Anbau von gentechnisch veränderten Pflanzen und aquatische Systeme: Exposition, Effekte und die Nutzung von higher-tier Ansätzen für die Risikobewertung

von M. Sc. Antonia Pott

geboren am 19.01.1989 in Moers

Angenommene Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat)

Fachbereich 7: Natur- und Umweltwissenschaften

Universität Koblenz-Landau

Berichterstatter:

Prof. Dr. Ralf Schulz, Landau

Jun.-Prof. Dr. Mirco Bundschuh, Landau

Tag der Disputation:

14.09.2022

Die kumulative Dissertation stützt sich auf folgenden Veröffentlichungen:

1) Pott, Antonia; Otto, Mathias; Schulz, Ralf (2018): Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment. In: *The Science of the total environment* 635, S. 687–698. DOI: 10.1016/j.scitotenv.2018.04.013 **(Anhang A.1)**

2) Pott, Antonia; Bundschuh, Mirco; Bundschuh, Rebecca; Otto, Mathias; Schulz, Ralf (2020): Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders - an attempt to establish dose-effect relationships through food-spiking. In: *Scientific reports* 10 (1), S. 5262. DOI: 10.1038/s41598-020-62055-2 **(Anhang A.2)**

3) Pott, Antonia; Bundschuh, Mirco; Otto, Mathias; Schulz, Ralf (2022): Assessing effects of genetically modified plant material on the aquatic environment using higher-tier studies. In: *Bulletin of Environmental Contamination and Toxicology* (in press) **(Anhang A.3)**

Inhaltsverzeichnis

Danksagung

Zu Beginn möchte ich mich bei allen bedanken, die mich beim Anfertigen meiner Dissertation auf die unterschiedlichste Weise unterstützt haben.

Mein besonderer Dank gilt:

Prof. Dr. Ralf Schulz für die Betreuung, die Unterstützung und für die Übernahme des Berichterstatters. Außerdem möchte ich mich für die wertvollen Ratschläge bedanken, dass ich mit Fragen immer zu ihm kommen konnte und seine Ermutigung meine Dissertation fertigzustellen.

Jun.-Prof. Dr. Mirco Bundschuh für die Betreuung, die Korrekturen sowie die Übernahme des Berichterstatters. Außerdem möchte ich mich für die wertvollen Anregungen bei all meinen Fragen bedanken.

Prof. Dr. Martin Entling für die Übernahme des Vorsitzes der Promotionskommission.

Dr. Mathias Otto für die viele Zeit, Gedult und Mühe, die er in meine Dissertation investiert hat. Vielen Dank für die unermüdliche Unterstützung und die vielen Gespräche.

Rebecca Bundschuh für ihre Hilfsbereitschaft und das Einarbeiten in die Laborarbeit.

Therese Bürgi und Simon Lüderwald für die Hilfe im Labor.

Der gesamten Arbeitsgruppe „Ökotoxikologie und Umwelt“ für die Hilfe und die angenehme Arbeitsathmosphäre.

Dem gesamten Fachgebiet „Bewertung gentechnisch veränderter Organismen/ Gentechnikgesetz“ am Bundesamt für Naturschutz für deren Hilfsbereitschaft, angenehme Antmosphäre und meine lehrreiche Zeit dort.

Dem Bundesamt für Naturschutz für die finanzielle Unterstützung, welche die Dissertation möglich gemacht hat.

Meiner Familie dafür, dass sie mich immer bei meiner Promotion unterstützt haben.

Meinem Partner Martin für die unendliche Unterstützung, die Besuche in Bonn und in Landau und dafür, dass er mich immer wieder aufgebaut und motiviert hat.

Zusammenfassung

Gentechnisch veränderte Pflanzen werden seit etwa 25 Jahren kommerziell angebaut. Besonders häufig werden Bt-Pflanzen verwendet, die Gene des Bakteriums *Bacillus thuringiensis* (Bt) enthalten und Cry-Proteine produzieren. Die Risikobewertung konzentrierte sich lange auf die terrestrische Umwelt. Erst mit der Veröffentlichung von Rosi-Marshall et al. im Jahr 2007 rückten mögliche Auswirkungen auf die aquatische Umwelt in den Fokus. In dem ersten Teil dieser Dissertation wurde die vorhandene Literatur zu lower-tier Effektstudien und Studien über den Verbleib ausgewertet, die die Auswirkungen von GV-Pflanzen auf die aquatische Umwelt untersuchen. Es zeigen sich potentielle Effekte auf aquatische Organismen. Einige Studien wiesen außerdem den Eintrag von GV-Pflanzenmaterial in die aquatische Umwelt sowie das Herauswaschen der Toxine in das Wasser nach.

Im zweiten Teil der Dissertation wird die Wirkung des Cry1Ab-Toxins auf zwei Arten an Köcherfliegenlarven (*Chaetopterryx* spec., *Sericostoma* spec.) untersucht. Trichopteren sind phylogenetisch nah verwandt mit Lepidopteren, die häufig die Zielorganismen von Cry-Toxinen sind. Um mehrere Konzentrationen verabreichen zu können wurde eine neue Spiking-Methode eingesetzt, bei der gelöstes Cry1Ab-Toxin auf Blätter der Schwarzerle (*Alnus glutinosa*) aufgetragen wird. Effekte zeigten sich ins Besondere bei sublethalen Endpunkten. Der Lipidgehalt der *Chaetopteryx* spec. Larven war nach zwölf Wochen geringer mit zunehmender Cry1Ab Konzentration. Die Verringerung des Lipidgehalts könnte auf eine Erhöhung des Energiebedarfs für Reparaturmechanismen hindeuten. Bei *Sericostoma* spec. zeigte sich nach sechs Wochen eine Verlangsamung der Larvalentwicklung in der höchsten Cry1Ab Konzentration, was zu einer späteren Emergenz und damit zu Auswirkungen auf die Nahrungskette führen könnte.

Im dritten Teil der Dissertation wurde die Bewertung von Auswirkungen von GV-Pflanzen auf die aquatische Umwelt anhand von higher-tier Studien untersucht. Da higher-tier Studien bei Pestiziden bereits häufig vorkommen, wurden diese mit den bereits durchgeführten higher-tier Studien mit GV-Pflanzen verglichen. Es zeigt sich, dass es keine Standardisierung von higher-tier Studien mit GV-Pflanzen gibt, was für die Qualitätssicherung und die Vergleichbarkeit von Studien notwendig ist. Außerdem bestehen große Schwierigkeiten damit verschiedene Versuchskonzentrationen herzustellen, was für die Untersuchung einer Dosis-Wirkungsbeziehung notwendig ist.

Insgesamt zeigt sich, dass es noch erheblichen Forschungsbedarf gibt, was die Auswirkungen von GV-Pflanzen auf die aquatische Umwelt angeht. Weitere Studien sind für eine umfangreiche und aussagestarke Risikobewertung unumgänglich.

Abstract

Genetically modified plants have been grown commercially for about 25 years. Bt plants, which contain genes from the bacterium *Bacillus thuringiensis* (Bt) and produce Cry proteins, are used particularly frequently. Risk assessment has long focused on the terrestrial environment. Only since the publication of Rosi-Marshall et al. in 2007 potential effects on the aquatic environment came into focus. The first part of this dissertation analyse the existing literature on lower-tier effect studies and fate studies examining the effects of GM plants on the aquatic environment. Potential effects on aquatic organisms are apparent. Some studies also demonstrate the entry of GM plant material into the aquatic environment and the leaching of toxins into the water.

The second part of the dissertation investigate the effects of Cry1Ab toxin on two species of caddisfly larvae (*Chaetopterryx* spec., *Sericostoma* spec.). Trichopterans are phylogenetically closely related to lepidopterans, which are often the target organisms of Cry toxins. In order to be able to create several concentrations, a new spiking method was used in which dissolved Cry1Ab toxin was applied on leaves of black alder (*Alnus glutinosa*). Effects were particularly evident at sublethal endpoints. The lipid content of *Chaetopteryx* spec. larvae was lower after twelve weeks with increasing Cry1Ab concentration. The reduction in lipid content may indicate an increase in energy requirements for repair mechanisms. *Sericostoma* spec. showed a slowdown in larval development at the highest Cry1Ab concentration after six weeks, which could lead to later emergence and thus effects on the food chain.

In the third part of the dissertation, the assessment of impacts of GM crops on the aquatic environment was investigated using higher-tier studies. Since higher-tier studies are already common with pesticides, these were compared to the higher-tier studies already conducted with GM crops. It is found that there is no standardization of higher-tier studies with GM crops, which is necessary for quality assurance and comparability of studies. In addition, there are great difficulties in establishing different test concentrations, which is necessary for the investigation of a dose-response relationship.

Overall, it is clear that there are still significant knowledge gaps of the effects of GM plants on the aquatic environment. Further studies are essential for a comprehensive and meaningful risk assessment.

1. Einleitung

1.1 Gentechnisch veränderte Organismen

Gentechnisch veränderte Organismen (GVO) gibt es seit etwa 25 Jahren. Darunter werden Pflanzen, Tiere oder Mikroorganismen, deren Gene durch gentechnische Verfahren verändert wurden, verstanden. Diese Verfahren unterscheiden sich von konventioneller Züchtung in der Form, dass auch Gene einer anderen Art in die DNA eingebaut werden können. In dieser Arbeit konzentriere ich mich ausschließlich auf gentechnisch veränderte (GV) Pflanzen, die sog. Grüne Gentechnik, da sie für unsere Fragestellung relevant sind. Bei GV-Pflanzen gibt es eine Vielzahl an gentechnischen Veränderungen, zwei Gruppen kommen aber hauptsächlich vor. Mit 45,1% der gentechnischen Veränderungen dominiert die Herbizidresistenz (HR), gefolgt von der Insektenresistenz (IR) mit 34,6% (Parisi et al. 2016). GV-Pflanzen mit einer Herbizidresistenz expremieren Enzyme, die Herbizide abbauen, wodurch die Pflanze höhere Dosierungen der Herbizidapplikation (z.B. Glyphosat) verträgt. Dies führt dazu, dass Beikräuter effektiver bekämpft werden können ohne die Nutzpflanze selbst zu schädigen. In insektizidresistenten GV-Pflanzen wurden Gene des Bodenbakteriums *Bacillus thuringiensis* eingefügt. Diese Gene expremieren Proteine, sog. Bt-Toxine, die im alkalischen Verdauungstrakt von Insekten aktiviert werden und zur Auflösung der Darmmembran und somit zum Tod des Insekts führen (Glare und O´Callaghan 2000). Durch den Einsatz von Bt-Pflanzen kann das Vorkommen von Fraßschädlingen eingedämmt werden. Bt-Pflanzen produzieren entweder kristalline Proteine (Cry) oder vegetative isektizide Proteine (Vip) (Palma et al. 2014). Gentechnisch verändert wurden vor allem Nutzpflanzen, die zur Lebens- und Futtermittelproduktion angebaut werden (z.B. Soja, Mais, Raps), aber auch andere Nutzpflanzen sind von Bedeutung (z.B. Baumwolle). Heute werden GV-Pflanzen vor allem in Nord- und Südamerika angebaut. Im Jahr 2014 waren 82% des weltweit angebauten Sojas gentechnisch verändert (James 2014). Bei Baumwolle waren es 68% und bei Mais 30% (James 2014). Die hohe landwirtschaftliche Nutzung von gentechnisch veränderten Pflanzen zeigt sich auch in der hohen Anzahl an zugelassenen GV Einzeltransformationsereignissen weltweit, welche im Jahr 2014 die Anzahl von 102 erreichte (Parisi et al. 2016).

1.2 Zulassung von GV-Pflanzen in der Europäischen Union

Gentechnisch veränderte Pflanzen unterliegen in der Europäischen Union (EU), genauso wie Pestizide, einem Zulassungsverfahren. In der EU untersucht die Umweltverträglichkeitsprüfung potenzielle schädliche Auswirkungen auf Nichtzielorganismen (EU 2001b). Die Wichtigkeit Insektizide mit in die Risikobewertung einzubeziehen ist

weitestgehend akzeptiert und zeigt sich in umfangreichen Studien (Stehle und Schulz 2015b). Dagegen gibt es für GV-Pflanzen nur in geringem Umfang Studien zur Risikobewertung von GV-Pflanzen (Pott et al. 2018). In den 10 Jahre nach dem ersten kommerziellen Anbau von GV-Pflanzen im Jahr 1995 (BfN 2022a) hat sich die Risikoforschung fast ausschließlich auf die Auswirkungen von GV-Pflanzen auf den Bereich der terrestrischen Umwelt konzentriert. Erst im Jahr 2007 kam mit der Studie von Rosi-Marshall et al. (2007), welche mögliche Effekte auf Köcherfliegenlarven zeigte, auch die aquatische Umwelt in den Fokus. Köcherfliegen (Trichoptera) haben aquatische Larvenstadien und sind phylogenetisch nah verwandt mit Schmetterlingen (Lepidoptera), welche häufig der Zielorganismus von Bt-Mais sind (z.B. Larven des Maiszünslers). Zusätzlich konnte in der Studie auch gezeigt werden, dass GV-Pflanzenmaterial in Gewässer eingetragen wird und somit ein potenzielles Risiko für aquatische Invertebraten besteht (Rosi-Marshall et al. 2007). Während manche die Schlussfolgerungen der Studie als übertrieben ansahen (Beachy et al. 2008; Parrott 2008), war dies jedoch der Anfang davon, dass die aquatische Umwelt auch in nachfolgenden Studien betrachtet wurde (Venter und Bøhn 2016). Auch die europäische Behörde für Lebensmittelsicherheit (EFSA) beschäftigte sich damals mit der Studie und kam jedoch zu dem Schluss, dass das Risiko von GV-Pflanzen auf die aquatische Umwelt vernachlässigbar sei (EFSA 2011a, 2011b).

Bei dem Zulassungsverfahren von GV-Pflanzen handelt es sich um ein EU-weites Verfahren, an dem alle Mitgliedsstaaten beteiligt sind. Die Zulassung wird in der Freisetzungs-Richtlinie 2001/18/EG (EU 2001a) geregelt. Bei der Genehmigung wird unterschieden zwischen dem Import und dem Anbau einer GV-Pflanze. In der EU ist der Bt-Mais MON810 seit 1998 zum kommerziellen Anbau zugelassen (BfN 2022a), welcher ins besondere in Spanien und Portugal stattfindet (BfN 2022b). Die gentechnisch veränderte Kartoffel Amflora, welche eine veränderte Stärkeproduktion besitzt, wurde bis 2012 in Deutschland angebaut und ist somit bis heute die letzte GV-Pflanze, die in Deutschland kommerziell angebaut wurde (BfN 2022a). Dagegen dürfen im Jahr 2022 etwa 90 verschiedene GV-Pflanzen in die EU importiert werden (EC 2022) und finden v.a. als Futtermittel ihre Anwendung.

1.3 GV-Pflanzen und aquatische Umwelt

Aquatischen Ökosysteme sind eng mit der terrestrischen Umwelt verknüpft. Aus dieser gelangen nicht nur Mineralien und organischer Eintrag in Gewässer (Vannote et al. 1980), sondern in landwirtschaftlich genutzten Gebieten neben Phosphaten und Stickstoff auch Pestiziden (Stehle und Schulz 2015a). Die Wasserrahmenrichtlinie (WRRL), welche 2000 in Kraft trat, legt den Rahmen fest für die Verbesserung des ökologischen Status von Gewässern

durch Wassermanagementmaßnahmen (EU 2000). Die Landwirtschaft ist ein großer Faktor, der bei vielen Gewässern der Erreichung eines guten ökologischen Statuses entgegensteht (Vörösmarty et al. 2010).

1.4 Risikobewertung von GV-Pflanzen in der aquatischen Umwelt: Experimenteller Ansatz

Um die Auswirkungen von GV-Pflanzen auf Gewässer experimentell untersuchen zu können müssen potentielle Expositionspfade ermittelt werden (Bundschuh et al. 2016). Da sich die Proteine, die aufgrund der gentechnischen Veränderung neu gebildet werden (z.B. Bt-Toxine) im Pflanzenmaterial befinden, sind Detritus fressende Organismen im besonderen Maße exponiert (Hilbeck et al. 2017). Eine wassergebundene Exposition sollte ebenfalls betrachtet werden (Englert et al. 2017c), ist aber vermutlich weniger relevant als die Exposition über das Futter. Die Untersuchung der Exposition über das Futter steht allerdings vor einer großen Herausforderung, denn die Konzentration des Bt-Toxins in der Pflanze ist abhängig von der gentechnischen Veränderung. Dies bedeutet, dass für Tests mit GV-Pflanzenmaterial nur eine Konzentration des Bt-Toxins zur Verfügung steht. Dies macht die Untersuchung einer Dosis-Wirkungsbeziehung, die auf verschiedenen Konzentrationen des Bt-Toxins im Pflanzenmaterial beruht, unmöglich. Eine mögliche Lösung ist die Anwendung eines Spiking-Verfahrens. Dabei wird Bt-Toxin in verschiedenen Konzentrationen gelöst und diese Lösungen auf Pflanzenmaterial aufgetragen. Der Vorteil ist, dass verschiedene Konzentrationen für Tests zur Verfügung stehen. Außerdem kann auch anderes Pflanzenmaterial, als das der GV-Pflanze, verwendet werden. So kann Pflanzenmaterial ausgewählt werden, dass einen hohen Nährstoffgehalt hat. Dies kann die Futterqualität erhöhen, was vor allem bei langen Expositionsdauern von hoher Bedeutung ist. Dieses Verfahren kann sowohl bei higher- als auch bei lower-tier Tests angewendet werden. Als Tier 1 Studien werden akute Labortest mit Standardtestorganismen verstanden. Lower-tier Studien stellen einen worst-case Ansatz unter festgelegten Testbedingungen dar. Laborstudien, die keine Standardorganismen verwenden oder eine verlängerte Testdauer haben werden Tier 2 Studien genannt. Komplexere Testdesigns werden als higher-tier Studien bezeichnen. Hier unterscheidet man zwischen Populationsstudien in Mikro- oder Mesokosmen (Tier 3) und Feldstudien oder Landschaftsmodellierungsansätzen (Tier 4) (EFSA 2013). In dieser Arbeit konzentrieren ich mich auf experimentelle higher-tier Studien (Mikro-, Mesoksomen, Feldstudien). Experimentelle higher-tier Studien können eingesetzt werden, wenn in lower-tier Studien bereits Effekte gefunden wurden, die nun unter realistischeren Bedingungen untersucht werden

sollen. Bei diesen Testansätzen können mehrere Organismengruppen gleichzeitig beobachtet werden und direkte und indirekte Effekte abgebildet werden (Brock et al. 2010; Preston 2002; EFSA 2013). Innerhalb von Nahrungsnetzten können sowohl Top-down als auch Bottom-up Effekte untersucht werden (Wieczorek et al. 2015). Außerdem ermöglicht die lange Versuchsdauer auch die Abbildung von chronischen Effekten sowie einer Wiedererholung (Wieczorek et al. 2017). In die Untersuchungen können außerdem verschiedene Matrizes, wie Wasser, Sediment und Pflanzen, miteinbezogen werden, was das Verhalten der Testsubstanz in der Umwelt realistischer abbildet (Hand und Oliver 2010). Für die Untersuchung der Auswirkungen von GV-Pflanzen auf Nichtzielorganismen ist ein gestufter Testansatz vorgesehen (EFSA 2010a, 2010b). Werden bei diesem Verfahren in lower-tier Studien negative Effekte gefunden, werden weitere higher-tier Studien durchgeführt. Für das Zulassungsverfahren von GV-Pflanzen in der EU sind bisher allerdings keine aquatischen higher-tier Studien notwendig.

2. Ziel der Dissertation

Das Ziel dieser Dissertation ist die Auswirkungen des Anbaus von GV-Pflanzen auf aquatische Ökosysteme zu untersuchen. Dabei wurde sowohl die Exposition der aquatischen Umwelt mit GV-Pflanzen als auch die Effekte von GV-Pflanzen auf aquatische Organismen betrachtet. Ein besonderes Augenmerk wurde außerdem auf die Nutzung von higher-tier Versuchsansätzen für die Abschätzung von Effekten auf die aquatische Umwelt gelegt. Im ersten Teil der Dissertation (Veröffentlichung 1; Anhang A.1) war es das Ziel den aktuellen Wissensstand über das Risiko, welches vom Anbau von GV-Pflanzen auf die aquatische Umwelt ausgeht, zu analysieren. Im zweiten Teil der Dissertaion (Veröffentlichung 2; Anhang A.2) wurden Fragestellungen bezüglich der konkreten Methodik von Versuchen zur Abschätzung der Effekte von GV-Pflanzen auf aquatische Organismen untersucht und entsprechende Experimente durchgeführt. Das Ziel war es die Machbarkeit einer Spiking-Methode zu prüfen, bei der das Bt-Toxin Cry1Ab auf Blätter der Schwarzerle (*Alnus glutinosa*) aufgetragen und somit verschiedene Konzentrationen erzeugt werden sollten. Im dritten Teil der Dissertation (Veröffentlichung 3; Anhang A.3) sollte schließlich die Möglichkeit der Nutzung von higher-tier Ansätzen betrachtet werden. In diesem Teil werden Besonderheiten und Schwierigkeiten analysiert die es zu beachten bzw. zu lösen gibt, wenn eine higher-tier Studie mit GV-Pflanzenmaterial durchgeführt wird. Da higher-tier Studien mit Pestiziden bereits etabliert sind, dienen diese als Referenz für den Vergleich mit higher-tier Studien mit GV-Pflanzenmaterial.

3. Layout der Dissertation

3.1 Aufteilung der Dissertation

Die Dissertation lässt sich in drei Teile aufteilen (Abbildung 1). Diese Teile können nach theoretischen und experimentellen Teilen sowie danach welche Versuchsstufe sie abdecken aufgeteilt werden. Bei dem ersten Teil handelt es sich um ein Datenreview, welches vorhandene Literatur der Stufen 1 und 2 analysiert. Teil II sind Experimente der Stufe 2, die auf den Erkenntnissen des ersten Teils aufbauen. Bei dem dritten Teil handelt es sich um eine Analyse, die die Nutzung von higher-tier Versuchen zur Risikobewertung von GV-Pflanzen auf die aquatische Umwelt bewertet. Diese Auswertung behandelt die Stufen 3 und 4 und baut auf Teil I und Teil II auf. Die Dissertation beinhaltet somit theoretische und experimentelle Teile und deckt mit diesen alle vier Versuchsstufen ab.

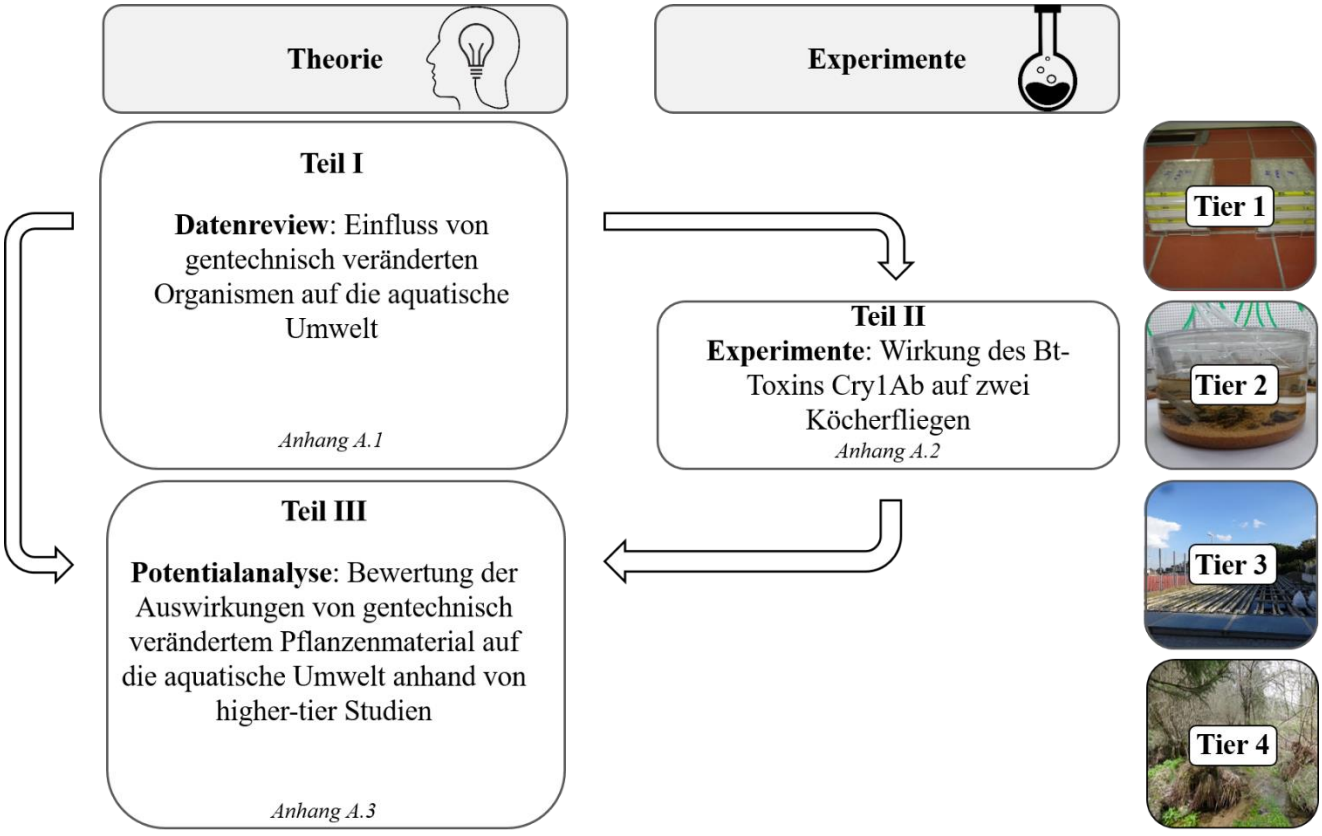


Abbildung 1: Aufteilung der Dissertation in drei Teile. Diese unterscheiden sich nach theoretischen und experimentellen Teilen. Außerdem wird aufgezeigt welche Versuchsstufe sie abdecken. Es wird auf die Anhänge verwiesen, in welchen die entsprechenden Veröffentlichungen zu finden sind. Bilder: pixabay_GDJ, pixabay_foobarbazbing.

3.2 Teil 1: Einfluss von gentechnisch veränderten Organismen auf die aquatische Umwelt

Im ersten Teil (Abbildung 1) wurde die vorhandene wissenschaftliche Literatur über den Verbleib von GV-Pflanzen in der aquatischen Umwelt und den Effekten von GV-Pflanzen auf aquatische Organismen untersucht. Dabei wurden die Entwicklung sowie der aktuelle Wissensstand über die Risikobewertung von GV-Pflanzen in der aquatischen Umwelt festgehalten und ausgewertet. Berücksichtigt wurden Tier 1 und Tier 2 Studien. Da es viele verschiedene Kombination aus verwendeter Pflanze, gentechnischer Veränderung und neu gebildetem Protein gibt und dies jeweils Auswirkungen auf die Risikobewertung haben kann, wurde nach diesen Parametern unterschieden. Zur Literatursuche wurden verschiedene Datenbanken herangezogen. Es wurden nur Veröffentlichungen in die Analyse miteinbezogen, die

- den Verbleib von GV-Pflanzen, einem neu gebildeten Protein, oder relevanten nicht GV-Pflanzen in der aquatischen Umwelt oder
- negative Effekte von GV-Pflanzen oder dem neu gebildeten Protein auf aquatische Nichtzielinvertebraten und Protisten in Single-species Tests untersuchen,
- im Peer-Review Verfahren veröffentlicht wurden und
- auf Englisch geschrieben sind.

Dadurch wurden Veröffentlichungen ausgeschlossen, die Studien zur Untersuchung aquatischer Gemeinschaften durchführen, Sprayfomulierungen von Bti (*Bacillus thuringiensis isaelensis*) verwenden, Bt Gene oder Vertebraten untersuchen sowie Studien die in terrestrischen oder ufernahen Habitaten durchgeführt wurden. Alle Studien wurden in die zwei Bereiche Effektstudien und Studien, die den Verbleib untersuchen, eingeteilt. Studien über den Verbleib wurden dann nochmal in Expositions- und Abbaustudien unterteilt.

3.3 Teil 2: Wirkung des Bt-Toxins Cry1Ab auf zwei Köcherfliegen – ein Versuch zur Ermittlung einer Dosis-Wirkungsbeziehungen durch Spiking

Im zweiten Teil wurde die Wirkung des Bt-Toxins Cry1Ab experimentell auf Köcherlfiegenlarven untersucht (Abbildung 1). Verwendet wurden dafür die beiden Arten *Sericostoma* spec. und *Chaetopteryx* spec. Diese Arten wurden ausgewählt, da sie in Europa verbreitet sind (Malicky 2005; Wagner 1990; Kučinić et al. 2013; Waringer und Malicky 2017; Wallace 1977; Friberg und Jacobsen 1999) und zum Laubabbau beitragen (Rumbos et al. 2010; Graça 2001). Die Versuchsdauer betrug bis zu 12 Wochen, weshalb der Versuch als Tier 2

Studie bewertet werden kann. Als Endpunkte wurden Mortalität, Fraß, Wachstum (Köcherweite) und das Larvenstadium (Kopfkapselweite) beobachtet. Zusätzlich wurde noch der Lipidgehalt der Larven gemessen, da dieser eine wichtige Energiereserve während der Metamorphose ist (Arrese und Soulages 2010).

Bei diesem Versuch wurde eine neue Spiking-Methode angewendet. Dafür wurde das Cry1Ab Protein in einer Pufferlösung gelöst und anschließend verschiedenen Konzentrationen hergestellt. Diese Lösungen wurden auf Laubscheiben der Schwarzerle (*Alnus glutinosa*) aufgetragen. Die Laubscheiben wurden kurz an der Luft trocknen gelassen und dann bei -18°C aufbewahrt bis sie im Fütterungsversuch zum Einsatz kamen.

Für die Fütterungsstudien wurden für jede Konzentration und jede Köcherfliegenlarve 10 Replikate angesetzt. Dafür wurde in Glasschalen Sediment und ein Medium gefüllt und diese mit fünf zufällig ausgewählten Köcherfliegenlarven besetzt. Die Köcherfliegenlarven wurden mit den gespikten Pflanzenmaterial über 6 Wochen (*Sericostoma* spec.) und über 12 Wochen (*Chaetopteryx* spec.) gefüttert. Jede Woche wurde das Medium und das gespikte Pflanzenmaterial ausgetauscht und die Mortalität notiert. Die Futterreste wurden getrocknet und gewogen, um den Fraß bestimmen zu können.

Um den Abbau des Cry1Ab Proteins auf den Laubscheiben untersuchen zu können, wurden gespikte Laubscheiben sieben Tage unter den gleichen Bedingungen wie in der Fütterrungsstudie verwahrt. An den Tagen 0, 3 und 7 wurden Proben entnommen, deren Cry1Ab Konzentration mit einem Enzymgebundenen-Immunosorbent Assay (ELISA) bestimmt wurde.

Die aufgetragene Cry1Ab Menge auf den Laubscheiben wurde mithilfe eines ELISAs quantifiziert (Zwahlen et al. 2003). Die Proben wurden dafür gemahlen und in einem Puffer gelöst. Anschließend wurden sie in eine mit Antikörpern beschichteten Wellplatte eingefüllt. Nach mehreren weiteren Schritten wurde eine Verfärbung der Platte sichtbar, welche mit einem Tecan Mikroplattenmessgerät quantifiziert wurde. Anhand einer Eichgeraden konnte auf die Cry1Ab Konzentrationen auf den gespikten Blättern geschlossen werden. Da sich teilweise große Unterschiede zwischen den nominalen und den gemessenen Konzentrationen zeigten, wird sich in den Ergebnissen auf die gemessenen Konzentrationen bezogen.

Um die Bioaktivität des verwendeten Cry1Ab-Toxins nachzuweisen, wurde ein Zielorganismentest durchgeführt. Dafür wurden Larven des Maiszünslers (*Ostrinia nubilalis*) verwendet. Laub der Schwarzerle wurde gemahlen und anschließend mit verschiedenen

Konzentrationen des gelösten Cry1Abs gespiked. Das Laubpulver wurde anschließen mit Aufzuchtfutter vermischt. Das gespikte Futter wurde über 7 Tage an die Larven verfüttert und die Mortalität alle 24 Stunden aufgenommen.

3.4 Teil 3: Bewertung der Auswirkungen von gentechnisch verändertem Pflanzenmaterial auf die aquatische Umwelt anhand von higher-tier Studien

Im dritten Teil wurde die Möglichkeit untersucht, experimentelle higher-tier Versuche zur Risikobewertung von GV-Pflanzen zu verwenden (Abbildung 1). Dieser Teil baut somit auf dem ersten und zweiten Teil auf. Als experimentelle higher-tier Versuche werden Tier 3 (Mikro-, Mesokosmen) und Tier 4 Studien (Feldstudien) bezeichnet. Zu Tier 4 Studien zählen auch komplexe Modellierungen, die in diesem Teil allerdings nicht betrachtet werden. Es werden Besonderheiten und Schwierigkeiten bei der Durchführung von higher-tier Studien, welche in der Zulassung von Pestiziden bereits üblich sind, bewertet. Die Grundlage für die Bewertung ist die Auswertung von Literatur sowie eigene Schlussfolgerungen aus der Durchführung von Versuchen, wie im zweiten Teil beschrieben. Dieser Teil zieht auch einen Vergleich zwischen higher-tier Versuchen zur Risikobewertung von GV-Pflanzen und higher-tier Versuchen zur Risikobewertung von Pestiziden und zeigt die Unterschiede und Gemeinsamkeiten auf. Wenn ein higher-tier Versuch durchgeführt wird, muss zu Beginn entschieden werden, ob für diesen GV-Pflanzenmaterial oder ein bakteriell hergestelltes reines Bt-Toxin verwendet wird. Beide Möglichkeiten werden in diesem Teil betrachtet.

4. Ergebnisse und Diskussion

In den folgenden Unterkapiteln werden die Ergebnisse und die Diskussion der drei Teile der Dissertationen dargestellt. Die kompletten Veröffentlichungen dieser Teilbereiche sind im Anhang (A.1-A.3) zu finden.

4.1 Einfluss von gentechnisch veränderten Organismen auf die aquatische Umwelt: Bewertung der verfügbaren Daten für die Risikobewertung

Insgesamt habe ich 39 Publikationen in die Auswertung miteinbezogen. In diesen Publikationen beschäftigen sich 84 Studien mit der Risikobewertung von GV-Pflanzen auf die aquatische Umwelt. Diese lassen sich in 31 Effekt- und in 53 Studien, die sich mit dem Verbleib in der Umwelt beschäftigen, unterteilen. Die letzteren unterscheiden sich wiederum in 22 Expositions- und 31 Abbaustudien. Im Vergleich zu terrestrischen Studien (Duan et al. 2008; Kostov et al. 2014; Lövei et al. 2009; Marvier et al. 2007; Naranjo 2009; Wolfenbarger et al. 2008) ist die Anzahl der Studien in der aquatischen Umwelt deutlich geringer. Die meisten Effektstudien untersuchen die Auswirkungen von Mais (55%), Reis (16%), Baumwolle (6%) und Soja (3%). Von Mais gibt es die meisten für den Anbau zugelassenen GV-Pflanzen (Parisi et al. 2016), was sich auch in den Studien zeigt. Am meisten wurden insektenresistente GV-Pflanzen untersucht (Abbildung 2), dagegen nur wenige herbizidresistente. Das Bt-Toxin Cry1Ab wurde am häufigsten für Studien verwendet. Andere Cry- oder Vip-Proteine kamen nur in geringer Anzahl in Studien vor. Nur zwei verschiedene gestackte GV-Pflanzen, d.h. mit mehrfacher gentechnischer Veränderung, wurden untersucht. Da immer mehr gestackte GV-Pflanzen vorkommen (Parisi et al. 2016), ist auch die Bewertung von kombinatorischen Effekten auf aquatische Organismen von zunehmender Bedeutung.

Ein weiterer wichtiger Aspekt bei Effektstudien ist die Wahl des Testorganismuses (Abbildung 3). Am meisten wurden Crustaceen (63%), insbesondere Daphnien, verwendet. Die Studien mit Daphnien zeigen, dass es eine mögliche Gefahr geben kann auch wenn nicht alle Studien Effekte auf die Mortalität zeigen. Neben der Exposition über das Futter in Form von GV-Pflanzenmaterial wurden die Daphnien in manchen Studien auch über das Wasser mit Bt-Toxinen exponiert. Als Endpunkte wurden beispielsweise die Mortalität, die Dichte und das Wachstum gemessen. Die Ergebnisse variieren allerdings und zeigen in mehreren Studien keine eindeutige Dosis-Wirkungsbeziehung (Raybould et al. 2014; Zhang et al. 2016). Auf den Endpunkt Fertilität zeigen sich verschiedenen Effekte nachdem die Daphnien mit MON810 Mais, der Cry1Ab produziert, gefüttert wurden (Bøhn et al. 2008; Bøhn et al. 2010; Holderbaum

et al. 2015), wobei Holderbaum et al. (2015) bei der kumulativen Fertilität und dem Alter bei der Reifung allerdings keine Effekte finden konnte. Bøhn et al. (2016) untersucht die Wirkung von Cry1Ab, Cry2Aa und dem Herbizid Roundup-Ready. Es zeigt sich, dass die beiden Toxine zu einer höheren Mortalität führen als nur die einzelnen Toxine.

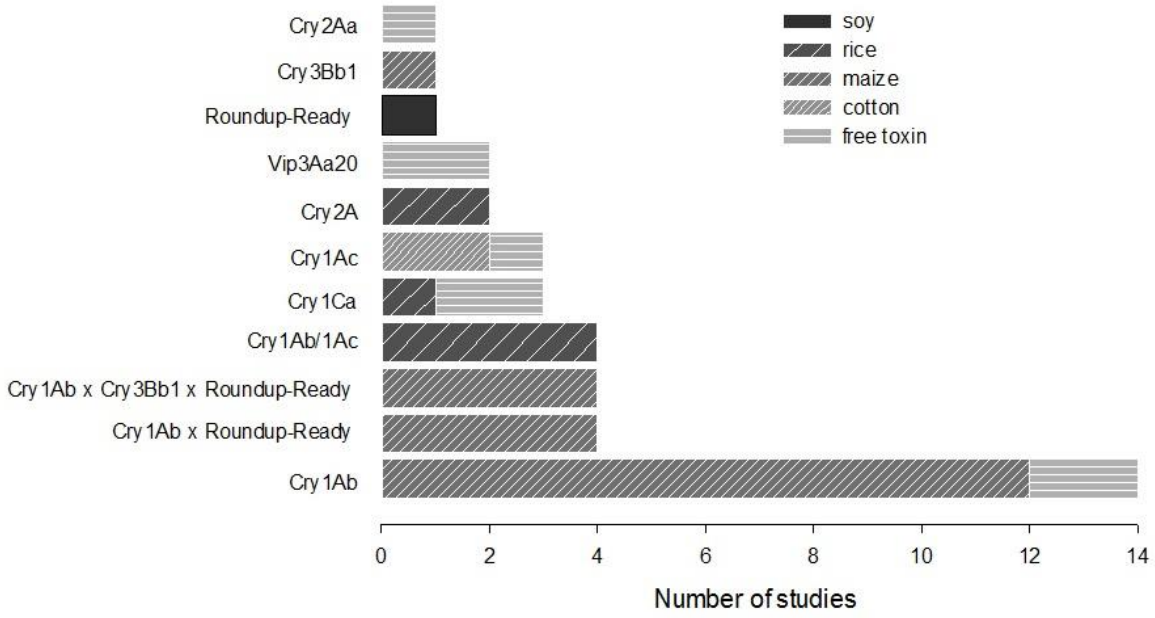


Abbildung 2: Neue Proteine (n = 39), die in 31 Effektstudien untersucht wurden (s. Anhang A.1). Die Unterteilung der Pflanzenarten (Soja, Reis, Mais oder Baumwolle) basiert auf dem in den Studien verwendeten Pflanzenmaterial, Extrakten oder aus Pflanzenmaterial ausgewaschenem Toxin.

Bei Insekten wurden vor allem Trichopteren (19%) für Versuche eingesetzt, welche eine wichtige Rolle als Zerkleinerer von eingetragenem Pflanzenmaterial in Gewässern spielen (Gonzalez und Graca 2003; Suter und Cormier 2015; Wallace und Webster 1996; Wantzen und Wagner 2006). Außerdem sind sie phylogenetisch eng verwandt mit den Zielorganismen von vielen Bt-Toxinen. Insgesamt zeigen die Studien mit Köcherfliegenlarven kein eindeutiges Bild. Bei Köcherlfliegenlarven der Art *Helicopsyche borealis* wurde eine höhere Mortalität gefunden nachdem sie mit Cry1Ab enthaltenen Maispollen gefüttert wurden im Vergleich zur Kontrolle mit nicht-GV Pollen (Rosi-Marshall et al. 2007). Mehrere Studien mit verschiedenen Köcherfliegenlarven (*Lepidostoma liba*, *Lepidostoma* sp., *Pycnopsyche* cf. *scrabipennis*) zeigten keine veränderte Mortalität im Vergleich zur Kontrolle nachdem sie mit GV-Pflanzenmaterial gefüttert wurden (Chambers et al. 2010; Jensen et al. 2010; Rosi-Marshall et al. 2007). Insgesamt zeigen die Studien mit Köcherfliegenlarven zwar kein eindeutiges Ergebnis, aber das eine Gefährdung potentiell bestehen könnte. Studien wurden bisher nur mit Köcherfliegenlarven durchgeführt, die Laub zerkleinern oder Biofilme fressen. Bislang wurden

keine Versuche mit Netze spinnenden Köcherfliegenlarven durchgeführt. Diese sind jedoch direkt mit Pflanzenmaterial und Pollen exponiert.

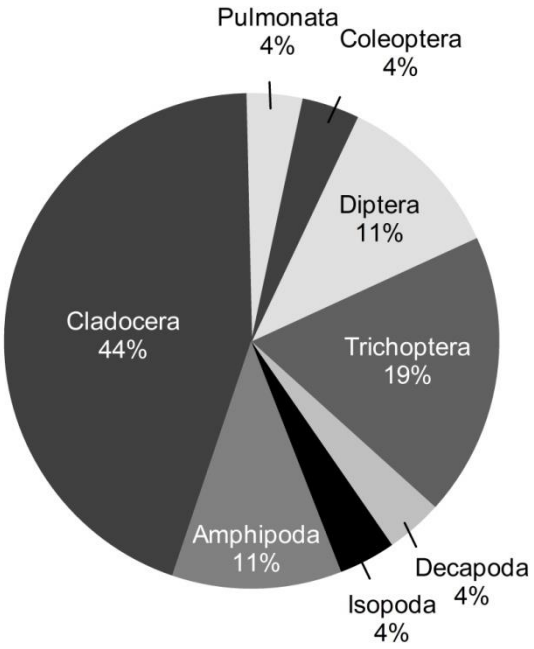


Abbildung 3: Invertebraten Taxa, die in 27 Effektstudien untersucht wurden (n = 27 Organismen) (s. Anhang A.1).

Neben Effektstudien wurden auch Expositionsstudien durchgeführt. Die Exposition der aquatischen Umwelt mit einem neuen Protein wurde in 59% der Studien und die Exposition mit Pflanzenmaterial in 41% der Studien untersucht. Die meisten Studien behandelten die Exposition mit Mais und wenige Studien mit Reis. Untersuchungen mit anderen GV-Pflanzen, wie Soja, Baumwolle oder Raps fehlen. Die Studien zeigen, dass die aquatische Umwelt mit GV-Pflanzen oder Bt-Toxinen exponiert ist. Es zeigten sich vier Eintragspfade:

- Grundwasser und Bodenporenwasser (Cry-Toxin) (Strain et al. 2014; Strain und Lydy 2015; Whiting et al. 2014);
- Oberflächenabfluss (Pflanzenmaterial, Cry-Toxin) (Strain et al. 2014; Strain und Lydy 2015; Whiting et al. 2014);
- Winddrift, z.B. währende der Ernte (Pflanzenmaterial)(Jensen et al. 2010; Kratz et al. 2010); und
- Drainage (Cry-Toxin) (Griffiths et al. 2017; Tank et al. 2010).

Auch wenn diese Eintragspfade bereits untersucht wurden, ist ihr Ausmaß noch unbekannt. Abbildung 4 verdeutlicht die verschiedenen Eintragspfade und fasst die gemessenen Werte zusammen. Insgesamt zeigen die Expositionsstudien, dass die größten Eintragspfade vermutlich Winddrift und Oberflächenabfluss von Wasser oder Sediment sind. Für eine umfassende Expositionsabschätzung sollten aber alle Pfade miteinbezogen werden. Außerdem

zeigen die Ergebnisse, dass die aquatische Umwelt nicht nur über einen langen Zeitraum durch GV-Pflanzenmaterial exponiert wird, sondern dass sich Pflanzenmaterial auch mit der Strömung bewegt und somit auch an Orten vorkommen kann, die weiter entfernt sind vom eigentlichen GV-Pflanzenanbau. Bt-Toxin konnte nicht nur im Wasser, sondern auch im Sediment nachgewiesen werden, wodurch sich auch eine mögliche Exposition von benthischen Organismen ergibt.

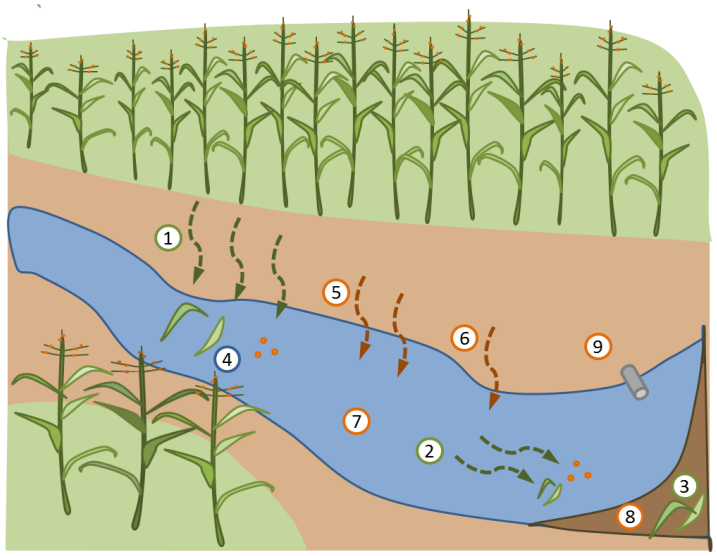


Abbildung 4: Exposition der aquatischen Umwelt mit GV-Pflanzen (s. Anhang A.1). Die nachstehenden tabellarischen Daten zeigen gemessene Werte, keine Modellierungsergebnisse **a** Douville et al., 2005, **b** Griffith et al. 2017, **c** Kratz et al., 2010, **d** Rosi-Marshall et al., 2007, **e** Strain and Lydy, 2015, **f** Tank et al., 2010.

Plant material		Ref.
1	Wind drift: 0.1-7.9 g AFDM/m²	d
	Harvest drift: 26 g dw/m² (edge of the field)	c
	Pollen drift: 0.1-1.0 g/m²	d
2	Transport of plant material in flowing water: 0.38-180 m	d
	Transport of pollen in flowing water: 20-60 m	d
3	Plant material in sediment: up to 6.4 AFDM/m²	d

Free toxin		Ref.
5	Run-off water: up to 130 ng/L	e
	Run-off sediment: up to 143 ng/g dw	e
6	Groundwater, soil pore water: 17.2 ng/L, 21.7 ng/L (single observations)	e
7	Toxin in water: 0.014 ng/ml (mean), 0.2 ppb (single observation), up to 60 ng/L	a,b, f
8	Toxin in sediment: 0.5-0.9 ppb	a
9	Drainage: up to 60 ng/L	b,f

Toxin in plant material		Ref.
4	Toxin in plant material in water: 95 ± 73 ng/g dw	e

Zur Bewertung wieviel GV-Pflanzenmaterial oder Bt-Toxine in der aquatischen Umwelt vorkommen ist neben der Abschätzung der Exposition auch die Ermittlung des Abbaus nötig. Abbaustudien wurden ins Besondere mit Mais (52%) durchgeführt. Außerdem wurde Reis (23%), Zitterpappel (6%) und Baumwolle (3%) untersucht. Studien mit Soja fehlen gänzlich.

In den meisten Studien wurde Cry1Ab verwendet. Neben Cry-Proteinen wurde in geringer Anzahl auch der Abbau von doppelsträngiger Ribonukleinsäure (dsRNA) untersucht. Nur wenige gestackte Pflanzen wurden in den Abbaustudien eingesetzt. Abbaustudien mit Bt-Toxinen zeigen eine Halbwertszeit von wenigen Stunden bis zu mehreren Tagen. In Pflanzenmaterial konnten Cry-Toxine allerdings auch noch nach langen Zeitspannen von bis zu 135 Tagen in Mais und 210 Tagen in Reis nachgewiesen werden (Li et al. 2007; Wang et al. 2007). Neben Abbau verringert sich der Gehalt an Bt-Toxinen in Pflanzen auch durch Auswaschung des Bt-Toxins ins Gewässer, was bis zu 70 Tage anhalten kann (Griffiths et al. 2009).

Insgesamt zeigt die Auswertung der Studien, dass es weder für Effekt-, noch Expositions-, noch Abbaustudien standardisierte Testmethoden gibt. Solche Testmethoden sind jedoch wichtig, um Versuche miteinander vergleichbar zu machen. So zeigten die Abbaustudien beispielsweise einen veränderten Abbau je nachdem welche Testbedingungen verwendet wurden (Temperatur, pH-Wert etc.). Außerdem zeigt die Auswertung, dass Studien ins Besondere mit GV-Pflanzen mit nur einer gentechnischen Veränderung durchgeführt wurden. Es gibt heute aber viele gestackte GV-Pflanzen, weshalb die Untersuchung von kombinatorischen Auswirkungen von hoher Bedeutung ist. Durch die Wirkung mehrerer Bt-Toxine können eventuell Effekte auftreten, die man bei einem Versuch mit nur einem Toxin, nicht erkennen würde. Die Effektstudien wurden bisher nur mit einer oder zwei Konzentrationen durchgeführt. Dadurch sind die Erstellungen einer Dosis-Wirkungskurve und die Berechnung von Werten wie einem LC_{50} oder einem EC_{50} nicht möglich. Diese Parameter sind aber notwendig, um verschiedene Studien vergleichen und das Risiko auf die aquatische Umwelt abschätzen zu können.

4.2 Wirkung des Bt-Toxins Cry1Ab auf zwei Köcherfliegen – ein Versuch zur Ermittlung einer Dosis-Wirkungsbeziehungen durch Spiking

Bei den *Chaetopteryx* spec. Larven ist die Mortalität über die Versuchsdauer in der Kontrolle und in allen Konzentrationen angestiegen (Abbildung 5). Obwohl die höchste Konzentration am Ende des Versuchs eine doppelt so hohe Mortalität zeigt wie in der Kontrolle, handelt es sich nicht um einen statistisch signifikanten Effekt. Bei den *Sericostoma* spec. Larven zeigte sich ebenfalls kein statistisch signifikanter Unterschied. Für beide Köcherfliegenlarven konnten außerdem keine signifikanten Effekte auf den Fraß beobachtet werden.

Der Lipidgehalt der Larven von *Chaetopteryx* spec. war nach zwölf Wochen am Ende der Fütterungsstudie geringer mit zunehmender Cry1Ab Konzentration (Abbildung 6). Ein

signifikanter Unterschied zur Kontrolle zeigte sich in der zweit höchsten Konzentration (17,2 ng Cry1Ab/mg). Bei *Sericostoma* spec. zeigte sich dagegen kein Unterschied.

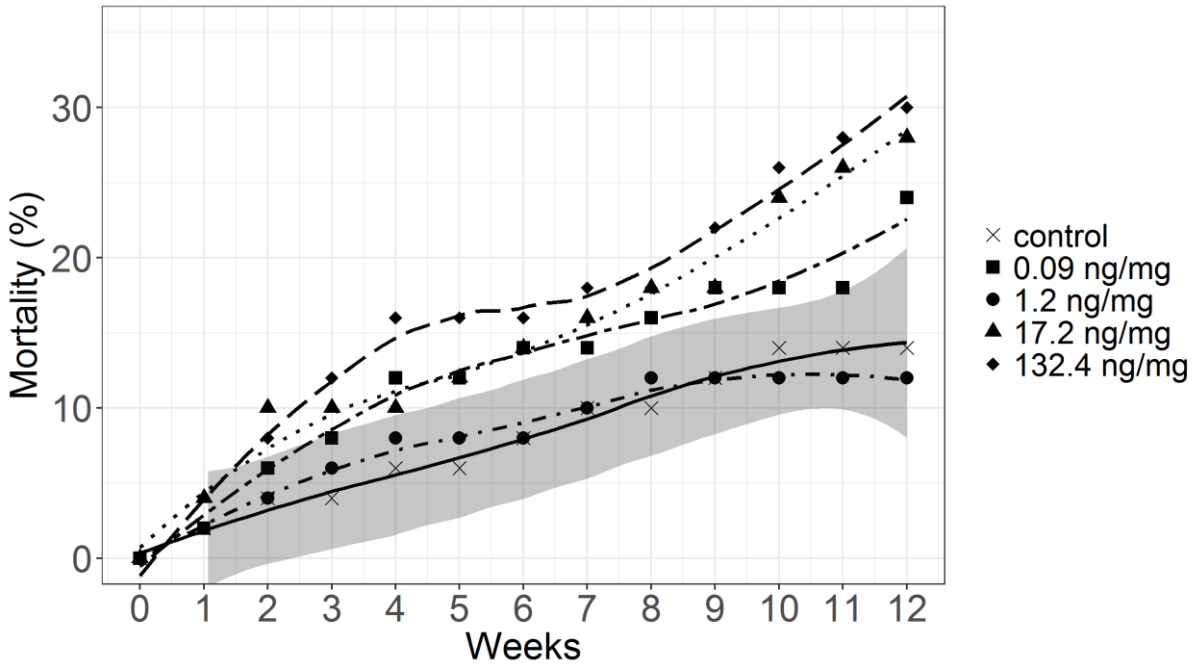


Abbildung 5: *Chaetopteryx* spec. Mortalität (%) über die gesamte Studiendauer von 12 Wochen der Fütterungsstudie (s. Anhang A.2). Verwendet wurden die Cry1Ab-Konzentrationen 0,09, 1,2, 17,2 und 132,4 ng/mg. Dargestellt sind Mittelwerte (n=10) und Regressionslinien. Der schattierte Bereich zeigt das 95%ige Konfidenzintervall des Mittelwerts der Kontrollen.

Zu Beginn der Fütterungsstudie mit *Sericostoma* spec. befanden sich die Larven alle in den Larvenstadien III + IV und V+VI (Abbildung 7). Nach sechs Wochen Fütterungsstudie zeigte sich jedoch eine Veränderung. In der Kontrolle haben sich signifikant mehr Larven in höhere Larvenstadien weiterentwickelt. In der höchsten Cry1Ab Konzentration zeigte sich eine Entwicklungsverzögerung. Bei den *Chaetopteryx* spec. Larven war keine solche Entwicklung zu beobachten.

Mehrere Studien zeigen bereits, dass es ein potentielles Risiko von Bt-Pflanzen auf Köcherfliegenlarven gibt. Diese Fütterungsstudie unterstreicht die vorhandenen Ergebnisse. Sie zeigt außerdem wie wichtig die Einbeziehung von sublethalen Endpunkten ist. Auf die Mortalität wurde in Studien bisher, wenn überhaupt, nur geringe Effekte gemessen. Sublethale Endpunkte zeigten jedoch bereits mehrfach Unterschiede (Chambers et al. 2010; Jensen et al. 2010) und können ebenfalls starke Auswirkungen auf aquatische Ökosysteme haben. Bt-Toxine treten in der aquatischen Umwelt eher in geringen Konzentrationen, aber über lange Zeiträume auf. Eine chronische Exposition mit Bt-Toxinen führt zwar zur Schädigung des Darms, ist jedoch nicht lethal (Erb et al. 2001; Gulzar und Wright 2015; Binning et al. 2014; Eizaguirre et al. 2005).

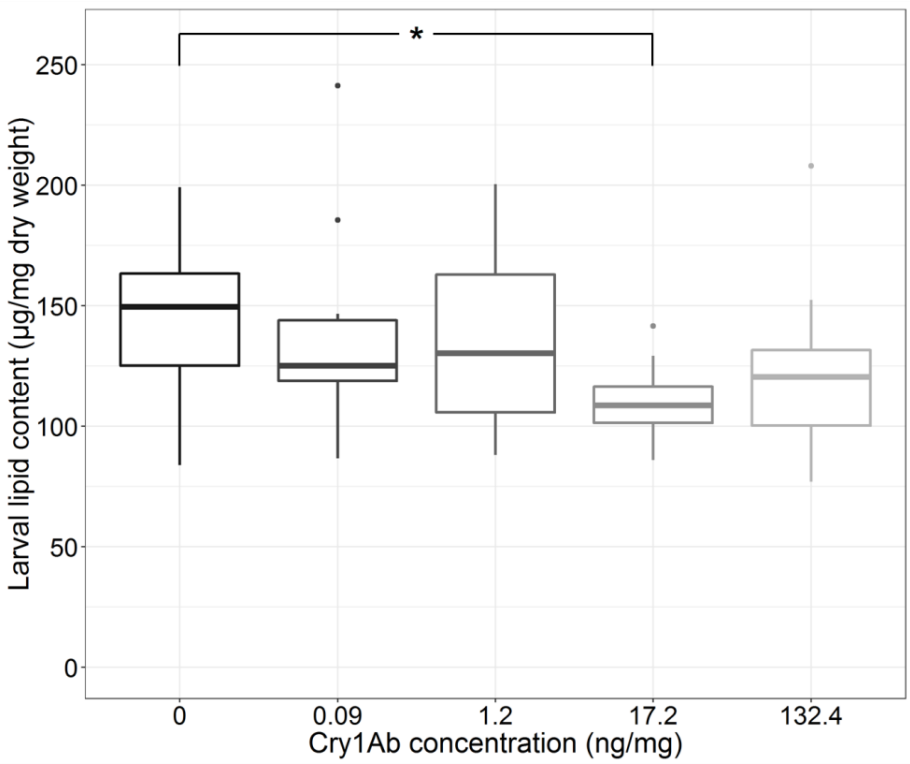


Abbildung 6: Lipidgehalt der Larven von *Chaetopteryx* spec. nach 12 Wochen Fütterung mit unterschiedlichen Konzentrationen (0,09, 1,2, 17,2, 132,4 ng/mg) von Cry1Ab-gespiketen Blattscheiben (s. Anhang A.2). Die dicken Linien in den Boxplots zeigen die Mediane (n=15), unteres und oberes Quartil sind durch das obere und untere Ende der Box abgedeckt. Effektgröße: 23,5 %. * zeigt einen signifikanten Unterschied zur Kontrolle ($p<0.05$)

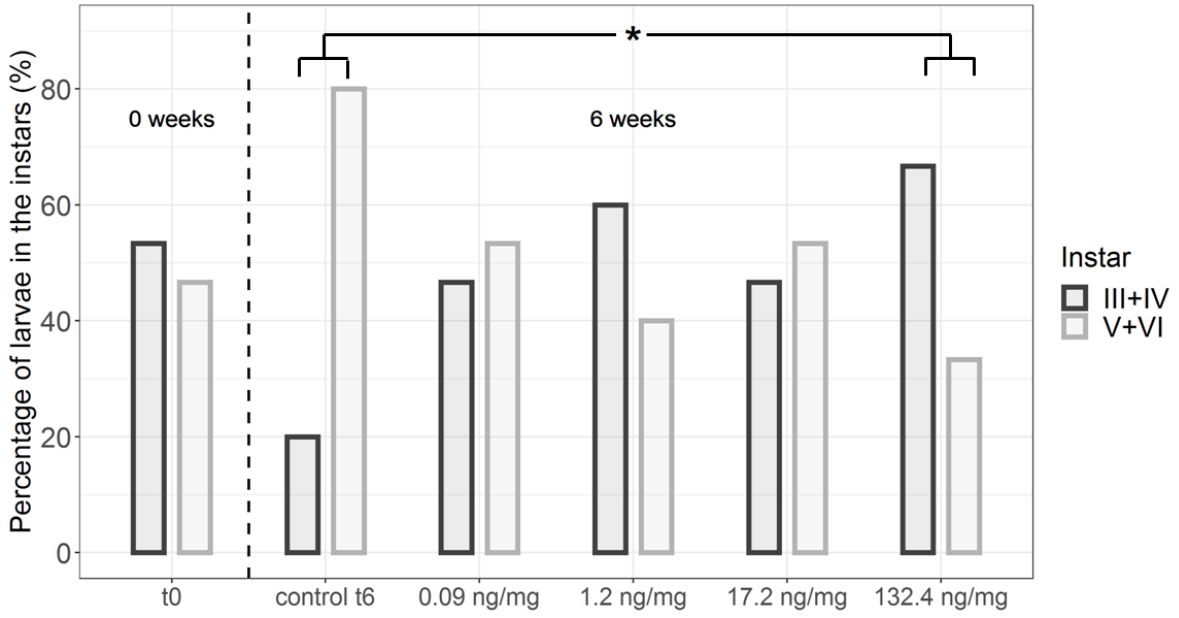


Abbildung 7: *Sericostoma* spec. Larvenstadium zu Beginn (t0) und am Ende (t6) des Versuchs in der Kontrolle (Kontrolle t6) bzw. in den verschiedenen Konzentrationen (n=15) (s. Anhang A.2). Die gestrichelte Linie trennt die Daten der Larvenstadien zu Beginn (0 Wochen) und am Ende (6 Wochen) des Versuchs. Larvenstadium III wurde in der Konzentration 17,2 ng/mg gefunden. Das Larvenstadium VI wurde in den Konzentrationen 0,09 und 1,2 ng/mg gefunden. * zeigt einen signifikanten Unterschied zur Kontrolle ($p<0,05$)

Der verringerte Lipidgehalt sowie die verlangsamte Larvenentwicklung, wenn auch in vielen Fällen nicht signifikant, könnte eine Folge dieser Schädigung sein. Die Verringerung des Lipidgehalts könnte auf eine Erhöhung des Energiebedarfs für Reparaturmechanismen hindeuten, während das Wachstum beibehalten wird (Konschak et al. 2019). Die Erhörung des Energiebedarfs kann jedoch längerfristige Folgen für aquatische Organismen haben, da eventuell für zukünftige wichtige Entwicklungsschritte, wie der Metamorphose oder der Fortpflanzung, nicht mehr genügend Energie zur Verfügung steht und diese Schritte daher beeinträchtigt sein könnten (Konschak et al. 2019).

Die verzögerte Entwicklung der *Sericostoma* spec. Larven könnte zu einer späteren Emergenz führen. Dies kann Auswirkungen auf Nahrungsnetzte haben, da das Auftreten von Beute und Predatoren eventuell nicht mehr zeitgleich stattfindet. Da Köcherfliegenlarven sowohl in der aquatischen Umwelt als auch als Adulte in der terrestrischen Umwelt vorkommen, könnte eine spätere Emergenz in beiden Nahrungsnetzten zu Bottom-up oder Top-down Effekten führen (Schulz et al. 2015). Eine Verzögerung der Larvalentwicklung wurde ebenfalls schon bei terrestrischen Lepidopterenlarven (Lang und Otto 2010; Rausell et al. 2000), die mit Cry1Ab exponiert waren, als auch bei Pestizid exponierten Köcherfliegenlarven beobachtet (Liess und Schulz 1996, 1999; Schulz und Liess 1995).

Die gefundenen Ergebnisse in dieser Fütterungsstudie sind häufig nicht signifikant, was vermutlich auf eine hohe Variabilität in den gemessenen Endpunkten zurückzuführen ist. Außerdem könnte auch die relativ geringe Anzahl an Replikaten und einer damit einhergehenden geringeren statistischen Trennschärfe sowie der schnelle Abbau des Cry1Ab-Toxins dazu geführt haben. Die gemessenen Cry1Ab Konzentrationen in dieser Fütterungsstudie ähneln den Konzentrationen in frischem Bt-Pflanzen (Griffiths et al. 2009; Böttger et al. 2015). Das Risiko könnte also bei Bt-Pflanzenmaterial, dass sich schon länger in Gewässern befindet und deutlich niedrigere Cry1Ab Konzentrationen aufweist (Tank et al. 2010), geringer sein.

Die neu entwickelte Spiking-Methode, die in dieser Fütterungsstudie angewendet wurde, konnte verschiedene Cry1Ab-Konzentrationen herstellen. Die verschiedenen Konzentrationen wurden auch durch die ELISA bestätigt. Die Methode ist also dazu geeignet Dosis-Wirkungsbeziehungen zu untersuchen. Die Zielorganismentests mit Larven des Maiszünslers (*Ostrinia nubilalis*) bewiesen außerdem, dass die Spiking-Methode die Testorganismen mit Cry1Ab exponiert. Zu erwähnen ist auch, dass die Methode noch weitere Entwicklung bedarf,

da die Quantifizierung zeigt, dass nur etwa 10% der nominalen Konzentration auf den Blättern gemessen werden konnte.

4.3 Bewertung der Auswirkungen von gentechnisch veränderten Pflanzenmaterial auf die aquatische Umwelt anhand von higher-tier Studien

Bisher wurden drei Arten von higher-tier Studien durchgeführt, die die Wirkung von GV-Pflanzen auf die aquatische Umwelt untersucht haben. Ein Studienansatz hat die Häufigkeit von aquatischen Organismen in Reisfeldern untersucht, die mit GV-Reis und mit nicht-GV Reis bepflanzt worden sind (Li et al. 2014; Liu et al. 2016; Wang et al. 2014). Zu verschiedenen Zeitpunkten wurden Wasserproben entnommen und die Abundanz von Zoo- und Phytoplankton bestimmt. Die Studien wurden mit Cry2A und mit einem gestackten Reis Cry1Ab/1Ac durchgeführt. Insgesamt zeigten sich nur geringe Effekte, die teilweise möglicherweise von der unterschiedlichen Wasserqualität herrührten (Liu et al. 2016).

Der zweite highe-tier Versuchsansatz ist die Durchführung einer Studie mit Litterbags (Swan et al. 2009; Chambers et al. 2010; Axelsson et al. 2010; Axelsson et al. 2011; Liu et al. 2017). Dabei wird GV- und nicht-GV Pflanzenmaterial in Netzbeutel gefüllt, die in Flüssen verankert werden. Nach verschiedenen Zeitintervallen werden die Netzbeutel entnommen, die vorhandenen Organismen bestimmt und gezählt sowie der Abbau des Pflanzenmaterials bestimmt. Bei Bt-Mais (Cry1Ab, Cry1Ab x Cry3Bb1) sowie einer GV-Zitterpappel (CAD) zeigte sich ein geringerer Laubabbau im Vergleich zu nicht-GV Pflanzen (Swan et al. 2009; Axelsson et al. 2010). In anderen gentechnische veränderten Zitterpappeln (COMT, Cry3Aa) und Cry1Ab/1Ac Reis konnte dieser Effekte jedoch nicht nachgewiesen werden (Axelsson et al. 2010; Axelsson et al. 2011; Liu et al. 2017).

Ein dritter Versuchsansatz ist die Entnahme eines benthischen Bohrkerns in Gewässern, die sich neben Feldern mit GV-Pflanzen befinden (Chambers et al. 2010). Die Organismen in dem Bohrkern werden bestimmt und gezählt. Die meisten untersuchten Endpunkte (z.B. Abundanz aller Taxa, Gesamtbiomasse) zeigten keine Unterschiede zwischen Bt-Pflanzen (Cry1Ab) und nicht-Bt Pflanzen. Nur bei einzelnen Taxa waren bei der Abundanz und der Biomasse Unterschiede zu beobachten.

Bei der Durchführung von higher-tier Studien ist die Auswahl von geeigneten Testorganismen besonders wichtig (Tabelle 1). Dabei sollte die vorhandene Exposition, die funktionelle Gruppe, gefährdete Arten und die Durchführbarkeit beachtet werden (Hilbeck et al. 2017). Die Zielorganismen vieler Bt-Toxine sind Lepidopteren. Da Köcherfliegen (Trichoptera)

phylogenetische eng verwandt sind mit Lepidopteren, ist es sinnvoll, sie als Testorganismen für higher-tier Studien mit GV-Pflanzen einzusetzen. Es wurden auch bereits in mehreren Studien Effekte auf Köcherfliegenlarven beobachtet (Pott et al. 2020; Rosi-Marshall et al. 2007). Bei der Verwendung von Köcherfliegenlarven ist es auch von besonderer Bedeutung lange Testdauern zu verwenden, um die gesamte Larvalentwicklung abzubilden und somit auch Effekte auf den Lebenszyklus zeigen zu können. Higher-tier Studien sind bei der Risikobewertung von Pestiziden bereits üblich, weshalb sie hier als Referenz dienen. Bei Mesokosmenstudien mit Pestiziden werden häufig natürliche Organismengemeinschaften verwendet und nicht einzelne Organismen ausgesucht (Cañedo-Argüelles et al. 2014; Wieczorek et al. 2016).

Pestizide gelangen in die aquatische Umwelt über Oberflächenabfluss, Winddrift, Drainage oder direkte Applikation (Flury 1996; Schulz et al. 2001; Wauchope 1978; Armbrust und Peeler 2002; Lamers et al. 2011; Starner und Goh 2012). Exposition aquatischer Organismen findet über das Wasser oder möglicherweise durch das Sediment statt. Diese Eintragspfade sind auch für GV-Pflanzen relevant. Der Oberflächenabfluss transportiert neben GV-Pflanzenmaterial auch gelöstes Bt-Toxin (Strain et al. 2014; Strain und Lydy 2015; Whiting et al. 2014), wohingegen der Eintragspfad der Winddrift nur für Pflanzenmaterial (z.B. während des Ernteprozesse) relevant ist (Jensen et al. 2010; Kratz et al. 2010). Über die Drainage kann gelöstes Bt-Toxin in Gewässer gelangen (Griffiths et al. 2017; Tank et al. 2010). Der Eintrag von GV-Pflanzenmaterial führt dazu, dass aquatische Organismen, vor allem Zerkleinerer, über das Futter mit GV-Pflanzen exponiert werden (Tabelle 1). Der Eintragspfad über das Futter ist auch möglich bei systemischen Pestiziden (Bundschuh et al. 2019; Englert et al. 2017a; Englert et al. 2017b; Englert et al. 2017c; Kreutzweiser et al. 2008) und Fungiziden (Newton et al. 2018; Zubrod et al. 2019). Systemische Pestizide und Fungizide verteilen sich während des Wachstums innerhalb der Pflanze und können daher über Laub in Gewässer gelangen. Bt-Toxin kann über Auswaschung aus dem Pflanzenmaterial in das Wasser übergehen, wodurch ebenfalls eine wassergebundene Exposition möglich ist. Da gelöstes Bt-Toxin in deutlich geringeren Konzentrationen vorkommt als in frischem GV-Pflanzenmaterial (Douville et al. 2005; Tank et al. 2010), ist dieser Expositionsweg vermutlich von geringere Bedeutung, sollte für eine umfangreiche Risikobewertung aber trotzdem bedacht werden (Englert et al. 2017b).

Für Studien mit Pestiziden wird üblicherweise der aktive Wirkstoff oder die Formulierung verwendet (Hanson et al. 2007; Beuter et al. 2019; van Wijngaarden et al. 2004), welche erworben werden können, was ebenfalls auf reines Bt-Toxin zutrifft (Pott et al. 2020). Dies

sieht bei GV-Pflanzenmaterial allerdings anders aus. GV-Saatgut wird nicht extra für die Forschung verkauft, sodass es nur mit Zustimmung der Hersteller erworben werden kann (Tabelle 1). Selbst dann sind die Nährstoffzusammensetzung sowie die Höhe der Expression des Bt-Toxins in GV-Pflanzen unterschiedlich und muss daher vor der Verwendung in einer Studie quantifiziert werden (Tabelle 1). Außerdem kann das Saatgut auch mit Saatgut anderer GV-Pflanzen oder Pestiziden verunreinigt sein. Daher ist es notwendig die Identität des GV-Saatguts zu kontrollieren.

Tabelle 1: Mögliche methodische Verbesserungen für aquatische higher-tier Effektstudien über die Auswirkungen von GV-Pflanzenmaterial oder Bt-Toxinen (weitere Einzelheiten siehe Text) (s. Anhang A.3). *In all diesen Fällen kann die Herstellung von eigenem gentechnisch verändertem (und nicht gentechnisch verändertem) Pflanzenmaterial erforderlich sein, was einen großen zusätzlichen Aufwand bedeutet und manchmal sogar unmöglich sein könnte.

Problem	**Verbesserung**
Relevante Artengruppen nicht gut vertreten	Einbeziehung von Trichoptera-Arten, Berücksichtigung der Larvenentwicklungszeit bei der Studiendauer
Expositionspfad über das Futter nicht angemessen dargestellt	Einbeziehung von (verschiedenen Arten) von Pflanzenmaterial, das das betreffende GV-Toxin enthält; ausreichend lange Expositionsdauer
Geringe Stabilität von frischem GV-Pflanzenmaterial	Einfrieren von gentechnisch verändertem Pflanzenmaterial, jedoch unter Berücksichtigung der möglichen Nebenwirkungen des Einfrierens
GV-Pflanzenmaterial schwer zu beschaffen	Gentechnisch veränderte Pflanzen und Referenzmaterial sollten für die unabhängige Forschung leichter verfügbar sein*
Reinheit und Identität von GV-Pflanzenmaterial oft unklar	Überprüfung der Identität und Reinheit von GV-Pflanzen durch analytische oder bioanalytische Verfahren*
Verwendung unterschiedlicher Dosen/Konzentrationen schwierig	Spiking von Nicht-GV-Pflanzenmaterial oder Herstellung unterschiedlicher Konzentrationen mit Nicht-GV-Pflanzenmaterial *
Keine vorhandene Negativkontrolle	Etablierung von Referenzsubstanzen*

In Pestizidstudien können verschiedene Konzentrationen verwendet werden, indem der aktive Wirkstoff oder die Formulierung entsprechend herunter verdünnt werden. Bei GV-Pflanzenmaterial ist es jedoch nicht möglich, da das Material nur eine Konzentration des Bt-

Toxins enthält (Tabelle 1). Dazu kommt, dass die Konzentration des Bt-Toxins vom Pflanzenteil und vom Entwicklungsstand der Pflanze abhängig ist (Griffiths et al. 2009; Böttger et al. 2015). Die Herstellung von verschiedenen Konzentrationen an Bt-Toxin ist durch die Anwendung einer Spiking-Methode möglich. Dabei werden verschiedene Konzentrationen von reinem Bt-Toxin hergestellt, die dann auf nicht-GV Pflanzenmaterial aufgetragen werden.

Bei allen Studien ist immer eine Negativkontrolle wichtig, um Effekte zu quantifizieren. Bei Pestiziden und auch bei der Verwendung von Bt-Toxinen ist dies kein Problem, da Versuchsansätze ohne Wirkstoff bzw. Toxin genommen werden können. Bei GV-Pflanzen ist dies jedoch nicht möglich (Tabelle 1). Es kann zwar hier ebenfalls eine nicht-GV Pflanze verwendet werden, allerdings unterscheiden sich GV-Pflanzen und nicht-GV Pflanzen nicht nur durch das neu gebildete Toxin sondern auch durch andere Parameter (Swan et al. 2009; Axelsson et al. 2010; Saxena und Stotzky 2001). Dies kann dazu führen, dass der Nährwertgehalt der Pflanzen unterschiedlich ist, was wiederum das Ergebnis einer Fütterungsstudie stark beeinflussen kann. Daher sollte bei der Auswahl einer nicht-GV Pflanze auf ein vergleichbares C/N-Verhältnis und einen ähnlichen Ligningehalt wie die GV-Pflanze geachtet werden (Rosi-Marshall et al. 2007). Zusätzlich könnten mehrere nicht-GV Pflanzen als Negativkontrolle verwendet werden, sodass Effekte aufgrund eines unterschiedlichen Nährstoffgehalts besser beobachtet werden können.

5. Schlussfolgerung und Ausblick

Alle drei Teile dieser Dissertation zeigen einheitlich, dass es für die Risikobewertung von GV-Pflanzen insgesamt eine geringe Datenlage gibt im Vergleich zu der Risikobewertung von Pestiziden. Die Anzahl der Studien, aber auch die verwendeten Pflanzen und gentechnischen Veränderungen sind niedrig und spiegeln bei weitem nicht die tatsächliche Verbreitung von GV-Pflanzen global und die Anzahl der mittlerweile verwendeten gentechnischen Veränderungen wider. Die fehlenden Daten betreffen alle untersuchten Bereiche, sowohl lower- (Teil I, Anhang A.1) und higher-tier Effektstudien (Teil III, Anhang A.3) als auch Expositionsstudien.

Methodisch zeigen die vorhandenen Studien verschiedene Schwierigkeiten auf. Besonders die fehlende Standardisierung von jeder Art von Studie ist hier zu nennen und hoch problematisch (Teile I-III, Anhänge A.1, A.2, A.3). Standardisierungen garantieren eine hohe Qualität der Studien sowie eine Vergleichbarkeit von Studienergebnissen. Diese beiden Faktoren sind von hoher Relevanz für eine zuverlässige und aussagestarke Risikobewertung.

Neben der fehlenden Standardisierung ist auch die Schwierigkeit verschiedene Konzentrationen herzustellen ein wichtiger Aspekt, den es zu beachten gibt (Teile II und III, Anhänge A.2, A.3). Dadurch, dass GV-Pflanzenmaterial nur eine Konzentration des neuen Proteins enthält, ist die Erstellung von Dosis-Wirkungskurven und Parametern wie dem LC_{50} und dem EC_{50} nicht möglich. Die Berechnung solcher Parameter ist für die Vergleichbarkeit von Studien unumgänglich. Auch für die Festlegung von Grenzwerten sind diese Parameter von hoher Bedeutung, da sie Aufschluss darüber geben, ab welcher Konzentration mit einer negativen Beeinflussung der aquatischen Umwelt zu rechen ist, die mit dem Vorsorgeprinzip nicht mehr zu vereinbaren wäre. Eine Möglichkeit, um verschiedene Konzentrationen herstellen zu können, bietet die Spiking-Methode. Dabei handelt es sich allerdings um eine junge Methode, welche in jedem Fall weiterer Verifizierung bedarf.

GV-Pflanzen können von aquatischen Organismen, neben anderen Expositionspfaden, im hohen Maße über die Nahrung aufgenommen werden (Rosi-Marshall et al. 2007). Ähnlichkeiten bestehen daher zu systemischen Pestiziden, wie zum Beispiel den Neonikotinoiden. Diese Art von Pesiziden verteilt sich während des Wachstums in der gesamten Pflanze und kommt daher im Pflanzenmaterial und nicht auf dessen Oberfläche vor.

Mit systemischen Pestiziden werden daher ebenfalls im Besonderen zerkleinernde aquatische Organismen exponiert (Englert et al. 2017c).

Die Zulassung von GV-Pflanzen in der EU beinhaltet zwar eine Umweltverträglichkeitsprüfung, in der negative Auswirkungen auf die Umwelt abgeschätzt werden sollen, für diese sind aquatische Tests bisher allerdings nicht obligatorisch. Eine gesetzliche Verankerung von aquatischen Tests würde ihre hohe Bedeutung für den Schutz der Umwelt herausstellen und auch die Forschung weiter vorantreiben. Nur mit der Abschätzung von negativen Auswirkungen auch auf die aquatische Umwelt, deckt die Umweltverträglichkeitsprüfung einen umfassenden und aussagekräftigen Bereich ab.

Die bisherige Forschung bezieht sich vor allem auf einzelne gentechnische Veränderungen und GV-Pflanzen (Teile I und III, Anhänge A.1, A.3), die mit „klassischen“ gentechnischen Methoden hergestellt worden sind. Die Entwicklung und Anwendung von gentechnischen Pflanzen ist aber bereits deutlich weiter. Gestackte GV-Pflanzen, also Pflanzen mit mehreren gentechnischen Veränderungen, sind weit verbreitet und wurden bisher nur in geringem Ausmaß untersucht. Aktuell sind bis zu sechsfach gestackte Maissorten in der EU zum Import zugelassen (EC 2022). Diese Maissorten expremieren mehrere Bt-Toxine und besitzen zusätzlich noch eine Herbizidresistenz. Die Auswirkungen solcher vielfach gestackter Pflanzen auf die aquatische Umwelt und auch mögliche kombinatorische Effekte sind weitesgehend unerforscht. Außerdem führen sogenannte „neue“ gentechnische Verfahren, wie z.B. CRISPR/Cas, zur schnelleren und gezielteren Veränderung von Genen, die bisher bei der Risikobewertung auf die aquatische Umwelt nicht betrachtet worden sind, aber potentiell Auswirkungen auf die Umwelt haben könnten. Insgesamt sind in der Risikobewertung von GV-Pflanzen auf die aquatische Umwelt aktuell große Lücken zu finden, die durch weitere Forschung verringert werden müssen, um negative Auswirkungen auf die Umwelt besser einschätzen und damit verhindern zu können.

6. Literaturverzeichnis

Armbrust, Kevin L.; Peeler, Harold B. (2002): Effects of formulation on the run-off of imidacloprid from turf. In: *Pest Manag Sci* 58 (7), S. 702–706. DOI: 10.1002/ps.518.

Arrese, Estela L.; Soulages, Jose L. (2010): Insect fat body: Energy, metabolism, and regulation. In: *Annu Rev Entomol* 55, S. 207–225. DOI: 10.1146/annurev-ento-112408-085356.

Axelsson, E. Petter; Hjältén, Joakim; LeRoy, Carri J.; Julkunen-Tiitto, Riitta; Wennström, Anders; Pilate, Gilles (2010): Can Leaf Litter from Genetically Modified Trees Affect Aquatic Ecosystems? In: *Ecosystems* 13 (7), S. 1049–1059. DOI: 10.1007/s10021-010-9373-y.

Axelsson, E. Petter; Hjältén, Joakim; LeRoy, Carri J.; Whitham, Thomas G.; Julkunen-Tiitto, Riitta; Wennström, Anders (2011): Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition. In: *J Appl Ecol* 48 (6), S. 1472–1479. DOI: 10.1111/j.1365-2664.2011.02046.x.

Beachy, Roger N.; Fedoroff, Nina V.; Goldberg, Robert B.; McHughen, Alan (2008): The burden of proof: A response to Rosi-Marshall et al. In: *Proc Natl Acad Sci USA* 105 (7), E9; author reply E11. DOI: 10.1073/pnas.0711431105.

Beuter, Liesa-Kristin; Dören, László; Hommen, Udo; Kotthoff, Matthias; Schäfers, Christoph; Ebke, Klaus Peter (2019): Testing effects of pesticides on macroinvertebrate communities in outdoor stream mesocosms using carbaryl as example test item. In: *Environ Sci Eur* 31 (1), S. 3290. DOI: 10.1186/s12302-019-0185-1.

BfN 2022a: Bundesamt für Naturschutz: Agrogentechnik. Online verfügbar unter https://www.bfn.de/agrogentechnik, zuletzt geprüft am 24.07.2022.

BfN 2022b: Bundesamt für Naturschutz: Anbau gentechnisch veränderter Pflanzen in Europa. Online verfügbar unter https://www.bfn.de/karten-und-daten/anbau-gentechnisch-veraenderter-pflanzen-europa, zuletzt geprüft am 24.07.2022.

Binning, Rachel R.; Coats, Joel; Kong, Xiaoxiao; Hellmich, Richard L. (2014): Susceptibility and aversion of *Spodoptera frugiperda* (Lepidoptera. Noctuidae) to Cry1F Bt maize and considerations for insect resistance management. In: *J Econ Entomol* 107 (1), S. 368–374. DOI: 10.1603/ec13352.

Bøhn, Thomas; Primicerio, Raul; Hessen, Dag O.; Traavik, Terje (2008): Reduced fitness of *Daphnia magna* fed a Bt-transgenic maize variety. In: *Arch Environ Contam Toxicol* 55 (4), S. 584–592. DOI: 10.1007/s00244-008-9150-5.

Bøhn, Thomas; Rover, Carina Macagnan; Semenchuk, Philipp Robert (2016): *Daphnia magna* negatively affected by chronic exposure to purified Cry-toxins. In: *Food Chem Toxicol* 91, S. 130–140. DOI: 10.1016/j.fct.2016.03.009.

Bøhn, Thomas; Traavik, Terje; Primicerio, Raul (2010): Demographic responses of *Daphnia magna* fed transgenic Bt-maize. In: *Ecotoxicology* 19 (2), S. 419–430. DOI: 10.1007/s10646-009-0427-x.

Böttger, Rita; Schaller, Jörg; Lintow, Sven; Gert Dudel, E. (2015): Aquatic degradation of Cry1Ab protein and decomposition dynamics of transgenic corn leaves under controlled conditions. In: *Ecotoxicol Environ Saf* 113, S. 454–459. DOI: 10.1016/j.ecoenv.2014.12.034.

Brock, Theo C. M.; Alix, Anne; Brown, Colin D.; Capri, Ettore; Gottesbüren, Bernhard F.F.; Heimbach, Fred et al. (Hg.) (2010): Linking aquatic exposure and effects. Risk assessment of pesticides. Boca Raton, London, New York: CRC Press, Taylor & Francis Group (Society of environmental toxicology and chemistry (SETAC)).

Bundschuh, Rebecca; Bundschuh, Mirco; Otto, Mathias; Schulz, Ralf (2019): Food-related exposure to systemic pesticides and pesticides from transgenic plants: evaluation of aquatic test strategies. In: *Environ Sci Eur* 31 (1), S. 3290. DOI: 10.1186/s12302-019-0266-1.

Bundschuh, Rebecca; Kuhn, Ulrike; Bundschuh, Mirco; Naegele, Caroline; Elsaesser, David; Schlechtriemen, Ulrich et al. (2016): Prioritizing stream types according to their potential risk to receive crop plant material - A GIS-based procedure to assist in the risk assessment of genetically modified crops and systemic insecticide residues. In: *Sci Total Environ* 547, S. 226–233. DOI: 10.1016/j.scitotenv.2015.12.124.

Cañedo-Argüelles, Miguel; Bundschuh, Mirco; Gutiérrez-Cánovas, Cayetano; Kefford, Ben J.; Prat, Narcís; Trobajo, Rosa; Schäfer, Ralf B. (2014): Effects of repeated salt pulses on ecosystem structure and functions in a stream mesocosm. In: *Sci Total Environ* 476-477, S. 634–642. DOI: 10.1016/j.scitotenv.2013.12.067.

Chambers, Catherine P.; Whiles, Matt R.; Rosi-Marshall, Emma J.; Tank, Jennifer L.; Royer, Todd V.; Griffiths, Natalie A. et al. (2010): Responses of stream macroinvertebrates to Bt maize leaf detritus. In: *Ecol Appl* 20 (7), S. 1949–1960. DOI: 10.1890/09-0598.1.

Douville, M.; Gagn, F.; Masson, L.; McKay, J.; Blaise, C. (2005): Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. In: *Biochem Syst Ecol* 33 (3), S. 219–232. DOI: 10.1016/j.bse.2004.08.001.

Duan, Jian J.; Marvier, Michelle; Huesing, Joseph; Dively, Galen; Huang, Zachary Y. (2008): A meta-analysis of effects of Bt crops on honey bees (Hymenoptera: Apidae). In: *PloS one* 3 (1), e1415. DOI: 10.1371/journal.pone.0001415.

EC 2022: Community register of GM food and feed. Online verfügbar unter https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm, zuletzt geprüft am 24.07.2022.

EFSA (2010a): Guidance on the environmental risk assessment of genetically modified plants. In: *EFS2* 8 (11), S. 1879. DOI: 10.2903/j.efsa.2010.1879.

EFSA (2010b): Scientific Opinion on the assessment of potential impacts of genetically modified plants on non-target organisms. In: *EFS2* 8 (11), S. 1877. DOI: 10.2903/j.efsa.2010.1877.

EFSA (2011a): Scientific Opinion on application (EFSA-GMO-CZ-2008-54) for placing on the market of genetically modified insect resistant and herbicide tolerant maize MON 88017 for cultivation under Regulation (EC) No 1829/2003 from Monsanto. In: *EFSA Journal* 9 (11), S. 2428. DOI: 10.2903/j.efsa.2011.2428.

EFSA (2011b): Scientific Opinion updating the evaluation of the environmental risk assessment and risk management recommendations on insect resistant genetically modified maize 1507 for cultivation. In: *EFSA Journal* 9 (11), S. 2429. DOI: 10.2903/j.efsa.2011.2429.

EFSA (2013): Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. In: *EFS2* 11 (7). DOI: 10.2903/j.efsa.2013.3290.

Eizaguirre, M.; Tort, S.; López, C.; Albajes, R. (2005): Effects of sublethal concentrations of *Bacillus thuringiensis* on larval development of *Sesamia nonagrioides*. In: *J Econ Entomol* 98 (2), S. 464–470. DOI: 10.1093/jee/98.2.464.

Englert, Dominic; Bakanov, Nikita; Zubrod, Jochen P.; Schulz, Ralf; Bundschuh, Mirco (2017a): Modeling Remobilization of Neonicotinoid Residues from Tree Foliage in Streams - A Relevant Exposure Pathway in Risk Assessment? In: *Environ Sci Technol* 51 (3), S. 1785–1794. DOI: 10.1021/acs.est.6b05213.

Englert, Dominic; Zubrod, Jochen P.; Link, Moritz; Mertins, Saskia; Schulz, Ralf; Bundschuh, Mirco (2017b): Does Waterborne Exposure Explain Effects Caused by Neonicotinoid-Contaminated Plant Material in Aquatic Systems? In: *Environ Sci Technol* 51 (10), S. 5793–5802. DOI: 10.1021/acs.est.7b00827.

Englert, Dominic; Zubrod, Jochen P.; Pietz, Sebastian; Stefani, Sonja; Krauss, Martin; Schulz, Ralf; Bundschuh, Mirco (2017c): Relative importance of dietary uptake and waterborne exposure for a leaf-shredding amphipod exposed to thiacloprid-contaminated leaves. In: *Scientific reports* 7 (1), S. 16182. DOI: 10.1038/s41598-017-16452-9.

Erb, S. L.; Bourchier, R. S.; van Frankenhuyzen, K.; Smith, S. M. (2001): Sublethal Effects of *Bacillus thuringiensis* Berliner subsp. *kurstaki* on *Lymantria dispar* (Lepidoptera. Lymantriidae) and the Tachinid Parasitoid *Compsilura concinnata* (Diptera: Tachinidae). In: *Environ Entomol* 30 (6), S. 1174–1181. DOI: 10.1603/0046-225X-30.6.1174.

EU (2000): Directive 2000/60/EC of European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

EU (2001a): Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms.

EU (2001b): Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms.

Flury, Markus (1996): Experimental Evidence of Transport of Pesticides through Field Soils—A Review. In: *J. environ. qual.* 25 (1), S. 25–45. DOI: 10.2134/jeq1996.00472425002500010005x.

Friberg, Nikolai; Jacobsen, Dean (1999): Variation in growth of the detritivore-shredder *Sericostoma personatum* (Trichoptera). In: *Freshwater Biol* 42 (4), S. 625–635. DOI: 10.1046/j.1365-2427.1999.00501.x.

Glare, T. R.; O´Callaghan, M. (2000): *Bacillus thuringiensis*: Biology, ecology and safety. Chichester: Wiley.

Gonzalez, J. M.; Graca, M.A.S. (2003): Conversion of leaf litter to secondary production by a shredding caddis-fly. In: *Freshwater Biol* 48 (9), S. 1578–1592. DOI: 10.1046/j.1365-2427.2003.01110.x.

Graça, Manuel A.S. (2001): The role of invertebrates on leaf litter decomposition in streams - a review. In: *Int Rev Hydrobiol* 86 (4-5), S. 383–393. DOI: 10.1002/1522-2632(200107)86:4/5<383::AID-IROH383>3.0.CO;2-D.

Griffiths, Natalie A.; Tank, Jennifer L.; Royer, Todd V.; Rosi, Emma J.; Shogren, Arial J.; Frauendorf, Therese C.; Whiles, Matt R. (2017): Occurrence, leaching, and degradation of Cry1Ab protein from transgenic maize detritus in agricultural streams. In: *Sci Total Environ* 592, S. 97–105. DOI: 10.1016/j.scitotenv.2017.03.065.

Griffiths, Natalie A.; Tank, Jennifer L.; Royer, Todd V.; Rosi-Marshall, Emma J.; Whiles, Matt R.; Chambers, Catherine P. et al. (2009): Rapid decomposition of maize detritus in agricultural headwater streams. In: *Ecol Appl* 19 (1), S. 133–142. DOI: 10.1890/07-1876.1.

Gulzar, Asim; Wright, Denis J. (2015): Sub-lethal effects of Vip3A toxin on survival, development and fecundity of *Heliothis virescens* and *Plutella xylostella*. In: *Ecotoxicology (London, England)* 24 (9), S. 1815–1822. DOI: 10.1007/s10646-015-1517-6.

Hand, Laurence H.; Oliver, Robin G. (2010): The behavior of isopyrazam in aquatic ecosystems: implementation of a tiered investigation. In: *Environ Toxicol Chem* 29 (12), S. 2702–2712. DOI: 10.1002/etc.337.

Hanson, Mark L.; Graham, David W.; Babin, Emmanuelle; Azam, Didier; Coutellec, Marie-Agnes; Knapp, Charles W. et al. (2007): Influence of isolation on the recovery of pond mesocosms from the application of an insecticide. I. Study design and planktonic community responses. In: *Environ Toxicol Chem* 26 (6), S. 1265–1279. DOI: 10.1897/06-248R.1.

Hilbeck, Angelika; Bundschuh, Rebecca; Bundschuh, Mirco; Hofmann, Frieder; Oehen, Bernadette; Otto, Mathias et al. (2017): Procedure to select test organisms for environmental risk assessment of genetically modified crops in aquatic systems. In: *Integr Environ Assess Manag* 13 (6), S. 974–979. DOI: 10.1002/ieam.1965.

Holderbaum, Daniel Ferreira; Cuhra, Marek; Wickson, Fern; Orth, Afonso Inácio; Nodari, Rubens Onofre; Bøhn, Thomas (2015): Chronic Responses of *Daphnia magna* under Dietary Exposure to Leaves of a Transgenic (Event MON810) Bt-Maize Hybrid and its Conventional Near-Isoline. In: *J Toxicol Environ Health* 78 (15), S. 993–1007. DOI: 10.1080/15287394.2015.1037877.

James, Clive (2014): Global Status of Commercialized Biotech/GM Crops: 2014. International Service for the Acquisition of Agri-biotech Applications (ISAAA).

Jensen, Peter D.; Dively, Galen P.; Swan, Christopher M.; Lamp, William O. (2010): Exposure and nontarget effects of transgenic Bt corn debris in streams. In: *Environ Entomol* 39 (2), S. 707–714. DOI: 10.1603/EN09037.

Konschak, Marco; Zubrod, Jochen P.; Baudy, Patrick; Englert, Dominic; Herrmann, Bastian; Schulz, Ralf; Bundschuh, Mirco (2019): Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder Chaetopteryx villosa (Trichoptera). In: *Aquatic toxicology (Amsterdam, Netherlands)* 206, S. 33–42. DOI: 10.1016/j.aquatox.2018.10.021.

Kostov, Kaloyan; Damgaard, Christian; Hendriksen, Niels; Sweet, Jeremy B.; Krogh, Paul (2014): Are population abundances and biomasses of soil invertebrates changed by Bt crops compared with conventional crops? A systematic review protocol. In: *Environ Evid* 3 (1), S. 10. DOI: 10.1186/2047-2382-3-10.

Kratz, Werner; Mante, Christina; Hofmann, Frieder; Schlechtriemen, Ulrich; Kuhn, Ulrike; Ober, Steffi; Vögel, Rudolph (2010): Exposure of maize harvest by-products to aquatic ecosystems and protected nature reserves in Brandenburg, Germany. In: *Theorie in der Ökologie* (16), S. 21–23.

Kreutzweiser, David P.; Good, Kevin P.; Chartrand, Derek T.; Scarr, Taylor A.; Thompson, Dean G. (2008): Are leaves that fall from imidacloprid-treated maple trees to control Asian longhorned beetles toxic to non-target decomposer organisms? In: *J Environ Qual* 37 (2), S. 639–646. DOI: 10.2134/jeq2007.0278.

Kučinić, Mladen; Szivák, Ildikó; Pauls, Steffen U.; Bálint, Miklós; Delić, Antun; Vučković, Ivan (2013): *Chaetopteryx bucari* sp. n., a new species from the *Chaetopteryx rugulosa* group from Croatia (Insecta, Trichoptera, Limnephilidae) with molecular, taxonomic and ecological notes on the group. In: *ZooKeys* (320), S. 1–28. DOI: 10.3897/zookeys.320.4565.

Lamers, Marc; Anyusheva, Maria; La, Nguyen; van Nguyen, Vien; Streck, Thilo (2011): Pesticide Pollution in Surface- and Groundwater by Paddy Rice Cultivation: A Case Study from Northern Vietnam. In: *Clean Soil Air Water* 39 (4), S. 356–361. DOI: 10.1002/clen.201000268.

Lang, A.; Otto, M. (2010): A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target Lepidoptera. In: *Entomol Exp Appl* 135 (2), S. 121–134. DOI: 10.1111/j.1570-7458.2010.00981.x.

Li, Guangsheng; Wang, Yongmo; Liu, Biao; Zhang, Guoan (2014): Transgenic *Bacillus thuringiensis* (Bt) rice is safer to aquatic ecosystems than its non-transgenic counterpart. In: *PloS one* 9 (8), e104270. DOI: 10.1371/journal.pone.0104270.

Li, Y.; Wu, K.; Zhang, Y.; Yuan, G. (2007): Degradation of Cry1Ac Protein Within Transgenic *Bacillus thuringiensis* Rice Tissues Under Field and Laboratory Conditions. In: *Environ Entomol* 36 (5), S. 1275–1282. DOI: 10.1093/ee/36.5.1275.

Liess, Mathias; Schulz, Ralf (1996): Chronic effects of short-term contamination with the pyrethroid insecticide fenvalerate on the caddisfly *Limnephilus lunatus*. In: *Hydrobiologia* 324 (2), S. 99–106. DOI: 10.1007/BF00018170.

Liess, Mathias; Schulz, Ralf (1999): Linking insecticide contamination and population response in an agricultural stream. In: *Environ Toxicol Chem* 18 (9), S. 1948–1955. DOI: 10.1002/etc.5620180913.

Liu, Yongbo; Jiang, Wanxiang; Liang, Yuyong; Zhao, Caiyun; Li, Junsheng (2017): No effect of Bt-transgenic rice litter on the meiobenthos community in field ditches. In: *Pest Manag Sci* 73 (6), S. 1213–1219. DOI: 10.1002/ps.4446.

Liu, Yongbo; Li, Junsheng; Luo, Zunlan; Wang, Huaru; Liu, Fang (2016): The fate of fusion Cry1Ab/1Ac proteins from Bt-transgenic rice in soil and water. In: *Ecotoxicol Environ Saf* 124, S. 455–459. DOI: 10.1016/j.ecoenv.2015.11.025.

Lövei, Gabor L.; Andow, David A.; Arpaia, Salvatore (2009): Transgenic Insecticidal Crops and Natural Enemies: A Detailed Review of Laboratory Studies. In: *Environ Entomol* 38 (2), S. 293–306. DOI: 10.1603/022.038.0201.

Malicky, Hans (2005): Ein kommentiertes Verzeichnis der Köcherfliegen (Trichoptera) Europas und des Mediterrangebiets. In: *Linzer Biol Beitr* 37 (1), S. 533–596.

Marvier, Michelle; McCreedy, Chanel; Regetz, James; Kareiva, Peter (2007): A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. In: *Science* 316 (5830), S. 1475–1477. DOI: 10.1126/science.1139208.

Naranjo, S. (2009): Impacts of Bt crops on non-target invertebrates and insecticide use patterns. In: *CAB Reviews* 4 (011). DOI: 10.1079/PAVSNNR20094011.

Newton, Kymberly; Zubrod, Jochen P.; Englert, Dominic; Lüderwald, Simon; Schell, Theresa; Baudy, Patrick et al. (2018): The evil within? Systemic fungicide application in trees enhances litter quality for an aquatic decomposer-detritivore system. In: *Environ Pollut* 241, S. 549–556. DOI: 10.1016/j.envpol.2018.05.069.

Palma, Leopoldo; Muñoz, Delia; Berry, Colin; Murillo, Jesús; Caballero, Primitivo (2014): *Bacillus thuringiensis* toxins: An overview of their biocidal activity. In: *Toxins* 6 (12), S. 3296–3325. DOI: 10.3390/toxins6123296.

Parisi, Claudia; Tillie, Pascal; Rodríguez-Cerezo, Emilio (2016): The global pipeline of GM crops out to 2020. In: *Nat Biotechnol* 34 (1), S. 31–36. DOI: 10.1038/nbt.3449.

Parrott, Wayne (2008): Study of Bt impact on caddisflies overstates its conclusions: response to Rosi-Marshall et al. In: *Proc Natl Acad Sci USA* 105 (7), E10; author reply E11. DOI: 10.1073/pnas.0711284105.

Pott, Antonia; Bundschuh, Mirco; Bundschuh, Rebecca; Otto, Mathias; Schulz, Ralf (2020): Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders - an attempt to establish dose-effect relationships through food-spiking. In: *Scientific reports* 10 (1), S. 5262. DOI: 10.1038/s41598-020-62055-2.

Pott, Antonia; Otto, Mathias; Schulz, Ralf (2018): Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment. In: *Sci Total Environ* 635, S. 687–698. DOI: 10.1016/j.scitotenv.2018.04.013.

Preston, Benjamin L. (2002): Indirect effects in aquatic ecotoxicology: implications for ecological risk assessment. In: *Environ Manag* 29 (3), S. 311–323. DOI: 10.1007/s00267-001-0023-1.

Rausell, C.; Martinez-Ramirez, A. C.; Garcia-Robles, I.; Real, M. D. (2000): A binding site for *Bacillus thuringiensis* Cry1Ab toxin is lost during larval development in two forest pests. In: *Appl Environ Microb* 66 (4), S. 1553–1558. DOI: 10.1128/AEM.66.4.1553-1558.2000.

Raybould, Alan; Burns, Andrea; Hamer, Mick (2014): High concentrations of protein test substances may have non-toxic effects on *Daphnia magna*: Implications for regulatory study designs and ecological risk assessments for GM crops. In: *GM Crops Food* 5 (4), S. 296–301. DOI: 10.4161/21645698.2014.950540.

Rosi-Marshall, E. J.; Tank, J. L.; Royer, T. V.; Whiles, M. R.; Evans-White, M.; Chambers, C. et al. (2007): Toxins in transgenic crop byproducts may affect headwater stream

ecosystems. In: *Proc Natl Acad Sci USA* 104 (41), S. 16204–16208. DOI: 10.1073/pnas.0707177104.

Rumbos, Christos I.; Stamopoulos, Dimitrios; Georgoulas, Giorgios; Nikolopoulou, Efi (2010): Factors affecting leaf litter cecomposition by *Micropterna sequax* (Trichoptera: Limnephilidae). In: *Int Rev Hydrobiol* 95 (4-5), S. 383–394. DOI: 10.1002/iroh.201011236.

Saxena, Deepak; Stotzky, G. (2001): Bt corn has a higher lignin content than non-Bt corn. In: *Am. J. Bot.* 88 (9), S. 1704–1706. DOI: 10.2307/3558416.

Schulz, Ralf; Bundschuh, Mirco; Gergs, René; Brühl, Carsten A.; Diehl, Dörte; Entling, Martin H. et al. (2015): Review on environmental alterations propagating from aquatic to terrestrial ecosystems. In: *Sci Total Environ* 538, S. 246–261. DOI: 10.1016/j.scitotenv.2015.08.038.

Schulz, Ralf; Liess, Mathias (1995): Chronic effects of low insecticide concentrations on freshwater caddisfly larvae. In: *Hydrobiologia* 299 (2), S. 103–113. DOI: 10.1007/BF00017562.

Schulz, Ralf; Peall, S. K.; Dabrowski, J. M.; Reinecke, A. J. (2001): Spray deposition of two insecticides into surface waters in a South African orchard area. In: *J. environ. qual.* 30 (3), S. 814–822. DOI: 10.2134/jeq2001.303814x.

Starner, Keith; Goh, Kean S. (2012): Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010-2011. In: *Bull Environ Contam Toxicol* 88 (3), S. 316–321. DOI: 10.1007/s00128-011-0515-5.

Stehle, Sebastian; Schulz, Ralf (2015a): Agricultural insecticides threaten surface waters at the global scale. In: *Proc Natl Acad Sci USA* 112 (18), S. 5750–5755. DOI: 10.1073/pnas.1500232112.

Stehle, Sebastian; Schulz, Ralf (2015b): Pesticide authorization in the EU - environment unprotected? In: *Environ Sci Pollut Res* (22), S. 19632–19647.

Strain, Katherine E.; Lydy, Michael J. (2015): The fate and transport of the Cry1Ab protein in an agricultural field and laboratory aquatic microcosms. In: *Chemosphere* 132, S. 94–100. DOI: 10.1016/j.chemosphere.2015.03.005.

Strain, Katherine E.; Whiting, Sara A.; Lydy, Michael J. (2014): Laboratory and field validation of a Cry1Ab protein quantitation method for water. In: *Talanta* 128, S. 109–116. DOI: 10.1016/j.talanta.2014.04.036.

Suter, Glenn W.; Cormier, Susan M. (2015): Why care about aquatic insects: Uses, benefits, and services. In: *Integr Enviro Assess Manage* 11 (2), S. 188–194. DOI: 10.1002/ieam.1600.

Swan, Christopher M.; Jensen, Peter D.; Dively, Galen P.; Lamp, William O. (2009): Processing of transgenic crop residues in stream ecosystems. In: *J Appl Ecol* 14, S. 711. DOI: 10.1111/j.1365-2664.2009.01728.x.

Tank, Jennifer L.; Rosi-Marshall, Emma J.; Royer, Todd V.; Whiles, Matt R.; Griffiths, Natalie A.; Frauendorf, Therese C.; Treering, David J. (2010): Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. In: *Proc Natl Acad Sci USA* 107 (41), S. 17645–17650. DOI: 10.1073/pnas.1006925107.

van Wijngaarden, René P. A.; Cuppen, Jan G. M.; Arts, Gertie H. P.; Crum, Steven J. H.; van den Hoorn, Martin W.; van den Brink, Paul J.; Brock, Theo C. M. (2004): Aquatic risk assessment of a realistic exposure to pesticides used in bulb crops: a microcosm study. In: *Environ Toxicol Chem* 23 (6), S. 1479–1498. DOI: 10.1897/03-80.

Vannote, Robin L.; Minshall, G. Wayne; Cummins, Kenneth W.; Sedell, James R.; Cushing, Colbert E. (1980): The River Continuum Concept. In: *Can J Fish Aquat Sci* 37 (1), S. 130–137. DOI: 10.1139/f80-017.

Venter, Hermoine J.; Bøhn, Thomas (2016): Interactions between Bt crops and aquatic ecosystems: A review. In: *Environ Toxicol Chem* 35 (12), S. 2891–2902. DOI: 10.1002/etc.3583.

Vörösmarty, C. J.; McIntyre, P. B.; Gessner, M. O.; Dudgeon, D.; Prusevich, A.; Green, P. et al. (2010): Global threats to human water security and river biodiversity. In: *Nature* 467 (7315), S. 555–561. DOI: 10.1038/nature09440.

Wagner, Rüdiger (1990): Influence of temperature, photoperiod and nutrition on growth and consumption of *Chaetopteryx villosa* (Trichoptera). In: *Ecography* 13 (3), S. 247–254. DOI: 10.1111/j.1600-0587.1990.tb00615.x.

Wallace, I. D. (1977): A key to larvae and pupae of *Sericostoma personatum* (Spence) and *Notidobia ciliaris* (Linne) (Sericostomatidae: Trichoptera) in Britain. In: *Freshwater Biol* 7 (1), S. 93–98. DOI: 10.1111/j.1365-2427.1977.tb01660.x.

Wallace, J. B.; Webster, J. R. (1996): The role of macroinvertebrates in stream ecosystem function. In: *Annu Rev Entomol* 41, S. 115–139. DOI: 10.1146/annurev.en.41.010196.000555.

Wang, Haiyan; Ye, Qingfu; Gan, Jay; Wu, Licheng (2007): Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils. In: *J Agric Food Chem* 55 (5), S. 1900–1904. DOI: 10.1021/jf062924x.

Wang, Jiamei; Chen, Xiuping; Li, Yunhe; Zhu, Haojun; Ding, Jiatong; Peng, Yufa (2014): Effect of straw leachates from Cry1Ca-expressing transgenic rice on the growth of *Chlorella pyrenoidosa*. In: *Environ Toxicol Chem* 33 (5), S. 1156–1162. DOI: 10.1002/etc.2535.

Wantzen, Karl M.; Wagner, Rüdiger (2006): Detritus processing by invertebrate shredders: A neotropical-temperate comparison. In: *J North Am Benthological Soc* 25 (1), S. 216–232. DOI: 10.1899/0887-3593(2006)25[216:DPBISA]2.0.CO;2.

Waringer, Johann; Malicky, Hans (2017): The larvae of *Sericostoma bergeri* Malicky, 1973 and *Sericostoma herakles* Malicky, 1999 (Trichoptera, Sericostomatidae). In: *ZooKeys* (695), S. 123–133. DOI: 10.3897/zookeys.695.14531.

Wauchope, R. D. (1978): The Pesticide Content of Surface Water Draining from Agricultural Fields - A Review. In: *J. environ. qual.* 7 (4), S. 459–472. DOI: 10.2134/jeq1978.00472425000700040001x.

Whiting, Sara A.; Strain, Katherine E.; Campbell, Laura A.; Young, Bryan G.; Lydy, Michael J. (2014): A multi-year field study to evaluate the environmental fate and agronomic effects of insecticide mixtures. In: *Sci Total Environ* 497-498, S. 534–542. DOI: 10.1016/j.scitotenv.2014.07.115.

Wieczorek, Matthias V.; Bakanov, Nikita; Lagadic, Laurent; Bruns, Eric; Schulz, Ralf (2017): Response and recovery of the macrophytes Elodea canadensis and Myriophyllum spicatum following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms. In: *Environ Toxicol Chem* 36 (4), S. 1090–1100. DOI: 10.1002/etc.3636.

Wieczorek, Matthias V.; Bakanov, Nikita; Stang, Christoph; Bilancia, Daniel; Lagadic, Laurent; Bruns, Eric; Schulz, Ralf (2016): Reference scenarios for exposure to plant

protection products and invertebrate communities in stream mesocosms. In: *Sci Total Environ* 545-546, S. 308–319. DOI: 10.1016/j.scitotenv.2015.12.048.

Wieczorek, Matthias V.; Kötter, Denise; Gergs, René; Schulz, Ralf (2015): Using stable isotope analysis in stream mesocosms to study potential effects of environmental chemicals on aquatic-terrestrial subsidies. In: *Environ Sci Pollut Res* 22 (17), S. 12892–12901. DOI: 10.1007/s11356-015-4071-0.

Wolfenbarger, L. LaReesa; Naranjo, Steven E.; Lundgren, Jonathan G.; Bitzer, Royce J.; Watrud, Lidia S. (2008): Bt crop effects on functional guilds of non-target arthropods: A meta-analysis. In: *PloS one* 3 (5), e2118. DOI: 10.1371/journal.pone.0002118.

Zhang, Li; Guo, Ruqing; Fang, Zhixiang; Liu, Biao (2016): Genetically modified rice Bt-Shanyou63 expressing Cry1Ab/c protein does not harm *Daphnia magna*. In: *Ecotoxicol Environ Saf* 132, S. 196–201. DOI: 10.1016/j.ecoenv.2016.06.011.

Zubrod, Jochen P.; Bundschuh, Mirco; Arts, Gertie; Brühl, Carsten A.; Imfeld, Gwenaël; Knäbel, Anja et al. (2019): Fungicides: An Overlooked Pesticide Class? In: *Environ Sci Technol* 53 (7), S. 3347–3365. DOI: 10.1021/acs.est.8b04392.

Zwahlen, C.; Hilbeck, A.; Gugerli, P.; Nentwig, W. (2003): Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. In: *Mol Ecol* 12 (3), S. 765–775. DOI: 10.1046/j.1365-294X.2003.01767.x.

7. Anhang

A.1: Pott, Antonia; Otto, Mathias; Schulz, Ralf (2018): Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment.

A.2: Pott, Antonia; Bundschuh, Mirco; Bundschuh, Rebecca; Otto, Mathias; Schulz, Ralf (2020): Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders - an attempt to establish dose-effect relationships through food-spiking.

A.3: Pott, Antonia; Bundschuh, Mirco; Otto, Mathias; Schulz, Ralf (2022, in press): Assessing effects of genetically modified plant material on the aquatic environment using higher-tier studies.

A.4: Eidesstattliche Erklärung

A.5: Lebenslauf

A.1 Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment

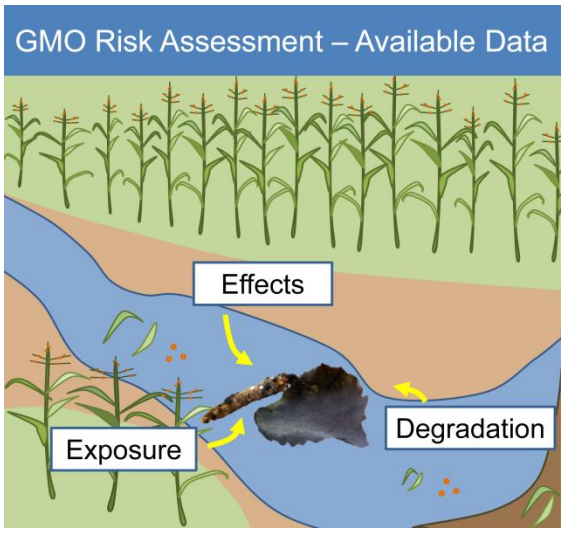
Antonia Pott[1,2*], Mathias Otto[1], Ralf Schulz[2]

[1] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[2] Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrassße 7, 76829 Landau, Germany

In: *The Science of the total environment* 635, S. 687–698

Graphical Abstract



Highlights

- Genetically modified (GM) crop material can enter aquatic environments.
- An analysis of available GM studies dealing with effects and fate is presented.
- Gaps should be addressed to improve risk assessment of GM crops.

Abstract

The aquatic environment is strongly connected to the surrounding agricultural landscapes, which regularly serve as sources of stressors such as agrochemicals. Genetically modified crops, which are cultivated on a large scale in many countries, may also act as stressors. Despite the commercial use of genetically modified organisms (GMOs) for over 20 years, their impact on the aquatic environment came into focus only 10 years ago. We present the status quo of the available scientific data in order to provide an input for informed aquatic risk assessment of GMOs. We could identify only 39 publications, including 84 studies, dealing with GMOs in the aquatic environment, and our analysis shows substantial knowledge gaps. The available information is restricted to a small number of crop plants, traits, events, and test organisms. The analysis of effect studies reveals that only a narrow range of organisms has been tested and that studies on combinatorial actions of stressors are virtually absent. The analysis of fate studies shows that many aspects, such as the fate of leached toxins, degradation of plant material, and distribution of crop residues in the aquatic habitat, are insufficiently investigated. Together with these research needs, we identify standardization of test methods as an issue of high priority, both for research and risk assessment needed for GMO regulation.

Keywords: Genetically modified crops, aquatic ecosystems, environmental risk assessment, non-target effects, Bt toxin

Background

Aquatic habitats are highly connected to the surrounding terrestrial ecosystems from which they receive minerals and organic input (Vannote et al., 1980). Agricultural ecosystems, comprising the largest terrestrial biome, contribute to this input into aquatic ecosystems and are substantial sources not only of phosphate and nitrogen but also of chemical stressors such as pesticides (Stehle and Schulz, 2015a). Stressors from agriculture, therefore, are the focus of efforts to limit damage to aquatic ecosystems such as rivers, lakes, or estuaries. With the Water Framework Directive (WFD) (EU, 2000), the European Union (EU) acknowledges the need for sustainable water management because of considerable pressures on the aquatic environment. Agriculture has been identified clearly as causing considerable problems for achieving the aim of good ecological status of all waterbodies (Vörösmarty et al., 2010).

In this review, we analyse the current knowledge on risks posed to aquatic environments by the cultivation of genetically modified (GM) crops. GM crops have now been grown commercially for over 20 years, with increasing rates of adoption, especially in North and South America. In 2014, 82% of the worldwide production of soybean was transgenic, followed by 68% of cotton and 30% of maize (James, 2014). The increasing adoption is reflected in the increasing number of authorized single GM transformation events. Their numbers reached 102 in 2014 (Parisi et al., 2016). Only two traits dominate GM crops: herbicide resistance (HR) (45.1%) and insect resistance (IR) (34.6%) (Parisi et al., 2016). Whereas HR is achieved by the expression of enzymes breaking down herbicides, IR is realized by the expression of insect toxins derived from the soil bacterium *Bacillus thuringiensis* (Bt). The toxins are incorporated into plant tissues and trigger the lysis of gut membranes which is then followed by death in target organisms (Glare and O´Callaghan, 2000).

As pesticides and GM crops contain biologically active compounds, and side effects have been recognized, both are subject to environmental risk assessment (ERA) and regulated in many countries. In the EU, the environmental risk assessment needs to identify potential adverse effects of the genetically modified organism (GMO) on the environment (EU, 2001). The relevance of insecticides, e.g. those sprayed as pesticides, for aquatic ecosystems is not questioned, and indeed is reflected in risk assessment (Stehle and Schulz, 2015b). However, risks from plant-incorporated insecticides via GM crops are considered poorly in risk assessments and biosafety research. For the first 10 years, assessment of risks of GMOs to

organisms or ecosystems almost exclusively focused on terrestrial habitats. An influential study on the potential effects of GMOs on aquatic insect larvae (Rosi-Marshall et al., 2007), put impacts on aquatic environments into focus. The researchers also measured environmental exposure of headwater streams to Bt maize and the insecticidal Cry1Ab toxin from this GM crop (Tank et al., 2010). Besides experimental data showing a potential hazard to caddisflies (Trichoptera), an insect group with aquatic larval stages phylogenetically closely related to the Lepidoptera, and thus, to the target insects of Bt maize, Rosi-Marshall et al. (2007) also highlighted the input of GM plant material into the aquatic environment and the potential risk of cultivation of GM crops to aquatic invertebrates. While some authors felt that the conclusions of Rosi-Marshall et al. were overstated (Beachy et al., 2008; Parrott, 2008), the issue of effects of Bt maize on aquatic ecosystems gained momentum and was addressed by other research groups and in another review (Venter and Bøhn, 2016). It was also picked up by regulatory bodies, such as the European Food Safety Authority (EFSA), dealing with the market release of GMOs. However, EU authorities concluded that the risks to aquatic ecosystems from the Bt crops analysed thus far were negligible (e.g. EFSA, 2011a, 2011b).

In this study, we analyse the available scientific literature on the hazard and fate of GMOs in the aquatic environment. Our objective is to describe the developments and the current state of knowledge of risk assessment of GMOs in the aquatic environment. As risk assessment in the EU is case specific, our analysis differentiates between crops, traits, and novel proteins.

Methods

The identification of relevant studies based on multiple sources. A literature search was carried out in CAB Abstracts (CABI Wallingford, UK) and in the databases ISI Web of Knowledge (Thomson Reuters, New York, USA), BIOSIS (Thomson Reuters, New York, USA), AGRICOLA (National Agricultural Library, Beltsville, USA), AGRIS (Food and Agricultural Organisation (FAO) of the United Nations, Rome, Italy), and BASE (Bielefeld University Library, Bielefeld, Germany). Two search strings were used for CAB Abstracts, i.e. [(*Bacillus thuringiensis*)] and [(*Bacillus thuringiensis*) and (aquatic*)], and one for ISI Web of Knowledge, BIOSIS, AGRICOLA, AGRIS, and BASE, i.e. [(*Bacillus thuringiensis*) and (aquatic*)]. Furthermore, the relevant publications identified were scanned for references not covered by the database searches. The literature search was performed up to December 2017 and all papers published online before this date were included.

We narrowed the results of the database search to the scope of these publications as follows: we included only publications that (1) investigated the fate of GM plants, the relevant novel

protein, or relevant non-GM plants in the aquatic environment; or (2) investigated adverse effects of GM plants or the relevant novel protein on non-target aquatic invertebrates and protists in single-species tests. Only experiments that were peer-reviewed (3) and published in English (4) were included. By doing so, we excluded publications that included community studies, spray formulations of Bti (*Bacillus thuringiensis israelensis*), Bt genes, or vertebrates, and studies carried out in terrestrial or riparian habitats. As some publications contained different numbers of studies, five criteria were used to differentiate them: (1) every publication consisted of at least one study; (2) already published data were not classified as an own study; (3) several experiments with exactly the same test design were counted as one study; (4) every experiment with a different test design, e.g. a different test species, counted as one study; and (5) one study could investigate several endpoints, several treatments, several GMOs, several sampling sites, and several sampling dates and dates of experimental procedure. Every study was analysed separately (see supplementary information).

All studies were separated thematically into two main categories: effect and fate studies. Effect studies were single-species studies that investigated adverse effects of a GMO on non-target aquatic organisms. Fate studies investigated the behaviour and distribution of GMOs in the aquatic environment. In this respect, fate studies were divided into two subcategories: exposure studies investigating the occurrence of GMO plant material or novel protein in the aquatic environment, as well as intake of GMO plant material or novel protein into the test organisms; and degradation studies containing experiments that dealt with the decay of the GMO or novel protein in the aquatic environment over time.

Results & Discussion

Overall number of studies published

We identified 39 publications dealing with transgenic crops or their novel proteins in aquatic environments. A total of 84 studies were derived from these publications and could be separated into 31 effect studies and 53 fate studies. Among fate studies, we discuss exposure and degradation aspects separately. The exposure of aquatic environments to GMOs was investigated in 13 publications that included 22 studies. Twenty-one publications comprising 31 studies considered degradation of GMOs in aquatic environments. Compared to terrestrial studies, on which several reviews and meta-analyses have been carried out (Duan et al., 2008; Kostov et al., 2014; Lövei et al., 2009; Marvier et al., 2007; Naranjo, 2009; Wolfenbarger et al., 2008), the total number of studies on aquatic environments is very small.

Effect studies

The published studies on the effects of GM crops on aquatic organisms (Table 1) have a clear focus on maize (55%, 17 studies), rice (16%, five studies), cotton (6%, two studies), and soy (3%, one study), reflecting the importance of maize which holds the largest share of single events currently authorized for cultivation (22.5%, Parisi et al., 2016). Some studies (19%, six studies) were carried out using a pure Bt toxin in their experiments and, therefore, could not be allocated to a specific plant. Effect studies with oilseed rape, another major GM crop, are missing and the small number of studies on cotton or soy does not reflect their large-scale cultivation. Effect studies on other GM applications (e.g. vegetables, fruits) are lacking.

IR was the trait most frequently investigated. HR was tested mainly in combination with IR (Figure 1). As Bt toxins do differ in their activity range, ideally information on each of the toxins should be available. However, apart from Cry1Ab, other Bt proteins were rarely studied, especially those targeting the Coleoptera. Besides single events, only one fusion protein (Cry1Ab/1Ac), one double-, and one triple-stacked event were investigated in the studies reviewed. However, there exist to date many multiple-stacked events (Parisi et al., 2016), and effect studies lag behind this development. In total eight different GMO events were used in effect studies, and in 19 of the cases the GM event remains unclear.

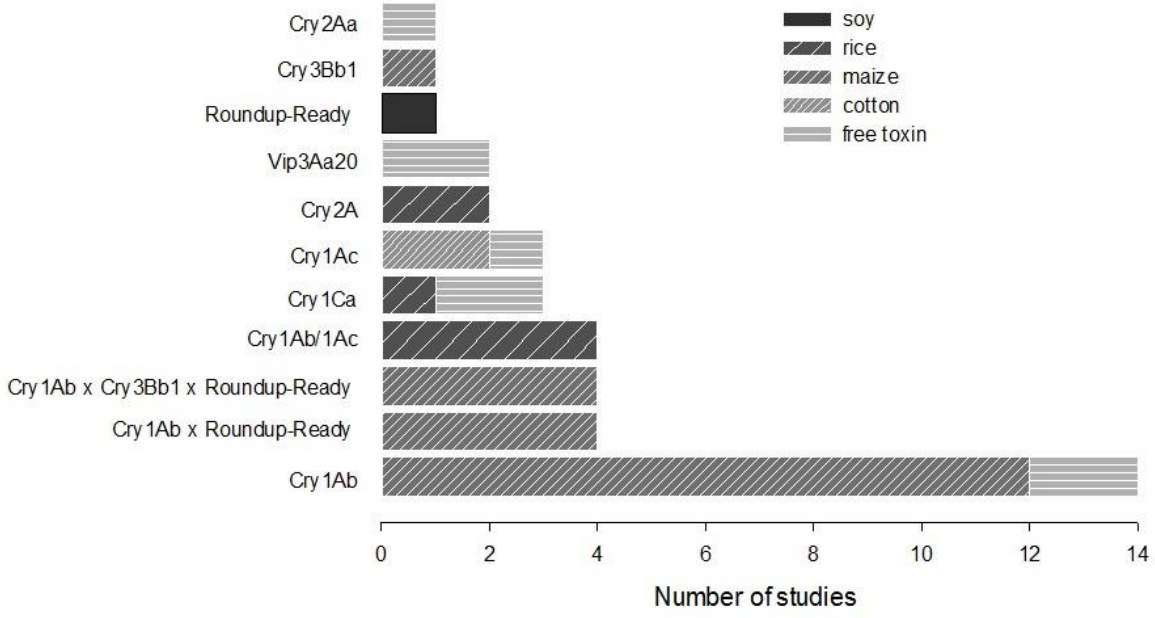


Figure 1: Novel proteins (n = 39) investigated in 31 effect studies. The classification of plant species (soy, rice, maize, or cotton) is based on the plant material used, extracts, or leachates in the studies.

Table 1: Overview of studies reporting on biological effects of genetically modified organisms on aquatic systems.([1] R-R = Roundup Ready)

Organism Order	Species	Test substrate	Plant	Novel Protein[1]	GMO-event	Results	Ref.
Cladocera	*Daphnia magna*	kernels	maize	Cry1Ab	MON810	Increased mortality (67 ± 27 SD ng Cry1Ab per gram dried grain tissue); no effect on growth; fewer individuals reaching maturity; lower age at maturation	a
Cladocera	*Daphnia magna*	kernels + predator smell	maize	Cry1Ab	MON810	Increased mortality (67 ± 27 SD ng Cry1Ab per gram dried grain tissue); reduced growth; reduced fecundity	b
Cladocera	*Daphnia magna*	kernels	maize	Cry1Ab	MON810	Reduced population growth	b
Cladocera	*Daphnia magna*	toxin	not specified	Cry1Ab, Cry2Aa	not specified	Increased mortality; high concn: reduced growth and fecundity; low concn: enhanced growth and fecundity; increase of early reproduction	c
Cladocera	*Daphnia magna*	soybean meal	soy	R-R	not specified	Increase mortality; reduced growth; reduced cumulative fecundity	e
Cladocera	*Daphnia magna*	leaves	maize	Cry1Ab	MON810	No effect on mortality; reduced growth; fewer juveniles per stage; more ephippia production; no effect on cumulative fecundity and age at maturation	f
Cladocera	*Daphnia magna*	leachate	rice	Cry2A, Cry1Ab/1Ac	not specified, Huahui-1	Increased mortality; no effect on growth; enhanced total reproduction per adult	i
Cladocera	*Daphnia magna*	toxin	not specified	Vip3Aa20	not specified	No effect on mortality; reduced growth in one low concentration; Increased fecundity	l
Cladocera	*Daphnia magna*	toxin	not specified	Vip3Aa20	not specified	no effect on mortality; enhanced growth in one higher concn; lower age at maturation; medium concn: enhanced fecundity; high concn: reduced fecundity	l

Cladocera	*Daphnia hyalina*	leachate	rice	Cry1Ab/1Ac	Bt-Minghui63, Bt-Shanyou63	No effect on population density	n
Cladocera	*Daphnia hyalina*	toxin	not specified	Cry1Ab, Cry1Ac	not specified	No effect on population density	n
Cladocera	*Daphnia magna*	rice seed meal	rice	Cry1Ab/1Ac	Bt-Shanyou63	Increased mortality at the lowest out of three concentrations (2.3 ± 0.33 SD µg Cry1Ab/1Ac per gram dried grain tissue); reduced growth at the lowest out of three concns; no effect on fecundity	r
Amphipoda	*Hyallela aszteca*	leaves	maize	Cry1Ab	not specified	No effect on mortality and growth	d
Amphipoda	*Hyallela aszteca*	seed extract	cotton	Cry1Ac	GK-12	Increased mortality	h
Amphipoda	*Hyallela aszteca*	leave discs	maize	Cry1Ab	not specified	No effect on mortality	q
Isopoda	*Ceacidotia communis*	leaves	maize	Cry1Ab x R-R, Cry1Ab x Cry3Bb1 x R-R	MON810 x NK603, MON810 x MON863 x NK603	Cry1Ab x R-R: increased mortality; reduced growth; Cry1Ab x Cry3Bb1 x R-R: no effect on mortality and growth	g
Decapoda	*Orconectes rusticus*	leaves, stalks, cobs	maize	Cry1Ab	not specified	Increased mortality; reduced growth; no effect on consumption	j
Trichoptera	*Lepidostoma liba*	leaves	maize	Cry1Ab	not specified	No effect on mortality; reduced growth	d
Trichoptera	*Lepidostoma* sp.	leaves	maize	Cry1Ab x R-R, Cry1Ab x Cry3Bb1 x R-R	MON810 x NK603, MON810 x MON863 x NK603	No effect on mortality and growth	g
Trichoptera	*Pycnospyche* cf. *srabripennis*	leaves	maize	Cry1Ab x R-R, Cry1Ab x Cry3Bb1 x R-R	MON810 x NK603, MON810 x MON863 x NK603	No effect on mortality; increased growth	g
Trichoptera	*Lepidostoma liba*	leaves	maize	Cry1Ab	not specified	No effect on mortality; reduced growth	m

Trichoptera	*Helicopsyche borealis*	pollen	maize	Cry1Ab	not specified	Increased mortality	m
Diptera	*Tipula (Nippo-tipula)* cf. *abdominalis*	leaves	maize	Cry1Ab x R-R, Cry1Ab x Cry3Bb1 x R-R	MON810 x NK603, MON810 x MON863 x NK603	Cry1Ab: no effect on mortality; reduced growth; Cry1Ab x Cry3Bb1: no effect on mortality and growth	g
Diptera	*Chirnomus dilutus*	seed extract	cotton	Cry1Ac	GK-12	Increased mortality	h
Diptera	*Chirnomus dilutus*	root extract	maize	Cry3Bb1	MON863	Increased mortality; no effect on growth	k
Coleoptera	*Ancyronyx* spp.	leave discs	maize	Cry1Ab	not specified	No effect on mortality	q
Pulmonata	*Gyraulus*	leaves	maize	Cry1Ab	not specified	No effect on mortality and growth	d
Peniculida	*Paramecium caudatum*	leachate	rice	Cry2A, Cry1Ab/1Ac	not specified, Huahui-1	Reduced density	i
Chlorellales	*Chlorella pyrenoidosa*	toxin	not specified	Cry1Ca	not specified	No effect on population growth	o
Chlorellales	*Chlorella pyrenoidosa*	leachate	rice	Cry1Ca	T1C-19	No effect on population growth; reduced enzym concentration (Malondialedahyde, T-SOD)	p
Chlorellales	*Chlorella pyrenoidosa*	toxin	not specified	Cry1Ca	not specified	No effect on population growth	p

a Bøhn et al. 2008, **b** Bøhn et al. 2010, **c** Bøhn et al. 2016, **d** Chambers et al. 2010, **e** Cuhra et al. 2015, **f** Holderbaum et al. 2015, **g** Jensen et al. 2010, **h** Li et al. 2013, **I** Li et al. 2014, **j** Linn & Moore 2014, **k** Prihoda & Coats 2008, **l** Raybould et al. 2014, **m** Rosi-Marshall et al. 2007, **n** Wang et al. 2013b, **o** Wang et al. 2014a, **p** Wang et al. 2014b, **q** Whiting & Lydy 2015, **r** Zhang et al. 2016

Another important aspect of effect studies is the range of invertebrate test organisms used (Figure 2). Crustaceans were the most frequently studied (63%), in particular daphnids (order Cladocera; 44%). The Amphipoda (11%), Isopoda (4%), and Decapoda (4%) were investigated to a small extent.

Insect studies (34% of effects studies; Table 1) were dominated by experiments with the Trichoptera, which play an important role as decomposers of allochthonous plant inputs in streams (Gonzalez and Graca, 2003; Suter and Cormier, 2015; Wallace and Webster, 1996; Wantzen and Wagner, 2006). Many of the Cry toxins (e.g. Cry1Ab, Cry1A.105, Cry1Ac, Cry1F, Cry2Ab2, and Vip3A) used in Bt plants target the Lepidoptera and, therefore, may also affect the phylogenetically close Trichoptera. Other insect groups, molluscs or protists were only studied less frequently (Table 1). However, tests with aquatic macrophytes or bacteria were missing, although the latter represent an important group of decomposers.

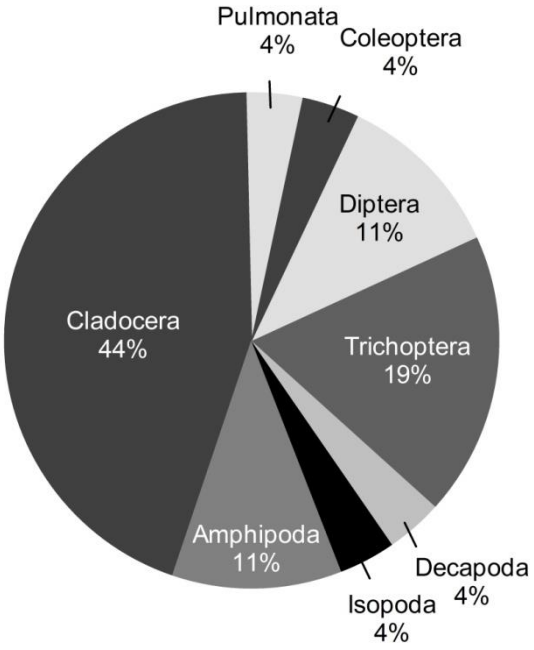


Figure 2: Invertebrate taxa investigated in 27 single-effect studies (n = 27 organisms).

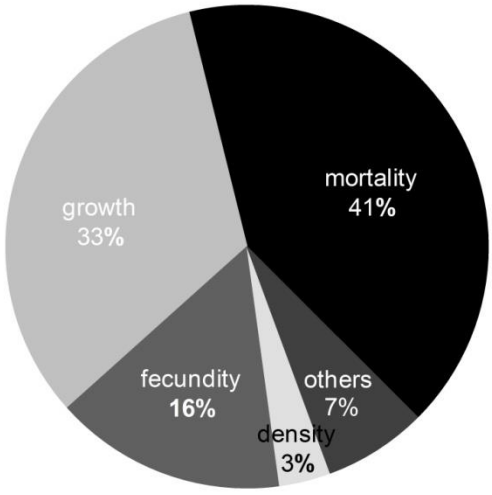


Figure 3: Endpoints in 27 single-species effect studies with invertebrates. 'Others' are further endpoints such as demography, biomass, consumption, or ephippia production (n = 58 endpoints).

In invertebrates, mortality and growth were the two most frequently used endpoints (Figure 3; Table 1). Besides the lethal endpoints, several sub-lethal endpoints were investigated, however, partly only in a small number of studies. We consider the fact that reproduction (e.g. individual fecundity, population fecundity, age at maturation, or stage fecundity) was used as an endpoint

in 16% of the invertebrate studies as positive; however, only one species (*D. magna*) was covered in these studies.

Effects on crustaceans

Overall, studies on crustaceans indicated a potential hazard of Bt toxins to daphnids (Table 1), yet some studies (e.g. Holderbaum et al., 2015) could not detect an effect on mortality. In addition to *in planta* studies, several experiments were carried out exposing *Daphnia* through the water phase to Bt proteins. Besides mortality and density, in other studies the growth of *Daphnia* was also assessed (Table 1), yet the outcomes varied. Several studies showed no clear dose-response relationship for *Daphnia* (Raybould et al., 2014; Zhang et al., 2016).

In some studies, different effects on the fecundity of *Daphnia* were observed after being fed Cry1Ab-containing maize (MON810): lower fecundity (Bøhn et al., 2010), lower total number of eggs (Bøhn et al., 2008; Bøhn et al., 2010), fewer individuals reaching maturity (Bøhn et al., 2008), lower age at maturation (Bøhn et al., 2008), fewer juveniles per stage (Holderbaum et al., 2015), and more ephippia production (Holderbaum et al., 2015) when fed transgenic maize compared to when fed non-transgenic maize. However, Holderbaum et al. (2015) showed no effect of Cry1Ab maize on cumulative fecundity and age at maturation. In *D. magna,* combinatorial effects of Cry1Ab, Cry2Aa and Roundup-Ready were investigated (Bøhn et al., 2016). They found that the combination of the two toxins Cry1Ab and Cry2Aa caused higher mortality than single toxins and that herbicide and insect resistance Bt toxins may interact. These experiments also provided evidence for possible trade-offs in life history parameters such as an increase in early reproduction at the cost of higher mortality in later life stages.

Only one study was carried out with the Isopoda (Jensen et al., 2010) and showed for some treatment groups significantly reduced body length, lower body mass, and lower survival, while a study using the amphipod *Hyalella azteca* fed Cry1Ab maize (unspecified event) did not show any effects in either mortality (Chambers et al., 2010; Whiting and Lydy, 2015) or growth (Chambers et al., 2010; Table 1).

Effects on insects

Despite their close taxonomic relationship to the Lepidoptera, caddisflies showed varied responses in effect studies, both for lethal and sub-lethal endpoints. The algal-scraping trichopteran *Helicopsyche borealis* had higher mortality when fed a high concentration of pollen from an unspecified Cry1Ab maize event (43%) than when fed the non-GM control (18%) (Rosi-Marshall et al., 2007; Table 1). Several laboratory feeding studies with

trichopterans (*Lepidostoma liba, Lepidostoma* sp., *Pycnopsyche* cf. *scabripennis*) found no statistically significant effects on mortality when fed on GM plant material (MON810 x NK603; MON863 x MON810 x NK603 or unspecified events) compared to when fed on a non-GM control (Chambers et al., 2010; Jensen et al., 2010; Rosi-Marshall et al., 2007).

A study exposed the larvae of the crane fly *Tipula (Nippotipula)* cf. *abdominalis* (Diptera) to Cry1Ab and Cry1Ab x Cry3Bb1 containing feed (Jensen et al., 2010). The Cry1Ab treatment showed no effect on mortality, but showed a growth reduction by 19.6% compared to the control. In contrast, the Cry1Ab x Cry3Bb1 treatment had no effect either on mortality or on growth. Because of the lack of effects of the stacked Bt treatment, the authors suggested that the Cry1Ab toxin was not the reason for reduced growth. This interpretation neglects the possibility of an antagonistic effect between Bt proteins. Larvae of the dipteran midge *Chironomus dilutus* were fed a root extract of Cry3Bb1-producing maize (event MON863; (Prihoda and Coats, 2008); in contrast to the results of Jensen et al. (2010), Cry3Bb1 triggered significantly higher mortality at higher concentrations than in the negative control without affecting larval growth. In another experiment (Li et al., 2013), *C. dilutus* was exposed to different concentrations of an extract of GM-cotton seeds (event GK-12) containing Cry1Ac, which represents, to our knowledge, the only case where an LC_{50} value has been determined for a GMO in an aquatic non-target organism (NTO). Overall, no studies with the dipteran-specific Cry2 and Cry4 proteins have been conducted, but results show that Cry1 and Cry3 proteins could also have effects on mortality and growth of dipterans.

Effects on other groups

Riffle beetle larvae (Elmidae: *Ancyronyx* spp.) showed no effects on mortality, and the pulmonate *Gyraulus* sp. showed no effects on mortality and growth after being fed Cry1Ab maize (Table 1). *Paramecium caudatum*, a unicellular organism, showed lower density after exposure to a leachate of GM rice lines producing Cry2A (unspecified event) or Cry1Ab/1Ac, while the green alga *Chlorella pyrenoidosa*, responded with altered biomarkers for oxidative stress (Table 1).

Despite the increasing number of stacked GMO events, including up to six different Bt toxins and combinations with several herbicide resistance traits, only two stacked events have been used in studies reviewed here. Even with no stacked events, combinatorial effects are highly relevant for risk assessment as aquatic systems may accumulate GMO residues from many fields and, therefore, from different GMOs. We know from terrestrial systems that

combinatorial effects, e.g. those between different Bt proteins, are poorly understood and difficult to predict (De Schrijver et al., 2016; Hilbeck and Otto, 2015); therefore, testing is the only way to provide reliable information for risk assessment. The lack of common methodology makes comparison of different studies difficult. We suggest that standardized test procedures are developed and aquatic ecotoxicity tests are part of the data provided for authorization of GMOs.

Based on the assumption that effects are most likely to occur in organisms of the same taxonomic order as the target organism (van Frankenhuyzen, 2009, 2013), we conclude that in some cases the test organism selected for aquatic testing of GMOs is not meaningful. For instance, Cry2A protein is active against both Lepidopteran and Dipteran, but was used only in tests with the Cladocera and Peniculida (Bøhn et al., 2016; Li et al., 2014). Further, the proteins Cry1Ca, Cry1Ab/1Ac confer lepidopteran-specific resistance but were only analysed in tests with the Chlorellales, Cladocera, and Peniculida, respectively. Overall, effect studies demonstrate that species selection has not been systematic and that most of the Bt toxins used in GM crops have not been tested systematically on aquatic species. Recently, a comprehensive method to assist the selection of aquatic habitats most likely at risk has been developed (Bundschuh et al., 2016). Hilbeck et al. (2008 and 2014) developed a procedure to use information from exposed environments to assist the selection of ecologically meaningful terrestrial test species in ecotoxicity tests, which has now been adapted for use in aquatic environments (Hilbeck et al., 2017).

Results of the studies with caddisflies are inconclusive but a hazard could be identified in some experiments. So far, only shredding and scraping caddisflies have been used; however, net spinning caddisflies are lacking, although, compared to biofilm feeders, they may be exposed in the most direct way to GM plant residues and pollen. Experiments with caddisflies are restricted to three genera (*Lepidostoma, Helicopsyche,* and *Pycnopsyche*); thus, information on most species is missing, although sensitivity to toxins may be expected to differ between species. Experiments so far have been carried out only with one or two different treatment concentrations and not with exposure via the water phase. Therefore, we recommend using several concentrations in experiments to create a dose response curve.

As crustaceans play an important role in many aquatic ecosystems, including those in proximity to maize cultivation, hazard related to these organisms may have ecosystem-wide consequences. Consistent with increasing reports on cross-order and cross-phylum specificity of Bt proteins (van Frankenhuyzen, 2009, 2013), experiments indicate that cross-activities may

also affect crustaceans. In particular, Bt proteins expressed in insect-resistant GMOs were observed to affect arthropod taxa different from the target organisms; for example, Cry1Ab, targeting the Lepidoptera, had effects on chironomids (Diptera) and Cry3Bb1, targeting the Coleoptera had effects on tipulids (Diptera: Tipulidae)(Jensen et al., 2010). Effects may manifest as lethal or sub-lethal. Fecundity endpoints in particular could reveal meaningful information on sub-lethal damage to *Daphnia*. Results indicate that changes in life history parameters, such as decreased age at maturation or increased fecundity in early life (e.g. Li et al., 2014; Raybould et al., 2014), may be useful for assessing sub-lethal effects. Because of chronic exposure patterns, we recommend further investigation of reproductive parameters on a regular basis in experiments, including consideration of potential population effects.

Overall, a number of effect studies provides evidence that hazard from Bt crops to aquatic NTOs do exist, and thus, should be considered in GMO risk assessment. To do so, the hazard has to be compared with the expected environmental concentrations of the toxin, e.g. in the form of a hazard quotient (Suter II, 2006). To date, not enough data are available to perform this comparison. More effort should be given to determining dose response curves with GMOs and aquatic organisms. Adequate data would enable benchmarks, such as LC_{50}, EC_{50} (concentration, which causes an effect in 50% of test organisms), NOEC (no observed effect concentration), and LOEC (lowest observed effect concentration), which ensure comparability of results.

Exposure studies

The exposure of the aquatic environment to novel proteins or plant residues was measured in 59% or 41%, respectively, of studies in the review. Bt proteins were the only novel proteins investigated. While all studies that measured Bt proteins specified the plant of origin, not all studies that measured the presence or input of GM plant residues clarified the type of Bt toxin. Mainly maize and, to some extent, rice have been used in exposure studies, while data on many other major GM crops, such as soy, cotton, or oilseed rape are missing. In total, four different Bt toxins (Figure 4) and six different GMO events (maize: MON810, MON88017 x MON810, MIR162; rice: Huahui-1, Bt-Minghui63, Bt-Shanyou63) were investigated in exposure studies. In 10 studies, however, the GM event was not provided.

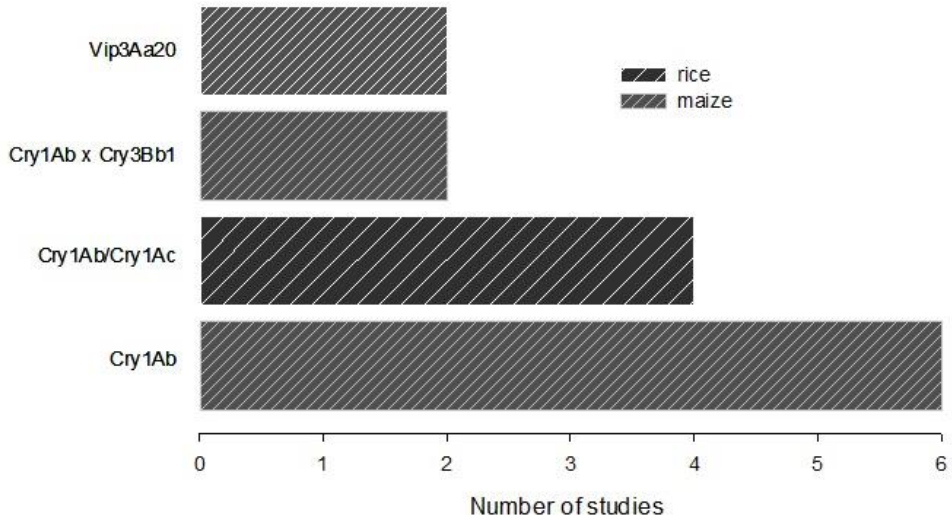


Figure 4: Novel proteins (n =14) investigated in 14 exposure studies. Data do not include studies with plant material, which did not determine the novel protein. In 11 studies, exposure to plant material or to free toxin in samplings near GM crops was measured. In three cases, exposure to Bt toxin in maize was modelled.

Different approaches were used to assess the exposure of the aquatic environment to the GMO (Table 2). The direct input of GM plant material from the field into the aquatic environment was assessed as well as the mobility of plant material in surface water. Only two studies included the spatial distribution of the GMO or its novel proteins in aquatic systems (Kratz et al., 2010; Rosi-Marshall et al., 2007). Besides measuring the amount of GM plant material, the amount of Bt toxins was analytically quantified directly in the aquatic environment, however, only one study directly tested and quantified the exposure of organisms themselves (Rosi-Marshall et al., 2007).

Studies that investigated the exposure of the aquatic environment to GMOs provide evidence that waterbodies are exposed to GM plant residues and free Bt toxin through multiple inputs. In total, four pathways exist for entry of GMO plant material or dissolved Cry toxin into the aquatic environment: (1) groundwater and soil pore water (Cry toxin) (Strain et al., 2014; Strain and Lydy, 2015; Whiting et al., 2014); (2) surface run-off (plant material, Cry toxin) (Strain et al., 2014; Strain and Lydy, 2015; Whiting et al., 2014); (3) wind drift, e.g. during harvesting (plant material)(Jensen et al., 2010; Kratz et al., 2010); and (4) drainage (Cry toxin)(Griffiths et al., 2017; Tank et al., 2010). All pathways were investigated to different degrees. However, the quantity and dynamics of the transport of GM crop residue input into plant residues within waterbodies are poorly understood. Figure 5 illustrates entry pathways and summarizes measured values from exposure studies.

Table 2: Description of approaches used in exposure studies (n=22).

Approach	Description	n	References
Environmental sampling	Taking and analysing several types of samples (plant residues, sediment, water, organisms).	10	a, b, e, g, h, i, j, k, m
Modelling	Modelling aquatic estimated environmental concentrations (EECs).	3	f, l
Standing stock	Recording the presence or absence of crop tissue in surface water at specific sites.	2	c, j
Litter trap	Placing cages beside surface water and measuring crop tissue input from the field.	3	c, d, g
Pollen trap	Investigating pollen input into surface waters using a trap.	1	g
Transport distance	Measuring transport distance of maize pollen, leaves and cobs in the stream.	1	g
Root exudates	Measuring the release of a Bt toxin from the roots of a GM plant.	1	k
Greenhouse experiments	Planting a Bt-plant in a cube container and measuring the amount of the Bt toxin in water and soil samples.	1	e

a Douville et al., 2005, **b** Griffith et al. 2017, **c** Jensen et al., 2010, **d** Kratz et al., 2010, **e** Liu et al., 2016, **f** Raybould and Vlachos, 2011, **g** Rosi-Marshall et al., 2007, **h** Strain et al., 2014, **i** Strain and Lydy, 2015, **j** Tank et al., 2010, **k** Wang et al., 2013a, **l** Wolt and Peterson, 2010, **m** Whiting et al., 2014

Tank et al. (2010) measured free Cry1Ab protein in water, with a mean concentration of 14 ± 5 ng/L, in 23% of all sampling sites near Bt-maize fields, whereas Douville et al. (2005) were able to obtain only one Cry1Ab measurement above the detection limit (200 ng/L) in 11 water samples near Bt-maize fields. A further study detected Cry1Ab in streams near Bt-fields in 73% of all sampling sites (Griffiths et al., 2017). The Cry1Ab toxin could be measured only rarely in groundwater and pore water samples of Bt-maize fields, with measured levels of 21.7 ng/L and 17.2 ng/L (Strain et al., 2014; Strain and Lydy, 2015; Whiting et al., 2014). Cry1Ab protein was also detected in tile drains from drainages (Griffiths et al., 2017; Tank et al., 2010) in a concentration of 21 ng/l (Tank et al., 2010)

Free Bt toxin (Cry1Ab) can move with surface run-off water and surface run-off sediment from the field into the aquatic environment (Strain et al., 2014; Strain and Lydy, 2015; Whiting et

al., 2014). This is supported by results that showed Cry1Ab concentrations in run-off water and run-off sediment from a GM-maize field up to 130 ng/L and 143 ng/g dw, respectively (Strain et al., 2014; Strain and Lydy, 2015; Whiting et al., 2014).

In view of the different GM traits, crop types, agricultural practices, and regional differences, quantitative information on the exposure of water bodies to GM plant residues is very limited and based only on headwater streams in two US maize growing regions (Indiana, Maryland) (Jensen et al., 2010; Rosi-Marshall et al., 2007; Tank et al., 2010) and one region in Germany (Kratz et al., 2010). The data summarized below, therefore, should be used with caution. Different methods and units make an appropriate comparison of test results difficult. Tank et al. (2010) found maize leaves, cobs or stalks in 86% of the investigated streams; they could detect Cry1Ab protein in maize residues in 13% of the sites, with a mean concentration of 95 ± 73 ng/g dry mass. Similar field surveys for other maize growing systems in different countries and for different types of aquatic habitats are missing. In the US, lateral maize debris input and the existence of maize material in a stream adjacent to a maize field were demonstrated by using litter traps and a standing stock study (Jensen et al., 2010). Jensen et al. (2010) detected maize debris input shortly after harvest in October; it peaked in February/March, with a maximal input of approximately 8 g AFDM/m/d, and stopped in April with new vegetation growth. Maize debris was found almost the whole year inside the stream channel, but showed high variation according to date and location (Jensen et al., 2010). Other data show that the annual input from maize residues in a stream ranged from 0.1 to 7.9 g AFDM/m^2 and the annual pollen input from 0.1 to 1.0 g/m^2 (Rosi-Marshall et al., 2007). Due to different units, data from the two publications are scarcely comparable. In Germany, plant material was shown to enter the aquatic environment during harvesting. The deposition of harvest by-products (maize) showed a distance-related gradient with a mean deposition of 26 g dw/m^2 at the edge of the field and 0.17 g dw/m^2 at a distance of 133 m, with fine particles dominating with increasing distance (Kratz et al., 2010). In headwater streams, Rosi-Marshall et al. (2007) recorded travelling distances of 0.38–180 m for maize leaves/cobs and 20–60 m for pollen.

Toxins were investigated not only in water but also in the sediment of waterbodies. Cry1Aab concentrations between 0.5 and 0.9 ppb (=0.5–0.9 ng/g) were measured in sediments in sampling sites near Bt-maize fields (Douville et al., 2005). Besides the toxin, maize residues also entered stream sediment in amounts up to 6.4 g AFDM/m^2 (Rosi-Marshall et al., 2007).

Special attention should be paid to GM rice, since rice fields are often flooded with water and, therefore, may represent an aquatic environment. This is the reason for data from field soil

measurements being included in this review only for rice and not for other GM crops. Greenhouse and field studies detected Cry1Ab/1Ac in the soil at concentrations of 14.4 and 23.5 ng/g dw at a distance of 5 cm and 3.7 ng/g dw at a distance of 25 cm from the rice plant and showed peak concentrations in the earing stage (Liu et al., 2016). The Cry1Ab/1Ac toxin concentration in soil of a Bt-SY63 (Bt-Shanyou63) rice field (1.264 – 2.125 ng/g) was significantly higher than that in non-Bt-SY63 rice plots (Wang et al., 2013a). However, the Bt concentration in Bt-MH63 (Bt-Minghui63) rice fields (0.816–1.655 ng/g soil) did not differ from that of conventional fields (Wang et al., 2013a). The authors assumed that the presence of the common soil bacterium *B. thuringiensis* led to a basal level of Cry1Ab/1Ac in the soil, which could also be measured in fields planted with non-Bt rice (Wang et al., 2013a). Results from a hydroponic culture with Bt-MH63 rice showed that Cry1Ab/1Ac is released via root exudates (0.06–0.09 ng/ml; Wang et al., 2013a). Therefore, inputs of Bt toxin into the soil of rice fields may be from both degradation of plant tissues and root exudates. In the water phase, Wang et al. (2013a) measured maximum concentrations of Cry1Ab/1Ac of 0.031 ng/ml and 0.023 ng/ml for rice events Bt-SY63 and Bt-MH63, respectively. Highest values were recorded during anthesis, indicating that toxin input via pollen may contribute to the recorded concentrations.

Besides the estimation of environmental concentrations, the actual intake of GMOs or Bt toxin by aquatic organisms is also an important aspect of exposure assessment. Direct intake of plant residues by caddisflies has been shown (Rosi-Marshall et al., 2007). About 50% of caddisflies collected during peak pollen shedding had pollen in their guts. Feeding on plant material potentially harbouring systemic pesticides is an important exposure pathway (Englert et al., 2017b) and should be investigated in more detail also in the context of GMOs.

As an alternative to analytical measurements, simulation models (GENEEC, FIRST), currently used to estimate the intake of pesticides into waterbodies have been applied to GMOs to estimate the environmental concentration (EEC) of Bt toxins in water. Model outputs predicted peak EECs of Cry1Ab from maize directly after harvest of 1.3, 1.2, and 7.2 µg/L for three scenarios, the GENEEC pond (a static pond of 20,000 m^3 volume, 2 m deep, draining a 10-ha field planted with maize), the FIRST index reservoir (a 144,000 m^3 water body, 2.74 m deep catching run-off from a 172.8 ha watershed with 56% of area planted with maize and with annual flow of twice the reservoir volume), and a semi-aquatic wetland (represented as a 3600 m^3 volume, 0.15 m deep, draining a 10 ha field planted with maize) (Wolt and Peterson, 2010), respectively. For the Vip3Aa20 protein from the maize event MIR162, calculations based on

the GENEEC model, with two different assumptions concerning the average concentration of Vip3Aa20 in plants, predicted EECs of 74.05 µg/L and 57.08 µg/L (Raybould and Vlachos, 2011).

In comparison, measurements of Bt toxins from surface water could be detected only in some samples and at concentrations one to two orders lower (14 ng/L, 200 ng/L; Douville et al., 2005; Tank et al., 2010) than the modelled concentrations. This may imply that the model calculations used are sufficiently precautionary for use in risk assessment. However, it should be noted that both GENEEC and FIRST were calibrated for soluble proteins and assumed that substances were homogenously distributed in the aquatic environment. Hence, accumulation of plant residues, which must be considered, was not included. In streams, sections with reduced flow velocity may cause sinks of accumulated plant tissues causing exposure much greater than predicted by average input (Bundschuh et al., 2016). The amount of GM crop residues in these sinks is difficult to predict and may be influenced by various factors such as the hydro-dynamics of the stream in combination with cropping intensity or the quality of riparian buffer zones. In addition, the catchment area may be influenced by regional geography. Valley locations and long-range transport of crop residues in running waters may also increase local exposure. Another factor for inclusion in worst-case estimates of exposure is the impact of heavy rain and storms leading to massive and peaked input of crop residues (Venter and Bøhn, 2016).

In the EEC value determination for Cry1Ab in an aquatic environment, Wolt and Peterson (2010) included a half-life of 1.3 days. Several publications show that the Cry1Ab protein in maize degrades by 50% in a few hours to several days, but the protein can still be apparent after weeks, showing slow degradation over a long time scale (Böttger et al., 2015; Griffiths et al., 2009). Including only the half-life of a Cry protein as a parameter in an EEC calculation potentially overestimates the degradation of the protein and underestimates its concentration in the aquatic environment. As discussed previously, Cry proteins enter the aquatic environment through four pathways, but in the EEC calculation, only direct input of plant residues was included. Not taken into account were inputs of free Cry proteins via groundwater/soil pore water, surface run-off, and drainage. Multiple inputs of Cry proteins into the aquatic environment make the estimation of their concentrations more complex (Griffiths et al., 2017). For reliable EEC estimation, more data are needed. Overall, the small number of studies providing measured values from the field is remarkable. More data are necessary to validate models.

Overall, results from exposure studies indicate that the most relevant exposure route for Bt toxin from the field into streams is probably via wind drift and surface run-off of water or sediment. However, for reliable exposure assessment, all entry routes should be considered. To this end, a better understanding of the complex carry-over of GM plant residues and Bt toxins from the terrestrial to the aquatic environment is needed. Findings indicate that the aquatic environment is not only exposed to GM plant material for a long period but also that plant residues may move in the stream with the current (Rosi-Marshall et al., 2007) and thus may be carried to locations more distant from GM plant fields. However, this aspect has hardly been quantified or included in exposure assessments so far.

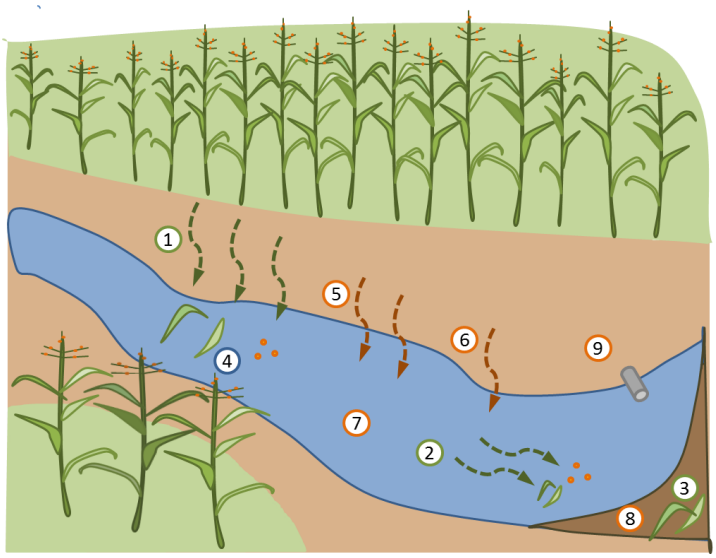


Figure 5: Pathways for exposure of the aquatic environment to GMOs. The tabulated data below show measured values, not modelling results. **a** Douville et al., 2005, **b** Griffith et al. 2017, **c** Kratz et al., 2010, **d** Rosi-Marshall et al., 2007, **e** Strain and Lydy, 2015, **f** Tank et al., 2010.

Plant material		Ref.
1	Wind drift: 0.1-7.9 g AFDM/m²	d
	Harvest drift: 26 g dw/m² (edge of the field)	c
	Pollen drift: 0.1-1.0 g/m²	d
2	Transport of plant material in flowing water: 0.38-180 m	d
	Transport of pollen in flowing water: 20-60 m	d
3	Plant material in sediment: up to 6.4 AFDM/m²	d

Free toxin		Ref.
5	Run-off water: up to 130 ng/L	e
	Run-off sediment: up to 143 ng/g dw	e
6	Groundwater, soil pore water: 17.2 ng/L, 21.7 ng/L (single observations)	e
7	Toxin in water: 0.014 ng/ml (mean), 0.2 ppb (single observation), up to 60 ng/L	a,b, f
8	Toxin in sediment: 0.5-0.9 ppb	a
9	Drainage: up to 60 ng/L	b,f

Toxin in plant material		Ref.
4	Toxin in plant material in water: 95 ± 73 ng/g dw	e

As well as in the aqueous matrix, Bt toxins and plant residues were also measured in the sediment and, thus, may lead to exposure of benthic organisms. Consequently, sediment effect

studies are also necessary. Five studies with test organisms commonly used for sediment toxicity testing (*C. dilutus, H. azteca*) have been conducted (Chambers et al., 2010; Li et al., 2013; Prihoda and Coats, 2008; Whiting and Lydy, 2015), but only Li et al. (2013) used exposure through the sediment.

More studies are necessary to quantify the actual intake of GM plant residues and Bt toxins into organisms. As no uniform procedures exist to measure exposure, we suggest that standardized test protocols are developed. We can conclude that the aquatic environment is exposed to GM plant material and Bt toxin, but so far, the precise extent of exposure and its spatial and temporal variability have not been quantified sufficiently.

Degradation studies

Degradation studies (N= 31 studies) were carried out with some important GM crop species (maize 52%, rice 23%, aspen-poplar 6%, cotton 3%) but did not include soy or rapeseed. In total, plant material from 11 different GMO events was deployed. Degradation studies strongly focus on maize and Cry proteins, in particular Cry1Ab. However, evidence for most Cry proteins is restricted to single studies. Apart from novel proteins dsRNA targeting pest insects was used in two studies (Albright et al., 2017; Fischer et al., 2017). Fig. 6 provides an overview over proteins and crop plants used in degradation studies.

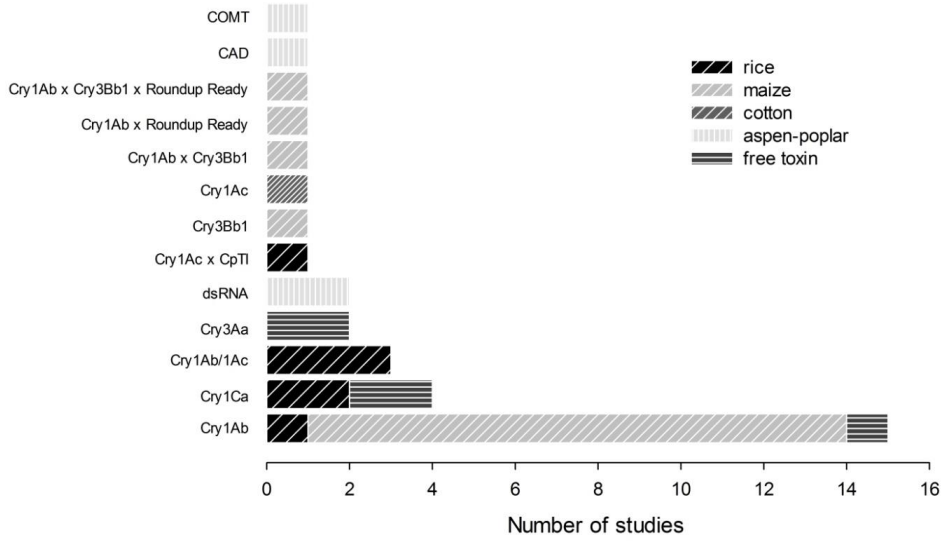


Figure 6: Novel genetic modification (n = 34) investigated in 31 degradation studies. Data do not include studies that did not determine the novel protein in the GM plant material. In 25 studies, plant material, extracts, and leachates were used. In one case, degradation of the Bt toxin in maize was modelled (Cry1Ab). CAD= Suppress cinnamyl alcohol dehydrogenas, COMT= Suppress caffeate/5-hydrogyfeulate O-mehtyltransferase, CpTI=cowpea trypsin inhibitor

A wide range of methods were used in experiments on the degradation of both the GM plant material and the respective novel proteins and on leaching of novel proteins from the plant matrix. A summary of the different approaches used in degradation studies are presented in Table 3. While in the laboratory spiking samples was the approach most commonly used field experiments were all carried out with litterbags. Modelling was deployed in one instance to predict degradation.

Table 3 Summary of approaches and results from degradation studies (n=31).

Material	Crop	Type	Method	n	References	Degradation
Laboratory						
Residues	maize	leaves	spiked samples	4	o	$t_{1/2}$ Cry1Ab: 2 h
Residues	maize	leaves	litter decomposition	1	d	$t_{1/2}$ Cry1Ab: 4 d; still 6% Cry1Ab after 21 d; no difference in dry weight between Bt and non-Bt maize after 21 d
Residues	maize	leaves	leaching	2	p, h	leaching over 10 d, after 70 d, still 1%
Residues	maize	leaves, stalks, roots	spiked samples	1	m	$t_{1/2}$ Cry3Bb1: 0.4-2.9 d
Residues	rice	leaves	spiked samples	1	i	after 135 d still 2.4% Cry1Ac in plant
Residues	rice	stems	leaching	1	t	leaching over 14 d
Residues	rice	straw	leaching	1	k	leaching at least over 7 d
Plant extract	cotton	Cry1Ac	spiked samples	1	j	$t_{1/2,\ sediment}$ Cry1Ac: 2.1-6.0 d, $t_{1/2,\ water}$ Cry1Ac: 11.0-15.8 d
Plant extract	rice	Cry1Ab	spiked samples	1	r	$t_{1/2}$ Cry1Ab: 82.5-210.0 d
Leachate	maize	Cry1Ab	spiked samples	3	h	microorganisms in water promote degradation
Leachate	rice	Cry1Ca	spiked samples	1	t	Cry1Ca detected in algae
Purified protein	-	Cry1Ab	spiked samples	1	e	$t_{1/2}$ Cry1Ab: 4.4-7 d
Purified protein	-	Cry1Ca	spiked samples	2	s	Cry1Ca detected in algae
Purified dsRNA	-	dsRNA (no biological activity), DvSnf7 dsRNA	spiked samples	2	c, f	$t_{1/2,\ water}$ dsRNA: 1.2-3 d $t_{1/2,\ sediment}$ dsRNA: 1.7-5.3 d
Field						
Residues	maize	leaves	litterbag	4	g, n, q	Mostly no or only small difference in degr. between Bt and non-Bt maize; difference only in single sites; after 1 h, still 39% Cry1Ab; after 70 d, still 20%

Residues	rice	leaves	litterbag	2	l	No difference in degr. between Bt and non-Bt rice
Residues	aspen - poplar	leaves	litterbag	2	a, b	no difference in degr. between GM and non-GM aspen-poplar
Mathematical model						
Purified protein	-	Cry1Ab	modelling	1	u	after 60 d still 8% Cry1Ab

a Axelsson et al., 2010, **b** Axelsson et al., 2011**, c** Albright et al., 2017, **d** Böttger et al., 2015, **e** Douville et al., 2005, **f** Fischer et al., 2016, **g** Griffith et al., 2009, **h** Griffith et al. 2017, **i** Li et al., 2007, **j** Li et al., 2013, **k** Liu et al., 2016, **l** Liu et al. 2017, **m** Prihoda and Coats, 2008, **n** Rosi-Marshall et al., 2007, **o** Strain and Lydy, 2015, **p** Strain et al., 2014, **q** Swan et al., 2009, **r** Wang et al., 2007, **s** Wang et al., 2014a, **t** Wang et al., 2014b, **u** Wolt and Peterson, 2010

Litterbag experiments as well as experiments involving microbial respiration, as an indicator of decomposition of plant material, suggest that the decomposition of GM crop material does not differ substantially from non-GM crop material (Table 3).

Degradation studies of Bt toxins in plant material show no uniform picture. Cry proteins were reported to show a half-life between few hours to several days (Table 3). At the same time studies indicate a long-term presence of Cry toxins in in plant residuals up to 135 and 210 days for Bt maize and Bt rice, respectively. A long-term presence was also predicted by modelling (Wolt and Peterson, 2010). The degradation of GM plant material or Bt toxin was influenced by experimental conditions, e.g. temperature (Li et al., 2013; Strain and Lydy, 2015), pH (Wang et al., 2007) and sterility of the matrix (Douville et al., 2005; Griffiths et al., 2017; Li et al., 2007; Li et al., 2013), whereas light showed no impact (Griffiths et al., 2017). Recently, GMO have been modified to express dsRNA to control insect pests. Degradation of dsRNA has been investigated so far in two studies which showed an initial lag phase and a subsequent rapid decline of dsRNA in water with a half-life of a few days.

Bt toxin concentration in plant leaves declines over time by leaching into the surrounding water. Leaching of Cry toxins (Cry1Ab, Cry1Ab/1Ac, Cry1Ca) from plant material (maize and rice) has been examined in four studies and been observed up to 25 days (approximately 7, 10, 14, 25 days; Griffiths et al., 2017; Liu et al., 2016; Strain et al., 2014; Wang et al., 2014b). Leached proteins may be absorbed to organic matter and surface-active particles (Prihoda and Coats, 2008; Strain and Lydy, 2015). In two studies by Wang et al. (2014b; 2014a) the green algae *Chlorella pyrenoidosa* absorbed the Bt toxin, Cry1Ca, when cultured in a medium containing either the pure toxin or a leachate of an T1C-19 rice event. The higher the exposure level to Cry1Ca, the higher its concentration was in algal cells. Venter and Bøhn (2016) speculated that the quantification of Bt toxins in water samples may be influenced by this absorption.

Summing up, degradation studies have included only a limited range of novel proteins and few GM events with a focus on insect resistant plants. Differences in methods and endpoints used are so broad that comparisons within crop/protein/endpoint combinations are hardly possible and evidence is often based on single studies. Several studies indicate a half-life of Bt toxins ranging from of few hours to several days, illustrating the uncertainties of present degradation estimates. Uncertainties are likely to be caused by a lack of standardization of methods (e.g. different temperature conditions) or methodological aspects related to the release of the active substance from plant tissue. Results show that microorganisms in surface water promote the degradation of Bt toxins within plant material and mixed in sediment. Furthermore, increasing temperature and decreasing pH values may lead to faster degradation. We therefore recommend that standardized test conditions are established, especially for temperature, pH, and sterility of the matrix, to ensure comparability between studies. As experiments should facilitate an estimate of environmental exposure over time, the risk assessment of a GMO would benefit from basic information on degradation of different plant tissues and from degradation experiments carried out under lower temperatures mimicking conditions in colder regions and over winter. In practical terms, estimates of the time for 90% of degradation and more information on the kinetics of degradation would be useful.

For a risk assessment non only degradation of novel protein in plant tissues but also leaching of proteins into the water phase is relevant as it represents a different exposure route. We assume that leaching is likely to lead to locations with high concentrations of free Bt toxins in the water due to accumulation of GM plant material in areas with low flow velocity. Higher concentrations of Bt toxins, in particular, could reach lakes with low water exchange. Algal adsorption of Cry toxins should be investigated in more detail to clarify whether feeding on such algae is an exposure pathway which should be included in the risk assessment.

Synthesis and outlook

Streams and lakes are substantially influenced by their terrestrial surroundings (Vannote et al., 1980). Allochthonous inputs are essential for many aquatic organisms, as direct sources of food or as indirect sources forming the basis of a detritivore food web. By summarizing the available experiments and results, we demonstrate that, by now, basic data exist on both the hazard of and exposure to GM crops, particularly Bt crops, for aquatic organisms and habitats. Therefore, risk assessment of GM crops for aquatic habitats is justified. However, this knowledge has thus far not led to wider risk analysis covering the main GM crops and relevant novel proteins.

In summary, these shortcomings may impair risk assessment, especially in the EU where authorization of GMO crop cultivation is linked to case-specific assessment (EU, 2001), which needs to be informed by data on the receiving environments. With the exception of one study (Kratz et al., 2010), no experiments have been conducted in the receiving environments of Europe. Results from US experiments, which were carried out in highly degraded headwater streams in the vicinity of intensively used agricultural areas (Jensen et al., 2010; Rosi-Marshall et al., 2007; Tank et al., 2010), can be extrapolated to the EU only to a limited extent. Results from these degraded headwater streams may have poor predictive value for aquatic habitats with good conservation status that accommodate organisms more sensitive to abiotic and biotic stressors. This situation is of high relevance in Europe where the WFD aims at good status for all ground and surface waters. Moreover, semi-natural and natural habitats may be more interspersed in Europe than in the sites studied in the US sites, thus increasing the likelihood of exposure of areas under nature protection.

Despite these issues, exposure estimates, such as those by Carstens et al. (2012), have been used in GMO risk assessment in Europe to argue that the risk for aquatic non-target organisms from cultivation of Bt maize can be considered negligible as exposure is anticipated to be very low (EFSA, 2011b). As we have described here, modelling assumptions, in particular the assumption of homogenous distribution of residues and/or novel proteins in aquatic environments, should be questioned. Based on our review, we believe that exposure will be context-dependent and, in many cases, spatially aggregated. Better data on the transport of crop residues, both from the field into waterbodies and, importantly, within waterbodies, play key roles for a fuller understanding of the exposure of habitats and organisms to GMOs and novel proteins.

It became apparent during this review that the lack of standardized methodologies to assess the fate and effects of novel proteins in or from plant residues impedes the comparability, and thus, the analysis of such data. A set of standardized methods or general guidance on methodology and endpoints of experiments with GM residues in aquatic environments would benefit risk assessment. Standards for aquatic ecotoxicity testing of GMOs should be developed in order to inform risk assessment better and to be able to handle the next generation of GMOs that may include plants expressing more bioactive compounds such as pharmaceuticals. New standards reflecting localization of active compounds within plant tissue will also be useful in assessment of systemic pesticides such as neonicotinoids (Englert et al., 2017b; Englert et al., 2017a). Both industry and regulators bear the responsibility to close identified knowledge gaps to improve our understanding of ecological effects of GM crop cultivation on aquatic ecosystems.

References

Albright VC, Wong CR, Hellmich RL, Coats JR. Dissipation of double-stranded RNA in aquatic microcosms. Environ Toxicol Chem 2017;36(5):1249–53.

Axelsson EP, Hjältén J, LeRoy CJ, Julkunen-Tiitto R, Wennström A, Pilate G. Can Leaf Litter from Genetically Modified Trees Affect Aquatic Ecosystems? Ecosystems 2010;13(7):1049–59.

Axelsson EP, Hjältén J, LeRoy CJ, Whitham TG, Julkunen-Tiitto R, Wennström A. Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition. J Appl Ecol 2011;48(6):1472–9.

Beachy RN, Fedoroff NV, Goldberg RB, McHughen A. The burden of proof: A response to Rosi-Marshall et al. Proc Natl Acad Sci USA 2008;105(7):E9; author reply E11.

Bøhn T, Primicerio R, Hessen DO, Traavik T. Reduced fitness of *Daphnia magna* fed a Bt-transgenic maize variety. Arch Environ Contam Toxicol 2008;55(4):584–92.

Bøhn T, Rover CM, Semenchuk PR. *Daphnia magna* negatively affected by chronic exposure to purified Cry-toxins. Food Chem Toxicol 2016;91:130–40.

Bøhn T, Traavik T, Primicerio R. Demographic responses of *Daphnia magna* fed transgenic Bt-maize. Ecotoxicology 2010;19(2):419–30.

Böttger R, Schaller J, Lintow S, Gert Dudel E. Aquatic degradation of Cry1Ab protein and decomposition dynamics of transgenic corn leaves under controlled conditions. Ecotoxicol Environ Saf 2015;113:454–9.

Bundschuh R, Kuhn U, Bundschuh M, Naegele C, Elsaesser D, Schlechtriemen U et al. Prioritizing stream types according to their potential risk to receive crop plant material - A GIS-based procedure to assist in the risk assessment of genetically modified crops and systemic insecticide residues. Sci Total Environ 2016;547:226–33.

Carstens K, Anderson J, Bachman P, De Schrijver A, Dively G, Federici B et al. Genetically modified crops and aquatic ecosystems: Considerations for environmental risk assessment and non-target organism testing. Transgenic Res 2012;21(4):813–42.

Chambers CP, Whiles MR, Rosi-Marshall EJ, Tank JL, Royer TV, Griffiths NA et al. Responses of stream macroinvertebrates to Bt maize leaf detritus. Ecol Appl 2010;20(7):1949–60.

Cuhra M, Traavik T, Bøhn T. Life cycle fitness differences in *Daphnia magna* fed Roundup-Ready soybean or conventional soybean or organic soybean. Aquacult Nutr 2015;21(5):702–13.

De Schrijver A, Devos Y, De Clercq P, Gathmann A, Romeis J. Quality of laboratory studies assessing effects of Bt-proteins on non-target organisms: Minimal criteria for acceptability. Transgenic Res 2016;25(4):395–411.

Douville M, Gagn F, Masson L, McKay J, Blaise C. Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. Biochem Syst Ecol 2005;33(3):219–32.

Duan JJ, Marvier M, Huesing J, Dively G, Huang ZY. A meta-analysis of effects of Bt crops on honey bees (Hymenoptera: Apidae). PloS one 2008;3(1):e1415.

EFSA. Scientific Opinion on application (EFSA-GMO-CZ-2008-54) for placing on the market of genetically modified insect resistant and herbicide tolerant maize MON 88017 for cultivation under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2011a;9(11):2428.

EFSA. Scientific Opinion updating the evaluation of the environmental risk assessment and risk management recommendations on insect resistant genetically modified maize 1507 for cultivation. EFSA Journal 2011b;9(11):2429.

Englert D, Bakanov N, Zubrod JP, Schulz R, Bundschuh M. Modeling Remobilization of Neonicotinoid Residues from Tree Foliage in Streams - A Relevant Exposure Pathway in Risk Assessment? Environ Sci Technol 2017a;51(3):1785–94.

Englert D, Zubrod JP, Link M, Mertins S, Schulz R, Bundschuh M. Does Waterborne Exposure Explain Effects Caused by Neonicotinoid-Contaminated Plant Material in Aquatic Systems? Environ Sci Technol 2017b;51(10):5793–802.

EU. Directive 2000/60/EC of European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy; 2000.

EU. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms; 2001.

Fischer JR, Zapata F, Dubelman S, Mueller GM, Uffman JP, Jiang C et al. Aquatic fate of a double-stranded RNA in a sediment-water system following an over-water application. Environ Toxicol Chem 2017;36(3):727–34.

Glare TR, O´Callaghan M. *Bacillus thuringiensis*: Biology, ecology and safety. Chichester: Wiley; 2000.

Gonzalez JM, Graca MAS. Conversion of leaf litter to secondary production by a shredding caddis-fly. Freshwater Biol 2003;48(9):1578–92.

Griffiths NA, Tank JL, Royer TV, Rosi EJ, Shogren AJ, Frauendorf TC et al. Occurrence, leaching, and degradation of Cry1Ab protein from transgenic maize detritus in agricultural streams. Sci Total Environ 2017;592:97–105.

Griffiths NA, Tank JL, Royer TV, Rosi-Marshall EJ, Whiles MR, Chambers CP et al. Rapid decomposition of maize detritus in agricultural headwater streams. Ecol Appl 2009;19(1):133–42.

Hilbeck A, Bundschuh R, Bundschuh M, Hofmann F, Oehen B, Otto M et al. Procedure to select test organisms for environmental risk assessment of genetically modified crops in aquatic systems. Integr Environ Assess Manag 2017;13(6):974–9.

Hilbeck A, Meier M, Benzler A. Identifying indicator species for post-release monitoring of genetically modified, herbicide resistant crops. Euphytica 2008;164(3):903–12.

Hilbeck A, Otto M. Specificity and combinatorial effects of Bacillus thuringiensis Cry toxins in the context of GMO Environmental Risk Assessment. Front Environ Sci 2015;3:467.

Hilbeck A, Weiss G, Oehen B, Römbke J, Jänsch S, Teichmann H et al. Ranking matrices as operational tools for the environmental risk assessment of genetically modified crops on non-target organisms. Ecological Indicators 2014;36:367–81.

Holderbaum DF, Cuhra M, Wickson F, Orth AI, Nodari RO, Bøhn T. Chronic Responses of *Daphnia magna* Under Dietary Exposure to Leaves of a Transgenic (Event MON810) Bt-Maize Hybrid and its Conventional Near-Isoline. J Toxicol Environ Health 2015;78(15):993–1007.

James C. Global Status of Commercialized Biotech/GM Crops: 2014: International Service for the Acquisition of Agri-biotech Applications (ISAAA); 2014.

Jensen PD, Dively GP, Swan CM, Lamp WO. Exposure and nontarget effects of transgenic Bt corn debris in streams. Environ Entomol 2010;39(2):707–14.

Kostov K, Damgaard C, Hendriksen N, Sweet JB, Krogh P. Are population abundances and biomasses of soil invertebrates changed by Bt crops compared with conventional crops?: A systematic review protocol. Environ Evid 2014;3(1):10.

Kratz W, Mante C, Hofmann F, Schlechtriemen U, Kuhn U, Ober S et al. Exposure of maize harvest by-products to aquatic ecosystems and protected nature reserves in Brandenburg, Germany. Theorie in der Ökologie 2010(16):21–3.

Li G, Wang Y, Liu B, Zhang G. Transgenic *Bacillus thuringiensis* (Bt) rice is safer to aquatic ecosystems than its non-transgenic counterpart. PloS one 2014;9(8):e104270.

Li Y, Wu K, Zhang Y, Yuan G. Degradation of Cry1Ac Protein Within Transgenic *Bacillus thuringiensis* Rice Tissues Under Field and Laboratory Conditions. Environ Entomol 2007;36(5):1275–82.

Li Y-L, Du J, Fang Z-X, You J. Dissipation of insecticidal Cry1Ac protein and its toxicity to nontarget aquatic organisms. J Agric Food Chem 2013;61(46):10864–71.

Linn MD, Moore PA. The effects of bt corn on rusty crayfish (*Orconectes rusticus*) growth and survival. Arch Environ Contam Toxicol 2014;67(3):436–43.

Liu Y, Jiang W, Liang Y, Zhao C, Li J. No effect of Bt-transgenic rice litter on the meiobenthos community in field ditches. Pest management science 2017;73(6):1213–9.

Liu Y, Li J, Luo Z, Wang H, Liu F. The fate of fusion Cry1Ab/1Ac proteins from Bt-transgenic rice in soil and water. Ecotoxicol Environ Saf 2016;124:455–9.

Lövei GL, Andow DA, Arpaia S. Transgenic Insecticidal Crops and Natural Enemies: A Detailed Review of Laboratory Studies. Environ Entomol 2009;38(2):293–306.

Marvier M, McCreedy C, Regetz J, Kareiva P. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 2007;316(5830):1475–7.

Naranjo S. Impacts of Bt crops on non-target invertebrates and insecticide use patterns. CAB Reviews 2009;4(011).

Parisi C, Tillie P, Rodríguez-Cerezo E. The global pipeline of GM crops out to 2020. Nat Biotechnol 2016;34(1):31–6.

Parrott W. Study of Bt impact on caddisflies overstates its conclusions: response to Rosi-Marshall et al. Proc Natl Acad Sci USA 2008;105(7):E10; author reply E11.

Prihoda KR, Coats JR. Aquatic fate and effects of *Bacillus thuringiensis* Cry3Bb1 protein: Toward risk assessment. Environ Toxicol Chem 2008;27(4):793–8.

Raybould A, Burns A, Hamer M. High concentrations of protein test substances may have non-toxic effects on *Daphnia magna*: Implications for regulatory study designs and ecological risk assessments for GM crops. GM Crops Food 2014;5(4):296–301.

Raybould A, Vlachos D. Non-target organism effects tests on Vip3A and their application to the ecological risk assessment for cultivation of MIR162 maize. Transgenic Res 2011;20(3):599–611.

Rosi-Marshall EJ, Tank JL, Royer TV, Whiles MR, Evans-White M, Chambers C et al. Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proc Natl Acad Sci USA 2007;104(41):16204–8.

Stehle S, Schulz R. Agricultural insecticides threaten surface waters at the global scale. Proc Natl Acad Sci USA 2015a;112(18):5750–5.

Stehle S, Schulz R. Pesticide authorization in the EU - environment unprotected? Environ Sci Pollut Res 2015b(22):19632–47.

Strain KE, Lydy MJ. The fate and transport of the Cry1Ab protein in an agricultural field and laboratory aquatic microcosms. Chemosphere 2015;132:94–100.

Strain KE, Whiting SA, Lydy MJ. Laboratory and field validation of a Cry1Ab protein quantitation method for water. Talanta 2014;128:109–16.

Suter GW, Cormier SM. Why care about aquatic insects: Uses, benefits, and services. Integr Enviro Assess Manage 2015;11(2):188–94.

Suter II GW. Ecological Risk Assessment, Second Edition. 2nd ed; 2006.

Swan CM, Jensen PD, Dively GP, Lamp WO. Processing of transgenic crop residues in stream ecosystems. J Appl Ecol 2009;14:711.

Tank JL, Rosi-Marshall EJ, Royer TV, Whiles MR, Griffiths NA, Frauendorf TC et al. Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. Proc Natl Acad Sci USA 2010;107(41):17645–50.

van Frankenhuyzen K. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr Pathol 2009;101(1):1–16.

van Frankenhuyzen K. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J Invertebr Pathol 2013;114(1):76–85.

Vannote RL, Minshall GW, Cummins KW, Sedell JR, Cushing CE. The River Continuum Concept. Can J Fish Aquat Sci 1980;37(1):130–7.

Venter HJ, Bøhn T. Interactions between Bt crops and aquatic ecosystems: A review. Environ Toxicol Chem 2016;35(12):2891–902.

Vörösmarty CJ, McIntyre PB, Gessner MO, Dudgeon D, Prusevich A, Green P et al. Global threats to human water security and river biodiversity. Nature 2010;467(7315):555–61.

Wallace JB, Webster JR. The role of macroinvertebrates in stream ecosystem function. Annu Rev Entomol 1996;41:115–39.

Wang H, Ye Q, Gan J, Wu L. Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils. J Agric Food Chem 2007;55(5):1900–4.

Wang J, Chen X, Li Y, Su C, Ding J, Peng Y. Green algae (*Chlorella pyrenoidosa*) adsorbs *Bacillus thurigiensis* (Bt) toxin, Cry1Ca insecticidal protein, without an effect on growth. Ecotoxicol Environ Saf 2014a;106:6–10.

Wang J, Chen X, Li Y, Zhu H, Ding J, Peng Y. Effect of straw leachates from Cry1Ca-expressing transgenic rice on the growth of *Chlorella pyrenoidosa*. Environ Toxicol Chem 2014b;33(5):1156–62.

Wang Y, Hu H, Huang J, Li J, Liu B, Zhang G. Determination of the movement and persistence of Cry1Ab/1Ac protein released from Bt transgenic rice under field and hydroponic conditions. Soil Biol Biochem 2013a;58:107–14.

Wang Y, Huang J, Hu H, Li J, Liu B, Zhang G. Field and laboratory studies on the impact of two Bt rice lines expressing a fusion protein Cry1Ab/1Ac on aquatic organisms. Ecotoxicol Environ Saf 2013b;92:87–93.

Wantzen KM, Wagner R. Detritus processing by invertebrate shredders: A neotropical-temperate comparison. J North Am Benthological Soc 2006;25(1):216–32.

Whiting SA, Lydy MJ. A site-specific ecological risk assessment for corn-associated insecticides. Integr Enviro Assess Manage 2015;11(3):445–58.

Whiting SA, Strain KE, Campbell LA, Young BG, Lydy MJ. A multi-year field study to evaluate the environmental fate and agronomic effects of insecticide mixtures. Sci Total Environ 2014;497-498:534–42.

Wolfenbarger LL, Naranjo SE, Lundgren JG, Bitzer RJ, Watrud LS. Bt crop effects on functional guilds of non-target arthropods: A meta-analysis. PloS one 2008;3(5):e2118.

Wolt JD, Peterson RKD. Prospective formulation of environmental risk assessments: Probabilistic screening for Cry1A(b) maize risk to aquatic insects. Ecotoxicol Environ Saf 2010;73(6):1182–8.

Zhang L, Guo R, Fang Z, Liu B. Genetically modified rice Bt-Shanyou63 expressing Cry1Ab/c protein does not harm *Daphnia magna*. Ecotoxicol Environ Saf 2016;132:196–201.

Supplementary Information for:

Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment

Antonia Pott[1,2*], Mathias Otto[1], Ralf Schulz[2]

[1] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[2] Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrassße 7, 76829 Landau, Germany

Die ergänzenden Informationen zu dieser Veröffentlichung sind auf der Homepage von dem Journal *Science of the Total Environment* unter folgendem Link zu finden: https://doi.org/10.1016/j.scitotenv.2018.04.013

A.2 Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders – an attempt to establish dose-effect relationships through food-spiking

Antonia Pott[1,2*], Mirco Bundschuh[1,3], Rebecca Bundschuh[1], Mathias Otto[2], Ralf Schulz[1]

[1] iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany

[2] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, 75007 Uppsala, Sweden

*Correspondence to pott@uni-landau.de

In: *Scientific reports* 10 (1), S. 5262.

Abstract

Genetically modified organisms (GMOs), which produce *Bacillus thuringiensis* (Bt) toxins, are widely used in agriculture in some parts of the world. Despite this, ecotoxicological methods, tailored to GMOs, are lacking to assess effects on aquatic environments. With the objective to investigate a food-related exposure pathway for aquatic shredders, we used a new food-spiking method while caddisfly larvae (*Chaetopteryx* spec., *Sericostoma* spec.) served as test species. Pure Cry1Ab toxins were spiked on black alder leaf discs and subsequently used in a feeding experiment. The toxin did not influence larval mortality compared to the control. The results, however, showed significant effects on larval lipid content (*Chaetopteryx* spec.) and development (*Sericostoma* spec.) at concentrations of 17.2 and 132.4 ng Cry1Ab/mg leaf, respectively. These changes are indicative for impacts in the fitness of the specimen and thus relevant in a risk assessment context. Ultimately, the food-spiking method allowed applying different Bt toxin concentrations leading to the establishment of dose-response relationships for various response variables. The use of long test durations and sublethal endpoints (consumption, lipid content, growth, larval instars) is, moreover, advisable when testing GMO effects.

Introduction

Genetically modified organisms (GMOs) have been cultivated for 20 years. An often applied genetic modification in agriculturally used crops is the expression of a synthetic *Bacillus thuringiensis* (Bt) toxin to achieve insect protection[1]. Bt crops integrate crystal proteins (Cry) or vegetative insecticidal proteins (Vip) in the plant tissues[2]. Target insects, which feed on Bt plants ingest the toxin, that is activated in the alkaline digestive tract and binds to the midgut epithelial membrane, which leads to pore formation and the death of target pest[2,3]. GMOs are regulated in many countries and are subject to an environmental risk assessment (ERA) including the assessment of effects from the GM-crop cultivation on non-target organisms[4]. In this respect ERA strongly focused on terrestrial effects. Only with the publication of Rosi-Marshall et al.[5], which showed adverse effects from GM-maize on detritus feeding caddisflies and quantified the entry of GM maize material into the aquatic environment, also freshwater ecosystems received more attention.

Consequently, only a small number of studies dealt with the effects of GM-crops on aquatic non-target organisms (NTO)[6]. Studies with daphnids, the most frequently assessed genus, for instance, suggested a potential hazard of Bt toxin[7–9]. Studies that address the impact of GM-

crop residues on detritus feeding caddisfly larvae (Trichoptera), which are taxonomically closely related to Lepidoptera targeted by some Cry toxins such as Cry1Ab, showed high variability in their responses[5,10,11]. This inconclusive picture with regards to the sensitivity of caddisfly larvae, suggests a complex interaction between toxins and the quality of plant material influencing the exposure of detritus feeding organisms and ultimately their responses.

In this context, it seems sensible to define potential exposure pathways based on which a testing strategy can be developed[12]. Waterborne exposure is likely of relatively low relevance for detritus feeding organisms as those organisms ingest plant material potentially containing Bt toxins, which likely reflects the major exposure pathway. Hence, the administration of those toxins as part of the food of these shredders[13] might be a truly worst case scenario. This requirement comes however with a substantial challenge, that is both the Bt toxin itself and its concentration are characteristic for a given GM event. These circumstances make the establishment of a dose response relationship based on the Bt concentration in the plant material hardly possible. A potential solution may be the artificial spiking of plant material with increasing Bt-toxin concentrations, while this spiked plant material serves as food for caddisfly larvae with the aim to derive a dose response relationship. At the same time this strategy allows to replace the crop plant material by a substrate that has a higher nutritious quality for shredders. Thereby, the wellbeing of the organisms during testing is improved, specifically in situations in which responses over long exposure durations are targeted.

Against this background, the present study aimed to investigate the feasibility of this testing strategy using Cry1Ab as a model Bt-toxin at increasing concentrations on black alder (*Alnus glutinosa*) leaf discs. As earlier studies suggested caddisfly larvae susceptible to Cry1Ab, the trichopteran species *Sericostoma* spec. and *Chaetopteryx* spec. were employed as model species (supported by own yet unpublished data). Their selection is motivated by a wide distribution throughout Europe[14–19] and their contribution to leaf litter decomposition[20,21]. Mortality, consumption, growth (case width), and larval instars (head capsule width) were recorded as response variables over a study duration of up to 12 weeks. In addition, the lipid content in caddisfly larvae was measured, as it is an important energy resource for insects during starvation and metamorphosis [22].

Material and Methods

Preparation of leaf discs

Black alder (*Alnus glutinosa*) leaves were collected in 2014 from trees near Landau, Germany (49°11` N; 8°05` E). After collection the leaves were stored in a freezer at -18°C. Some of the leaves were placed in a stream for 2 weeks to establish a natural microbial community[23]. Those leaves were later used for the conditioning (=colonization by heterotrophic microorganisms) of the leaf material used as food during the experiments. For the conditioning, leaf discs with 2 cm in diameter were cut from frozen leaves with a cork borer avoiding the main vein. These leaf discs were conditioned with leaves preconditioned in a natural stream for 10 days in medium[24]. The medium consisted of 100 mg $CaCl_2$ x $2H_2O$, 10 mg $MgSO_4$ x $7H_2O$, 500 mg morpholino propane sulfonic acid (MOPS), 100 mg KNO_3 and 5.5 mg K_2HPO_4 per litre. By using NaOH the pH was adjusted to 7.00±0.05. Afterwards the leaf discs were dried at 60°C for 24 h. The discs were weighted and six to eight leave discs were combined per replicate. The number of leaf discs per replicate was increased with study duration, due to the enhanced food requirements of the caddisfly larvae with their growth.

Food-spiking method

A trypsin activated Cry1Ab protein (Marianne Carey, Case Western Reserve University, Cleveland, USA) was dissolved in a buffer. The buffer contained 0.55 g CAPS-buffer and 125 µl Tween-20 (both Carl Roth, Karlsruhe, Germany) per 250 ml. The pH was adjusted to 10.5±0.05 using NaOH. To dissolve the Cry1Ab protein, buffer was added in small steps to the protein, while each of these steps was followed by ultrasonication to increase dissolution. The resulting solution (538.0 ng/µl or 539.6 ng/µl, depending on the batch) was used as stock solution which was further diluted using buffer.

Before spiking, the leave discs were rehydrated in distilled water for 24 h, to prevent the discs from floating on the water surface and thus to guarantee their accessibility for the test organisms. Afterwards, the water was decanted and the leaves were gently dried with paper tissue and spiked with Cry1Ab to achieve a nominal concentration of 0, 1, 10, 100 and 1000 ng/mg leaf disc. The spiked leaf discs were let air dried for an hour and stored at -18 °C until further use.

Test organisms

Larvae of *Sericostoma* spec. and *Chaetopteryx* spec were collected one week before the start of the respective experiment in spring 2015 from a near natural stream (49°5' N; 7°37' E) in Rhineland-Palatine, Germany. The larvae were kept in aerated stream water collected from the source stream at 16°C on a 12:12 light:dark cycle and were fed naturally colonized black alder leaves.

Caddisfly test

For each treatment 10 replicates were set up using 500 ml crystallizing dishes containing five randomly selected caddisfly larvae. Each dish was filled with 300 ml SAM-5S medium[25] and 100 g natural loamy sand – the latter serving as a building material for the cases of the test organisms. SAM-5S medium contains 147 mg $CaCl_2$ x $2H_2O$, 85.5 mg $NaHCO_3$, 61.5 mg $MgSO_4$ x $7H_2O$, 3.8 KCL and 1.03 mg NaBr per litre[25]. The sediment was collected at the same site as the larvae and was muffled for four hours at 450 °C to eliminate sediment organic material which may have served as alternative food source for the caddisfly larvae interfering with the response variables assessed. The caddisfly larvae were fed spiked leaf discs of the respective treatment over 6 (*Sericostoma* spec.) and 12 (*Chaetopteryx* spec.) weeks. In the experiment with *Sericostoma* spec. the mortality in the control after 6 weeks was already relatively high, so that we decided to terminate it. The feeding test lasted from 23. April to 16. July 2015 (*Chaetopteryx* spec.) and from 27. May to 8. July 2015 (*Sericostoma* spec.), while the environmental conditions were kept at 16°C and on a 12:12 light:dark cycle. Every week the medium and the leaf discs were renewed and each replicate was checked for mortality. The remaining leaf discs were dried at 60°C and weighted to the nearest 0.01 mg to determine the consumption on a weekly basis. Three additional crystallising dishes with spiked leaf discs but without caddisflies were used in order to correct the weekly consumption for microbial leaf mass loss.

To estimate the growth and the instar of the caddisfly larvae, case width and head capsule width were measured from digital images taken at the start and termination of the experiment. The measurements were realised using Axiovision (Zeiss, Jena, Germany). The larval instars were determined by the head capsule width according to Wagner[15,26]. At the start and after termination of the experiment, 15 larvae of each treatment were removed from their cases and were frozen in liquid nitrogen. Afterwards, these larvae were freeze-dried and weighted to the nearest 0.01 mg. They were kept in a -18 °C freezer until further analysis.

The lipid contents of these larvae were measured at the beginning and termination of the experiment using the vanillin-phosphor method as proposed by van Handel[27] and modified by Zubrod et al.[28]. Briefly, larvae were placed for 72 h in a 1:1 chloroform:methanol solution. They were grinded with mortars and centrifuged and the supernatant were used for the lipid analysis. In a water bath the solvents were vaporised at 95°C. Afterwards 200 µL sulphuric acids (95%) were added and the samples placed in a water bath for 10 min. The samples were let to cool down to room temperature. Five mL of vanillin-phosphor reagent were added and extinction was measured photometrical with a microplate reader (Tecan, Männedorf, Switzerland) at a wavelength of 490 nm. A 7-point calibration curve was prepared using commercial soybean oil (Sojola, Herford, Germany).

Degradation Experiment

To investigate how the Cry1Ab concentration on the spiked leaf discs developed between food renewals, a degradation experiment was performed. Therefore, spiked leaf discs were kept under the same conditions as reported for the feeding test for seven days. After day 0, 3 and 7, subsamples were removed, leaf discs were freeze-dried, weighted to the nearest 0.01 mg and stored in a -18 °C freezer until Cry1Ab quantification. For each treatment and each sampling date 5 replicates were set up.

Cry1Ab quantification

The Cry1Ab protein concentrations of the spiked leaves from the caddisfly experiments, samples from the degradation experiment and the spiked leaf powder from the European corn borer (ECB) biotest (Fig. S8) were quantified using an enzyme-linked immunosorbent assay (ELISA)[29]. All samples were freeze-dried and if needed grounded using a mixer mill. PBST buffer was added to the sample and shaken in the mixer mill. The PBST buffer contained 8 g sodium chloride, 1.15 g sodium phosphate, 0.2 g potassium phosphate, 0.2 g potassium chloride, 0.5 g Tween-20 per litre and the pH was adjusted to 7.4±0.05 with HCl. The samples were centrifuged and placed on ice. The supernatant was removed and diluted with buffer. Every sample was analysed in triplicate in an antibody coated 96-well plate (Agdia, Elkhart, USA). The well plate was shaken for 1.5 h. Then, the well plate was washed three times with the PBST buffer and an enzyme conjugate was added. The well plate was shaken for 1.5 h and washed again. A substrate solution was added. The colorimetric response was measured at the wavelength of 650 nm using a Tecan microplate reader. To quantify the Cr1Ab concentration the measured absorbance was compared with a 13-point standard curve ranging from 0 to 6.8 ng Cry1Ab/ml. The measured Cry1Ab concentrations were normalised to the sample weight.

The ELISA analyses of the spiked leaves in the caddisfly feeding test revealed Cry1Ab concentrations of 0.09, 1.2, 17.2 and 132.4 ng/mg. As a consequence of the partly high deviation between the nominal and measured concentration, the present paper refers to the measured concentration in the following.

Statistical Analysis

Mortality was assessed through a time to event analysis, i.e. a log-rank Kaplan-Meier test, using SigmaPlot version 12 (Sysstat, Erkrath, Germany). For all other endpoints R software version 3.4.3 was used. Consumption per individual was measured by dividing the consumed food through the number of living larvae. In weeks, where larvae died they were counted only half assuming those individuals have been active for at least half of the week in which they died. Consumption was calculated as mg consumed leaf per larvae and week. Microbial decomposition (Md) and consumption (C) were calculated as shown in equation (1) and (2).

$$Md = 1 - \frac{(L_b - L_a)}{L_b} \quad (1)$$

$$C = \frac{(L_b * Md - L_a)}{S} \quad (2)$$

L_b and L_a is the leaf weight before and after feeding and S are the surviving larvae. Consumption was analyzed using two-way repeated measures ANOVA with experimental duration and treatment as independent factors. Mean consumption (MC) was expressed as the mean of mg consumed leaf per larvae averaged over the whole study duration. Mean consumption of all weeks, case width gain and the larval lipid content were assessed for significant differences by a one-way independent ANOVA with treatment as an independent factor followed by a Dunnett Post-hoc test. Effect sizes are expressed as a percent deviation between the control and the respective treatment. The larval instars were assessed by using a Chi^2-Test with adjusted alpha levels (Bonferroni correction). The significance level was set at $p<0.05$. For the degradation experiment means are plotted with a regression line and a 95% confidence interval.

Results

Mortality

The mortality of *Chaetopteryx* spec. larvae increased in all treatments with study duration (Fig. 1, Table S1). The results showed, however, no statistically significant difference ($p>0.05$) among treatments. A 2-fold higher mortality was, nonetheless, reported at the highest

concentration (30±25.4%) relative to the control (14±19%) suggesting a concentration-dependent response. In *Sericostoma* spec. no significant differences regarding mortality was reported (Fig. S1).

Consumption

The consumption of leaf material by *Chaetopteryx* spec. larvae increased over the study duration independent of the treatment (Fig. 2, Table S2). In the control the consumption rose from 6.7±1.3 mg/ind/week to 16.3±2.4 mg/ind/week at the end of the experiment. In contrast to *Chaetopteryx* spec., the consumption of *Sericostoma* spec. remained approximately the same until test termination (Fig. S2). In the control the consumption was 5.9±1.6 mg/ind/week at the beginning and 6.3±5.0 mg/ind/week at termination. We found no significant difference ($p>0.05$) in consumption among treatments, neither for *Chaetopteryx* spec. nor *Sericostoma* spec.

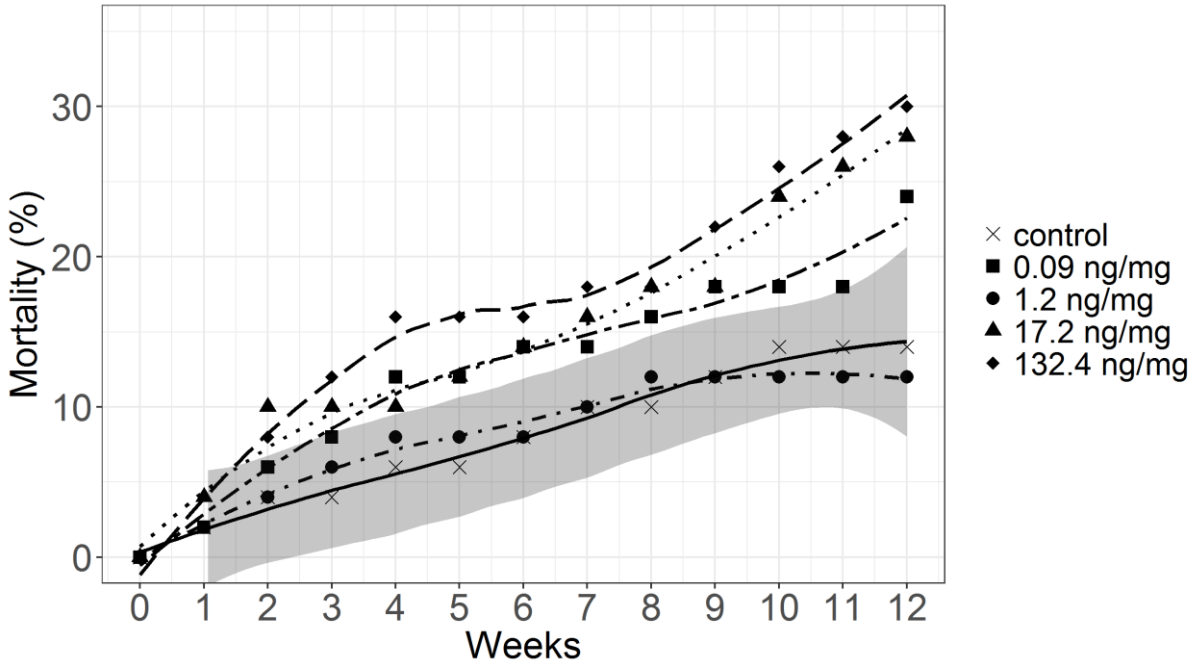


Figure 1 *Chaetopteryx* spec. mortality (%) over the entire study duration of 12 weeks of feeding test. Shown are means (n=10) and regression lines. Shaded area depicts the 95% confidence band of the controls' mean

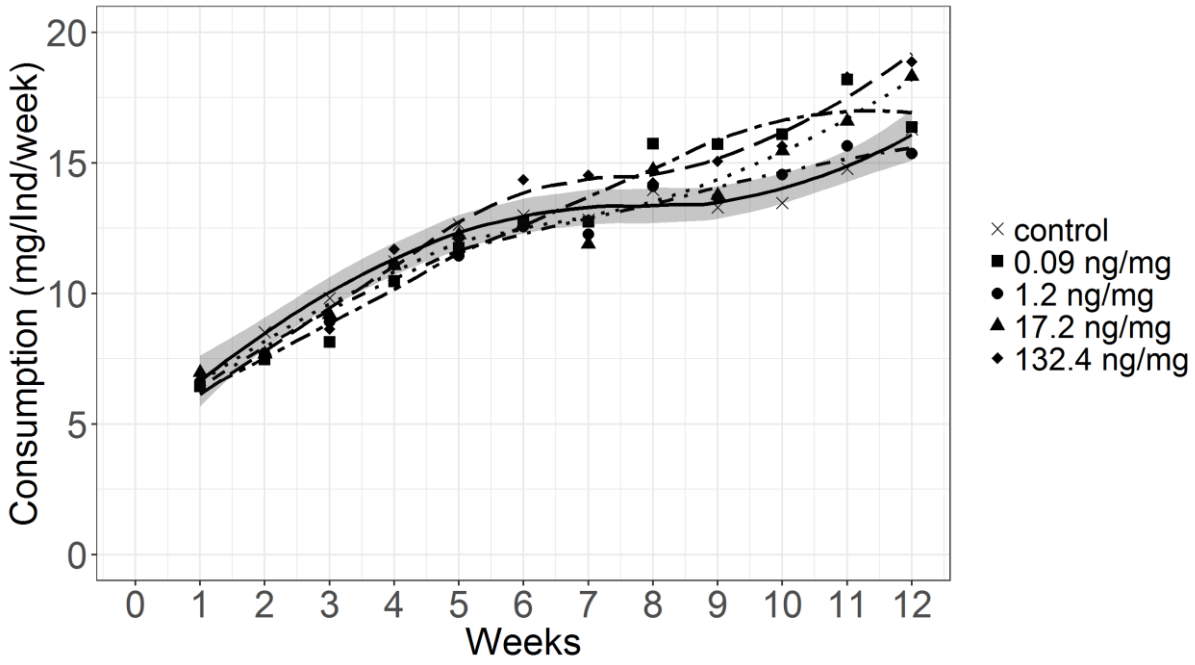


Figure 2 *Chaetopteryx* spec.'s leaf consumption (mg dry weight/individual/week) over the entire study duration of 12 weeks of feeding test. Shown are means (n=10) and regression lines. Shaded area depicts 95% confidence band of the control regression

By calculating the mean consumption of all weeks, we investigated, if the caddisflies changed their feeding rate which could be the result of damage in the digestive tract (Fig. S3). The mean consumption of all weeks of *Chaetopteryx* spec. and *Sericostoma* spec. larvae showed no significant difference between treatments and control (effect sizes between 1.8% and 7.5% for *Chaetopteryx* spec. and between 0.7% and 13.1% for *Sericostoma* spec.). The results revealed no dose-response relationship.

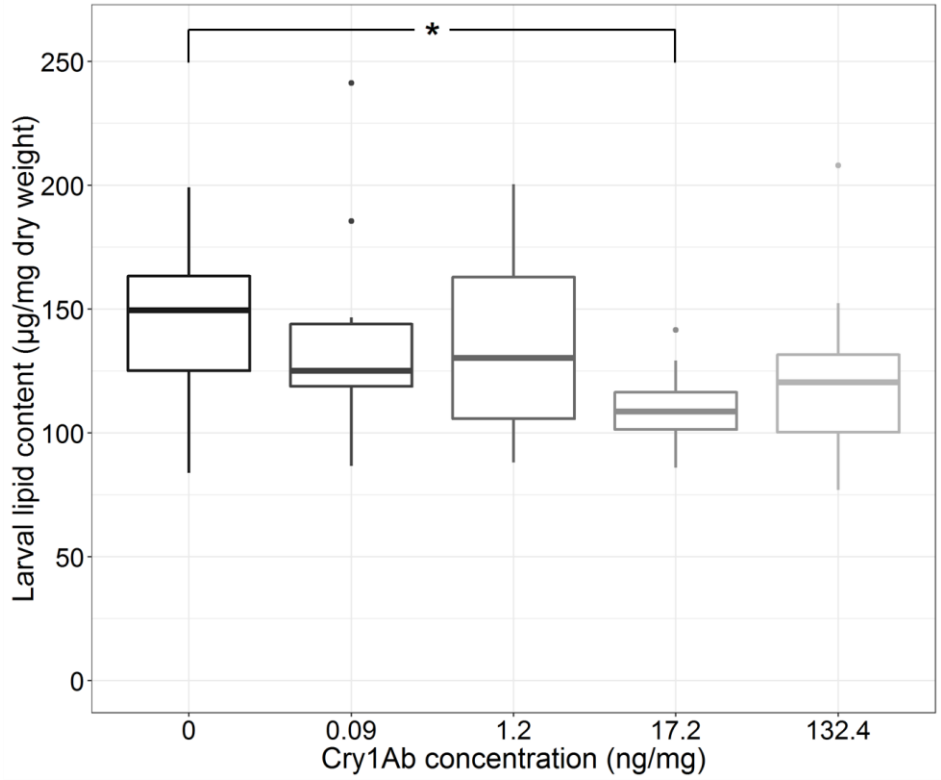


Figure 3 *Chaetopteryx* spec. larval lipid content after 12 weeks of feeding with Cry1Ab spiked leaf discs. Thick lines in the boxplots show medians (n=15), lower and upper quartile are covered by the upper and lower end of the box. Effect size: 23.5%. * shows significant difference to control ($p<0.05$)

Lipid content and case width

The lipid content of *Chaetopteryx* spec. was 244±60 µg/mg at the beginning of the experiment. At the termination of the experiment, the lipid content decreased with increasing Cry1Ab concentration, while only for the 17.2 ng Cry1Ab/mg treatment a significant reduction relative to the control was observed (effect size of approximately 25%; Fig. 3, Table S3). This observation was not confirmed for *Sericostoma* spec (Fig. S4).

The case width of the *Chaetopteryx* spec. larvae increased in all treatments over the experimental duration. The results showed a significant lower case width gain ($p<0.05$) in the highest Cry1Ab treatment relative to the control (Fig. S5). Moreover, at the termination of the experiment, the caddis width differed less than 5% among treatments. Similarly, there was no effect on *Sericostoma* spec. detectable (Fig. S6).

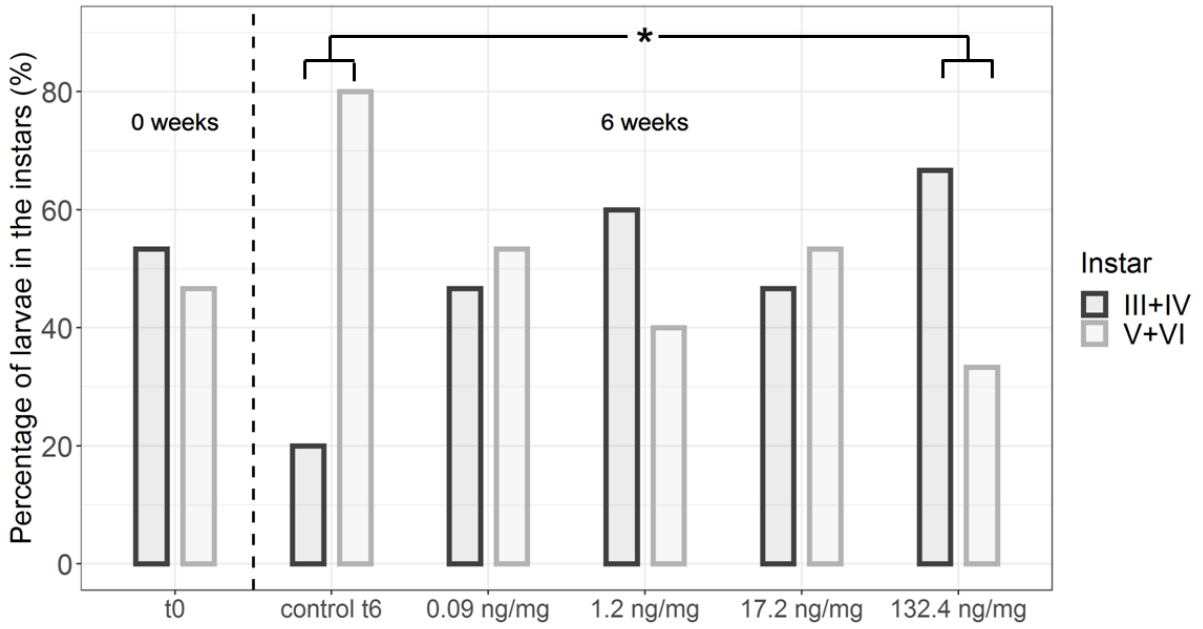


Figure 4 *Sericostoma* spec. larval stage at the start (t0) and the termination (t6) of the experiment in the control (control t6) and the various treatments, respectively (n=15). The dashed line separates the data of the larval instars at the beginning (0 weeks) and at the end (6 weeks) of the experiment. Larval stage III was found in the 17.2 ng/mg treatment. Larval stage VI was found in the 0.09 and 1.2 ng/mg treatment. * shows significant difference to control ($p<0.05$)

Larval instars

At the beginning of the caddisfly experiment, the *Sericostoma* spec. larvae in the control were almost equally distributed among instars ≤IV and ≥V (Fig. 4). The Chi^2-Test showed a significant difference ($p>0.05$) in larval instars after 6 weeks, i.e. larval development was delayed, between the highest concentration and the control (Table S3). This pattern in larval instars could not be confirmed by the experiments with *Chaetopteryx* spec (Fig. S7).

Degradation experiment

The degradation experiment showed a rapid decline of the Cry1Ab concentration on leaves during one week (Fig. 5). At the highest concentration (132.4 ng/mg), for instance, a decrease to 4.01 ng/mg was observed after 7 days. No Cry1Ab toxin was measured in the control.

Discussion

Bt effects on caddisflies

Despite their close taxonomic relationship to Lepidoptera, which are targeted by a variety of Bt toxins expressed in transgenic crops, only few studies examined the hazard of Bt toxins on caddisflies[6]. Our results add to that knowledge and suggest that at least some groups of caddisfly larvae may indeed be sensitive to the Cry1Ab toxin when exposed through their food over an

extended period of time. Lethal effects of Bt toxins on caddisflies have been so far only shown in Rosi-Marshall et al.[5] for the scraping caddisfly *Helicopsyche borealis* when fed pollen from a Cry1Ab maize event. Most of the other studys[10,11] demonstrated sublethal effects on caddisfly larvae after exposure to Bt toxins. Observations of Chambers et al.[11] and Rosi-Marshall et al.[5] highlight a significantly lower growth of *Lepidostoma liba* larvae when fed Cry1Ab containing maize leaves compared to non-Bt maize. Divergent observations in other Trichopteran species such as *Pycnopsyche* cf. *scrabripennis* document an increase in biomass when larvae were exposed to Cry1Ab x Cry3Bb1 x Roundup Ready maize leaves compared to the exposure to either Roundup Ready maize or Cry1Ab maize[10]. In fact a uniform response of all Trichopteran species to Bt toxin, or a special class of Bt toxin, cannot be expected as Lepidoptera show a large inter-specific variation in their sensitivity [30].

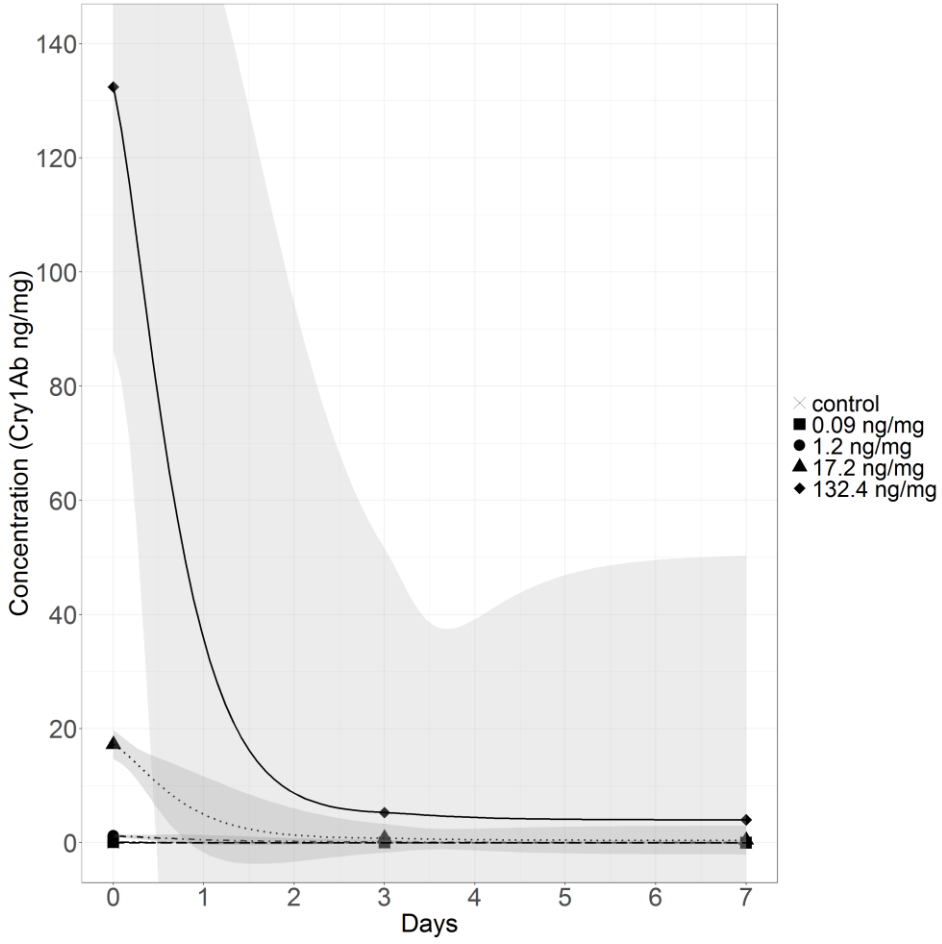


Figure 5 Cry1Ab concentration on spiked leaf discs during 7 days stored in medium. Shown are means (n=5), regression line and 95% confidence interval. No Cry1Ab toxin was measured in the control

For Trichoptera, we suggest that sublethal responses can indeed be more informative for the risk assessment relative to mortality, because especially at chronic low exposure, the damage of the gut membrane caused by Bt toxins is known not to be lethal[31–34]. In our case decreasing larval lipid content and retarded insect development indicated impairments, though often not

statistically significant, this may be mainly related to the rather high variability in those variables and the relatively low number of replications and thus statistical power and the quick degradation of the spiked toxin.

The trend to a lower larval lipid content in *Chaetopteryx* spec. with increasing Cry1Ab concentrations may indicate a reallocation of energy towards defence and repair mechanisms while maintaining growth[35]. The significant lower case width gain in the highest Cry1Ab concentration may be mainly driven by the significant differences in the case width at the start. A reallocation of energy may, however, have negative implications in the ability of the organism to withstand future challenges such as metamorphoses and their ability to reproduce in the terrestrial life stage[35]. In contrast to *Chaetopteryx* spec., the significant difference in the larval stages of *Sericostoma* spec. among treatments at the termination of the experiment indicates – although variability could not be defined – together with non-substantial changes in their lipid content that ensuring sufficient energy reserves for future investments (e.g. number of offspring) is favoured over the speed of development. The slower development of the larval instars in some of the Cry1Ab treatments, likely postpones emergence which might temporarily disconnect the provisioning of prey from aquatic recourses to terrestrial food webs potentially inducing bottom-up or top-down directed effects[36]. However, it has to be mentioned that in our study the control and the highest concentration obviously contained already different *Sericostoma* spec. instars at the beginning of the experiment. A delay of the development of larval instars by Cry1Ab toxin was also reported for terrestrial lepidopteran larvae[30,37] and for caddisfly larvae when exposed to pesticides[38–40]. Consequently, alterations in the physiology (i.e., lipid reserves) and larval instars of insects as a consequence of exposure to stress in general, and Cry1Ab in particular, seems a common response suggesting that the subtle effects observed in the present study warrant further research.

The Cry1Ab concentrations in leaves of MON810 and a not specified event are on average approx. 5 ng/mg[41] and 10.2 ng/mg (values between 7.3 and 15.1 ng/mg)[42], respectively. Maize debris collected in the riparian zone outside the water show lower Cry1Ab concentrations (mean 0.20 ± 0.34 ng/mg)[43]. Cry1Ab leaches from maize detritus into the water, so concentrations in maize leaves collected in a stream can be expected to be lower than in the riparian zones. Cry1Ab in maize detritus in streams showed concentrations of Cry1Ab in average of approx. 1 ng/mg, and 0.095 ± 0.073 ng/mg[41,43]. For the lipid content we found significant effects at a concentration of 17.2 ng/mg, which are similar to Cry1Ab concentrations found in fresh maize leaves. At a concentration of 132.4 ng/mg the larval instars showed significant sublethal effects and, thus, approximately tenfold above Cry1Ab concentrations in fresh Bt-maize leaves. Our

sublethal effects were found at concentrations ten- to thousand folds above the Cry1Ab concentrations in maize detritus from the stream or the riparian zone. The results indicate that significant effects may occur if fresh Bt-maize leaves are present. The risk seems lower if looking at Bt-maize material from the stream or the riparian zone. However, SmartStax, a stacked GM maize expressing six Cry toxins shows clearly higher concentrations in leaves (total Cry toxin concentration 1518 ng/mg)[44]. Moreover, risk assessment should cover possible uncertainties arising from species differences and the extrapolation from acute to chronic tests. For this purpose, assessment factors (e.g. 10, 100 or 1000) are used especially for the determination of Predicted No Effect Concentrations (PNEC). Furthermore, due to the limited data, this assessment is subject to uncertainty. This illustrates the need for further verification studies, especially because of the specific mode of action of the toxins.

Relevance of the food-spiking method

With the food spiking method, it was ensured that the observed effects are triggered by the Bt toxin and not by the quality of the leaf material, as the latter was prepared in a standardised manner. To highlight the strength and limitations we discuss the proposed food spiking method in the context of other published methods involving GMO and caddisflies[6]. In contrast to our study, recently published studies often did not quantify the Bt toxin concentration in the applied plant material and were restricted to one or two treatment concentrations[5,10,11]. However, different concentrations are necessary to obtain dose-response relationship and abstract related effect thresholds, which are important input data for risk assessment. The food-spiking method, as applied here, allowed establishing different concentrations that were supported via ELISA and it is thus suitable to investigate dose-response relationships. Indeed the present study allowed identifying concentrations of Cry toxins that affected the test species in a sublethal manner. Those effect concentrations can be compared to concentrations actually measured in crop plants ultimately informing risk assessment. In the following we look at further aspects that highlight the relevance of the proposed approach.

Moreover, our additional experiments involved biotests with *Ostrinia nublilalis*, a target insect pest of Cry1Ab maize. These biotests clearly demonstrated that the spiking method is suitable to expose the test organisms and thus to assess food related effects on aquatic shredders. As shredders are an important functional group in aquatic systems the method can be a valuable tool for the assessment of effects of GMO cultivation on aquatic environments[12].

To compare the GM-plant and its isogenic control line is the basis of the assessment in many studies[7,10,45,46]. Feeding studies, particularly those involving a non-GM isogenic line as a control, may fail of providing equally nutritious food for shredders in both the treatments and the control, complicating any conclusion on potential environmental risks of the toxin. Studies measured differentially expressed proteins in a GM-maize relative to its non-transgenic isogenic line, however, the biological relevance of such changes is still unknown[47,48]. Because nutritional quality of food leaves is the same both in the control and treatments groups, our food-spiking method strongly suggests that the responses of caddisfly shredders are largely triggered by the toxin. The GM-plant material used in some recently published studies was conditioned, i.e. microbially colonized for 3, 7 or 14 days[5,10,11]. We agree that the conditioning is essential for shredding organisms as it improves the food quality. At the same time, conditioning can lead to uncontrolled degradation and leaching of the Bt toxin into the water phase[49]. In our food-spiking method leaf conditioning took place prior to spiking, thus, we excluded the risk of a Bt toxin degradation during the conditioning process. Nonetheless degradation and dilution during the feeding experiment cannot be avoided and the spiking method itself requires further verification as we detected only roughly 10% of the nominal concentration at test initiation.

Studies reported in the literature were often limited to test durations of up to 30 days. The entry of maize debris into the water starts in October after harvest, is highest in February and stops after growth of new vegetation in April[10]. Aquatic organisms are thus exposed for a long time period to low Bt toxin concentrations in the environment[41,42,50]. In order to realistically represent the conditions in the environment, we chose an exposure time of 6 and 12 weeks. We conclude that using such prolonged exposure time is more closely reflecting the actual field scenario and thus consider this as an advantage when investigating Bt toxins.

The degradation experiment showed a rapid decrease of the Bt toxin concentration on the leaf discs during one week in the water. This resembles the real exposure in a stream, which is probably also not constant. To achieve a more even Bt toxin level, the spiked leaf discs may be exchanged more often.

To conclude, we showed significant effects on some endpoints and our data suggest concentration-dependent responses. Effects were found, however, at higher concentrations and were not consistent in all species and all endpoints. We, nonetheless, showed that long test durations and the use of sublethal endpoints are reasonable and may assist assessment of risks. From our results we concluded that the food-spiking method is able to realise a food-related

exposure of shredders which could inform risk assessment, but this is only a first step and additional studies would be beneficial to develop this method further.

References

1. Parisi, C., Tillie, P. & Rodríguez-Cerezo, E. The global pipeline of GM crops out to 2020. *Nature biotechnology* **34,** 31–36; 10.1038/nbt.3449 (2016).

2. Palma, L., Muñoz, D., Berry, C., Murillo, J. & Caballero, P. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. *Toxins* **6,** 3296–3325; 10.3390/toxins6123296 (2014).

3. Glare, T. R. & O'Callaghan, M. *Bacillus thuringiensis*. *Biology, ecology and safety* (Wiley, Chichester, 2000).

4. EU. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms (2001).

5. Rosi-Marshall, E. J. *et al.* Toxins in transgenic crop byproducts may affect headwater stream ecosystems. *P Natl Acad Sci USA* **104,** 16204–16208; 10.1073/pnas.0707177104 (2007).

6. Pott, A., Otto, M. & Schulz, R. Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment. *Sci Total Environ* **635,** 687–698; 10.1016/j.scitotenv.2018.04.013 (2018).

7. Bøhn, T., Primicerio, R., Hessen, D. O. & Traavik, T. Reduced fitness of *Daphnia magna* fed a Bt-transgenic maize variety. *Arch Environ Con Tox* **55,** 584–592; 10.1007/s00244-008-9150-5 (2008).

8. Cuhra, M., Traavik, T. & Bøhn, T. Life cycle fitness differences in *Daphnia magna* fed Roundup-Ready soybean or conventional soybean or organic soybean. *Aquacult Nutr* **21,** 702–713; 10.1111/anu.12199 (2015).

9. Holderbaum, D. F. *et al.* Chronic responses of *Daphnia magna* under dietary exposure to leaves of a transgenic (event MON810) Bt-maize hybrid and its conventional near-isoline. *J Toxicol Env Health* **78,** 993–1007; 10.1080/15287394.2015.1037877 (2015).

10. Jensen, P. D., Dively, G. P., Swan, C. M. & Lamp, W. O. Exposure and nontarget effects of transgenic Bt corn debris in streams. *Environ Entomol* **39,** 707–714; 10.1603/EN09037 (2010).

11. Chambers, C. P. *et al.* Responses of stream macroinvertebrates to Bt maize leaf detritus. *Ecol Appl* **20,** 1949–1960; 10.1890/09-0598.1 (2010).

12. Bundschuh, R. *et al.* Prioritizing stream types according to their potential risk to receive crop plant material-A GIS-based procedure to assist in the risk assessment of genetically modified crops and systemic insecticide residues. *Sci Total Environ* **547,** 226–233; 10.1016/j.scitotenv.2015.12.124 (2016).

13. Hilbeck, A. *et al.* Procedure to select test organisms for environmental risk assessment of genetically modified crops in aquatic systems. *Integr Environ Asses* **13,** 974–979; 10.1002/ieam.1965 (2017).

14. Malicky, H. Ein kommentiertes Verzeichnis der Köcherfliegen (Trichoptera) Europas und des Mediterrangebiets. *Linzer Biol Beitr* **37,** 533–596 (2005).

15. Wagner, R. Influence of temperature, photoperiod and nutrition on growth and consumption of *Chaetopteryx villosa* (Trichoptera). *Ecography* **13,** 247–254; 10.1111/j.1600-0587.1990.tb00615.x (1990).

16. Kučinić, M. *et al. Chaetopteryx bucari* sp. n., a new species from the *Chaetopteryx rugulosa* group from Croatia (Insecta, Trichoptera, Limnephilidae) with molecular, taxonomic and ecological notes on the group. *ZooKeys,* 1–28; 10.3897/zookeys.320.4565 (2013).

17. Waringer, J. & Malicky, H. The larvae of *Sericostoma bergeri* Malicky, 1973 and *Sericostoma herakles* Malicky, 1999 (Trichoptera, Sericostomatidae). *ZooKeys,* 123–133; 10.3897/zookeys.695.14531 (2017).

18. Wallace, I. D. A key to larvae and pupae of *Sericostoma personatum* (Spence) and *Notidobia ciliaris* (Linne) (Sericostomatidae: Trichoptera) in Britain. *Freshwater Biol* **7,** 93–98; 10.1111/j.1365-2427.1977.tb01660.x (1977).

19. Friberg, N. & Jacobsen, D. Variation in growth of the detritivore-shredder *Sericostoma personatum* (Trichoptera). *Freshwater Biol* **42,** 625–635; 10.1046/j.1365-2427.1999.00501.x (1999).

20. Rumbos, C. I., Stamopoulos, D., Georgoulas, G. & Nikolopoulou, E. Factors affecting leaf litter cecomposition by *Micropterna sequax* (Trichoptera: Limnephilidae). *Int Rev Hydrobiol* **95,** 383–394; 10.1002/iroh.201011236 (2010).

21. Graça, M. A.S. The role of invertebrates on leaf litter decomposition in streams - a review. *Int Rev Hydrobiol* **86,** 383–393; 10.1002/1522-2632(200107)86:4/5<383::AID-IROH383>3.0.CO;2-D (2001).

22. Arrese, E. L. & Soulages, J. L. Insect fat body: Energy, metabolism, and regulation. *Annu Rev Entomol* **55,** 207–225; 10.1146/annurev-ento-112408-085356 (2010).

23. Bundschuh, M., Zubrod, J. P. & Schulz, R. The functional and physiological status of *Gammarus fossarum* (Crustacea; Amphipoda) exposed to secondary treated wastewater. *Environ Pollut* **159,** 244–249; 10.1016/j.envpol.2010.08.030 (2011).

24. Dang, C. K., Chauvet, E. & Gessner, M. O. Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. *Ecol Lett* **8,** 1129–1137; 10.1111/j.1461-0248.2005.00815.x (2005).

25. Borgmann, U. Systematic analysis of aqueous ion requirements of *Hyalella azteca*: A standard artificial medium including the essential bromide ion. *Arch Environ Con Tox* **30,** 356–363; 10.1007/BF00212294 (1996).

26. Wagner, R. A laboratory study on the life cycle of *Sericostoma personatum* (Kirby & Spence), and light dark-dependent food consumption. *Hydrobiologia* **208,** 201–212; 10.1007/BF00007785 (1990).

27. van Handel, E. Rapid determination of glycogen and sugars in mosquitoes. *J Am Mosquito Contr* **1,** 299–301 (1985).

28. Zubrod, J. P., Bundschuh, M., Feckler, A., Englert, D. & Schulz, R. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environ Toxicol Chem* **30,** 2718–2724; 10.1002/etc.679 (2011).

29. Zwahlen, C., Hilbeck, A., Gugerli, P. & Nentwig, W. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Mol Ecol* **12,** 765–775; 10.1046/j.1365-294X.2003.01767.x (2003).

30. Lang, A. & Otto, M. A synthesis of laboratory and field studies on the effects of transgenic *Bacillus thuringiensis* (Bt) maize on non-target Lepidoptera. *Entomol Exp Appl* **135,** 121–134; 10.1111/j.1570-7458.2010.00981.x (2010).

31. Erb, S. L., Bourchier, R. S., van Frankenhuyzen, K. & Smith, S. M. Sublethal Effects of *Bacillus thuringiensis* Berliner subsp. *kurstaki* on *Lymantria dispar* (Lepidoptera. Lymantriidae) and the Tachinid Parasitoid *Compsilura concinnata* (Diptera: Tachinidae). *Environ Entomol* **30,** 1174–1181; 10.1603/0046-225X-30.6.1174 (2001).

32. Gulzar, A. & Wright, D. J. Sub-lethal effects of Vip3A toxin on survival, development and fecundity of *Heliothis virescens* and *Plutella xylostella*. *Ecotoxicology (London, England)* **24,** 1815–1822; 10.1007/s10646-015-1517-6 (2015).

33. Binning, R. R., Coats, J., Kong, X. & Hellmich, R. L. Susceptibility and aversion of *Spodoptera frugiperda* (Lepidoptera. Noctuidae) to Cry1F Bt maize and considerations for insect resistance management. *J Econ Entomol* **107,** 368–374; 10.1603/ec13352 (2014).

34. Eizaguirre, M., Tort, S., López, C. & Albajes, R. Effects of sublethal concentrations of *Bacillus thuringiensis* on larval development of *Sesamia nonagrioides*. *J Econ Entomol* **98,** 464–470; 10.1093/jee/98.2.464 (2005).

35. Konschak, M. *et al.* Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder *Chaetopteryx villosa* (Trichoptera). *Aquat Toxicol* **206,** 33–42; 10.1016/j.aquatox.2018.10.021 (2018).

36. Schulz, R. *et al.* Review on environmental alterations propagating from aquatic to terrestrial ecosystems. *Sci Total Environ* **538,** 246–261; 10.1016/j.scitotenv.2015.08.038 (2015).

37. Rausell, C., Martinez-Ramirez, A. C., Garcia-Robles, I. & Real, M. D. A binding site for *Bacillus thuringiensis* Cry1Ab toxin is lost during larval development in two forest pests. *Appl Environ Microb* **66,** 1553–1558; 10.1128/AEM.66.4.1553-1558.2000 (2000).

38. Liess, M. & Schulz, R. Chronic effects of short-term contamination with the pyrethroid insecticide fenvalerate on the caddisfly *Limnephilus lunatus*. *Hydrobiologia* **324,** 99–106; 10.1007/BF00018170 (1996).

39. Liess, M. & Schulz, R. Linking insecticide contamination and population response in an agricultural stream. *Environ Toxicol Chem* **18,** 1948–1955; 10.1002/etc.5620180913 (1999).

40. Schulz, R. & Liess, M. Chronic effects of low insecticide concentrations on freshwater caddisfly larvae. *Hydrobiologia* **299,** 103–113; 10.1007/BF00017562 (1995).

41. Griffiths, N. A. *et al.* Rapid decomposition of maize detritus in agricultural headwater streams. *Ecol Appl* **19,** 133–142; 10.1890/07-1876.1 (2009).

42. Böttger, R., Schaller, J., Lintow, S. & Gert Dudel, E. Aquatic degradation of Cry1Ab protein and decomposition dynamics of transgenic corn leaves under controlled conditions. *Ecotox Environ Safe* **113,** 454–459; 10.1016/j.ecoenv.2014.12.034 (2015).

43. Tank, J. L. *et al.* Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. *P Natl Acad Sci USA* **107,** 17645–17650; 10.1073/pnas.1006925107 (2010).

44. Hilbeck, A. & Otto, M. Specificity and Combinatorial Effects of *Bacillus thuringiensis* Cry Toxins in the Context of GMO Environmental Risk Assessment. *Front. Environ. Sci.* **3,** 467; 10.3389/fenvs.2015.00071 (2015).

45. Li, G., Wang, Y., Liu, B. & Zhang, G. Transgenic *Bacillus thuringiensis* (Bt) rice is safer to aquatic ecosystems than its non-transgenic counterpart. *PloS one* **9,** e104270; 10.1371/journal.pone.0104270 (2014).

46. Swan, C. M., Jensen, P. D., Dively, G. P. & Lamp, W. O. Processing of transgenic crop residues in stream ecosystems. *J Appl Ecol* **14,** 711; 10.1111/j.1365-2664.2009.01728.x (2009).

47. Agapito-Tenfen, S. Z., Guerra, M. P., Wikmark, O.-G. & Nodari, R. O. Comparative proteomic analysis of genetically modified maize grown under different agroecosystems conditions in Brazil. *Proteome Sci* **11,** 46; 10.1186/1477-5956-11-46 (2013).

48. Agapito-Tenfen, S. Z. *et al.* Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. *BMC Plant Biol* **14,** 346; 10.1186/s12870-014-0346-8 (2014).

49. Strain, K. E., Whiting, S. A. & Lydy, M. J. Laboratory and field validation of a Cry1Ab protein quantitation method for water. *Talanta* **128,** 109–116; 10.1016/j.talanta.2014.04.036 (2014).

50. Li, Y., Wu, K., Zhang, Y. & Yuan, G. Degradation of Cry1Ac protein within transgenic *Bacillus thuringiensis* rice tissues under field and laboratory conditions. *Environ Entomol* **36,** 1275–1282; 10.1093/ee/36.5.1275 (2007).

Acknowledgement

We thank Therese Bürgi, Simon Lüderwald for laboratory assistance and Annette Herz for providing European corn borer eggs.

Author contributions

A.P., M.B., M.O. and R.S. conceived the experiments. A.P. and R.B. performed the experiments. A.P. wrote the first version of the manuscript. All authors reviewed the manuscript.

Competing interests

R.S. is managing director of a small consultancy working in the field of ecotoxicology and environmental risk assessment. The authors, however, do not feel a conflict of interest as a consequence of this situation.

Supplementary Information for:

Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders – an attempt to establish dose-effect relationships through food-spiking

Antonia Pott[1,2*], Mirco Bundschuh[1,3], Rebecca Bundschuh[1], Mathias Otto[2], Ralf Schulz[1]

[1] iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany

[2] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, 75007 Uppsala, Sweden

Table S4 *Chaetopteryx* spec. mortality (%) during the 12 weeks of feeding test. Shown are means (n=10) and standard deviation (SD).

	Bt concentration (ng Cry1Ab/mg leaf disc DW)									
Week	**control mean**	**control SD**	**0.09 mean**	**0.09 SD**	**1.2 mean**	**1.2 SD**	**17.2 mean**	**17.2 SD**	**132.4 mean**	**132.4 SD**
1	2.0	6.3	2.0	6.3	2.0	6.3	4.0	8.4	2.0	6.3
2	4.0	8.4	6.0	13.5	4.0	8.4	10.0	14.1	8.0	16.9
3	4.0	8.4	8.0	14.0	6.0	9.7	10.0	14.1	12.0	21.5
4	6.0	9.7	12.0	16.9	8.0	10.3	10.0	14.1	16.0	20.7
5	6.0	9.7	12.0	16.9	8.0	10.3	12.0	14.0	16.0	20.7
6	8.0	10.3	14.0	21.2	8.0	10.3	14.0	13.5	16.0	20.7
7	10.0	10.5	14.0	21.2	10.0	14.1	16.0	15.8	18.0	22.0
8	10.0	10.5	16.0	20.7	12.0	14.0	18.0	17.5	18.0	22.0
9	12.0	14.0	18.0	19.9	12.0	14.0	18.0	17.5	22.0	22.0
10	14.0	19.0	18.0	19.9	12.0	14.0	24.0	20.7	26.0	25.0
11	14.0	19.0	18.0	19.9	12.0	14.0	26.0	19.0	28.0	27.0
12	14.0	19.0	24.0	22.7	12.0	14.0	28.0	16.9	30.0	25.4

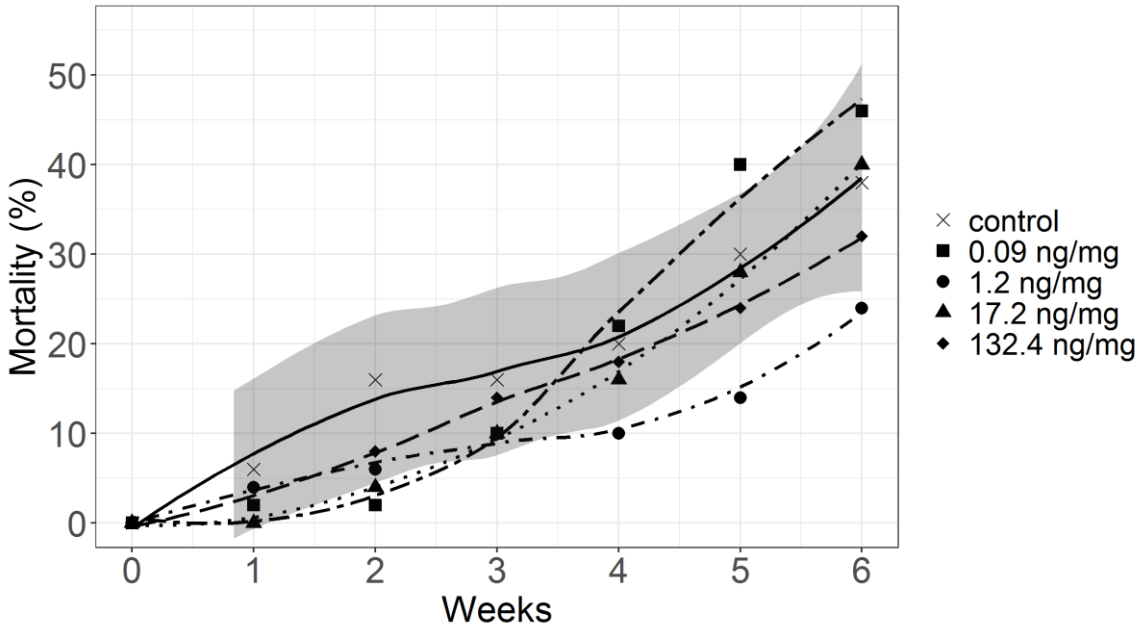


Figure S1 *Sericostoma* spec. mortality (%) over the entire study duration of 6 weeks of feeding test. Shown are means (n=10) and regression lines. Shaded area depicts the 95% confidence band of the controls' mean

Table S2 *Chaetopteryx* spec. consumption (mg leaf DW/individual/week) during the 12 weeks of feeding test. Shown are means (n=10) and standard deviation (SD).

	Bt concentration (ng Cry1Ab/mg leaf disc DW)									
Week	**control mean**	**control SD**	**0.09 mean**	**0.09 SD**	**1.2 mean**	**1.2 SD**	**17.2 mean**	**17.2 SD**	**132.4 mean**	**132.4 SD**
1	6.7	1.3	6.4	1.6	6.6	1.2	7.0	2.7	6.4	2.0
2	8.6	1.3	7.6	2.5	7.8	1.4	7.9	1.9	7.6	1.7
3	10.0	1.8	8.3	2.0	9.0	1.8	9.6	3.0	9.2	3.4
4	11.2	1.5	10.6	2.4	11.2	1.7	11.1	2.9	11.8	3.0
5	12.8	1.6	12.2	3.3	11.6	1.3	12.2	3.1	12.4	2.6
6	13.0	1.4	12.7	2.7	12.5	1.7	12.8	3.0	14.4	4.4
7	13.0	1.8	13.1	2.6	12.3	1.8	12.0	3.6	14.5	4.0
8	14.1	1.4	15.7	4.7	14.4	3.1	15.0	3.2	14.4	3.1
9	13.3	1.5	15.9	5.0	13.8	3.1	14.0	3.7	15.1	4.6
10	13.7	1.6	16.3	4.2	14.5	3.6	15.5	2.9	16.1	4.5
11	15.2	3.7	18.2	6.6	15.6	3.0	17.6	17.6	19.3	7.9
12	16.3	2.4	16.4	4.3	15.3	3.6	18.5	3.5	19.4	7.0

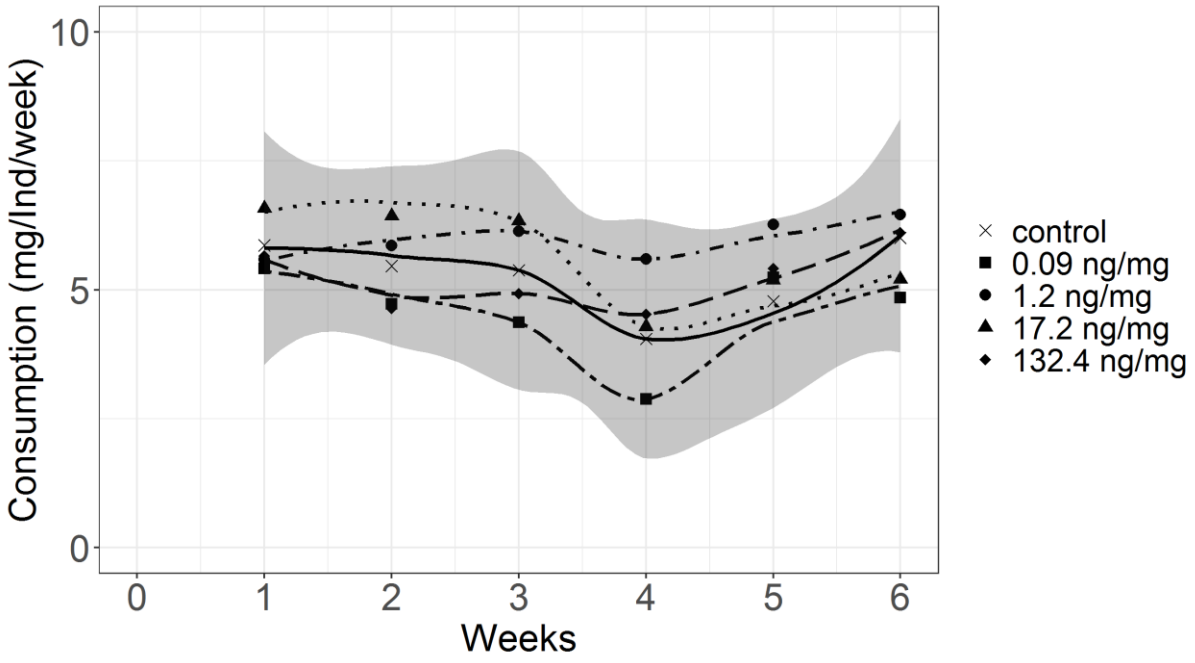


Figure S2 *Sericostoma* spec.'s leaf consumption (mg dry weight/individual/week) over the entire study duration of 6 weeks of feeding test. Shown are means (n=10) and regression lines. Shaded area depicts 95% confidence band of the control regression

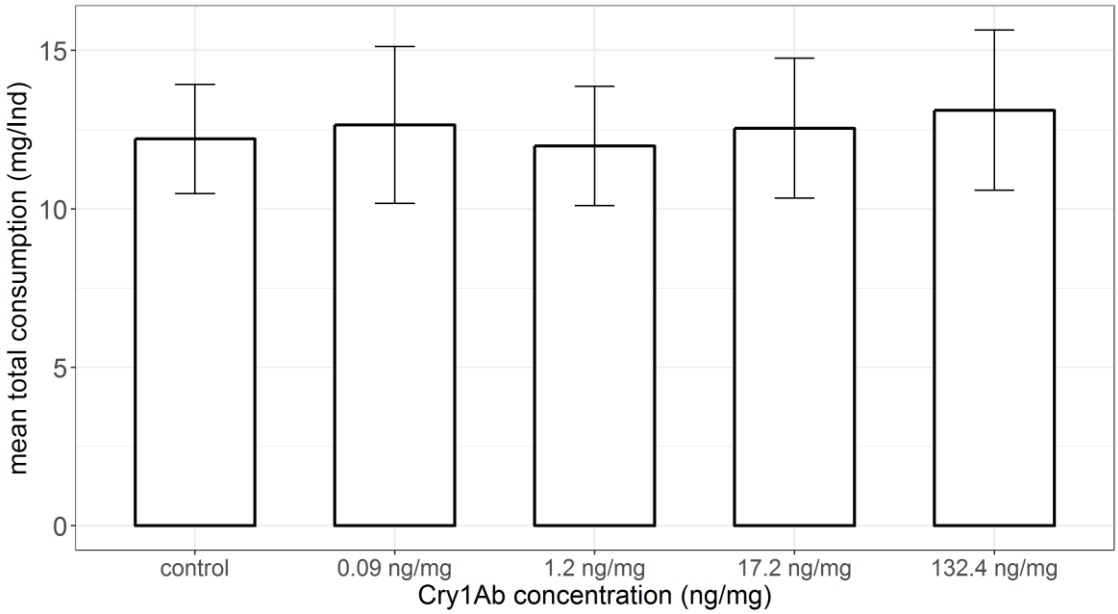


Figure S3 *Chaetopteryx* spec. mean consumption per week (mg dry weight/individual) in the feeding test. Shown are means (n=12) and standard deviation

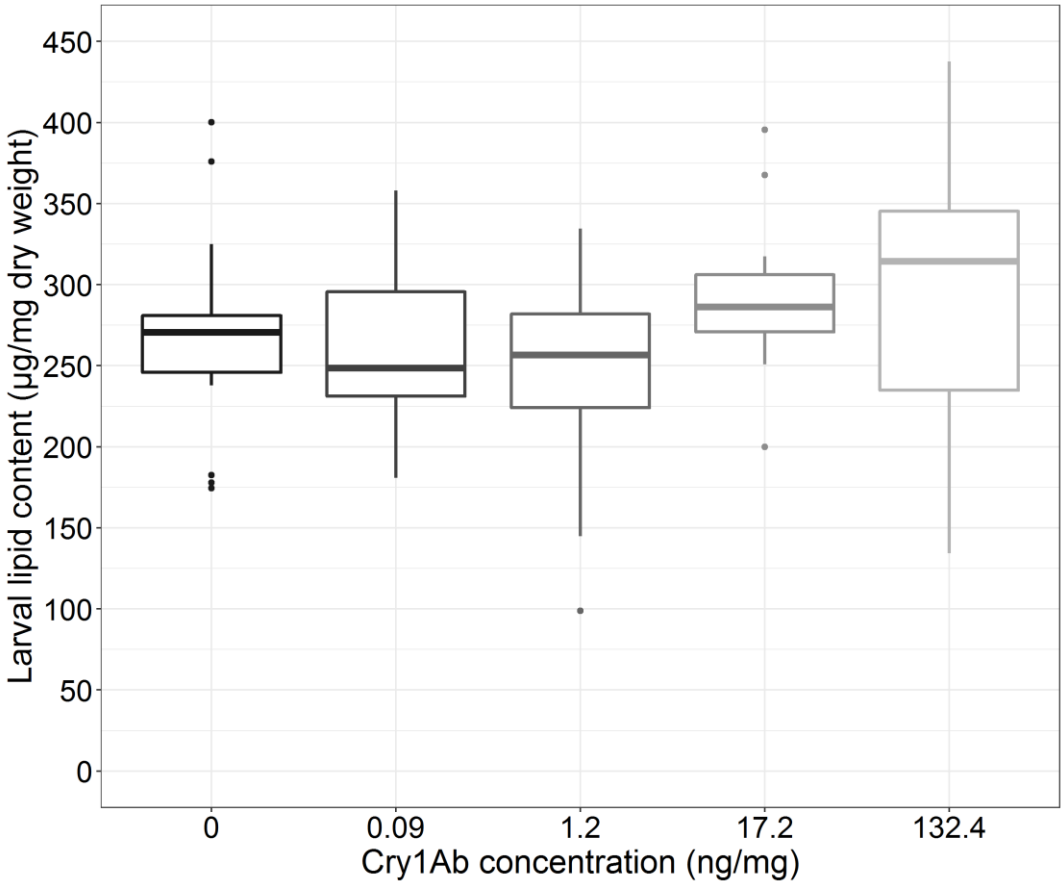


Figure S4 *Sericostoma* spec. larval lipid content after 6 weeks of feeding with Cry1Ab spiked leaf discs. Thick lines in the boxplots show medians (n=15), lower and upper quartile are covered by the upper and lower end of the box.

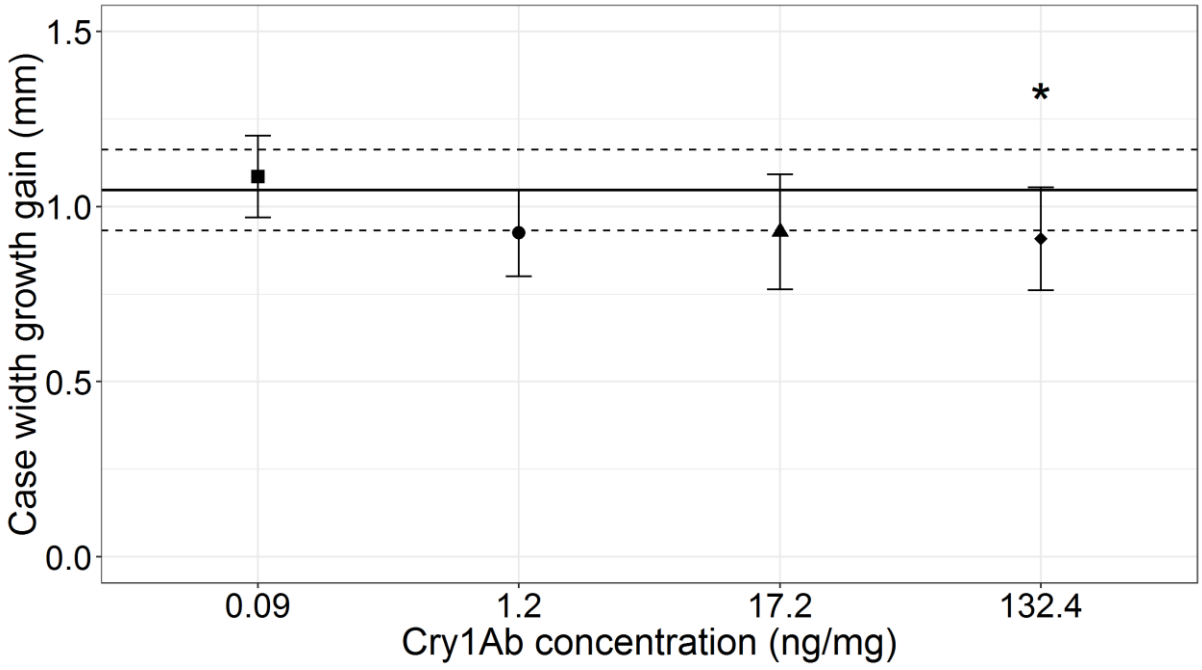


Figure S5 *Chaetopteryx* spec. growth gain of the caddis width after 12 weeks feeding with Cry1Ab spiked leave discs. Shown are medians (n=10) and 95% confidence interval. The solid and dashed lines show the median and the 95% confidence interval of the control. Effect size: 25.8%

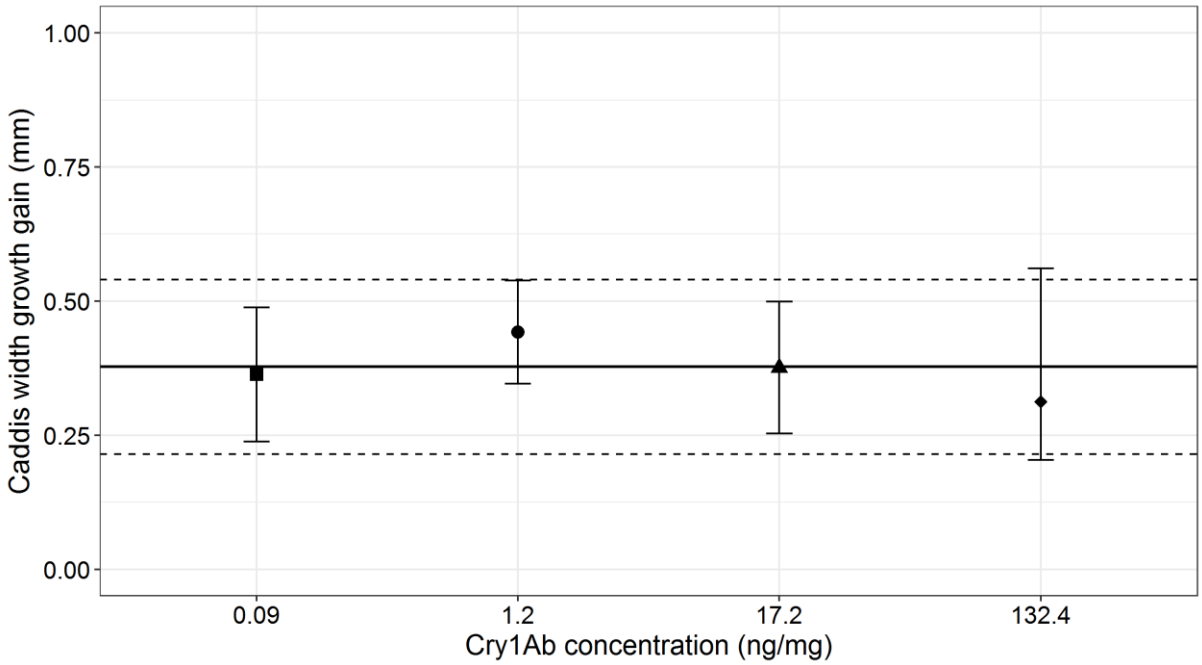


Figure S6 *Sericostoma* spec. growth gain of the caddis width after 6 weeks feeding with Cry1Ab spiked leave discs. Shown are medians (n=6-10) and 95% confidence interval. The solid and dashed lines show the median and the 95% confidence interval of the control.

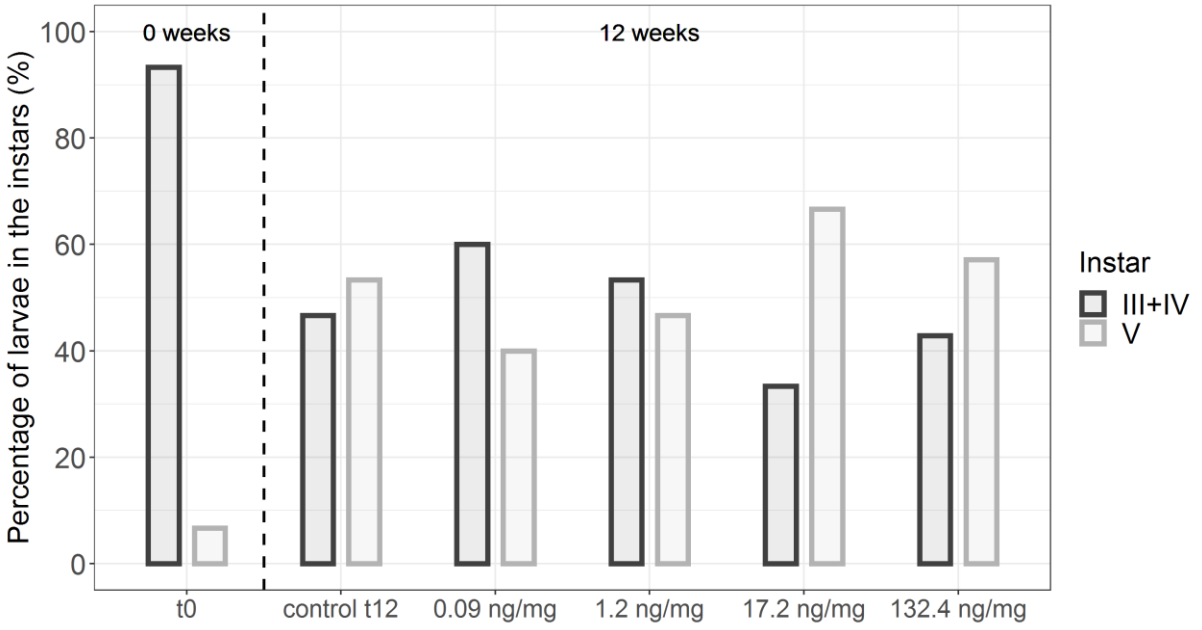


Figure S7 *Chaetopteryx* spec. larval stage at the start (t0) and the termination (t12) of the experiment in the control (control t12) and the various treatments, respectively (n=14-15). The dashed line separates the data of the larval instars at the beginning (0 weeks) and at the end (12 weeks) of the experiment. Larval stage III was found in control t12.

Table S3 Results of the statistical analysis. Shown are p-valuse and effect sizes for significant effects between Cry1Ab concentrations and the control. For the larval instars of *Sericostoma* spec. effect sizes are shown for the instars III+IV and V+VI.

Species	Endpoints	Concentration(ng/mg)	p-value	Effect size (%)
Chaetopteryx spec.	lipid content	17.2	0.0152	23.5
Chaetopteryx spec.	growth (case width)	132.4	0.0178	25.84
Sericostoma spec.	larval instars	132.4	0.0099	-233,3/ 58,3

European corn borer biotest

The European corn borer (*Ostrinia nubilalis*) is a target organism of Cry1Ab protein. In order to investigate, if the Cry1Ab protein used in the caddisfly experiment is bioactive and reaches its target site, biotests with larvae of the European corn borer (ECB) (Crambidae; *Ostrinia nubilalis*) (Annette Herz, Julius Kühn Institute, Darmstadt, Germany) were carried out. ECB eggs were reared in a climate chamber at 25±1°C, a relative humidity of 60% and a 16:8 L:D cycle. The procedure followed Conradin[1]: After hatching, the larvae were fed rearing diet ad libitum[2]. The diet contained 390 ml distilled water, 10 g agar, 25 g corn semolina, 25 g wheat germ, 25 g yeast powder, 0.9 g benzoic acid, 0.9 g nipagin and 2.25 g ascorbic acid per 0.5 l. At an age between 24 and 48 h, each larva and a small piece of moistened paper tissue were placed separately in rearing trays (C-D International, INC. Pitman, NJ, USA) to run the bioassay under conditions as detailed for rearing. To prepare the respective food, black alder leaves were dried at 60°C for 24 h and ground. Subsequently, 200 mg leaf powder was filled in vessels and Cry1Ab was added at increasing concentrations from its respective stock. The Cry1Ab spiked leaf powder was mixed with rearing diet targeting seven concentrations (0.49, 1.95, 7.81, 31.25, 125, 500, 2000 ng Cry1Ab/g). A control with diet and pure plant material and a control with diet only were also used. Subsamples from the spiked leaf powder were taken to analyze the Cry1Ab concentration and were stored in a -18 °C freezer. The bioassay duration was 7 days and the mortality was recorded every 24 h. The larvae were considered dead if after gentle touches with a brush no movement was observed. Although the spiking methods and the environmental conditions differed among experiments with ECB and the caddisfly species, we argue that the insights with regards to the biological activity of the Cry1Ab toxin applied through this method is not affected substantially.

The dose-response curve for *Ostrinia nubilalis* was prepared using a log-logistic model in the drc package[3] in R. The model that fits the data best were selected based on the model's AIC (Akaike information criterion).

The Cry1Ab concentrations on the spiked leaf powder as used during the ECB biotest uncovered measured concentrations of 0.09, 0.4, 2.6, 11.4, 58.8, 228.8 and 1087.7 ng/g. The ECB showed an increasing mortality with increasing Cry1Ab concentration and, thus, revealed a clear dose-response curve (Fig. S8). The calculated LC_{50} of 1.011 ng Cry1Ab/g is clearly lower than in another study with LC_{50} values between 100 and 2120 ng/g[4]. The mortality of the European corn borer in our study shows that the Cry1Ab toxin reached the target site in the larva. This finding could be transferred to the caddisfly test and verifies the bioactivity of the used Cry1Ab toxin. For tests with GMO a positive control often does not exist, which makes the proof of the bioactivity even more important.

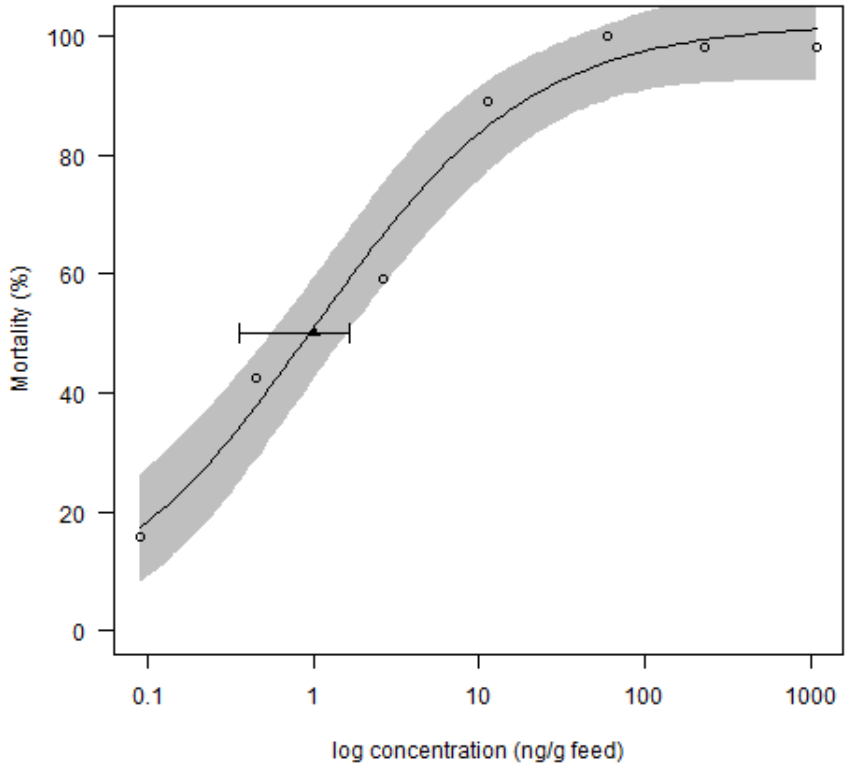


Figure S8 European corn borer mortality after 7 days of feeding with Cry1Ab spiked feed (n=3). Shown are means (n=3), dose-response curve and the 95% confidence interval. Triangle: LC_{50}=1.011 ng/g, 95% CI lower level: 0.359, upper level: 1.66. LC_{90}=28.499 ng/g, 95% CI lower level: -14,933, upper level: 71.931

References

1. Conradin, C. *Effect of antibiotics on the insecticidal activity of Bt-corn and DELFIN® toward the European corn borer Ostrinia nubilalis*. Semester thesis (2010).
2. Ivaldi-Sender, C. Simple techniques for a permanent breeding of oriental fruit moth *Grapholita molesta* Lepidoptera Torticidae on artificial diet. *Ann Zool Ecol Anim* **6,** 337–343 (1974).
3. Ritz, C., Baty, F., Streibig, J. C. & Gerhard, D. Dose-Response Analysis Using R. *PloS one* **10,** e0146021; 10.1371/journal.pone.0146021 (2015).
4. Saeglitz, C. *et al.* Monitoring the Cry1Ab susceptibility of European Corn Borer in Germany. *J Econ Entomol* **99,** 1768–1773; 10.1093/jee/99.5.1768 (2006).

A.3 Assessing effects of genetically modified plant material on the aquatic environment using higher-tier studies

Antonia Pott[1,2*], Mirco Bundschuh[1,3], Mathias Otto[2], Ralf Schulz[1]

[1] iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany

[2] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Lennart Hjelms väg 9, 75007 Uppsala, Sweden

*Correspondence to pott@uni-landau.de

In: *Bulletin of Environmental Contamination and Toxicology* (in press).

Abstract

Genetically modified organisms are used extensively in agriculture. To assess potential side effects of genetically modified (GM) plant material on aquatic ecosystems, only a very small number of higher-tier studies have been performed. At the same time, these studies are particularly important for comprehensive risk assessment covering complex ecological relationships. Here we evaluate the methods of experimental higher-tier effect studies with GM plant material (or Bt toxin) in comparison to those well-established for pesticides. A major difference is that nominal test concentrations and thus dose-response relationships cannot easily be produced with GM plant material. Another important difference, particularly to non-systemic pesticides, is that aquatic organisms are exposed to GM plant material primarily through their feed. These and further differences in test requirements, compared with pesticides, call for a standardisation for GM-specific higher-tier study designs to assess their potentially complex effects in the aquatic ecosystems comprehensively.

Key words: Genetically modified organisms, Bt toxin, higher-tier, aquatic ecosystems, non-target effects

Introduction

Genetically modified (GM) plants have been cultivated since 1996 and transgenic maize, soy and cotton contribute to the worldwide adoption of the technology (Fausti et al. 2012; James 2019; Schulz et al. 2021). Next to herbicide resistance, insect resistance is one of the main traits in GM plants (Parisi et al. 2016). The main way to achieve insect resistance is to insert genes from bacterial origin mostly from *Bacillus thuringiensis* (Bt), leading to the expression of insecticidal proteins within the plant. A range of Bt and vegetative insecticidal proteins (VIP) have been modified to adapt to plant expression and to enhance toxicity against different pest taxa (Gill et al. 1992; Schnepf et al. 1998; Sharma et al. 2004). Bt toxins expressed in GM plant material mainly target Lepidoptera and Coleoptera. During the first 10 years, the environmental risk assessment of Bt plants focused on the terrestrial environment (Pott et al. 2018; Venter und Bøhn 2016). This changed as evidence accumulated that also aquatic ecosystems are exposed to Bt toxins from GM plant cultivation, for instance, with plant material from run-off or by Bt toxin leached from agricultural soil (Tank et al. 2010; Strain und Lydy 2015; Jensen et al. 2010). As some studies showed adverse effects from GM plants on aquatic organisms (Bøhn et al. 2010; Pott et al. 2020; Prihoda und Coats 2008; Rosi-Marshall et al. 2007), this points to a potential risk to aquatic ecosystems (Rosi-Marshall et al. 2007).

In the context of the European chemical risk assessment, which serves in this paper as reference framework, test approaches are divided in four tiers. The test approaches become more complex as the tiers increase, but also more realistic. Acute laboratory tests with standard test organisms fall under the category of Tier 1 studies. Tier 1 studies represent a worst-case situation under controlled test conditions and investigate specific endpoints. Until now, many studies investigating the effects of GM plants on aquatic organisms, are laboratory single-species tests (Carstens et al. 2012; De Schrijver et al. 2016; Pott et al. 2018). Laboratory tests, which have a longer exposure time or do not use standard test organisms are classified as a Tier 2 study. Approximately 32 out of a total of 33 evaluated studies carried out Tier 2 studies (Pott et al. 2018; Pott et al. 2020; Bundschuh et al. 2019). Tier 1 and Tier 2 tests are commonly grouped under the term ´lower-tier studies´ (for a list of lower-tier studies see supplementary information in Pott et al. 2018). According to the European Food Safety Authority (EFSA) aquatic guidance document for pesticides (EFSA 2013) further and more complex test designs are categorized as Tier 3 (population- or community studies in micro or mesocosms) and Tier 4 (field scale or landscape modeling approaches) studies, often covered under the general term ´higher-tier studies´. In the present paper, we refer to experimental (micro-, mesocosm, field) higher-tier effect studies. Experimental higher-tier studies are useful, if adverse effects that have been found in lower-tier studies, should be assessed under more field relevant conditions. They enables the investigation of diverse groups of organisms considering both direct and indirect effects (Preston 2002; Brock et al. 2010; EFSA 2013). The inclusion of different matrices (water, sediment, plants), as possible options in higher-tier studies, allows for a more realistic fate and behavior of the tested chemical (Hand und Oliver 2010). Experimental higher-tier studies can reveal both top-down and bottom-up effects in the food webs (Wieczorek et al. 2015) and extended study durations supports the assessment of chronic endpoints as well as recovery (Wieczorek et al. 2017).

Effects on non-target organisms induced by GMO are also assessed on the basis of tiered testing (EFSA 2010a, 2010b; Wolt et al. 2010). In this tiered system, higher tiers are foreseen if hazards were identified in the initial tiers. Higher-tier studies with aquatic organisms are yet not a prerequisite in the European Union for the approval of a GMO. It follows that until now only few non-standardized higher-tier studies with GMO exist, despite these studies being well established for pesticides (Sanderson 2002; Brock et al. 2010).

Here we evaluate specifics and difficulties in carrying out higher-tier studies with GM plant material (or Bt toxin) through a comparison with pesticide higher-tier approaches. We consider

for the GM studies whether GM plant material or a pure bacterially produced Bt toxin is used. We focus on GM plant material and do not include here the ecological consequences of Bti applications against biting midges, which were reviewed, for example, by Brühl et al. (2020).

Existing higher-tier studies using GM-plant material

Up to now, there are three approaches that have been used for higher-tier studies investigating the effects of GM plants on the aquatic environment (Table S1). One approach measured the abundance of aquatic organisms in the water of rice fields planted with GM rice and non-GM rice (Li et al. 2014; Liu et al. 2016; Wang et al. 2013). At several time points water samples were taken and the number of zoo- or phytoplankton were counted. The respective studies showed only minor effects on zoo- and phytoplankton. A Cry1Ab/1Ac producing rice (Bt MH63, Bt SY63) had no impact on the abundance of rotifers, cladocerans and copepods (Wang et al. 2013). In contrast, another study showed significantly higher abundances of zooplankton (rotifers, cladocerans, copepods) in a Cry1Ab/1Ac (Huahui-1) and a Cry2A (variety of Bt MH63) producing rice treatment than in non-Bt rice plots (Li et al. 2014). Since the authors found no effects on zooplankton in a previous study, they conclude that the significant effects may be caused by the use of pesticides on the plots. Liu et al. (2016) also found no significant difference in the abundance, species number and biodiversity indices of zooplankton between Bt (Cry1Ab/1Ac, Huahui-1) and non-Bt rice fields in two sampling years. Although the phytoplankton did not show any differences in 2013, significant higher abundances, number of species and biodiversity indices were observed in 2014 in Bt rice fields compared to the non-Bt treatment (Liu et al. 2016). However, the statistical analysis showed that the difference was mainly driven by water quality parameters differing among treatments (Liu et al. 2016).

A second approach used GM plant material in litterbags to measure the abundance of aquatic organisms on decomposition of the plant material (Swan et al. 2009; Chambers et al. 2010; Axelsson et al. 2010; Axelsson et al. 2011; Liu et al. 2017). GM plant material and a non-GM control were placed into mesh bags, which were attached to the stream bottom. After consecutive time periods, the aquatic organisms present on the leaves were counted, the species identified and the remaining plant material was quantified. From the differences between GM-treatment and control conclusions could be derived.

At two out of ten sites in the USA the Bt maize producing Cry1Ab and the stacked maize, which produces Cry1Ab and Cry3Bb1 had significant lower breakdown rate than the non-Bt maize (Swan et al. 2009). Similar results were shown by another study, which measured a slower

decomposition in genetically modified aspen-poplar (CAD) than in a non-GM control (Axelsson et al. 2010). However, aspen-poplars with other genetically modifications (COMT, Cry3Aa) did not show such effect (Axelsson et al. 2010; Axelsson et al. 2011). Furthermore, Bt rice (Cry1Ab/1Ac) showed also no significant different decomposition compared to a non-Bt control (Liu et al. 2017).

A few taxa showed significantly lower abundance in single Bt maize (Cry1Ab) and stacked Bt maize plants (Cry1Ab x Cry3Bb1, Cry1Ab x Cry3Bb1 x Roundup Ready) compared to a non-Bt control (Swan et al. 2009). Overall, Swan et al. (2009) showed only at a few sites and in a few taxa (e.g. *Pycnopsyche* sp.) significant differences. Chambers (2010) also confirmed minor effects. The authors conducted a litterbag study on 12 streams in the USA with Bt (Cry1Ab) and non-Bt maize and found no significant effects in diversity, invertebrate biomass and functional feeding group composition. Also a study in Sweden showed no significant effects concerning the abundance of invertebrates in a litterbag experiment between two genetically modified aspen-poplars (CAD, COMT) and a non-GM aspen-poplar (Axelsson et al. 2010). Most likely, the toxin concentrations in the leaves were too low to produce direct effects. In contrast to the aforementioned studies, other genetically modified aspen-poplars, which produce Cry3Aa (Bt17, Bt27), led to a significant increase in average abundance of EPT (Ephemeroptera, Plecoptera, Trichoptera) fauna and Coleopterans compared with a non-GM control (Axelsson et al. 2011). However, this study showed no differences in total abundance, abundance of orders, functional feeding groups and species richness. Also, no significant difference in the meiobenthos abundance were found between Bt rice (Cry1Ab/1Ac) and non-Bt rice in a litterbag study conducted in China (Liu et al. 2017).

In a third approach, benthic cores were collected in streams whose banks are covered with either non-Bt or Bt maize. Invertebrates were removed from the samples and the species identified. No significant differences in abundance of all taxa, total biomass, functional feeding group composition, diversity, richness and EPT richness between Bt (Cry1Ab, event not specified) and non-Bt streams were found (Chambers et al. 2010). Only single taxa showed significantly different abundance and biomass in Bt streams compared to non-Bt streams. However, the authors explain these effects, despite the similar morphology of the streams, with differences in stream physical characteristics (e.g. velocity) and food resource availability (e.g. prey availability).

Overall, the currently available higher-tier GM plant material studies found little or no evidence for effects on aquatic organisms. This either reflects the real situation or methodological aspects

specific for higher-tier studies with GM plant material which leads to difficulties in evaluating effects in comparison to dissolved substances, which will be discussed using the comparably well-established higher-tier approaches for pesticides as a reference.

Higher-tier studies on GM plant material versus pesticides

Higher-tier studies with pesticides usually aim at representing a variety of aquatic species with a diversity of ecological traits (EFSA 2013). When selecting suitable test organisms for any kind of higher-tier study, criteria such as exposure situation, functional role, endangered species status, and practicability may be considered (Hilbeck et al. 2017). As a key point, the mode of action of the test substance should be considered. Those GM traits predominantly used focused on Lepidoptera, therefore caddisflies (Trichoptera), which are phylogenetically closely related (Hilbeck et al. 2017), should consequently be considered for testing (Table 1). In fact, GM effects have already been found in Trichoptera (Pott et al. 2020; Rosi-Marshall et al. 2007). However, only shredding and scraping caddisfly larvae have been studied to date (Pott et al. 2018). Studies with net-spinning Trichoptera do not exist, although their nets may trap GM plant debris.

Ensuring a sufficient variety of traits of Trichoptera is thus considered important, particularly when natural communities are used, as sometimes done in mesocosm studies (Cañedo-Argüelles et al. 2014; Wieczorek et al. 2016). In this context, it is important to ensure study durations that are sufficiently long to allow the full larval development of caddisflies to also detect effects that occur later in the life cycle (Schulz und Liess 1995).

Pesticides are introduced into the aquatic environment as a result of edge-of-field runoff, spray drift, via drainage or following direct application (Flury 1996; Schulz et al. 2001; Wauchope 1978; Armbrust und Peeler 2002; Lamers et al. 2011; Starner und Goh 2012). It follows that the exposure is regularly via water and potentially via sediment. Edge-of-field runoff and drainage are relevant pathways also for GM material into the aquatic environment when the toxin itself is considered (Tank et al. 2010; Strain und Lydy 2015; Jensen et al. 2010). However, the situation is different when GM plant material is concerned. Here, the entry route via the surface run-off and aerial transport, for example, during harvest, likely are key routes of entry (Bundschuh et al. 2016; Bundschuh et al. 2019; Pott et al. 2018).

Most importantly, the entry of GM plant material also leads to an entirely different exposure of the aquatic organisms (Table 1). This plant material, which contains Bt toxins, may serve as food (Bundschuh et al. 2019). Not only the input of GM plant material into waterbodies (Pott

et al. 2018), but also the direct uptake of maize plant material, for example, by Trichoptera has already been shown (Rosi-Marshall et al. 2007). The exposure to GM plant material is, however, relevant for all kinds of shredding organisms, including insect larvae or crustaceans (Zubrod et al. 2014). It is in this context of interest which stream types are particularly exposed, since this may affect exposure conditions (Bundschuh et al. 2016). In running waters, the leaf material may be transported downstream (Rosi-Marshall et al. 2007) and accumulate in areas with low current, where high concentrations of GM plant material may occur (Bundschuh et al. 2016). This is particularly relevant in natural waterbodies and highlights the variability in exposure scenarios in the field (Bundschuh et al. 2016) – a fact which requires attention in designing higher-tier studies.

It should be noted that the entry pathway via plant material is also relevant for systemic insecticides (neonicotinoids)(Bundschuh et al. 2019; Englert et al. 2017a; Englert et al. 2017b; Englert et al. 2017c; Kreutzweiser et al. 2008) or fungicides, though the latter has not yet been studied well (Newton et al. 2018; Zubrod et al. 2019). Systemic pesticides are distributed throughout the plant as it grows. Plants treated with systemic pesticides are therefore similar to GM plant material with regard to their environmental distribution (Bundschuh et al. 2016) and uptake pathway (Bundschuh et al. 2019). Interestingly, the exposure via food is usually not considered even in higher-tier pesticide studies.

In addition to the entry and exposure pathway, the entry frequency is also important when designing higher-tier studies. Many insecticides are applied once or few times per season and, therefore, re-occurring transient, short-term exposure scenarios are typical (Brown et al. 2002; Stehle et al. 2013). On the other hand, many fungicides and herbicides, yet also systemic insecticides, such as neonicotinoids, occur in surface waters for chronic, long-term time periods (Brown et al. 2002; Zubrod et al. 2019; Morrissey et al. 2015). Similarly, GMOs are likely to occur in the field for extended periods of time (up to weeks or months, Jensen et al. (2010). These exposure characteristics require appropriate consideration in setting up higher-tier effect studies with GM plant material (Table 1). Another temporal aspect is, however, that the amounts of toxin in GM plant material can be degraded (Li et al. 2007; Böttger et al. 2015; Pott et al. 2020). Dispersion, sorption, for example, to sediments (Douville et al. 2005), and degradation, as also shown for pesticides (Stang et al. 2013; Stang et al. 2014; Wieczorek et al. 2018), may furthermore reduce the bioavailability of GM toxins over time.

Studies have shown that Bt toxins can leach from leaf material (Griffiths et al. 2017). Through leaching, the toxins exposure profile may shift to water-borne exposure (Wang et al. 2014; Liu

et al. 2016; Englert et al. 2017c), again comparable to systemic neonicotinoid insecticides (Englert et al. 2017c). This clearly extents the exposure pathway via food as described above, by a rather direct water-borne exposure and justifies studies using suspended or solved Bt toxin as the test item. However, since Bt toxins occur in water at significantly lower concentrations than in fresh GM-plant material and degrade rapidly in water (Brandão-Dias et al. 2021; Douville et al. 2005; Tank et al. 2010), this route of exposure may be less important.

The test substance used in mesocosm studies with pesticides is the active ingredient of the pesticide or the formulated product (Hanson et al. 2007; Beuter et al. 2019; van Wijngaarden et al. 2004) as commercially available. Unlike the Bt toxin, which can be manufactured using bacteria and which thus is readily available for ecotoxicity studies (Pott et al. 2020), the situation for GM plant material is more complicated. In these studies, pollen, cob and stalk may be used in addition to leaves. These materials are not sold specifically for scientific purposes but have to be grown from seed. As the risk assessment of GMO takes place before market authorization, seeds cannot be obtained at this stage without consent of the GMO developer. This situation is a key challenge for any sort of GM plant-related ecotoxicity study from the public domain (Table 1). Even after commercialisation obtaining seeds is difficult because buying seeds is linked to licence agreements which may not foresee to use seeds for research purposes. Once seed is obtained, the variability in plant composition and in the expression of novel proteins due to different genetic backgrounds must be accounted for in ecotoxicity tests. Efforts prior to any higher tier study needs to be taken to produce a rather standardized GM plant material.

Another aspect directly related, is the identity and purity of the test material available for higher-tier studies. Unlike the situation for pesticides or for Bt toxins, GM plant material may contain traces of other GM plants along with the presence of unwanted other pollutants such as (systemic) pesticides. Methods for the detection of GM material are described, for example, in the DIN EN ISO series of standards “foodstuffs – Methods for analysis for the detection of genetically modified organisms and derived products” (DIN EN ISO 24276, 21569, 21570). The concentrations of the novel protein should also be measured, for example, by using an enzyme-linked immunosorbent assay (Pott et al. 2020). In case of the Bt toxin, it is, however, also important to estimate the bioactivity of the specific protein (or charge) in question. Bioactivity is usually checked by using bioassays with the target organism (Pott et al. 2020). However, the role of breakdown products of Bt toxins is unclear (Latham et al. 2017). Furthermore, appropriate follow-up measurements of the GM plant material or more precisely

the Bt toxin therein, are required throughout the course of a higher-tier study, to characterise exposure (Strain et al. 2014).

An aspect that is common practice in higher-tier pesticide studies, namely the usage of various test concentrations, poses critical problems, when it comes to GM plant material. Usually, only plant material with one GM toxin concentration is available, which makes it impossible to obtain different concentrations (Table 1). This is a fundamental drawback for the subsequent risk assessment and further regulatory steps. The provision of different test concentrations is not easily possible. Theoretically, it is possible to mix GM plant material with non-GM plant material. Such a procedure, however, greatly depends on the availability of non-GM plant material as comparable as possible to the GM plant material. Otherwise, feeding preferences of aquatic organisms, which have been repeatedly reported (Bundschuh et al. 2009; Konschak et al. 2019), may affect the exposure conditions. Although the toxin concentration can vary greatly depending on the plant part and stage of development (Griffiths et al. 2009; Böttger et al. 2015), since these different developmental stages differ in various characteristics and can therefore not simply be used to provide treatments with intermediate toxin levels. While even in higher-tier pesticide studies, one high exposure concentration may be used as a positive control, the difficulty to provide different exposure concentrations for GM plant material, also applies to the simple provision of positive control treatments.

Lastly, a negative control, i.e. a treatment without the toxicant present, yet all other conditions being the same, is another cornerstone of any ecotoxicological study including higher-tier experiments. While this is again fairly easy to establish in studies with pesticides (or the pure Bt toxin), similar conditions as described previously, may cause severe problems with regard to a negative control in a higher-tier study with GM plant material (Table 1). Simply using a non-GM plant may not be sufficient, since the genetically modified plant may differ from the non-GM plant by other parameters than just the Bt toxin (Swan et al. 2009; Axelsson et al. 2010; Saxena und Stotzky 2001). This could, as briefly mentioned above, for example, result in differences in the nutritional value of the plant material, which then affects the outcome of the study. Therefore, when selecting suitable plant material for a negative control, care should be taken to ensure, for example, a comparable C/N ratio and lignin content to the GM plant material (Rosi-Marshall et al. 2007). Ideally more than one non-GM control plant materials with similar composition may be employed, to better understand the role of plant composition parameters on the ecotoxicological endpoints.

All the problems with regard to the establishment of different treatment concentrations for GM plant materials, do not apply that strictly when the pure GM toxin is used. On the other hand, these studies may not sufficiently represent the exposure pathway via food. This may be circumvented by using a spiking method (Pott et al. 2020). i.e. in applying pure Bt toxin to non-GM plant material, which is then used as food. However, this is still a relatively new approach requiring further attention and refinement in future.

In contrast to pesticides, there is no standardisation or guidance for GM higher-tier effect studies. However, this aspect is important in order to define suitable test conditions that allow comparability of different studies and therefore increase their applicability in the risk assessment. The need to develop guidance for GM higher-tier effect studies is also apparent from the existing guidance documents, for example, from EFSA (EFSA 2010a, 2010b) which foresees a tiered testing approach including higher-tier testing for GMO. Higher-tier aquatic studies with GMO can only play a realistic role in risk assessment on the premise that suitable methods will be developed in the future. Since the development of insecticidal proteins, or the usage of DS-RNAi constructs continues at a fast pace (Boeckman et al. 2019; Schellenberger et al. 2016; Anderson et al. 2018), the discussion around adequate higher-tier studies will likely gain traction.

References

Anderson, Jennifer A.; Staley, Jamie; Challender, Mary; Heuton, Jamie (2018): Safety of Pseudomonas chlororaphis as a gene source for genetically modified crops. In: *Transgenic research* 27 (1), S. 103–113. DOI: 10.1007/s11248-018-0061-6.

Armbrust, Kevin L.; Peeler, Harold B. (2002): Effects of formulation on the run-off of imidacloprid from turf. In: *Pest management science* 58 (7), S. 702–706. DOI: 10.1002/ps.518.

Axelsson, E. Petter; Hjältén, Joakim; LeRoy, Carri J.; Julkunen-Tiitto, Riitta; Wennström, Anders; Pilate, Gilles (2010): Can Leaf Litter from Genetically Modified Trees Affect Aquatic Ecosystems? In: *Ecosystems* 13 (7), S. 1049–1059. DOI: 10.1007/s10021-010-9373-y.

Axelsson, E. Petter; Hjältén, Joakim; LeRoy, Carri J.; Whitham, Thomas G.; Julkunen-Tiitto, Riitta; Wennström, Anders (2011): Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition. In: *J Appl Ecol* 48 (6), S. 1472–1479. DOI: 10.1111/j.1365-2664.2011.02046.x.

Beuter, Liesa-Kristin; Dören, László; Hommen, Udo; Kotthoff, Matthias; Schäfers, Christoph; Ebke, Klaus Peter (2019): Testing effects of pesticides on macroinvertebrate communities in outdoor stream mesocosms using carbaryl as example test item. In: *Environ Sci Eur* 31 (1), S. 3290. DOI: 10.1186/s12302-019-0185-1.

Boeckman, Chad J.; Huang, Emily; Sturtz, Kristine; Walker, Carl; Woods, Rachel; Zhang, John (2019): Characterization of the Spectrum of Insecticidal Activity for IPD072Aa: A Protein Derived from Pseudomonas chlororaphis with Activity Against Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae). In: *Journal of economic entomology* 112 (3), S. 1190–1196. DOI: 10.1093/jee/toz029.

Bøhn, Thomas; Traavik, Terje; Primicerio, Raul (2010): Demographic responses of *Daphnia magna* fed transgenic Bt-maize. In: *Ecotoxicology* 19 (2), S. 419–430. DOI: 10.1007/s10646-009-0427-x.

Böttger, Rita; Schaller, Jörg; Lintow, Sven; Gert Dudel, E. (2015): Aquatic degradation of Cry1Ab protein and decomposition dynamics of transgenic corn leaves under controlled conditions. In: *Ecotoxicol Environ Saf* 113, S. 454–459. DOI: 10.1016/j.ecoenv.2014.12.034.

Brandão-Dias, Pedro F. P.; Rosi, Emma J.; Shogren, Arial J.; Tank, Jennifer L.; Fischer, David T.; Egan, Scott P. (2021): Fate of Environmental Proteins (eProteins) from Genetically Engineered Crops in Streams is Controlled by Water pH and Ecosystem Metabolism. In: *Environmental science & technology* 55 (8), S. 4688–4697. DOI: 10.1021/acs.est.0c05731.

Brock, Theo C. M.; Alix, Anne; Brown, Colin D.; Capri, Ettore; Gottesbüren, Bernhard F.F.; Heimbach, Fred et al. (Hg.) (2010): Linking aquatic exposure and effects. Risk assessment of pesticides. Boca Raton, London, New York: CRC Press, Taylor & Francis Group (Society of environmental toxicology and chemistry (SETAC)).

Brown, Colin D.; Bellamy, Pat H.; Dubus, Igor G. (2002): Prediction of pesticide concentrations found in rivers in the UK. In: *Pest management science* 58 (4), S. 363–373. DOI: 10.1002/ps.466.

Brühl, Carsten A.; Després, Laurence; Frör, Oliver; Patil, Chandrashekhar D.; Poulin, Brigitte; Tetreau, Guillaume; Allgeier, Stefanie (2020): Environmental and socioeconomic effects of mosquito control in Europe using the biocide Bacillus thuringiensis subsp. israelensis (Bti). In: *The Science of the total environment* 724, S. 137800. DOI: 10.1016/j.scitotenv.2020.137800.

Bundschuh, Mirco; Hahn, Torsten; Gessner, Mark O.; Schulz, Ralf (2009): Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. In: *Environmental toxicology and chemistry* 28 (1), S. 197–203. DOI: 10.1897/08-075.1.

Bundschuh, Rebecca; Bundschuh, Mirco; Otto, Mathias; Schulz, Ralf (2019): Food-related exposure to systemic pesticides and pesticides from transgenic plants: evaluation of aquatic test strategies. In: *Environ Sci Eur* 31 (1), S. 3290. DOI: 10.1186/s12302-019-0266-1.

Bundschuh, Rebecca; Kuhn, Ulrike; Bundschuh, Mirco; Naegele, Caroline; Elsaesser, David; Schlechtriemen, Ulrich et al. (2016): Prioritizing stream types according to their potential risk to receive crop plant material - A GIS-based procedure to assist in the risk assessment of genetically modified crops and systemic insecticide residues. In: *Sci Total Environ* 547, S. 226–233. DOI: 10.1016/j.scitotenv.2015.12.124.

Cañedo-Argüelles, Miguel; Bundschuh, Mirco; Gutiérrez-Cánovas, Cayetano; Kefford, Ben J.; Prat, Narcís; Trobajo, Rosa; Schäfer, Ralf B. (2014): Effects of repeated salt pulses on ecosystem structure and functions in a stream mesocosm. In: *The Science of the total environment* 476-477, S. 634–642. DOI: 10.1016/j.scitotenv.2013.12.067.

Carstens, Keri; Anderson, Jennifer; Bachman, Pamela; De Schrijver, Adinda; Dively, Galen; Federici, Brian et al. (2012): Genetically modified crops and aquatic ecosystems: Considerations for environmental risk assessment and non-target organism testing. In: *Transgenic Res* 21 (4), S. 813–842. DOI: 10.1007/s11248-011-9569-8.

Chambers, Catherine P.; Whiles, Matt R.; Rosi-Marshall, Emma J.; Tank, Jennifer L.; Royer, Todd V.; Griffiths, Natalie A. et al. (2010): Responses of stream macroinvertebrates to Bt maize leaf detritus. In: *Ecol Appl* 20 (7), S. 1949–1960. DOI: 10.1890/09-0598.1.

De Schrijver, Adinda; Devos, Yann; De Clercq, Patrick; Gathmann, Achim; Romeis, Jörg (2016): Quality of laboratory studies assessing effects of Bt-proteins on non-target organisms: Minimal criteria for acceptability. In: *Transgenic Res* 25 (4), S. 395–411. DOI: 10.1007/s11248-016-9950-8.

DIN EN ISO 21569:2013-08, Lebensmittel - Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Qualitative auf Nukleinsäuren basierende Verfahren.

DIN EN ISO 21570:2013-08, Lebensmittel- Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Quantitative auf Nukleinsäuren basierende Verfahren.

DIN EN ISO 24276:2013-10, Lebensmittel - Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Allgemeine Anforderungen und Definitionen.

Douville, M.; Gagn, F.; Masson, L.; McKay, J.; Blaise, C. (2005): Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. In: *Biochem Syst Ecol* 33 (3), S. 219–232. DOI: 10.1016/j.bse.2004.08.001.

EFSA (2010a): Guidance on the environmental risk assessment of genetically modified plants. In: *EFS2* 8 (11), S. 1879. DOI: 10.2903/j.efsa.2010.1879.

EFSA (2010b): Scientific Opinion on the assessment of potential impacts of genetically modified plants on non-target organisms. In: *EFS2* 8 (11), S. 1877. DOI: 10.2903/j.efsa.2010.1877.

EFSA (2013): Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. In: *EFS2* 11 (7). DOI: 10.2903/j.efsa.2013.3290.

Englert, Dominic; Bakanov, Nikita; Zubrod, Jochen P.; Schulz, Ralf; Bundschuh, Mirco (2017a): Modeling Remobilization of Neonicotinoid Residues from Tree Foliage in Streams - A Relevant Exposure Pathway in Risk Assessment? In: *Environ Sci Technol* 51 (3), S. 1785–1794. DOI: 10.1021/acs.est.6b05213.

Englert, Dominic; Zubrod, Jochen P.; Link, Moritz; Mertins, Saskia; Schulz, Ralf; Bundschuh, Mirco (2017b): Does Waterborne Exposure Explain Effects Caused by Neonicotinoid-Contaminated Plant Material in Aquatic Systems? In: *Environ Sci Technol* 51 (10), S. 5793–5802. DOI: 10.1021/acs.est.7b00827.

Englert, Dominic; Zubrod, Jochen P.; Pietz, Sebastian; Stefani, Sonja; Krauss, Martin; Schulz, Ralf; Bundschuh, Mirco (2017c): Relative importance of dietary uptake and waterborne exposure for a leaf-shredding amphipod exposed to thiacloprid-contaminated leaves. In: *Scientific reports* 7 (1), S. 16182. DOI: 10.1038/s41598-017-16452-9.

Fausti, Scott W.; McDonald, Tia Michelle; Lundgren, Jonathan G.; Li, Jing; Keating, Ariel Ruth; Catangui, Mike (2012): Insecticide use and crop selection in regions with high GM adoption rates. In: *Renew. Agric. Food Syst.* 27 (4), S. 295–304. DOI: 10.1017/s1742170511000561.

Flury, Markus (1996): Experimental Evidence of Transport of Pesticides through Field Soils—A Review. In: *J. environ. qual.* 25 (1), S. 25–45. DOI: 10.2134/jeq1996.00472425002500010005x.

Gill, S. S.; Cowles, E. A.; Pietrantonio, P. V. (1992): The mode of action of Bacillus thuringiensis endotoxins. In: *Annual review of entomology* 37, S. 615–636. DOI: 10.1146/annurev.en.37.010192.003151.

Griffiths, Natalie A.; Tank, Jennifer L.; Royer, Todd V.; Rosi, Emma J.; Shogren, Arial J.; Frauendorf, Therese C.; Whiles, Matt R. (2017): Occurrence, leaching, and degradation of Cry1Ab protein from transgenic maize detritus in agricultural streams. In: *Sci Total Environ* 592, S. 97–105. DOI: 10.1016/j.scitotenv.2017.03.065.

Griffiths, Natalie A.; Tank, Jennifer L.; Royer, Todd V.; Rosi-Marshall, Emma J.; Whiles, Matt R.; Chambers, Catherine P. et al. (2009): Rapid decomposition of maize detritus in agricultural headwater streams. In: *Ecol Appl* 19 (1), S. 133–142. DOI: 10.1890/07-1876.1.

Hand, Laurence H.; Oliver, Robin G. (2010): The behavior of isopyrazam in aquatic ecosystems: implementation of a tiered investigation. In: *Environmental toxicology and chemistry* 29 (12), S. 2702–2712. DOI: 10.1002/etc.337.

Hanson, Mark L.; Graham, David W.; Babin, Emmanuelle; Azam, Didier; Coutellec, Marie-Agnes; Knapp, Charles W. et al. (2007): Influence of isolation on the recovery of pond mesocosms from the application of an insecticide. I. Study design and planktonic community responses. In: *Environmental toxicology and chemistry* 26 (6), S. 1265–1279. DOI: 10.1897/06-248R.1.

Hilbeck, Angelika; Bundschuh, Rebecca; Bundschuh, Mirco; Hofmann, Frieder; Oehen, Bernadette; Otto, Mathias et al. (2017): Procedure to select test organisms for environmental risk assessment of genetically modified crops in aquatic systems. In: *Integr Environ Assess Manag* 13 (6), S. 974–979. DOI: 10.1002/ieam.1965.

James, Clive (2019): Global Status of Commercialized Biotech/GM Crops: 2019. International Service for the Acquisition of Agri-biotech Applications (ISAAA).

Jensen, Peter D.; Dively, Galen P.; Swan, Christopher M.; Lamp, William O. (2010): Exposure and nontarget effects of transgenic Bt corn debris in streams. In: *Environ Entomol* 39 (2), S. 707–714. DOI: 10.1603/EN09037.

Konschak, Marco; Zubrod, Jochen P.; Baudy, Patrick; Englert, Dominic; Herrmann, Bastian; Schulz, Ralf; Bundschuh, Mirco (2019): Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder Chaetopteryx villosa (Trichoptera). In: *Aquatic toxicology (Amsterdam, Netherlands)* 206, S. 33–42. DOI: 10.1016/j.aquatox.2018.10.021.

Kreutzweiser, David P.; Good, Kevin P.; Chartrand, Derek T.; Scarr, Taylor A.; Thompson, Dean G. (2008): Are leaves that fall from imidacloprid-treated maple trees to control Asian longhorned beetles toxic to non-target decomposer organisms? In: *Journal of environmental quality* 37 (2), S. 639–646. DOI: 10.2134/jeq2007.0278.

Lamers, Marc; Anyusheva, Maria; La, Nguyen; van Nguyen, Vien; Streck, Thilo (2011): Pesticide Pollution in Surface- and Groundwater by Paddy Rice Cultivation: A Case Study from Northern Vietnam. In: *Clean Soil Air Water* 39 (4), S. 356–361. DOI: 10.1002/clen.201000268.

Latham, Jonathan R.; Love, Madeleine; Hilbeck, Angelika (2017): The distinct properties of natural and GM cry insecticidal proteins. In: *Biotechnology & genetic engineering reviews* 33 (1), S. 62–96. DOI: 10.1080/02648725.2017.1357295.

Li, Guangsheng; Wang, Yongmo; Liu, Biao; Zhang, Guoan (2014): Transgenic *Bacillus thuringiensis* (Bt) rice is safer to aquatic ecosystems than its non-transgenic counterpart. In: *PloS one* 9 (8), e104270. DOI: 10.1371/journal.pone.0104270.

Li, Yunhe; Wu, Kongming; Zhang, Yongjun; Yuan, Guohui (2007): Degradation of Cry1Ac Protein Within Transgenic *Bacillus thuringiensis* Rice Tissues Under Field and Laboratory Conditions. In: *Environ Entomol* 36 (5), S. 1275–1282. DOI: 10.1093/ee/36.5.1275.

Liu, Yongbo; Jiang, Wanxiang; Liang, Yuyong; Zhao, Caiyun; Li, Junsheng (2017): No effect of Bt-transgenic rice litter on the meiobenthos community in field ditches. In: *Pest management science* 73 (6), S. 1213–1219. DOI: 10.1002/ps.4446.

Liu, Yongbo; Li, Junsheng; Luo, Zunlan; Wang, Huaru; Liu, Fang (2016): The fate of fusion Cry1Ab/1Ac proteins from Bt-transgenic rice in soil and water. In: *Ecotoxicol Environ Saf* 124, S. 455–459. DOI: 10.1016/j.ecoenv.2015.11.025.

Morrissey, Christy A.; Mineau, Pierre; Devries, James H.; Sanchez-Bayo, Francisco; Liess, Matthias; Cavallaro, Michael C.; Liber, Karsten (2015): Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. In: *Environment international* 74, S. 291–303. DOI: 10.1016/j.envint.2014.10.024.

Newton, Kymberly; Zubrod, Jochen P.; Englert, Dominic; Lüderwald, Simon; Schell, Theresa; Baudy, Patrick et al. (2018): The evil within? Systemic fungicide application in trees enhances litter quality for an aquatic decomposer-detritivore system. In: *Environmental pollution (Barking, Essex : 1987)* 241, S. 549–556. DOI: 10.1016/j.envpol.2018.05.069.

Parisi, Claudia; Tillie, Pascal; Rodríguez-Cerezo, Emilio (2016): The global pipeline of GM crops out to 2020. In: *Nat Biotechnol* 34 (1), S. 31–36. DOI: 10.1038/nbt.3449.

Pott, Antonia; Bundschuh, Mirco; Bundschuh, Rebecca; Otto, Mathias; Schulz, Ralf (2020): Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders - an attempt to establish dose-effect relationships through food-spiking. In: *Scientific reports* 10 (1), S. 5262. DOI: 10.1038/s41598-020-62055-2.

Pott, Antonia; Otto, Mathias; Schulz, Ralf (2018): Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment. In: *The Science of the total environment* 635, S. 687–698. DOI: 10.1016/j.scitotenv.2018.04.013.

Preston, Benjamin L. (2002): Indirect effects in aquatic ecotoxicology: implications for ecological risk assessment. In: *Environmental Management* 29 (3), S. 311–323. DOI: 10.1007/s00267-001-0023-1.

Prihoda, Kelsey R.; Coats, Joel R. (2008): Aquatic fate and effects of *Bacillus thuringiensis* Cry3Bb1 protein: Toward risk assessment. In: *Environ Toxicol Chem* 27 (4), S. 793–798. DOI: 10.1897/07-300.1.

Rosi-Marshall, Emma J.; Tank, J. L.; Royer, T. V.; Whiles, M. R.; Evans-White, M.; Chambers, C. et al. (2007): Toxins in transgenic crop byproducts may affect headwater stream ecosystems. In: *Proc Natl Acad Sci USA* 104 (41), S. 16204–16208. DOI: 10.1073/pnas.0707177104.

Sanderson, Hans (2002): Pesticide studies: replicability of micro/mesocosms. In: *Environmental science and pollution research international* 9 (6), S. 429–435. DOI: 10.1007/BF02987597.

Saxena, Deepak; Stotzky, G. (2001): Bt corn has a higher lignin content than non-Bt corn. In: *Am. J. Bot.* 88 (9), S. 1704–1706. DOI: 10.2307/3558416.

Schellenberger, Ute; Oral, Jarred; Rosen, Barbara A.; Wei, Jun-Zhi; Zhu, Genhai; Xie, Weiping et al. (2016): A selective insecticidal protein from Pseudomonas for controlling corn rootworms. In: *Science (New York, N.Y.)* 354 (6312), S. 634–637. DOI: 10.1126/science.aaf6056.

Schnepf, E.; Crickmore, N.; van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J. et al. (1998): Bacillus thuringiensis and its pesticidal crystal proteins. In: *Microbiology and molecular biology reviews : MMBR* 62 (3), S. 775–806. DOI: 10.1128/MMBR.62.3.775-806.1998.

Schulz, Ralf; Bub, Sascha; Petschick, Lara L.; Stehle, Sebastian; Wolfram, Jakob (2021): Applied pesticide toxicity shifts toward plants and invertebrates, even in GM crops. In: *Science (New York, N.Y.)* 372 (6537), S. 81–84. DOI: 10.1126/science.abe1148.

Schulz, Ralf; Liess, Mathias (1995): Chronic effects of low insecticide concentrations on freshwater caddisfly larvae. In: *Hydrobiologia* 299 (2), S. 103–113. DOI: 10.1007/BF00017562.

Schulz, Ralf; Peall, S. K.; Dabrowski, J. M.; Reinecke, A. J. (2001): Spray deposition of two insecticides into surface waters in a South African orchard area. In: *J. environ. qual.* 30 (3), S. 814–822. DOI: 10.2134/jeq2001.303814x.

Sharma, Hari C.; Sharma, Kiran K.; Crouch, Jonathan H. (2004): Genetic Transformation of Crops for Insect Resistance: Potential and Limitations. In: *Critical Reviews in Plant Sciences* 23 (1), S. 47–72. DOI: 10.1080/07352680490273400.

Stang, Christoph; Elsaesser, David; Bundschuh, Mirco; Ternes, Thomas A.; Schulz, Ralf (2013): Mitigation of biocide and fungicide concentrations in flow-through vegetated stream mesocosms. In: *J. environ. qual.* 42 (6), S. 1889–1895. DOI: 10.2134/jeq2013.05.0186.

Stang, Christoph; Wieczorek, Matthias Valentin; Noss, Christian; Lorke, Andreas; Scherr, Frank; Goerlitz, Gerhard; Schulz, Ralf (2014): Role of submerged vegetation in the retention processes of three plant protection products in flow-through stream mesocosms. In: *Chemosphere* 107, S. 13–22. DOI: 10.1016/j.chemosphere.2014.02.055.

Starner, Keith; Goh, Kean S. (2012): Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010-2011. In: *Bulletin of environmental contamination and toxicology* 88 (3), S. 316–321. DOI: 10.1007/s00128-011-0515-5.

Stehle, Sebastian; Knäbel, Anja; Schulz, Ralf (2013): Probabilistic risk assessment of insecticide concentrations in agricultural surface waters: a critical appraisal. In: *Environmental monitoring and assessment* 185 (8), S. 6295–6310. DOI: 10.1007/s10661-012-3026-x.

Strain, Katherine E.; Lydy, Michael J. (2015): The fate and transport of the Cry1Ab protein in an agricultural field and laboratory aquatic microcosms. In: *Chemosphere* 132, S. 94–100. DOI: 10.1016/j.chemosphere.2015.03.005.

Strain, Katherine E.; Whiting, Sara A.; Lydy, Michael J. (2014): Laboratory and field validation of a Cry1Ab protein quantitation method for water. In: *Talanta* 128, S. 109–116. DOI: 10.1016/j.talanta.2014.04.036.

Swan, Christopher M.; Jensen, Peter D.; Dively, Galen P.; Lamp, William O. (2009): Processing of transgenic crop residues in stream ecosystems. In: *J Appl Ecol* 14, S. 711. DOI: 10.1111/j.1365-2664.2009.01728.x.

Tank, Jennifer L.; Rosi-Marshall, Emma J.; Royer, Todd V.; Whiles, Matt R.; Griffiths, Natalie A.; Frauendorf, Therese C.; Treering, David J. (2010): Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. In: *Proc Natl Acad Sci USA* 107 (41), S. 17645–17650. DOI: 10.1073/pnas.1006925107.

van Wijngaarden, René P. A.; Cuppen, Jan G. M.; Arts, Gertie H. P.; Crum, Steven J. H.; van den Hoorn, Martin W.; van den Brink, Paul J.; Brock, Theo C. M. (2004): Aquatic risk assessment of a realistic exposure to pesticides used in bulb crops: a microcosm study. In: *Environmental toxicology and chemistry* 23 (6), S. 1479–1498. DOI: 10.1897/03-80.

Venter, Hermoine J.; Bøhn, Thomas (2016): Interactions between Bt crops and aquatic ecosystems: A review. In: *Environ Toxicol Chem* 35 (12), S. 2891–2902. DOI: 10.1002/etc.3583.

Wang, Jiamei; Chen, Xiuping; Li, Yunhe; Zhu, Haojun; Ding, Jiatong; Peng, Yufa (2014): Effect of straw leachates from Cry1Ca-expressing transgenic rice on the growth of *Chlorella pyrenoidosa*. In: *Environ Toxicol Chem* 33 (5), S. 1156–1162. DOI: 10.1002/etc.2535.

Wang, Yongmo; Huang, Jiacheng; Hu, Huawei; Li, Jianhong; Liu, Biao; Zhang, Guoan (2013): Field and laboratory studies on the impact of two Bt rice lines expressing a fusion protein Cry1Ab/1Ac on aquatic organisms. In: *Ecotoxicol Environ Saf* 92, S. 87–93. DOI: 10.1016/j.ecoenv.2013.02.018.

Wauchope, R. D. (1978): The Pesticide Content of Surface Water Draining from Agricultural Fields - A Review. In: *J. environ. qual.* 7 (4), S. 459–472. DOI: 10.2134/jeq1978.00472425000700040001x.

Wieczorek, Matthias V.; Bakanov, Nikita; Bilancia, Daniel; Szöcs, Eduard; Stehle, Sebastian; Bundschuh, Mirco; Schulz, Ralf (2018): Structural and functional effects of a short-term

pyrethroid pulse exposure on invertebrates in outdoor stream mesocosms. In: *The Science of the total environment* 610-611, S. 810–819. DOI: 10.1016/j.scitotenv.2017.08.048.

Wieczorek, Matthias V.; Bakanov, Nikita; Lagadic, Laurent; Bruns, Eric; Schulz, Ralf (2017): Response and recovery of the macrophytes Elodea canadensis and Myriophyllum spicatum following a pulse exposure to the herbicide iofensulfuron-sodium in outdoor stream mesocosms. In: *Environmental toxicology and chemistry* 36 (4), S. 1090–1100. DOI: 10.1002/etc.3636.

Wieczorek, Matthias V.; Bakanov, Nikita; Stang, Christoph; Bilancia, Daniel; Lagadic, Laurent; Bruns, Eric; Schulz, Ralf (2016): Reference scenarios for exposure to plant protection products and invertebrate communities in stream mesocosms. In: *The Science of the total environment* 545-546, S. 308–319. DOI: 10.1016/j.scitotenv.2015.12.048.

Wieczorek, Matthias V.; Kötter, Denise; Gergs, René; Schulz, Ralf (2015): Using stable isotope analysis in stream mesocosms to study potential effects of environmental chemicals on aquatic-terrestrial subsidies. In: *Environmental science and pollution research international* 22 (17), S. 12892–12901. DOI: 10.1007/s11356-015-4071-0.

Wolt, Jeffrey D.; Keese, Paul; Raybould, Alan; Fitzpatrick, Julie W.; Burachik, Moisés; Gray, Alan et al. (2010): Problem formulation in the environmental risk assessment for genetically modified plants. In: *Transgenic research* 19 (3), S. 425–436. DOI: 10.1007/s11248-009-9321-9.

Zubrod, Jochen P.; Baudy, Patrick; Schulz, Ralf; Bundschuh, Mirco (2014): Effects of current-use fungicides and their mixtures on the feeding and survival of the key shredder Gammarus fossarum. In: *Aquatic toxicology (Amsterdam, Netherlands)* 150, S. 133–143. DOI: 10.1016/j.aquatox.2014.03.002.

Zubrod, Jochen P.; Bundschuh, Mirco; Arts, Gertie; Brühl, Carsten A.; Imfeld, Gwenaël; Knäbel, Anja et al. (2019): Fungicides: An Overlooked Pesticide Class? In: *Environmental science & technology* 53 (7), S. 3347–3365. DOI: 10.1021/acs.est.8b04392.

Table 1: Potential methodological or design improvements for aquatic higher-tier effect studies with GM plant material or Bt toxins (see text for further details).

Problem	**Improvement**
Relevant species groups not represented well	Inclusion of different traits of Trichoptera species, consideration of larval development time in study duration
Exposure pathway via food not adequately represented	Inclusion of (various types) of plant material containing GM toxin of interest; sufficiently long exposure duration
Low stability of fresh GM plant material	Freezing of GM plant material, yet consideration of potential side effects of freezing
GM plant material difficult to obtain	GM plant and reference material should be easier available for independent research*
Purity and identity of GM plant material often unclear	Verification of GM identity and purity through analytical or bioanalytical procedures*
Use of different doses/concentrations not easy	Spiking of non-GM plant material or creating different concentrations thereof with non-GM plant material*
No available negative control	Use of non-GM plant material with similar nutrient content

* In all these cases, the production of own test-specific GM (and non-GM) plant material may be required, which causes a lot of additional effort and might sometimes even be impossible.

Supplementary Information for:

Assessing effects of genetically modified plant material on the aquatic environment using higher-tier studies

Antonia Pott[1,2*], Mirco Bundschuh[1,3], Mathias Otto[2], Ralf Schulz[1]

[1] iES Landau, Institute for Environmental Sciences, University of Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany

[2] Federal Agency for Nature Conservation (BfN), Konstantinstrasse 110, 53179 Bonn, Germany

[3] Department of Aquatic Sciences and Assessment, Swedish University of Agricultural Sciences, Lennart Hjelms väg 9, 75007 Uppsala, Sweden

Tabel S1: Overview of studies reporting on effects of genetically modified plants on aquatic organisms using higher-tier studies.

Nr.	Author	Year	Title	Journal	Organism Order	farthest determination	Endpoint	Method	Test substrate	Plant	Novel Protein	GMO-event
1	Axelsson et al.	2010	Can leaf litter from genetically modified trees affect aquatic ecosystems?	Ecosystems	Ephemeroptera, Plecoptera, Trichoptera	38 species were identified, e.g. *Nemoura cinerea, Leptophlebia marginata, Nemoura flexuosa*.	total abundance, abundance of Ephemeroptera, Plecoptera and Trichoptera separately, abundacne of predators and detrivores separately, species richnessof the EPT fauna	litterbag	plant material (leaves)	aspen-poplar (*Populus tremula x Populus alba*)	CAD, COMT	not-specified
2	Axelsson et al.	2011	Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition	Journal of Applied Ecology	Ephemeroptera, Plecoptera, Trichoptera, Coleoptera	27 species, e.g. *Amphinemura borealis, Taeniopteryx nebulose, Protonemura meyeri*. Full list see Supporting Material Appendix S1	total abundance, abundance of orders, functional feeding group and species richness, community composition	litterbag	plant material (leaves)	aspen-poplar (*Populus tremula x Populus tremuloides*)	Cry3Aa	Bt17, Bt27
3	Chambers et al.	2010	Responses of stream macroinvertebrates to Bt maize leaf detritus	Ecological Applications	57 taxa, e.g. Tricladida, Nematoda, Hirudinea, Oligochaeta, Amphipoda, Copepoda, Cambaridae, Ostracoda, Coleoptera, Diptera, Ephemeroptera, Hemiptera, Odonata, Plecoptera, Trichoptera. Not all identified species are listed in the paper.	57 taxa, e.g. Tricladida, Nematoda, Hirudinea, Oligochaeta, Amphipoda, Copepoda, Cambaridae, Ostracoda, Coleoptera, Diptera, Ephemeroptera, Hemiptera, Odonata, Plecoptera, Trichoptera.	abundance, biomass, richness, EPT richness, community composition, diversity, functional feeding group composition	benthic core	plant material (whole plant)	maize	Cry1Ab	not-specified

	Chambers et al.	2010	Responses of stream macroinvertebrates to Bt maize leaf detritus	Ecological Applications	Oligochaeta, Chironomidae, Trichoptera, Plecoptera. Not all identified species are listesd in the paper.	For example *Allocapnia spec.*	community composition, diversity, biomass, functional feeding group composition	litterbag	plant material (leaves)	maize	Cry1Ab	not-specified
4	Li et al.	2014	Transgenic *Bacillus thuringiensis* (Bt) rice is safer to aquatic ecosystems than ist non-transgenic counterpart	Plos One	Rotifera (phylum), Cladocera (order), Copepoda (subclass)	33 species, e.g. *Brachionus capsuliflorus, Alona guttata, Mesocylops sp*. Full list see Supporting Material Appendix S2.	abundance, diversity	field study	plant material (whole plant)	rice	Cry1Ab/1Ac, Cry2A	Bt MH63
5	Liu et al.	2016	Effects of Bt-transgenic rice cultivation on planktonic communities in paddy field and adjacent ditches	Science of the Total Environment	307 phytoplanton species, e.g. *Dictyosphaeria cavernosa, Navicula dicephala*. 43 zooplankton species, e.g. *Stentor polymorphrus, Platyias militaris*. Not all identified species are listed in the paper.	307 phytoplanton species, e.g. *Dictyosphaeria cavernosa, Navicula dicephala*. 43 zooplankton species, e.g. *Stentor polymorphrus, Platyias militaris*.	abundance, diversity	field study	plant material (whole plant)	rice	Cry1Ab/Cry1Ac	Huahui-1
6	Liu et al.	2017	No effect of Bt-transgenic rice litter on the meiobenthos community in field ditches	Pest Mangement Science	Diptera, Oligochaeta, Hirudinea, Gastropoda, Turbellaria, Trichoptera	7987 specimens, e.g. *Chronomus ochreatus, Limnodrilus hoffmeisteris, Dero digitata*.	community composition, abundance	litterbag	plant material (leaves)	rice	Cry1Ab/Cry1Ac	Huahui-1

7	Swan et al.	2009	Processing of transgenic crop residues in stream ecosystems	Journal of Applied Ecology	Amphipoda, Decapoda, Isopoda, Diptera, Plecoptera, Trichoptera, Gastropoda (class)	Amphipoda, Decapoda, Isopoda, Tipulidae, *Taeniopteryx sp*, Hydropsychidae, Lepidostoma sp., *Frenesia sp.*, *Pycnopsyche sp.*, Gastropoda	abundance	litterbag	plant material (leaves)	maize	Cry1Ab, Cry1Ab x Cry3Bb1	MON810, MON810 x MON 863
	Swan et al.	2009	Processing of transgenic crop residues in stream ecosystems	Journal of Applied Ecology	Amphipoda, Isopoda, Plecoptera, Trichoptera, Gastropoda (class)	Amphipoda, Isopoda, *Taeniopteryx sp*, Hydropsychidae, *Pycnopsyche sp*., Gastropoda	abundance	litterbag	plant material (leaves)	maize	Cry1Ab x Roundup Ready, Cry1Ab x Cry3Bb1x Roundup Ready	MON810 x NK603, MON810 x MON 863 x NK603
8	Wang et al.	2013	Field and laboratory studies on the impact of two Bt rice lines eypressing a fusion protein Cry1Ab/1Ac on aquatic organism	Ecotoxicology and Environmental Safety	Rotifera (phylum), Cladocera (order), Copepoda (subclass)	Rotifera, Cladocera, Copepoda	abundance	field study	plant material (whole plant)	rice	Cry1Ab/Cry 1Ac	Bt MH63, Bt SY63

A.4 Eidesstattliche Erklärung

Hiermit versichere ich, dass

- Ich die eingereichte Dissertation selbstständig verfasst habe und alle von mir für die Arbeit benutzten Hilfsmittel und Quellen in der Arbeit angegeben sowie die Anteile etwaig beteiligter Mitarbeiterinnen oder Mitarbeiter sowie anderer Autorinnen oder Autoren klar gekennzeichnet sind;
- Ich nicht die entgeltliche Hilfe von Vermittlungs- oder Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen habe;
- Ich die Dissertation nicht in gleicher oder ähnlicher Form als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung im In- oder Ausland eingereicht habe;
- Ich nicht die gleiche oder eine andere Abhandlung in einem anderen Fachbereich oder einer anderen wissenschaftlichen Hochschule als Dissertation eingereicht habe;
- mir bewusst ist, dass ein Verstoß gegen einen der vorgenannten Punkte den Entzug des Doktortitels bedeuten und ggf. auch weitere rechtliche Konsequenzen haben kann.

(Datum)	(Antonia Pott)

A.5 Lebenslauf

Persönliche Daten

	Antonia Pott Peterstraße 32, 50321 Brühl Geboren am 19. Januar 1989 in Moers Staatsangehörigkeit: deutsch

Berufserfahrungen

seit 02/2019	**Referentin Umweltzeichen** RAL gGmbH, Bereich RAL Umwelt, Bonn
seit 05/2014	**Promotion** Universität Koblenz-Landau, Institut für Umweltwissenschaften
05/2014 – 05/2018	**Wissenschaftliche Mitarbeiterin** Bundesamt für Naturschutz, Fachgebiet Bewertung gentechnisch veränderter Organismen/Gentechnikgesetz, Bonn
10/2011 – 03/2014	**Masterstudium RWTH Aachen University** Masterarbeit: A new method for the detection of microplastics in the North Sea brown shrimp (*Crangon crangon*) by Fourier Transform Infrared Spectroscopy (FTIR) (Note: 1,7) **Abschluss:** Master of Science Ökotoxikologie (Note: 1,5)
10/2008 – 09/2011	**Bachelorstudium RWTH Aachen University** Vertiefungsmodul: Umweltwissenschaften Bachelorarbeit: Exposure of hydrophobic and fugitive substances in the Fish Edmbryo Toxicity Test (Note: 1,7) **Abschluss:** Bachelor of Science Biologie (Note: 2,2)

1999 – 2008	**Norbert-Gymnasium Knechtsteden, Dormagen** **Abschluss:** Allgemeine Hochschulreife (Note: 1,8)

Paper

06/2022	**Pott, A**.; Bundschuh, M.; Otto, M.; Schulz, R. (2022): Assessing effects of genetically modified plant material on the aquatic environment using higher-tier studies. Bulletin of Environmental Contamination and Toxicology (submitted)
03/2020	**Pott, A**., Bundschuh, M., Bundschuh, R., Otto, M., Schulz, R. (2020) Effect of Bt toxin Cry1Ab on two freshwater caddisfly shredders – an attempt to establish dose-effect relationships through food-spiking, Scientific Reports, 10: 5262
04/2018	**Pott, A.**, Otto, M., Schulz, R. (2018) Impact of genetically modified organisms on aquatic environments: Review of available data for the risk assessment, Science of the Total Environment, 635: 687-698

Vortrag

05/2017	**Pott, A.**, Bundschuh, M., Bundschuh, R., Otto, M., Schulz, R. (2017) Assessing effect of the Bt toxin Cry1Ab on trichopterans with a food-spiking method, 27. Setac Europe Tagung, Brüssel

Poster

05/2012	**Pott A**., Peddinghaus S., Hollert H., Keiter S. (2012) Different test conditions for hydrophobic and fugitive substances in the Fish Embryo Toxicity Test for a reliable risk assessment, 6. SETAC World Tagung und 22. Jahrestagung der SETAC Europe, Berlin
09/2011	**Pott A**., Peddinghaus S., Hollert H., Keiter S. (2011) Exposure of hydrophobic and fugitive substances in the Fish Embryo Toxicity Test, 16. Jahrestagung der SETAC GLB, Landau

(Datum) (Antonia Pott)