product, such

as *E. coli*, or through the production of toxins. Therefore, applicants must show that the applied production method does not impact human health. To exclude the presence of toxins, it is possible to use a microorganism that is safe for human consumption, such as yeast. Apart from the presence of toxins, the presence of viable microorganisms should also be excluded. When viable microorganisms are present, the applicant would have to apply for approval of a genetically modified microorganism. This lies beyond the scope of the plant protection product legislation and should be assessed under a different legislative framework. Finally, when the production method is based on genetically engineered microorganisms, the presence of microbial contaminants needs to be excluded. More information on what data needs to be submitted to address these concerns can be found in chapter 6.3.

5.3 Impact of formulation on the human health risk assessment

Unformulated dsRNA is not stable enough to ensure a long-lasting efficacy after foliar or soil application, in particular for use in the field. Moreover, for certain applications, such as the control of plant viruses, entry into cells is required, which does not occur for unformulated/unmodified dsRNA (Dietz-Pfeilstetter et al., 2021). It is therefore expected that certain co-formulants will be applied in dsRNA plant protection products to increase stability of dsRNA and to enhance cellular uptake to increase efficacy (see also chapter 3).

This could impact the systemic uptake in humans. For example, nanomaterials can be used in formulations for the purpose of enhancing cellular uptake of dsRNA (Mendelsohn et al., 2020). Attempts have also been made to increase the oral availability of non-viral nucleic acid-based therapeutics. However, even with these optimised formulations the oral bioavailability remained very low and their use seemed to be mainly for local administration, rather than systemic use (O'Driscoll et al., 2019). Although optimising the formulation of dsRNA remains challenging, it must be assumed that the increased systemic bioavailability and cellular uptake may increase the toxicity of these dsRNA products.

Consideration should be made on the intended formulation and additional data on adverse effects specifically related to the dsRNA, such as sequence specific effects and immunostimulatory effects, may be needed to conduct the risk assessment. Specific considerations of the formulation method itself may also be needed, e.g. in the case of nanoparticle carriers. Chapter 6.3 provides more guidance on how these issues could be addressed.

5.4 Exposure to dsRNA during and after application

5.4.1 Non-dietary exposure to operators, bystanders, workers and residents

Operator exposure

The exposure routes to dsRNA-containing products for operators are not expected to be different from those to conventional chemical products. The main routes are dermal exposure and inhalation exposure.

For dermal exposure the risk to pure dsRNA is expected to be limited. This is due to the limited systemic bioavailability, high degradation and

elimination, and low cellular uptake, as described in section 5.1.2. For formulated products where the formulation does not impact the systemic bioavailability or cellular uptake, no risks due to exposure to the dsRNA active substance are expected either. However, the situation becomes a bit more complex for dsRNA which has been formulated or modified to improve efficacy, in such a way that it does increase the systemic bioavailability and cellular uptake.

In the case of inhalation exposure, potential risks for unformulated/unmodified dsRNA consist of the immunostimulatory effects of dsRNA. The publication by Rodrigues and Petrick (Rodrigues and Petrick, 2020) recommends that operators use appropriate respiratory protection (RPE) to limit inhalation exposure. While this would be a useful measure to mitigate risks, wearing RPE not just during mixing and loading, but for a full working day puts a strain on operators. Moreover, the potential health risk after inhalation exposure to dsRNA for bystanders and residents, as indicated below, cannot be addressed by wearing RPE. No information could be found in public literature on to what extent respiratory exposure to dsRNA takes place during spray application. Considering the dilution factor in the spray tank and the generally large size of spray droplets, the actual inhalation exposure to dsRNA during spraying may be too limited to induce an adverse effect. However, since no information could be retrieved no clear conclusion can be drawn. Therefore, it would be advisable to address these concerns about immunostimulatory effects after inhalation by conducting additional hazard studies or studying empirical data on the particle size distribution of spray droplets to exclude health effects from inhalation exposure (see chapter 6.2), instead of prescribing RPE as proposed by Rodrigues and Petrick (2020).

Worker exposure
The main exposure route for workers is via dermal contact with treated crops. Environmental factors such as rain and UV-light play an important factor in worker exposure to dsRNA. UV-light is known to degrade dsRNA in the environment (Christiaens et al., 2020). A study conducted by the industry, in which dsRNA was applied by foliar spray application in fields, showed rapid degradation with a 95% reduction 3 days after treatment and an almost 99% reduction 7 days after application (Bachman et al., 2020). The DT50 values were between 0.5-0.7 days, with a DT90 between 1.9-2.3. Negligible amounts of dsRNA (ng/g) were detected at the time of harvest which included a treatment that took place 7 days prior to harvest. Another study showed that controlled conditions mimicking a rain event readily washed away the applied dsRNA. This would mean that for most applications of dsRNA in the field worker exposure is expected to be limited to inspection activities and not harvest activities, provided that the pre-harvest interval is sufficiently long (e.g. 7 days).

In greenhouse conditions the biodegradation on plants may be slower. One experiment, which looked at the efficacy of insecticidal dsRNA applied to potato leaves, observed an efficacy of up to 28 days, which was explained by the absence of UV-light. It should be mentioned that no quantification was performed in this study.

However, even if the biodegradation on plants is lower due to greenhouse conditions, there is no expected risk for workers from unformulated/unmodified dsRNA, since the dermal uptake of dsRNA is very limited, as described in section 5.1.2.

As was highlighted in the OECD document (OECD 2023b), the availability of data on the environmental fate of modified and formulated dsRNA is limited, and therefore no general conclusion can be made on the impact of formulation on dermal exposure to dsRNA.

Bystander/resident exposure
Following the EFSA Guidance on the assessment of exposure of operators, workers, residents and bystanders in the risk assessment of plant protection products, the relevant exposure routes for bystanders and residents are dermal exposure through contact with surface deposits and entry into fields with treated crops, and inhalation exposure due to spray drift and vapour. Oral exposure is a relevantroute for children, due to hand-to-mouth contact with residues (EFSA 2022). As indicated in the sections on exposure for workers, operators and consumers (see section 5.4.2 below), their risk due to dermal or oral exposure to unformulated/unmodified dsRNA is expected to be limited. In the case of inhalation exposure the potential immunostimulatory effect of dsRNA should be addressed by conducting additional hazard studies or studying empirical data on the particle size distribution of spray droplets.
In case of formulated or modified dsRNA insufficient information is available to draw conclusions on the potential risks of exposure for bystanders and residents.

5.4.2 Dietary exposure to consumers

As indicated in section 5.4.1, the foliar degradation of dsRNA is high, in particular in field conditions. This will limit consumer exposure to dsRNA-based plant protection products. Greenhouse conditions may result in a lower degradation rate. Nevertheless, no risk to consumers from unformulated dsRNA is expected, because humans have long been exposed to large amounts of dsRNA via the consumption of virus-infected plants, without any adverse effects. This is likely due to rapid degradation of dsRNA by nucleases in the gastrointestinal tract. Regulatory bodies have previously concluded that the human health risk for consumers after oral uptake of unformulated/unmodified dsRNA in plants is limited (see section 5.1.2).

In case of formulated or modified dsRNA insufficient information is available to determine the exposure to and risks for consumers.

6 Adjustment of risk assessment data requirements for chemical substances

Plant protection products must be compliant with European regulations (Regulation (EC) No. 1107/2009). Only authorised plant protection products may be sold and used. For plant protection products to receive an authorisation, the applied active substance(s) in the product must be approved. For approval of the active substance the data requirements of Regulation (EU) No. 283/2013 have to be met. After an assessment of the risks to the environment and human health and its safety and efficacy, the product can receive an authorisation. The data requirements for the products can be found in Regulation (EU) No. 284/2013.

6.1 Fate and behaviour in the environment

This chapter mainly discusses the data requirements that we believe can (or should) be met in a different manner for dsRNA as an active substance. This concerns Section 7 (Fate and behaviour in the environment) and Section 8 (Ecotoxicology) of the data requirements in Regulation 283/2013. On some topics we also discuss the data requirements for formulated products, with the caveat that little is currently known about the formulations used and the purpose of the formulation (e.g. stabilisation of dsRNA and/or enhanced cell penetrability).

6.1.1 Fate and behaviour in soil

Data requirements in Regulation EU 283/2013 prescribe measurements of the degradation of unformulated/unmodified dsRNA in the soil. These measurements are not considered meaningful, because of the fast degradation of dsRNA in the soil. The data requirements in Regulation EU 284/2013 for degradation of dsRNA are regarded as useful, because formulation can contribute to slower degradation of the dsRNA, causing the product to remain intact and active for a longer period. However, at this moment it is still unclear to what extent formulation slows down the degradation of dsRNA. Regulation EU 284/2013 for products contains data requirements equivalent to those in Regulation EU 283/2013 for the active ingredient. Tests like the OECD Test Guideline 307: 'Aerobic and anaerobic transformation in soil' can be used for formulated products.

In the OECD report it is noticed that laboratory studies with conventional chemical pesticides require the use of radioactive labelled test material. It is discussed that this is not relevant for dsRNA, because the nucleotides will be incorporated by the organisms in the soil and tangle the half-life measurements. We agree with this conclusion, and we observe that both labelled and unlabelled test material can be used for OECD Test Guideline 307. However, it is unclear whether unlabelled dsRNA can be isolated from the soil. Therefore this guideline does not seem entirely applicable for dsRNA and we recommend looking further into this.

Another observation regarding the OECD Test Guideline 307 is that it focuses on the soil. It may, however, also be relevant to analyse the degradation of dsRNA on plant material, because the product is sprayed onto the plant and is - to a certain extent - available to other organisms.

6.1.2 Fate and behaviour in water and sediment

It is possible that a dsRNA product reaches water and sediment by means of drift and produces an effect on non-target organisms in those environments. Tests for the degradation of the unformulated dsRNA seem redundant, because of the rapid degradation of the dsRNA. However, degradation of the formulation can be tested because the formulated dsRNA product remains intact longer than the unformulated dsRNA.
We are of the opinion that the shake flask test can be used to indicate whether a formulation with dsRNA degrades rapidly in water or not. If rapid degradation is demonstrated, further analyses might not be necessary for other dsRNA sequences. We believe that a one-time set of experiments with a number of different formulations can lead to a statement which can be referenced to in every dossier. In our opinion, only if tests demonstrate that a relevant concentration of stabilized dsRNA is expected in water via drift and only if relevant non-target organisms are present in water or sediment, testing with non-target organism may be required. This applies only to formulations aimed at stabilizing the dsRNA and not to formulations that target simultaneously enhanced penetrability into the cell.

6.1.3 Fate and behaviour in air

A dsRNA product may also end up outside the field it is sprayed on and have an effect on non-target organisms there. Tests for unformulated dsRNA seem irrelevant to us here, because of the fast degradation of the dsRNA. In the case of formulations though, oral testing on non-target organisms might be necessary to measure effects, depending on the stability of the product. However, given the specificity of the products we believe these tests may not be relevant.
We believe that a single research on the photodegradation in air for different types of products can be sufficient. It is then no longer necessary to perform this test for every product. This may only be required when special persistent properties are attributed to the formulation. Based on the existing data, no effects are expected from the concentrations of dsRNA applied for agricultural use. However, it may be desirable to obtain a better understanding of the level of exposure and its possible effects, for example in the form of an immune response.

6.2 Ecotoxicology

It is still uncertain how many and which species should be tested for dsRNA products. The following suggestions have been made, but no consensus has been found:

- Testing a key species that is common in the agricultural crop field. The drawback of this option is that this species may not be amenable for testing in the laboratory. For this reason, it was suggested at the OECD Conference to use an existing list of well-known species which can be tested in the laboratory.

- Choosing species closely related to the pest insect, as this is where the most effect can be expected. These species should come from the existing list of amenable species.
- Choosing species with important ecosystem services (such as pollinators, predators, decomposers, etc.). These species are already on the aforementioned standard set of organisms to be tested.
- Using the species which were tested for RNA-producing plants.

Another recommendation made in the OECD-report is to first determine whether organisms are actually exposed to the dsRNA spray. Testing is not necessary if this is not the case. When tests are performed though, it is recommended that only oral tests are used and that these tests are not carried out at high (unrealistic) doses, as this will lead to highly variable results. The choice of which non-target insects to be tested should be more focused on a case-by-case basis, as it is done for RNA-producing plants or Bt-producing plants, and it should focus more on species closely related to the target organism.

The following paragraphs discuss various organisms required in the ecotoxicology studies section of the data requirements. Not all organisms from that section are discussed; organisms for which testing with the active dsRNA ingredient is not considered necessary are not mentioned.

6.2.1 *Effects on non-targets: birds, mammals (other vertebrates)*

An effect of orally exposed dsRNA as an active substance on birds, mammals and other vertebrates is not expected, given that these organisms have barriers through which the dsRNA cannot pass (Bachman et al., 2016, Rodrigues and Petrick 2020). We therefore presume that these organisms are not sensitive to dsRNA. This assumption is also made in the OECD-report and is supported by the fact that current knowledge and experience with RNAi drugs show that it is very difficult to apply the drugs to humans (O'Driscoll et al., 2019). However, the OECD-report comments that there must be no sequence similarity with the dsRNA in birds, mammals and other vertebrates. The risk of sequence similarity is estimated very low by us, but this should be substantiated with data by the applicant. In addition, the OECD-report states that the dsRNA can be ingested indirectly by a predator through eating prey. However, only little data on specific barriers of uptake across non-target taxa are available (Chan and Snow 2017, Dávalos et al., 2019). The risk of ingestion via prey is considered to be negligible (Romeis and Widmer 2020).

6.2.2 *Effects on non-targets: aquatic invertebrates*

Daphnia (water fleas) also have barriers through which the dsRNA cannot pass, with the result that no effects are expected in this organism. In the OECD-report *Daphnia* are mentioned as a species that could potentially be included in a standard set of organisms, which is already used for chemical pesticides. However, testing a standard set of non-target organisms for dsRNA products is not always considered useful (Romeis and Widmer 2020). This will be discussed in more detail later.

6.2.3 *Effects on non-target arthropods: bees*

Tests with honeybees are in principle only needed if the bees are related to the target organism that is controlled with the dsRNA spray (and therefore probably also contain the 'target' sequence for the dsRNA). However, feeding studies of sequence unspecific dsRNA triggered upregulation of immune response related genes in honeybees (Flenniken and Andino 2013, Brutscher, Daughenbaugh and Flenniken 2017). Only the oral test would be suitable. The acute contact test is not suitable because the oral route of exposure is the most important one (Romeis and Widmer 2020). However, as a pollinator the honeybee is listed as a test species in the standard set of organisms. There are also wild bee species in the European Union that play a role in pollination. Those species may be exposed to the dsRNA and may be related to the target species. Tests for these species are often missing. This should be considered when selecting non-target organisms to be tested.

6.2.4 *Effects on non-target arthropods: other than bees*

Tests with the two standard species *Aphidius rhopalosiphi* and *Typhlodromus pyri* are not considered useful unless they are closely related to the pest to be controlled. These two species were initially selected as indicator species because of their sensitivity to chemical pesticides, but are unlikely to be the most sensitive species for the major part of dsRNA products (Romeis and Widmer 2020). In the discussion in which non-target species need to be tested, it is sometimes recommended to test representatives of ecosystem services like, predators, pollinators and decomposers. However, this view is not shared by all parties.

6.2.5 *Effects on terrestrial non-target higher plants*

Plants have effective barriers, like the plant cuticle and the cell walls, to reduce the uptake of dsRNA. Besides that, microbial communities on the plant surface contribute to the rapid degradation of dsRNA, and therefore to the reduced uptake by the plant. Nevertheless, evidence has been found that uptake and transport of dsRNA in the vascular system of plants sometimes occurs (Koch et al., 2016). High pressure spraying (Dalakouras et al., 2016) and the use of particular carriers as formulations (Mitter et al., 2017b) can further enhance the uptake of dsRNA by the plant.

6.3 Human health

The data requirements to assess the human health risk of chemical active substances are laid down in section 5 of Regulation (EU) 283/2013. For plant protection products the data requirements are laid done in section 7 of Regulation (EU) 284/2013. In general, it can be concluded that the specific studies requested in this section are not suited for dsRNA, as they are tailored towards conventional chemicals. The sections below describe in detail which studies are considered relevant for dsRNA.

6.3.1 *Unformulated dsRNA*

In the case of unformulated/unmodified dsRNA it is considered unnecessary to conduct oral animal studies. As indicated in chapter 5, the bioavailability and cellular uptake of unformulated dsRNA is

extremely limited. This conclusion is in line with the outcome of an international scientific workshop on the risk assessment for RNAi-based GM plants, organised by EFSA (EFSA 2014). The report of this workshop states that "*animal studies with RNAi molecules (e.g. a 28-day toxicity study) were considered of doubtful relevance to identifying hazards. Based on the current knowledge, gained in pharmaceutical research and development, RNAi molecules show limited bioavailability, quick turn-over (for further reading please refer, for example, to Ballarín-González et al., 2013) and no adverse effects following oral gavage (even for formulations specifically designed to maximise their effects).*" Similarly, the OECD-report on the environmental risk assessment of spray dsRNA based products concludes that "*available evidence suggests that the likelihood of systemic exposure of mammals to RNA molecules in the field as pesticides is very low, assuming no modification or addition of other products ingredients to facilitate pesticidal action in the target organism*" (OECD, 2020). Therefore, for unformulated/unmodified dsRNA it is not considered necessary to conduct oral toxicity studies, such as the 90-day, chronic or reproductive/development toxicity studies.

For inhalation exposure, the possibility of an immune stimulatory response cannot be fully excluded based on the currently available information. If inhalation exposure is expected to occur, a toxicity study via the inhalation route may be needed. Concerning the duration of exposure, it should be noted that the OECD-report on the environmental risks of dsRNA states that it may take dsRNA-based plant protection products longer to be effective than conventional pesticides (OECD 2023a). Taking this into consideration, the OECD-report on the human health risks notes that the protocols used may require a longer study observation period. No exact recommendation is made, but it is clear that an acute inhalation toxicity study would not be sufficient to address the concern.

The data requirements under Regulation (EU) 283/2013 also include provisions for adverse effects such as skin sensitisation and eye and skin irritation. No references indicating a potential concern for these endpoints were found in literature, although immunostimulatory responses after ocular exposure were mentioned in the OECD-report (OECD 2023). Currently, no OECD test guideline is available that would allow for the detection of such effects.

Regarding genotoxicity, very little information could be found. The OECD-report on the human health risk of dsRNA mentions that potential genotoxicity has been excluded for mRNA-based therapeutics, but that mRNA has a different mode of action of mRNA is different than that of dsRNA and that implications for genotoxicity may also be different (OECD 2023b). The OECD-report concludes that genotoxicity will likely need to be considered on a case-by-case basis.

6.3.2 Formulated dsRNA

Formulated dsRNA may increase systemic bioavailability and cellular uptake of the dsRNA. As indicated by Jackson and Linsley (2010), there is currently insufficient information on the immunogenicity or other adverse effects of these delivery approaches to draw conclusions

regarding their safety. Typically, the hazard assessment for conventional chemical plant protection product formulations is addressed under Regulation (EC) 284/2013. However, these data requirements are restricted to acute toxicity testing, and would not address the potential risks of formulated dsRNA as the measured endpoints in these studies are too limited and the exposure duration is too short.

As a first step the impact of the applied modification or formulation on the systemic availability of dsRNA could be addressed using absorption, distribution, metabolism and elimination (ADME) studies, as required under Regulation 283/2013 for active substances. If the applied modification/formulation does not impact the systemic availability, this could be used to waive further toxicity testing. However, further testing is needed if the systemic availability is increased.

To address the potential for sequence specific effects, bioinformatics may be used to determine homology to humans (see also chapter 4.2.2). If the target sequence of the dsRNA is not present in humans, no effects are expected. It should be noted that sequence identicality does not mean that the dsRNA would provoke an RNAi response, but should serve as a caution for potential unintended interaction and should be considered an indication for further review (USEPA, 2014; (OECD 2023b)).

To address other adverse effects of formulated dsRNA, the conduct of at least some repeated oral toxicity dose studies may be needed. For animal welfare reasons, it is proposed that not the full set of animal studies required under Regulation 283/2013 are conducted, but that a tiered approach is applied. For example, if the formulated dsRNA does not show any adverse effect at the limit dose in a 90-day oral toxicity study, this could be used as an argument to waive higher tier (chronic) studies. The applicant should provide justification why studies using other exposure routes, such as dermal exposure, are not needed. In determining the appropriate test strategy, the specific formulation method should also be considered. For example, the use of nanoparticle carriers in the formulation does mean certain specific considerations are needed in the risk assessment. EFSA has previous published a guidance document on how to address nanotechnologies in the risk assessment of chemicals, which describes a specific stepwise approach on how the hazard identification of these chemicals can be tackled (EFSA 2018b).

When more information on the potential effects of formulated dsRNA becomes available, the impact of formulation methods and the need for further testing should be reviewed to see if some general conclusions on their safety can be drawn.

6.3.3 Production method

In case genetically engineered microorganisms are used as a production method, additional information may be needed to address the potential presence of toxins, viable microorganisms and microbial contaminants. Data requirements and test methods in place for microbial plant protection products could be used to address these concerns. To address the presence of toxins, a GLP-compliant 5-batch analysis should be conducted, measuring the known toxins of the microorganism used in

the production method. The Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substance (SANCO/2020/12258) can be used as a guideline to identify if any toxins might be produced. In this 5-batch analysis microbial contaminants should be measured according the Working Document on Microbial Contaminant Limits for Microbial Pest Control Products (SANCO/12116/2012 –rev. 0). The presence of viable microorganisms can also be measured in this analysis. All analytical methods used in the 5-batch analysis should be validated, and the validation study should be provided by the applicant and checked by risk assessors.

7 Discussion

Earlier research on the use of plant protection products based on dsRNA has shown promising results. Many of these studies focused on the use of RNAi expressed by genetically modified plants, although the interest in topical applications has also grown. Many developments are taking place on the formulation of dsRNA-based products, in order to increase the efficacy of topical applications of dsRNA, e.g. by increasing the stability in the field.

Concerning the environmental risks, this report focuses on dsRNA-sprays to control pest insects that eat the crop species. dsRNA products with other purposes, such as weed control, suppression of resistance to chemical plant protection products and growth promotion are not discussed, just like dsRNA which is applied in different application manners, e.g. powders, soil sprays end seed treatments. Depending on the type of product or the method of application a partially different set of data requirements is needed, as well as different knowledge to assess these data. The recommendations indicated in the human health risk assessment below can be applied in a broader context, as these are less related to the target organism or application method.

Assessing the risks for dsRNA requires a different approach than for conventional chemical plant protection products. Potential elements to consider in the risk assessment of dsRNA are sequence similarity and immune stimulation. The risk is expected to be lower for unformulated dsRNA, as it rapidly degrades in the environment and shows low systemic availability. However, formulation methods may increase the stability and increase the need for information on their potential risks. The production method of the dsRNA can also lead to additional concerns that need to be considered in the risk assessment. Based on these specific risks and on the high specificity of dsRNA it is clear that the standard data requirements for chemical plant protections products as laid down in Regulation (EU) 283/2013 for active substances and Regulation (EU) 284/2013 for products are not suitable for (un)formulated dsRNA.

In assessing the environmental risks, bioinformatics can play an important role in choosing the non-target species to test, based on the sequence similarity with the target gene of the dsRNA. This tool may possibly lead to reduced testing in the future. Testing of species closely related to the target organism and that have a corresponding target sequence on a case-by case basis is more useful. If no effects are found, the probability of effects on unrelated species is small. Depending on the formulation, additional testing may be required.
However, some adjustments to the standardised protocols may be needed to make them suitable for dsRNA products, such as study duration and the mode of exposure. The data requirements for the environmental risk assessment for chemical plant protection products are not always applicable to products with RNAi as an active substance. Part of this concern can be resolved by conducting the assessment at

(formulated) product level, rather than at active ingredient level, in view of the rapid degradation of unformulated dsRNA.

In case genetically engineered microorganisms are used in the production method, the potential presence of toxins, viable microorganisms and microbial contaminants needs to be considered. Data requirements already in place for microbial plant protection products under part B of Regulation (EU) 283/2013 can be applied to address these concerns.

For the human health assessment, EFSA and OECD have previously concluded that conducting oral or dermal *in vivo* animal studies is of dubious relevance for unformulated/unmodified dsRNA, due to its limited bioavailability and rapid degradation and elimination. For inhalation exposure, the potential risk of immunostimulatory effects needs to be addressed by either an inhalation study or it needs to be shown by the applicant that the inhalation exposure is expected to be negligible. Concern for genotoxicity needs to be addressed on a case-by-case basis. There is a general lack of information on adverse effects for formulated dsRNA to draw conclusions on their safety, Therefore, additional toxicity studies are needed compared to unformulated/unmodified dsRNA. It is recommended that a tiered approach is applied to address the potential risks of these formulated dsRNA products, starting with an absorption, distribution, metabolism and elimination (ADME) study or a short-term oral toxicity study. Once more information on the potential effect of formulated dsRNA becomes available, the need for further testing should be reviewed to determine if general conclusions on their safety can be drawn. This can be done after dsRNA-based plant protection active substances have been approved under Regulation 1107/2009, based on the information available in the draft assessment reports.

8 Conclusions and recommendations

Based on the available information it is clear that the current data requirements for plant protection products are not directly applicable to dsRNA-based products. Moreover, although the OECD-reports on the environmental and human health risks provide some indication of the potential risks to be considered, no clear set of requirements is defined in these documents. It is therefore recommended to develop specific data requirements for dsRNA within the EU.

Recommendations on how to assess the potential environmental and human health risk are provided below. These recommendations provide guidance to risk assessors on how to evaluate dsRNA-based plant protection products, and can be used in the development of dsRNA specific data requirements.

Environment:

- Adjustment of testing of distribution and persistence of dsRNA in the environment.
- A different approach to choosing non-target organisms to test possible effects of dsRNA-products, like choosing species closely related to the pest insect. Lessons may be learned from the case-by-case selection of non-target insects to be tested in RNA-producing plants or Bt-producing plants.
- The use of bioinformatics as a tool for the selection of non-target organisms to be tested based on sequence similarity. Mapping the extent to which bioinformatics may replace testing on non-target organisms in the future.
- Modifications of test protocols when testing non-target organisms, such as adjusting the duration of the study, the level of the dose, the administration route, and the life stage of the organism.
- Testing the formulated product instead of just the active substance, because of the potential stabilisation and increased effect of the formulated product.
- Additional recommendation: exploring the adaptation of data requirements for other dsRNA products (herbicides, fungicides).

Human health:

- For dsRNA-based plant protection products produced via genetically modified organisms, applicants should provide information on the presence of toxins, viable microorganisms and microbial contaminant via GLP-compliant five batch analysis, using validated analytical methods. Existing data requirements for microbial active substances under Regulation 283/2013 can be applied.
- For unformulated dsRNA, no risk to humans is expected following dermal or oral exposure, and no toxicity studies for these exposure routes are required. An inhalation toxicity study is needed to address potential immunostimulatory responses in cases where inhalation exposure is expected to occur.

- For formulated or modified dsRNA, additional toxicity studies must be requested if the applicant cannot show that the formulation or modification has no impact on the absorption, systemic degradation and cellular uptake. A tiered approach is recommended, starting with an oral short-term study. If no effects are observed at the limit dose, other oral toxicity studies may be waived. Argumentation should be provided by the applicant as to why studies using other exposure routes are not needed.
- The specific formulation method should be considered, e.g. in the case of nanoparticle carries. The EFSA guidance document on how to address nanotechnologies in the risk assessment of chemicals (EFSA 2018b) can be used to determine the appropriate test strategy for this particular formulation method.
- When sufficient formulated/modified dsRNA-based plant protection products are approved, a review of these dossiers is recommended to determine the impact of formulation/modification on the risks for human health risk and to see if continued animal testing is required.

9 Acknowledgement

The researchers would like to thank J.W.A. Scheepmaker and D.C.M. Glandorf for their contribution to the preparation of this report.

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Published by:

National Institute for Public Health and the Enviroment, RIVM
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

January 2024

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