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# Considerations and regulatory approaches in the USA and in the EU for dsRNA-based externally applied pesticides for plant protection

**Antje Dietz-Pfeilstetter[1*], Achim Gathmann[2], Dominik Klinkenbuß[2], Mike Mendelsohn[3]**

[1]Institute for Biosafety in Plant Biotechnology, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Braunschweig, Germany
[2]Federal Office of Consumer Protection and Food Safety, Department of Plant Protection Products, Unit Environment, Braunschweig, Germany
[3]Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, United States Environmental Protection Agency, Washington, DC, United States

*** Correspondence:**
Antje Dietz-Pfeilstetter
antje.dietz@julius-kuehn.de

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**Abstract**

**Increasing pest and pathogen challenges as well as having fewer conventional pesticides to employ require innovative and sustainable solutions for plant protection. One group of pesticides that is in the pipeline and is expected to be subject to regulation and risk assessment procedures in the near future, is based on the natural gene silencing mechanism RNA interference (RNAi). These dsRNA-based products can be highly specific for a target organism due to the sequence-specific interaction between effective small interfering RNAs (siRNAs) and a complementary target RNA. General regulatory frameworks for pesticide authorization in the U.S. and in the EU are presented. In addition, production and application procedures and specific characteristics of dsRNA-based pesticides relevant for risk assessment and regulation are considered.**

## 1 Introduction

Increasing food demands of a growing world population in combination with new plant pests and diseases due to climate change (Chakraborty and Newton, 2011) call for improved solutions for crop protection. The future potential of current pest and pathogen control in crops based on chemical pesticides is limited because fewer approved pesticides are available, especially in Europe. Actually, it is expected that the "Green Deal" recently initiated in the European Union (EU) will result in a further reduction of active pesticidal substances for the control of insect pests and fungal pathogens. Therefore innovative and sustainable approaches are required to combat current and future pest problems.

One possible solution is the cultivation of genetically modified (GM) crops with built-in plant protection. Examples are GM crops expressing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) that specifically kill certain pest insects (e.g. Perlak et al., 1990) and GM maize expressing double-stranded (ds) RNA resulting in the sequence specific degradation of messenger RNA (mRNA) coding for an essential insect protein by an RNA interference (RNAi) mechanism

(Bachman et al., 2016). GM crops with such plant-incorporated protectants (PIP) have been authorised for cultivation in the USA several years ago (Mendelsohn et al., 2003; Head et al., 2017). In the EU, however, there is only cultivation of one insect resistant Bt maize line on small hectarages in Spain and Portugal (Gómez-Barbero et al., 2008; ISAAA, 2019).

RNAi, which is a natural gene silencing mechanism in plants and animals (Fire et al., 1998; Baulcombe, 2004) can not only be used for plant-incorporated protection in GM crops by means of host-induced gene silencing (HIGS), but can also serve as a new mode of action for exogenously applied pesticides. There have been various reports of RNAi molecules as plant protection compounds (San Miguel and Scott, 2016; Worrall et al., 2019) since the first successful foliar applications of dsRNAs for plant virus control (Tenllado and Diaz-Ruiz, 2001). Fungal pathogens as well as different insect pests are susceptible to RNAi and can be controlled by topical dsRNA applications (Taning et al., 2020; Fletcher et al., 2020). RNAi is a fast-growing technology with increasing commercial interest, which is reflected by global research trends and the patent situation (Jalaluddin et al., 2019). It is expected that RNAi-based plant protection products, which are much more specific than conventional pesticides and less persistent in the environment, will be available in the near future for practical uses and will need to undergo appropriate safety evaluation and authorization procedures.

## 2 United States (U.S.) Regulatory framework for pesticides

In the U.S., a pesticide is defined as: (1) any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest; (2) any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant; and (3) any nitrogen stabilizer. Pesticide products must obtain a U.S. Environmental Protection Agency (EPA) registration before manufacture, transport, and sale (Leahy et al., 2014). Field testing prior to a registration requires an Experimental Use Permit. EPA assesses pesticide end-use and manufacturing-use products for registrations and evaluates the active ingredient as well as the actual products. Additionally, states or territories within the U.S. require pesticide product registration as well. While most states or territories rely on the review of data and safety findings of the U.S. federal government, there are several states, e.g. California, that also require data to be submitted to them, which they review and also do their own assessment.

EPA primarily regulates the use of pesticides under the legal basis of two federal statutes enacted by the U.S. Congress: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Other statutes that play roles in the regulation of pesticides in the U.S. include the Food Quality Protection Act of 1996 (FQPA), the Pesticide Registration Improvement Act (PRIA), the Endangered Species Act (ESA), the Migratory Bird Treaty Act, and the Clean Water Act. FIFRA is the pesticide licensing law and provides the basis for registration, sale, distribution and use of pesticides in the U.S. FIFRA authorizes EPA to assess and register pesticides for specific uses. EPA also has the authority to suspend or cancel the registration of a pesticide if subsequent information shows that continued use would pose unreasonable risks. Pesticide registration is based on a risk/benefit standard. Through the registration process, EPA regulates pesticide use through labelling, packaging, composition, and disposal.

EPA must make a finding of no unreasonable adverse effects to man and the environment from use of the pesticide in order to support its registration decision under FIFRA. The Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to set maximum residue levels, or tolerances, for pesticides used in or on foods or animal feed. Under FFDCA and amendments to both FFDCA and



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FIFRA under the FQPA, EPA must make a similar finding of a reasonable certainty of no harm if the use of such agents results in residues in food or feed. If the submitted information supports this safety finding, EPA may establish a numerical tolerance or an exemption from the requirement of a tolerance regarding those residues.

Data requirements for assessing pesticide products are laid down in the regulations enacted by EPA in Title 40 of the Code of Federal Regulations (CFR) Part 158. These data requirements support evaluation of both the pesticide active ingredients and products. In addition to data requirements for conventional chemical pesticides, 40 CFR Part 158 lists requirements for biochemical and microbial pesticides which are usually inherently less toxic than conventional chemical pesticides. Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances that interfere with mating, such as insect sex pheromones, as well as various scented plant extracts that attract insect pests to traps. Microbial pesticides consist of a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient. Guidance for conducting the studies listed exist in EPA's Test Guidelines for Pesticides and Toxic Substances, https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances. (Leahy et al., 2014).

## 3 European Union (EU) Regulatory framework for plant protection products

The authorization process of plant protection products is already outlined in Schenkel and Gathmann (2020). The general principles of the European authorisation process are again described hereafter to ease the comparison between the U.S. and the EU in this paper.

In the EU, in general, any plant protection product (PPP) – i.e. a pesticide that protects crops or other useful plants - placed on the market needs an authorization. The legal framework is defined in the Regulation (EC) No 1107/2009 (EC, 2009). The authorization process is divided in approval of the active substance and in the authorization of the PPP. A precondition for the authorization of a PPP is an approval of the active substance (a.s.) by the EU Commission based on a risk assessment led by the European Food Safety Authority (EFSA). A PPP containing an approved active substance is assessed and authorised by the Member States (MS). To enhance the efficiency of the authorization process, the EU is divided in three zones, the Northern, Central and Southern zone (EC, 2009). The risk assessment of a PPP is conducted by one Member State (zonal rapporteur Member State (zRMS)) for the whole zone. In principle, MS of the same zone are forced to take over the results of the assessment and the decision of the zRMS. However, MS can make claims on national ecological or agricultural specificities to decide on dividing risk management options for their country.

Data requirements for assessing active substances are laid down in Regulation (EC) No 283/2013 (EC, 2013a) and for PPPs in Regulation (EC) No 284/2013 (EC, 2013b), respectively. Uniform principles for evaluation and authorisation of plant protection products are laid down in the implementing Regulation (EU) No 546/2011 (EC, 2011a). The goal of the regulations is to achieve a high level of protection of human and animal health as well as the environment in all Member States. The regulations are complemented by guidance documents produced by OECD, EPPO and EFSA to describe the methodological requirements for the risk assessment of active substances and pesticide products.

In principle the Regulation (EC) No 1107/2009 (EC, 2009) differentiates between PPPs based on chemicals and microorganisms, where microorganism means any microbiological entity, including



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124 lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic
125 material. Specific data requirements for the assessment of microorganisms are laid down for active
126 substances in Part B of Regulation (EC) No 283/2013 (EC, 2013a) and for PPP in Part B of
127 Regulation (EC) No 284/2013 (EC, 2013b), respectively. Additionally, further categories of PPPs are
128 defined in the Regulation (EC) No 1107/2009 (EC, 2009). PPPs classified as low-risk products have
129 to fulfil strict criteria in particular low toxicological potential (and low persistence in the environment
130 (for details see Article 22 Annex II of EC (2009)). Furthermore, no specific risk mitigation measures
131 should be required following the risk assessment for low-risk PPPs and they have to be sufficiently
132 effective. In practice only a few a.s. are authorised in the EU as low-risk PPPs (see EU, 2021).
133 Another category of PPPs are basic substances. A basic substance is not predominantly used for plant
134 protection purpose but nevertheless it is useful in plant protection either directly or in a product
135 consisting of the substance and a simple diluent. It does not have any inherent capacity to cause
136 endocrine disrupting, neurotoxic or immunotoxic effects and it is not a substance of concern (Article
137 23, EC, 2009), which means it has no capacity to cause an adverse effect on humans (Article 3, EC,
138 2009). Currently 23 basic substances are approved in the EU for example beer, *Allium cepa* extraxt or
139 L-cysteine (EU, 2021). A category "biopestides" with divergent data requirements as in the U.S.
140 does not exist in the EU. PPPs based on dsRNA do not fit in any of these categories.

141 The initial focus of the regulations was set on chemicals as active substances. However, Article 77 of
142 the Regulation (EC) No 1107/2019 (EC, 2009) offers the possibility that "the Commission may …
143 adopt or amend technical and other guidance documents such as explanatory notes or guidance
144 documents on the content of the application concerning microorganisms, pheromones and biological
145 products, for the implementation of this regulation. The Commission may ask the Authority to
146 prepare or to contribute to such guidance documents." The rationale behind Article 77 might be that
147 data requirements for these product classes deviate from those for chemicals. Such specific adaptions
148 on the data requirement are already implemented for microorganisms, pheromones and botanicals
149 (EC, 2011b, 2014a, b, 2016). However, no specific guidance documents defining the data
150 requirements for the authorisation of dsRNA-based PPPs are in sight, but first considerations and
151 recommendations were presented by OECD (2020).

152 In summary PPPs based on dsRNA currently have to be considered as chemical PPPs.

## 153 4 Production and application methods of dsRNA-based pesticides

154 For successful commercial application of RNAi for crop protection, it is necessary that products are
155 cost-efficient, safe and can be reliably delivered to the site of action in the target organism. There are
156 several ways of production for sequence specific dsRNA. While chemical synthesis as well as *in*
157 *vitro* transcription is rather costly, dsRNA production in microorganisms is much less expensive
158 (Taning et al., 2020; Dalakouras et al., 2020). However, considerations for microbial production
159 include possible by-products from fermentation and the added issue of genetically modified
160 organisms (GMO). In addition to the usual quality control to ensure dsRNA purity, bacterial
161 production systems require particular care in order to exclude potential contaminants and living
162 GMOs. Recently cell-free platforms for dsRNA synthesis have been established which enable the
163 inexpensive GMO-free production of large dsRNA amounts sufficient for usage in agricultural
164 applications (Taning et al., 2020).

165 It has been found repeatedly that naked dsRNA is not stable long enough after foliar or soil
166 application to ensure long-lasting plant protection (Mitter et al., 2017; Qiao et al., 2021), especially
167 under field conditions where UV light, rain-fall and microbial degradation account for its rapid



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168 degradation and inactivation (Parker et al., 2019; Bachman et al., 2020). Therefore, stabilizing
169 formulations may be necessary for successful usage of dsRNA in topical spray-induced gene
170 silencing (SIGS) applications. Mitter et al. (2017) used positively charged layered double hydroxyde
171 (LDH) clay nanosheets as dsRNA carrier (BioClay) in order to protect dsRNA from degradation after
172 foliar application. Other potential dsRNA carriers that were found to increase the persistence of
173 dsRNA in soil are cationic polymers (Whitfield et al., 2018).

174 Depending on the target pest or pathogen there are different ways of exogenous application of
175 dsRNA (Dalakouras et al., 2020). Most straightforward is SIGS that was shown to be effective for
176 the control of leaf-feeding insects and against certain phytopathogenic fungi (San Miguel and Scott,
177 2015; Koch et al., 2016; Wang et al., 2016), when dsRNA uptake into plant cells is not required. If
178 xylem sap-feeding insects or xylem-residing pathogens are the target, more laborious application
179 techniques are required. Dalakouras et al. (2018) demonstrated that double-stranded hairpin (hp)
180 RNA could be delivered into the xylem of woody plants by trunk injection, where it was systemically
181 transported. With conventional SIGS and trunk injection, dsRNA is not taken up by plant cells and
182 thus is not processed into effective small interfering RNAs (siRNAs) within the plant. For the control
183 of plant pathogenic viruses, however, dsRNA or effector siRNAs need to be delivered into plant cells
184 in order to enter the plant RNA silencing machinery. Entry into plant cells is usually not easily
185 achieved due to the cuticule and the plant cell wall, which act as physical barriers for nucleic acids.
186 For successful delivery of RNAi inducers, mechanical wounding of plant tissues is required. This can
187 be achieved by high-pressure spraying, as was shown by Dalakouras et al. (2016) or by special
188 carrier molecules facilitating cellular uptake. Examples for carrier molecules successfully used for
189 plant cell delivery are positively charged peptides (Numata et al., 2014), cationic fluorescent
190 nanoparticles (Jiang et al., 2014), DNA nanostructures (Zhang et al., 2019) and carbon nanotubes
191 (Demirer et al., 2020). These carriers also provide some protection against RNase degradation inside
192 the plant.

193 Besides carrier molecules or formulations for the enhancement of environmental stability and plant
194 cellular uptake, additional RNA modifications and/or carrier compounds like chitosan, carbon
195 quantum dots (CQD) or silica nanoparticles may be used to enhance cellular intake by insects (Das et
196 al., 2015). Thereby the range of RNAi-based pesticide control may be extended to pest organisms
197 that are less susceptible to RNAi.

## 198 5 Specific characteristics of dsRNA-based pesticides

199 There are several factors to consider regarding the data needed to support dsRNA-based pesticides/
200 PPPs due to their mode of action. In this section we highlight some aspects which might alter data
201 needs for the risk assessment and decision making. A detailed overview about the state of the art is
202 given in the OECD Working paper "Considerations for the Environmental Risk Assessment of the
203 Application of Sprayed or Externally Applied ds-RNA-Based Pesticides" (OECD, 2020).

204 Due to the sequence-specific interaction of siRNAs with complementary target RNA in RNAi (Fire
205 et al., 1998; Baulcombe, 2004), this mode of action can be highly specific for a target organism or a
206 group of target organisms. Yet, potential effects on non-target organisms (NTOs) due to partial
207 sequence homologies of dsRNA and transcription products in NTOs have to be considered
208 (Christiaens et al, 2018). There may be differences between organisms in terms of how the RNAi
209 machinery functions in relation to base pair mismatches and there are scientific uncertainties about
210 the rules determining interactions between siRNA and mRNA. However, current research on RNAi
211 mechanisms, generation of genomic data libraries for relevant species, and design of algorithms to

212 make reliable predictions are expected to increase the usability of bioinformatics data in the
213 prediction of potential effects on NTOs. Under these conditions, bioinformatics models and
214 programmes that can access a database with a large selection of key sequences can help in identifying
215 off-target sequences to minimise the risk of potential off-target hits. However, bioinformatics can
216 only be one component of the consideration. Risk assessment is also strongly influenced by
217 biological assay data and an overall understanding of the biological and ecological systems in which
218 the dsRNA will be used (Fletcher et al., 2020). Sequence information and bioinformatics can inform
219 the selection and prioritization of non-target species for toxicity and effects testing. Bioinformatics
220 should be augmented by an empirical approach – to introduce dsRNA (that is perfectly
221 complementary to the selected gene in a target organism) to a range of other organisms, starting with
222 close relatives and then moving outwards, to see how more phylogenetically-distant organisms
223 respond (Bachman et al., 2013; Romeis and Widmer, 2020).

224 Non-Target organism toxicity study protocols for addressing risk with dsRNA-based products require
225 some revisions compared to how they are carried out for biochemical pesticides because dsRNA-
226 based pesticides often take longer to display efficacy. Any evaluation needs to account for this time
227 lag by extending the study observation period. For organisms that have been demonstrated to be
228 responsive to environmental RNA, consideration of life cycle studies (growth, development and
229 reproduction) and studies on other non-lethal effects should be considered. The importance of sub-
230 lethal endpoints in addition to mortality has been stressed by Romeis and Widmer (2020), who
231 suggest an approach on how to conduct non-target studies for dsRNA-based plant protection
232 products.

233 In the evaluation of environmental risks from the application of dsRNA-based pesticides, the
234 distribution, fate, stability and persistence of the dsRNA following product application in the
235 environment will be important (OECD, 2020). The study by Dubelman et al. (2014) shows a rapid
236 degradation of the DvSnf/ dsRNA transcript derived from a genetically modified maize variety in
237 different agricultural soils. Bachman et al. (2020) report a rapid decline in the concentration of foliar
238 applied dsRNA under field conditions with 95% reduction after 3 days. In a recent study, Parker et al.
239 (2019) found that decrease of dsRNA in soil was due to both adsorption to soil and to chemical and
240 microbial degradation. However, the data regarding the environmental fate of dsRNA are still
241 somewhat limited. According to USEPA (2013) the distribution and fate of dsRNA in the
242 environment will depend on the following factors: application rate of active ingredient, application
243 timing, application method, number of applications, off-site movement of applied dsRNA and the
244 stability and persistence of exogenously applied dsRNA following application.

245 Some insects, especially of the order Coleoptera (beetles), have been shown to be very sensitive to
246 dsRNA (Baum and Roberts, 2014). Therefore, even small amounts of ingested dsRNA with target
247 sequence homology can induce RNAi which may cause insect mortality. However, there are large
248 differences in the response of insects to ingested dsRNA. Insects of the order Diptera (flies and
249 mosquitoes), Hemiptera (aphids, hoppers, stinkbugs), and Lepidoptera (moths and butterflies),
250 respond to dsRNA with greater variability compared to beetles (Cooper et al., 2019). Additionally,
251 differences in the response can also be found within the same order between species, life stages,
252 genes and tissues (reviewed in Christiaens et al., 2020). The current knowledge suggests that dsRNA
253 based products might be very specific for an insect genus or species, respectively. The mechanisms
254 triggering the sensitivity of different species to RNAi are not fully understood yet. The potential use
255 of RNAi technology for pest and plant pathogen control is influenced by the cellular uptake of
256 dsRNA (Cooper et al., 2019; Wytinck et al., 2020). Starting with the uptake process and followed by
257 the behaviour of the dsRNA in the organism there are many steps that will affect RNAi efficiency.



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Cooper et al. (2019) collected different hypotheses which were proposed to explain the observed differences in RNAi efficiency among insects. These factors are instability of dsRNA, incomplete dsRNA internalization, deficient core RNAi machinery, impaired systemic spreading of the RNAi signal and refractory target genes.

The problems identified for insects can be similarly transferred to other fields of dsRNA-based PPP applications. While it has been well known for some time that certain fungi like *Ustilago maydis* and *Saccharomyces cerevisiae* lack core RNAi components (Drinnenberg et al., 2011), recent findings by Qiao et al. (2021) suggest large differences in dsRNA uptake between fungal pathogens. To counter some of the issues, producers are likely to use carriers and formulations containing synergists or co-formulants that stabilize dsRNA in the environment and enhance transport into the cells of target pests. Special attention should be paid to how barriers to uptake are proposed to be overcome for the target organism. Information and/or studies on the impact of carriers and formulations on uptake and environmental persistence are important to characterize exposure to the dsRNA (OECD, 2020).

Due to their relevance for gene regulation and virus defense in plants and animals, dsRNAs as well as their processing products are natural components of food and feed (Dávalos et al., 2019). Therefore, dsRNAs have a long history of safe consumption by humans and other vertebrates (OECD, 2020). Exposition of humans and farm animals after oral uptake of dsRNA or siRNA has been reported to be negligible because of degradation and multiple barriers in the gastrointestinal tract of mammals (O´Neill et al., 2011; Petrick et al., 2013). As is stated in the OECD Working paper (2020), the common occurrence of effective physiological and biochemical barriers among mammals and probably among other vertebrates is likely a consequence of the widespread presence of RNAs in the environment.

## 6 Classification and authorization procedure of dsRNA-based pesticides for plant protection according to U.S. and EU regulations

While data requirements specific to sprayed or externally applied dsRNA-based pesticides have not been enacted in the U.S., EPA bases its requirements for these pesticides on the biochemical pesticide requirements, outlined in Subpart U - Biochemical Pesticides of 40 CFR Part 158.[1] Often, the technical grade material of the pesticide is tested in mammalian and non-target organism toxicity studies along with product formulations for acute mammalian toxicity and irritation studies. Some of this data may be appropriate to waive according to 40 CFR Part 158.45.[2] Further, data not listed in Subpart U for Biochemical Pesticides that is more specific to sprayed or externally applied dsRNA-based pesticides may be required according to 40 CFR Part 158.75.[3] Where specific product formulations impact barriers to and uptake of the dsRNA, such additional data might include product-specific formulation non-target organism toxicology testing to better characterize the potential for hazard.

If a living genetically engineered organism is used in the manufacturing process to produce a sprayed or externally applied dsRNA-based pesticide, but is not viable in the final product, it would be



[1] [ HYPERLINK "https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=sp40.26.158.u&rgn=div6" ].

[2] [ HYPERLINK "https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=se40.26.158_145&rgn=div8" ].

[3] [ HYPERLINK "https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=se40.26.158_175&rgn=div8" ].

295 considered a pesticide intermediate in the US under the Toxic Substances Control Act (TSCA).
296 Pesticide intermediates are subject to oversight under TSCA and would likely require submission of a
297 Microbial Commercial Activity Notice (MCAN) before initiating manufacture (Wozniak et. al.,
298 2013).

299 As outlined above, it can be expected that dsRNA-based PPPs will have different properties than the
300 chemicals mostly used as active substances in PPPs until now. Adaptations of the EU data
301 requirements for the risk assessment might be reasonable for different reasons (see previous chapter).
302 In the EU applications of PPPs with dsRNA as active substance are expected in the coming years.
303 Therefore, the Competent Authorities in the EU should start intensive discussion how the risk of such
304 products will be adequately assessed in the future. One cannot expect that specific guidance
305 documents will be in place when first applications are submitted in the EU. Experiences show that
306 the development and implementation of such guidelines take several years. Until then, the same data
307 requirements are requested as for chemical PPPs. Possible waivers or adjustments of the risk
308 assessment for specific areas of concern might be decided on case-by-case basis.

309 In the EU additional regulatory requirements might be taken into account depending on the used
310 production system. In the case of production systems based on GM microorganisms an authorisation
311 according to Directive (EC) 2001/18 is needed if it cannot be guaranteed that the GM
312 microorganisms are completely inactivated (EC, 2001). It is important to note that GMO-derived
313 products which do not contain a living organism and are not to be used as food or feed, like topically
314 applied PPPs, are not regulated under GMO regulations within the EU (see also Schenkel &
315 Gathmann, 2021).

316 **7 Conclusions**

317 The thoughts we highlight in this paper are pertinent for assessing the risk of sprayed or externally
318 applied dsRNA-based pesticides for plant protection. Further discussion of these regulatory and
319 biosafety issues would help better clarify the risk assessment and risk management framework for
320 these new and innovative products. Sprayed or externally applied dsRNA-based pesticides are in the
321 pipeline and the regulatory authorities will likely be having to assess their risk in the near future.

322 **8 Conflict of Interest**

323 *The authors declare that the research was conducted in the absence of any commercial or financial*
324 *relationships that could be construed as a potential conflict of interest.*

325 **9 Author Contributions**

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