



Pressure from insect-resistant maize on protected butterflies and moths

Mathias Otto¹  | Phillip Papastefanou²  | Lorenz Fahse³

¹Federal Agency for Nature Conservation (BfN), Bonn, Germany

²TUM School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany

³Institute for Environmental Sciences, University of Koblenz-Landau, Renamed Rhineland-Palatinate Technical University Kaiserslautern, Landau, Germany

Correspondence

Mathias Otto, Federal Agency for Nature Conservation (BfN), Konstantinstraße 110, 53179 Bonn, Germany. Email: mathias.otto@bfm.de

Current address: Phillip Papastefanou, Max Planck Institute for Biogeochemistry, Hans-Knöll-Str. 10, 07745 Jena, Germany.

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Abstract

Intensification in agriculture affects many insect species, including butterflies. Insect-resistant crops, such as Bt (*Bacillus thuringiensis*) maize, which produces a toxin active against Lepidoptera, are an alternative to insecticide sprays. Genetically modified crops are regulated in most countries and require an environmental risk assessment. In the European Union, such assessments include the use of simulation models to predict the effects on nontarget Lepidoptera (NTL). To support the assessment of protected NTL, we extended an individual-based, stochastic, spatially explicit mathematical model (LepiX) to include a wider range of exposure scenarios, a species-sensitivity distribution, and an option for repeated exposure of individuals. We applied the model to transgenic maize DAS-1507, which expresses a high concentration of Bt toxin in pollen that may be consumed by NTL larvae on their host plants nearby. Even in the most conservative scenario without repeated exposure, mortality estimates for highly sensitive species ranged from 41% to 6% at distances of 10–1000 m from the nearest maize field. Repeated exposure can cause additional mortality and thus is relevant for the overall risk assessment. Uncertainties in both exposure and ecotoxicity estimates strongly influenced the predicted mortalities. Care should be taken to include these uncertainties in the model scenarios used for decision-making. In accordance with other modeling results, our simulations demonstrated that mean mortality may not be safe for protected species. With its high pollen expression, DAS-1507 maize may pose risks to sensitive and protected butterfly and moth species that may be difficult to manage. High expression of Bt toxin in pollen is unnecessary for controlling target pests. Consequently, we suggest that Bt maize with high pollen expression not be cultivated in regions where protected butterflies are to be conserved.

KEYWORDS

Bt maize, genetically modified organism, GMO, Lepidoptera, mathematical modeling, nature conservation, risk assessment

Resumen

La intensificación en la agricultura afecta a muchas especies de insectos, incluyendo a las mariposas. Los cultivos resistentes a los insectos, como el maíz Bt (*Bacillus thuringiensis*), el cual produce una toxina activa contra los lepidópteros, son una alternativa a los insecticidas. Los cultivos genéticamente modificados (GM) están regulados en la mayoría de los países y requieren de una evaluación de riesgo ambiental. En la Unión Europea (EU), dichas evaluaciones incluyen el uso de modelos de simulación para pronosticar los efectos sobre los lepidópteros no objetivo (LNO). Para apoyar a la evaluación de LNO protegidos, extendimos un modelo matemático espacialmente explícito, estocástico y basado en el individuo

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(LepiX) para incluir una mayor gama de escenarios de exposición, una distribución de la sensibilidad de las especies y una opción para la exposición repetida de los individuos. Aplicamos el modelo al maíz transgénico DAS-1507, el cual expresa una alta concentración de toxina Bt en el polen que puede ser consumido por las larvas de LNO en una planta hospedera cercana. Incluso en el escenario más conservador sin una exposición repetida, las estimaciones de mortalidad para las especies altamente sensibles variaron entre el 41% y el 6% en distancias de 10-1000 m a partir del campo de maíz más cercano. La exposición repetida puede causar mortalidad adicional y por lo tanto es relevante para la evaluación general del riesgo. La incertidumbre en las estimaciones de la exposición y la ecotoxicidad influyeron fuertemente sobre la mortalidad pronosticada. Se debe tener cuidado de incluir estas incertidumbres en los escenarios modelados usados para la toma de decisiones. De acuerdo con los resultados de otros modelos, nuestras simulaciones demostraron que la mortalidad media podría no ser segura para las especies protegidas. Con su alta producción de polen, el maíz DAS-1507 podría representar un riesgo difícil de manejar para las especies de mariposas y polillas sensibles y protegidas. No se necesita una expresión elevada de la toxina Bt en el polen para controlar a las plagas. En consecuencia, sugerimos que no se cultive el maíz Bt con una alta producción de polen en las regiones en donde se busca conservar a las mariposas protegidas.

Presión del maíz resistente a insectos sobre mariposas y polillas protegidas

PALABRAS CLAVE

conservación de la naturaleza, evaluación de riesgo, Lepidoptera, maíz Bt, modelo matemático, organismo genéticamente modificado, OGM

INTRODUCTION

Testing pesticides and genetically modified (GM) crops that may produce their own pesticides (Glare & O'Callaghan, 2000; Koul & Dhaliwal, 2004) is linked to risk assessment procedures in the European Union (EU) and many other countries. The assessment provides protection for human health, animal welfare, and the environment. Conservation issues are part of the environmental risk assessment and include effects on protected species (EFSA, 2016; NRC, 2013). The importance of this aspect is highlighted by reports of drastic insect decline (Brühl & Zaller, 2019; Hallmann et al., 2017).

Bacillus thuringiensis (Bt) maize is one of the main GM crops developed to protect against insect pests (Parisi et al., 2016). Insect resistance in Bt crops is achieved by splicing genes derived from strains of *Bacillus thuringiensis* into the plant genome, enabling the expression of toxic Bt proteins (Höfte & Whiteley, 1989; Palma et al., 2015). Typically, Bt proteins are expressed in all plant tissues, including pollen. Wind-pollinated plants, such as maize and other grasses, produce large amounts of pollen that spread across the surrounding landscape over considerable distances (Hofmann et al., 2014; Klein et al., 2006). Consequently, natural areas, including sites of conservation concern, are exposed to pollen and thus Bt toxins (Hofmann et al., 2010). Therefore, herbivores, especially insects, may consume toxic pollen originating from GM crops when feeding on their host plants. Nontarget Lepidoptera (NTL) are probably affected by the cultivation of Bt maize because maize pollen deposition and larval phenology overlap in many species (Lang & Otto, 2010). Furthermore, the number and distribution of European butterflies and moths, including common and widespread

species (van Dyck et al., 2009), have decreased dramatically over the last decades (European Environment Agency, 2013; Habel et al., 2016; van Strien et al., 2019). For butterflies and likely many other insects (Hallmann et al., 2017), loss of habitat and increased pesticide use due to the intensification of agriculture are major factors in this decline. Resulting off-field effects mediated by spray drift, pesticide runoff, or toxic pollen need to be investigated to inform risk management and take appropriate actions for specific protection goals.

In the EU (Reg. [EC] 1829/2003, Directives 2001/18/EC and 2018/350) and many other countries, genetically modified organisms (GMOs) are regulated and have to undergo an environmental risk assessment before market release. Potential effects on nontarget organisms (NTOs) are an important aspect of this assessment. Moreover, effects on NTLs are one of the most obvious risks to investigate for Bt plants with resistance against pest Lepidoptera. Nevertheless, authorities initially overlooked such effects. Concern only gained momentum after Losey et al. (1999) provided evidence that larvae of the monarch butterfly (*Danaus plexippus*) may be at risk from ingesting Bt maize pollen on their host plants. These results triggered a new, comprehensive assessment of the effects of Bt maize cultivation on monarch butterflies (Sears et al., 2001), including a full research program to obtain the necessary biological background information for the assessment (Anderson et al., 2005; Dively et al., 2004; Hellmich et al., 2001; Jesse & Obrycki, 2003; Mattila et al., 2005). Wolt et al. (2005) also carried out a detailed assessment of the pale grass blue butterfly (*Pseudoxizeeria Maba*) in Japan; however, such assessments are rare.

Modeling is recognized as a valuable tool to support risk assessment of NTLs from the cultivation of Bt maize because

of the high complexity and limited field data. Effects may depend on many factors, including the sensitivity of the species, phenology of the species, overlap with the maize flowering period, feeding behavior of the larvae, distribution and amount of pollen on host plants, and adoption rate of Bt maize cultivation (Baudrot et al., 2021; Lang & Otto, 2010; Perry et al., 2012). Since 2009, several models differing in complexity have been proposed for use in assessing the effects of Bt maize pollen on NTLs (Baudrot, Walker, et al., 2021; EFSA, 2015; Fahse et al., 2018; Holst et al., 2013; Perry et al., 2012; Perry et al., 2010; Walker et al., 2017). The European Food Safety Authority (EFSA) assesses GM crops and is about to replace its existing model with a more advanced solution (Baudrot, Lang, et al., 2021). Models may improve understanding of landscape biodiversity effects (Leclerc et al., 2017; Perry et al., 2012; Perry et al., 2010) or effects on local butterfly populations (Fahse et al., 2018; Holst et al., 2013).

For rare and protected species models, estimating local (i.e., a small spatial scale [Perry et al., 2012]) effects is appropriate because single populations or individuals are protected. Consequently, local populations play an important role, and protected areas with management for insect conservation need to be considered in risk assessment.

Fahse et al. (2018) built LepiX 1.0, a simulation model to assist risk estimation for local populations of butterflies and moths. In this initial assessment, Bt maize Mon810 and *Inachis io* were used in a test case, and the case highlighted the importance of reliable estimates for toxicity parameters and of reliable mortality estimates. We made LepiX more generic and addressed additional research questions related to the uncertainties of mortality estimates. We included a sensitivity estimate based on a species-sensitivity distribution (SSD), similar to Perry et al. (2012), because only a few NTL species have been tested (Lang & Otto, 2010; Lang et al., 2019) and species of conservation concern cannot be tested because of ethical standards. In the current simulation, we also included several scenarios for the strength of pollen deposition, the duration of exposure, and, for the first time to our knowledge, an option to allow the repeated exposure of larvae during their development.

Although most Bt maize events express toxins in pollen, the concentration of Bt toxin in pollen can vary substantially between events. We simulated the effects of pollen from maize DAS-O15O7-1 (DAS-1507), which expresses a high concentration of Bt toxin in pollen and may, therefore, have a more profound impact on protected areas compared with the cultivation of MON810 maize.

METHODS

LepiX-1.1

LepiX is an individual-based, object-orientated model programmed in C# that is used to analyze and estimate mortality in lepidopteran larvae after exposure to Bt maize pollen on their host plants. The model consists of five modules (Figure 1). The individual modules are described in detail in Fahse et al.

TABLE 1 Sensitivity classes of Lepidoptera larvae used for analysis of susceptibility to toxins present in the pollen of Bt maize.

	SSD ^a percentile							
	0.5	1	2	5	10	20	50	70
LC ₅₀ ^b (Pollen / cm ²)	1.6	4.7	13.5	56.7	176	600	4900	17685
log ₁₀	0.20	0.67	1.13	1.75	2.25	2.78	3.69	4.25

^aSSD: Species sensitivity distribution.

^bLC₅₀: Concentration eliciting 50% mortality.

(2018). Amendments to LepiX 1.0 in version 1.1 are described below. The full source code and model input and output used are publicly accessible (<https://zenodo.org/records/8188901>; <https://zenodo.org/records/8435077>).

Estimating species sensitivity by assuming SSD

Although LepiX-1.0 uses a species-specific dose–response curve to calculate mortalities, LepiX-1.1 offers the option of estimating mortality based on SSD (Posthuma et al., 2002). The concept of SSD is based on the assumption that interspecies differences in toxicity to a stressor can be described by a statistical distribution, which in turn is valid for the chosen group or community of organisms. The SSDs are used in combination with a chosen level of protection. This approach has the advantage that the level of protection defines the likelihood that a certain percentage of the species is protected (Forbes & Carlow, 2002). This concept was adopted by policymakers in the 1990s, (Aldenberg et al., 2002) and has subsequently been used to assist in the assessment of risks from pesticides to endangered species (Raimondo et al., 2008). In 2011, an SSD was implemented in a risk assessment model and risk management of Bt maize to derive different sensitivity classes of NTLs (EFSA, 2011).

In our SSD approach, we used a ranked distribution of species sensitivities (Figure 2, Table 1) based on an ordered ascending list *L* containing LD₅₀ or LC₅₀ (dose or concentration at which 50% of larvae die) of *n* species. We first derived a set *X* containing the log₁₀-transformed values of *L*: $X = \log_{10}(L)$ and a second set *Y* consisting of the hazen (Posthuma et al., 2002) position of each species:

$$Y = \frac{P - 0.5}{n}, \tag{1}$$

where *P* is a list containing the position of each species in *L*. Together, *X* and *Y* form a set of points that we used as input data to fit the sigmoidal SSD curve. We used a logit function for the SSD curve:

$$\text{logit}(x) = \frac{1}{1 + e^{ax + b}}. \tag{2}$$

The logit function was chosen to be consistent with the EFSA (EFSA, 2011; Perry et al., 2012) and was based on the same species data set (see Table 2 EFSA, 2011; most data

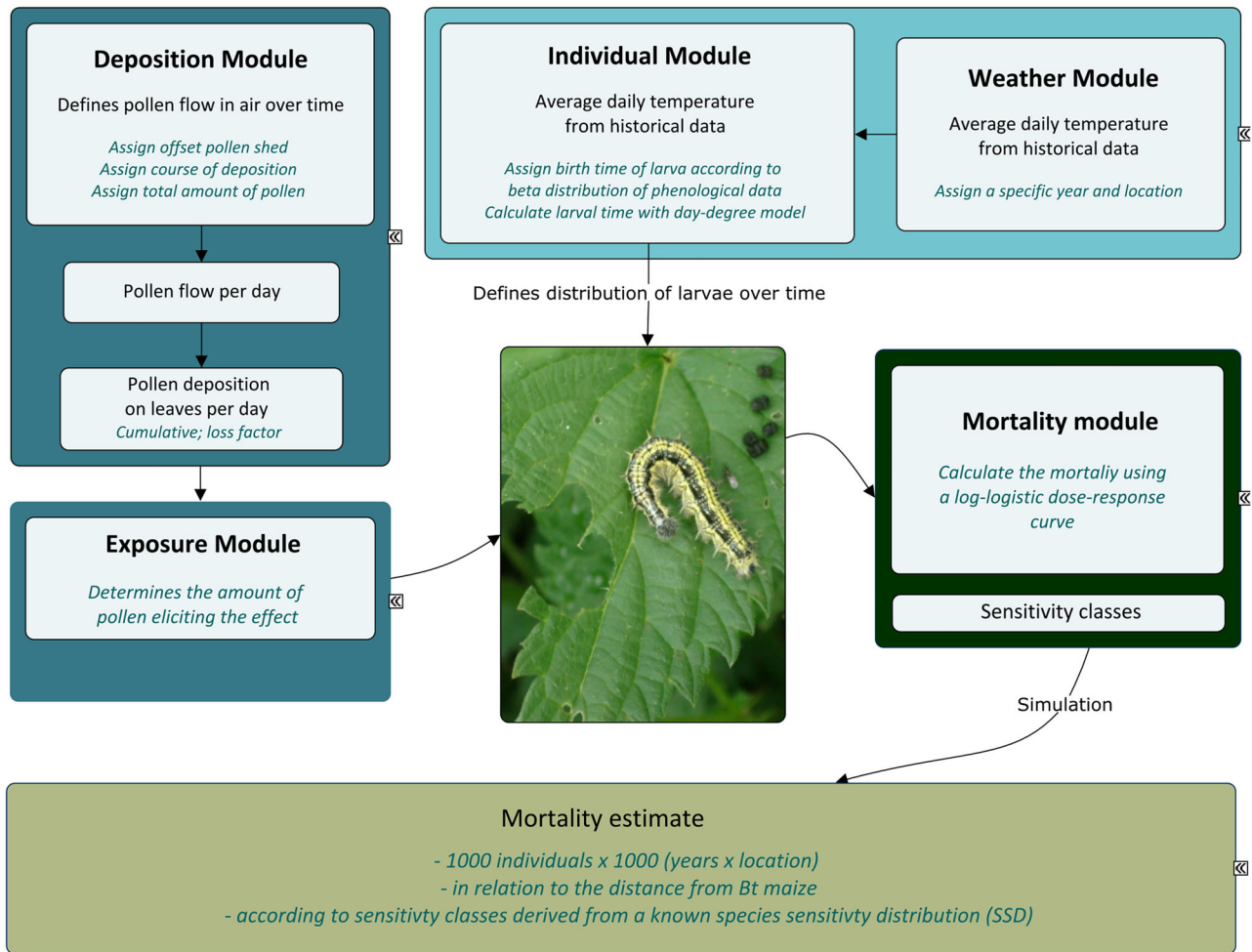


FIGURE 1 Schematic model overview of LepiX-1.1 for the estimation of the effects of Bt maize pollen on nontarget lepidopteran species feeding on host plants.

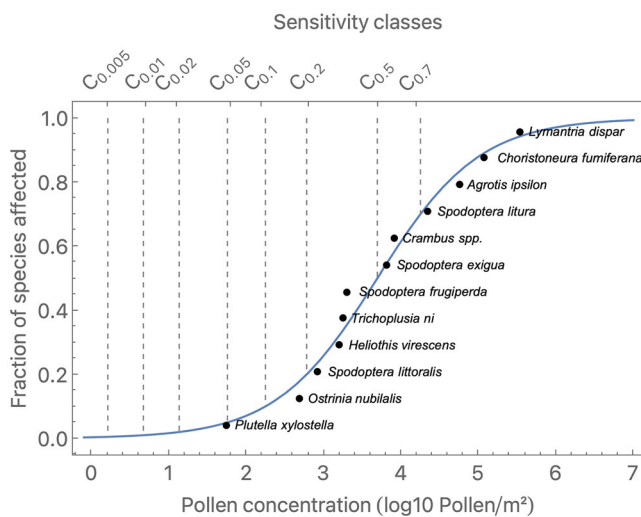


FIGURE 2 Species-sensitivity distribution for butterfly species exposed to Cry1F protein (C_i, sensitivity class used for model scenarios; single points, LC₅₀ [concentration eliciting 50% mortality] for different species). Toxicity data after Wolt et al. (2005) cf. Perry et al. (2012).

TABLE 2 Necessary distances (m) to the Bt maize field to fall short of a 1% mortality threshold for mean population mortality.

Larval sensitivity class ^c	Slope ^a -1.76			Slope -0.84		
	Deposition scenario ^b			Deposition scenario		
	low	standard	high	low	standard	high
0.5	1200	> 2000	> 2000	2000	> 2000	> 2000
1	160	> 2000	> 2000	300	> 2000	> 2000
2	30	2000	> 2000	40	> 2000	> 2000
5	2	120	160	5	180	300
10	0	20	30	0.5	0.5	30
20	0	2	5	0	2	5
50	0	0	0	0	0	0
70	0	0	0	0	0	0

^aSlope of the dose-response curve; values: -1.76 and -0.84 from Holst et al. 2013 and Perry et al. 2010, respectively.

^bDeposition scenario: Different assumption on the quantity of pollen deposition on host plants (see methods).

^cLarval sensitivity class according to a species sensitivity distribution (SSD); values denote the percentiles of the distribution.

derived from Wolt et al., 2005). Because risk assessment aims to protect sensitive species, we chose values below the average sensitivity (50th percentile [Table 1]).

Repeated exposure

The overlap between larval phenology and maize pollen availability on host plant leaves defines the possible Bt toxin exposure period for larvae. In LepiX, we defined the exposure A_L as the mean pollen deposition on leaves over period m , where m was set to a 4-day interval representative of the exposure time that elicits effects in larvae from Bt pollen uptake.

In the field, the length of the larval period depends on species and weather conditions, and longer larval periods under unfavorable weather conditions must be considered. Moreover, the pollen-shedding period may last up to 3 weeks in a field and over 5 weeks in a region (Hofmann et al., 2013). Therefore, larval phenology and the presence of pollen may overlap for several weeks (see also Baudrot et al., 2021), and repeated exposure is plausible, which may influence the magnitude of the effects. Consequently, we developed a model option in which larvae may experience a second round of exposure. A second round of exposure must be smaller than the first because it is calculated from the remaining values of the temporal distribution of pollen deposition.

Mathematically, we identified the second-highest exposure interval I_2 of the same exposure time (m) within the total larval period, leading to a second exposure A_{L2} of the larvae. We restricted the exposure interval overlap; hence, intervals I_1 and I_2 did not intersect. Using the dose–response relationship from the mortality module, we assigned mortality rates p_1 and p_2 for each of the two exposure intervals. The resulting mortality p of the larvae was estimated using the converse probability: $p = 1 - (1 - p_1)(1 - p_2)$.

Deposition scenarios

Pollen deposition on host plant leaves is cumulative and fluctuates considerably over time and between regions (Hofmann et al., 2011). To date, a standardized methodology is missing. Estimates of leaf pollen deposition are the most direct information defining exposure and have a substantial influence on any modeling approach dealing with mortality effects from Bt maize cultivation on NTLs. Therefore, uncertainties connected with these estimates are highly debated (EFSA, 2015; EFSA, 2016; Hofmann et al., 2014; Kruse-Plass et al., 2017; Perry et al., 2017), and, currently, no scientific procedure has been agreed upon for its calculation (Arpaia et al., 2018). In LepiX 1.0, the absolute amount of pollen deposition was derived from field data obtained by passive technical pollen samplers (PMF) (Hofmann et al., 2014). These data provide the distance relationship and information on the variability of single measures

of pollen flow that is to be expected under field conditions. This variability is caused by many factors, including different seed varieties, seeding dates, weather conditions, and the position of the acceptor relative to the maize field. Therefore, using the mean expected pollen deposition (standard deposition) does not represent the worst-case situations.

To consider the uncertainties related to leaf pollen deposition, we introduced three scenarios. In the low-deposition scenario, we reduced the factor measured by Hofmann et al. (2016) to correlate pollen deposition measured by PMF to the respective pollen leaf deposition on the host plant, in our case nettle. This was achieved by reducing the pollen air-to-leaf parameter (γ°) by a factor of 10. In the standard-deposition scenario, pollen deposition was as described in Fahse et al. (2018). In the worst-case deposition scenario, we used the upper 80% CI of a single observation from the distance relationship to account for instances of relatively high pollen deposition (Hofmann et al., 2014).

Case study of maize event DAS-1507 and *I. io* type phenology

We parameterized the simulation for DAS-1507 maize, which expresses the Cry1F protein targeting Lepidoptera. This maize is licensed for cultivation in North and South America as a single event and as part of a stacked GM varieties. In Europe, the application for cultivation is pending, but the product is approved for import and processing.

The Cry1F protein in DAS-1507 maize has an estimated protein concentration of 32 ng/mg DW Cry1F protein expression in pollen (EFSA, 2011), which is about 350 times higher than that of Cry1Ab in pollen of MON810 (Fahse et al., 2018). Pollen dispersal is independent of the Bt toxin content, consequently, high expression of Bt events is well suited for analyzing the potential effects on NTLs in adjacent habitats. Furthermore, there are a sufficient number of ecotoxicity tests for Cry1F, allowing the calculation of an SSD.

The individual model was parameterized to reflect the phenology of *I. io*, as described by Fahse et al. (2018). All simulations refer to regions where *I. io* is bivoltine and, therefore, partly exposed to maize pollen during the larval stage (Fahse et al., 2018).

Mortality threshold and risk management

A threshold mortality of 1% has been defined by EFSA as biologically relevant for protected butterfly species (EFSA, 2011, 2015). To meet this protection level, buffer distances between Bt maize cultivation and larval habitats can be prescribed as part of risk management. Because the distances are defined by mortality thresholds, uncertainties in the ecotoxicity parameters are important (Fahse et al., 2018).

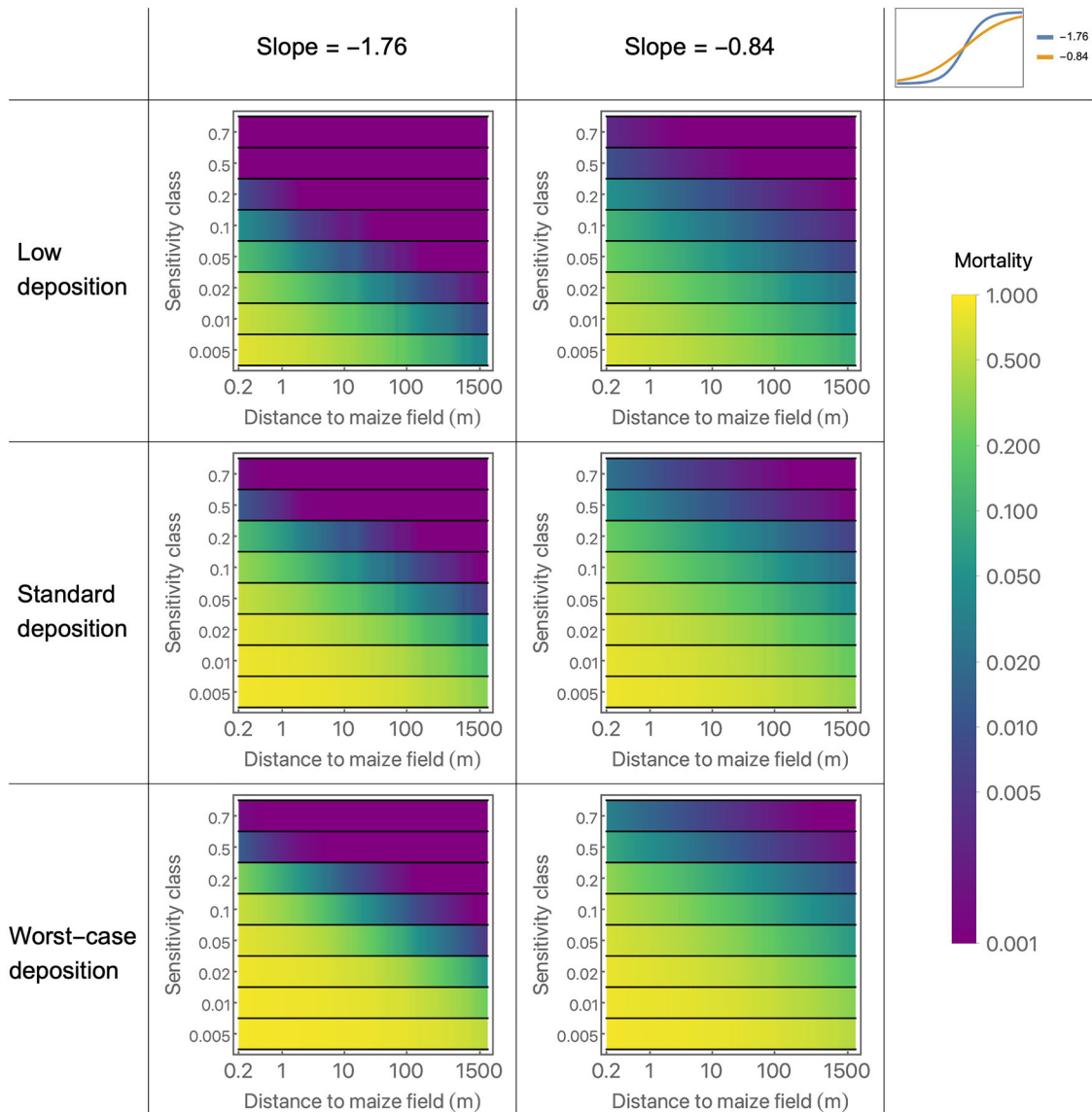


FIGURE 3 Estimated mortality of larvae when feeding on host plants at different distances from fields cultivated with DAS-1507 maize expressing the Cry1F toxin in pollen. Mortality estimates result from a single exposure interval during the larval stage; high, standard, and low refer to the respective scenario for deposition. Slopes -1.76 and -0.84 based on Holst et al. (2013) and Perry et al. (2010), respectively.

RESULTS

Larval mortality and deposition scenarios

Larval mortality caused by Bt pollen was predicted to range from 0% to 76% for all deposition and slope combinations (Figure 3). High mortality occurred near the maize field (0–10 m) and at distances beyond the 1-km range, even for the low-deposition scenario and a single exposure. Both ecotoxicity parameters (slope and LC_{50} of the dose–response curve) exerted a strong influence on the prediction of larval mortality (Figure 3). In the low-deposition scenario, buffer zones that achieved a $< 1\%$ protection level varied from 0 m for the lowest- to 1200 m for the highest sensitivity class when a conservative slope estimate was used (Table 2). Using a less conservative

slope estimate did not affect the larvae with a low sensitivity to the Bt toxin; rather, it shifted the buffer zone for the chosen protection level from 1200 to 2000 m.

Effect of repeated exposure

The additional, second exposure event, which was not included in our overall risk estimates (Figure 3), led to a moderate but substantial increase in overall mortality (Figure 4). Combined with the overall average, its effect may be greater in some populations, as can be seen from the shift in quartiles in Figure 4a. The effect was more pronounced when looking at the 25% most affected (upper quartile) individuals of all populations (Figure 4b).

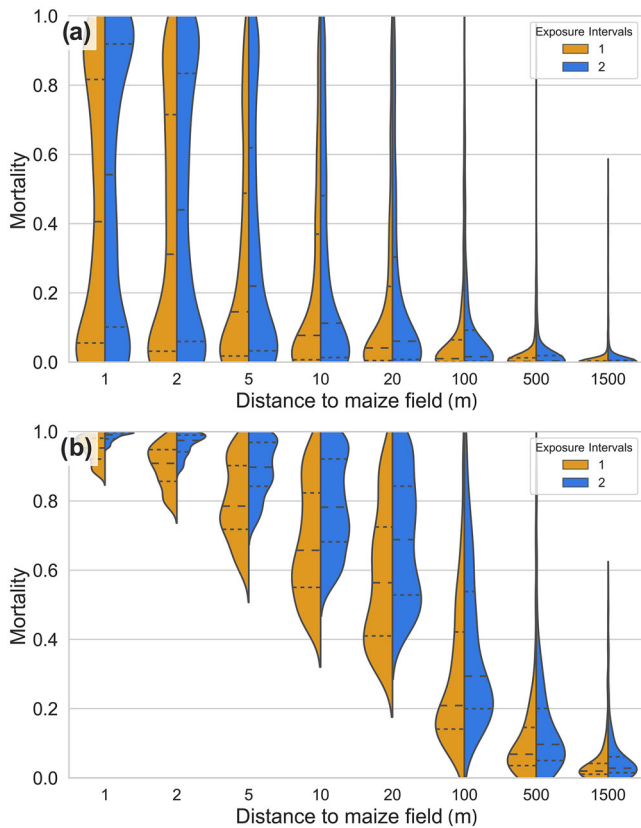


FIGURE 4 Mortality per population depending on the distance to the maize field considering 1 (orange) or 2 (blue) intervals of exposure: (a) all 1000 simulated populations and (b) only the 25% most affected (upper quartile) of individuals per population (dashed lines, quartiles [25th median and 75% quantile]). Model settings: low deposition scenario, slope = -1.76 , sensitivity class of larvae = C2 (2nd percentile of the species-sensitivity distribution).

Average mortality versus mortality of individual populations

In contrast to the average, a single population will be subject to lower variability (e.g., conditions in one specific year and site). We found that the average population mortality may underestimate the mortality risk for some populations (Figure 5). The median mortality over all populations was estimated to be 4%; 115 (of 1000 simulated) populations experienced mortalities above 50%, and 14 populations experienced mortalities above 90%.

DISCUSSION

Compared with Fahse et al. (2018), who used MON810 maize as a case study, DAS-1507 maize expresses a similar toxin. However, the higher quantities of toxin in the pollen caused a much higher risk for NTLs, even in the low-deposition scenario. Although Cry1F has been assumed to be less toxic by a factor of 5, at least to some Lepidoptera species, DAS-1507 maize pollen includes a 350 times higher amount of Bt toxin than MON810 maize (Hellmich et al., 2001; Wolt et al., 2005).

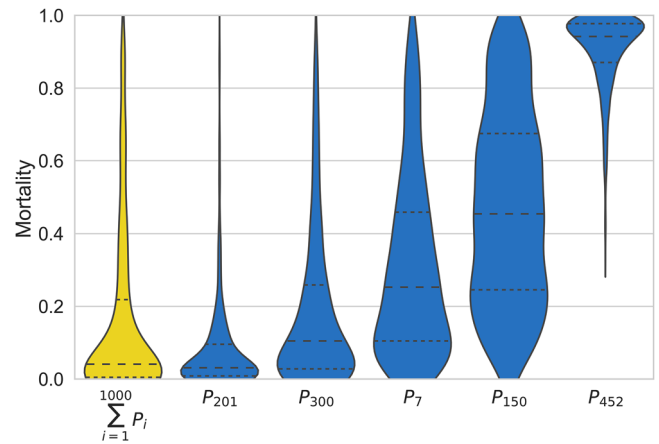


FIGURE 5 Comparison between mean mortality of 1000 populations (yellow) and mortality of selected populations (blue). The P_n assigns the n th population simulated. Model settings: low deposition scenario, slope = -1.76 , sensitivity class C2 (2nd percentile of the species-sensitivity distribution), distance to the maize field 20 m.

In this respect, DAS-1507 maize is conspicuous, exceeding the pollen concentration of Bt176 maize, which was phased out in 2001 after debates on negative effects on the monarch butterfly (Hellmich et al., 2001).

Irrespective of the slope used or the deposition scenario, our results, even without a second exposure interval (Figure 3), indicated that DAS-1507 maize may pose a substantial risk to Lepidoptera larvae with a sensitivity lower than the 5th percentile of the SSD. The mean mortality of these species ranged from 50%–100% at the field edge to 0%–20% at distances beyond 1 km. Similar to Fahse et al. (2018), our mortality estimates were strongly influenced by toxicity parameters, such as the slope of the dose–response relationship and the underlying deposition scenario. For LepiX 1.1, the uncertainty of the input data sets and parameterization are the main reasons for limitations in modeling estimates and the resulting risk assessment.

Ecotoxicological data and SSD

Toxicity parameters of the hazard and exposure combined allowed assessment of the risk to NTOs. Similar to Fahse et al. (2018), our simulation showed that ecotoxicological parameters, such as LC_{50} and the slope of the dose–response curve, had a strong influence on the risk estimates and management measures (Figure 3 & Table 2). Although the importance of reliable data has been emphasized previously (Arpaia et al., 2018; Lang & Otto, 2010; Lang et al., 2019; Perry et al., 2010), the pertinent data are rare and limited in methodology and the species range tested.

Lang et al. (2019) reviewed the methodology of studies submitted in GMO applications and concluded that nonstandardized test designs have several shortcomings that negatively affect the reliability of results and cause uncertainties in risk assessment. This study also updated a previous review (Lang

& Otto, 2010), confirming that the taxonomic range of the Lepidoptera tested heavily leans toward pest-target species. Data available for Cry1F showed a similar pattern: the SSD included 17 species, of which 15 (82%) were pests and 2 (12%) were true nontarget species. The consequences of this selection bias are unknown, and representative species should be assessed to obtain plausible sensitivity estimates (Forbes & Carlow, 2002).

For the assessment of NTLs, the criteria to derive the SSD should include a broad taxonomic range of species and different larval sizes (Lang et al., 2020) because the latter will influence the intake dose (fewer pollen grains represent a higher dose in small larvae). The LC₅₀ values used in the current case were identical to those used for the EU risk assessment (EFSA, 2011). Most of the data were produced by Dow AgroScience (Wolt et al., 2005) and are the result of bioassays without pollen but an *Escherichia coli*-produced Cry1F protein incorporated in an artificial diet. The resulting LC₅₀ values had to be recalculated from the µg Cry1F/mL diet to Cry1F in the pollen/cm² leaf area. This complicated recalculation is based on rough estimates, such as diet uptake and leaf thickness; the calculation has so far not been validated, nor has the uncertainty of the calculation been expressed in the risk assessment. Although the LC₅₀ values influence the values for the sensitivity classes, there are no data regarding the slope of the dose–response relationship, which is an important model parameter in this and in the present EU model and the assessments based on this model (EFSA, 2009, 2011, 2012, 2015).

An SSD approach is useful to define limits of concern for protected species, which otherwise could not be subjected to ecotoxicity testing. However, to implement the approach, authorities should demand better ecotoxicity data for more nontarget species during the authorization process of Bt maize (Lang et al., 2019).

Pollen deposition on host plant leaves

Pollen deposition is one of the main drivers of any model addressing the risks from Bt maize to NTLs. However, the estimation of pollen deposition on host plants and larval exposure is challenging because it relates to the process of temporal and spatial pollen release, transport of pollen in the air under thermic conditions, deposition of pollen on a receptor surfaces, loss from these surfaces over time, and uptake of pollen by larvae. Different approaches for estimating exposure have been discussed, but there is no scientific consensus (Hofmann et al., 2010; Hofmann et al., 2016; Hofmann et al., 2011; Hofmann et al., 2014; Kruse-Plass et al., 2017; Lang et al., 2015; Perry et al., 2013; Perry et al., 2017; Perry et al., 2012). We agree with other authors that this issue is highly relevant for risk assessment (Arpaia et al., 2018; Lövei et al., 2020), and additional data, including longer distances from the maize field, will clarify this issue (Lang et al., 2015). To obtain reliable data representing the large variability in pollen deposition on host plants, we strongly suggest using comparable methods that measure pollen densities directly on host plants, ideally in parallel with standardized measurements of pollen flow in the air. Data from

several regions with high replication in locations and measurements over the entire flowering period are required to include high natural variability. Unless such data are available, any risk assessment should be informed by scenario building, reflecting the expected uncertainty in pollen deposition and the temporal loss of pollen on leaves.

Effective exposure

The Bt toxins cause delayed effects, and the time span between exposure and the observation of effects, as well as the duration of exposure, are relevant for the quantification of nontarget effects (Lang et al., 2019). Therefore, the exposure in the field and laboratory should be similar. However, exposure in the field is neither constant over time nor in duration. The significance of these differences is rarely acknowledged in modeling. Lang and Otto (2010) pointed out that studies with longer exposure times were more likely to detect the adverse effects of Bt toxins on Lepidoptera. This is corroborated by the findings on Bt sprays against target Lepidoptera. Fast and Régnière (1984) demonstrated that extending the exposure time from 3 days to continuous exposure resulted in an approximately 60-fold smaller LC₅₀ value in spruce budworms (Tortricidae: *Choristoneura fumiferana*). Data are also missing when comparing studies with constant exposure to effects from a variable exposure over time, which is the normal condition in the field. The 4-day interval to calculate the effective exposure is a compromise. Longer periods would decrease and shorter periods increase the values used to determine mortality in LepiX_1.1. More research is necessary to understand the relationship between the total amount of exposure (or toxin intake) and its period.

Repeated exposure

Maize flowering may be interrupted by unfavorable weather conditions and may, therefore, occur in several bouts of pollen emission over a period longer than 14 days (Hofmann et al., 2013). Depending on the definition of effective exposure, an individual may be exposed to Bt pollen several times during its larval stage in the field. Currently, LepiX-1.1 is the only model addressing this aspect. In the overall population the effect of an additional second exposure was moderate (Figure 3) because the magnitude of the first exposure was higher in our model by definition (see Methods). However, for single populations or individuals, the added mortality can make a significant difference. Thus, the inclusion of repeated exposures in a risk scenario is valuable for protecting endangered or legally protected species.

Relevance of between- and within-population variation

Similar to Fahse et al. (2018), we observed high between- and within-population variation in mortality. This variation can be explained by the stochastic elements used for the deposition,

weather, and larval phenology in the LepiX model. Generally, stochastic elements are preferable over deterministic elements to account for variability widely observed in natural systems. An individual-based approach allows the use of one model for different goals, such as the overall decline of butterflies or harm to rare or protected species. Individual-based approaches are best suited when considering potential harm to species (Grimm & Railsback, 2005). To protect single populations or individuals, mean estimates of larval mortality over all populations cannot be considered adequate (Baudrot et al., 2021; Fahse et al., 2018).

Here, we showed a mortality of 4% over all populations, whereas the mortality of single populations ranged up to 95%. Consequently, the current regulatory models assessing the effects on Lepidoptera from Bt maize cultivation (EFSA, 2015) should ideally shift from a purely deterministic to an individual-based, more stochastic model in order to distinguish between the average expected mortality and the likelihood of higher mortality risks for single populations.

Sublethal effects and additional stressors not considered in present model approaches

Sublethal effects and additional stressors can add uncertainty to risk assessment based on any of the available models. Sublethal effects, such as prolonged developmental time and decreases in pupal weight or adult size, have been described for target and NTLs (Felke et al., 2002; Lang & Vojtech, 2006; Paula et al., 2014; Sedaratian et al., 2013). Sublethal effects may have negative impacts on individual fitness or population growth. Furthermore, sublethal effects may be enhanced or translated into mortality when larvae simultaneously (or later) encounter additional stressors to their exposure to Bt toxins. Furthermore, acute and sublethal effects encountered under favorable conditions for the test species in the laboratory may underestimate the effects of exposure to Bt toxins in the field (Lang & Otto, 2010). Because most Bt maize varieties are also herbicide resistant (Parisi et al., 2016), herbicide applications during the maize growing period may reduce host plants, limiting butterfly populations (Lang et al., 2019; Lövei et al., 2020). To summarize, there are good reasons to assume that our results tend to underestimate the mortality risks for nontarget species.

Consequences of high toxin expression in Bt maize

The DAS-1507 variant of Bt maize expresses a 350-fold higher toxin concentration than MON810 in pollen, thus markedly increasing the exposure of off-field larvae to the Bt toxin. Our findings indicate that such high pollen-expressing events may pose a significant threat, particularly to rare and protected butterfly species. Using estimates for overall population effects may not be sufficient to protect single populations or individuals of endangered species. Sublethal effects of Bt proteins or combinatorial effects with other stressors, such as pesticides or

pathogens, remain an unresolved issue in modeling the effects of Bt maize on NTLs.

The high expression of the toxin in DAS-1507 maize is unnecessary for controlling the target pests, stem borers. Also, the magnitude of the expression of Bt in pollen is higher than in other commercially available Bt maize events. From an environmental perspective, Bt expression in pollen constitutes a superfluous exposure of the environment and NTOs to a toxin. This is not only relevant for the single event DAS-1507 maize but also for all respective breeding stacked events (i.e., crosses of other GMO with DAS-1507). In the EU, many protected areas are closely interconnected with agroecosystems and farmland, which make up to 40% of the total area of Natura 2000 sites (European Commission, 2018). Therefore, we advise waiving the cultivation of Bt plants with high pollen expression in areas where rare butterfly or moth species are protected. If such high pollen-expressing GMOs are to be cultivated in the EU, managing risks with sufficiently large isolation distances and a strict monitoring program is recommended.

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ORCID

Mathias Otto  <https://orcid.org/0000-0003-3161-5625>

Phillip Papastefanou  <https://orcid.org/0000-0002-4613-2565>

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