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Do genetically engineered plants affect aquatic non-target invertebrates? – a critical review

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ABSTRACT

Environmental risk assessments (ERAs) of genetically engineered (GE) plants rely on robust, high-quality, and relevant ecotoxicological studies on non-target invertebrates. Although ERAs also consider evidence from published literature, the quality of these studies and their suitability for ERA are often unclear. This review focuses on aquatic invertebrates and comprises 51 articles identified through systematic literature searches. The review provides a detailed critical appraisal of studies and synthesizes recommendations for future research.

Studies using purified *Bt* proteins reported effects on aquatic species only at very high concentrations, which possibly reflect non-specific toxicity rather than *Bt* protein-specific effects. Adverse effects reported for *Bt* plant material were frequently inconsistent. Methodological shortcomings indicate plant-background effects or experimental artifacts rather than *Bt* protein effects. Studies reporting no effects are difficult to interpret when exposure levels are unknown or test durations are short. Artifacts may arise when test substances are insufficiently characterized, when negative control treatments differ from test treatments in aspects other than the test compound, or when results are inconsistent across treatments and temporal repetitions. For laboratory studies involving GE plants other than *Bt*, as well as field studies, the available evidence does not indicate adverse effects, although methodological limitations were also identified for those kind of studies. Future research using plant material should be designed to disentangle effects of the gene product from plant background effects. The critical appraisal scheme developed in this review provides guidance for robust test protocols using purified proteins and GE plants and is also applicable to studies on terrestrial non-target species.

1. Introduction

Before genetically engineered (GE) plants can be released, regulatory approval is required worldwide (SCBD, 2000). Environmental risk assessment (ERA) is part of the regulatory dossier, alongside molecular characterization of the novel plant and food and feed safety assessment. Potential risks to beneficial species, the ecosystem services they provide, and other valued elements of biodiversity are a key part of the ERA.

Similar to risk assessments for plant protection products, ERAs of GE plants consider the toxicity (hazard) of novel gene products, such as insecticidal proteins from the bacterium *Bacillus thuringiensis* (*Bt*), as well as the exposure of organisms to potentially hazardous concentrations in the environment. A tiered risk assessment approach is followed (Romeis et al., 2008). In lower-tier hazard identification studies, single surrogate test species from various taxonomic groups are exposed to high concentrations of the novel substance. Such studies are conducted in the laboratory with purified test substances mixed into (artificial) diets or media. If potential risk cannot be excluded, more realistic assessments using GE plant material are performed. In the European Union, *in-planta* tests are recommended by the authorities in any case as the GE plant itself is considered the entity that may pose a risk (stressor of concern) and not only the novel substance it produces (EFSA, 2010). Laboratory experiments can simulate worst-case exposure conditions while minimizing confounding factors. In contrast, higher tier semi-field and field experiments with GE plants allow the assessment of ecological effects in a realistic exposure scenario including species interactions and with the possibility of direct as well as indirect effects occurring. However, the multitude of confounding factors and high variability of environmental conditions limit the effect sizes that are detectable under those conditions.

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In contrast to the risk assessment of plant protection products, where standardized protocols with specified validity criteria need to be followed, no ring-tested protocols are available for the ERA of GE crops. This allows more flexibility to tailor experimental test designs specifically to the properties of the novel transgene but may also challenge risk assessors when evaluating study quality.

While non-target studies conducted to support regulatory dossiers of GE plant products are submitted by applicants to the regulatory authorities, they are often not published. In contrast, many studies from public research institutions have become available that are usually not part of the regulatory dossiers. Nevertheless, they are also reviewed and evaluated during the ERA process. The EU directive for the placement of genetically modified organisms on the market foresees a so-called safeguard clause in the case of new scientific evidence. For example, early laboratory studies that claimed potential hazards of *Bt* plants for non-target species have contributed to the banning of MON810 maize cultivation in several countries, such as Germany (BVL, 2009). The importance of research findings on GE crops in a political context also sparked discussions in the scientific community (Beachy et al., 2008; Parrott, 2008; Rauschen, 2010; Ricroch et al., 2010), which centered around robustness and transparency of study design and the conclusions that can be drawn from the results of laboratory studies with a given setup (Romeis et al., 2013). For commercial transformation events that passed the regulatory risk assessment successfully, emerging public studies showing adverse effects have the potential to trigger a risk re-assessment. Because such studies may also attract high media attention and impact political decisions, it is evident that the study design is under particular scrutiny. In contrast, emerging studies that corroborate the outcome of the risk assessment get less attention, even though the methodology may be equally flawed.

Study quality criteria for ecotoxicological studies of chemicals have been developed, discussed and refined for almost 30 years (Klimisch et al., 1997; Kase et al., 2016; Hanson et al., 2017). For studies involving GE plants, similar quality criteria have been proposed (Romeis et al., 2011; De Schrijver et al., 2016). Nevertheless, the quality of publicly available studies for GE plants and thus their usefulness for ERA, often remains unclear.

While most previous reviews on the non-target effects of GE plants focus on terrestrial species, this work is limited to studies with aquatic invertebrates. Since 2007, when the first aquatic non-target studies were published, research has progressed and a considerable amount of data has become available.

In the present review, we introduce several important features: 1) The review captures literature until 2026; 2) Published articles on all GE crops are included (although the majority of data derived from *Bt* crops); 3) Relevant studies were identified through systematic literature search; 4) Each study is evaluated against a set of quality and relevance criteria. The review covers laboratory studies with purified insecticidal proteins and GE plant material as well as field studies with GE plants. Reported study results are discussed in the light of the study quality assessment and in the context of realistic exposure of aquatic organisms to GE plant products. Finally, recommendations for the design of future studies to be meaningful for risk assessment are provided.

2. Material & methods

2.1. Review question

This review targets the following question: "Which evidence exists on effects of GE plants or material from such plants on aquatic non-target invertebrates?"

In lower-tier risk assessment studies, bacteria-produced, purified proteins are often mixed into the food or medium of aquatic non-target invertebrates instead of plant material. Therefore, the following sub-question was formulated: "How do insecticidal proteins, genetically engineered to be produced in plants, affect aquatic non-target invertebrates when administered directly in purified form?" In the context of commercial GE plants, most relevant are insecticidal *Bt* proteins, although novel proteins derived from plants or other bacteria have emerged recently.

2.2. Limits of the review

The review is limited to studies on GE plant material and *Escherichia coli*-produced, purified insecticidal proteins used in the context of GE plants. Other substances that have no practical relevance are not considered. Studies using formulations of mass-produced spores of *Bt* strains or crystals extracted from spore material are also not considered. Such studies are often conducted in the context of developing potential biological control products, not in the context of GE plant ERA.

The review focuses on comparisons of clearly specified GE and non-GE plant material without pesticide treatment or with similar treatment in both groups. Studies with different herbicide applications in the context of herbicide-tolerant plants and studies with different insecticide treatments as part of the experimental design in the context of insect-resistant plants are not considered. However, we do consider studies with plant material collected from commercial fields that had a different, and often unknown, history of pesticide applications.

"Non-target" is defined as invertebrates other than the pests intended to be reduced by the respective GE plant. For example, Lepidoptera for *Bt* plants producing Cry1, Cry2, and VIP3-class proteins, and *Diabrotica* spp. (Coleoptera: Chrysomelidae) for *Bt* plants producing Cry3-class proteins. Because Cry2-class proteins are known to show activity against certain mosquito larvae (Diptera) (Glare and O'Callaghan, 2000), studies with purified Cry2 and aquatic Diptera larvae are considered only when a clear context of GE plants is given.

2.3. Searches in literature databases

Three major bibliographic databases were searched: Web of Science (Clarivate Analytics, Philadelphia, USA), Scopus (Elsevier B.V.), and China National Knowledge Infrastructure CNKI (China Academic Journals, Electronic Publishing House Co., Ltd). The search terms, consisting of three different parts, are listed in **Table S1** (**Supplementary Material 1**). The first part (#1) searched for specific invertebrate taxa that occur mainly in aquatic environments. The second part (#2) searched for higher taxonomic groups in combination with terms related to aquatic environments. The third part (#3) aimed at references about GE plants or insecticidal proteins expressed in *Bt* plants. The three parts were connected as follows: (#1 OR #2) AND #3. Database searches were conducted on April 12, 2022 and updated on April 15, 2026. The references identified in Scopus and Web of Science were downloaded to Endnote20 (Clarivate Analytics). Duplicates were removed automatically by first screening for similarity in title, year, and authors, and then by screening for similarity in title, year, and page numbers. References identified in CNKI were screened online. Relevant references, including full texts, were downloaded and integrated into the Endnote library.

2.4. Literature screening

Original experimental studies conducted either in the laboratory or in the field were identified in a screening process of titles and abstracts with predefined inclusion (eligibility) criteria:

1) The test substance consists of specified **GE higher plants** (studies on GE bacteria, algae, fungi, and animals were not eligible) or clearly defined *E. coli* produced and **purified insecticidal proteins** with clear relationship to GE plants (studies on formulations from mass-produced *Bt* bacterial strains or spore products were not eligible).
2) The studied organisms are **aquatic invertebrates**, either recorded in the context of GE crops cultivated outdoors or tested in the laboratory or greenhouse.

3) A comparison with **non-GE control** plants or diet without purified *Bt* protein is available.
4) Measurement endpoints are clearly defined.

Duplicate references that remained after the automatic duplicate removal procedure were manually removed. The full texts of potentially useful references were obtained and screened using the same eligibility criteria as for the title and abstract screening. If similar data were available in two articles (e.g., peer-reviewed paper and Master or PhD thesis), the peer reviewed article was preferred, and a note was added to the other article. In some cases, more detailed information from the thesis that was not available in the peer-reviewed article was used and labelled accordingly.

After full text screening, 58 original articles were identified as eligible for this review. Of those articles, 7 were excluded later because they contained data that were published elsewhere (**Table S2, Figure S1**).

2.5. Pilot study

When designing the search strings for the three literature databases, the retrieved references were checked against an internal list of known original studies, which was compiled for the purpose of a PhD thesis (Chen, 2021). Twenty-three previously known original studies were retrieved by the systematic search. One additional study listed in the PhD thesis (Mendelsohn et al., 2003) is a summary of data collected for regulatory risk assessment, not specific to aquatic organisms, without an abstract, and with the short title "Are *Bt* crops safe". This article was not found in the literature searches but nevertheless retrieved because it was cited in several other articles.

2.6. References from other sources

Nine relevant reviews were identified during the literature screening process, i.e., reviews on effects of GE plants on non-target invertebrates or broader reviews with a detailed discussion of effects on aquatic invertebrates (**Table S2**). Those reviews were screened for additional references to original experimental results. In addition, the reference sections of all relevant original articles were screened. This screening identified 7 additional original articles, of which 5 were included in the review while the others contained data that were published elsewhere (**Table S2**).

2.7. Critical appraisal

The benefit of laboratory experiments compared with field experiments is the possibility to control as many factors as possible so that, ideally, only the effect of the stressor of concern, i.e., the insecticidal protein or the GE plant, on a non-target organism can be tested. In practice, however, laboratory studies also are subject to a range of confounding factors that can influence the results and may lead to erroneous conclusions if overlooked (Romeis et al., 2013). Based on an earlier compilation of study quality criteria (Romeis et al., 2011), a critical appraisal catalogue was developed and applied to all studies identified in this review (**Supplementary Material 2**). The catalogue consists of specific questions that can be answered with yes ("green", not problematic), no ("red", potentially problematic), or no rating possible because information is lacking ("blue") (**Table S3**). For field studies, the critical appraisal catalogue developed for a database on non-target effects of *Bt* maize (Meissle et al., 2022) was adapted and simplified by combining some of the questions (**Table S4**). In the following, we report critical appraisal criteria for laboratory studies with purified protein (P), plant tissue (T), or field studies (F):

- Cohort (P, T): Did the test organisms derive from the same cohort of a laboratory colony?
- Equivalence (P): Was the purified protein (test substance) equivalent to the plant-expressed protein?
- Purity (P): Is the purity of the purified protein known?
- Formulation (P): Was the formulation of the purified protein free from additional substances that may have affected the results?
- Expression (T, F): Did the GE plant express the introduced gene and contain the respective gene product?
- Intactness (P, T): Can intactness of the test substance as used in the bioassay be assumed?
- Biological activity (P, T): Was biological activity of the test substance given in the applied experimental design?
- Dose (P, T): Is the dose of the test substance known?
- Homogeneity (P): Was the test substance homogenously distributed in the medium or food?
- Continuous exposure (P, T): Were the test organisms continuously exposed to the test substance?
- Ingestion (P, T): Did the test organisms ingest the test substance?
- Negative/non-GE control (P, T, F): Were appropriate negative controls applied in the experiment?
- Control mortality (P, T): Was the control mortality <20% throughout the test?
- Positive control (P, T, F): Was a positive control included in the test confirming that effects can be detected with the applied experimental setup?
- Replicates (F): Did the study include a sufficient number of replicates?
- Replicate dispersal (F): Were the replicates equally or randomly dispersed within the field or the landscape?
- Management (F): Were GE and non-GE plots or fields managed in the same way?
- Duration (F): Was the duration of the experiment appropriate and covered with repeated recordings?
- Methodology (F): Was the applied sampling methodology appropriate for the collected taxa?
- Natural variation (T, F): Were different unrelated non-GE varieties included to assess observed results for GE- and non-GE controls in the context of natural variation?
- Multiple lines (T, F): Were multiple GE- and non-GE controls included to assess consistency of observed effects?
- Data availability (P, T, F): Did the authors provide the full dataset (replicate-to-replicate data)?
- Other concerns (P, T, F): Are there any other concerns regarding accuracy, integrity, or transparency of the methods and resulting data?

For a full list of critical appraisal criteria and a detailed description, see **Supplementary Material 2.** Based on the critical appraisal and the results of this review, a table highlighting important potential confounding factors in non-target studies was created and solutions for robust future studies were suggested (Table 1).

3. Literature review

The literature search revealed 51 relevant articles with original experimental data on effects of GE plants or purified insecticidal proteins on non-target aquatic invertebrates (**Figure S1**). The identified data were assigned to the following categories: (1) laboratory studies using purified insecticidal proteins (Fig. 1); (2) laboratory studies using *Bt* plant material (Fig. 2); (3) laboratory studies using GE plant material other than *Bt* (Fig. 3); and (4) field studies with GE plants (Fig. 4). In this review, "study" is defined as experiments with one invertebrate species derived from one article (except for field studies which recorded invertebrate communities). A study can comprise different experiments, e.g., different experimental durations or different proteins. Independent laboratory experiments covering multiple invertebrate species are assigned to separate studies, even if they are presented in the same

Table 1
Confounding factors and potential solutions to avoid artifacts in non-target studies of GE plants, either in the laboratory (lab) or in the field.

Area of concern	Relevance	Confounding factor	Suggested solution
Test organisms	Lab	High variability in response of tested specimens	Use specimens from the same cohorts of established laboratory colonies or lab-reared offspring from field-collected adults. If not possible, acclimatize field-collected specimens to lab conditions and ensure (statistically) equal distribution of males and females and/or uniform size and weight among all treatments
Test substance & negative controls	Lab	*Purified proteins*: effects caused by the formulation (e.g., buffer in which test substance is dissolved)	Apply same amount of buffer to the negative control treatment or confirm absence of buffer-effects in separate study
	Lab	*Purified proteins*: unspecific effects caused by the added amount of test substance (e.g., protein)	Add same amount of another, non-toxic substance, e.g., bovine serum albumin, or denatured test substance as one negative control treatment
	Lab	*Purified proteins*: functional equivalence to plant-expressed substance is unclear	Confirm equivalence by molecular analysis
	Lab	*Purified proteins:* effects caused by contamination in test substance (low purity)	Use test substance with high purity, or include treatment with material that was produced with the same production platform as the test substance (e.g., *E. coli*), but without the active ingredient, as one negative control treatment, or demonstrate that impurities do not cause effects
	Lab & field	*Plant material*: expression of GE trait unclear	Confirm expression levels by, e.g., enzyme-linked immunosorbent assay (ELISA) or Western blots for GE proteins or polymerase chain reaction (PCR) for any gene product
	Lab & field	*Plant material:* unspecific effects caused by plant background or transformation-related effects	Use near-isolines or lines as closely related to the GE line as possible as negative control treatment. If effects between GE and non-GE control are observed, • use the same transformation event in different plant backgrounds, or • use different transformation events for the same trait (e.g., Cry protein), or • use different tissues of the plant and correlate toxin concentration with effects For the interpretation of observed effects, establish natural range of variation that is not considered harmful by including several conventional varieties
Positive control	Lab & field	Unclear if • experimental setup ensures ingestion • statistical power is sufficient to show effects • test organisms react similarly in different repetitions of the experiment	Include positive control with similar mode of action as test substance and known effect on the test organism
Exposure	Lab	Test substance inactivated by damaging treatments before the start of the experiment (conditioning in water; heat treatment, etc.)	Determine concentrations of test substance, e.g., by ELISA or high performance liquid chromatography (HPLC). Use test substance directly and avoid damaging treatments. If conditioning or processing is necessary for the test setup, confirm concentration in test substance after the conditioning or processing steps; biological activity of insecticidal proteins can be confirmed by sensitive insect assays, protein length can be analyzed with Western blots
	Lab	Unequal distribution of test substance	When spiked food or artificial diets are used, ensure homogeneous distribution, e.g., by measuring substance in subsamples of the prepared food or by mixing food coloring into medium or diet
	Lab	Instability of test substance during the test	Change medium and food with test substance regularly to ensure continuous exposure (for *Bt* proteins in water: every 1-2 days)
	Lab	Ingestion of test substance unclear	Confirm uptake of diet with test substance (e.g., measure test substance in test organisms, confirm ingestion of plant material microscopically)
Endpoints & test duration	Lab	High control mortality	Terminate experiment when control mortality reaches 20%; redesign experiment to reduce control mortality (e.g., provide additional high-quality food; acclimatize test organisms to experimental conditions; improve water quality); discard single experimental repetitions if control mortality exceeds 20%. If high control mortality is unavoidable, ensure high statistical power and confirm consistency by repeating the assay multiple times with high number of replicates.
	Lab	Duration of test too short to reveal effects based on mode of action of the test substance	Ensure test duration is long enough according to the mode of action of the test substance and the life cycle of the test organism. For *Bt* proteins, tests should last >5 days
Replication & statistical power	Lab & field	Variability in response data is high and differences among treatments are not statistically significant	Use replication as recommended in standard protocols; calculate detectable effect sizes from control data; include positive controls
Consistency & data availability	Lab & field	Consistency is unknown as experiment was only conducted once with one GE/non-GE comparison and one concentration, or results are inconsistent among repetitions or treatments, or no dose-response relationship is evident	Conduct experiments multiple times and, if possible, with multiple GE/non-GE lines and multiple concentrations to be able to detect inconsistencies; if inconsistencies occur and cannot be explained, plant background effects or experimental artifacts are likely (see recommendations under "negative controls")
	Lab & field	Reliability and data quality cannot be assessed by peers because full datasets are not available	Provide the replicate-to-replicate data that were used for any statistical analyses as supplementary materials or via external data repository. This increases transparency and trust and allows inclusion in future meta-analyses and systematic reviews
Tritrophic studies	Lab & field	Reduced nutritional quality of impaired prey leads to effects on the predator	Monitor potential effects on prey and use only healthy and unaffected prey for tritrophic studies
Interpretation of results	Lab	Relevance of effects observed with a tested dose is unclear	Discuss results in the context of realistic environmental exposure

article. There were no laboratory studies investigating species assemblages.

3.1. Studies with purified insecticidal proteins

A total of 18 studies, extracted from 12 articles, have assessed the impact of purified insecticidal proteins on aquatic non-target species in the laboratory (Fig. 1, **Table S5**). The majority of studies (11) used Cry1-class *Bt* proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C) as test substance, followed by Cry2A (5) and VIP3A (2). Recent studies included a plant-derived insecticidal protein (IPD083Cb) and a protein from *Pseudomonas* bacteria (IPD072Aa). Studies were conducted with a total of 10 species from 4 families in 3 taxonomic orders. For none of the laboratory studies with purified insecticidal proteins, the full dataset (replicate-to-replicate data used for statistics) was available.

Eight studies were performed with water fleas (*Daphnia* spp., Cladocera: Daphniidae). Toxicity of insecticidal protein dissolved in medium was tested over 7 to 78 days. In seven studies with *Daphnia magna*, <24 h old juveniles from laboratory cultures were exposed to the insecticidal proteins dissolved in medium. Survival as well as sublethal parameters including growth, development, and fecundity were recorded (Bøhn et al., 2016; Chen et al., 2018b; Ibory et al., 2025; Raybould et al., 2014; Raybould and Vlachos, 2011; Spegar et al., 2025; Wang, 2014). In one study on *Daphnia hyalina*, field-collected adults were used and the population size after 9 days of exposure was recorded (Wang et al., 2013). Lethal and sub-lethal effects were reported from six studies with *D. magna* at protein concentrations ≥ 500 µg/L. Raybould et al. (2014) suggested that observed effects on growth and reproduction at high concentrations might have been caused by the addition of protein to the medium rather than by specific toxicity of *Bt* proteins, because they observed comparable adverse effects when using bovine serum albumin as a non-toxic protein control. In fact, none of the other studies on purified proteins and *D. magna* (or other test species) included inactivated protein or another non-toxic protein as a negative control.

In the studies with *D. magna*, there were no concerns regarding quality of the test substance (equivalence, purity), formulation, intactness, biological activity, dosing, or homogeneity, although biological activity remains unknown in the study by Bøhn et al. (2016).

Three studies confirmed *Bt* protein in the test medium and/or test organisms by enzyme-linked immunosorbent assays (ELISA) (Chen et al., 2018b; Raybould and Vlachos, 2011; Wang, 2014) and test medium was replaced every one or two days ensuring constant exposure during the test period. Bøhn et al. (2016), however, replaced the medium only every three days and did not measure Cry1Ab concentrations. The same study was conducted until the last specimen died. Laboratory experiments are generally most reliable if the mortality in the control treatment remains low (<20%) over the entire experimental period (Romeis et al., 2011). As a positive control treatment, potassium dichromate (Chen et al., 2018b; Wang, 2014) or potassium chloride (Ibory et al., 2025) was used. In the study with field-collected *D. hyalina*, the quality of the test substance and its biological activity remained unclear, the medium was not changed over the experimental period of 9 days, and the population sizes decreased steadily in both *Bt* and control treatments (Wang et al., 2013). Field-collected test organisms are not adapted to laboratory rearing conditions or may carry other fitness deficits, which may lead to high control mortalities.

In two studies with caddisfly species (Trichoptera), lasting 6 and 12 weeks, respectively, effects on sub-lethal parameters were evident when larvae fed on leaf discs spiked with Cry1Ab (Pott et al., 2020). *Sericostoma* sp. showed delayed larval development at a measured concentration of 132 µg/L (nominal 1000 µg/L) spiked to alder leaves and *Chaetopteryx* sp. showed reduced lipid content at a measured concentration of 17.2 µg/L (nominal 100 µg/L). Large variation was introduced into these test systems by using field-collected larvae and control mortality for *Sericostoma* sp. was relatively high. In addition, effects on case width of *Chaetopteryx* sp. were discussed as mainly driven by significant differences in case width and larval instars among treatments at the start of the experiment (Pott et al., 2020). Furthermore, effects on lipid content of *Chaetopteryx* sp. were observed at medium concentration, but not at high concentration (no clear dose-response relationship). The overall level of exposure of caddisflies to Cry1Ab was likely low given the fact that the medium and food were changed weekly and the Cry proteins degraded quickly under the test conditions (as reported by the authors).

Experiments with midges (Diptera: Culicidae) were conducted with 3 to 6 days old 3rd to 4th instars from laboratory colonies. Larvae were exposed short-term (24 or 48 h) to Cry1 or Cry2 proteins dissolved in medium. A total of 6 midge-species were studied (Sims, 1995, 1997; Smith et al., 1996). LC_{50} values varied among the species and the tested Cry proteins. Highest sensitivity to Cry2A was observed in *Anopheles quadrimaculatus* (LC_{50} = 400 µg/L after 24 h), lowest in *Culex pipiens* (>200'000 µg/L after 48 h) (Sims, 1997). Similarly, Cry1C lead to LC_{50} values between 90'000 µg/L in *Aedes aegypti* and 143'000 µg/L in *Aedes gambiae* after 48 h (Smith et al., 1996). No acute toxicity was reported for Cry1Aa and Cry1Ac in *A. aegypti* (Sims, 1995; Smith et al., 1996). In the studies on midges, details on test species origin, purity of the test substance, tested dose-range, and control mortality were often lacking. Purity of Cry2A was reported as 60% (Sims, 1997), which indicates that substances other than the Cry protein may have influenced the results. *Bt* proteins typically act within several days after ingestion. Therefore, it is unclear how representative acute toxicity tests with very high doses that are terminated after only 24 or 48 h are for the field situation, where *Bt* protein concentrations are much lower and larvae are potentially exposed throughout their developmental period. It is also unclear whether the mosquito larvae ingested the *Bt* proteins within the short test period, when no food was provided.

3.2. Studies with Bt plant material

In total, 30 studies were available from 22 articles with test organisms being exposed to material from *Bt*-transgenic plants under laboratory conditions (Fig. 2, **Table S6**). In the majority of studies, material from maize (19 studies) was used, followed by rice (9) and cotton (2). The most tested plant material was leaves (16), followed by flour (5), pollen (2), root or seed extract (3), straw leachate (2), water from *Bt* rice fields (2), or seedlings (2) (note that multiple materials were tested in some studies). Many studies (26) were conducted with *Bt* plants containing Lepidoptera-active Cry1 and Cry2 proteins. One study was conducted with material from plants producing a Coleoptera-active Cry3 protein, and material from stacks containing a combination of Lepidoptera- and Coleoptera-active proteins was tested in seven studies (some studies included single- and stacked-gene plants). *Bt* protein concentrations in plant material were reported by most authors (for a summary see **Supplementary Material 3, Table S9**). For maize, highest levels were reported for leaf tissue, lowest for pollen and grains (flour). The organisms studied were diverse with a total of 15 species from 12 families in 8 taxonomic orders. The largest number of studies (10) was done with *D. magna*. The full dataset for laboratory studies with *Bt* plant material was available in only two studies.

3.2.1. Daphnia spp.

In the studies with neonate *D. magna* (10 studies) and adult *D. hyalina* (1), effects on survival and different sublethal parameters were recorded over an experimental period of 13-60 days (Bøhn et al., 2008; Chen et al., 2018a, 2021; Deng, 2018; Holderbaum et al., 2015; Li et al., 2014; Wang, 2014; Wang et al., 2013; Zhang et al., 2016, 2018). In one study, acute toxicity after 48 h was tested (Oh et al., 2011). When flour, leaf powder, or pollen of maize or rice containing a single Cry1 protein was suspended in the medium, no differences for *D. magna* between the *Bt* and non-*Bt* treatments in the measured parameters was observed in three studies (Oh et al., 2011; Wang, 2014; Zhang et al., 2018). In another three studies, adverse, but inconsistent effects on

Taxon	Life stage	Test substance	Delivery	Duration	Cohort	Equivalence	Purity	Formulation	Intactness	Biol. activity	Dose	Homogeneity	Con. exposure	Ingestion	Neg. control	Control mort.	Pos. control	Data avail.	Oth. concerns	Results	Reference
Cladocera: Daphniidae																					
Daphnia magna	juveniles	Cry1Ab; Cry2Aa	diss. in medium	78 d																↓ [≥0.75 mg/L]	Bøhn et al. (2016)
		Cry1B.34 Cry1B.61.1, IPD083Cb	diss. in medium	21 d																↓ [≥30 mg/L] 0 [≤3 mg/L]	Iboyi et al. (2025)
		Cry1C	diss. in medium	21 d																0 [≤0.5 mg/L]	Chen et al. (2018b)
		Cry1Ca	diss. in medium	7/21 d																↓ [0.5 mg/L 21d]	Wang (2014)
		VIP3A	diss. in medium	10 d																↓ [0.75 mg/L]	Raybould & Vlachos (2011)
				21 d																(↓) [≥0.75 mg/L]	Raybould et al. (2014)
		IPD072Aa	diss. in medium	21 d																0 [15 mg/L]	Spegar et al. (2025)
Daphnia hyalina	adults	Cry1Ab; Cry1Ac	diss. in medium	9 d																0 [2.5 mg/L]	Wang et al. (2013)
Trichoptera: Sericostomatidae																					
Sericostoma sp.	larvae	Cry1Ab	spiked leaf discs	6 wk																↓ [130 mg/kg]	Pott et al. (2020)
Trichoptera: Limnephilidae																					
Chaetopteryx sp.	larvae	Cry1Ab	spiked leaf discs	12 wk																(↓) [≥17 mg/kg]	Pott et al. (2020)
Diptera: Culicidae																					
Aedes aegypti	larvae	Cry2A	diss. in medium	48 h																↓ [LC50=37 mg/L]	Sims (1997)
		Cry1Ac	diss. in medium	24 h																0 [1 mg/L]	Sims (1995)
		Cry1Aa, Cry1Ac Cry1C	diss. in medium	24/48 h																0 [≤1000 mg/L] ↓ [LC50=90 mg/L]	Smith et al. (1996)
Aedes gambiae	larvae	Cry1C	diss. in medium	48 h																↓ [LC50=143 mg/L]	Smith et al. (1996)
Aedes triseriatus	larvae	Cry2A	diss. in medium	48 h																↓ [LC50=2.8 mg/L]	Sims (1997)
Anopheles quadrimaculatus	larvae	Cry2A	diss. in medium	24 h																↓ [LC50=0.4 mg/L]	Sims (1997)
Culex pipiens	larvae	Cry2A	diss. in medium	48 h																0 [LC50>200 mg/L]	Sims (1997)
Culex quinquefasciatus	larvae	Cry1C	diss. in medium	24 h																↓ [LC50=126 mg/L]	Smith et al. (1996)

Fig. 1. Laboratory studies exposing aquatic non-target invertebrates to purified insecticidal proteins. In addition to basic information about the study and the reference, an overview of the critical appraisal is provided. Green fields indicate nonproblematic criteria, red fields problematic ones, and blue fields indicate that no information was available (see **Supplementary Material 2, Table S3**). For detailed information, see **Table S5**. Results are summarized as follows: ↓ adverse effect with test substance compared with control; 0 no difference. Parenthesis indicate that results were not consistent for the different treatments or endpoints measured. Values in brackets inform about lowest observed effects concentrations, no observed effect concentrations, or LC_{50} concentrations.

survival and/or sublethal parameters were reported, when *D. magna* were fed maize or rice material containing Cry1Ab (Bøhn et al., 2008; Zhang et al., 2016) or material from SmartStax maize containing 6 Cry proteins (Chen et al., 2021). In one of those studies, effects of *Bt* maize were compared to effects of an unrelated non-*Bt* hybrid grown in the field under potentially different conditions, which makes effects due to differences in plant composition likely (Bøhn et al., 2008). In addition, effects on survival and fecundity were only observed in one of three repetitions in this study. In another study, adverse effects were reported when *D. magna* was fed flour from seeds with unknown history of production and low *Bt* protein content, but not when leaves or pollen were provided that contained higher concentrations of Cry proteins and were produced side-by-side in the glasshouse (Chen et al., 2021). In the third study, effects were only observed at the lowest of three concentrations (Zhang et al., 2016). Finally, in one study, adverse effects of *Bt* maize leaf powder produced in the climate chamber were reported on *D. magna* (Holderbaum et al., 2015). The impact of rice (Cry1C) straw leachate on *D. magna* was evaluated by Chen et al. (2018a). While the addition of rice straw leachate *per se* caused adverse effects, no differences between *Bt* and non-*Bt* rice were observed. One study reported the performance of *D. magna* in water collected from *Bt* and non-*Bt* rice fields that had received different insecticide treatments (Li et al., 2014). A positive effect was observed in the *Bt* treatment, most likely due to lower amounts of insecticides in *Bt* fields compared with the conventional rice. Another study revealed no effect in *D. magna* performance in water from greenhouse-grown plants (Deng, 2018). In all studies with *D. magna*, test organisms from clonal populations were used, which ensured homogeneous genetic properties. Expression in *Bt* plants was confirmed by ELISA, except for Li et al. (2014), and intactness of the test substance can be assumed for studies where plant material was fed directly to the test organisms. Biological activity of the used plant material was generally not tested, except for Deng (2018). Doses were known in most cases and continuous exposure was ensured by adding or replacing food and medium regularly (≤ 3 d). When only plant material was provided as food, ingestion can be assumed because the test organisms developed and grew. In addition, the presence of the Cry proteins in *D. magna* was demonstrated by ELISA (Chen et al., 2018a; Deng, 2018) or the presence of plant material in the gut was demonstrated microscopically (Chen et al., 2021; Holderbaum et al., 2015). With one exception (Bøhn et al., 2008), the near isoline or parental plant material was used as the non-*Bt* control. One problem of all long-term studies with material from crop plants being fed to *Daphnia* spp. is high control mortality (>20%). While the natural food for *Daphnia* spp. is green algae, crop plant powder as exclusive food provides insufficient nutrition for the species (Chen et al., 2021). Assays with high control mortality generally face a high risk for generating non-reproducible artifacts, as effects of the test substance overlay with non-specific effects of food quality, handling, test organism fitness, etc. This may reduce statistical power and warrants cautiousness in interpreting results. If algae would be provided in addition to crop plant powder in future studies, survival in the control treatment may be increased, with the downside of a lower dose of ingested GE product. Potassium dichromate positive controls were included in three studies with *D. magna* (Chen et al., 2018a; Oh et al., 2011; Wang, 2014). The natural variation among conventional varieties was addressed by Chen et al. (2021), who included one local maize landrace in their study with two *Bt*/non-*Bt* maize pairs and considered the natural variation among five conventional varieties established in a previous study for data interpretation. For this study, also the full dataset has been published.

In the study with *D. hyalina* no difference in population density was detected when field-collected specimens were kept on Cry1Ab/Ac rice straw leachate as compared to the control (Wang et al., 2013). While rice

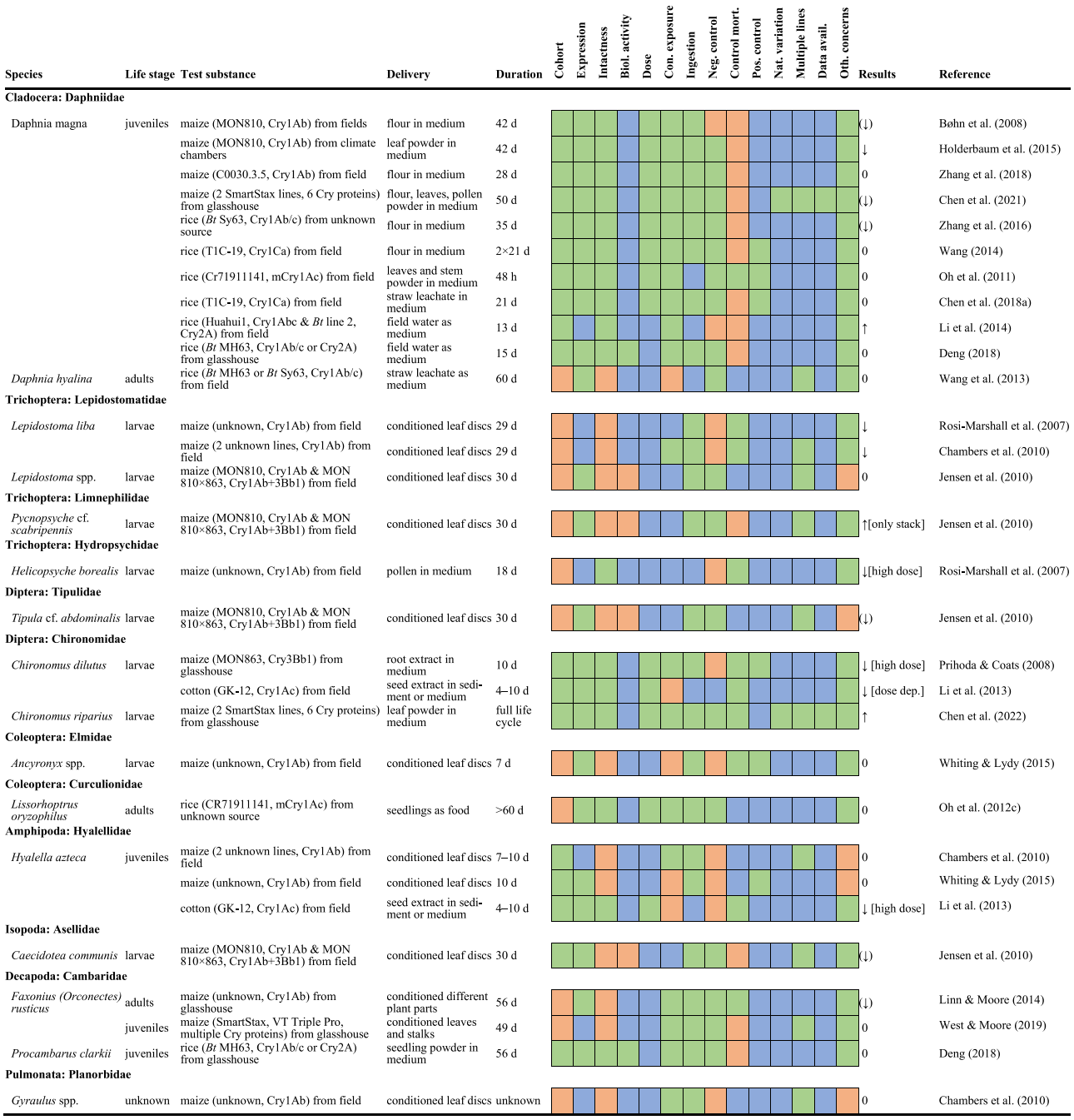

Fig. 2. Laboratory studies exposing aquatic non-target invertebrates to *Bt* plant material as test substance. In addition to basic information about the study and the reference, an overview of the critical appraisal is provided. Green fields indicate nonproblematic criteria, red fields problematic ones, and blue fields indicate that no information was available (see **Table S3**). For detailed information see **Table S6**. Results are summarized as follows: ↓ adverse effect in *Bt* treatment compared with non-*Bt* control; 0 no difference; ↑ positive effect in *Bt* treatment compared with non-*Bt* control. Parenthesis indicate that results were not consistent for the different treatments or endpoints measured. Information in brackets details specific treatments where effects were observed.

straw was soaked for 20 days in water before introducing *D. hyalina*, no Cry1Ab/c measurements in the leachate after this incubation period were reported and straw and water were not replaced during the 60 days of the experiment. Thus, degradation of the *Bt* protein before the start of the experiment is likely, biological activity, administered dose, and ingestion of the *Bt* protein are unknown, and constant exposure is not given.

3.2.2. *Trichoptera*

Five studies have been conducted with larvae of different caddisfly species belonging to three families (Chambers et al., 2010; Jensen et al., 2010; Rosi-Marshall et al., 2007). Survival was recorded in all five studies and additional sublethal parameters in four of them. The test species were all collected in the field and fed either senescent and conditioned leaves or pollen from Cry1Ab-producing maize. Two studies also comprised stacked maize producing Cry1Ab and Cry3Bb1 (Jensen et al., 2010). In three studies, adverse effects in the *Bt* treatment were reported (Chambers et al., 2010; Rosi-Marshall et al., 2007). In all three cases, an unrelated non-*Bt* variety had been selected as control. Because early studies reported differences in lignin content and C:N ratio among *Bt* and non-*Bt* maize lines (Saxena and Stotzky, 2001), the rationale of Rosi-Marshall et al. (2007) and Chambers et al. (2010) was to compare

maize lines that were unrelated but showed similar lignin contents and C:N ratios. By using unrelated maize lines as comparators, however, a multitude of other plant compounds, plant responses, or physiological processes may have been different and relating results to effects of the *Bt* protein is impossible. Effects of maize pollen were evident when high doses were applied, but not at realistic input rates (Rosi-Marshall et al., 2007). Jensen et al. (2010) observed higher dry mass when *Pycnopsyche* sp. were fed with stacked *Bt* maize compared with either non-*Bt* maize or Cry1Ab-producing maize. The four studies using leaf material were conducted with conditioned leaves that had remained in stream water for 3 days up to 2 weeks before being fed to the caddisflies. Because *Bt* protein concentrations in aqueous environments decrease quickly (see **Supplementary Material 3, Table S10**), intactness of the *Bt* protein at the beginning of the experiment was likely limited and the authors did not measure *Bt* concentrations after the conditioning process. Furthermore, in two studies, biological activity was confirmed with sensitive insects pre-conditioning, while no biological activity was detected after the 2-week conditioning time (Jensen et al., 2010). In the three other studies with caddisflies, *Bt* protein content in the test substance and biological activity were not addressed at all (Chambers et al., 2010; Rosi-Marshall et al., 2007). In addition, leaf material was likely not changed during the course of the experiment in two studies (Jensen et al., 2010) and the interval of changing was not reported in another study (Rosi-Marshall et al., 2007). Therefore, exposure (dose) was uncertain and probably low in all studies with caddisflies. Control mortality was above 50% in the study with *Pycnopsyche* sp. (Jensen et al., 2010). Positive controls were not used in the studies with caddisflies, natural variation was not addressed, and full datasets are not available.

3.2.3. Diptera

For the order of Diptera, one study with *Tipula* sp. (Jensen et al., 2010) and three studies with *Chironomus* spp. are available (Chen et al., 2022; Li et al., 2013; Prihoda and Coats, 2008). When field-collected *Tipula* larvae (age and size unknown) were fed with senescent and conditioned leaves from Cry1Ab or stacked Cry1Ab and Cry3Bb1-producing maize, sublethal effects were observed with the Cry1Ab-producing maize compared with the near-isoline, but not with the stacked maize line, indicating that the effects were likely not caused by the Cry protein (Jensen et al., 2010). Like the studies with caddisflies, the provided dose remains unknown, but likely low because of the conditioning procedure (reduced intactness of the *Bt* protein), the lack of biological activity after conditioning, and because leaf material was not changed during the experimental period. Furthermore, mortality data were not reported, so control mortality remains unknown. Different test substances were used in the studies with 2-11 days old *Chironomus* spp. larvae, including maize root extract containing Cry1Ab (Prihoda and Coats, 2008), cotton seed extract containing Cry1Ac (Li et al., 2013), and leaf powder from two SmartStax maize hybrids (Chen et al., 2022). In the two studies using *Bt* plant extract, adverse effects on the larvae were observed at high concentrations (Li et al., 2013; Prihoda and Coats, 2008). Matrix effects are likely since one study (Prihoda and Coats, 2008) did not include a non-*Bt* extract as a control and the other study (Li et al., 2013) did not specify the concentration of the non-*Bt* extract in the culture medium. While Li et al. (2013) reported that medium was changed twice a day in the 10 days study with spiked sediment, no replacement took place in the 4 days study with spiked water. The study using SmartStax leaf powder and the nearest non-transformed control line revealed a positive effect (shortened larval development) with *Bt* maize (Chen et al., 2022). Among the studies with Diptera, this study was the only one that established the natural range of variation by including four conventional maize lines and provided replicate-to-replicate data in the supplementary material. One study with Diptera used diflubenzuron as a positive control (Prihoda and Coats, 2008).

3.2.4. Coleoptera

Two studies were conducted with field-collected Coleoptera (Elmidae larvae or Curculionidae adults) that were fed with conditioned maize leaves (Whiting and Lydy, 2015) or rice seedlings (Oh et al., 2012c) producing Cry1 proteins. In both studies, no adverse effects on beetle performance were reported. In the study by Whiting and Lydy (2015), intactness of the plant material after the conditioning process was likely reduced and consequently biological activity and dose remain unclear. In addition, the authors did not mention that plant material was changed during the 7 days of the test, and they did not specify the non-*Bt* control. They did, however, use a positive control (tefluthrin and clothianidin). Oh et al. (2012c) fed the beetles for >60 days with *Bt* rice seedlings and measured sensitivity to clothianidin thereafter. Biological activity and control mortality were not addressed, and no positive control was used. In both studies on Coleoptera, natural variation was not addressed, only one *Bt* line was used, and data were not made available.

3.2.5. Amphipoda, Isopoda, Decapoda

Three studies were conducted with juvenile (6-14 days old) *Hyalella azteca* (Amphipoda). In two studies senescent and conditioned maize leaves containing Cry1Ab were used as test substance (Chambers et al., 2010; Whiting and Lydy, 2015). Both studies reported no effects on survival and/or the sublethal endpoint measured. An unrelated (Chambers et al., 2010) or unknown (Whiting and Lydy, 2015) hybrid was used as the non-*Bt* control and the level of exposure to Cry1Ab after the conditioning of leaves remains unclear (intactness of the test substance, biological activity, dose). Furthermore, no data are presented, thus control mortality remains unclear in both studies. Leaf material and water were not changed by Whiting and Lydy (2015). One study tested cotton (Cry1Ac) seed extract and reported decreased survival with increasing concentration (Li et al., 2013). A matrix effect cannot be excluded since a single unknown concentration of pure extract was tested as negative control.

One study was conducted with juvenile isopods (*Caecidotia communis*) using maize leaves (senescent, conditioned) that contained Cry1Ab or stacked Cry1Ab and Cry3Bb1 as test substance (Jensen et al., 2010). Similar to the results with Tipulidae from the same article, a significant reduction of survival and growth was reported for the Cry1Ab-producing maize but not for the stack when compared with the non-*Bt* control treatment. Exposure was likely low because bioactivity of the Cry protein could not be confirmed in the plant material after the 2-week conditioning process. This, together with the inconsistency in the results, points to plant background rather than *Bt* protein effects. In addition, control mortality was 45% by the end of the 30-day experiment. To be able to work with a homogeneous population of test specimens, Jensen et al. (2010) collected adults in the field, kept them in the laboratory until they produced offspring, and distributed the offspring equally among treatments for the actual experiments.

Three studies are available for field-collected Decapoda (Deng, 2018; Linn and Moore, 2014; West and Moore, 2019). Adults (Linn and Moore, 2014) and juveniles (West and Moore, 2019) of *Faxonius rusticus* were fed various senescent maize materials after 1-week conditioning. The plant materials either contained Cry1Ab or a combination of Cry1, Cry2 and Cry3 proteins, respectively. Test specimens were field-collected and thus of unknown age, but the authors reported mean carapace length. Lower survival and growth of adults were reported compared to the near-isogenic treatment when fed Cry1Ab-containing maize material, but low survival was also seen when non-*Bt* maize was mixed with sycamore leaves, while high survival was observed when *Bt* material was mixed with sycamore leaves. Thus, results are inconsistent and seem random, which suggests artifacts rather than *Bt* effects (Linn and Moore, 2014). Similarly, no consistent differences were observed when material from two different stacked *Bt* maize lines were fed to juveniles (West and Moore, 2019). Exposure in both studies through the conditioned material is once more unclear (intactness, biological activity, dose),

particularly because plant material was dried at 80°C in addition to the conditioning process. Control mortality for maize treatments was >90% in the study with juveniles and results seemed random with high variation.

Two months old juveniles of *Procambarus clarkii* from aquaculture were fed with rice powder containing Cry1Ab/Ac or Cry2A or with powder from near-isoline plants (Deng, 2018). While control mortality was >20% after 6 weeks, no adverse effects on survival or sublethal parameters were observed.

In none of the studies with Amphipoda, Isopoda, and Decapoda, natural variation was addressed nor were full datasets provided.

3.2.6. Pulmonata

One study examined the performance of field-collected *Gyraulus* spp. snails on senescent and conditioned maize leaves containing Cry1Ab (Chambers et al., 2010). Leaf discs were renewed regularly and no effects on survival or growth were observed when compared to material from an unrelated non-*Bt* maize hybrid. However, data were not presented (only statistics) and test duration, control mortality, expression levels, and intactness, biological activity, and dose after the conditioning process remain unclear.

3.3. Studies with GE plant material other than Bt

Aquatic test organisms were exposed to material from transgenic plants containing traits other than insect-resistance provided by *Bt* proteins in 10 studies (10 articles) conducted under laboratory conditions (Fig. 3, **Table S7**). Most studies used material from rice (6 studies), followed by soybean (3), and cotton (1). The traits included drought-tolerance (Nam et al., 2007; Oh et al., 2016a), disease resistance (Oh et al., 2014), altered composition (Oh et al., 2012a, 2012b, 2016b; Richardson et al., 2016), biological compound production (Moon et al., 2013; Oh et al., 2020), and herbicide tolerance (Cuhra et al., 2015). ELISA has been used to confirm expression of PAT or BAR marker proteins, or proteins from the *Theoredox* gene or the *γ-tocopherol methyltransferase* gene (Oh et al., 2016a, 2012a, 2014; Oh et al., 2020, 2016b). Carotinoids (Oh et al., 2012a, 2012b) and gossypol (Richardson et al., 2016) have been measured by high performance liquid chromatography (HPLC).

The majority of studies (7) were conducted with neonate *D. magna*. Typically, immobilization was recorded after 48 h (Oh et al., 2016a, 2014, 2012b; Oh et al., 2020, 2016b). In two longer studies, lasting 15/20 or 42 days, respectively (Cuhra et al., 2015; Nam et al., 2007), fecundity and population growth was assessed. In all studies, effects of the plant material *per se* was found to affect the measurement endpoints. None but one study reported differences between GE and non-GE material (Cuhra et al., 2015). In this study, differences (both positive and negative) between herbicide-tolerant GE soybeans and non-GE soybeans were observed. The compared GE and non-GE groups, however, consisted of a blend of different varieties cultivated on different fields and treated with different pesticides. In fact, chemical analyses revealed differences in composition and pesticide contamination of the plant materials. This likely caused the effects observed on the test organism, which appeared random and inconsistent. In the study by Nam et al. (2007), high control mortality was reported from the drought-tolerant rice treatments while little information on the used materials and the growing conditions was provided.

One study was conducted with field-collected adults of the weevil *Lissorhoptrus oryzophilus* (Oh et al., 2012a). The beetle's sensitivity to clothianidine was unaffected after feeding on carotenoid-fortified rice seedlings for >60 days. No information on mortalities during the 60 days feeding period is available. Rice seedlings containing a stilbene synthase gene did not result in effects on survival or growth of juvenile *Pomacea canaliculate* gastropods after a feeding period of 30 days (Moon et al., 2013). However, expression was not reported and no information was provided whether food was replaced during the 28 days of the experiment. Finally, a positive effect was observed in a study with 12 days old postlarvae of the decapod *Litopenaeus vannamei*. When feeding on cottonseed from two ultra-low gossypol lines, a higher feed-efficacy ratio and protein efficiency ratio as compared with conventional cottonseed with high gossypol content was observed (Richardson et al., 2016).

Positive controls (potassium dichromate) were applied in two studies with non-*Bt* plant material (Oh et al., 2016a, 2020). Two GE lines with the same trait and two unrelated conventional lines were used by Richardson et al. (2016). The full dataset was available in six studies.

3.4. Field studies

Altogether 13 studies (13 articles) have focused on the impact of GE plants on aquatic invertebrates under field conditions (Fig. 4, **Table S8**). With the exception of one study (Axelsson et al., 2010), all field studies included insect-resistant *Bt* plants. Most studies were conducted in rice (10 studies), followed by maize (2 studies) and aspen-poplar hybrids (2 studies). In the field studies, various invertebrate groups, such as Annelida, Nematoda, Tricladida, Crustaceae, Insecta, and Mollusca were collected. Taxa were analyzed either on species level, or on higher taxonomic level. Most field studies looked at abundance and community composition of the invertebrates.

All studies with rice focused on Lepidoptera-active *Bt*-transgenic lines producing Cry1 or Cry2 proteins. Invertebrate communities were observed directly in the flooded rice paddies. Studies focused on various non-target groups including Crustacea, Insecta, Annelida, and/or Mollusca. Either water samples (Bai et al., 2006; Chen et al., 2003, 2017; Hu, 2010; Li et al., 2014; Liu et al., 2016; Wang et al., 2013; Zhang, 2013) or benthic cores (Gui et al., 2015) were collected from *Bt* or non-*Bt* rice plots or litter bags with *Bt* or non-*Bt* leaf material were exposed in a ditch (Liu et al., 2017) to analyze colonization and degradation. Studies typically reported species abundance and diversity. With two exceptions, no differences between *Bt* and non-*Bt* rice were reported. In the first exception, a higher abundance and diversity of invertebrates was recorded in water from *Bt* rice (Li et al., 2014). This was attributed to higher pesticide residue levels measured in water from non-*Bt* rice fields, because fields were treated with pesticides as agronomically needed. In the second exception, an increased abundance of Ephemeroptera species in *Bt* rice in the late tillering stage was observed (Bai et al., 2006). Presence of *Bt* proteins was confirmed in *Bt* rice material (Hu, 2010; Liu et al., 2017, 2016; Wang et al., 2013; Zhang, 2013) and in soil (Liu et al., 2016), but not, or only in traces, in water (Chen et al., 2017; Hu, 2010; Liu et al., 2017, 2016; Wang et al., 2013).

In both studies with maize, no consistent effects were observed on the abundance and composition of invertebrates in leaf-litter bags exposed to streams (Chambers et al., 2010; Swan et al., 2009) and in benthic cores (Chambers et al., 2010). While one study included unrelated hybrids as comparators for the *Bt* treatments (Chambers et al., 2010), the second study included the near-isogenic non-GE line (Swan et al., 2009). In addition, Chambers et al. (2010) worked in different farmer fields, which were likely subject to different management. Expression of the *Bt* protein was confirmed in both studies.

In the studies with aspen-poplar hybrids, either two *Bt*-transgenic lines (Axelsson et al., 2011) or two lines that had been altered for lignin characteristics were used (Axelsson et al., 2010). In both studies, leaf litter was exposed in litter bags to streams and near-isoline material was used as controls. Besides the decomposition of the plant material, abundance, community, and species richness of selected aquatic insect taxa colonizing the leaf litter were recorded. In the study with lines altered for lignin characteristics, no effect on the measurement endpoints were observed (Axelsson et al., 2010). In the study with two *Bt* lines, insect community assemblages differed between the two *Bt* lines and were increased in the *Bt* lines compared with the non-*Bt* control (Axelsson et al., 2011). Expression was addressed in the study with *Bt* protein producing lines, but not in the study with lignin-altered lines.

Species	Life stage	Test substance	Delivery	Duration	Cohort	Expression	Intactness	Dose	Con. exposure	Ingestion	Neg. control	Control mort.	Pos. control	Nat. variation	Multiple lines	Data avail.	Oth. concerns	Results	Reference
Cladocera: Daphniidae																			
Daphnia magna	juveniles	rice (drought-tolerant) from unknown source	plant extract in medium	15/20 d														0	Nam et al. (2007)
		rice (drought-tolerant) from field	leaf & stem powder in medium	48 h														0	Oh et al. (2016a)
		rice (disease-resistant) from field	leaf & stem powder in medium	48 h														0	Oh et al. (2014)
		rice (carotenoid-fortified) from field	leaf & stem powder in medium	48 h														0	Oh et al. (2012b)
		soybean (herbicide-tolerant) from field	meal in medium	42 d														(↓)	Cuhra et al. (2015)
		soybean (medical application) from field	whole plant powder in medium	48 h														0	Oh et al. (2020)
		soybean (vitamin E-fortified) from field	leaf & stem powder in medium	48 h														0	Oh et al. (2016b)
Coleoptera: Curculionidae																			
Lissorhoptrus oryzophilus	adults	rice (carotenoid-fortified) from field	leaf & stem powder in medium	>60 d														0	Oh et al. (2012a)
Architaenioglossa: Ampullariidae																			
Pomacea canaliculata	juveniles	rice (health promoting) from field	seedlings as food	28 d														0	Moon et al. (2013)
Decapoda: Penaeidae																			
Litopenaeus vannamei	juveniles	cotton (low gossypol seeds) from field	Seed meal as food	64 d														↑	Richardson et al. (2016)

Fig. 3. Laboratory studies exposing aquatic non-target invertebrates to GE plant material other than *Bt* plants. In addition to basic information about the study and the reference, an overview of the critical appraisal is provided. Green fields indicate nonproblematic criteria, red fields problematic ones, and blue fields indicate that no information was available (see **Table S3**). For detailed information see **Table S7**. Results are summarized as follows: (↓) inconsistent adverse effect in GE treatment compared with non-GE control; ↑ positive effect in GE treatment compared with non-GE control; 0 no difference.

Taxa	GE plant(s)	Experimental design	Expression	Non-GE control	Positive control	Replicates	Rep. dispersal	Management	Duration	Methodology	Nat. variation	Multiple lines	Data avail.	Oth. concerns	Results	Reference
Maize																
57 macroinvertebrate taxa	different hybrids, Cry1Ab	benthic cores & litterbag colonization in streams flowing through maize fields													0	Chambers et al. (2010)
10 macroinvertebrate taxa	MON810, Cry1Ab, MON810& MON863, Cry1Ab+Cry3Bb1	litterbag colonization in streams													0	Swan et al. (2009)
Aspen																
38 insect species	two GE lines with altered lignin characteristics	litterbag colonization in streams													0	Axelsson et al. (2010)
27 insect species	2 lines (*Bt*17, *Bt*27), Cry3Aa	litterbag colonization in stream													↑	Axelsson et al. (2011)
Rice																
33 zooplankton species	Huahui No. 1 (Cry1Ab/c), *Bt* line 2 (Cry2A)	zooplankton counted in water from rice plots (diff. insecticide sprays than control)													↑	Li et al. (2014)
43 zooplankton species	Huahui No. 1 (Cry1Ab/c)	zooplankton counted in water from rice plots (1 or 2 insecticide sprays)													0	Liu et al. (2016)
21 meiobenthos species	Huahui No. 1 (Cry1Ab/c)	litterbag colonization in stream													0	Liu et al. (2017)
Rotifera, Cladocera, Copepoda	*Bt* MH63, *Bt* SY63 (Cry1Ab/c)	zooplankton counted in water from rice plots													0	Wang et al. (2013)
Chironomidae, Coleoptera, Ephemeroptera	Huachi B6, Cry1Ab	Invertebrates counted in benthic cores from rice paddies													↑	Bai et al. (2006)
36 zoobenthos taxa	Huahui No. 1, Cry1Ab/c	benthic cores in rice paddies													0	Gui et al. (2015)
29 zooplankton species	*Bt* MH63, Cry1Ab/c	zooplankton counted in water from rice plots													0	Zhang (2013)
17 zooplankton species	T1C-19 (Cry1C), T2A-1 (Cry2A)	zooplankton counted in water from rice plots													0	Chen et al. (2017)
Insecta, Gastropoda	Kangchong1, Kangchong2, Huahui No. 1, Cry1Ab/c	aquatic species collected from water from rice plots													0	Chen et al. (2003)

Fig. 4. Field studies sampling aquatic non-target invertebrates in experiments involving GE and non-GE plants. In addition to basic information about the study and the reference, an overview of the critical appraisal is provided. Green fields indicate nonproblematic criteria, red fields problematic ones, and blue fields indicate that no information was available (see **Table S4**). For detailed information see **Table S8**. Results are summarized as follows: ↑ positive effect in GE treatment compared with non-GE control; 0 no difference.

Field studies mostly did not include positive controls, e.g., plots treated with insecticides, except Liu et al. (2016) and Liu et al. (2017). This, combined with low numbers of replicates and high environmental variability, may have resulted in low statistical power to detect potential effects. Multiple GE lines with the same trait were used in several studies (Axelsson et al., 2010, 2011; Chen et al., 2003; Hu, 2010; Swan et al., 2009; Wang et al., 2013). While most authors of field studies reported at least 4 sampling events throughout the season, some studies comprised only 2 or 3 samplings (duration). None of the studies used different non-related varieties to establish the natural range of variation, and replicate-to-replicate data are not available for any of the studies.

3.5. Relationship of tested concentrations in experimental studies to realistic exposure

In an ERA context, it is important to know the hazard of novel substances and the environmental exposure of organisms to the substance in the anticipated area of application. Laboratory studies often simulate worst-case exposure conditions, such as high concentrations of purified proteins or plant tissue with high concentrations of insecticidal protein fed directly, continuously, and exclusively to the test organisms. If no adverse effects are observed in such studies, one can conclude that the risk under environmental conditions is likely low (given the experiment has no flaws that led to false negative results). If effects of the

insecticidal protein cannot be excluded in worst-case experiments, however, it is important to discuss the findings in the context of realistic environmental exposure. The basis for all exposure calculations is the expression level in the GE plant. Aquatic organisms may consume GE plant products directly by feeding on plant debris or indirectly via leachates (Carstens et al., 2012; Venter and Bøhn, 2016). Exposure of predators via prey is not discussed further due to the lack of such studies with aquatic species. Because of the instability of insecticidal proteins, exposure via soluble protein in water and sediment is less potent than ingestion of material directly derived from GE plants. For a detailed discussion on modelled exposure values, different exposure routes, degradation dynamics, and published evidence on measured concentrations in plant tissue, water, and sediment, see **Supplementary Material 3**.

Taking *Bt* rice as an example and considering an average concentration of 10 mg Cry1Ab/Cry1Ac per kg dry weight of plant material, modelled worst-case exposure to soluble *Bt* protein would be between 3 and 100 μg/L, depending on the model. In studies where aquatic invertebrates were continuously exposed to purified *Bt* proteins, (consistent) effects were evident at concentrations well above 100 μg/L. In acute toxicity studies with mosquitoes, significant effects were observed at concentrations even in the mg/L range. We thus conclude that the concentrations of soluble protein resulting in effects on aquatic invertebrates in laboratory tests exceed the modelled worst-case concentrations. They even more exceed the concentrations measured in water bodies in the field, at least by a factor of 1000.

For particle-feeders and shredders, the worst-case exposure scenario would be food comprised of 100% green *Bt* plant tissue. Realistic exposure, however, can be considered much lower, because of the rapid degradation processes of *Bt* proteins in water.

4. Conclusions and recommendations

In this comprehensive review, we discuss non-target effects of purified *Bt* proteins and GE plants on aquatic invertebrates. Altogether 25 aquatic taxa from different functional groups have been tested in the laboratory, including filter feeders (*Daphnia* spp., Culicidae larvae), shredders (Trichoptera larvae, Amphipoda, Isopoda, Tipulidae larvae, Decapoda), gathering collectors (*Chironomus* spp. larvae, Elmidae), scrapers (*Helicopsyche* larvae, aquatic snails), and herbivores (Curculionidae beetles). Most attention was paid to *Bt* crops, where studies with purified *Bt* proteins and studies with plant material as test substance were reviewed. Studies with purified Lepidoptera-targeting *Bt* proteins (Cry1A, Cry1C, Cry2A, VIP 3A) dominated the literature. Similarly, studies with plant material were conducted mainly with Lepidoptera-targeting *Bt* crops, although some studies with Coleoptera-targeting *Bt* maize and stacked *Bt* maize were available. In comparison with studies on terrestrial non-target organisms, data of GE plant effects on aquatic non-target invertebrates are limited. Interestingly, no studies with GE plants and aquatic non-target species that belong to the known activity spectrum of the expressed proteins were identified, such as aquatic beetles and Coleoptera-active Cry3-class proteins, or aquatic Diptera and Cry2A, which is known to affect Lepidoptera and Diptera. It is understandable that field studies concentrate on *Bt* rice that is commonly grown in an aquatic environment. Only two studies with *Bt* maize and none with *Bt* cotton investigated potential effects on aquatic non-target communities.

To be able to interpret the published results, we critically appraised all studies according to schemes adapted and/or developed for this review (Fig. 1–5). To our knowledge, this has not been done comprehensively for aquatic non-target studies of GE crops. Studies with identified critical issues are labelled in red, while studies with unknown information are labelled in blue. Such unknowns may result from a lack of experimentation (e.g., no inclusion of positive controls or no measurement of insecticidal protein in test materials) or from reporting deficiencies (e.g., no reporting of control data or no availability of full datasets). For such unknowns, it remains unclear whether a critical issue is present or not. Overall, we did not weigh different critical appraisal criteria, as each of them may point to important flaws, artifacts, and difficulties for interpretation and relevance of results. All criteria are summarized in Fig. 5 and the most important ones are discussed in the following. Most non-target studies were conducted over a time period longer than 4 days. Some acute studies, however, are also available, in particular for purified *Bt* proteins and GE plant material other than *Bt*. Those studies are difficult to interpret because biological molecules, such as insecticidal proteins, often act within several days, and not within 24 or 48 hours (unless very high concentrations are applied). Nevertheless, several studies with purified insecticidal proteins, either short- or long-term, showed dose-dependent adverse effects on aquatic non-target species when high concentrations were applied. As demonstrated for *D. magna*, there is evidence that such effects may have been unspecific and caused by the presence of high concentrations of protein rather than by the specific mode of action of the expressed insecticidal proteins. Studies with purified protein commonly used medium or water as a comparator instead of denaturized protein or other non-toxic protein.

For studies with *Bt* plant material, expression levels of the *Bt* proteins in plant tissue were commonly available. Similarly, most studies with GE plant material other than *Bt* provided evidence of transgene expression. In some studies, however, plant material was conditioned for up to two weeks in stream water before being used for feeding. In two studies, *Bt* maize material was even dried at 80°C before starting a conditioning process. In none of these studies the authors measured *Bt* protein concentrations in the material they actually provided to their test organisms. Given the rapid degradation dynamics of *Bt* protein in plant tissue in water and the heat-sensitivity of *Bt* proteins, it is unclear how much intact protein remained in the conditioned plant material at the beginning of the exposure period. Biological activity of *Bt* proteins in plants was addressed by only few authors and remained unclear in the remaining studies. While biological activity was confirmed for *Bt* rice (Deng, 2018) and *Bt* maize before a conditioning process, no bioactivity was found in maize material after conditioning for two weeks in stream water (Jensen et al., 2010), which provides evidence that the administered dose was low. Future studies using conditioned test material should therefore ensure that test protein concentration and biological activity is confirmed in the material actually fed to the test organisms. For continuous exposure, most, but not all authors changed the test substance, i.e., plant material or medium with purified insecticidal protein, every 1-3 days. While untransformed near-isolines were used as negative controls in most studies with plant material, in some studies, GE plants were compared to unrelated plant material or no information on the relationship between GE and non-GE comparators and how they were produced was provided. Most laboratory studies used synchronized juveniles from laboratory cultures. Studies using field-collected test organisms, however, did not provide statistical evidence of similarity among the test specimens assigned to the different treatments. All long-lasting experiments, and particularly some experiments with field-collected specimens, revealed high control mortalities, which poses problems for data interpretation and reliability of the study set-up. Positive controls (potassium dichromate or other insecticidal compounds) were only used in few studies.

For experiments conducted with one GE line and one non-GE comparator, there is no reason for concern, if there is no difference in the response of a non-target species (assuming no flaws in experimental design that would mask an effect). For the experiments that do show differences, however, it is difficult to distinguish between specific effects of the GE trait (e.g., the production of an insecticidal protein), and unspecific effects of the plant background. Few authors used two different *Bt* lines producing the same proteins, stacked as well as single gene plants, and/or different materials from the same plant lines. Inconsistencies among those treatments indicate unknown differences in the plant lines (plant-background effects) rather than *Bt*-specific effects.

Fig. 5. Critical appraisal of laboratory studies with purified *Bt* proteins, *Bt* plant material, and non-*Bt* plant material, and field studies. For each criterion, the percentages of studies labelled red, blue, and green are given for each study type. Criteria not relevant remain blank.

Future research should thus address the potential influence of plant background effects in more depth. Consistency should also be present when experiments are repeated multiple times. To be able to interpret the size of differences in response to GE plants compared to the nearest non-GE plants, several unrelated conventional varieties had been included in non-target experiments and the natural range of variation among (conventional) varieties was established. This methodology, which was suggested in two recent studies with aquatic species, however, has not yet been applied by other authors of studies on GE plants and aquatic species (Chen et al., 2021, 2022).

Full datasets were only available for few of the reviewed studies, which is understandable for older studies published at a time when no online data repositories were available. Availability of data, however, allows quality control as well as performance of meta-analyses in the future. With funding agencies, companies, public institutions, and publishers following FAIR (findable, accessible, interoperable, and reusable) data principles, data availability is expected to improve for future studies (Wilkinson et al., 2016).

In summary, reported effects of *Bt* plants on aquatic non-target invertebrates are often inconsistent and methodological shortcomings suggest that plant-background effects and other unspecific effects rather than effects caused by the *Bt* protein may have played a role. Similarly, studies reporting no effects often showed methodological shortcomings, such as unknown exposure concentrations and short study duration, which prevented detection of potential *Bt*-specific effects. Artifacts are particularly likely when test substances were not well characterized, no appropriate controls were used, only one GE/non-GE pair was compared, and consistency of results could not be demonstrated. In any

case, environmental exposure of non-target organisms in the field is often orders of magnitude lower than the hazardous concentrations applied in laboratory studies.

In a scientific field that is of high political, economic, and societal importance, the quality of published studies should comply with the highest standards. In contrast to ecotoxicological testing of chemicals, there are no standardized, harmonized and ring tested protocols available for testing GE plants and their products on non-target species. This allows more freedom in designing studies but requires particular attention to rigorous study designs that address relevant risk hypotheses. While this is evident for studies submitted to support the regulatory dossiers, it is also important for public studies, which have an impact in the scientific community, the society, and regulatory decision making. It is recommended that clear guidance on robust test protocols for GE plants and their expressed traits is developed. Guidelines for designing and conducting high quality ecotoxicological studies as well as reporting standards exist, not only for chemical testing (Klimisch et al., 1997; Kase et al., 2016; Hanson et al., 2017), but also for GE plant testing (Romeis et al., 2011; De Schrijver et al., 2016). Such guidelines should be rigorously followed to produce meaningful and trustworthy results. To facilitate this, we here provide a list of potential confounding factors and recommendations on how to avoid them (Table 1). Although this review focused on aquatic invertebrates, the recommendations for experimental design and data interpretation are also applicable to studies with terrestrial non-target species.

CRediT authorship contribution statement

Yi Chen: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Jörg Romeis:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Michael Meissle:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Yi Chen reports financial support was provided by Sanya Yazhou Bay Science and Technology City. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2026.100726.

Data availability

No data was used for the research described in the article.

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