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Federal Agency for Nature Conservation

BfN – Skripten 236

2008

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Cover pictures: Angelika Hilbeck (lady bird, maize field)
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The Project (FKZ 805 64 005) was completed in November 2006.

This publication is included in the literature database "DNL-online" (www.dnl-online.de)

BfN-Skripten are not available in book trade but can be downloaded in a pdf version from the internet at: http://www.bfn.de/0502_skripten.html

Publisher: Bundesamt für Naturschutz (BfN)
Federal Agency for Nature Conservation
Konstantinstrasse 110
53179 Bonn, Germany
URL: http://www.bfn.de

Printed by the printing office of the Federal Ministry of Environment, Nature Conservation and Nuclear Safety.

Printed on 100% recycled paper.

Bonn, Germany 2008

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Zusammenfassung

Bevor eine gentechnisch veränderte Pflanze (GVP) in der EU in Verkehr gebracht werden darf, muss diese gemäß EU-Freisetzungsrichtlinie 2001/18/EG oder der EG-Verordnung Nr. 1829/2003 über gentechnisch veränderte Lebens- und Futtermittel einer Umweltverträglichkeitsprüfung unterzogen werden. Ziel der Umweltverträglichkeitsprüfung ist es, von Fall zu Fall etwaige direkte, indirekte, sofortige oder spätere schädliche Auswirkungen von GVP auf die menschliche Gesundheit und die Umwelt, die bei der absichtlichen Freisetzung oder dem Inverkehrbringen von GVP auftreten können, zu ermitteln und zu evaluieren (Anhang II A der Richtlinie 2001/18/EG). Ein wichtiger Teil dieser Umweltverträglichkeitsprüfung basiert auf ökotoxikologischen Studien, in denen die Auswirkungen der GVP auf die belebte Umwelt überprüft werden.

Bis heute werden die ökotoxikologischen Untersuchungen für die Zulassungsverfahren von GVP in der Regel mittels Tests durchgeführt, die ursprünglich für die Chemikalienprüfung entwickelt und standardisiert worden sind. Analog zur Chemikalienprüfung erfolgen die Tests in einem stufenweisen, hierarchischen Verfahren. Getestet werden selten die ganzen Pflanzen, sondern meistens mikrobiell hergestellte, gereinigte Transgenprodukte. Aus rechtlicher Sicht genügt dieses Verfahren nicht den Anforderungen der EU-Freisetzungsrichtlinie. Bis heute existiert in der EU keine abgestimmte, einheitliche Methodik für die Auswahl relevanter ökotoxikologischer Tests und Testorganismen. Der Entwicklung und Etablierung eines solchen Standards kommt demnach eine hohe Priorität zu.

Ziel dieses Projektes war es, den aktuellen Forschungs- und Wissensstand zu ökotoxikologischen Testmethoden und -strategien aus der Chemikalienprüfung zu analysieren und hinsichtlich deren Eignung für die Umweltverträglichkeitsprüfung von GVP zu bewerten. Außerdem waren spezifische Anforderungen an Teststrategien und Testmethoden abzuleiten, die an die ökotoxikologische Untersuchung von GVP zu stellen sind, damit diese den gesetzlichen Anforderungen der EU-Freisetzungsrichtlinie entsprechen. Daraus resultierte ein Vorschlag für ein Konzept zur ökotoxikologischen Risikoabschätzung von GVP. Dieses Konzept wurde mit dem heute gängigen Verfahren aus der Chemikalien-/ Pestizidzulassung abgeglichen, um bezüglich der Anzahl durchzuführender Tests zu einem vergleichbaren Aufwand zu kommen und um vergleichbare Aussagen zu erlauben. Schließlich war es auch Aufgabe dieses Projektes, das vorgeschlagene Konzept für die ökotoxikologische Risikoabschätzung von GVP anhand eines konkreten Fallbeispiels zu veranschaulichen.

Die innerhalb dieses Projektes gemachten Analysen zeigen auf, dass das den Zulassungsanträgen von GVP bis heute zugrundeliegende Konzept der ökotoxikologischen Risikoabschätzung in mehrfacher Hinsicht Defizite aufweist. Das Hauptproblem liegt dabei in der Übertragung und Anwendung eines Ansatzes, der ursprünglich für andere Zwecke entwickelt wurde. So untersucht die Ökotoxikologie die Umweltwirkungen von Einzelsubstanzen (Chemikalien, u.a. Pestizide). Übertragen auf GVP führte dies dazu, dass die ökotoxikologischen Untersuchungen in der Regel auf neuartige stoffliche Eigenschaften beschränkt werden, die durch die eingeführten Genkonstrukte vermittelt werden (z.B. die Expression eines bestimmten insektiziden Bt-Proteins). Im Fall von GVP liegt aber nicht eine Einzelsubstanz, sondern ein lebender Organismus vor, der als Gesamtorganismus auf vielfältige Weise – und nur zum Teil über Stoffe – mit seiner Umwelt interagiert. Im speziellen Fall, in dem eine GVP neue bioaktive Proteine exprimiert (z.B. Bt-Proteine), besteht zwar eine ausgeprägte stoffliche Komponente, deren Umweltwirkungen, wenn auch nicht abschließend, zumindest bis zu einem gewissen Grad mit den bisher durchgeführten merkmalsbasierten Untersuchungen abgeklärt werden können. Wenn aber eine GVP keine neuartigen bioaktiven Proteine exprimiert, sondern zum Beispiel „lediglich“ einen veränderten Stoffwechsel aufweist (z.B. Kartoffel mit veränderter Stärkezusammensetzung), greift das Konzept der nur auf das neuartige Transgenprodukt basierenden Untersuchung zu kurz. Es käme

der ‚a priori'-Annahme gleich, dass solche Pflanzen keine negativen Auswirkungen auf die belebte Umwelt haben könnten. Veränderungen im Stoffwechsel, wie zum Beispiel die Zusammensetzung primärer oder sekundären Pflanzeninhaltsstoffe, können aber durchaus signifikanten Einfluss auf assoziierte Nahrungsketten haben und wichtige ökologische Funktionen beeinträchtigen. So werden mit den bisherigen ökotoxikologischen Test aus der Chemikalienprüfung bei der Anwendung auf GVP eine Reihe von gesetzlich verlangten Risikoaspekten nicht berücksichtigt, wie zum Beispiel unterschiedliche Expositionsszenarien, strukturelle und ggf. auch funktionelle Unterschiede zwischen bioaktiven Proteinen, die in GVP exprimiert werden und solchen, die mikrobiell hergestellt werden, sowie unerwartete Effekte (Pleiotropie). Folglich werden unerwartete Effekte und Effekte, die aufgrund der Interaktionen der veränderten Gesamtpflanze mit der Umwelt zu Stande kommen, mit solchen Tests nicht erfasst.

Die in der EU-Freisetzungsrichtlinie 2001/18/EG vorgeschriebene Umweltverträglichkeitsprüfung für GVP schreibt explizit eine Fall-zu-Fall Beurteilung vor. In Anhang II der Richtlinie wird ausgeführt, was unter einem Fall zu verstehen ist. Demnach setzt sich ein Fall aus folgenden 3 Elementen zusammen:

1. der Empfängerpflanze (Biologie, Ökologie, Agronomie)
2. der transgenen Eigenschaft (Zielwirkung) und der phänotypischen Ausprägung (die GVP)
3. der Umwelt, in der die Pflanze freigesetzt wird ('Aufnahmemilieu') und ihre Anwendung (‚intended use')

Da die Ergebnisse von ökotoxikologischen Untersuchungen zur Umweltverträglichkeitsprüfung der GVP herangezogen werden, müssen sie ausgehend von der ‚Fall'-Definition der EU-Freisetzungsrichtlinie abgeleitet werden. Dies schliesst so auch GVP mit ein, die keine neuartigen bioaktiven Transgenprodukte exprimieren, sondern z.B. einen veränderten Stoffwechsel aufweisen oder in Verbindung mit dem Einsatz gewisser Chemikalien (z.B. Herbizide) angebaut werden. Ein Konzept zur ökotoxikologischen Untersuchung von GVP sollte deshalb auf der ‚Fall'-Definition aufbauen, um den gesetzlichen Anforderungen gerecht zu werden und fachlich auf dem aktuellen Stand der Wissenschaft zu sein.

Dass die ökotoxikologischen Test aus der Chemikalien-/ Pestizidprüfung nur eingeschränkt auf GVP anwendbar sind, hat auch die im Rahmen dieses Projektes durchgeführte Evaluation von 126 bestehenden Testmethoden ergeben. Die Analyse zeigte auf, dass nur wenige Tests ohne Modifikation für die Risikobeurteilung von GVP geeignet sind, da nur wenige Tests die GVP-spezifischen Expositionswege abdecken. Für die Mehrheit der Tests werden Anpassungen empfohlen, die von Fall zu Fall aber unterschiedlich ausfallen können. Darüber hinaus ist es unerläßlich, neue, auf den spezifischen Fall abgestimmte Tests zu entwickeln und, soweit möglich, zu standardisieren.

Gestützt auf die Ergebnisse der im Projekt durchgeführten Analysen, schlagen wir ein Konzept zur ökotoxikologischen Risikoabschätzung vor, das den Besonderheiten von GVP Rechnung trägt. Kernstück dieses Konzeptes ist ein Auswahlverfahren für die zu untersuchende Testorganismen und ökologischen Prozesse sowie der zu verwendenden Testmethoden. Das Auswahlverfahren ermöglicht es, eine fall-spezifische ökotoxikologische Teststrategie festzulegen, in der neben dem Transgenprodukt und der GVP auch die Umwelt berücksichtigt wird, in die die Freisetzung erfolgen soll. Das Auswahlverfahren für die ökotoxikologische Prüfung wird eingebettet in einen Rahmen zur Umweltverträglichkeitsprüfung von GVP, der im Folgenden kurz umrissen wird. Er konkretisiert die Vorgaben der EU-Freisetzungsrichtlinie und setzt sich aus 4 Komponenten zusammen.

In Komponente I, der Gefährdungsidentifikation, sollen diejenigen Eigenschaften von GVP identifiziert werden, die zu negativen Effekten führen könnten. Hierbei werden auch die möglicherweise betroffenen Biodiversitätsfunktionen und die für sie relevanten Organismen identifiziert. Dies liefert die Grundlage für die Auswahl fallspezifischer Testorganismen und -strategien und legt den Rahmen und die Grenzen der Umweltverträglichkeitsprüfung fest.

In Komponente II soll die Expositionssituation abgeschätzt werden. Dazu wird für die in Komponente I ausgewählten Testorganismen ermittelt, ob und in welchen Konzentrationen sie der GVP, ihren Transgenprodukten und Metaboliten ausgesetzt sind. In Fällen, in denen eine GVP keine neuen bioaktiven Proteine exprimiert, sondern einen veränderten Stoffwechsel aufweist, ist die Exposition der Umwelt gegenüber Ausprägungen des veränderten Stoffwechsels Gegenstand der Untersuchungen. Analog sind im Fall von GVP, die zusammen mit der Anwendung einer Chemikalie (z.B. Herbizide) ihre Wirkung entfalten, diese Chemikalien in ihrer neuen Anwendungsform in der Expositionsabschätzung zu berücksichtigen.

In Komponente III sollen die Effekte einer GVP bestimmt werden. Dazu wird das für den jeweiligen Fall erarbeitete ökotoxikologische Testprogramm durchgeführt. Analog zu den Chemikalien, erfolgt die Durchführung der Tests in einem stufenweisen Verfahren mit dem entscheidenden Unterschied, dass nicht ein immer gleiches, standardisiertes Testprogramm (von Testorganismus bis experimentelles Protokoll) durchlaufen wird. Nach dem von uns vorgeschlagenen Verfahren werden Testorgansimen ausgewählt, die im betreffenden Ökosystem, in dem die GVP freigesetzt bzw. angebaut werden soll, vorkommen. Diese Testorganismen werden anhand von wissenschaftlich realistischen Szenarien zu möglichen negativen Effekten bis zur Verwerfung oder Annahme der abgeleiteten Hypothesen untersucht.

In Komponente IV, der Risikocharakterisierung, erfolgt eine Synthese und Bewertung der in den vorgeschalteten Schritten gewonnenen Erkenntnisse. Die Konsequenzen möglicher negativer Effekte werden abgeschätzt.

Innerhalb des 4-teiligen Rahmenkonzepts zur Umweltverträglichkeitsprüfung von GVP kommt Komponente I eine zentrale Bedeutung zu. Hier werden die für einen bestimmten Fall relevanten Testorganismen mittels wissenschaftlicher Kriterien herausgefiltert und entsprechende Testmethoden abgeleitet. Wir schlagen ein klar strukturiertes, transparentes und nachvollziehbares Auswahlverfahren für Testorganismen vor, welches sämtliche drei Punkte die einen ‚Fall' definieren (GVP, transgene Eigenschaft und Aufnahmemilieu), berücksichtigt. Es gliedert sich in 6 Schritte:

Schritt 1: Identifikation von funktionellen Organismengruppen
Schritt 2: Priorisierung der Organismen und Funktionen
Schritt 3: Herleitung möglicher Expositionspfade
Schritt 4: Anwendung von Praktikabilitätskriterien
Schritt 5: Bestimmung von Negativ-Effekt-Szenarien
Schritt 6: Ausformulierung von Testhypothesen und -protokollen ausgehend von den Negativ-Effekt-Szenarien

In Schritt 1 werden die wichtigsten ökologischen Funktionen identifiziert, die nicht negativ von einer GVP betroffen sein sollen. Diese ökologischen Funktionen können sowohl von einzelnen Organismen bzw. -gruppen ausgeübt werden (funktionelle Gruppen wie Bestäuber, Herbivoren, natürliche Feinde), als auch spezifische Prozesse beinhalten, die von verschiedenen Organismen in Wechselwirkung mit biotischen und abiotischen Faktoren ausgeübt oder kontrolliert werden.

Durch den Fokus auf ökologische Funktionen kann die Anzahl an Testorganismen in den ökotoxikologischen Tests auf die ökologisch relevanten Organismen konzentriert werden. Aus Sicht der ökotoxikologischen Risikoabschätzung ist es wichtig, dass bei der Auswahl der ökologischen Funktionen eine große Bandbreite an ökologischen Dienstleistungen abgedeckt wird, die potenziell negativ betroffen sein könnten. Aufgrund der Fallspezifität werden die ausgewählten ökologischen Funktionen zwischen verschiedenen (transformierten) Kulturpflanzen, transgenen Eigenschaften und geographischen Regionen variieren.

In Schritt 2 werden anhand von Expertenwissen zu jeder funktionellen Kategorie die bekannten (Nicht-Ziel)Organismen, die im betreffenden Agrarökosystem vorkommen, aufgelistet. Oft werden einige Arten in mehreren funktionellen Kategorien erscheinen (z.B. Marienkäfer: natürlicher Gegenspieler und Bestäuber). Diese Arten sollten in den nachfolgenden Auswahlschritten prioritär behandelt werden. Schließlich werden sämtliche gelisteten Arten anhand ökologischer Kriterien priorisiert (z.B. geographische Verbreitung, Abundanz, Habitatspezialisierung, etc.), um die relevantesten Arten herauszufiltern und die Artenliste einzuengen.

In Schritt 3 wird für die verbleibenden Arten aus Schritt 2 eine Expositionsanalyse durchgeführt, aus der hervorgeht, ob und zu welchem Grad eine Art mit einer GVP bzw. deren Stoffwechselprodukten oder komplementär eingesetzten Pflanzenschutzmitteln in Kontakt kommt. Ziel dieses Schrittes ist die Einteilung der gelisteten Arten in solche, die mit großer Wahrscheinlichkeit exponiert sind und solche, bei denen eine Exposition eher unwahrscheinlich ist. Am Ende von Schritt 3 liegt eine Liste mit all jenen Arten vor, die ökologisch von hoher Relevanz sind und gegenüber einer bestimmten GVP am stärksten exponiert sind. Es wird empfohlen, lediglich diese Arten für die ökotoxikologische Testung in Betracht zu ziehen. Beim Fehlen einer geeigneten Testmethode kann die Entwicklung einer oder mehrerer neuer Testmethoden erforderlich werden.

In Schritt 4 werden für die Auswahl der Testorganismen und der Testentwicklung die Arten aus Schritt 3 nach Praktikabilitätskriterien weiter gefiltert, unter anderem um die Reproduzierbarkeit in den Tests zu gewährleisten. Als Kriterien, deren Gewichtung unterschiedlich ausfallen kann, werden vorgeschlagen:

- Leichte Haltung und Züchtbarkeit unter Laborbedingungen
- Kurzer Generationszyklus
- Enger Kontakt mit Boden, Pflanzen oder Pflanzenresten
- Mittlere Sensitivität gegenüber Stressfaktoren
- Geringe Sensitivität gegenüber schwankenden Umweltbedingungen
- Weite Verbreitung in verschiedenen Habitaten

In Schritt 5 werden für die in den vorangehenden Schritten ausgewählten und nach Praktikabilitätskriterien gefilterten Arten alle möglichen Negativ-Effekt-Szenarien identifiziert. Die Szenarien leiten sich über kausale Wirkungsketten ab. Dabei können auch Wissenslücken erkannt werden, die mit spezifischen Experimenten zu schließen sind oder als solche in der Risikobewertung berücksichtigt werden müssen.

In Schritt 6 werden zu den verschiedenen Negativ-Effekt-Szenarien ökotoxikologische Test-Hypothesen formuliert, für die schließlich Experimente ausgewählt bzw. entwickelt werden. Wichtige Punkte, die bei der Auswahl und Entwicklung von Experimenten beachtet werden müssen, umfassen die Testmethodik, über die eine Exposition simuliert wird, die Messendpunkte sowie die Berücksichtigung der realen ökologischen Gegebenheiten. Letztere beinhalten u.a. den Einbezug der gesamten

GVP in den Experimenten und die Berücksichtigung von Expositionspfaden, wie sie im Feld auftreten. Die Untersuchung der gesamten GVP ermöglicht es auch, unerwartete Effekte verschiedenster Art mit zu erfassen (Pleiotropie).

Bei der Auswahl bzw. Entwicklung der Experimente kommen auch an dieser Stelle Praktikabilitätskriterien zur Anwendung, die unterschiedlich zu gewichten sind. Diese beinhalten Punkte wie:

- Standardisierung
- Praktische Durchführbarkeit
- Anwendbarkeit
- Validitätskriterien
- Bestehende Erfahrung mit einem Test
- Anzahl und Sensitivität der Messparameter
- Statistische Auswertungsmethoden
- Reproduzierbarkeit
- Wiederholbarkeit
- Ressourcenbedarf (Zeitaufwand, Material- und Personalkosten)
- Analytische Nachweisbarkeit der Exposition
- Wohlergehen der Testorganismen

Ziel des oben beschriebenen Auswahlverfahrens für Testorganismen und -methoden ist es, zu einer handhabbaren Anzahl an Tests zu kommen. Der Vergleich mit der Umweltrisikoprüfung für Pestizide hat gezeigt, dass die Anzahl der ausgewählten Tests und die für deren Durchführung benötigten Ressourcen etwa im selben Rahmen liegen. Des Weiteren kann davon ausgegangen werden, dass eine gewisse Anzahl an Testorganismen und -methoden für die Prüfung mehrerer GVP angewendet werden kann. Diese Testmethoden sollten in der näheren Zukunft über Ringversuche standardisiert werden.

Das vorgeschlagene Auswahlverfahren für Testorganismen und -methoden wurde anhand des Fallbeispiels der gentechnisch veränderten Amylopectin-Kartoffel veranschaulicht. Die Anwendung des Auswahlverfahrens beschränkte sich auf die im Zusammenhang mit einer Freisetzung der GV Amylopectin-Kartoffel als wichtig eingestufte Biokontrollfunktion. So ist es denkbar, dass Blattläuse auf den veränderten Stoffwechsel in der Kartoffel reagieren, was wiederum Auswirkungen auf die natürlichen Gegenspieler der Blattläuse haben kann. Im vorliegenden Fall wurden als wichtige Gegenspieler der Blattläuse 5 Coccinelliden-Arten identifiziert, von denen nach Durchlaufen der Schritte 1 bis 4 des Auswahlverfahrens lediglich noch *Coccinella septempunctata* als geeignete Testspezies vorgeschlagen wurde. Die weitere Analyse ergab, dass keine der bestehenden Testmethoden für den vorliegenden Fall direkt anwendbar wäre und deshalb entweder die bestehenden Methoden modifiziert oder neue Testmethoden entwickelt werden müssten.

Summary

Before a genetically modified plant (GMP) can be placed on the market in the EU an environmental risk assessment has to be conducted according to EU-Directive 2001/18/EC or the Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed. Important elements of the environmental risk assessment are ecotoxicological tests investigating adverse effects of a GMP on the living environment.

To date, the environmental risk assessment in the application dossiers usually relies on ecotoxicological tests originally developed and standardised for chemicals. In these tests frequently not the whole GMPs are tested but only specific transgene products. Although this ecotoxicological testing concept is widely used in the dossiers of GMPs seeking regulatory approval it does not fulfil the requirements of the Directive 2001/18/EC. No harmonised concept for the ecotoxicological testing of GMPs is available today that considers the characteristics of whole GMPs. Therefore, the development of such a testing concept is urgent. This project makes a significant contribution to that goal.

The aim of this project was, firstly, to analyse existing ecotoxicological methods and strategies developed for the testing of chemicals and evaluate their suitability for the risk assessment of GMPs. Secondly, specific requirements for a ecotoxicological testing strategy and testing methods for GMP were elaborated guided by the legal requirements of the Directive 2001/18/EC. The result was a proposal for an ecotoxicological testing concept for GMPs that leads to a comparable number of tests as used for the testing of chemicals like pesticides. Finally, the proposed testing concept was applied to a case example.

Our analyses showed that the main shortcoming in the current risk assessment is the use of an ecotoxicological concept that was developed to assess environmental effects of chemicals. By using this concept for the assessment of GMPs ecotoxicological tests are restricted to substantial characteristics of the GMP only. However, a GMP is not a substance but a living organism that is able to reproduce and propagate itself. By that it interacts in many different ways with its environment.

The Directive 2001/18/EC explicitly demands an environmental risk assessment on a case-by-case basis. A case is described in Annex II of the Directive by the following 3 elements:

- the crop plant (its biology, ecology and agronomy)
- the novel trait relating to its intended effect and phenotypic characteristics of the GM crop plant (the GMO)
- the receiving environment relating to the intended use of the GMP

Only an ecotoxicological testing concept for GMP that integrates this case definition will fulfil the legal requirements and deliver the relevant data for the environmental risk assessment. Based on this fact we propose a concept for the ecotoxicological testing of GMPs where test organisms and methods are selected on a case-by-case basis. A detailed and step-wise species and methods selection procedure is outlined that considers the GMP and its environment and by that the ecological context of the plant. To place the selection procedure in a broader risk assessment context it is embedded in a risk assessment framework derived from the specifications in the Directive 2001/18/EC. This framework consists of 4 components:

In Component I, the hazard identification, all available information related to a GMP is compiled in order to define and characterise the respective traits that can lead to adverse effects for which the risk assessment is required. Here, potentially affected biodiversity functions and the relevant organisms

executing this function are identified. Main outcome of this component is a testing plan including test species and testing methods. This determines the scope and limits of the risk assessment.

In Component II, the exposure of the organisms and functions selected in component I to the GMP is assessed.To do this, the concentration of transgene products and its metabolites must be determined, including their bioactivity. In cases where there are no novel transgene products expressed but a significant change of primary or secondary compounds is caused, the exposure towards these compounds will be at the center of the assessment. Analogous, for GMPs that require the application of a chemical for the transgenic trait to unfold its effect, these chemicals in context with the GMP will be included in the exposure assessment. This will help to determine the probability of the occurrence of a potential adverse effect for the set of test species identified in Component I. The activities in Component II can include testing at various levels of ecological realism (laboratory, greenhouse, semi-field, and field) in order to verify and/or quantify exposure to transgene products or metabolites of the set of test species identified in Component I.

The aim of Component III, the effect determination, is to measure whether the GMP, its use, or the transgene product can affect structural (i.e., related to individual species) or functional (i.e., related to services provided by the whole community) endpoints. These activities can include testing at various levels of ecological realism (laboratory, greenhouse, semi-field, and field) in order to either verify and/or falsify adverse effects observed in previous steps or to confirm the lack thereof.

In Component IV, the risk characterisation, the risk originating from the GMP is estimated by combining, and comparing the information gained and data obtained in the previous three components in a quantitative way.

Within the risk assessment framework outlined above, Component I is of great importance, because in this component the relevant test organisms and methods are selected for a certain case using scientific criteria. The proposed selection procedure for testing organisms consists of the following 6 steps:

step 1 Identification of functional groups of species
step 2 Ranking of species or functions
step 3 Determination of possible exposure pathways
step 4 Applying practicability criteria
step 5 Development of adverse effects scenarios
step 6 Formulating adverse effects scenarios as testable hypotheses.

Step 1 of the species selection procedure involves the identification of the most important ecological functions that must not be affected by the introduction of the GMP and its properties that can cause adverse effects in the given cropping system and receiving environment. By using ecological functions inappropriate conclusions associated with the test species used in current ecotoxicitiy testing can be avoided. Furthermore, the use of ecological functions allows to focus testing on critical ecological processes and to limit the number of species that must be tested to those that are ecologically relevant.

In step 2, all information and expertise available is used to list under each selected functional category from step 1 the known non-target species that actually occur in the crop ecosystems referring to the case.

In step 3, for the remaining species or functions identified from the previous step, an exposure analysis is conducted to determine whether and to what degree the species comes into contact with the transgene products (incl. their metabolites), any other altered composition of metabolic compounds or the

corresponding measures necessary for the intended effect of the GMP. The goal of this step is to differentiate candidate species into those that are possibly exposed and those unlikely to be exposed.

In step 4, practicability criteria regarding the suitability for ecotoxicological testing are applied to the list of step 3 to remove those species that are not appropriate to obtain reproducible test results.

In step 5, possible adverse effect scenarios are identified. This step ends with the formulation of a testable adverse effect hypothesis for which experiments / tests can be selected or developed.

In step 6, adverse effect hypotheses are formulated using the information from steps 3 and 5. The information and data synthesised during the previous steps also guide the development of ecologically meaningful experiments in terms of protocols, feeding strategies, food types to be used, etc..

With the outlined species selection procedure, the limited resources and available time for research can be allocated to those species and processes that are at the highest risk identified and – if adversely affected – can induce severe consequences. From the developed adverse effect scenarios meaningful testable risk hypotheses, most important data gaps and experimental designs can be derived for regulatory ecotoxicological testing. One of the main goals of the proposed test species and methods selection procedure is to end up with a number of tests that can be practically handled. Comparisons with the risk assessment of pesticides showed that the numbers of test produced by the selection procedure and the resources needed to conduct the tests are within the same range.

The selection procedure is in detail illustrated using the example of the GM-amylopectin potato – a GMP with an altered metabolism. In applying the selection procedure we focused only on the identification of possibly affected biodiversity functions and the relevant species and processes. Within the biodiversity functions biocontrol was identified as an important function in the respective case. For example, it is possible that aphids feeding on the GM-amylopectin potato will react to the altered metabolism and by that influencing natural enemies feeding on the aphids. Five coccinellid species were identified as important natural enemies in the case of the GM-amylopectin potato. After applying steps 1 to 4 of the selection procedure only *Coccinella septempunctata* remained as suitable test species. Further analyses revealed that none of the already existing test methods for *C. septempunctata* would be appropriate for the case of the GM-amylopectin potato. For this reason either the existing methods would have to be modified or new test methods would have to be developed.

1 Introduction

Since the publication of Rachel Carson's 'Silent Spring', public concerns over environmental degradation from chemical pollution, including the massive use of synthetic pesticides and fertilisers in agriculture, has stimulated the development of legislation resulting in extensive regulations on pre-release testing of environmental chemicals. Since almost two decades now, chemical environmental stressors such as pesticides are subjected to a number of standardised ecotoxicity tests for regulatory approval prior to their environmental release (e.g., EU 1991). Based on the data from ecotoxicity testing, the chemical substances are classified into toxicity categories that are in turn associated with specifications for use (possibly restricting their use) and recommendations for safe handling and use of these substances. The development of agreed testing procedures for chemicals has a long and on-going history in which the OECD took a leading role regarding their international harmonization (OECD 1981-2006).

Genetically modified organisms (GMOs) in general and genetically modified plants (GMPs) in particular were a contentious issue since the advent of the technology and, very quickly, it was agreed that they are subject to regulations. Potential harmful effects caused by GMOs were described and discussed in the scientific literature already in the eighties of the 20th century (Cairns & Pratt 1986a; Cairns & Pratt 1986b; Regal 1986; Tiedje et al. 1989).

However, while legislation exists in most countries regulating the release of GMOs, no agreed standardised pre-release testing procedures exist up to today. This is despite the fact that GMPs are grown already on a large scale in some countries since 10 years. As of today, the applicants follow largely the OECD guidelines for environmental chemicals (Andow & Hilbeck 2004). This includes some self-determined ecotoxicity testing in cases where a pesticidal compound is expressed in the plants (e.g., Bt-toxins) and some feeding trials for human health assessments also following the chemical paradigm.

However, plants are not chemicals and regulations and scientifically sound testing procedures should account for the differences:

- In GMPs, the plant-expressed transgene product is an integral component of the plant and coupled to its metabolism. This leads to variable expression levels of the transgene product that is additionally modulated by abiotic conditions of the environment and the seasonal changes in temperature, moisture and light. On the other hand, due to the use of universally functioning viral promotors and terminators, the transgene products of most if not all currently commercially available GMPs are expressed essentially in all plant parts throughout the entire growing season. When comparing with pesticides, this is equivalent to a long persistence of the pesticidal substance and an almost complete coverage of the plant.
- GMOs are capable of self-reproduction. This is a fundamental difference to chemicals. Because of this capability, biological organisms can increase in the environment and potentially spread and exist for – by human standards – largely unlimited time. In contrast, chemicals cannot reproduce and, thus, their absolut amount will at best (or worst) remain stable for a long time but over time always decline. Most disappear within human conceivable time periods due to degradation.
- GMOs can actively spread and with them their transgene products.
- In addition, all passive mechanisms of spread as for chemicals also apply to transgene products released into the environment from the living GMO (e.g., exudates, leaching from living and dead material).

For these reasons, it is extraordinarily more difficult – if not impossible – to determine the exact exposure concentrations of transgene products in a given environmental compartment than for chemicals. Due to the potential of irreversible longterm effects, longterm testing in microcosms and the field is critical, possibly more so than for chemicals (van der Meer 1993). Hence, scientifically sound testing strategies and methodologies for the required case-specific risk assessment of GMOs should account for the whole organism and treat a GMO as an integrated biological system consisting – in the case of a GMP – of the plant, the novel trait and the receiving environment. For a detailed discussion of the legal basis for assessing the risks of the whole GMO see the following Boxes (Box 1: German and Box 2: English)

Box 1: Juristische Stellungnahme

Ausgangsfrage
Was die Bewertung von Risiken gentechnisch veränderter Organismen anbelangt, stehen sich in der Ökotoxologie zwei unterschiedliche Konzepte gegenüber, standardisierte Tests für bestimmte Konstrukte und ein deutlich breiterer, auf den gesamten GVO bezogener Ansatz. Aus rechtlicher Sicht stellt sich die Frage, ob eine merkmalsbasierte, auf die durch das jeweilige Konstrukt vermittelte Eigenschaft (z.B. ein bestimmtes Bt-Toxin) beschränkte Untersuchung den Anforderungen der RL 2001/18/EG genügt, oder ob nicht vielmehr in der Risikoprüfung jeweils der gesamte gentechnisch veränderte Organismus zu betrachten ist.

Stellungnahme
Nach § 15 Abs. 1 S. 2 Nr. 4 GenTG ist dem Antrag auf Genehmigung einer Freisetzung gentechnisch veränderter Organismen (GVO) eine Risikobewertung nach § 6 Abs. 1 GenTG beizufügen. Diese erfolgt gemäß § 5 Abs. 1 Nr. 4 GenTVfV nach Maßgabe des Anhangs II der RL 2001/18/EG sowie der Entscheidung der Kommission vom 24.Juli 2004 über Leitlinien zur Ergänzung des Anhangs II (Entscheidung 2002/623/EG) auf der Grundlage der nach Anhang III A. Nr. II bis IV der RL 2001/18/EG vorzulegenden Informationen. Für das Inverkehrbringen von GVO gilt gemäß § 15 Abs. 3 S. 3 Nr. 4 GenTG, § 6 Abs. 1 Nr. 3 GenTVfV entsprechendes.

Das Ziel der Risikoprüfung besteht nach Anhang II A. RL 2001/18/EG darin, *von Fall zu Fall* etwaige direkte, indirekte, sofortige oder spätere schädliche Auswirkungen von GVO auf die menschliche Gesundheit und die Umwelt, die bei der absichtlichen Freisetzung oder dem Inverkehrbringen von GVO auftreten können, zu ermitteln und zu evaluieren. Das auch in Art. 4 Abs. 3 der Richtlinie verankerte sog. case by case-Prinzip schreibt nicht nur die Betrachtung eines jeden Gen-Konstrukts vor, sondern fordert für jede neue Verwendung oder die Verwendung in einem neuen Aufnahmemilieu eine neue Untersuchung (vgl. Palme in: Eberbach/Lange/Ronellenfitsch, GenTR/BioMedR, Einl zur FreisetzungsRL Rn.73).

Dies entspricht dem Grundansatz des am Vorsorgeprinzip orientierten europäischen Gentechnikrechts, wonach die Gestattungswirkung der Freisetzungs- oder Inverkehrbringensgenehmigung stets nur konkrete GVO umfasst, nicht aber pauschal eine durch ein bestimmtes Gen-Konstrukt vermittelte Eigenschaft. (Auch bei der Zulassung im differenzierten Verfahren nach Art. 7 RL 2001/18/EG erfolgt die Verfahrensvereinfachung nicht generell, sondern nur in Bezug auf bestimmte GVO in bestimmten Ökosystemen.)

Anhang II C.1 der Richtlinie 2001/18/EG benennt die bei der Risikobewertung zu berücksichtigenden Merkmale, dazu gehören die „genetische(n) Veränderung(en), sei es Einfügung oder Deletion genetischen Materials sowie die relevanten Informationen über den Vektor und den Spenderorganismus“, der GVO sowie die vorgesehene Freisetzung oder Verwendung und das Aufnahmemilieu. Die Richtlinie unterscheidet somit explizit zwischen der genetischen Veränderung als solcher und dem genetisch veränderten Organismus. Nach Anhang II C.2 Nr.1 sind in der Risikobewertung „*alle Merkmale der GVO*, die mit der genetischen Veränderung in Verbindung stehen und schädliche Auswirkungen auf die menschliche Gesundheit oder die Umwelt haben können“ zu ermitteln. Die Richtlinie betont weiterhin, dass sich diese schädlichen Auswirkungen, etwa Auswirkungen auf die Populationsdynamik von Arten im Aufnahmemilieu, von Fall zu Fall unterscheiden können. Der Schluss bei einem Gen-Konstrukt „einmal schädlich = immer schädlich“ oder „einmal unschädlich = immer unschädlich“ wäre somit eine unzulässige Verkürzung der Risikobewertung (vgl. Palme a.a.O).

Box 2: Legal basis

Legal question
Regarding the evaluation of risks from genetically modified organisms, two different concepts currently exist: standardized tests for particular constructs and a significantly broader approach including the whole GMO.
From a legal perspective the question arises whether a narrow property-based assessment focussing only on the construct (e.g., a particular Bt-toxin) suffices the requirements put forward in the Directive 2001/18/EC, or whether a risk assessment has to consider the entire genetically modified organism.

Statement
According to Article 15 paragraph 1 second sentence no. 4 Genetic Engineering Act (GenTG) an application dossier for regulatory approval of release of genetically modified organisms (GMO) has to contain a risk evaluation based on Article 6 paragraph 1 GenTG . The risk evaluation follows Article 5 paragraph 1 no. 4 Regulation on application and notification documents and the permission and notification procedures according to the Genetic Engineering Act (GenTVfV) which is based on the provisions put forward in Annex II of the Directive 2001/18/EC and the Commission Decision of 24 July 2002 establishing guidance notes supplementing Annex II (2002/623/EC) on the information required in Annex II A. No. II - IV of Directive 2001/18/EC. Similar applies for placing on the market of GMOs following Article 15 paragraph 3 third sentence no. 4 GenTG, , Article 6 paragraph 1 no. 3 GenTVfV.

The goal of the risk assessment is according to Annex II A. Directive 2001/18/EC to evaluate on a case-by-case basis the direct, indirect, immediate or delayed adverse effects of a GMO on human health and the environment that can result from the intentional release or placing-on-the-market of a GMO.
The case-by-case principle, which is anchored in 'Art. 4 Abs. 3' of the Directive, not only requires to consider each ('trans')gene construct but requires for each new use or use in a new receiving environment a new investigation (compare with Palme in: Eberbach/Lange/Ronellenfitsch, GenTR/BioMedR, Einl zur FreisetzungsRL Rn. 73).

This corresponds to the principles of the European Gene Technology legislation anchored in the precautionary principle. According to this legislation the permitted effect of a field release or placing on the market always includes the whole GMO and not only the particular (trans-)gene construct.
(Also in the differentiated approval procedure according to Art. 7 of the Directive 2001/18/EC, the procedural simplification is not permitted generally but only in relation to a particular GMO in specified ecosystems.)
Annex II C.1 of the Directive 2001/18/EC lists the characteristics of GMOs that have to be taken into account in the risk assessment: 'the genetic modification(s), be it inclusion or deletion of genetic material, and relevant information on the vector and the donor', the GMO and the intended release or use and the receiving environment. The Directive therefore explicitly distinguishes between the genetic modification as such and the genetically modified organism.

Annex II C.2 Nr. 1 specifically requires that for the risk assessment 'any characteristics of the GMOs linked to the genetic modification that may result in a adverse effects on human health or the environment shall be identified'. The Directive further emphasizes that the adverse effects, e.g., on the population dynamics of species in the receiving environment, can differ from case to case. The conclusion for a (trans-)gene construct 'once damaging = always damaging' or 'once not damaging = always not damaging' would be a not acceptable reduction of the risk assessment (see Palme cited above).

The goal of this research & development project commissioned by the BfN is:

1. The **compilation and evaluation of existing ecotoxicological testing methodologies** and – **strategies for chemicals regarding their suitability for risk assessment of transgenic plants**.
2. The **evaluation of the currently used ecotoxicological testing procedures for GMPs**.

For the second step, two case example GMPs are used: 1507 Bt-maize, registered and grown already in the USA and the conventional hybrid produced from crossing two GM maize varieties: NK603 (herbicide-resistant) and MON810 (Bt). Based on these analyses, **recommendations for alternative strategies and improvements** are made. These include the formulation of specific provisions for ecotoxicological testing strategies for GMPs in order to be in compliance with the legal requirements

put forward in the relevant legislation. Lastly, we **present an improved testing strategy and risk assessment framework for GMPs** and, finally, conduct a short test run with an amylopectin-producing GM potato.

2 Analysis of the current practice in application dossiers of GM crop plants

Current environmental risk assessment (ERA) of GM crop plants relies on the chemicals testing model. This model is based on data obtained through ecotoxicological testing of environmental chemicals such as pesticides. The reliance of ERA of GM crop plants on the chemicals testing model has been repeatedly criticized and its shortcomings have been described (Wolfenbarger & Phifer 2000; Marvier 2002; Andow et al. 2004). Here, a summary of the main points of the criticism will be provided followed by a detailed analysis of the resulting methodological shortcomings of current GMO safety tests.

2.1 Risk assessment procedures used for chemicals

The term *environmental risk assessment* can be defined as „simply a systematic means of developing a scientific basis for regulatory decision making" (Barnthouse et al. 1992). The concept of ERA was developed in the USA during the late seventies of the 20th century (Fava et al. 1987). It was firstly used for anthropogenic stress factors with potential impacts on the environment. In the eighties, the risk of chemicals were prospectively assessed (EPA 1992). Shortly afterwards this concept was also adapted by European authorities for harmonising the registration of pesticides (1991) and the notification of industrial chemicals (EU 2003).

For ERA, fate and effects of the substance to be assessed in the environment are of highest importance. In this context it is not important whether the respective chemical is used as a pesticide or as a plasticiser. For historical reasons, there are some differences in the EU guidelines covering the different chemical groups (e.g., concerning the nomenclature used). However, the use, the amount and the pathways of the chemical determine the exposure of organisms (Box 3). It is therefore important whether a chemical is routinely applied at a crop site or whether it reaches the environment by diffuse emission after combustion. Basically, the tests required for the registration of GMPs in the European Union (EU 2001) follow the same principles as the tests performed with chemicals. In the following, important similarities and differences between the risk assessment of chemicals and GMPs will be discussed.

2.1.1 Important elements of the environmental risk assessment of chemicals

Depending on the exposure situation, the ERA has to be carried out for each environmental compartment (water, sediment, soil and air) separately. The crucial element of most assessments is the determination of the concentration expected in the environment (Predicted Environmental Concentration: PEC) and the concentration without an expected effect in the environment (Predicted No Effect Concentration: PNEC) (Leeuwen & Hermens 2001) (see Box 3).

A determination of the **PEC** for „new" chemicals is done by using complex models based on the physico-chemical properties of the substances, how and when they reach the environment (i.e., use pattern, amount etc.) and, to a certain extent, environmental variables. The PECs of existing chemicals can be measured directly in the environment using the respective proven analytical detection methods.

The determination of the **PNEC** is usually performed by measuring the effects of a chemical on individual species (e.g., the mortality, growth or reproduction) in laboratory tests. In this respect, an indication of the potential longterm effects is the lipophilicity of the chemical because libophil chemicals

(high log Pow value) can accumulate in the fat tissues of organisms and along the food chain. This may result in delayed, secondary poisoning of species on higher trophic levels. Using "safety" or "assessment factors" each effect concentration (determined in tests or modelled based on substance properties) will be extrapolated to the concentration expected to have no effect in the field (PNEC).

In the case of environmental chemicals the quantitative characterisation of risk is done by dividing PEC by PNEC (Fig. 1 and Box 3). If the resulting quotient is ≤ 1 this indicates that there is no concern for the environment when using this substance. In this case the ERA is completed. PEC/PNEC quotients > 1 indicate a risk for the environment. In this case the result can be refined by using test methods more relevant for the field (e.g., semi-field approaches) or by re-modelling exposure by using actual values from the area where the test substance will be used. Using these new PEC/PNEC data the risk is assessed again (refined ERA). The process of refining the ERA has to be repeated until it is clear whether there is concern or not. In case of concern, measures to decrease the risk are necessary.

In general, the ERA for pesticides is performed very similar to the assessment of industrial chemicals. However, due to historical reasons, the quotient (called TER (=Toxicity Exposure Ratio)) is calculated just the other way around (EC 2002a). Assuming that the pesticide is used according to the principles of 'good agriculture practice' and depending on the comparison of the TER and certain "safety" or "assessment factors" it has to be decided whether the use of the pesticide can be considered safe or not (for pesticides, these "safety" or "assessment factors" are applied after the TER calculation while for industrial chemicals they are already used when calculating the PNEC). If such a safe use cannot be assumed, the authorities can require safety measures (e.g., buffer zones between the treated area and surface waters or a lower application rates). If risks cannot be avoided by safety measures the pesticide can be banned completely.

Another difference between industrial chemicals and pesticides is that for the former the ERA is done for each environmental compartment while the risk of pesticides is assessed for each organism group separately (in particular in the terrestrial compartment).

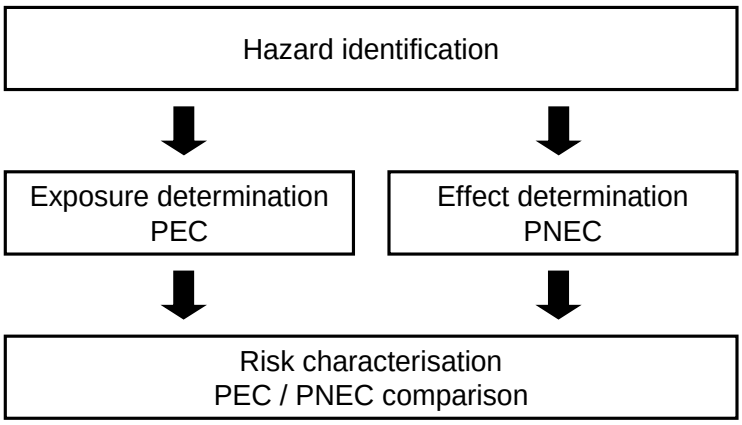

Fig. 1: Scheme of the most important components of the environmental risk assessment for chemicals.

Independently from the legal requirements like notification of industrial chemicals or registration of pesticides it is possible to use the results of ecotoxicological (in particular aquatic) tests for the hazard classification of chemicals (= Risk- and Safety-phrases). Such information, usually given on the label or in the MSDS (= Material Safety Data Sheet) allows to use the chemical in a safe way (e.g., by re-

quiring protection measures like the use of gloves or gas masks). In addition, risk phrases cover also environmental concern: For example, R-50 means "very toxic for aquatic organisms" or S-61 "Avoid entry into the environment". Most noteworthy is that the hazard classification is not based on an ERA but shows the intrinsic properties of a chemical (i.e., no comparison between effect and exposure was made).

Box 3: Environmental Risk Assessment (ERA) for chemicals

Part I: Hazard identification
Use, amount and pathways of a chemical determine the exposure of organisms.
Depending on the exposure situation ERA has to be done for each environmental compartment separately (water, sediment, soil, air). For pesticides also different groups of organisms have to be considered.

Part IIa: Exposure determination
Determination of the concentration expected in the environment (Predicted Environmental Concentration: PEC) is usually carried out by applying models based on i) physico-chemical properties of the substance, ii) the intended use pattern, and iii) environmental variables. Alternatively, exposure also can be measured directly in the environment using chemical methods.

Part IIb: Effect determination
Determination of the concentration that has no statistically significant effect ($p<0.05$) on the respective endpoint within a given exposure period when compared with the control (Predicted No Effect Concentration: PNEC).
Performed by measuring the effects of the chemical on individual species (e.g., mortality, reproduction) in laboratory, semi-field and field tests (the latter mainly for pesticides).
Extrapolation from effect concentration to PNEC by applying "safety" or "assessment factors".

Part III: Risk characterisation
Industrial chemicals:
Ratio PEC to PNEC: resulting quotient of values ≤ 1 indicate no concern for the environment. PEC/PNEC quotients of > 1 indicate risk for the environment.
A refined ERA is possible through more relevant field tests or re-modelling exposure with more realistic assumptions. The process is going to be repeated until it is clear whether concern exists or not.

Pesticides:
Calculation of Toxicity Exposure Ration (TER = toxicity concentration/PEC) and comparison with trigger values.
If safe use cannot be proven, measures (e.g., buffer zones, lower application rates) can be required or the pesticide may be banned.

Principles of effect testing
With few exceptions, standard testing guidelines published by OECD and ISO are used.
Use of these guidelines follows a hierarchical (tiered) order.
Tier 1 covers simple, short-term, lower-cost tests with a limited number of species under assumed worst-case conditions.
Depending on the type of ERA higher-tier (e.g., semi-field, field) tests may be performed (or literature data are included).
Final aim of tiered strategy is to ensure that only substances with a high potential for causing risk in the environment are tested with a high demand for resources.
Test requirements as well as tiered strategy are widely accepted by all stakeholders (industry, agencies, universities).

2.1.2 Principles of effect testing

Ecotoxicological tests methods for chemicals were developed within the last 30 years in order to tackle problems occurring when chemicals enter the environment. In the mid-seventies of the 20th century the ‚Chemicals Testing Program' was initiated by OECD in order to harmonize test requirements and avoid trade barriers. One of the main tasks of this program was to harmonise and to standardise the various test methods, which had been developed in several industrial countries (Forbes & Forbes 1994; Kitano 1992). In the early eighties, OECD published a set of 51 guidelines for testing the fate and effects of chemicals (OECD 1984a). Up to now, this number increased to 55, growing constantly. Many countries have adopted the OECD guidelines in their national legislation (Fent 1998). In addition, national test methods are used in parallel to OECD methods (most noteworthy in the USA (EPA 1996)).

The use of standardised guidelines for the testing of chemicals follows a hierarchical (tiered) order. The first tier consists of simple, short-term and low-cost single species tests. Tests are performed under assumed worst-case conditions and are designed to determine the effects of one or repeated applications of the test substance over a wide range of concentrations. The test result is usually summarised as the LC_{50}- or LD_{50}-value (LC = Lethal Concentration; LD = Lethal Dose); i.e., the concentration or dose at which 50% of the test organisms die.

Depending on the type of ERA performed with such a value (i.e., after comparison with an estimated or measued exposure) higher-tier tests become necessary. Such tests, either performed in the laboratory, semi-field or field, are often more complex, long-lasting and expensive (Cairns Jr. 1981; Bradbury et al. 2004). If no significant effects occur in the tested concentration range (usually, the highest test concentration required in an OECD test is 1'000 mg/kg soil or 1'000 mg/L water), no further tests are required.

The final aim of the tiered test strategy is to ensure that only substances with a high potential for causing an adverse effect in the environment are tested in complex and resource demanding tests (in terms of time and costs) (Bradbury et al. 2004). In other words, the use of test guidelines in a tiered test strategy is an example for a compromise between environmental and economical requirements (Baird et al. 1996; Forbes & Forbes 1994). The advantage of the initial acute tests is their efficient identification of toxic effects of the test substance. However, the disadvantage of the tiered approach is the inability to determine ecological effects under field conditions (Chapman 2002; Kareiva et al. 1996). A tiered strategy is only adequate to ensure the protection of the environment if the criteria when to stop testing (or to proceed to a higher tier) are scientifically sound. In this context, a wrong decision not to proceed with testing (Type II error) must clearly be avoided since otherwise serious consequences for the environment could occur (Forbes & Forbes 1994).

Due to the difficulties to extrapolate results gained on the first tier of the test strategy to higher tiers, the use of multi-species-tests were recommended already 25 years ago (Cairns Jr. 1981). The results of complex aquatic mesocosm studies or field tests with earthworms clearly are of higher ecological relevance. However, at the same time mesocosm studies are often difficult to interpret. Moreover, results from mesocosm studies are usually difficult to compare among each other because test conditions, such as weather are rarely reproducible and, thus, test results are highly variable. At higher tier testing, the results of scientific studies published in the open literature are often included.

2.1.3 Examples for ecotoxicological test methods

For testing chemicals according to a tiered test strategy, standardised test methods are necessary. Most of the test species used in these standard tests are easy to cultivate, genetical uniform and of medium sensitivity towards a wide range of chemicals. The ecological relevance of the test organisms has a secondary role (Table 1). The first tier requires a minimum set of test species for industrial chemicals and pesticides alike. For the aquatic compartment (surface water), this test set contains three species from three trophic levels: one algae species, water flea (*Daphnia magna*) and one fish species (mostly rainbow trout; Bradbury et al. 2004). At higher tiers, further species of these three groups are required as well as additional organism groups like higher plants. In addition, sediment organisms can be included in the test set if exposure of this compartment is possible.

Depending on the chemical group assessed, the set of tests becomes more complex when investigating the terrestrial compartment. Industrial chemicals (depending on the production volume) can be tested with birds (quails, ducks), earthworms (*Eisenia fetida*) and plants (e.g., oat and turnip). Pesticides have to be tested with the same species plus micro-organisms and several species of non-target arthropods (e.g., parasitic wasps and predatory mites, but also beetles, spiders or green lacewings; even though green lacewing tests have not been standardised by OECD so far (EC 2002b)). The risk of the pesticide to be assessed is evaluated for each organism group independently; i.e., there is no overall assessment of the pesticide for the whole terrestrial compartment.

Tab. 1: Standardised ecotoxicological test guidelines* required for the registration of pesticides in Europe according to Guideline 91/414/EC (1991).

Test organism	Test	Duration	Guideline
Aquatic compartment – lower tier tests			
Algae (*Desmodesmus subspicatus*)	Chronic toxicity Growth rate	4 days	(OECD 1984b)
Water fleas (*Daphnia* spp.)	Acute toxicity (immobilisation)	2 days	(OECD 2004a)
Water fleas (*Daphnia* spp.)	Chronic toxicity (reproduction)	21 days	(OECD 1998a)
Fish spp., e.g., rainbow trout (*Oncorhynchus mykiss*)	Acute toxicity (mortality)	4 days	(OECD 2003)
Fish spp., e.g., trout (*Oncorhynchus mykiss*)	Chronic toxicity (juvenile growth)	28 days	(OECD 2000a)
Fish spp., e.g., trout (*Oncorhynchus mykiss*)	Bioaccumulation	42 days	(OECD 1996)
Aquatic compartment – potential higher tier studies			
Fish spp., e.g., trout (*Oncorhynchus mykiss*)	Chronic toxicity (juvenils)	60 days (post-hatch)	(OECD 1992)
Higher plants (*Lemna gibba*)	Chronic test (growth, biomass)	7 days	(OECD 2002)
Midge larvae (sediment) (*Chironomus riparius*)	Emergence of larvae	28 days	(OECD 2004c) / (OECD 2004d)
Aquatic mesocosm (whole community)	Semi-field test (abundance, species diversity)	Variable	Not specified
Terrestrial compartment – lower tier tests			
Birds spp., e.g., quail (*Coturnix japonica*)	Acute toxicity (Dietary toxicity)	8 days	(OECD 1984c)
Bees (*Apis mellifica*)	Acute toxicity (oral / contact exposure)	2 days	(OECD 1998b) / (OECD 1998c)

* Note that depending on usage class (e.g., herbicide, acaricide), frequency of applications, persistence and, mostly, observed effects the type and number of tests can vary (see EU 2002). In addition, several additional tests (in particular with non-target arthropods) can be required under specific circumstances.

Tab. 1: continued.

Test organism	Test	Duration	Guideline
Terrestrial compartment – lower tier tests			
Non-target arthropod: Predatory mite (*Typhlodromus pyri*)	Acute toxicity (mortality, reproduction)	14 days	(Candolfi et al. 2000)
Non-target arthropod: Predatory mite (*Aphidus rhopalosiphi*)	Acute toxicity (mortality, reproduction)	15 days	(Candolfi et al. 2000)
Compost worm (*Eisenia fetida/andrei*)	Acute toxicity (mortality)	14 days	(OECD 1984e)
Compost worm (*Eisenia fetida/andrei*)	Chronic toxicity (reproduction)	56 days	(OECD 2004f)
Micro-organisms (whole community)	Acute toxicity (carbon and nitrogen mineralisation)	28 days	(OECD 2000b) / (OECD 2000c)
Plants (6 – 10 species) (e.g., *Avena sativa*)	Seedling emergence and biomass	14 – 21 days	(OECD 2004b) / (OECD 2004g)
Bacterial community (sewage sludge)	Acute toxicity (respiration rate)	0.125 days	(OECD 1984f)
Birds spp., e.g., quail (*Coturnix japonica*)	Chronic toxicity (reproduction)	About 32 days	(OECD 1984d)
Bees (*Apis mellifica*)	Semi-field (tent test) (mortality, behaviour)	7 days	(EPPO 2000)
Non-target arthropod: Predatory mite (*Typhlodromus pyri*)	Chronic (extended) toxicity (mortality, reproduction)	14 days	(Candolfi et al. 2000)
Non-target arthropod: Predatory mite (*Aphidus rhopalosiphi*)	Chronic (extended) toxicity (mortality, reproduction)	14 days	(Candolfi et al. 2000)
Non-target arthropod: Ground beetle (*Poecilus crupreus*)	Acute toxicity (mortality, feeding rate)	14 days	(Candolfi et al. 2000)
Non-target arthropod: Staphylinid beetle (*Aleochara bilineata*)	Chronic toxicity (mortality, reproduction)	18 – 20 days	(Candolfi et al. 2000)
Non-target arthropod Predatory mite (*Typhlodromus pyri*)	Field test (abundance)	28 days	(Candolfi et al. 2000)
Non-target arthropod (arthropod community)	Field test (abundance, species diversity)	Variable	Not specified
Earthworms (whole community)	Field test (abundance, biomass, species diversity)	180 – 365 days	(ISO 1999b)
Springtail (*Folsomia candida*)	Chronic toxicity (reproduction)	28 days	(ISO 1999a)
Pot worms (*Enchytraeus albidus*)	Chronic toxicity (reproduction)	42 days	(OECD 2004e)
Predatory mite (*Hypoaspis aculeifer*)	Chronic toxicity (reproduction)	16 days	(OECD 2006)
Organic matter degradation (litter bags)	Field test (organic matter mass loss)	180 – 365 days	(Römbke et al. 2003)

In the following a typical example for the number of tests contained in a pesticide dossier of an existing substance submitted to the registration authorities is given (less non-standard tests are provided for newly registered compounds).

Properties of the example pesticide:

History:	Production for about 25 years
Use:	Insecticide in pomiculture
Application rate:	Low
Application frequency:	Once per growing season
Persistency:	High
Metabolization:	3 soil metabolites
Aquatic toxicity:	High
Avian toxicity:	High

In addition to the specifically conducted tests about 20 publications on the terrestrial ecotoxicity (partly written by company associates, partly by university members) were submitted with the dossier.

Number of soil tests submitted:

	Standard	Non-standard
Earthworms:	3	4
Microorganisms:	2	2
Other soil animals:	0	0
Litter decomposition:	0	0
Plants:	12	0

Number of valid arthropod tests submitted:

Bees:	Standard	Non-standard
Laboratory:	5	4
Semi-field:	3	4
Field:	1	8
Other arthropods:	Standard	Non-standard
Laboratory:	7	24
Semi-field:	1	5
Field:	0	9

Field studies were conducted worldwide.

Total number of tests:

	Standard	Non-standard
Soil tests:	17	6
Arthropod tests:	17	54

Remarks:

1. The high number of non-standard arthropod tests is caused by the fact that this compound is an insect growth regulator.
2. The non-standard tests mainly origin from the time before the harmonized EU registration.
3. The dossier and the number of tests may not be regarded as unusual.
4. Many tests were conducted on initiative of the company.

2.1.4 Adopting ecotoxicological tests for chemicals to GMPs

Today, when testing insecticidal GMPs like Bt-maize for regulatory approval applicants follow largely the OECD guidelines for testing of environmental chemicals (pesticide model). This results in the testing of a standard set of species exposed to the microbially produced toxin. Analogous to pesticides, these microbially produced Bt-toxins are fed directly to testing organisms (bitrophic exposition) in an experimental set-up originally developed to assess acute toxicity. In fact, the applied test methods do not go much beyond the methods presented in Table 1 since only these have been standardised and validated sufficiently. Even for chemical testing, it is problematic to use test organisms of higher trophic levels because the test substance is often not ingested directly as parent compound by these organisms but is ingested via one or several intoxicated prey species (multitrophic exposition). These prey species may contain the test substance or metabolites thereof in unknown concentrations.

From research with DDT, we know that persistent insecticides such as DDT can accumulate along the food chain. In organisms at the end of the food chain it reaches concentrations that are multi-fold above the levels originally introduced into the ecosystem (Steinberg et al. 1995; Woodwell et al. 1967). For chemicals, the possibility of secondary poisonings has been addressed in several EU directives either by direct measures (e.g., measuring the bioaccumulation of chemicals in fish (OECD 1996)) or by modelling accumulation in fish, birds, and mammals. Analogous tests for the terrestrial compartment using earth worms are currently being standardised (Egeler et al. 2005).

Usually, in tests carried out for ecotoxicological testing of GMPs high concentrations of the test substance are applied. In the case of Bt-toxins, these concentrations exceed by far the concentrations expressed in GMPs. Further, the significance of such tests is very limited since bio-chemically the Bt-toxin expressed in GMPs can be quite different from the mirobially derived toxin. For example, the Bt-toxin of the Cry1-class used in the tests is either derived from the original *Bacillus* or from genetically modified *Escherichia coli*. After the microbial synthesis the toxin is in its inactive form, a protoxin of 130 kDa in size (Kumar et al. 1996; Müller-Cohn et al. 1996; Höfte & Whiteley 1989). Before used in the tests, the protoxin is cleaved by Trypsin and cut into the toxic fragment of 65 kDa size.

However, in transgenic Bt-plants, fragments of different sizes of the Cry1-class Bt-toxins are produced within different events. For example, the Bt-corn event MON810 expresses a 91 kDA fragment whereas Bt-corn event 176 expresses a 64 kDa fragment (Andow & Hilbeck 2004). From other events, it is known that the Bt-toxins degrade within the plant to fragments of even smaller size (36, 40, 55, 60 kDa) (Andow & Hilbeck 2004; AGBIOS 2006). How the different fragment sizes affect the bioactivity of the Bt-toxin and its spectrum of targeted organisms can only be determined experimentally. However, many answers to these questions remain unclear. From investigations with a Dipteran species it is known that this species is able to degrade a Bt-protoxin of the Cry1-class considered to be specific towards Lepdiopteran species into a 55 kDa fragment that is toxic to Dipteran species (Haider et al. 1986). In conclusion, this means that the Bt-toxins expressed in GMPs may vary significantly in size and activity from the test substances used in ecotoxicological testing.

Further, many questions regarding the environmental fate of the Bt-toxins expressed in GMPs still remain unanswered. Investigations have been carried out on the degradation processes of Bt-toxins from GMPs in soil (Zwahlen et al. 2003a) and to a far lesser extent in the digestive system in farm animals (Lutz et al. 2005). Lutz et al (2005) found Bt-toxin in the excrements of cows fed with Bt-corn (event 176). However, the original Bt-toxin (Cry1Ab) had been degraded into two fragments of 17 and 34 kDa. Obviously, these fragments still had retained their reactive portions of the molecule and, therefore, they could be detected with regular test-kits originally developed for Cry1Ab-toxin of the size of 65 kDa, as it is expressed in some GMPs. By deploying cow dung the Bt-toxins are brought back again into the environment. To our knowledge, the bioactivity of the 17 and 34 kDa fragment of the Cry1Ab-toxin has not been investigated so far.

With GMPs the environmental interactions are even more complex than with chemicals. As a novel compound the transgene product (e.g., Bt-toxin) is an integral part of the plant and, therefore, coupled to the plants' metabolism. Expression of the transgene product varies with metabolic and seasonal changes. By using universal viral promoters for transgene expression in current GMPs the transgene product is expressed in all plant parts during the entire growing season. Compared with pesticides, this represents a high persistence and in biocontrol terms an almost perfect surface and systemic distribution of an active ingredient on and in a plant. Beyond the environmental effects possibly caused by transgene products, the transformation process may also alter metabolic processes of the GMP (Saxena & Stotzky 2001b), which may independently or together with the transgene product affect non-target organisms (Birch et al. 2002).

Another important difference between the risk assessment for chemicals and for GMPs is the use of “safety” or “assessment factors”. For chemicals they are applied in order to cover uncertainties caused a) by the use of (few) standard species, b) by differences between laboratory and field situation and c) by different endpoints. However, in order to apply them a concentration-response relationship has to be established in the tests. This is not possible when testing whole plants (GMPs), because the concentration of a toxin in a plant cannot be higher or lower as the specific expression level which is highly variable as explained above. Hence, the chemical approach is only justified for testing the isolated toxin which, when left at that, is not complying with the legal requirements for GMP.

Because of the differences between chemicals and plants expressing biochemicals as integral component, acute ecotoxicological testing with microbial derived transgene products will only deliver limited information relevant to the environmental safety of GMPs. With such tests unexpected effects and effects due to the interaction of the plant with its environment are entirely omitted. The use of standard test species from chemical testing does not take into account the required case-specific approach of the risk assessment of GMP and ignores the receiving environment a GMP always interacts with. In short: A GMP is not a chemical and any environmental testing should account for the difference. Therefore, test strategies for case-specific risk assessment of GMPs should include the transgene product, the transformed plant and the environment of deployment as an integrated system. This is even more true for GMPs that do not express a toxin but have for instance an altered metabolism (e.g., amylopectin potatoes). In these cases adoption of test principles from the chemical testing is even more problematic because environmental effects of these GMPs may become evident on other levels. Following the logic for strict ecotoxicity testing of a chemical would result in no testing at all for those GMPs that do not express a novel toxin, which would not comply with the EU Directive that requires that all GMPs are tested for direct, indirect, short- and longterm and cumulative delayed effects.

2.2 Methodologies and species used for ecotoxicity testing of chemicals – evaluation of their possible suitability for GM crop plants testing

2.2.1 Tabular listing of test methods of potential use for the risk assessment of transgenic plants

In the following compilation 126 individual test methods used for ecotoxicity testing of chemicals were analysed and described in a uniformly structured way (Appendix C). The goal was to evaluate existing tests for their potential suitability for the risk assessment of GMPs. It should be noted that it was not the aim of this project to present a complete list of all tests methods ever recommended. However, it is assumed that with the exception of non-target arthropod (NTA)-tests the majority of available methods is given here. A method had to be not only used in ecotoxicological studies but it had to be also – more or less – standardised to be included in this compilation.

Each method presented as a table in Appendix C was classified according to its investigation level (laboratory, semi-field or field). In addition, eluate tests using soil organisms, a selection of aquatic test methods and ecotoxicological test methods using birds are presented in separate subchapters of Appendix C. Within each group of methods the individual tests are classified according to the respective taxonomic group and the year of publication in chronological order. All tables follow this same outline (cf. Römbke et al. 1995, Løkke & Van Gestel 1996). The listed categories (see below) include information on the ecological function, the trophic level of the test organism, experimental conditions and any other information that was deemed of importance for the risk assessment of GMPs. Initially, it was also intended to include anthropogenic categories like "conservation concern" or "cultural concern". However, none of these criteria were relevant for those species used in the test methods reviewed so far (e.g., no soil invertebrate species has been classified in a Red List as far as we know). Therefore, no anthropogenic categories were considered. In detail, the tables in Appendix C contain information for the following categories:

Principle Classification of the test method (e.g., laboratory test) and aim for which the test was developed

Guideline Formal status: e.g., the test is published as an internationally validated and standardised guideline or is proposed as a new test idea in the scientific literature

Test species Taxonomic description of the test species

Ecology Assignment of organisms to trophic levels according to Groot & Dicke (2002) and TGD (2003), preferred food and typical habitat

Test design Number of test organisms per test vessel (including its description)

Substrate Composition of the test medium

Parameter Listing of the measurement endpoints

Duration Duration of the test and number of examinations

Application Description of the exposure to the test substance

Concentration Number and spacing of the concentrations to be tested

Performance Important test conditions (e.g., temperature, moisture) and specific methodological details (e.g., extraction methods)

Reference substance Name of the reference substance, how often such a test should be performed and, if possible, concentrations to be used and results to be expected

Validity criteria Description which conditions (usually in the controls) must be fulfilled in order to evaluate the test as valid

Assessment Statistical methods for the evaluation of the "raw" data in order to get assessment criteria like LC_{50}- or NOEC-values

Notes All other information that might be interesting to understand the test method.

Testing of GMP? Information if the test or test organism have already been used in the testing of GMP.

The information provided in the 126 individual tables of Appendix C can be evaluated in several ways. In the following, the compiled tests are classified according to 6 different criteria:

1. compartment tested,
2. investigation level (i.e., level of ecological complexity),
3. taxonomic group
4. ecological group
5. status of standardisation
6. used for GMPs.

Criteria 1:

'Compartment tested':	**Terrestrial tests:**	**Aquatic tests**
	113	13

Most of the listed tests focus on the terrestrial compartment. Only a relative limited number of aquatic tests (the most important ones) were included here. For example, out of a two-digit number of possible fish tests only the acute laboratory test is cited, because it is part of the "base-set", which is used for the hazard classification of chemicals (together with the algae and acute *Daphnia* test). Also some terrestrial organisms which have been used in water or eluate tests are listed.

Criteria 2:

'Investigation level':	**Laboratory (aquatic):**	**Laboratory (terrestrial)**
	13	94
	Semi-field:	**Field:**
	13	6

Historically, ecotoxicology started with relatively simple laboratory tests. In addition, current legal requirements mainly ask for data from laboratory tests, because they are quick, cheap and relatively easy to interpret, whereas field tests are more expensive and due to their complexity more difficult to interpret. Semi-field means that the respective test is located somewhere between the two extremes: closed laboratory and open field. Some of these tests are in fact extended laboratory trials while others cover nearly the whole complexity of field trials. In reality, the vast number of tests is performed on the laboratory scale.

Criteria 3: 'Taxonomic group'

In the following, only terrestrial tests (113) will be considered:

'Taxonomic group':	Laboratory	Semi-field	Field
• Microbes:	11	0	0
• Plants:	13	0	0
• Nematodes:	7	0	0
• Oligochaetes:	15	0	1
• Insects:	26	5	2
• Crustaceans:	3	1	0
• Arachnides:	8	0	1
• Birds:	8	0	0
• Others:	3	0	0
• Multi-species:	0	7	2

Since the groups listed here are inconsistent in their taxonomic representation, these numbers are of limited value. For example, while all plants are summarised in one category, animals are divided into six groups. However, the above listing repflects the way these tests are compiled in legal or standardisation documents. The number of tests is mainly governed by legal requirements, in particular those from the area of pesticide testing. For example, many oligochaete tests were developed because large earthworms are not only widely distributed and ecologically relevant, but also because they were accepted by the public and in the scientific community as the most important soil invertebrates. Together with their easy handling and testing they seemed to be the perfect surrogate for the vast number of soil invertebrate species.

Accordingly, the test development of the many insect tests was driven by the implementation of integrative plant protection measures. In order to distinguish between those pesticides, which had side-effects on "beneficial arthropods" (mainly predators or parasitoids of pest species) and those having none, there was a need for appropriate methods. Finally, the number of bird tests increased only recently. This increase can be explained by the protection of birds becoming an important nature conservation goal in some countries of the European Union.

Criteria 4:

'Ecological group':	Laboratory	Semi-field	Field
• Primary producers:	13	0	0
• Decomposers:	30	1	1
• Consumers 1. ord. (herbivores):	18	0	0
• Consumer 2. ord. (predators):	18	0	1
• Consumer 3. ord. (parasitoids):	2	3	0
• Pollinators:	3	2	2
• Not assignable:	10	7	2

These numbers clearly reveal a bias for decomposers. It seems that primary producers are underrepresented while the number of decomposers is high (mainly due to the many oligochaete tests). Also the number of tests with "true" herbivores is lower than one would expect given their ecological impor-

tance. Within the group of first order consumers, only few species are truly living on green plant material; many are in fact bacterial feeders like the nematodes included here. Also, there are more than two tests with parasitoids proposed in the literature but none was ever standardised. Finally, the tests which could not be assigned to a group are either covering whole communities or the respective test species is feeding on different food sources (e.g., Collembola).

Criteria 5:

'Standardisation status':	**Laboratory**	**Semi-field**	**Field**
• OECD standard	14	0	1
• ISO standard	16	0	1
• IOBC standard	10	1	0
• ASTM / EPA / FDA standard	10	1	0
• Environment Canada standard	6	0	0
• National (BBA etc.) standard	7	2	2
• Literatur proposal	31	8	1
• EPPO standard	0	1	1

Out of the 113 terrestrial test methods described nearly one third (40) are proposals from the open literature, which usually are only acceptable as additional information for the environmental risk assessment of chemicals. Experiences gained in some of the research projects described in the open literature have been incorporated into test guidelines. In addition, it is possible to use a method from literature for higher tier testing, i.e., in cases where specific situations do occur. Usually not acceptable are guidelines published by national organisations like the German BBA, more or less for formal reasons. This is in particular interesting in the case of BBA guidelines, which often formed the basis for latter ISO or OECD guidelines (at least data gained according to BBA guidelines in the past will be accepted until today but new studies should not be started based on these guidelines).

Criteria 6:

'Used for GMPs':

The following methods have been used for the assessment of GMPs (see Appendix C for details):

- Potential ammonium oxidation (ISO 15685)
- Microbial soil respiration (ISO 17155)
- Earthworm test with *Aporrectodea caliginosa* (Kula & Larink 1998)
- Several earthworm tests with *Eisenia fetida* (OECD 207; ISO 11268-2; ISO 17512-1)
- Collembolan reproduction test with *Folsomia candida* (ISO 11267)
- Litter-bag test (OECD-Guidance Document 56)
- Water flea test with *Daphnia magna* (ISO 6341)
- Bird test with *Colinus virginianus* (OECD 205)

The 10 test methods listed under criteria 6 clearly reflect the legal requirements from the ERA of pesticides. However, it was not always possible to clearly identify, which test method had really been used in a specific GMP-study (e.g., because it was a mixture of several ones or, more often, because it was modified considerably due to the different questions aimed to answer for GMPs), especially in tests with earthworms and birds.

The following test systems, for which standardised test protocols exist, have been used for the assessment of GMPs but in non-standardised tests (see Appendix C for details):

- Dehydrogenase activity
- Soil protozoa
- *Lumbricus terrestris*
- *Orius* spp.
- *Apis mellifera*
- *Porcellio scaber*
- *Crysoperla carnea*

The lack of standardised tests for the assessment of GMPs illustrates the necessity for specifically adapted test protocols. From the compilation in Appendix C it can be concluded that most often aboveground arthropods have been used for testing of nontarget effects of GMPs, probably because these organisms are expected to be exposed either by directly feeding on the GMPs or by preying on herbivores that were feeding on the GMPs. Arthropods are followed by decomposers (i.e., oligochaetes and crustaceans), mainly earthworms, as the second most used test organisms, which feed on dead plant tissue. We also concluded from the evaluation of the compiled test methods (see also Chapter 2.2.3, which covers soil invertebrates) that only in few cases a test method developed for chemicals can be used for testing of GMPs without modifications (e.g., testing the surrogate toxin on individual species). Such adaptations should urgently be developed and validated (e.g., in order to secure a proper exposure).

2.2.2 Applicability for the assessment of GMP

A careful analysis of all possible exposure routes of a non-target organism to GMPs is essential for selecting the proper test species (see Chapter 3.1). Exposure is highly dependent on the characteristics of the novel trait of the GMP (e.g., toxin expression, herbicide resistance) and its expression patterns. Current testing of GMPs has been focused on Bt-plants, for which potential exposure routes are relatively easy to predict. In the future, cultivation of GMPs may well lead to additional exposure routes not considered so far. Non-target organisms may be exposed to GMP material through the following routes:

- direct feeding of living (e.g., roots, tubers) or dead (e.g., plant litter, roots) GMP material (on the soil surface or after incorporation by for example ploughing)
- exposure to novel proteins through soil particles or pore water after degradation of GMP material
- exposure to root exudates through soil particles or pore water
- secondary exposure to the novel proteins through feeding on other organisms that have incorporated GMP material (including also decomposing GMP residues in soils).

General exposure scenarios covered in the standardised guidelines compiled in Appendix C are:

- (contaminated/spiked) (field) soil
- spiked aqueous medium / eluate
- spiked dung (veterinary pharmaceuticals)
- spray application on plants (bees)
- spray application on glas plates (NTA tests)
- spiked food / oral dose
- direct contact (bees)

Considering the above mentioned exposure routes of non-target organims to GMPs, it can be concluded that test methods with the following exposure scenarios may be applicable to the assessment of GMPs requiring little or no modification:

- field soil, e.g., from GMP cultivation (or spiked in the lab)
- eluate from field soil with GMP cultivation
- dung from animals fed with GMP material
- direct contact to GMPs (bee tests)
- feeding with GMP material (e.g., birds)

Some test methods might become applicable to GMP assessment after suitable modification such as:

- incorporation of GMP material into the soil
- mesocosms planted with GMPs
- eluate/extraction of transgene products from GMP material
- direct contact to GMP material (e.g., nectare, pollen, etc.)
- feeding with GMP material
- see also suggestions of Hund-Rinke et al. (2004)

Some test methods will be difficult or impossible to modify in order to achieve a realistic exposure scenario, e.g.:

- spiked aqueous medium
- spray application on plants (plant test)
- spray application on glass plates (NTA tests)

Thus, it can be concluded, that from the large number of compiled test methods (Appendix C), which reflect the majority of available standardised tests, only very few can be adopted for the assessment of GMPs without modifications (bird tests, bee tests; semi-field and field tests). For the majority of test methods adequate modifications appear feasible but will be strongly dependent on the given case. Especially for NTAs, new methods are needed but existing experience (e.g., cultivation methods) can be used for their development. New test methods will have to take the variable properties of GMPs into account, e.g., by providing flexibility in the choice of the exposure scenario.

2.2.3 Literature review on laboratory testing of GMPs using soil invertebrates

In this chapter, we review laboratory studies published in the "open" literature that were carried out in the context of effect determination of GMPs using true soil inhabiting invertebrate species and actual GMP material (i.e., whole plant approach). No tests using e.g., microbial expressed Bt-toxin were included. Furthermore, the test results had to be published in the scientific literature, i.e., no confidential data from application dossiers or extracts of such dossiers in internet case-studies are presented here (see Chapter 2.3). To date, only few laboratory tests have been described in the scientific literature that assess the effects of GMPs on single species of soil invertebrates. In the following, these published tests are summarised for the individual soil invertebrate groups and species and discussed afterwards in relation to exposure routes, observed endpoints, test parameters (e.g., duration), practicability and selection of species/functional groups. Table 2 gives an overview of the study design of each test reviewed.

Earthworms

Eisenia fetida

Ahl Goy et al. (1995) exposed *E. fetida* to leaf extracts of ECB (European corn borer) tolerant maize, corresponding to 0.35 mg CryIA(b)/kg soil, in artificial soil for 14 days, hence, probably following the standard OECD guideline 207 (OECD 1984e). They assumed the concentration being 785 times higher than the expected concentration in the soil, when maize plants will be incorporated into the soil after harvest. No effects on survival or weight gain were observed in comparison to a non-specified control maize. No details are given concerning the extraction method of the Bt-toxin from the leaves and no estimation can be made as to how the toxin may be comparable to a situation where Bt-toxin enters the soil from decaying leaf material in the field.

Lumbricus terrestris

Saxena & Stotzky (2001) performed tests with commercially purchased *L. terrestris* in a natural field soil planted with Bt (NK4640Bt) or isogenic non-Bt maize for 40 days and in soil amended with ground, air-dried biomass (leaves, stems, and roots) of Bt or non-Bt maize (1% plant material in 500 g soil) for 45 days. No significant differences in mortality and earthworm weight were observed. The presence of the Bt-toxin in the soil from the earthworms' guts was verified at test end through immunological assays and bioassays. However, the amount of the Bt-toxin was not quantified.

Zwahlen et al. (2003) fed adult field-collected *L. terrestris* with N4640Bt and isogenic maize leaf litter in field soil for 200 days. The plant material was not incorporated into the soil but put on the soil surface according to the feeding habits of *L. terrestris*. The initial Cry1Ab toxin concentration in the transgenic Bt leaves was 15.5 µg/g dw leaf. The toxin concentration decreased to 1.2 µg/g dw leaf during the first 40 days of the trial but remained at a level of 0.2 to 0.7 µg/g dw leaf until the end of the trial. No lethal effects were observed. No statistically significant differences in relative weights were observed during the first 160 days of the trial, but after 200 days adult *L. terrestris* had a statistically significant weight loss of 18% of their initial weight when fed Bt maize litter compared to a weight gain of 4% in non-Bt maize fed earthworms.

Aporrectodea caliginosa

Vercesi et al. (2006) performed various tests with field-collected *A. caliginosa* in natural soil. Finely ground leaves of MEB307 Bt and near-isogenic maize were incorporated into the soil up to concentrations of 5 g dry mass/kg soil. Cow dung was supplied as additional food source. The content of the Bt-toxin Cry1Ab was determined to be 9.6 µg/g in MEB307 (dry leaves). Adult and juvenile earthworms were exposed for 28 days and 14 weeks, respectively. The fungicide benomyl served as a positive control but did not always show statistically significant effects. No effects on survival, growth, development and cocoon production were observed in the Bt maize treatments. However, a slight but statistically significant effect on cocoon hatchability was observed with a NOEC of 3 g dry mass/kg and an EC10 of 4.2 g dry mass/kg soil. Growth of juvenile *A. caliginosa* was unaffected when the earthworms were kept in pots with a growing Bt maize plant for 28 days.

Collembolans

Folsomia candida

Yu et al. (1997) fed leaf discs or milled leaves of transgenic cotton lines #81 and #249 (control: parent variety Coker 312) and transgenic and non-transgenic potato leaves to *F. candida* on a field soil for 7 to 8 weeks. For the transgenic cotton lines expression rates were up to 0.1% soluble Cry1Ab protein or 10 to 25 µg protein per gram of fresh weight plant tissue. Transgenic potato leaves had an expected expression of 0.1% soluble Cry3A protein or 10 to 20 µg protein per gram of fresh weight of plant. Bean leaves soaked with cadmium nitrate served as a positive control. No effects on body length and reproduction parameters of *F. candida* were observed in the Bt treatments.

Romeis et al. (2003) fed dried root material of "Greina" and "Golin" KP4- (killer protein) transgenic and non-transgenic (isolines) wheat varieties to *F. candida* on plaster of Paris and activated charcoal. The animals were exposed individually and in groups of 10 until after the third oviposition and for eight weeks, respectively. Food material was provided ad libitum in a 1:10 mixture with baker's yeast on small pieces of filter paper and renewed every week. No effects on life-history parameters mortality, oviposition, cluster size of oviposition bouts, skipping of oviposition bouts, insect weight after third egg laying, and egg viability were observed.

Protaphorura armata

Heckmann et al. (2006) investigated the effects of feeding dried ground root tissue of two Bt maize varieties (Cascade and MEB307) and their isogenic varieties (Rivaldo and Monumental) to laboratory-cultured *P. armata* on plaster of Paris and activated charcoal for four weeks. The amount of Cry1Ab expressed in the root tissue was determined to be 1.37 and 1.01 µg/g for varieties Cascade and MEB307, respectively. No effects on mortality and body surface area were observed.

Nematodes

Saxena and Stotzky (2001) observed no effect on the number of nematodes in a natural field soil planted with Bt (NK4640Bt) or isogenic non-Bt maize for 40 days and in soil amended with ground, air-dried biomass (leaves, stems, and roots) of Bt or non-Bt maize (1% plant material in 500 g soil) for 45 days. The experiments were conducted in parallel to tests performed with the earthworm *L. terrestris* (see above).

Isopods

Porcellio scaber

Escher et al. (2000) fed pre-decomposed X4335-EPR Bt maize and isogenic maize leaves to field-collected *P. scaber* on plaster of Paris and activated charcoal for 8 days in food-choice experiments and for 7 months in a reproduction trial. No differences in consumption of either Bt or non-Bt maize were found. There was also no difference in the number of juveniles per female. Differences in juvenile mortality and adult and juvenile weight gain were found to be related to higher food quality of Bt maize due to a slightly lower C:N ratio, a lower lignin content, and a higher content of soluble carbohydrates. No measurements on the level of toxin expression were performed.

Wandeler et al. (2002) performed 20-day feeding experiments with field-collected *P. scaber*, two Bt maize varieties (Max88 and N4640Bt), and six conventional varieties (N4640 being isogenic to N4640Bt) on plaster of Paris. Analysis of the two Bt maize varieties by enzyme-linked immunosorbent assay (ELISA) indicated an initial Cry1Ab toxin concentration of 19.7 and 2.9 µg/g dw in N4640Bt and Max88, respectively. After 20 days, the toxin concentration decreased to 15.5 and 1.1 µg/g, respectively. The presence of the Bt-toxin in the isopods' gut was verified after the experiment by ELISA. *P. scaber* fed statistically significant less from N4640Bt leaves than from its control N4640. Max88 was consumed statistically significant more than N4640Bt but there was no statistically significant difference to N4640. Within the six non-transgenic maize varieties, a wide range of consumption was detected. The transgenic maize variety N4640Bt equalled the poorly consumed varieties, Max88 was one of the most consumed varieties.

Oribatid mites

Oppia nitens

Yu et al. (1997) fed leaf discs or milled leaves of transgenic cotton lines #81 and #249 (control: parent variety Coker 312) leaves to *O. nitens* on a field soil for 7 weeks. For the transgenic cotton lines expression rates were up to 0.1% soluble Cry1Ab protein or 10 to 25 µg protein per gram of fresh weight plant tissue. No effects on population growth rates of *O. nitens* were observed in the Bt treatments.

Tab. 2: Study design of the soil invertebrates tests reviewed.

GMP	Trait	Test organism	Expression	Exposition	Concentration / Dose	Duration	Endpoints	Effect	Reference
ECB tolerant maize	Cry1A(b) toxin	*Eisenia fetida*	Leaves	Artificial soil	0.35 mg a.i./kg	14 days	Mortality Body weight	none none	Ahl Goy et al. 1995
NK4640Bt	Cry1A(b) toxin	*Lumbricus terrestris*	Root exudates	Soil	3 Plants	40 days	Mortality Body weight	none none	Saxena & Stotzky 2001
NK4640Bt	Cry1A(b) toxin	*Lumbricus terrestris*	Plants	Soil	1% plant material in 500 g soil	45 days	Mortality Body weight	none none	Saxena & Stotzky 2001
N4640Bt maize	Cry1A(b) toxin	*Lumbricus terrestris*	Leaves	Food	15.5 µg/g dw	200 days	Mortality Weight gain	none LOEC ≤ 15.5 µg/g dw	Zwahlen et al. 2003
MEB307 maize	Cry1A(b) toxin	*Aporrectodea caliginosa*	Leaves	Soil	1, 2, 3, 4, 5 g DM[1] leaves/kg	28 days	Mortality Body weight Cocoon production Cocoon hatchability	none none none EC10 = 4.2 g DM[1] leaves/kg	Vercesi et al. 2006
MEB307 maize	Cry1A(b) toxin	*Aporrectodea caliginosa*	Leaves	Soil	1, 2, 3, 4, 5 g DM[1] leaves/kg	14 weeks	Mortality Growth Maturation	none none none	Vercesi et al. 2006
MEB307 maize	Cry1A(b) toxin	*Aporrectodea caliginosa*	Plants	Plants + soil	1 Plant	28 days	Growth	none	Vercesi et al. 2006

[1] DM = dry matter

Tab. 2: continued.

GMP	Trait	Test organism	Expression	Exposition	Concentration / Dose	Duration	Endpoints	Effect	Reference
Golin TR wheat	KP4 protein	*Folsomia candida*	Roots	Food	Root powder:Baker's yeast 10:1	Until after 3rd oviposition	Mortality Body weight Duration between emergence and first and third oviposition Skipping of oviposition bouts Number of eggs Duration of egg development Egg viability	none none none none none none none	Romeis et al. 2003
Greina TR wheat	KP4 protein	*Folsomia candida*	Roots	Food	Root powder:Baker's yeast 10:1	Until after 3rd oviposition	Mortality Body weight Duration between emergence and first and third oviposition Skipping of oviposition bouts Number of eggs Duration of egg development Egg viability	none none none Lower percentage compared to control (14 vs. 38%) none none none	Romeis et al. 2003

Tab. 2: continued.

GMP	Trait	*Test organism*	Expression	Exposition	Concentration / Dose	Duration	Endpoints	Effect	Reference
Golin TR wheat	KP4 protein	*Folsomia candida*	Roots	Food	Root powder:Baker's yeast 10:1	8 weeks	Mortality Body weight	none none	Romeis et al. 2003
Greina TR wheat	KP4 protein	*Folsomia candida*	Roots	Food	Root powder:Baker's yeast 10:1	8 weeks	Mortality Body weight	none none	Romeis et al. 2003
Line #81 cotton	Cry1A(b) toxin	*Folsomia candida*	Leaves	Food	10-25 µg/g	7-8 weeks	Start of oviposition Number of eggs Body length	none none none	Yu et al. 1997
Line #249 cotton	Cry1A(c) toxin	*Folsomia candida*	Leaves	Food	10-25 µg/g	7-8 weeks	Start of oviposition Number of eggs Body length	none none none	Yu et al. 1997
Bt potato	Cry3A toxin	*Folsomia candida*	Leaves	Food	10-20 µg/g	7-8 weeks	Start of oviposition Number of eggs Body length	none none none	Yu et al. 1997
Cascade maize	Cry1A(b) toxin	*Protaphorura armata*	Roots	Food	1.37 µg/g	4 weeks	Mortality Body surface area	none none	Heckmann et al. 2006
MEB307 maize	Cry1A(b) toxin	*Protaphorura armata*	Roots	Food	1.01 µg/g	4 weeks	Mortality Body surface area	none none	Heckmann et al. 2006
NK4640Bt	Cry1A(b) toxin	Nematodes	Root exudates	Soil	3 Plants	40 days	Number	none	Saxena & Stotzky 2001
NK4640Bt	Cry1A(b) toxin	Nematodes	Plants	Soil	1% plant material in 500 g soil	45 days	Number	none	Saxena & Stotzky 2001

Tab. 2: continued.

GMP	Trait	Test organism	Expression	Exposition	Concentration / Dose	Duration	Endpoints	Effect	Reference
X4335-EPR maize	Cry1A(b) toxin	*Porcellio scaber*	Leaves	Food	X4335-EPR maize leaves	8 days	Consumption	none	Escher et al. 2000
X4335-EPR maize	Cry1A(b) toxin	*Porcellio scaber*	Leaves	Food	X4335-EPR maize leaves	7 months	Reproduction	none	Escher et al. 2000
Max88 maize	Cry1A(b) toxin	*Porcellio scaber*	Leaves	Food	2.9 µg/g	20 days	Food consumption	none	Wandeler et al. 2002
N4640Bt maize	Cry1A(b) toxin	*Porcellio scaber*	Leaves	Food	19.7 µg/g	20 days	Food consumption	LOEC ≤ 19.7 µg/g	Wandeler et al. 2002
Line #81 cotton	Cry1A(b) toxin	*Oppia nitens*	Leaves	Food	10-25 µg/g	7 weeks	Number of juveniles	none	Yu et al. 1997
Line #249 cotton	Cry1A(c) toxin	*Oppia nitens*	Leaves	Food	10-25 µg/g	7 weeks	Number of juveniles	none	Yu et al. 1997

Discussion

Exposure

In the tests published for soil invertebrates so far (Table 2), mainly exposure through direct feeding on dead GMP material has been assessed. Generally, this is a reasonable approach for an initial assessment of toxin expressing GMPs. In the case of earthworms, two studies assessed exposure through direct feeding by mixing GMP material into the soil (Saxena & Stotzky 2001a; Vercesi et al. 2006). The same two studies also tried to address exposure to root exudates (Saxena & Stotzky 2001a; Vercesi et al. 2006).

Studies using GMP material often estimated exposure by quantifying the amount of Bt-toxin present in the plant material. A much more accurate procedure to determine exposure is by measuring the presence of the toxin in the test animal. This was only done in one study (Saxena & Stotzky 2001a). Care must be taken when feeding animals with plant material that would not normally be their preferred food-source in the GMP-receiving environment (e.g., Yu et al. 1997; Romeis et al. 2003). The influence of food quality may mask detrimental effects of the GMP or may produce false positive results.

One study was performed using Bt-toxin extracted from the GMP and incorporated into the test soil (Ahl Goy et al. 1995). The methodology of extraction may impact the structure of the toxin and lead to an altered exposure situation compared to the one to be expected under field conditions. While using Bt-toxins extracted from transgenic Bt-plant material is still a better approximation of a realistic exposure than using microbial surrogate toxin, a validated methodology for extraction should be developed including verification of the bioactivity.

Tests investigating the effects of secondary poisoning of soil organisms (i.e., tests with organisms of higher trophic levels like predatory mites) are missing to date. After all, the plausibility and realism of the exposure route is critical although laboratory tests are always simplified approximations of realistic exposure scenarios compared to the field. A battery of test organims for the assessment of GMPs should cover all relevant exposure routes.

Endpoints

When considering possible endpoints of ecotoxicological testing of GMPs, ideally one would want to cover all relevant life-cycle parameters of a certain test species. Realistically, laboratory testing will have to concentrate on those endpoints that are most likely to be sensitive to an expected impact of the GMP. Those are often sub-lethal endpoints like feeding-behaviour, reproduction, or growth. Acute lethal effects should of course be investigated but not be the focus. Functional parameters like organic matter decomposition may partly be covered in laboratory experiments, e.g., through feeding trials, but will most likely be better investigated in the field, e.g., by performing litter bag studies (Römbke et al. 2003) as for example carried out by Cortet et al. (2006). In the laboratory studies listed in Table 2 the above mentioned endpoints have been covered, mostly concentrating on sub-lethal parameters but not involving entire life-cycle studies.

Test parameter

Test parameters like duration, temperature, light regime, moisture and test substrate should be chosen in respect to the specific GMP, the test organism, the intended exposure route, and the observed endpoints. Ideally a situation should be created that resembles realistic field conditions as close as possi-

ble while retaining the intended advantages of laboratory trials like short duration, practicability, controllability, low variability, and repeatability. This means that, for example, natural soils should be favored although in certain cases, the use of artificial substrates like OECD artificial soil or plaster of Paris might be more appropriate. In the studies listed in Table 2, artificial soil was used in one study (Ahl Goy et al. 1995) while four studies were carried out with natural soils (Yu et al. 1997; Saxena & Stotzky 2001a; Zwahlen et al. 2003b; Vercesi et al. 2006). The remaining four studies – all using arthropod test organisms – used plaster of Paris and activated charcoal (Escher et al. 2000; Wandeler et al. 2002; Romeis et al. 2003; Heckmann et al. 2006). In most cases, when choosing natural soils as testing substrates one must again consider the demands of the test species as well as the potential receiving environment of the GMP. In this respect, soil classification concepts like REFESOL (Kördel et al. 2005), BBSK (Römbke et al. 2000; Römbke et al. 2002), and EURO-Soils (Römbke & Amorim 2004) could provide useful assistance. The choice of an appropriate control is crucial. Hence, when assessing a certain GMP, the control material should originate from the isogenic variety of the GMP and be treated exactly like the GMP material. This was usually the case in the above mentioned studies. The duration of the evaluated studies varied between 8 days and 7 months (Escher et al. 2000). Most studies had a duration of 4 to 8 weeks which can be considered as a reasonable time-frame for a laboratory assessment of GMP.

Practicability

A limiting factor for the use of ecologically relevant species for the assessment of GMPs will be difficulties in breeding and handling of a certain species due to its specific biology and ecological requirements. Species that will not easily reproduce in the laboratory on a reasonable time-scale will obviously not be a suitable subject for reproduction or life-cycle assays. In some cases it may be acceptable to collect animals from the field but this has the obvious disadvantage that availability of test animals might be limited and vary strongly between seasons and ecological regions. Also the quality (e.g., age, individual fitness) of the test animals will strongly vary in space and time, impacting the comparability and reproducibility of studies. Selection and quality criteria for field-collection of test species would have to be very well defined and strictly followed. In the studies listed in Table 2 some species were field-collected (*L. terrestris*, *A. caliginosa*, *P. scaber*) while others originated from laboratory cultures (*E. fetida*, *F. candida*, *P. armata*, *O. nitens*). Generally, any test system proposed for an ERA of GMPs should potentially be able to meet the requirements of ISO and OECD standardisation and quality standards of GLP to allow for a transparent, repeatable and justiciable evaluation of GMP.

Selection of species/functional groups

In the studies reviewed (Table 2) saprophagous groups are relatively well covered while predatory soil organisms have not been tested. The selection of species and functional groups strongly depends on the characteristics of the GMP and is closely linked to exposure routes. However, some soil invertebrate groups, such as earthworms, will almost always be involved in any environmental assessment of GMP due to their close association with and high relevance for agricultural habitats.

Being the most commonly used test organisms for ERA of chemicals, *E. fetida* and *F. candida* are an obvious first choice for conducting ecotoxicological tests with GMP. However, these are standard test organisms that were selected primarily because of their amenability to laboratory culturing and sensitivity to a wide range of chemicals (e.g., heavy metals) but do not usually occur in agricultural habitats

and, hence, are not necessarily ecologically relevant (Jänsch et al. 2005). Hence, more ecologically relevant species should definitely be included in the ERA of GMPs (see below).

L. terrestris is a deep-burrowing (anecic) species and ecologically highly important "ecosystem engineer" in central European agricultural soils. It feeds on soil surface plant litter. It has repeatedly been used in ecotoxicological assays but is difficult to handle due to its size and long life-cycle, and it cannot easily be cultured in the laboratory on a mass-scale. For this reason, it has not been included as standard test species in the laboratory assessments of pesticides in Europe but its occurrence and the effect on this species is an important parameter for the performance and evaluation of the standardised earthworm field trial (ISO 1999b). The same is true for *A. caliginosa*, a horizontal-burrowing inhabitant of the upper mineral soil (endogeic).

Terrestrial isopods (e.g., *Porcellio scaber*) belong to the soil macrofauna and live mainly close to the soil surface or even in the litter layer. While they usually have a minor role in central or northern European regions, their importance is clearly higher in the Mediterranean. Especially at sites with often dry soils they are important decomposers which, together with millipeds or ants (and termites in the tropics), can take over the role of earthworms more or less completely (Garcia 2004). Some species such as *P. scaber* can be kept and bred in the laboratory quite well. They have been increasingly used when investigating the importance of exposure pathways via food as well as in studies looking at bioaccumulation of chemicals.

The ecological relevance of other soil invertebrate species is less well investigated. For example, the influence of nematodes and oribatid mites on soil processes and functions is certainly important when considering their extremely high numbers in many soils (Petersen & Luxton 1982). However, it is very problematic if not impossible to identify individual species responsible for these activities because of their high taxonomic diversity (at one site easily more than hundred species and subspecies can occur). In addition, only parasitic (and, thus, economically relevant) nematode species have been investigated in laboratory or field tests studying the consequences of anthropogenic stress. In the case of oribatid mites, the situation is even worse: because of their disputed taxonomy very few data sets concerning their ecological relevance as well as their reaction to anthropogenic stress are available.

Recommendations

Based on the soil invertebrates test reviewed here, it is recommended to select test species from organism groups that are ecologically relevant for the respective receiving environments as well as testable. In addition, they should cover different exposure routes, as well as different taxonomic and physiological groups (see Chapter 3.4 for a more detailed discussion). Currently, earthworms, collembolans and isopods are the most likely candidates. The main challenge will be to increase the number of species from these groups beyond the "typical" ecotoxicological test species in order to account for differences of the receiving environments, behavioural types and exposure pathways (mainly via feeding) more adequately.

2.3 Detailed critical appraisal of the methodologies and species used for ecotoxicity testing for regulatory purposes of GM crop plants

We compiled the list of test species from two application dossiers made available to us by the German Federal Agency for Nature Conservation (BfN), that were subjected to testing for the regulatory approval process of GM crop plants in the EU. These case example plants were chosen to allow the BfN to benefit from this research & development project for the on-going evaluation of these GMPs. One dossier covered the pending approval for cultivation of the 1507 maize expressing the Bt-toxin Cry1F in the EU. Since 2005/06, this GM maize is already approved for food and feed in the EU but not for environmental release. Hence, an environmental risk assessment is now required. In the US, the 1507 maize was deregulated for commercial production in 2001. The second dossier covered the pending approval for import and use as food and feed (no cultivation) of a hybrid obtained through conventionally cross-breeding of two GM maize varieties, the NK603 resistant against glyphosate and MON810 expressing the Bt-toxin Cry1Ab. Consequently, no formal environmental risk assessment is required at this time. However, possible environmental consequences resulting from unintentional release during transport and processing should be considered. The hybrid NK603 x MON810 has not been registered anywhere yet. However, the individual single-trait parents are both registered in the US and the EU:

- MON810 since 1995/96 for all purposes in the US and since 1998 for all purposes in the EU
- NK603 since 2000 for all purposes in the US and 2004 for feed (marketing) only in the EU

Further, we conducted a data base research to compile the list of test species used in pre-release biosafety testing of GMPs contained in the application dossiers (AGBIOS, etc.).

For the two GMPs for which the submitted dossiers were available to us (Maize 1507 and NK603xMON810), a total of 10 species had been subjected to first tier testing for 1507 Maize and 8 species for MON810 (Table 3 and Table A.1 (Appendix A)). However, only data of a selection of these were actually included and provided in the submitted dossiers to the competent authorities in Europe/Germany (Table 3). Except for the broiler chicken studies, the tests listed in Table 3 had been conducted quite some time ago (some as early as 1992) using mostly surrogate proteins produced in transgenic *Escherichia coli* or *Pseudomonas fluorescence*. In some instances Bt-maize pollen was also used as test substance (e.g., honey bee tests). This data has been used for regulatory purposes in all countries of the world where permission for import or cultivation has been seeked. We further noted that the ecotoxicity tests for nontarget effects provided in the dossier NK603xMON810 all stemmed from the original MON810 application submitted during the early 1990ies to the US authorities. No new or additional tests were conducted for the new hybrid varities. Hence, no potential interaction effects or unexpected pleiotropic effects of the new hybrid were tested.

The NK603xMON810 dossier contained only the brief summaries of the ecotoxicity studies for 4 test species plus a broiler chicken study (Table 3). However, from our database search, we found that data for two more standard test species, a ladybird beetle (*Hippodamia convergens*) and *Brachymeria intermedia* (a parasitoid of the housefly), had been obtained and submitted in the original single application of MON810 (Table 3). All of these studies are company-internal documents and have not been published.

The dossier of the 1507 maize contained data on 6 test species, including the same as for the MON810 maize and 2 additional ones, the monarch butterfly and *Eisenia fetida*. Testing of the monarch butterfly has been added to the standard testing routine since the highly publicized report by Losey et al. (1999) had come out. Following that report, an extensive research program was launched in 2000

wherein a large amount of information was collected that allowed an in-depth risk analysis of this species. The dossier of 1507 maize also contained a broiler chicken study where the same comments apply than for the NK603xMON810 hybrid (see above).

Tab. 3: Overview of test species used for regulatory approval for 1507 maize and NK603 x MON810.

	Dossier	
Test organisms	**1507**	**(NK603 x) MON810**
Chrysoperla carnea (Green lacewing)	√	√ (1992)
Hippodamia convergens (Ladybird beetle)	√	√(1992)
Nasonia vitripennis (Parasite of housefly)	√	--
Brachymeria intermedia (Parasite of house fly)	--	√(1992)
Eisenia fetida (Compost worm)	√	√* n.s.
Apis mellifera (Honey bees)	√	√(1994)
Folsomia candida (Springtails)	√* n.s.	√* n.s.
Daphnia magna (Water flea)	√* n.s.	√* n.s.
Colinus virginifera (Northern Bobwhite Quail)	√* n.s.	√* n.s.
Danaus plexipus (Monarch butterfly)	√	n.s.[1]
*Oncorhynchus mykiss (*Rainbow trout)	√* n.s.	n.s.[2]
Broiler Chicken	√	√

n.s. = test conducted but data not submitted in dossier
√ = test conducted and data submitted in dossier
√*n.s. = test conducted but no data submitted in dossier – data found elsewhere (AGBIOS website, BRAD documents)
-- = no test performed

In the following, we provide an in-depth evaluation of the applied testing methodologies for the 5 arthropod test species used in both applications NK603xMON810 and 1507 maize (*C. carnea*, *H. convergens*, *A. mellifera*, *F. candida*, and *D. magna*) and the annelid species *E. fetida* also used in both applications (Table 3). Details on the applied methodologies were compiled in Table A.1 (Appendix A) and the relevant ecotoxicity pesticide testing strategy indicated (also compare Chapter 2.2 and Appendix C). Where appropriate, we provide recommendations for improvement based on recent scientific findings.

[1] MON810 maize pollen and other Bt pollen and proteins were studied for their effects on monarch butterfly during the early 2000s as a reaction to the publication of the paper by Losey et al. (1999). For example, see Hellmich et al. (2001),
[2] no information found elsewhere

1. Test species

Species name: *Daphnia magna* (Water fleas)

Test description: 48-hour static renewal toxicity of pollen from modified maize to water fleas (*Daphnia magna*). Derived from OECD Guideline No. 202.

Results: FONSI[3]

Scientific evaluation of the applied methodology:

Since *Daphnia magna* is extremely sensitive to metal ions like copper and zinc, pesticides, detergents, bleaches, and other dissolved toxins, it is a widely used species in ecotoxicological tests to assess and indicate water quality. The toxicity test with *D. magna* is designed for substances soluble in water. However, pollen and the contained Bt-protein do not or only insufficiently dissolve in water. In order to exert any activity, Bt-proteins must be ingested via pollen uptake. Daphnia's natural food source are various groups of bacteria, yeast, microalgae, detritus, and dissolved organic matter. All these food groups are of the size of 1 to 5 µm in diameter. Corn pollen on the other hand has a size of around 70 µm in diameter. For daphnids of the family *Cladocera* it is known that by filtering their food, bigger or nutritional inadequate particles are excreted unprocessed via abdomen. Therefore, it firstly should be experimentally established that *D. magna* can actually ingest insufficiently dissolved pollen or pollen fragments and hence any Bt-toxin with it, before conclusions based on this testing procedure can be drawn. Such key tests are missing to our knowledge to date.

Further, an exposure time of 48 hours is designed to test for acute toxicity at best. But even in susceptible target pest insects, mortality due to the ingestion of Bt-toxin is reliably measurable only after 48 hours. However, the NOEC value is given, which is a value typically provided for chronic test. Therefore, this test is unsuitable for regulatory safety testing of transgenic Bt-maize.

Recommendation: According to the OECD guidelines for testing of chemicals, a chronic toxicity study on *D. magna* exists that requires an exposure time of at least 14 days (Guideline no. 202, Reproduction Test). This would be the minimum requirement to be fullfilled for a scientifically acceptable test for GMPs.

Further, a plausible exposure route and hazard scenario should be provided as in the case of corn pollen, toxicity tests with *D. magna* seem to be of minor ecological relevance. Only daphnids in water bodies very close to corn fields might be exposed.

2. Test species

Species name: *Eisenia fetida* (earthworm, compost worm)

Test description: Single dose test evaluating toxicity to earthworms (*Eisenia fetida*) using Cry1Ab enriched maize leaf protein. OECD guideline no. 207

Results: FONSI

Scientific evaluation of the applied methodology:

Eisenia fetida is a standard test organism for ecotoxicity testing of industrial pollutants and synthetic pesticides. *E. fetida* prefers habitats that contain high amounts of decomposing organic matter. Hence, they are widely commercially sold as compost worms to enhance compost decomposition. Because of the relatively low content in organic matter, *E. fetida* is not able to survive for a long time in most field soils. Therefore, *E. fetida* is of minor ecological relevance in corn fields. Aside of the fact that it is a marginally relevant species for most agricultural settings, it needs to be demonstrated first whether *E. fetida* ingests any Bt-protein in this test. *E. fetida*, a typical epedaphic species, does not feed through soil but ingests concentrated organic debris. By mixing corn leaf protein powder into a test soil sub-

[3] Finding of no signficiant impact

strate (consisting of peat, clay and industrial sand, OECD guideline no. 207), the study tests reliably for contact toxicity but it needs to be proven that it is an adequate system to test for adverse effects due to ingestion of corn plant residues as it occurs in the field.

Recommendations: If possible, those earthworm species that actually occur in the receiving environment should be tested. Further, appropriate feeding habits and exposure routes must be accounted for in an ecologically meaningful way. Zwahlen et al. (2003b) tested and proposed such improved testing methodologies. Improvements included a) longer exposure times (200 days) during juvenile development of earthworms, because typically these are the most affected lifestages by Bt-toxins, b) using realistic exposure routes (plant material in soil) and c) measuring sublethal effects in addition to lethal effects since the Bt-toxins persist for fairly long periods of time in soil. From Zwahlen et al. (2003b) it can be concluded that exposure times should exceed 200 days. Considering the longterm exposure and long life span of earthworms, at minimum, chronic toxicity test should be conducted modified for GMPs from OECD guideline no. 222 and ISO Guideline No. 11267 (Table A.1 (Appendix A)).

3. Test species

Species name: *Apis mellifera* L. (honeybee)

Test description: Effect of Bt maize pollen on larval honeybee (*Apis mellifera* L.) development.

Results: FONSI

Scientific evaluation of the applied methodology:

The test is designed as an acute oral toxicity test analogous to toxicity testing of synthetic pesticides to larval honey bees. Honeybee larvae receive a single serving of 20 mg of undigested pollen either from Bt-maize or isogenic maize plus 1 droplet of a sucrose solution to "liquify the pollen and flow down to the mouth of the larva". Following the administration of the pollen/sucrose mix, frames were kept for 30 minutes under a moist towel. "After at least 30 minutes under the moistened towel, during which time the larvae were expected to consume the treatment material, the frames were returned to their original hive." A one-time 30-minute uptake exposure period of undigested pollen seems short and of limited relevance considering the foraging and feeding behaviour of bees (repeated visits of the same pollen source by worker bees). Pollen has to be pre-digested by nurse bees in order to be digestible for larval honeybees (Wittmann 1982). In their hypopharyngeal glands, nurse bees produce the protein-rich jelly also called brood food, which is fed to the young larvae (Crailsheim 1992). The pollen contained in this jelly has been broken down in the nurse bees (Wittmann 1982). Larvae older than 3 days (3^{rd} to 4^{th} instars) receive brood food containing some unprocessed pollen (Haydak 1970) but the significance of this pollen is not known and it is not an essential constituent of the food of worker bee larvae (Haydak 1970). Therefore, it is questionable whether the unprocessed pollen is properly digested and the Bt-toxin released in the larval gut as expected.

Interestingly, even in the arsenate treatment, a known acute toxin for honey bees and used in the test as positive control, only in one replication (of a total of 3), mortality was indeed noteworthy. In the other 2 replicates survival averaged 88% (in 1 replicate all larvae survived!) and was only little lower than in the Bt-toxin treatment without pollen (92%). This seems to confirm that the methodology of undigested pollen and added test substance may indeed not lead to the uptake as expected. All other treatments, Bt-maize pollen, isogenic pollen and control food allowed 99-100% survival.

Recommendations: The development of improved protocols for testing of honey bees are underway. An improved testing methodology would allow nurse bees to pre-digest collected Bt-pollen and/or nectar and administer it to honeybee larvae in a realistic way. Such more realistic feeding methodologies and exposure routes were extensively investigated in a research program funded by the German 'Bundesministerium für Bildung und Forschung'. A group of researchers under the leadership of Prof.

Hans-Heinrich Kaatz at the Martin-Luther-Universität in Halle investigated and developed various strategies for longterm chronic testing and field testing of exposure to Bt-maize pollen of honeybees for 3-4 years. These research projects yielded important data and valuable improvements for proper testing of honeybees.
No significant adverse effects of Bt-maize were observed on the tested honeybees, except for one instance where an unexpected high infestation of microsporidia adversely affected all honeybees. This effect was more distinct for the Bt-maize fed honeybees than for the isogenic maize pollen fed honeybees. The scientists speculated that this might indicate an interaction of the pathogen and the Bt-toxin. Obviously, this should be further investigated. A summary of the findings can be found at http://www.biosicherheit.de/de/sicherheitsforschung/68.do-ku.html.

4. Test species
Species name: *Folsomia candida* (springtails)
Test description: 28-day survival and reproduction study in collembola (*Folsomia candida*) using Cry1Ab-enriched maize leaf protein.
Results: FONSI - only mentioned but no details on methodologies and results provided in the dossier
Scientific evaluation of the applied methodology:
Soil is amended with a mixture of microbially produced Bt-toxin and brewer's yeast to satisfy this species needs for fungal food. Usually, this kind of test is used to assess chronic toxic effects of pesticides. However, the proper food for *F. candida* is saprophytic fungi growing on and living from decaying plant matter. Consequently, the laboratory experiment is a poor simulation of a realistic exposure route as it would occur in the receiving environments. Bt-toxins enter the soil ecosystem via various routes during the growing season. Post-harvest residues like corn stalks and roots slowly release the Bt-toxin in the soil for many months (Zwahlen et al. 2003a). Bt-toxins can be detected in soil organisms in maize fields up to two years after the last Bt-crop has been grown in a field (Zwahlen & Andow 2005). Sims & Holden (1996) showed that Bt-proteins in post-harvest plant material from transgenic Bt-corn were active for 2 to 120 days. In order to investigate the toxic potential of Bt-corn on *F. candida*, first tests should be carried out verifying whether the saprophytic fungi on transgenic plant material contains Bt-toxin and whether it passes the toxin to *F. candida*.
Recommendations: Testing of *Folsomia candida* should involve a tri-trophic rather than a bi-trophic exposure route or at least involve GMP material in addition to the yeast-Bt-toxin mixture. Certainly, uptake of Bt-toxin through either exposure must be verified and quantified before this test can be considered reliable.

5. Test species
Species name: *Hippodamia convergens* (ladybird beetle)
Test description: Single dose test evaluating toxicity to adult beetles (*Hippodamia convergens*) using microbial Bt-toxin mixed in honey. Procedures outlined in Series 885 of the US Environmental Protection Agency's microbial pesticide registration guidelines, OPPTS Number 885.4340 (1).
Results: FONSI
Scientific evaluation of the applied methodology:
Microbially produced Bt-toxin was added to a honey solution at a given concentration. This solution was then fed to adult ladybird beetles. Main problem with this experiments is that adults were used for testing. Bt-toxins are widely known for their larvicidal activity but have very little if any effect at all

on adult stages of insects, including the most susceptible target insects. Thus, here simply the wrong life stage is being used.
Recommendation: Larval stages should be used for testing. Tri-trophic testing in addition to bi-trophic ecotoxicity test should be conducted.

6. Test species
Species name: *Chrysoperla carnea* (Green Lacewings)
Test description: Single dose test evaluating toxicity to lacewing larvae (*Chrysoperla carnea*) using microbially produced Bt-toxin coated meal moth eggs. Carried out for 1507 maize (Cry1F-toxin) and MON810 (Cry1Ab-toxin). Procedures outlined in Series 885 of the US Environmental Protection Agency's microbial pesticide registration guidelines, OPPTS Number 885.4340 (1)
Scientific evaluation of the applied methodology:
Green lacewing larvae are piercing sucking predatory larvae. They pierce with their forceps-like mouthparts through skins and shells of prey or eggs, inject enzymes that pre-digest and liquify the prey content and ingest the content by sucking out the prey bodies leaving their skins and shells as intact remnants. With the methodology of this test, the Bt-toxin solution is applied externally onto the surface of the meal moth eggs. Thus, it is highly questionable whether the lacewing larvae are ingesting any of the externally applied toxin when only ingesting the internal content of the eggs. Experimental proof of uptake and, consequently, exposure of the larvae to the tested substance should be demonstrated before this methodology can be accepted as a valid testing procedure for transgenic plants containing the Bt-toxin in their tissue and reaching a predatory insect via prey that has ingested the plant material. Until then there is reasonable concern that this testing methodology is inappropriate.
No statistical analysis was performed. In the Bt-treatment mortality was 6% higher (9 died, 30%) than in the control (7 died, 24%). Control mortality was very high with 24% during 13 days. Hilbeck et al. (1998) conducted a similar study with green lacewing larvae also including a control treatment consisting of a meal moth egg diet that resulted in less than 10% total mortality for the entire immature life stage lasting about 3 weeks. After the comparable time period of 14 days, less than 8%, roughly a third of that in this regulatory trial, had died. This high control mortality further indicates suboptimal rearing conditions that can mask a potential treatment effect. In addition to the inappropriate bioassay methodology, the trials were not repeated and no statistical analysis could be carried out to determine whether the observed higher mortality in the treatment group was real or not. Hence, no conclusions in either direction can be drawn from such an experiment. Therefore, the test should be dismissed.
Recommendations: The case of the most appropriate lacewing testing system will be discussed in depth in Chapter 2.5 as an example because it has received much more scientific attention than any other nontarget organism aside of the monarch butterfly (Lövei & Arpaia 2005).

Conclusions

All of the above reviewed ecotoxicity tests of GMOs were conducted with a pre-defined standard set of 6 a total of 11 test species used for regulatory purposes of chemicals, in particular pesticides. In addition, both dossiers also contained results of a feeding study with broiler chicken typically used as testing for food and feed safety but often also considered for ecotoxicological purposes. The experimental designs were all minimal, with small sample sizes, few if any replications and short duration only allowing to detect strong, acute toxic effects – as for pesticides. Consequently, statistical analyses were rudimentary if not missing altogether. For a detailed critique of the statistical analyses see also

Marvier (2002). Further tests with the Monarch butterfly are irrelevant for Europe because this species does not occur there.

For the hybrid NK603xMON810 data from ecotoxicity studies of only 5 test species were provided in the current dossier before the German authorities (Table 3). Four of them, involving the typical standard insect species (i.e., honey bees, lacewings, ladybirds, housefly parasite), were well over 10 years old, dating as far back as 1992. These tests had been conducted for the registration of the single parent MON810 in 1996. Since the current testing paradigm is based solely on the testing of the isolated surrogate protein produced by transgenic microbes such as *Escherichia coli*, no additional or new studies were conducted using actually the NK603xMON810 hybrid plant material. It is unclear why the other ecotoxicity tests on 4 more test species including *E. fetida*, *D. magna*, *F. candida* and the Northern Bobwhite quail were not submitted. The data of these tests are meanwhile widely available and posted on the AGBIOS website as 'MON810 Environmental Risk Assessment Case Study'[4] (Table 3) as well as in the BRAD documents posted by the Environmental Protection Agency of the USA (www.epa.gov/oppbppd1/biopesticides/ingredients).

Only the broiler chicken study was indeed conducted using the kernels from the new hybrid and published in the open, peer-reviewed scientific literature (Taylor et al. 2003). However, such tests are primarily submitted for documenting human and animal health impact although they can deliver evidence for related wildlife animals such as farmland birds.

Further, while for the NK603xMON810 maize only approval for food and feed purposes is requested, a good quality application dossier should base its conclusions on the totality of data produced in-house and published in the peer-reviewed literature and should consider environmental impacts resulting from unintentional releases during transport and processing. For the MON810 part of the hybrid, a summary of the in-house (Monsanto-) produced ecotoxicity trials from 1994 (2 years before the first Bt-plants were commercialised) were delivered but none for the herbicide-resistant NK603 part of the hybrid. Herbicide resistance is packaged with the application of the corresponding broad spectrum herbicide, in this case glyphosate. Recently, scientists reported significant adverse effects of glyphosate on amphibians (Relyea 2005; Relyea et al. 2005).

At best, all studies qualify as initial first tier toxicity testing and have limited if any relevance for environmental safety/risk assessments. Basing the assessment of environmental impacts solely on such limited rudimentary tests is in disagreement with several provisions put forward by the relevant regulations. Firstly, no longterm direct effects, no short or longterm indirect effects, and no delayed effects are tested (Annex II, EU Directive 2001/18). Secondly, since hardly any GMP material was used for testing, also no conclusions regarding unexpected and pleiotropic effects resulting from the genetic modification or the interaction of the transgene product with other plant-produced primary or secondary metabolites were tested as is required by EU Directive 2001/18/EC. This means that the risk assessment is neither based on 'sound science' (as requested by the Cartagena Protocol) nor reliable for that matter. This calls for serious and quick improvements!

2.4 Review and summary of published studies on nontarget effects of Bt-plants

In Table B.1 (Appendix B) a total of 39 laboratory studies are listed chronologically that were published on nontarget effects of Bt crop plants on arthropods in international peer-reviewed scientific journals (see Table 4 for a summary). While we do not claim that this list is comprehensive, we are

[4] http://www.agbios.com/cstudies.php?book=ESA&ev=MON810

convinced that it covers most papers. However, we are aware that a number of articles were published outside of this kind of international, peer-reviewed literature that is difficult for us to access, read and evaluate for language reasons (e.g., studies from China).

Altogether a total of 23 herbivore species from 5 insect orders and one spidermite were tested (Table 4). Further, 20 natural enemy species from 4 insect orders and 2 predatory mites were tested. Of these 20 natural enemy species 7 were hymenopteran parasitoids. Additionally, 1 macro soil organism (*Lumbricus terrestris*), 3 detrivorous or fungivorous species (*Folsomia candida, Porcellio scaber, Blattella germanica*), some undetermined nematodes and protozoa were investigated. The vast majority of these organisms were tested only in one study (Table 4). Only 3 herbivore species (*Apis mellifera* (4 studies), monarch butterfly (4 studies), *Rhopalosiphum padi* (3 studies)) were tested in more than two different studies. For the natural enemies, these were the predators *Chrysoperla carnea* (10 studies) and *Coleomegilla maculata* (4 studies).

Tab. 4: Overview of tested species in international, peer-reviewed 'open' literature (also see Appendix B1).

	Number of studies conducted on different trophic levels (feeding type)		
Species name	**1st – herbivores**	**2nd – predators, parasitoids**	**Other (fungivore, saproph.)**
Lepidoptera			
Danaus plexippus	4		
Papilio polyxenes	1		
Spodoptera littoralis	2		
Manduca sexta	1		
Autographa gamma	1		
Pieris brassicae	1		
Pieris rapae	1		
Plutella xylostella	1		
Galleria mellonella	1		
Acherontia atropos	1		
Coleoptera			
Coleomegilla maculata		4	
Hippodamia convergens		2	
Leptinotarsa decemlineata	2		
Anthonomus grandis	1		
Diabrotica undecimpunctata	1		
Hymenoptera			
Cotesia flavipes		1	
Cotesia plutellae		1	
Cotesia marginiventris		1	
Athalia rosae	1		
Apis mellifera	4		
Aphidius nigripes		1	
Parallorhogas pyralophagus		1	
Nasonia vitripennis		1	
Copidosoma floridanum		1	
Neuroptera			
Chrysoperla carnea		10	
Diptera			
Aedes aegypti			1 (blood)

Tab. 4: continued.

	Number of studies conducted on different trophic levels (feeding type)		
Species name	**1st – herbivores**	**2nd – predators, parasitoids**	**Other (fungivore, saproph.)**
Collembola			
Folsomia candida			1 (fungi/detrivore)
Homoptera			
Aphis fabae	1		
Macrosiphum avenae	1		
Macrosiphum euphorbiae	1		
Rhopalosiphum padi	3		
Nilaparvata lugens	1		
Myzus persica	1		
Heteroptera			
Orius insidiosus		2	
Orius tristicolor		2	
Orius majusculus		1	
Geocoris punctipes		2	
Geocoris pallens		1	
Lygus hesperus	1		
Nabis spp.		2	
Cyrtorhinus lividipennis		1	
Zelus renardii		1	
Blattodea			
Blattella germanica			1
Others:			
Acari			
Tetranychus urticae	2		
Phytoseiulus persimilis		1	
Oppia nitens		1	
Annelidae			
Lumbricus terrestris			1 (earthworm)
Nematodes			1 (unclear, various)
Isopoda			
Porcellio scaber			2 (detrivore)
Protozoa			1 (various, unclear)
TOTAL: 48 species, 2 organisms/groups from 9 insect orders 6 others	**23 species 5 insect orders 1 other**	**20 species 4 insect orders 1 other**	**7 organisms/groups 3 insect order 4 other**

The reasons for the selection of the different test species in the reviewed laboratory studies and the different number of studies conducted are arbitrary and not necessarily based on an ecological reasoning. Further, only very few species are relevant for subtropical or tropical agroecosystems (e.g., *Cyrtorhinus lividipennis* and *Parallorhogas pyralophagus*). The vast majority of tested species are relevant for northern, temperate production systems. But in any case, even for the most simplified monoculture, the number of tested species are few and the selection not based on transparent hazard identification procedures or risk hypotheses.

The majority of experiments tested GMP parts, many used GM-maize pollen. Few studies used the microbially produced surrogate Bt-protein (Table B.1, Appendix B). The vast majority of the studies measured the impact of transgene products on mortality of the test species. Other often measured parameters included development time and relative weight gain.

Overall, the published studies as of today are inconsistent, and no coherent and predictable understanding of the observed Bt-effects on nontarget arthropods is emerging yet. In about 13 to 15 studies,

the authors reported either both, negative and no effects or only no effects. In about half a dozen studies only negative effects on the tested non-target organisms were reported. Positive effects were almost non-existent (1 study).

Regarding the type and degree the observed effects were often unpredictable. Only the fact that Bt-maize pollen containing the lepidopteran-specific Cry1Ab toxin adversely affected the caterpillars of the Monarch butterfly and other Lepidoptera species was hardly surprising. However, the exposure route was the actual surprise that had been overlooked until the publication by Losey et al. (1999). Since then Monarch butterfly caterpillars became a standard test species for regulatory approval of Bt-plants in the USA (see above).

Most researchers expressed the opinion that an extrapolation of laboratory results to field situations is difficult and requires more research. Obviously, this is true for all results, no effects, positive effects and negative effects. On the other hand, the published data do not allow for the conclusion that no effects in the ecosystems will occur. In fact, a substantial number of studies provide experimental evidence that give rise to concerns that also in the field effects should be expected and investigated. However, we are currently not in a position to estimate the ecosystem consequences of such effects, neither positive nor negative. The scope of the effects will be a function of the scale of production of GM crops in space and time. It cannot be excluded that repeated, large scale production of some crops will affect some species, possibly significantly, in the long run. But the totality of the measured data on development time, weight and other sublethal parameters, suggest that many effects could be chronic and subtle and go unnoticed for many years.

History teaches us that chronic and subtle effects should not be underestimated and can cumulate to drastic consequences in the long-run (Harremoës et al. 2002). This re-enforces the importance of continued surveillance programs that would allow to detect subtle longterm consequences, e.g., shifts in species composition and abundances, in a timely fashion. For such monitoring programs (as they are required under the EU Directive 2001/18/EC), laboratory experiments can provide important information that would allow to make an educated decision on which species to include in the monitoring program and what to measure.

2.4.1 Stumble stones on the path to improved understanding

The various research programs of the past years yielded a number of studies delivering important data. The vast majority of them are isolated one-time studies (Table 4) following quite different methodologies. Further, the results of those studies that investigated a few organisms repeatedly (e.g., lacewing larvae, Chapter 2.5) did not lead to a scientific consensus regarding the kind of impact Bt-plants might exert and the possible mode of action at work in the observed nontarget effects. Quite in contrast, as for the *C. elegans* case described by Crickmore (2005), it complicated the situation further by rather differing lines of interpretation.

An exception of the above mentioned isolated studies with diverging results presents the case of the monarch butterfly. Here, a series of coordinated investigations in which many scientists participated led to a more solid increase in our understanding and consensus of the possible spatio-temporal exposure of monarch butterfly larvae to Bt-maize pollen. The likelihood of adverse effects on monarchs was further decreased with the company's withdrawal of the Bt-maize Event 176 in the US[5] that had the highest expression of Bt-toxin in the pollen. This high concentration caused significant mortality in

[5] http://www.oznet.ksu.edu/swao/Entomology/Bt_Folder/Bt%20Corns2.html

monarch butterfly larvae even at low pollen concentrations. However, in the EU Event 176 has not yet been withdrawn by the company. As a result of the monarch controversy in the US, this topic got also on the research agenda in Europe and lead to increased testing of other butterfly species who could be similarly at risk as the monarch butterfly (Table B.1, Appendix B). The monarch does not yet occur in Europe.

Despite the published data summarised above, great scientific dissens continues to exist regarding the understanding that can be derived from these data. The same data set leads scientists to arrive at opposing conclusions for a number of reasons. In part this results from the fact that the majority of the studies are non-coordinated isolated experiments with limited comparability. This will be evaluated in detail in the following subchapter.

2.4.2 Limited comparability of studies

Despite superficial similarities in terms of test species and stated testing objectives, the studies listed in Appendix B really are more 'apples and pears'. The analysis of the studies is further complicated by the fact that a detailed evaluation of some supposedly similar studies require a great deal of detailed expertise regarding the testing organisms and applied methodologies. This will be explained in more detail in Chapter 2.5 using one of the few cases where a particular nontarget species was investigated repeatedly, the green lacewings (see also Andow & Hilbeck 2004). The chosen methodologies often also reflect a differing understanding of the possible effects and sometimes an implicit hierarchy regarding the importance and interpretation of the obtained data. Some scientists for example place great importance on the capability to detect and identify the effect of the isolated transgene product only while they pay less attention to the prey-mediated effect confounding complex GMP-prey interactions. An illustrative example again is the case of the green lacewings (see Chapter 2.5).

Other examples involve lectin-producing GM crops that were not included in the tables of Appendix B that only dealt with Bt-plants. In these studies, natural enemies were only fed with GMP-fed prey of the same size and weight as were observed in the non-GM control. (e.g., Riddick & Barbosa 1998; Down et al. 2000; Couty et al. 2001a; Couty et al. 2001b). This selection of same-sized prey was based on the assumption that these prey individuals were apparently of comparable nutritional quality and, thus, represented the proper treatment. For example, Down et al. (2000) could show that no effects occurred in the tested natural enemy if they fed them selected same-sized aphids raised on GNA[6]-plants when compared with aphids raised on non-GNA plants. Others had shown earlier that effects did occur when raised on mixed size prey fed with GNA-plants (Birch et al. 1999). For parasitoids, the argument for using same-size prey might be relevant as it was shown that the size of the host aphid can influence the parasitation rate (host finding and acceptance for oviposition) and fitness of the emerging parasitoid (Stadler & Mackauer 1996). Parasitoids emerging from smaller hosts are often less fit than those emerging from larger hosts.

For predaceous insects, however, feeding of same-sized prey is less important and would only matter if the number of prey is limited and thus smaller prey consistitute shortage of prey. Otherwise predators simply compensate feeding on smaller prey by feeding on more prey. In laboratory trials this can easily be adjusted by offering prey *ad libitum*. However, offering selected prey only means to cut back on ecological realism when offering only a selected part of the entire prey spectrum a natural enemy would encounter in a field situation. Conclusions for a field situation are limited from such studies because only a well-fed fraction of a natural prey/host population is offered that for whatever reason

[6] GNA=Galanthus nivalis Agglutinin

did not ingest much of the transgene product or is naturally already more resistant towards the novel transgene product. In the field, natural enemies encounter prey/hosts that is/are unpredictably more or less affected and of differing sizes from various age classes and life stages. They all can contain highly variable concentrations of the transgene product (Harwood et al. 2005). Selective feeding behaviour in the context of Bt-plants is little investigated (Meier & Hilbeck 2001) and will likely differ due to annual, seasonal and regional variability of prey spectra and – prey densities. In the field, most insects and animals for that matter are forced to adapt their consumption to the given availability of prey.

Other scientists thought it to be more important to study the totality of interactions a natural enemy can face in the field including the complex interactions of novel toxins with other primary or secondary metabolites in plants as well as in the prey they ingest. Ecological realism was the guiding principle for designing the experiments rather than isolated effects that hardly ever occur as such in the field (Baur & Boethel 2003; Hilbeck et al. 1998a; Hilbeck et al. 1999; Hilbeck 2001; Hilbeck 2002). For polyphagous predators such as the green lacewings what will happen in the field will depend upon the given prey spectrum, which varies not only between seasons but quite strongly between years as well.

While experiments with isolated, microbially produced transgene products deliver important initial toxicological data, they do not allow for conclusions on ecological effects in the field. Multitrophic experiments using whole plants and mixed prey are necessary to obtain data on ecological impacts. To differentiate the isolated effect of the plant-produced transgene product only in such ecologically more realistic trials with higher trophic level organisms is rather impossible due to the prohibitively high number of possible interaction effects (Andow & Hilbeck 2004). There are simply too many exposure and effect pathways that are all confounded in a tri- or multitrophic experiment. Andow & Hilbeck (2004) estimated that more than 250 possible exposure pathways exist by which a third or higher trophic level organism can come in contact with the transgene product or any of its metabolites. Only very few involve the exposure to only the original plant-produced transgene product. For an environmental risk assessment it is critical to understand which organisms are important for key functions in a given receiving environment that should not be adversely affected by the totality of all combined effects they could realistically encounter in that receiving environment. Such organisms and functions need to be tested in a as realistic fashion as possible.

2.5 Method matters – A case example of Green Lacewing studies

Five studies published on the effects of Bt-toxins are often portrayed as supposedly contradictory while in reality the differences in the results can clearly be explained through the differences in the methodologies used and the underlying questions asked. In 2 studies direct (bi-trophic) effects of microbially produced Bt-toxins were tested (Hilbeck et al. 1998b; Romeis et al. 2004) and in 3 other studies the effects of prey-mediated (tri-trophic) exposure to Bt-toxins from Bt-maize (Hilbeck et al. 1998a; Dutton et al. 2002) or microbially produced Bt-toxins und -protoxins (Hilbeck et al. 1999) were tested (Figure 2 and Table D-1, Appendix D). This case was analysed in detail in a recent publication by Hilbeck & Schmidt (2006). Here, we will present an overview of the main studies.

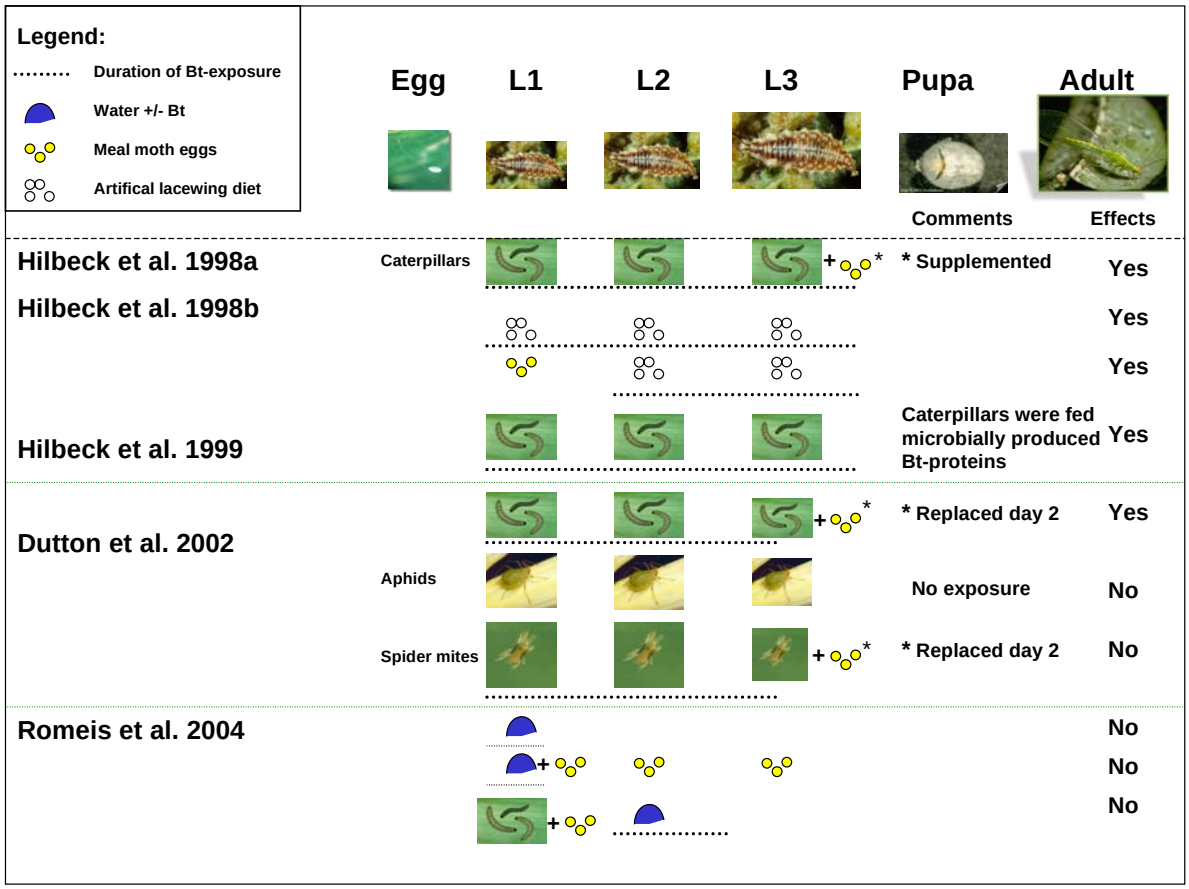

Fig. 2: Overview of the 5 studies on ecotoxicity testing of Bt-toxins and *C. carnea* larvae.

2.5.1 Bi-trophic effects

Despite the different types of artificial diets chosen and parameters measured (Figure 2), some components of the two studies by Hilbeck et al. 1998b and Romeis et al. 2004 are comparable and yielded indeed similar results. Hilbeck et al. (1998b) detected a significant direct lethal effect of Bt-toxins that began to manifest itself during the second larval stage but not during the first larval stage. Also, Romeis et al. (2004) could not observe adverse effects due to exposure to Bt-toxin during the first larval stage alone (Table 5: artificial diet 2.1, 5.2 and 5.3; Figure 2).

Tab. 5: Comparison of the individual similar components of the 5 studies on nontarget effects of Bt-toxins and Bt-fed prey on Green Lacewing larvae.

Study no.		**1.1**	**4.1**	**1.1**	**4.1**	**5.4**	**2.3**	**5.4**	**2.1**	**5.2**	**2.1**	**5.2**
Instar	**Parameter**	**Bt-prey (*S. littoralis*)**		**Control (*S. littoralis*)**		**?**	**Mealmoth eggs**		**Bt-artificial diet**		**Control artificial diet**	
L1	mortality	24%	50%	10%	10%	27.8%	1-2%	1%	6%	---	6%	---
	development time (days)	5	5	4.5	3	5.7	4.5	3.7	7	---	7	---
	days until death									9.5		9.5
L2	mortality	40%	60%	21%	35%	---	---	---				
	development time (days)	6.5	8	6.5	6	---	---	---				
L1 – A	mortality	60%	80%	37%	40%	---	---	---				
	development time (days)	31	24	31	21	---	---	---				

1.1: Hilbeck et al. (1998a)
2.1 + 2.3: Hilbeck et al. (1998b)
4.1: Dutton et al. (2002)
5.2 + 5.4: Romeis et al. (2004)

L1 = first larval instar
L2 = second larval instar
L3 = third larval instar
A = adult lacewing

Hilbeck et al. (1998b) used an artificial diet that was specifically developed for commercial mass production of lacewing larvae and allows continuous and complete development of the larvae from egg hatch to adult eclosion. This artificial diet was amended with Bt-toxin (100µg/ml) and fed to the lacewing larvae during their entire juvenile feeding stage until pupation. They measured stage-specific mortality and development time. From the second instar on, lacewings exhibited a significantly higher mortality in the Bt-treatment than in the control (2.1 in Table 4) and additionally a significantly longer stage-specific development time.

Romeis et al. (2004) used sucrose solution as artificial diet in their trials. This diet does not allow continuous and complete development of the predaceous lacewing larvae. Development of the larvae is arrested with sucrose diet but it allows them to survive periods of lack of prey better than when sustaining themselves on water only (Limburg & Rosenheim 2001). Lacewing larvae remained in the same larval stage for up to 6 days longer than when being provided with water only. Parameter measured was the time it took the insects to die. Romeis et al. (2004) added Bt-toxins to the sucrose solution to see whether this caused faster death or not. All test insects starved to death at the same speed regardless whether Bt-toxin was added to the sucrose solution or not (5.2 in Table 4)). Also the exposure to Bt-sucrose solution during only a part of the first instar – 6 out of 11 days – did not result in a difference when provided untreated mealmoth eggs. This quite optimal food did allow for recovery of the larvae without sustained consequences.

2.5.2 Tri-trophic effects

Both, Hilbeck et al. (1998a) and Dutton et al. (2002) fed lacewing larvae with caterpillars that either had fed on Bt-maize or isogenic maize (Figure 2). Hilbeck et al. 1998a continued to feed lacewings with this prey until pupation (3 larval stages) while Dutton et al. (2002) did this only until first half of the third instar. During the last half of this most intensive feeding stage only untreated mealmoth eggs were provided while Hilbeck et al. (1998a) supplemented meal moth eggs in addition to caterpillar prey (Figure 2). Despite these differences, in both studies significantly more lacewing larvae died when they were raised with prey caterpillars that contained Bt-toxin in both studies. Dutton et al. (2002) further conducted similar feeding studies with other types of prey, aphids and spider mites

(Figure 2). For both of these prey types, lacewing larvae developed at similar rates and mortality was also similar regardless whether their prey had fed on Bt- or isogenic maize (Table D-1, Appendix D). For aphids this can be explained because as strict phloem-feeders they did not contain Bt-toxin. Raps et al. (2001) and Head et al. (2001) did not detect any Bt-toxin in the phloem of Bt-maize or aphids feeding on it. In contrast, spider mites did ingest the Bt-toxin from the Bt-maize but this did not induce higher mortality in the lacewing larvae. However, no data on the biochemical processing of the Bt-toxin in the spider mites such as degradation to smaller fragments and their bioactivity were conducted that could explain the lack of an effect.

Hilbeck et al. (1999) conducted further experiments where they fed lacewing larvae with prey caterpillar that had fed on artificial diet containing different concentrations of microbially produced Bt-toxins (Figure 2). Again, signficantly higher mortality rates in lacewings were observed that increased as the concentration of the Bt-toxin in the diet for their prey increased (Table D-1, Appendix D). While the prey caterpillars only showed signficantly higher mortality of 42% at the highest Bt-toxin concentration, lacewing larvae exhibited a lethal effect at all concentrations reaching over 70% when their prey had fed on the highest concentration diet. At the lower concentrations caterpillars only exhibited sublethal effects, such as reduced weight when feeding on the diet for several days. As designated as food, caterpillars were only allowed to feed for 12-24 hours on the Bt-diet.

2.5.3 Different interpretations of results

From their results on direct and prey-mediated Bt feeding trials, Romeis et al. (2004) and Dutton et al. (2002) concluded that the observed mortality is exclusively due to the sublethal effects on the prey. They argue that these effects reduced the prey's nutritional value to the point that it induced the observed high mortality in the predators and that Bt-toxin had no role in it.

In contrast, Hilbeck et al. (1998a,b; 1999) interprete the totality of data as further convincing evidence for the complex interaction of the crop plant, the expressed Bt-toxin and the prey organism. The direct effects of the Bt-toxin feeding study clearly document the sensitivity of *C. carnea* larvae, certainly at higher concentrations (Hilbeck et al 1998b). The direct feeding trials by Romeis et al. (2004) complement the findings by Hilbeck et al. (1998b) in as much as they document that short term or intermittent exposure to Bt containing diet did not lead to measurable adverse effects in lacewing larvae, in particular, when followed by optimal diet. The studies by Romeis et al. (2004) further document that Bt-toxins did not accelerate the starvation times and no lag effects remain when the insects are fed again with optimal diet. In fact, the totality of data on lacewings and Bt reconfirm earlier conclusions (Hilbeck 2001; Hilbeck 2002; Andow & Hilbeck 2004) that complex interactions are at work possibly involving other modes of action and altered biochemistry of Bt-toxins when firstly expressed in a plant and secondly passing through the gut of a herbivore prey organisms. Possibly, all of the following effects could be involved:

- possibly altered nutritional prey quality,
- toxicity of the Bt-toxin or its metabolites,
- toxicity of natural plant secondary metabolites interacting with the Bt-toxin/metabolites.

It is impossible to keep these processes apart experimentally as too many possible interactions can be involved (Andow & Hilbeck 2004). The control mortalities of all three studies by Hilbeck et al. (1998a,b und 1999) confirm this. Highest control mortality (37%) was observed in tritrophic studies using Bt-maize plants and feeding them to the caterpillar prey that in turn was used to raise the green

lacwing larvae. Dutton et al. (2002) reported an even higher control mortality of 40% using a similar set-up (Table D-1, Appendix D). The lowest control mortality was observed in tritrophic studies where caterpillar prey that had fed on optimized artificial diet was fed to lacewing larvae (26%). Green lacewing larvae that were raised with artificial food only died at a rate of 30%. This indicates that the maize plant by itself is a suboptimal diet when fed via prey than any optimized artificial diet either fed via prey or directly to lacewing larvae. Reasons likely include the absence of secondary plant compounds such as DIMBOA in maize, which in additian can conceivably interact in one form or another with the novel transgene product, the Bt-toxin. Absence of effects when using different prey species in a tritrophic set-up with Bt-maize confirms this hypothesis as Bt-toxins can be processed and altered in different ways in different insects leading to differing biological activities (Haider et al. 1986) or the loss thereof. Most recently, Broderick et al. (2006) reported that the presence of certain midgut bactria is required for Bt toxins to unfold its activity in the investigated tartet insect. We argue that this might also help to explain some of the peculiar effects observed with nontarget organisms such as the green lacewing.

3 Proposal for an improved environmental risk assessment concept for GMPs concerning the prerelease ecotoxicity testing of nontarget organisms

3.1 Introduction

The proposed concept is focused on the market release of GMPs in Europe and is based on the requirements put forward by the EU and other international legislation and regulation. The two most relevant legally binding documents in this context are:

Directive 2001/18/EC on delibarate release into the environment of genetically modified organisms (EU 2001): The EU Directive foresees a case-by-case and step-by-step risk assessment and exceeds in this respect the chemical testing model. The concept of testing a standard set of organisms based on their indicator value for a wide range of pollutants in model environmental compartments (e.g., aquatic, terrestrial, etc.) is replaced by the provision to focus on organisms/functions with ecological relevance for the receiving environment.

Cartagena Protocol on Biosafety: The Cartagena Protocol on Biosafety under the Convention on Biodiversity (CBD 2000) identifies the need for comprehensive, transparent, and scientific methodologies for pre-release testing of transgenic plants to ensure that they do not adversely affect biodiversity and the sustainable use of biodiversity. The Cartagena Protocol on Biosafety also requires a case-by-case risk assessment. Similar to the Directive 2001/18/EC, the Cartagena Protocol requires an assessment with a focus on biodiversity relevant to the receiving environment (CBD 2000, see Annex III).

In contrast to the protection goals and guidance given, both regulations do not detail what data need to be submitted or which methods should be applied in the ERA. The European Food Safety Athority (EFSA) published a guidance document for the risk assessment of GMP and derived food and feed (EFSA 2004). However, with regard to the risk assessment for nontraget organisms this guidance document hardly specify the rather general requirements of the Directive 2001/18/EC. This project aims to fill this gap and proposes a risk assessment concept that maintains an ecotoxicology focus but devises a methodology that allows to tailor the risk assessment to the respective receiving environment and increases ecological realism.

In the following, we will detail this new methodology embedded in the **risk assessment framework** as outlined in Directive 2001/18/EC and subsequently define criteria for the **selection of species and methods**. For the latter it is important to consider other standards for ecotoxicological test methods, in particular those by OECD (e.g., OECD 2005). Because many of these methods refer to experiences from the assessment of pesticides, they are discussed together with the specific requirements of GMP testing. However, as mentioned earlier, the environmental risk assessment of pesticides and GMPs differ from each other: While for pesticides it has to be shown – starting with worst-case laboratory tests and only moving to higher tiers if there is concern for risk – that a "safe use" is possible. In the case of GMPs, the burden of proof is different. For GMPs it has to be demonstrated at each level (laboratory, semi-field and field) that no risk for the environment is apparent. This difference in the legal documents accounts for the fact that GMPs can reproduce and propagate while pesticides will be degraded and disappear from the environment in the long run.

In order to use the available resources most efficiently, the discussion in the following focuses on the terrestrial compartment (both above and below ground), since this is the one most likely to be affected by GMPs. At this point, it should be noted that ecotoxicological testing of GMPs is only one part of

the overall environmental risk assessment. Before a GMP can be approved other risk aspects such as consequences of gene flow, dispersion of the GMP in the environment, human and animal health risks, ect., have to be considered as well. Further, the risk of a GMP should also be weighed against potential alternative solutions.

3.2 Improved environmental risk assessment (ERA) framework

The general risk assessment frameworks used for any other anthropogenic factor causing stress in the environment and the ones presented in the Cartagena Protocol on Biosafety and the Directive 2001/18/EC do not differ much in their basic components.

The frameworks from the Cartagena Protocol on Biosafety and the Directive 2001/18/EC consist of the distinct components presented in Figure 3 where different activities are carried out that build on each other similar to the schemes used for the assessment of chemicals. In Figure 3, for each component a short description is provided following the wording of EU Directive 2001/18/EC. Additionally, our interpretation of the content of the individual component is provided (italic letters).

The environmental risk assessment *sensu strictu* is completed with the risk characterisation (component IV in Figure 3). The following components V and VI in Figure 3 address aspects of risk management and are not covered in this report. Risk management seeks measures to limit the risks of GMPs in the environment including not only ecological but also socio-economical and political criteria (cost/benefit).

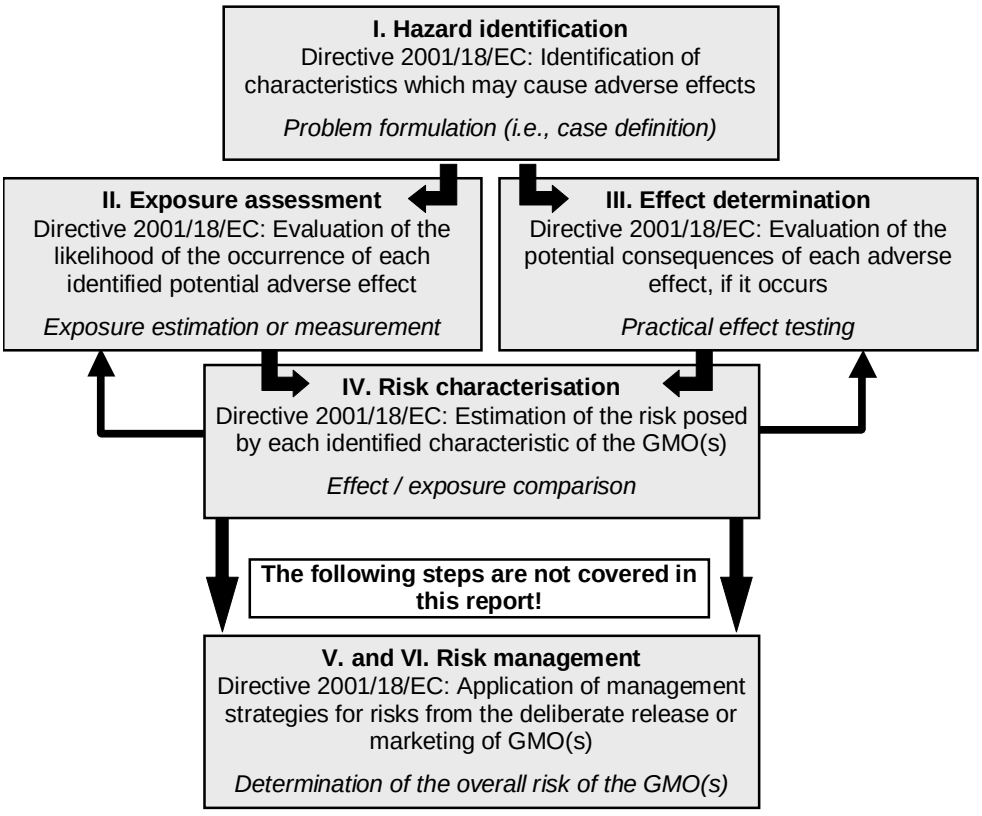

Fig. 3: General components in the risk assessment frameworks of the Cartagena Protocol on Biosafety and the Directive 2001/18/EC underlying the selection of test species and methods.

A detailed description of components I to IV of the ERA framework (Figure 3) is outlined in the following Chapter 3.2.1. In Chapter 3.2.2 a hierarchical (=tiered) scheme is presented how to carry out the ecotoxicological testing process and how to arrive at a final decision.

3.2.1 Detailed description of the improved risk assessment framework

Component I. – Hazard identification

While the risk assessment as outlined in EU Directive 2001/18/EC and, accordingly, in the Cartagena Protocol on Biosafety begins immediately with identifying properties that can have harmful effects, we propose to start the risk assessment process with a first component called hazard identification (Figure 4). The aim of this first component is to formalise and operationalise the identification of the properties that can lead to harmful effects through contextualisation and determination of the scope of the risk assessment. Component I corresponds to '...the identification of characteristics which may cause adverse effects' in the Directive 2001/18/EC.

It is in this component that all available information related to the GMP is compiled in order to define and characterise the respective cases for which the risk assessment is required (Figure 4).

Based on the provisions given by the Directive 2001/18/EC and similarly by the Cartagena Protocol on Biosafety, a case is described by the 3 following elements:

- the crop plant (its biology, ecology and agronomy),
- the novel trait relating to its intended effect and and phenotypic characteristics of the GM crop plant (the GMO)
- the receiving environment relating to the intended use of the GM crop plant.

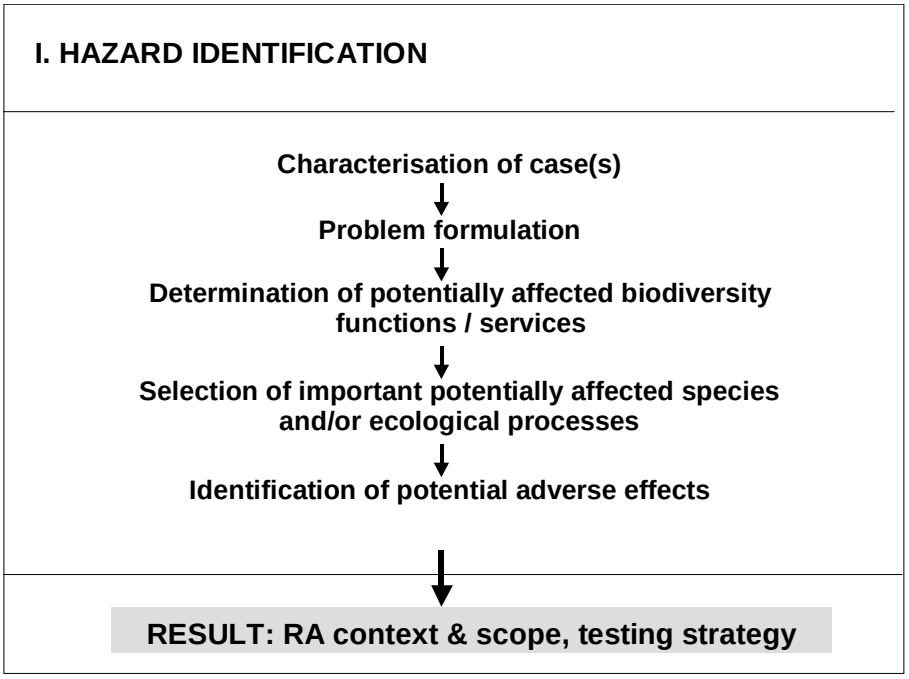

Fig. 4: Determining the scope and context of the risk assessment and laying out the testing strategy for regulatory purposes.

The result of Component I of the ERA framework is the identification of the most relevant biodiversity functions that are potentially adversely affected by the introduction of the GMP and the identification of its properties that can cause adverse effects. These properties differ between different GMPs. In the case where a GMP expresses bioactive protein(s) (such as Bt-proteins), adverse effects will primarly result from these proteins. For GMPs that are designed to be used with a corresponding chemical (e.g., glyphosate) or GMPs whose primary and/or secondary metabolism is significantly altered (e.g., shift in starch biosynthesis), adverse effects may primarly result from effects of respective chemicals (and their metabolites) or from possible consequences due to the altered metabolism respectively.

Having identified the properties which may cause adverse effects, the most important set of tests and test species for ecotoxicity testing can be determined using the guidance tables developed for this task (see Chapter 3.3 for details). Finally, by analysing the compiled information of Component I, potential adverse effects can be identified.

We recommend to include all relevant stakeholders already in Component I of the ERA framework. Having characterised the respective case and contextualised the problems/hazards that may occur, the outcome of Component I is a strategy for performing Components II and III, which can include both practical testing as well as modelling efforts. The advantage of Component I is to focus the risk assessment on those hazards that are most relevant and, hence, makes the ERA process more efficient.

Component II – Exposure assessment

In Component II of the ERA framework (Figure 5), the probability of the occurrence of a potential adverse effect is determined for the set of test species identified in Component I referring to the 'evaluation of the likelihood of the occurrence of each identified potential adverse effect' in the Directive 2001/18/EC.

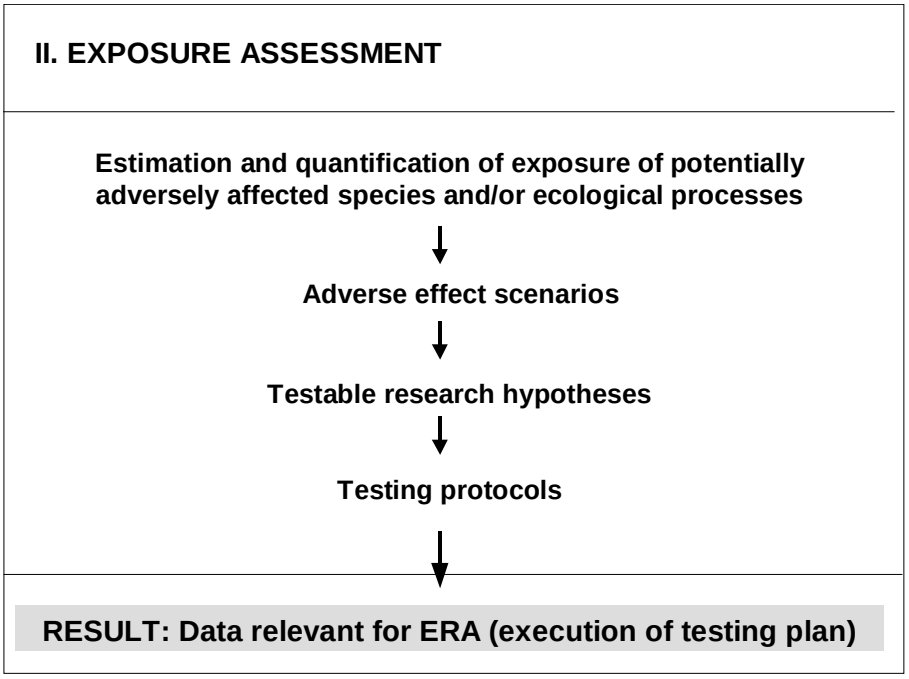

Fig. 5: Determination of the probability of occurrence of potential adverse effects.

The activities in Component II of the ERA framework can include testing at various levels of ecological realism (laboratory, greenhouse, semi-field, and field) in order to verify and/or quantify exposure to transgene products or metabolites. The question whether exposure is possible has already been answered in Component I (hazard identification). Assuming that a substantial amount of practical experience with GMPs will be compiled in the near future, exposure modelling could assist the assessment.

Based on the exposure assessment, adverse effect scenarios can be investigated. For transgenic plants that do not express novel bioactive compounds but require the application of corresponding chemicals (e.g., glyphosate), differences in exposure to these chemicals compared with the conventional farming of the same crop would be the focus of the assessment. In the case of a transgenic plant with altered metabolism, focus of the assessment would be the exposure of the receiving environment to characteristics of the altered metabolism.

The main outcome of the exposure assessment is data that are, combined with data from Component III, used for the risk characterisation (Component IV) to determine the likelihood of occurrence of all possible adverse effect scenarios. During the analysis it may be possible to eliminate some adverse effect scenarios already at an early stage when it can be demonstrated that exposure is highly unlikely. At this point, further testing of adverse ecotoxicological effects would not be necessary. Component II also helps to further minimise the testing efforts by focussing in the experiments on the most relevant species, which are likely to be exposed and fullfill important ecological functions.

Component III – Effect determination

The main activity of Component III of the ERA framework, the effect determination, is the implementation of the ’testing plan’ (Figure 6) developed during Component I (hazard identification). This Component correspondes to the ‘evaluation of the potential consequences of each adverse effect, if it occurs’ in the Directive 2001/18/EC. The aim of Component III is to measure whether the GMP, its use, or the transgene product can affect structural (i.e., related to individual species) or functional (i.e., related to services provided by the whole community) endpoints. Testing follows the systematic hierarchical scheme outlined in Figure 8.

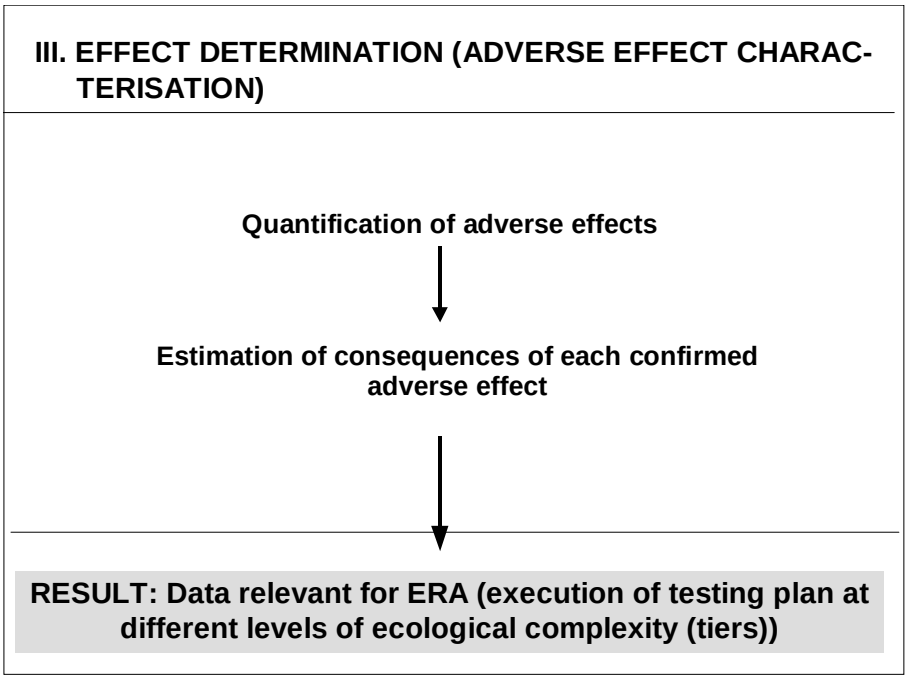

Fig. 6: Evaluation of the consequences of adverse effects.

Component IV – Risk characterisation

In Component IV of the ERA framework, the risk characterisation**,** the risk originating from the GMP is estimated by combining and comparing the information gained and data obtained in the previous three components in a quantitative way (Figure 7). This component refers to the 'estimation of the risk posed by each identified characterisitc of the GMO(s)' in the Directive 2001/18/EC. If at a realistic exposure level significant effects can occur, a risk for the environment is probable. Several outcomes are possible: For example, high risk can occur by combining strong effects with moderate/low exposure or vice versa. Low toxicity and limited exposure can result in low risk, while the opposite is true when strong effects and significant exposure levels do occur.

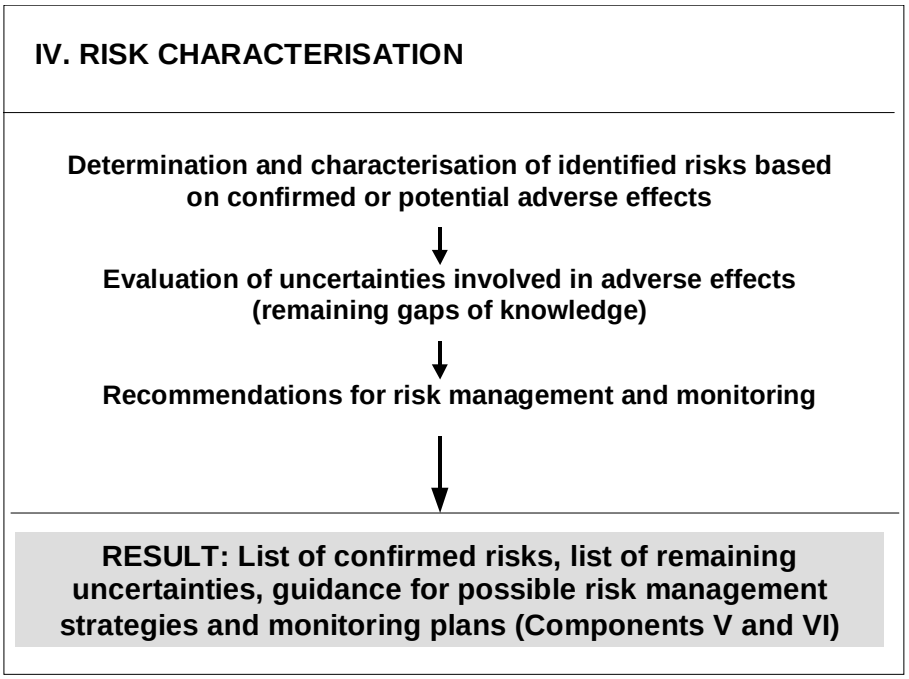

Fig. 7: Combination and comparison of the information gained and data obtained in the previous three components.

3.2.2 Ecotoxicological testing process and decision-finding scheme

Altough laboratory tests give valuable input for the risk assessment we do not advise to assess the ecotoxiocological risk of a GMP completely with data from laboratory tests. Test results and/or the exposure in the environment could be either variable or difficult to quantify (see also Chapter 3.5). It may experimentally difficult to mimic a worst-case szenario with a realistic exposure path in a laboratory setting. Because of multiple interactions between both abiotic and biotic factors in the receiving environment laboratory tests can only be one aspect of the assessment of GMO effects in the environment. This means, lab results will always need to be supplemented by semi-field and field experiments (Figure 8). As mentioned before, GMPs have the potential to reproduce and propagate in the environment and, as a consequence, may not be retrieved. Therefore, a high level of certainty about the risks of a GMP in the field is essential.

To account for the properties of GMPs within an ecotoxicological testing program, we propose to proceed according to the hierarchical (= tiered) approach shown in Figure 8. Since it should be evident that GMPs do not cause an adverse effect in the environment (see Chapter 3.1), one or several tests will be required at different levels (laboratory testing, semi-field testing, and field testing). Especially, if significant uncertainties remain at one level, it is necessary to proceed to higher tier tests conducted at higher levels of ecological complexity, (e.g., in terrestrial model ecosystems or directly in the field). In contrast to the tiered approach used for chemicals, this means that even if no effects can be observed in laboratory tests, semi-field- and field-tests are needed to test for unexpected, indirect, long-term, and cumulative effects (see Annex II Directive 2001/18/EC).

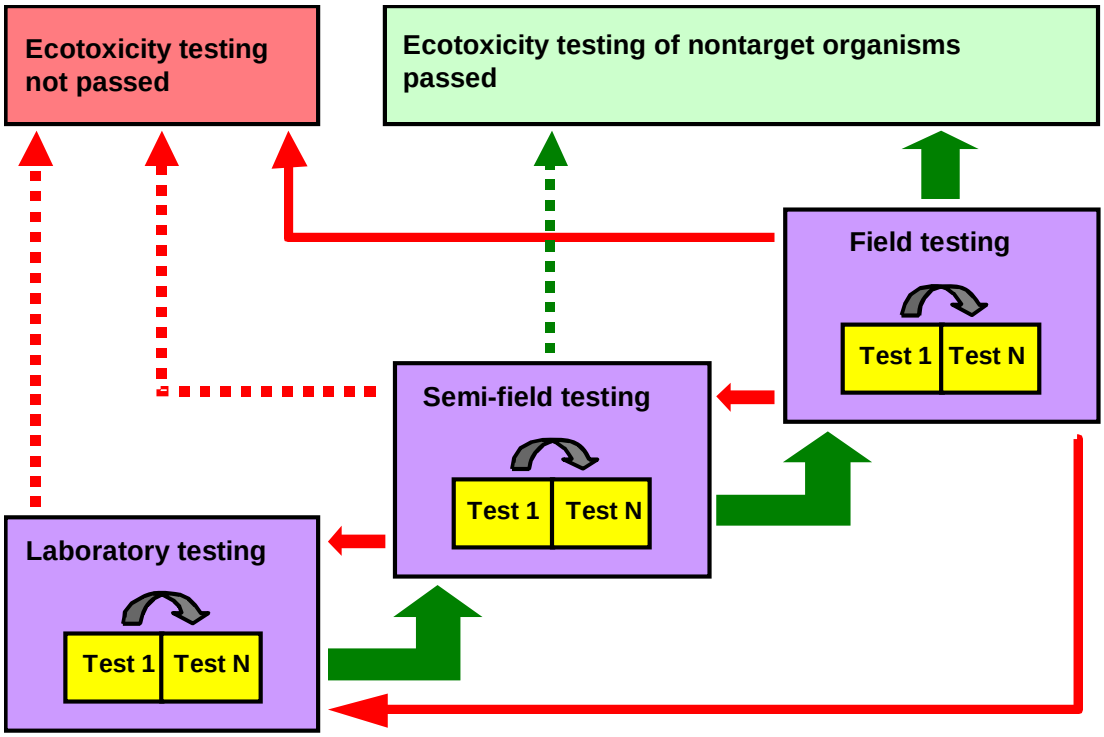

Fig. 8: Flow-chart of an efficient "tiered" ecotoxicological testing process and decision-finding scheme: If already at a low tier (laboratory) a high, not manageable risk is determined, no further testing may be necessary. N = Maximum number of required tests. The main procedure includes early indication at the lower tiers (laboratory and greenhouses) followed by additional tests in the field. If field data do not support the findings in the laboratory, additional laboratory test with modified test protocols may be necessary.

The data gained in laboratory tests are used for a first risk assessment. If no risk is identified, additional test on a higher level should be carried out to support the initial test results. The new results have to be fed again into the ERA framework for re-evaluation. Where uncertainties remain and no effects are observed, further experiments at the field level are necessary (operationalisation of precaution). Data obtained at the lower tiers determine the scope, type of experiment, and the extensiveness of the required field experiments. Where environmental risks are identified, no further ecotoxicological testing is conducted for that particular risk scenario. In such cases, risk management options (not covered in this report) must be considered: can the risk be mitigated or limited? Is it the only risk identified or are there more? Moreover, the risk must be weighed against expected benefits of the GMOs and other aspects of risk assessment.

3.3 Selection of ecotoxicological test species and test methods

The selection of the ecologically most relevant processes and test species for an individual case is a key process of hazard identification (Component I of the ERA framework) and is also linked to the exposure assessment (Component II of the ERA framework) (see Chapter 3.2.1). The selection of test species is followed by the development of proper experimental designs and exposure protocols for testing.

The selection procedure is based on the characterisation of the respective case: the crop plant, the GMP trait and its intended effect, and its intended use in the receiving environment (Chapter 3.2.1). At the same time, it should be ensured that the outcome of the species selection is practical, meaning that the number of tests and, thus, the efforts and resources spent, are well-balanced with the expected outcome of the testing.

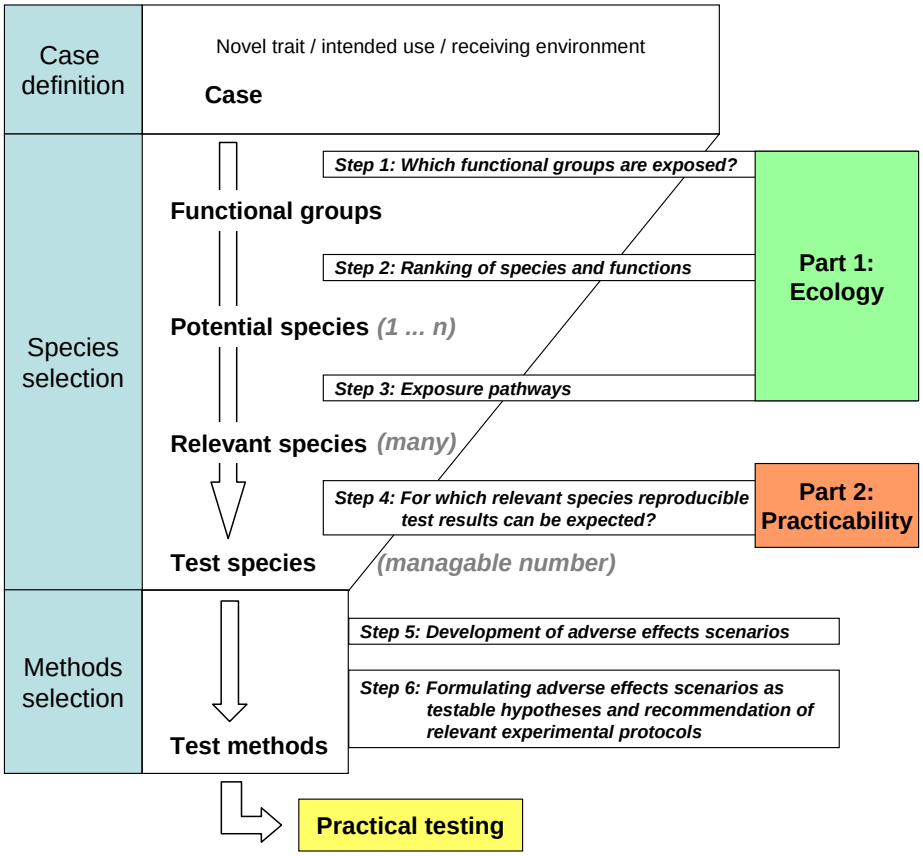

Fig. 9: Species and methods selection procedure for ecotoxicity testing of GMPs

Figure 9 gives an overview of the proposed selection procedure for test species and methods. The procedure consists of a number of questions that allow, in a funnel-like process, to reduce the (potentially quite high) number of candidate test species or functions in a systematic, transparent, and step-wise fashion to a relevant (Chapter 3.3.2) and also practical number (Chapter 3.3.3) of test species/processes. The outcome of the species selection is a battery of tests including the species and/or processes to be tested. Test results can be used to assess the environmental risk of the case. The final determination of the testing methods is guided by practical criteria (Chapter 3.3.6). The inclusion of

the criterion practicability providing justifiability of test results is expected to improve the willingness to adopt the proposed procedures by the stakeholders.

3.3.1 Species selection: Experiences from pesticide testing

Five general criteria for the design of a testing strategy are listed below and consist of various testing methods arranged at different hierarchical tiers (Keddy et al. 1995; Römbke et al. 1996; Torstensson & Petterson 1996 and Løkke et al. 2002). The criteria are valid regardless of the respective environmental compartment. In order to fulfill these criteria, possible effects are tested at different levels of realism – laboratory, semi-field, and field:

1. **Coverage of various taxonomic/physiological groups:** Are ecologically relevant physiological and/or taxonomic units (e.g., arthropods, oligochaetes) covered by the test strategy?
2. **Coverage of various trophic levels:** Can the respective test method be considered representative of a particular trophic (e.g., saprophagous, predatory, etc.) level? Are all relevant trophic groups represented in the test strategy?
3. **Coverage of various exposure pathways:** Are different exposure pathways and/or application scenarios covered? Are bioaccumulation and/or biomagnification also included in the proposed strategy?
4. **Extrapolation of results:** Is it possible, to use the same species (or at least groups) on different investigation levels (laboratory, semi-field, field)? If yes, such extrapolation would facilitate clearly the evaluation of the results.
5. **Coverage of various structural and functional endpoints:** Can the test results be extrapolated from the individual and/or population level to the ecosystem level? Accordingly, are different endpoints taken into consideration?

While the first four criteria are generally regarded as being relevant for the elaboration of a test strategy (Chapter 2.1.3 and Appendix C; see accepted test standards for the aquatic medium), the fifth criteria requires an explanation. Up to now, for the testing of chemicals nearly all efforts for the terrestrial ecosystem are focused on standardised test methods with structural endpoints (i.e., effects on individuals, which are subsequently extrapolated to populations or species). However, chemicals can also cause functional effects (i.e., ecosystem services like organic matter decomposition are impacted). Usually, functional effects can be traced back to a structural effect on key species. In contrast, small, usually not detected effects on various species or their interactions can cause a functional effect on a higher level of biological organisation.

Example of Benomyl: The fungicide Benomyl is often applied in apple plantations where it can cause severe side-effects on earthworms (especially the „key species“ *Lumbricus terrestris*). Consequently, distinct, in some cases even lasting effects on litter degradation were observed (e.g., Kennel 1990). In the long run, the fungicide applications even proved to be counterproductive to the extent that leaves covered with spores of the target fungi were not consumed and therefore the fungi not inactivated any more by the earthworms. As a result fungi could grow in much higher numbers than previously without the application of the fungicide. These experiences support the approach of using a broad range of test species (“test battery”) and to work with adverse effect scenarios and hypotheses involving entire processes or functions rather than individual standard species.

3.3.2 Proposed species selection procedure for GMP testing: Ecology (Part 1)

Currently, similar species as for pesticide testing (Chapter 3.3.1) are used for the risk assessment of GMPs with minor changes proposed (Dutton et al. 2003, Romeis et al. 2006). However, due to the specific properties of living organisms like GMPs, the use of a standard set of test species is insufficient and not compliant with the legal requirements for case-specificity as put forward by the Directive 2001/18/EC and the Cartagena Protocol on Biosafety.

A case-specific risk assessment is guided by the three main factors constituting a 'case':

- the crop plant (its biology, ecology and agronomy)
- the novel traits relating to its intended effect and phenotypic characteristics of the GM crop plant (the GMO), and
- the potential receiving environment including the farming practises (i.e., intended use).

Using this definiton of a case (also see Chapter 3.2.1) as a starting point, the questions that arise first are as follows:

- What species or ecological functions should be tested?
- Which criteria should guide the identification and selection of the most appropriate test species/ecological processes?
- How should they be tested (exposure, protocols, methodologies)?

This being recognised, some procedures for selecting non-target testing organisms relevant for the respective receiving environments have been proposed (Groot & Dicke 2002; Birch et al. 2004; Schmitz et al. 2003). However, the most detailed selection procedure of test species has been developed within an international project by a group of scientists of a global IOBC working group[7] (Birch et al. 2004; Hilbeck et al. 2006). In the following, we first describe the different steps of the species selection procedure in general. Then, we illustrate the selection procedure by describing two case studies that were conducted based on these procedures (Bt-maize in Kenya and Bt-cotton in Brazil, Chapter 3.3.4) and, finally, the procedure is applied to the case example of this project, the GM amylopectin-potato (Chapter 3.6). While the following procedure has been developed primarily with GMPs like Bt-crops in mind, it can be adapted for assessing potential adverse effects of other GMPs that either require the use of a corresponding chemical (e.g., herbicides) or exhibit altered composition of primary and/or secondary metabolic compounds (e.g., starches).

[7] IOBC – International Organisation for Biological Control. The global WG on 'Transgenic Organisms in Biocontrol and IPM'

The step-wise species selection procedure begins with a first set of three steps (Figure 9, Part 1):

step 1 Identification of functional groups of species

step 2 Ranking of species or functions

step 3 Determination of possible exposure pathways.

The species selection procedure begins broadly with considering all known species relevant to selected important ecological functions that were identified for a given cropping system in a given receiving environment (step 1). Based on a defined set of ecological ranking criteria (see Chapter 3.6.2), the extensive list of species is narrowed down in a stepwise fashion to a manageable and testable number of species (step 2). The reduction process also includes the systematic identification of all possible exposure routes (step 3), which further serves as basis for the development of hazard scenarios and experimental protocols. This approach combines a functional and species approach for practicability reasons.

With the outlined species selection procedure, the limited resources and available time for research can be allocated to those species and processes that are at the highest risk identified and – if adversely affected – can induce severe consequences.

Step 1: Identification of functional groups of species

Step 1 of the species selection procedure involves the identification of the most important ecological functions that must not be affected by the introduction of the GMP and its properties that can cause adverse effects in the given cropping system and receiving environment. Ecological functions relate to ecosystem processes. It should be noted that the term "function" is used here in two ways:

- Either it is used for species having the same ecological role ("functional group"). Species groups with ecological functions include for example nontarget primary consumers, secondary consumers (natural enemies) or pollinators (Andow & Hilbeck 2004).

- Or the term function is used for a specific process which is performed or controlled by different species in close interaction with biotic and abiotic ecosystem properties (for instance, the decomposition of organic matter in soil). This latter function depends on the interaction of several groups of micro-organisms, invertebrates and plants and is also influenced by climate, soil properties or litter quality and quantity etc. (Swift et al. 1979).

By using ecological functions inappropriate conclusions associated with the test species used in current ecotoxicitiy testing can be avoided. Furthermore, the use of ecological functions allows to focus testing on critical ecological processes and to limit the number of species that must be tested to those that are ecologically relevant.

For the purposes of risk assessment, it is most important that the functional groups encompass a broad range of possible environmental services they deliver and that might be affected by the introduction of transgenic crops. The selected functional categories considered for risk assessment will vary among different crop types and crop production regions even for the same transgenic trait. For example, if the crop is a legume, nitrogen fixation is a function of utmost importance regardless of the novel trait. For non-legumes, this function is less important. Or if the novel trait confers resistance to a pest or disease through the expression of a novel toxic protein, potentially most affected functions are those that are executed by organisms that are associated with both the crop and the target pest or disease. Hence, these would include for example organisms that either feed on the crop and unintentionally also ingest

the toxic protein or those that were potentially controlling the herbivores (target and non-target) or disease to a certain extent, i.e., accomplish a biocontrol function.

Step 2: Ranking of species or functions

Using all information and expertise available, the known non-target species that actually occur in the crop ecosystems should be listed under each selected functional category. It is important to list the species known to exist in a broad range of production systems for the given crop, including organic and integrated crop production systems. The existing species community in cropping systems where pesticides are frequently used is likely to be heavily biased towards 'survivors' of the 'pesticide treadmill'. Therefore, species found in low-input and/or small-scale intercropping systems should also be considered so that the risk assessment is also relevant for other than conventional farming practises. This is particularly important if the release of a transgenic crop targets low-input, subsistence or organic cropping systems. Some species are likely to be listed in more than one functional category. For example, some ladybird species function as a predaceous natural enemy of potential crop pests as well as a pollen feeder. Such species may be key species, and would be examined carefully in the subsequent steps of the process. The selected species are then systematically evaluated and ranked based on relevant ecological criteria. For this ranking process an assessment tool in form of matrices was developed that are described in detail in Chapter 3.6.2.

Step 3: Determination of possible exposure pathways

For the remaining species or functions identified from the previous step, an exposure analysis is conducted to determine whether and to what degree the species comes into contact with the transgene products (incl. their metabolites), any other altered composition of metabolic compounds (intended or unintended, e.g., starch or carbohydrate composition) or the corresponding measures necessary for the intended effect of the GMP (e.g., application of chemicals). This analysis is case-specific to the transgenic crop and requires information on the phenotypic pattern of transgene expression and any induced pleiotropic changes in the various parts of the transgenic plant over the whole growing season (Grossi-de-Sa et al. 2006) and the pattern of application of the corresponding chemical, if appropriate. The goal of this step is to differentiate candidate species into those that are possibly exposed and those unlikely to be exposed to the transgene products (incl. their metabolites), any other altered composition of metabolic compounds or to the corresponding measures necessary for the intended effect of the GMP (e.g., application of chemicals).

Exposure can be bitrophic via exposure to the transgenic plant or plant parts, including residues and secretions that contain the transgene product, or exposure can occur through higher trophic level exposure to the transgene product or metabolites or corresponding chemicals in organisms that have been exposed to these (added or altered) compounds. Moreover, the plant parts and transgene products can move separately from the transgenic crop, leading to exposure in other parts of the environment, e.g., pollen, nectar, seeds or plant residues. The transgene may move via gene flow (pollen, seed, horizontal) to other related plants that may then express the transgene, thereby causing exposure. For a Bt-toxin, which acts as a gut toxin, exposure must occur via consumption to have any effect. Additionally, it must be anticipated that the transgene products or their metabolites might interact with existing plant compounds, and that the result of this interaction might affect the non-target species (Birch et al. 2002; Andow & Hilbeck 2004), or might affect the quality of the transgenic plant tissue thereby affecting non-target species (Saxena & Stotzky 2001b; Birch et al. 2002).

For example, nectar feeding in parasitoids and plant feeding in *Orius* spp. or *Chrysoperla* spp. could be considered important to adult longevity in the absence of hosts or prey. Further, candidate species may be exposed via multiple routes within a single life stage. For example, *Chrysoperla carnea* larvae, that are generalist predators, feed additionally on various sources of plant liquid, including nectar, exudates from trichomes and plant sap leaking from wounds (Limburg & Rosenheim 2001; Schmidt & Hilbeck, personal observations). Some coccinellid larvae are known to feed on pollen in addition to their typical herbivore prey, and could therefore experience combined exposure through bitrophic and tritrophic routes. Also in this step, uncertainty can be identified in a systematic fashion when conducting the exposure analysis. This helps to determine research priorities and understand the limitation of either existing data or data aimed to obtain through experiments.

The species are systematically evaluated and again ranked based on relevant ecological criteria for this step and developed as an assessment tool (matrices) described in Chapter 3.6.3 in detail. The product of the species selection procedure is a list of selected test species and ecological functions that were determined to be of greatest ecological importance and most extensively exposed to the GMP and its novel transgene product(s) or affected by novel means of application. It is this list that should be subjected to a carefully performed practicability check (see next chapter).

3.3.3 Species selection procedure for GMP testing: Practicability (Part 2)

In order to get reproducible and justifiable test results with the identified species from part 1 of the species selection procedure (Chapter 3.3.2) they have to fulfill additional criteria which are listed in below (for a detailed discussion of these criteria see e.g., Edwards (1988)). Within the species selection procedure these criteria are considered in step 4 (Figure 9, Part 2).

- **Easy to keep and breed:** Are the organisms easy to breed? It is assumed that no catches in the field are necessary. Is regular and standardised food available or can be developed with 'reasonable' efforts?
- **Quick succession of generations:** Are the prerequisites for mass breeding given throughout the year? Is the organisms' individual development so fast that results are yielded within „reasonable" time (which naturally varies, depending on the species involved)?
- **Live in close contact with the soil, plants or plant residues:** How are the organisms exposed? Is an uptake (as a precondition for an effect) only possible via the body surface, or also via respiration or food? In which environmental (sub)compartment does the species primarily occur (e.g., in soil pore water, in the soil atmosphere or on the soil surface)?
- **Moderate sensitivity to stress factors:** Do the organisms react sensitively to different stress factors (e.g., transgene products, plant residues, pesticides)? A broad sensitivity spectrum is more important than a high sensitivity to individual stress factors.
- **Low sensitivity to fluctuations in environmental conditions:** Do the organisms strongly react to fluctuations of environmental factors, e.g., soil properties, temperature, moisture, etc., which cannot be avoided even if the test system is standardised?
- **Wide distribution in various environments:** For example, is the species found in different soil types, and, if possible, even in more than one geographical region? How close is the relationship to certain soil factors (and thus the testing in different field soils)?

Step 4: Applying practicability criteria

In step 4 the above criteria are used as a "filter" in order to remove those species from the list of Part 1 of the species selection procedure that are not suitable for obtaining reproducible test results. Certainly, the different criteria have to be weighed differently. For example, due to practicability and comparability reasons, organisms cultured in the laboratory should be used in the tests. However, in exceptional situations (i.e., very high ecological relevance of one species, e.g., being an ecosystem engineer (Lavelle et al. 1997) in the case to be assessed) the use of field catches might have to be considered.

Referring to previous experiences with chemicals, tests in which field catches have been used are rare. For example, the compost worm *Eisenia fetida*, has been proposed as the standard soil test species more than 20 years ago (OECD 1984). Immediately afterwards, it was criticised as a non-soil-inhabitant and as less sensitive than "field" species. However, despite great efforts (e.g., Løkke & van Gestel 1998), no "true" earthworm species like the ecologically highly relevant species *Lumbricus terrestris*, got acceptance as a test species due to two reasons: firstly, it is extremely difficult to test because of its long life cycle (one year) and secondly, there is no "most sensitive" species – sensitivity depends always on the stress factor and the test conditions. In the case of earthworms, the difference in sensitivity between *E. fetida* and other species is nearly always smaller than a factor of 10, often less than 5 (Römbke 1997). In summary, the environmental risk of, in this case, chemicals is best assessed by using *E. fetida*.

3.3.4 Feasability of the species selection process: Two case studies

The species selection procedure described in Chapter 3.3.2 for generating the scientific data basis for environmental risk assessment was tested within a capacity building project using two case examples in two countries: Bt-maize in Kenya and Bt-cotton in Brazil (Hilbeck & Andow 2004; Hilbeck et al. 2006). Using these two case studies, the three steps (a-c)of the species selection procedure are illustrated below (please note that the focus of these two examples is on the ecological relevance of the selected species while criteria or practicability were not taken into account yet).

Step 1: Identification of functional groups of species

In both case examples (Bt-maize and Bt-cotton) species and ecological processes from 4 functional categories were evaluated (soil invertebrates were not considered due to a lack of knowledge of these inconspicuous organisms in both countries). The choices of the functional categories varied between the two countries.

In Kenya, the 5 selected functional categories for Bt-maize were: herbivorous pests (target and nontarget pests), flower-visiting insects, natural enemies (i.e., beneficial predators and parasitoids of the listed herbivorous pests), weeds and soil processes. For Bt-cotton in Brazil, also 5 functional categories were selected but they differed somewhat in kind and detail: herbivorous pests (target and nontarget pests) were considered similarly important in both countries. Flower-visiting insect were evaluated in more detail in Brazil with a stronger emphasis on pollinating species than in Kenya. Weeds were not considered in Brazil but natural enemies were analysed with more detail than in Kenya and differenciated into predators and parasitoids. For soil processes, different ones were emphasised in the two countries. Some of these differences reflected the differing state of knowledge and understanding, the

agricultural and socio-economic as well as the ecological importance of the functional categories in the two countries.

Step 2 & 3: Ranking of species or functions and determination of possible exposure pathways

In Kenya a total of 56 species and species' groups and 7 ecological soil processes went into the analysis (Table 6). Of these, 18 species and species groups were proposed as testing organisms for biosafety studies. This represents a reduction of roughly 70% of the original listed species and processes. Further, there was agreement that the impact of Bt-maize on 4 ecological soil processes should be studied in detail prior to commercialisation (Table 6).

In Brazil, a total of 117 species and species groups and 25 ecological soil processes went into the analysis (Table 6). Of these, 22 species were selected as testing organisms and proposed for biosafety studies. This represented an 80% reduction from the original list. Further, the experts determined 5 ecological soil processes to be studied in biosaftey tests prior to commercialisation of Bt-cotton.

The initial lists of organisms subjected to the evaluation were compiled by scientific experts of the respective countries. They are based on information published in the scientific literature and the knowledge of the relevant experts. In both countries, most knowledge was available for those arthropods that have either been reported to be damaging under current agricultural production practises or being capable to influence damaging pests. Significantly less information and knowledge was available on species with pollinating functions or other functions in the ecosystem.

Tab. 6: Summary and overview of number of species and ecological processes entering the analysis and finally being selected and recommended for pre-release biosafety testing of Bt-crops.

	Kenya						**Brazil**					
	Pest	**Poll**	**NE**	**We**	**Ecol soilp**	**Tot* (%)**	**Pest**	**Poll**	**Pred**	**Par**	**Ecol soilp**	**Tot* (%)**
Number of organisms entering the analysis	26	---	9	21	*7*	**56**	38	22	42	14	*25*	**117**
Number of selected organisms and processes for biosafety testing	9	4	8	2	*4*	**18** (32)	4	4	2	5	*8 + 1 species***	**21** (18)
Experiments and testing protocols	2 + 1***	1	2	2	*4*	**8***	3	2	2	2	*5 + 1 species***	**10***

***without soil processes; ** earth worm; ***Saprovore; (%) = percent of number entering

Pest=herbivorous pest; Poll=pollinator; NE=Natural Enemy (containing predators + parasitoids); Pred=Predator; Par=Parasitoid; We=Weeds; Ecol soilp=ecological soil processes; Tot= Total

Herbivorous pests

In both countries, herbivorous pest was the functional category that ended up with the most comprehensive species' list (26 in Kenya and 38 in Brazil) and information available including expert knowledge. The relevant scientific experts of the two countries could be recruited for this process and were the most competent source of information. Therefore, knowledge gaps were not considered limiting factors for the analysis. In both countries, the experts reached unanimous decisions on which are the most important herbivorous species that should be subjected to pre-release testing. In Kenya, these were 9 species and in Brazil 4 species.

Weeds

While in Kenya weeds are one of the most important limiting factors for production of maize in the typcial subsistence production context, they are also a serious but solvable problem in the industrialised cotton production in Brazil. In Brazil, cotton farmers have a large arsenal of chemicals at their disposal to control the most serious weeds. In contrast, in Kenya, the infamous parasitic weed *Striga* spp. is in many cases the biggest problem in maize production and can lead to a total loss of the harvest, i.e., food for the family. Hardly any external inputs for control are available to a typical Kenyan subsistence farmer.

Pollinators

Pollination via insects is essential for cotton production but much less so for maize production. Maize is primarily pollinated by wind. Therefore, pollinators were evaluated with more detail in Brazil than in Kenya. However, in both countries serious knowledge gaps surfaced during the evaluation. In Brazil, the identified knowledge gaps resulted in the formulation of a research project that aimes at closing some of the most important informations gaps. This project succeeded in receiving funding and the relevant research is currently on-going.

Natural Enemies

The functional category of natural enemies was considered very important in both countries. The experts of both countries agreed that any future agricultural technology should aim at preserving this important ecological service and should be evaluated in that regard prior to commercialisation. The distinction between predatory species and parasitoids in the Brazil analysis represents primarily a refinement of the methodologies developed in Kenya.

Ecological soil processes

In Kenya and Brazil, 7 and 25 ecological processes were evaluated, respectively (Table 6). Four and eight of these were ranked as important for risk analysis in Kenya and Brazil, respectively. In both countries, soil processes of the two functional categories (‚biogeochemical cycling' and ‚degradation of GMP material') were determined as most important. However, the selected processes differed in both countries as outlined below.

In both countries cellulose-degradation and carbon cycling were considered important soil processes for maize and cotton production, while in Brazil the experts additionally prioritized the macrobial impact on degradation of plant residues as a most important process in cotton production. Further, the persistence of the transgene product (Bt-toxin) in soils was identified as an important biosafety issue to be investigated prior to commercialisation of these GM-crops. This represented largely a further development of the methodologies that had begun in Kenya rather than a difference in judegment between the experts of the two countries.

However, while in Kenya nitrogen fixation and the uptake of phosphorus and micro nutrients were considered to be key processes in the category of 'bio-geochemical cycling', these were nitrification and ammonification in Brazil. This selection reflects the agronomic reality in both countries. The soils in Africa are mostly poor in phosphorous and micro nutrients which are, in addition, difficult to mobi-

lise by the plants. Nitrogen fixation and mobilization is a key process in particular for traditional subsistence farming since farmers have often no means to add fertilisers. Therefore, it is of critical importance that any novel GM crop is capable of utilizing the little nutrients that are available in those soils. In contrast, in Brazil, cotton is produced industrially with enormous amounts of fertilisers added. Therefore, the Brazilian experts determined the efficiency of nitrogen cycling to be of critical importance for cotton production that should not be adversely affected by Bt-cotton.

3.3.5 Scientific ecological principles for the development of testing protocols

For the selected species that resulted from the selection procedure of steps 1 to 4 (see Chapter 3.3.2 and 3.3.3) the procedure continues with two additional steps 5 and 6 for the selection of test methods:

step 5 **Development of adverse effects scenarios**

step 6 **Formulating adverse effects scenarios as testable hypotheses (what question(s) to answer) and recommendation of relevant experimental protocols (how to do proper testing for answering the question(s))**

Step 5: Development of adverse effects scenarios

In steps 1 to 4 of the selection procedure for test species (Chapter 3.3.2 and 3.3.3), existing data and information were gathered and synthesised and gaps of knowledge identified to determine those candidate test species and ecological functions that might be most at risk from the introduction of a GMP. The selected species and functions should be subjected to pre-release biosafety testing. The goal of step 5 is to identify all possible adverse effect scenarios. This step ends with the formulation of a testable adverse effect hypothesis for which experiments / tests can be developed (if necessary) and undertaken to confirm or refute it. The bi- and multi-trophic exposure pathways from step 3 of the species selection procedure are used to guide the development of adverse effect scenarios for each candidate species or function. These adverse effect scenarios are possible causal pathways or chain of events by which a significant adverse effect could occur

The knowledge gap(s) associated with the adverse effect scenarios should be identified and, in step 6, experiments / tests to address these gaps can be proposed. This will also allow to quasi rank the gaps of knowledge identifying those that are critical for an adverse effect scenario to realise. For example, an adverse risk scenario involves specialist natural enemies to be affected when feeding on their preferred nontarget aphid prey that feeds in turn on GMPs expressing the insectidical compound Bt. A critical knowledge gap would for example relate to understanding whether or not the Bt-toxin occurs in the phloem sap of the GMP, the primary food source of aphids (see below). This knowledge gap would determine whether or not an aphid specialist natural enemy would be at risk and should be included in a testing program. From the developed hazard scenarios meaningful testable risk hypotheses, most important data gaps and experimental designs can be derived for regulatory ecotoxicological testing.

Step 6: Formulating adverse effects scenarios as testable hypotheses and recommendation of relevant experimental protocols

Step 6 uses the information from steps 3 (identification of exposure pathways) and 5 (adverse effect scenarios) to construct hypotheses that can be supported or refuted by appropriate laboratory, greenhouse or field experiments. When considering the entire hypothetical causal chain of events identified during the previous step, it should be possible to identify specific hypotheses that can be supported or refuted relatively easily. The identified adverse effect hypotheses are at the center of this testing approach. A hypothesis might be supported or refuted by conducting one small experiment (Test 1, Figure 8, Chapter 3.2.2; e.g., determine whether or not Bt-toxin is present in crop plant phloem) or it may be necessary to conduct a series of experiments in an interativ process (Test 1-N, Figure 8, Chapter 3.2.2). The information and data synthesised during the previous steps also guide the development of ecologically meaningful experiments in terms of protocols, feeding strategies, food types to be used, etc. To use again the example from above, for phloem-sap sucking insects like aphids, a suspected exposure route would be that the transgene product, e.g., a Bt-toxin, is present in the phloem of the transgenic plant and ingested by aphids when feeding on the phloem. A lack of Bt-toxin in the phloem of maize plants implies that no sap-sucking insect is exposed to Bt-toxin on the transgenic maize events tested (Raps et al. 2001). Hence, there can be no risks that extend from this pathway, and any other experiments related to this adverse effect pathway are superfluous. In contrast, Bernal et al. (2002a) found Bt-toxin in the honeydew of the brown plant hopper feeding on Bt rice, which would confirm this as a suspected exposure pathway. Here, proper experimental protocols would include testing for the presence or absence of the transgene product in the relevant food/prey items of the predator or parasitoid.

Experiments that support or refute adverse effect scenarios

Several issues are critical to the sound design of experiments to evaluate adverse effect scenarios: exposure methodologies, measurement endpoints and ecological realism. Ecological realism requires the use of the whole transgenic plant in the experiments. Classical ecotoxicological tests as conducted for environmental chemicals can only serve as initial or supporting evidence but not constitute the sole basis of biosafety testing for transgenic organisms. It would leave all GMPs that do not express a novel bioactive compound entirely untested, which is not in compliance with the EU regulations.

Exposure: Two kinds of exposure methodologies are necessary (Andow & Hilbeck 2004; Birch et al. 2004). Firstly, a "**whole plant" methodology** is required: the whole transgenic plant, not just the transgene product, has to be evaluated including the application of the required corresponding chemical where applicable (see Boxes 1 and 2). This is the only scientifically practical way to include the great majority of possible exposure or impact pathways allowing the detection of any potential effects due to altered secondary metabolism, pleiotropic effects and the various possible interactions between these individual components in the GMP when growing in its normal environment. Secondly, conventional ecotoxicology methodologies can be adapted to allow an assessment of the effects of **isolated transgene products**.

Endpoints for species assessments: Endpoint for an experiment are the parameters that provide a measure of the adverse effect. An appropriate endpoint for pre-release experiments is generational relative fitness or some component of relative fitness (Andow & Hilbeck 2004; Birch et al. 2004). Generational relative fitness is the lifetime survival and reproduction of the non-target species throughout one entire generation of a test species, often including physiologically very distinct life stages. For a holometabolous insect, this includes the egg, larva, pupa and adult. Generational relative fitness is a particularly useful endpoint, because it relates directly to the possible adverse effect, e.g.,

reduced survival. Hence, survival experiments should estimate survival through all of the developmental stages of the non-target species, and adult life stage parameters should be measured, including age-specific mortality and female fecundity.

Including ecological realism: In principle, the duration of the experiment should correspond to the time the non-target species would be exposed to the transgenic plant, plant parts and residues in relation to the temporal pattern of expression and persistence of the transgene product and its metabolites in the cropping situation. These factors should also be considered when designing surrogate experiments with microbially-produced transgene products. While an integrated testing program following this methodology can start with simple ecotoxicological tests using microbially produced transgene products (termed 'surrogate' proteins; (Freese & Schubert 2004)), it is imperative that the whole plant is also presented to the test species to mimic the way the species would come into contact with the plant under field conditions. Raising plants and test species in controlled environments requires carefully designed growing conditions. If appropriate care is not taken, the experimental plants could grow atypically, e.g., etiolated with low specific leaf weights, and not represent typical primary and secondary plant metabolism. This is particularly important for GMPs whose metabolism was altered. Excised plant tissues quickly change metabolically, so laboratory bioassays using excised plant material should be either short (24-48 hours maximum) or newly excised plant material should be supplied every 24 hours. However, such experiments are only an approximation of growing a transgenic crop in the field. A more detailed discussion on the whole plant approach to assessing transgenic plants is provided by Andow & Hilbeck (2004) and Birch *et al.* (2004).

3.3.6 Additional criteria for the selection of test methods

Additional criteria for further evaluation of the methods beyond ecological realism and exposure verification that should be followed are described here. It should be noted that for the sake of simplicity several criteria were grouped together (e.g., „Practicability" also covers aspects of the inputs and costs).

- **Standardisation:** Is the method published as a validated (international) guideline? Are the experimental conditions defined precisely enough to allow other laboratories to achieve comparable results? Are there any reservations from the standpoint of applying the GLP (Good Laboratory Practice) principles? Is the test accepted by authorities for legal purposes?
- **Practicability:** Is the respective method easy to use? Is the test application limited by high demands on laboratory personnel (e.g., taxonomic knowledge of certain animal species)?
- **Applicability:** Is the test only possible by using artificial substrates (like OECD artificial soil) or can natural substrates like field soils (and if yes, which ones) be used? Are tests available for "special" cases like acid soils or the litter layer?
- **Rejection standards:** Can criteria be defined for the validity of the test method? The use of negative controls and/or the regular testing of reference substances are recommended. The selection of the latter should be based on the criteria of the particular test system (e.g., clear-cut, reproducible effect) as well as on other criteria (e.g., avoidance of human toxicological properties, persistence of the reference compound) (see (Yeardley et al. 1995)).
- **Documentation/Experience:** Up to what point is the test organism's behaviour known under conditions with and without stress? Are enough data known from a particular test method to evaluate new results?

- **Number and sensitivity of measurement parameters (endpoints):** Can several parameters be measured in the same test? Is the test system sufficiently sensitive to react in a field-relevant way (e.g., at realistic concentrations of a toxine)?
- **Statistical basis:** Do acceptable statistical methods exist for the evaluation and interpretation of the data acquired?
- **Reproducibility:** How large is the variability when conducting the test at various points in time in a laboratory and/or in various laboratories?
- **Replicability:** How large is the variability when conducting the test with different individual units (replicates)?
- **Input/Time requirement/Costs/Technical and personnel potential:** How costly is the test? How much time is required for conducting the test? Are the required test components (e.g., test organisms, soils, and equipment for the application of chemicals, for example) easily available?
- **Analytical verification of the exposure:** Can/will the concentration of the GMP toxine in the test substrate (e.g., soil or food) and/or the organism (CBR = Critical body residues) be measured? For the ecotoxicological testing of chemicals, this is not done regularly so far, but in some pesticide tests (e.g., (OECD 2004b) and (OECD 2004g)) at least the verification of the stock solutions is already required.
- **Animal welfare:** Are the test organisms treated according to international standards? Can the number of animals be lower without losing information? Is it possible to gain the same kind of information from a test already performed, i.e., is a new test really necessary?

Again, these criteria are of different importance. As already stated, they cannot be easily scored but should be handled in a flexible way (i.e., a high degree of expert knowledge is required). However, assuming that – at least in the medium to long run – several species and methods will be used in a more or less routine way, due to the fact that they represent important functions or species that will repeatedly be selected, these methods have to be standardised by an international organization (e.g., OECD). Such a standardisation process is quite formal and includes experts form many countries, representing usually very different backgrounds and experiences. Therefore, the (ecological and legal) relevance of a test method will be investigated very thorough before implementation.

3.4 Summary and recommendations

The described species selection procedure for GMP testing is in compliance with the relevant regulations (in particular EU Directive 2001/18/EC) but also considers experiences gained within the ecotoxicological testing of pesticides (e.g., EU Council Directive 91/414/EEC). The number of tests and amount of work does probably not differ drastically from the requirements in other areas of environmental risk assessment (e.g., pesticides). However, for environmental risk assessment of GMOs testing of the GM-organism as a whole is central! For specific questions (e.g., optimisation of plant testing or hazard classification), ecotoxicity testing using the purified transgene product only (in the case where a GMP expresses a novel bioactive compound) can deliver complementary or initial information. However, if there is proof that no exposure to the test item or parts of it is possible, knock-on effects resulting from significant changes in biodiversity functioning due to indirect effects should be evaluated before a conclusion of safety can be reached with confidence. This will likely require at least some adverse effect hypotheses to be refuted in the laboratory and the field.

3.4.1 Selection of test species / functions

Focus on ecological relevance:

- For each case, the most important functional groups and ecosystem functions should be identified.
- Species from different trophic levels and taxa units representing these functional groups and ecological functions have to be ranked according to their significance.
- When doing so, different exposure pathways (e.g., food, skin) have to be covered too.
- Experience available so far indicates that only about 20 – 30% of all species considered in the initial evaluation (step 1) end up to be proposed for pre-release biosafety testing.

Focus on practicability:

- Species / functions identified as important for the specific case have to be evaluated in terms of practicability (culture possible? quick life cycle? medium sensitivity towards GMPs but low towards environmental factors? relevant (i.e., chronic) endpoints?).
- Only those species / functions passing most if not all these criteria will be tested.

3.4.2 Selection of test methods

The selection of test methods is based on a defined set of criteria and relies on "expert knowledge" while at the same time provides transparent decision making. Ecological realism is central to the methodologies, e.g., as far as possible chronic endpoints (preferably life-cycle parameter) have to be used. The same (and/or the most relevant) exposure pathways as in the field have to be tested. Each test method for GMPs should fulfill the same criteria as required for other methods concerning standardisation (available as an OECD or ISO document), justifiability (gives reproducible results), quality assurance (concerning documentation or rejection standards) or resources needed (time, costs, equipment).

Concerning exposure, it is highly recommended to use a "whole plant" methodology: the whole transgenic plant, not just the surrogate transgene product, has to be evaluated. In addition, conventional ecotoxicology methodologies can be adapted to allow a more ecologically relevant assessment of the effects of exposure to the transgene products.

3.4.3 Outlook

While in Chapter 3.3.4 case studies are presented in more detail, a first evaluation indicates that in Western and Central Europe for a given GMP:

- between 3 to 5 cases can be expected for each GMP which is going to be assessed in the European Union;
- a number greater than one thousand species can easily occur at one given agricultural site (below and above ground);
- the number of selected species using the proposed selection scheme can range between 10 to 20 per case;
- the number of selected processes using the proposed selection scheme can range between 5 to 10 per case;

- right now, it cannot be determined how many of these test species can be drawn from the pool of already standardised test species and methodologies (in the medium to long run this percentage will likely increase).

The above numbers are estimates, but practical experience in other parts of the world have shown that these numbers can be realistic (see Chapters 3.3.4 and 3.5). More importantly, the numbers are in the same order of magnitude as the numbers of species used for pesticide registration. It is expected that in the foreseeable future due to the manageable number of cases in Europe, at least those species relevant for the most important cases will be standardised after ring testing, e.g., according to OECD format. The criteria for selecting test species were tested for practicability using the case examples of the GM amylopectin-potato as requested by the German Federal Agency for Nature Conservation (BfN). The results of this exercise are presented in the following chapter.

3.5 The case example – GM amylopectin-potato

In this chapter, the above outlined species selection procedure for GMP testing will be explained in more detail by applying it to the case example chosen for this project: a GM amylopectin-potato with an increased level of amylopectin due to a strong reduction of amylose. For this GM crop, an application for field release has been submitted. In the following, we focus only on the identification of possibly affected biodiversity functions and the relevant species and processes. For a risk analysis, data on these processes and species should be delivered. We will briefly outline how proper risk scenarios and research questions for testing can be developed most efficiently. We made an effort to use recent data and information for our illustrative analysis. However, we do not claim that this data is either comprehensive nor the most up-to-date information. For a full risk assessment for regulatory purposes based on our proposed model, the provided information should be verified and completed and the scope extended beyond the one of this project that focussed only on ecotoxicity aspects.

GM amylopectin-potato event EH92-527-1

This GM potato was developed by inhibition of the synthesis of the second starch component, amylose, typically co-occurring with amylopectin in conventional potatoes roughly in a ratio of 80% amylopectin and 20% amylose. Both components, amylose and amylopectin, are used for industrial purposes already today, but separating both components is cost-intensive. This technology offers a possibility for reducing these costs by producing potatoes that only synthesise one of the 2 starch components. It is assumed that the genetic engineering process did not affect any other process of the plant except the specific processes involved in the production of starch from sugar in the potato tubers only. However, besides the analysis of the chemical composition of tuber samples no data verifying this were delivered within the application. Based on many years experience with genetic engineering of plants, including potato, unintended changes due to the transformation process must be expected (Birch et al. 2002). The property that can cause potential adverse effects is therefore the altered primary metabolism and any unintentional effect due to the transformation process, for example on glucoalkoid content (secondary metabolism) as described by Birche et al. (2002).

3.5.1 Step 1: Identification of functional groups of species

In order to conduct the identification process of the possibly affected biodiversity functions for the GM amylopectin-potato in a systematic and transparent way, guidance tables were developed (Table 7 and 8). The identification of the important ecological functions for the cropping system listed in Table 7 and 8 is guided by the biology of the crop and its agronomic requirements for production and the receiving environmental conditions.

The components and criteria of guidance Table 7 extend beyond those relevant for ecotoxicology testing only while Table 8 lists only all considered categories in the case example. Even though Table 7 extends the scope of exotoxoicological testing, a case-specific, comprehensive risk analysis of GMPs should consider potential adverse effects on organisms and processes also of agronomic, socio-economic and possibly other relevance. This would for example include potential land use changes and agricultural intensification (Table 7). Sensitivity to diseases and weeds could arguably be included in ecotoxicity testing (Table 8). All of these clearly have important environmental implications when ignored or 'bad' agricultural practises (e.g., intensification of chemical use) are applied. In the context of ecotoxicity, however, we focussed on those functions only that are mainly of ecological relevance like degradation and recycling of plant residues, biocontrol and pollination (Table 8).

The exercise is primarily meant for illustrative purposes rather than claiming completeness of most current information. For a full risk assessment, the questions posed in the guidance tables should be completed with all scientific expertise available. Based on the experience from the GMO ERA project (Hilbeck & Andow 2004; Hilbeck et al. 2006), this is best achieved by gathering relevant experts of a country around a table and achieve consensus views on the listed issues.

While, as pointed out above, for a comprehensive risk assessment for regulatory approval of GMPs, all components listed in Table 7 need to be evaluated, in the scope of this project, we focus on those functions only that are relevant for ecotoxicology testing. These still include 19 components mainly dealing with the associated biota of the GM potatoes (Table 8).

Tab. 7: Guidance table for case-specific selection of important, potentially affected biodiversity functions for environmental risk assessment: Case example – GM amylopectin-potato.

Main criteria	Characteristics	Associated ecological function/agricultural practise	Affected organisms/process
I. Crop biology			
Harvested product?	Tuber	Below-ground herbivory, pest management	Tuber feeders
Symbiosis with nitrogen-fixing microbes?	No	---	---
Type of reproduction?	Vegetative multiplication	Below-ground herbivory	Below-ground tuber feeders
	Seed reproduction for certified disease-free seed potatoes	Special needs	Above ground plant and seed feeders
Sensitive to diseases?	Yes, highly sensitive	Pest management	Fungal and viral pathogens including their vectors (e.g., aphids)
Sensitive growth stage?	Early stages	Plant competition, pathogens	Weeds, diseases
Sensitive growing conditions?	Likes cool growing conditions BUT sensitive to frost	Temperate regions: Frost-free period Tropical regions – elevated areas	Frost protection
	Sensitive to logged soil water	Soil melioration	Soil cultivation measures
Input routes of transgenic plant parts and transgene products			
What plant residues are expected and in what quantities *before* harvest? Do they contain transgenes or transgene products	Some leaf, stem and root material, few flowers Unclear, transgenes likely, no novel transgene product, altered metabolic compounds	Plant material decomposition, nutrient recycling	Detrivores (macro- and microorganisms)
What plant residues are expected and in what quantities *after* harvest? Do they contain transgenes or transgene products	Whole plants incl. leaves > stem > roots Larger quantities Unclear, transgenes likely, no novel transgene product, altered metabolic compounds	Plant material decomposition, nutrient recycling	Detrivores (macro- and microorganisms)
What plant excretions/ exudates possibly containing transgene products are expected?	No novel transgene product, altered metabolic compounds	Rhizosphere, Mycorhiza	Root colonizing micro- and meso-fauna and fungi, mycorhiza microbes
Potato-associated valued species?	No	---	---

Tab. 7: continued.

Main criteria	Characteristics	Associated ecological function/agricultural practise	Affected organisms/process
II. Trait – intended effect			
Novel transgene product expressed? If yes, which?	No	---	---
Metabolite eliminated or significantly reduced?	Yes, Amylose	Herbivory and food chain effects (digestability)	Above-ground herbivores and associated natural enemies (biocontrol organisms)
Metabolite significantly increased?	Yes, Amylopectin	Herbivory and food chain effects (digestability)	Above-ground herbivores and associated natural enemies (biocontrol organisms)
Intended effect?	No amylose, amylopectin yield increased	Non-food/feed, industrial use	Herbivores
Application of corresponding chemical required? If yes, which?	No	---	---
Antibiotics resistance gene present?	Yes	Legal compliance issue	Legal compliance issue
		Horizontal gene transfer (HGT)	Microbes prone to HGT
III. Receiving environment – intended use			
a. Region			
Landscape structure? Fragmented hilly to uniform plain	Grows everywhere	Undemanding	---
Climate type? temperate to tropical	Temperate	Frost protection	Altered production cycle?
Number of potential different production regions?	Large potato production regions of Germany	Main potato production related functions	Biota of large potato production regions
b. Farming system			
How many crop production cycles?	Temperate regions: 1	Crop rotation	Crops per season
Intended/anticipated scale of release	Large scale	Land use	Area planted
Replacing other crops (loss, shift, addition)?	Possibly if successful	Land use	Area planted (compared to previously)
Expanding agricultural production zones (to what degree)?	Unlikely	---	---
Cropping system? Large to small, subsistence	Large scale for industrial purposes	Change in ag-practise - intensification	Competition for food potatoes
Farming practise? Chemical intensive, integrated, organic?	Industrial, non-food – chemical intensive	Changes due to intensification	Indicators for intensification
Pest management type?	Industrial, non-food - chemical intensive	Changes due to intensification	Virus and fungal diseases, insect pests, nematodes

Tab. 7: continued.

Main criteria	Characteristics	Associated ecological function/agricultural practise	Affected organisms/process
III. Receiving environment – intended use			
b. Farming system			
Use of harvested product	Tubers for starch production for industrial use		
Recycling of plant residues after use	Possible	compost	Compost organisms
c. Soil type			
Soil type (heavy to light) ?	Medium-light soils because of below-ground tubers	Soil processes influenced by light soils	Organic matter decomposition rates Soil moisture retention, etc.
		Soil diseases and pests typical for light soils	Nematodes, certain fungi, etc.
Organic matter content? High to low	Undemanding	Degradation of plant material that is rich in amylopectin	Plant residue decomposition, microbial and macrofaunal degradation
Prone for soil erosion?	If light soils - yes	Intensification – increase in monoculture field size	Erosion indicators

Tab. 8: Selected important, potentially affected biodiversity functions relevant for ecotoxicity testing: Case example – GM amylopectin-potato.

Main criteria	Characteristics	Associated ecological function/agricultural practise	Affected organisms/process
I. Crop biology			
Harvested product?	Tubers	Below-ground (tuber) herbivory, pest management	Tuber feeders
Symbiosis with nitrogen-fixing microbes?	No	---	---
Type of reproduction?	Vegetative multiplication	Below-ground (tuber) herbivory	Tuber feeders
	Seed reproduction for certified disease-free seeds?	Special needs	Above ground plant and seed feeders
Sensitive to diseases?	Yes, highly sensitive to important fungal and vector-transmitted viral pathogens	Pest management	Fungal and viral pathogens including their vectors (e.g., aphids)
Sensitive growth stage?	Early plant stages	Plant competition, pathogens	Weeds, diseases
Sensitive growing conditions?	Likes cool growing conditions but sensitive to frost	Temperate regions: frost-free period of temperate Tropical regions: elevated areas	Frost protection
Input routes of transgenic plant parts and transgene products			
What plant residues are expected and in what quantities before harvest? **Do they contain transgenes or transgene products**	Some leaf, stem and root material, few flowers Unclear, transgenes likely, no novel transgene product, altered metabolic compounds	Plant material decomposition, nutrient recycling	Detrivores (macro- and microorganisms)
What plant residues are expected and in what quantities after harvest? **Do they contain transgenes or transgene products**	Whole plants incl. leaves > stem > roots Larger quantities Unclear, transgenes likely, no novel transgene product, altered metabolic compounds	Plant material decomposition, nutrient recycling	Detrivores (macro- and microorganisms)
What plant excretions/ exudates possibly containing transgene products are expected?	No novel transgene product, altered metabolic compounds	Rhizosphere, Mycorhiza	Root colonizing micro- and meso-fauna and fungi, mycorhiza microbes
Potato-associated valued species?	No	---	---

Tab. 8: continued.

Main criteria	Characteristics	Associated ecological function/agricultural practise	Affected organisms/process
II. Trait – intended effect			
Novel transgene product expressed? If yes, which?	No	---	---
Metabolite eliminated or significantly reduced?	*Yes, Amylose*	Herbivory and food chain effects	Above- and below-ground herbivores and associated natural enemies **(bio-control)**
Metabolite significantly increased?	*Yes, Amylopectin*	Herbivory and food chain effects	Above- and below-ground herbivores and associated natural enemies **(bio-control)**
Intended effect?	No amylose, increased amylopectin yield	Non-food/feed, industrial use	Above- and below-ground herbivores and associated natural enemies **(bio-control)**
Application of corresponding chemical required? **If yes, which?**	No	---	---
III. Receiving environment – intended use			
a. Region			
b. Farming system			
Farming practise (chemical intensive, integrated, organic)?	Non-food – possibly more chemical intensive than previous crops	Changes due to intensification	Indicators for intensification (secondary pests)
Pest management type?	Non-food - possibly chemical intensive	Changes due to intensification	Virus and fungal diseases, secondary insect pests & **bio-control**
c. Soil type			
Soil type (heavy to light)?	Medium-light soils	Soil processes influenced by soil type	Organic matter decomposition rates; soil moisture retention, etc.
		Soil diseases and pests typical for soil type	Nematodes, certain fungi, etc.
Organic matter content (high to low)?	Undemanding	Degradation of plant material that is potentially rich in amylopectin (tuber residues)	Plant residue decomposition, microbial and macrofaunal degradation

3.5.2 Step 2: Ranking of non-target species and functions

Listing non-target species

The data provided by the applicant in an attempt to fullfil the requirements for pre-release risk assessment are a valuable source of information for this step. The applicant has conducted biodiversity surveys in a number of potato fields in 3 different potentially receiving environments in Europe: Germany, Netherlands and Sweden. From these field inventories, the list of species executing identified important functions can be derived for the particular receiving environments.

The following taxonomic groups were documented in the applicant's trial in the German potato field: 25 spider species, 25 Coleoptera families (6 species identified) caught in pitfall traps and 9 families (2 species identified) were caught in blue sticky traps. Of these, 28 Carabidae species and 22 Staphylinidae species were further identified. In addition, 33 Diptera families (6 species identified) were caught in pitfall traps and 31 Dipteran families (4 species identified) in yellow sticky traps. Further, 25 Hymenoptera families (23 species identified) were caught in pitfall traps, 25 Hymenopteran families in yellow sticky traps and 20 Hymenopteran families in blue sticky traps.

For none of the orders, families, and species an analysis on number of overlapping families or summary of the number of total families and species caught across all trap types could be found in the documents. Hence, before using the data in a risk analysis, the relevant information extracted. Based on that, the detected species can be classified in functional categories und their roles in the ecosystem identified. From this, a species and family list for all identified relevant functions can be developed that can then be subjected to further analysis. However, so far only above-ground organism groups or those living directly on the soil surface have been considered while true soil inhabiting groups like earthworms or collembolans are missing from the biodiversity inventory.

Tab. 9: Coccinellid species from applicant's biodiversity inventory.

	Number of individuals on different potato varieties			
Coccinellid species	**GM potato**	**Kuras**	**Seresta**	**Total**
Coccinella septempunctata	80	84	83	247
Hippodamia variegata	11	11	9	29
Oenopia conglobata	1	1	0	2
Propylea quatuordecimpunctata	13	11	23	47
Tytthaspis sedecimpunctata	0	0	1	1
Total	**105**	**107**	**116**	**326**

For illustrative purposes, we continue the selection process by choosing one function identified to be important: natural enemies (biocontrol). The analysis aims at identifying possible effects of the identified property, i.e., altered metabolism of GM potatoes, on relevant aphid-based foodchains. Aphids are identified to be important pests by themselves but, more importantly, are vectors for detrimental potato virus diseases. Further, aphids are known for their remarkable sensitivity to changes in the metabolism of their host plants. It is therefore reasonable to expect that any unintended or intended changes in metabolism – primary or secondary – will be reflected in their fitness. This in turn could impact their

status as virus vector. Natural enemies will modulate any such effect for the better or worse. For illustrative purposes, we continue the next steps with the coccinellid species listed in Table 9.

Ranking using a species screening matrix

In this second part of step 2, the listed species or processes are ranked according to a defined set of criteria relating to their potential exposure to the crop plant and the significance a possible adverse effect would have on their ecological functions (Boxes 4 and 5). The goal is first to identify those species/processes with the greatest likelihood of being exposed to the crop plant, and therefore of being potentially affected. Second, the goal is to identify the species/functions most likely to have a significant role in the crop ecosystem, which if disturbed, would result in the greatest adverse environmental effect.

Box 4: Species Selection Process - Pathways of Exposure to Crop (adapted from Hilbeck et al. (2006)): Non-target species x spatio-temporal crop coincidence (preliminary estimate of likelihood of effect)

Geographic distribution
The degree of overlap in the geographic distribution of the crop and the non-target species at the country or region or agro-ecological zone scale (depending on what spatial scale has been chosen for the analysis).

Habitat specialization
The degree of association between the non-target species and the crop habitat. The crop habitat is defined as the crop field and its margins and includes all of the species associated with the field and its margins, including the crop, any intercrop and weeds. A habitat specialist occurs only in the crop habitat; a habitat generalist occurs in many other habitats.

Abundance
The average or typical density where the species is present. Assessment of abundance requires good field expertise with the sampling methods used to measure density and knowledge of the typical population fluctuations of the species. Density measures can be difficult to compare across species when different sampling methods are used. Moreover, species may be difficult to compare because of vast differences in size and biology. For example, cotton aphids are small and can occur in high numbers while *Helicoverpa armigera* (Lepidoptera) is larger and occurs in smaller numbers. Field expertise is needed to compare the relative densities of such species.

Phenology
Degree of temporal overlap of non-target species with the crop plant.

a) From the non-target species' perspective: What proportion of the non-target species' life cycle takes place while the crop is alive?
b) From the crop' perspective: What proportion of the crop growing cycle is covered by the non-target species life cycle?

Linkage
For species: degree of specialisation to a particular food. For herbivores this would be the degree of feeding specialisation to the crop (host range) and/or for higher trophic level species this would be the degree of feeding specialisation to the prey/host associated with the crop. Linkage might also be called feeding specialisation and focuses on trophic relations. It can also specify what lifestage of the non-target species feeds on the crop plant.

Association
For functions: degree of association with relevant plant tissues, parts, residues and secretions. For soil functions, this should include association with roots, plant parts that fall onto the soil (pollen, flowers, residue), plant residue incorporated into the soil, and root exudates.

Box 5: Species Selection Process - Pathways of Exposure to Crop (adapted from Hilbeck et al. 2006): Nontarget species/process x crop trophic relationship (relates to functional significance for cropping system)

For species:

- functional significance in relation to the functional group (see step 1) in the cropping system. Examples: for predators and parasitoids, importance as a biological control agent is ranked; for herbivores, importance as potential secondary pest is ranked.
- other significance in the agro-ecosystem, functional role to be specified and its significance ranked. Example: for herbivores, the role as a disease vector, seed disperser, decomposer, plant biological control agent, or other possible functions is considered (see step 1).

For soil ecosystem processes:

- importance as an indicator of soil health. If possible, indicator organisms that are appropriate to the case study should be specified.
- the significance of the process for the cropping system (system of crop rotation, intercropping and multiple cropping).
- how directly does the ecological process affect crop development (do other ecosystem processes intervene to mitigate such effects)? This requires consideration of the coincidence between the seasonal pattern of variation in the rate of the function and the development of the crop.

The criteria listed in Boxes 4 and 5 can be systematically aligned in a screening matrix that allows an efficient and transparent prioritisation process. Using these matrices, the five coccinellid species from the applicant's biodiversity inventory (Table 9) were evaluated for their suitability as testing candidates for risk assessment.

Firstly, some general information on each listed nontarget species is required as listed systematically in Table 10. Next, the spatio-temporal coincidence of the listed nontarget species with the crop is systematically ranked (Table 11). All coccinellids are relevant biocontrol organisms, occur in all of Germany but are not particularly associated to the potato habitat (Table 11). Their occurrence in potato fields is entirely dependent on whether or not their prey or foods occur there. Given the presence of aphids, coccinellid adults will oviposit on potato leaves and the hatching larvae will likely spent their entire immature life stage in the potato field due to their limited mobilility. Only those larvae whose eggs were deposited near the field margin have a chance to migrate out of the field and find other prey if aphid abundance becomes too low to allow complete development of the larvae.

Tab. 10: Matrix I – General information.

Species or species group	**Order and family**	**Life cycle stage with natural enemy function**	**Main prey**
Coccinella septempunctata	Coccinellidae	all	*aphids, fungi, thrips, pollen*
Hippodamia variegata	Coccinellidae	all	*aphids, pollen*
Oenopia conglobata	Coccinellidae	all	*aphids, psylla, pollen (probably)*
Propylea quatuordecimpunctata	Coccinellidae	all	*aphids, pollen*
Tytthaspis sedecimpunctata	Coccinellidae	all	*pollen, fungi, mites, thrips*

Tab. 11: Matrix I – Part A: Nontarget species x spatio-temporal crop coincidence (preliminary estimate of likelihood of effect).

Species	**Geographic distribution (widely distributed?)**	**Habitat specialization: degree of association with potato habitat**	**Abundance: on potato crop**	**Phenology: how much of species life cycle is on potato?**	**Phenology: how much of potato season is species present?**	**Linkage: degree of feeding specialization on species on potato**	**OVERALL RANK: overall estimate (in words)**	**OVERALL RANK (rounded): overall estimate as mean (rank 1-3) no decimals**
Coccinella septempunctata	all (1)	low (many crops where aphids appear) (3)	variable (dependent on prey) (2)	can be partial or all (2)	can be partial to all (dependent on presence of aphids) (2)	low (3)	medium	2
Hippodamia variegata	all (1)	low (dependent on presence of aphids) (3)	variable (dependent on prey) (2)	can be partial or all (2)	can be partial to all (dependent on presence of aphids) (2)	low (3)	medium	2
Oenopia conglobata	all (1)	medium (trees, shrubs) (2)	low (3)	probably partial or transient (3)	probably partial or transient (3)	low (3)	low	3
Propylea quatuordecimpunctata	all (1)	medium (prefers small grains where aphids appear) (2)	variable (dependent on prey) (2)	can be partial or all (2)	can be partial to all (dependent on presence of aphids) (2)	low (3)	medium	2
Tytthaspis sedecimpunctata	all (1)	medium (gramineae and oil crops) (2)	Low (3)	can be partial or all (2)	can be partial to all (2)	low (3)	medium	2

Following the same principle as for the ranking of the spatio-temporal coincidence the trophic relationship of the Coccinellids with the crop plant is evaluated as a preliminary estimate for the significance of a potential environmental effect, i.e., is an effect, if observed, of ecological meaning or not (Table 12). Again, information on a number of ranking criteria has to be provided and systematically evaluated as outlined in Box 5.

Four of the coccinellid species listed in Table 10 are well-known predators of aphids, which is what they are valued for. One of them, *Tytthaspis sedecimpunctata*, feeds on fungi, predominantly mildew. However, most of them were also observed to feed on other prey.

Coccinella septempunctata (Seven-spot ladybird) and *Propylea quatordecimpunctata* (Fourteen-spot ladybird) are among the most well-known and most voracious aphid predators in Germany. They are very common in many crops. *C. septempunctata* was the most abundant species and always occured in higher densities than *P. quatordecimpunctata* (Table 9). Cannibalism occurs in *C. septempunctata* but is rare for adults (Box 6).

Box 6. What Do Sevenspotted Lady Beetle Adults Really Eat?

"Although their primary food source is **aphids**, they do feed on other foods as well. During a two year study, adult sevenspotted lady beetles were collected throughout the year in wheat, oat and rye fields at three locations in Germany. The different food types ingested was determined using gut dissection. During the course of the year the composition of the gut contents changed as the lady beetles went through their seasonal cycle. The alimentary canal was generally empty when the beetles came out of hibernation. In the spring, as the lady beetles began dispersing, **soil particles, fungal spores, and aphids** were found most frequently in the gut. Feeding on **pollen** was also observed in the spring, but only 6% of the beetles dissected had pollen in their gut. During the summer, when the beetles were reproducing, aphid remains were present in over 85% of the individuals. Surprisingly, fungal spores occurred more frequently than remains of other arthropods during this time, with half of the beetles dissected containing fungal spores. In late summer and autumn, fungal spores occurred most frequently. Aphids had been eaten by only 37% of the lady beetles. **Pollen**, primarily from goldenrod, was also an important food in late summer as the beetles prepared to enter hibernation again in the fall. Sevenspotted lady beetle adults showed very uniform food preferences. Other than aphids, nearly all other arthropods found were **thrips**; only a **few fly or beetle larvae** remains were found. **Cannibalism** was rare (it is more common for larvae). Nearly all the spores found were of two types: ***Alternaria*** sp. (80%) and uredospores of ***Puccinia*** sp. These spores were a dominant food component during the whole active life period. Spores were consumed from April until September, in the absence and presence of aphids. Fungal spores may be an obligatory food source for these lady beetles. " Source: (Triltsch 1997)

P. quatordecimpunctata is most common in small grain fields. However, it is unclear to what extent pollen-feeding occurs for both species. An internet based search did only yield few reports on confirmed pollen feeding by species of both genera (Triltsch 1997; Bai et al. 2005). Therefore, we presumed pollen feeding to be possible in principle but certainly not of prime importance for survival. *C. septempunctata* and *P. quatordecimpunctata* can easily be reared solely on aphids. Similar is true for *Hippodamia variegata* (Adonis Ladybird), which is also a known aphid predator. However, compared to the two previous species, *H. variegata* is less abundant and voracious. Pollen-feeding of this species is not well-studied but established for this species[8] (Ferran & Dixon 1993).

[8] http://www.cnr.berkeley.edu/biocon/BC%20Class%20Notes/129-132%20Conservation.pdf.

Tab. 12: Matrix I – Part B: Nontarget species (NT) x trophic relationship with crop plant (preliminary estimate for significant of environmental effect).

Species	**Significance as biological control agent in potato**	**Significance as a food for other natural enemies**	**Significance in association with other crops**	**Significance in natural areas**	**OVERALL RANK: MAXIMUM PRELIMINARY SIGNIFICANCE**	**RANK: NT[9] x spatio-temporal coincidence PART A**	**RANK: NT[9] x trophic relationship (significance) PART B**	**PRELIMINARY RISK RANK**
Coccinella septempunctata	high (1)	unclear but relevant at some level (2)	high (1)	1	1	2	1	**2**
Hippodamia variegata	probably medium (2)	unclear but relevant at some level (2)	high (1)	1	2	2	2	**2**
Oenopia conglobata	probably low (3)	unclear but relevant at some level (2)	high (1)	1	2	3	2	**3**
Propylea quatuordecimpunctata	high (1)	unclear but relevant at some level (2)	high (1)	1	1	2	1	**1**
Tytthaspis sedecimpunctata	possibly high (1)	unclear but relevant at some level (2)	high (1)	1	1	2	1	**1**

[9] NT = non target species

Oenopia conglobata is a well-known predator of pistachio psylla (*Agonoscena pistaciae*) in Iran and is reported to occur mainly on trees in orchards (e.g., pistachios (Mehrnejad & Jalali 2004)) and shrubs (Burgio et al. 2004). In fact, even in the abundant presence of aphids, this species preferred psylla in pistachio orchards and developed faster with higher survival rates than when raised on aphids (*Aphis gossypii*) only (Mehrnejad & Jalali 2004).

The diet of the tiny *T. sedecimpunctata* (Sixteen-spot Ladybird) is poorly understood, but mildew and pollen are presumed to be constituents. However, this species was also reported to feed on mites and thrips. In fact, Ricci et al. (1983) state that when all food kinds are available these ladybirds prefer pollen and arthropods while fungi are eaten after hay-making and after the flowering of Gramineae and oil crops.

All species listed in Table 10 are also important regulating organisms in adjacent vegation and natural or semi-natural areas. They occur in many different habitats ranging from cultivated crop fields, weed of field margins, hedgerows, shrubs and trees (Burgio et al. 2004). For example, *T. sedecimpunctata* is reported to live primarily on Gramineae like *Lolium perenne*, *L. temulentum* and *Triticum aestivum* (wheat), on Compositae like *Carthamus tinctorius*, *Chamomilla recutita* and *Pulicaria vulgaris* and on Convolvulaceae like *Convolvulus arvensis* (Ricci et al. 1983). Habitat management around agricultural lands can significantly improve biocontrol within the agricultural lands with regard to most if not all coccinellid species (Sengonca et al. 2002). They are not associated closely with any particular habitat but follow their preferred food items to many different places.

Finally, a rank is assigned to each candidate species or process that summarises the entire evaluation process of direct and higher trophic exposure to the crop. If there is sufficient information available, this ranking should be done for each region, agro-ecological zone or cropping system being considered. The ranks for likelihood of exposure to the crop and for its significance can be summed to give a final rank for each species/process. It can be quantitative or qualitative, and is a relative rank; the species or processes are compared for each criterion, using published information, supplemented with available expert knowledge. This provides that a process is transparent and the evaluations are more readily defendable. The gaps in knowledge in the screening matrix can be identified by question marks. This will allow to understand the extent and quality of the associated uncertainty and also inform the next steps identifying data needs and setting research priorities.

After ranking, in the case of the coccinellids associated with the GM potato, 4 species were identified to be of medium to high importance (Table 12) and will be retained for the next steps of the assessment process. One species, *O. conglobata*, was evaluated to be of minor importance for this function and will therefore be excluded from the further analysis.

3.5.3 Step 3: Trophically mediated exposure to transgenic plant and transgene products

The transgene products of the GM amylopectin potato is the altered enzyme composition resulting in an altered carbohydrate metabolism, presumably, but not demonstrated yet, only occurring in the potato tubers. However, it is conceivable that through feedback mechanisms or pleiotropic effects the carbohydrate metabolism of the entire plant is unintentionally also affected in one way or another. Further also other unintended effects can occur as was demonstrated for GM potatoes befores (e.g., glycoalkaloid content, Birch et al. 2002). On the basis of the available data, i.e., phenotypic and agronomic analysis and the analysis of the chemical composition of the tuber and the pulp unintended changes in metabolism can not be excluded completely. In addition, side effects of intended altered metabolism on non target species are unknown. The analysis will be conducted under the precaution-

ary assumption that the metabolism has been altered. Furthermore this example illustrates how to proceed, when unknown / unspecific effects should be investigated.

The analysis begins with an evaluation of a possible **bitrophic exposure** and its pathways (Table 13). For one species only, *T. sedecimpunctata*, pollen feeding has been established as important food source. For all other coccinellid species, pollen feeding is not well investigated but occasionally observed. However, it is likely not a prime and obligatory food source as all species can for example easily be reared on an arthropod diet only (see Step 2). Additionally, altered sugar contents in pollen due to the genetic modification is not thought to be likely nor to impact coccinellid feeding adversely even if it were detectable. Therefore, all coccinellids were ranked low for bitrophic exposure to transgene products and metabolites (Table 13), although, this is untested.

Tab. 13: Matrix II – Part A: Bitrophic exposure pathways.

INFORMATION FROM PREVIOUS TABLE				**BITROPHIC EXPOSURE**			**RANK**	**Question**	**RANK**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**Life cycle stage with natural enemy function**	**Main prey**	**Growth stage of potato when present (early, mid, reproductive)**	**List the plant tissues or secretions on which it feeds**	**Which tissues/ secretions fed upon express transgene product?**	**Is this feeding important for the predator?**	**Is bitrophic exposure possible?**	**Are transgene product or metabolites detectable after feeding on plant tissue or secretion?**	**Does bitrophic exposure occur?**
Coccinella septempunctata	all	*aphids, fungi, thrips, pollen*	all	pollen	None*	medium likelihood (2)	**low likelihood (3)**	**no***	**medium likelihood (2)**
Hippodamia variegata	all	*aphids, pollen*	all	pollen	None*	low likelihood (3)	**low likelihood (3)**	**no***	**low likelihood (3)**
Propylea quatuordecimpunctata	all	*aphids, pollen? (in other species at least)*	all	probably pollen, else none	None*	low likelihood (3)	**low likelihood (3)**	**no***	**low likelihood (3)**
Tytthaspis sedecimpunctata	all	*pollen, fungi, mites, thrips*	all	pollen	None*	low likelihood (3)	**low likelihood (3)**	**no***	**high likelihood (1)**

*untested

Next, **tritrophic exposure via feeding on herbivore products** or prey that feed on the GM potato plant was evaluated. For none of the remaining four species a significant level of feeding on products produced by herbivores, such as frass, honeydew etc., could be established based on a brief literature and internet-based data search. Hence, all were ranked low for these criteria (Table 14a). Three of the remaining coccinellid predators are known aphid predators. However, an analysis of the likely exposure pathway yielded a number of open questions that will determine whether or not this exposure scenario will be realised or not.

The production of the starch component amylose in the GM amylopecting-potato is suppressed using antisense GM technology. The transformation aimed at altering exclusively the transformation of starch from sugars in the tubers only. Whether or not this tranformation also alters the sugar contents in quantity or quality in the phloem sap, which in turn would be expected to alter the performance of aphids feeding on these plants is currently unknown. Aphids have a fine sensory system for sugar content and composition of their host plants and are known to react to them in their fitness parameters (fecundity, fertility, development time, survival, etc.). Since they are not only pests by themselves but also vectors for important virus diseases, increased or decreased fitness of aphids on GM potatoes are of prime interest in the context of sustainable agricultural production and ecotoxicology as an increased problem with certain aphids would inevitably lead to more pesticide use. Thus, for all aphid feeding coccinellids, exposure to new or altered GM products is determined to be high (Table 14b). For *T. sedecimpunctata,* a high likelihood of exposure arises not only through feeding on pollen and its prey arthropods but also via feeding on fungi living on the GMPs. It is unknown whether or not mildew fungi will exhibit altered fitness on amylopectin potatoes and to what degree they might convey the possible effects of the altered primary metabolite composition of the host plant to their predators (in this case the fungi-feeding *T. sedecimpunctata*). Hence, also this species was ranked high in its likelihood to be exposed to the GM products (Table 14b).

Tab. 14a: Matrix II – Part B: Tritrophic exposure pathways via feeding on herbivore products.

Part B - TRITROPHIC EXPOSURE VIA FEEDING ON HERBIVORE PRODUCTS				**RANK**	**Question**	**RANK**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**Does the predator feed on prey products/excretions (e.g., honeydew, frass, faeces)?**	**Do any of these herbivore products have detectable transgene products or metabolites?**	**Are herbivore products an important part of the predator diet?**	**Is tritrophic exposure via feeding on herbivore products possible?**	**Are transgene product or metabolites detectable in predator after feeding on herbivore products?**	**Does tritrophic exposure via feeding on herbivore products occur?**
Coccinella septempunctata	low likelihood (3)	-	low likelihood (3)	**low likelihood (3)**	-	**low likelihood (3)**
Hippodamia variegata	low likelihood (3)	-	low likelihood (3)	**low likelihood (3)**	-	**low likelihood (3)**
Propylea quatuordecimpunctata	low likelihood (3)	-	low likelihood (3)	**low likelihood (3)**	-	**low likelihood (3)**
Tytthaspis sedecimpunctata	low likelihood (3)	-	low likelihood (3)	**low likelihood (3)**	-	**low likelihood (3)**

Tab. 14b: Matrix II – Part B: Tritrophic exposure pathways via feeding on herbivore prey.

Part B - TRITROPHIC EXPOSURE VIA FEEDING ON PREY				**RANK**	**Question**	**RANK**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**Does the predator feed on prey that feed on the transgenic plant tissues? (see column D)**	**Is the prey likely to be exposed to transgene product or metabolites when eaten by the predator? (consult herbivores group)**	**Is this prey an important part of the predators diet?**	**Is tritrophic exposure via feeding on prey possible?**	**Are transgene product or metabolites detectable in natural enemy after feeding on prey?**	**Does tritrophic exposure occur through prey?**
Coccinella septempunctata	high likelihood (1)	probably (2)*	high likelihood (1)	**high likelihood (1)**	**unnot expected*** **	**high likelihood (1)**
Hippodamia variegata	high likelihood (1)	probably (2)*	high likelihood (1)	**high likelihood (1)**	**not expected*** **	**high likelihood (1)**
Propylea quatuordecimpunctata	high likelihood (1)	probably (2)*	high likelihood (1)	**high likelihood (1)**	**not expected*** **	**high likelihood (1)**
Tytthaspis sedecimpunctata	high likelihood (1)	probably (2)**	high likelihood (1)	**high likelihood (1)**	**not expected*** **	**high likelihood (1)**

* prey feed on phloem sap which might differ in its contents of sugar for GM potatoes. Altered sugar contents in amylopectin potatoes vs. conventional potatoes is unclear

** unclear to what degree such altered sugar composition and contents might also be present in pollen or fungi growing on GM potatoes

***untested

Finally, **exposure via feeding on higher trophic level organisms** including its own species (i.e., cannibalism) was considered (Table 15a). For all coccinellids it was considered to be of low likelihood.

Other impact routes could occur through **changes in feeding behaviour** such as increased or decreased consumption rates which for biocontrol organisms translates into biocontrol efficacy. The conceivable impact of the GM potatoes on herbivores due to the changes in primary metabolite production (sugars and starch(es)), in particular on phloem-feeding aphids (increase or decrease in density or altered nutritional qualities), can lead to changes in the biocontrol efficacy of their primary natural enemies, including coccinellids. Hence, the likelihood of changes in predation and biocontrol efficacy to occur due to GM-trait induced changes in the behaviour of the natural enemies is considered possible and ranked medium (Table 15b).

Lastly, additional exposure to transgene products that are expressed in **recipient plants of gene flow** is considered in the case of the GM amylopectin-potato to be low due to the lack of such recipient plants and the limited pollen-production and pollen-flow of currently cultivated potato varieties (Table 15b).

Tab. 15a: Matrix II – Part B: Higher trophic exposure pathways via feeding on organisms of higher trophic levels.

Part B - HIGHER TROPHC LEVEL EXPOSURE VIA CANNIBALISM OR INTRAGUILD FEEDING				**RANK**	**Question**	**RANK**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**Does the predator cannibalise its own species or eat other intraguild foods (prey that are natural enemies themselves)?**	**Is this species possibly exposed?**	**Are any of the intraguild foods significant food sources for the natural enemy?**	**Is higher trophic level exposure possible via cannibalism or intraguild feeding?**	**Are transgene product or metabolites detectable in the natural enemy after cannibalism or intraguild feeding?**	**Does higher trophic level exposure occur via cannibalism or intraguild feeding?**
Coccinella septempunctata	low likelihood (3)	low likelihood (3)	low likelihood (3)	**low likelihood (3)**	**untested but probably not**	**low likelihood (3)**
Hippodamia variegata	low likelihood (3)*	low likelihood (3)	low likelihood (3)	**low likelihood (3)**	**untested but probably not**	**low likelihood (3)**
Propylea quatuordecimpunctata	low likelihood (3)*	low likelihood (3)	low likelihood (3)	**low likelihood (3)**	**untested but probably not**	**low likelihood (3)**
Tytthaspis sedecimpunctata	low likelihood (3)*	low likelihood (3)	low likelihood (3)	**low likelihood (3)**	**untested but probably not**	**low likelihood (3)**

* probably mainly eggs of own or other coccinellid species

Tab. 15b: Matrix II – Part C: Exposure via changes in feeding behaviours or after gene flow.

Part C - BEHAVIOURAL MODIFICATION OF EXPOSURE			**RANK**	**EXPOSURE AFTER GENE FLOW**	**RANK**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**What feeding preferences or other behaviour could increase or decrease exposure?**	**Does natural enemy avoid eating exposed prey?**	**Are behaviours likely to increase or decrease exposure?**	**Could the predator eat prey on plants that have received the transgene because of gene flow? (see gene flow section)**	**Is exposure via gene flow recipents possible?**
Coccinella septempunctata	if fitness of aphids changes --> immi- or emigration adults	**not tested (?)**	**possible (2)***	low likelihood	**low likelihood (3)**
Hippodamia variegata	if fitness of aphids changes --> immi- or emigration adults	**not tested (?)**	**possible (2)**	low likelihood	**low likelihood (3)**
Propylea quatuordecimpunctata	if fitness of aphids changes --> immi- or emigration adults	**not tested (?)**	**possible (2)**	low likelihood	**low likelihood (3)**
Tytthaspis sedecimpunctata	if fitness of prey or fungi changes --> immi- or emigration adults	**not tested (?)**	**possible (2)**	low likelihood	**low likelihood (3)**

* must be tested

When summarising all partial ranks (Table 16), all species ended with a medium value for overall exposure in this step. Except for *C. septempunctata,* all other coccinellid species were detected in the biodiversity inventory by the applicant in much lower numbers (Table 9). For a full risk assessment, the importance of all species as biocontrol organisms should be validated and their typcial abundance levels confirmed. For the purposes of this study, at minimum *C. septempunctata* was selected as candidate for pre-release testing with a high functional importance and medium likelihood to be exposed to transgene products and metabolites unless it can be experimentally confirmed that no changes in primary and secondary metabolism beyond the tuber occurred.

Tab. 16: Matrix II – Overall estimate of exposure likelihood resulting from all possible pathways.

	RANK BI-TROPHIC	**RANK TRI-TROPHIC VIA HERBIVORE PRODUCTS**	**RANK TRI-TROPHIC VIA PREY**	**RANK HIGHER TROPHIC LEVELS**	**RANK BEHAVIOUR**	**RANK EXPOSURE AFTER GENE FLOW**	**OVERALL EXPOSURE**	**OVERALL EXPOSURE**
Candidate species (retain only the highest ranked species from the previous step (Table 12): here rank 2	**Rank: Does bitrophic exposure occur?**	**Does tritrophic exposure via feeding on herbivore products occur?**	**Does tritrophic exposure occur through prey?**	**Does higher trophic level exposure occur via cannibalism or intraguild feeding?**	**Are behaviours likely to increase or decrease exposure?**	**Is exposure via gene flow recipents possible?**	**AVERAGE RANK VALUES**	**EXPOSURE LIKELIHOOD**
Coccinella septempunctata	low likelihood (3)	low likelihood (3)	high likelihood (1)	low likelihood (3)	mediun likelihood (2)	low likelihood (3)	2.50	medium likelihood
Hippodamia variegata	low likelihood (3)	low likelihood (3)	high likelihood (1)	low likelihood (3)	medium likelihood (2)	low likelihood (3)	2.50	medium likelihood
Propylea quatuordecimpunctata	low likelihood (3)	low likelihood (3)	high likelihood (1)	low likelihood (3)	medium likelihood (2)	low likelihood (3)	2.50	medium likelihood
Tytthaspis sedecimpunctata	low likelihood (3)	low likelihood (3)	high likelihood (1)	low likelihood (3)	medium likelihood (2)	low likelihood (3)	2.50	medium likelihood

3.5.4 Criteria for the selection of ecotoxicological test methods

In the light of additional criteria for the selection of ecotoxicological test methods, the selected species *Coccinella septempunctata* is further discussed focussing on the biological properties of the potential test species (see Chapter 3.3.3 and Table 17). It should be noted that the criterion "wide distribution in the environment" is not evaluated here since it was already covered in the previous chapter as part of the evaluation of ecological relevance for certain regions (i.e., those where it is planned to cultivate the GM amylopectin-potato).

Tab. 17: Evaluation of biological properties of the selected ecologically relevant potential test species in terms of practical testing.

Species	Breeding	Life-cycle	Exposure	Sensitivity	Conditions
Coccinella septempunctata (Appendix C, Table 58)	Yes, easy to medium complex	Several generations per year	Several exposure pathways	High, based on pesticide testing	High temp. (25°C) necessary
NEXT SPECIES					

After the evaluation of the biological properties of *C. septempunctata*, the test method available for the potential test species is evaluated focusing on the practical performance of testing (see Chapter 3.4.2 and Table 18). It should be noted that the criteria "analytical verification of exposure" as well as "animal welfare" are not important in this context. The verification of exposure had already been analysed for *C. septempunctata* in order to reach this step. Animal welfare is not relevant for *C. septempunctata*, it is not an endangered species of the "Red List".

Tab. 18: Evaluation of practical performance of the test available for the selected ecologically relevant potential test species.

Species	Standardisation	Practicability	Applicability	Validity criteria	Experience	Statistics	Ressources
C. septempunctata (Appendix C, Tab. 58)	Yes, by IOBC	Medium complex	Depends on modification	Yes, defined	Very high (IOBC)	Good, medium variabil.	Medium amount
NEXT SPECIES							

Recommendation:

C. septempunctata is a suitable species for the testing of GMPs provided that the appropriate modifications of the test method are made (no pesticide application through spraying but, for example, use of GMP material as a food source; direct: e.g., pollen; indirect: e.g., prey previously exposed to GMP material). In this case this will probably mean, that a largely new testing protocol will have to be developed to ensure the exposure of *C. septempunctata* since the current IOBC guideline tests only at contact exposure of spray applications. This diminishes to a certain extend the advantage of an existing testing guideline. However, some advantages of an already completed standardisation process are the existence of laboratory breeding methods, it's the species' sensitivity to chemical stressors and the existence of validity criteria.

3.5.5 Risk scenarios and adverse effect hypotheses to be tested

From the previous analyses, the following risk scenarios can be derived:

The **impact of the GM amylopectin-potato on its main aphid pests** should be established experimentally, possibly also on other important herbivores like the Colorado potato beetle (*Leptinotarsa decemlineata*). These analyses could not be conducted in this project.

The following risk scenarios are possible:

i) If aphids' fitness increases, they will become more serious pests, which will likely also increase their efficacy in spreading virus diseases.
ii) If aphids' fitness decreases, they will become less serious pests which will likely also decrease their efficacy in spreading virus diseases.

Examples of adverse effect hypotheses to be tested:

1. Aphid fitness is increased.
2. Aphid capability of transmitting virus diseases is increased.

Endpoints to measure include aphid fecundity, fertility, survival and development times. Additionally, their efficacy in spreading important plant viruses should be measured to ensure that their vector capacity is not changed for better or worse even if the aphids seem to be unaffected. Viruses are too serious a disease to be ignored.

Those aphid species exhibiting changes in fitness should subsequently be used for feeding studies with ***C. septempunctata*** aiming at determining their **biocontrol efficacy**.

If aphid fitness increases, their population density increases possibly attracting more coccinellids. Changes in fitness of aphids due to changes in primary or secondary metabolite composition in their host plants might extend to their natural enemies in unexpected ways. This could exacerbate a possible fitness increase of the aphids or vice versa, reenforcing a possible fitness decrease in aphids and allowing more efficient biocontrol unless coccinellids exhibit an altered feeding behaviour on aphids feeding on the GM potatoes.

Examples of an adverse effect hypothesis:

1. *C. septempunctata*'s fitness is decreased.
2. *C. septempunctata* feeds less on aphids raised on GM potato.

None of these hypotheses can be predicted but only measured experimentally.

Endpoints to measure would at minimum include comparative consumption rates in a no-choice and choice situation and relative generational fitness parameters (e.g., survival, reproduction). One experiment might already be enough to reject the adverse effect hypotheses. Possibly several might have to be conducted, including some in a field setting, until the hypotheses can be refuted or confirmed with confidence.

If no aphid species exhibits a change in any fitness parameter when feeding on the GM amyplopectin-potatoes, at least one feeding study with *C. septempunctata* feeding on a selected main virus vector aphid species should be conducted to ensure that no unexpected changes occur at the third trophic level. Such unexpected changes might impact the aphids ability of survival and virus transmission.

Endpoints to measure are the virus transmission rate and success of the aphids with and without the presence of *C. septempunctata*.

In a full risk assessment, all risk scenarios should be developed carefully. Graphical display can greatly facilitate this task. The illustrative application of the proposed species selection procedure on the GM amylopectin-potato is by no means comprehensive. Other biocontrol organisms might turn out to be more important also feeding on aphids and other pest species. However, any documentation of a pre-release risk assessment for regulatory purposes should at minimum contain data supporting a conclusion regarding fitness of aphids, their virus transmission efficacy and biocontrol efficacy of selected natural enemies like coccinellids. A selected natural enemy could likely be *C. septempunctata*, but this species needs to be confirmed by including more data and expertise on main coccinellid species in potato fields in Germany than those from the applicants field surveys.

4 Main challenges of ecotoxicology testing of GMOs in respect to environmental risk assessment

There are a number of obstacles (see Chapter 2) posing significant challenges to the adoption of improved testing methodologies that should be considered by the competent authorities. Potential strategies to overcome these obstacles for an improved environmental risk assessment should be developed pro-actively. If at all possible, potential strategies should involve or aim for an European consensus in order to be effective.

4.1 Adoption by industry

The improvements and adjustments within ecotoxicology testing of GMPs proposed in this report (see Chapter 3) guarantee for a risk assessment according to the state-of-the-art of science and technology. They are essential to ensure that only thoroughly tested and safe GMPs are released to the environment. However, the wide implementation of the improved ecotoxicology testing will strongly depend on their adoption by industry. Industries are likely to adopt innovative methodologies if they can recognize an added value. In this regard it will be helpful to point out the benefits of the improved ecotoxicology testing leading to a higher environmental safety and helping to regain farmers‘ and consumers‘ trust.

So far, the industries are used to the ecotoxicology testing concept based on the chemical testing paradigm now in use since essentially two decades. Advantages of the chemical testing paradigm are:

- longterm experience with testing principles and methodologies
- established procedures and production of testing materials
- harmonized methodologies usable in all countries
- standardised test methods
- focused and known testing regimes
- costs can be estimated with reasonable reliability.

Most of the above mentioned advantages will also apply if the proposed concept for the selection of test species and methods for ecotoxicological testing of GMPs (Chapters 3.3.3) is widely adopted. An additional advantage is that tests species and methods are selected case-specific and on a scientific basis. This will improve the overall risk assessment of GMPs and increase their biosafety. It should be in the interest of all stakeholders involved that the release of GMOs neither leads to direct nor indirect short or long-term environmental effects.

Innovative methodologies – regardless how few and how cost effective – always mean changes in procedure and conceptual understanding requiring training of personell and changes in laboratory handling, all of which may result at least temporarily in uncertainty about costs arising and reliability of outcomes. Thus, industries will not easily adopt innovative methodologies unless all industries are going to be requested to do so and are convinced that it will ease the application procedure on the long run and strengthen the reliability of the outcome of the approval process. Harmonization of the procedures and recommendations at least within the EU will be a prerequisite.

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OECD (1984d): OECD-guideline for testing of chemicals No. 206. Avian reproduction test. - Paris (Organisation for Economic Co-Operation and Development).

OECD (1984e): OECD-guideline for testing of chemicals No. 207. Earthworm acute toxicity test. - Paris (Organisation for Economic Co-Operation and Development).

OECD (1984f): OECD-guideline for testing of chemicals No. 209. Activated sludge, respiration inhibition test. - Paris (Organisation for Economic Co-Operation and Development).

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Glossary

Case:	Crop plant + novel trait and phenotypic characteristic (intended effect) + receiving environment + farming practice (intended use including scale of release)
EC_{50}	Effect concentration, 50%: concentration of a compound where 50% of its effect is observed (term originally used in the environmental risk assessment for chemicals)
ECB	European corn borer. Target organism of transgenic Cry1Ab-corn
ECx/ERx	Effect concentration or effect rate, x% (value not specified; can be between 1 and 99: concentration of a compound where x% of its effect is observed (term originally used in the environmental risk assessment for chemicals)
Ecological function	Role a species or organism group plays in an ecosystem; often expressed in terms of feeding habitats (e.g., predators, herbivores etc.)
Event	Successfully transformed plant cell used to develop a transgenic plant
Functional category	For example, feeding habits organized in groups
Functional groups	Groups of species with, for example, the same feeding habit (e.g., predators)
GMO	Genetically modified organism
GMP	Genetically modified plant
Indicator organism	Organism that signals particular conditions, e.g., whose presence or absence in an environment indicates conditions such as its oxygen level or the presence of a contaminating substance
KP4	Killer protein. Protein expressed by the kp4-gen, inducing fungal resitance
LC_{50}	(Lethal concentration). Median lethal concentration is the concentration of a test substance that is statistically likely to kill 50% of exposed test organisms within a given time period. The LC_{50} is expressed as a mass of test substance per dry mass of the test soil (term originally used in the environmental risk assessment for chemicals)
LOEC	(Lowest observed effect concentration) is the lowest test substance concentration that has a statistically significant effect after a prolonged (subacute, subchronic or chronic) exposure for instance on reproduction ($p < 0.05$), expressed for example in terms of the number of juveniles produced within a given exposure period, when compared with the control. The LOEC is expressed as a mass of test substance per dry mass of the test soil. All test concentrations above the LOEC must have an effect that is statistically different from the control. (term originally used in the environmental risk assessment for chemicals)
NOEC	(No observed effect concentration) is the test substance concentration immediately below the LOEC. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) for example on the number of juveniles produced within a given exposure period when compared with the control (term originally used in the environmental risk assessment for chemicals).
Non-target species	Species not intended to be affected by a plant protection product (PPP; pesticide); in contrast to target (= pest) species

PEC	Predicted environmental concentration (term originally used in the environmental risk assessment for chemicals)
PNEC	Predicted no effect concentration (term originally used in the environmental risk assessment for chemicals)
Pre release testing	Test requirements before the substance to be tested is allowed to be marketed
TER	Toxicity exposure ratio (=toxicity concentration / PEC) (term originally used in the environmental risk assessment for chemicals)
(Hierarchical) tiers	Term used in two meanings: - Stepwise procedure of testing efforts: lowest tier = laboratory; medium tier = semi-field; highest tier: field - Different levels of biological organization: starting from the molecule level to the ecosystem
Trait	In the context of transgenic organism trait refers to the feature(s) of the organism obtained by genetic engineering (e.g., insect resistance, herbicide resistance, etc.)

Appendix A – Compilation of methodologies

Tab. A.1: Methodologies used for ecotoxicity testing for regulatory purposes of GM crop plants.

Study ID	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**2**
Source	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier
GMP	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize
Test organism	*Chrysoperla carnea*	*Chrysoperla carnea*	*Chrysoperla carnea*	*Chrysoperla carnea*	*Chrysoperla carnea*	*Hippodamia convergens*	*Hippodamia convergens*	*Hippodamia convergens*
Trivial	Green lacewing	Green lacewing	Green lacewing	Green lacewing	Green lacewing	Ladybird beetle	Ladybird beetle	Ladybird beetle
Life stage	larvae	larvae	larvae	larvae	larvae	adult	adult	adult
Guideline	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	microbial	microbial	microbial	microbial	microbial	microbial	microbial	microbial
Control	untreated	untreated	untreated	untreated	untreated	untreated	untreated	untreated
Exposition	food	food	food	food	food	food	food	food
Food	*Sitotroga sp.* eggs + water	*Sitotroga sp.* eggs + water	*Sitotroga sp.* eggs + water	*Sitotroga sp.* eggs + water	*Sitotroga sp.* eggs + water	commercial honey	commercial honey	commercial honey
Air temperature	20.9 - 21.3	20.9 - 21.3	20.9 - 21.3	20.9 - 21.3	20.9 - 21.3	26.4 - 26.9	26.4 - 26.9	26.4 - 26.9
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity	54 - 90%	54 - 90%	54 - 90%	54 - 90%	54 - 90%	52 - 92%	52 - 92%	52 - 92%
Soil moisture								
Concentration / Dose	480	480	480	480	480	480	480	480
Unit	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.
Replicates	30	30	30	30	30	3	3	3
Organisms per replicate	1	1	1	1	1	25	25	25
Duration	13	13	13	13	13	29	29	29
Unit	d	d	d	d	d	d	d	d
Observation	mortality	mortality	pupation	behaviour	clinical signs of toxicity	mortality	mortality	behaviour
Endpoint	LC50	NOEC	NOEC	NOEC	NOEC	LC50	NOEC	NOEC
Exact	>	>=	>=	>=	>=	>	>=	>=
Value	480	480	480	480	480	480	480	480
Unit	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.
Reference	Wildlife International Ltd. Project No. 354-115A	Wildlife International Ltd. Project No. 354-115A	Wildlife International Ltd. Project No. 354-115A	Wildlife International Ltd. Project No. 354-115A	Wildlife International Ltd. Project No. 354-115A	Wildlife International Ltd. Project No. 354-113B	Wildlife International Ltd. Project No. 354-113B	Wildlife International Ltd. Project No. 354-113B
Remarks								

Tab. A.1: continued.

Study ID	2	3	3	3	3	4	4	4
Source	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier
GMP	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize
Test organism	*Hippodamia convergens*	*Nasonia vitripennis*	*Nasonia vitripennis*	*Nasonia vitripennis*	*Nasonia vitripennis*	*Eisenia fetida*	*Eisenia fetida*	*Eisenia fetida*
Trivial	Ladybird beetle	Parasitic hymenopteran	Parasitic hymenopteran	Parasitic hymenopteran	Parasitic hymenopteran	Earthworm	Earthworm	Earthworm
Life stage	adult	adult	adult	adult	adult	adult	adult	adult
Guideline	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OPPTS 885.4340	OECD 207	OECD 207	OECD 207
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	microbial	microbial	microbial	microbial	microbial	microbial	microbial	microbial
Control	untreated	untreated	untreated	untreated	untreated	untreated	untreated	untreated
Exposition	food	food	food	food	food	artificial soil	artificial soil	artificial soil
Food	commercial honey	commercial honey	commercial honey	commercial honey	commercial honey	none	none	none
Air temperature	26.4 - 26.9	26.2 - 27.0	26.2 - 27.0	26.2 - 27.0	26.2 - 27.0	20.0 - 20.5	20.0 - 20.5	20.0 - 20.5
Substrate temperature						21.0 - 22.0	21.0 - 22.0	21.0 - 22.0
pH-value						7.75 - 7.87	7.75 - 7.87	7.75 - 7.87
Organic matter						0.1	0.1	0.1
Texture						Sandy	Sandy	Sandy
Rel. air humidity	52 - 92%	59 - 86%	59 - 86%	59 - 86%	59 - 86%			
Soil moisture						31.1 - 32.2% dw	31.1 - 32.2% dw	31.1 - 32.2% dw
Concentration / Dose	480	320	320	320	320	1.7	1.7	1.7
Unit	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	mg a.i./kg	mg a.i./kg	mg a.i./kg
Replicates	3	3	3	3	3	4	4	4
Organisms per replicate	25	25	25	25	25	10	10	10
Duration	29	12	12	12	12	14	14	14
Unit	d	d	d	d	d	d	d	d
Observation	clinical signs of toxicity	mortality	mortality	behaviour	clinical signs of toxicity	mortality	mortality	body weight
Endpoint	NOEC	LC50	NOEC	NOEC	NOEC	LC50	NOEC	NOEC
Exact	>=	>	>=	>=	>=	>	>=	>=
Value	480	320	320	320	320	1.7	1.7	1.7
Unit	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	ppm a.i.	mg a.i./kg	mg a.i./kg	mg a.i./kg
Reference	Wildlife International Ltd. Project No. 354-113B	Wildlife International Ltd. Project No. 354-114D	Wildlife International Ltd. Project No. 354-114D	Wildlife International Ltd. Project No. 354-114D	Wildlife International Ltd. Project No. 354-114D	Wildlife International Ltd. Project No. 354-112	Wildlife International Ltd. Project No. 354-112	Wildlife International Ltd. Project No. 354-112
Remarks								

Tab. A.1: continued.

Study ID	4	4	5	5	6	6	6	7
Source	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier
GMP	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize
Test organism	*Eisenia fetida*	*Eisenia fetida*	*Apis mellifera*	*Apis mellifera*	*Folsomia candida*	*Folsomia candida*	*Folsomia candida*	*Colinus virginianus*
Trivial	Earthworm	Earthworm	Honeybee	Honeybee	Collembolan	Collembolan	Collembolan	Northern bobwhite quail
Life stage	adult	adult	larvae	larvae				juvenile
Guideline	OECD 207	OECD 207	OPPTS 885.4380	OPPTS 885.4380				
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	microbial	microbial	1507 maize pollen	microbial	microbial	microbial	Microbial	1507 maize grain
Control	untreated	untreated	non-genetically modified pollen	untreated				
Exposition	artificial soil	artificial soil	food	food	food	food	food	food
Food	none	none	pollen in 30% sucrose	30% sucrose	brewer's yeast	brewer's yeast	brewer's yeast	grain
Air temperature	20.0 - 20.5	20.0 - 20.5	22.0 - 28.0	22.0 - 28.0				
Substrate temperature	21.0 - 22.0	21.0 - 22.0						
pH-value	7.75 - 7.87	7.75 - 7.87						
Organic matter	0.1	0.1						
Texture	Sandy	Sandy						
Rel. air humidity			27 - 37%	27 - 37%				
Soil moisture	31.1 - 32.2% dw	31.1 - 32.2% dw						
Concentration / Dose	1.7	1.7	2	5.6	79-, 388, 1560-fold	79-, 388, 1560-fold	79-, 388, 1560-fold	100000
Unit	mg a.i./kg	mg a.i./kg	mg Pollen/10 µl sucrose	µg/larvae	field exposure rate	field exposure rate	field exposure rate	mg 1507 maize grain/kg diet
Replicates	4	4	4	4				
Organisms per replicate	10	10	20	20				
Duration	14	14	16	16	28	28	28	5
Unit	d	d	d	d	d	d	d	d
Observation	behaviour	clinical signs of toxicity	adult emergence	adult emergence	mortality	mortality	reproduction	mortality
Endpoint	NOEC	NOEC	NOEC	NOEC	LC50	NOEC	NOEC	LC50
Exact	>=	>=	>=	>=	>	>=	>	>
Value	1.7	1.7	2	5.6	1560-fold	1560-fold	1560-fold	100000
Unit	mg a.i./kg	mg a.i./kg	mg Pollen/10 µl sucrose	µg/larvae	field exposure rate	field exposure rate	field exposure rate	mg 1507 maize grain/kg diet
Reference	Wildlife International Ltd. Project No. 354-112	Wildlife International Ltd. Project No. 354-112	California Agricultural Research Inc. Study No. CAR 172-99	California Agricultural Research Inc. Study No. CAR 172-99	Halliday 1998a	Halliday 1998a	Halliday 1998a	Gallagher et al. 1999
Remarks								

Tab. A.1: continued.

Study ID	7	7	7	7	8	8	8	8
Source	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier	1507 maize dossier
GMP	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize	1507 maize
Test organism	*Colinus virginianus*	*Colinus virginianus*	*Colinus virginianus*	*Colinus virginianus*	*Daphnia magna*	*Daphnia magna*	*Daphnia magna*	*Daphnia magna*
Trivial	Northern bobwhite quail	Northern bobwhite quail	Northern bobwhite quail	Northern bobwhite quail	Water flea	Water flea	Water flea	Water flea
Life stage	juvenile	juvenile	juvenile	juvenile	neonate	neonate	neonate	neonate
Guideline								
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	1507 maize grain	1507 maize grain	1507 maize grain	1507 maize grain	1507 maize pollen	1507 maize pollen	microbial	microbial
Control								
Exposition	food	food	food	food	water	water	water	water
Food	grain	grain	grain	grain				
Air temperature								
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	100000	100000	100000	100000	100	100	100	100
Unit	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg Pollen/L	mg Pollen/L	mg/L	mg/L
Replicates								
Organisms per replicate								
Duration	5	5	5	5	48	48	48	48
Unit	d	d	d	d	h	h	h	h
Observation	mortality	clinical signs of toxicity	body weight	food consumption	mortality	mortality	mortality	mortality
Endpoint	NOEC	NOEC	NOEC	NOEC	LC50	NOEC	LC50	NOEC
Exact	>=	>	>	>	>	>=	>	>=
Value	100000	100000	100000	100000	100	100	100	100
Unit	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg 1507 maize grain/kg diet	mg Pollen/L	mg Pollen/L	mg/L	mg/L
Reference	Gallagher et al. 1999	Gallagher et al. 1999	Gallagher et al. 1999	Gallagher et al. 1999	Drottar & Krueger 1999	Drottar & Krueger 1999	Drottar & Krueger 1999	Drottar & Krueger 1999
Remarks								

Tab. A.1: continued.

Study ID	9	9	10	10	11	11	11	11
Source	MON 810 dossier	MON 810 dossier	MON 810 dossier	MON 810 dossier	Agbios case study	Agbios case study	Agbios case study	Agbios case study
GMP	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize
Test organism	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Colinus virginianus*	*Colinus virginianus*	*Colinus virginianus*	*Colinus virginianus*
Trivial	Honeybee	Honeybee	Honeybee	Honeybee	Northern bobwhite quail	Northern bobwhite quail	Northern bobwhite quail	Northern bobwhite quail
Life stage	adult	adult	larvae	larvae	juvenile	juvenile	juvenile	juvenile
Guideline	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380				
Test substance	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein	B.t.k. HD-1 protein
Expression	microbial	microbial	microbial	microbial	MON 80187 maize grain	MON 80187 maize grain	MON 80187 maize grain	MON 80187 maize grain
Control	untreated	untreated	untreated	untreated	MON 80087 maize grain	MON 80087 maize grain	MON 80087 maize grain	MON 80087 maize grain
Exposition	food	food	water	water	food	food	food	food
Food	50:50 honey:water	50:50 honey:water	protein in water	protein in water	game bird ration and maize meal	game bird ration and maize meal	game bird ration and maize meal	game bird ration and maize meal
Air temperature	24.0 - 27.0	24.0 - 27.0						
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity	30 - 80%	30 - 80%						
Soil moisture								
Concentration / Dose	20	20	20	20	50000, 100000	50000, 100000	50000, 100000	50000, 100000
Unit	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
Replicates	3	3	3	3	1	1	1	1
Organisms per replicate	40	40	50	50	10	10	10	10
Duration	9	9			8	8	8	8
Unit	d	d			d	d	d	d
Observation	mortality	mortality	mortality	mortality	mortality	mortality	body weight	behaviour
Endpoint	LC50	NOEC	LC50	NOEC	LC50	NOEC	NOEC	NOEC
Exact	>	>=	>	>=	>	>=	>=	>=
Value	20	20	20	20	100000	100000	100000	100000
Unit	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
Reference	MRID No. 43439203	MRID No. 43439203	MRID No. 43439202	MRID No. 43439202	MRID No. 45020112	MRID No. 45020112	MRID No. 45020112	MRID No. 45020112
Remarks								

Tab. A.1: continued.

Study ID	11	12	12	13	13	13	14	14
Source	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study
GMP	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize
Test organism	*Colinus virginianus*	*Daphnia magna*	*Daphnia magna*	*Eisenia fetida*	*Eisenia fetida*	*Eisenia fetida*	*Folsomia candida*	*Folsomia candida*
Trivial	Northern bobwhite quail	Water flea	Water flea	Earthworm	Earthworm	Earthworm	Collembolan	Collembolan
Life stage	juvenile	neonate	neonate	adult	adult	adult	juvenile	juvenile
Guideline								
Test substance	B.t.k. HD-1 protein	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin
Expression	MON 80187 maize grain	Bt11 maize pollen	Bt11 maize pollen	microbial	microbial	microbial	MON 810 plant tissue	MON 810 plant tissue
Control	MON 80087 maize grain	non-genetically modified pollen	non-genetically modified pollen	untreated	untreated	untreated	MON 823 plant tissue	MON 823 plant tissue
Exposition	food	water	water	artificial soil	artificial soil	artificial soil	food	food
Food	game bird ration and maize meal			none	none	none	brewer's yeast and lyophilised plant powder	brewer's yeast and lyophilised plant powder
Air temperature							24	24
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	50000, 100000	100	100	200	200	200	0.5, 5, 50	0.5, 5, 50
Unit	ppm	mg Pollen/L	mg Pollen/L	mg/kg	mg/kg	mg/kg	% of diet	% of diet
Replicates	1	3	3	4	4	4	4	4
Organisms per replicate	10	10	10	10	10	10	10	10
Duration	8	48	48	14	14	14	28	28
Unit	d	h	h	d	d	d	d	d
Observation	food consumption	mortality	mortality	mortality	mortality	body weight	mortality	mortality
Endpoint	NOEC	LC50	NOEC	LC50	NOEC	NOEC	LC50	NOEC
Exact	>=	>	>=	>	>=	>=	>	>=
Value	100000	100	100	200	200	200	50	50
Unit	ppm	mg Pollen/L	mg Pollen/L	mg/kg	mg/kg	mg/kg	% of diet	% of diet
Reference	MRID No. 45020112	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study	Agbios case study
Remarks								

Tab. A.1: continued.

Study ID	14	15	15	16	16	16	16	16
Source	Agbios case study	MON 810 dossier	MON 810 dossier	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481
GMP	MON 810 maize	MON 810 maize	MON 810 maize	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn
Test organism	*Folsomia candida*	*Chrysoperla carnea*	*Chrysoperla carnea*	*Gallus domesticus*	*Gallus domesticus*	*Gallus domesticus*	*Gallus domesticus*	*Gallus domesticus*
Trivial	Collembolan	Green lacewing	Green lacewing	Broiler chickens	Broiler chickens	Broiler chickens	Broiler chickens	Broiler chickens
Life stage	juvenile	larvae	larvae					
Guideline								
Test substance	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	MON 810 plant tissue	microbial	microbial	1507 maize grain	1507 maize grain	1507 maize grain	1507 maize grain	1507 maize grain
Control	MON 823 plant tissue	untreated	untreated	non-genetically modified grain	non-genetically modified grain	non-genetically modified grain	non-genetically modified grain	non-genetically modified grain
Exposition	food	food	food	food	food	food	food	food
Food	brewer's yeast and lyophilised plant powder	*Sitotroga sp.* eggs + water	*Sitotroga sp.* eggs + water	commercial cornsoy type ration	commercial cornsoy type ration	commercial cornsoy type ration	commercial cornsoy type ration	commercial cornsoy type ration
Air temperature	24							
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	0.5, 5, 50	16.7	16.7	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03
Unit	% of diet	ppm	ppm	% of diet	% of diet	% of diet	% of diet	% of diet
Replicates	4	30	30	5	5	5	5	5
Organisms per replicate	10	1	1					
Duration	28	7	7	6	6	6	6	6
Unit	d	d	d	w	w	w	w	w
Observation	reproduction	mortality	mortality	mortality	mortality	mean body weight	mean daily weight gain	mean food conversion
Endpoint	NOEC	LC50	NOEC	LC50	NOEC	NOEC	NOEC	NOEC
Exact	>=	>	>=	>	>=	>=	>=	>=
Value	50	16.7	16.7	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03	54.21 - 57.03
Unit	% of diet	ppm	ppm	% of diet	% of diet	% of diet	% of diet	% of diet
Reference	Agbios case study	MRID No. 43468003	MRID No. 43468003	MRID No. 45622001	MRID No. 45622001	MRID No. 45622001	MRID No. 45622001	MRID No. 45622001
Remarks								

Tab. A.1: continued.

Study ID	17	17	17	17	17	17	18	18
Source	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481
GMP	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn
Test organism	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Apis mellifera*	*Danaus plexippus*	*Danaus plexippus*
Trivial	Honeybee	Honeybee	Honeybee	Honeybee	Honeybee	Honeybee	Monarch butterfly	Monarch butterfly
Life stage	larvae	larvae	larvae	larvae	larvae	larvae	larvae	larvae
Guideline	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4380	OPPTS 885.4340	OPPTS 885.4340
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin
Expression	pollen	pollen	pollen	microbial	microbial	microbial	microbial	microbial
Control	control diet	control diet	control diet	control diet	control diet	control diet	control diet	control diet
Exposition	food	food	food	food	food	food	food	food
Food								
Air temperature								
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	64	64	64	640	640	640	10000	10000
Unit	ng Cry1F in 2 mg pollen/larva	ng Cry1F in 2 mg pollen/larva	ng Cry1F in 2 mg pollen/larva	ng Cry1F protein/larva	ng Cry1F protein/larva	ng Cry1F protein/larva	ng/mL	ng/mL
Replicates								
Organisms per replicate								
Duration							7	7
Unit							d	d
Observation	mortality	mortality	behaviour	mortality	mortality	behaviour	mortality	mortality
Endpoint	LC50	NOEC	NOEC	LC50	NOEC	NOEC	LC50	NOEC
Exact	>	>=	>=	>	>=	>=	>	>=
Value	64	64	64	640	640	640	10000	10000
Unit	ng Cry1F in 2 mg pollen/larva	ng Cry1F in 2 mg pollen/larva	ng Cry1F in 2 mg pollen/larva	ng Cry1F protein/larva	ng Cry1F protein/larva	ng Cry1F protein/larva	ng/mL	ng/mL
Reference	MRID No. 45020109	MRID No. 45020109	MRID No. 45020109	MRID No. 45020109	MRID No. 45020109	MRID No. 45020109	MRID No. 45131102	MRID No. 45131102
Remarks								

Tab. A.1: continued.

Study ID	18	19	19	20	20	20	21	21
Source	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	BRAD 006481	MON 810 dossier	MON 810 dossier
GMP	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	Bt Cry1F corn	MON 810 maize	MON 810 maize
Test organism	*Danaus plexippus*	*Eisenia fetida*	*Eisenia fetida*	*Onchorhynchus mykiss*	*Onchorhynchus mykiss*	*Onchorhynchus mykiss*	*Brachymeria intermedia*	*Brachymeria intermedia*
Trivial	Monarch butterfly	Earthworm	Earthworm	Rainbow trout	Rainbow trout	Rainbow trout	Parasitic hymenopteran	Parasitic hymenopteran
Life stage	larvae	adult	adult	juvenile	juvenile	juvenile		
Guideline	OPPTS 885.4340	OECD 207	OECD 207	OPPTS 885.4200	OPPTS 885.4200	OPPTS 885.4200		
Test substance	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1F toxin	Cry1Ab toxin	Cry1Ab toxin
Expression	microbial	microbial	microbial	microbial	microbial	microbial	microbial	microbial
Control	control diet	untreated	untreated	untreated	untreated	untreated	untreated	untreated
Exposition	food	artificial soil	artificial soil	food	food	food	food	food
Food				standard fish diet	standard fish diet	standard fish diet	honey/water	honey/water
Air temperature								
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	10000	2.26	2.26	100	100	100	20	20
Unit	ng/mL	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	ppm	ppm
Replicates		4	4					
Organisms per replicate		10	10					
Duration	7	14	14	8	8	8	30	30
Unit	d	d	d	d	d	d	d	d
Observation	growth	mortality	mortality	mortality	mortality	sublethal effects	mortality	mortality
Endpoint	LOEC	LC50	NOEC	LC50	NOEC	NOEC	LC50	NOEC
Exact	<=	>	>=	>	>=	>=	>	>=
Value	10000	2.26	2.26	100	100	100	20	20
Unit	ng/mL	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	ppm	ppm
Reference	MRID No. 45131102	MRID No. 45020106	MRID No. 45020106	MRID No. 45044201, 46019306	MRID No. 45044201, 46019306	MRID No. 45044201, 46019306	MRID No. 43468004	MRID No. 43468004
Remarks								

Tab. A.1: continued.

Study ID	21	22	22	22	23	23	23	24
Source	MON 810 dossier	MON 810 dossier	MON 810 dossier	MON 810 dossier	NK 603 x MON 810 dossier	NK 603 x MON 810 dossier	NK 603 x MON 810 dossier	NK 603 x MON 810 dossier
GMP	MON 810 maize	MON 810 maize	MON 810 maize	MON 810 maize	NK 603 corn	NK 603 corn	NK 603 corn	NK 603 x MON 810 corn
Test organism	*Brachymeria intermedia*	*Hippodamia convergens*	*Hippodamia convergens*	*Hippodamia convergens*	*Gallus domesticus*	*Gallus domesticus*	*Gallus domesticus*	*Gallus domesticus*
Trivial	Parasitic hymenopteran	Ladybird beetle	Ladybird beetle	Ladybird beetle	Broiler chickens	Broiler chickens	Broiler chickens	Broiler chickens
Life stage								
Guideline								
Test substance	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	Cry1Ab toxin	CP4 EPSPS	CP4 EPSPS	CP4 EPSPS	CP4 EPSPS & Cry1Ab toxin
Expression	microbial	microbial	microbial	microbial	NK 603 maize grain	NK 603 maize grain	NK 603 maize grain	NK 603 x MON 810 maize grain
Control	untreated	untreated	untreated	untreated	non-genetically modified grain	non-genetically modified grain	non-genetically modified grain	non-genetically modified grain
Exposition	food	food	food	food	food	food	food	food
Food	honey/water	honey/water	honey/water	honey/water	corn supplemental with commercial dehulled soybean meal	corn supplemental with commercial dehulled soybean meal	corn supplemental with commercial dehulled soybean meal	corn supplemental with commercial dehulled soybean meal
Air temperature								
Substrate temperature								
pH-value								
Organic matter								
Texture								
Rel. air humidity								
Soil moisture								
Concentration / Dose	20	20	20	20	55 - 60	55 - 60	55 - 60	55 - 60
Unit	ppm	ppm	ppm	ppm	% of diet	% of diet	% of diet	% of diet
Replicates					10	10	10	10
Organisms per replicate					10	10	10	10
Duration	30	9	9	9	42	42	42	42
Unit	d	d	d	d	d	d	d	d
Observation	clinical signs of toxicity	mortality	mortality	clinical signs of toxicity	live weight	feed intake	feed conversion	live weight
Endpoint	NOEC	LC50	NOEC	NOEC	NOEC	NOEC	NOEC	NOEC
Exact	>=	>	>=	>	>=	>=	>=	>=
Value	20	20	20	20	55 - 60	55 - 60	55 - 60	55 - 60
Unit	ppm	ppm	ppm	ppm	% of diet	% of diet	% of diet	% of diet
Reference	MRID No. 43468004	MRID No. 43468005	MRID No. 43468005	MRID No. 43468005	Taylor et al. 2003	Taylor et al. 2003	Taylor et al. 2003	Taylor et al. 2003
Remarks								

Tab. A.1: continued.

Study ID	**24**	**24**
Source	NK 603 x MON 810 dossier	NK 603 x MON 810 dossier
GMP	NK 603 x MON 810 corn	NK 603 x MON 810 corn
Test organism	*Gallus domesticus*	*Gallus domesticus*
Trivial	Broiler chickens	Broiler chickens
Life stage		
Guideline		
Test substance	CP4 EPSPS & Cry1Ab toxin	CP4 EPSPS & Cry1Ab toxin
Expression	NK 603 x MON 810 maize grain	NK 603 x MON 810 maize grain
Control	non-genetically modified grain	non-genetically modified grain
Exposition	food	food
Food	corn supplemente l with commercial dehulled soybean meal	corn supplemente l with commercial dehulled soybean meal
Air temperature		
Substrate temperature		
pH-value		
Organic matter		
Texture		
Rel. air humidity		
Soil moisture		
Concentratio n / Dose	55 - 60	55 - 60
Unit	% of diet	% of diet
Replicates	10	10
Organisms per replicate	10	10
Duration	42	42
Unit	d	d
Observation	feed intake	feed conversion
Endpoint	NOEC	NOEC
Exact	>=	>=
Value	55 - 60	55 - 60
Unit	% of diet	% of diet
Reference	Taylor et al. 2003	Taylor et al. 2003
Remarks		

Appendix B – Ecotoxicological laboratory studies used for the risk assessment of Bt-plants

Tab. B.1: Chronological survey of ecotoxicological laboratory studies on nontarget effects of microbial Bt-formulations and/or genetically modified Bt-plants.

Year	**Nontarget - Herbivores**	**Test substance**		**Effects**	**Natural Enemies**	**Test substance**		**Effects**	**Natural Enemies**	**Test substance**		**Effects**	**Soil organisms**	**Test substance**		**Effects**
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
1995	*Anthonomus grandis* *Diabrotica undecimpunctata* *Leptinotarsa decemlineata* *Myzus persicae* *Apis mellifera* *Blattella germanica* (Sims 1995)		Bt (Cry1Ac)	0 0 0 0 0 0	*Aedes aegypti* *Hippodamia convergens* *Chrysoperla carnea* *Nasonia vitripennis* (Sims 1995)		Bt (Cry1Ac) (bi-troph)	0 0 0 0								
1996				0, --	*Hippodamia convergens* (Dogan et al. 1996)	Bt (CryIII) (tri-troph)		0								
	Apis mellifera (Arpaia 1996)		Bt (Cry3B)	0												

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
1997				--	Coleomegilla maculata *Chrysoperla carnea* *Orius insidiosus* (Pilcher et al. 1997)	Bt (Cry1Ab) Pollen (bi-troph)		0 0 0					*Folsomia Candida* *Oppia nitens* (Yu et al. 1997)	Bt (Cry1Ac and Cry1Ab)		0 0
1998				?	*Chrysoperla carnea* (Hilbeck et al. 1998b)	Bt (Cry1Ab) (tri-troph)		--								
					Chrysoperla carnea (Hilbeck et al. 1998a)		Bt (Cry1Ab) (bi-troph)	--								
	Rhopalosiphum padi (Lozzia et al. 1998)	Bt (Cry1Ab)		0	*Chrysoperla carnea* (Lozzia et al. 1998)	Bt (Cry1Ab) (tri-troph)		0								

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
1998					*Coleomegilla maculata* (Riddick & Barbosa 1998)	Bt (Cry3A) (tritroph)		0								
1999	*Danaus plexippus* (Losey et al. 1999)	Bt (Cry1Ab) Pollen		--	*Chrysoperla carnea* (Hilbeck et al. 1999)		Bt (Cry1Ab, Cry2A) (tritroph)	--	*Cotesia plutellae* (Schuler et al. 1999)	Bt (Cry1Ac) (tritroph)		0				
	Ostrinia nubilalis, Acherontia atropos, Manduca sexta, Autographa gamma, Leptinotarsa decemlineata, Aphis fabae, Macrosiphum avenae (Deml et al. 1999)		CryIA(c) CryIIIA	-- 0,0 --,-- --,-- 0,0 --,0 0,0				--								

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
2000	*Danaus plexippus* (Jesse & Obrycki 2000)	Bt (Cry1Ab) Pollen		--	*Orius tristicolor* *Geocoris punctipes* *Geocoris pallens* *Lygus hesperus* *Nabis* spp. (Armer et al. 2000)	Bt (Cry3) (bi-troph)		0 0 0 0 0								
													Porcellio scaber Mikroben (Escher et al. 2000)	Bt (Cry1Ab)		0; + --
	Papilio polyxenes (Wraight et al. 2000)	Bt (Cry1Ab) Pollen		0, --	*Orius majusculus* (Zwahlen et al. 2000)	Bt (Cry1Ab, tri-troph)		0								

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
2001	*Danaus plexippus* (Hellmich et al. 2001)	Bt (Cry1Ab ; Cry1Ac ; Cry9C ; Cry1F) pollen	Bt (Cry1Ab ; Cry1Ac ; Cry9C ; Cry1F)	0; --	*Orius insidiosus* (Al-Deeb et al. 2001)	Bt (Cry1Ab) (tri-troph)	Bt (Dipel; mixed Cry Toxine) (tri-troph)	0					*Lumbricus terrestris* Nematoden Protozoa Mikroben (Saxena & Stotzky 2001b)	Bt (Cry1Ab) Wurzel-exudate, Pfl.reste		0
	Rhopalosiphum padi (Meier & Hilbeck 2001)	Bt (Cry1Ab)		0	*Chrysoperla carnea* (Meier & Hilbeck 2001)	Bt (Cry1Ab) (tri-troph)		0; --								
	Apis mellifera (Malone et al. 2001)		Cry1BaTI (Aprotinin)	0 --												
	Macrosiphum euphorbiae (Ashouri et al. 2001)	Bt (Cry3A) PI (OCI)		-- 0					*Aphidius nigripes* (Ashouri et al. 2001)	Bt (Cry3A) PI (OCI) (tri-troph)		-- 0				

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
2002	*Pieris brassicae* *Pieris rapae* *Plutella xylostella* (Felke et al. 2002)	Bt (Cry1Ab) Pollen		-- -- --	*Coleomegilla maculata* (Lundgren & Wiedemann 2002)	Bt (Cry3Bb) Pollen (bi-troph)		0								
					Coleomegilla maculata (Duan et al. 2002)	Bt (Cry3Bb1) Pollen (bi-troph)		0								
	Athalia rosae (Howald 2002)	Bt (Cry1Ac)		0	*Orius tristicolor* *Geocoris punctipes* *Zelus renardii* *Nabis* spp. (Ponsard et al. 2002)	Bt (Cry1Ac) (tri-troph)		-- -- 0 0								

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
2002													*Porcellio scaber* (Wandeler et al. 2002)	Bt (Cry1Ab)		0; --
	Nilaparvata lugens (Bernal et al. 2002a)	Bt (Cry1Ab, Cry1Ac)		0	*Cyrtorhinus lividipennis* (Bernal et al. 2002a)	Bt (Cry1Ab, Cry1Ac) (tri-troph)		0	*Parallorhogas pyralophagus* (Bernal et al. 2002b)	Bt (Cry1Ab) (tri-troph)		--, 0				
	Rhopalosiphum padi *Tetranychus urticae* *Spodoptera littoralis* (Dutton et al. 2002)	Bt (Cry1Ab)		0 0 --	*Chrysoperla carnea* (Dutton et al. 2002)	Bt (Cry1Ab) (tri-troph)		0; --								
2003	*Apis mellifera* *Galleria mellonella* (Hanley et al. 2003)	Bt (Cry1Ab Cry1F)		0 --												

Tab. B.1: continued.

Year	Nontarget - Herbivores	Test substance		Effects	Natural Enemies	Test substance		Effects	Natural Enemies	Test substance		Effects	Soil organisms	Test substance		Effects
		GMP	**Protein**		**Predators**	**GMP**	**Protein**		**Parasitoids**	**GMP**	**Protein**			**GMP**	**Protein**	
2003									*Cotesia marginiventris* *Copidosoma floridanum* (Baur & Boethel 2003)	Bt (Cry1Ac) (tritroph)		--				
2004	*Danaus plexippus* (Anderson et al. 2004)	Bt (Cry1Ab) Anthere n		--; 0	*Chrysoperla carnea* (Romeis et al. 2004)		Bt (Cry1Ab) (bitroph)	0								
									Cotesia flavipes (Prütz & Dettner 2004)	Bt (Cry1Ab) (tritroph)		--				
2005	*Tetranychus urticae* (Rovenska et al. 2005)	Bt (Cry3Bb)		--, 0	*Phytoseiulus persimilis* (Rovenska et al. 2005)	Bt (Cry3Bb) (tritroph)		--								

*different from Bernal et al. working on *Nilapavarta lugens* & *Cyrtorhinus lividipennis*

Effects: + = positive; 0 = none; -- = negative

Appendix C – Tabular listing of test methods

C1 Terrestrial laboratory methods

C1.1 Soil microorganisms

Table 1:	**Soil respiration**
Principle:	Influence of chemicals and heavy metals on the abundance, activity and vitality of the microflora in forest soils
Guideline:	Swedish Environmental Protection Agency guideline: Soil Biological Variables in Environmental Hazard Assessment Editor L. Torstensson 'MATS' 1993 (ISO Standard under development)
Test species:	Microorganisms present in the test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	CO_2 evolution (respiration) from unamended soil as well as decomposition of easily degradable growth-sustaining substrate (glucose + ammonium) is monitored every h. From CO_2 evolution data microbial parameters can be obtained: Basal respiration rate, SIR, Lag time (= time from addition of glucose until exponential growth starts); test vessel: appropriate container of a respirometer
Substrate:	Field soil treated according to ISO 10381-6 (2006a); the sub-samples should contain 1 g of organic matter. If mineral soils are used the sub-samples should not be less than 20 g
Parameter:	Basal respiration, substrate induced respiration, lag time
Duration:	ca. 5 d
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 20°C; intrinsic pH of the soil; < 400% of the OM content
Validity criteria:	Ammonium-oxidising activity of soil 200 to 800 ng N/g soil /h
Reference substance:	None
Assessment:	Mean values for each sample. The microbial parameters should be plotted against the concentration of the contaminating substance and evaluated by regression analysis; in tests of chemicals EC10, EC50
Notes:	Test can be used in field- and laboratory studies and is suitable for the Ao or more layer of podzolic forest and arable soils. For mineral soils, complementary studies of suitable moisture content and sample size will have to be made. It can be also used for soils of unknown quality and soils sampled along contamination gradients. In contaminated soils the quotient of basal respiration/substrate induced respiration is much higher and the lag times much longer than in uncontaminated soils.
Testing of GMP?	Soil respiration has been used, but using a different protocol (Hund-Rinke et al. 2004)

Table 2:	**Mineralization and nitrification**
Principle:	Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
Guideline:	ISO 14238 (1997)
Test species:	Microbial organisms present in a test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	The rates or extent of N-mineralization in aerobic soils are determined by measuring the concentrations of ammonium, nitrite and nitrate released during mineralization of nitrogen contained in the soil organic matter, or during mineralization of an added nitrogenous organic compound; test vessel: appropriate container; soil layer < 3 cm.
Substrate:	Field soil treated according to ISO 10381-6 (2006a); 50 to 100 g recommended; or bulk incubation with sub-sampling
Parameter:	Mineralization rate, nitrification rate
Duration:	28 d
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 20 ± 2°C; intrinsic pH of the soil; permanent dark; moisture 40 to 60% WHC or ca. 0.02 MPa suction pressure
Validity criteria:	None
Reference substance:	None
Assessment:	Concentration of mineral N; Inhibitory dose (ID %); regression analysis
Notes:	The International Standard describes laboratory procedures in different soils, or for comparison of N-mineralization in one soil collected at different times of the year. To determine the influence of chemicals on N-mineralization a simplified test design can be used allowing for the establishment of dose-response relationships. The experience on monitoring the soil quality of polluted soils is limited. Care must be taken to collect unpolluted control soil.
Testing of GMP?	No

Table 3:	**Biomass – SIR method**
Principle:	Soil quality – Determination of soil microbial biomass – Part 1: Substrate induced respiration method
Guideline:	ISO 14240-1 (1997)
Test species:	Microbial organisms present in a test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Soil is amended with a series of increasing concentrations of glucose until a maximum respiration rate is reached. From this rate, the active biomass is estimated; test vessel: an appropriate container of a respirometer
Substrate:	Field soil treated according to ISO 10381-6 (2006a)
Parameter:	Respiration / CO_2 evolution
Duration:	6 h
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 22 ± 1°C; intrinsic pH and moisture of the soil
Validity criteria:	None
Reference substance:	None
Assessment:	Soil microbial carbon
Notes:	The International Standard for the determination of microbial biomass offers different incubation systems. ISO 14240-1 gives a method for the estimation of active microbial biomass in soil.
Testing of GMP?	Soil respiration has been used, but using a different protocol (Hund-Rinke et al. 2004)

Table 4:	**Biomass – FE method**
Principle:	Soil quality – Determination of soil microbial biomass – Part 2: Fumigation – extraction method
Guideline:	ISO 14240-2 (1997)
Test species:	Microbial organisms present in a test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Through fumigation of the soil sample, intact microbial cells are lysed and the microbial organic matter released. The organic carbon extracted is determined for fumigated and unfumigated samples. The difference is used to determine microbial biomass; test vessel: glass beaker or petri dish
Substrate:	Field soil treated according to ISO 10381-6 (2006a); 25 to 50 g (dry mass)
Parameter:	Extractable organic carbon
Duration:	22 to 24 h
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 25 ± 2°C; intrinsic pH of the soil; moisture min. 30% WHC
Validity criteria:	None
Reference substance:	None
Assessment:	Soil microbial carbon
Notes:	ISO 14240-2 gives a method for the estimation of microbial biomass of soils by measurement of total biomass of extractable organic material mainly from freshly killed microorganisms. The $CHCl_3$-fumigation also effects soil fauna. But the contribution of carbon from these organisms can be neglected (< 5%) and therefore it is referred to as microbial biomass. The method is applicable to aerobic and anaerobic (e.g. water logged or paddy) soil over the whole range of soil pH. Biomass can be also measured in soils containing actively decomposing substrates and soils supersaturated with K_2SO_4 solution.
Testing of GMP?	No

Table 5:	**Soil Microorganisms: Nitrogen Transformation Test**
Principle:	Long-term effects of chemicals on nitrogen transformation activity of soil microorganisms
Guideline:	OECD 216 (2000a)
Test species:	Microorganisms present in the test soil, carbon content at least 1% of soil total organic carbon
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Incubation of samples in appropriate containers with sufficient headspace to avoid development of anaerobic conditions
Substrate:	One single soil; sand content 50 to 75%; pH: 5.5 to 7.5; Corg 0.5 to 1.5%
Parameter:	Nitrate content
Duration:	28 d, if effect of ≥ 25% extension to max. 100 d; assessment after 0, 7, 14, and 28 d, afterwards every 14 d
Application:	In water or quartz sand (e.g., after evaporation of organic solvent)
Concentration:	For agrochemicals at least two, lower conc. reflects at least the maximum amount expected under practical conditions, higher conc. 5 times the lower conc.; for non-agrochemicals series of at least 5 conc.; at least 3 replicates
Performance:	Temperature 20 ± 2°C; permanent dark; moisture 40 to 60% of WHCmax; soil amended with 5 g/kg soil dw of suitable organic substrate with C/N ratio between 12/1 and 16/1
Validity criteria:	Variation between replicate control samples < 15%
Reference substance:	None
Assessment:	Inhibition ≥ 25%?; calculation of ECx using regression model
Notes:	-
Testing of GMP?	No

Table 6:	**Soil Microorganisms: Carbon Transformation Test**
Principle:	Long-term effects of chemicals on carbon transformation activity of soil microorganisms
Guideline:	OECD 217 (2000b)
Test species:	Microorganisms present in the test soil, carbon content at least 1% of soil total organic carbon
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Incubation of samples in appropriate containers with sufficient headspace to avoid development of anaerobic conditions
Substrate:	One single soil; sand content 50 to 75%; pH: 5.5 to 7.5; Corg 0.5 to 1.5%
Parameter:	Glucose-induced respiration rate
Duration:	28 d, if effect of ≥ 25% extension to max. 100 d; assessment after 0, 7, 14, and 28 d, afterwards every 14 d
Application:	In water or quartz sand (e.g., after evaporation of organic solvent)
Concentration:	For agrochemicals at least two, lower conc. reflects at least the maximum amount expected under practical conditions, higher conc. 5 times the lower conc.; for non-agrochemicals series of at least 5 conc.; at least 3 replicates
Performance:	Temperature 20 ± 2°C; permanent dark; moisture 40 to 60% of WHCmax; addition of 2 to 4 g glucose/kg soil dw per assessment date
Validity criteria:	Variation between replicate control samples < 15%
Reference substance:	None
Assessment:	Inhibition ≥ 25%?; calculation of ECx using regression model
Notes:	-
Testing of GMP?	Soil respiration has been used, but using a different protocol (Hund-Rinke et al. 2004)

Table 7:	**Ammonium oxidation – Rapid test**
Principle:	Soil quality – Ammonium oxidation – a rapid method to test potential nitrification in soil
Guideline:	ISO 15685 (2001)
Test species:	Autotrophic ammonium oxidising bacteria present in the test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Autotrophic ammonium oxidising bacteria in soil are exposed to ammonium sulphate in a soil slurry buffered at pH 7.2. The accumulation rate of the nitrite during 6 h of incubation is taken as an estimate of the activity; test vessel: glass flasks (of appropriate volume) on an oscillating table
Substrate:	Soil slurry; soil treated according to ISO 10381-6 (2006a); 25 g moist soil in 100 ml medium
Parameter:	Rate of ammonium oxidation
Duration:	6 h
Application:	No information available
Concentration:	2 replicates
Performance:	Temperature 25°C; pH approx. 7.2
Validity criteria:	Ammonium-oxidising activity of soil 200 to 800 ng N/g soil /h
Reference substance:	None
Assessment:	Mean, standard dev.; in tests of chemicals EC10, EC50
Notes:	The test is a rapid method to determine the potential rate of ammonium oxidation, the first step in the autotrophic nitrification in nitrifying soils. The measurement can be taken as an assessment of the potential activity of nitrifying populations at the time of sampling. It can be used as a rapid screening test for monitoring of soil quality, and is suitable for testing the effects of both chemical substances in soil and the effects of cultivation methods. Test substances with limited water solubility require special attention.
Testing of GMP?	Yes (Hund-Rinke et al. 2004)

Table 8:	**Laboratory methods for determination of microbial soil respiration**
Principle:	Microbial soil respiration of aerobe, unsaturated soils
Guideline:	ISO 16072 (2002a)
Test species:	Microorganisms present in the test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Various measuring methods possible: measurement of O_2-consumption by electrolytic O_2-generation; determination of CO_2-release by titration; coulometric determination of CO_2-release by gas analyzer; determination of CO_2-release by infrared gas analyzer; determination of CO_2-release by gas chromatograph; determination of soil respiration by measurement of pressure difference
Substrate:	Field soil treated according to ISO 10381-6 (2006a); amount dependent on measuring method
Parameter:	Soil respiration
Duration:	Depending on measuring system
Application:	No information available
Concentration:	Natural soil
Performance:	Constant temperature between 20 and 30°C; pore water pressure -0.01 to -0.03 MPa or 40 to 60% of WHCmax; specific parameters depending on measuring system
Validity criteria:	No information available
Reference substance:	None
Assessment:	Calculation of soil respiration depending on measuring system
Notes:	-
Testing of GMP?	Soil respiration has been used, but using a different protocol (Hund-Rinke et al. 2004)

Table 9:	**Determination of abundance and activity of soil microflora using respiration curves**
Principle:	Determination of the activity of the active aerobe heterotrophic microbial biomass in soils
Guideline:	ISO 17155 (2002b)
Test species:	Microorganisms present in the test soil; pre-incubation for 3 to 4 d at 20°C
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Respiration analyzer, e.g. according to ISO 16072 (2002a)
Substrate:	Field soil treated according to ISO 10381-6 (2006a), characterised by texture, water content, WHC, pH and Corg
Parameter:	Basal soil respiration; SIR; respiration activation quotient; lag-phase, growth rate, time until maximum peak
Duration:	Until occurrence of constant respiration rates; measurement once per h
Application:	In water, in organic solvent or in quartz sand
Concentration:	Natural soil; with chemical 5 concentrations and 3 replicates
Performance:	Temperature: 20°C; pore water pressure -0.01 to -0.03 MPa or 40 to 60% of WHCmax; for SIR addition of substrate consisting of 84% glucose, 14% diammoniumsulfate and 2% KH_2PO_4 (for organic content >5% 0,2 g substrate per g organic substance; for organic content <5% 1 g substrate per 100 g soil soil dw).
Validity criteria:	No information available
Reference substance: None	
Assessment:	Evaluation measurement parameter (higher activation quotient, longer lag-phase, longer time until maximum peak?)
Notes:	-
Testing of GMP?	Yes (Hund-Rinke et al. 2004)

Table 10:	**Dehydrogenase activity in soils – Method using TTC**
Principle:	Determination of dehydrogenase activity of soils with triphenyltetrazolium chloride (TTC)
Guideline:	ISO-Draft guideline 23753-1 (2004c)
Test species:	Microorganisms present in the test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Soil material gets spiked with TTC and incubated in 30 mL test tubes. The released triphenyl formazan (TPF) is extracted with acetone and measured photometrically at 485 nm
Substrate:	Field soil treated according to ISO 10381-6 (2006a); 5 g per test vessel
Parameter:	Release of TPF
Duration:	16 h
Application:	No information available
Concentration:	Natural soil
Performance:	Temperature 25 ± 1°C; natural moisture
Validity criteria:	No information available
Reference substance:	None
Assessment:	Dehydrogenase activity determined by calibration curve
Notes:	-
Testing of GMP?	Dehydrogenase activity has been measured using TTC but following a different protocol (Wu et al. 2004)

Table 11:	**Dehydrogenase activity in soils – Method using INT**
Principle:	Determination of dehydrogenase activity of soils with iodotetrazolium chloride (INT)
Guideline:	ISO-Draft guideline 23753-2 (2004d)
Test species:	Microorganisms present in the test soil
Ecology:	Decomposer: Mineraliser (organic matter); in pore water, on particles
Test design:	Soil material gets spiked with INT and incubated in 30 mL test tubes. The released iodonitrotetrazolium formazan (INTF) is extracted with tetrahydrofuran or acetone and measured photometrically at 485 nm
Substrate:	Field soil treated according to ISO 10381-6 (2006a); 2 to 5 g per test vessel
Parameter:	Release of TPF
Duration:	18 h
Application:	No information available
Concentration:	Natural soil
Performance:	Temperature 30 ± 1°C; natural moisture
Validity criteria:	No information available
Reference substance:	None
Assessment:	Dehydrogenase activity determined by calibration curve
Notes:	-
Testing of GMP?	Dehydrogenase activity has been measured using INT but following a different protocol (Griffiths et al. 2000)

C1.2 Plants

Table 12:	**Effects on various higher plants**
Principle:	Acute laboratory test with various higher plants using several endpoints
Guideline:	EPA Pesticide Assessment Guidelines Subdivision J: Hazard Evaluation: non-target Plants. EPA 540-9-82-020 (1982); (Holst & Ellwanger 1982)
Test species:	10 plant crop species possible (e.g. corn, soy); seeds are taken from commercial seed producers
Ecology:	Primary producers; in arable soils
Test design:	10 seeds or 5 plants per test vessel (not specified)
Substrate:	Natural field soils (characterization: organic content, amount of fine soil particles and pH-value)
Parameter:	Growth of the overground shoot, germination and emergence of the seedlings
Duration:	Germination part of the test: 5 d, emergence or growth: at least after two weeks
Application:	Application of the test substance with a volatile solvent on quartz sand. Mixing of the contaminated sand (after evaporation of the solvent) into the field soil
Concentration:	5 concentrations with 3 replicates
Performance:	Optimal conditions for growth and germination of the selected test species (e.g. growth chambers or greenhouse)
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	EC50 or LC50
Notes:	Also useful for soil quality assessment
Testing of GMP?	No

Table 13:	**Phytotoxicity**
Principle:	Acute laboratory test for early growth stages of higher plants
Guideline:	Method proposal published by BBA (BBA 1984a)
Test species:	*Avena sativa* (L.), oat; *Brassica rapa ssp. rapa*, turnip; seeds are taken from commercial seed producers
Ecology:	Primary producers; in arable soils
Test design:	6 pre-germinated seeds per test vessel (7 cm diameter); worked in approx. 1.5 cm (oat) or 0.5 cm (turnip) depth; pre-germination is done using moist filter paper for 36 to 48 h
Substrate:	Sieved field soil, stored for 14 d at 40% max. WHC; characterization: Org. content < 3%, particles < 20 µm 10 to 20% DW; pH 5 to 7.5
Parameter:	Growth reduction (weight); inhibition of emergence compared to control; phytotoxic effects
Duration:	Harvest of the plants not earlier than 14 d after emergence of 50% of the seedlings in the control (approx. after. 17 d)
Application:	Mixing of the test substance into the field soil (once at the beginning of the test)
Concentration:	No Range-Finding; Definitive Test: 0, 1, 10, 100, 1000 mg/kg DW; 4 replicates each
Performance:	Incubation at 20°C, 16/8 h light-dark cycle, at least 7000 Lux; soil moisture 80% WHCmax with *A. sativa*, 60% WHCmax with *B. rapa*
Reference substance:	No information available
Validity criteria:	Emergence of at least 5 out of 6 control plants; minimum weight per individual control plant 800 mg
Assessment:	Graphical assessment of concentration/response relationship; EC50-determination using standard methods (e.g. Finney 1971); determination of the coefficient of variance
Notes:	Very similar to the OECD Acute Plant Test
Testing of GMP?	No

Table 14:	**"Life cycle"-test using *Arabidopsis thaliana***
Principle:	Sub-lethal laboratory test using the higher plant *Arabidopsis thaliana*
Guideline:	Method proposal from literature (Ratsch et al. 1986)
Test species:	*Arabidopsis thaliana* (Cruciferae), "Ackerschmalwand"; origin not specified
Ecology:	Primary producers; in arable and other soils
Test design:	Seed surfaces have to be sterilised; sowing of at least 5 seeds per test vessel (not specified); ≥ 400 plants per replicate necessary (≈ 1 m²)
Substrate:	Clay mineral Montmorillonit
Parameter:	Number and length of leaves; duration till flowering, length of shouts, reproduction success (others possible)
Duration:	Up to 8 weeks
Application:	Application of the test substance with a volatile solvent on quartz sand. Mixing of the contaminated sand (after evaporation of the solvent) into the field soil
Concentration:	No information available
Performance:	20 to 30°C; light-dark cycle 16/8 h; 300 to 400 µE/s/m²
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	No information available
Notes:	Test substrate is artificial
Testing of GMP?	No

Table 15:	**Growth reduction of *Avena sativa* and *Brassica rapa***
Principle:	Acute laboratory test using oat and turnip
Guideline:	Method proposal for Level 2 of the German Chemical Law (Günther & Pestemer 1990)
Test species:	*Avena sativa* L. (Poaceae); oat; *Brassica rapa* (DC.) Metzg. (Cruciferae); turnip; origin not specified
Ecology:	Primary producers; in arable soils
Test design:	7 seedlings (5 to 6 d old) of *A. sativa* or *B. rapa* per test vessel ("Neubauer-Schale")
Substrate:	Variable: natural field soil (characterization: pH-value, organic content, grain size distribution) or Vermiculite
Parameter:	Growth and bioavailability of the test substance in the soil
Duration:	10 d for *B. rapa* and 14 d for *A. sativa*
Application:	Natural field soil: mixing of the test substance as an aqueous solution in the soil, storage of the treated soil for one night. Vermiculite: Moistening with the aqueous test solution using a semi-automatic system using wicks
Concentration:	9 concentrations with 4 replicates each
Performance:	Temperature: 23°C day/15°C night; light-dark cycle 16/8 h; additional light; moistening is done using a semi-automatic wick system
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Statistical assessment using non-linear regression analysis
Notes:	-
Testing of GMP?	No

Table 16:	**Effects on root elongation**
Principle:	Acute laboratory test using one higher plant
Guideline:	ISO-Guideline 11269-1 (1992)
Test species:	*Hordeum vulgare* (L.), wheat; recommended variety: CV Triumph; other species with "straight" roots are also possible; origin not specified
Ecology:	Primary producers; in arable soils
Test design:	6 seedlings (after pre-germination (approx. 36 to 48 h) on moist filter paper per test vessel (vessels approx. 11 cm high and 8 cm in diameter, filled with approx. 500 g DW soil); planting of the seedlings approx. 1 cm below the soil surface (compaction must be avoided)
Substrate:	Control: washed industrial sand with defined particle size (> 0.6 mm 10%, > 0.2 mm 80%, < 0.2 mm 10%); test and control soils should have a "good quality"; characterization not specified
Parameter:	Determination of root elongation (minimal: 0.5 mm precision)
Duration:	Stop of the test after 5 d
Application:	Variable, according to the exposure situation: e.g. mixing of a water-soluble substance in the soil
Concentration:	No information available; 3 replicates are recommended
Performance:	Temperature: 20 ± 2°C at day, 16°C at night; Light-dark cycle: 12:12 or 16:8 h at 25,000 lumens/m²; soil moisture 70 ± 5% WHCmax
Reference substance:	No information available.
Validity criteria:	No information available
Assessment:	NOEC; statistical comparison of test and control plants (t-test)
Notes:	Also possible for soil quality assessment
Testing of GMP?	No

Table 17:	**Plant-life-cycle bioassay**
Principle:	Sub-lethal laboratory test using higher plants
Guideline:	Method proposal from the literature (Sheppard et al. 1993)
Test species:	*Brassica rapa* (DC.) Metzg. (Cruciferae); turnip; rapid-cycling type)
Ecology:	Primary producers; in arable soils
Test design:	Sowing of 10 seeds per test vessel ("pot"); installation of a passive watering system; after emergence thinning of the number of plants to 5 plants per "pot"
Substrate:	Homogenized and sieved soil; characterisation: pH-value, texture, org. content (without specification)
Parameter:	Emergence, number of flowering plants, height and fresh weight of shouts, number of pod, seed weight
Duration:	After riping of seeds; i.e. after. approx. 40 to 50 d
Application:	No information available. If secondary ions are occurring, their concentration has to be adjusted in the control soil
Concentration:	5 to 7 concentrations with 3 replicates each
Performance:	Constant temperature and light (not specified)
Reference substance:	No information available
Validity criteria:	Control: More than 5 seedlings per vessel; equal reaction of the plants in the treated vessels
Assessment:	NOEL, EC10, EC50; determination visually or graphically; several proposal for suitable statistical methods
Notes:	Especially useful for soil quality assessment
Testing of GMP?	No

Table 18:	**Effects on the growth of seedlings**
Principle:	Acute laboratory test using seedlings of higher plants
Guideline:	ASTM-Guideline E 1598-94 (1994)
Test species:	24 plant crop species, at least 5 species of them have to be tested (in special cases other species are possible); supply from commercial seed producers; storage of the seeds at 4 ± 2°C
Ecology:	Primary producers; in arable soils
Test design:	Variable: e.g. 3 seeds per test vessel (pots made of glass or steel)
Substrate:	Variable: Artificial Soil, natural field soil, quartz sand including nutrient solution or commercially available garden soil (as long as they are suitable for plant growth and are uncontaminated; pH-value 6.0 to 7.5)
Parameter:	Height of the seedlings, dry weight of the overground parts, length of the roots and morphological changes
Duration:	Harvest at the latest 28 d after application (at least 21 d after the emergence of 50% of the seedlings in the control)
Application:	Variable: depending from the exposure pathway, e.g. mixing in the test substrate directly or via quartz sand or spraying of an aqueous solution on the soil surface
Concentration:	Range-Finding-Test: 2 or more concentrations in decimal steps; Definitive Test: at least 5 concentrations; Limit test: 1 concentration; number of replicates not specified
Performance:	Temperature: 20 to 30°C; lighting according to the requirements of the selected test species (> 300 µmol/m²/s), light-dark cycle at least 14:10 h; air humidity approx. 50%; daily watering and horizontal rotation of the test vessels; in general: growth of the plants must be “sufficient”
Reference substance:	No information available
Validity criteria:	Control: Survival of at least 90% and a normal growth of the seedlings
Assessment:	NOEC, LC50 and LC25; the phytotoxicity can be assessed qualitatively
Notes:	Also for soil quality assessment and the testing of waste water possible
Testing of GMP?	No

Table 19:	**Seedling emergence, screening test with lettuce**
Principle:	Effects of contaminated soils or other contaminated samples on the germination of seeds
Guideline:	ISO-Draft Guideline 17126 (2003b)
Test species:	*Lactuca sativa* L. (Asteraceae): lettuce
Ecology:	Primary producers; in arable soils
Test design:	40 seeds per test vessel; lower parts of plastic petri dishes (diameter 15 cm) are used as test containers
Substrate:	100 g per vessel test substrate plus growth medium (washed, fine quartz sand, e.g. with grain size 0.4 to 0.8 mm); 90 g cover material (washed, coarser quartz sand, e.g. with grain size 0.7 to 1.2 mm, possibly 0.8 to 1.4 mm)
Parameter:	Number of emerged seedlings
Duration:	Until complete emergence in control dishes, usually 120 h
Application:	Mixture of test soil with growth medium
Concentration:	At least five mixture ratios with spacing-factor ≤ 2; should include concentrations where 0 and 100% emergence are expected; 3 replicates
Performance:	Temperature: 18 to 24 ± 2 °C; during first 48 h complete darkness; light-dark cycle 16/8 h; 4300 ± 430 Lux fluorescent light with a timer to maintain diurnal cycling; moisture 85% of WHCmax
Reference substance: None	
Validity criteria:	Seedling emergence at least 90%
Assessment:	ECx-calculation using probit analysis or other applicable statistical methods
Notes:	Can also be used for the testing of chemicals
Testing of GMP?	No

Table 20:	**Effects on emergence and growth of higher plants**
Principle:	Acute laboratory test using several endpoints of various higher plants
Guideline:	ISO-Draft Guideline 11269-2 (2004a)
Test species:	One mono- and one dicotyledonous species have to be selected out of a list of 18 plant crop species; origin not specified
Ecology:	Primary producers; in arable soils
Test design:	10 seeds per test vessel (pots with an internal diameter of approx. 8.5 to 9.5 cm; daily control for the maintenance of the different moisture required by the various species
Substrate:	Each soil, which fulfils the following conditions: C_{org} < 1.5% (< 3% org. content), fine particles < 20% DW; pH-value: 5 to 7.5. Natural field soils can be mixed with sand
Parameter:	Emergence and early growth of seedlings; visual assessment of phytotoxic effects (e.g. chlorosis)
Duration:	Stop of the test not earlier than 14 d and not later than 21 d, after 50% of the control seedlings are emerged
Application:	Application of the test substance in water, with a volatile solvent on quartz sand or as dry substance. Mixing of the solution/contaminated sand (after evaporation of the solvent)/substance into the substrate; no surface application
Concentration:	Range-Finding-Test: 0, 1, 10, 100, 1000 mg/kg DW; Definitive Test: sufficient number for the determination of an ECx and/or LOEC ("spacing factor": ≤ 2); 4 replicates each
Performance:	Approval of a "normal" growth, e.g. by using a phytotron: Temperature: variable depending on the species; light-dark cycle 16:8 h with a minimum of 7000 Lux; soil moisture 60 to 80% WHCmax
Reference substance:	Sodium-trichloroacetate or boric acid
Validity criteria:	Control: 7 “healthy” seedlings per vessel should emerge
Assessment:	Statistical determination of NOEC/LOEC or ECx values
Notes:	Also possible for soil quality assessment
Testing of GMP?	No

Table 21:	**Chronic toxicity in higher plants**
Principle:	Effect of (contaminated) soils on higher plants in a chronic laboratory test using oat and turnip
Guideline:	ISO-Draft Guideline 22030 (2004b)
Test species:	*Avena sativa* L. (Poaceae): oat; *Brassica rapa* CrGC syn. Rbr Metzg. (Cruciferae): turnip
Ecology:	Primary producers; in arable soils
Test design:	10 seeds per test vessel, thinned to 8 plants after emergence (pots with a surface of 73.5 cm²), harvest of all but four plants after 14 d
Substrate:	Natural field soils (characterization: pH-value, organic content, grain size distribution, WHCmax, salt content, water soluble content of K, N and P); control: reference or standard soil (organic substance ≤ 5%; particles < 20 µm ≤ 20%) or OECD artificial soil
Parameter:	Growth and biomass after 14 d; growth, biomass and reproduction at test end
Duration:	5 to 6 weeks for *B. rapa* and 7 to 8 weeks for *A. sativa*
Application:	Mixture of test soil with control soil
Concentration:	At least five mixture ratios: 0%, 12.5%, 25%, 50% and 100% or other spacing-factor < 2
Performance:	Temperature: 23 ± 3 °C; light-dark cycle 16/8 h; 13000 ± 2000 lx; pH 5.0 to 7.5; moistening is done using a semi-automatic wick system
Reference substance:	Zinc sulphate, boric acid, or sodium-trichloroacetate
Validity criteria:	At least 75% emergence; growth of "healthy" plants; no more than one dead plant
Assessment:	Statistical determination of NOEC/LOEC; ECx-calculation using logistic regression or probit analysis
Notes:	Can also be used for the testing of chemicals
Testing of GMP?	No

Table 22:	**Soil toxicity using terrestrial plants**
Principle:	Emergence/growth of terrestrial plants exposed to contaminants in soil
Guideline:	EC EPS 1/RM/45 (2005a)
Test species:	5 monocotyledon and 7 dicotyledon crop species
Ecology:	Primary producers; in arable soils
Test design:	Depending on species 5 or 10 seeds/vessel: polypropylene cups (1 L), covered for 7 d or until plants reach top of container
Substrate:	Field-collected soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, and texture) or artificial soil if testing site soils; recommend artificial soil for tests with chemicals or chemical products spiked in soil; identical wet wt, equivalent to a volume of ~500 mL; 350 g dry wt if artificial soil
Parameter:	Number of emerged seedlings at end of test in each test vessel; shoot/root length and dry mass at test end; number of surviving plants at test end showing an atypical appearance
Duration:	Depending on species 14 or 21 d
Application:	Field-collected contaminated soil; if spiking chemical/soil mixtures prepared manually or by mechanical agitation; test substance added as measured quantities in solution (i.e., in water or an organic solvent) or as solid material comprised of the test substance; ensure homogeneity
Concentration:	≥ 5 replicates if single-concentration test; ≥ 4 replicates if multi-concentration test (minimum 9, recommend 11)
Performance:	Moisture: optimal percentage of WHC if field-collected soil, ~70% of WHC if artificial soil; during test, hydrate to saturation; air temperature: daily range, constant 24 ± 3°C; alternatively, day: 24 ± 3°C, night: 15 ± 3°C; humidity ≥ 50%; lighting: full spectrum fluorescent: mimic natural light spectrum; 300 ± 100 µmol/(m²*s) adjacent to the level of the soil surface; photoperiod 16L:8D
Reference substance:	Boric acid
Validity criteria:	Depending on species min. 60-90% emergence; mean survival of emerged control seedlings 90%; mean percentage of control seedlings exhibiting phytotoxicity or anomalies < 10%; depending on species different minimum root and shoot length
Assessment:	Mean percent emergence, length of longest shoots and roots, and dry wt of shoots and roots at test end; multi-concentration test: EC50 for inhibition of emergence; ICx for mean shoot and root length and dry wt of individual plants surviving in each concentration at test end
Notes:	-
Testing of GMP?	No

Table 23:	**Seedling emergence and early growth of higher plants**
Principle:	Terrestrial plant test: seedling emergence and seedling growth test
Guideline:	OECD-Draft Guideline 208 (2005a)
Test species:	32 plant crop species, a number of potential non-crop species are also suggested; origin not specified. The number of species to be tested is dependent on relevant regulatory requirements
Ecology:	Primary producers; mainly in arable soils
Test design:	Sowing of at least 5 undressed seeds per test vessel (size and form not specified)
Substrate:	Field soil with up to 1.5% organic carbon (characterization: type and texture, % organic carbon, pH and salt content) or artificial substrate
Parameter:	Mortality; % emergence and biomass of surviving plants
Duration:	14 to 21 d after emergence of 50% of the control plants
Application:	Application of the test substance in water, with a volatile solvent on quartz sand or as dry substance. Mixing of the solution/contaminated sand (after evaporation of the solvent)/substance into the substrate or surface application
Concentration:	5 concentrations with at least 4 replicates, range-finding or limit test
Performance:	Test conditions should allow a sufficient growth of the plants
Reference substance:	No information available
Validity criteria:	Emergence of at least 70% of the control seedlings; at least 90% survival of emerged control seedlings; the control plants should grow ”normally”
Assessment:	NOEC/LOEC, ECx, ERx; statistical assessment using regression analysis
Notes:	-
Testing of GMP?	No

Table 24:	**Effects on leaves and above-ground portions of plants**
Principle:	Terrestrial plant test: vegetative vigour test
Guideline:	OECD-Draft Guideline 227 (2005b)
Test species:	32 plant crop species, a number of potential non-crop species are also suggested; origin not specified. The number of species to be tested is dependent on relevant regulatory requirements. Plants are grown from seeds to the 2 to 4 true leaf stage before application
Ecology:	Primary producers; mainly in arable soils
Test design:	Sowing of at least 5 undressed seeds per test vessel (size and form not specified)
Substrate:	Field soil with up to 1.5% organic carbon (characterisation: type and texture, % organic carbon, pH and salt content) or artificial substrate
Parameter:	Mortality; biomass of surviving plants
Duration:	21 to 28 d after treatment
Application:	Spraying on plant and leaf surfaces
Concentration:	5 concentrations with at least 4 replicates, range-finding or limit test
Performance:	Test conditions should allow a sufficient growth of the plants
Reference substance:	No information available
Validity criteria:	Emergence of at least 70% of the control seedlings; at least 90% survival of emerged control seedlings; the control plants should grow ”normally”
Assessment:	NOEC/LOEC, ECx, ERx; statistical assessment using regression analysis
Notes:	-
Testing of GMP?	No

C1.3 Nematodes

Table 25:	**Toxicity with nematodes**
Principle:	Acute laboratory test using nematodes
Guideline:	Method proposal from literature (Donkin & Dusenberry 1993)
Test species:	*Caenorhabditis elegans* var. Bristol (strain N2) (Rhabditidae); laboratory mass culture
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	20 worms per test vessel (Pyrex Petri disks (60 * 15 mm) with lid, filled with 7 g sea sand and 3 mL of test solution
Substrate:	Variable: soil solutions made of field soils which have to be adjusted concerning the concentration of several salts
Parameter:	Mortality
Duration:	24 h
Application:	Mixing of the test substance in the test solution (K-medium containing 0.05 M NaCl plus 0.03 M KCl); no other specifications
Concentration:	Variable; 5 replicates
Performance:	Temperature: 20°C; feeding with a bacterial suspension (*Escherichia coli*); microscopical examination at the end of the test after extraction of the nematodes from soil particles using a centrifuge
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	LC50, e.g. using Probit Analysis
Notes:	Up to now the test was only rarely performed. Actually, it has been transformed into ASTM guideline No. E 2172-01 (2001)
Testing of GMP?	No

Table 26:	**Laboratory soil toxicity tests with nematodes**
Principle:	Evaluation the adverse effects of chemicals associated with soil to nematodes
Guideline:	ASTM E 2172-01 (2001)
Test species:	*Caenorhabditis elegans* (Rhabditidae); 3 to 4 d old from age-synchronized laboratory mass culture
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	10 individuals per test vessel (35 x 10 mm petri dishes, made of materials that minimize the sorption and leaching of test compounds and not affect the survival, growth, and reproduction of the test organism adversely), filled with 2.33 g soil and 1.5 mL solution volume
Substrate:	Field-collected soil (characterized by pH, organic matter, CEC, N, texture, moisture)
Parameter:	Mortality
Duration:	24 h
Application:	Field-contaminated soil or spiking according to ASTM E 1676-04 (2004a) and E 1706-05 (2005); 7 d before start of the test the test, control and reference soil is placed into the test containers; 3 replicates
Concentration:	Variable, should bracket predicted LC50 (e.g., according to range-finding test)
Performance:	Temperature 20°C; pH 3.1 to 11.9; no feeding
Reference substance:	No information available
Validity criteria:	≥ 80% recovery; ≥ 90% control survival
Assessment:	Pair wise comparison; EC/LC50, e.g. using Probit Analysis
Notes:	-
Testing of GMP?	No

Table 27:	**Toxic effect of sediment and soil samples on nematodes**
Principle:	Toxicity of environmental samples on growth, fertility and reproduction of *Caenorhabditis elegans*
Guideline:	ISO-Working Draft (Höss 2006)
Test species:	Juvenile *Caenorhabditis elegans* (Rhabditidae) Maupas, 1899 strain N2 from laboratory mass culture
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	10 individuals per test well (12 well multi dish), 0.5 g test material and 0.5 mL food medium per well
Substrate:	Environmental soil and sediment samples, sieved to 2 mm
Parameter:	Growth, fertility and reproduction
Duration:	96 h
Application:	Field-contaminated soil/sediment
Concentration:	3 replicates
Performance:	Temperature: 20 ± 0.5°C; moisture: minimal water content of 40%; food: *Escherichia coli* (OP50; uracile-deficient strain)
Reference substance:	Cadmium chloride
Validity criteria:	≥ 80% recovery; sediment fertility ≥ 80% in control
Assessment:	% inhibition of test parameter in comparison to respective control
Notes:	-
Testing of GMP?	No

Table 28:	**Toxicity test using predatory nematodes**
Principle:	Acute and chronic laboratory test using predatory nematodes
Guideline:	Method proposal from literature (Wilms 1992)
Test species:	*Seinura tenuicaudata* or *Monobutlerius degrissei* (Nematoda); field catches; *Panagrellus redivivus* (Nematoda); laboratory mass culture
Ecology:	Consumer 2. order: Predators (preferably nematods); in soil pore water
Test design:	Acute test: ca. 1000 worms per test vessel (Petri dish (diameter: 5 cm), filled with 3 mL of tap water; Chronic test: unknown number of nematodes per test vessel (plastic pots (diameter: 8 cm), filled with 150 test substrate
Substrate:	Acute test: water; chronic test: field soil/sand mixture (moisture: 20%)
Parameter:	Mortality, population growth
Duration:	Acute test: 24 h; chronic test: 28 d; examination at the end of the test
Application:	Acute test: mixing of the test substance in the test solution (tap water); chronic test: spraying of an aqueous solution of the test substance on the soil surface
Concentration:	Variable; no details given
Performance:	Temperature: 21°C; continuous dark; feeding with other nematodes (*P. redivivus*) only in the chronic test; acute test: microscopical examination of an aliquot (100 individuals); chronic test: counting of the worms after their extraction from soil particles using a centrifuge
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Acute test: LC50; chronic test: comparison between treated and control vessels
Notes:	The test was developed for pesticides, but has been used only rarely so far.
Testing of GMP?	No

Table 29:	**Chronic toxicity test with nematodes**
Principle:	Chronic laboratory test using nematodes
Guideline:	Method proposal from literature (Niemann & Debus 1996)
Test species:	*Panagrellus redivivus* (Panagrolaimidae, Rhabditida); laboratory mass culture
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	Approx. 50.000 ± 3.500 worms per test vessel (Petri disks (5 cm diameter) with lid, filled with 6 to 7 mL of test solution)
Substrate:	Variable: mixture of soil and liquid nutrient agar (ratio 2:1 (percentage of weight))
Parameter:	Abundance
Duration:	7 d
Application:	Mixing of the test substance in the test soil
Concentration:	Variable; 3 replicates
Performance:	Temperature: 21 ± 2°C in the dark; microscopical examination at the end of the test after extraction of the worms using a modified wet extraction method
Reference substance:	No information available
Validity criteria:	The reproduction factor (VF = number of worms at the end divided by the number at the start) should be between 2.0 and 5.0
Assessment:	VF = 1.0 to 2.0: moderately toxic; VF = 0 to 1.0: strongly toxic
Notes:	Up to now only rarely used (e.g. Lindane and PCB 52); also for soil quality assessment possible.
Testing of GMP?	No

Table 30:	**"Life-history-strategy" test using nematodes**
Principle:	Sub-lethal laboratory test using nematodes
Guideline:	Formal method proposal from literature (Kammenga et al. 1996)
Test species:	*Plectus acuminatus* (Bastian, 1865); (Plectidae); laboratory mass culture on agar plates
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	100 adult *P. acuminatus* females (4 weeks old) per test vessel (6 cm Petri disks with 5.0 g DW Artificial Soil)
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water; 70% moisture DW, pH-value 5.5
Parameter:	Ratio between the number of juveniles and adults at the end of the test
Duration:	3 weeks
Application:	Depending on the solubility of the test substance: as an aqueous solution, using organic solvents or mixing with quartz sand
Concentration:	Not specified in detail; for example 5 concentrations, but not higher than 1000 mg/kg (spacing factor: 3.2); 2 replicates
Performance:	Incubation at 20°C; in the dark; feeding with soil bacteria (*Acinetobacter johnsonion* ($2*10^9$ cells/g) at the start of the test; counting is done by a combination of wet extraction and sieving using 45 µm sieves
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	NOEC using ANOVA; EC50 using non-linear regression analysis
Notes:	Residue analysis of the test substance in Artificial Soil and/or pore water is recommended.
Testing of GMP?	No

Table 31:	**Sub-lethal effects on the competition behaviour of two nematode species**
Principle:	Sub-lethal laboratory test using two nematode species
Guideline:	Formal method proposal from literature (Kammenga & Riksen in: Løkke & Van Gestel 1998)
Test species:	*Plectus acuminatus* (Bastian 1865); *Heterocephalobus pauciannulatus* (Marinari, 1967); (Plectidae); laboratory cultures with 2- 3 weeks of acclimatisation
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	50 *P. acuminatus* (21 d old) and 10 *H. pauciannulatus* (8 d old) - only parthenogenetic females - per test vessel (30 mm Petri disks)
Substrate:	Artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water; 75% of the WHC_{max}
Parameter:	Ratio of the number of the two species at the end of the test
Duration:	2 weeks
Application:	Depending on the solubility of the test substance: as an aqueous solution, using organic solvents or mixing with quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg; Definitive Test: 5 concentrations, but not higher than 1000 mg/kg; 5 replicates
Performance:	Temperature: 20 ± 1°C; permanent dark; feeding with soil bacteria (*Acinetobacter johnsonion* ($2*10^8$ cells/g)); permanent addition; extraction is done by a modified Oostenbrink-method; sieving with 1000 µm and 25 µm sieves
Reference substance:	No information available
Validity criteria:	Control: population growth rate equal or higher than 6; ratio between *P. acuminatus*/*H. pauciannulatus*: 1/5.
Assessment:	EC50, EC10 and LC50
Notes:	One of the few examples of a two-species test laboratory system.
Testing of GMP?	No

C1.4 Earthworms

Table 32:	**Sub-lethal toxicity test with the earthworm *Aporrectodea caliginosa***
Principle:	Sub-lethal laboratory test with several endpoints using an ecologically important earthworm species
Guideline:	Formalised guideline proposal (Kula & Larink in Løkke & Van Gestel 1998)
Test species:	*Aporrectodea caliginosa* (Savigny, 1826); (Lumbricidae); some worms should be kept in alcohol to allow species verification; laboratory culture; acclimatisation period: 1 to 7 d; if this period is elongated, the use of field collected animals seems to be possible
Ecology:	Decomposer: Destruents (dead organic matter); in mineral soil (endogeic)
Test design:	6 adult worms per plastic container (1 L), filled with 500 g DW of soil (depth: 5 to 6 cm)
Substrate:	Standard Lufa field soil 2.2; organic content: ≈ 4%; pH: 6.0 ± 0.5; moisture 60% WHCmax
Parameter:	Mortality, biomass, morphological and behavioural changes of the adult worms; number of cocoons at the end of the test
Duration:	Min. 4 weeks after application; examination at end of the test period
Application:	According to solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 g quartz sand
Concentration:	Preliminary test: 0, 1, 10, 100 and 1000 mg/kg DW (one replicate); Final Test: several concentrations (spacing factor < 2) plus control (4 replicates); not higher than 1000 mg/kg DW
Performance:	Temperature: 15 ± 1°C; without light; constant moisture; feeding with finely ground cattle manure (0.5 g/worm/week); cocoons are selected using a "suitable" method whereas adult worms are sorted out by hand
Reference substance:	Benomyl or Carbendazim: 2.0 to 2.7 mg/kg a.i./kg DW should reduce the number of cocoons produced by at least 30%
Validity criteria:	Control: mortality after 14 d ≤ 10%; decrease of biomass ≤ 20%; at least 18 cocoons per test vessel at the end of the test; coefficient of variance of cocoon numbers < 50%
Assessment:	Determination of LOEC, NOEC, EC10 and EC50 using suitable statistical methods
Notes:	The low reproduction rate of this ecologically very important species (a world-wide distributed soil dweller) is a problem, since results gained with field collected animals might be difficult to compare.
Testing of GMP?	Yes (similar protocol; Vercesi et al. 2006)

Table 33:	**Earthworm, acute mortality test**
Principle:	Acute laboratory test using compost worms (Oligochaeta)
Guideline:	OECD 207 (OECD 1984a); related guidelines show only slight modifications (e.g. BBA 1984, NEN 1988, ISO 1993)
Test species:	*Eisenia fetida* (Savigny, 1826) or *E. andrei* Bouché, 1972; compost worm (Lumbricidae); laboratory mass culture
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	10 worms per test vessel (e.g. 2 L glass); approx. 3 months old (300 - 600 mg FW)
Substrate:	Artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Mortality, biomass, morphological and behavioural changes
Duration:	2 weeks; examinations after 0, 7 and 14 d after application
Application:	According to the solubility of the test substance: Dissolved in water or, if not water-soluble, mixing with quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Final Test: 5 concentrations (spacing factor up to 1.8), but not higher than 1000 mg/kg DW
Performance:	temperature: 20 ± 2°C; moisture: 40 to 60% of the WHC_{max}; permanent light (400 to 800 Lux); pH: 6.0 ± 0,5; no feeding
Reference substance:	Chloroacetamide (LC50: 20 - 40 mg/kg DW according to literature; 20 to 80 mg/kg DW according to ISO)
Validity criteria:	Control: mortality after 14 d ≤ 10%
Assessment:	Determination of the LC50, e.g. using Probit Analyse
Notes:	A filter-paper-test, described in the same guideline, is not used anymore.
Testing of GMP?	Yes (Ahl Goy et al. 1995)

Table 34:	**Earthworm sub-acute toxicity test**
Principle:	Extended laboratory test using earthworms (Oligochaeta)
Guideline:	US Food + Drug Administration 4.12 (FDA 1988)
Test species:	*Eisenia fetida* (Savigny, 1826), compost worm (Lumbricidae); other species such as *Lumbricus terrestris* or *L. rubellus* are also possible; the latter are rarely used since they cannot obtained from mass cultures
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic), also in vertical burrows of mineral soil (anecique)
Test design:	10 adult worms per test vessel (e.g. 2.5 L glass); approx. 2 months old (300 to 600 mg FW)
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water; pH: approx. 7.0; moisture: 25% DW
Parameter:	Mortality, biomass, morphological and behavioural changes (daily documentation) of the worms and analytical determination of the actual concentration of the test substance in soil
Duration:	28 d; examinations after 0, 7, 14, 21 and 28 d after application
Application:	Homogenous mixing of the test substance either using water or quartz sand; preferably together with the food
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Final Test: 5 concentrations (spacing factor up to 1.8)
Performance:	Temperature: 20 ± 2°C (13 ± 2°C in the case of *L. terrestris*); 24/0 h light-dark cycle (400 to 800 Lux); feeding with dried, finely ground cattle manure.
Reference substance:	Chloroacetamide
Validity criteria:	Control: mortality after 14 d ≤ 10%
Assessment:	Determination of LC50 (e.g. with Probit Analysis) and NOEC biomass (e.g. using ANOVA)
Notes:	The residue analysis of the test substance in Artificial Soil, i.e. by using radioactive labelled substances, is required.
Testing of GMP?	*E. fetida* and *L. terrestris* have been used, but different protocols (Ahl Goy et al. 1995; Saxena & Stotzky 2001; Zwahlen et al. 2003; Hund-Rinke et al. 2004)

Table 35:	**Earthworm sub-chronic toxicity test**
Principle:	Extended laboratory test using earthworms (Oligochaeta)
Guideline:	EPA OPPTS 850.6200 (1996a)
Test species:	*Eisenia fetida andrei* (Bouche, 1972), compost worm (Lumbricidae); acclimated for a minimum of 7 d prior to testing
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	10 adult worms per test vessel (e.g. 1 pt glass canning jars filled with 200 g DW Artificial Soil); with a biomass of 300 to 600 mg FW
Substrate:	Artificial Soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water; pH: 6.5 ± 0.5; moisture: 35% DW
Parameter:	Mortality, biomass, behavioural and pathological symptoms (daily documentation) of the worms; analytical determination of the actual concentration of the test substance in soil (only Definitive Test; in each test vessel after 0, 7, 14, 21, and 28 d as minimum); determination of total carbon (TC)
Duration:	28 d for Range-Finding and Definitive Test, respectively; examinations 0, 7, 14, 21 and 28 d after application
Application:	Homogenous mixing of the test substance either using water or quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: 5 or more concentrations (spacing factor 1.5 to 2.0); at least 3 replicates
Performance:	Temperature: 20 ± 2°C; relative humidity above 85%; continuous light (400 Lux measured at the soil surface); all parameters should be recorded weekly; no feeding
Reference substance:	No information available
Validity criteria:	Control: mortality after 28 d > 20%; decline of mean weight > 30%
Assessment:	Determination of LC50, EC50 and NOEC, LOEC (biomass) by using appropriate statistical methods (e.g. Probit Analysis, ANOVA, moving average and binomial, including 95% confidence intervals)
Notes:	One of the few terrestrial tests in which the residue analysis of the test substance in Artificial Soil, i.e. by using radioactive labelled substances, is required. If in the Range-Finding Test the LC50 > 1000 mg/kg DW, the Definitive Test does not have to be done. The worms may be harmed since they are not fed during the test period.
Testing of GMP?	*E. fetida* has been used, but different protocols (Ahl Goy et al. 1995; Hund-Rinke et al. 2004)

Table 36:	**Sub-lethal effects on earthworms**
Principle:	Sub-lethal laboratory test with various endpoints using earthworms
Guideline:	Method proposal in the literature (Gibbs et al. 1996)
Test species:	*Eisenia fetida* (Savigny, 1826); compost worm (Lumbricidae); laboratory mass culture
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	Two worms per test vessel (punctured plastic bags (approx. 16.5 * 15 cm); filled with 100 g DW of test substrate)
Substrate:	Artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Mortality, biomass, morphological and behavioural changes of the adult worms; number of cocoons and of hatched juvenile worms
Duration:	2 months; examinations after 21 and 56 d after application
Application:	According to the solubility of the test substance; no further details given
Concentration:	No information available; 20 to 30 replicates
Performance:	Incubation at 20 ± 2°C; light-dark cycle 12:12 h; feeding with fermented Alfalfa-meal (commercially available; 2.0 g/test vessel). Adult worms are counted and weighed (DW determination after removal of gut content) after three weeks. Juveniles (by heat extraction) and cocoons (by suspension and hand-sorting) are counted after additional 35 d.
Reference substance: Chloroacetamide is possible	
Validity criteria:	No information available
Assessment:	Determination of NOEC and LC50 using the "Spearman-Karber" method or ANOVA
Notes:	Also useful for soil quality assessment
Testing of GMP?	*E. fetida* has been used, but different protocols (Ahl Goy et al. 1995; Hund-Rinke et al. 2004)

Table 37:	**Reproduction and growth test with the earthworm *Eisenia fetida***
Principle:	Sub-lethal laboratory test using earthworms
Guideline:	ISO 11268-2 (1998); related guidelines show only slight modifications, e.g. BBA 1994b; Kula & Larink in Løkke & Van Gestel 1998; OECD 222 (2004)
Test species:	*Eisenia fetida* (Savigny, 1826) or *E. andrei* Bouché, 1972; compost worm (Lumbricidae); synchronized laboratory mass culture
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	10 worms per test vessel (e.g. Bellaplast container); 2 to 12 months old (250 to 600 mg FW); acclimatisation period: 1 to 7 d
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water (or Standard field soil Lufa 2.2)
Parameter:	Mortality, biomass, morphological and behavioural changes of the adult worms; number of juveniles at the end of the test
Duration:	2 months; examinations 0, 28 and 56 d after application
Application:	Spraying of the test substance on top of the test substrate (including worms) using a laboratory spraying advice (BBA) or by mixing (ISO)
Concentration:	Pesticides: 1 * and 5 * of the maximum recommended application rate; other chemicals: Range-Finding-Test: 0, 1, 10, 100 and 1000 mg/kg DW (one replicate); Final Test: several concentrations (spacing factor < 2) (4 replicates); not higher than 1000 mg/kg DW
Performance:	Temperature: 20 ± 2°C; light-dark cycle 8:16, 12:12 or 16:8 h at 400 to 800 Lux; feeding with finely ground cattle manure; extraction of the juveniles by means of a water bath, by sieving or via hand-sorting.
Reference substance:	BBA: Carbendazim or Benomyl (concentration according to 750 g a.i./ha); ISO: LOEC of Carbendazim (1 to 5 mg a.i./kg soil DW)
Validity criteria:	Control: mortality after 14 d ≤ 10%; decrease of biomass ≤ 20%; at least 30 juveniles per test vessel at the end of the test; coefficient of variation of average juvenile numbers < 50% (BBA) or 30% (ISO)
Assessment:	Evaluation with "suitable" statistical methods (e.g. Williams-Test)
Notes:	A detailed description of the test development and the results of two ringtests is given in Riepert & Kula (1996). In the ISO guideline adaptations for soil quality assessment are described.
Testing of GMP?	Yes (Hund-Rinke et al. 2004)

Table 38:	**Laboratory soil toxicity test with a lumbricid earthworm**
Principle:	Acute and sub-lethal laboratory test using earthworms
Guideline:	ASTM E 1676-04 (2004a)
Test species:	*Eisenia fetida* (Savigny, 1826), compost worm (Lumbricidae); preferably the subspecies *Eisenia fetida andrei* (Bouche, 1972); laboratory mass cultures; theoretically other lumbricid species are also possible but no specific information is given for these
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	10 adult clitellate worms per test vessel (e.g. glass 473 mL canning jars filled with 200 g DW soil)
Substrate:	Variable: e.g. artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water (pH = 7.0 ± 0.5) or field soils, e.g. from potentially contaminated sites or from reference sites (mixtures of field soil with artificial or reference soils are possible); field soil characterization: pH-value, cation exchange capacity, percent organic matter, total nitrogen, texture, percent water content
Parameter:	Mortality, biomass, morphological and behavioural changes of the worms (growth and reproduction can be used in long-term tests); analytical determination of actual concentration of the test substance in soil
Duration:	Variable: 7 to 28 d (typically 14 d); e.g. examinations after 0, 7, 14, 21 and 28 d after application; if growth and reproduction are used as endpoints, the duration has to be extended
Application:	Variable: Homogenous mixing of the test substance either using water or quartz sand
Concentration:	Range-Finding-Test (not specified); Final Test: at least 5 concentrations and a negative control with at least 3 replicates
Performance:	Temperature: 19 to 15°C; 24/0 h light-dark cycle with at least 400 Lux; moisture level: 35 to 45% DW of soil; no feeding in tests with a duration of less than 28 d
Reference substance:	No information available (reference soils which represent the test soil as much as possible can be used in soil quality assessment)
Validity criteria:	Mean survival > 90% in the control
Assessment:	Determination of LC50 (e.g. using Probit Analysis) and NOEC biomass (e.g. using ANOVA)
Notes:	This guideline is in many respects very general; e.g. it is suited for determining chemical toxicity as well as for soil quality assessment.

Testing of GMP?	*E. fetida* has been used, but different protocols (Ahl Goy et al. 1995; Hund-Rinke et al. 2004)

Table 39:	**Lethality test for soil toxicity using earthworms**
Principle:	Test for toxicity of contaminated soil to earthworms
Guideline:	EC EPS 1/RM/43 (2004)
Test species:	*Eisenia andrei*, *E. fetida*, or *Lumbricus terrestris*; juveniles or sexually mature adults with clitellum; if *E. andrei/fetida*, individual wet wt 250-600 mg; if *L. terrestris*, individual wet wt 3 to 10 g
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic), also in vertical burrows of mineral soils (anecique)
Test design:	5 worms/test chamber if *E. andrei/fetida* and 3 worms/test chamber if *L. terrestris*; test chamber 500-mL jar; perforated translucent or transparent cover, secured with a rubber band recommended as cover
Substrate:	Field-collected soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, and texture) or artificial soil if testing site soils; recommend artificial soil for tests with chemicals spiked in soil; identical wet wt, equivalent to a volume of ~350 mL; 200 g dry wt if artificial soil
Parameter:	No. of live worms in each test chamber on d 0, 7 (optional), and 14; no. of worms seen on surface of soil in each test chamber on d 0 (i.e., at 1 h), 7 (optional), and 14; pathological symptoms or distinct behavioural abnormalities for worms on d 0, 7 (optional), and 14
Duration:	14 d
Application:	Field-collected contaminated soil or soils spiked with a chemical; test substance may be added as measured quantities in solution (i.e., in water or an organic solvent) or as solid material comprised partly or completely of the test substance; ensure homogeneity
Concentration:	Single or multiple concentrations; ≥ 5 replicates if single-concentration test; ≥ 3 replicates if multi-concentration test using *E. andrei/fetida*; ≥ 5 replicates if multi-concentration test using *L. terrestris*
Performance:	Moisture: hydrate to the optimal percentage of WHC if field-collected soil, or to ~70% of WHC if artificial soil; temperature: daily average, 20 ± 2°C; instantaneous, 20 ± 3°C; lighting: incandescent or fluorescent; intensity, 400 to 800 Lux adjacent to surface of soil in test chamber; fixed photoperiod (e.g., 16L:8D or 12L:12D); do not feed
Reference substance:	Boric acid
Validity criteria:	invalid if mean 14-d survival in control soil < 90%
Assessment:	Percent survival on d 7 (optional) and 14; 7-d LC50 (optional) and 14-d LC50 if multi-concentration test
Notes:	-

Testing of GMP?	*E. fetida* has been used, but different protocols (Ahl Goy et al. 1995; Hund-Rinke et al. 2004)

Table 40:	**Effects of prolonged exposure to contaminated soil on the survival, reproduction, and growth of earthworms**
Principle:	Test for toxicity of contaminated soil to earthworms
Guideline:	EC EPS 1/RM/43 (2004)
Test species:	Cultured *Eisenia andrei*, sexually mature adults with clitellum; individual wet wt 250-600 mg; one week acclimatization in control soil
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	5 worms/test chamber; test chamber 500-mL jar; perforated translucent or transparent cover, secured with a rubber band recommended
Substrate:	Field-collected soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, and texture) or artificial soil if testing site soils; recommend artificial soil for tests with chemicals spiked in soil; identical wet wt, equivalent to a volume of ~350 mL; 200 g dry wt if artificial soil
Parameter:	No. of live adult worms on d 0 and 28; presence of uneaten food on the soil surface at each feeding date; no. of live juvenile worms on d 56; obvious pathological symptoms or distinct behavioural abnormalities
Duration:	≥ 56 d
Application:	Field-collected contaminated soil or soils spiked with a chemical; test substance added as measured quantities in solution (i.e., in water or an organic solvent) or as solid material comprised of the test substance; ensure homogeneity
Concentration:	Single or multiple concentrations; 10 replicates
Performance:	Moisture: optimal % of WHC if field-collected soil, or ~70% of WHC if artificial soil; temperature: daily average, 20 ± 2°C; instantaneous, 20 ± 3°C; lighting: incandescent or fluorescent; intensity, 400 to 800 Lux; fixed photoperiod (e.g., 16L:8D or 12L:12D); food: cooked oatmeal; 5 mL per test chamber each feeding on d 0, 14, 28, and 42
Reference substance:	Boric acid
Validity criteria:	Invalid if: mean 28-d survival in control < 90%; mean reproduction rate for adults in control < 3 live juveniles/adult; invalid if mean dry wt of individual live juveniles in control soil at test end < 2.0 mg
Assessment:	Mean percent survival of adults on d 28; mean number and dry wt of live juveniles on d 56; if multi-concentration test: 28-d LC50 for adult worms, ICx for numbers of live juveniles produced during 56-d test, ICx for mean dry wt of individual worms surviving at test end
Notes:	-

Testing of GMP?	*E. fetida* has been used, but using different protocols (Ahl Goy et al. 1995; Hund-Rinke et al. 2004)

Table 41:	**Avoidance-response test using earthworms**
Principle:	Sub-lethal laboratory test with a behavioural endpoint using earthworms
Guideline:	Method proposal in the literature (Stephenson et al. 1998)
Test species:	*Eisenia fetida* (Savigny, 1826); compost worm (Lumbricidae); laboratory mass culture or *Lumbricus terrestris* (Linne, 1756; night crawler (Lumbricidae); laboratory keeping after purchase
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic), also in vertical burrows of mineral soils (anecique)
Test design:	1, 5 or 10 worms per testing apparatus (circular plexiglass unit consisting of a (soil-free) central chamber and six pie-shaped chambers, all connected to each other (outer diameter: 25.5 cm), the six were filled with approx. 300 g DW test substrate (contaminated or spiked soil)
Substrate:	Potentially contaminated or spiked field soils or reference soils (Artificial Soil according to OECD (1984a) can be used as negative control); soils have to be characterized (minimum not specified)
Parameter:	Stay of the worms at the end of the test; mortality,
Duration:	Variable; 24 h up to 21 d; examination only at the end of the test
Application:	Variable; e.g. dilution series between contaminated and reference soils; spiking of artificial or reference soils is also possible (no details given)
Concentration:	Diluted soils: 0 to 100% of test soil or spiked soil (no details given)
Performance:	Incubation at 20 ± 2°C (*E. fetida*) or 15 to 18°C (*L. terrestris*); moisture: approx. 70% of WHC_{max} (= 30 to 40% DW soil); a light is placed above the central chamber; no feeding; determination of the stay of the worms after separation of the individual chambers
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	LC50 determination by Spearman-Karber methods; LOAEL/NOAEL for lethality by ANOVA; otherwise it was checked whether the distribution of the worms among the chambers is random using chi-square procedures
Notes:	Mainly useful for soil quality assessment (comparable test idea: Hund 1998)
Testing of GMP?	*E. fetida* has been used, but different protocol (Hund-Rinke et al. 2004)

Table 42:	**Acute sublethal test for the effect of contaminated soil on the avoidance behaviour of earthworms**
Principle:	Test for toxicity of contaminated soil to earthworms
Guideline:	EC EPS 1/RM/43 (2004)
Test species:	*Eisenia andrei*, *E. fetida*, or *Lumbricus terrestris*; sexually mature adults with clitellum; individual wet wt ranging within 250 to 600 mg if *E. andrei*, 250 to 800 mg if *E. fetida*, or 3 to 10 g if *L. terrestris*
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic), also in vertical burrows of mineral soils (anecique)
Test design:	10 worms per test unit: circular container with central chamber (ID, ~5.4 cm) and six pie-shaped interconnecting compartments, fitted lid; stainless steel or plexiglass; OD, ~23 cm, height, ~9 cm, 1.0-cm holes in bottom of central chamber (two/compartment) and sides of compartments (three/side) for worm movement; 3 compartments per unit with same treatment, alternating in neighbouring compartments
Substrate:	Field soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, texture), artificial soil if testing site soils; recommend artificial soil for tests with chemicals spiked in soil; none in central chamber; per test compartment: identical wet wt, equivalent to a vol. of ~350 mL
Parameter:	Compartment (treatment) entered by each worm at start of test; at test end number of live and dead worms in the soil or on soil surface in each test compartment; obvious pathological symptoms or distinct behavioural abnormalities in each test compartment at test end
Duration:	48 h if *E. andrei/fetida*; 72 h if *L. terrestris*
Application:	Field-collected contaminated soil; if spiking, chemical/soil mixtures prepared manually or by mechanical agitation; test substance added as measured quantities in solution (i.e., water or organic solvent) or as solid material comprised of the test substance; ensure homogeneity
Concentration:	Single or multiple concentrations; ≥ 5 replicates
Performance:	Moisture: optimal percentage of WHC if field-collected soil, or ~70% of WHC if artificial soil; temperature: daily average, 20 ± 2°C; instantaneous, 20 ± 3°C; lighting: continuous darkness; do not feed
Reference substance:	Boric acid
Validity criteria:	invalid if mean 14-d survival in any test unit < 90%
Assessment:	Percent of live worms per treatment in each test unit at test end; ECx for avoidance if multi-concentration test
Notes:	-
Testing of GMP?	*E. fetida* has been used, but using a different protocol (Hund-Rinke et al. 2004)

Table 43:	**Avoidance test for testing the quality of soils and effects of chemicals on behaviour of earthworms**
Principle:	Sub-lethal laboratory test with a behavioural endpoint using earthworms
Guideline:	ISO-Draft guideline 17512-1 (2006b)
Test species:	*Eisenia fetida* (Savigny, 1826) or *E. andrei* Bouché, 1972; non-synchronized laboratory mass culture (individual weight 300 to 600 mg)
Ecology:	Decomposer: Destruents (dead organic matter); in organic-rich soil (epigeic)
Test design:	10 worms per test vessel. Two section chamber: 1 to 2 L; cross-sectional area of ~200 cm²; soil depth 5 to 6 cm. Six section chamber: circular test unit, central chamber with 6 pie-shaped interconnecting compartments into which the test soil is placed; interconnecting holes along bottom of compartment walls (3/side) and bottom of central chamber (2/side). Vessels wrapped to eliminate lateral light
Substrate:	Field-collected (sieved to 2 mm) or artificial soil
Parameter:	Mean number of live individuals in the test soil
Duration:	48 h
Application:	Dilution of test soil with control soil or spiking of chemical into the soil; according to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Soil dilution series or spiking of chemicals; 5 replicates (with six section chamber in multi-concentration test at least 2)
Performance:	Temperature 20 ± 2°C; moisture: 60% of WHCmax; light regime: 400 to 800 Lux at controlled light/dark cycle between 12 h/12 h and 16 h/8 h; no feeding
Reference substance:	Boric acid (400 to 800 mg/kg soil dw)
Validity criteria:	Invalid if number of dead or missing worms > 10% per treatment, distribution of worms in vessel with control soil only within range of 60%:40%
Assessment:	Differences in the mean number of individuals between test and control soil by pair wise comparison; calculation of percent avoidance; if appropriate calculation of ECx by Spearman-Kärber or probit procedures
Notes:	-
Testing of GMP?	Yes (Hund-Rinke et al. 2004)

C1.5 Enchytraeids

Table 44:	**Enchytraeid behavioural test**
Principle:	Sub-lethal laboratory test using saprophagous annelids (Enchytraeidae)
Guideline:	Proposal from literature (Achazi et al. 1996)
Test species:	*Enchytraeus albidus*; *E. crypticus* (Enchytraeidae); laboratory mass culture
Ecology:	Decomposer: Destruents (dead organic matter); in soil pore water
Test design:	10 worms per g soil in the outer chamber of a two part test vessel (diameter 5 cm, filled with 60 g FW reference soil); in the inner chamber (diameter 2 cm filled with contaminated or treated soil), which is connected to the outer chamber, no worms are inserted
Substrate:	Reference soil: Lufa St. 2.2; test soil: variable
Parameter:	Behaviour of the worms; i.e. whether they move into the treated soil or not; reproduction (number of juveniles)
Duration:	14 to 28 d; examinations: variable
Application:	Variable: Mainly contaminated soil, but mixing of the test substance in the test substrate (without worms) is possible
Concentration:	Variable, no details given; 7 replicates
Performance:	No details given; probably at the end of the test the final number of worms as well as the place where they were found is recorded
Reference substance:	EC50 (reproduction) for Carbendazim: 1.2 ± 0.8 mg a.i./kg DW
Validity criteria:	No information available
Assessment:	No information available
Notes:	Up to now only rarely used; the test is still under development. However, recently it was used for chemicals (Amorim et al. 2005).
Testing of GMP?	No

Table 45:	**Enchytraeid reproduction test (ERT)**
Principle:	Sub-lethal laboratory test using saprophagous annelids (Enchytraeidae)
Guideline:	Guideline following OECD 220 (2003a), ISO 16387 (2003a) and ASTM E 1676-04 (2004a) standards
Test species:	*Enchytraeus albidus*; *Enchytraeus sp.* (Enchytraeidae)
Ecology:	Decomposer: Destruents (dead organic matter); in soil pore water
Test design:	10 adult worms (as identified by visible eggs in the clitellum region) per test vessel (e.g. 0.2 to 0.25 L glass with lid)
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Range-finding-test: mortality, behaviour Definitive Test: number of juveniles
Duration:	Range-Finding Test: 2 weeks Definitive Test: 6 weeks. Removal of the adult worms after 3 weeks; counting of the juveniles hatched after 3 more weeks. For faster reproducing *Enchytraeus sp.*, test duration can be reduced to 3 weeks
Application:	Mixing of the test substance in the test substrate (without worms); if not water-soluble, mixing with quartz sand
Concentration:	Range-Finding-test: Control, 0, 0.1, 1, 10, 100, 1000 mg/kg (1 replicate); Definitive Test, NOEC design: 5 concentrations plus control (4 replicates) (spacing factor 1.8); ECx design: 12 concentrations (2 replicates)
Performance:	Room temperature (20 ± 2°C); weekly feeding (oats strewn onto the soil surface); permanent light; moisture: 40 to 60% of the WHC_{max}; Counting of the hatched juveniles after staining with Bengal red.
Reference substance:	: EC50 (reproduction) for Carbendazim: 1.2 ± 0.8 mg a.i./kg DW
Validity criteria:	Control: Mortality < 10% after 3 weeks; number of juveniles per test vessel > 25 after 6 weeks; Coefficient of variance (reproduction) less than 50%.
Assessment:	Determination of the No-Observed-Effect-Concentration (NOEC) by ANOVA or determination of the ECx using regression analysis
Notes:	The test was validated in an international ringtest (Römbke & Moser 2002).
Testing of GMP?	No

Table 46:	**Sub-lethal toxicity test with the enchytraeid worm *C. sphagnetorum***
Principle:	Acute/sub-lethal laboratory test using an enchytraeid species
Guideline:	Formalised guideline proposal (Rundgren & Augustsson in: Løkke & Van Gestel 1998)
Test species:	*Cognettia sphagnetorum* (Vejdovsky, 1878) (Enchytraeidae); laboratory synchronized cultures of field collected animals
Ecology:	Decomposer: Destruents (dead organic matter); in the litter layer
Test design:	1 worm per test vessel (5 cm³ glass vials, filled with 2 g of soil); the worms should have a similar size (e.g. 30 ± 2 segments).
Substrate:	Modified standard soil (Lufa St. 2.2): 75% sieved peat (*Sphagnum*) plus 25% St. 2.2 soil; 80% WHC_{max}
Parameter:	Numbers of unfragmented worms and fragments (incl. their number of segments); survival and growth of the fragments after their transfer to treated soil; mortality and pathological or behavioural changes
Duration:	Ten weeks; examinations are done weekly
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test: 4 concentrations plus control (5 replicates); Final test: unspecified number plus a control (30 replicates)
Performance:	Incubation at 15 ± 1°C; permanent light; relative humidity in the test vessels 100%; feeding with 1% dried algae (*Pleurococcus* sp.) or 0.2 g baker's yeast per 100 g DW of soil; counting of worms by wet extraction
Validity criteria:	Growth of the control worms: ≥ 1 segment/week; mortality of the adult worms in the control < 10%
Reference substance:	Copper chloride
Assessment:	Determination of NOEC, LOEC, EC10 and EC50 by means of suitable statistical methods
Notes:	Especially suitable for acid forest soils
Testing of GMP?	No

C1.6 Collembolans

Table 47:	**Acute "Beneficial Arthropod"-test with collembolans**
Principle:	Acute laboratory test using Collembola and three different exposure scenarios: 1. Exposure on the surface of an aqueous solution; 2. Exposure on moist filter-paper on top of an aqueous solution; 3. Exposure on more (forest soil) or less (quartz sand) natural substrate.
Guideline:	IOBC-Guideline proposal (Kiss & Bakonyi 1992)
Test species:	*Folsomia candida* (Willem, 1902); (Isotomidae), springtail (at least 2 other species are possible); laboratory mass culture
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	15 adults per test vessel (reagent test tubes; when testing volatile substances the animals are placed in smaller tubes which are closed by a fine mesh; these will then be put into the test tubes
Substrate:	Variable: aqueous solution, sand or forest soil (sieved and moistened to 25% moisture); when testing volatile substances coming out of an aqueous solution the springtails are kept in glass tubes laid out with moist filter paper which are fixed above the vessel with the test solution
Parameter:	Mortality
Duration:	Examination after 72 h
Application:	The test substances are applied according to agricultural practice; i.e. sprayed as an aqueous solution or strewn on the surface of the substrate
Concentration:	Only the highest recommended application rate of a pesticide
Performance:	Temperature: approx. 25°C; 80 to 90% rel. air humidity
Reference substance:	No information available
Validity criteria:	Control: mortality usually less than 2 to 3%
Assessment:	According to IOBC-criteria
Notes:	Probably very rarely used
Testing of GMP?	*F. candida* has been used but following different protocols (Yu et al. 1997; Romeis et al. 2003; Hund-Rinke et al. 2004)

Table 48:	**Sub-lethal toxicity test with the springtail *Folsomia candida***
Principle:	Sub-lethal laboratory test using springtails
Guideline:	Formalised guideline proposal (Wiles & Krogh in: Løkke & Van Gestel 1998)
Test species:	*Folsomia candida* (Willem, 1902); (Isotomidae), springtail; laboratory mass culture; 2 to 5 d acclimatisation is necessary
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	10 juvenile animals (10 to 12 d old, 0.7 to 0.8 mm long) per test vessel (glass tube; 2.5 cm diameter; 5 cm high), filled with 5 g soil FW
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water or Lufa Standard field soil 2.2; each with 50% WHC_{max}; in total an amount of 150 g DW (= 4 ± 0.1 g DW per test vessel)
Parameter:	Measurement of the body length of the juveniles using a PC-based system; number per juveniles per test vessel and per surviving adult animal; mortality of adults; assessment of food
Duration:	8 weeks plus 3 weeks for the incubation of the laid eggs; weekly examinations of mortality and reproduction
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test: 0.1, 1, 10, 100, 1000 mg/kg DW; Final test: at least 5 concentrations ≤ 1000 mg/kg DW; at least 3 replicates
Performance:	Temperature: 15 ± 0.5°C (also possible at 20°C); light-dark cycle: 16:8 h or 12:12 h at < 1000 Lux; air humidity 70 to 90%; weekly feeding with 3 granules of baker's yeast per test vessel; weekly counting of springtails by using a hand-held air aspirator; afterwards they are placed back in fresh treated soil. Incubation of the "old" soil until egg hatch is completed; afterwards counting of juveniles by wet flotation
Reference substance:	No information available
Validity criteria:	Control mortality of parent springtails ≤ 20% at the end of the test; number of juveniles per surviving adult produced per week at least 10
Assessment:	EC50 and EC10 (e.g. using ANOVA) incl. 95% confidence intervals
Notes:	Residue analysis of the test substance in the test substrate is recommended. Also useful for soil quality assessment.
Testing of GMP?	*F. candida* has been used but following different protocols (Yu et al. 1997; Romeis et al. 2003; Hund-Rinke et al. 2004)

Table 49:	**Reproduction toxicity test with Collembola**
Principle:	Sub-lethal laboratory test using one springtail species
Guideline:	ISO Guideline 11267 (1999) based on a draft published by BBA (Riepert 1991; Riepert and Kula 1996)
Test species:	*Folsomia candida* (Willem, 1902); (Isotomidae), springtail; synchronized laboratory mass culture
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	10 individuals (10 to 12 d old) per test vessel (100 mL glass vessels with lid (diameter 5 cm)), filled with 30 g FW of test substrate
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Mortality and reproduction
Duration:	Examination after 4 weeks
Application:	According to solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test (optional): 4 concentrations (e.g. 1, 10, 100, 1000 mg/kg) and a control; Definitive test (not higher than 1000 mg/kg DW): NOEC approach: at least 5 concentrations, organised in a geometrical row (spacing factor not exceeding 2), 5 replicates; ECx approach: 12 concentrations, spacing factor might be variable, 2 replicates (control: 5 replicates)
Performance:	Temperature: 20 ± 2°C; light-dark cycle of 12:12 h or 16:8 h with 400 to 800 Lux; moisture: 40 to 60% WHC_{max} (compensation necessary if the loss exceeds 2%); feeding with 2 mg of granulated dry yeast; determination of springtails by adding water to the test vessels and counting of the animals floating at the surface (e.g. by using a counting grid or on a projected slide)
Reference substance:	LOECs: E 605 forte (a.i. Parathion 507.5 g/L): 0.10 to 0.32 mg/kg DW or Betanal plus (a.i. Phenmedipham 160 g/L): 100 to 200 mg/kg
Validity criteria:	Control: mortality ≤ 20%; minimal reproduction of 100 juveniles per replicate; CV (reproduction) ≤ 30%
Assessment:	NOEC, LOEC and ECx by using "suitable" statistical methods (e.g. multiple t-Test)
Notes:	The history and the validation of this test is documented in detail in Riepert and Kula (1996). It can also be used in soil quality assessment.
Testing of GMP?	Yes (Hund-Rinke et al. 2004)

Table 50:	**Sub-lethal toxicity test with the springtail *Folsomia fimetaria***
Principle:	Sub-lethal laboratory test using springtails
Guideline:	Formalised guideline proposal (Wiles & Krogh in: Løkke & Van Gestel 1998)
Test species:	*Folsomia fimetaria* Linné, (Isotomidae), springtail; laboratory culture from field catches
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	10 male and 10 female adults per test vessel (glass cylinder; 6 cm diameter; 5.5 cm high), filled with 30 g soil FW
Substrate:	Artificial Soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water or Lufa Standard field soil 2.2; each with 50% WHC_{max}
Parameter:	Mortality and growth of the adults and the number of juveniles (reproduction); extraction of the animals using a dry extraction method (25 to 40°C during 12 h)
Duration:	Examination after 3 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test: 0.1, 1, 10, 100, 1000 mg/kg DW; Final test: not specified number of concentrations for determination of an EC10 but not higher than 1000 mg/kg DW; at least 4 replicates
Performance:	Temperature: 20 ± 1°C; light-dark cycle: 12:12 h with 400 to 800 Lux; constant moisture (± 10%); feeding of 15 mg dried baker's yeast at d 0 and after 14 d
Reference substance:	Insecticide Dimethoate: EC50 (reproduction) 1.5 to 2.5 mg/kg DW in Standard field soil Lufa 2.2
Validity criteria:	Control: mortality not higher than 20% and at least 200 juveniles per test vessel at the end of the test
Assessment:	EC50, EC10 and LC50 determined by using "suitable" methods
Notes:	Some specimens should be preserved in 70% ethanol for future reference.
Testing of GMP?	No

Table 51:	**Effects of exposure to contaminated soil on the survival and reproduction of *Folsomia candida* and *Folsomia fimetaria***
Principle:	Sublethal soil toxicity test using collembolans
Guideline:	EC Draft guideline (2005b)
Test species:	*Folsomia candida* and *F. fimetaria*: synchronized laboratory cultures; 10 to 12 (*F. candida*) and 23 to 26 d (*F. fimetaria*) after eclosion
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	*F. candida* 10, *F. fimetaria* 20 (10 females and 10 males) individuals/test vessel: 100- to 125-mL glass jar (~5-8 cm diam.), covered; metal lid secured with metal or plastic screw-top ring
Substrate:	Field-collected soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, and texture); recommend artificial soil for tests with chemicals or chemical products spiked in soil; 30 g wet wt
Parameter:	No. of live adult springtails and no. of progeny at the end of the test
Duration:	*F. candida*: 21 d; *F. fimetaria*: 28 d
Application:	Field-collected contaminated soil; if spiking chemical/soil mixtures prepared manually or by mechanical agitation; test substance added as measured quantities in solution (i.e., in water or an organic solvent) or as solid material comprised of the test substance; ensure homogeneity
Concentration:	Single or multiple concentrations; ≥ 3 replicates/treatment, controls ≥ 5
Performance:	Moisture: optimal percentage of WHC if field-collected soil, or ~70% of WHC if artificial soil; during test, hydrate as necessary; temperature: daily average and instantaneous, 20 ± 2°C; lighting: incandescent or fluorescent; intensity, 400 to 800 Lux; fixed photoperiod (e.g., 16L:8D or 12L:12D); feeding: granulated dry yeast; *F. candida*: ~10 mg per test vessel on d 0 and ~20 mg on d 20; *F. fimetaria*: ~3 mg per test vessel on d 0 and 7; sprinkled onto soil surface
Reference substance:	Boric acid
Validity criteria:	Mean survival of adults in control soil at test end ≥ 70% for *F. candida* in natural and ≥ 80% in artificial soil; ≥ 80% for *F. fimetaria*, regardless of soil type; mean reproduction rate for adults in control soil ≥ 100 live progeny/vessel for *F. candida* and ≥ 200 for *F. fimetaria*
Assessment:	Mean survival of adults and mean number of live progeny in each treatment; if multi-concentration test: LC50 for adult springtails and ICx for mean number of live progeny produced per concentration
Notes:	-
Testing of GMP?	*F. candida* has been used but following different protocols (Yu et al. 1997; Romeis et al. 2003; Hund-Rinke et al. 2004)

Table 52:	**Sub-lethal effects on predatory mites and springtails**
Principle:	Sub-lethal laboratory test using a two-species system: a predatory mite and springtails serving as food
Guideline:	Formalised guideline proposal (Krogh and Axelsson in: Løkke and Van Gestel 1998)
Test species:	*Hypoaspis aculeifer* Canestrini (Gamasina), predatory mite; *Folsomia fimetaria* L. (Isotomidae), springtail; synchronized laboratory culture
Ecology:	*H. aculeifer*: Consumer 2. order: Predators (microarthropods); in the uppermost soil and litter layer; *F. fimetaria*: Decomposer or consumer 2. order (dead organic matteror nematodes); in the soil air space
Test design:	10 male and 5 female adults of *H. aculeifer* and 100 individuals of *F. fimetaria* (16 to 19 d old at the start of the test) per test vessel (glass cylinder; 6 cm diameter; 5.5 cm high)
Substrate:	Artificial soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water or Lufa Standard field soil 2.2; each with 50% WHC_{max} (modifications according to the requirements of the animals are possible); in total an amount of 60 g FW
Parameter:	Predatory mites: mortality and growth of the adults and the number of juveniles (reproduction)
Duration:	Examination after 3 weeks
Application:	According to solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 4 replicates
Performance:	Temperature: 20 ± 1°C; light-dark cycle: 12:12 h with 400 to 800 Lux; feeding of collembolans with 15 mg baker's yeast at d 0 and d 14; mites feed on the springtails; dry extraction method; counting either manually under a stereo microscope or by using digital image processing
Reference substance:	Insecticide Dimethoate: EC50 (reproduction of the mites) 2.0 to 3.0 mg/kg DW Standard field soil Lufa 2.2 (at approx. 1 mg/kg DW a hormesis effect is sometimes observed)
Validity criteria:	Control: mortality of the female mites not higher than 10% and at least 20 juvenile mites per test vessel at the end of the test
Assessment:	EC50, EC10 and LC50 using "suitable" statistical methods

Notes:	One of the few examples of a two-species test laboratory system.
Testing of GMP?	No

Table 53:	**Sub-lethal toxicity test with the springtail *Isotoma viridis***
Principle:	Sub-lethal laboratory test using springtails
Guideline:	Formalised guideline proposal (Wiles & Krogh in: Løkke & Van Gestel 1998)
Test species:	*Isotoma viridis* Bourlet, 1839; (Isotomidae), springtail; laboratory culture from field catches; 2 to 5 d of acclimatisation necessary
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	20 juvenile animals (5 to 7 d old, 0.5 to 0.6 mm long) per test vessel (glass cylinder; 2.5 cm diameter; 5 cm high), filled with 5 g soil FW
Substrate:	Artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water or Lufa Standard field soil 2.2; each with 50% WHC_{max}; in total an amount of 150 g DW (= 4 ± 0.1 g DW per test vessel)
Parameter:	Measurement of the body length of the springtails using a PC-based system; mortality of the adults (using hand-sorting)
Duration:	8 weeks; weekly examinations of mortality and growth
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test: 0.1, 1, 10, 100, 1000 mg/kg DW; Final test: at least 5 concentrations but not higher than 1000 mg/kg DW; at least 3 replicates
Performance:	Temperature: 15 ± 0.5°C (20°C is also possible); light-dark cycle: 16:8 h at < 1000 Lux; air humidity 70 to 90%; weekly feeding with 3 granules of baker's yeast per test vessel; weekly counting of springtails by using a hand-held air aspirator; afterwards they are placed back in fresh treated soil
Reference substance:	No information available
Validity criteria:	Control: mortality not higher than 50% at the end of the test
Assessment:	EC50 and EC10 (e.g. using ANOVA)
Notes:	Residue analysis of the test substance in the test substrate is recommended. The low survival rate in the control is problematic. Some specimens should be preserved in 70% ethanol for future reference. Also useful for soil quality assessment.
Testing of GMP?	No

Table 54:	**Effects of exposure to contaminated soil on the survival and reproduction of *Orthonychiurus folsomi***
Principle:	Sublethal soil toxicity test using collembolans
Guideline:	EC Draft guideline (2005b)
Test species:	Laboratory cultured adult *Orthonychiurus folsomi*; males usually range from ~1 to 1.5 mm and females > 2 mm, with large rounded abdomens
Ecology:	(Probably) decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	10 individuals/test vessel (target 5 males and 5 females): 100- to 125-mL glass jar (~5-8 cm diam.), covered; metal lid secured with metal screw-top ring or plastic screw-top lid recommended as cover
Substrate:	Field-collected soil (characterized by moisture, WHC, pH, conductivity, TOC, OM, and texture) or artificial soil if testing site soils; recommend artificial soil for tests with chemicals or chemical products spiked in soil; 30 g wet wt
Parameter:	Total number of live adult springtails and total number of progeny in each test vessel at the end of the test
Duration:	35 d
Application:	Field-collected contaminated soil or soils spiked with a chemical; test substance may be added as measured quantities in solution (i.e., in water or an organic solvent) or as solid material comprised partly or completely of the test substance; ensure homogeneity
Concentration:	Single or multiple concentrations; 10 replicates/treatment incl. controls
Performance:	Moisture: hydrate to the optimal percentage of WHC if field-collected soil, or to ~70% of WHC if artificial soil; during test, hydrate as necessary; temperature: daily average, 20 ± 2°C; instantaneous, 20 ± 2°C; lighting: incandescent or fluorescent; intensity, 400 to 800 Lux adjacent to surface of soil in test chamber; fixed photoperiod (e.g., 16L:8D or 12L:12D); feeding: granulated dry yeast; 5 mg per test vessel sprinkled onto soil surface on d 7, 14, 21, and 28 only
Reference substance:	Boric acid
Validity criteria:	Mean 35-d survival of adults in control soil ≥ 80%; mean reproduction rate for adults in control soil ≥ 70 live progeny/adult
Assessment:	Mean survival of adults and mean number of live progeny in each treatment, on d 35; if multi-concentration test: LC50 for adult springtails and ICx for mean number of live progeny produced per concentration at test end
Notes:	-
Testing of GMP?	No

C1.7 Beetles

Table 55:	**Acute effects on staphylinid beetles**
Principle:	Acute laboratory test for pesticides
Guideline:	Modified guideline proposal according to IOBC-criteria (Samsoe-Peterson 1987; modified by Römbke & Vickus (pers. comm.))
Test species:	*Aleochara bilineata* (Gyll.); (Staphylinidae), rove beetle; laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	9 coupled females (1 to 4 weeks old); 1 beetle per test vessel (small glass Petri disk with glass lid)
Substrate:	Approx. 5 g moistened quartz sand per test vessel
Parameter:	Mortality, feeding rate and behavioural changes of the adult beetles (after 5 d); number of the eggs laid per female at the end of the test as well as the number of hatched juveniles
Duration:	Daily examinations during the first 5 d after application; afterwards sieving of the laid eggs and counting of the hatched beetle larvae 9 and 12 d later
Application:	Spraying of the test substance on top of the sand surface (including beetles) in a laboratory device in analogy to agricultural practice; substances not soluble in water are strewn on the sand surface
Concentration:	Highest recommended application rate
Performance:	Temperature: approx. 22 ± 2°C; light-dark cycle 16:8 h with 50 to 75 Lux; minimal air humidity 50%; daily feeding with 30 eggs of the onion fly (*Delia antiqua*)
Reference substance:	Fungicide Afugan (active ingredient: Pyrazophos) with 1 L/ha 100% mortality
Validity criteria:	Control: mortality < 11% (= not more than 1 animal); number of eggs: 50 ± 10; feeding rate: 50 to 100 fly eggs per beetle after 5 d
Assessment:	According to IOBC-criteria
Notes:	Only rarely required by registration agencies
Testing of GMP?	No

Table 56:	**Reproductions toxicity test with staphylinids**
Principle:	Sub-lethal and chronic laboratory test for pesticides (generation-test)
Guideline:	BBA VI, 23 - 2.1.10 (BBA 1994c)
Test species:	*Aleochara bilineata* (Gyll.); Staphylinidae (rove beetle); laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	10 pairs (2 to 3 d after pupation) of adult *A. bilineata* per test vessel (glass large Petri disk; approx. 5 cm high filled) together with approx. 500 pupae of the onion fly *Delia antiqua*
Substrate:	600 mL moistened quartz sand per test vessel
Parameter:	Mortality of the adult beetles (after 4 weeks); number of the juvenile beetles hatched from the parasitized fly larvae
Duration:	In total approx. 90 d; 1, 2, and 3 weeks after application addition of *Delia*-pupae; after 4 weeks removal of the adult beetles; the final end of the test depends from the length of the hatching period
Application:	Spraying of the test substance on top of the sand surface (including beetles) in a laboratory device in analogy to agricultural practice; substances not soluble in water are strewn on the sand surface
Concentration:	Highest recommended application rate
Performance:	Temperature: approx. 21 ± 2°C; light-dark cycle 16:8 h with 600 to 1200 Lux; feeding of the adult beetles with midge larvae 5 times per week
Reference substance:	No information available (maybe possible: Afugan (active ingredient: Pyrazophos))
Validity criteria:	Control: parasitizing rate at least 25%
Assessment:	According to IOBC-criteria
Notes:	-
Testing of GMP?	No

Table 57:	**Chronic effects on *Aleochara bilineata* Gyllenthal**
Principle:	Test for evaluating chronic effects of plant protection products on rove beetles under laboratory and extended laboratory conditions
Guideline:	Method proposal from literature (Grimm et al. in: Candolfi et al. 2000)
Test species:	*Aleochara bilineata* Gyllenhal (Coleoptera: Staphylinidae); laboratory culture or commercial supplier; 1 to 7 d old
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	10 pairs of male and female adult beetles per test vessel (glass or plastic container with minimum ground surface of 150 cm² with at least 4 cm deep (min. 600 cm³) layer of moist quartz sand or sandy soil covered with lid with opening covered with fine mesh nylon netting), age 1 to 7 d and approx. 1500 onion fly, *Delia antiqua* (Meigen) as hosts for larvae
Ecology:	Secondary consumers
Substrate:	Quartz sand; extended conditions: sandy field soil (e.g., LUFA 2.1)
Parameter:	Mortality of adults after 28 d (optional); adult beetle emergence from pupae
Duration:	approx. 11 to 13 weeks; after 4 weeks adults are removed
Application:	Spraying or incorporated directly into substrate
Concentration:	On the basis of a volume application rate of 400 L/ha; minimum of 4 replicates
Performance:	Temperature: 20 ± 2°C; 16-h photoperiod (< 2000 Lux); relative air humidity 60 to 90%; Moisture: quartz sand: sand:water = 10:1, field soil: 35 ± 5% of WHCmax; feeding every 1 to 3 d (red mosquito larvae, yellow mealworm larvae, *Tenebrio molitor* or raw mince meat), after 7, 14 and 21 d adding of approx. 500 onion fly pupae and mixing with substrate
Validity criteria:	Average number of beetles above 400 in control; reduction of 50% reproductive capacity relative to control in reference item treatment
Reference substance:	Pyrazophos EC 300 (1 L/ha) or Dimethoate EC 400 (1.1 L/ha)
Assessment:	Determination of mean number of offspring per beetle, treatment observations expressed as percent reduction relative to control; for rate-response relationships probit regression analysis is recommended
Notes:	-
Testing of GMP?	No

Table 58:	**Effects of veterinary pharmaka on dung beetles**
Principle:	Test for evaluating effects of veterinary pharmaka on *Aphodius constans* in cattle dung
Guideline:	Method proposal from literature (Hempel et al. 2006)
Test species:	*Aphodius constans* Duft.; first-stage larvae
Ecology:	Decomposer/Consumer 1. order/Non-target herbivores/Destruents (organic matter and microbes); in dung pats
Test design:	One larva per well or tube
Substrate:	Recommended: formulated dung (fresh dung is possible)
Parameter:	Survival of the larvae after 3 weeks (biomass or morphological changes of the adults after 9 weeks, if appropriate)
Duration:	3 weeks (may be prolonged to 12 weeks depending on the test substance)
Application:	When testing a water-insoluble substance, it should be applied in 15 mL solvent (e.g. acetone) per 10 g dung (d.w.)
Concentration:	Range-finding test: 0.1; 1.0; 10; 100; 1000 mg a.i./kg plus water control; definitive test: NOEC-design: 5 concentrations plus water control depending on the range-finder-test (spacing factor 2 -3); when using a solvent, an additional solvent control is necessary
Performance:	Temperature: 20 ± 2°C; moisture adjustment of wells or tubes with aqua demin. once per week; no feeding
Validity criteria:	<20 % larval mortality in the controls when using formulated dung; <30 % if fresh dung is used in the test
Reference substance:	Ivermectin
Assessment:	NOEC/EC50
Notes:	-
Testing of GMP?	No

Table 59:	**Acute and sub-lethal effects on *Coccinella septempunctata***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on the plant dwelling insect *Coccinella septempunctata*
Guideline:	Method proposal from Schmuck et al. in: Candolfi et al. (2000)
Test species:	*Coccinella septempunctata* L. (Coleoptera: Coccinellidae); laboratory culture or commercial supplier
Ecology:	Consumer 2. order: Predators (aphids); on plant (leaf) surfaces
Test design:	Pre-imaginal mortality assessment: At least 40 larvae per treatment in test units (sprayed glass plates covered with safety glass with recesses of 5 ± 1 cm, safety glass cylinders of 5 ± 1 cm diameter and 4 cm height treated with Fluon or Talcum placed in each recess; sealed with fine mesh gauze) until they have ecdysed. Adult beetles are transferred to a maintenance container Assessment of reproductive performance: When ≥ 90% of viable pupae have hatched in control and test item group, beetles are sexed and transferred to breeding containers (max. 5 pairs/container: glass or plastic lined with filter paper, closed by nylon netting, containing paper cylinders for egg laying, exchanged daily). Assessment of reproduction commences 1 w after control beetles started to lay eggs. All eggs laid in subsequent 2 w are collected and checked for fertility (larval hatch). Mortality of adults is checked daily.
Substrate:	Glass plates
Parameter:	Pre-imaginal mortality assessment: Larval and pupal death, abnormal behaviour Assessment of reproductive performance: Number of eggs, mortality of beetles
Duration:	20 to 30 d
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha
Performance:	Temperature: 25 ± 2°C; 16L:8D photoperiod (≥ 1000 Lux); relative air humidity 60 to 90%; food: various aphid species
Validity criteria:	Average pre-imaginal mortality of larvae ≤ 30%; number of eggs laid by control females above lower limit of historical data base (2.0 fertile eggs per viable female per d); reference mortality ≥ 40%
Reference substance:	Afugan 30 EC (a.i. pyrazophos; 10 mL/ha in 200 L water/ha)
Assessment:	Percentage of pre-imaginal mortality (Abbott's corrected), average number of eggs per female per d, percentage of fertile eggs
Notes:	-
Testing of GMP?	No

Table 60:	**Acute and sub-lethal effects on ear-wigs**
Principle:	Acute and sub-lethal laboratory test developed for pesticides using two ages stages of ear-wigs (larvae = sensitive stage; adults = robust stage)
Guideline:	Guideline proposal analogous to IOBC-criteria (Sauphanor et al. 1992)
Test species:	*Forficula auriculata* L.; (Dermaptera), ear-wig; laboratory culture (start of the culture by taking adults from the field)
Ecology:	Consumer 2. order: Predators (e.g. aphids); in the litter or lower vegetation layer
Test design:	Larvae of different ages (10 per test vessel (glass Petri disks)) and adult animals (5 pairs)
Substrate:	Moistened sand
Parameter:	Larvae: mortality and feeding rate; adults: number of eggs
Duration:	Final examination after 15 d; intermediate examinations every 2 d (for the first time after 24 h).
Application:	Spraying of the test substance on top of the sand surface (including beetles) in a laboratory device in analogy to agricultural practice; substances not soluble in water are strewn on the sand surface
Concentration:	Highest recommended application rate; 5 replicates
Performance:	Temperature: approx. 18 ± 1°C; light-dark cycle 16:8 h (larvae) and 12:12 h (adults); relative air humidity 75 ± 5%; feeding with artificially made mixture of Pollen every 2 d. The animals are put on the test substrate 2 h after application.
Reference substance:	No information available
Validity criteria:	Comparison with untreated controls (water)
Assessment:	According to IOBC-criteria
Notes:	Usually the test is done with the sensitive stage; only when effects are occurring the test with adult animals has to be performed.
Testing of GMP?	No

Table 61:	**Sub-lethal toxicity test with adult staphylinids**
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Formalised guideline proposal (Metge & Heimbach in: Løkke & Van Gestel 1998)
Test species:	*Philonthus cognatus* Stephenson 1832 (Staphylinidae), rove beetle; laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	Exposure period: 15 male and 15 female beetles per concentration; one beetle per test vessel (glass cylinder with 35 cm³ volume and 2.8 cm diameter; 7 cm high), filled with 20 g DW of soil; reproduction period: 10 * 10 * 6 cm plastic vessel, up to a height of 2 cm filled with clay balls; 3 pairs of beetles per test vessel (in total 5 replicates)
Substrate:	Lufa Standard field soil 2.1 or 2.2 moistened to 50% WHC_{max}
Parameter:	Number of eggs per female per week, hatching rate of the juveniles; mortality of the adults during the egg-laying period
Duration:	Exposure period: 7 d; reproduction period: 6 to 10 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 5 replicates
Performance:	Exposure period: temperature: 15 ± 1°C; light-dark cycle: 16:8 h; relative air humidity: 85 to 100%; feeding with dissected, unfrozen fly pupae (*Calliphora sp.*), one half of a pupa after 0, 2, 4 and 6 d; reproduction period: temperature: 20 ± 1°C
Reference substance:	No information available
Validity criteria:	Control: mortality of females not higher than 30% in the first 4 weeks of the 4 weeks of the reproduction period
Assessment:	ECx and LCx using "accepted" statistical methods
Notes:	-
Testing of GMP?	No

Table 62:	**Sub-lethal toxicity test using staphylinid larvae**
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Formalised guideline proposal (Metge & Heimbach in: Løkke & Van Gestel 1998)
Test species:	*Philonthus cognatus* Stephenson 1832 (Staphylinidae), rove beetle; laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	Exposure period: 15 male and 15 female beetles per concentration; one beetle per test vessel (glass cylinder with 35 cm³ volume and 2.8 cm diameter; 7 cm high), filled with 20 g DW of soil; reproduction period: 10 * 10 * 6 cm plastic vessel, up to a height of 2 cm filled with clay balls; 3 pairs of beetles per test vessel (in total 5 replicates)
Substrate:	Lufa Standard field soil 2.1 or 2.2 moistened to 50% WHC_{max}
Parameter:	Mortality, weight of the hatched beetles, developmental time, mortality in winter; reproduction
Duration:	Exposure period: > 14 d; artificial winter: long day conditions for 2 to 4 weeks, afterwards short-day conditions for 6 to 10 weeks, finally again long-day conditions for 1 week; reproduction period: after the period of artificial winter long-day conditions for 7 d followed by 6 to 10 weeks observation time and 2 weeks during which the larvae hatch
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 4 replicates
Performance:	Exposure period: temperature: 15 ± 1°C; light-dark cycle: 16:8 h; relative air humidity: 85 to 100%; feeding with dissected, unfrozen fly pupae (*Calliphora sp.*), one half of a pupa after 0, 2, 4 and 6 d; reproduction period: temperature: 20 ± 1°C
Reference substance:	No information available
Validity criteria:	Control: mortality of the larvae not higher than 20%
Assessment:	ECx and LCx
Notes:	Very complex test system, which serves also as a Range-Finding-Test for semi-field tests
Testing of GMP?	No

Table 63:	**Acute toxicity test using carabid beetles**
Principle:	Acute laboratory test developed for pesticides
Guideline:	BBA VI, 23-2.1.8 (BBA 1991a)
Test species:	*Poecilus cupreus* (L.); ground beetles (Carabidae); laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae and pupae); in the uppermost soil and the litter layer
Test design:	3 males and 3 females, respectively (2 to 10 weeks old) per test vessel (Bellaplast-vessels)
Substrate:	Moistened quartz sand (70% WHC_{max})
Parameter:	Mortality, feeding rate (number of consumed pupae) and behavioural changes
Duration:	2 weeks; examinations 2, 4 and 6 h as well as 1, 2, 4, 7, 11 and 14 d after application of the test substance; if more than beetles have died in the second week of the test the duration is increased to 4 weeks
Application:	Spraying of the test substance on top of the sand surface (including beetles) in a laboratory device in analogy to agricultural practice; substances not soluble in water are strewn on the sand surface
Concentration:	Highest recommended application rate
Performance:	Temperature: 20 ± 2°C; light-dark cycle 16:8 h at 500 to 2000 Lux; feeding: 1 fly pupa per living beetle every 2 to 3 d; in parallel moistening of the sand
Reference substance:	Afugan (active ingredient: Pyrazophos); concentration: 1 L/ha
Validity criteria:	Control: mortality not higher than 10% at the end of the test
Assessment:	Statistical assessment of the feeding rate (e.g. by using ANOVA)
Notes:	The test is less often used nowadays due to the low sensitivity of the adult beetles.
Testing of GMP?	No

Table 64:	**Effects on carabid beetle larvae**
Principle:	Acute and sub-lethal laboratory test for pesticides
Guideline:	Guideline proposal acc. to IOBC (Heimbach 1998); cf. Kegel (1989)
Test species:	*Poecilus cupreus* (alternatively *P. versicolor, P. lepidus)* (Carabidae), ground beetles; laboratory culture (individually kept)
Ecology:	Consumer 2. order: Predators (e.g. fly pupae); in the mineral soil
Test design:	Larvae (12 to 48 h old); 1 animal per test vessel (glass tubes (inner diameter 2.5 cm; height 7 cm; vol. 35 mL), filled with 25 g DW soil); 35 to 40 tubes are kept together in a ventilated box with a moist bottom
Substrate:	Lufa St. 2.1 (> 80% sand; organic content < 1%); Kegel (1989) proposed an air-dried field crop soil (sieved and moistened (13% moisture), 15 g (tube); pH-value 3.9 to 4.0)
Parameter:	Mortality, uncoordinated movements or other behavioural changes, developmental duration, hatching rate and male/female ratio
Duration:	Until hatching of adult beetles (20°C: 40 to 50 d; 15°C: 60 to 70 d); 2 to 3 examinations/week until larvae begin to pupate, afterwards daily
Application:	Spraying of the test substance on top of the soil surface (including beetles) in a laboratory device in analogy to agricultural practice (also pipettes could be used); if the test substance has to be mixed into the test substrate, water-soluble substances are added together with moistening water; substances not soluble in water like granules are mixed into the soil immediately before it is filled into the tubes
Concentration:	Highest recommended application rate with at least 40 replicates (limit test); for dose-response testing (at least 3 concentrations with mortality > 20% and less than 100% mortality) 20 replicates per concentration
Performance:	Temperature: 20°C (alternatively: 15°C); continuous dark; moisture up to 35 of WHC_{max} (has to be kept constant during the test); feeding with dead fly pupae (cut into halves, 1 pupa per larva 2 to 3 times per week), feeding has to be stopped when the larvae begin to pupate; hatched beetles are weight immediately after changing their colour to black
Reference substance:	Alternatively a product containing the a.i. lambda-cyhalothrin (10 g a.i./ha) or an a.i. dimethoate (35 g a.i./ha); both concentrations should result in a mortality rate of approximately 65 ± 35%
Validity criteria:	Mortality in the control: < 20%
Assessment:	According to IOBC-criteria; limit tests: e.g. t-Tests, dose-response tests: Probit-Analysis, Williams test
Notes:	When other species than *P. cupreus* are used, the test conditions (e.g. an increase of the duration) have to be changed accordingly.
Testing of GMP?	No

Table 65:	**Acute and sub-lethal effects on *Poecilus cupreus* – Laboratory test**
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on carabid beetles
Guideline:	Method proposal from literature (Heimbach et al. in: Candolfi et al. 2000)
Test species:	*Poecilus cupreus* L. (Coleoptera: Carabidae); laboratory culture or commercial supplier; 3 d acclimatization
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	3 male and 3 female beetles per replicate (plastic, glass or metal containers with substrate surface area of 180 ± 20 cm² and 6 ± 1 cm depth with ventilated transparent lids and walls treated with Fluon or Talcum), on d 0 test item is applied on substrate surface and beetles (if sprayed).
Substrate:	250 ± 1 g dw quartz sand or LUFA 2.1 standard soil (extended laboratory test)
Parameter:	Mortality, behaviour and food consumption
Duration:	2 to 3 weeks, examinations on d 1, 2, 4, 7, 10/11, 14, (17 and 21)
Application:	Spraying or incorporation into substrate
Concentration:	On the basis of a volume application rate of 400 L/ha; 5 replicates
Performance:	Water content 70 ± 5% (quartz sand) or 55 ± 5% (soil) of WHC, temperature: 20 ± 2°C; 16L:8D photoperiod (200 to 2000 Lux); relative air humidity 60 to 90%; food: pupae of *Musca* spp. or *Delia* spp.
Validity criteria:	Mortality ≤ 6.7% after 14 d, 13.3% after 21 d; reference mortality 65 ± 35% after 14 d (Abbott's corrected)
Reference substance:	Pyrazophos (294 g a.i./ha; 1 L/ha Afugan) or parathion (4.5 g a.i./ha; 9 mL/ha E 605 forte)
Assessment:	Mortality of beetles (Abbott's corrected), mean food consumption per live beetle per assessment period; total food consumption
Notes:	-
Testing of GMP?	No

C1.8 Bees

Table 66:	**Effects of pesticides on honeybees**
Principle:	Inhalation, contact, and oral toxicity of pesticides to honeybees
Guideline:	BBA 23-1 (1991)
Test species:	*Apis mellifera* (honeybee); bees caught at hive entrance or brushed off the honeycombs
Ecology:	Consumer 1. order: Pollinators (pollen); in the (flowering) vegetation layer
Test design:	10 bees per cage: 10 x 5.5 x 8.5 cm steel cages with front glass window, holes in the top for feeding and in the bottom for ventilation
Substrate:	-
Parameter:	Mortality; abnormal behaviour; signs of toxicity
Duration:	24 h, if showing toxicity in one exposure route extension to 72 h, continuous observation during initial 30 min., afterwards after 1 h, on the first evening and following morning and after 24, 48 and 72 h
Application:	Inhalation toxicity: petri-dish (diam. 9.2 cm, height 1.7 cm) half-way filled with application solution, cage placed on edge of petri-dish to allow gaseous components to enter the cage through the perforated bottom Permanent contact toxicity: 150 cm^2 paper soaked with application solution, air-dried (25°C) and put into cages Contact toxicity through spraying: spraying of bees through wire mesh (in place of front glass window) with 1 mL application solution per cage, afterwards transfer of bees to untreated cage Oral toxicity: 1% product in 50% sucrose solution; individual (10 mm^3 = 100 µL product) or group feeding (200 mm^3), after 3 h feeding with uncontaminated sucrose solution
Concentration:	Twice the intended application concentration (except with oral toxicity: 1%-solution); 3 replicates and 2 repetitions
Performance:	In months May to October; temperature at least 25°C; relative humidity 40 to 60%; permanent dark; food: 50% sucrose solution
Reference substance:	No information available
Validity criteria:	Control mortality ≤ 15% after 72 h
Assessment:	X% mortality after X minutes or hours; for oral toxicity (individual feeding) determination of LD50
Notes:	Guideline outdated
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

Table 67:	**Honeybees, Acute Oral Toxicity Test**
Principle:	Oral toxicity of test compounds to adult worker honeybees
Guideline:	OECD 213 (1998a); EPPO PP 1/170(3) (2000)
Test species:	*Apis mellifera* (honeybee); young adult worker bees of same race, similar age, feeding status collected from adequately fed, healthy, as far as possible disease-free, queen-right colonies with known history and physiological status
Ecology:	Consumer 1. order: Pollinators (pollen); in the (flowering) vegetation layer
Test design:	10 bees per cage: easy to clean, well-ventilated cages of appropriate size and material, 50 mm long and 10 mm wide glass tube (open end narrowed to about 2 mm) for feeding
Substrate:	-
Parameter:	Mortality after 4, 24 and 48 h, if prolonged in 24 h intervals; amount of diet consumed per group; abnormal behaviour
Duration:	48 h after test solution has been replaced with sucrose solution alone; if mortality continues to rise by more than 10% after first 24 h, duration should be extended to max. 96 h (as long as control mortality < 10%)
Application:	Depending solubility of test substance: in 50% sucrose solution in water, with substances of low water solubility, 1% vehicles (e.g., organic solvent, emulsifier, dispersant) of low toxicity to bees may be used; 100 to 200 µL of 50% sucrose solution in water per replicate, after consumption or after max. 6 h replaced with sucrose solution alone
Concentration:	Five doses in geometric series, with factor not exceeding 2.2; limit test: 100 µg a.i./bee; 3 replicates
Performance:	Temperature 25 ± 2°C; relative humidity 50 to 70%; permanent dark; food: sucrose solution in water with final concentration of 500 g/L (50% w/v), after given test doses, food provided *ad libitum*
Reference substance:	E.g., dimethoate (LD50-24h 0.10 to 0.35 µg a.i./bee)
Validity criteria:	Control mortality ≤ 10%; LD50 of toxic standard meets specified range
Assessment:	LD50 at each recommended observation time by appropriate statistical methods, Abbott's correction
Notes:	-
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

Table 68:	**Honeybees, Acute Contact Toxicity Test**
Principle:	Contact toxicity of test compounds to adult worker honeybees
Guideline:	OECD 214 (1998b); EPPO PP 1/170(3) (2000)
Test species:	*Apis mellifera* (honeybee); young adult worker bees of same race, similar age, feeding status collected from adequately fed, healthy, as far as possible disease-free, queen-right colonies with known history and physiological status
Ecology:	Consumer 1. order: Pollinators (pollen); in the (flowering) vegetation layer
Test design:	10 bees per cage: easy to clean, well-ventilated cages of appropriate size and material, 50 mm long and 10 mm wide glass tube (open end narrowed to about 2 mm) for feeding
Substrate:	-
Parameter:	Mortality after 4, 24 and 48 h, if prolonged in 24 h intervals; amount of diet consumed per group; abnormal behaviour
Duration:	48 h; if mortality continues to rise by more than 10% after first 24 h, duration should be extended to max. 96 h (as long as control mortality < 10%)
Application:	As solution in carrier (organic solvent or water solution with wetting agent; 1 µL of solution applied to dorsal side of thorax of each anaesthetized bee
Concentration:	Five doses in geometric series, with factor not exceeding 2.2; limit test: 100 µg a.i./bee; 3 replicates
Performance:	Temperature 25 ± 2°C; relative humidity 50 to 70%; permanent dark; food: sucrose solution in water with final concentration of 500 g/L (50% w/v), provided *ad libitum*
Reference substance:	E.g., dimethoate (LD50-24h 0.10 to 0.30 µg a.i./bee)
Validity criteria:	Control mortality ≤ 10%; LD50 of toxic standard meets specified range
Assessment:	LD50 at each recommended observation time by appropriate statistical methods, Abbott's correction
Notes:	-
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

C1.9 Isopods

Table 69:	**Sub-lethal consumption test with isopods**
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Proposal from literature (Drobne & Hopkin 1994, Drobne 1997)
Test species:	*Porcellio scaber* (Latr. 1814); Porcellionidae; or *Oniscus asellus* (Oniscidae) woodlouse; field collection or laboratory culture; acclimatisation not specified
Ecology:	Decomposer: Destruents (dead organic matter); in the litter layer
Test design:	12 males and non-gravid females (each approximately 40 mg FW) per test vessel (Petri dishes, diameter 9 cm); individually held together with one maple leaf of known weight
Substrate:	Except the maple leaves: none
Parameter:	Amount of faecal pellets; bioaccumulation
Duration:	6 weeks (examination of 50% of test organisms after 4 and 6 weeks, respectively); in the last 2 weeks the remaining animals are transferred to untreated leaves
Application:	Variable, no details given; the test substance is applied topically on the maple leaves (nominal concentrations per g DW)
Concentration:	At least 3 concentrations plus a control; 12 replicates
Performance:	Temperature: 20°C; light-dark cycle 16:8 h; relative air humidity 100%; feeding with air-dried and re-hydrated field-collected maple leaves; daily collection of faecal pellets produced during the previous 24 h; at the end of the test weighing of the pellets and the leaves remains; accumulation of the test chemical can be measured in dissected animals
Reference substance:	Zinc salt (either $ZnCl_2$, $Zn(NO_3)_2$ or ZnO), to be tested in regular intervals
Validity criteria:	No information available
Assessment:	Calculation of assimilation efficiency and consumption rate (is directly proportional to faecal production)
Notes:	Extension of the test by contaminating soil probably possible
Testing of GMP?	*P. scaber* has been used but following different protocols (Escher et al. 2000; Wandeler et al. 2002)

Table 70:	**Growth test with isopods**
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Formalised guideline proposal (Fischer et al. in: Løkke & Van Gestel 1998)
Test species:	*Porcellio scaber* (Latr. 1814); Porcellionidae (woodlouse); laboratory culture; acclimatisation not necessary
Ecology:	Decomposer: Destruents (dead organic matter); in the litter layer
Test design:	10 males and females per test vessel (plastic boxes, diameter 8 cm, 4 cm high); 10 to 14 d old with a weight of 20 to 30 mg FW
Substrate:	Lufa Standard field soil St. 2.2; < 50% WHC_{max}
Parameter:	Mortality, growth
Duration:	4 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW (3 replicates); Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 5 replicates
Performance:	Temperature: 20 ± 2°C; light-dark cycle 16:8 h with at least 500 Lux; relative air humidity > 80%; feeding twice weekly with a complex mixture (50% maple litter, 40% rabbit food, 10% potato pancake); weekly examinations of mortality and biomass
Reference substance:	Insecticide Dimethoate
Validity criteria:	Control: mortality not higher than 20%
Assessment:	EC10, EC50 and LC50
Notes:	Extension of the test by using contaminated food possible
Testing of GMP?	*P. scaber* has been used but following different protocols (Escher et al. 2000; Wandeler et al. 2002)

Table 71:	**Reproduction test with isopods**
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Formalised guideline proposal (Fischer et al. in: Løkke & Van Gestel 1998)
Test species:	*Porcellio scaber* (Latr. 1814); Porcellionidae (woodlouse); laboratory culture; acclimatisation not necessary
Ecology:	Decomposer: Destruents (dead organic matter); in the litter layer
Test design:	6 males and 12 females per test vessel (plastic boxes, diameter 12 cm, 8 cm high); 12 to 16 weeks old with a weight of approx. 40 mg FW
Substrate:	Lufa Standard field soil St. 2.2; < 50% WHC_{max}
Parameter:	Mortality, reproduction
Duration:	Maximal 10 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW (3 replicates); Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 5 replicates
Performance:	Temperature: 20 ± 2°C; light-dark cycle 16:8 h with at least 500 Lux; relative air humidity > 80%; feeding twice weekly with a complex mixture (50% maple litter, 40% rabbit food, 10% potato pancake); weekly examinations of mortality and reproduction
Validity criteria:	Control: mortality not higher than 20%; number of juveniles per 100 mg FW of adult animals: at least 50
Reference substance:	Insecticide Dimethoate
Assessment:	EC10, EC50 and LC50
Notes:	Extension of the test by using contaminated food possible
Testing of GMP?	*P. scaber* has been used but following different protocols (Escher et al. 2000; Wandeler et al. 2002)

C1.10 Mites

Table 72:	**Reproduction test with the predatory mite *H. aculeifer***
Principle:	Sub-lethal laboratory test developed for chemicals using food as the main exposure route
Guideline:	Method proposal from literature (Schlosser & Riepert 1992a+b)
Test species:	*Hypoaspis aculeifer* (Canestrini, 1883); Gamasina (predatory mites); laboratory culture
Ecology:	Consumer 2. order: Predators (microarthropods); in the uppermost soil and the litter layer
Test design:	Individually held adult females (100 mL glass cylinder; 22.5 mm diameter; 5 cm high; filled with 30 g FW); exposure of the mites via their food, which also are exposed via their food (peas in the case of collembolans) or Agar (in the case of enchytraeids)
Substrate:	Artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Number of laid eggs (egg production); larval development (10 accidentally selected eggs are observed individually) and population dynamics (observation of all laid eggs)
Duration:	30 (feeding with collembolans) or 22 (feeding with enchytraeids) d if larval development is the main test parameter or up to 24 weeks in the case of measuring population dynamics
Application:	Depending on the water solubility of the test substance; measured as concentration in food (mg/kg peas or mg/L Agar)
Concentration:	3 concentrations; not higher than 1000 mg/kg DW; 5-6 replicates
Performance:	Temperature: 21 ± 1°C; permanent dark; feeding (2 to 3 times per week) with collembolans or enchytraeids (*Onychiurus fimatus, Enchytraeus albidus*); counting of eggs or animals using a stereomicroscope
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Determination of the concentration where a significant difference to the control is found by using commercial statistical packages
Notes:	One of the few examples in which the test organisms are exposed via their food. However, the exposure concentration of the test organisms is not known since only the food organisms are directly exposed to the chemicals. Up to now only rarely performed (e.g. with Lindane and Potassium dichromate).
Testing of GMP?	No

Table 73:	**Sub-lethal toxicity test with the predatory mite *H. aculeifer***
Principle:	Sub-lethal laboratory test developed for chemicals
Guideline:	Method proposal from literature (Krogh in: Løkke 1995)
Test species:	*Hypoaspis aculeifer* (Canestrini); Gamasida (predatory mites); laboratory culture
Ecology:	Consumer 2. order: Predators (microarthropods); in the uppermost soil and the litter layer
Test design:	10 female and 5 male mites per test vessel (glass cylinder; 6 cm diameter; 5 cm high; filled with 30 g FW soil)
Substrate:	Sieved field soil (sandy loam); characterization: organic content, grain size distribution, pH-value (without any organisms due to drying or freezing); shortly before start of the test inoculation with fresh soil eluate
Parameter:	Adult mortality and number of laid eggs (reproduction)
Duration:	3 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture in mg/kg food (algae paste)
Concentration:	5 concentrations, but not higher than 1000 mg/kg DW; 4 replicates
Performance:	Temperature: 20°C; feeding with collembolans (*Folsomia fimetaria, F. candida*), approx. 650 springtails per week; extraction of the mites by means of a dry extraction method
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	NOEC and LOEC
Notes:	The reproduction can be increased at low concentrations.
Testing of GMP?	No

Table 74:	**Predatory mite reproduction test in soil**
Principle:	Effects of chemical substances in soil on the reproductive output of the soil mite species *Hypoaspis (Geolaelaps) aculeifer*
Guideline:	OECD-Draft Guideline (2005)
Test species:	*Hypoaspis (Geolaelaps) aculeifer* Canestrini (Acari: Laelapidae); Gamasina (predatory mites); laboratory culture
Ecology:	Consumer 2. order: Predators (microarthropods); in the uppermost soil and the litter layer
Test design:	10 adult females per test vessel (glass; 3 to 5 cm diameter; height of soil ≥ 1.5 cm)
Substrate:	20 g artificial soil according to OECD (1984a): Quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water
Parameter:	Number of surviving females and number of offspring per test vessel
Duration:	Until first offspring have reached deutonymph stage (16 d at 20°C)
Application:	Mixed into the soil as aqueous solution or in quartz sand (use of organic solvent or insoluble substances)
Concentration:	NOEC-design: 5 concentrations, 4 replicates (8 controls); ECx-Design: 12 concentrations, 2 replicates (six controls); Combined approach: 8 concentrations, 4 replicates (8 controls); spacing factor ≤ 1.8; limit-test
Performance:	Temperature: 20 ± 2°C; light-cycle 16:8 h at 400 to 800 Lux; moisture 40 to 60% of WHCmax; feeding *ad libitum* with cheese mites (*Tyrophagus putrescentiae*), collembolans (e.g. *Folsomia fimetaria*), enchytraeids (e.g. *Enchytraeus crypticus*) or nematodes (e.g. *Turbatrix silusiae*)
Reference substance:	Dimethoate
Validity criteria:	Mortality ≤ 25%; at least 100 juveniles per test vessel; 10 to 90% effect in reference substance
Assessment:	NOEC or ECx estimation by appropriate statistical analysis
Notes:	-
Testing of GMP?	No

Table 75:	**Sub-lethal toxicity test with the oribatid mite *Platynothrus peltifer***
Principle:	Acute/sub-lethal laboratory test with oribatid mites
Guideline:	Formalised guideline proposal (Van Gestel & Doornekamp in: Løkke & Van Gestel 1998)
Test species:	*Platynothrus peltifer* (Koch, 1839); Oribatida (beetle mites); field captures with at least 2 weeks of acclimatisation
Ecology:	Consumer 1. order: Non-target herbivores (algae, dead organic matter); in the litter layer or uppermost soil layer
Test design:	20 adult mites (optional: tritonymph juveniles) per test vessel (plastic cylinder; 5 cm diameter; 3.5 cm high; filled with 7.5 g soil DW)
Substrate:	Artificial Soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water with 30 to 40% WHC_{max} (optionally the standard field soil Lufa St. 2.2 is possible)
Parameter:	Adult mortality and number of juveniles (reproduction)
Duration:	Preliminary test: 2 weeks; final test: 12 weeks; examinations after 2, 6 and 12 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Preliminary test: 1, 10, 100, 1000 mg/kg DW; Final test: 5 concentrations, but not higher than 1000 mg/kg DW, plus a control; 5 replicates
Performance:	Temperature: 15 ± 1°C; light-dark cycle 12:12 h with approximately 400 Lux; constant soil moisture; average air humidity 75%; feeding with green algae (*Desmococcus* sp.), approx. 0.01 g/2 weeks; extraction of the mites by means of a dry extraction method (e.g. Tullgren apparatus)
Reference substance:	Copper chloride; in OECD soil the LC50 should be approximately 320 mg/kg DW (in Lufa 2.2: 242 to 315 mg/kg DW)
Validity criteria:	Control: mortality not higher than 20% (after 2 weeks) and at least 100 juveniles per test vessel (after 12 weeks)
Assessment:	NOEC, EC10, EC50 and LC50 using suitable statistical methods
Notes:	Due to difficulties in culturing this species the test has not been presented to OECD for standardisation.
Testing of GMP?	No

Table 76:	**Sub-lethal toxicity test with the oribatid mite *Platynothrus peltifer* on plaster of Paris with dietary exposure**
Principle:	Acute/Sub-lethal laboratory test with dietary exposure
Guideline:	Formalised guideline proposal (Van Gestel & Doornekamp in: Løkke & Van Gestel 1998)
Test species:	*Platynothrus peltifer* (Koch, 1839); Oribatida (beetle mites); sampled in the field with an acclimatisation period of at least 2 weeks
Ecology:	Consumer 1. order: Non-target herbivores (algae, dead organic matter); in the litter layer or uppermost soil layer
Test design:	10 adult mites (optionally: tritonymph juveniles) per test vessel (plastic cylinder; diameter of 1.5 cm; height of 3.0 cm)
Substrate:	"Plaster of Paris" at the bottom of the test vessel
Parameter:	Mortality of adults but mainly number of eggs produced (reproduction); maybe the number of juveniles hatched from the eggs after another 4 to 6 weeks is more suitable; optionally number of faecal pellets
Duration:	10 weeks (optionally 14 to 116 weeks); weekly examinations (including transfer of adults to new test vessels)
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture in mg/kg food (algae paste)
Concentration:	Preliminary test: 1, 10, 100, 1000 mg/kg DW of dried algal substrate; Final test: 5 concentrations but not higher than 1000 mg/kg DW plus a control; 5 replicates
Performance:	Temperature: 15 ± 1°C; light-dark cycle of 12:12 h with approximately 400 Lux; relative air humidity: 75%; feeding with green algae (*Desmococcus* sp.) approx. 0.01 g/2 weeks; extraction of the mites by means of a dry extraction method (e.g. Tullgren apparatus)
Reference substance:	Copper chloride; in OECD soil the LC50 should be approximately 320 mg/kg DW (in Lufa 2.2: 242 to 315 mg/kg DW)
Validity criteria:	Control: mortality not higher than 20% after two weeks:
Assessment:	NOEC, EC50 and LC50
Notes:	Due to difficulties in culturing this species the test has not been presented to OECD for standardisation.
Testing of GMP?	No

Table 77:	**Acute and sub-lethal effects on *Typhlodromus pyri***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on phytoseiid mites
Guideline:	Method proposal from literature (Blümel et al. in: Candolfi et al. 2000)
Test species:	*Typhlodromus pyri* Scheuten (Acari: Phytoseiidae); laboratory culture or commercial supplier; 24 h after moulting of larvae
Ecology:	Consumer 2. order: Predators (preferably aphids); on (plant) leaf surfaces
Test design:	20 protonymphs per test unit: Open method: two horizontally fixed glass cover slides (24 x 50 to 60 x 0.13 to 0.17 mm), split for water supply by capillary forces, on permanently moistened filter paper and bordered by barrier of non-drying glue gel. Coffin cell method (mortality assessment only): bottom glass plate (100 x 50 x 3 mm; three 6 mm holes), top glass plate (100 x 50 x 15 mm), central frame of Teflon (3 mm) with slanting edges (lower surface 74 x 23 mm; upper 80 x 30 mm), two connecting tubes in outside holes and cotton taper in middle hole for water supply. Island method: round cover glass (diam. 45 mm; thickness 0.1 mm) in glass petri dish lid (diam. 54 mm) with central bottom hole of 6 mm, placed on bench situated in tray constantly filled with water resulting in flotation of cover glass.
Substrate:	Glass plates
Parameter:	Juvenile mortality; reproduction
Duration:	14 d; three evaluations d 7 to 14
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha; 5 replicates
Performance:	Temperature: 25 ± 2°C; 16 h photoperiod; relative air humidity 60 to 90%; food: various plant pollen
Validity criteria:	Arithmetic mean mortality ≤ 20%; cumulative mean number of eggs per female ≥ 4; cumulative mean mortality of 50 to 100% in reference
Reference substance:	Dimethoate 400 EC (9 to 15 mL product/ha)
Assessment:	Relative mortality of juveniles (Abbott's corrected) after 7 d; cumulative number of eggs per female (d 7 to 14)
Notes:	-
Testing of GMP?	No

C1.11 Spiders

Table 78:	**Acute toxicity test with wolf spiders**
Principle:	Acute laboratory test developed for pesticides
Guideline:	BBA No. VI, 23-2.1.9 (BBA 1994a)
Test species:	*Pardosa sp.*; Lycosidae (wolf spiders); field catches with an acclimatisation period in the laboratory of at least 7 d
Ecology:	Consumer 2. order: Predators (microarthropods); on the soil surface and in the vegetation letter
Test design:	1 female or 1 male (sub-adult to adult) per test vessel (e.g. glass with a volume of approx. 200 mL)
Substrate:	Moistened quartz sand (70% WHC_{max})
Parameter:	Mortality, feeding rate (number of fed flies), behavioural changes
Duration:	2 weeks; examinations after 2, 4 and 6 h as well as 1, 2, 3, 4, 7, 9, 11 and 14 d after application of the test substance
Application:	Spraying of the test substance on top of the soil surface (including beetles) in a laboratory device in analogy to agricultural practice; substances not soluble in water are strewn on the soil surface
Concentration:	Highest recommended application rate
Performance:	Temperature: 20 ± 2°C; light-dark cycle 16:8 h with 500 to 2000 Lux; feeding with 5 *Drosophila* flies (strain without wings) per spider per examination (in parallel moistening of the sand)
Reference substance:	Not definitely decided e.g. Karate (active ingredient: lambda-Cyhalothrin, 2 to 4 g/ha); expected mortality: 50 to 80%
Validity criteria:	Control: mortality < 15% at the end of the test
Assessment:	Statistical comparison of feeding rates (e.g. using ANOVA)
Notes:	Some modifications are possible: e.g. due to the high natural mortality of the males in certain periods of the year.
Testing of GMP?	No

Table 79:	**Acute and sub-lethal effects on *Pardosa spp.***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on spiders
Guideline:	Method proposal from literature (Heimbach et al. in: Candolfi et al. 2000)
Test species:	*Pardosa spp.* (Araneae: Lycosidae); collected from the field; 7 d acclimatization
Ecology:	Consumer 2. order: Predators (microarthropods); on the soil surface and in the vegetation layer
Test design:	3 d before start of the test 30 spiders per treatment are placed into the test units (plastic, glass or metal containers with substrate surface area of 90 ± 20 cm² and 6 ± 1 cm depth with ventilated transparent lids and walls treated with Fluon or Talcum), on d 0 test item is applied on substrate surface and spiders (if sprayed).
Substrate:	125 ± 1 g dw quartz sand
Parameter:	Mortality, behaviour, food consumption, and moulting
Duration:	2 to 3 weeks; examinations on d 1, 2, 3, 4, 7, 10, and 14
Application:	Spraying or incorporation into substrate
Concentration:	On the basis of a volume application rate of 400 L/ha
Performance:	Water content 70 ± 5% of WHC, temperature: 20 ± 2°C; 16L:8D photoperiod (500 to 1500 Lux); relative air humidity 60 to 90%; food: wingless *Drosophila* spp. or palatable species of aphid
Validity criteria:	Mortality ≤ 6.7% after 14 d, 13.3% after 21 d; reference mortality 65 ± 35% (Abbott's corrected)
Reference substance:	Karate (a.i. lambda-cyhalothrin; 2 to 4 g a.i./ha), Thiodan 35 EC (a.i. endosulfan; 30 to 40 g a.i./ha), or Dimethoate EC (600 to 900 g a.i./ha)
Assessment:	Mortality of spiders (Abbott's corrected), mean food consumption per live spider per assessment period; total food consumption
Notes:	-
Testing of GMP?	No

C1.12 Further terrestrial laboratory methods

Table 80:	**Test with soil-protozoa**
Principle:	Acute and sub-lethal laboratory test using soil protozoa
Guideline:	Method proposal from literature (Ekelund et al. in: Løkke 1995)
Test species:	Natural protozoa coenosis, isolated from crop field soil
Ecology:	Consumer 1. order/Decomposer: Non-target herbivores/Destruents (e.g. bacteria, protozoa, dead organic matter); in the pore water
Test design:	Inoculum (not specified) per test vessel (116 mL Serum flasks, filled with 5 g sterilised, sieved soil (25% moisture))
Substrate:	Variable: e.g. field soils; characterization: grain size distribution, organic content, pH-value, WHC_{max}
Parameter:	mortality, soil respiration (CO_2)
Duration:	7 weeks; examination three times per week (destructive sampling)
Application:	Mixing of the test substance as an aqueous solution in the test substrate
Concentration:	Variable; 5 replicates
Performance:	Temperature: 10 or 15°C; permanent dark; quantification of the Protozoa using the "Most probable number (MPN)"-method with microtiterplates
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Comparison with the control
Notes:	The development of this test method has been stopped due to unknown reasons. A comparable method was recently published by Berthold & Jakl (2002).
Testing of GMP?	Soil-protozoa have been investigated but following different protocol (Saxena & Stotzky 2001) or in the field (Griffiths et al. 2000; 2005)

Table 81:	**Sub-lethal toxicity test with centipedes**
Principle:	Laboratory test for the determination of the sub-lethal effects of chemicals on centipedes; performance in 2 test approaches
Guideline:	Formalised guideline proposal (Laskowski et al. in: Løkke & Van Gestel 1998)
Test species:	*Lithobius mutabilis* (Koch, 1852); Chilopoda (centipedes); keeping of field catches in the laboratory; at least 2 weeks of acclimatisation
Ecology:	Consumer 2. order: Predators (microarthropods); in the uppermost soil and the litter layer
Test design:	"Individual" test: 1 animal per test vessel (plastic box 11 * 7.5 * 4.5 cm); "population" test: 3 males plus 3 females per test vessel (plastic box 16 * 11 * 6 cm); at least 2 years old, weight 25 to 35 mg FW
Substrate:	Artificial Soil according to OECD (1984a): quartz sand, kaolin clay, *Sphagnum* peat, calcium carbonate and water with 50% WHC_{max}
Parameter:	Adult mortality, growth, respiration rate and mobility of the juveniles; hand-sorting of the animals as well as using an image analysis system and a respiration measurement device
Duration:	12 weeks in the case of persistent chemicals; 4 weeks in the case of quick degradation of the test substance; examination of mortality after 1, 2, 3, 4, 5,, 7, 10, 14, 21, and 28 d, afterwards weekly; growth rate at the beginning and the end of the test; respiration rate: after 1, 2, 4 and 8 weeks; mobility: during one week after the end of the test
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 6 replicates
Performance:	Temperature: 15 ± 1°C; light-dark cycle 16:8 h with 300 to 600 Lux; relative air humidity > 80%; feeding: 1 deep frozen pupae of the house fly *Musca domestica* twice per week per 1 *L. mutabilis*
Reference substance:	Insecticide Dimethoate (LC50 (14 d): 19.3 mg/kg a.i. DW)
Validity criteria:	Control: mortality not higher than 10% (after 4 weeks)
Assessment:	EC20, EC50 and LC50
Notes:	Extension of the test by using contaminated food or by determination of the biomagnification factor possible.
Testing of GMP?	No

Table 82:	**Sub-lethal toxicity test using diplopods**
Principle:	Sub-lethal laboratory test developed for chemicals using several endpoints
Guideline:	Formalised guideline proposal ((Tajovsky in: Løkke & Van Gestel 1998)
Test species:	*Brachydesmus superus* (Latzel, 1884); Polydesmidae, diplopods; keeping of field catches in the laboratory; at least 2 weeks of acclimatisation
Ecology:	Decomposer: Destruents (organic matter); on the soil surface and in the litter layer
Test design:	1 male and 1 female per test vessel (plastic boxes 8 * 10 * 4 cm); adult animals with a body length of 8 to 10 mm
Substrate:	Lufa Standard field soil St. 2.2
Parameter:	Adult mortality, number of the nests built by the females, number of eggs and juveniles per nest, duration of the individual development; in addition behavioural changes, post-embryonic development as well as occurrence of food in the gut
Duration:	10 weeks
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand; if insoluble in water and organic solvents, mixed with 10 finely ground quartz sand
Concentration:	Range-Finding-Test: 0.1, 1, 10, 100, 1000 mg/kg DW; Definitive Test: not specified number of concentrations for determination of an ECx but not higher than 1000 mg/kg DW; at least 5 replicates
Performance:	Temperature: 15 ± 1°C; light-dark cycle 16:8 h with nearly no light; relative air humidity > 80%; feeding with litter from the site where the animals were caught (dried, pulverized and, potentially, mixed with the test substance (by adding water pellets can be formed)), in addition after 3, 6 and 9 weeks 10 mg of baker's yeast; weekly examination of mortality and number of nests; all other parameter only at the end of the test; extraction of the animals by means of hand-sorting
Reference substance:	No information available
Validity criteria:	Control: no mortality; other parameter no information available
Assessment:	ECx, EC50, NOEC and LC50
Notes:	Extension of the test by using contaminated food or by determination of the biomagnification factor possible.
Testing of GMP?	No

Table 83:	**Acute and sub-lethal effects on *Orius laevigatus***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on predatory bugs
Guideline:	Method proposal from literature (Bakker et al. in: Candolfi et al. 2000)
Test species:	*Orius laevigatus* (Fieber) (Heteroptera: Anthocoridae); laboratory culture or commercial supplier
Ecology:	Consumer 2. order: Predators (aphids, thrips, spider mites); in the lower vegetation layer
Test design:	Mortality assessment: Juvenile bugs exposed in groups of 10 in test units (glass plates held apart by treated stainless steel or PTFE frame with slanting edges, bottom plate with 3 holes, 2 connecting tubes in outer holes and cotton taper in middle. Cell placed over demin. water bath allowing cotton taper to hang into bath, one tube connected to lower pressure pump for ventilation) for a min. of 9 d and until 80% of control bugs are adult. Assessment of reproductive performance: Fecundity assessment 4 d after 80% criterion was satisfied. Females transferred to oviposition substrates (untreated leaf discs on agar or wet cotton wool kept in plastic Petri-dishes fitted with 80 µm nylon gauze) for two subsequent periods of 2 d and number of eggs is determined. Hatching success is determined from first batch of eggs after 5 d.
Substrate:	Glass plates
Parameter:	Mortality assessment: Mortality/escape Assessment of reproductive performance: No. of eggs/bug; egg hatch
Duration:	appr. 21 d
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha; 8 replicates
Performance:	Temperature: 25 ± 2°C; 16L:8D photoperiod (200 to 3000 Lux); relative air humidity 60 to 90%; wind speed in cells avoiding build-up of vapour; food: eggs of *Ephestia* sp.
Validity criteria:	Mortality ≤ 25%; reference mortality ≥ 40%; min. egg production of 2 per female per d; ≤ 5 bugs producing zero values; egg hatch ≥ 70%
Reference substance:	Dimethoate EC 400 (2.5 mL in 200 L water/ha)
Assessment:	Percentage of mortality/escape, number of eggs per bug, percentage of egg hatch; relative performance of sub-lethal endpoints as % reduction
Notes:	-
Testing of GMP?	No; other species of the genus *Orius* have been used in different testing protocols (Pilcher et al. 1997; Armer et al. 2000; Zwahlen et al. 2000; Al-Deeb et al. 2001)

Table 84:	**Acute and sub-lethal effects on *Aphidius rhopalosiphi***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on parasitic wasps
Guideline:	Method proposal from literature (Mead-Briggs et al. in: Candolfi et al. 2000)
Test species:	*Aphidius rhopalosiphi* (DeStephani-Perez) (Hymenoptera: Braconidae, Aphidiinae); laboratory culture or commercial supplier
Ecology:	Consumer 3. order: Parasitoids (aphids); in the vegetation layer
Test design:	10 wasps (at least 5 females) per test unit (treated glass plates held apart by shallow untreated frame, 8 to 12 cm diameter, 1 to 2 cm tall, frame with drilled holes covered with fine-gauge mesh for ventilation), after 48 h a minimum of 15 female wasps per treatment placed in fecundity areas (confined over pots of aphid-infested cereal plants using clear-walled, ventilated cylinders) for 24 h; number of aphid mummies that develop is recorded 10 to 12 d later
Substrate:	Glass plates
Parameter:	Mortality/immobility of adults after 48 h, number of mummies developing after 10 to 12 d for each wasp
Duration:	approx. 13 d
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha; 4 replicates
Performance:	Temperature: 20 ± 2°C; 16-h photoperiod (400 to 3000 Lux; fecundity assessment: 4000 to 20000 Lux); relative air humidity 60 to 90%
Validity criteria:	Mortality should not exceed 5 out of 40 wasps (13%); minimum of 5 mummies per female
Reference substance:	Dimethoate EC 400 (0.3 mL in 200 L water/ha)
Assessment:	Percentage of adult mortality/immobility (Abbott's corrected), mean number of mummies produced per wasp; for rate-response relationships probit regression analysis is recommended
Notes:	-
Testing of GMP?	No

Table 85:	**Acute and sub-lethal effects on *Trichogramma cacoeciae***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on parasitic wasps
Guideline:	Method proposal from literature (Hassan et al. in: Candolfi et al. 2000)
Test species:	*Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae); laboratory culture; appr. 24 h old.
Ecology:	Consumer 3. order: Parasitoids (preferably eggs of Lepidoptera); in the vegetation layer
Test design:	At least 50 female wasps per test unit (treated glass plates fitted onto aluminium or stainless steel frame (13 x 13 cm, walls 1.5 cm high, 1 cm wide) with holes for ventilation (covered with black, tightly stretched, porous material) and introduction starting population, food and host eggs (closed with removable adhesive tape)) are exposed to a total of 9000 host eggs over a period of 7 d. Afterwards collection and incubation of host eggs.
Substrate:	Glass plates
Parameter:	Mortality (only if 100%), capacity of parasitism
Duration:	Minimum of 13 d; evaluation after d 3, 10, 11, and 13
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha; minimum of 4 replicates
Performance:	Temperature: 25 ± 2°C; continuous light (2500 Lux first 3 h, afterwards 500 Lux); relative air humidity 60 to 90%; food mixture of 3 g gelatine, 100 mL distilled water and 200 g honey
Validity criteria:	Total number of parasitized eggs per female > 15; total mortality or 50% reduction of parasitisation in reference
Reference substance:	Dimethoate 40 EC (0.013 to 0.025 g a.i./ha)
Assessment:	Mean number of parasitized eggs per female
Notes:	-
Testing of GMP?	No

Table 86:	**Acute and sub-lethal effects on *Chrysoperla carnea***
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on green lacewings
Guideline:	Method proposal from literature (Vogt et al. in: Candolfi et al. 2000)
Test species:	*Chrysoperla carnea* (Neuroptera: Chrysopidae); laboratory culture or commercial supplier; 2 to 3 d old
Ecology:	Consumer 2. order: Predators (microarthropods and their eggs); in the vegetation layer
Test design:	Mortality assessment: Larvae placed in test units (sprayed glass plates, glass or acrylic cover with holes of min. 4 cm diam. and rings of glass, PS or acrylic glass of min. 1.5 cm height treated with Fluon or Talcum fitted in holes). Min. 5 d after formation cocoons are collected and put in rearing box (plastic, glass or PS box, cotton or nylon gauze cover) Assessment of reproductive performance: Min. 3 females and 2 males. Test starts earliest 1 w after first egg laying. Two 24-h egg samples taken within 1 w and incubated for hatching. After hatching (~6 d) percentage of hatched eggs is determined and unusual observations are noted. If egg production is < 15 eggs per female or hatching rate < 70% test is prolonged for at least two further assessments over 24 h periods
Substrate:	Glass plates
Parameter:	Mortality assessment: Survival of larvae, pupation and hatching of adults, behavioural abnormalities; Assessment of reproductive performance: Cumulative mortality, egg production and hatching rate
Duration:	5 to 6 weeks
Application:	Spraying
Concentration:	On the basis of a volume application rate of 200 L/ha
Performance:	Temperature: 25 ± 2°C; 16-h photoperiod (≥ 1000 Lux); relative air humidity 60 to 90%; larval food eggs of *Sitotroga cerealella* or *Ephestia kuehniella*; adult food mixture of condensed milk, egg, honey, fructose, dried brewer's yeast, wheatgerm and, water
Validity criteria:	Max. cumulative mortality ≤ 20%; mean number of eggs per female per d ≥ 15; mean hatching rate ≥ 70%; reference mortality ≥ 50%
Reference substance:	Dimethoate EC 400 (30 to 45 mL/ha in 200 L water/ha)
Assessment:	Percentage of larval mortality (Abbott's corrected), average number of eggs per female per d, percentage of fertile eggs
Notes:	-
Testing of GMP?	*C. carnea* has been used but following different protocols (Pilcher et al. 1997; Hilbeck et al. 1998a+b; 1999; Lozzia et al. 1998; Meier & Hilbeck 2001; Dutton et al. 2002; Romeis et al. 2004)

C2 Terrestrial semi-field tests

Table 87:	**NATEC plant metabolism box**
Principle:	Closed laboratory model ecosystems (soil, air, plants, and animals (earthworms)) developed for fate studies with chemicals
Guideline:	Proposal from literature (Figge et al. 1983, modified according to Kühle 1986)
Ecology:	Not applicable. Different trophic layers and soils can be covered.
Test species:	Microorganisms (natural coenosis), animals (partly natural coenosis (e.g. Protozoa), partly introduced (e.g. earthworms), plants (seedling); introduced organisms: either laboratory culture or field catches
Test design:	Extraction from the field as an undisturbed core
Substrate:	Various natural field soils; characterization as in laboratory degradation tests: grain size distribution, pH-value, organic content
Parameter:	Analytical determination of he amount of test substance in the various system compartments (soil (various layers separately), air, plants, animal, (species specific), leachate).
Duration:	Sampling is variable; e.g. after 30 d
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand (as close to agricultural practice as possible)
Concentration:	Depending on the test substance
Performance:	Aerobe (regularly exchange of air) incubation under controlled environmental conditions (temperature, light, air movement, precipitation)
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Mass balance and specific values like DT_{50}-value or the bioconcentration factor; use of these data in fate models (volumes of the various compartments in the box are exactly known)
Notes:	^{14}C-radio-labelled chemicals (in exceptional cases without labelling)
Testing of GMP?	No

Table 88:	The TME-System ("Microcosm")
Principle:	Investigation of terrestrial model ecosystems (soil, plants, animals (e.g. earthworms, enchytraeids)) in the greenhouse in order to determine the fate and the effects of chemicals.
Guideline:	Draft Guideline according to Van Voris et al. (1985) modified by Knacker et al. (1990, 1991, 2004) and UBA (1994); a comparable paper was published as ASTM E 1197-87 (ASTM 2004b)
Test species:	Natural biocoenosis of micro-organisms, animals and plants; addition of organisms (e.g. planting of oat seeds) is possible
Ecology:	Not applicable. Different trophic layers and soils can be covered.
Test design:	Undisturbed soil cores from the field in 40-cm long HD-PE tube (17.5 cm diameter), a HDPE bottom plate with drilled holes covered with inert gauze, PVC-tubing to 1 L PE bottle to collect leachate
Substrate:	(Nearly) any natural soil (e.g. EuroSoils); characterisation analogous to other soil tests (e.g. laboratory degradation studies)
Parameter:	Fate endpoints: determination of chemical residues in soil layers (0 to 5cm; 5 to 15 cm), in leachate and plants; functional endpoints: nutrient cycling, microbial activity, decomposition; structural endpoints: abundance, diversity and community structure of flora and fauna
Duration:	Variable; e.g. 16 weeks with destructive sampling after 1 (only control, highest and lowest treatment level), 8, and 16 weeks
Application:	Depending on the properties of the test substance: dropping, sprinkling, spraying or mixing into the soil (if possible according to real field conditions); 6 control and 4 treatment replicates per sampling date
Concentration:	Depending on the test substance and exposure scenario
Performance:	Temperature: 12 ± 2°C around TMEs, 23 ± 5°C air temp.; rel. humidity 50 to 80%; day/night cycle 16/8 h; illumination 8000 to 12000 Lux; irrigation with artificial rain water up to 4 times/week via “rain-head”
Reference substance:	Carbendazim is a possibility
Validity criteria:	Not available
Assessment:	Absolute amount or concentration of the test substance in the various environmental compartments; comparison of the biotic parameters in the treated TMEs with untreated control soil cores (NOEC, ECx)
Notes:	Tested up to now: Chemicals (partly ^{14}C-labelled), waste material (e.g. fly ash) (e.g. Moser et al. 2004a+b; Römbke et al. 2004).
Testing of GMP?	No

Table 89:	**Artificial model ecosystem of the BBA Berlin**
Principle:	Closed model ecosystems (soil, air, plants, animals) in the greenhouse developed for fate investigations of chemicals (partly effects)
Guideline:	Method proposal from literature (Heise et al. 1988)
Test species:	Plants (sowed species representative for an urban meadow coenosis); addition of animals (earthworms, isopods, carabids); laboratory culture or field catches
Ecology:	Not applicable. Different trophic layers and soils can be covered.
Test design:	Homogenous mixture (gnotobiotic system); number of organisms' variable
Substrate:	Various natural field soils; characterization as in laboratory degradation tests: grain size distribution, pH-value, organic content
Parameter:	Analytical determination of the amount of the test substance in the individual system compartments (soil, air, plants, animals (species specific), leachate)
Duration:	Sampling variable; e.g. after 23 to 26 d
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand (as close to agricultural practice as possible)
Concentration:	Depending on the test substance
Performance:	Aerobe (regularly exchange of air) incubation under controlled environmental conditions (temperature, light, air movement, precipitation)
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Determination of the overall turnover rate, mineralisation, volatilization, species-specific differences in the uptake rate, residues in soil and leachate (including differentiation of metabolites); general assessment following an own index system
Notes:	^{14}C-radiolabelled chemicals are recommended.
Testing of GMP?	No

Table 90:	**Terrestrial multispecies-method**
Principle:	Use of model ecosystems (soil, plants, animals) for the assessment of the effects of chemicals
Guideline:	Method proposal from literature (Mothes-Wagner et al. 1992)
Test organisms:	Natural coenosis of microorganisms and animals; addition of enchytraeids, nematodes, predatory mites and beans possible
Ecology:	Not applicable. Different trophic layers and soils can be covered.
Test design:	Extraction as a whole soil core from the field or use of sieved soil; addition of approx. 2000 *Enchytraeus coronatus*, approx. 25.000 *Pelora strongyloides* and approx. 200 adult, female *Tetraychus. urticae*; sowing of 50 *Phaseolus vulgaris* seeds) per test vessel (container: 30 * 46 * 20 cm (= 0.138 m² with a depth of 20 cm); filled with approx. 25 kg of soil)
Substrate:	Variable: various natural or artificial soils; characterization: pH-value, WHC_{max}, nitrate- and phosphate concentration
Parameter:	Variable: e.g. enzyme distribution and activity, cell pathology and digesting enzymes of the test organisms, mortality, reproduction, phytotoxicity, behavioural changes, male/female ratio, population density, age stages etc.
Duration:	Examination variable; e.g. up to 90 d
Application:	Variable: as close to real field situations as possible
Concentration:	Depending on test substance and exposure scenario
Performance:	Temperature: 25 to 28°C (day), 18 to 20°C (night); light-dark cycle 16:8 h; air humidity 40 to 50%; in the laboratory or greenhouse
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Comparison of the development of the biological components in the various treatments with the control
Notes:	Comparison with field situations has been performed.
Testing of GMP?	No

Table 91:	**Gnotobiotic soil-microcosms**
Principle:	Use of microcosms for the investigation of the effects of chemicals on populations of nematodes and microarthropods
Guideline:	Method proposal from literature (Parmelee et al. 1993; 1997)
Test species:	Natural coenosis of nematodes and microarthropods
Ecology:	Consumer 1. order: Non-target herbivores (organic matter and microbes); in mineral soil
Test design:	Artificial mixture of 150 m³ soil per test vessel (plastic tubes: 4 cm diameter, 20.7 cm long)
Substrate:	Variable: sieved top field soil or artificial soils; characterization: grain size distribution, cation exchange capacity, pH-value
Parameter:	Residue analysis; number of nematodes (differentiated in fungivorous, bacteriovorous, herbivorous and omnivorous-predatory organisms as well as juveniles); number of microarthropods (differentiated in various mite groups (Prostigmata, Mesostigmata, Oribatida), Collembola and "other" insects
Duration:	7 d (up to 56 d possible)
Application:	According to the solubility of the test substance: dissolved in water or in an organic solvent in a mixture with finely ground quartz sand (Artificial Soil) or dried field soil; the chemicals are added when the test substrate is mixed
Concentration:	Variable: e.g. 4 concentrations; 6 replicates
Performance:	Temperature: 18 to 21°C; maintenance of initial moisture by adding sufficient water periodically; extraction of nematodes with Baermann funnels and of microarthropods with high-gradient Tullgren funnels
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Concentration of the test substance in soil; number of organisms in comparison to the untreated control (e.g. by ANOVA)
Notes:	It can also be used in soil quality assessment.
Testing of GMP?	No

Table 92:	**Acute effects on arthropods**
Principle:	Acute semi-field test developed for pesticides using various arthropod species
Guideline:	Guideline proposal according to IOBC (Wiles & Jepson 1992)
Test species:	Variable: e.g. the collembolans (springtails) *Isotoma viridis, Isotomurus palustris, Sminthurus viridis* (field catches) or *Folsomia candida* (laboratory mass culture)
Ecology:	Decomposer or consumer 2. order (dead organic matter or nematodes); in the soil air space
Test design:	Adult individuals: 20 (*F. candida*) or 10 (field catches) of similar size or weight per test enclosure (plastic ring)
Substrate:	Untreated field soil, e.g. a sandy Standard field soil (Lufa St. 2.2; organic substance 4.5%, pH 5.9) or site specific soils; approx. 50 g are filled in a plastic ring (with smooth surfaces) (diameter 9 cm)
Parameter:	Mortality; in parallel residue analyses of the test substance in soil
Performance:	Documentation of the natural meteorological conditions; per site 3 replicates per test substance or control in normal crop fields
Duration:	Examination after every 24 h (new addition of animals); total study duration e.g. 15 d after application
Application:	Spraying of the test substances as an aqueous solution on the field crop sites including the plastic ring.
Concentration:	Highest recommended application rate of a pesticide
Validity criteria:	No information available
Reference substance:	No information available
Assessment:	Determination of the DAT_{50} (= age of the pesticide residue, at which 50% of the test organisms are dying) using Probit Analysis
Notes:	The test results can be affected by unusual climatic conditions (e.g. strong cold).
Testing of GMP?	No

Table 93:	**"Field-Microcosms"**
Principle:	Use of microcosms for the investigation of the effects of chemicals on added microarthropods
Guideline:	Method proposal from literature (Petersen & Gjelstrup in: Løkke 1995)
Test species:	Natural coenosis of microorganisms; addition of microarthropods (e.g. the collembolans *Folsomia fimetaria* and *Isotoma notabilis* or the predatory mite *Hypoaspis aculeifer*); laboratory culture
Ecology:	Not applicable. Different trophic layers and soils can be covered.
Test design:	Adding of 30 just adult (16 to 23 d) collembolans (partly also 5 predatory mites) per test vessel (plastic tubes with a diameter of approx. 10 cm and a length of 10 to 30 cm); putted differently deep into the soil
Substrate:	Variable: natural, defaunated soil; characterization: grain size distribution, cation exchange capacity, pH-value
Parameter:	Number of collembolans
Duration:	1 to 7 d in the laboratory; afterwards exposure in the field for about 12 weeks (variable); examination for acute effects after 2 weeks and for chronic effects after 6 to 10 weeks
Application:	In the case of pesticides: Spraying in analogy to agricultural practice
Concentration:	Variable; e.g. maximum recommended rate in the case of pesticides; 10 replicates
Performance:	Laboratory: temperature: 5 to 10°C; field: natural environmental conditions (monitoring of climatic parameters); extraction of the microarthropods using dry extraction methods
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Determination of EC50; number of organisms in comparison to an untreated control
Notes:	In field samples taken close to the microcosms the number of collembolans was clearly lower than in the microcosms.
Testing of GMP?	No

Table 94:	**Sub-lethal toxicity of staphylinids in the semi-field**
Principle:	Determination of the lethal and sub-lethal effects of chemicals on larval staphylinids in the semi-field
Guideline:	Formal guideline proposal (Metge & Heimbach in: Løkke & Van Gestel 1998)
Test species:	*Philonthus cognatus* Stephenson 1832 (Staphylinidae); rove beetle; laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	Exposure phase: 9 approximately 1 to 2 d old larvae per test vessel (galvanised tin-vessels; 26 * 23,5 * 12 cm high, filled with 2500 g DW soil), the larvae are individually separated by plastic rings; reproduction phase: Input of the pupae taken from the field in glass cylinders (volume: 35 cm^3; 2.8 cm diameter; 7 cm high), filled with 20 g DW soil (see Table 61 for further details).
Substrate:	Lufa Standard soil 2.1 or 2.2 (50% WHC_{max})
Parameter:	Determination of the mortality of the larvae, their biomass at the time of hatching and the average duration of development; in addition, observation of the mobility of the larvae.
Duration:	Exposure phase: not determined (depending from field conditions); reproduction phase: see Table 61
Application:	Depending on the solubility of the test substance: either as an aqueous solution, by using organic an organic solvent or mixing in with quartz sand.
Concentration:	Results from laboratory tests are used for the determination of 4 to 5 concentrations; but not higher than 1000 mg/kg
Performance:	Exposure phase: field conditions (e.g. temperature: > 10 and < 28°C; reproduction phase: see Table 61. Daily feeding in the field with unfrozen, dissected *Calliphora sp.* pupae
Reference substance:	No information available
Validity criteria:	Control: mortality of the larvae of 30% at the maximum
Assessment:	Determination of LOEC, ECx and LCx
Notes:	This test is a direct extension of the laboratory test described in Table 61.
Testing of GMP?	No

Table 95:	**Acute effects on carabid beetles under semi-field conditions**
Principle:	Determination of the acute effects of pesticides on carabids under semi-field conditions (enclosures at agricultural sites)
Guideline:	Guideline proposal analogous to requirements of BBA and IOBC (Dohmen et al. 1996)
Test species:	*Poecilus cupreus* (L.); (Carabidae), ground beetle; laboratory culture
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	Adult beetles (1 to 2 months old); 12 animals (6 males + 6 females) per field enclosure (steel frame with a size of 50 * 50 * 30 cm)
Substrate:	Untreated field soil; e.g. a silty sand (org. substance: 1,8%, pH 7.1)
Parameter:	Mortality, feeding rate and behavioural changes
Duration:	Assessment after 14 d; examinations every 3 d.
Application:	Like in agriculture the test substance has to sprayed as an aqueous solution on the substrate or has to be strewn on the soil surface.
Concentration:	Highest recommended application rate; 4 replicates per substance and control
Performance:	Documentation of the natural climatic conditions; sealing of the frames with a wire mesh in order to avoid escaping of the animals as well entering of predators; between the frames normal crops should grow; feeding every 2 to 3 d (per beetle one fly pupa); on the last day of the test sieving of the soil to get also beetles dug into the soil
Reference substance:	Hostathion (a.i.: Triazophos); 2.5 L/ha
Validity criteria:	Control: mortality < 10%; feeding rate: 1.5 to 3.5 fly pupae per beetle
Assessment:	Assessment after IOBC-criteria
Notes:	Test- as well as control results (validity criteria!) can be influenced strongly by climatic events (e.g. coldness).
Testing of GMP?	No

Table 96:	**Acute and sub-lethal effects on *Poecilus cupreus* – Semi-field test**
Principle:	Test for evaluating acute and sub-lethal effects of plant protection products on carabid beetles
Guideline:	Method proposal from literature (Heimbach et al. in: Candolfi et al. 2000)
Test species:	*Poecilus cupreus* L. (Coleoptera: Carabidae); laboratory culture or commercial supplier; 24 h acclimatization
Ecology:	Consumer 2. order: Predators (e.g. fly larvae); in the uppermost soil and the litter layer
Test design:	5 male and 5 female beetles per enclosure (50 x 50 cm metal frames, inner surface painted with Fluon, appr. 25 cm deep, pushed into soil with appr. 10 cm extruding, covered by large gauge mesh).
Substrate:	Homogeneous, bare sandy soil field with low organic matter content
Parameter:	Mortality, behaviour and food consumption
Duration:	14 d, examinations on d 1, 2, 4, 7, 10/11, and 14
Application:	Should reflect normal use, spraying or incorporation into substrate
Concentration:	4 replicates
Performance:	Temperature ≥ 10°C during first 2 d after application, dry sites may be moistened with water before introduction of beetles; food: pupae of *Musca* spp. or *Delia* spp.
Validity criteria:	70% recovery of beetles; reference mortality plus non-recovery of ≥ 35%
Reference substance:	Parathion-methyl (300 g a.i./ha; 750 g/ha ME 605 Spritzpulver) or triazophos (1000 g a.i./ha; 2.5 L/ha Hostathion)
Assessment:	Mortality of beetles (Abbott's corrected), mean food consumption per feeding date; total food consumption
Notes:	-
Testing of GMP?	No

Table 97:	**Effects of pesticides on honeybees – Tent test**
Principle:	Bees foraging on sprayed flowering crop in field tents
Guideline:	BBA 23-1 (1991)
Test species:	*Apis mellifera* (honeybee); colonies with max. 3 full frames (ca. 5000 bees)
Ecology:	Consumer 1. order: Pollinators (pollen); in (flowering) vegetation
Test design:	At least 10 to 12 bees/m²; tents: base area 2 x 3, better 3 x 4 m, height 2 m, wire mesh or gauze, mesh size ≤ 3.5 mm
Substrate:	Flowering *Phacelia tanacetifolia* Benth., alternatively rape, borage or mustard
Parameter:	Mortality; flight intensity; abnormal behaviour
Duration:	72 h, max. extension to 7 d (in case of effect or for systemic pesticides); continuous observation during first hour after application, afterwards every 2 h on d 1, at least 3 times on d 2 and 3
Application:	According to intended use; during bee flight
Concentration:	Twice the intended application concentration; 2 repetitions on different occasions
Performance:	Measuring of temperature, humidity, precipitation, % cloud cover; wind speed max. 1 to 2 m/sec.; water supply
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Daily mortality, flight intensity, behaviour, assessment of bees in hive and brood
Notes:	Guideline outdated
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

Table 98:	**Side-effects on honeybees – Cage test**
Principle:	Bees foraging on sprayed flowering crop in field cages
Guideline:	EPPO PP 1/170(3) (2000)
Test species:	*Apis mellifera* (honeybee); small colonies
Ecology:	Consumer 1. order: Pollinators Pollinators (pollen); in the (flowering) vegetation layer
Test design:	One small healthy queen-right colony per cage, of at least three full frames, or a nucleus; cages min. 40 m², max. mesh size 3 mm
Substrate:	Suitable test crops are *Borago*, *Brassica*, *Phacelia*, *Sinapis* and other flowering plants attractive to bees
Parameter:	Foraging activity and behaviour of bees; number of dead bees in dead-bee traps and rest of the cage; other assessments appropriate to type of product
Duration:	7 d, assessments after 0, 1, 2, 4, and 7 d
Application:	Spraying during daytime; only formulated products; spraying of cage walls should be avoided
Concentration:	Highest dose specified for intended use; sufficient number of replicates to enable appropriate statistical analysis and risk assessment
Performance:	feeding may be necessary and water should be offered
Reference substance:	E.g., parathion, dimethoate
Validity criteria:	Tests should be repeated where control mortality is considerable in comparison with toxic standard and where mortality in toxic standard is low
Assessment:	Appropriate statistical analysis of mortality and other data relevant to properties of product
Notes:	Can be modified for specific tests, e.g. repellence or evaluation of the hazard of pesticides to honeybees foraging on aphid honeydew
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

Table 99:	**The isopod-litter-method**
Principle:	Investigation of the interaction of litter decomposition and woodlice in a microcosm treated with chemicals
Guideline:	Guideline proposal published in literature (Van Wensem 1993)
Test species:	Natural microbial coenosis inhabiting poplar leaves; *Porcellio scaber* (Latr. 1814); woodlouse (Porcellionidae); laboratory culture
Ecology:	Decomposer: Destruents (dead organic matter); in the litter layer
Test design:	Artificial, open microcosm system composed of a sand layer (thickness: 4 cm), 4 g litter and 8 adult isopods with comparable biomass (20 to 30 mg); introduction of the animals after 4 weeks) per test vessel (plastic cylinders; diameter 6 cm, height 12 cm)
Substrate:	Sand (moistened up to WHC_{max}) and poplar leaves (*Populus x canadensis*); approx. 2 weeks old, dissected in pieces of approx. 4 cm²
Parameter:	Mineralisation of carbon, nitrogen and phosphorus; continual measurement of CO_2; mortality and biomass of the isopods
Duration:	8 weeks; sampling variable; e.g. after 4 and 8 weeks
Application:	Mixing of the water-soluble test substance in dried poplar leaves
Concentration:	5 concentrations with a "spacing factor" of √10, but not more than 1000 mg/kg; the highest test concentration should not cause any mortality of the isopods; 4 replicates per examination (= 12 replicates in total per concentration)
Performance:	Temperature: 20 ± 2°C; light-dark cycle: 16:8 h with low light intensity; weekly checks in order to keep soil and litter moisture constant
Reference substance:	No information available
Validity criteria:	All microcosms should similar soil and litter moisture values.
Assessment:	Determination of NOEC, e.g. by using ANOVA
Notes:	This test has not been used in a routine way.
Testing of GMP?	No

C3 Terrestrial field studies

Table 100:	**Field test with earthworms**
Principle:	Chronic field test with the natural earthworm coenosis of a meadow or field
Guideline:	BBA VI, 2-3 (BBA 1994d); very similar is ISO 11268-3
Test species:	Natural earthworm coenosis (usually several species; in Germany obligatory: *Lumbricus terrestris*); in total at least 100 Ind/m² should be present at the chosen meadow site
Ecology:	Decomposer: Destruents (organic matter); in the mineral soil, vertical burrows and in the litter layer (endogeic, aneciques, epigeic)
Test design:	10 * 10 m plots (6 * 6 m core area + 2 m border strips); a crop site as well as a meadow are possible
Substrate:	Undisturbed field soil which has to be characterized as follows: Soil type, grain size distribution, org. content, pH-value, $WHC_{max.}$, vegetation type and climatic conditions during the test period
Parameter:	Mortality, biomass and species spectrum; sampling method: formol or electrical extraction; the efficiency of the chosen method has to be examined once using hand-sorting (4 replicates)
Duration:	1 year; examinations after 1, approx. 5 and 12 months
Application:	As usually in agriculture; e.g. by spraying or strewing on the soil surface
Concentration:	Highest recommended rate and four times this concentration; 4 replicates each
Performance:	As normally done in agriculture; e.g. mulching of meadows
Reference substance:	Benomyl (2 kg/ha)
Validity criteria:	Comparison with an untreated control (application of water) and with a positive control (application of the reference substance)
Assessment:	Use of "suitable" statistical methods
Notes:	Despite the fact that crop sites could be used for this test they are often not recommendable since the number of earthworms is too low.
Testing of GMP?	No

Table 101:	Effects of chemicals on the breakdown of organic matter in soil
Principle:	Breakdown of organic matter in litter bags buried in field soil
Guideline:	OECD-Draft guideline (2006)
Test species:	Natural saprophagous soil biocoenosis
Ecology:	Directly (e.g. decomposer: destruents) or indirectly (consumer 2. order: predators) the whole soil organism community is involved.
Test design:	Litter bags of non-degradable material with mesh size of 5 to 10 mm, size ca. 10 x 20 cm, bags filled with 4 g dm of wheat straw in a thin and even layer; plots 5 x 5 m, randomly distributed, plots separated by untreated 3 m wide strips, bags evenly distributed with in each plot, no litter bags within 1 m of plot border, 8 random bags per sampling date
Substrate:	Arable land under cultivation (characterized by texture, pH, WHCmax, OM content, moisture, CEC, vegetation type and cover, history of crop cultivation and pesticide applications)
Parameter:	Organic matter mass loss and breakdown rate
Duration:	At least 6 up to 12 months (if 60% mass loss not reached after 6 months in controls or statistically significant differences between control and treatment); at least three sampling dates within first 6 months, first sampling after about 1 month
Application:	Plateau concentration: mechanical incorporation evenly distributed into the top 10 cm 1 to 2 weeks before burying the litter bags; annual application within 1 week of burying the bags according to proposed agricultural use and relevant use conditions; homogeneous application across entire test plot; soil analysis for test substance; 6 replicates
Concentration:	Calculated plateau concentration and annual cumulative application rate; at least one treatment rate and a control
Performance:	Season according to intended use pattern of substance; site free of vegetation during application period; sowing of plants after application of plateau concentration but before burying of litter bags; at least 10 mm of precipitation within 3 d after spray application (irrigation if necessary); use of fertilisers or other pesticides should be avoided; no harvesting of the crop
Validity criteria:	At least 60% mass loss in controls at end of study; CoV max. 40% within first 6 months of test
Reference substance: None	
Assessment:	Statistically significant differences in organic matter mass loss between control and treatment
Notes:	-
Testing of GMP?	Yes (Cortet et al. 2006)

Table 102:	**Testing of arthropods in crop fields**
Principle:	Determination of short-term effects of insecticides on "Non-Target-Arthropods" (except bees) in crop fields
Guideline:	National Guideline, Working Document 7/7 (MAFF 1993)
Test species:	Natural arthropod coenosis (carabids, staphylinids and spiders); preferably groups, which were also tested in the laboratory;
Ecology:	Consumer 2. order: Predators (preferably microarthropods); on the soil surface and lower vegetation layer
Test design:	Two approaches are possible: 1. Non separated large areas (> 1 ha); 2. Small enclosed areas (10 * 10 m). The colonisation of each area should be examined by using 20 pitfall-traps before starting the test.
Substrate:	Untreated field soil of wheat fields (without insecticide or molluscicide application in the same year); documentation of meteorological parameters
Parameter:	Number and species spectrum of the arthropod groups listed above
Duration:	Pitfall-traps: Sampling in approach 1: twice before 2 and 4 d and afterwards weekly until approx. 7 weeks after application; approach 2: 5 and 2 d before application as well as 2 and 4 and afterwards weekly until approx. 7 weeks after application; mesh catches in approach 1: 5, 10, 15 and 30 d after application; exceptional samples are taken in order to determine the density of aphids
Application:	The test substances are applied like in agricultural practice as an aqueous solution in the field at a date, when concerning exposure a worst-case situation is given (e.g. in England: June - July).
Concentration:	Highest recommended application rate, one untreated control as well as a positive control and maybe a negative control; 4 replicates per treatment, respectively (preferably in two years).
Performance:	Only very general hints are given.
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Determination of significant differences by using the ANOVA method.
Notes:	-
Testing of GMP?	No

Table 103:	**Testing of predatory mites in vineyards and orchards**
Principle:	Evaluation of short and long term effects of plant protection products on population dynamics of phytoseiid mites in vineyards and orchards
Guideline:	Method proposal from literature (Blümel et al. in: Candolfi et al. 2000)
Test species:	Natural predatory mite population (Acari: Phytoseiidae; e.g., *Typhlodromus pyri* Scheuten)
Ecology:	Consumer 2. order: Predators (preferably aphids); on (plant) leaf surfaces
Test design:	Plot size large enough to minimise edge effects, sample size at least 30 mites per sample/plot; homogeneous agricultural unity; minimum of 15 grapevines or 8 slender spindle bush trees (at least 3 to 4 years old; sufficiently dense foliage) per replicate per treatment
Substrate:	Leaves of grapevines or orchard trees
Parameter:	Population density
Duration:	Until 28 d after last application, continued sampling in 4 week intervals if effect > 50%; evaluation up to the 3rd treatment before each successive treatment (but not later than 14 d after the previous treatment), after more than 3 treatments every second treatment (but not later than 14 d after previous treatment), post application samples 7 and 28 d after last treatment
Application:	Spraying or mist blowing
Concentration:	According to the intended use; minimum of 5 replicates (at least 2 for reference)
Performance:	Field conditions; wind speed should not exceed 3 m/sec at application
Validity criteria:	> 50% effect in reference after one or more applications
Reference substance:	According to study purpose a fungicide, acaricide or insecticide
Assessment:	Mean number of phytoseiid mites/leaf relative to control
Notes:	-
Testing of GMP?	No

Table 104:	**Effects of pesticides on honeybees – Field test**
Principle:	Inhalation, contact, and oral toxicity of pesticides to honeybees
Guideline:	BBA 23-1 (1991)
Test species:	*Apis mellifera* (honeybee); healthy, normally developed colonies with at least 2 full frames
Ecology:	Consumer 1. order: Pollinators (pollen); in (flowering) vegetation layer
Test design:	4 colonies in one row per plot: at least 0,25 ha, distance to other attractive flowering crops at least 3 km, colonies on homestead serve as control
Substrate:	Phacelia or rape in full bloom
Parameter:	Mortality; flight intensity; abnormal behaviour (at hive entrance)
Duration:	1 week, continuous assessment on day of application until the evening, on the following d 3 assessments, afterwards daily
Application:	With good weather in the morning; according to intended use; during bee flight
Concentration:	Twice the intended application concentration; no reference and control plots; 2 repetitions on separate occasions
Performance:	Measuring of temperature, humidity, precipitation
Reference substance:	None
Validity criteria:	No information available
Assessment:	Mortality, population development, behaviour at hive entrance, brood status, flight intensity
Notes:	Guideline outdated
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

Table 105:	**Side-effects on honeybees – Field tests**
Principle:	Bee colonies in or on the edge of large test fields of flowering crops
Guideline:	EPPO PP 1/170(3) (2000)
Test species:	*Apis mellifera* (honeybee); healthy, well-fed, queen-right colonies in normal condition, containing at least 10000 to 15000 bees, at least 10 to 12 frames and 5 to 6 brood frames
Ecology:	Consumer 1. order: Pollinators (pollen); in (flowering) vegetation layer
Test design:	At least 3 colonies per treatment in or on the edge of flowering crop; plot size at least 1500 m², well separated to avoid bees foraging on wrong plot, not close to other attractive flowering crops
Substrate:	Crop on which use of test product is proposed; if not possible, rape, mustard, phacelia or other crop attractive to bees; crop should be in full flower
Parameter:	Foraging activity and behaviour of bees; number of dead bees in dead-bee traps
Duration:	Up to 3 months, assessments after 0, 1, 2, 4, 7, 14, and 28 d at same time of day
Application:	Spraying during daytime; only formulated products; treatments applied simultaneously (within at most 2 h); according to intended use
Concentration:	Highest dose specified for intended use; replication often not feasible because of requirements of separation
Performance:	Temperature and humidity should be recorded throughout the trial period. Rainfall and sunshine or cloud cover should also be reported
Reference substance:	Should be registered for similar intended use of test product
Validity criteria:	Tests should be repeated where exposure at time of application cannot be convincingly demonstrated and where control mortality is > 15%
Assessment:	Appropriate statistical analysis of mortality and other data relevant to properties of product
Notes:	-
Testing of GMP?	*A. mellifera* has been used in different protocols (see Malone & Pham-Delègue 2001 for a review)

C4 Eluatetests with soil organisms

Note: here only eluatetests using soil organisms are compiled. For further eluatetests see Chapter C5. “Aquatic test methods” and ISO guideline 15799 (2003c).

Table 106:	**Effects on protozoans in liquid medium**
Principle:	Laboratory test for the determination of the toxicity of chemicals on protozoans in a liquid medium
Guideline:	Method proposal from literature (Ekeland et al. in: Løkke 1995)
Test species:	Genera *Cercomonas sp.* (Flagellata), *Acanthamoeba sp.* (Amoebae) and *Colpoda sp.* (Ciliata); isolated from natural field protozoa coenosis in crop soils; laboratory culture
Ecology:	Consumer 1. order/Decomposer: Non-target herbivores/Destruents (e.g. bacteria, protozoa, dead organic matter); in the pore water
Test design:	50 µL protozoan-inoculum per test vessel (Microtiter "wells", after dissolution filled with 100 µL test solution including food, respectively)
Substrate:	Aqueous test solution; characterization not specified
Parameter:	Number of protozoans
Duration:	4 d
Application:	Mixing of the test substance in the aqueous test solution
Concentration:	Variable
Performance:	No information available; quantification of the protozoans by counting of 12 places of 4 "wells" of the microtiterplates, respectively
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Comparison with the control (%); EC10, EC50 and EC10, hormesis (= increase of the number of test organisms)
Notes:	Except the well-known *Caenorhabditis elegans* probably the genus *Cercomonas* has the highest potential in terms as a new "Single-Species-Test".
Testing of GMP?	No

Table 107:	**Acute toxicity for nematodes**
Principle:	Acute laboratory test for the determination of the toxicity of dissolved chemicals to nematodes
Guideline:	Guideline proposal in analogy to BBA- or OECD-formats after Debus & Niemann (1994), based on the work of Samoiloff et al. (1980)
Test species:	*Panagrellus redivivus* (Panagrolaimidae, Rhabditida); Laboratory mass culture
Ecology:	Consumer 1. order: Non-target herbivores (microbes); in soil pore water
Test design:	2 mL of a culture suspension with an unknown number of nematodes of various developmental stages per test vessel ("test flasks")
Substrate:	Solution of Na_2HPO_4, KH_2PO_4, NaCl and $MgSO_4$ in distilled water
Parameter:	Mortality
Duration:	4 d; examination 24, 48, 72 and 96 h after application
Application:	Mixing of the test substance in the test solution (without worms); if only partly water-soluble, use of a solvent (maximal 2 Vol.-%) is possible
Concentration:	Variable; in the Range-Finding-Test with large spacing factors and in the Definitive Test up to the factor 5 in order to determine the LC50
Performance:	Temperature: 21 ± 2°C; microscopical examination at the end of the test
Reference substance:	No information available
Validity criteria:	No information available
Assessment:	Determination of the LC50, e.g. by using the Probit Analysis
Notes:	Also useful for soil quality assessment
Testing of GMP?	No

Table 108:	**Aquatic test with enchytraeids**
Principle:	Acute laboratory test in the aquatic medium using enchytraeids
Guideline:	Method proposal published in the literature (Römbke & Knacker 1989)
Test species:	*Enchytraeus albidus* (Henle, 1847) (Enchytraeidae) or other species from the genus *Enchytraeus* coming from a mass breeding
Ecology:	Decomposer: Destruents (dead organic matter); in soil pore water
Test design:	10 worms per test vessel (100 mL glass, filled with 50 mL of reconstituted water
Substrate:	Reconstituted water as described in aquatic OECD tests
Parameter:	Mortality, behavioural changes
Duration:	4 d; daily examinations
Application:	Dilution of water-soluble substances in the test substrate
Concentration:	5 concentrations with 3 to 4 replicates
Performance:	Temperature: 12 ± 1°C; permanent dark; high air moisture; no feeding during the test; counting and observation using a stereomicroscope
Validity criteria:	No information available
Reference substance:	Potassium dichromate (LC50 approx. 1.9 mg/L)
Assessment:	LC50 (e.g. using Probit Analysis)
Notes:	The test could be useful for the influence of the bioavailability of a substance since the same species can be tested in water or soil.
Testing of GMP?	No

Table 109:	**Toxicity for *E. bigeminus***
Principle:	Laboratory test for the determination of the effects of chemicals on the mortality and the growth of enchytraeids
Guideline:	Method proposal from literature (Christensen & Jensen in: Løkke 1995)
Test species:	*Enchytraeus bigeminus* (Nielsen & Christensen) (Enchytraeidae); laboratory mass culture (asexual reproduction)
Ecology:	Decomposer: Destruents (dead organic matter); in soil pore water
Test design:	20 worms per test vessel (glass-Petri dishes with 5 cm diameter, filled with 1 mL water); length approx. 25 segments
Substrate:	Water (not specified)
Parameter:	Mortality, growth rate (growth of new segments)
Duration:	7 d
Application:	Variable; e.g. as an aqueous solution
Concentration:	Variable: several concentrations with 5 replicates
Performance:	Temperature: 21°C; permanent dark; high air humidity; feeding once with 0.01 mg rolled oats per test vessel; counting of individuals fixed in alcohol by means of a stereomicroscope
Validity criteria:	No information available
Reference substance:	No information available
Assessment:	Determination of EC10 and LC50 (e.g. by using linear regression methods)
Notes:	Proposal: the endpoints biomass or length are probably better suited as test parameter number of segments (which is however more easy to determine).
Testing of GMP?	No

Table 110:	**Toxicity of soil eluates on *Enchytraeus sp.***
Principle:	Laboratory test for the determination of effects of chemicals (including acids) on the mortality of enchytraeids in aquatic substrates
Guideline:	Method proposal published from literature (Graefe 1991)
Test species:	*Enchytraeus minutus* (Nielsen & Christensen) or *E. lacteus* (Nielsen & Christensen) (Enchytraeidae); laboratory mass culture
Ecology:	Decomposer: Destruents (dead organic matter); in soil pore water
Test design:	10 worms per test vessel (not specified; filled with 10 mL water)
Substrate:	Soil eluate or leaching water; e.g. from acidified forest soils; characterization: pH-value, Ca- and Al-concentration
Parameter:	Mortality, wounds (e.g. loss of individual segments)
Duration:	Variable: 24 to 48 h up to 10 d; daily examinations
Application:	Direct use of soil eluates
Concentration:	1 concentration; number of replicates not specified
Performance:	No information available; no feeding during the test; counting using a stereomicroscope
Validity criteria:	No information available
Reference substance:	No information available
Assessment:	No information available
Notes:	Test is specific for field soil eluates.
Testing of GMP?	No

C5 Aquatic test methods

Note: here only tests methods with aquatic organisms are compiled. For eluatetests using soil organisms see Chapter C4. “Eluatetests with soil organisms”.

Table 111:	***Vibrio fischeri* – Luminescent bacteria test**
Principle:	Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) (Part 1,2,3)
Guideline:	ISO 11348 (1998b)
Test species:	*Vibrio fischeri* NRRL B-11177 Freshly prepared 1, liquid-dried 2, freeze-dried 3; inoculum from culture
Ecology:	Decomposer: Mineraliser (organic matter); in pore water and on particles
Test design:	Short term inhibition of effect of toxicants on bacterial luminescence; test vessel: test tubes
Substrate:	1 mL salt water
Parameter:	Inhibition of luminescence
Duration:	15 and 30 min.
Application:	No information available
Concentration:	5 concentrations × 3 replicates
Performance:	Temperature 15 ± 1°C; pH 7.0 ± 0.2; Light intensity/quality: obscurity
Validity criteria:	No information available
Reference substance:	3,5-dinitrophenol, $ZnSO_4$, or $K_2Cr_2O_7$
Assessment:	LC50; regression
Notes:	Coloured substances may interfere with luminescence. This test can be performed with bacteria from different origins. The standard is divided in three parts for that purpose.
Testing of GMP?	No

Table 112:	**Freshwater algal growth inhibition test**
Principle:	Water Quality – Freshwater algal growth inhibition test with *Scenedesmus subspicatus* and *Pseudokirchneriella subcapitata*
Guideline:	ISO 8692 (2004e)
Test species:	Unicellular algae *Scenedesmus subspicatus* or *Pseudokirchneriella subcapitata*; inoculum from culture
Ecology:	Primary producers; limnic surface water
Test design:	Effect on unicellular algae growth; test vessel: 250 mL erlenmeyer flasks
Substrate:	~ 100 mL water
Parameter:	Growth rate or biomass integral
Duration:	72 h
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 23°C; pH 8.3; light intensity/quality 35 to 70×10^{18} photons/m²/s (400 to 700 nm); continuous light; food: mineral culture medium
Validity criteria:	Control population increase > 16 within 72 h
Reference substance:	$K_2Cr_2O_7$: EC50 growth rate *Scenedesmus* 0.84; EC50 growth rate *Pseudokirchneriella* 1.19
Assessment:	NOEC or ECx (x = 10, 20, 50); multisample comparison or regression
Notes:	Chemicals absorbing light in the range 400 to 700 nm may interfere with algal growth for physical reasons rather than by toxic action. Metals may not be bio-available by complexation with EDTA from the test medium. Volatile substances may be stripped by aeration in the tests flasks. See the ISO 14442 (2006c) for information on difficult substances management.
Testing of GMP?	No

Table 113:	**Marine algal growth inhibition test**
Principle:	Water quality – Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*
Guideline:	ISO 10253 (2006d)
Test species:	Unicellular algae; *Skeletonema costatum* or *Phaeodactylum tricornutum*; inoculum from a population
Ecology:	Primary producers; marine surface water
Test design:	Algal population growth inhibition; test vessel: 250 mL
Substrate:	Approx. 100 mL seawater
Parameter:	Population growth inhibition
Duration:	72 h
Application:	No information available
Concentration:	3 replicates (6 for control)
Performance:	Temperature 20 ± 1°C; pH 8 ± 0.2; light intensity/quality 35 to 70 × 10^8 photons/m²/s (400 to 700 nm); continuous light; food: nutritive medium
Validity criteria:	Control growth rate 0.1 h^{-1} (S.c.) 0.072 h^{-1} (P.t.)
Reference substance:	$K_2Cr_2O_7$ or 3,5-dichlorophenol
Assessment:	NOEC and ECx; comparison and regression
Notes:	Chemicals absorbing light in the range 400 to 700 nm may interfere with algal growth for physical reasons rather than by toxic action. Metals may not be bio-available by complexation with EDTA from the test medium. Volatile substances may be stripped by aeration in the tests flasks. See the ISO 14442 (2006c) for information on difficult substances management.
Testing of GMP?	No

Table 114:	***Lemna minor* – Growth inhibition test**
Principle:	Testing water - Determination of the inhibitory effect on the growth of *Lemna minor*
Guideline:	AFNOR XP T90-337 (1996)
Test species:	*Lemna minor*; age of test organism 14 d (an OECD ringtest has shown that this period is too short!)
Ecology:	Primary producers; limnic surface water
Test design:	Determination of effect on growth of the aquatic plant *Lemna minor*; test vessel: 250 mL
Substrate:	100 mL water
Parameter:	Growth
Duration:	4 d
Application:	No information available
Concentration:	3 replicates
Performance:	Temperature 25 ± 1°C; pH 7.5 ± 1; light intensity/quality 35 to 70 × 10^8 photons/m²/s (400 to 700 nm); continuous light; food: nutritive mineral medium
Validity criteria:	Growth rate within 0.25 to $0.35j^{-1}$
Reference substance:	$K_2Cr_2O_7$; IC_{50} within 10 to 30 mg/L
Assessment:	IC_{50}; regression
Notes:	An OECD Test is in preparation; substances absorbing light might interfere with growth for non toxic reasons, EDTA within the medium might cause non bioavailability of the effective substance.
Testing of GMP?	No

Table 115:	***Daphnia magna* – Inhibition of mobility**
Principle:	Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)
Guideline:	ISO 6341 (1996a)
Test species:	*Daphnia magna* Straus; age < 24 h
Ecology:	Consumer 1. order: Non-target herbivores (algae); limnic surface water
Test design:	Determination of the effect of toxicants on mobility of young daphnids; 5 daphnids per test vessel (20 mL)
Substrate:	10 mL freshwater
Parameter:	Immobilisation
Duration:	48 h
Application:	No information available
Concentration:	3 replicates
Performance:	20 ± 0.2°C; pH 7.8 ± 0.2; permanent darkness
Validity criteria:	Control mortality < 10%
Reference substance:	$K_2Cr_2O_7$: LC50 0.9 to 2 mg/L
Assessment:	LC50; regression
Notes:	-
Testing of GMP?	Yes (not in open literature)

Table 116:	***Daphnia magna* reproduction test**
Principle:	Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera: Crustacea)
Guideline:	ISO 10706 (2000)
Test species:	*Daphnia magna* Straus at least third generation obtained by acyclical parthenogenesis; age < 24 h
Ecology:	Consumer 1. order: Non-target herbivores (algae); limnic surface water
Test design:	Inhibition of reproduction and survival of *Daphnia magna*; test vessel: 100 to 200 mL beakers; 1 animal per vessel
Substrate:	50 to 100 mL aqueous test medium
Parameter:	Mortality of adults, inhibition of reproduction or growth
Duration:	21 d
Application:	No information available
Concentration:	5 concentrations × 10 replicates
Performance:	Temperature within 18 to 22°C, variations within less than 2°C; pH 7.8 ± 0.2; light intensity/quality < 1200 Lux; photoperiod 16 h light
Validity criteria:	Mortality of adults or living males < 20% in the control, mean number of offspring per parent > 60 in the control
Reference substance:	The daphnid culture may be controlled using acute $K_2Cr_2O_7$ test
Assessment:	NOEC and ECx; Dunnett or Williams test and regression
Notes:	This test is mainly used for pure substances, short term alternatives exist, for instance using *Ceriodaphnia dubia*.
Testing of GMP?	No

Table 117:	**Marine copepods – Acute toxicity test**
Principle:	Water quality - determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea)
Guideline:	ISO 14669 (1999c)
Test species:	Marine copepods, *Acartia tonsa* (Dana), *Tisbe battagliai* (Volkmann-Rocco), *Nitocra spinipes* (Bœck); age of test organism At. Stage 5 or adults, T.b. copepodids 6 ± 2 d, N.s. Adults 3 to 4 weeks
Ecology:	Consumer 1. order: Non-target herbivores (organic matter, microplancton); in marine surface waters
Test design:	Determination of effects of toxicants on survival of marine copepods; test chamber size depending on the number of animals per vessel (recommended 5)
Substrate:	Natural or synthetic seawater; volume A.t. 5 mL per animal, others 0.5 mL/animal
Parameter:	Mortality of animals
Duration:	48 h
Application:	No information available
Concentration:	5 replicates
Performance:	Temperature 20 ± 0.2°C; pH 8.0 ± 0.3; photoperiod 16-h daylight
Validity criteria:	Dissolved oxygen at end of test > 4 mg/L, control mortality < 10%
Reference substance:	$K_2Cr_2O_7$
Assessment:	LC50; regression
Notes:	-
Testing of GMP?	No

Table 118:	**Freshwater fish acute toxicity test**
Principle:	Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton Buchanan (Teleostei, Cyprinidae)]
Guideline:	ISO 7346 (1996b)
Test species:	Adult *Danio rerio* Hamilton-Buchanan
Ecology:	Consumer 2. order: Predators (omnivorous, mainly microcrustaceans); in limnic surface waters
Test design:	Effect on survival of *Danio rerio*; test vessel: content up to 10 L; at least 7 fish per vessel
Substrate:	1 L fresh water per g of fish
Parameter:	Mortality
Duration:	96 h
Application:	No information available
Concentration:	1 replicate
Performance:	Temperature 23 ± 1°C; pH 7.8 ± 0.2; normal laboratory illumination; photoperiod 12 to 16 h day light
Validity criteria:	Dissolved 02 > 60% saturation, control fish mortality < 10%, no abnormal behaviour
Reference substance:	$K_2Cr_2O_7$
Assessment:	LC50; regression
Notes:	*Danio rerio* is the new name of zebra fish and replaces *Brachydanio rerio*.
Testing of GMP?	No

C6 Birds

Table 119:	**Avian Acute Oral Toxicity Test**
Principle:	Test for evaluating the acute oral toxicity of chemical substances and mixtures to northern bobwhite and mallard.
Guideline:	EPA OPPTS 850.2100 (1996b)
Test species:	Northern Bobwhite quail *(Colinus virginianus)*, Mallard duck *(Anas platyrhynchos)*; young adults, at least 16 weeks old, initial weight: 180 g for bobwhite and 900 g for mallard
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes (quail) or on the surface of limnic surface waters (duck)
Test design:	Minimum 10 birds for each dose level and the control, divided into two pens of 5 birds; floor area: ≥ 500 cm^2/bird for bobwhite and ≥ 1000 cm^2/bird for mallard; height: ≥ 24 cm for bobwhite and ≥ 32 cm for mallard
Parameter:	Mortality (LD_{50} + 95% confidence limits)
Duration:	Observation period of 14 d (may be extended to at least 21 d or until mortality or signs of intoxication are not observed for 72 h)
Application:	Single oral dose either by gavage or capsule
Concentration:	Minimum 5 dose levels (spaced geometrically, factor < 1.67) and a control; Limit test concentration: 2000 mg a.s./kg bw
Performance:	Temperature: 15 - 27°C; Photoperiod: 8/16 h light/dark; Ventilation: 10 - 15 air changes per h; Relative humidity: 45 - 70%
Validity criteria:	Mortality in the control within the test period ≤ 10%
Reference substance:	Not required
Assessment:	Birds should be monitored closely for the first 60 - 120 minutes after dosing; individual body weights: start of the test and weekly thereafter; feed consumption: weekly throughout the test; gross pathology: at least 2 or 3 birds dying at each dose level and all control birds that died
Notes:	Feed should be withheld from all test groups for a minimum of 15 h prior to administration. Doses are to be based on the individual body weight of each bird.
Testing of GMP?	*C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 120:	**Avian Acute Oral Toxicity Test**
Principle:	Test for estimating the acute oral toxicity of substances to birds.
Guideline:	OECD-Guideline proposal 223 (2002). Proposals A and B.
Test species:	Northern bobwhite quail (*Colinus virginianus*), Japanese quail (*Coturnix coturnix japonica).* Also additional species like mallard *(Anas platyrhynchos)*, pigeon *(Columba livia)*, zebra finch *(Poephila guttata)*, budgerigar *(Melopsittacus undulates)*; acclimatisation and diet prior to dosing should be at lest 14 d for cage reared birds.
Ecology:	Quail: Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes
Test design:	Limit test: design consists of dosing 5 animals simultaneously at the limit dose. If mortality occurs, several options can be considered. Full test (LD_{50}-only and dose-response): for LD_{50}-only min. 2 dosing stages. Dose-response test is an extension to 3 or more stages. At each stage, birds are given single doses of test substance into crop or proventriculus. In 1st dosing stage, each bird is given a different dose to bracket the best available LD_{50} estimate. From the results, doses for the 2nd stage are determined. If there is an estimate of LD_{50} from limit test, the full test starts with this stage. Process continues to 3rd stage in the dose-response test or if added precision is needed in estimating LD_{50}. Floor area: pigeon: ≥ 3000 cm^2/bird, quail: ≥ 1000 cm^2/bird, mallard: ≥ 2000 cm^2/bird, budgerigar and zebra finch: ≥ 500 cm^2/bird
Parameter:	Mortality (LD_{50} + 95% confidence limits)
Duration:	Observation period of 14 d after dosing (may be extended)
Application:	One oral dose (gavage or capsule). Birds fasted 12 - 15 h before dosing.
Concentration:	Limit test: 2000 mg/kg bw. Full test: equally spaced, no controls.
Performance:	Individual caging is preferred. Temperature: 15 to 27°C; Photoperiod: 8/16 h light/dark for quail and mallard, 10/14 h light/dark for other species; Ventilation: at least 10 air changes per h
Validity criteria:	-
Reference substance:	-
Assessment:	Birds observed individually during first 2 h after dosing, on at least 3 more regular occasions during first 24 h and at least daily thereafter (regurgitation, signs of intoxication and remission, abnormal behaviour, mortality, time to death). Body weights: start and end of study. Feed consumption: 1, 3, 7 and 14 d after dosing. Gross pathology: all birds.
Notes:	-

Testing of GMP?	*C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 121:	**Avian Dietary Toxicity Test**
Principle:	Test for evaluating the dietary toxicity of chemical substances and mixtures to northern bobwhite and mallard.
Guideline:	EPA OPPTS 850.2200 (1996c)
Test species:	Northern Bobwhite quail *(Colinus virginianus)*, Mallard duck *(Anas platyrhynchos)*; age of birds: 10 to 14 d (bobwhite), 5 to 10 d (mallard)
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes (quail) or on the surface of limnic surface waters (duck)
Test design:	Minimum 10 birds for each dose level and 20 birds for the control; floor area: ≥ 300 cm^2/bird for bobwhite and ≥ 600 cm^2/bird for mallard
Parameter:	Mortality (LC50 + 95% confidence limits)
Duration:	5 d of exposure to test substance in the diet (exposure period) and 3 d of additional observation (post exposure period) while birds receive untreated diet. May be extended until 2 successive mortality-free days and one day free of toxic signs occur, or until 21 d after beginning the test.
Application:	The test substance should be mixed thoroughly and evenly into the diet.
Concentration:	Min. 5 dose levels (spaced geometrically, factor < 1.67) and a control
Performance:	Temperature: 22 to 38°C; Photoperiod: 14/10 h light/dark; Relative humidity: 45 to 70%
Validity criteria:	Invalid if > 10% mortality in control birds. There must be evidence that the test substance concentration has been satisfactorily maintained in the diet (it should be at least 80% of the nominal concentration) throughout the first 5 d of test period. Lowest treatment level should not result in compound-related mortality or other observable effects.
Reference substance:	Not required
Assessment:	Birds should be monitored minimum 3 times on the first day of exposure and daily thereafter. Average body weights: start of the test and after 3-d post exposure period (for each pen); Feed consumption: daily in controls, 2nd lowest and 2nd highest concentration. For all other pens for the exposure period and the post exposure period. Weekly throughout the test. Gross pathology: not required.
Notes:	Chemical analyses should be conducted at beginning of exposure from high, middle, and low concentrations. If test substance is known or found to be volatile or labile to the extent ≥ 25% loss over a 5–d period, then a second series of analyses of the same concentrations previously analyzed should be conducted at the end of the exposure period.

Testing of GMP?	*C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 122:	**Avian Dietary Toxicity Test**
Principle:	Test for evaluating the dietary toxicity of substances to birds.
Guideline:	OECD 205 (1984c)
Test species:	Bobwhite quail *(Colinus virginianus)*, mallard duck *(Anas platyrhynchos)*, pigeon *(Columba livia)*, Japanese quail *(Coturnix coturnix japonica)*, ring-necked pheasant *(Phasianus colchicus)*, red-legged partridge *(Alectoris rufa)*; age of birds: species-specific recommendations are given.
Ecology:	Quail: Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes
Test design:	Each group consists of 10 birds. 5 or 10 birds per pen (pigeons should be housed individually); floor area: species-specific recommendations are given.
Parameter:	Mortality (LC50 + 95% confidence limits) and signs of toxicity
Duration:	5 d of exposure to the test substance in the diet (exposure period) and 3 d of additional observation (post exposure period) while the birds are receiving an untreated diet. May be extended until 2 successive mortality-free days and one day free of toxic signs occur, or until 21 d after beginning the test.
Application:	The test substance should be mixed uniformly into the diet.
Concentration:	Minimum 5 dose levels (spacing factor $\leq$ 2), two control groups and one treatment group for each dose level; Limit test: 5000 mg/kg bw.
Performance:	General: Photoperiod: 12-16 h light/dark; good ventilation; Species-specific environmental conditions are given for temperature and relative humidity.
Validity criteria:	The mortality in the controls should not exceed 10% at the end of the test. There must be evidence that the concentration of the substance being tested has been satisfactorily maintained in the diet (at least 80% of nominal) throughout the first 5 d of the test. The lowest treatment level should not result in compound-related mortality or other toxic effects.
Reference substance:	Not required
Assessment:	Observations: signs of toxicity and other abnormal behaviour: twice on d 1, daily thereafter; mortality: twice on d 1, daily thereafter; body weights: d 0, 5, 8 and at the end of the test (if extended); food consumption: d 0-5, 5-8 and 8-end of the test (if extended)
Notes:	Birds should be acclimated for a minimum of 7 d. During the 72-h period preceding testing, the health should be monitored.
Testing of GMP?	Yes (*C. virginianus*; not in the open literature)

Table 123:	**Avian Reproduction Test**
Principle:	Test for evaluating reproductive effects of chemical substances and mixtures to northern bobwhite and mallard.
Guideline:	EPA OPPTS 850.2300 (1996d)
Test species:	Northern Bobwhite quail *(Colinus virginianus)*, Mallard duck *(Anas platyrhynchos)*; age of birds: 1st breading season, at least 7 months old
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes (quail) or on the surface of limnic surface waters (duck)
Test design:	12 replicate pens containing 1 male and 1 female; floor area: ≥ 5000 cm^2/bird for bobwhite and ≥ 10000 cm^2/bird for mallard
Parameter:	NOEC + any statistically significant effect levels
Duration:	Initial phase (exposure to test substance): 6 - 8 weeks; second phase (pre egg-laying, photostimulation): 2 - 4 weeks; Final phase (egg-laying): 8 - 10 weeks; A withdrawal period of 3 weeks may be added if reduced reproduction is observed. The birds should be exposed for at least 10 weeks prior to the onset of egg laying.
Application:	The test substance should be mixed into the diet in a manner that will ensure even distribution of the test substance throughout the diet.
Concentration:	3 treatment groups and a control
Performance:	Temperature: 21°C; Ventilation: 4/15 (winter/summer) air changes per h; Photoperiod: initial phase: 7 to 8 h light/day, thereafter: 6 to 17 h light/day; Relative humidity: 55%
Validity criteria:	A test is unacceptable if bobwhite chick or mallard duckling productivity in control groups does not average 12 or 10, respectively, 14-d old survivors per pen over a 10–week period. A test is unacceptable if the average eggshell thickness in control groups is less than 0.19 mm for bobwhite and 0.34 mm for mallards. A test is unacceptable if more than 10 percent of the adult control birds die during the test.
Reference substance:	Not required
Assessment:	<u>Adults</u> Observations on mortality and signs of toxicity should be made daily. Body weight: For each bird at the beginning of treatment period, at 14-d intervals until onset of egg-laying, and at termination of treatment. Feed consumption: By pen as often as body weights are measured prior to the onset of egg laying and at least biweekly throughout the rest of the study. Gross pathology: On all birds that died during the test and for all survivors at the end of the test.

Eggs

Eggs should be collected daily and stored at 16°C and 55-80% relative humidity. Stored eggs should be turned daily. At weekly or biweekly intervals eggs should be removed from storage and candled to detect eggshell cracks. During the incubation period, eggs should be maintained at 37.5 °C and approximately 70 percent relative humidity. Eggs should be candled again on d 11 of incubation to determine fertility and early death of embryos. A final candling should be done on d 18 to measure embryo survival. On d 21, eggs should be removed to a separate incubator or hatcher. Hatching will normally be complete by the end of d 24. During hatching the temperature and relative humidity should be 37.5 °C and 70 percent, respectively. Temperature in brooder pens should be measured at 2.5 to 4 cm above the pen floor.

Chicks/ducklings

By d 24 or 27 of incubation, the hatched bobwhite chicks and ducklings, respectively, should be removed from the hatcher or incubator. Chicks or ducklings should be either housed according to the appropriate parental pen group or individually marked. A temperature gradient in the pen from 22 - 35°C. Chicks and ducklings should be maintained until an age of 14 d. Photoperiod should be 14 h light/day with a 15-30 min transition at dawn and dusk. Eggshell thickness: Every 2 weeks all eggs newly laid that day.

Notes: Samples of treated diets should be analysed to confirm proper dietary concentrations of the test substance. Analyses should be conducted at all test substance concentrations at the beginning of the test period and again 10 to 12 weeks later.

Testing of GMP? *C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 124:	**Avian Reproduction Test**
Principle:	Test for evaluating reproductive effects of substances to birds.
Guideline:	OECD 206 (1984d)
Test species:	Bobwhite quail *(Colinus virginianus)*, Mallard duck *(Anas platyrhynchos)* or Japanese quail *(Coturnix coturnix japonica)*; age of birds: 9-12 months ± 2 weeks for mallard and 20-24 weeks ± 1 week for bobwhite. Japanese quail be proven breeders before use in the test (± 1/2 weeks).
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes (quail) or on the surface of limnic surface waters (duck)
Test design:	Birds may be kept in pens as pairs or as groups of one male and two (bobwhite and Japanese quail) ort three (mallard) females. 12 pens per test concentration and control for tests with pairs, 8 pens of mallard and 12 pens of bobwhite or Japanese quail for tests with groups. Floor area: ≥ 2500 cm^2/bird for bobwhite, ≥ 10000 cm^2/bird for mallard, ≥ 1500 cm^2/bird for Japanese quail
Parameter:	NOEC + any statistically significant effect levels
Duration:	Not less than 20 weeks.
Application:	The test substance should be uniformly mixed into the diet.
Concentration:	3 treatment groups and a control. The concentrations should be based on the results of a dietary LC50 test. The maximum recommended test concentration is 1000 mg a.s./kg bw.
Performance:	Adult birds should be maintained with good ventilation at 22 ± 5°C and 50 to 75% relative humidity. A 15 to 30 minutes transition period at dawn and dusk is recommended. Species-specific environmental conditions are given for eggs and young birds.
Validity criteria:	The mortality in the controls should not exceed 10% at the end of the test. The average number of 14-d-old survivors per hen in the controls should be at least 14, 12 and 24 for mallard, bobwhite and Japanese quail, respectively. The average egg shell thickness for the control group should be at least 0.34, 0.19 and 0.19 mm for mallard, bobwhite and Japanese quail, respectively. There must be evidence that the concentration of the substance being tested has been satisfactorily maintained in the diet (at least 80% of nominal) throughout the test period.
Reference substance:	Not required
Assessment:	Birds are induced by photoperiod manipulation, to lay eggs. Eggs are collected over a ten-week period, artificially incubated and hatched,

and the young maintained for 14 d. Mortality of adults, egg production, cracked eggs, egg shell thickness, viability, hatchability and effects on young birds are compared with the control. Observations: mortality and signs of toxicity: daily; body weights of adults: at start of exposure, prior to onset of egg laying and at the end of the study; body weights of young: at 14 d of age; food consumption of adults: intervals of 1 or 2 weeks; food consumption of young: 1st and 2nd week after hatching; gross pathological examination of all adult birds.

Adults

Birds should be held under short-day conditions (7-8 h light/day) for 8 weeks after start of the tests. The photoperiod is then lengthened to 16-18 h light/day to bring the birds into breeding conditions. Egg-laying should begin 2-4 weeks after photoperiod is lengthened. The test should be continued for 8-10 weeks after egg-laying begins.

Eggs

Eggs should be collected daily and stored and set weekly for incubation. Prior to incubation all eggs should be candled to detect cracks. Eggs set for incubation should be candled again after 6 to 11 d to determine viability. Eggs should be transferred to hatching conditions on d 23 for mallard ducks, d 21 for bobwhite quail and d 16 for Japanese quail. Hatching should be completed by d 25 to 27 for mallard ducks, d 23 to 24 for bobwhite quail and d 17 to 18 for Japanese quail.

Hatchlings

Hatchlings should be maintained on appropriate diets without test substance for 14 d. Lightning should be on a diurnal basis (e.g. 14/10 h light/dark) with a 15 minute transition at dawn and dusk.

Notes: The birds should be acclimated for at least 14 d prior to testing.

Testing of GMP? *C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 125:	**Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite**
Principle:	Test for evaluating the effects of pesticides and other chemicals upon avian health and reproduction.
Guideline:	OECD-Draft guideline (2000c)
Test species:	Northern bobwhite *(Colinus virginianus)* or Japanese quail *(Coturnix coturnix japonica)* approaching their first breeding season.
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes
Test design:	Adult birds should be housed in pairs (one male/one female). The number of pairs (e.g. 20) should be sufficient to ensure that there are 16 breeding pairs in the control group at the end of the treatment period. Floor area: age-related housing conditions are recommended.
Parameter:	NOEC for all health and reproductive parameters evaluated. The number of 14-d old survivors per hen is the integrated biological endpoint.
Duration:	6-week treatment (total test period: 13-14 weeks)
Application:	The test substance should be mixed into the diet.
Concentration:	3 treatment groups and a control. The concentrations should be based on the results of a range-finding study or other avian tests. The maximum recommended test concentration is 1000 mg a.s./kg bw.
Performance:	Adult birds should be maintained with good ventilation (8-15 air changes per h). Age-related housing conditions are recommended. Photoperiod: 16-17 h light and 7-8 h dark (minimum light intensity 10 Lux).
Validity criteria:	The test substance concentration in the diet should be maintained within losses of ≤ 20% of initial concentrations. All mortalities in the control group should be explained. At least 16 breeding pairs of control birds that have produced eggs must be available at the end of the 6-week treatment period.
Reference substance:	Not required
Assessment:	Japanese quail: The test begins with the start of the pre-treatment period. The birds should be at peak egg production at the start of the pre-treatment period. Northern bobwhite: The test begins with the start of the pre-treatment period. The onset of egg laying will occur during the acclimation period. The pre-treatment period lasts two weeks.

	Adults Daily observations to detect overt signs of toxicity and other clinical signs. Food consumption (per pair) should be recorded at least weekly, food spillage should be noted. Body weights should be determined at start of pre-treatment, at start of treatment and at the end of treatment period. All adult birds are to undergo necropsy and gross pathology assessment. Offspring - Eggs During pre-treatment and treatment period, all eggs (except those that are damaged) are set, incubated and allowed to hatch. All offspring are maintained on untreated diet until 14 d after hatching. Eggs are collected at least daily. Before placing the eggs in the incubator, they are candled to check for cracks. Conditions for egg storage, incubation and hatching are recommended. Certain eggs are used for measurement of eggshell thickness and eggshell strength. Fertility and embryo viability are checked by candling (time-points are given). Hatchlings Hatchlings are weighted and held for 14 d and observed daily for clinical signs. After 14 d hatchlings are weighted again and killed.
Notes:	-
Testing of GMP?	*C. virginianus* has been used but following different testing protocols (not in the open literature)

Table 126:	**Avian Repellency Test**
Principle:	Test for evaluating the avoidance response of birds to food containing potentially toxic substances. Birds are fed on a diet containing different concentrations of the test substance. In a choice test birds are also offered untreated food; in a no-choice test only the control group is given untreated food.
Guideline:	OECD-Draft guideline (2003)
Test species:	Japanese quail *(Coturnix coturnix japonica)* is the preferred species.
Ecology:	Consumer 1. order: Non-target herbivores (mainly seeds and grass); in the lower vegetation layer of open landscapes
Test design:	Diets offered *ad libitum* for a 24-h feeding period. Each of the test and control groups consists of 10 adult birds (for no-choice test). The birds are housed individually in facilities suitable for the test species.
Parameter:	Calculation of a repellency factor from dietary studies such as LC50 tests, or - if food avoidance occurs - calculation of avoidance factors.
Duration:	At least 11 d (7 d acclimatisation, 24 h treatment period, 3 d post-treatment period).
Application:	The test substance should be mixed into the diet.
Concentration:	Normally 3 treatment groups and a control.
Performance:	21 ± 3°C, 50-75% relative humidity, standard diet *ad libitum*, photoperiod of 8/16 h light/dark.
Validity criteria:	During acclimatisation period, mortality should be ≤ 5%. Test design is not appropriate if birds refuse untreated diet during acclimatisation. In the no-choice test, it should be demonstrated, that food intake rate of treated and control groups is similar during acclimatisation period when all birds have access to untreated food only.
Reference substance:	Not required
Assessment:	Signs of intoxication and abnormal behaviour: observations in intervals of 2 h over a period of 8 h during exposure and once a day for the following 3 d Body weights: at the beginning of acclimatisation period (d -7), on d -4 and -1 of acclimatisation period, on the day of treatment (d +1) and at the end of the test (d +3), always in the morning. Food consumption: measured over 24-h periods: every day after the fourth day of acclimatisation (d -3) until the end of the test (d +3). Gross necropsy of birds that die during the test.
Notes:	The results of the test can be used as a factor in risk assessment and are not intended for determining the hazard of the substance.
Testing of GMP?	No

C7 Literature

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Appendix D – Studies investigating effects of Bt-toxin on lacewing larvae

Tab. D-1: Comparative overview of the 5 studies investigating the effects of Bt-toxins on lacewing larvae.

Study material	**Treatments**	**First instar (L1)**	**Secon instar (L2)**	**Third instar (L3)**	**Pupa (non-feeding)**	**Entire juvenile stage (L1-Adult)**
1. Hilbeck et al. (1998a) Tri-troph., Bt-maize	*1.1:* Replications: 4 Treatments: 2 Larvae/trt: 50 N = 400	Bt-caterpillars (NTO)	Bt-caterpillars (NTO)	Bt-caterpillars (NTO)		
	Duration Bt-exposure	Entire stage	Entire stage	Entire stage		
	Parameter Mortality: Development time:	 Bt: 24% Co: 10% Bt: 5.0 days Co: 4.5 days	 Bt: 40% Co: 21% Bt: 6.5 days Co: 6.5 days	 Bt: 11% Co: 7% Bt: 7.3 days Co: 7.8 days	 Bt: 0% Co: 2% Bt: 12.5 days Co: 12.5 days	 Bt: 60% Co: 37% Bt: 31 days Co: 31 days
	1.2: Replications: 4 Treatments: 2 Larvae/trt: 50 N = 400	Bt-caterpillars (TO)	Bt-caterpillars (TO)	Bt-caterpillars (TO)		
	Duration Bt-exposure	Entire stage	Entire stage	Entire stage		
	Parameter Mortality: Development time:	 Bt: 29% Co: 10% Bt: 5.8 days Co: 5.1 days	 Bt: 45% Co: 20% Bt: 7.5 days Co: 5.1 days	 Bt: 11% Co: 7% Bt: 7.5 days Co: 6.5 days	 Bt: 8.0% Co: 2.5% Bt: 12.5 days Co: 12.5 days	 Bt: 66% Co: 38% Bt: 32 days Co: 29 days

Tab. D-1: continued.

Study material	**Treatments**	**First instar (L1)**	**Secon instar (L2)**	**Third instar (L3)**	**Pupa (non-feeding)**	**Entire juvenile stage (L1-Adult)**
2. Hilbeck et al. (1998b) Bi-troph.; MO-Bt (100µg/ml)	*2.1:* Replications: 5 Treatments: 2 Larvae/trt.: 30 N = 300	Bt-lacewing diet	Bt-lacewing diet	Bt-lacewing diet		
	Duration Bt-exposure	Entire stage	Entire stage	Entire stage		
	Parameters: Mortality Development times	 Bt: 6% Co: 6% Bt: 7 days Co: 7 days	 Bt: 26% Co: 8%* Bt: 11 days Co: 10 days	 Bt: 22% Co: 12%* Bt: 12 days Co: 10 days	 Bt: 34% Co: 14% Bt: 12 days Co: 12 days	 Bt: 57% Co: 30% Bt: 37.5 days Co: 37.5 days
	2.2: Replications: 5 Treatments: 2 Larvae/trt.: 30 N = 300	Meal moth eggs	Bt-lacewing diet	Bt-lacewing diet		
	Duration Bt-exposure	none	Entire stage	Entire stage		
	Parameter: Mortality Development times	 Bt: 2 % Co: 2% Bt: 4.5 days Co: 4.5 days	 Bt: 15% Co: 6% Bt: 4.3 days Co: 4.0 days	 Bt: 7.5% Co: 5.0% Bt: 7.5 days Co: 7.5 days	 Bt: 7.5% Co: 4.0% Bt: 12 days Co: 12 days	 Bt: 29% Co: 17% Bt: 28.0 days Co: 27.5 days
	2.3: Replikations: 5 Treatments: 1 Larvae/trt: 30 N = 150	Meal moth eggs	Meal moth eggs	Meal moth eggs		
	Duration Bt-exposure	Entire stage	Entire stage	Entire stage		
	Parameters: Mortality Development times	 ca. 1% 4.5 days	 0 % 3.2 days	 0.5 % 4.3 days	 5% 12 days	 8% 23 days

Tab. D-1: continued.

Study Material	Treatments	First instar (L1)	Secon instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-Adult)
3. Hilbeck et al. (1999) Tri-troph; MO-Bt	*3.1:* Replications: 4 Treatments: 8 Larvae/trt: 30 N = 960	Bt-caterpillars (NTO)	Bt-caterpillars (NTO)	Bt-caterpillars (NTO)		
	Duration Bt-exposure	Entire stage	Entire stage	Entire stage		
	Parameters: Mortality Cry1Ab Toxin: 100, 50, 25 Cry1Ab Protoxin: 200, 100, 50 Cry2A Control	 35, 18, 10% 12.5, 12, 17.5% 10% 6%	 25, 12.5, 17.5% 16, 15, 20% 14% 4%	 35, 46, 31% 33, 25, 41% 24% 13%	 40, 35, 24% 14, 9, 20% 15% 10%	 78, 69, 55% 56, 46, 62% 47.5% 26%
4. Dutton et al. (2002) Tri-troph.; Bt-Mais (Bt 11)	4.1: Replications: 2 Treatments: 2 Larvae/trt: 30 N=120	Bt-caterpillars (NTO)	Bt-caterpillars (NTO)	Bt-caterpillars (NTO) + meal moth eggs		
	Duration Bt-exposure	Entire stage	Entire stage	2 days		
	Parameters: Survival (mortality) Development times Weight	 Co: 90% (10%) Bt: 50% (50%) Co: 3 days Bt: 5 days ---	 Co: 65% (35%) Bt: 40% (60%) Co: 6 days Bt: 8 days Co: ca. 1mg Bt: < 1mg	 Co: 95% (5%) Bt: 90% (10%) Co: 5 days Bt: 5 days Co: 2 mg Bt: 2 mg	 =/< 2%	 Co: 60% (40%) Bt: 20% (80%) Co: 21 days Bt: 24 days Co: 10 mg Bt: 9 mg

Tab. D-1: continued.

Study Material	**Treatments**	**First instar (L1)**	**Secon instar (L2)**	**Third instar (L3)**	**Pupa (non-feeding)**	**Entire juvenile stage (L1-Adult)**
	4.2: Replications: 2 Treatments: 2 Larvae/trt: 30 N=120	Bt-spider mites	Bt-spider mites	Bt-spider mites + meal moth eggs		
	Duration Bt-exposure	Entire stage	Entire stage	2 days		
	Parameters: Survival (Mortality) Development times Weight	 Co: 95% (5%) Bt: 95% (5%) Co: 3 days Bt: 3 days ---	 Co: 95% (5%) Bt: 100% (0%) Co: 3 days Bt: 3 days Co: ca. 1 mg Bt: ca. 1 mg	 Co: 95% (5%) Bt: 95% (5%) Co: 4 days Bt: 4 days Co: ca. 3 mg Bt: ca. 3 mg	 =/< 2%	 Co: 90% (10%) Bt: 95% (5%) Co: 20 days Bt: 19 days Co: ca. 8 mg Bt: ca. 8 mg
	4.3: Replications: 2 Treatments: 2 Larvae/trt: 30 N=120	Bt-aphids	Bt-aphids	Bt-aphids		
	Duration Bt-exposure	none	none	none		
	Parameters: Survival (Mortality) Development times Weight	 Co: 96% (4%) Bt: 96% (4%) Co: 3 days Bt: 3 days ---	 Co: 98% (2%) Bt: 98% (2%) Co: 3 days Bt: 3 days Co: ca. 1 mg Bt: ca. 1 mg	 Co: 100% (0%) Bt: 98% (2%) Co: 4 days Bt: 4 days Co: ca. 2 mg Bt: ca. 2 mg	 =/< 2%	 Co: 92% (8%) Bt: 95% (5%) Co: 20 days Bt: 20 days Co: ca. 9 mg Bt: ca. 9 mg

Tab. D-1: continued.

Study Material	Treatments	First instar (L1)	Secon instar (L2)	Third instar (L3)	Pupa (non-feeding)	Entire juvenile stage (L1-Adult)
5. Romeis et al. (2004) Bi-troph., MO-Bt (Cry1Ab)	*5.1:* Replications: 1 Treatments: 2 Larvae/trt: 40 N=80	Bt-sugar solution	---	---	---	---
	Duration Bt-exposure	30 minutes	---	---	---	---
	Parameters: consumption (% weight gain)	 Bt: 15.7% Co: 14.7%	---	---	---	---
	5.2: Replications: 6 Treatments: 5 (4 Bt-Concentrations + Ko) Larvae/trt: 10 N=300	Bt-sugar solution	---	---	---	---
	Duration Bt-exposure	Offered until death	---	---	---	---
	Parameters: Duration until death (days, arrested larval development)	 4 Bt-conc: 9-10 d Control : 9.5 d	---	---	---	---
	5.3: Replications: 3 Treatments: 2 Larvae/trt: 20 N=120	Bt-sugar solution + meal moth eggs	Meal moth eggs	Meal moth eggs	---	---
	Duration Bt-exposure	6 days	none	none	---	---
	Parameters: Survival (mortality) Development times Dry weight L3	 Bt: 87.9% (12.1%) Co: 84.7% (15.3%) Bt: 5.1 days (+6 Bt) Co: 5.0 days (+6 Bt) ---	 Bt: 96.1% (3.9%) Co: 96.0% (4.0%) Bt: 3.4 days Co: 3.4 days --- ---	 --- --- Bt: 1252 µg Co: 1139 µg	 --- ---	---

Tab. D-1: continued.

Study Material	**Treatments**	**First instar (L1)**	**Secon instar (L2)**	**Third instar (L3)**	**Pupa (non-feeding)**	**Entire juvenile stage (L1-Adult)**
	5.4: Replications: 3 Treatments: 6 Larvae/trt: 10 (Mme) 20 (*S.l.*) N= 90 (Mme) N= 180 (*S.l.*)	caterpillars (*S. littoralis* (S.l.), NTO) or meal moth eggs (Mme)	Bt-sugar solution Sugar solution Water	Bt-sugar solution Sugar solution Water	---	---
	Duration exposure	none	Yes; all – duration unclear and variable	Unclear (short) – few individuals	---	---
	Parameters: survival (mortality): Mme (L1) *S. littoralis* (L1) Development times: *E. kühniella* (L1) *S. littoralis* (L1) Duration until death: *E. kühniella* (L1) *S. littoralis* (L1) Percent until L3 *E. kühniella* (L1) *S. littoralis* (L1)	 Co: 98.9% (1.1%)* Co: 72.2% (27.8%) Co: 3.7 days* Co: 5.7 days --- --- --- --- --- --- --- ---	 --- --- --- --- 46 – 47 days 46 – 47 days 5.6 days (Water) 20 – 21 days 20 – 21 days 2.1 days (Water) --- ---	 --- --- --- --- not distinguished (incl. L2) not distinguished (incl. L2) 37.9 – 46.7% 0-2.4% (Water)	---	---

*If numbers were not stated explicitly in text or tables, they were estimated from the figures of the publications and, therefore, can be associated with slight inaccuracies
Co = Kontrolle; Bt = Bt-treatment; NTO = Non-target organisms; TO = Target organism