Insect Model for Toxicokinetics: Foundation for Species Selectivity

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Prologue

Dear Knowledge Seekers and Fact Finders,

I have been working on the topic of the dissertation presented here for the last years and I am excited to be able to share my findings with you now.

This work was carried out at Syngenta Crop Protection, Stein, Switzerland, in collaboration with the Helmholtz Centre for Environmental Research, Leipzig, Germany, in the Department of Cell Toxicology between 2020 and 2024. The thesis was written in monographic form, also includes content from manuscripts intended or already submitted, and is therefore also partly based on the following research manuscripts:

Manuscript 1: RÖMER C. I., ASHAUER R., ESCHER B. I., HÖFER K., MUEHLEBACH M., SADEGHI-TEHRAN P., SHERBORNE N., BUCHHOLZ A. (2024) Journal of Economic Entomology (10;117(3):982-992): Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model.

Manuscript 2: RÖMER C. I., ASHAUER R., ESCHER B. I., HOLLENDER J., BURKHARD R., HÖFER K., MUEHLEBACH M., BUCHHOLZ A. (in submitted process): Comparison of absorption and excretion of test compounds in sucking versus chewing pests.

Manuscript 3: RÖMER C. I., SHERBORNE N., ESCHER B. I., HOLLENDER J., HÖFER K., MUEHLEBACH M., BUCHHOLZ A., ASHAUER R., (in preparation): Comparative toxicokinetics of insecticide scaffolds and their putative biotransformation in target and non-target species.

Illustrations and text have therefore been taken or adapted from the manuscripts (or publication drafts), which are original parts of my dissertation, without further indication. The original manuscripts are attached to this at the end.

Although written in scientific language, my aim is to make my research and its results accessible to a wider audience. Therefore, additional information boxes are used to explain complex topics quickly and in understandable language. The concepts are summarized clearly and as far as possible without using scientific terminology.

I hope you enjoy reading this report and thank you for your interest!

List of abbreviations

Abbreviation	Deffinition
°C	Degrees celsius
µL	Microlitre
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolism and Excretion
BAF	Bioaccumulation factor
BYOM	Build Your Own Model
CAS	Chemical Abstract Service (Registry Number)
CHC	Cuticular hydrocarbons
Co.KG	Compagnie Kommanditgesellschaft
Da	Dalton
DEBtox	Dynamic Energy Budget of toxic effects
e.g.	*Exempli gratia* / for example
ESI	Electrospray Ionization Source
et al.	*Et alii* / and others
g/mol	Gram atomic mass
GABA	Gamma-aminobuyric acid
GmbH	Gesellschaft mit beschränkter Haftung
h	Hour(s)
HSS	High Strength Silic
i.e.	*Id est* / that is
IGR	Insect growth regulator
IPM	Integrated pest management
kV	Kilovolt
L	Litre
L/h	Litre per minute
ln	Natural logarithm
Log P	Partition coefficient (abbreviation P is defined as a particular ration of the concentrations of solute between two solvents)
Lux	Unit of illumination
MATLAB	Program language of matrix laboratory

mg	Milligram
w.wt	Wet weight
min	Minute
mL/min	Millilitre per minute
mm	Millimeter
mm^2	Square millimeter
MT	Microtiter plate
n	Number of objects in a sample
ND	Not detected
Ø size	Diameter
Ø	Average
ODE	Ordinary differential equations
OECD	Organization for Economic Cooperation and Development
PPP	Plant Protection Product
R	Program language of the R Project for Statistical Computing
RH	Relative humidity
rpm	Rounds per minute
SIR	Single ion recording
T	Temperature
t	Time
TD	Toxicodynamics
TK	Toxicokinetics
TKTD	Toxicokinetic-Toxicodynamic
TM	Trademark
UHPLS-MS	Ultra-High Performance Liquid Chromatography coupled with Mass Spectrometry
V	Volt

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Figure Cover Page: Scientific drawing of insects © E. Römer
Figure Acknowledgement: Drawing of insects © E. Römer

Summary

The study presented here deals with the fate, toxicokinetics and bioaccumulation of incorporating structural motifs similar to agrochemicals on selected target and non-target insect species against the background of the currently observed global decline of most insect populations caused by habitat loss and chemical pollution.

In standardised bioassays the biological responses of three test organisms to seven inactive test compounds and Coumarin as reference compound were investigated. The assays were specially designed and adapted to the requirements for the development of toxicokinetic (TK) models, the processes of uptake and excretion of that test substances were investigated. The insect species included in the experiments represent different ecological adaptation types in terms of their habitat selection, their type of food intake and different agricultural importance: *Spodoptera littoralis* (leaf chewing larval pest), *Myzus persicae* aphids (sap sucker pest) and *Chironomus riparius* mosquito larvae (aquatic non-target).

The assays included an exposure phase with treated food or contaminated water, followed by an elimination phase with untreated food or water. Three of the compounds tested proved lethal at the dose tested, one of them against *M. persicae*, two even against *C. riparius.* No changes in behavior (movement, feeding and excretion) were monitored in treated compared to untreated *S. littoralis*.

Aphids have an about threefold higher ratio of food uptake to body mass than *S. littoralis* larvae, but the total quantity of compounds absorbed in the aphid bodies remained lower. Hydrophilic compounds were detected at higher quantities in insect excretion products (feces and honeydew) than in respective insect bodies at the end of the exposure period. In contrast, the quantities of the more lipophilic compounds in the insect body increased.

Two TK models were applied to the *S. littoralis* data, the first considering only the internal and external concentrations of the test compounds. As this was not sufficient to sufficiently describe the TK, the second TK model (applied to *S. littoralis* and *C. riparius*) additionally included the increase in body mass and the biotransformation products of these compounds by the tested individuals.

S. littoralis has a faster uptake and elimination kinetics and a more pronounced biotransformation for the test chemicals, which may be of relevance for pest control. In contrast, the elimination kinetics of *C. riparius* were found to be slower, raising concerns about bioaccumulation and possible effects on the food web. Aphids had to be excluded from

modelling due to their reproduction which makes proper mathematical calculations of compound quantities per body mass impossible. The combination of data from TK bioassay and modelling disclosed different metabolic profiles for the tested species: *S. littoralis* and *C. riparius* processed compounds via both oxidation and demethylation pathways, whereas *M. persicae* only utilized oxidation pathways.

This thesis provides new insights, but also highlights limitations in the representation of species-specific differences in TK, which could become relevant for the development of selective insecticides. The differences observed in the uptake, biotransformation and excretion of the administered test substances underline the complexity of biological reactions of insects to them. The study also illustrates that our ecological knowledge is not yet sufficient to satisfactorily predict the selective effects compounds on individual insect species and their long-term effects on insect populations when additional stress factors resulting from climate change, occur.

The differences in the biotransformation capacity between the species revealed by the study are discussed against the background of a possible release of environmental toxins in an environment that is changing due to climate change. In addition, possibilities and requirements for the application of toxicokinetic models to improve selectivity for the development of future insecticides for the protection and conservation of insect diversity are shown.

Zusammenfassung

Die vorliegende Studie befasst sich mit den selektiven Wirkungen von Insektiziden auf ausgewählte Insektenarten vor dem Hintergrund des derzeit zu beobachtenden weltweiten Rückgangs der meisten Insektenpopulationen, der durch Lebensraumverlust und chemische Verschmutzung verursacht wird.

In standardisierten Biotests wurden die biologischen Reaktionen von drei Testorganismen auf sieben inaktive Strukturvarianten die ähnliche strukturelle Motive wie Agrochemikalien enthalten und Cumarin als Refernenzverbindung untersucht.

In den speziell entwickelten und an die Anforderungen für die Entwicklung toxikokinetischer (TK) Modelle angepassten Biotests wurden die Prozesse der Aufnahme und Ausscheidung der Testsubstanzen untersucht. Die in die Versuche einbezogenen Insektenarten repräsentieren unterschiedliche ökologische Anpassungstypen in Bezug auf ihr natürliches Habitat, ihre Art der Nahrungsaufnahme und ihre unterschiedliche landwirtschaftliche Bedeutung: Mottenlarven der Art *Spodoptera littoralis*, Blattläuse der Art *Myzus persicae* und Zuckmückenlarven der Art *Chironomus riparius*. Die Tests umfassten eine Expositionsphase mit Testsubstanz behandelter Futter oder kontaminiertem Wasser, gefolgt von einer Eliminationsphase mit unbehandeltem Futter oder Wasser.

Drei der getesteten Verbindungen erwiesen sich bei der getesteten Dosis als tödlich, eine davon bei *M. persicae*, sogar zwei bei *C. riparius*. Behandelte *S. littoralis* zeigten keine Veränderungen im Verhalten (Bewegung, Fressen und Ausscheidung) im Vergleich zu unbehandelten. Die Blattläuse wiesen ein etwa dreifach höheres Verhältnis von Nahrungsaufnahme zu Körpermasse auf als die Mottenlarven, aber die Gesamtmenge der in den Blattlauskörpern aufgenommenen Verbindungen blieb geringer. Hydrophilere Verbindungen wurden am Ende des Expositionszeitraums in höheren Mengen in den Ausscheidungsprodukten der Insekten (Kot und Honigtau) als in den jeweiligen Insektenkörpern selbst nachgewiesen. Die Mengen der lipophileren Verbindungen im Insektenkörper nahmen hingegen zu.

Zwei aufeinander aufbauende TK-Modelle wurden auf die Expositionsdaten von *S. littoralis* angewandt, wobei das erste nur die internen und externen Konzentrationen der Testverbindungen berücksichtigte. Da dies nicht ausreichte um die TK zufriedenstellend zu beschreiben wurden in das zweite Modell (angewand an *S. littoralis* und *C. riparius*) zusätzlich die Zunahme der Körpermasse und die Biotransformationsprodukte dieser Verbindungen durch die getesteten Individuen mit aufgenommen. *S. littoralis* hat eine schnellere Aufnahme- und

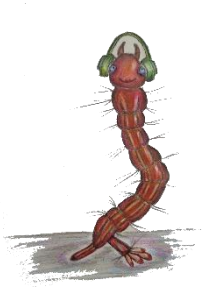
Eliminationskinetik und eine ausgeprägtere Biotransformation für die Testchemikalien, was für die Schädlingsbekämpfung bedeutsam sein kann. Im Gegensatz dazu erwies sich die Eliminationskinetik von *C. riparius* als langsamer, was Bedenken hinsichtlich der Bioakkumulation und möglicher Auswirkungen auf das Nahrungsnetz weckt.

Blattläuse mussten von der Modellierung ausgeschlossen werden, da sie sich im Laufe der Experimente vermehrten, was eine korrekte mathematische Berechnung der Verbindungsmengen pro Körpermasse unmöglich macht. Die Kombination der Daten aus dem TK- Biotests und der Modellierung ergab unterschiedliche Stoffwechselprofile für die getesteten Arten: *S. littoralis* und *C. riparius* verarbeiteten Verbindungen sowohl über Oxidations- als auch Demethylierungswege, während *M. persicae* nur Oxidationswege nutzte.

Die Studie liefert neue Erkenntnisse, zeigt aber auch die Grenzen bei der Darstellung von artspezifischen Unterschieden in der TK-Modellierung auf, die für die Entwicklung selektiver Insektizide relevant werden könnten. Die beobachteten Unterschiede bei der Aufnahme, Biotransformation und Ausscheidung der verabreichten Testsubstanzen unterstreichen die Komplexität der biologischen Reaktionen von Insekten auf diese Substanzen. Die Studie zeigte auch, dass unser ökologisches Wissen noch nicht ausreicht, um die selektiven Wirkungen von Umweltgiften auf einzelne Insektenarten und ihre langfristigen Auswirkungen auf Insektenpopulationen bei zusätzlichen Stressfaktoren ausgelöst durch den Klimawandel zufriedenstellend vorherzusagen.

Die durch die Studie verdeutlichen Unterschiede in der Biotransformationskapazität zwischen den Arten werden vor dem Hintergrund einer möglichen Freisetzung von Umweltgiften in einer sich durch den Klimawandel verändernden Umwelt diskutiert. Darüber hinaus werden Möglichkeiten und Anforderungen bei der Anwendung toxikokinetischer Modelle zur Verbesserung der Selektivität für die Entwicklung zukünftiger Insektizide zum Schutz und Erhalt der Insektenvielfalt aufgezeigt.

Acknowledgments



I would like to take this opportunity to express my gratitude to all of those who have accompanied and supported me on my journey toward the completion of this thesis. It is very important to me to express my sincere appreciation and deep gratitude. I apologize if I have inadvertently left anyone out. At this point, I would like to thank my former department of Chemical Ecology at the Bielefeld University under the direction of **Caroline Müller**. Without you I would never have come to the point of becoming an independent scientist. In particular, **Thorben Müller** and **Lisa Johanna Tewes**, who have known me since my first semester as a young scientist, have supported me throughout this journey and have become wonderful friends. I am pretty sure that I would not be a scientist today without my first positive experiences in the lab and the trust of all of you. Thank you for bringing me to the dark side of infinite science.

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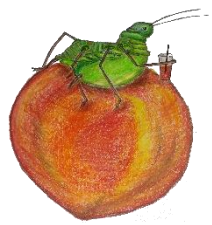

I would particularly like to thank my PhD family at Syngenta, where I have been based for the last few years, where I was welcomed as an intern in the past and where I was supported in my daily experimental work during my PhD. I would especially like to thank you, **Anke Buchholz**, for your constant mentoring, the countless scientific discussions and the opportunities you have provided me from day one. I still remember my first interview with you. It was from a research cabin in Sweden with a poor internet connection. Since then, I have been very happy and

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Thank you, **Melanie Krinke**, my favorite lab partner from my university days. You were truly the highlight of my biology studies. Our late-night visits to the university, all the coffee breaks and the countless hours we spent studying and growing up together over the years were invaluable. You have become like a sister to me and through our shared academic choices we have shared many unforgettable moments over the past eight years.

I seize this moment to express my gratitude to my brother, **Leonard Römer**. You were by my side from the very first day of our studies, and we started the first week of biology side by side. It's quite unexpected that I was the biologist between us, especially considering your collection of horrible pets. Since then, you've been the perfect companion, offering me a respite from the daily grind with great drink and even better music.

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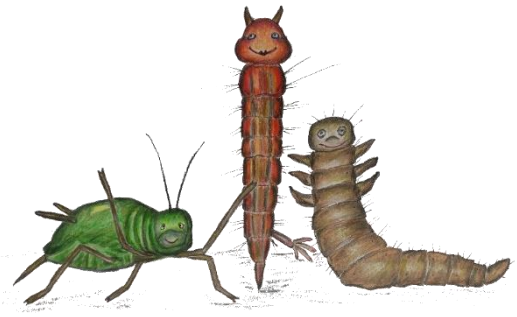
Last but not least I would like to thank you, **Götz Gladisch**, for your support, your love and for being such a wonderful partner by my side. You always had my back and lifted my spirits at the right moments. Without you and Bibi, it would not have been possible to write a thesis without tears of despair. You are my home.



Introduction

Insects and the environment

Insects are known to represent the largest group of animals on earth today, exceeding more than one million scientifically described species (MORA *et al.* 2011; KEITH *et al.* 2015; SCHMIDT *et al.* 2023) (Figure 1). It has recently been estimated that insects represent just about one third to quarter of all species on the globe (MORA *et al.* 2011). Recent methodological advances in ecological research have shed light on the complex interactions between insects and other components of the ecosystem. These studies reveal intricate relationships that underscore the central role of insects in maintaining ecological balance (HOCHKIRCH *et al.* 2018; SÁNCHEZ-BAYO & WYCKHUYS 2019; HOCHKIRCH *et al.* 2023).

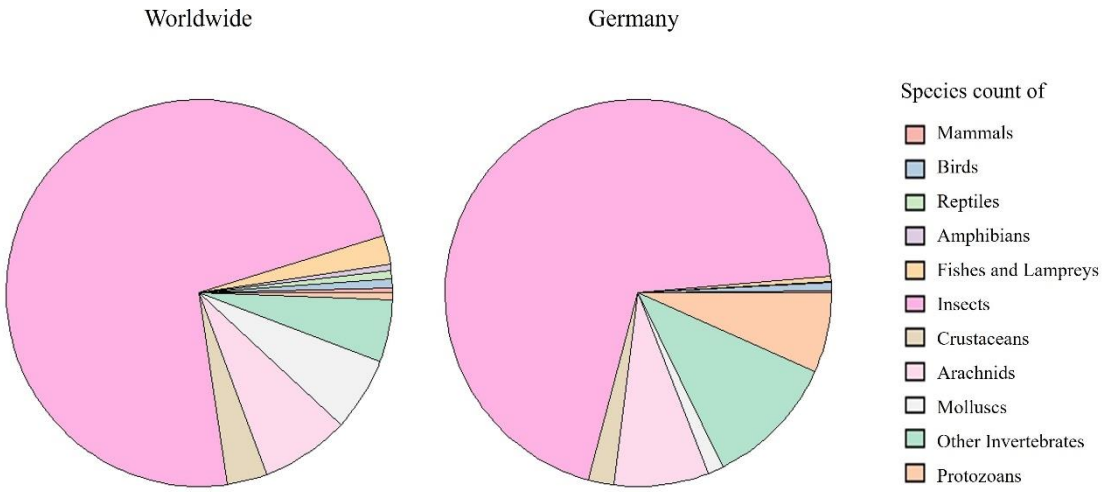


Figure 1: Count of described species worldwide and in Germany. The number of species identified and named in each taxonomic group in 2022. As many species are yet to be described worldwide, the total number is largely underestimated. Adapted from Bundesamt für Naturschutz 2024. (LUDWIG *et al.* 1996; WISSKIRCHEN & HAEUPLER 1998*;* KOPERSKI 2000; VÖLKL *et al.* 2004; CHAPMAN 2009; HAUPT *et al.* 2009; BINOT-HAFKE *et al.* 2011; MORA *et al.* 2011; SCHNITTLER *et al.* 2011; RACHOR *et al.* 2013; KEITH *et al.* 2015). Figures created using R (version 3.5.3; R Core Team, 2020).

From a human perspective, pests that cause crop losses are considered target insects, while beneficial organisms are considered non-target insects. This traditional classification is becoming increasingly problematic as it ignores the fact that the species in question can change the status of their classification, are not necessarily restricted to certain habitats, but can spread from there to other areas and are thus part of the overall connectivity of ecosystems (MÜLLER 2018). To effectively conserve insect biodiversity, it seems more appropriate to consider insects holistically including organisms currently designated as pests.

Insects are not only an integral part of most terrestrial and freshwater ecosystems, but also vital to their functioning and health. They provide critical services such as pollination (RITCHIE 2021). About 80% of wild plants depend on insect pollinators essential for plant reproduction and survival, biodiversity, as well as agricultural productivity (RITCHIE 2021). As a prime example for a process, which not only underpins diverse ecosystems but also secures human global food production, bees are critical in pollinating a wide range of flowering plants (POTTS *et al.* 2016).

Beyond pollination, insects significantly contribute to nutrient cycling and soil enrichment through the decomposition of organic matter. Their role is crucial to maintain soil health and fertility (CULLINEY 2013) supporting plant growth and ecosystem resilience. But even more evident, insects are also a key component within the food webs. Approximately 60% of bird species, many of them endangered today (KEITH *et al.* 2015), rely on them for food (HALLMANN *et al.* 2017; HALLMANN & VON TIEDEMANN 2019), exemplarily highlighting the importance of insects in maintaining biodiversity.

However, current trends indicate a worrying decline in insect populations. In protected ecosystems in Germany for example, the biomass of flying insects has declined by as much as 75% in the last 25 years alone (HALLMANN *et al.* 2017; HOCHKIRCH *et al.* 2018). This alarming trend is likely to be even more pronounced in unprotected areas, especially where conventional agriculture and the use of insecticides are widespread (RADER *et al.* 2014; UCHIDA *et al.* 2016; RITCHIE 2019).

The role of insects as crop pests

Ecological interaction of insects with their man-made habitats on agricultural land are complex and mainly induced by human use. On one hand, as described above, especially insect pollination plays a fundamental role for functioning of agriculture. On the other hand, however, they represent important pest organisms and can thus be also detrimental to human agricultural practices (RIBEIRO *et al.* 1994; TONNANG *et al.* 2022). Pests and diseases account for a significant proportion of the estimated global annual crop losses of around 52 % in 2015 (BVL,

2015), with insects, beetles, aphids, and Lepidopteran caterpillars accounting for about 15 % of it.

The diverse destructive patterns of behavior of pest insects, often referred to as agricultural pests, can have a wide range of significant damaging impact on crop health and yield (RIBEIRO *et al.* 1994; PANIZZI 1997; PATOLE 2017). Grasshoppers, for example, can drastically reduce crop yield and quality by consuming leaves, stems and fruits, affecting food security and agricultural economies worldwide (KAMIL USMANI & USMANI 2018; LECOQ 2022). A thorough understanding of the nature and extent of this type of damage is essential for an effective pest management (RAUSHER 1983; TONNANG *et al.* 2022). Insects also serve as vectors for plant diseases, spreading pathogens that lead to crop devastation (NG & PERRY 2004). In addition, cosmetic damage caused by insects can reduce the market value of crops, especially fruits and vegetables, where its appearance is critical for human consumers (CAPINERA 2020).

There are two mostly relevant feeding strategies employed by insects feeding on leaves of crops. First of all, chewing and biting insects equipped with mandibles cut pieces of leaf material; notable examples include Lepidoptera larvae (caterpillars) (KRENN 2019). This physical damage leads to reduced photosynthetic capacity, stunted growth and, in severe cases, to total crop loss. The other prominent feeding type of insects investigated here is sucking and piercing from a food source. This group of insects possess specialized feeding organs such as stylets, which allow the ingestion of cell liquids and plant saps. Hemipteran species, such as aphids, are so called phloem feeders, i.e. their stylets take the sap from the plant vascular tissue (xylem and phloem) (TALEVI 2022). The resulting damage may be caused less by the direct ingestion of sap than by the transmission of plant viruses and other diseases (NG & PERRY 2004). With both feeding strategies insects are known to be able to completely destroy the harvest on farmland invaded by them, often resulting in critical situations for supplying local human populations with the necessary amounts of food, especially in third world countries.

The role of insecticides in agriculture plant protection

To prevent comparable negative humanitarian effects or situations, agriculture is at the nexus of several of the United Nations' Sustainable Development Goals (BEXELL & JÖNSSON 2017). High yields and productivity are key to achieve 'Zero Hunger' (BLESH *et al.* 2019), whilst understanding and minimizing the environmental impacts of agriculture is required to reach the goals to protect 'Life on Land' and ‘Life below water’(KERTON 2023). To maintain and improve crop yields by keeping pest populations below economic thresholds, the use of insecticides has been established as an essential tool in effective pest management (BUNTIN 2000; HAILE 2000; TONNANG *et al.* 2022). Insecticides also help to ensure food security by reducing post-harvest

losses caused by pest damage during storage and transport (CARVALHO 2006; SEUFERT *et al.* 2012). However, in contrast to pest species which are target for insecticides, non-target organisms, apart from species not affecting agriculture, include beneficial species such as essential pollinators and predatory species, which are integral to Integrated Pest Management (IPM) systems. Maintaining a balance between the consequences of pest control on non-target insects and the positive and beneficial effects of the latter species on crops is and remains a complex challenge for agriculture.

Worldwide approximately 590,000 tons of insecticides are applied each year (DE *et al.* 2014). With the growing global human population, projected to reach about 9.8 billion by 2050 (NATIONS 2015; LEESON 2018; DORLING 2021), demands for food resources and agricultural land will rise, too. Associated with this, remaining crucial for crop protection, the use of insecticides is expected to increase as well (AKAMATSU 2011; SHELTON & LAHM 2015; SPARKS & LORSBACH 2017; ZHANG *et al.* 2018; MAIENFISCH & MANGELINCKX 2021). But on the other hand, their successful use is increasingly undermined by challenges such as pest biology, crop growth cycles, the development of insecticide-resistances by pests and by target-species shifts in crops (STEVENS *et al.* 1988; BONAVENTURE 2012; CORSI & LAMBERTH 2015; JESCHKE 2016; LOSO *et al.* 2017; SPARKS & LORSBACH 2017). It is therefore imperative that the strategies and compounds used for insect control in the future are carefully selected, taking into account the biology of the pests and the growth cycles of the plants during their development in order to maximize crop protection (STEVENS *et al.* 1988; BONAVENTURE 2012). Such optimized Plant Protection Products (PPPs), including insecticides, can increase and ensure productivity and yield through their effectiveness against the target species, i.e., agricultural pests (BUNTIN 2000; CARVALHO 2006; SEUFERT *et al.* 2012), but also carry the risk of unintentional poisoning of non-target species and contamination of other parts of the environment, such as freshwater ecosystems.

The environmental impact of plant protection products depends on their proper use, environmental fate, and toxicology profile. This is especially the case in insecticides, which usually are actively brought into the environment to protect crops from damage by herbivores insects and plant parasites. Optimizing selectivity, i.e. maximising efficacy against targets whilst, at the same time, minimizing toxicity against non-targets to reduce environmental risks of PPPs, is an important scientific challenge. The overall aim to make such progress is to achieve sustainable food production and the aims of the above-mentioned United Nations' Sustainable Development Goals as well (BEXELL & JÖNSSON 2017).

Application and distribution of insecticides in agricultural land

To protect cultivated plants using agro-chemicals, they need to be applied evenly distributed on the farmland under risk of pest damage. Typically, PPPs are therefore applied to the plants by spraying onto the leaves dissolved in a water matrix (WANG & LIU 2007; ANGGRIANI *et al.* 2020). There are two principal scenarios for the application, a curative or a preventive treatment (COURSHEE 1960; HILZ & VERMEER 2013). For a curative treatment pests are directly exposed to the wet spray solution, whereas in a preventative treatment, pests get exposed to a spray deposit dried on the foliage (WANG & LIU 2007) (Figure 2). While preventive treatment focuses on exposure prior to pest damage, curative treatment has multiple exposure pathways.

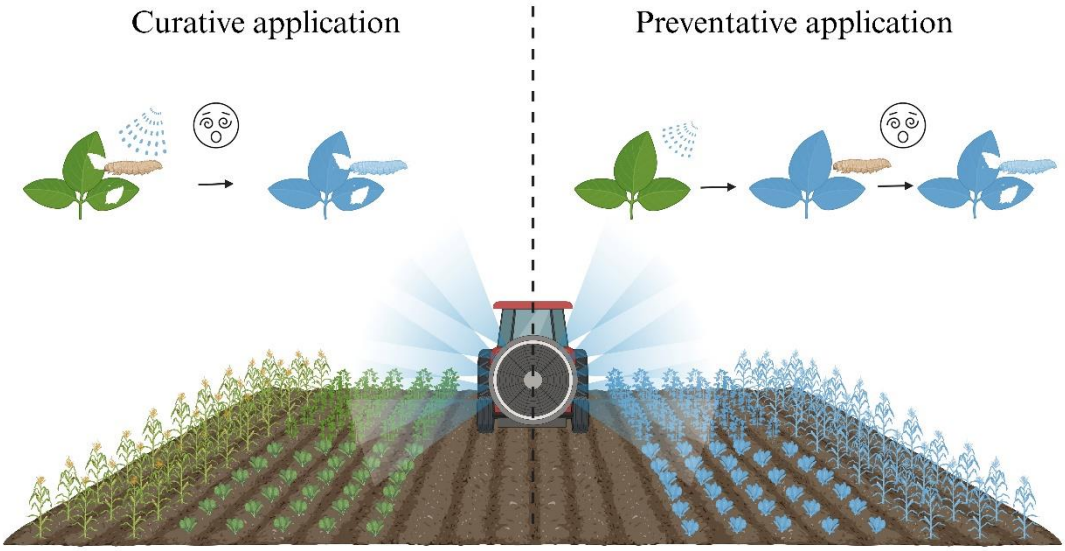


Figure 2: Foliar application of compounds. Curative *versus* preventative application in crop protection. (Created using BioRender.com)

Insecticides entering the environment

Despite the attempt to restrict the application as targeted, a significant proportion of insecticides may miss the intended leaves and drop on the ground or become airborne and drift off the target area during crop treatment (BALLSCHMITER 1992; AL HEIDARY *et al.* 2014). In addition, active ingredients and their formulation compounds, are subject to degradation over time in soil, water, and various environmental matrices, including metabolism by organisms like bacteria, fungi, plants, and animals (MENN 1978; G SOUZA *et al.* 2010; KAUR *et al.* 2021). The process of degradation can unintentionally introduce the chemicals of concern into ecosystems through various pathways such as run-off from target sites, leaching and atmospheric deposition (TIRYAKI & TEMUR 2010).

Runoff and leaching are of particular concern, because they can easily transport agrochemicals from treated fields to groundwater and nearby waterways, posing a risk to aquatic life and water quality (WAGNER & LÖTTERS 2013; MINGO *et al.* 2016; KUMAR *et al.* 2021). In addition, apart from farming activities, industrial activities can contribute to the release of these harmful chemicals and/or their components into the environment, where they can settle on terrestrial or aquatic habitats (ANJUM *et al.* 2017) (Figure 3). Volatile chemicals pose a further challenge, as they can evaporate into the atmosphere and be transported over varying distances (GLOTFELTY *et al.* 1990). Once in the air, they are subject to complex atmospheric dynamics, which can lead to their eventual deposition in different ecosystems, far from the original application site (NORSTROM *et al.* 1988; BEDOS *et al.* 2002) (Figure 3).

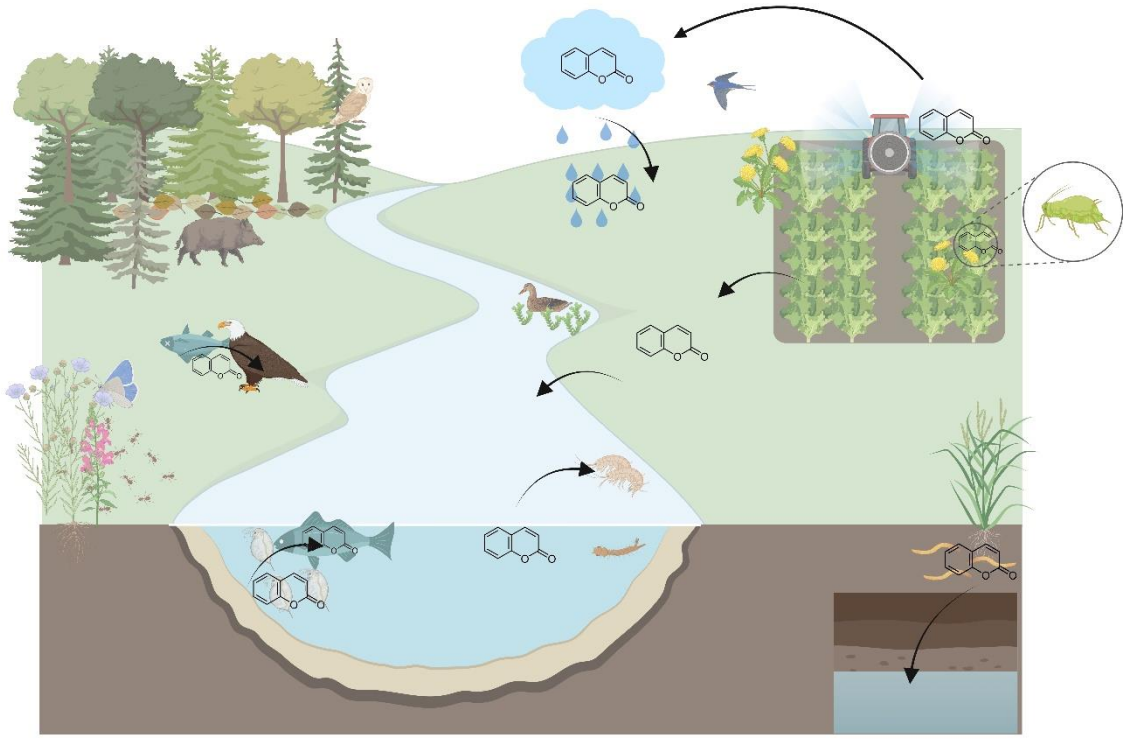


Figure 3: Environmental fate of compounds. Distribution of compounds (shown here for the example of Coumarin) after release into the environment. Examples are given for the main environmental compartments (air, water, flora and fauna, soil). (Created with BioRender.com)

This atmospheric journey and subsequent deposition can lead to unintended exposure of non-target species to these compounds. Once there, uptake and distribution of chemicals within the ecosystem depends on the specific properties of the chemical and the nuances and composition of the local ecosystem (CLEMENTS *et al.* 1994; HANSLIK *et al.* 2020). They may be taken up by any type of organism of various food web levels and accumulate in its tissue over time

(CONRAD 1977). This bioaccumulation can lead to biomagnification, where chemicals become concentrated in organisms of the higher trophic levels of the food web (CONRAD 1977; THOMANN 1989; MCLACHLAN 1995; GRAY 2002; KELLY *et al.* 2007) (Figure 3).

Insecticide classes and their market share

Insecticides are designed to control or eliminate populations of insects and do so by a variety of mechanisms. While there are numerous classes of insecticides, each with their own unique chemical structures and modes of action, the following only focuses on the major classes currently dominating the global market. They represent the most widely used and researched insecticidal compounds in pest management (Figure 4).

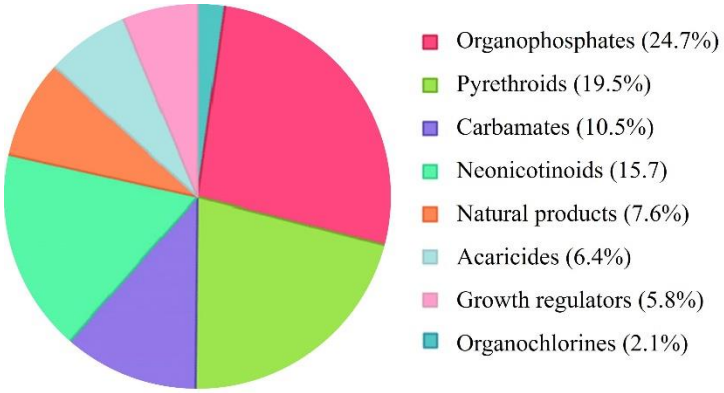


Figure 4: Main classes of insecticides and their market share. Source modified and combined from (LABBÉ *et al.* 2011) and (NAUEN 2006).

Organophosphates, which account for about 24.7% of the products on the insecticide market, are known for the mechanism of inhibiting acetylcholinesterase (NAUEN 2006; LABBÉ *et al.* 2011). This leads to an accumulation of acetylcholine at nerve endings, resulting in permanent stimulation of the nervous system, paralysis, and eventually death in insects (ADEYINKA *et al.* 2023; ARAÚJO *et al.* 2023). Organophosphates are also known to be toxic to a number of other organisms, including for example fish (ROHANI 2023). Pyrethroids, which account for 19.5% of the products on the markets, are synthetic analogs of the natural pyrethrins found in chrysanthemum flowers (NAUEN 2006; LABBÉ *et al.* 2011). They act on the voltage-gated sodium channels in nerve cells, disrupting normal bioelectrical activity and leading to paralysis and death of the insect (GAJENDIRAN & ABRAHAM 2018). Neonicotinoids account for 15.7% of the market (NAUEN 2006; LABBÉ *et al.* 2011). They are a newer class of systemic insecticides derived from the naturally occurring alkaloid nicotine that selectively target the nicotinic acetylcholine receptors in insects, resulting in lethal overstimulation of their nervous system

(JESCHKE & NAUEN 2008; MATSUDA *et al.* 2020). Carbamates, with a market share of 10.5%, are chemically related to organophosphates and also act as acetylcholinesterase inhibitors, although they generally have a faster degradation rate and lower environmental persistence (FUKUTO 1990; NAUEN 2006; LABBÉ *et al.* 2011). Natural products, with a market share of 7.6% (NAUEN 2006; LABBÉ *et al.* 2011), include a diverse range of compounds derived from plants, microbes and other natural sources, each with a specific mode of action that can be harnessed for pest control caterpillars (NAVON 2000; BRAVO *et al.* 2011). Insect growth regulators (IGRs), which account for 5.8% of the market (NAUEN 2006; LABBÉ *et al.* 2011), interfere with the hormonal control of insect development and reproduction, resulting in ineffective molting, abnormal development, or reduced fertility (OBERLANDER & SILHACEK 2000; TUNAZ & UYGUN 2004). Organochlorines, now only having a small market share of 2.1% (NAUEN 2006; LABBÉ *et al.* 2011), were once widely used because of their long-lasting effects (BLUS 2002; TURUSOV *et al.* 2002). They interfere with the function of GABA-gated chloride channels, leading to uncontrolled nerve activity (ENSLEY 2018).

These major classes of insecticides, readily available on the market, are central to pest management strategies, but it is important to note that there are other classes and compounds in use, which contribute to the overall insect control landscape (SPARKS 2013; NAUEN *et al.* 2019). The selection and use of these insecticides must take into account efficacy, resistance management, environmental impact and regulatory status (NAUEN *et al.* 2019; SPARKS *et al.* 2021).

The role of insecticide concentration for the effects on organisms

Once modulated by multiple abiotic and biotic factors, residues of insecticides released into the environment are found at varying concentrations. But the interaction of this factors also produces complex spatial and temporal distribution patterns of insecticides (LALOUETTE *et al.* 2016). Even sublethal concentrations of toxins, may induce significant physiological or behavioral changes in organisms exposed to it (DESNEUX *et al.* 2007). However, the response to an insecticide is nuanced and influenced by genetic factors, overall fitness and environmental variables (VAN TOOR *et al.* 2008; BIJLSMA & LOESCHCKE 2012). The variability of the response in combination with fluctuating insecticide concentrations makes it particularly difficult to distinguish between lethal and sublethal effects. In addition, some insecticide formulations can exacerbate the risks due to their additive, potentiating, or synergistic effects and prolonged persistence in the environmental (COX & SURGAN 2006; ZHU *et al.* 2014; ANSANTE *et al.* 2017).

Thus each application may result in sublethal effects at certain times or on certain organisms, highlighting the need for comprehensive studies going beyond mere toxicity covering a range of

life history traits at different insecticide concentrations (KATTWINKEL *et al.* 2011; WAGNER & LÖTTERS 2013; BERGER *et al.* 2018; MÜLLER 2018).

Uptake and fate of compounds in insects

The first fundamental step in ensuring the effectiveness of any insecticide is the uptake or absorption by the target organisms. However, the major differences in the feeding habits, physiology and life cycles of insects pose a considerable challenge in the development of insecticides. Understanding the fate of compounds within an organism as influenced by chemical processes is reflected by the broad concept of Absorption, Distribution, Metabolism and Excretion (ADME). Specifically, the potential effects of agrochemicals, particularly inactive insecticides, applied preventatively to foliage and exposed to several representative invertebrate species were to be investigated in this study.

There are two dominant routes of uptake of insecticides into the target organism; via oral uptake and via contact with body surfaces (EBELING 1974; CLARKE & JEWESS 1990; MISSNER & POHL 2009) (Figure 5). Piercing sucking Hemipteran insects, such as aphids and leafhoppers, can take up systemic insecticides orally while sucking the sap from the vascular system of the plant that had ingested these compounds (GEROLT 1983). Herbivorous Lepidopteran larvae, on the other hand, ingest the insecticides when they consume the entire treated foliage (HALLMANN & VON TIEDEMANN 2019). Insecticides attached to the surface of the plant can also be ingested by the caterpillars during crawling on the forage plant, either through direct contact exposure with the applied products or by ingestion (oral) at the site of application (GEROLT 1983). In contrast to this, sucking herbivores take up chemicals exclusively tarsal and by probing, but not through ingestion (TAPPERT *et al.* 2017) (Figure 5). This route of uptake by the insect is primarily determined by the effective dose applied to the leaf surface (GEROLT 1983).

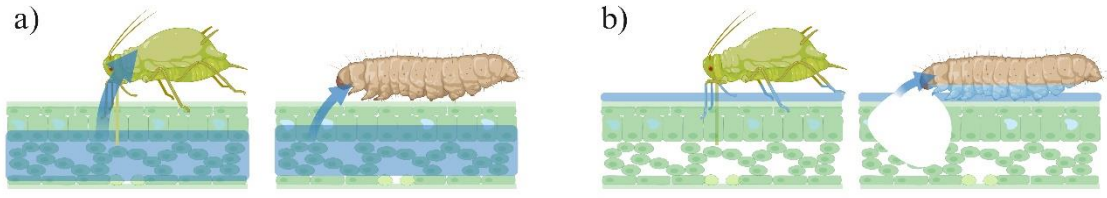


Figure 5: Schematic overview of insecticide absorption. **a)** Oral absorption by active sucking or feeding **b)** contact (aphid) absorption from surface by passive diffusion, caterpillar contact and oral absorption. Compound and action site marked in blue. (Figure adapted from MÜLLER (2018); created with BioRender.com).

Oral absorption by active feeding (Figure 5a) and contact absorption by passive diffusion over the body surface (Figure 5b) are influenced by life stage and feeding behavior of the insects. The subsequent internal distribution in the insect tissue could be due to several permeation steps through different biomembranes (WAN *et al.* 2022). The effective dose at internal target sites or structures, such as the nervous system or endocrine glands, depends on the duration of exposure (EVANS 1999). Insecticides act in the organism primarily at highly specific binding sites such as acetylcholine receptors, acetylcholine esterase or ion channels in nerve cell membranes (CASIDA & DURKIN 2013).

However, insect metabolism (or biotransformation) of parent compounds by enzymes or deposition in fat can also detoxify the organism from these compounds and potential metabolites (CONRAD 1977; RHARRABE *et al.* 2007; ROY *et al.* 2016). Phytophagous insects create different excretion products, like feces or honeydew according to their diet. Excretion may include different metabolic processes and rates of compounds. Further elimination or detoxification strategies are enhanced excretion rates, regurgitation or shown in egg-laying (BROADBENT 1951; CONRAD 1977; O'DONNELL 2008). Overall, detoxification is directly related to the kinetics of compound elimination, due to combined metabolic processes and excretion mechanism (SMITH 1955; FEBVAY *et al.* 1988). Therefore, in this context the terminus elimination does not only refer to excretion, but also includes the metabolization of a compound.

Potential ecosystematical effects of insecticides

Insecticide exposure can induce several sub-lethal up to lethal effects to be taken into account in the analysis of the interaction between insecticides and insects, as they can have influence on a multitude of biological performance parameters of exposed individuals (MÜLLER *et al.* 2017; MÜLLER *et al.* 2019). Performance parameters indicative of individual fitness of organisms are, for example, feeding behavior, body mass and reproductive output (GUTSELL & RUSSELL 2013; SCHUIJT *et al.* 2021). If the observed insecticide effects on some of such parameters are intended, we treat them as aspects of efficacy. In contrast, the same or similar effects on non-target organisms are considered as unintended toxicity. Today, many insecticides are known to not only harm the target organisms, but as mentioned above, also have significant negative impact on populations of several other non-target species. Therefore, designing insecticides selective for the targeted species, i.e., optimizing for efficacy and for minimum environmental toxicity at the same time, appears to be the key for sustainable food production and biodiversity protection. Various methods are established in industrial optimization of agrochemical design to reduce the potential number of compounds to be synthesized and tested (DUDEK *et al.* 2006; GICHERE *et al.* 2021) to achieve this.

In addition, insecticides may not only act at the primary target sites (for example the nervous system), but also interact with secondary sites of action, leading to sub-lethal effects influencing the physiology, behavior, ecology and communication of an insect (DESNEUX *et al.* 2007). While research often focuses on development and mortality, fewer studies consider the effects on insect behavior and modification of chemical phenotypes, being important for intraspecific communication and mating (MÜLLER *et al.* 2017; MÜLLER *et al.* 2019). In this context, non-target organisms that do not develop into crop pests are of particular concern. They highlight the ecological risks of insecticide contamination, as they are highly important food sources for both invertebrates and vertebrates (GEIGER *et al.* 2010). The classification of herbivorous insects as pests or non-target species can vary depending on the context. And the given environment plays a critical role in this classification, with the status of the insects in quest changing depending on their interaction(s) with crop or non-crop plants. For example, the green peach aphid, *Myzus persicae* (SULZER, 1776), is a well-known agricultural pest, but also inhabits non-agricultural plants (VAN EMDEN *et al.* 1969; BLACKMAN & EASTOP 2008). Likewise, the mustard leaf beetle, *Phaedon cochleariae* (FABRICIUS, 1792), which is labeled as pest in agriculture, represents a non-target organism in natural Brassicaceae habitats (DE PAIVA 1977; UDDIN *et al.* 2012; MÜLLER & MÜLLER 2016). Therefore, distinguishing between targeted and non-targeted insects is crucial to efficiently control crop losses and maintain beneficial interactions as well as ecosystem functioning at the same time.

The potential effects of insecticides on insect development are sex-specific.

Research has repeatedly shown that insecticides can negatively affect key life history traits such as the number of offspring, survival rates and intrinsic growth rates in a variety of insects, including natural enemies of crop pests and pollinators (BAYRAM *et al.* 2010; AMARASEKARE *et al.* 2016; MÜLLER *et al.* 2017; ALMASI *et al.* 2018; MÜLLER *et al.* 2019). The influence of insecticides on these vital traits is not only species-specific, but even varies at different developmental stages within the same species (NIELSEN *et al.* 2000; LEONOVA & SLYNKO 2004). For example, in predatory beetles such as *Eriopis connexa* (GERMAR, 1824), the larval stages can experience either prolonged or shortened developmental times depending on the type and dosage of insecticide, which in turn affects their life cycle and ecological role (RIMOLDI *et al.* 2017).

In addition, studies on the developmental speed of male and female insects have shown that the sex-specific responses to insecticides are different (WAGNER & LÖTTERS 2013; BAIER *et al.* 2016; DEBECKER *et al.* 2016). In the damselflies of the genus *Ischnura* CHARPENTIER, 1840 for example, the females showed longer development times when exposed to insecticides, whereas the males developed faster (DEBECKER *et al.* 2016). Such differences in development and

potential resulting shifts in the local or temporary sex ratios can have significant effects on population dynamics and reproductive behavior within species (COSTA *et al.* 2014; MÜLLER *et al.* 2017; NAVARRO-ROLDÁN & GEMENO 2017).

Insect endosymbionts and insecticide resistance

Entomological research on the complex interactions between insecticides and insect symbioses has shown that endosymbiotic microbiota are important for insect adaptation, nutrition, digestion, detoxification, and resistance to insecticides (KIKUCHI *et al.* 2012; XIA *et al.* 2013; GUEDES *et al.* 2016; CLAVE *et al.* 2022). Genomic sequencing could identify specific strains of gut bacteria to be responsible for the resistance and susceptibility of *Plutella xylostell*a (L., 1758) (GUO *et al.* 2013; XIA *et al.* 2013). A symbiont degrading insecticides in the fly *Bactrocera dorsalis* (HENDEL, 1912), was found to be inherited via the egg surface in another microbiome study (GUO *et al.* 2013). Ongoing and future research is likely to identify more symbionts that degrade insecticides, responsible for the development of resistance mechanisms in pests (VAN DEN BOSCH & WELTE 2017). A database enabling to exploit such data on symbiotic relationships could support pest management to counter resistance and develop highly specific insecticides (GRESSEL 2018; MÜLLER 2018).

Insecticide effects on chemical communication

The effect of insecticides on chemical communication in insects is a critical aspect in research so far, as exposure to insecticides has been shown to significantly disrupt communication pathways essential for their interaction, mating, and survival (MÜLLER *et al.* 2017; MÜLLER *et al.* 2019). Several studies confirmed that all levels of interspecific and intraspecific communication are affected, altering essential behaviors including mating rituals and predator-prey dynamics (CLAVER *et al.* 2003; DESNEUX *et al.* 2007; TAPPERT *et al.* 2017; WANG *et al.* 2018).

Intraspecific chemical communication is particularly affected, as sublethal concentrations of insecticides have been shown to affect both, production and perception of pheromones crucial for insect mating (WANG *et al.* 2018). In addition to volatile pheromones, mating behavior, as well as other social interactions within species, are fundamentally determined by communication via cuticular hydrocarbons (CHCs), compounds that cover the insect surface and act as short-range contact signals. Environmental factors, such as food quality or pesticide exposure, are known to modify CHC profiles, resulting in changes in mating behavior and thus possibly the reproductive success of species (GEISELHARDT *et al.* 2009; MÜLLER *et al.* 2017). All this can affect population dynamics, species interactions, and even influence the evolutionary trajectory of the species in quest. Besides negative effects on non-target organisms in on and off-site ecosystems, disruption of chemical communication can have direct

consequences for non-target beneficials in the agricultural system at the application site, such as natural enemies of target species and biocontrol agents. In the exemplary case of *Trichogramma chilonis* ISHII, 1941, a parasitoid of several Lepidopteran pests, even low levels of certain insecticides had a significant negative impact on mating behavior (WANG *et al.* 2018). Furthermore, acting as neuronal information disruptors, insecticides can reduce olfactory responses of predators or parasitoids to their prey or host (CLAVER *et al.* 2003; TAPPERT *et al.* 2017). Moreover, prey recognition and acceptance, for which CHC profiles play a crucial role, may be impaired by alteration of these compounds, known to be influenced by insecticide exposure. Impairing the ability of predators and parasitoids to locate and effectively control pest populations would potentially raise their mortality, disrupt the ecological balance and, counter to the intended effect, lead to increases in pest populations.

The current and still developing knowledge on such complex limitations for the use of insecticides should be included for developing pest management strategies that minimize potential negative ecological impacts and preserve the intricate chemical communication networks vital to insect life and ecosystem functioning (GEISELHARDT *et al.* 2009; MÜLLER *et al.* 2017; TAPPERT *et al.* 2017; MÜLLER 2018; WANG *et al.* 2018)

Hormetic effects of insecticides on insects

The focus in research on insecticide effects is often tending and pronouncing their harmful effects, but there are also well documented cases, in which sublethal concentrations of insecticides have been shown to have beneficial effects on insect populations, a phenomenon known as hormesis (MATTSON 2008; TRICOIRE-LEIGNEL *et al.* 2012). For example, in the green peach aphid [*Myzus persicae* (SULZER, 1776)], a hormesis effect was shown in response to Imidacloprid, leading to stimulation of reproduction at low-dose exposure, while causing inhibition and toxicity in high doses (CUTLER *et al.* 2009; CUTLER 2013). The phenomenon of hormetic responses demonstrates the sensitivity and complexity of the interaction between insect physiology and exposure to chemicals (WANG *et al.* 2017). Similar effect of hormetic responses to very low concentrations of insecticides were found in Lepidopteran species, in which they increased sensitivity and responsiveness to sex pheromones, potentially improving mating success (LALOUETTE *et al.* 2016).

Such responses generally indicate insect populations in fields that have been inadequately treated with insecticides may increase more than in untreated fields, contrary to application intentions, posing a critical challenge to pest management practices.

Insect mechanisms to cope with chemical stress induced by insecticides

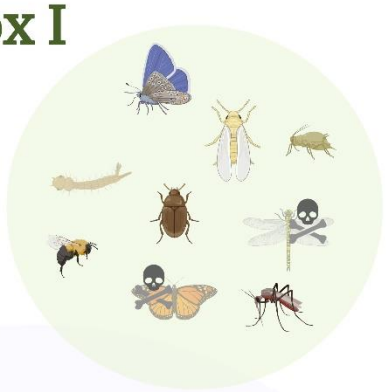
Insects have developed a number of mechanisms to cope with chemical stress caused by insecticide exposure. These coping mechanisms can be broadly categorized into three phases: immediate compensation, recovery after exposure, and long-term transgenerational adaptation (FORGASH 1985; HAMMOND 1996; REED *et al.* 2001; SAKAMOTO *et al.* 2006; GUEDES *et al.* 2016).

Generally, insects respond to chemical stressors by immediate adjustment of their metabolic processes. The body mass of an insects, a dynamic measure, is constantly determined by energy expenditure caused by metabolism and energy uptake by food consumption. Stressors, such as insecticides, are known to alter the metabolic rate of insects (FORGASH 1985; HAMMOND 1996; REED *et al.* 2001; SAKAMOTO *et al.* 2006). As a direct response, the uptake of food typically increases, rapidly compensating energy losses. The direct correlation between food uptake and changes in body mass allows measuring of such compensatory feeding and its effects on the metabolism (REED *et al.* 2001; MÜLLER & MÜLLER 2016). In several studies, an increased food consumption was suggested to be a robust indicator of the presence of physiological effects, as well as the extent of metabolic compensation after exposure to insecticides (MÜLLER & MÜLLER 2016; WOLZ *et al.* 2021).

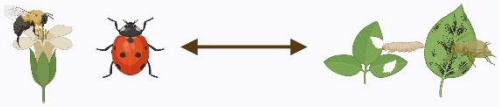
In addition to such immediate response, insects have a remarkable ability to recover from stress induced by insecticide exposure. The recovery time can vary greatly between species, taking from days to several generations. For example, individual of the bedbug (*Cimex lectularius* L., 1758) have been shown to recover from Pyrethroid stress within two weeks (FELDLAUFER *et al.* 2013). But on the other hand, in aquatic species such as *Daphnia magna* (STRAUS, 1820), recovery from insecticide stress needs one to two generations (LIESS *et al.* 2006). Furthermore, there also is a potential for long-term transgenerational adaptation in the response of insects to chemical stressors (LIESS & FOIT 2010). Studies suggest that exposure to insecticides can occasionally have beneficial transgenerational effects, as for example, the offspring of Colorado potato beetles (*Leptinotarsa decemlineata* SAY, 1824), which had been exposed to insecticides, revealed improved survival rates and greater body mass as adults, suggesting an evolutionary adaptation to insecticide stress (MARGUS *et al.* 2019).

Overall, research into the development of must carefully consider a wide range of ecological and physiological phenomena in insects. These range from specific metabolic responses of individuals to complex intra- and interspecific interactions of communities and food webs. The fundamental challenge of protecting crop yields in agricultural systems from pests while minimizing interference with the fragile ecosystem requires well-designed and comprehensive experimental studies., A crucial point is therefore the selection of a variety of representative model organisms to gain insight into different uptake pathways, the variation of effects on the individual and its interactions with the environment, and the short- and long-term adaptation mechanisms.

Information box I

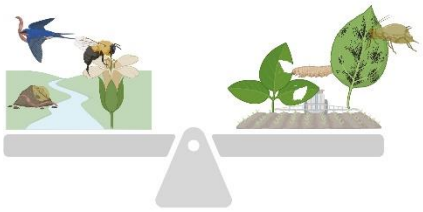


- Insects are known to represent the largest group of animals on earth today.
- They are an integral part of most terrestrial and freshwater ecosystems.
- Current trends indicate a worrying decline in insect populations.
- Insects, in this context of agriculture, can be both, beneficial as well as detrimental to human agricultural practices.



- Leaf-feeding insects, often referred to as agricultural pests, can cause a wide range of damage to crops.
- In contrast, non-target organisms have no measurable negative impact on agriculture.
- Insecticides are key for effective pest management, helping to keep populations below economic thresholds and maintain crop yields.
- They can (unintentionally) be distribute in the ecosystem and be present in different concentrations.
- Uptake of insecticides can be described by Absorption, Distribution, Metabolism, Excretion.

- Insecticides can have different intended and unintended effects.
- This depends on the classification of insects as target (pest) or non-target.
- Therefore, effects need to be monitored and understood to ensure balanced protection of non-target species and control of agricultural pests.



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[1] For further details and references, please refer to the introduction sections before. Created with Bio.Render.com

Study organisms

Insects are considered excellent model organisms for scientific research due to a number of factors, especially their high reproductive rate and short life cycle. This allows to observe multiple generations within short periods of time, which is particularly useful for studies in genetics and evolutionary biology, especially as there is a wide range of genetic tools available for insects (REED *et al.* 2017). In addition, many insects have a relatively simple genetic structure, but share significant genetic material with more complex organisms, including humans, making them valuable for studying genetic diseases and basic developmental processes. Despite their small size, insects exhibit complex behaviors and social systems, making them also suitable models for the study of neuroscience, ethology and sociobiology (LEONELLI & ANKENY 2013).

Another advantage of using insects in research is that they are easy to maintain. They need minimal space and resources, making them cost-effective for laboratories. The ability to breed and keep them in large numbers is beneficial for later on statistical analysis, reducing variability in experimental results. In addition, insects are of great global importance to human health and agriculture as vectors of diseases, as pollinators in natural habitats and as pests in agriculture. Studying them has already lead to important breakthroughs in medicine, conservation and pest control having direct applications to human concerns (LEONELLI & ANKENY 2013; MATTHEWS & VOSSHALL 2020).

From both legislative and regulatory perspective, insect research often raises fewer ethical concerns than vertebrate research. This generally makes it easier to obtain research permits. In many regions, regulations governing the use of insects in research are less stringent, which reduces bureaucratic hurdles and allows studies to begin far more quickly (MÜLLER & GROSSNIKLAUS 2010). The combination of these biological and regulatory advantages make insects highly valuable and accessible for a wide range of scientific research, contributing to their status as excellent model organisms (BUSCHMANN 2013).

In order to carry out a comprehensive comparative study of insects, it is essential to examine a sufficiently broad spectrum of species. This includes both target organisms that are specifically targeted in agriculture and non-target organisms that may be unintentionally affected. In addition, for assessing the impact of pollutants the study of insects from different habitats, including terrestrial and aquatic environments is essential. The species selected had to fulfill certain criteria: They should be able to be reared quickly and reliably, even under the restricted working conditions enforced by the COVID-19 pandemic; be available for testing throughout the year and independent of seasonal variations; be suitable for the development of the research

objectives and allow the adequate performance of bioassay or data analysis; be recognized indicator species in ecotoxicological or efficacy research; and last but not least, be quantifiable to the extent of exposure to the test compounds in the experiment. Two polyphagous terrestrial insects and one aquatic insect, each fulfilling the established criteria for meaningful comparative analysis, were carefully selected as three indicator species.

Spodoptera littoralis (BOISDUVAL, 1833)

Spodoptera littoralis, also referred to as the (African or Egyptian) Cotton leafworm, is a polyphagous moth species from the species-rich family Noctuidae (NOCTUIDAE 1833). In the past it was often confused with *Spodoptera litura* (FABRICIUS, 1775), a fact only recent recently disclosed (HEALTH 2015; HEALTH *et al.* 2019).[2] The distribution of *Spodoptera littoralis* spans three continents (Figure 6), with populations actually established in diverse terrestrial habitats in Africa, southern Europe and parts of Asia (BROWN & DEWHURST 1975; POGUE 2002).

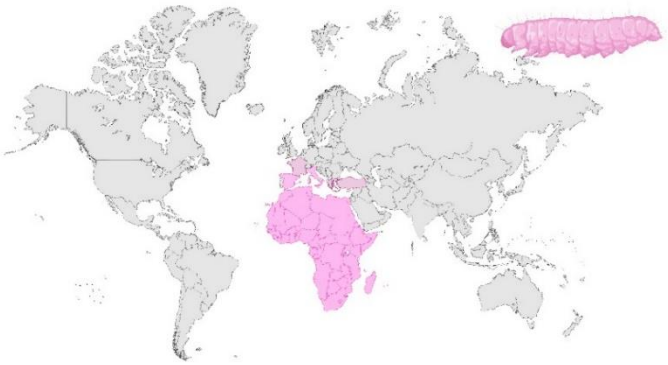


Figure 6: Distribution of *Spodoptera littoralis* (HEALTH 2015). (Created with Bio.Render.com)

S. littoralis caterpillars are known for their ability to feed on a multitude of plant species (SALAMA *et al.* 1971; HEALTH 2015). Their extensive host range allows them to establish and thrive in a wide range of environments (SALAMA *et al.* 1971). This moth´s life cycle (Figure 7) is characterized by the capability of producing several generations per year, with the number of generations and time for development highly depending on temperature (YONES *et al.* 2012). The optimal temperature for the reproduction of the species is around 25°C (BHATT & BHATTACHARYA 1976; SIDIBE & LAUGE 1977; OCETE 1984). The female moth lays clusters of eggs, typically on the undersides of leaves. Initially cream-colored, the eggs darken as the larvae develop inside. After hatching, the caterpillars go through several so called “instar” larval stages, feeding voraciously, and growing rapidly (BROWN & DEWHURST 1975; POGUE 2002).

[2] This leads to the situation, that older literature published dealing with studies on *Spodoptera littoralis* may in fact represent studies on *Spodoptera litura*, and thus, making data taken from those publications cover different species and incomparable.

Once fully grown, the caterpillar pupates, usually in the soil or near the ground in a cocoon. In this pupa the larva transforms to the adult moth which emerges from it. The nocturnal moths begin to mate and lay eggs shortly after hatching, starting the cycle all over again (MELZER 2011). Larvae, particularly later instars, leave the host plant during the day and return at night to feed, a strategy that may reduce the risk of predation (SINAKEVITCH *et al.* 2008).

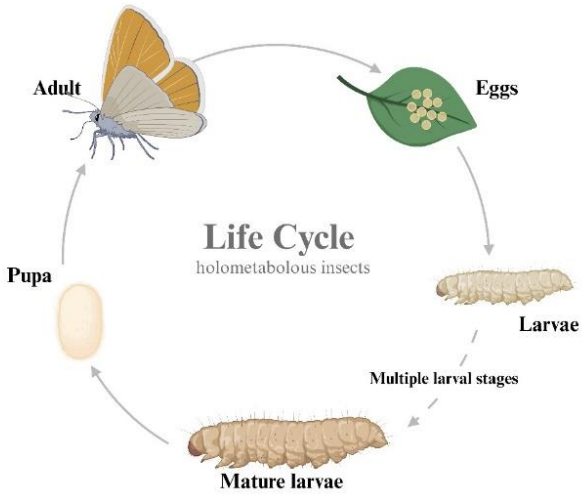


Figure 7: Life cycle of the cotton leaf worm *Spodoptera littoralis* (OCETE 1984). (Created with Bio.Render.com)

S. littoralis provides important ecological functions in their natural habitat. As herbivores, *S. littoralis* caterpillars feed on numerous plant species, which can have influence on the dynamics of plant communities by potentially affecting the population sizes and dominance of certain plants (HUNTLY 1991). The moth and its caterpillars also serve as food source for various predators and parasitoids, including birds, bats, spiders, wasps and beetles, and thus play a crucial role in the food web (PAINE 1966; RICHARD 2013).

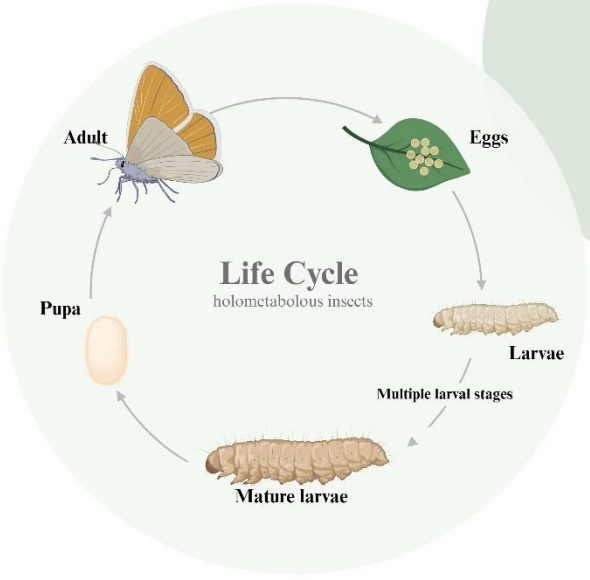
S. littoralis larvae can infest a host range that includes over 87 crops from 40 different plant families, and can therefore have a devastating impact on diverse agricultural systems, (SALAMA *et al.* 1971; HEALTH 2015). The species tends to damage economically important crops, especially such as cotton, soybean, maize and tomato (SMITH *et al.* 1997; HEALTH 2015). The larvae of *S. littoralis* use to cut off leaves with their mandibles and swallow them, which physically damages the plant and reduces its photosynthetic capacity, inhibits growth and, in extreme cases, can lead to complete defoliation (KRENN 2019). In agriculture, such feeding patterns can lead to significant yield and quality losses, including total crop loss. In addition, the species has shown remarkable adaptability and increasing resistance to many insecticides, which poses a major challenge for crop health management (MOUSTAFA *et al.* 2023).

Egyptian Cotton Leafworm

Spodoptera littoralis

Spodoptera littoralis (Boisduval, 1833)

- Commonly known as the Egyptian or African Cotton Leafworm, belongs to the family of Noctuidae.
- This moth species is found throughout Africa, southern Europe and parts of Asia.
- The caterpillars are capable of feeding on a wide range of plant species.



- The life cycle consists of four distinct stages: egg, larva, pupa and adult.
- Both larvae and adults are a source of food for various predators, such as birds, and contribute significantly to the food web.

- Larvae cause physical damage to host plants by using their mandibles to cut and consume leaf fragments.
- In agricultural settings, the feeding habits larvae can lead to significant reductions in crop yield and quality, potentially culminating in complete crop failure.

3

[3] For further details and references, please refer to the species description before. Created with Bio.Render.com

Myzus persicae (SULZER, 1776)

Myzus persicae, commonly known as the green peach aphid, is a highly polyphagous terrestrial species of the globally distributed family Aphididae (VAN EMDEN *et al.* 1969). *Myzus persicae* has a widespread distribution (Figure 8) and particularly thrives in regions where its host plants are extensively cultivated (COCU *et al.* 2005; MARGARITOPOULOS *et al.* 2009).

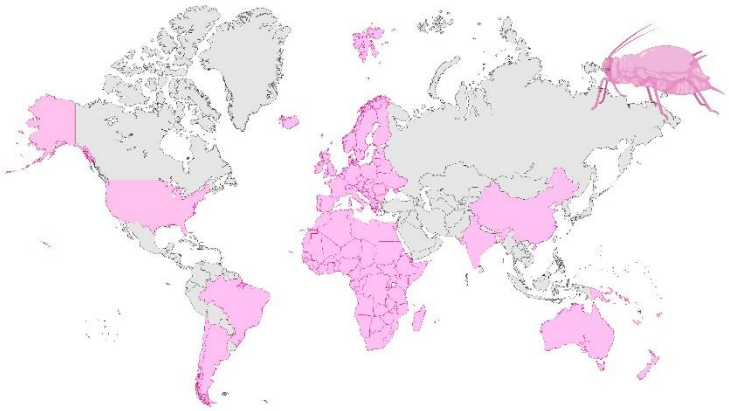


Figure 8: Distribution of the green peach aphid *Myzus persicae* (COCU *et al.* 2005; MARGARITOPOULOS *et al.* 2009). (Created with Bio.Render.com)

Green peach aphid have a wide range of host plants, including name-giving peach trees, other fruit trees, vegetables and ornamental plants (ANNIS *et al.* 1982). The aphids are equipped with specialized piercing and sucking mouthparts, stylets, which facilitate the extraction of cell fluids and plant saps, namely from the plant's vascular tissues, namely xylem and phloem. Therefore they are classified as phloem feeders (TALEVI 2022).

Optimal temperatures for *M. persicae* reproduction range between 20°C and 25°C, but they can reproduce over a wider span of temperatures, which contributes to their success as a species (DIXON 1977). Green peach aphids have a complex life cycle that, depending on environmental conditions, can involve both sexual and asexual reproduction (Figure 9). During the growing season, females can reproduce parthenogenetically, giving birth to live nymphs without the need for males (DIXON 1977). This allows rapid population growth under favorable conditions (CAPINERA 2001).

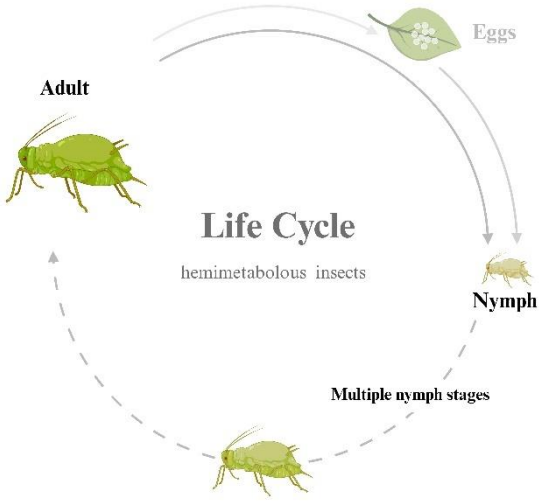


Figure 9: Life Cycle of *Myzus persicae* (CAPINERA 2001; FERICEAN et al. 2011). (Created with Bio.Render.com)

Behaviorally, *M. persicae* shows a preference for the underside of leaves and favors new growth areas on plants they feed on (VAN EMDEN *et al.* 1969; FERICEAN *et al.* 2011). In natural ecosystems, *M. persicae* serves as a food source for a variety of small predators and parasitoids, such as birds like tits and insects like ladybirds, lacewings, or parasitoid wasps, playing an important role in the biological control of aphid populations (EMDEN & HARRINGTON 2017).

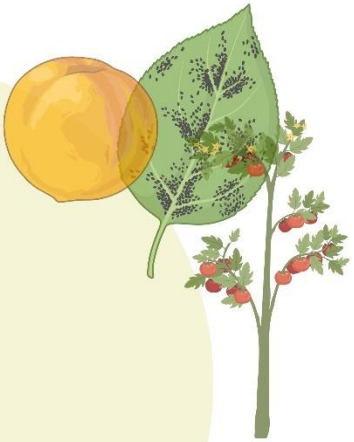
The adaptability of *M. persicae* to survive in a wide range of environmental conditions and its high rate and speed in reproduction, as well as its ability to rapidly colonize host plants (DIXON 1977), make it a difficult pest to control. It can infest over 500 plant species and is known to cause significant economic losses in agricultural systems, both through direct feeding damage and through its role as a vector for numerous plant viruses (NG & PERRY 2004; BLACKMAN & EASTOP 2008; DEDRYVER *et al.* 2010). Green peach aphids feed on plant sap and cause stunted growth and other physical deformities in plants by depriving them from sap. Their feeding behavior is not only directly harmful to host plants, but also facilitates the spread of insect-borne viruses. With 275 out of 600 known cases of virus transmissions, almost half of this are due to the activity of aphids (AUCLAIR 1963; NAULT 1997; GOMEZ *et al.* 2006). They can develop resistance to pesticides, which make them even more difficult to control (DEDRYVER *et al.* 2010).

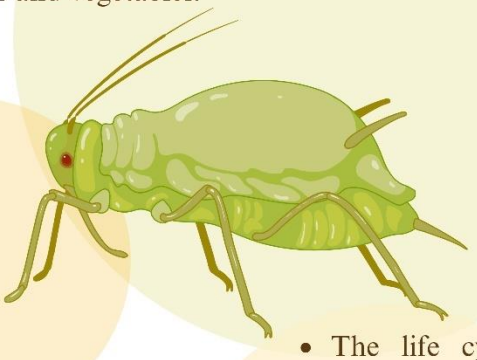
Green Peach Aphid

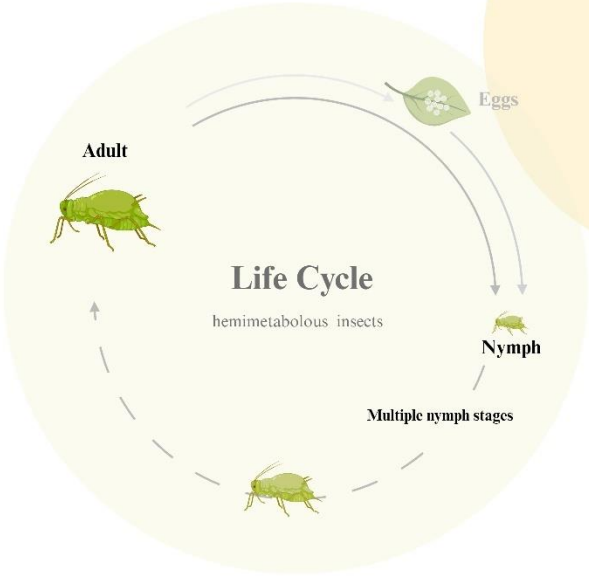
Myzus persicae

Myzus persicae (Sulzer, 1776),

- Commonly known as the Green Peach Aphid, belongs to Aphididae family.
- This aphid species is found throughout temperate regions globally.
- This species feeds on a wide variety of plants, including peach trees and vegetables.







- The life cycle is complex, with reproduction varying according to environmental conditions. Females can reproduce asexually, giving birth to nymphs.
- They are a food source for many predators, such as ladybirds.

- Aphids are equipped with specialized mouthparts, namely stylets, which facilitate the extraction of cell fluids and plant saps.
- Due to their direct feeding damage and their role as vectors of many plant viruses, these aphids are classified as pests in agricultural fields.

4

[4] For further details and references, please refer to the species description before. Created with Bio.Render.com

Chironomus riparius MEIGEN, 1804

Chironomus riparius, commonly known as the non-biting midge or the harlequin-fly, is a small semi-aquatic species of the dipteran family Chironomidae. The species is commonly found in and around freshwater ecosystems, including rivers, streams and lakes (FERRINGTON 2008).
The geographical distribution of *C. riparius* populations (Figure 10) covers North America, Europe and parts of mainly southern Asia, indicating its adaptability to a wide range of environmental conditions (FERRINGTON 2008). The species is particularly associated with sediment-rich freshwater habitats and known for its tolerance to water pollution (PINDER 1986; STIEF *et al.* 2005).

The behavior of *C. riparius* larvae includes the construction of tubes in the sediment which serve as a habitat and site to forage for food. The larvae have specialized mouthparts that allow them to filter or collect fine organic particles, and by that, they contribute to the decomposition of organic matter and to the recycling of nutrients within the aquatic system (PINDER 1986).

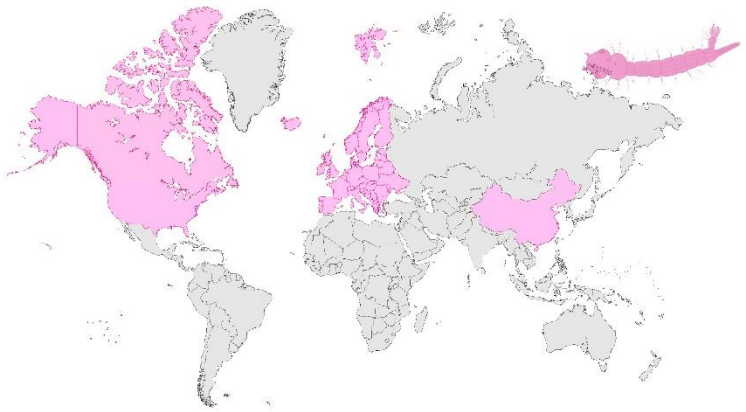


Figure 10: Distribution of *Chironomus riparius* (FERRINGTON 2008). (Created with Bio.Render.com)

The life cycle of *Chironomus riparius* comprises four distinct stages: egg, larva, pupa, and adult (Figure 11). The eggs are laid in gelatinous formations on the water surface or on aquatic vegetation (PINDER 1986). After hatching, the larvae, commonly called "bloodworms" due to their red hemoglobin coloration, burrow into the sediment, where they built tubular burrows (NATH 2018). They play a crucial role in nutrient cycling and sediment turnover webs (PÉRY *et al.* 2002; PÉRY & GARRIC 2006; BERTIN *et al.* 2014). The development of the larvae over several stages can take several weeks to months, depending on water temperature and food availability. When they are mature, the larvae pupate in their tubes and finally hatch as adult

mosquitoes, which neither bite nor feed and are only concerned with reproduction (PINDER 1986; ARMITAGE 1995).

In addition, larvae are an important food source in the ecosystem for a variety of fish and other aquatic predators. Adults are also important in terrestrial ecosystems. They are important prey for bats and some birds (PAINE 1966; CLEMENTS *et al.* 1994; FERRINGTON 2008).

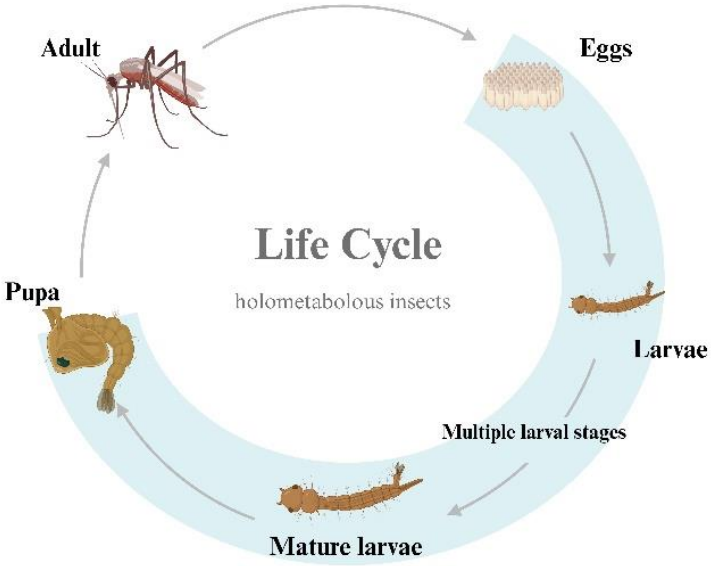


Figure 11: Life cycle of *Chironomus riparius* (PINDER 1986). Blue: aquatic stages. (Created with Bio.Render.com)

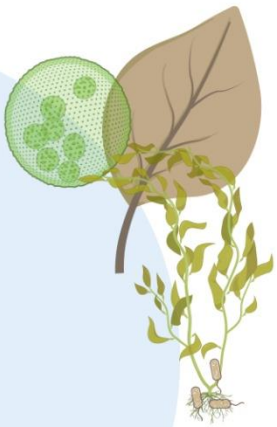
C. riparius has become a model organism in ecotoxicology due to its sensitivity to pollutants, its ease of cultivation in the laboratory and its well-understood life history. The species is therefore used in standardized bioassays to assess the toxicity of sediment and water samples (CHOI 2004; OECD 2011). The presence and abundance of *C. riparius* serves as an indicator of the condition of freshwater habitats, and its study provides valuable insights into the impact of anthropogenic activities on aquatic ecosystems (BAZZANTI & BAMBACIGNO 1987; OECD 2011; BUSCHMANN 2013).

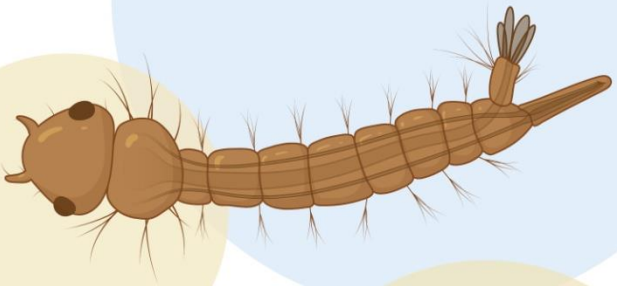
Non-Biting Midge

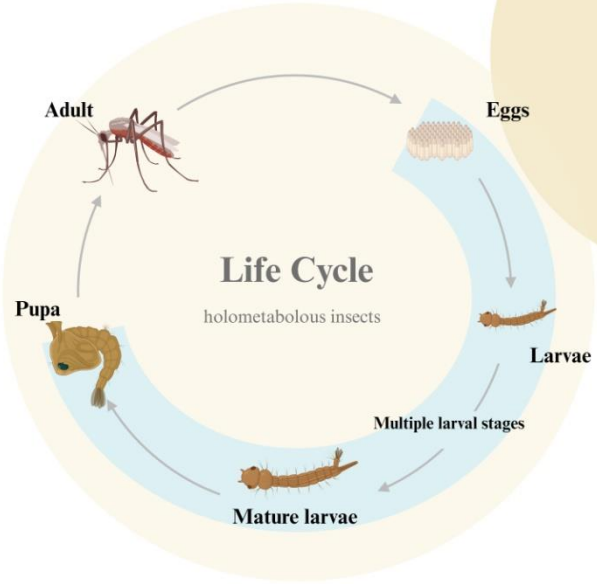
Chironomus riparius

Chironomus riparius Meigen, 1804

- Commonly known as Non-Biting Midge or Harlequin Fly, belongs to the Chironomidae family.
- This species is widespread in freshwater habitats such as rivers, streams and lakes.
- Larvae build tubes in the sediment, which they use for housing and feeding.







- The life cycle consists of four stages: egg, larva, pupa (in water) and adult.
- Both larvae and adults are a source of food for various predators, such as fish (larvae), or bats (adults) and contribute significantly to the food web.

- The presence and abundance of *C. riparius* is a biological indicator of the health of freshwater environments.

5

[5] For further details and references, please refer to the species description before. Created with Bio.Render.com

How chemical analysis helps

A vast variety of insects have been classified by man as pest organisms requiring control measures. For this, industrially developed and produced insecticides are used on a large scale, which carry the risk of more or less extensive contamination of the environment. Logically chemical analytics plays a key role in assessing the (potential) environmental impact of insecticides in use or under development, allowing to monitor their transformation and fate in organisms and ecosystems (TIRYAKI & TEMUR 2010; KOWALSKA *et al.* 2020; HOCHKIRCH *et al.* 2023). In this context quantitative analytical approaches are essential to accurately monitor the uptake of compounds and their potential metabolites, effectively translating bioassay results into accurate data. Such analysis could generate a deeper understanding of biochemical interactions and helps to identify species-specific selectivity of compounds in use. Examining and comparing the metabolic profiles reveal unique biological pathways and mechanisms of different species, allowing a more targeted approach to species-specific research.

One of the most advanced analytic techniques is used in this study, the ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) (LÓPEZ-RUIZ *et al.* 2019; KAUFMANN 2020). UHPLC-MS is excellent at detecting chemical compounds. The process starts by dissolving analytes in a mobile phase. These are then rapidly separated in a high-performance column under ultra-high pressure, a method that provides faster separation and higher resolution compared to traditional liquid chromatography.

After separation, the compounds are ionised, often by methods such as electrospray ionization, converting them into charged gaseous particles. These ions are then introduced into a mass spectrometer where they are sorted according to their mass-to-charge ratio. The mass spectrometer acts as an ultra-sensitive detector, identifying molecules by their unique masses (DONG 2019).

The precision of UHPLC-MS goes beyond simply quantifying trace amounts of insecticides in environmental samples; it also enables the observation of their putative metabolites (TOLSTIKOV 2009; JOHNSON & GONZALEZ 2012). The detection of these metabolites provides insights into the degradation pathways of insecticides to researchers, allowing a thorough assessment of their potential environmental impact and effects on living organisms.

The integration of bioassays, chemical analysis, and modeling

To fully understand the effects of insecticides, it is not sufficient to rely on bioassays alone, which often provide only snapshots of toxicity under specific conditions. Mechanistic modeling complements these experimental approaches by placing bioassay data in a broader context,

simulating and understanding the effects on single insects or populations and/ or the behavior of insecticides in different environments. The intricate relationship between chemical analysis, bioassays and modeling provides a comprehensive method for studying the effects of insecticides on organisms. Techniques such as UHPLC-MS allow precise detection and measurement of insecticides and their putative metabolites (KAUFMANN 2020). However, knowledge gained from chemical analysis and bioassays has inherent limitations due to factors such as biological variability, sample preparation methods, chromatographic and ionization techniques, or the bioavailability of the analytes (BADAWY 2018; LÓPEZ-RUIZ *et al.* 2019; KAUFMANN 2020). Despite these limitations, the combination of these methods provides a more complete contaminant profile of a sample, with each method compensating for the limitations of the other. However, it is essential that models are critically examined and evaluated to avoid misinterpretation or potential errors that can arise from inaccurate modeling assumptions (JAGER & ASHAUER 2018).

In addition, direct observation of organisms through imaging and behavioral studies provides critical biological context (GUTSELL & RUSSELL 2013). This approach can reveal a spectrum of responses, such as changes in feeding, excretion, movement, and potential symptoms of exposure, providing crucial insights into biological effects of insecticides in real-world scenarios (from *in vitro* to *in vivo*) (TIEMANN 2008; SAHU & CASCIANO 2009; BALE *et al.* 2014; FABIAN *et al.* 2019). These observations, when integrated with analytical data, greatly enhance our understanding (ASHAUER & ESCHER 2010; JAGER 2020).

Modeling plays an essential role in this integrative approach. It synthesizes data from chemical analyses and biological observations and, in this study, uses computational models to combine detected compound quantities or concentrations with their known biological effects. This helps to predict cumulative effects on organisms and ecosystems and translates specific measurements into broader environmental impacts (JAGER & ASHAUER 2018). This comprehensive approach, combining the precision of chemical analysis, biological insights of direct observation, and the predictive power of modeling, provides a nuanced understanding of environmental interactions (THOMANN 1989; ASHAUER *et al.* 2010; ASHAUER & ESCHER 2010; MADDEN *et al.* 2014; GERGS *et al.* 2015; JAGER & ASHAUER 2018).

Toxicokinetic-Toxicodynamic models

Toxicokinetic-Toxicodynamic (TKTD) models are an integral part of ecotoxicology and serve as mathematical tools to elucidate the interaction between chemical exposure and biological effects in organisms (RUBACH *et al.* 2010; NYMAN *et al.* 2014). These models are divided into two primary components: toxicokinetics (TK), which describes the uptake, distribution, metabolism and excretion of chemicals in an organism, and toxicodynamics (TD), which focuses on the

resulting toxic effects at different biological levels (ASHAUER & ESCHER 2010; JAGER *et al.* 2011; NYMAN *et al.* 2014; JAGER & ASHAUER 2018) (Figure 12).

In practice, TKTD models follow a pathway typically starting with external exposure to a chemical, followed by internal processing of the chemical (TK), interaction with biological targets (TD), and finally leading to damage or adverse effects over time (JAGER & ASHAUER 2018; PRODUCTS *et al.* 2018). These models are particularly valuable for predicting the ecological risks posed by chemicals, as they take into account the actual dynamics of exposure and the sensitivity of the organism (JAGER & ASHAUER 2018). It is essential to first dissect and understand the toxicokinetic component in order to fully exploit the capabilities of TKTD models. In this primary phase, the focus is on the behavior of chemicals, specifically what the organism does with the chemical, rather than the effects of the chemical on the organism. Understanding TK is a prerequisite for moving to a more complex, comprehensive approach where the dynamics of chemical action and biological response are integrated. Studies of TK in non-target organisms, and the corresponding experimental designs, are well established in environmental toxicology (RUBACH *et al.* 2010; NYMAN *et al.* 2014). However, studies on TK in target insect species are rare in the scientific literature. As a result, there is currently no published standardized experimental design and data analysis workflow for the toxicokinetics of chemicals in target insects.

Species-specific differences in TK between target and non-target insects are particularly important because they can help maximize the efficacy of insecticides against pests while minimizing the negative impact on the environmental, and because TK related properties can be altered by targeted chemical design (ASHAUER *et al.* 2012; NYMAN *et al.* 2014). However, it is crucial to first determine and understand the toxicokinetic characteristics of research compounds in these organisms before using them in chemistry optimisation of new insecticides. Currently this understanding is hindered by the lack of comparative TK studies with target and non-target species. Such comparisons are further complicated by the very different biology and resulting uptake and elimination routes of different insect species. To fill this knowledge gap, specific bioassays are combined with analytical approaches to measure internal concentrations of compounds and their putative biotransformation. This information is used to construct quantitative toxicokinetic models. Finally, the TK models are used to elucidate interspecific patterns and to quantitatively explain inherent differences.

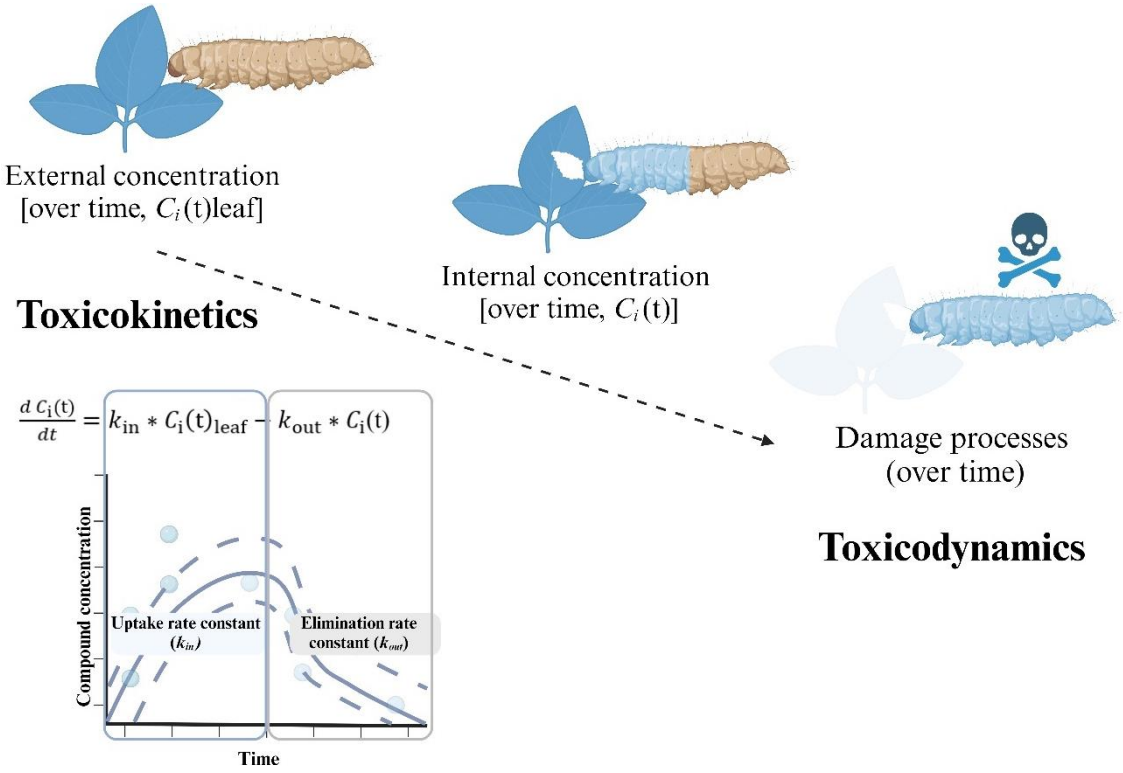
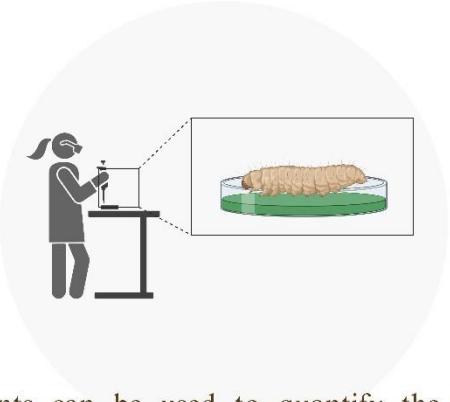
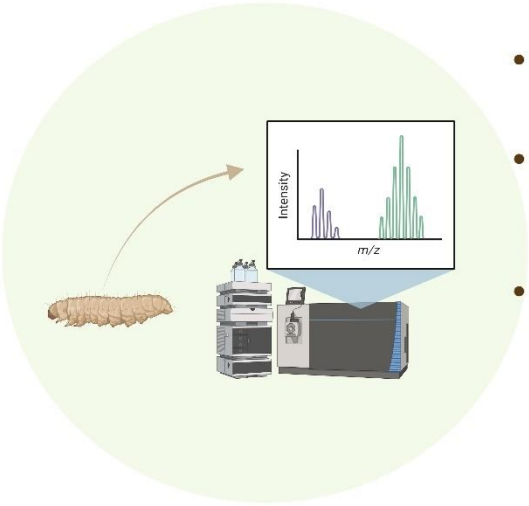


Figure 12: Toxicokinetic and Toxicodynamic approach. Time-dependent approach of toxicokinetic and toxicodynamic models from external concentration to internal concentration. This is then translated into time-dependent effects on the organism. Figure adapted from the DEBtox information site, www.debtox.info.

Information box II

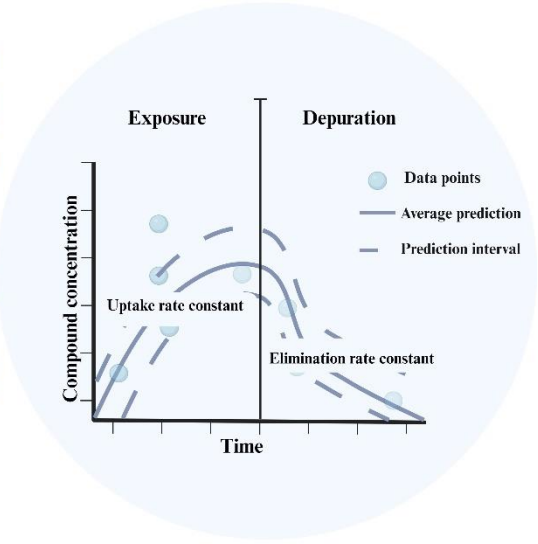
- The effects of insecticides can be observed in individual specimens, for example through changes in physiology, behaviour or mortality.
- These direct observations provide important information on the toxicity and mechanism of action of insecticides.





- Experiments can be used to quantify the effects of insecticides on organisms.
- They provide experimental data on the sensitivity of different species and can help to understand dose-response relationships.
- Chemical analysis, such as high performance liquid chromatography (HPLC), can be used to measure the concentrations of insecticides that lead to the observed effects.

- These concentrations can then be used in toxicokinetic (TK) models to simulate the distribution, metabolism and excretion of insecticides in the insect body.
- Species-specific differences in TK between target and non-target insects are particularly important as they can help maximize efficacy against pests while minimizing adverse environmental impacts.



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[6] For further details and references, please refer to the introduction sections before. Created with Bio.Render.com

Scientific context and relevance

The decline in insects is currently a frequent topic of discussion in the media. Numerous very different factors are cited as being responsible for this (JARVIS 2018; CARDOSO *et al.* 2020). Intense agriculture and progressing urbanization have led to structural fragmentation or destruction of natural habitats for insects, leaving them with fewer places to forage, nest, and mate (CARDOSO *et al.* 2020; HOCHKIRCH *et al.* 2023). As agricultural farmlands (crop fields) cover approximately 12% of the planet's total land area, human activities on it have a direct impact on a significant proportion of habitat changes. Habitat changes have recently been reported to be one of the main drivers of insect declines with approximately 49.7 % over the past 40 years (reviewed in SÁNCHEZ-BAYO & WYCKHUYS (2019) (Figures 13-14). The prevalence of large-scale monocultures in agriculture significantly reduces composition and availability of food sources for insects, resulting in a decline of their diversity and numbers (ALTIERI 2009; HOCHKIRCH *et al.* 2023). This circumstance has for example been claimed to probably be the most important driver of the global decline of birds (CHAMBERLAIN & FULLER 2000).

Further drivers, such as artificial night lighting, destroy the natural bio-rhythms of nocturnal insects, causing disorientation and disrupt species interaction, also leading to measurable changes in insect communities (OWENS & LEWIS 2018; GRUBISIC & VAN GRUNSVEN 2021; KEHOE *et al.* 2022). This light pollution is particularly harmful to such species, which navigate or communicate using natural light cues (KNOP *et al.* 2017). As insects are often highly sensitive to changes in temperature and humidity, this may additionally affect their survival and reproduction (KHALIQ *et al.* 2014; SÁNCHEZ-BAYO & WYCKHUYS 2019). On the long term, climate change effects on weather patterns also have to be of growing concern, as extreme weather events, such as droughts and floods, altering the living conditions of insects can lead to population declines (KIRITANI 2013). Nevertheless Sánchez-Bayo & Wyckhuys (2019) reported, that climate change may support declines in insects, but are not the main driver. Favored changing climate conditions, invasive species (plants and animals) can have additional negative influence on native insects. Such plants may, for example, competitively reduce autochthonous species or represent poisonous food resources, while introduced animals may either compete for, or prey on resources important for local insect populations, all in all leading to severe disruption in ecological balances (MCNEELY 2001; MOLLOT *et al.* 2017). Even worse, facilitated by global trade and travel, introduction and expansion of parasites and diseases, to which native species have no resistance, can also result in massive declines of their populations (MOLLOT *et al.* 2017; HULME 2021). In recent studies introduced devastating pathogens have been made responsible for reductions of approximately 10.7 % of insect populations, which may

not have immunological defense mechanisms against it (SÁNCHEZ-BAYO & WYCKHUYS 2019; ANDERSON-TEIXEIRA *et al.* 2021).

This multitude of causes besides chemical pollution, which are responsible for the decline in insect populations and biodiversity, illustrates that this process is a complex problem to be solved (Figures 13-14). If we want to protect and preserve insect diversity, we need a coordinated approach as well as a comprehensive basic understanding of the relevant influencing factors. To combat insect decline, it is essential that politicians and the public are informed, work together on the various causes and promote sustainable solutions to preserve insect diversity (SAMWAYS *et al.* 2020).

However, an effective overall strategy for the conservation of biodiversity as a whole (not just that of insects) also includes close monitoring of environment chemical pollution and the assessment of the effects and consequences for specific groups of organisms. Available studies on the drivers of biodiversity losses are complex and encompass a wide range of research methods and disciplines (HALLMANN *et al.* 2017; SÁNCHEZ-BAYO & WYCKHUYS 2019; HOCHKIRCH *et al.* 2023). Scientists use field and laboratory studies and experiments, as well as long-term ecological monitoring, to understand the effects of various stressors on insect populations (JAGER & ASHAUER 2018; MONTGOMERY *et al.* 2020). For example, studies on pollination have shown (sub-)lethal effects on honeybees (MÜLLER 2018; HOCHKIRCH *et al.* 2023). As part of integrated pest management (IPM), natural predators have received considerable attention in field and laboratory studies (BAYRAM *et al.* 2010; TAPPERT *et al.* 2017). Research often focuses on quantifying the extent of decline, identifying the most endangered species and understanding the mechanisms driving this trend (HALLMANN *et al.* 2017; SÁNCHEZ-BAYO & WYCKHUYS 2019; HOCHKIRCH *et al.* 2023).

However, the widespread use of pesticides on arable and pasture land over the past six decades has been shown to have a significant negative impact on a wide range of wildlife, from insects to birds and bats (MINEAU & CALLAGHAN 2018; CARDOSO *et al.* 2020; SÁNCHEZ-BAYO 2021). Quantitative analysis has shown that chemical pollution from fertilizers, pesticides and industrial chemicals is the second most important factor associated with declines in insect taxa. (Figure 13) (SÁNCHEZ-BAYO & WYCKHUYS 2019). The application of multivariate and correlative statistical methods disclosed that pesticides have a stronger impact on biodiversity than other practices (e.g. harvesting technics) associated with intensive agriculture (GIBBS *et al.* 2009; MINEAU & CALLAGHAN 2018). For example, HALLMANN *et al.* (2017) found that 80% of the decline in biomass of flying insects in Germany could not be attributed to agricultural expansion, deforestation, urban development, or climate change. But nevertheless, the authors suggest a still unidentified relationship to the use of pesticides (HALLMANN *et al.* 2017). As

residues from agricultural and urban sources accumulate in aquatic ecosystems, they have also been shown to be one of the main critical factors in the loss of biodiversity in various taxonomic groups (MORA *et al.* 2011; BEKETOV *et al.* 2013; WESTON *et al.* 2014).

But a considerable amount of the research results available to date has been devoted to investigating the effects of "old" insecticides, many of which have already been withdrawn from the market under current regulations. An overview of the sublethal effects of insecticides can be found, for example, in MÜLLER (2018). In contrast, investigations on effects of novel insecticidal agents on insects still appear to be comparatively sparse. In addition, there are significant gaps in our basic understanding of how invertebrates, including insects, take up and process such insecticides. Only very few studies available investigate whether there are differences in the uptake and metabolism of insecticides between target and non-target species at all (e.g., in RUBACH, ASHAUER *et al.* 2010, TALEVI 2022).

Few studies on metabolism have addressed special/specific parts of the ADME processes associated with insecticides, focusing on pathways for detoxification and metabolism in insects (TALEVI 2022), particularly for example highlighting the function of P450 enzymes (LE GOFF *et al.* 2006; GUENGERICH 2008). Furthermore, cutting-edge omics technologies and high-throughput screening methods have shed some light on the molecular base of insecticide action, highlighting the importance of evaluating both laboratory and field-collected samples (LALOUETTE *et al.* 2016).

Industry-led research, on the other hand, focuses on possibilities to control classified target species (pest insects). This includes screening methods to discover new possible insecticides or efficacy studies to develop strategies that accurately control pest populations (ALLENZA & ELDRIDGE 2007; BUCHHOLZ *et al.* 2015; DENT & BINKS 2020). In contrast, risk assessment methods focus primarily on non-target organisms to estimate the extent of the impact of potential new (or existing) insecticides (OECD-Guidelines).

Overall, although there are different perspectives on the effects of active ingredients depending on the focus of the work, both academic and industrial research teams have a common goal, which is to fully understand the effects of insecticides on the organisms exposed to them. Another common goal should be to develop strategies that improve the selectivity of active compounds used in different species in such a way that their impact on the environment is minimized.

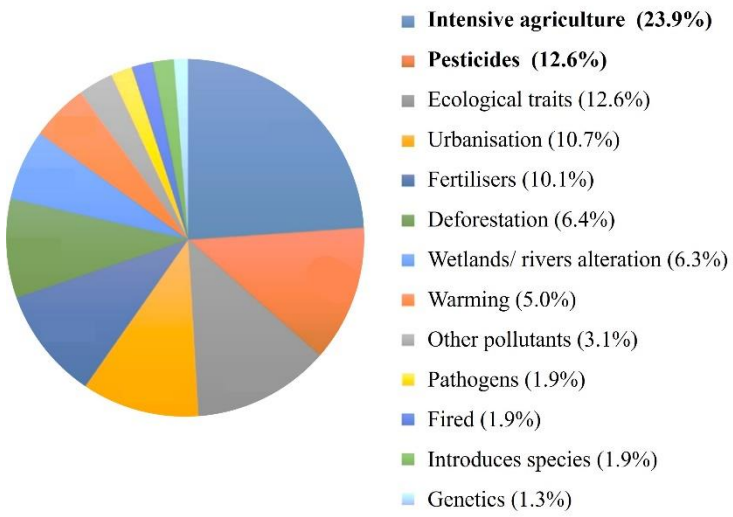


Figure 13: Main factors associated with declines in insect population. (With friendly permission taken from: © SÁNCHEZ- BAYO & WYCKHUYS 2019).

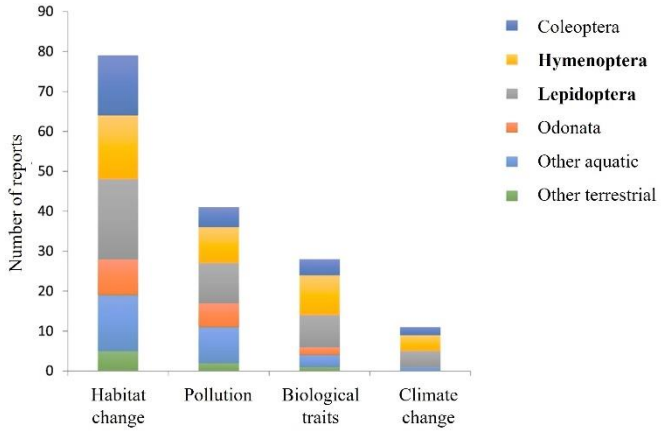


Figure 14: Top four factors responsible for decline of selected taxonomic groups of insects.

(With friendly permission taken from: © SÁNCHEZ- BAYO & WYCKHUYS 2019).

Aim of doctoral thesis

Central intention of my thesis is to at least partially fill some parts of the knowledge gap addressed in detail above including that it is unknown

- if bioassays for non target-species can be used for target species.
- how pathways to species-specific effects can be detect.
- what is the fate and kinetic of inactive compounds (structural mofits).

Systematic laboratory studies will shed light on physiological interactions of different insect species with selected chemical compounds, combining the perspectives of bioaccumulation assessment for non-target species and pest management. My research takes a novel approach by transferring and adapting bioaccumulation assessment methods originally developed for non-target insects to the pest or target species. This approach aims to highlight potential similarities and differences in the impact of compounds on both groups.
The species selected for this exemplary study are thus representing the two distinct non-systematical groups of insects, the 'target' and 'non-target' species (OECD 2011; HEALTH 2015; SINGH *et al.* 2021). It has to be kept in mind, that the classification of a species as pest is not static, as it can vary depending on geographical and other contextual factors (BLACKMAN & EASTOP 2008; UDDIN *et al.* 2012; MÜLLER & MÜLLER 2016). Three insect species from different taxonomic groups were used, allowing the agricultural perspective of plant protection to be taken into account, which determines whether these insects are classified as target or non-target organisms: the cotton leafworm (here as a biting pest), the green peach aphid (here as a sucking pest) and the aquatic non-biting midge of the harlequin fly (here as a non-target organism).

In a stepwise approach, ranging from the study of individual species to the comparison of different pest species, the species-specific responses and vulnerabilities to the effects of exposure to the test chemicals were be investigated, culminating in a comprehensive analysis contrasting the responses of these species (Figure 15).

There were a number of technical challenges to overcome along the way, including **I)** the development of a framework for species comparisons (including bioassays, chemical analytics, and modeling approaches), **II)** the evaluation of species selection as study organisms, and **III)** the possibility of applying existing non-target risk assessment methods to targets.

Therefore, the following questions were intended to be answered:

a) Does the amount of the initial compound taken up into the insect body differ from the amount of the excreted products? (mass balance)

b) Does the measurable total amount of a compound ingested per individual depend on the species tested? (individual bioaccumulation)

c) Are there differences in the toxicokinetics of the various tested starting compounds **I.)** within a single insect species and **II.)** between different insect species? (intra- and interspecific toxicokinetics)

d) Does the biotransformation of the test compounds differ in the animal species tested? (species-specific biotransformation)

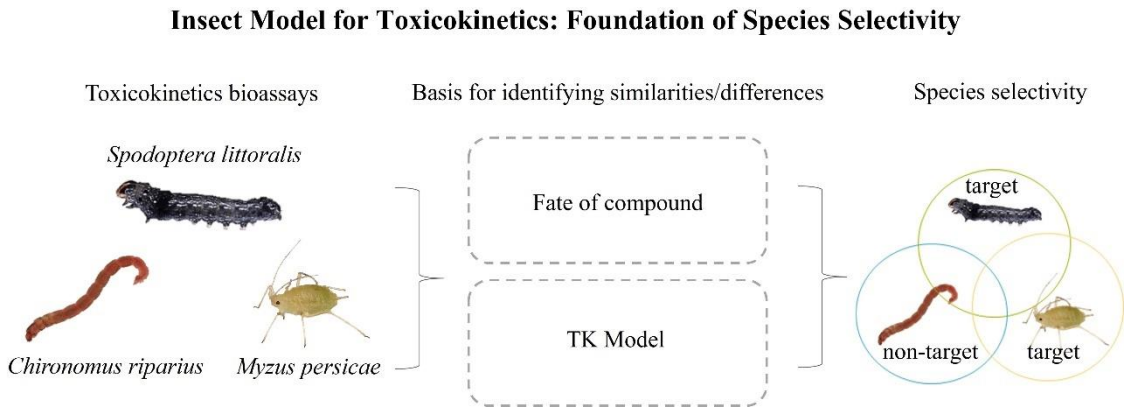


Figure 15: Insect model for toxicokinetics study: foundation of species selectivity.

Material and Methods

The experiments in this study involved a time-staggered measurement of the amounts of test compounds ingested in the body of three selected insect species, their putative metabolites and their excretion over the period of exposure (uptake) and excretion (depuration) period. During the exposure period, terrestrial insects were exposed to test compounds through the food source provided (either leaf disks or artificial diet), aquatic insects through the water they were kept in. After exposure, all tested insects were transferred to untreated food sources or water for a subsequent depuration period. Over the whole time of the running experiments samples of all insect test groups including the control, the exposed source (food or water), and if possible, of the insects excretion products were frequently taken. This was followed by solvent extraction from the samples and analytical chemistry of the extracts to quantify the parent compound quantities and their putative metabolites therein. A TK model was then created using the data obtained (Figure 16).

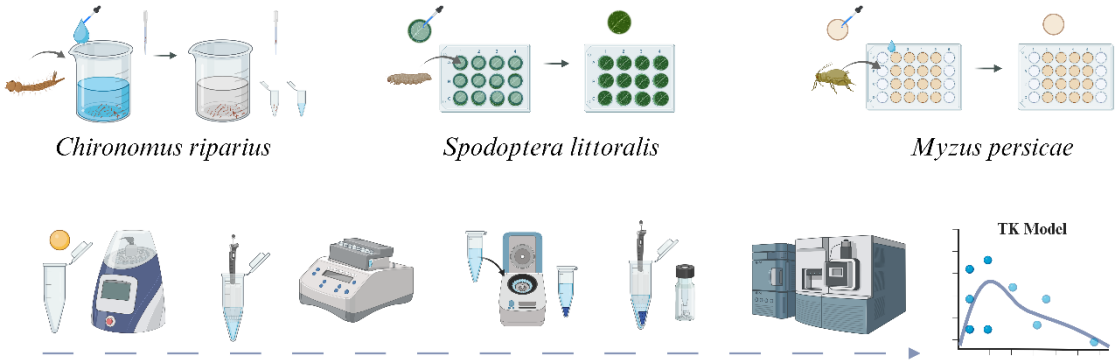
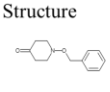
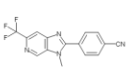
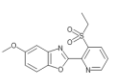
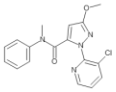
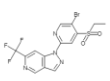


Figure 16: Design of toxicokinetic assay. Individuals were exposed to compound treated water, leaf disks or diet during exposure period, followed by a depuration period. Sample preparation. First samples were macerated, followed by extraction and centrifugation, finally clear supernatant was used for residue measurements by Ultra-High Performance Liquid Chromatography-Mass Spectrometry. (Figure created with BioRender.com)

Test compounds

Seven representative insecticidally inactive test compounds were synthesized in-house (≥ 95% purity). They, to some degree, incorporate structurally scaffolds or fragments of insecticide-likeness. Additionally, Coumarin [CAS 91-64-5, (≥ 95% purity, Merck KGaA, Darmstadt, Deutschland) Table 3] was used as a reference for a commercially available compound. They ranged in hydrophobicity (Log P 1.43 to 4.7) molecular mass (146 to 433 g/mol, Table 1 & 3). Octanol Log P was measured using an in-house chromatographic method. Compounds were dissolved at **(1)** 2000 mg/L in water, **(2)** at 100 mg/L artificial diet, **(3)** at 100 mg/L in water containing 15 % acetonitrile (ACN) (gradient grade for analytics 99.9 %). These high rates of 0.1 mg compound per leaf disk, diet or water was chosen to ensure good analytical detection of compounds in all biological matrices (insects and excreta) and should also increase the possibility of detecting putative metabolites.

Table 1: Compound properties (chemical structure, Log P, molecular weight, and water solubility) of tested compounds.

Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Coumarin
Structure							
Log P							
1.58	1.50	2.24	3.57	1.84	2.03	4.70	1.43
Molecular mass (g/mol)							
205.25	262.62	302.25	380.68	318.35	342.78	435.22	146.14
Water solibility (mg/L)							
1413	589	10	6.46	120	166	0.26	1050

Toxicokinetic bioassays

Spodoptera littoralis bioaccumulation assay

Food plant preparation

Soybean plants, *Glycine max* (L.) MERR (1997) cv. Toliman, were used in the *S. littoralis* feeding-contact assay. Four soybean seeds were germinated and grown per pot (Ø size 6.5 cm) filled with white peat growth medium. Plants were grown in a greenhouse under controlled conditions [14-hours light (27 Lux., 22 °C) and 8-hours dark (18 °C) cycle, 65 ± 5 % relative

humidity (RH)] and used in the assay after 14 days of germination. Two leaf disks (Ø size 20 mm) were cut from two fully developed true leaves and stored on wet filter paper to minimize desiccation.

Experimental design

Egyptian cotton leafworms (*Spodoptera littoralis*) were reared in the laboratory under standardized conditions (23 ± 1 °C, 55 ± 10 % RH), including an in-house artificial diet for both adults and larvae. This laboratory strain had not previously been exposed to insecticides.

The larvae stages were synchronized by transferring the derived second larval stage (L2) from artificial diet to an empty Petri dish (Ø 12 cm). The dish contained only dry filter paper and was covered with a cotton filter. Within two hours, the larvae moulted into the early third larval stage (L3).

The assay combined an exposure phase of 24 hours and a follow-up depuration phase of 24 hours under standardized conditions (25 ± 1 °C, 55 ± 10 % RH, 16-hour light/8-hour dark cycle, Figure 17 & 21). *S. littoralis* larvae were exposed to treated leaf disks, reflecting a preventative bioassay with oral and contact uptake of test compounds.

Before exposure, 50 µL of test solutions, containing 0.1 mg of test compounds, were evenly distributed on the leaf disks (Ø 20 mm) by shaking for 20 seconds at 300 rpm using a pipetting robot (Fluent® Automation Workstation, Tecan Group Ltd, Männedorf, Switzerland). For the control group, a 15 % ACN-water solution was prepared. After 30 min of evaporation of the test solution, the leaf disks were placed in a 12-well microtiter plate lined with moist filter paper to retain moisture (12-MTP, FalconTM, Northfield, Minnesota, USA).

A single freshly moulted *S. littoralis* larva (L3) was placed on the leaf disk in each individual microtiter plate well and then covered with a transparent film with evaporation pores. The larvae were exposed to treated leaf disks for 24 hours and then transferred to a microtiter plate containing untreated leaf disks where they were allowed to degrade (excrete) the test compounds for the follow-up 24-hours (Figure 16).

Sampling

S. littoralis larvae samples were collected during the exposure period (T 0-24 hours) and the depuration period (T 24-48 hours). As a reference, leaf samples were collected from a parallel assay without larvae, and therefore no real mass balance can be established. At the end of the exposure period, the larvae always completely consumed the leaf disks used in the bioassays. The result of the leaf disk concentration comes from two parallel bioassays. Sampling time points were chosen to cover early time points and end of both periods (exposure and depuration)

in accordance to working hours. Samples were taken after 0, 1, 5, 24, 25, 29, 48 hours (*S. littoralis* sample: one larva per time point (experimental replicates: n=12); soybean samples: 3 g (n=133), leaf disks pooled per time point (n=3). Feces samples were taken at the end of the exposure (after 24 hours) and of the depuration phase (after 48 hours). All samples were transferred to 2.5 mL tubes (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop metabolism (Figure 16).

Larval performance parameters

Behavior and performance parameters (larval size, food consumption, excretion, and movement) of the larvae were recorded during the exposure period by taking images in 1-hour-intervals (Figure 17). Larval size (measured in mm along the longitudinal body axis), their food consumption (as percentage of leaf area), conversion of food to feces (indicated by the number of feces pellets) and movement (by frame to frame comparison) were recorded visually using the method described in literature (SADEGHI-TEHRAN *et al.* 2017; SADEGHI-TEHRAN *et al.* 2019; WOLZ *et al.* 2021).

Myzus persicae bioaccumulation assay

In contrast to the larval toxicokinetic assay, an artificial diet was prepared for the aphid (sachet) assay to provide different test compounds in an imbibable manner and at comparable exposure quantities. A leaf disk assay would carry the risk of compound specific foliar penetration rates and therefore unknown exposure quantities at the aphid feeding sites, the plant vascular tissues.

Diet preparation

The aqueous artificial diet solution prepared according to FEBVAY *et al.* (1988) contained sucrose, minerals and amino acids. For the exposure period, 1 mL of this artificial diet spiked with 0.1 mg of the solubilized test compounds was added to 12 wells of a 24-well microtiter plate (MT-plate, FalconTM, Northfield, Minnesota, USA). For the control groups, an equivalent amount of solvent was added to their artificial diet. The MT-plates were then covered with a layer of stretched Parafilm™ and a perforated plate that prevented aphids from moving between the separate wells (Figure 18-19). The identical MT-plates used in the extinction phase were prepared in the same way, but no test compounds were added to the artificial food.

Experimental design

The green peach aphids (*Myzus persicae*) used in the experiments originate from an asexual and wingless laboratory strain previously not exposed to any insecticides before. Aphids were reared in a mixed age population on pea seedlings under standardized conditions (20 ± 1 °C, 60 ± 10 %

RH, 16-hour light/8-hour dark cycle). The tips of the infested pea seedlings were cut off and placed in a Petri dish with a dry filter paper, which was covered with a cotton filter (Ø size 14.5 cm). Aphids of all life stages were allowed to migrate independently from the drying pea seedling onto the filter paper within two hours. Only the aphids selected as vital using this method were used for the experiments. Aphid populations of 15-30 individuals were transferred to the individual wells of the MT plates (Figures 18b & 19). The aphids immediately began to suck diet solution through the Parafilm™ membrane. The MT plate containing the test animals was then covered with cardboard and turned upside down. After a waiting time of 15 minutes, the cardboard and all non-feeding aphids were removed. An empty MT plate was placed under the infested MT plate to collect the honeydew. The aphids were exposed to treated diet for 72 hours and then transferred to a microtiter plate containing untreated diet where they were allowed to degrade (excrete) the test compounds for the follow-up 72-hours (Figure 16). As the experimental groups contained aphids of mixed ages, adult females were able to produce nymphs so that the number of individuals per well could increase over the course of the experiment.

Sampling

As the number of aphids therefore fluctuated from well to well and over time, the MT plates were photographed before insect sampling to record the number of individuals by counting. Sampling time points were chosen to cover three time points of both periods (exposure and depuration). Aphid (experimental replicates: n= 8) and food samples were collected at 0, 24, 48, 72 (end of exposure), 75, 80 and 144 hours (end of depuration). Honeydew was collected at the end of the exposure (after 72 hours) and excretion phase (after 144 hours) by dissolving it from the plate with 1000 µL ACN. Here, samples from all replicates (8 wells) per treatment were pooled. All sample types were transferred to 2.5 mL tubes (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop any metabolism.

Chironomus riparius bioaccumulation assay

Harlequin fly larvae (*Chironomus riparius*) were reared in the laboratory under standardized conditions (20 ± 1 °C, 60 ± 10 % relative humidity (RH), 16-hour light/8-hour dark cycle) and the larval stages served with powder of fish food (10 mg, TetraMin, Tetra GmbH, Melle, Germany) according to OECD (2011). Adults were fed with a honey-water solution. Larvae derived from a laboratory strain (provided by IES Switzerland) previously not been exposed to insecticides.

Larvae were selected by transferring egg clusters to water beakers (100 mL), where the larvae hatched within four days. Their first larval instar (3 days) was used for the bioassay.

Experimental design

The toxicokinetic bioassay was adapted from the acute toxicity test recommended in the OECD guidelines (OECD 2011). The experiments were performed under standardized conditions (20 ± 1 °C, 55 ± 10 % RH, 16-hours light/8-hours dark cycle). The larvae of *C. riparius* were exposed to the test compounds for 24-hours in 100 mL glass beakers, each containing 0.1 mg of the test compound. The test solution was evenly distributed by manual stirring for 20 seconds. In addition, 10 mg of food was added to each beaker. The larvae were exposed to the test compounds in the treated water for a period of 24 hours. Afterwards they were transferred to clean compound-free beakers containing the same amount of food (10 mg) for the subsequent 48-hours depuration (detoxification) phase (Figure 16).

Sampling

Larval samples (experimental replicates: n=3), each containing 3 individuals, and water samples (500 µL) were collected during the exposure and depuration periods. They were collected with a single use pipette and rinsed in clean tab water for one minute. Sampling time points were chosen to cover early and as many as possible time points and end of both periods (exposure and depuration) in accordance to working hours. Samples were collected at 0, 2, 4, 6, 24, 26, 28, 30, 48, 50, 52, 54, 56 and 72 hours. Each sample was separately transferred to 2.5 mL tubes (n= 3 per sampling time), MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop metabolism.

Analytical workflow

Sample preparation

The same chemical analytical method was applied to all biological matrices to quantify compound concentrations, but extraction differed for the exposure matrix (leaf, diet, or water), insect bodies and excretion products. As all samples were processed as a total mass of the given biological matrix, it therefore is not possible to distinguish whether the detected compound(s) were absorbed internally or fixed on the surface (Figure 18).

For each insect, the total body wet weight (w.wt) was measured after thawing using a Sartorius-balance (BCE124I-1S Entris® II, Data Weighing Systems, Inc., Wood Dale, IL, USA). Leaf, insect and feces samples were each homogenized using a macerator (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) with a ceramic ball (Ø size 6.35 mm, MP Biomedicals™ zirconium oxide-coated beads, Lucerne Chem AG, Lucerne, Switzerland). Next, 500 µL ACN were added to each sample, before shaking for 3-hours at 300

rpm and 20 °C using an Eppendorf ThermoMixer® (Merck & Cie, Schaffhausen, Switzerland). After shaking, samples were centrifuged at 9000 rpm for 2 min. Preparations of leaf samples involved addition of 30 mL ACN and a cleaning buffer step before centrifugation (buffer I 8g mixture: 450 g magnesium sulfate, 115 g sodium acetate). After centrifugation leaf, diet, water, honeydew and feces samples were filtered through a 0.20 µm pore size filter (CHROMAFIL®Xtra PET-20/13, Macherey-Nagel GmbH & Co.KG, Düren, Germany).

Supernatants (150 µL) of each sample were transferred to analytical glass vials, with a 200 µL glass insert (Vials N11, with 0.2 mL insert, Macherey-Nagel GmBH & CO.KG, Düren, Germany).

Chemical analysis

Ultra High-Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) was performed using ACN as solvent. Spectra for parent compounds and their putative metabolites were recorded from all samples on a Mass Spectrometer Xevo TQ-XS Triple Quadrupole Mass Spectrometer) from Waters Corporation (Milford, USA) equipped with an Electrospray Ionization Source (ESI) (Figure 16).

Parent compounds and metabolites in samples were chromatographically separated on an Acquity Iclass Plus system with an Acquity UPLC High Strength Silica (HSS) column (T3, 2.1 x 30 mm, particle size 1.8 µm) using a water solution (A) (90 % water, 10 % methanol, and 0.1 % formic acid) and (B) ACN (0.1 % formic acid). Samples were measured in gradient elution mode with fluctuating flow rates. The gradient flow consisted of following steps: initial flow rate of 1.0 mL/min of 80 % A/20 % B until 0.10 min, then from 0.10 min to 0.20 min to 25 % B, followed by a flow rate change to 0.750 mL/min till 1.20 min with 30 % B, from 1.20 min to 1.45 min to 100 % B, then until 1.45 min to 20 % eluent B, and finally, from end of the run at 2 min with a flow rate of 0.050 mL/min and 50 % B in an isocratic mode. Column temperature was maintained at 60 ± 5 °C, and sample injection volume was 2 µL. For MS detection conditions, the desolvation Gas Flow was set at 1000 L/h at temperature of 500 °C. The flow rate of the cone gas was set at 150 l/h, with capillary voltage of 3 kV, source temperature of 150 °C, and cone voltage ranging from 15 to 60 V. Detection of parent compounds was performed by single ion recording (SIR) in a Mass Range of 120 to 1000 Da. The parent compound was quantified using a calibration series.

Comparison of compound quantities in pest organisms

a) Quantities of compound in insect bodies as a function time

Total compound quantities were measured for individual replicates. They were expressed either as quantity per compound for one individual *S. littoralis* larvae or for one 'aphid equivalent' each. The data of the measurement results from the insect samples in the *M. persicae* aphid assay had to be standardized by dividing the measured quantity of compounds by the counted number of aphids at the time of sampling.

$$\text{aphid equivalent} = \frac{\text{mg}_{\text{compound}}}{\text{n}_{\text{aphids per sample}}} \quad (1)$$

This was relevant because the individual experimental approaches (wells) in all cases contained a variable number of individuals due to the aphid´s prolific reproduction. However, this approach does not take into account the differences in the timing of their reproduction and the age and size of the individual aphids at the time of sampling. The resulting data on the quantities of compounds in the insects will be used to compare their distribution patterns over time for each species investigated.

b) Comparison of compound quantity in insect or excretion

At the end of the exposure and depuration (excretion) period, the quantities of the parent compound measured in the insect body and in the excretion products (feces or honeydew) were compared. Feces samples were collected and measured for each experimental replicate (*S. littoralis* larva) and each time-point. The data from the honeydew samples represent the total quantity of parent compounds in the total amount of this excretion product from each treatment group at the end of the exposure and depuration period. These data were correlated with the number of aphids recorded in all twelve replicates at the time of sampling. In this way, similar comparability is achieved as for the quantities of compound in aphid equivalents.

c) Calculation of diet uptake to body mass ratio

To better understand the relationship between the quantities of compounds in the insects and the ingestion rate of the food, the amount of ingested food m_{food} (leaf disk or artificial food) was calculated in relation to the body mass m_{insect} of the insects. This should provide information on whether one insect species or experimental group in the experiment had a higher food uptake in relation to its own body mass than another. This ratio is referred to as ‘diet uptake to body mass ratio’.

$$\text{diet uptake to body mass ratio}_{\text{ end of exposure}} = \frac{\text{m}_{\text{food}}}{\text{m}_{\text{insect}}} \quad (1)$$

For *S. littoralis* it was determined at the end of the exposure period (24 h) when the larvae had consumed the entire leaf disk (average weight Ø 23 mg). For the calculation the average weight of the *S. littoralis* larvae (Ø 17.14 mg) at 24 hours was used.

It was not possible to determine the uptake rate of *Myzus persicae* in my experiment. Therefore, the ingestion rate of 0.022 µL h^{-1} reported by RHODES *et al.* (1996) for pea aphids (*Acyrthosiphon pisum* HARRIS, 1776) feeding on an artificial diet was used as an approximation. The ratio of diet uptake to body mass were calculated by dividing the volume thus postulated for the uptake after 72 hours (1.58 µL) by the average wet weight of an aphid equivalent (Ø 0.42 mg).

Toxicokinetic model

As part of the study, two consecutive toxicokinetic models were created. The first basic **TK models (1)** were used to apply the model to only one pest species (*Spodoptera littoralis)* to determined uptake and elimination rates by calibrating a one-compartment first-order toxicokinetic (TK) model to the measured concentration in the insect (internal concentration data) and exposure source (leaf disk, external concentration data).

The subsequently developed "second level" **TK model (2)** was used to simulate the uptake, biotransformation, and elimination of test compounds in an organism for a pest species (*Spodoptera littoralis)* and one non target species (*Chironomus riparius*), taking into account the growth of the organism over time to map growth dilution in the form of a series of ordinary differential equations (ODEs). The model is calibrated separately for each species-compound pairing. The growth of the organism is modeled as a function of the current size of the organism (DE GRAAF & PREIN 2005).

TK model 1 (basic)

The TK models were built in MATLAB (version R2021a) using the Build Your Own Model[7] (BYOM, version 60_beta5) platform. Uptake and elimination rates were determined by calibrating a one-compartment first-order toxicokinetic (TK) model to the measured concentration in the larvae and leaf disks (internal concentration data).

The toxicokinetic model can be represented mathematically as follows:

$$\frac{d\,C_i(t)}{dt} = k_{in} * C_e(t) - k_e * C_i(t) \qquad (2)$$

[7] (https://www.debtox.info/byom.html)

where $C_i(t)$ represents the internal concentration of the initial parent compound i [$mg_{compound}/mg_{wet\_weight\ (w.wt)}$] in and on the organism (whole body residue), t represent is time (h), $C_e(t)$ the external concentration (mg_i/mg_{leaf}) in and on the leaf disk, k_{in} [$mg_{leaf}/(mg_{w.wt}$ *h)] the uptake rate constant and k_e (1/h) the elimination rate constant.

Equation 2 was applied separately for each compound, yielding compound-specific uptake and elimination rate constants. Ordinary differential equations (ODEs) were implemented using maximum likelihood estimation with a normal likelihood function for model calibration by minimizing the likelihood difference between measured and modelled internal body concentrations Confidence intervals were calculated for the uptake and elimination rate constants with likelihood profiling, with the BYOM platform setting an upper limit of 100, which can be equated to infinity (Table 2, Figure S4).

TK Model 2 (second level)

TK models using Build Your Own Model (BYOM, version 64) were built in MATLAB (version R2021a) (Table 2). The TK model simulates the uptake, biotransformation, and elimination of test compounds within an organism as a series of ordinary differential equations (ODEs) as well as the growth of the organism over time to account for growth dilution.

$$\frac{dW_w}{dt} = b(W_m{}^{\frac{1}{3}}W_{wt}{}^{\frac{2}{3}} - W_{wt}) \quad (4)$$

Where W_{wt} is the wet weight ($mg_{w.wt}$), W_m is the maximum mass if the organism ($mg_{w.wt}$) and b is the growth rate constant (1/h). The change in internal concentration (whole body residue), adjusted for growth dilution, is calculated as follows:

$$\frac{dC_{in}}{dt} = k_{in}C_e - k_eC_{in} - \sum_{i=1:n} k_{m_{f,i}}C_{in} - C_{in}W_{wt}\frac{dW_{wt}}{dt} \quad (5)$$

where C_{in} is the internal concentration ($mg/mg_{w.wt}$), k_{in} the uptake rate constant (1/h), for *S. littoralis* and *C. riparius*, respectively, C_e the external concentration ($mg/mg_{w.wt}$ or mg/l, for *S. littoralis* and *C. riparius*, respectively), k_e the elimination rate constant (1/h) and $k_{m_{f,i}}$ the formation rate constant of metabolite *i* (1/h). The metabolite concentration Met_i ($mg/mg_{w.wt}$) of metabolite *i*, adjusted for growth dilution, is calculated as follows:

$$\frac{dMet_i}{dt} = k_{m_{f,i}}C_{in} - k_{m_{e,i}}Met_f - Met_f\frac{1}{W_{wt}}\frac{dW_{wt}}{dt} \quad (6)$$

where $k_{m\_e,i}$ is the elimination rate constant of metabolite *i* (1/h). Equations (3-6) are integrated numerically (Table S2).

Model calibration

The TK model was calibrated using the parameter space explorer algorithm developed by JAGER (2021), which also generates parameter confidence intervals and stores a parameter sample. Resampling then facilitates creation of prediction intervals around simulated state-variables (e.g. internal concentrations) taking parameter covariance into account. For each combination of species and test compound, all model parameters were jointly optimized by minimizing the minus log-likelihood. If the elimination rate constant (k_e) could not be well identified (i.e., if the confidence interval included parameter boundaries) it was fixed. To fix k_e, the time at which 95% of steady state is reached ($t_{95\%}$) was used to calculate the parameter as follows:

$$k_e = -\frac{\ln 0.05}{t_{95\%}} \quad (7)$$

In case that 95% of steady state was reached at the first sampling time, the $t_{95\%}$ was estimated directly from the data (e.g., $t_{95\%}$ = 1 hour).

Bioaccumulation factor

The bioaccumulation factor (BAF) is the ratio of the concentration of the test compound within the organism compared to the concentration in the external source at steady state, which means the concentration in *Spodoptera* larvae compared to the concentration in the treated soybean leaf disk or the concentration in *Chironomus* larvae compared to the concentration in water.

BAF in TK models 1

The BAF can be calculated as the ratio between the uptake rate constant (k_{in}) and elimination rate constant (k_e):

$$BAF = \frac{k_{in}}{k_e} \quad (8)$$

The BAF value can indicate whether the concentration of the test compound in the organism is higher compared to the concentration in the external source.

BAF in TK models 2

The BAF can be calculated as the ratio between the uptake rate constant (k_{in}) and the sum of elimination rate constant (k_e) and all metabolite formation rate constants ($k_{m_{f,i}}$):

$$BAF = \frac{k_{in}}{k_e + \sum k_{m_{f,i}}} \quad (9)$$

In order to calculate the confidence intervals of the two BAF taken into account the parameter covariation, the model was run with a constant concentration (set as 1) up to steady state. The resulting confidence interval of the internal concentration corresponds to the confidence interval of the BAF (ASHAUER *et al.* 2010).

Experimental overview

Fate of synthetic chemicals and toxicokinetic model in *Spodoptera littoralis*

In the first experiment, a reduced number of test compounds were used to describe the fate of compounds in *Spodoptera littoralis* and to test whether the data obtained in the experiment could be used to apply a TK model on pest insects. Therefore, selected performance parameters of the larval were recorded during and at the end of the experimental exposure period, the quantities of compound in the leaf, insect and feces were investigated and basic TK models (1) were developed.

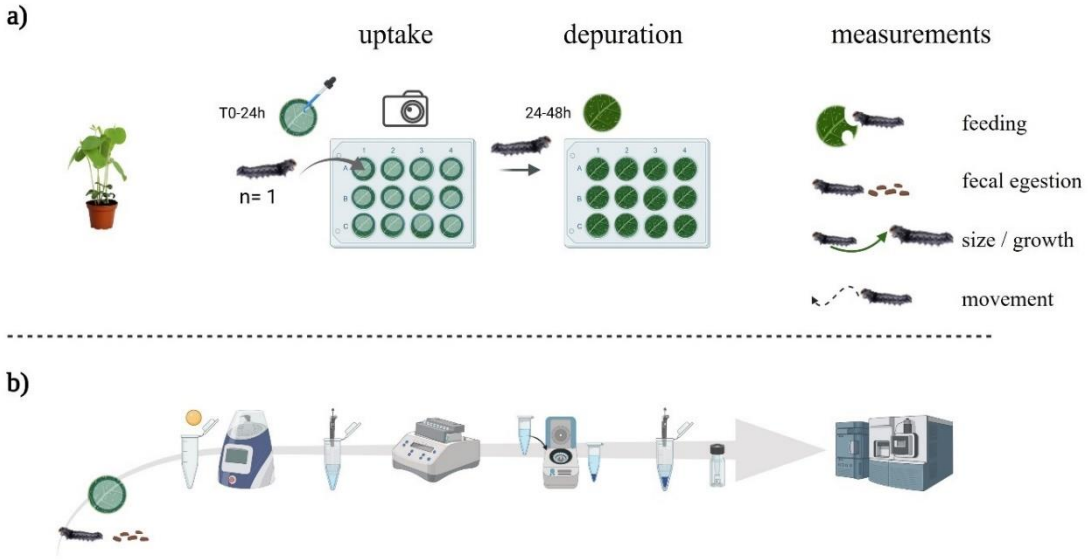


Figure 17: Toxicokinetic assay design: **(a)** Individual *Spodoptera littoralis* larvae were exposed to compound treated leaf disks. Feeding contact assay with a 24-hours exposure period (including imaging) followed by a 24-hours depuration period. **(b)** Schematic sample preparation. Biological samples were macerated. After extraction and centrifugation, the clear supernatant was used for residue measurements by Ultra-High Performance Liquid Chromatography-Mass Spectrometry. (Created with BioRender.com)

Comparison of absorption and excretion of test compounds in sucking versus chewing pests

In the following experiment, the test compounds A-G were used to investigate how such diverse compounds are absorbed and excreted by the two pest species (*Spodoptera littoralis* and *Myzus persicae*). The aim is to describe and compare the chemical fate of the test compounds in the course of absorption, metabolism (biotransformation) and excretion. The aim is to determine whether *Myzus persicae* is still suitable as a reference organism in TK models.

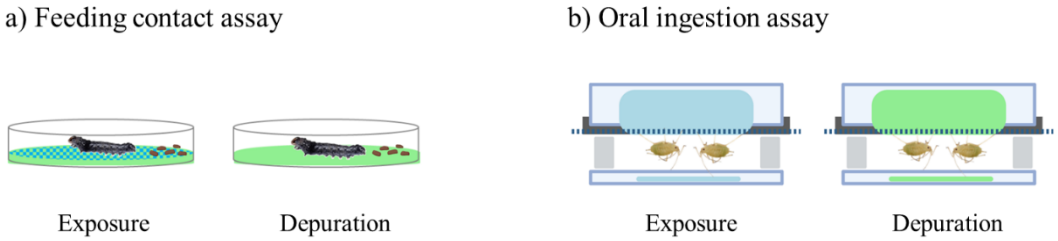


Figure 18: Structure of experimental assays. **a)** Schematic overview of the *Spodoptera littoralis* feeding contact assay in a 12-well microtiter plate with either compound-treated or untreated leaf disk. **b)** Schematic overview of the *Myzus persicae* oral ingestion assay in a 24-well microtiter plate, with either compound-treated or untreated diet. Blue = exposure preventative pipetted on leaf disk or in artificial diet, green = no treatment with compound. (Created with BioRender.com)

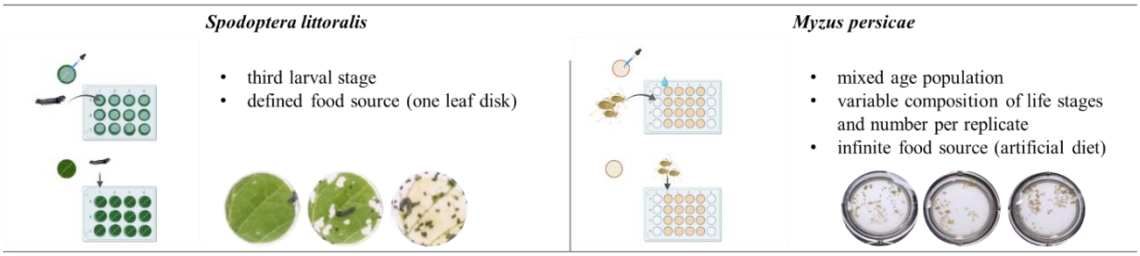


Figure 19: Bioassay conditions for *Spodoptera littoralis* and *Myzus persicae* highlighting the key differences. (Created with BioRender.com)

Comparative toxicokinetics of insecticide scaffolds and their putative biotransformation in target and non-target species

All compounds were investigated in a comparative toxicokinetics approach in caterpillars of *Spodoptera littoralis* and larvae of *Chironomus riparius*. The overall aim of these experiments was to elucidate differences between species in terms of toxicokinetics and biotransformation, is achieved by applying a uniform toxicokinetic modeling framework to all combinations of species and compounds.

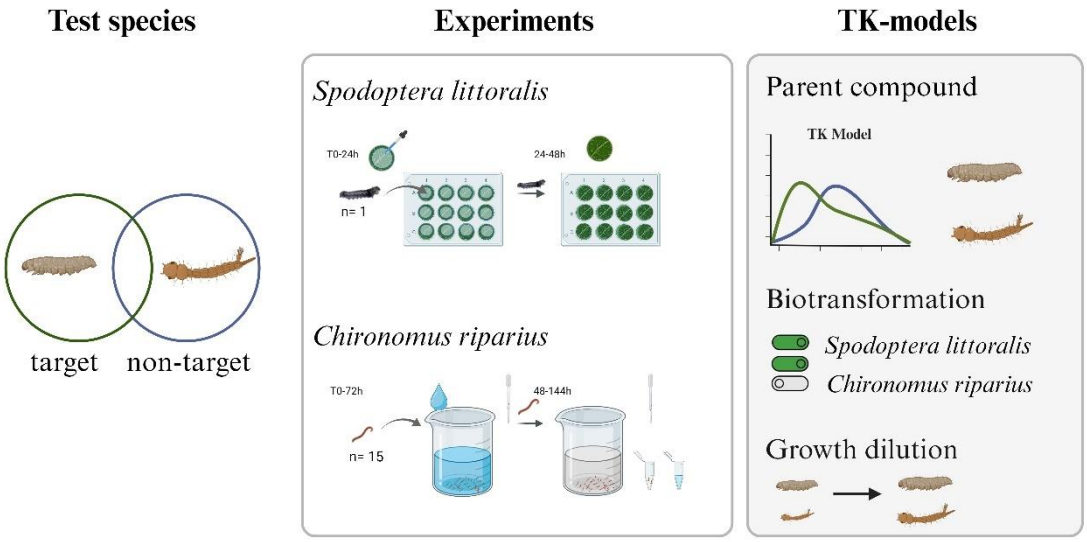


Figure 20: Toxicokinetic bioassay design: Individual *Spodoptera littoralis* larvae were exposed to compound treated leaf disks. Feeding contact assay with a 24-hour exposure period followed by a 24-hour depuration period. *Chironomus riparius* larvae (n=3, pooled) individuals were exposed to compound treated water with 10 mg food: 24h exposure period followed by a 48h depuration period. (Created using BioRender.com)

Results

Fate of synthetic chemicals and toxicokinetic model in *Spodoptera littoralis*

Larval performance parameters

The data on the performance parameters of the larval behavior with regard to food intake, excretion, larval size and movement did not differ from the untreated controls during the 24-hour exposure (Figures 21-22). Over this period the larvae in all groups continued feeding without detectable influence of day and night shift and all had consumed almost the entire leaf disk at the end of the exposure (Figures 21-22 & S3). The movement of the *S. littoralis* larvae between image frames was constant all throughout the exposure (Figure 21). In average larva grew up to an average length of 55.9 mm (Figures 22 & S2) and had transformed one leaf disk (Ø size 20 mm) into 33 feces pellets (Figures 3 & S3). Feces dropping (defecation) usually begins about 4 to 5 hours after the larvae have been placed on the leaves (Figure S3). Thus, exposure to test compounds did not disrupt normal food consumption, defecation, growth, and movement pattern of L3-larvae.

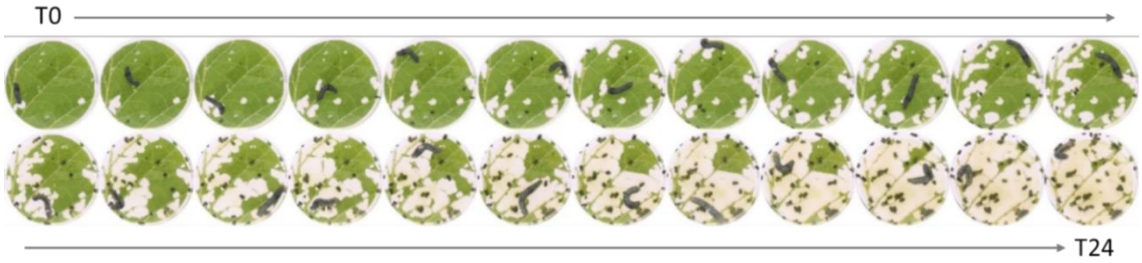


Figure 21: Larva feeding pattern of *Spodoptera littoralis* on a single leaf disk in the toxicokinetic assay. Image frames of a representative example (compound D) observed over 24 hours of exposure.

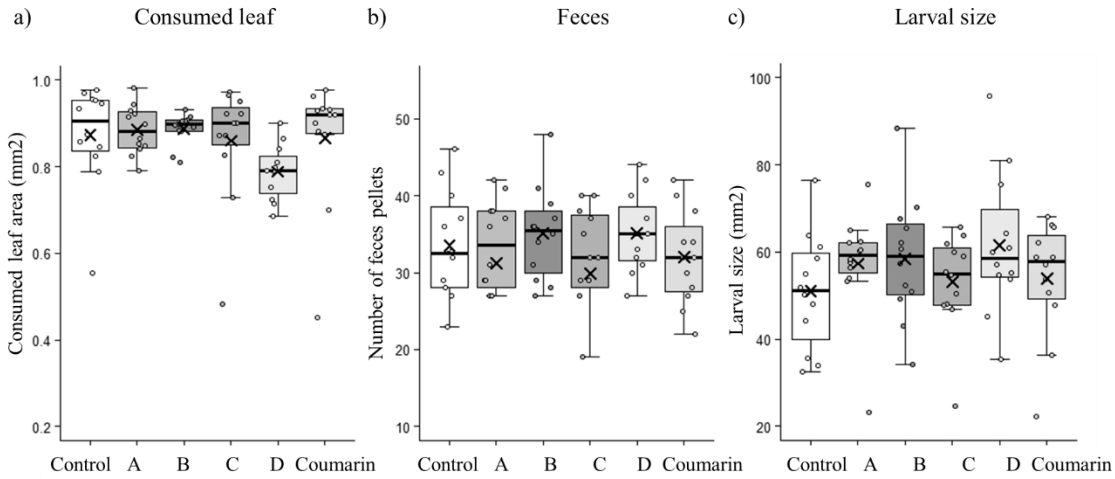


Figure 22: Physiological parameters of *Spodoptera littoralis* after 24 hours of exposure. **a)** Consumed leaf area (Proportion = pixel per mm^2), **b)** number of feces pellets, and **c)** larval size, shown for all test groups (control, test compounds A-D, Coumarin). Boxplots show interquartile ranges, medians (black lines), and means (×). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Individual data points (n = 12), including outliers, are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

Quantity of compound in samples

A separate bioassay without larvae feeding on leaves was used for the leaf disk measurements of compounds, as all leafs were completely eaten during the exposure period. Therefore no direct mass balance of leaf disk related to larva and feces can be performed. The quantities of compounds A, C and D were consistent on the leaf disks during the 24-hour exposure, demonstrating stable exposure profiles (Figures 23a & S24, Table 6). In contrast, the amount of compound B decreased from 0.1 mg to 0.05 mg of the parent compound per leaf disk sample within the first five hours of the experiment, a decrease of more than 50 % compared to the amount initially administered during the exposure period. Coumarin levels began to decrease after one hour and reached 0.005 mg after 24 hours (Figures 23a & S24, Table 6).

The time-course of compound quantities in larvae differed between the compounds and also during exposure and depuration periods (Figures 23b & S24, Table 6). Quantities of compounds A, C and D increased during the 24-hour exposure period and decreased during depuration period (Figure 23b). The amount of compounds C and D reached maximum levels (median 100 % of the parent compound quantity on treated leaf disk) in larval bodies, but compound A only 30 % (median) after 24 hours of exposure, while the level of compound B reached 30 % already after 5 hours. The level of Coumarin showed a maximum quantity of 60 % (median) of the initial dosage of the compound on the leaf disk within the larva after 1 hour of exposure, which further decreased to 15 % at the end of the 24-hour exposure (Figure 23b).

Feces samples include all feces pellets dropped by individual *S. littoralis* larvae and were either collected after exposure (interval T0-24 hour) or after depuration (interval T24-48 hour) respectively. At the end of the exposure period, the highest detected fractions in the feces were those of the compounds A and Coumarin with about 60 % (median) of the initially applicated compound dose, whereas detected amounts of compound B, C, and D reached 15-25 % (median) of that dose (Figure 23c). In the depuration period, with the exception of compound D, detectable quantities in feces pellets remained below 5 % (median) for all compounds. For compound D the detected quantities in feces increased up to 30 % (median) of the applicated parent compound dose (Figure 23c).

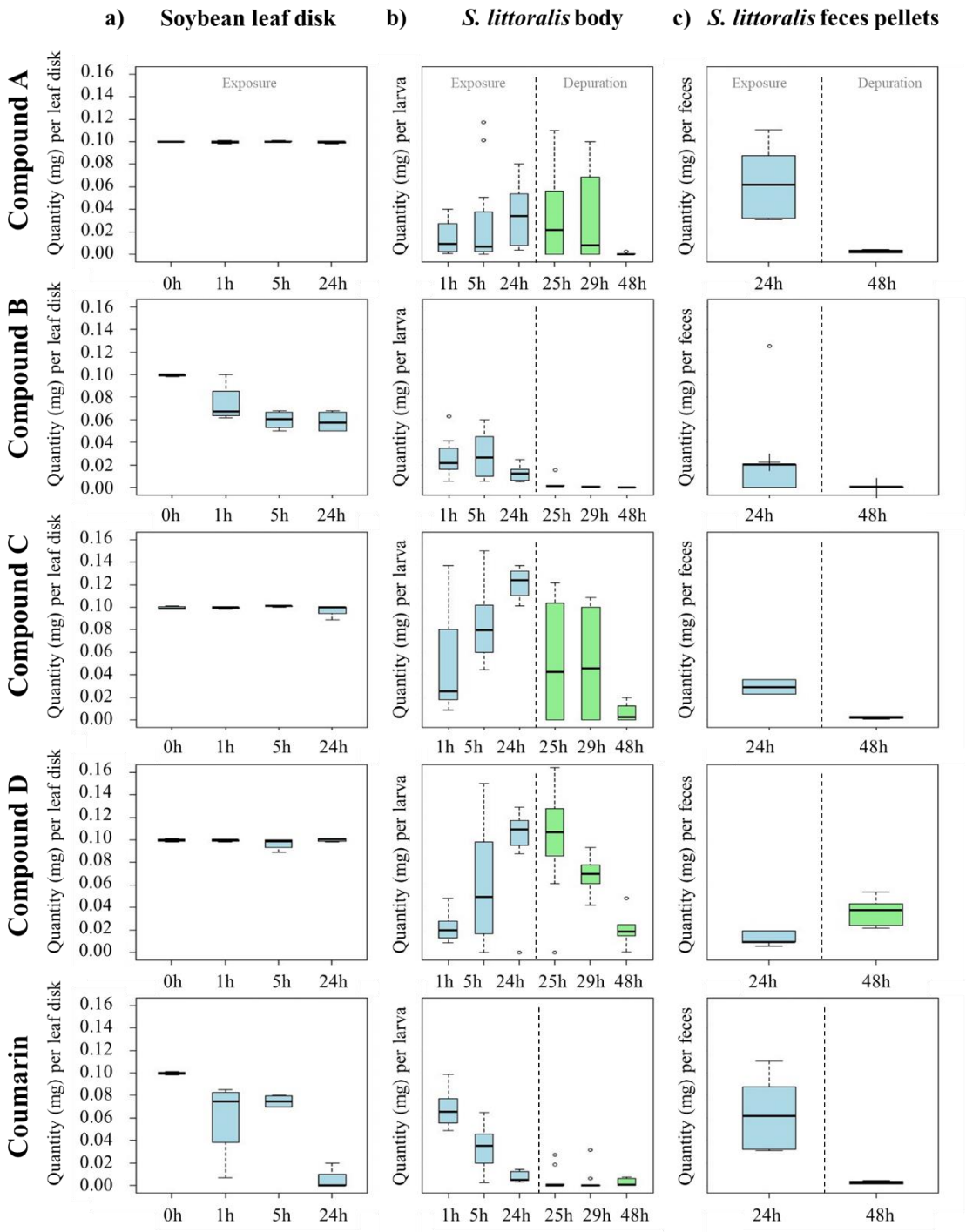


Figure 23: Quantities of compounds (mg) detected in soybean **a)** leaf disk (n=3), **b)** *Spodoptera littoralis* larvae (body, experimental replicates n=12) and **c)** feces pellets (pooled in experimental replicates n=12). Exposed larvae fed on treated leaf disk for 24-hours, thereafter, transferred to and fed on non-treated leaf disks for a follow-up depuration over 24-hours. A separate bioassay without larvae feeding on leaves was used for the leaf disk (a) measurements of compounds. Therefore no real mass balance of leaf, larva, and feces can be performed. Boxplots show interquartile ranges and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

Toxicokinetic model 1

Overall, the measurement results of the treatments with the test compounds compared using the model showed clear differences in the concentrations of the starting compounds in the larvae and the resulting constants for the uptake and elimination rates, as well as for the bioaccumulation factors (Table 2). The TK model reflects the concentration after 24-hours and the elimination period rather than the concentration observed in the first 5-hours (sampling time point 1 and 2) (Figure 24). All compounds and Coumarin showed higher uptake than elimination rate constants, resulting in bioaccumulation that led to concentrations in larvae above the values in the leaf disks (Table 2). The curves of the TK model (Figure 24) show further differences between the compounds, particularly in the time course of their uptake and elimination. In the exposure phase, the concentrations at the first sampling point were already at a similar concentration level as at the subsequent sampling point (5 hours). Calibration of the model for compounds A, B, and C resulted in parameters reaching a limit (k_{in} at upper limit) (Figure S3). Coumarin had a rapid uptake and a steep decline as a result of exposure decline (Figure 24) and the model parameters converged with confidence intervals, which were well-identified (closed parameter likelihood plot Figure S3). The model of compound D also converged with closed confidence intervals (Figure S3).

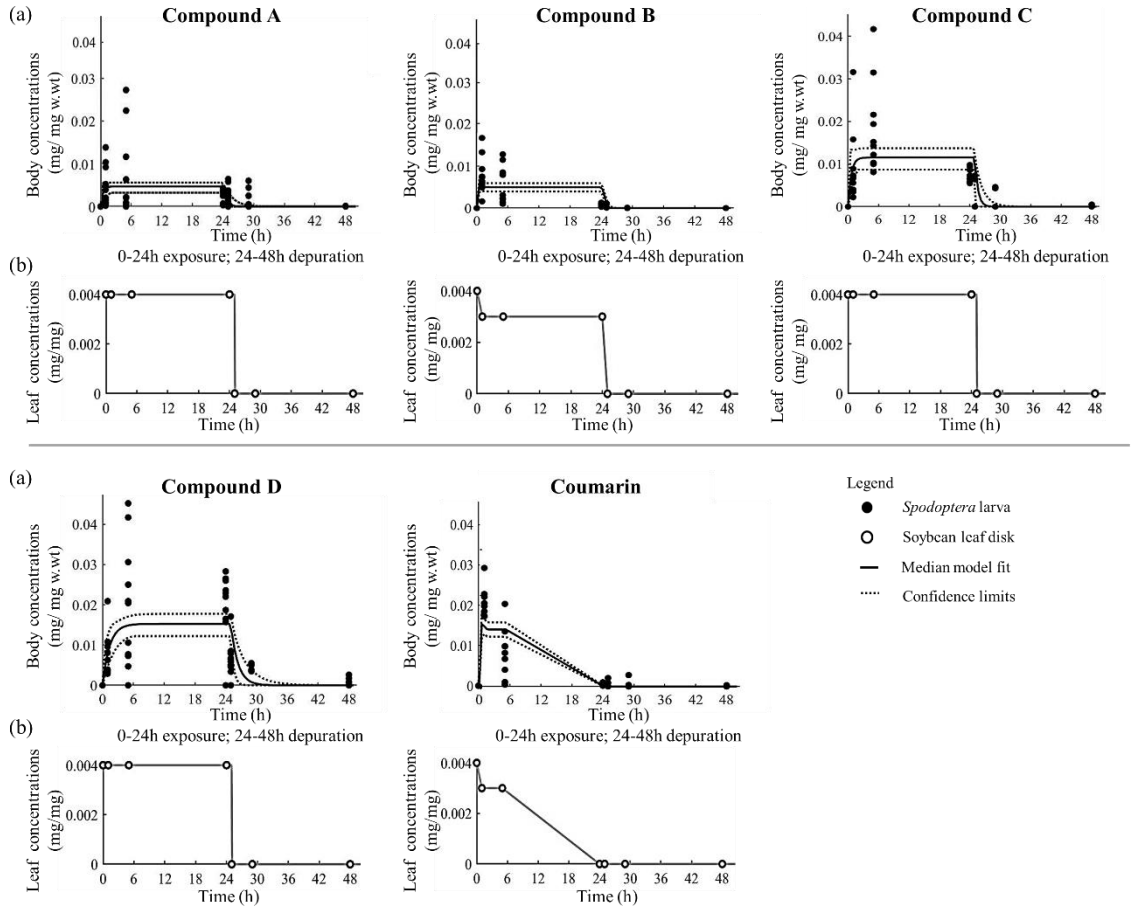


Figure 24: Concentrations of test compounds (A-D, Coumarin) in leaf disk and bodies of *Spodoptera littoralis* larvae. Exposed larvae (experimental replicates n=12) fed on treated leaf disks for 24-hours, followed by transfer to untreated leaf disks for depuration for 24-hours. **a)** TK-Model 1 for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 2) and 95 % confidence limits (dotted) of model fit represented by the lines. Dots indicate measured data. **b)** Exposure scenarios in feeding contact assay: soybean leaf disk (n=3) concentrations during exposure and depuration time. Created using MATLAB (Version R2021a, Build Your Own Model).

Table 2: Toxicokinetic model 1 parameters for *Spodoptera littoralis* larvae: uptake and elimination rate constants, bioaccumulation factors for and chemical descriptors (Log P, molecular weight) of tested compounds.

Compound	Log P	Molecular weight (g/mol)	Uptake rate constant (k_{in}) [$mg_{leaf}/(mg_{w.wt}*h)$]	Elimination rate constant (k_e) (1/h)	Bioaccumulation factor (BAF) ($mg_{leaf}/mg_{w.wt}$)
A	1.58	205.25	2.63 [0.61;>100]	2.24 [0.68;>100]	1.17 [0.78;1.58]
B	1.50	262.62	8.89 [3.31;>100]	5.43 [2.10;72.45]	1.64 [1.29;1.99]
C	2.24	302.25	4.86 [1.89;>100]	1.68 [0.63;45.21]	2.89 [2.26;3.85]
D	3.57	280.68	2.79 [1.26; 6.79]	0.73 [0.33;1.74]	3.82 [3.53;4.92]
Coumarin	1.43	146.14	18.71 [10.64;72.71]	4.52 [2.58;17.11]	4.16 [2.64;3.83]

Absorption and excretion of test compounds in sucking compared to chewing pests

Compound quantities in the two tested pest species

Notable differences in parent compound quantities were measured in the entire larval of *S. littoralis* and bodies of *M. persicae* (Figures 25-26) during the exposure and depuration periods of respective bioassays.

The amount of all compounds measured in the larvae of *S. littoralis*, with the exception of compound B, increased during the exposure and decreased during the depuration period (Figure 25). All compound reached their maximum quantities at different sampling times during exposure or depuration. Compound F reached this level after one hour of exposure of the larva to the treated leaf disk, followed by compound B after five hours, and compounds A, C, D, and E after 24 hours, i.e., at the end of the exposure period. Compound G, on the other hand, only reached its maximum quantity-level after 25 hours, i.e., one hour after the start of the depuration phase. Compound C reached the highest (0.085 mg per larva) and compound F the lowest (0.029 mg per larva) measured total amount in the larval bodies (Figure 25). Only compounds C and D were still above the detection limit in the larval bodies at the end of the depuration phase after 48 hours (Figure 25).

In *M. persicae* aphids (calculated equivalent based on aphid counts), no generic time courses of the measured amounts of the parent compounds were observed (Figure 26). The values for compounds C and D showed an increase in the aphid bodies during the 72-hour exposure period to the treated food, followed by a decrease during the depuration phase. The quantity-levels of

the parent compound G already decreased during the exposure period, with the maximum measured being reached at 48 hours. The initial compounds A, E, and F were present in the aphids in only comparably low quantities during the entire measuring period. Compound D had the highest total quantity (0.021 mg per aphid equivalent) in *M. persicae* aphids (Figure 26), in contrast, compounds A, B, and E had the lowest. Even at the end of the depuration and measurement period (144 h), compounds C, D, E, and G were still detectable in aphids (Figure 26).

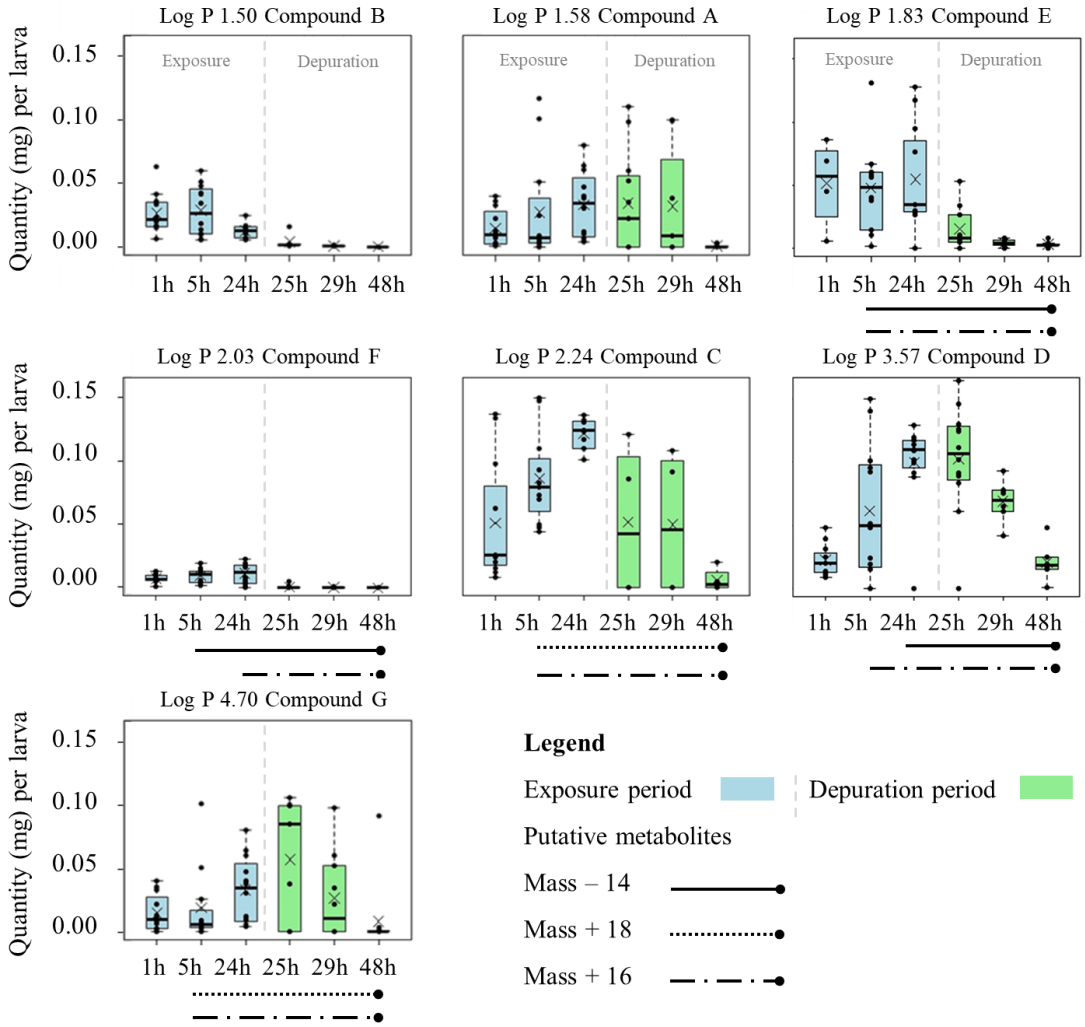


Figure 25: Compound quantities (mg) in larval bodies of *Spodoptera littoralis* over time. Compounds arranged in order of increasing Log P (Table 1). Larvae (experimental replicates n=12) fed on treated leaf disk within 24-hour of exposure, thereafter, transferred to untreated leaf disks for subsequent 24-hour of depuration. Over time occurrence of putative metabolites (mass changes, Table 3) represented as different line types below graphs, respectively. Boxplots show interquartile ranges, raw data points, mean (X), and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. (The data of the compound quantities (A-D) were studied in the first application of the TK model and in a test of the influence of the performance parameters, Figure 24, Table 2). Figures created using R (version 3.5.3; R Core Team, 2020).

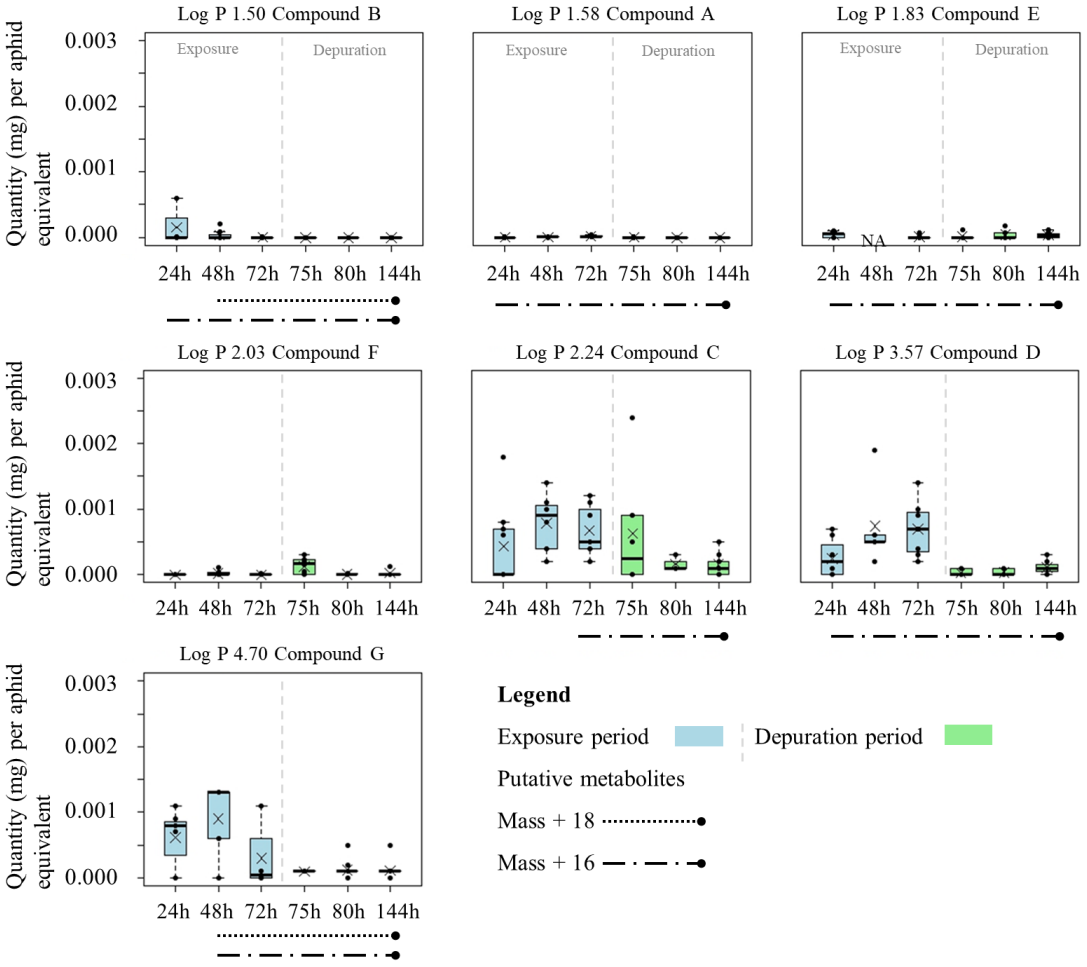


Figure 26: Compound quantities (mg) over time in bodies of *Myzus persicae* aphids (calculated aphid equivalent based on aphid counts). Compounds are arranged in order of increasing Log P (Table 3). Aphids (n= 8 experimental replicates; aphid population per sampling time) fed on treated artificial diet within 72-hours exposure, thereafter, transferred to untreated diet for subsequent 72-hours depuration. Over time occurrence of putative metabolites (mass changes, Table 1) represented as different line types below graphs, respectively. Boxplots show interquartile ranges, raw data points, mean (X), and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

Quantities of excreted parent compounds

The quantities of the compounds measured in the bodies of *S. littoralis* and *M. persicae* and their excretion products (feces and honeydew) differed between exposure and depuration periods (Figure 28).

a) *Spodoptera littoralis*

At the end of the exposure period (after 24 hours), compounds E, F, C, D, and G were present in the bodies of the insects in relatively higher quantities than in their excreted feces. Conversely, compounds A and B were present in higher quantities in the feces than in the insect bodies. Compound C reached the highest measurable total quantity in the body of the examined insects, whereas compound A reached the highest quantity of the parent compound in the feces. Compared to their excretion products, higher quantities of compounds E and G were still detected in the insects` bodies at the end of the depuration period (after 48 hours), while compounds C and F showed relatively higher measured quantities in the feces (Figure 27a).

b) *Myzus persicae*

At the end of the exposure period (after 72 hours), compounds C, D, E, and F showed higher quantities in the bodies if the insects compared to their excretion product, whereas higher quantities for compounds A and B were found in the honeydew (Figure 27b). Compound D had the highest quantity measured in the insect body (aphid equivalent) at the end of the exposure period, followed by compounds C and G. In contrast, compound E had the overall highest amount in honeydew at the end of the exposure period, followed by compound D.

Relatively higher quantities of compounds A, C, and D were found in the bodies of the insects at the end of depuration period (after 144 hours), whereas relatively higher quantities of compound E were measured in the honeydew (Figure 27b).

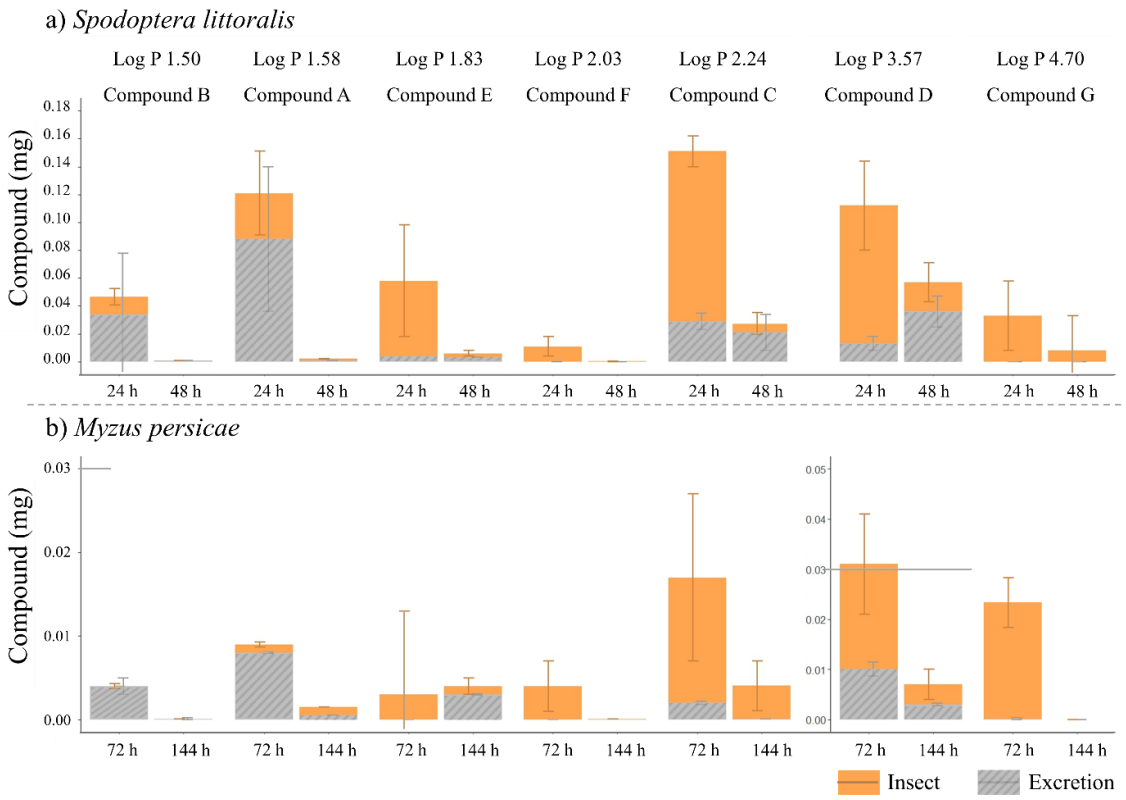


Figure 27: Distribution of parent compounds measured in insect bodies and excretion products. **a)** Quantity of compound (mg) per *Spodoptera littoralis* larva (n= 12 experimental replicates) at the end of exposure (0-24 hours) and depuration (24-48 hours) period. Larvae fed one treated leaf disk for 24-hours, thereafter one untreated leaf disks in the subsequent 24-hours. **b)** Quantity of compound (mg) per *Myzus persicae* aphid (n= 8 experimental replicates) (calculated aphid equivalent based on aphid counts) at the end of the exposure (0-72 hours) and depuration (72-144 hours) period, respectively. Aphids fed on treated artificial diet for 72 hours, followed by 72-hour feeding period on untreated diet. (Horizontal grey line indicates the different y-axis scale for compound D). Figures created using R (version 3.5.3; R Core Team, 2020).

Comparative toxicokinetics of test compounds and their putative biotransformation in *Spodoptera littoralis* and *Chironomus riparius*

Toxicokinetic model 2

Second level TK modeling could be successfully developed for all tested compounds in *S. littoralis* larvae (Figures 28-29, S11-12 & S14-18, Table 2 & S1,). But due to their lethal effect on *C. riparius*, such toxicokinetic bioassays could not be run for compounds C and G with that species (Figure 30, Table 5).

TK analysis of both species clearly showed that the amount and concentrations of the different parent compounds and their metabolites varied significantly (Figures 29-31, Tables 3-4).

The elimination rate constants (k_{out}) of compound C, F and G for *S. littoralis* could not be identified from the data and were therefore fixed, as otherwise the confidence intervals would approach infinite confidence intervals (Table 4, Figures S11 & S19- 24). The *S. littoralis* model accurately captures the concentration dynamics at both the end of the 24 hours of exposure, as well as during the elimination phase, but captures less well the concentrations observed in the initial sampling intervals (1 h, 5 h), as previously shown by TK model 1 for those of compound A-D (Figures 28-29 & 24). This was also detected for the larvae of *C. riparius* exposed to compound D (Figure 30). In both species investigated, its internal concentrations were increased during the exposure period, followed by a decrease in the subsequent depuration phase (Figures 28-29). The only exception was observed in *S. littoralis* larvae exposed to Coumarin (Figures 29 & S9), which was externally caused by the decrease of its concentration on the treated leaf disk.

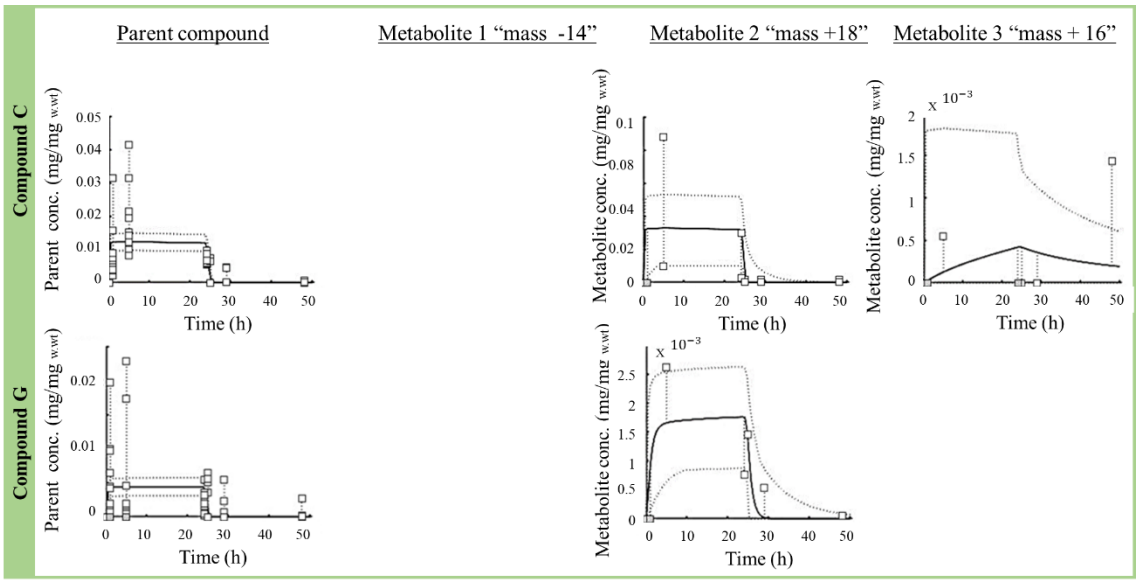


Figure 28: Compounds C and G and their metabolites (mg/mg$_{w.wt}$) in the larvae of *Spodoptera littoralis* (Measured data: squares, n= 12 experimental replicates). Exposed larvae fed on treated leaf disks for 24 hours, followed by a depuration period of 24 hours on untreated leaf disks. TK-Model (best fit model: solid line, 95 % confidence limits: dotted lines): parent compound uptake and elimination, as well as formation and elimination of metabolites. See Table 4 for model parameter values. TK models of parent compound concentrations (C) already investigated in model 1 (Figure 24, Table 2). Created using MATLAB (Version R2021a, Build Your Own Model).

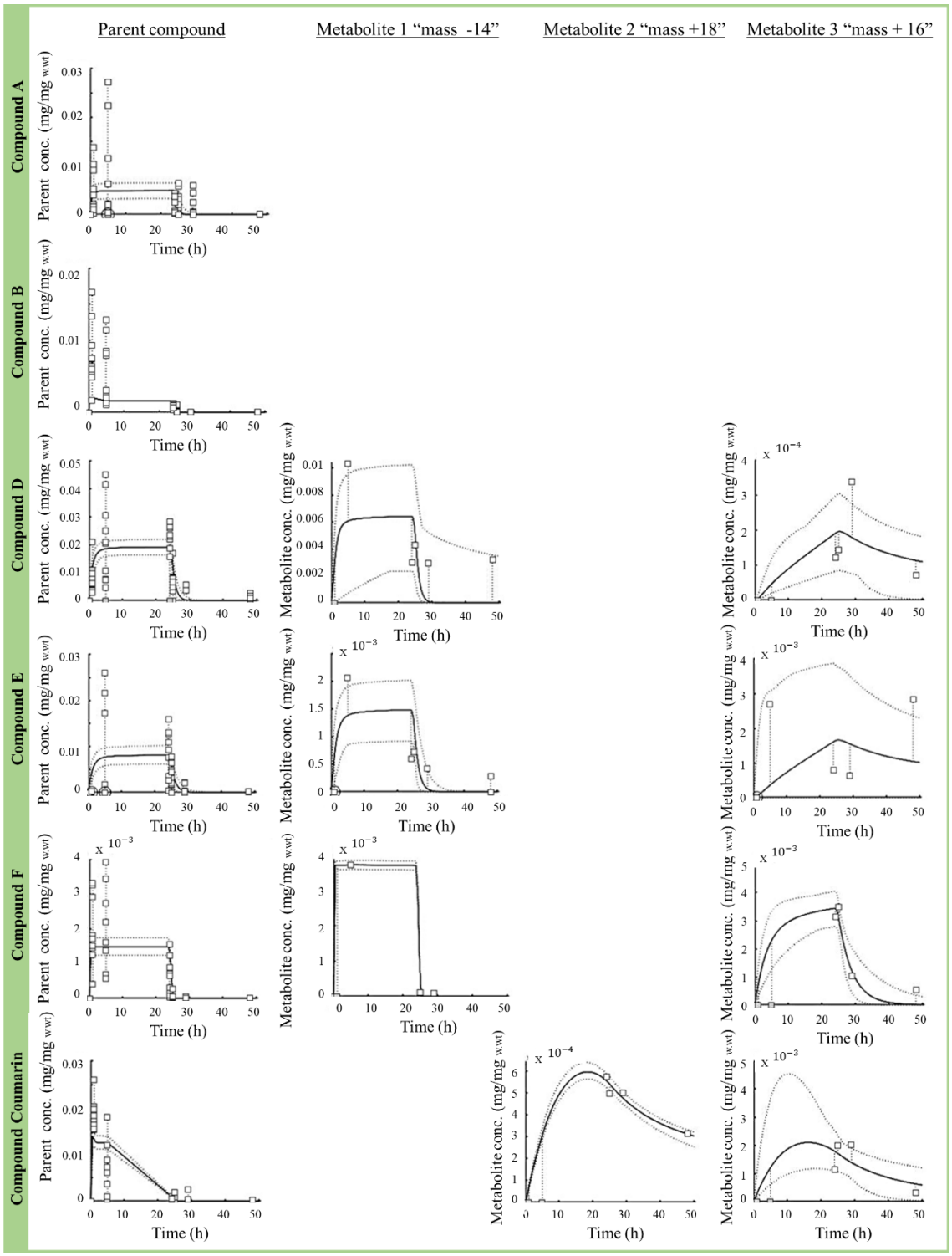


Figure 29: Body concentrations (mg/mg$_{w.wt}$) of compound A, B, D-F, Coumarin in *Spodoptera littoralis* larvae (measured data: squares, n= 12 experimental replicates). Exposed larvae fed on treated leaf disks for 24 hours, followed by a depuration period of 24 hours on untreated leaf disks. TK-Model (best fit model: solid line, 95 % confidence limits: dotted lines: parent compound uptake and elimination, as well as formation and elimination of metabolites. See Table 4 for model parameter values. TK models of parent compound concentrations (A-D) already investigated in model 1 (Figure 24, Table 2). Created using MATLAB (Version R2021a, Build Your Own Model).

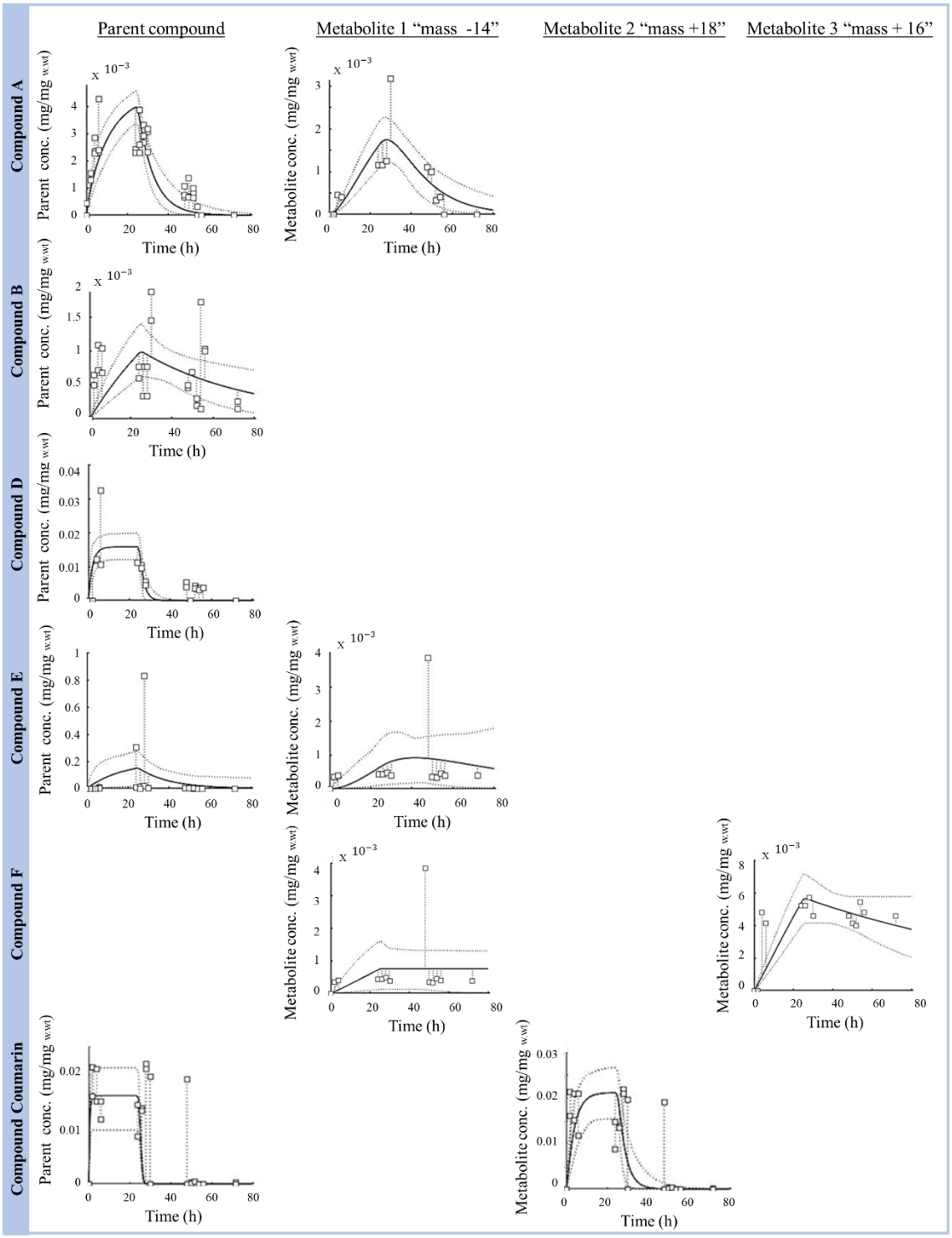


Figure 30: Concentrations of compound A, B, D-F, Coumarin ($mg/mg_{w.wt}$) in larvae of *Chironomus riparius* (measured data: squares, n= 3 experimental replicates). Larvae were exposed in water for 24 hours, followed by transfer to clean water for a depuration period of 48 hours. TK-Model (best fit model: solid line, 95 % confidence limits: dotted lines): parent compound uptake and elimination, as well as formation and elimination of metabolites. See Table 4 for model parameter values. Created using MATLAB (Version R2021a, Build Your Own Model).

Uptake and elimination rate constants

Uptake (k_{in}) and elimination (k_e) rate constants were generally higher in *S. littoralis* compared to in *C. riparius*, which was consistent for all tested compounds, with the exception of compound D, which had a higher elimination rate constant in *C. riparius* (Figures 31-32). The bioaccumulation factor was consistently higher in *C. riparius* (Figures 31-32).

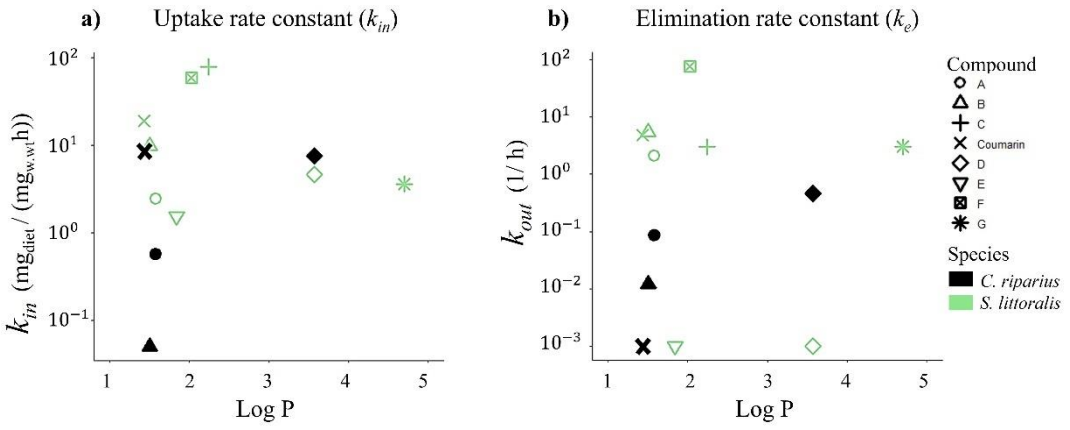


Figure 31: Toxicokinetic model parameters of parent compounds in relation to Log P for *Spodoptera littoralis* (n=12 experimental replicates) and *Chironomus riparius* (n= 3 experimental replicates). **a)** Uptake and **b)** elimination rate constants of parent compounds. Compounds C, F and G were only detected and modeled in *S. littoralis*. Figures created using R (version 3.5.3; R Core Team, 2020).

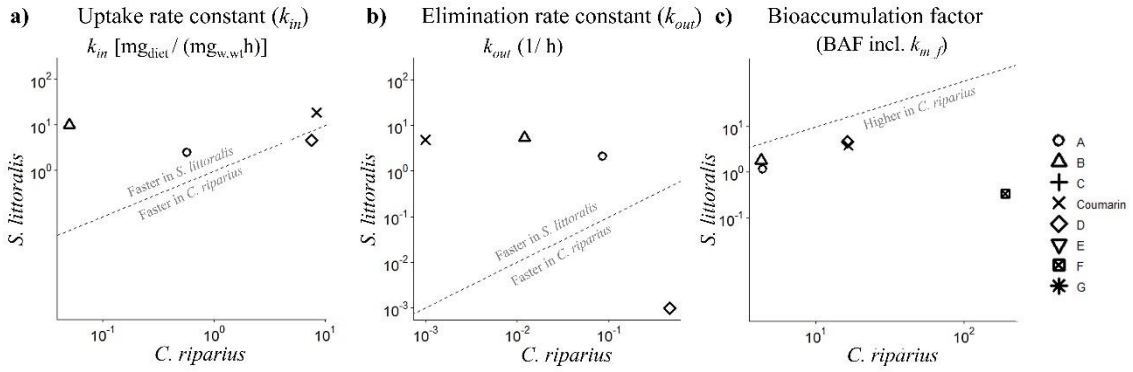


Figure 32: Correlation of toxicokinetic model parameters of the initial parent compounds for *Spodoptera littoralis* (n=12 experimental replicates) and *Chironomus riparius* (n= 3 experimental replicates). **a)** Uptake and **b)** elimination rate constants of parent compounds, and **c)** bioaccumulation factor. Note: compounds C, F and G were only detected and modeled in *S. littoralis*. 1:1 line dashed. Figures created using R (version 3.5.3; R Core Team, 2020).

Putative metabolism (biotransformation)

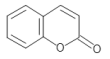
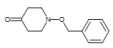
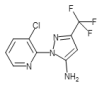
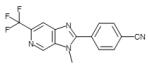
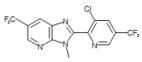
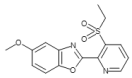
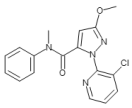
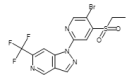
The species examined in the study each showed unique biotransformation profiles characterized by changes in the mass of the compounds tested (Tables 3-4). Three conspicuous mass shifts were observed: a decrease of 14 Da and increases of 16 and 18 Da (Table 3, Figure 34).

The putative metabolites observed in *Spodoptera littoralis* can be described and identified via the measured mass changes (-14, +18, +16 Da) of test compounds over time (Table 3, Figure S13). Metabolites with a decrease in the respective mass of -14 Da were detected for compounds E and F five hours after exposure of the larva and for compound D after 24 hours exposure to treated leaf disks, another metabolite with a mass increase of +18 Da was observed for compounds C and G after five hours exposure (Table 3). Five hours after the start of exposure, mass increases of +16 Da were observed for compounds C, D, E and G, and after 24 hours for compound F. No mass changes were observed for compounds A and B (Table 3).

The putative metabolites observed in *Chironomus riparius* can also be described as mass changes (-14, +18, +16 Da) of the test compounds over time (Table 3, Figure S13). Metabolite with a mass decrease of -14 Da each were detected after two hours for compounds F, compound E after six hours and compound A after 24 hours of exposure; a further metabolite with a mass increase of +18 Da was observed for compound Coumarin two hours after exposure (Table 3). A mass change of +16 Da was only detected for compound F after 4 hours. No mass changes were observed for compounds B and D (Table 3).

Different mass changes (+18, +16 Da) were observed in *Myzus persicae* over time (Table 3). Metabolites with a mass change of + 18 Da were observed for compounds B and G 48 hours after exposure to the treated food. A mass change of + 16 Da was observed for compounds A, B, D and E after 24 h, for compound G after 48 h, and for compound C only after 72 hours of exposure (Table 3).

Table 3: Earliest time of detection of putative metabolites in samples of *Spodoptera littoralis*, *Chironomus riparius*, and *Myzus persicae*. Putative metabolites of compounds (A-G), and Coumarin detected by mass difference (in Dalton) to given parent compound.

Parent compound		Putative metabolites								
		Spodoptera littoralis (larvae)			*Myzus persicae* (aphids)			*Chironomus riparius* (larvae)		
Chemical structure	Compound	Mass -14	Mass +18	Mass +16	Mass -14	Mass +18	Mass +16	Mass -14	Mass +18	Mass +16
	Coumarin	-	24 h	24 h	not observed			-	2 h	-
	A	-	-	-	-	-	24 h	24	-	-
	B	-	-	-	-	48 h	24 h	-	-	-
	C	-	5 h	5 h	-	-	72 h	not observed		
	D	24 h	-	5 h	-	-	24 h	-	-	-
	E	5 h	-	5 h	-	-	24 h	6 h	-	-
	F	5 h	-	24 h	-	-	-	2 h	-	4 h
	G	-	5 h	5 h	-	48 h	48 h	not observed		

Biotransformation in TK Model

The rate constants (k_{m_f}) in the larvae of *S. littoralis* for metabolite formation in compound E (+ 16 Da) and F (mass change of -14 and +16 Da) were fast compared to *C. riparius* (Figures 33a & S13). The formation rate constants of Coumarin metabolites were higher in *C. riparius* (Figure 34a, Table 4). The elimination rate constants (k_{m_e}) of biotransformation products with a mass change of -14 (compound E and F) were higher in *S. littoralis*. In contrast, the elimination rate constants of biotransformation products with a mass change of + 16 or + 18 for the compound F and Coumarin were higher in *C. riparius* (Figure 33b, Table 4).

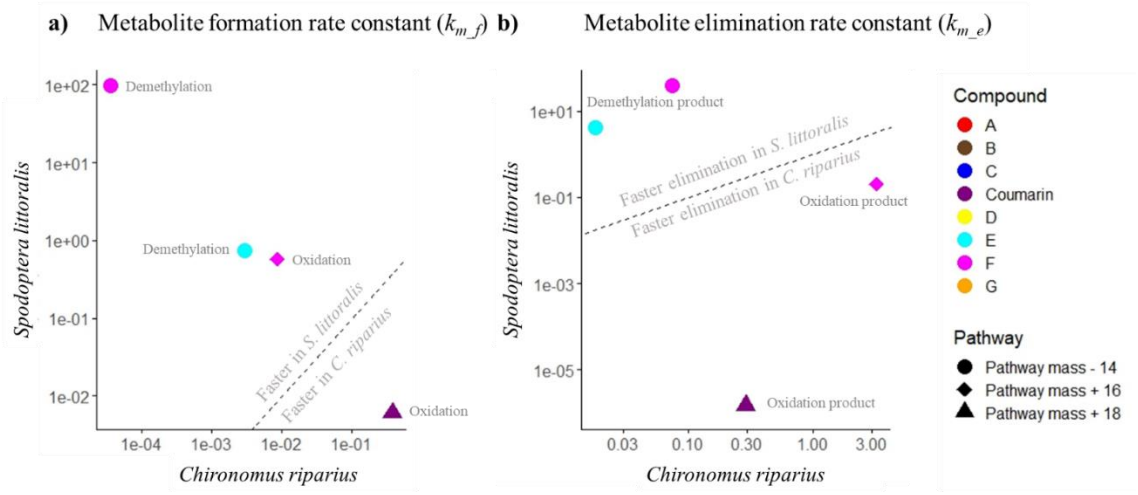


Figure 33: Biotransformation parameters of metabolites quantified in *Spodoptera littoralis* (n=12 experimental replicates) and *Chironomus riparius* (n= 3 experimental replicates). **a)** Metabolite formation rate constants and **b)** metabolite elimination rate constants for both species (Table 4). Metabolites refer to a mass decrease of 14 Da (demethylation) and an increase of 16 or 18 Da (oxidation) (Table 3). 1:1 line dashed. Figures created using R (version 3.5.3; R Core Team, 2020).

Table 4: Toxicokinetic model parameters (uptake and elimination rate constants, metabolite formation and elimination rate constants, and bioaccumulation factors) **(a)** for *Spodoptera littoralis* and (b) *Chironomus ripasirus* larvae of tested compounds (A-G, Coumarin) **(**ND= not detected).

a)

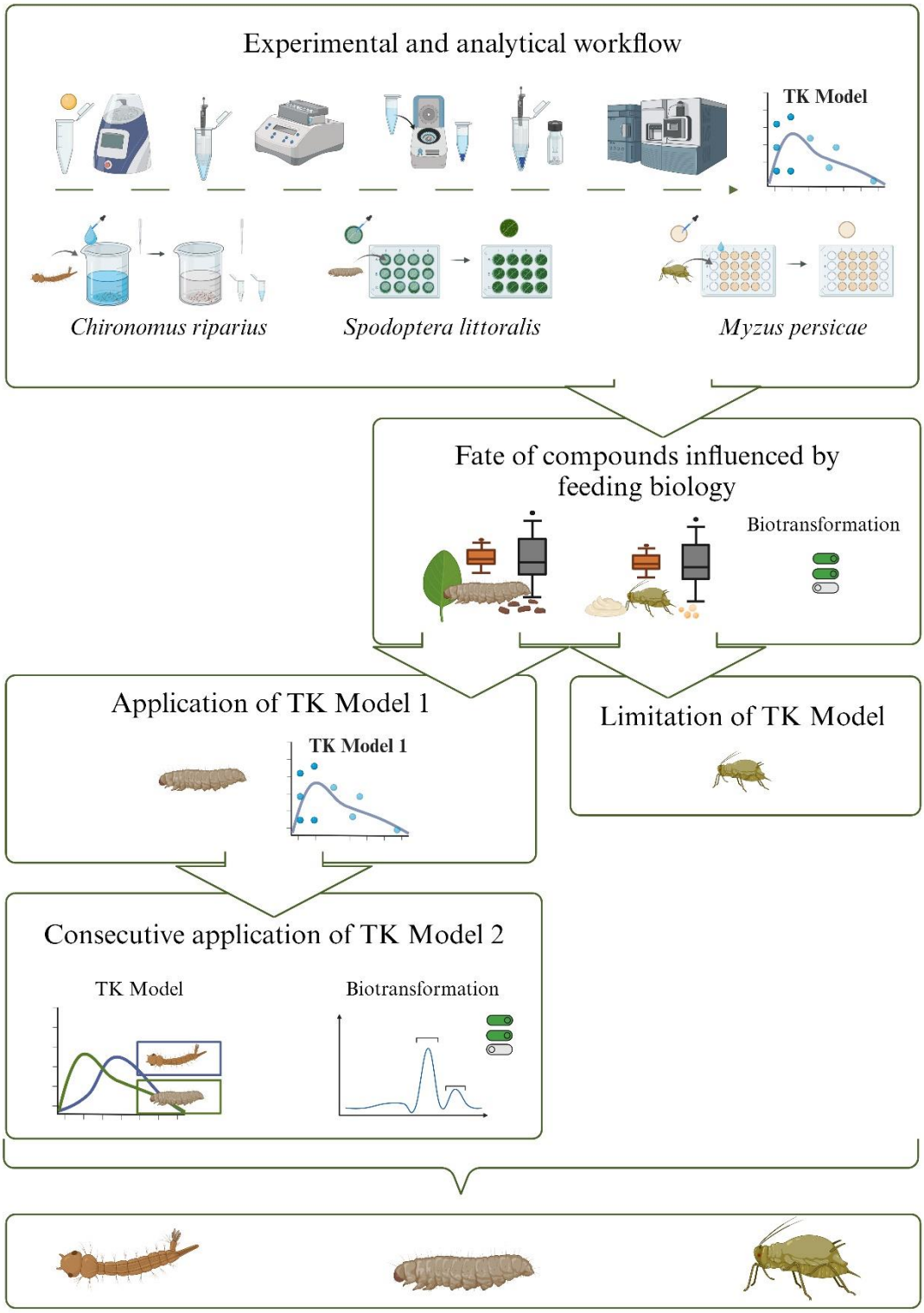
Species	Compound	k_{in} [mg_{diet} / ($mg_{w.wt}$h)]	k_{out} (1/h)	$k_{m1\_f,i}$ (1/h)	$k_{m1\_e,i}$ (1/h)	$k_{m2\_f,i}$ (1/h)	$k_{m2\_e,i}$ (1/h)	BAF (mg_{diet} / ($mg_{w.wt}$)
S. littoralis	A	2.445	2.117	ND	ND	ND	ND	1.15
		[0.782;100.0]	[0.6774;100]					[0.71;1.43]
	B	9.869	5.417	ND	ND	ND	ND	1.82
		[3.697;100.0]	[2.027;65.96]					[0.90;1.40]
	C	68.690	0.038	24.20	9.031	0.002	1.31E-06	2.833
		[3.337;100.0]	[0.001;38.19]	[0.3317;42.94]	[0.201;34.88]	[1.000e;06;15.85]	[1.000e;06;100.0]	[2.30;3.58]
	C fixed	78.580	2.996	24.80	9.195	0.003	1.56E-06	2.816
		[9.594;100.0]		[0.3906;39.74]	[0.2412;32.12]	[1.000e;06;14.53]	[1.000e;06;100.0]	[2.30;3.58]
	D	4.636	0.001	0.986	2.913	0.001	0.004	4.64
		[2.692;8.254]	[0.001;1.507]	[0.01223;1.812]	[1.000e;06;8.457]	[0.0003;0.003]	[1.000e;06;0.21]	[1.37;2.30]
	E	1.531	0.001	0.746	4.041	0.015	1.00E-06	2.00
		[0.802;3.198]	[0.001;1.094]	[0.079;1.676]	[0.434;9.005]	[1.000e;06;1.07]	[1.000e;06;100]	[1.36 ;2.28]
	F	58.850	75.870	96.00	38.350	0.586	0.203	0.341
		[18.32;80.22]	[0.001;100]	[42.38;100]	[17.28;47.09]	[0.248;1.211]	[0.064;0.424]	[0.28;0.40]
	F fixed	35.45	2.996	99.84	40.07	0.575	0.200	0.342
		[18.960;41.58]		[51.62;100]	[20.78;47.18]	[10.248;1.209]	[0.064;0.422]	[0.28;0.40]
	G	2.478	1.651	0.624	1.567	0.052	0.019	1.06
		[0.819;100]	[0.001;100]	[0.051;7.466]	[0.118;19.02]	[0.025;0.154]	[1.000e;06;0.186]	[0.64;1.30]
	G fixed	3.608	2.996	0.399	0.994	0.053	0.019	1.04
		[2.452;9.217]		[0.047;6.044]	[0.104;14.90]	[0.025 ;0.153]	[1.000e;06;0.176]	[0.64;1.30]
	Coumarin	18.890	4.835	0.006	0.000	0.026	0.020	3.90
		[10.20;100]	[2.577;26.80]	[0.005;0.007]	[1.000e;06; 0.008]	[0.012 ;0.091]	[1.000e;06;0.134]	[3.64; 4.69]

Table 4: Toxicokinetic model parameters (uptake and elimination rate constants, metabolite formation and elimination rate constants, and bioaccumulation factors) (a) for *Spodoptera littoralis* and **(b)** *Chironomus ripasirus* larvae of tested compounds (A-G, Coumarin) **(**ND= not detected).

b)

Species	Compound	k_{in} [mg_{diet} / ($mg_{w.wt}$·h)]	k_{out} (1/h)	$k_{m1\_f,i}$ (1/h)	$k_{m1\_e,i}$ (1/h)	$k_{m2\_f,i}$ (1/h)	$k_{m2\_e,i}$ (1/h)	BAF (mg_{diet} / ($mg_{w.wt}$)
C. riparius	A	0.569 [0.341;0.886]	0.086 [0.001;0.17]	0.045 [0.023;0.100]	0.069 [0.031;0.184]	ND	ND	4.34 [3.694; 5.65]
	B	0.050 [0.0282;0.088]	0.012 [0.002;0.04]	ND	ND	ND	ND	4.25 [2.030 >500]
	D	7.542 [4.222;21.12]	0.462 [0.2686;1.331]	ND	ND	ND	ND	16.32 [10.86 ;19.75]
	E	12.610 [1.219;73.08]	0.066 [0.001;0.451]	0.0003 [4.829e;05;0.051]	0.018 [1.000e;06;9.722]	ND	ND	190.51 [41.76; >500]
	F	ND	ND	0.058 [0.011;0.208]	0.000001 [1.000e;06;0.077]	0.480 [0.3070;0.7599]	0.008 [1.000e;06;0.022]	ND
	Coumarin	8.325 [4.392 ; 17.00]	0.001 [0.001;0.557]	0.409 [0.171;0.789]	0.419 [0.194;0.749]	ND	ND	16.65 [14.61;26.85]

Pictorial summary of results



8

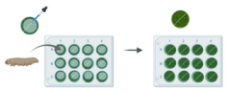
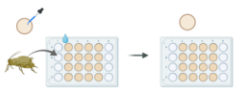
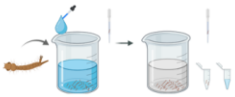
8 Results summarised in pictograms. See results section for detailed information (Created with BioRender.com)

Discussion

The comparative studies presented here are based on the successful development of uniform standardized bioassays for the three study organisms used, although they differ greatly in their life history. The analysis carried out in this study using the standardized method confirmed a critical aspect known from previous studies, namely that the species-specific response to the different compounds can vary considerably [for example compare NYMAN *et al.* (2014), or the review by MÜLLER (2018)].

Due to some compound-specific effects this could unexpectedly be confirmed right at the beginning of the study, as it turned out, that not all compounds could be used in all species (Table 5). All seven test compounds and Coumarin could be tested on *Spodoptera littoralis*. But on *Myzus persicae* and *Chironomus riparius* it was not possible to test the full set of test compounds. During the first exposure tests in *Myzus persicae* Coumarin, and in *Chironomus riparius* the compounds C and G proved to be lethal and therefore could not further be used in the follow-up experiments. These compounds obviously possess a highly specific toxicity. Lower concentrations were not intended to be used, as the high doses were chosen to ensure good analytical detection of parent compound and potential metabolites.

Table 5: Compounds tested on selected insect species in toxicokinetic bioassays. (Created with BioRender.com)

Compound tested in bioassays	*Spodoptera littoralis* 	*Myzus persicae* 	*Chironomus riparius* 
Coumarin	YES	lethal	YES
A	YES	YES	YES
B	YES	YES	YES
C	YES	YES	lethal
D	YES	YES	YES
E	YES	YES	YES
F	YES	YES	YES
G	YES	YES	lethal

The variation of observable species-specific effects is often related to differences in metabolic reactions between species, especially the abundance and activity of detoxifying enzymes (NAVARRO-ROLDÁN & GEMENO 2017). Such enzymatic variation not only determines the susceptibility of different species to specific compounds (such as potential insecticides), but also influences the resilience and recovery of their insecticide responses following any type of exposure (SMITH 1955; RHARRABE *et al.* 2007; CABRERA-BRANDT *et al.* 2010).

Changes in fecundity or the behavior of affected individuals or groups of organisms are often the first indicator of the effects of compounds or metabolic processes triggered by intoxication. This has consistently been shown for a wide range of insects, as insecticides have significant negative impact on key life history traits such as the number of offspring, survival rate and intrinsic growth rate (BAYRAM *et al.* 2010; AMARASEKARE *et al.* 2016; MÜLLER *et al.* 2017; ALMASI *et al.* 2018; MÜLLER *et al.* 2019). Apart from effects on development and reproduction, several effects on behavioral traits such as feeding, locomotion and navigation have been documented (HAYNES 1988). The effects resulting from insecticides on the nervous system can also have an impact on activities that are crucial for the survival of insects and their ecological functioning via behavioral changes (DESNEUX *et al.* 2007).

In a first step analysis, possible effects of the tested chemical compounds on the behavior of *S. littoralis* larvae were examined (Figure 17). All larvae examined in this study showed normal behavior during the complete observed exposure period. There were no deviations in their behavioral patterns compared to the control groups (Figures 21-22 & S 2-3). In the case of *Myzus persicae*, it was not yet technically possible to take pictures every hour until the end of this study, but the evaluation of photos taken at the time of sampling showed normal fluctuations in the observed parameter(s). In observation of *Chironomus riparius* individuals according to OECD guidelines, no changes in movement patterns were observed induced by exposition to the compounds tested. It can therefore be concluded that, on the one hand, no altered behavior is responsible for the observed differences in the amounts of compounds ingested in the biological matrices studied (KINGSOLVER & HUEY 2008; ANKLEY *et al.* 2010), and on the other hand, that the compounds ingested in the experimental did not trigger any changes in behavior during the experimental period.

A(D)ME of test compounds in two pest species

The different biological characteristics of the two species studied had a significant impact on the uptake of the test compounds in the experiment. The primary absorption pathways observed are emblematic of the conditions in the field under which pests come into preventive contact with pesticides applied via the leaf (Figures 18a & 34).

The larvae in the bioassay of *S. littoralis* fed on contaminated leaf disks and thus combining the supply of the test compounds via active oral uptake (larvae cut leaf pieces with their mandibles) with passive contact uptake (larvae crawl on spray deposits on leaf surfaces). In contrast, the *M. persicae* test was dominated by oral ingestion, as the aphids were fed with an artificial food imitating plant sap through an uncontaminated film on which they were moving without being exposed to the compounds (Figures 18a & 34).

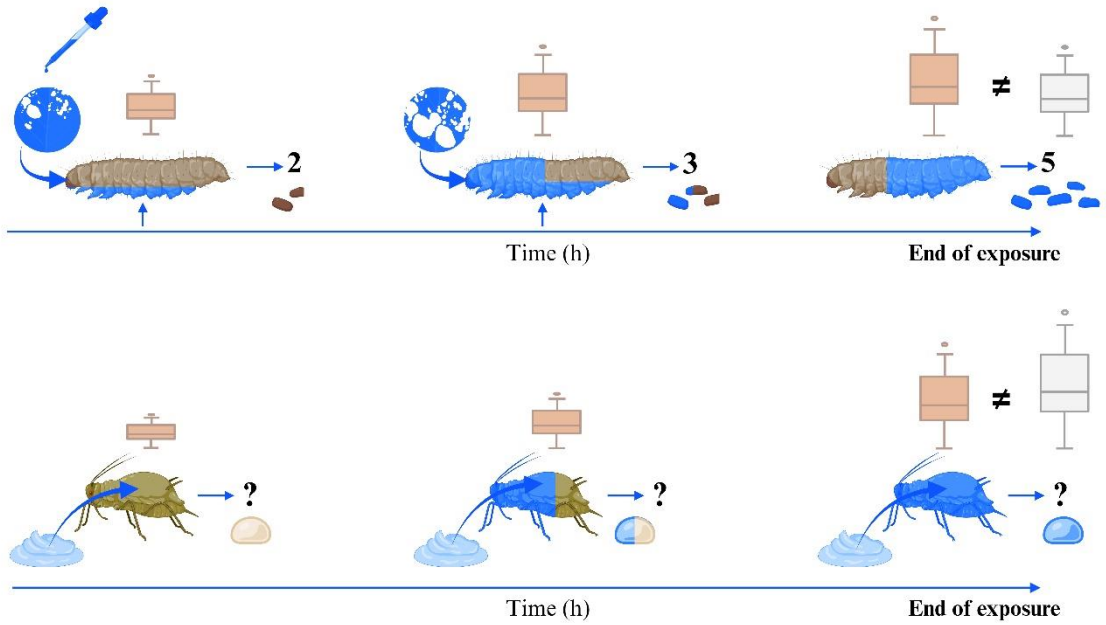


Figure 34: Absorption pathways in *Spodoptera* caterpillar and *Myzus* aphid. Absorption in *Spodoptera littoralis* is limited to oral and contact ingestion, in *Myzus persicae* to oral ingestion. The limitations of quantifying the number of honeydew droplets are illustrated as well as those of quantifying the quantity of precipitated compounds. (Created with BioRendder.com)

a) Does the amount of the initial compound taken up into the insect body differ from the amount of the excreted products?

In the initial experiments on *S. littoralis* and *M. persicae*, different patterns of compound quantities were detected in the bodies, the excretion products and the feces or honeydew (Figures 25-27). Predominantly, in both species' compounds with Log P values lower than 1.58, explicitly A and B, were already excreted at the end of the exposure period. In contrast, high quantities of compounds with relative higher Log P values (C: 2.24 and D: 3.57) were detected in feces of *S. littoralis* larvae only at the end of the depuration period (Figure 27a). These results are consistent with other studies on *S. littoralis*, in which consumed compounds remained unaltered at excretion (BEN-AZIZ *et al.* 1976).

The quantities of compound D in the honeydew of *M. persicae* were substantially higher at the end of the exposure period compared to those at the end of the depuration period, whereas the opposite was detected for compound E (Figure 27b). This suggests that compound D could already be excreted during its exposure, whereas compound E may have a longer residence time and get gradually excreted during the subsequent depuration period. Interestingly, compounds F and G, regardless of the time of sampling, could not be detected in the excretion products of the two species (Figure 27). This strongly indicates that this compounds were completely metabolized and therefore not present any more as parent compounds in feces or honeydew (Figure 27, Table 3), and that processes of biotransformation work as effective elimination pathway (SMITH 1955). The predominant detection of large quantities of the initial compounds in the excretion products, on the other hand, may indicate either a limited absorption potential in the insect body and/or a rapid excretion process.

In particular for *S. littoralis*, the compound quantity of feces measured in compound A-D showed substantial variation over time in larval bodies during the exposure period (Figure 23b). As the measurable excretion of compounds begins with the first dropping of feces pellets after roughly four to five hours of feeding, and the feces pellets contain a substantial quantity of test compounds, the highest variation in body tissue concentration variation was also observed in this time frame (Figures 23-24 & S2-3). The variation in the amounts of compounds in the larval bodies are also clearly reflected in the increased fluctuations of amounts observed in feces (Figure 23c). The uptake of a compound into the larval body could lead to high biotransformation in this species, which then leads to the reduced amounts in its feces. This rapid and quantitatively high excretion via the feces also provides an explanation for the fact that only a limited quantity of compounds was systemically absorbed into larval bodies. In addition, some of the compounds could even pass through the intestine without being absorbed.

For stable chemicals one can expect 100 % detectability of the parent compound across all matrices, and that the apportionment between the different matrices describes the fate of chemicals over time in plant and larval tissues in the given assay. The data from control experiments, except compounds B and Coumarin, exclude an abiotic degradation of the compound on the leaf disks and larvae for all compounds, (Table 6, Figures S5 & S9-10). The low detection quantity within the caterpillars and the decrease in the compound quantities thus most probably has to be explained by excretion or biotransformation.

Table 6: Detectability of stable compound in different sources (leaf, artificial diet, or water). For exposure profiles over time see Figures S5 & S9- S10.

Compound stable in bioassays	Leaf disk	Artificial diet	Water
Coumarin	no	not tested	YES
A	YES	YES	YES
B	no	no	YES
C	YES	YES	not tested
D	YES	YES	YES
E	YES	YES	YES
F	YES	YES	YES
G	YES	YES	not tested

In fact, the compounds C and D demonstrated chemical stability on the leaf disk (Figure 23). And indeed, once the entire leaf disks were eaten up (end of exposure period), highest quantities of it were measured in the bodies, but only low quantities in the feces of *S. littoralis* (Figures 23 & 27). This strongly suggests that these test compounds remained unchanged in these plant and insect matrices. Compound B, on the other hand, reached the maximum levels measured in the larval bodies after only five hours (Figures 23 & 25). This compound did not show full stability on the leaf disk. As the leaf disks were always eaten up at the end of the exposure phase, it has to be assumed, that if a compound is stable on the leaf disk, it must have been 100% absorbed or passed through the larval body during the experiment. However, maximum uptake could not be estimated from feeding observations for both species, because this indicator is not available for aphids, which feed *ad libitum* on liquid food available.

Metabolism (biotransformation) of compounds

Both, the mechanisms of absorption, and excretion, in insect species such as *S. littoralis* and *M. persicae*, are fundamentally influenced by their general physiology, their specific feeding biology, and of course, the properties of ingested chemical compounds. Internal metabolic processes have an impact on the residence time of compounds within the insect, which in turn affects the quantity of compounds found in excretion products (Figure 35). It is very likely that chemical compounds that undergo no or less extensive metabolic transformation are excreted faster and therefore have an overall shorter retention time. The quantity of compounds detected in the excretion products may therefore depend on the residence time of the compounds and the metabolic processes taking place during this time, and thus, at least without contradiction, be explained by the different A(D)ME processes taking place in the insect.

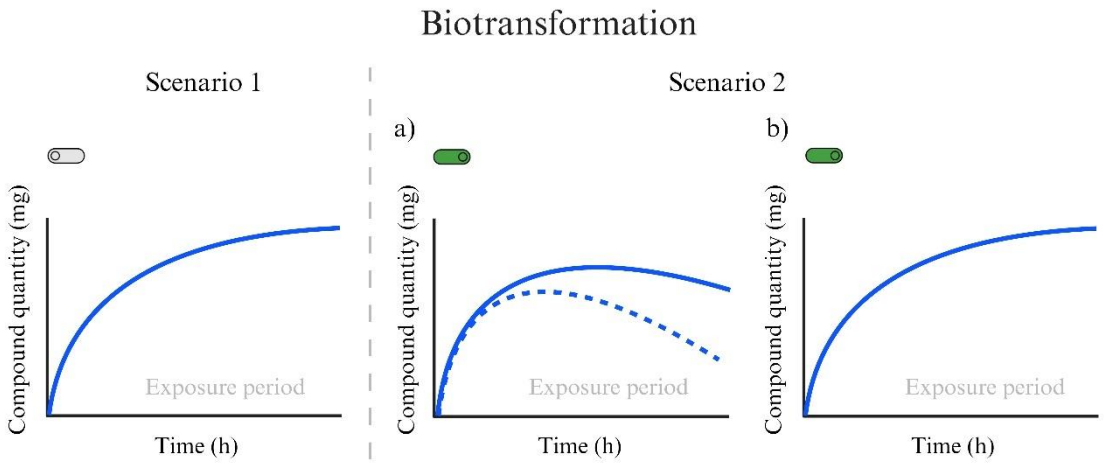


Figure 35: Putative scenarios of the effect of biotransformation on the accumulation and detectability of chemical compounds in insect tissues during (*ad libitum*) exposure. Scenario 1: In the absence of biotransformation, a steady accumulation of compounds is observed. Scenario 2a: When biotransformation occurs, it may result in differently reduced levels of detectable compounds in the insect body, suggesting metabolic processing. Scenario 2b: Alternatively, biotransformation may not measurably affect the rate of uptake of compounds, also resulting in an observable steady increase in compound levels despite metabolic activity (details see text).

Some parent compounds can decisively be reduced by metabolic processes degrading them in the body of certain insect species, which leads (or at least can lead) to metabolites with a different molecular mass (Da) and reduces its detectable amounts in the excretion products of the animals (feces or honeydew) (Figure 35).

In this study, three particularly striking changes in the mass of the analyzed compounds were observed, differences, which can most probable be attributed to different modifications in their molecular structures explainable by metabolic processes. Unique in *S. littoralis* larvae, mass reductions of -14 Da were observed for the test compounds D, E, and F, suggesting a species-specific metabolic process, possibly of demethylation, which could result in more hydrophilic compound derivates which are easier to excrete (Figure 25, Table 3). This pathway was not detected in the aphid *M. persicae* investigated in this study, may be either due to the different food offered to the two species or due to the fact that metabolites were below the detection limit (Figure S3). Nevertheless, RUP *et al.* (2006) described demethylation as biotransformation pathway for the mustard aphid, *Lipaphis erysimi* (KALTENBACH, 1843), feeding on radish plants. During chemical analysis run in this study on *S. littoralis* and *M. persicae*, mass increases of +16 Da and +18 Da were detected, both indicative for metabolites resulting from oxidation processes such as hydroxylation, but not necessarily for each compound (Table 3). Oxidation metabolites of compounds A and B were detected exclusively in *M. persicae* (Figure

26, Table 3). At this stage, however, it seems important to point out that these results can only be regarded as approximate values due to possible limitations in the analysis methodology.
S. littoralis showed different biotransformation processes for compounds C, D, E, F and G (Figure 25, Table 3). Remarkably, after five hours, along with a transient decrease in the amount of the parent compound, metabolites of compound E appeared before increasing again with prolonged exposure. This absorption indicates continuous absorption in parallel to the active biotransformation. Other than this, amounts of parent compounds C, D and G consistently increased in *S. littoralis* larvae irrespective to continuous metabolism. In contrast, the amount of compound F did not increase once metabolic processes were detected, indicating a higher biotransformation rate compared to the uptake rate.

In aphids, once a second metabolite appeared, the absorption of compound B seemed to decrease (Figure 26, Table 3). Compounds A and E were continuously metabolized from the beginning of the exposure, and no increase in parent compounds was observed in aphid bodies. The onset of metabolism of compounds C and G correlated well with the decrease of parent compounds. This indicates a faster biotransformation of the compounds compared to their oral uptake. However, the absorption of compound D into the aphid bodies persisted throughout the exposure period regardless of an early onset of metabolism.

These findings emphasize the complexity of the dynamic interactions between compound absorption and metabolic processes occurring in different insect species and suggest that biotransformation is not always sufficient for effective elimination of compounds from their bodies. To confirm this and to better understand the dynamics between absorption and biotransformation rates, which ultimately determine the resulting amounts of compounds in the insect body, a thorough analysis and systematic identification of the resulting metabolites seems essential (Figures 25-26 & 35, Table 3) (TOLSTIKOV 2009).

b) Does the measurable total amount of a compound ingested per individual depend on the species tested?

The consumption of leaf tissues provides the insect larvae with a complex mixture of nutrients, including carbohydrates, proteins and fats, as well as secondary plant metabolites such as potentially toxic alkaloids, terpenes, phenols, polyphenols or glycosides (CHOWN & NICOLSON 2004). These compounds in many cases act as chemical defense mechanism by e.g. disrupting the digestion of herbivores (JAMIESON *et al.* 2017). As an evolutionary result of regular contact with such compounds in nature, widespread polyphagous pests such as *Spodoptera* are known to have evolved superfamilies of detoxification genes that arm them against plant toxins and xenobiotics (AMEZIAN *et al.* 2021). The diet of aphids sucking from the vascular tissue is

typically rich in carbohydrates, but often deficient in the essential amino acids (unfavorable Carbon-Nitrogen ratio), which affects ingestion rates and population dynamics (LESCANO *et al.* 2022).

In order to better understand the effects of the physiological differences of the feeding mechanisms between the two pest species studied, the total food intake over the exposure period (larvae 24 hours, aphids 72 hours) was calculated in relation to the respective body mass. The resulting ratio between food intake to body mass is 1.3 for *S. littoralis* larvae and 3.7 for the *M. persicae* aphids (Figure 36). That aphids generally consume 2.8 times more food than *S. littoralis* larvae can be explained primarily by the considerable differences in the quality of the food consumed, resulting in different residence time of the diet in the respective gut system of the two insect species. The relatively higher food intake also implies a faster gut passage in the aphids, which influence both, uptake into the internal insect tissues and exposure to metabolic processes. This could contribute to the overall low levels of compounds measured in aphids, less diverse biotransformation compared to *S. littoralis*, and the tendency for higher absorption for more lipophilic test compounds. The almost three times higher throughput rate of the diet also means that aphids have a correspondingly higher probability of coming into contact with the test compounds contained in the diet offered *ad libitum*, which is why they are likely to be more intensively exposed to them.

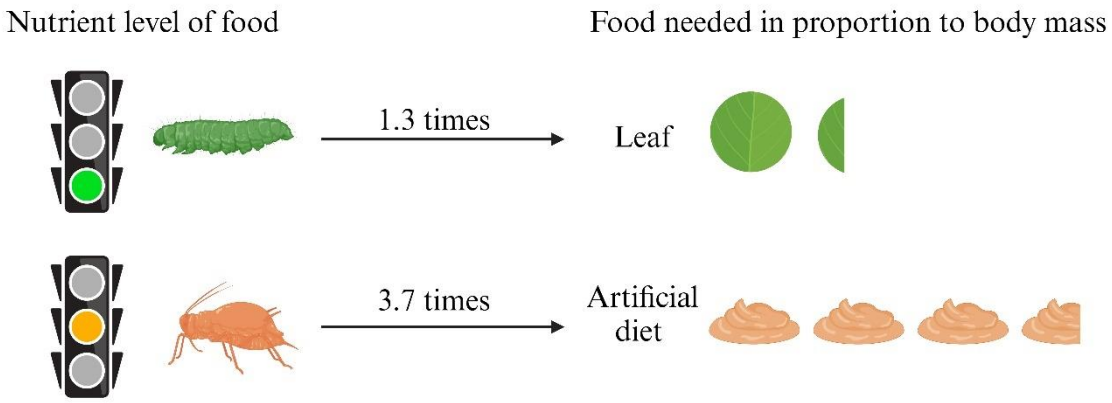


Figure 36: Schematic diet uptake to body mass ratio. Larvae consume 1.3 times in relation to their body mass, while aphid needs to consume 3.7 times in relation to their body mass.

The species-specific biological differences in the diet quantity and composition observed in this study, as well as other factors not investigated here such as gut digestive processes, may have a greater impact on the metabolic fate of chemical compounds than their intrinsic chemical properties. The unique feeding preferences and metabolic processes of individual species

suggest that the biological context is an important determinant of how a compound is ingested or absorbed, metabolized and utilized by different insects. This is not only relevant in a controlled experimental environment, but certainly also under natural field conditions where such biological diversity is the norm. Consequently, the bioavailability and uptake of chemical compounds by organisms must first be investigated from the perspective of the biological characteristics of the individual species in order to then also fully decipher the ecological effects of the compounds under investigation.

Application of TK existing for risk assessment in classified pest species

In principle, models can offer an excellent opportunity to depict complex and confusing processes or relationships in a simplified and comprehensible way. In this respect, toxicokinetic models (TK) are also suitable instruments that can contribute to the understanding of the observed differences in uptake kinetics, bioaccumulation, and the role of metabolism of the species under investigation. Standardization of the assays improves their reliability and the reproducibility of the data generated (BONTA 2002; JAGER & ASHAUER 2018). Under these conditions, comprehensive assessments of non-target risks and well-founded decisions on environmental management are made possible by the generated TK models (ASHAUER & ESCHER 2010; HOMMEN *et al.* 2015).

Toxicokinetics Model in caterpillars

c) Are there differences in the toxicokinetics of the various tested starting compounds
I.) within a single insect species?

In order to test its suitability for the exemplary relationships investigated here (compound uptake and elimination), some results obtained on *Spodoptera littoralis* were first used to model a single-compartment first-order TK model. Data from experiments with a reduced number of compounds (A-D) and Coumarin were used to demonstrate the basic feasibility of such TK modeling first.

The modeling showed that the observed differences in the quantities of the compounds in the larval bodies can be explained by different kinetic rate constants for uptake and excretion. Between the compounds, the constant for the uptake rate varied by more than ten orders of magnitude, while the constant for the excretion rate varied by a factor of only about two (Figure 24, Table 2). This variation confirms previous studies on other organisms, such as annelids (BELFROID *et al.* 1993; ŠMÍDOVÁ *et al.* 2021). TK models have already been shown to be able to predict the toxicokinetics of chemical compounds for a range of organisms (NYMAN *et al.* 2014), but the study presented here now also demonstrates, that the TK modeling approach can also be usefully adapted to target organisms.

The analytical results on the experimental uptake curves and their variability show that samples are to be taken more frequently at the beginning of the exposure period and the excretion phase in order to be able to describe the course of the processes in the model more closely (Figures 24 & S4). More data points would help to better understand critical parts of the toxicokinetics, in particular the curvature of the modelled internal concentration, and contribute to a better and more robust fit of the model.

However, the simple TK model used here is not able to explain all patterns in the observed body tissue concentration data. With the current test design, it is not possible to differentiate between the uptake of test compounds via surface contact and via oral uptake (Figures 4, 18, & 34). Therefore, the modeling of compound uptake over time was carried out without distinguishing between the two main uptake routes. Nevertheless, this different uptake pathways could be an important tool to differentiate between target and non-target species, for example due to different biological parameters such as the type of diet (Figure 36). At the very least, the compounds that are degraded in the insect should have undergone some systemic uptake, unless the gut microbiome, which has also not been considered, also contributes to biotransformation (CRISTOFOLETTI *et al.* 2003; ORTEGO 2012). Thus, the TK model (Figure 25, Table 3) unfortunately also inadequately captures the observed uptake of compound A, B and C, presumably due to the variable onset of fecal pellet excretion which appears to be the major route of elimination. Nevertheless, under these given experimental conditions, the TK model adequately captures the basic patterns of TK in *S. littoralis* L3 larvae for five different test compounds (Table 2). While the TK model generally reflected the concentrations of compounds in the larval body, the amounts of compounds measured in feces were not taken into account in the model. Additionally, the larvae of *S. littoralis* increased their body mass by a factor of 4 during the exposure because they ate the entire leaf disk (Figure S2). The possible reduction of the internal active compound concentrations due to this increase in mass over time is not taken into account. Despite these shortcomings, the applicability of this method to target species in general could be adequately demonstrated using the simplest available model. In order to understand relationships between hidden factors in the biology of organisms such as performance parameters or internal concentrations and total amounts determined by more frequent measurements, more complex TK models contribute to the necessary uncovering of the presumed starting point of detoxification by biotransformation.

Many phytophagous species, especially pest species such as of the Lepidoptera, have been shown to have a variety of enzymatic degradation pathways and detoxification mechanisms, such as excretion, to prevent bioaccumulation (DOW 1992; SCHULZ 1998; ROBERTS & HUTSON 1999; PERIĆ-MATARUGA *et al.* 2019). Since molecules with higher Log P values tend to have a

fundamentally greater affinity for biological membranes (HOFSTETTER *et al.* 2018), their bioaccumulation potential was also found to be higher (HAWKER & CONNELL 1985; ESSER 1986). These findings are confirmed in my study, as the bioaccumulation factor (BAF) generally increased (with the exception of Coumarin, which is putatively metabolized) with increasing hydrophobicity (Table 2, Table 3). However, the residence time of an initial compound in the larval body can not only significantly influence the toxic effect on a Lepidopteran pest species, but has been shown to increase the resistance potential to this compound due to enzymatic processes in pest species (WING *et al.* 1998; SIEGFRIED & SCHARF 2001). In my experiments, compounds A, B, E, F, G and Coumarin were completely eliminated from the larval bodies within the excretion period, which is why they should largely escape resistance-forming mechanisms in the tested insects (Figure 25, Table 3).

Toxicokinetics Model in aphids

The creation of a TK model for *M. persicae* was more complicated due to the biology of aphids. In particular, asexual reproduction, which is almost impossible to control, led to complex population dynamics that affected both the wet weight of the collected samples and the number of individuals collected. This complicated the use of the data for TK modeling in so far, as, although the bioassays contributed to the understanding the fate of the compounds in insect and in excretion, they showed methodological problems, especially in data integrity, which hindered the construction of a species-specific TK model. Particular challenges included the variability in individual age and population size of the aphid population and transgenerational exposure (Figures 19 & 37), which repeatedly challenged the assumption of a constant compound to wet weight ratio, which is fundamentally required for a simple TK model (mg $_{\text{compound}}$ / mg $_{\text{aphid wet weight}}$).

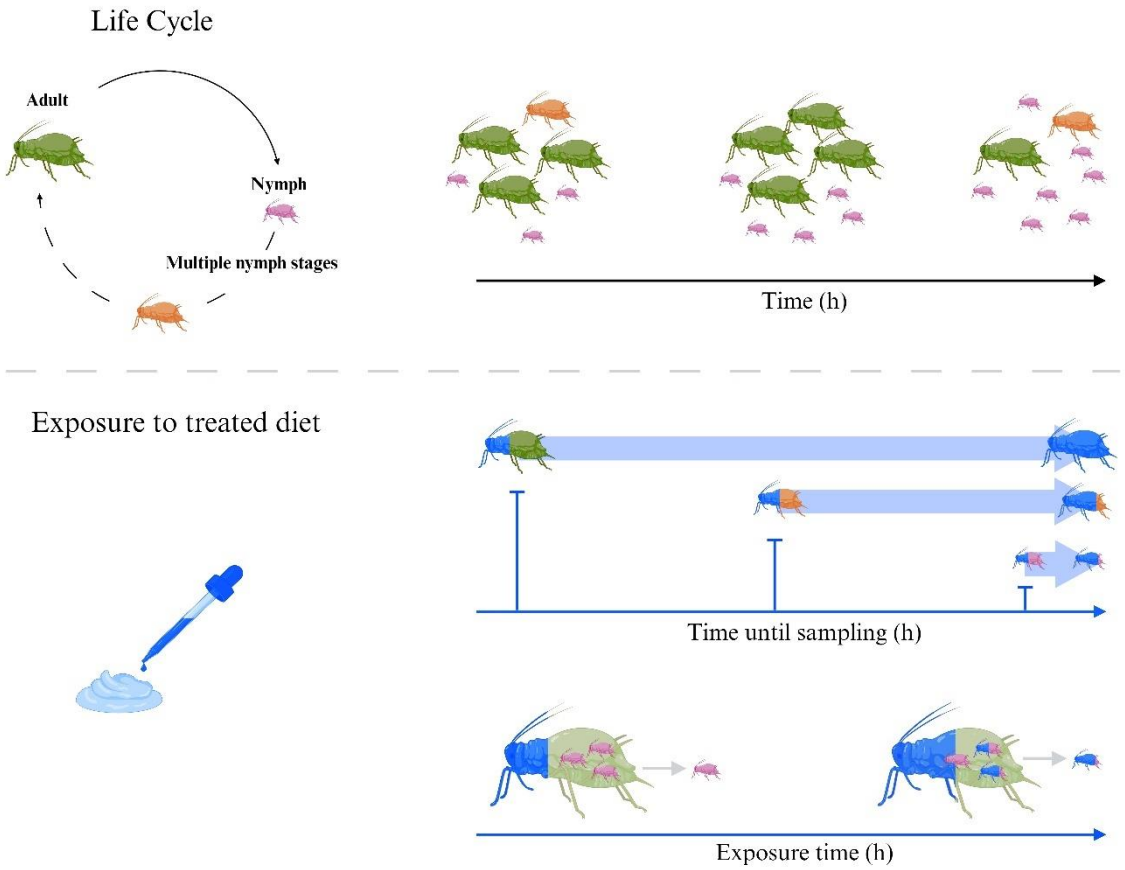


Figure 37: Limitations for the use of aphids as reference species in toxicokinetic modeling: Reproduction, variability in exposure time, and transgenerational exposure. The figure shows how the asexual reproduction of aphids influenced changes in population dynamics over the period of the bioassay. It also highlights the variability in age and number of aphids, which in turn affects the precise duration of exposure to the compounds tested for individual aphids. In addition, the graph shows the potential for transgenerational exposure, where offspring may be indirectly exposed to compounds through adult aphids (mothers). Created with BioRender.com

Given the challenges identified, it is clear that constructing such a simplistic toxicokinetic model for aphid populations is untenable and could lead to misinterpretations (Figure 37). In contrast, more complex models that account for reproduction, changing population numbers and variable exposure times could provide a more comprehensive understanding of toxicokinetic processes in aphid populations (JAGER 2021; JAGER *et al.* 2023). However, a revision of the current bioassay methodology is essential to improve the required data quality and consistency. By synchronizing adult aphids, collecting new nymphs daily and focusing measurements on adult specimens, the variability of body mass data could be reduced. These methodological improvements would rationalize the data and thus significantly improve the reliability and interpretability for the TK models. Overall, aphids appear to be suitable study and reference organisms in a simple TK model study to understand the fate and effects of active ingredients, to

monitor reproductive performance and population dynamics as an indicator of effects of active ingredients.

In order to develop a more accurate model-based understanding of toxicokinetics in aphid populations, not only will bioassay protocols need to be optimized, but far more complex modeling procedures will need to be developed. These concerted efforts would allow us to achieve a higher level of precision and insight. As this was not yet feasible under the actual working conditions, I decided to exclude the current aphid data from the comparative analysis of the TK model in target and non-target species. However, this does not mean that the aphid data will be permanently excluded from the analysis; they may be revisited in future research to complete the understanding of species selectivity in TK studies or to refine the modeling.

Are target and non-target species comparable?

After demonstrating that TK-modeling works in principle with the data sets from the bioassays used in my experiments, a second “level” comparative TK Model (2) was developed that describes the differences between target (*Spodoptera littoralis*) and non-target (*Chironomus riparius*) species and focused on the toxicokinetics and biotransformation of seven test compounds and Coumarin.

Since the size, growth, and body mass of an organism are crucial components in describing the uptake of a compound over time, they were therefore implemented into the second TK Model for both species. During the growth of an organism, the concentration of a compound continuously ingested via food is more or less continuously diluted by its growth-related mass increase, which is described by the factor called "growth dilution” (Figure 38). In the basic TK Model 1 it was shown that the larvae of *S. littoralis* can grow by a factor of 4 within the exposures (Figure S14). In contrast the larvae of *C. riparius* hardly grew within this time (Figure S15). The application of the factor for the growth dilution in the current model contributed to a better model fit in comparison to the TK- model not including growth related mass changes (Figures 14-18, Table S1). This clearly suggests that accounting for growth dilution is a critical factor for accurate modeling of toxicokinetics in developing organisms. The previous experiment also showed the role of fecal excretion, but this will not be introduced into the model at this stage to see if the models can only be improved through biotransformation and growth.

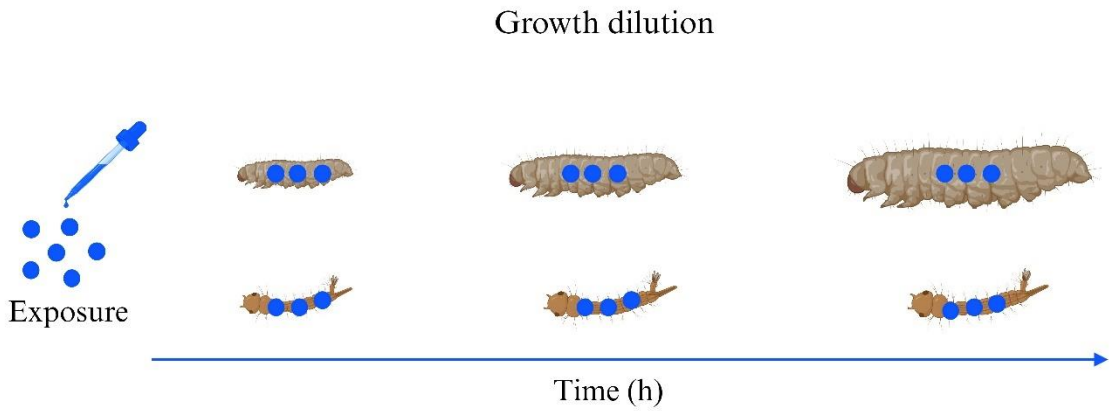


Figure 38: Growth dilution over time. Schematic overview of exposure and dilution by growth of a caterpillar and mosquito larva over time. Created with BioRender.com.

On this basis, TK models could be successfully developed for all tested compounds for the *S. littoralis* larvae, for *C. riparius* except for the in this species lethal compounds C and G (Table 5). The elimination rate constants (k_e) of compound C, F and G for *S. littoralis* could not be determined from the data and were therefore fixed (Figures 28-29, Table 4). It turned out, that better TK descriptions were obtained when biotransformation and larval growth data were included, as this allowed a more detailed mapping of TK specific differences between the two species (Figures S4 & S11). For *C. riparius*, no adjustment of the TK model by setting elimination rate constants (k_e) was required, which may be due to the data available for a larger number of sampling points in the *C. riparius* bioassay (Figure 30, Table 4).

The *S. littoralis* model, which is similar to the basic TK model (1), accurately captures both the concentration dynamics at the end of the 24 h exposure and over the elimination phase, but captures the concentrations observed in the initial sampling intervals less well. The same applies for *C. riparius* larvae exposed to compound D (Figure 28-30).

c) Are there differences in the toxicokinetics of the various tested starting compounds

II.) between different insect species?

TK analysis of both species showed clear differences between the concentrations of the parent compounds. The comparison of the species shows that the uptake (k_{in}) and elimination (k_e) constants were generally higher in *S. littoralis* than in *C. riparius*, with the exception for compound D (Figures 31-32). The highest uptake rate constants were detected for compounds with a Log P value between 2 and 2.5 (Figure 31, Table 4). This could indicate a more efficient uptake and distribution of the compounds within individuals of the species *S. littoralis*, possibly indicating a higher bioavailability of the compounds, which is desirable in terms of pest control efficacy. In particular, the models for compounds A, B, C, F and G showed fast kinetics,

indicating rapid uptake and processing in the organism (Figures 28-30, Table 7). A flat, angular shape of the modeled curve for the uptake of the compounds into the absorbing body facilitates the rapid achievement of a steady state for the compounds (Figures 28-30, Table 4), whereby this pattern was influenced for compounds C, F and G by the fixing of the elimination rate constants. In contrast to *S. littoralis*, Coumarin was the only compound in *C. riparius* to show fast kinetics, while all others showed slow kinetics (Figure 30, Table 7). The finding that the modeling of the uptake rate constants of the sampled compounds splits the investigated species into those with fast and slow kinetics contradicts the findings from the sampling intervals. For *S. littoralis* exclusively fast kinetics (except compound E), for *C. riparius* only slow kinetics (but except Coumarin) were found. Therefore, the time of sampling for *C. riparius* could critically influence the result of the modeling that can be read from the curve. The kinetics of metabolites are not further subdivided into fast or slow, as they always depend on four factors. These are the availability or uptake of the parent compound, its retention time, excretion and metabolism. Three of these factors always depend on the kinetics of the parent compound and are therefore directly linked to each other.

Nevertheless, the fact that in the *S. littoralis* body the concentration of many compounds reaches a near steady-state condition within only one hour after the start of exposure indicates fast kinetics and a real difference between the species. This rapid uptake could be attributed to the possible presence of two simultaneous pathways facilitating compound uptake (Figure 34).

Table 7: Kinetics of parent compounds. Assumed fast kinetics defined as reaching steady state within the first two sampling times within the exposure, slow kinetics include those not clearly reaching steady state in this period.

Compound	*Spodoptera littoralis*	*Chironomus riparius*
A	fast	slow
B	fast	slow
C	fast	–
D	fast	slow
E	slow	slow
F	fast	–
G	fast	–
Coumarin	fast	fast

Previous research has already demonstrated the utility of TK models in predicting the dynamics of compound uptake and elimination in a wide range of organisms (HENDRIKS *et al.* 2001; GRECH *et al.* 2017). Studies have also highlighted species-specific TK differences (NYMAN *et al.* 2014), for example in annelids (BELFROID *et al.* 1993; ŠMÍDOVÁ *et al.* 2021) or the

insecticide Chlorpyrifos in 15 freshwater arthropod species (RUBACH *et al.* 2010). With this study it could be demonstrated that the uptake and elimination rate constants for the tested pest and non-pest species varied to different ranges and extend. *S. littoralis* exhibited equal variability (four orders of magnitude) in uptake and elimination rates, whereas *C. riparius* displayed lower and asymmetric variability, with uptake rates varying by three orders of magnitude and elimination rates varying by only two (Table 4). These results suggest that *S. littoralis* may have a broader range of kinetic capabilities than to *C. riparius*, which appears to have a more consistent and potentially lower overall excretion capacity.

d) Does the biotransformation of the test compounds differ in the animal species tested?

Differences in biotransformation were observed between the species *S. littoralis* and *C. riparius*, each of which exhibited unique biotransformation profiles characterized by changes in the molecular mass of the compounds tested (Figures 28-30, Table 3). Three conspicuous mass shifts detected, a decrease of 14 Da and increases of 16 and 18 Da, indicate specific biotransformation reactions taking place in the organisms: a demethylation reaction (decrease of 14 Da), leading to a more hydrophilic metabolite that can be more easily be eliminated, and oxidative transformations such as hydroxylation, characterized by the increases of 16 and 18 Da (TESTA & KRÄMER 2006; GUENGERICH 2008).

Biotransformation is an important physiological process, involving the enzymatic conversion of compounds into more hydrophilic, readily eliminable compounds, or in some cases to activation of intended or unintended effects. This process plays a key role in preventing bioaccumulation, facilitating detoxification, and ultimately influencing the toxicity of compounds (ASHAUER *et al.* 2012; ROSCH *et al.* 2016). Understanding factors that influence biotransformation, such as life stage, sex, diet, and environmental conditions of insects, is essential for predicting the effects and fate of pesticides (BUCHWALTER *et al.* 2004; LE GOFF *et al.* 2006; WATERS & HARRISON 2012; SHAKOUR *et al.* 2022). In addition, biotransformation is closely linked to the development of insecticide resistance, which is a growing problem in the development and implementation of pest control strategies. By analyzing biotransformation processes in detail, researchers could potentially identify critical specific metabolic patterns responsible for this. This knowledge is crucial for monitoring species-specific metabolic responses to compounds, such as putative insecticides, and for recognizing differences in biotransformation capabilities of different species (NYMAN *et al.* 2014).

The study presented here confirms this, as the biotransformation profiles developed therein show that metabolites of the parent compounds are present at different times and in different concentrations (Figures 28-30, Table 3). No metabolites were detected for compound B in either

species, indicating no biotransformation had occurred or that metabolites were present at most below the detection limit (Table S3). Only three compounds, namely compound E, F and Coumarin produced the same four putative mass changes in both species across the entire range of compounds tested (Figure 33), namely, two metabolites for compound F, one metabolite for compound E and Coumarin. A wide range of metabolites for compounds C, D, E and G was detected for *S. littoralis*, suggesting a more complex enzymatic system for detoxification (Table 3, Figure S13). In contrast, *C. riparius* showed a more limited biotransformation capacity, indicating a comparatively lower ability to effectively detoxify and excrete compounds. However, the limited biotransformation capacity of this species may be an artefact of our limited quantification capabilities (lower detection limit in *S. littoralis* than in *C. riparius*) rather than due to the biological capacities of the species itself (Table S3). *S. littoralis* consistently produced two metabolites for all compounds, whereas *C. riparius* produced two metabolites for compound F and only one otherwise.

Compared to *C. riparius*, the constants for metabolite formation (k_{m_f}) from compounds E (+ 16 Da) and F (mass change of -14 and +16 Da) were higher for *S. littoralis* larvae (Figure 33a). The constants for the rate of formation of Coumarin metabolites were also higher in *C. riparius* (Figure 33a). The elimination rate constants (k_{m_e}) of biotransformation products with a mass change of -14 (compound E and F) were higher in *S. littoralis*, suggesting a species-specific efficiency in these elimination processes (Figure 33b). In contrast, the elimination rate constants of biotransformation products with a mass change of + 16 or + 18 (compounds F & Coumarin) were higher in *C. riparius* (Figure 33b), suggesting their more efficient elimination mechanisms or greater detoxification capacity (Table 8).

Table 8: Biotransformation pathways for compounds tested in both species.

	Spodoptera littoralis			*Chironomus riparius*		
Compound	Number of metabolites	Metabolite formation rate constant (k_{m_f})	Metabolite elimination rate constant (k_{m_e})	Number of metabolites	Metabolite formation rate constant (k_{m_f})	Metabolite elimination rate constant (k_{m_e})
E	2	Demethy-lation faster	Demethy-lation faster	1		
F	2	Demethy-lation faster	Demethy-lation faster	2		
		Oxidation faster				Oxidation faster
Coumarin	2			1	Oxidation faster	Oxidation faster

A previous study on the activity of detoxification enzymes in *Spodoptera frugiperda* (SMITH, 1797), a species closely related to *S. littoralis*, demonstrated that several enzymes as part of the detoxification activities, including microsomal oxidases (such as hydroxylase, N-demethylase or O-demethylase) and hydrolases, were more concentrated in an insecticide-resistant experimental group than in a susceptible strain (YU *et al.* 2003). This indicates a complex interaction with the compounds and possible biotransformation leading to detoxification of the compounds. Similarly, NOVOSELOV *et al.* (2015) and others have found that *S. littoralis* can detoxify certain insecticides with neuronal mode of action through amino acid conjugation, suggesting an adaptive detoxification mechanism (HEMMATI *et al.* 2022). Thus, the biotransformation capabilities of this species combined with its feeding mechanism, digestive physiology and the biochemical composition of its diet could facilitate faster elimination and enable adaptation to plant toxins and trigger insecticide resistance (HILLIOU *et al.* 2021). This poses significant challenges for control of this species as a pest. Finally, there is a need to investigate the metabolic pattern of *S. littoralis* in detail as part of future comparative studies on resistant and still sensitive strains to gain further insights into its biotransformation capabilities associated with resistance.

Such insights into biotransformation could be instrumental in the development and use of targeted pesticides and help minimize their impact on the environment. Systematic identification of compounds with the lowest possible bioaccumulation potential in non-target species would provide a realistic opportunity to develop more environmentally friendly pest control solutions. The described differences in the biotransformation capabilities of *S. littoralis* and *C. riparius* are good examples in this respect, as they can be used to describe the direct effects of the persistence of compounds in different ecological contexts. The different biotransformation rate constants for one and the same metabolite occurring in both species may indicate which of the species has a higher biotransformation capacity. Biotransformation pathways that are more pronounced in non-target species can either lead to detoxification or unintentionally increased toxicity. It therefore seems important to emphasize here that potential detoxification mechanisms should or must be investigated not only in the *C. riparius* studied here, but also in the diversity of non-target species in general.

Bioaccumulation of test compounds

The rate of uptake (k_{in}) and elimination (k_e) were generally found to be higher in *S. littoralis* than in *C. riparius*, which was true for all compounds with the exception of compound D. The Bioaccumulation factor was consistently higher in *C. riparius* (Figures 31-32). However, the BAF in *C. riparius* does not appear to be the result of higher uptake *per se*, but rather of slower elimination kinetics (k_e). This is supported by the differences in the biotransformation between the two species. According to my results, elimination seems to be the most important indicator

of increased BAFs and species differences. The higher BAF in *C. riparius* could be due to a difference in the elimination or biotransformation capacity of the two species (Figures 31-32). This may lead to biomagnification in aquatic food chains where *C. riparius* is abundant, and also depends on chemical uptake efficiency across the gut of predators, predator diet composition and feeding rate, as well as the characteristics of the food-web (FRANKE *et al.* 1994; ARNOT & GOBAS 2004). Further studies of such potentially active compounds in the test organism *C. riparius* are needed to better understand the role of their biotransformation, which have already been shown to be relevant in other aquatic invertebrates e.g., *Gammarus pulex* (L., 1758) (ASHAUER *et al.* 2012; JAGER 2018).

Differences in TK between species are critical for bioaccumulation in organisms and possible biomagnification of harmful compounds in food chains (THOMANN 1989; MCLACHLAN 1995; GRAY 2002; KELLY *et al.* 2007). As *C. riparius* is an important food source for freshwater fish, amphibians and various water birds and playing a central role in aquatic food webs (BERTIN *et al.* 2014; HANSLIK *et al.* 2020), any concentration of contaminants in them could have far-reaching ecological consequences (CLEMENTS *et al.* 1994; GRAY 2002; ARMITAGE & GOBAS 2007; KELLY *et al.* 2007). The pest species *S. littoralis* is also a similarly important food source for predatory insects, reptiles, birds and small mammals and thus plays an important role in terrestrial food webs (SALAMA & ZAKI 1984; MOHAGHEGH *et al.* 2012).

Limitations of toxicokinetic models

The use of toxicokinetic (TK) models to predict the behavior of compounds in biological systems is a powerful tool in environmental toxicology. However, the current model, applied here to two different species, *S. littoralis* and *C. riparius*, still has some limitations that need to be taken in account when interpreting the results:

First of all, the two species have inherently different uptake routes for active ingredients due to their biology (behavioral ecology). The terrestrial chewing-biting insect *S. littoralis* takes up the active ingredients both by contact via tarsi or prolegs and by oral uptake when it crawls and feeds on preventatively treated leaf disks. In contrast, the aquatic insect *C. riparius* is mainly exposed by passive uptake by contact with its complete bodies' surface to the surrounding medium. These differences in uptake pathways may influence the distribution and biotransformation of compounds within each species and, as an inevitable consequence, the results of the TK model.

Secondly, the model represents the total amount of compounds contained in the body as an "insect homogenate", which also includes any suspected compound on the exterior of the insect body. Thus, the approach may not accurately reflect the actual internal concentrations that are biologically relevant in toxicodynamics studies (but see specially adapted treatment of aquatic *C. riparius*). Furthermore, the model may not adequately cover early time points of

transformation processes for precursors with fast kinetics, which must lead to an underestimation of the initial uptake. To solve this problem and to accurately represent kinetics during the critical early uptake period, additional sampling should be performed at earlier stages of exposure with higher frequency than at later stages of the experiment. This sampling scheme should also be adopted at the beginning of the elimination phase for the same reason.

Finally, it was assumed that mass changes of detected chemical substances indicate the presence of metabolites without being able to identify them direct. This can lead to inaccuracies in the model if these detected mass changes do not correspond to the assumed metabolites actually formed in the organisms. This also includes the possibility that some metabolites cannot be detected because their concentrations do not exceed the detection limit. This applies in particular to analyses of *C. riparius*-samples, as their body mass is lower than that of *S. littoralis* larvae (Ø 17.15 mg). While the concentration of metabolites (expressed in mg/mg wet weight) could theoretically be the same for both species, the absolute mass of such compounds accumulated by *C. riparius* could be below the detection limit required for analysis due to its lower total biomass (Ø 2.4 mg). In other words, despite potentially identical concentrations, the lower absolute total amount of accumulated compounds in *C. riparius* compared to *S. littoralis* could pose a particular technical challenge for metabolite detection at the end of the exposure period that remains to be solved (Table S4). In my study, the limit of quantification of metabolites in *S. littoralis* was found to be about one order of magnitude lower. Therefore, the probability of metabolite detection is higher in *S. littoralis* than in *C. riparius*, as lower concentration could be detected (Table S3).

In summary, it must be stated that both (simple one-compartment first-order) TK models can only explain the patterns observed in the body tissue concentration data to a limited extent. Furthermore, these models cannot distinguish between exclusive intestinal passage and systemic absorption. Despite these, limitations the advanced second-level TK model was able to adequately capture the basic toxicokinetic patterns for various tested compounds in both investigated species within the experimental framework and provide information on the kinetics.

Evaluation of species selection as TK model organisms

Overall, the results of this study show that well-established approaches in environmental toxicology and non-target species risk assessment can be adapted to facilitate or enable comparisons between target and non-target organisms (Table 9). Adaptation not only allows species-specific differences to be highlighted or recognized that are relevant to the challenges and limitations in bioassay design, but also provides insights into the criteria and importance of selecting appropriate toxicokinetic (TK) models to capture and map toxicokinetic behaviors and

biotransformation processes. Such strategic adaptations have the potential to contribute to high selectivity in pest control measures.

The species studied organisms proved to be well-suited study models by providing valuable insights into the fate of compounds within a bioassay, thus also illustrating the role of excretion in pest species.

However, the applicability of TK models is not the same across all species, with particular variability observed for organisms such as aphids. The effectiveness of these models for asexually reproducing homologous pest species has yet to be fully demonstrated, raising the question of their broad applicability. The application of TK models may also require significant adaptations or the development of new complex bioassays, which may increase the time and analytical resources required for the experiments. Therefore, it seems advisable to select and establish a wide range of target and non-target species as reference organisms for specific studies to improve the efficacy of TK models, particularly for compound screening.

In the next analytical step, species with analogous absorption pathways should also be compared, as their specific biology - including factors such as food sources, uptake rates and excretion mechanisms - has or at least can have a significant impact on the selectivity of species to toxic compounds. In the final step, the observed species differences should then be tested experimentally with real insecticides and their potential effects.

Table 9: Factors determining suitability for modeling TK based on the experimental results.

Requirements	*S. littoralis*	*M. persicae*	*C. riparius*
Recognized as Indicator Species	toxicity studies	toxicity studies	risk assessment
Gain permissions to perform bioassays	yes	yes	yes
Rearing and handling are easy to control	yes	yes	yes
Species are well descried in literature	yes	yes	yes
Research Objective Suitability			
Quantifiable Exposure	yes	yes	yes
Clear exposure routes	no	yes	yes
Fate of compounds	yes	yes	yes
TK Model	yes	no	yes
Growth in different life stages	yes	yes	yes
Observation of single life stages	yes	no	yes
Image observation possible	yes	no	no
Reproduction	no	yes	no
Analytics allows observation of single individuals	yes	no	no
Possible alternative endpoints (exposure effects)	growth, movement, food consumption, excretion	reproduction	growth & movement

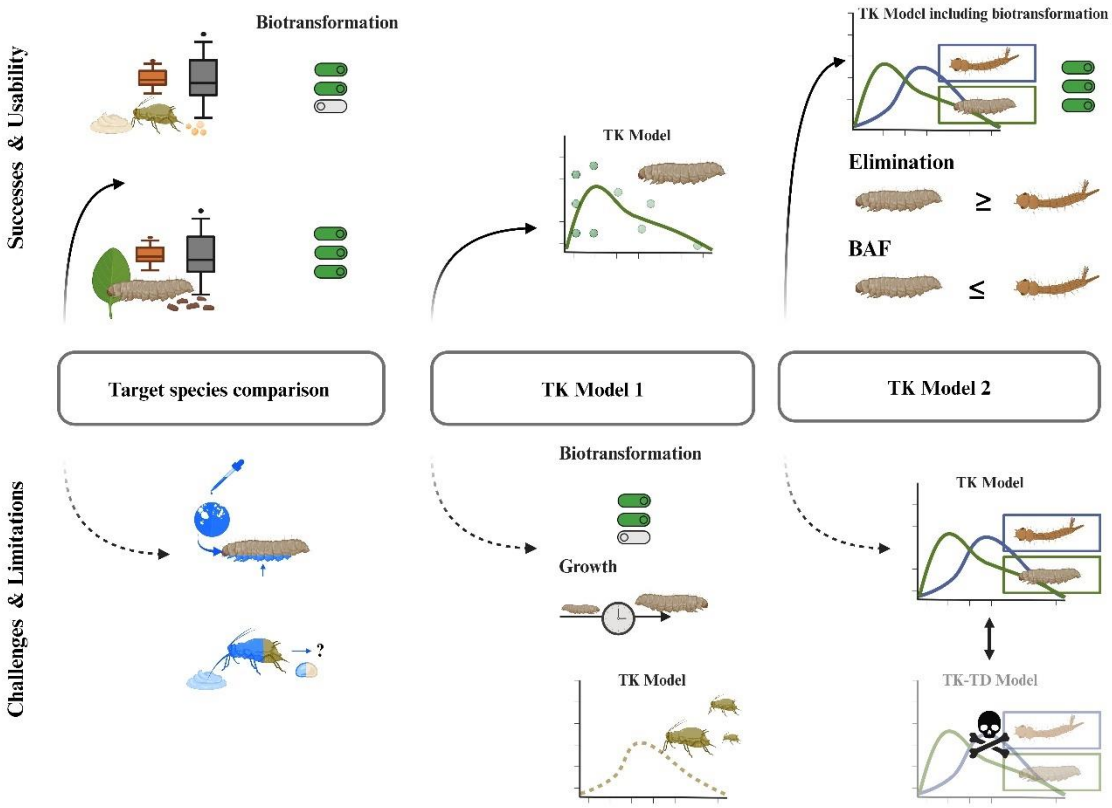


Figure 39: Summary of study successes and usability, as well as challenges and limitations.

Conclusion

The central topic of this study were to figure out whether selected chemical compounds have species-specific effect on selected insect species and whether TK models can be developed and used to depict these effects (Figure 39).

The first unexpected result was that, in addition to Coumarin, which was originally include in the study as extraction standard, the compounds (C and G), turned out to be toxic for two out of three organisms on they were tested, which is critical, but they were anyway not intended to be used in pest control. The risk potential for Coumarin was already known from the literature (PAVELA *et al.* 2021).

The exact reasons why the above-mentioned compounds in spite of not possessing a toxicofor are toxic to the insect species in the test is unknown, as is the background of their biotransformation. Fact is, that the insects studied have different biotransformation processes with unique pathways, timing and concentrations. This might not only pose a threat in terms of their pesticide sensitivity but could provide opportunities for the development of improved species-selective compounds that could be used as selective insecticides. The species-specific biotransformation differences could help to increase their efficacy against target pests while reducing their impact on non-target species. Insecticidal substances that combine high uptake and elimination potential in target insects with minimal excretion capacity in non-target insects would probably tend to lead to a higher bioaccumulation and thus pose ecological risks and should therefore be excluded from further use, just like shown for the inactive test compounds in *C. riparius*. Substances that show the opposite in bioassays could possibly proof to be optimal insecticides, as the risk they pose to the environment appears to be comparatively low. These findings could or should be the starting point for a refinement of available active ingredients, either by increasing of elimination rate in non-target insects or by insecticides that areas specifically activated by the biotransformation pathways in the target insects. Such tailored approaches could significantly improve the precision and sustainability of inevitable pest control measures.

Third, the measured amounts of compounds in the insect bodies do not reflect their true turnover in the system. This means that any measurement of the amount of chemical compounds chemically analyzed from biological samples must take into account that the values may underestimate the amounts actually present in the system if a mass balance (as in this study) cannot be determined. This problem can be partially solved by the standardized inclusion of excretion products in the analysis, but this still does not take into account the source of a potential contaminant. This is relevant in situations where such analysis needs to be performed as part of standardized environmental monitoring. If the sources of contaminants in diet are not

taken into account, which in some cases (e.g., honeydew from aphids for ants or honey bees) are excretion products, this may lead to an incorrect assessment of the observed situation (CALVO-AGUDO *et al.* 2022). Such inaccurate results can then even lead to necessary protective or remedial measures not being taken at the investigated sites due to the actual exposure, with potentially critical consequences. In some cases, the monitoring or data evaluation standards would therefore have to be adapted to these findings in the future.

The use of TK models seems appropriate to identify and justify methodological changes for the analysis of environmental changes on the one hand and for the development of insecticides that are more environmental friendly for the environment and particularly for non-target organism on the other (HOMMEN *et al.* 2015; JAGER & ASHAUER 2018; PRODUCTS *et al.* 2018). They can serve as a source for evaluating the potential effectiveness of changes in methods or effects of new substances even prior to their registration. This is also consistent with RUSSELL (2005), who developed the ethical principle of the three Rs: Replacement, Reduction and Refinement. According to this principle, the use of animals must be reduced or replaced by other methods as early as the design stage of animal experiments. TK models offer the insights required by RUSSELL, particularly with regard to the range of simulation alternatives, which can also reduce the costs and time required for research. For example, they can be used to predict required compound concentrations over time, independent of species, number of individuals tested and sampling, taking into account dose-response.

The use of models thus also has an impact on another social aspect, as they reduce the costs of developing new insecticides, their necessary approval by the responsible institutions as part of the risk assessment and their subsequent monitoring in the environment by research institutions and non-governmental organizations (NGOs). Models also provide profound data for legislative and administrative institutions to decide on approvals or restrictions for (further) use and cost-effective monitoring of toxic substances by humans in the field. Irrespectively of all the limitations outlined above, the use of models at the present time appears to be not only appropriate but also necessary as the best possible interim solution.

The basic problem formulated by RUSSELL (2005) is exacerbated by an aspect he did not consider, which has meant that insects have often not been included in conservation efforts. The principle formulated by George ORWELL in his book Animal Farm "*All animals are equal, but some animals are more equal than others*" describes an emotionally based condition that has historically led conservation efforts to clearly favor vertebrates, a bias known as "institutional vertebratism" (LEATHER 2013; ORWELL 2021). Nevertheless, there is no justifiable reason why insects should be given a lower priority than birds or mammals in terms of their worthiness for protection and conservation (CARDOSO *et al.* 2011). This bias is also likely to have contributed

significantly to the fact that the decline of insect had received and partially continues to receive too little attention and consideration compared to other more highly developed taxonomic groups (CARDOSO *et al.* 2020).

This has now been recognized, and several comprehensive associations of numerous scientists have recently drawn attention to the problem in large and developed and presented proposals for solutions (CARDOSO *et al.* 2020; SAMWAYS *et al.* 2020; HARVEY *et al.* 2023).

According to these authors, major economic and socio-political transformations seem inevitable if insects and biodiversity as a whole are to be protected at all (HARVEY *et al.* 2023), especially in the key areas they cite in RIPPLE *et al.* (2022): Reduction of fossil fuel consumption, reduction of short-lived pollutants, restoration of ecosystems, conversion to plant-based diet, transition to a circular economy and stabilization of human population.

According to SAMWAYS *et al.* (2020), halting the loss of insect requires the conservation, restoration and creation of habitats that provide all the essential eco-functional characteristics necessary for the long-term maintenance of sustainably reproducing insect populations and species. This requires large, high-quality areas that actually enable the necessary dynamics of natural ecosystems. The state that should be achieved as a reference point on the areas corresponds to the state that prevailed before the start of human intervention in the industrial age (ULICSNI *et al.* 2016). It is essential to monitor the effectiveness of all conservation measures, even if promising strategies have already been successfully applied in various habitats and ecosystem (SAMWAYS *et al.* 2020).

In addition to the protection of natural areas, ecologically compatible land use is of paramount importance in the context of development and agriculture. This means that current methods of soil cultivation, sowing, harvesting and pest control must be further developed and adapted to the requirements of conservation objectives. As changes in these areas take time, measures to control harmful insects using insecticides are particularly important as they must help to ensure the global food supply until alternative methods for food security are found. In this context, the development of highly specific substances for agricultural use is necessary as long as, for example, wheat, millet and other crops are cultivated in large monocultures sensitive to damage by pest invasions. One of the most impressive global examples of pest invasions are those of crickets: The Desert Locust *Schistocerca gregaria* (FORSSKÅL, 1775) regularly destroys the harvest of the African population (FAO 2024). When the migratory swarms of this grasshoppers, which are often more than 100 km^2 in size and consist of billions of individuals, reach cultivated areas, it only takes a few minutes to completely destroy the cultivated crops by defoliation (clear-feeding). In the last decade, the mass reproduction of locusts has been triggered by increasingly frequent rainfall, which means that local people are going hungry due to the

increasingly frequent plagues of locusts (KESPER 2021). In addition to the increase in rainfall in arid regions triggered by climate change, the lack of affordable and appropriate insecticides for locust control is one of the reasons making for this crisis in preventing crop losses and famine (KESPER 2021; FAO 2024).

On the other hand, this conflict could be alleviated somewhat if the local human population could or would use the crickets more frequently as food (EGONYU *et al.* 2021). The dilemma of making crickets unfit for consumption through the use of presently available insecticides could be at least partially solved by the development and use of insecticides that have rapid species-specific toxicity and low persistence, which would also be of advantage in environmental protection - an interesting and challenging development task for scientists and the chemical industry.

Regardless of such situations, in which human nutrition must be secured, it is important to protect insects population to ensure that they can maintain their often crucial role in global ecosystems (HOCHKIRCH *et al.* 2018).

Apart from reducing the use of pesticides and fertilizers, or the even better the use of alternatives, the creation of habitat networks is necessary (GURR *et al.* 2016; BERNHARDT *et al.* 2017). Protective measures do not always have to be large-scaled and institutionalized; in urban areas, individuals can also make an important contribution by designing their private or business habitat, be it gardens, balconies, windowsill or roofs, in such a ways that it offers pesticide-free refuges, water sources and food for insects and other animals (DEGUINES *et al.* 2020). In principle, pesticides have no place in private gardens and should only be used if absolutely necessary to ensure human nutrition. Insect-friendly gardening in urban areas can now make an important contribution to insect conservation (COSQUER *et al.* 2012; MACDONALD *et al.* 2015).

However, the example of the migratory grasshoppers not only shows that there are technical limits to the influence of human on insect populations, but also that our ecological background knowledge is far from sufficient to be able to estimate how the development of insect populations will be influenced by climate changes in the long term. It also remains unclear whether the changed ecological conditions will also affect the effectiveness and applicability of plant protection products. In view of the paramount role of insect in their natural habitats and – for better or for worse – in human nutrition, it is abundantly clear that research into the fundamentals of ecosystem interactions and possible ways of sustainably combating or protecting them, although worthwhile in many respects, is still insufficient. The most important goal that can be achieved for humanity, namely the preservation of biodiversity, is well worth the effort!

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Appendix

The following supporting figures and tables are included in the appendix.

Table S1: Toxicokinetic model parameters: Wet Weight growth rate (b, (1/h)) and Maximum Organism Size ($W_{m,}$ in $mg_{wet\ weight}$) for *Chironomus riparius* and *Spodoptera littoralis* larvae.

Table S2: Toxicokinetic model parameter symbols, names, and dimensions/ units.

Table S3: Comparative toxicokinetic parameters for *Spodoptera littoralis* and *Chironomus riparius.*

Table S4: Chemical structure of test compounds.

Figure S1: Performance parameters of *Spodoptera littoralis* larvae: consumed leaf area during exposure period.

Figure S2: Performance parameters of *Spodoptera littoralis* larvae: larval size over time.

Figure S3: Performance parameters of *Spodoptera littoralis* larvae: number of feces pellets.

Figure S4: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* feeding contact leaf disk assay for the five test compounds (A-D, Coumarin).

Figure S5: Exposure profiles: a) compound quantities (mg) in leaf disks over time. b) Compound quantities (mg) in artificial diet over time.

Figure S6: Parent compounds (sorted by Log P) in insects and excretion

Figure S7: Parent compounds in insects and excretion. Quantified per *Myzus persicae* aphids (body) and honeydew.

Figure S8: Parent compound quantities (mg) *Spodoptera littoralis* (larvae) and *Myzus persicae* (calculated aphid equivalent).

Figure S9: Exposure scenarios in the *Spodoptera littoralis* toxicokinetic study: soybean leaf disk concentrations during exposure (0- 24 h) and depuration time (24- 48 h).

Figure S10: Exposure scenarios in the Chironomus riparius toxicokinetic study: water concentrations during exposure (0- 24 h) and depuration time (24- 72 h) (mg/L).

Figure S11: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* toxicokinetic assay for the five test compounds (A-G, Coumarin).

Figure S12: TK model likelihood profile for predicted parameters for the *Chironomus riparius* toxicokinetic assay for the five test compounds (A-F, Coumarin).

Figure S13: Biotransformation of all metabolites per species (Table 1, Table 2) in (a) *Spodoptera littoralis* and (b) *Chironomus riparius*.

Figure S14: Wet weight (mg) of *Spodoptera littoralis* individuals during the toxicokinetic study.

Figure S15: Wet weight (mg) of *Chironomus riparius* individuals during the toxicokinetic study.

Figure S16: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (A-D).

Figure S17: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (E-G, Coumarin).

Figure S18: TK model likelihood profile for predicted parameters for the *Chironomus riparius* individuals during the toxicokinetic study for the test compounds (A-D).

Figure S19: Body concentrations in *Spodoptera littoralis* larvae exposed to test compound C.
Figure S20: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds C.
Figure S21: Body concentrations in *Spodoptera littoralis* larvae exposed to test compound F.
Figure S22: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compound F.
Figure S23: Body concentrations in *Spodoptera littoralis* larvae exposed to test compound G.
Figure S24: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compound G.

The following toxicokinetic model scripts are included in the appendix.

TK Model 1

Derivatives 1

Call_deri 1

Bioaccumulation Factor

TK Model 2

Derivatives 2

Call_deri 2

The following main data tables (raw) are included in the appendix.

Data *Spodoptera littoralis* – TK Model 1 and 2

Data *Chironomus riparius* – TK Model 2

Data *Spodoptera littoralis* feces– Compound quantities

Data *Spodoptera littoralis* – Compound quantities

Data *Myzus persicae* honeydew– Compound quantities

Data *Myzus persicae*– Compound quantities

Please contact me for further information and data if it is not provided in the supporting information, or if more data on organisms or sample individuals are required for the genetic identification of species (isis.roemer@web.de).

Tables and Figures

Table S1: Toxicokinetic model parameters: Wet Weight growth rate (b, (1/h)) and Maximum Organism Size ($W_{m,}$ in $mg_{wet\ weight}$) for *Chironomus riparius* and *Spodoptera littoralis* larvae.

Species	Compound	b	[conficence interval]	Wm	[conficence interval]
Spodoptera littoralis	A	0.096	[0.067;0.130]	41.430	[32.41;60.03]
	B	0.094	[0.065;0.127]	43.410	[33.57;64.27]
	C	0.038	[0.020;0.059]	151.400	[79.37;500*]
	C fixed	0.038	[0.020;0.060]	151.400	[79.37;500*]
	D	0.094	[0.065;0.127]	43.410	[33.57;64.27]
	E	0.086	[0.057;0.120]	46.970	[35.12;75.04]
	F	0.037	[0.020;0.056]	159.600	[84.99;500*]
	F fixed	0.037	[0.018;0.056]	159.600	[84.99;611.7*]
	G	0.078	[0.051;0.108]	52.280	[38.42;86.99]
	G fixed	0.078	[0.051;0.108]	52.280	[38.42;86.99]
	Coumarin	0.081	[0.059;0.108]	54.440	[42.43;78.33]
Chironomus riparius	A	0.508	[0.055;1.000*]	2.760	[2.577;4.367]
	B	0.001	[0.001;0.097]	500.000	[3.585;500*]
	D	0.078	[0.001;0.227]	3.277	[3.048;500*]
	E	1.000	[0.001;1.000*]	2.484	[2.734;500*]
	F	1.000	[0.001;1.000*]	2.484	[2.734;500*]
	Coumarin	0.000	[1.000e;06*;500*]	500.000	[1.000e;06*;500*]

Table S2: Toxicokinetic model parameter symbols, names, and dimensions/ units.

Parameter symbol	Parameter Name	Dimensions/units
C_i	Internal parent concentration	Mass of parent / body mass
C_e	External parent concentration	Mass of parent / mass leaf / volume water
W_{wt}	Wet weight	Body mass
Met_i	Metabolite concentration	Mass of metabolites / body mass
b	Growth rate	Time-1
k_{in}	Uptake rate	mass leaf/ body mass /time
k_{out}	Elimination rate	Time-1
k_{mf}	Metabolite formation rate	time-1
k_{me}	Metabolite elimination rate	time-1

Table S3: Comparative toxicokinetic parameters for *Spodoptera littoralis, Myzus persicae* and *Chironomus riparius.* This table presents the main toxicokinetic parameters: number of individuals per sample, body mass, internal concentration after exposure, external concentration, recovery percentage and limit of quantification adjusted for relative recovery.

Species	Test compounds	Number of individuals per sample	Ø Body mass per individual	Ø Internal concentration (mg/mg wet weight)*	Ø External concentration (mg/mg or mg/ml)	Ø Recovery (%)**	LoQ (mg/mg wet weight)***
S. littoralis	A	1	16.230	0.003	0.004	86	0.00003
	B	1	16.600	0.002	0.003	86	0.00003
	C	1	18.300	0.005	0.004	86	0.00002
	D	1	16.600	0.005	0.004	86	0.00003
	E	1	16.350	0.003	0.004	86	0.00003
	F	1	18.120	0.002	0.004	86	0.00002
	G	1	17.550	0.003	0.004	86	0.00002
	Coumarin	1	17.440	0.001	0.000	86	0.00002
M. Persicae	A	33	0.388	0.002	0.004	89	0.00111
	B	11	0.136	0.000	0.003	89	0.00316
	C	27	0.024	0.600	0.004	89	0.01792
	D	30	0.140	0.153	0.004	89	0.00307
	E	28	0.315	0.010	0.004	89	0.00137
	F	30	0.339	0.013	0.004	89	0.00127
	G	18	0.080	0.156	0.004	89	0.00538
	Coumarin	ND	ND	ND	ND	ND	ND
C. riparius	A	2	2.630	0.002	0.001	87	0.00017
	B	2	2.550	0.001	0.001	87	0.00017
	C	ND	ND	ND	ND	ND	ND
	D	2	2.550	0.011	0.001	87	0.00017
	E	2	2.000	0.156	0.001	87	0.00022
	F	2	2.030	ND	0.001	87	0.00021
	G	ND	ND	ND	ND	ND	ND
	Coumarin	2	3.020	0.012	0.001	87	0.00014

* Average internal concentration at the end of the exposure period
** Relative recovery including matrix effects
*** Limit of Quantification (LoQ) with consideration of the relative recovery

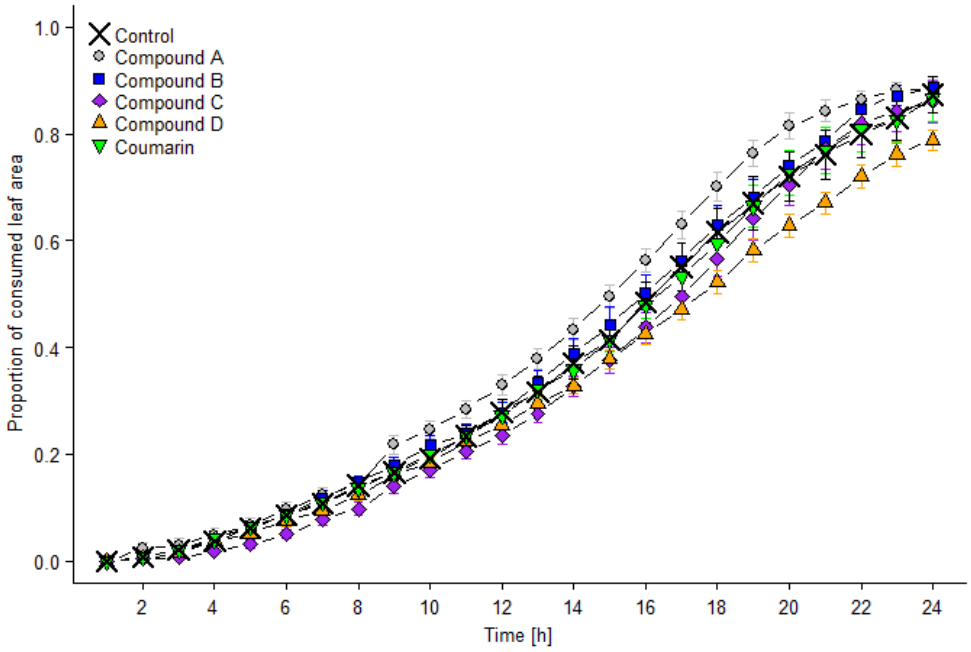


Figure S1: Performance parameters of *Spodoptera littoralis* larvae (n=12 experimental replicates): consumed leaf area during exposure period. Larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Figures created using R (version 3.5.3; R Core Team, 2020).

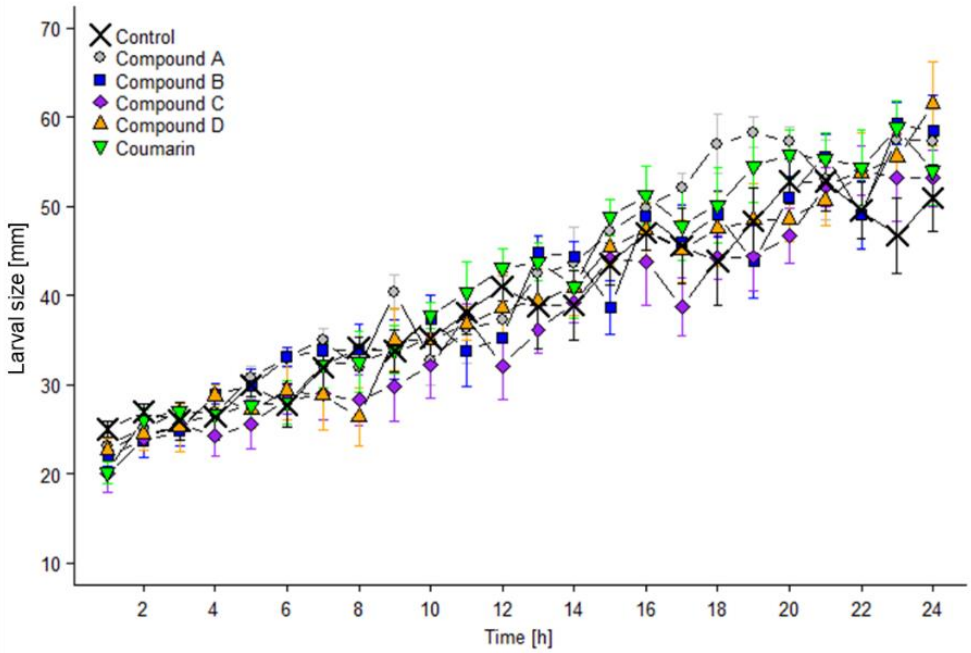


Figure S2: Performance parameters of *Spodoptera littoralis* larvae (n=12 experimental replicates): larval size over time. Larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Figures created using R (version 3.5.3; R Core Team, 2020).

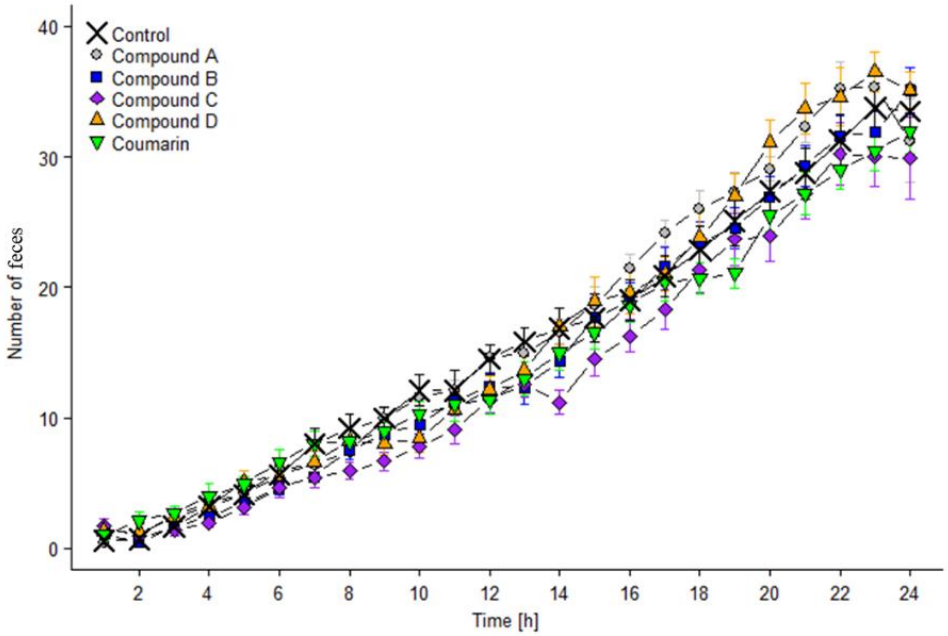


Figure S3: Performance parameters of *Spodoptera littoralis* larvae (n=12 experimental replicates): number of feces pellets. Larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Figures created using R (version 3.5.3; R Core Team, 2020).

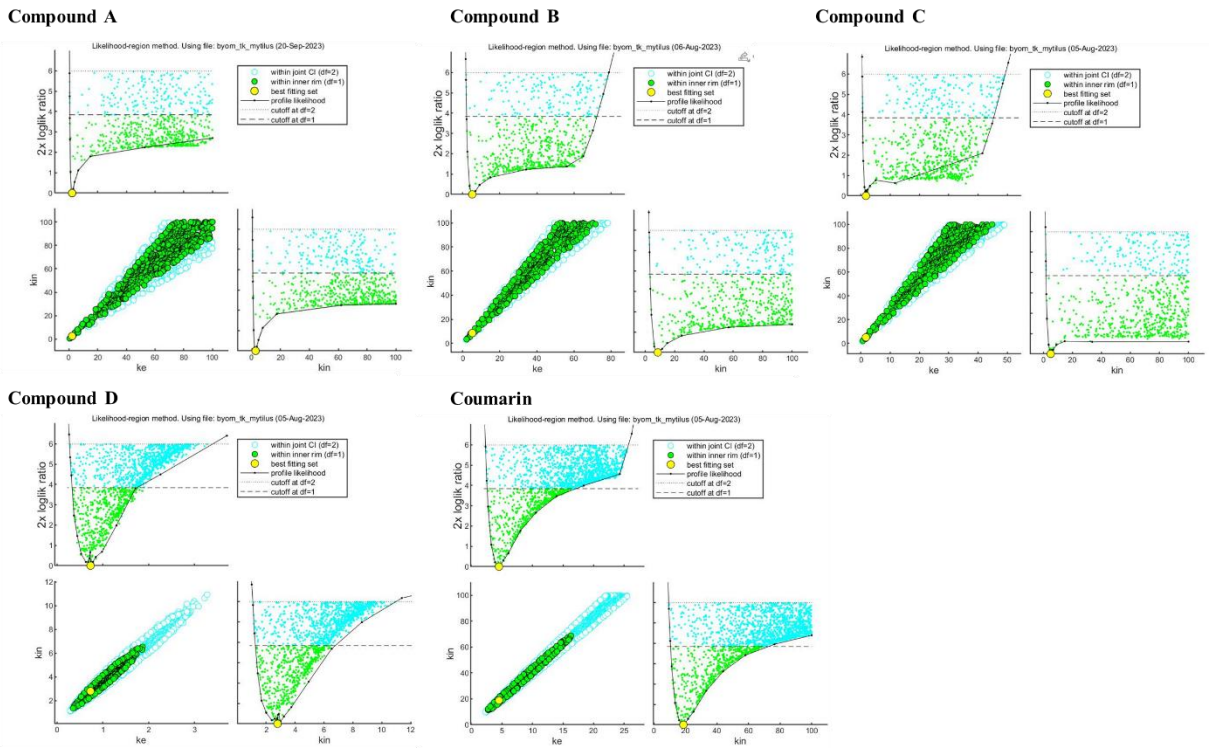


Figure S4: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* feeding contact leaf disk assay for the five test compounds (A-D, Coumarin). Likelihood profile for uptake (k_{in}) and elimination (k_e) rate constants

during a 24-hour exposure period and a 24-hour depuration period. Created using MATLAB (Version R2021a, Build Your Own Model).

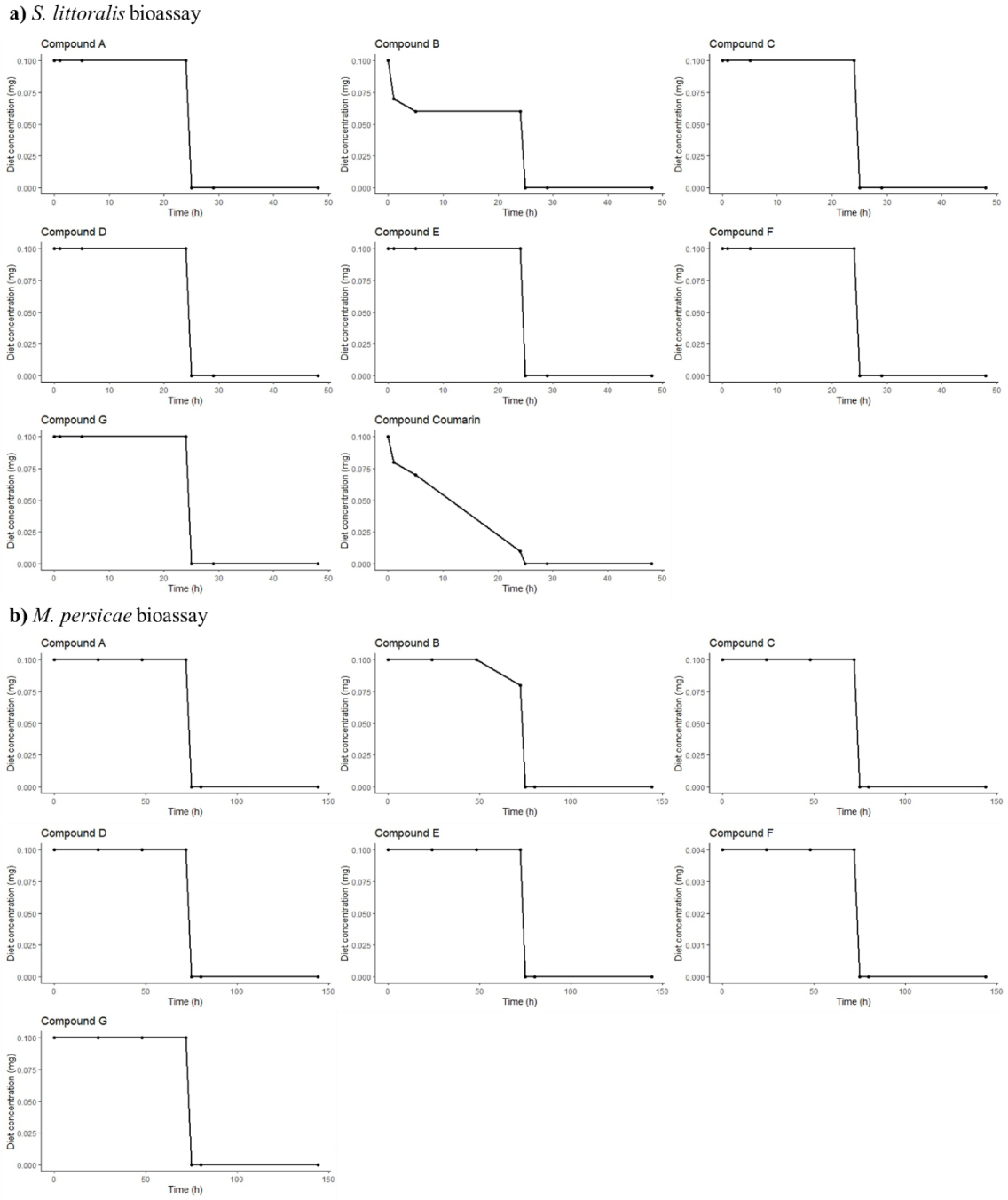


Figure S5: Exposure profiles: a) compound quantities (mg) in leaf disks (n=3 experimental replicates) over time. b) Compound quantities (mg) in artificial diet (n=3 experimental replicates) over time. Created using MATLAB (Version R2021a, Build Your Own Model).

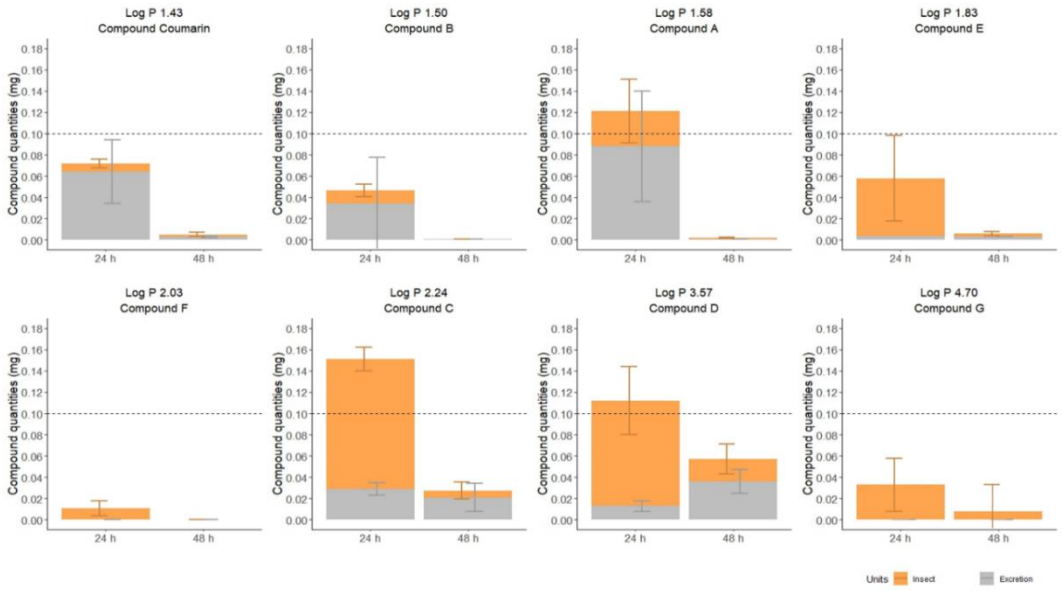


Figure S6: Parent compounds (sorted by Log P) in insects and excretion. Quantified per *Spodoptera littoralis* larvae (insect) and feces pellets (excretion). Exposed larvae (n=12 experimental replicates) fed on treated leaf disk for 24 hours, immediately after exposure interval larvae were transferred and fed on non-treated leaf disk for a follow-up depuration time of 24 hours. Parent compound quantities (A-D). Figures created using R (version 3.5.3; R Core Team, 2020).

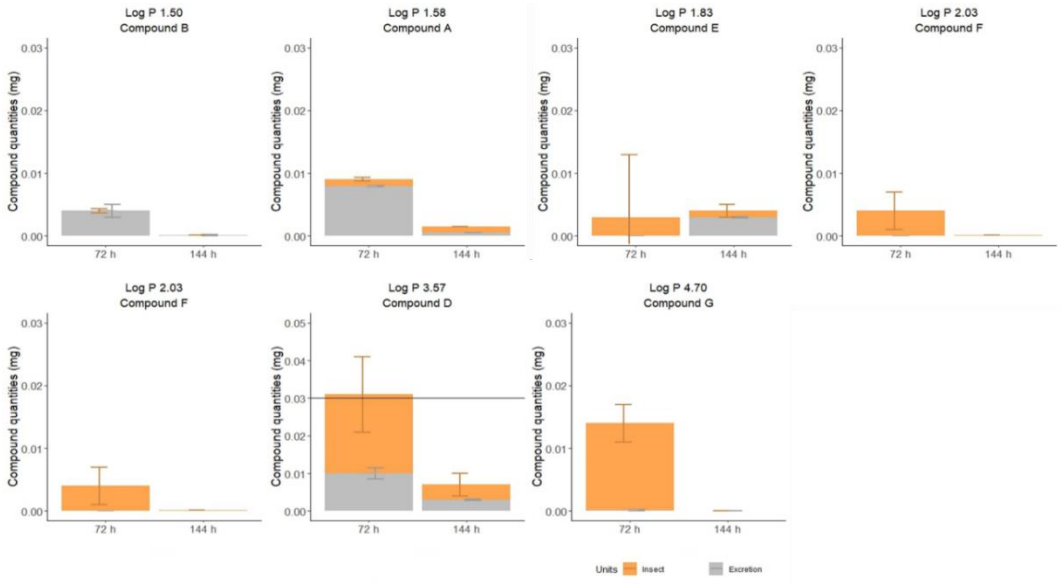


Figure S7: Parent compounds in insects and excretion. Quantified per *Myzus persicae* aphids larvae (n=8 experimental replicates) (body) and honeydew. Exposed aphids fed on treated diet for 72 hours, immediately after exposure interval aphids were transfers and fed on nontreated diet for a follow-up depuration time of 72 hours. Figures created using R (version 3.5.3; R Core Team, 2020).

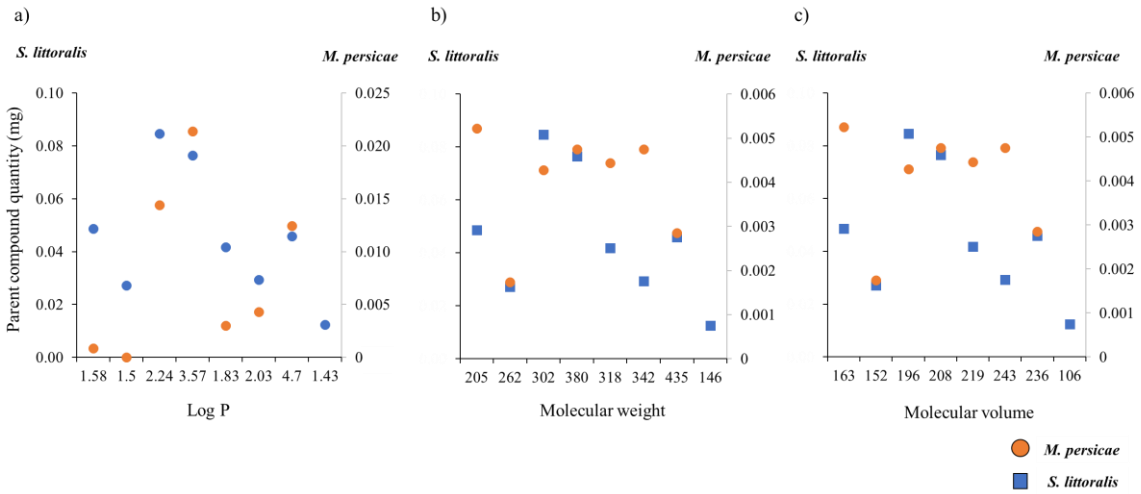


Figure S8: Parent compound quantities (mg) *Spodoptera littoralis* (larvae, n=12 experimental replicates) and *Myzus persicae* (calculated aphid equivalent, n= 8 experimental replicates). Dots represent the average of parent quantities (mg per insect) at the end of the exposure period; plotted against Log P (a), molecular weight (b), and molecular volume (c) (Table 1).

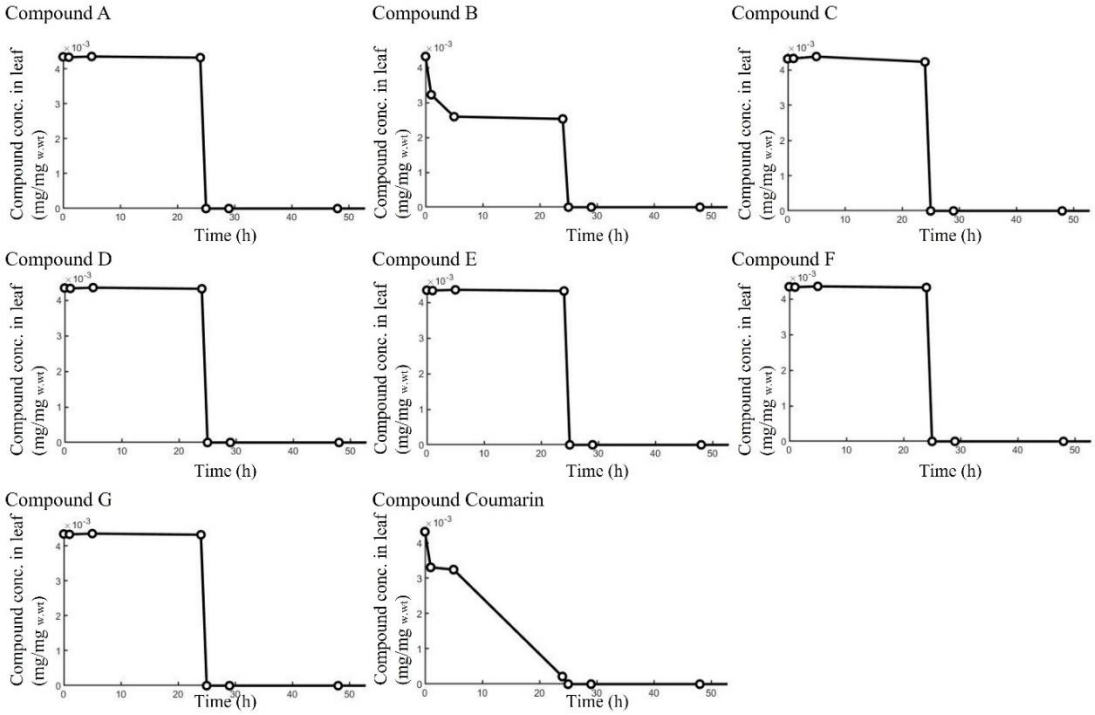


Figure S9: Exposure scenarios in the *Spodoptera littoralis* toxicokinetic study: soybean leaf disk (n=4 experimental replicates) concentrations during exposure (0- 24 h) and depuration time (24- 48 h) (mg/mg wet weight). Created using MATLAB (Version R2021a, Build Your Own Model).

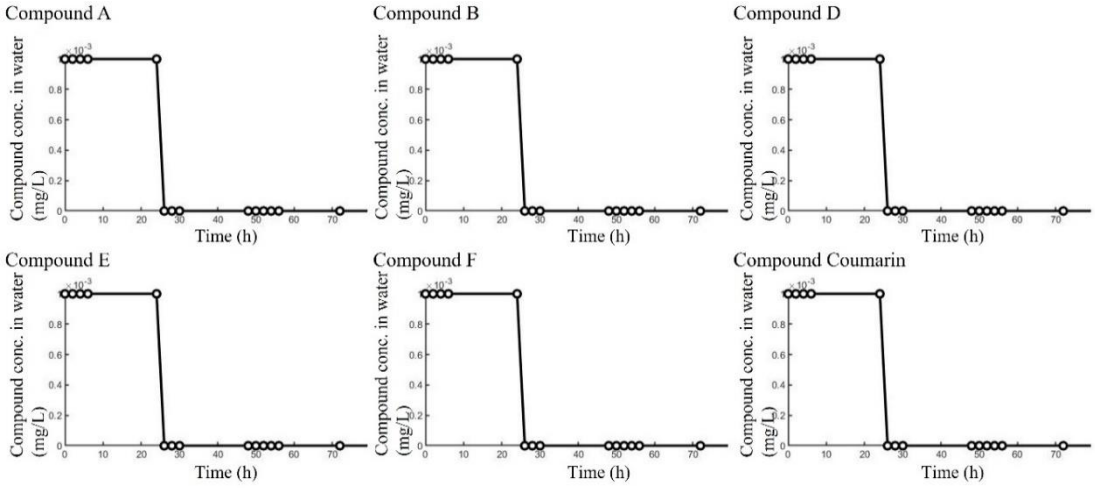


Figure S10: Exposure scenarios in the *Chironomus riparius* toxicokinetic study: water (n=2 experimental replicates) concentrations during exposure (0- 24 h) and depuration time (24- 72 h) (mg/L). Created using MATLAB (Version R2021a, Build Your Own Model).

TK model likelihood profile

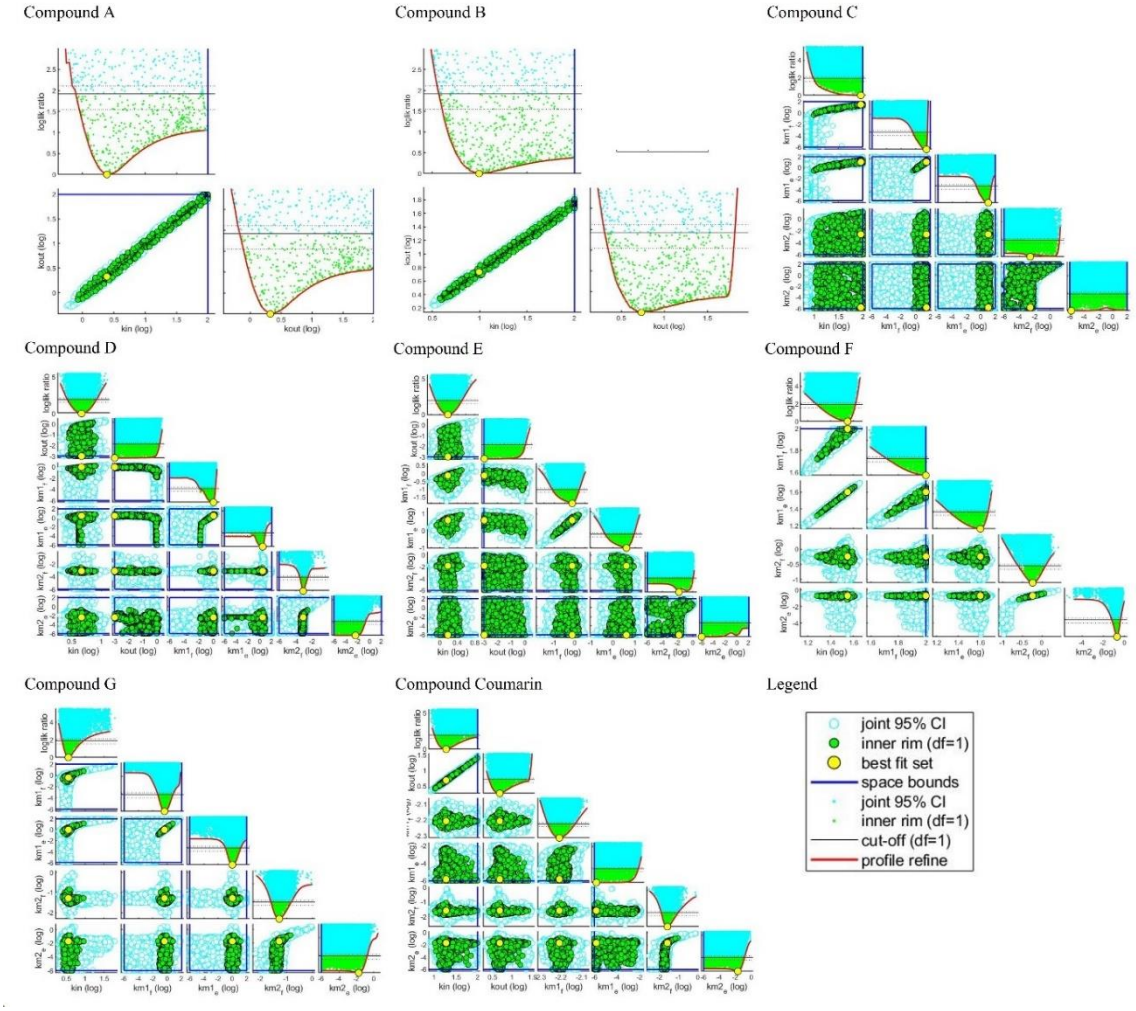


Figure S11: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* toxicokinetic assay for the five test compounds (A-G, Coumarin). Likelihood profile for uptake (k_{in}), elimination (k_e), metabolite formation (k_{m_f}), and metabolite elimination (k_{m_e}) rate constants during a 24 h exposure period and a 24 h depuration period. Created using MATLAB (Version R2021a, Build Your Own Model).

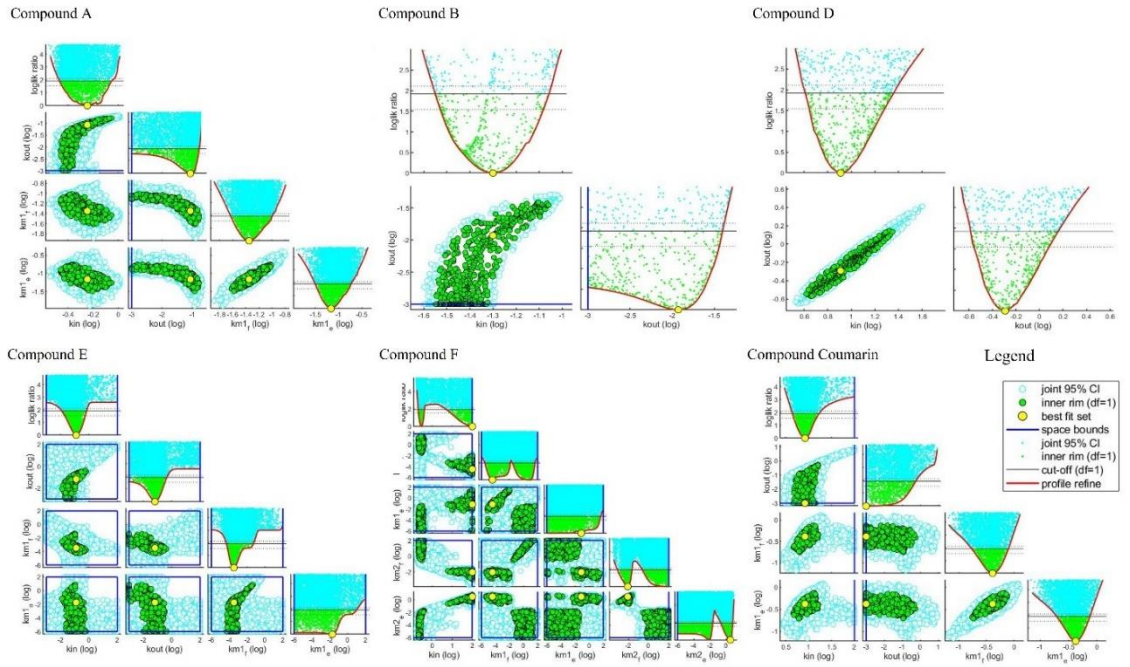


Figure S12: TK model likelihood profile for predicted parameters for the *Chironomus riparius* toxicokinetic assay for the five test compounds (A-F, Coumarin). Likelihood profile for uptake (k_{in}), elimination (k_e), metabolite formation (k_{m_f}), and metabolite elimination (k_{m_e}) rate constants during a 24 h exposure period and a 48-h depuration period. Created using MATLAB (Version R2021a, Build Your Own Model).

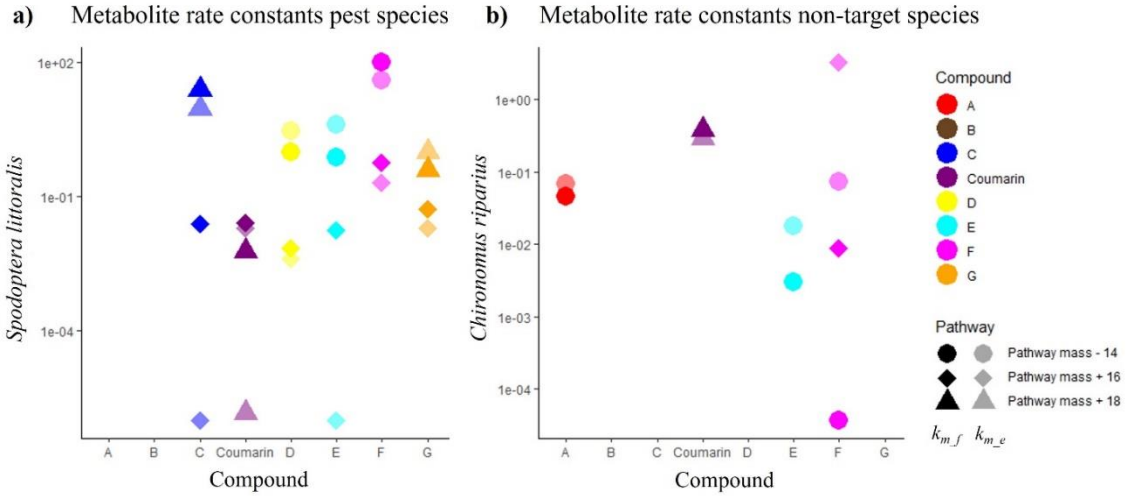


Figure S13: Biotransformation of all metabolites per species (Table 3) in (a) *Spodoptera littoralis* (n=12 experimental replicates) and (b) *Chironomus riparius* (n=3 experimental replicates). Metabolite fomation rate constants (k_{m_f}) shown as filled symbols, elimination rate constants (k_{m_e}) as empty symbols. Metabolites referred to by mass changes of decrease of 14 Da (demethylation) and increase of 16 or 18 (oxidation). Figures created using R (version 3.5.3; R Core Team, 2020).

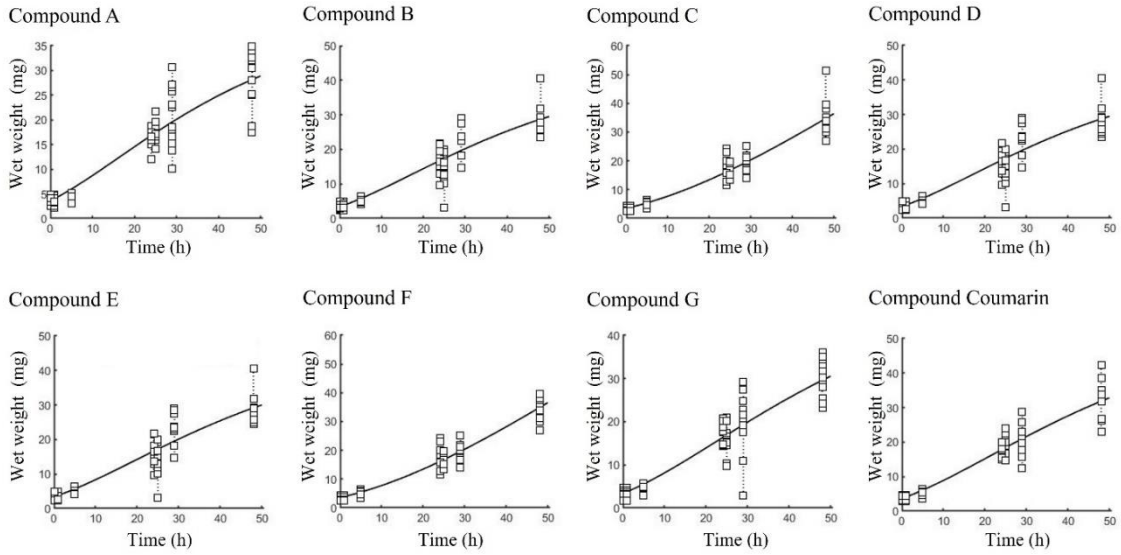


Figure S14: Wet weight (mg) of *Spodoptera littoralis* (n=12 experimental replicates) individuals during the toxicokinetic study. Created using MATLAB (Version R2021a, Build Your Own Model).

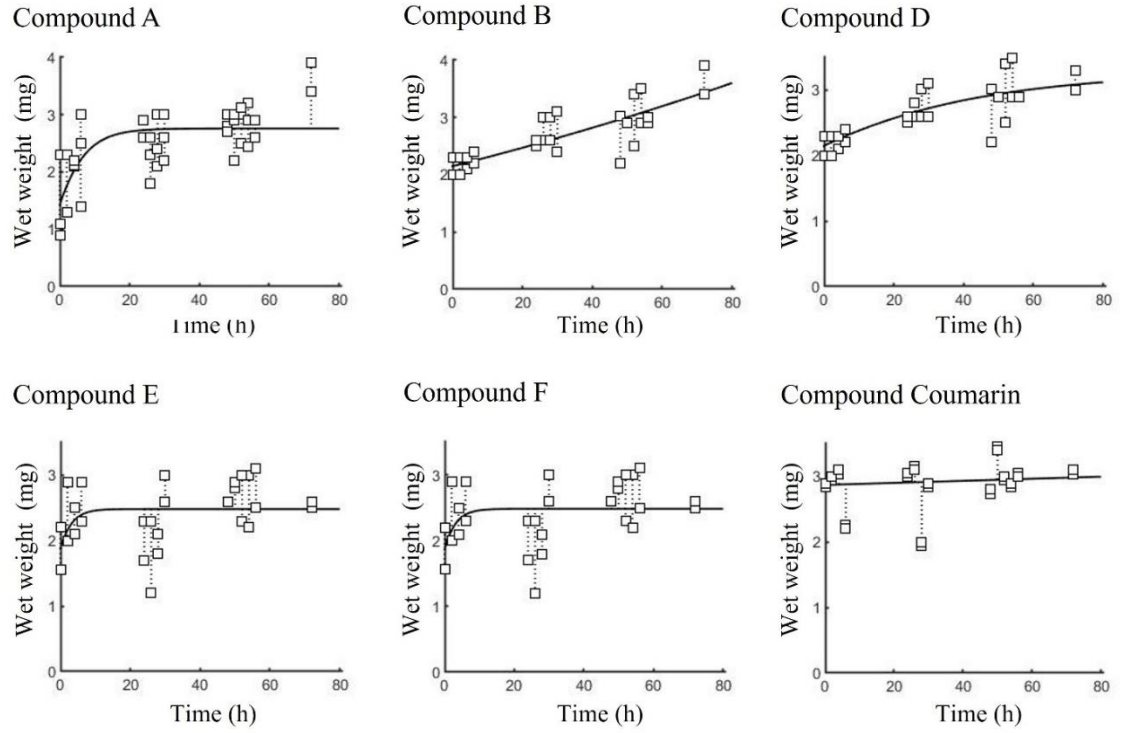


Figure S15: Wet weight (mg) of *Chironomus riparius* (n=3 experimental replicates) individuals during the toxicokinetic study. Created using MATLAB (Version R2021a, Build Your Own Model).

TK model parameters wet weight: likelihood profile

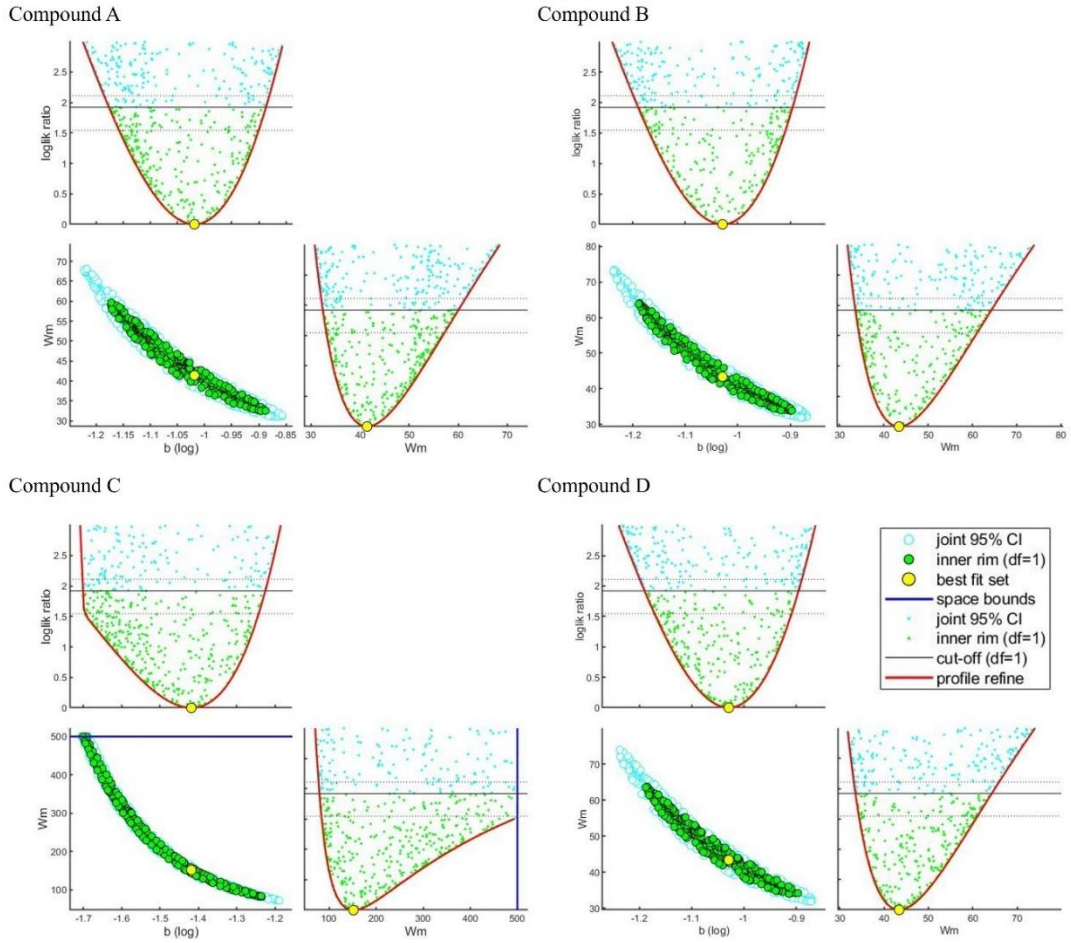


Figure S16: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (A-D). Likelihood profile Wet Weight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 48 h). Created using MATLAB (Version R2021a, Build Your Own Model).

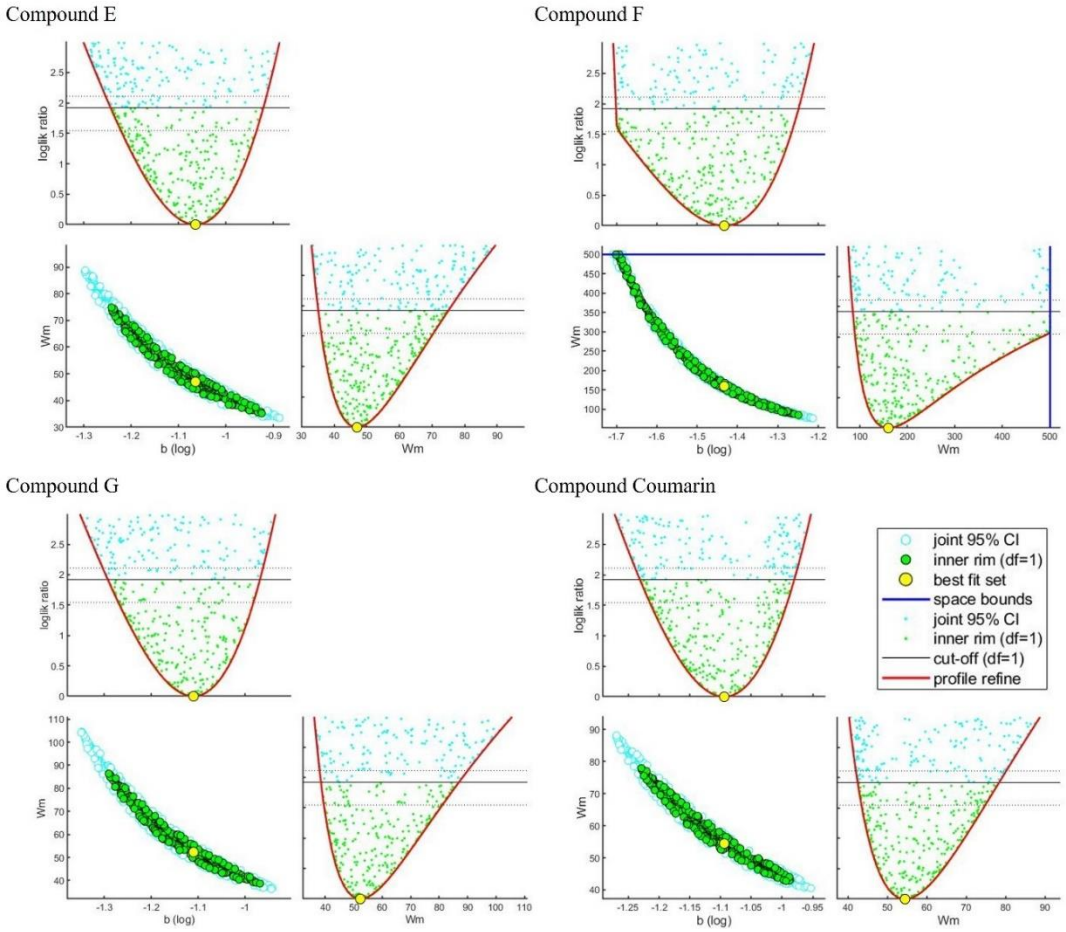


Figure S17: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (E-G, Coumarin). Likelihood profile Wet Weight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 48 h). Created using MATLAB (Version R2021a, Build Your Own Model).

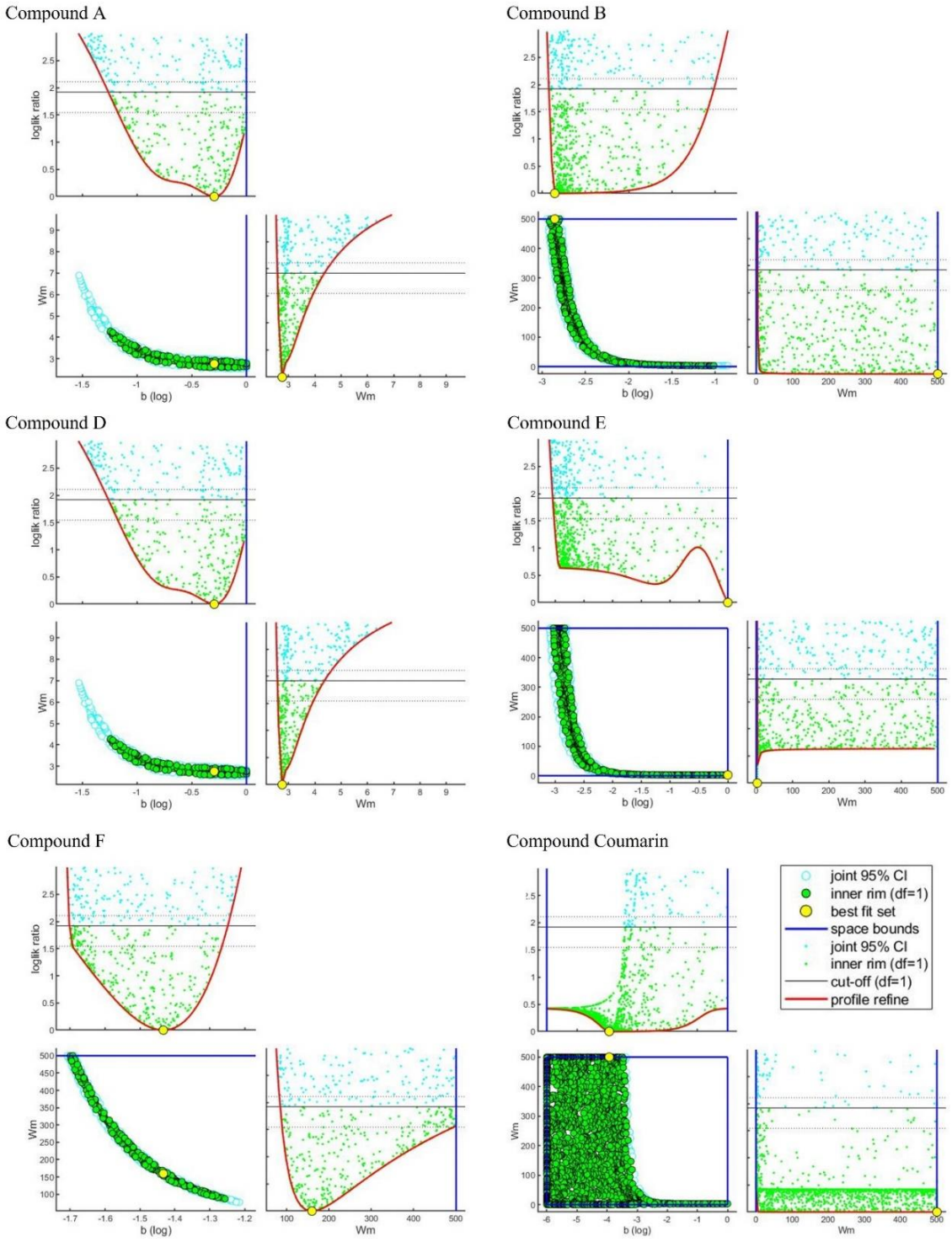


Figure S18: TK model likelihood profile for predicted parameters for the *Chironomus riparius* individuals during the toxicokinetic study for the test compounds (A-D). Likelihood profile Wet eight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 72 h). Created using MATLAB (Version R2021a, Build Your Own Model).

TK model – not fixed compounds: likelihood profile

Compound C

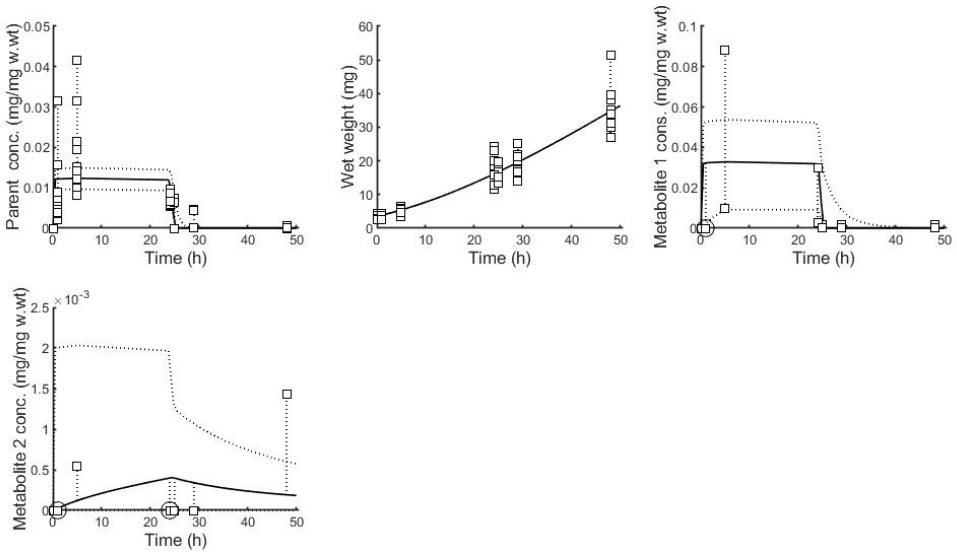


Figure S19: Body concentrations in *Spodoptera littoralis* larvae (n=12 experimental replicates) exposed to test compound C. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 4) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data. Created using MATLAB (Version R2021a, Build Your Own Model).

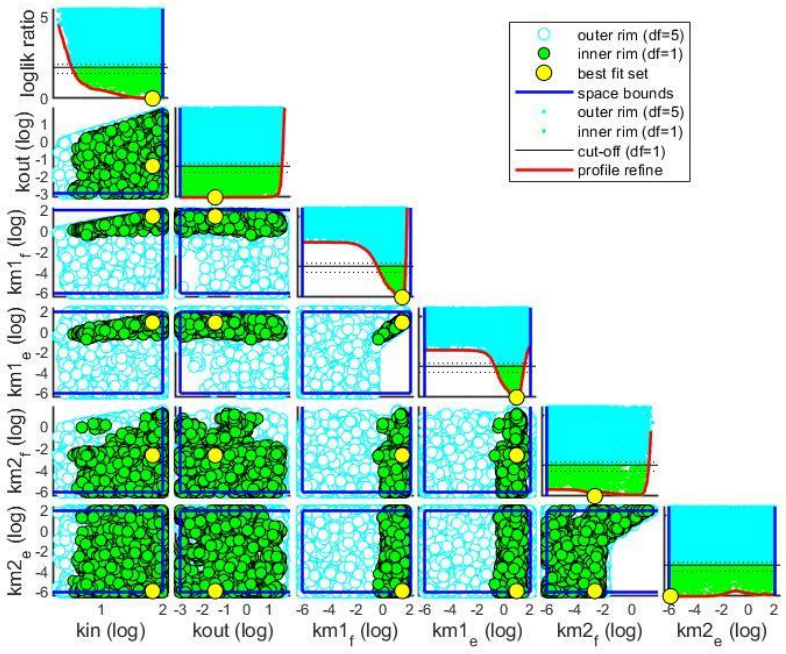


Figure S20: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compound C. Created using MATLAB (Version R2021a, Build Your Own Model).

Compound F

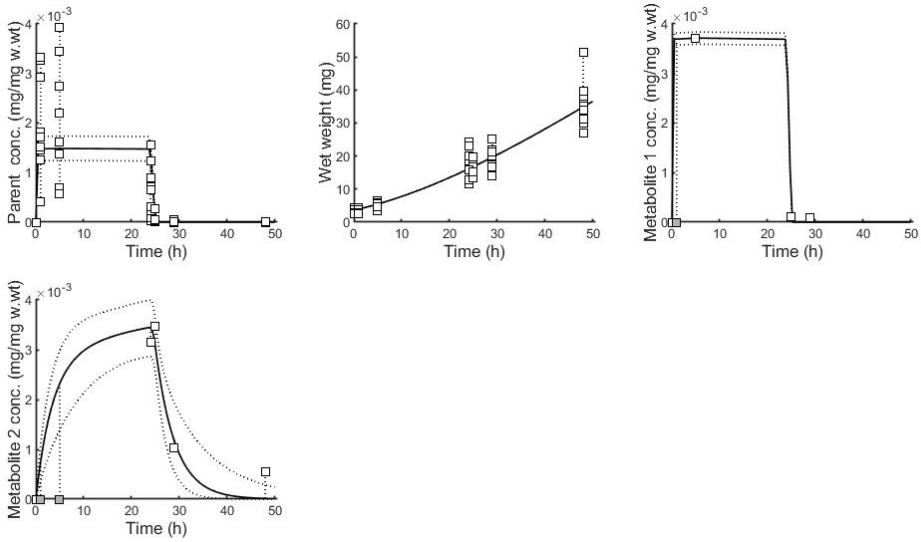


Figure S21: Body concentrations in *Spodoptera littoralis* larvae (n=12 experimental replicates) exposed to test compound F. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 4) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data. Created using MATLAB (Version R2021a, Build Your Own Model).

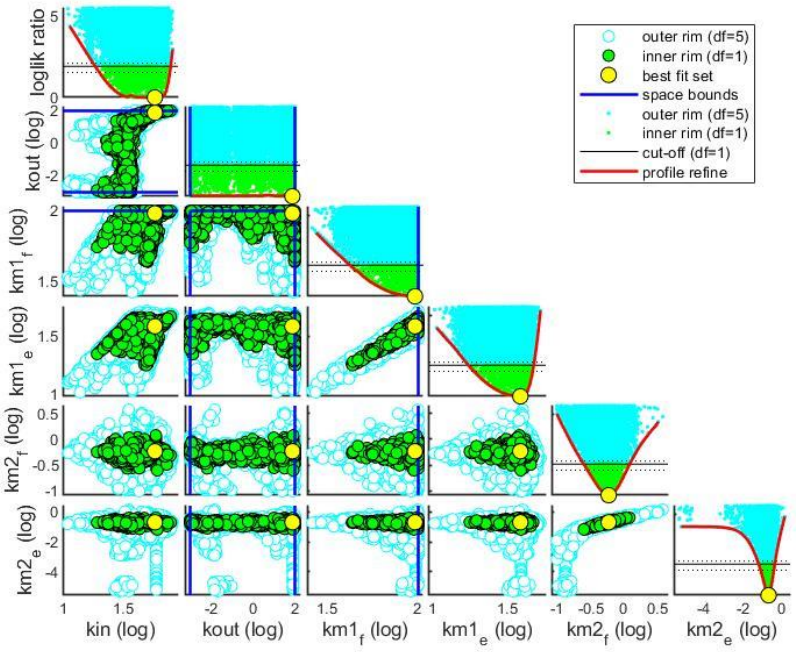


Figure S22: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compound F. Created using MATLAB (Version R2021a, Build Your Own Model).

Compound G

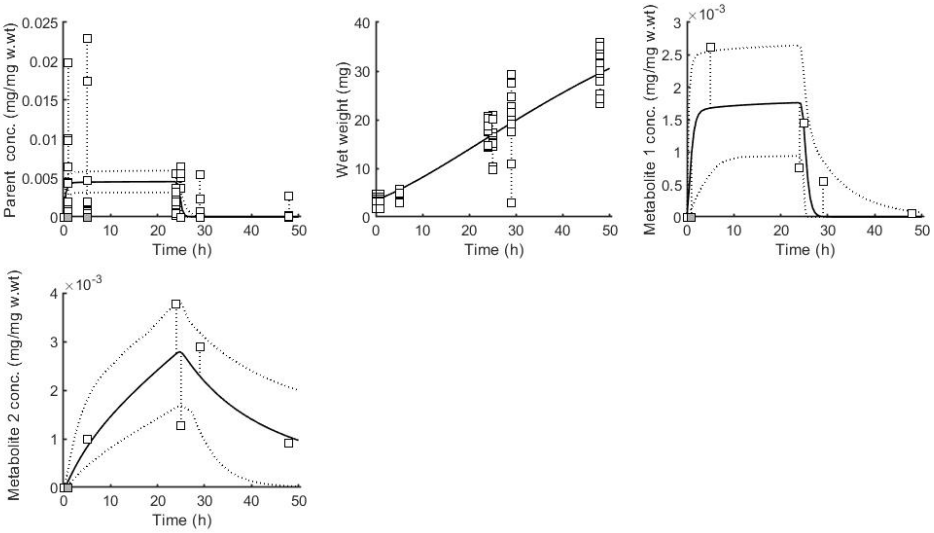


Figure S23: Body concentrations in *Spodoptera littoralis* larvae (n=12 experimental replicates) exposed to test compound G. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table X) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data. Created using MATLAB (Version R2021a, Build Your Own Model).

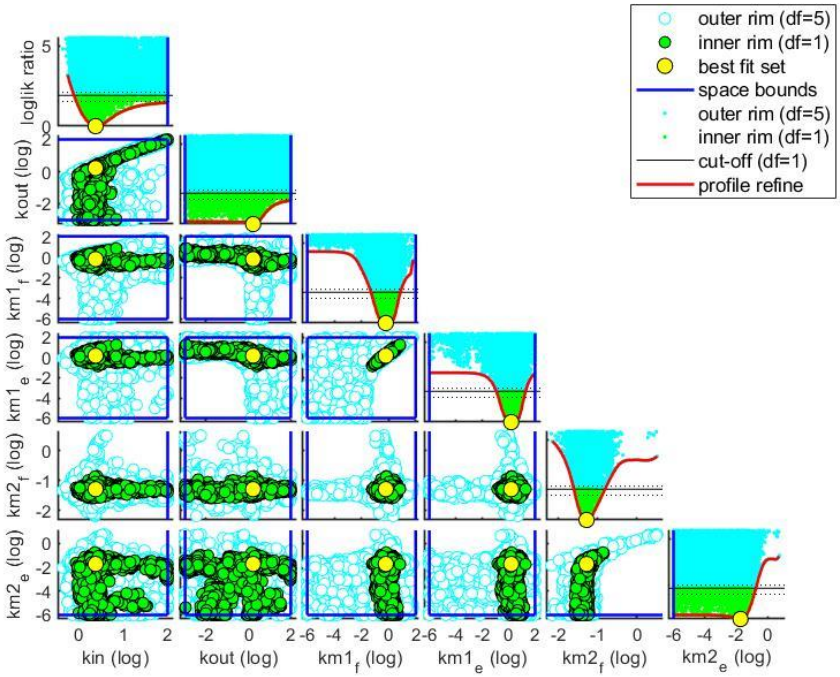
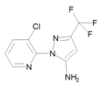
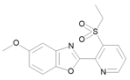
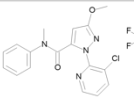
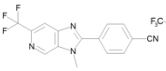
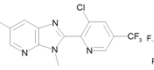
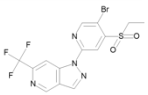


Figure S24: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compound G. Created using MATLAB (Version R2021a, Build Your Own Model).

Table S4: Chemical structure of test compounds and Coumarin.

Parent compound							
Coumarin	B	A	E	F	C	D	G
							

Toxicokinetic model scripts

Within the PhD study two toxicokinetic model using the Build Your Own Model (BYOM, version 60 TK model 1 or 64 TK model 2) platform was built in MATLAB (version R2021a). Copyrights are owned by Tjalling Jager. This source code is licensed under the MIT-style license found in the % LICENSE.txt file in the root directory of BYOM. The scripts were adapted by me (Clara Isis Römer) under the supervision of Neil Sherborne and Roman Ashauer.

TK Model 1

```
%%% This Script were used to analyze TK in a target species in the PhD Thesis of
Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne

% Copyright (c) 2012-2021, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.

%% Initial things
% Make sure that this script is in a directory somewhere *below* the BYOM
% folder.

clear, clear global % clear the workspace and globals
global DATA W X0mat % make the data set and initial states global variables
global glo % allow for global parameters in structure glo
diary off % turn off the diary function (if it is accidentaly on)
% set(0,'DefaultFigureWindowStyle','docked'); % collect all figure into one window
with tab controls
set(0,'DefaultFigureWindowStyle','normal'); % separate figure windows

pathdefine(0) % set path to the BYOM/engine directory (option 1 uses parallel
toolbox)
glo.basenm = mfilename; % remember the filename for THIS file for the plots
glo.saveplt = 0; % save all plots as (1) Matlab figures, (2) JPEG file or (3) PDF
(see all_options.txt)

%% The data set
% Data are entered in matrix form, time in rows, scenarios (exposure
% concentrations) in columns. First column are the exposure times, first
% row are the concentrations or scenario numbers. The number in the top
% left of the matrix indicates how to calculate the likelihood:

% * -1 for multinomial likelihood (for survival data)
% * 0 for log-transform the data, then normal likelihood
% * 0.5 for square-root transform the data, then normal likelihood
% * 1 for no transformation of the data, then normal likelihood

% observed % Ci cons
DATA{1} = [
 ];
% observed % Ci of metabolites
DATA{2} = [];
% observed % Cl (conc. in leaf disk)
Cl = [ ];
make_scen(4,Cl); % prepare as block-pulse scenario

% if weight factors are not specified, ones are assumed in start_calc.m

%% Initial values for the state variables

X0mat = [1 % the scenario(s) to run
 0
 0]; % initial values state 2: metabolite at t=0

%% Initial values for the model parameters
```

```
par.ke = [ 2.24 1 0 100]; % elimination rate
par.kin = [2.63 1 0 100]; % AI uptake contact
%par.Kil = [80 1 0 1e6]; % partition coefficient
par.km = [0 0 0 1e6]; % formation rate of the metabolite
par.kem = [0.1 0 0 100]; % elimination rate of the metabolite

%% Time vector and labels for plots
glo.t = linspace(0,50,100)%% (min/max Ylab) time
% specify the y-axis labels for each state variable
glo.ylab{1} = 'Body residue (mg/wg wwt)';
glo.ylab{2} = 'Metabolite';
% specify the x-axis label (same for all states)
glo.xlab = 'Time (h) 0-24h exposure; 24-48h depuration';
glo.leglab1 = 'A'; % legend label before the 'scenario'
glo.leglab2 = 'Parent Compound'; % legend label after the 'scenario' number

prelim_checks % script to perform some preliminary checks and set things up
% Note: prelim_checks also fills all the options (opt_...) with defauls, so
% modify options after this call, if needed.

%% Calculations and plotting

% Options for the plotting can be set using opt_plot (see prelim_checks.m).
% Options for the optimsation routine can be set using opt_optim. Options
% for the ODE solver are part of the global glo. For the demo, the
% iterations were turned off (opt_optim.it = 0).

opt_optim.fit = 1; % fit the parameters (1), or don't (0)
opt_optim.it = 1; % show iterations of the optimisation (1, default) or not (0)
glo.useode = 1; % calculate model using ODE solver (1) or analytical solution (0)

opt_plot.statsup = [2]; % vector with states to suppress in plotting fits, to
exclude plots of a scenario out of output (here 2 = scen2.)
%
% optimise and plot (fitted parameters in par_out)
par_out = calc_optim(par,opt_optim); % start the optimisation
calc_and_plot(par_out,opt_plot); % calculate model lines and plot them
glo.locC = 1;

% Calculate and plot specific plots for TKTD models
opt_tktd.min = 0; % set to 1 to show a dotted line for the control (lowest)
treatment
glo.locC = 1; % tell plot_tktd which state the concentrations are = make plots with
error bars incluedes means with SD
plot_tktd(par_out,opt_tktd,[]);

%% Likelihood region

opt_prof.detail = 2; % detailed (1) or a coarse (2) calculation
opt_prof.subopt = 0; % number of sub-optimisations to perform to increase
robustness
opt_prof.brkprof = 2; % when a better optimum is located, stop (1) or automatically
refit (2)

par_better = calc_likregion(par_out,500,opt_likreg,opt_prof,opt_optim);
% Second entry is the number of accepted parameter sets to aim for. Use -1
% here to use a saved set.

if ~isempty(par_better) % if the profiling found a better optimum ...
 calc_and_plot(par_better,opt_plot); % calculate model lines and plot them
 par_out = par_better; % use the new parameter structure for further analyses below
end

%% Profiling the likelihood
% Options for profiling can be set using opt_prof (see prelim_checks.m).

opt_prof.detail = 2; % detailed (1) or a coarse (2) calculation
opt_prof.subopt = 0; % number of sub-optimisations to perform to increase
robustness

% UNCOMMENT LINE(S) TO CALCULATE
par_better = calc_proflik(par_out,'all',opt_prof,opt_optim); % calculate a profile
```

```
% % Note: if a better optimum is found, the parameter structure is returned
% % in par_better. That structure could then be used in calc_and_plot, for
% % example.

%% Plot results with confidence intervals

% Use opt_conf.type to tell calc_conf which sample to use:
% -1) Skips CIs (zero does the same, and an empty opt_conf as well).
% 1) Bayesian MCMC sample (default); CI on predictions are 95% ranges on
% the model curves from the sample
% 2) parameter sets from a joint likelihood region using the shooting
% method (limited sets can be used), which will yield (asymptotically) 95%
% CIs on predictions
% 3) as option 2, but using the parameter-space explorer

opt_conf.type = 2; % make intervals from 1) slice sampler, 2) likelihood region
shooting, 3) parspace explorer
opt_conf.lim_set = 2; % use limited set of n_lim points (1) or outer hull (2,
likelihood methods only) to create CIs
opt_conf.sens = 1; % type of analysis 0) no sensitivities 1) corr. with state, 2)
corr. with state/control, 3) corr. with relative change of state over time
glo.locC = 1;
out_conf = calc_conf(par_out,opt_conf); % calculate confidence intervals on model
curves
calc_and_plot(par_out,opt_plot,out_conf); % call the plotting routine again to plot
fits with CIs
%glo.locC = 1; % tell plot_tktd which state the concentrations are = make plots
with error bars incluedes means with SD

% out_conf = calc_conf(par_out,opt_conf); % calculate confidence intervals on model
curves
% calc_and_plot(par_out,opt_tktd, out_conf); % call the plotting routine again to
plot fits with CIs
```

Derivatives 1

```
%%%% This Script were used to analyze TK in a target species in the PhD Thesis of
Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne
 %% BYOM function derivatives.m (the model in ODEs)
%
% Syntax: dX = derivatives(t,X,par,c,glo)
%
% This function calculates the derivatives for the model system. It is
% linked to the script files <byom_bioconc_extra.html byom_bioconc_extra.m>
% and <byom_bioconc_start.html byom_bioconc_start.m>. As input,
% it gets:
%
% * _t_ is the time point, provided by the ODE solver
% * _X_ is a vector with the previous value of the states
% * _par_ is the parameter structure
% * _c_ is the external concentration (or scenario number)
% * _glo_ is the structure with information (normally global)
%
% Note: _glo_ is now an input to this function rather than a global. This
% makes the code considerably faster.
%
% Time _t_ and scenario name _c_ are handed over as single numbers by
% <call_deri.html call_deri.m> (you do not have to use them in this
% function). Output _dX_ (as vector) provides the differentials for each
% state at _t_.
%
% * Author: Tjalling Jager
% * Date: November 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% Copyright (c) 2012-2023, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.
```

```
%% Start
% Note that, in this example, variable _c_ (the scenario identifier) is not
% used in this function. The treatments differ in their initial exposure
% concentration that is set in X0mat. Also, the time _t_ and structure _glo_ are
not
% used here.

function dX = derivatives(t,X,par,c,glo)

%% Unpack states
% The state variables enter this function in the vector _X_. Here, we give
% them a more handy name.
if glo.timevar(1) == 1
 Cl = read_scen(-1,c,t,glo);
else
 Cl = c;
end

Cin = X(1); % state 1 is the external concentration
%% Unpack parameters
% The parameters enter this function in the structure _par_. The names in
% the structure are the same as those defined in the byom script file. The
% 1 between parentheses is needed for fitting the model, as each parameter
% has 4-5 associated values.

kin = par.kin(1); % degradation rate constant, d-1
kout = par.kout(1); % elimination rate constant, d-1
%% Calculate the derivatives
dX = kin*Cl - kout*Cin;
```

Call_deri 1

```
%%% This Script were used to analyze TK in a target species in the PhD Thesis of
Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne

% BYOM function call_deri.m (calculates the model output)
%
% Syntax: [Xout,TE,Xout2,zvd] = call_deri(t,par,X0v,glo)
%
% This function calls the ODE solver to solve the system of differential
% equations specified in <derivatives.html derivatives.m>, or the explicit
% function(s) in <simplefun.html simplefun.m>. As input, it gets:
%
% * _t_ the time vector
% * _par_ the parameter structure
% * _X0v_ a vector with initial states and one concentration (scenario number)
% * _glo_ the structure with various types of information (used to be global)
%
% The output _Xout_ provides a matrix with time in rows, and states in
% columns. This function calls <derivatives.html derivatives.m>. The
% optional output _TE_ is the time at which an event takes place (specified
% using the events function). The events function is set up to catch
% discontinuities. It should be specified according to the problem you are
% simulating. If you want to use parameters that are (or influence) initial
% states, they have to be included in this function. Optional output Xout2
% is for additional uni-variate data (not used here), and zvd is for
% zero-variate data (not used here).
%
% * Author: Tjalling Jager
% * Date: September 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% Copyright (c) 2012-2021, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.

%% Start

function [Xout,TE,Xout2,zvd] = call_deri(t,par,X0v,glo)
Xout2 = []; % additional uni-variate output, not used in this case
```

```
zvd = []; % additional zero-variate output, not used in this case
% Note: if these options are not used, these variables must be defined as
% empty as they are outputs of this function.

%% Initial settings
% This part extracts optional settings for the ODE solver that can be set
% in the main script (defaults are set in prelim_checks). The useode option
% decides whether to calculate the model results using the ODEs in
% <derivatives.html derivatives.m>, or the analytical solution in
% <simplefun.html simplefun.m>. Using eventson=1 turns on the events
% handling. Also modify the sub-function at the bottom of this function!
% Further in this section, initial values can be determined by a parameter
% (overwrite parts of X0), and zero-variate data can be calculated. See the
% example BYOM files for more information.

useode = glo.useode; % calculate model using ODE solver (1) or analytical solution
(0)
eventson = glo.eventson; % events function on (1) or off (0)
stiff = glo.stiff; % set to 1 or 2 to use a stiff solver instead of the standard
one
% Unpack the vector X0v, which is X0mat for one scenario
X0 = X0v(2:end); % these are the intitial states for a scenario
%% Calculations
% This part calls the ODE solver (or the explicit model in <simplefun.html
% simplefun.m>) to calculate the output (the value of the state variables
% over time). There is generally no need to modify this part. The solver
% ode45 generally works well. For stiff problems, the solver might become
% very slow; you can try ode15s instead.

c = X0v(1); % the concentration (or scenario number)
t = t(:); % force t to be a row vector (needed when useode=0)

TE = 0; % dummy for time of events

if useode == 1 % use the ODE solver to calculate the solution
 % Note: set options AFTER the 'if useode == 1' as odeset takes
 % considerable calculation time, which is not needed when using the
 % analytical solution. Also note that the global _glo_ is now input to
 % the derivatives function. This increases calculation speed.

 % specify options for the ODE solver; feel free to change the
 % tolerances, if you know what you're doing (for some problems, it is
 % better to set them much tighter, e.g., 1e-9)
 reltol = 1e-4;
 abstol = 1e-7;
 options = odeset; % start with default options
 if eventson == 1
 options = odeset(options,'Events',@eventsfun,'RelTol',reltol,'AbsTol',abstol); %
add an events function and tigher tolerances
 else
 options = odeset(options,'RelTol',reltol,'AbsTol',abstol); % only specify
tightened tolerances
 end
 % options = odeset(options,'InitialStep',max(t)/1000,'MaxStep',max(t)/100); %
specify smaller stepsize

 % call the ODE solver (try ode15s for stiff problems, and possibly with for pulsed
forcings)
 if isempty(options.Events) % if no events function is specified ...
 switch stiff
 case 0
 [~,Xout] = ode45(@derivatives,t,X0,options,par,c,glo);
 case 1
 [~,Xout] = ode113(@derivatives,t,X0,options,par,c,glo);
 case 2
 [~,Xout] = ode15s(@derivatives,t,X0,options,par,c,glo);
 end
 else % with an events functions ... additional output arguments for events:
 % TE catches the time of an event, YE the states at the event, and IE the number
of the event
 switch stiff
 case 0
```

```
 [~,Xout,TE,YE,IE] = ode45(@derivatives,t,X0,options,par,c,glo);
 case 1
 [~,Xout,TE,YE,IE] = ode113(@derivatives,t,X0,options,par,c,glo);
 case 2
 [~,Xout,TE,YE,IE] = ode15s(@derivatives,t,X0,options,par,c,glo);
 end
 end
else % alternatively, use an explicit function provided in simplefun
 Xout = simplefun(t,X0,par,c,glo);
end

if isempty(TE) || all(TE == 0) % if there is no event caught
 TE = +inf; % return infinity
end

%% Output mapping
% _Xout_ contains a row for each state variable. It can be mapped to the
% data. If you need to transform the model values to match the data, do it
% here.

% Xout(:,1) = Xout(:,1).^3; % e.g., do something on first column, like cube it ...

% % To obtain the output of the derivatives at each time point. The values in
% % dXout might be used to replace values in Xout, if the data to be fitted
% % are the changes (rates) instead of the state variable itself.
% % dXout = zeros(size(Xout)); % initialise with zeros
% for i = 1:length(t) % run through all time points
% dXout(i,:) = derivatives(t(i),Xout(i,:),par,c,glo);
% % derivatives for each stage at each time
% end
%% Events function
% Modify this part of the code if _eventson_=1. This subfunction catches
% the 'events': time points where the derivative is undefined. This
% function should be adapted to the problem you are modelling. You can
% catch more events by making a vector out of _values_.
%
% Note that the eventsfun has the same inputs, in the same sequence, as
% <derivatives.html derivatives.m>.

function [value,isterminal,direction] = eventsfun(t,X,par,c,glo)

% Note: the events function is NOT used for these calculations. It is left
% in so this code can be easily modified to suit other problems.

a = par.a(1); % extract value for a parameter (here: a)
nevents = 1; % number of events that we try to catch

value = zeros(nevents,1); % initialise with zeros
value(1) = X(1) - a; % here: check whether the first state variable equals a
isterminal = zeros(nevents,1); % do NOT stop the solver at an event
direction = zeros(nevents,1); % catch ALL zero crossing when function is increasing
or decreasing
```

Bioaccumulation Factor

```
%%%% This Script were used to analyze BAF in a target species in the PhD Thesis of
Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne
 %% BYOM, byom_bioconc_extra.m, a detailed example

% * Author: Tjalling Jager
% * Date: November 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% BYOM is a General framework for simulating model systems in terms of
% ordinary differential equations (ODEs). The model itself needs to be
% specified in <derivatives.html derivatives.m>, and <call_deri.html
% call_deri.m> may need to be modified to the particular problem as well.
% The files in the engine directory are needed for fitting and plotting.
% Fitting relies on the multinomial likelihood for survival and independent
% normal distributions (if needed after transformation) for continuous
```

```
% data. Results are shown on screen but also saved to a log file
% (results.out).
%
% In this example file (byom_bioconc_extra), we will walk step-by-step
% through a byom script file, explaining what happens and showing the
% outputs.
%
% *The model:* An organism is exposed to a chemical in its surrounding
% medium. The animal accumulates the chemical according to standard
% one-compartment first-order kinetics, specified by a bioconcentration
% factor (_Piw_) and an elimination rate (_ke_). The chemical degrades at a
% certain rate (_kd_). When the external concentration reaches a certain
% concentration (_Ct_), degradation stops. This is useful to demonstrate the
% events function in call_deri.m, which catches this discontuity
% graciously. In the form of ODE's:
%
% $$ \frac{d}{dt}C_w=-k_d C_w \quad \textrm{as long as } C_w>C_t $$
%
% $$ \frac{d}{dt}C_i=k_e(P_{iw}C_w-C_i) $$
%
% *This script:* byom_bioconc_extra demonstrates fitting using replicated
% data, with different concentration vectors in both data sets.
% Furthermore, several options are explained, such as the inclusion of
% zero-variate data and priors for Bayesian analysis. More support in the
% BYOM manual downloadable from <http://www.debtox.info/byom.html here>. A
% stripped version (less text, less options) can be found as
% <byom_bioconc_start.html byom_bioconc_start.m>.
%
% Copyright (c) 2012-2023, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.

%% Initial things
% Before we start, the memory is cleared, globals are defined, and the
% diary is turned off (output will be collected in the file results.out).
% The function pathdefine.m makes sure that the engine directory is added
% to the path. Make sure that this script is in a directory somewhere
% *below* the BYOM folder.

clear, clear global % clear the workspace and globals
global DATA W X0mat % make the data set and initial states global variables
global glo % allow for global parameters in structure glo
diary off % turn off the diary function (if it is accidentaly on)
% set(0,'DefaultFigureWindowStyle','docked'); % collect all figure into one window
with tab controls
set(0,'DefaultFigureWindowStyle','normal'); % separate figure windows

pathdefine(1) % set path to the BYOM/engine directory (option 1 to use parallel
toolbox, if available)
glo.basenm = mfilename; % remember the filename for THIS file for the plots
glo.saveplt = 0; % save all plots as (1) Matlab figures, (2) JPEG file or (3) PDF
(see all_options.txt)

%% The data set
% Data are entered in matrix form, time in rows, scenarios (exposure
% concentrations) in columns. First column are the exposure times, first
% row are the concentrations or scenario numbers. The number in the top
% left of the matrix indicates how to calculate the likelihood:
%
% * -1 for multinomial likelihood (for survival data)
% * 0 for log-transform the data, then normal likelihood
% * 0.5 for square-root transform the data, then normal likelihood
% * 1 for no transformation of the data, then normal likelihood
%
% For each state variable there should be a data set in the correct
% position. So, _DATA{1}_ should be the data for state variable 1, etc. The
% curly braces indicate that this is a 'cell array'. If there are no data
% for a state, use _DATA{i}=0_ (if you forget, this will be automatically
% done in the engine script prelim_checks.m). Use NaN for missing data.
%
% Multiple data sets for each state can now be used. The first data set for
% state 1 becomes _DATA{1,1}_ and the second _DATA{2,1}_, etc. Note that
```

```
% each data set is treated as completely independent. For continuous data
% that means that each data set has its own error distribution (either
% treated as 'nuisance parameter' or provided by the user in _glo.var_).
% You can enter replicated data by adding columns with the same scenario
% number. The scenario number should occur only once in the initial values
% matrix _X0mat_.
%
% *Note:* for using survival data: enter the survival data as numbers of
% survivors, and not as survival probability. The model should be set up to
% calculate probabilities though. Therefore, the state variable is a
% probability, and hence the initial value in _X0mat_ below should be a
% probability too (generally 1). This deluxe script automatically
% translates the data into probabilities for plotting the results. For
% survival data, the weights matrix has a different meaning: it is used to
% specify the number of animals that went missing or were removed during
% the experiment (enter the number of missing/removed animals at the time
% they were last seen alive in the test).
%
DATA{1} = []; % Dummy dataset - possibly unnecessary

glo.timevar = 0; % constant conc, will take 1 as conc

%% Initial values for the state variables
% For each state variable, we need to specify initial values for each
% scenario that we want to fit or simulate in the matrix _X0mat_. The first
% row specifies the scenarios (here: exposure treatments) that we want to
% model. Here, there are 4 scenarios in _X0mat_, but each data set has only 3
% of them. Watch the plot to see how BYOM deals with that situation.
% If you do not want to fit certain scenarios (exposure treatments) from
% the data, simply leave them out of _X0mat_.
%
% Note: if you do *not* want to start at _t_=0, specify the exact time in the
% global variable _glo.Tinit_ here (e.g., _glo.Tinit_ = 100;). If it is not
% specified, zero is used. The _X0mat_ thus defines the states at
% _glo.Tinit_, and not necessarily the value at the first data point.
% Plotting also always starts from _glo.Tinit_.
%
% Initial states, scenarios in columns, states in rows. First row are the
% identifiers of all scenarios (here: nominal concentrations). Second row
% is external concentration in mg/L, and third row body residues. For
% replicated data, scenarios should occur only once in _X0mat_.

X0mat = [1
 0]; % initial values (internal concentrations)

%% Initial values for the model parameters
% Model parameters are part of a 'structure' for easy reference. This means
% that you can address parameters by their name, instead of their position
% in a parameter vector. This makes the model definition in derivatives.m a
% lot easier. For each parameter, provide the initial value, whether you
% want to fit it or fix it to the initial value, the minimum bound, the
% maximum bound, and whether to fit the parameter on log10-scale or on
% normal scale. Fitting on log-scale is advisable for parameters that can
% span a very wide range; the optimisation routine can search this range
% more effectively if it is on log-scale. If you do not include this last
% value, a one will be filled in by prelim_checks.m (which means fitting on
% normal scale).

% syntax: par.name = [startvalue fit(0/1) minval maxval optional:log/normal scale
(0/1)];
% par.kin = [0.09 1 1e-3 10 0]; % degradation rate constant, d-1
% par.kout = [0.13 1 1e-3 10 0]; % elimination rate constant, d-1

% % Optionally, include an initial state as parameter (requires changes in
call_deri.m too)
% par.Ci0 = [100 0 0 1e6]; % initial internal concentration (mg/L)
load('')
conf_type = select_pred(2);

%% More options for the analysis
% Optionally, global parameters can be used in the structure glo (not
% fitted). However, see the text file reserved_globals.txt for names to
```

```
% avoid.

% % specify globals for parameters that are never fitted
% glo.Ct = 5; % the threshold could be made into a fixed global (also modify
derivatives.m)
% % Special globals to modify the optimisation (used in transfer.m)
% glo.wts = [1 10]; % different weights for different data sets (same size as DATA)
% glo.var = [10 20]; % supply residual variance for each data set, after
transformation (same size as DATA)

%% Time vector and labels for plots
% Specify what to plot. This also involves the time vector for the model
% lines (which may be taken differently than the time vector in the data
% set). If time vector glo.t is not specified, a default is constructed,
% based on the data set. You can specify the text that you would like to
% use for the axes, and the text used for the legend (which always includes
% the identifier for the scenario: the values in the first row of _X0mat_).

glo.t = linspace(0,50,100); % time vector for

% specify the y-axis labels for each state variable
glo.ylab{1} = 'external concentration (mg/mg)';
glo.ylab{2} = 'internal concentration (mg/mg)';
% specify the x-axis label (same for all states)
glo.xlab = 'time (h)';
glo.leglab1 = 'conc. '; % legend label before the 'scenario' number
glo.leglab2 = 'mg/mg'; % legend label after the 'scenario' number

prelim_checks % script to perform some preliminary checks and set things up
% Note: prelim_checks also fills all the options (opt_...) with defauls, so
% modify options after this call, if needed.

%% Calculations and plotting
% Here, the functions are called that will do the calculation and the
% plotting. Note that calc_plot can provide all of the plotting information
% as output, so you can also make your own customised plots. This section,
% by default, makes a multiplot with all state variables (each in its own
% panel of the multiplot). When the model is fitted to data, output is
% provided on the screen (and added to the log-file results.out). The
% zero-variate data point is also plotted with its prediction (although the
% legend obscures it here).
%
% Options for the plotting can be set using opt_plot (see prelim_checks.m).
% Options for the optimisation routine can be set using opt_optim. Options
% for the ODE solver are part of the global glo.
%
% You can turn on the events function there too, to smoothly catch the
% discontinuity in the model. For the demo, the iterations were turned off
% (opt_optim.it = 0).

glo.eventson = 0; % events function on (1) or off (0)
glo.useode = 1; % calculate model using ODE solver (1) or analytical solution (0)
opt_optim.it = 1; % show iterations of the optimisation (1, default) or not (0)
opt_plot.annot = 1; % extra subplot in multiplot for fits: 1) box with parameter
estimates, 2) overall legend
opt_plot.bw = 1; % if set to 1, plots in black and white with different plot
symbols
opt_optim.type = 4; % optimisation method 1) simplex, 4) parameter-space explorer
opt_optim.fit = 0; % fit the parameters (1), or don't (0)
opt_optim.it = 1; % show iterations of the optimisation (1, default) or not (0)

opt_optim.ps_plots = 1; % when set to 1, makes intermediate plots to monitor
progress of parameter-space explorer
opt_optim.ps_profs = 1; % when set to 1, makes profiles and additional sampling for
parameter-space explorer
opt_optim.ps_rough = 0; % set to 1 for rough settings of parameter-space explorer,
0 for settings as in openGUTS
opt_optim.ps_saved = 1; % use saved set for parameter-space explorer (1) or not
(0);
% optimise and plot (fitted parameters in par_out)
% par_out = calc_optim(par,opt_optim); % start the optimisation
% return % stop here, and run analyses below manually later
```

```
%% Plot results with confidence intervals
% The following code can be used to make a standard plot (the same as for
% the fits), but with confidence intervals. Options for confidence bounds
% on model curves can be set using opt_conf (see prelim_checks).
%
% Use opt_conf.type to tell calc_conf which sample to use:
% -1) Skips CIs (zero does the same, and an empty opt_conf as well).
% 1) Bayesian MCMC sample (default); CI on predictions are 95% ranges on
% the model curves from the sample
% 2) parameter sets from a joint likelihood region using the shooting
% method (limited sets can be used), which will yield (asymptotically) 95%
% CIs on predictions
% 3) as option 2, but using the parameter-space explorer

opt_conf.type = 3; % make intervals from 1) slice sampler, 2) likelihood region
shooting, 3) parspace explorer
opt_conf.lim_set = 0; % use limited set of n_lim points (1) or outer hull (2,
likelihood methods only) to create CIs
opt_conf.sens = 0; % type of analysis 0) no sensitivities 1) corr. with state, 2)
corr. with state/control, 3) corr. with relative change of state over time
opt_plot.annot = 1; % extra subplot in multiplot for fits: 1) box with parameter
estimates, 2) overall legend

out_conf = calc_conf(par,opt_conf); % calculate confidence intervals on model
curves
calc_and_plot(par,opt_plot,out_conf); % call the plotting routine again to plot
fits with CIs
```

TK Model 2

```
%%%% This Script were used to analyze TK in 2 different species (target
%%%% and non-target species)in the PhD Thesis of Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne
%%%BYOM, byom_bioconc_extra.m, a detailed example
% * Author: Tjalling Jager
% * Date: November 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% BYOM is a General framework for simulating model systems in terms of
% ordinary differential equations (ODEs). The model itself needs to be
% specified in <derivatives.html derivatives.m>, and <call_deri.html
% call_deri.m> may need to be modified to the particular problem as well.
% The files in the engine directory are needed for fitting and plotting.
% Fitting relies on the multinomial likelihood for survival and independent
% normal distributions (if needed after transformation) for continuous
% data. Results are shown on screen but also saved to a log file
% (results.out).
%
% In this example file (byom_bioconc_extra), we will walk step-by-step
% through a byom script file, explaining what happens and showing the
% outputs.
%
% *The model:* An organism is exposed to a chemical in its surrounding
% medium. The animal accumulates the chemical according to standard
% one-compartment first-order kinetics, specified by a bioconcentration
% factor (_Piw_) and an elimination rate (_ke_). The chemical degrades at a
% certain rate (_kd_). When the external concentration reaches a certain
% concentration (_Ct_), degradation stops. This is useful to demonstrate the
% events function in call_deri.m, which catches this discontuity
% graciously. In the form of ODE's:
%
% $$ \frac{d}{dt}C_w=-k_d C_w \quad \textrm{as long as } C_w>C_t $$
%
% $$ \frac{d}{dt}C_i=k_e(P_{iw}C_w-C_i) $$
%
% *This script:* byom_bioconc_extra demonstrates fitting using replicated
% data, with different concentration vectors in both data sets.
% Furthermore, several options are explained, such as the inclusion of
% zero-variate data and priors for Bayesian analysis. More support in the
% BYOM manual downloadable from <http://www.debtox.info/byom.html here>. A
% stripped version (less text, less options) can be found as
```

```
% <byom_bioconc_start.html byom_bioconc_start.m>.
%
% Copyright (c) 2012-2023, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.

%% Initial things
% Before we start, the memory is cleared, globals are defined, and the
% diary is turned off (output will be collected in the file results.out).
% The function pathdefine.m makes sure that the engine directory is added
% to the path. Make sure that this script is in a directory somewhere
% *below* the BYOM folder.

clear, clear global % clear the workspace and globals
global DATA W X0mat % make the data set and initial states global variables
global glo % allow for global parameters in structure glo
diary off % turn off the diary function (if it is accidentaly on)
% set(0,'DefaultFigureWindowStyle','docked'); % collect all figure into one window
with tab controls
set(0,'DefaultFigureWindowStyle','normal'); % separate figure windows

pathdefine(1) % set path to the BYOM/engine directory (option 1 to use parallel
toolbox, if available)
glo.basenm = mfilename; % remember the filename for THIS file for the plots
glo.saveplt = 0; % save all plots as (1) Matlab figures, (2) JPEG file or (3) PDF
(see all_options.txt)

%% Section to load Data file - here with Example Names

file = 'XXX.xlsx';
sht = 'X';
[Cl, DATA] = load_data(file, sht);

make_scen(4, Cl);

%% Initial values for the state variables
% For each state variable, we need to specify initial values for each
% scenario that we want to fit or simulate in the matrix _X0mat_. The first
% row specifies the scenarios (here: exposure treatments) that we want to
% model. Here, there are 4 scenarios in _X0mat_, but each data set has only 3
% of them. Watch the plot to see how BYOM deals with that situation.
% If you do not want to fit certain scenarios (exposure treatments) from
% the data, simply leave them out of _X0mat_.
%
% Note: if you do *not* want to start at _t_=0, specify the exact time in the
% global variable _glo.Tinit_ here (e.g., _glo.Tinit_ = 100;). If it is not
% specified, zero is used. The _X0mat_ thus defines the states at
% _glo.Tinit_, and not necessarily the value at the first data point.
% Plotting also always starts from _glo.Tinit_.
%
% Initial states, scenarios in columns, states in rows. First row are the
% identifiers of all scenarios (here: nominal concentrations). Second row
% is external concentration in mg/L, and third row body residues. For
% replicated data, scenarios should occur only once in _X0mat_.

% initial values, add one extra row per metabolite, Ww0 and L0 will be overwritten
X0mat = [1; zeros(size(DATA,2),1) ]; % zeros for all, initial weight overwritten in
call_deri.m

%% Initial values for the model parameters
% Step 1: only fit b and Wm

% syntax: par.name = [startvalue fit(0/1) minval maxval optional:log/normal scale
(0/1)];
% Keep these in this order for adaptive scripts to work!
par.kin = [1.9 0 1e-3 100 0]; % degradation rate constant, d-1
par.kout = [0.13 0 1e-3 100 0]; % elimination rate constant, d-1

par.Ww0 = [mean(DATA{2}(2,2:end)) 0 1e-6 5 1]; % Initial weight, can be fixed if we
have good data at t=0
par.b = [0.08829 1 1e-6 1 0]; % growth rate
par.Wm = [48.68 1 1e-6 500 1]; % maximum wet weight
```

```
% Each metabolite has one formation and one elimination parameter, entered
% as a pair before moving to the next
par.km1_f = [0.101 0 1e-6 100 0]; % metabolite 1 formation
par.km1_e = [0.1516 0 1e-6 100 0]; % metabolite 2 elimination

par.km2_f = [0.171 0 1e-6 100 0]; % metabolite 1 formation
par.km2_e = [0.1 0 1e-6 100 0]; % metabolite 2 elimination

% % Optionally, include an initial state as parameter (requires changes in
call_deri.m too)
% par.Ci0 = [100 0 0 1e6]; % initial internal concentration (mg/L)

%% More options for the analysis
% Optionally, global parameters can be used in the structure glo (not
% fitted). However, see the text file reserved_globals.txt for names to
% avoid.

% % specify globals for parameters that are never fitted
% glo.Ct = 5; % the threshold could be made into a fixed global (also modify
derivatives.m)
% % Special globals to modify the optimisation (used in transfer.m)
glo.wts = zeros(1, length(X0mat) - 1);
glo.wts(2) = 1;
% glo.var = [10 20]; % supply residual variance for each data set, after
transformation (same size as DATA)

%% Time vector and labels for plots
% Specify what to plot. This also involves the time vector for the model
% lines (which may be taken differently than the time vector in the data
% set). If time vector glo.t is not specified, a default is constructed,
% based on the data set. You can specify the text that you would like to
% use for the axes, and the text used for the legend (which always includes
% the identifier for the scenario: the values in the first row of _X0mat_).

glo.t = linspace(0,50,100); % time vector for the model curves in days
glo.timevar = 1;
% specify the y-axis labels for each state variable
glo.ylab{1} = 'Internal concentration (mg/mg)';
glo.ylab{2} = 'wet weight';
glo.ylab{3} = 'metabolite 1 concentration';
% specify the x-axis label (same for all states)
glo.xlab = 'time (h)';
glo.leglab1 = 'conc. '; % legend label before the 'scenario' number
glo.leglab2 = 'mg/mg'; % legend label after the 'scenario' number

prelim_checks % script to perform some preliminary checks and set things up
% Note: prelim_checks also fills all the options (opt_...) with defauls, so
% modify options after this call, if needed.

%% Calculations and plotting
% Here, the functions are called that will do the calculation and the
% plotting. Note that calc_plot can provide all of the plotting information
% as output, so you can also make your own customised plots. This section,
% by default, makes a multiplot with all state variables (each in its own
% panel of the multiplot). When the model is fitted to data, output is
% provided on the screen (and added to the log-file results.out). The
% zero-variate data point is also plotted with its prediction (although the
% legend obscures it here).
%
% Options for the plotting can be set using opt_plot (see prelim_checks.m).
% Options for the optimisation routine can be set using opt_optim. Options
% for the ODE solver are part of the global glo.
%
% You can turn on the events function there too, to smoothly catch the
% discontinuity in the model. For the demo, the iterations were turned off
% (opt_optim.it = 0).

glo.eventson = 0; % events function on (1) or off (0)
glo.useode = 1; % calculate model using ODE solver (1) or analytical solution (0)
opt_optim.it = 1; % show iterations of the optimisation (1, default) or not (0)
opt_plot.annot = 1; % extra subplot in multiplot for fits: 1) box with parameter
estimates, 2) overall legend
```

```
opt_plot.bw = 1; % if set to 1, plots in black and white with different plot
symbols
opt_optim.type = 4; % optimisation method 1) simplex, 4) parameter-space explorer
opt_optim.fit = 1; % fit the parameters (1), or don't (0)
opt_optim.it = 1; % show iterations of the optimisation (1, default) or not (0)

opt_optim.ps_plots = 1; % when set to 1, makes intermediate plots to monitor
progress of parameter-space explorer
opt_optim.ps_profs = 1; % when set to 1, makes profiles and additional sampling for
parameter-space explorer
opt_optim.ps_rough = 1; % set to 1 for rough settings of parameter-space explorer,
0 for settings as in openGUTS
opt_optim.ps_saved = 0; % use saved set for parameter-space explorer (1) or not
(0);

% Fit the model for the first time:
par_out = calc_optim(par,opt_optim); % start the optimisation
calc_and_plot(par_out,opt_plot);

par = par_out; % Get growth pars into par struct
% par = rmfield( par , 'tag_fitted' ); % remove fitted tag
% Now turn on fitting for all others
par.b(2) = 0; par.Wm(2) = 0; % turn off fitting for these
par.kin = [1.9 1 1e-3 100 0]; % degradation rate constant, d-1
par.kout = [0.13 1 1e-3 100 0];
par.km1_f = [0.51 1 1e-6 100 0]; % metabolite 1 formation
par.km1_e = [1.16 1 1e-6 100 0]; % metabolite 2 elimination

par.km2_f = [0.071 1 1e-6 100 0]; % metabolite 1 formation
par.km2_e = [0.01 1 1e-6 100 0]; % metabolite 2 elimination

glo.wts = ones(1, length(X0mat) - 1); % adjust wts to incorporate all DATA equally

par_out = calc_optim(par,opt_optim); % start the optimisation
calc_and_plot(par_out,opt_plot);
return
%% Plot results with confidence intervals
% The following code can be used to make a standard plot (the same as for
% the fits), but with confidence intervals. Options for confidence bounds
% on model curves can be set using opt_conf (see prelim_checks).
%
% Use opt_conf.type to tell calc_conf which sample to use:
% -1) Skips CIs (zero does the same, and an empty opt_conf as well).
% 1) Bayesian MCMC sample (default); CI on predictions are 95% ranges on
% the model curves from the sample
% 2) parameter sets from a joint likelihood region using the shooting
% method (limited sets can be used), which will yield (asymptotically) 95%
% CIs on predictions
% 3) as option 2, but using the parameter-space explorer

opt_conf.type = 3; % make intervals from 1) slice sampler, 2) likelihood region
shooting, 3) parspace explorer
opt_conf.lim_set = 0; % use limited set of n_lim points (1) or outer hull (2,
likelihood methods only) to create CIs
opt_conf.sens = 0; % type of analysis 0) no sensitivities 1) corr. with state, 2)
corr. with state/control, 3) corr. with relative change of state over time
opt_plot.annot = 1; % extra subplot in multiplot for fits: 1) box with parameter
estimates, 2) overall legend

out_conf = calc_conf(par_out,opt_conf); % calculate confidence intervals on model
curves
calc_and_plot(par_out,opt_plot,out_conf); % call the plotting routine again to plot
fits with CIs
```

Derivatives 2

```
%%%% This Script were used to analyze TK in 2 different species (target
%%%% and non-target species)in the PhD Thesis of Clara Isis Römer
%% The script were adapted in cooperation of Clara Isis Römer and Neil Sherborne

%% BYOM function derivatives.m (the model in ODEs)
```

```
% Syntax: dX = derivatives(t,X,par,c,glo)
% This function calculates the derivatives for the model system. It is
% linked to the script files <byom_bioconc_extra.html byom_bioconc_extra.m>
% and <byom_bioconc_start.html byom_bioconc_start.m>. As input,
% it gets:
%
% * _t_ is the time point, provided by the ODE solver
% * _X_ is a vector with the previous value of the states
% * _par_ is the parameter structure
% * _c_ is the external concentration (or scenario number)
% * _glo_ is the structure with information (normally global)
%
% Note: _glo_ is now an input to this function rather than a global. This
% makes the code considerably faster.
%
% Time _t_ and scenario name _c_ are handed over as single numbers by
% <call_deri.html call_deri.m> (you do not have to use them in this
% function). Output _dX_ (as vector) provides the differentials for each
% state at _t_.
%
% * Author: Tjalling Jager
% * Date: November 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% Copyright (c) 2012-2023, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.

%% Start
% Note that, in this example, variable _c_ (the scenario identifier) is not
% used in this function. The treatments differ in their initial exposure
% concentration that is set in X0mat. Also, the time _t_ and structure _glo_ are
not
% used here.

function dX = derivatives(t,X,par,c,glo)

%% Unpack states
% The state variables enter this function in the vector _X_. Here, we give
% them a more handy name.
if glo.timevar(1) == 1
 Cl = read_scen(-1,c,t,glo);
else
 Cl = c;
end

Cin = X(1); % state 1 is the internal concentration
Ww = X(2); % wet weight, not actually an ODE
%% Unpack parameters
% The parameters enter this function in the structure _par_. The names in
% the structure are the same as those defined in the byom script file. The
% 1 between parentheses is needed for fitting the model, as each parameter
% has 4-5 associated values.

kin = par.kin(1); % uptake rate constant,
kout = par.kout(1); % elimination rate constant, d-1

b = par.b(1);
Wm = par.Wm(1); % maximum length

if length(X) >2
 % get all metabolite entries:
 parMat = struct2cell(par);
 if size(parMat,1) > 2*(length(X) - 2) + 5 % stops fitted tag disrupting things
when profiling (if ps_rough = 0)
 parMat(size(parMat,1),:) = [];
 end
 parMat = cell2mat(parMat); % convert parameter entries to matrix
 formations = parMat(6:2:end,1); % column vector of all metab. formations
 eliminations = parMat(7:2:end,1); % column vector of all metab. eliminations
else
```

```
 formations = 0;
 eliminations = 0;
end

%% Calculate the derivatives
% This is the actual model, specified as a system of two ODEs:
dWw = b*(Wm^(1/3)*Ww^(2/3) - Ww); % wet weight
dCin = kin*Cl - kout*Cin - sum(formations)*Cin - Cin*dWw/Ww; % subtract metabolite
formation and dilution by growth
% dCin = kin*Lref/L*Cl - kout*Lref/L*Cin - sum(formations)*Cin - Cin*dWw/Ww;; %
Option for
% size dependent uptake and elimination - to be discussed

% All metabolites handled in a column vector
dM_all = formations.*Cin - eliminations.*X(3:end) - X(3:end).*dWw/Ww; % Now
including dilution by growth

dX = [dCin; dWw; dM_all]; % Add extra metabolites to the end
```

Call_deri 2

```
%%% This Script were used to analyze TK in 2 different species (target
%%%% and non-target species)in the PhD Thesis of Clara Isis Römer
%% BYOM function call_deri.m (calculates the model output)
% Syntax: [Xout,TE,Xout2,zvd] = call_deri(t,par,X0v,glo)
%
% This function calls the ODE solver to solve the system of differential
% equations specified in <derivatives.html derivatives.m>, or the explicit
% function(s) in <simplefun.html simplefun.m>. As input, it gets:
%
% * _t_ the time vector
% * _par_ the parameter structure
% * _X0v_ a vector with initial states and one concentration (scenario number)
% * _glo_ the structure with various types of information (used to be global)
%
% The output _Xout_ provides a matrix with time in rows, and states in
% columns. This function calls <derivatives.html derivatives.m>. The
% optional output _TE_ is the time at which an event takes place (specified
% using the events function). The events function is set up to catch
% discontinuities. It should be specified according to the problem you are
% simulating. If you want to use parameters that are (or influence) initial
% states, they have to be included in this function. Optional output Xout2
% is for additional uni-variate data (not used here), and zvd is for
% zero-variate data (used in <byom_bioconc_extra.html
% byom_bioconc_extra.m>).
%
% * Author: Tjalling Jager
% * Date: November 2021
% * Web support: <http://www.debtox.info/byom.html>
% * Back to index <walkthrough_byom.html>
%
% Copyright (c) 2012-2023, Tjalling Jager, all rights reserved.
% This source code is licensed under the MIT-style license found in the
% LICENSE.txt file in the root directory of BYOM.
%% Start
function [Xout,TE,Xout2,zvd] = call_deri(t,par,X0v,glo)

% initialise extra outputs as empty for when they are not used
Xout2 = []; % additional uni-variate output
zvd = []; % additional zero-variate output

% Note: if these options are not used, these variables must be defined as
% empty as they are outputs of this function.

% if needed, calculate model values for zero-variate data from parameter
% set; these lines can be removed if no zero-variate data are used
if ~isempty(glo.zvd) % if there are zero-variate data defined (see
byom_bioconc_extra)
 zvd = glo.zvd; % copy zero-variate data structure to zvd
 zvd.ku(3) = par.Piw(1) * par.ke(1); % add model prediction as third value in zvd
else % if there are no zero-variate data defined (as in byom_bioconc_start)
 zvd = []; % additional zero-variate output, output defined as empty matrix
```

```
end

%% Initial settings
% This part extracts optional settings for the ODE solver that can be set
% in the main script (defaults are set in prelim_checks). The useode option
% decides whether to calculate the model results using the ODEs in
% <derivatives.html derivatives.m>, or the analytical solution in
% <simplefun.html simplefun.m>. Using eventson=1 turns on the events
% handling. Also modify the sub-function at the bottom of this function!
% Further in this section, initial values can be determined by a parameter
% (overwrite parts of X0), and zero-variate data can be calculated. See the
% example BYOM files for more information.

useode = glo.useode; % calculate model using ODE solver (1) or analytical solution
(0)
eventson = glo.eventson; % events function on (1) or off (0)
stiff = glo.stiff; % set to 1 or 2 to use a stiff solver instead of the standard
one

% Unpack the vector X0v, which is X0mat for one scenario
X0 = X0v(2:end); % these are the intitial states for a scenario
% % if needed, extract parameters from par that influence initial states in X0
X0(2) = par.Ww0(1);

%% Calculations
% This part calls the ODE solver (or the explicit model in <simplefun.html
% simplefun.m>) to calculate the output (the value of the state variables
% over time). There is generally no need to modify this part. The solver
% ode45 generally works well. For stiff problems, the solver might become
% very slow; you can try ode15s instead.

c = X0v(1); % the concentration (or scenario number)
t = t(:); % force t to be a row vector (needed when useode=0)

TE = 0; % dummy for time of events

if useode == 1 % use the ODE solver to calculate the solution
 % Note: set options AFTER the 'if useode == 1' as odeset takes
 % considerable calculation time, which is not needed when using the
 % analytical solution. Also note that the global _glo_ is now input to
 % the derivatives function. This increases calculation speed.

 % specify options for the ODE solver; feel free to change the
 % tolerances, if you know what you're doing (for some problems, it is
 % better to set them much tighter, e.g., both to 1e-9)
 reltol = 1e-4;
 abstol = 1e-7;
 options = odeset; % start with default options
 if eventson == 1
 options = odeset(options,'Events',@eventsfun,'RelTol',reltol,'AbsTol',abstol); %
add an events function and tigher tolerances
 else
 options = odeset(options,'RelTol',reltol,'AbsTol',abstol); % only specify
tightened tolerances
 end
 % options = odeset(options,'InitialStep',max(t)/1000,'MaxStep',max(t)/100); %
specify smaller stepsize

 % call the ODE solver (try ode15s for stiff problems, and possibly with for pulsed
forcings)
 if isempty(options.Events) % if no events function is specified ...
 switch stiff
 case 0
 [~,Xout] = ode45(@derivatives,t,X0,options,par,c,glo);
 case 1
 [~,Xout] = ode113(@derivatives,t,X0,options,par,c,glo);
 case 2
 [~,Xout] = ode15s(@derivatives,t,X0,options,par,c,glo);
 end
 else % with an events functions ... additional output arguments for events:
 % TE catches the time of an event, YE the states at the event, and IE the number
of the event
```

```
 switch stiff
 case 0
 [~,Xout,TE,YE,IE] = ode45(@derivatives,t,X0,options,par,c,glo);
 case 1
 [~,Xout,TE,YE,IE] = ode113(@derivatives,t,X0,options,par,c,glo);
 case 2
 [~,Xout,TE,YE,IE] = ode15s(@derivatives,t,X0,options,par,c,glo);
 end
 end
else % alternatively, use an explicit function provided in simplefun
 Xout = simplefun(t,X0,par,c,glo);
end

if isempty(TE) || all(TE == 0) % if there is no event caught
 TE = +inf; % return infinity
end

%% Output mapping
% _Xout_ contains a row for each state variable. It can be mapped to the
% data. If you need to transform the model values to match the data, do it
% here.

% Xout(:,1) = Xout(:,1).^3; % e.g., do something on first column, like cube it ...

% % To obtain the output of the derivatives at each time point. The values in
% % dXout might be used to replace values in Xout, if the data to be fitted
% % are the changes (rates) instead of the state variable itself.
% % dXout = zeros(size(Xout)); % initialise with zeros
% for i = 1:length(t) % run through all time points
% dXout(i,:) = derivatives(t(i),Xout(i,:),par,c,glo);
% % derivatives for each stage at each time
% end
%% Events function
% Modify this part of the code if _eventson_=1. This subfunction catches
% the 'events': in this case, it looks for the external concentration where
% degradation stops. This function should be adapted to the problem you are
% modelling (this one matches the byom_bioconc_... files). You can catch
% more events by making a vector out of _values_.
%
% Note that the eventsfun has the same inputs, in the same sequence, as
% <derivatives.html derivatives.m>.

function [value,isterminal,direction] = eventsfun(t,X,par,c,glo)

Ct = par.Ct(1); % threshold external concentration where degradation stops
nevents = 1; % number of events that we try to catch

value = zeros(nevents,1); % initialise with zeros
value(1) = X(1) - Ct; % thing to follow is external concentration (state 1) minus
threshold
isterminal = zeros(nevents,1); % do NOT stop the solver at an event
direction = zeros(nevents,1); % catch ALL zero crossing when function is increasing
or decreasing
```

Data

Data *Spodoptera littoralis* – TK Model 1 and 2

Time is always in hours expressed in columns replicates are in rows.

Compound A – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0043	0.0043	0.0043
1	0.0043	0.0043	0.0044	0.0043
5	0.0044	0.0043	0.0043	0.0043
24	0.0043	0.0043	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0103	0.0039	0.0052	0.0092	0.0048	0.0009	0.0015	0.0002	0.0006	0.0020	0.0138	0.0003
5	0.0020	0.0020	0.0004	0.0116	0.0224	0.0008	0.0022	0.0000	0.0272	0.0009	0.0000	0.0063
24	0.0004	0.0025	0.0037	0.0037	0.0003	0.0007	NaN	0.0003	0.0043	NaN	0.0007	0.0031
25	0.0000	0.0064	0.0000	NaN	0.0021	0.0000	0.0000	0.0014	0.0030	0.0037	NaN	0.0058
29	0.0000	0.0004	0.0060	0.0005	0.0044	0.0000	0.0000	0.0025	NaN	NaN	NaN	NaN
48	NaN	0.0000	0.0001	0.0000	0.0000	NaN	0.0001	0.0000	0.0000	0.0000	NaN	0.0000

time	weight (mg) wet larva											
0	4.2	4.8	3.5	3.6	2.5	3	4.4	3.5	3.3	4.2	2.9	3.1
1	3.5	3.3	4.2	3.6	2.5	2.2	4.6	4.2	4.8	3.5	2.9	3.1
5	3	4.5	5.2	4.4	4.5	4.9	3.7	4	4.3	4.7	4	4
24	16.6	16	16.7	17.5	16.5	15.1	NaN	15.9	18.8	NaN	17.1	12.1
25	21.7	17.2	14.1	NaN	16.6	19.6	18.8	15.9	17.2	16.1	NaN	17
29	27.2	23.2	16.7	18.5	22.6	26.6	25.7	15.2	13.8	10.1	17.1	30.7
48	NaN	32.6	35	25.3	25	NaN	18.7	28	17.5	30.5	33.7	32.1

Compound B – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0043	0.0043	0.0043
1	0.0030	0.0028	0.0027	0.0043
5	0.0022	0.0028	0.0030	0.0024
24	0.0022	0.0028	0.0030	0.0022
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0060	0.0093	0.0064	0.0061	0.0075	0.0073	0.0016	0.0049	0.0056	0.0052	0.0167	0.0133
5	0.0082	0.0031	0.0115	0.0021	0.0014	0.0079	0.0023	0.0128	0.0010	NaN	0.0085	0.0085
24	0.0007	NaN	0.0010	0.0008	0.0004	0.0011	0.0005	0.0003	NaN	0.0006	0.0008	0.0013
25	NaN	0.0001	0.0001	0.0002	0.0001	0.0011	NaN	NaN	NaN	NaN	NaN	NaN
29	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	NaN	NaN	NaN	NaN	NaN	NaN
48	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	NaN	NaN	0.0000	0.0000	NaN

time	weight (mg) wet larva											
0	4.9	2.3	3.2	2.5	2.6	3.6	3.6	3.1	2.9	4.9	3.8	3.1
1	2.6	3.6	3.2	3.3	4.8	2.3	3.6	3.1	4.1	4.5	3.8	3.1
5	4.2	4.5	4.2	5.1	4.3	6.5	4.2	4.7	5.5	NaN	5	4.9
24	21.7	13.6	16.4	18.3	18.2	15.8	21.5	15.3	16.4	9.6	13	19.4
25	NaN	16.8	19.9	12.9	11.9	15.1	19.1	10.1	3.1	15.3	16.3	12.8
29	23.6	28.5	14.7	23.1	23.1	18.3	22.4	28.9	27.4	NaN	23.8	27.5
48	25.8	25.1	29.1	29.3	40.5	31.8	26.9	27.7	NaN	24.6	25.8	23.4

Compound C– Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0044	0.0043	0.0043
1	0.0043	0.0043	0.0043	0.0043
5	0.0044	0.0044	0.0044	0.0043
24	0.0043	0.0039	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)										
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0089	NaN	NaN	0.0040	0.0073	0.0067	0.0035	0.0022	0.0056	0.0158	0.0316
5	0.0194	0.0081	0.0100	0.0315	0.0103	0.0143	0.0417	0.0152	0.0216	0.0152	0.0122
24	0.0066	0.0086	0.0098	0.0073	0.0059	0.0057	0.0094	0.0055	0.0065	NaN	NaN
25	0.0000	0.0000	0.0064	0.0073	NaN	NaN	NaN	NaN	NaN	NaN	NaN
29	0.0000	0.0043	0.0000	0.0046	NaN	NaN	NaN	NaN	NaN	NaN	NaN
48	0.0002	0.0005	0.0000	0.0000	NaN	NaN	NaN	NaN	NaN	NaN	NaN

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0005
24	0.0000
25	0.0000
29	0.0000
48	0.0014

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0005
24	0.0000
25	0.0000
29	0.0000
48	0.0014

time	weight (mg) wet larva										
0	2.7	2.8	3.8	3.1	3.5	3.9	4.4	4	3.6	4	3.1
1	2.7	3.8	3.8	3.1	3.5	3.9	4.4	4	3.6	4	3.1
5	4.8	5.5	5	4.7	4.6	5.8	3.6	4.8	5.1	4.6	6.5
24	20.1	15.9	12.7	16.1	18.6	23.1	11.7	18.6	20.1	n/a	24.4
25	19.8	15.1	13.5	16.6	17.3	15	17.4	15.3	16.6	18.2	15.2
29	21.3	25.3	16.6	20	22	16.6	17.2	14	18.7	16.4	13.9
48	27.1	39.5	33.9	31.3	31.4	35	30.1	38.2	31.3	51.3	34.2

Compound D– Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0044	0.0043	0.0043
1	0.0043	0.0043	0.0043	0.0043
5	0.0044	0.0044	0.0044	0.0043
24	0.0043	0.0039	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	0.0000	0.0000	0.0000
1	0.0029	0.0209	0.0063	0.0036	0.0096	0.0108	0.0039	0.0081	0.0034	0.0063	0.0032	NaN
5	0.0073	0.0000	0.0047	0.0306	0.0106	0.0209	0.0417	0.0077	0.0000	0.0204	0.0250	0.0452
24	0.0236	0.0000	0.0283	0.0229	0.0265	0.0157	0.0260	0.0187	0.0165	NaN	0.0220	0.0263
25	0.0067	0.0000	0.0079	0.0050	0.0034	0.0065	0.0059	0.0081	0.0051	0.0171	0.0085	0.0046
29	NaN	0.0055	0.0038	0.0050	0.0035	0.0052	NaN	NaN	NaN	NaN	NaN	NaN
48	0.0008	0.0006	0.0017	0.0000	0.0006	0.0026	NaN	NaN	NaN	NaN	NaN	NaN

time	mg/mg (metabolite1)
0	0.0000
1	0.0000
5	0.0104
24	0.0030
25	0.0043
29	0.0029
48	0.0032

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0000
24	0.0001
25	0.0001
29	0.0003
48	0.0001

time	weight (mg) wet larva											
0	4.9	2.3	3.2	2.5	2.6	3.6	3.6	3.1	2.9	4.9	3.8	3.1
1	2.6	3.6	3.2	3.3	4.8	2.3	3.6	3.1	4.1	4.5	3.8	3.1
5	4.2	4.5	4.2	5.1	4.3	6.5	4.2	4.7	5.5	NaN	5	4.9
24	21.7	13.6	16.4	18.3	18.2	15.8	21.5	15.3	16.4	9.6	13	19.4
25	NaN	16.8	19.9	12.9	11.9	15.1	19.1	10.1	3.1	15.3	16.3	12.8
29	23.6	28.5	14.7	23.1	23.1	18.3	22.4	28.9	27.4	NaN	23.8	27.5
48	25.8	25.1	29.1	29.3	40.5	31.8	26.9	27.7	NaN	24.6	25.8	23.4

Compound E – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0044	0.0043	0.0043
1	0.0043	0.0043	0.0043	0.0043
5	0.0044	0.0044	0.0044	0.0043
24	0.0043	0.0039	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)										
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0002	0.0001	0.0003	0.0001	0.0002	0.0001	0.0001	0.0001	NaN	0.0001	0.0000
5	0.0173	0.0017	0.0216	0.0261	NaN	NaN	NaN	NaN	NaN	NaN	0.0000
24	0.0026	0.0131	0.0036	0.0110	0.0093	0.0003	0.0160	0.0128	NaN	NaN	0.0076
25	0.0044	0.0020	0.0018	0.0064	0.0016	0.0000	0.0016	0.0050	0.0078	0.0031	0.0027
29	NaN	0.0003	0.0004	0.0021	0.0000	0.0023	NaN	NaN	NaN	NaN	NaN
48	0.0002	0.0001	0.0003	0.0001	0.0002	0.0001	0.0001	NaN	NaN	NaN	NaN

time	mg/mg (metabolite1)	
0	0.0000	0.0000
1	0.0000	0.0000
5	0.0021	0.0021
24	NaN	0.0006
25	NaN	0.0007
29	0.0004	0.0004
48	0.0000	0.0003

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0027
24	0.0008
25	NaN
29	0.0006
48	0.0028

time	weight (mg) wet larva										
0	4.9	2.3	3.2	2.5	2.6	3.6	3.6	3.1	2.9	4.9	3.8
1	2.6	3.6	3.2	3.3	4.8	2.3	3.6	3.1	4.1	4.5	3.8
5	4.2	4.5	4.2	5.1	4.3	6.5	4.2	4.7	5.5	NaN	5
24	21.7	13.6	16.4	18.3	18.2	15.8	21.5	15.3	16.4	9.6	13
25	NaN	16.8	19.9	12.9	11.9	15.1	19.1	10.1	3.1	15.3	16.3
29	23.6	28.5	14.7	23.1	23.1	18.3	22.4	28.9	27.4	NaN	23.8
48	25.8	25.1	29.1	29.3	40.5	31.8	26.9	27.7	NaN	24.6	25.8

Compound F – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0043	0.0043	0.0043
1	0.0043	0.0043	0.0044	0.0043
5	0.0044	0.0043	0.0043	0.0043
24	0.0043	0.0043	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	NaN	NaN	0.0029	0.0033	0.0017	0.0013	0.0015	0.0033	NaN	0.0015	0.0004	0.0018
5	0.0022	0.0006	0.0039	0.0027	0.0035	0.0007	0.0006	NaN	NaN	0.0014	0.0016	NaN
24	0.0003	0.0002	0.0006	0.0001	0.0009	0.0007	0.0016	0.0012	0.0000	NaN	0.0008	NaN
25	0.0003	0.0001	0.0000	0.0000	0.0000	0.0000	NaN	0.0000	NaN	0.0000	0.0000	NaN
29	0.0000	0.0001	0.0000	NaN	0.0000	NaN	0.0000	0.0000	0.0000	0.0000	NaN	0.0000
48	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	0.0000	0.0000	NaN	0.0000	0.0000	0.0000

time	mg/mg (metabolite1)
0	0.0000
1	0.0000
5	0.0037
24	NaN
25	0.0001
29	0.0001
48	NaN

time	mg/mg (metabolite2)
0	0.00000
1	0.00000
5	0.00000
24	0.00315
25	0.00348
29	0.00103
48	0.00054

time	weight (mg) wet larva											
0	2.7	2.8	3.8	3.1	3.5	3.9	4.4	4	3.6	4	3.1	4.2
1	2.7	2.8	3.8	3.1	3.5	3.9	4.4	4	3.6	4	3.1	4.2
5	4.8	5.5	5	4.7	4.6	5.8	3.6	4.8	5.1	4.6	6.5	6.1
24	20.1	15.9	12.7	16.1	18.6	23.1	11.7	18.6	20.1	n/a	24.4	18.1
25	19.8	15.1	13.5	16.6	17.3	15	17.4	15.3	16.6	18.2	15.2	17.1
29	21.3	25.3	16.6	20	22	16.6	17.2	14	18.7	16.4	13.9	18.5
48	27.1	39.5	33.9	31.3	31.4	35	30.1	38.2	31.3	51.3	34.2	36.5

Compound G – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0043	0.0043	0.0043
1	0.0043	0.0043	0.0044	0.0043
5	0.0044	0.0043	0.0043	0.0043
24	0.0043	0.0043	0.0043	0.0043
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0198	0.0043	0.0064	0.0098	0.0044	0.0007	0.0020	0.0003	0.0006	0.0014	0.0100	0.0000
5	0.0019	0.0018	0.0004	0.0174	0.0229	0.0007	0.0017	0.0000	0.0046	0.0011	0.0000	NaN
24	0.0004	0.0020	0.0032	0.0036	0.0002	0.0006	0.0027	0.0002	0.0055	0.0015	0.0008	0.0027
25	0.0000	NaN	0.0056	0.0065	NaN	0.0051	0.0061	0.0000	0.0000	0.0036	NaN	NaN
29	0.0000	NaN	0.0000	0.0000	NaN	0.0000	0.0000	0.0007	0.0023	0.0055	NaN	NaN
48	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0027	0.0000

time	mg/mg (metabolite1)
0	0.0000
1	0.0000
5	0.0026
24	0.0008
25	0.0015
29	0.0005
48	0.0001

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0010
24	0.0038
25	0.0013
29	0.0029
48	0.0009

time	weight (mg) wet larva											
0	1.8	3.1	3.4	3.4	2.7	3.3	3.7	3.2	4.2	4.7	4	3.2
1	1.8	3.1	3.4	3.4	2.7	3.3	3.7	3.2	4.2	4.7	4	3.2
5	3	4.9	4.7	2.9	4.4	5.8	4.9	3.6	5.5	3.6	5.5	3.7
24	14.7	20.3	18.7	17.7	20.2	17.9	17.6	20.8	14.5	19.6	14.4	14.3
25	20.2	9.7	15.2	15.4	15.7	16.6	16.4	17.4	21	10.3	NaN	14.5
29	27.5	3	17.5	19.2	NaN	21.6	19.9	29.3	22.8	11	24.8	NaN
48	30.2	35.1	24.3	36	34.5	28	25.4	23.3	31.6	28.9	33.3	23.7

Compound Coumarin – Leaf disk toxicokinetic bioassay

time	mg/mg leaf			
0	0.0043	0.0043	0.0043	0.0043
1	0.0030	0.0037	0.0030	0.0035
5	0.0030	0.0034	0.0030	0.0035
24	0.0000	0.0009	0.0000	0.0000
25	0.0000	0.0000	0.0000	0.0000
29	0.0000	0.0000	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0000

time	mg/mg w.wt (internal cons_larva)											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0196	0.0203	0.0155	NaN	0.0183	0.0153	0.0259	NaN	NaN	NaN	0.0176	0.0164
5	0.0037	NaN	0.0181	0.0010	0.0121	NaN	0.0004	0.0089	0.0060	NaN	0.0074	0.0088
24	0.0006	0.0003	0.0009	0.0002	0.0002	0.0002	0.0003	0.0003	0.0008	0.0005	0.0003	0.0009
25	0.0019	NaN	0.0008	NaN	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	0.0000	0.0001
29	0.0003	0.0000	0.0000	NaN	0.0025	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
48	0.0002	0.0001	0.0003	0.0000	0.0000	0.0000	NaN	0.0000	0.0002	0.0000	0.0000	0.0002

time	mg/mg (metabolite1)
0	0.0000
1	0.0000
5	0.0000
24	0.0006
25	0.0005
29	0.0005
48	0.0003

time	mg/mg (metabolite2)
0	0.0000
1	0.0000
5	0.0000
24	0.0011
25	0.0020
29	0.0020
48	0.0003

time	weight (mg) wet larva											
0	4.5	3.2	4.3	3	3.6	3.2	3.8	4.7	4.2	3	3	3.6
1	4.5	3.2	4.3	3	3.6	3.2	3.8	4.7	4.2	3	3	3.6
5	5.4	4.7	3.6	5.6	3.8	NaN	6.3	4	5.5	NaN	5	5.7
24	19.9	15.5	14.9	18.1	17.6	18	16.6	17.5	18	19.2	18.3	15.7
25	14.6	NaN	23.9	20	20.5	22.1	20.1	22.3	19	18.7	23.1	16.7
29	17.6	28.8	25.8	NaN	12.5	19.7	15.8	16.5	22.9	21.8	16.6	21.4
48	38.6	23.1	26.8	26.2	26.2	33.7	NaN	42.4	35.1	34	31.7	33.1

Data *Chironomus riparius* – TK Model 2

Time is always in hours expressed in columns replicates are in rows.

Compound A – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water		
0	0.001	0.001	0.001
2	0.001	0.001	0.001
4	0.001	0.001	0.001
6	0.001	0.001	0.001
24	0.001	0.001	0.001
26	0	0	0
28	0	0	0
30	0	0	0
48	0	0	0
50	0	0	0
52	0	0	0
54	0	0	0
56	0	0	0
72	0	0	0

time	mg/mg w.wt (internal cons_larva)		
0	0.000	0.000	0.000
2	0.002	0.002	0.001
4	0.002	0.002	0.003
6	0.002	0.004	0.002
24	0.002	NaN	0.002
26	0.002	0.003	0.004
28	0.003	0.003	0.003
30	0.003	0.002	0.003
48	0.001	0.001	0.001
50	0.001	0.001	0.001
52	0.001	0.001	0.001
54	0.000	0.000	0.000
56	0.000	0.000	NaN
72	0.000	0.000	NaN

time	mg/mg (metabolite1)		
0	0.000	NaN	NaN
2	0.000	NaN	NaN
4	0.000	NaN	NaN
6	0.000	NaN	NaN
24	0.001	NaN	NaN
26	0.001	NaN	NaN
28	0.001	NaN	NaN
30	0.003	NaN	NaN
48	0.001	NaN	NaN
50	0.001	NaN	NaN
52	0.000	NaN	NaN
54	0.000	NaN	NaN
56	0.000	NaN	NaN
72	0.000	NaN	NaN

time	weight (mg) wet larva		
0	1.1	0.9	2.3
2	1.3	1.3	2.3
4	2.2	2.12	2.1
6	2.5	1.4	3
24	2.6	2.4	2.9
26	2.6	2.3	1.8
28	2.4	2.1	3
30	2.2	3	2.6
48	2.7	3	2.8
50	3	2.9	2.2
52	3.12	2.5	3
54	2.45	2.9	3.2
56	2.6	2.9	NaN
72	3.9	3.4	NaN

Compound B – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg w.wt (internal cons_larva)	
0	0.0000	0.0000
2	0.0005	0.0007
4	0.0011	0.0007
6	0.0010	0.0007
24	0.0008	0.0006
26	0.0003	0.0008
28	0.0003	0.0008
30	0.0015	0.0019
48	0.0005	0.0005
50	0.0007	0.0007
52	0.0003	0.0002
54	0.0001	0.0017
56	0.0010	0.0010
72	0.0003	0.0001

time	weight (mg) wet larva	
0	2	2.3
2	2	2.3
4	2.3	2.1
6	2.4	2.2
24	2.6	2.5
26	3	2.6
28	3.01	2.6
30	3.1	2.4
48	3.02	2.2
50	2.9	2.9
52	3.4	2.5
54	3.5	2.9
56	3	2.9
72	3.9	3.4

Compound C – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg w.wt (internal cons_larva)	
0	0.0000	0.0000
2	NaN	0.1550
4	NaN	NaN
6	0.2923	0.2661
24	0.7583	NaN
26	0.0103	0.1952
28	0.0414	0.0526
30	0.0028	0.0598
48	0.0253	0.2345
50	0.0000	0.1300
52	0.0298	NaN
54	0.0000	NaN
56	0.0026	0.0001
72	NaN	NaN

time	weight (mg) wet larva	
0	1.3	2.3
2	2.1	2
4	0.9	2
6	2.2	2.3
24	2.4	2.3
26	3	2.1
28	2.9	1.9
30	2.7	3.1
48	3	2.9
50	3.12	3.4
52	2.45	2.4
54	2.6	2.6
56	3.9	3
72	3.2	2.7

Compound D – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg w.wt (internal cons_larva)	
0	0.000	0.000
2	0.000	0.000
4	0.012	0.012
6	0.033	0.010
24	0.011	0.011
26	0.010	0.010
28	0.004	0.006
30	NaN	NaN
48	0.004	0.005
50	0.000	0.000
52	0.004	0.004
54	0.003	0.004
56	0.004	0.004
72	0.000	0.000

time	weight (mg) wet larva	
0	2	2.3
2	2	2.3
4	2.3	2.1
6	2.4	2.2
24	2.6	2.5
26	2.8	2.6
28	3.01	2.6
30	3.1	2.6
48	3.02	2.2
50	2.9	2.9
52	3.4	2.5
54	3.5	2.9
56	2.9	2.9
72	3.3	3

Compound E – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg w.wt (internal cons_larva)	
0	0.0000	0.0000
2	0.0000	0.0000
4	0.0000	0.0000
6	0.0007	0.0052
24	0.3048	0.0069
26	0.0000	0.0142
28	0.8333	0.0183
30	0.0024	0.0030
48	0.0046	0.0002
50	0.0002	0.0002
52	0.0001	0.0000
54	0.0000	0.0000
56	0.0000	0.0000
72	0.0000	0.0000

time	mg/mg (metabolite1)	
0	0.0000	NaN
2	0.0003	NaN
4	0.0004	NaN
6	NaN	NaN
24	0.0004	NaN
26	0.0004	NaN
28	0.0005	NaN
30	0.0004	NaN
48	0.0038	NaN
50	0.0003	NaN
52	0.0003	NaN
54	0.0005	NaN
56	0.0004	NaN
72	0.0004	NaN

time	weight (mg) wet larva	
0	2.2	1.56
2	2.9	2
4	2.5	2.1
6	2.9	2.3
24	2.3	1.7
26	2.3	1.2
28	2.1	1.8
30	2.6	3
48	2.6	2.6
50	2.9	2.8
52	3	2.3
54	2.2	3
56	2.5	3.1
72	2.6	2.5

Compound F – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg (metabolite1)	
0	0.0000	NaN
2	0.0003	NaN
4	0.0004	NaN
6	NaN	NaN
24	0.0004	NaN
26	0.0004	NaN
28	0.0005	NaN
30	0.0004	NaN
48	0.0038	NaN
50	0.0003	NaN
52	0.0003	NaN
54	0.0005	NaN
56	0.0004	NaN
72	0.0004	NaN

time	mg/mg (metabolite2)	
0	0.00000	NaN
2	0.00000	NaN
4	0.00480	NaN
6	0.00414	NaN
24	0.00522	NaN
26	0.00522	NaN
28	0.00571	NaN
30	0.00462	NaN
48	0.00462	NaN
50	0.00414	NaN
52	0.00400	NaN
54	0.00545	NaN
56	0.00480	NaN
72	0.00462	NaN

time	weight (mg) wet larva	
0	2.2	1.56
2	2.9	2
4	2.5	2.1
6	2.9	2.3
24	2.3	1.7
26	2.3	1.2
28	2.1	1.8
30	2.6	3
48	2.6	2.6
50	2.9	2.8
52	3	2.3
54	2.2	3
56	2.5	3.1
72	2.6	2.5

Compound Coumarin – Water (OECD adapted) toxicokinetic bioassay.

time	mg/mL water	
0	0.001	0.001
2	0.001	0.001
4	0.001	0.001
6	0.001	0.001
24	0.001	0.001
26	0	0
28	0	0
30	0	0
48	0	0
50	0	0
52	0	0
54	0	0
56	0	0
72	0	0

time	mg/mg w.wt (internal cons_larva)	
0	0.00	0.00
2	0.02	0.02
4	0.02	0.02
6	0.02	0.01
24	0.01	0.01
26	0.01	0.01
28	0.02	0.02
30	0.02	0.00
48	0.02	0.00
50	0.00	0.00
52	0.00	0.00
54	0.00	0.00
56	0.00	0.00
72	0.00	0.00

time	mg/mg (metabolite1)	
0	0.000	0.000
2	0.021	0.016
4	0.021	0.015
6	0.021	0.012
24	0.015	0.009
26	0.013	0.014
28	0.021	0.022
30	0.020	0.000
48	0.019	0.000
50	0.000	0.000
52	0.000	0.000
54	0.000	0.000
56	0.000	0.000
72	0.000	0.000

time	weight (mg) wet larva	
0	2.9	2.84
2	3	2.94
4	3.1	3.04
6	2.2	2.26
24	3.05	2.99
26	3.1	3.16
28	2	1.94
30	2.9	2.84
48	2.8	2.74
50	3.4	3.46
52	3	2.94
54	2.9	2.84
56	3	3.06
72	3.1	3.04

Data *Spodoptera littoralis* feces– Compound quantities

Time is always in hours.

Compound A – Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.125	0
2	0.125	0
3	0.104	0
4	0.104	0
5	0.146	0.001
6	0.146	0.001
7	0.063	0.001
8	0.063	0.001
9	0.000	0.001
10	0.000	0.001
11	NA	NA
12	NA	NA

Compound B – Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.021	0.001
2	0.021	0.001
3	0.000	0.000
4	0.000	0.000
5	0.000	0.000
6	0.021	0.001
7	0.021	0.001
8	0.021	0.001
9	0.125	0.001
10	0.125	NaN
11	0.021	NaN
12	NaN	NaN

Compound C– Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.036	0.031
2	0.036	0.031
3	0.023	0.020
4	0.023	0.000
5	NaN	NaN
6	NaN	NaN
7	NaN	NaN
8	NaN	NaN
9	NaN	NaN
10	NaN	NaN
11	NaN	NaN
12	NaN	NaN

Compound D – Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.019	0.024
2	0.019	0.024
3	0.010	0.053
4	0.010	0.054
5	0.006	0.041
6	NaN	0.041
7	NaN	0.023
8	NaN	0.022
9	NaN	0.035
10	NaN	0.033
11	NaN	0.043
12	NaN	0.044

Compound E– Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.004	0
2	0.002	0
3	0.006	0
4	0.001	0.001
5	0.004	0.001
6	NA	NA
7	NA	NA
8	NA	NA
9	NA	NA
10	NA	NA
11	NA	NA
12	NA	NA

Compound F – Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	NaN	NaN
2	NaN	NaN
3	NaN	NaN
4	NaN	NaN
5	NaN	NaN
6	NaN	NaN
7	NaN	NaN
8	NaN	NaN
9	NaN	NaN
10	NaN	NaN
11	NaN	NaN
12	NaN	NaN

Compound G– Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	NaN	NaN
2	NaN	NaN
3	NaN	NaN
4	NaN	NaN
5	NaN	NaN
6	NaN	NaN
7	NaN	NaN
8	NaN	NaN
9	NaN	NaN
10	NaN	NaN
11	NaN	NaN
12	NaN	NaN

Compound Coumarin – Leaf disk toxicokinetic bioassay: Feces

	mg per feces total	
	time	
replicate	0-24 h	0-48 h
1	0.035	0.001
2	0.032	0.001
3	0.031	0.001
4	0.031	0.001
5	0.088	0.004
6	0.083	0.003
7	0.062	0.003
8	0.061	0.003
9	0.105	0.004
10	0.111	0.005
11	NaN	NaN
12	NaN	NaN

Data *Spodoptera littoralis* – Compound quantities

Compound A – Leaf disk toxicokinetic bioassay: total body

time (h)	mg per larva											
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0.036	0.013	0.022	0.033	0.012	0.002	0.007	0.001	0.003	0.007	0.04	0.001
5	0.006	0.009	0.002	0.051	0.101	0.004	0.008	0	0.117	0.004	0	0.025
24	0.006	0.04	0.061	0.064	0.005	0.01	0.047	0.004	0.08	0.03	0.012	0.038
25	0	0.11	0	0	0.035	0	0	0.022	0.052	0.06	NaN	0.098
29	0	0.0085	0.1	0.0085	0.099	0	0	0.038	NaN	NaN	NaN	NaN
48	0.001	0.001	0.003	0	0	0	0.001	0.001	0	0	NaN	0

Compound B – Leaf disk toxicokinetic bioassay: total body

time	mg per larva											
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0.0156	0.0336	0.0205	0.02	0.0361	0.0169	0.0059	0.0152	0.023	0.0233	0.0633	0.0413
5	0.0344	0.0138	0.0481	0.0106	0.0059	0.0513	0.0097	0.0601	0.0057	0.0185	0.0425	0.0417
24	0.0151	NaN	0.0161	0.0146	0.0067	0.0169	0.0104	0.0051	NaN	0.0057	0.0107	0.0247
25	0.0014	0.0012	0.0019	0.002	0.001	0.0159	NaN	NaN	NaN	NaN	NaN	NaN
29	0.0008	0.0007	0.0011	0.0008	0.0006	0.0005	NaN	NaN	NaN	NaN	NaN	NaN
48	0.0002	0.0001	0	0	0	0	NaN	NaN	0.0002	0.0002	0.0001	NaN

Compound C – Leaf disk toxicokinetic bioassay: total body

time	mg per larva										
0	0	0	0	0	0	0	0	0	0	0	0
1	0.024	0.1369	0.1337	0.0124	0.0257	0.026	0.0156	0.0087	0.02	0.0631	0.0981
5	0.0933	0.0447	0.0502	0.1479	0.0473	0.0829	0.15	0.0729	0.1101	0.07	0.0796
24	0.1325	0.1367	0.1242	0.1172	0.1104	0.132	0.1103	0.1014	0.1311	NaN	NaN
25	0	0	0.086	0.1213	NaN	NaN	NaN	NaN	NaN	NaN	NaN
29	0.00001	0.1084	0.00001	0.0919	NaN	NaN	NaN	NaN	NaN	NaN	NaN
48	0.0049	0.0202	0	0	NaN	NaN	NaN	NaN	NaN	NaN	NaN

Compound D – Leaf disk toxicokinetic bioassay: total body

time (h)	mg per larva											
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0.014	0.048	0.02	0.009	0.025	0.039	0.014	0.025	0.01	0.031	0.012	NaN
5	0.019	0	0.015	0.101	0.051	0.048	0.15	0.024	0	0.092	0.095	0.14
24	0.099	0	0.119	0.117	0.114	0.102	0.109	0.088	0.091	0.117	0.11	0.129
25	0.146	0	0.13	0.091	0.061	0.102	0.126	0.124	0.083	0.164	0.111	0.089
29	0.061	0.093	0.075	0.065	0.042	0.078	NaN	NaN	NaN	NaN	NaN	NaN
48	0.019	0.018	0.025	0.001	0.015	0.048	NaN	NaN	NaN	NaN	NaN	NaN

Compound E – Leaf disk toxicokinetic bioassay: total body

time	mg per larva											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0450	0.0060	0.0690	0.0860	NaN	NaN	NaN	NaN	NaN	NaN		NaN
5	0.0110	0.0590	0.0150	0.0560	0.0400	0.0020	0.0670	0.0600	NaN	0.1310	0.0380	NaN
24	0.0950	0.0270	0.0290	0.1170	0.0300	0.0000	0.0350	0.0760	0.1280	0.0300	0.0350	NaN
25	0.0090	0.0050	0.0070	0.0270	0.0000	0.0340	0.0530	0.0080	0.0110	NaN	0.0000	NaN
29	0.0030	0.0030	0.0020	0.0000	0.0070	0.0060	0.0050	0.003	0.0080	NaN	0.0040	NaN
48	0.0040	0.0030	0.0080	0.0030	0.0080	0.0030	0.0020	0.0030	0.0030	0.0030	0.0000	NaN

Compound F – Leaf disk toxicokinetic bioassay: total body

time	mg per larva											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	NaN	NaN	0.0111	0.0103	0.0060	0.0049	0.0067	0.0131	NaN	0.0058	0.0013	0.0076
5	0.0106	0.0031	0.0196	0.0129	0.0159	0.0040	0.0022	NaN	NaN	0.0063	0.0105	NaN
24	0.0061	0.0037	0.0082	0.0015	0.0167	0.0163	0.0183	0.0231	0.0006	NaN	0.0197	NaN
25	0.0053	0.0009	0.0004	0.0002	0.0003	0.0003	NaN	0.0005	NaN	0.0002	0.0005	NaN
29	0.0002	0.0014	0.0002	NaN	0.0002	NaN	0.0001	0.0000	0.0002	0.0002	NaN	0.0002
48	0.0001	0.0002	0.0003	0.0000	0.0001	NaN	0.0001	0.0003	NaN	0.0002	0.0003	0.0001

Compound G – Leaf disk toxicokinetic bioassay: total body

time (h)	mg per larva											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0357	0.0132	0.0218	0.0332	0.0120	0.0024	0.0073	0.0011	0.0025	0.0068	0.0401	0.0000
5	0.0058	0.0086	0.0019	0.0505	0.1008	0.0043	0.0084	0.0000	0.0255	0.0041	0.0000	NaN
24	0.0058	0.0404	0.0605	0.0642	0.0046	0.0100	0.0470	0.0043	0.0800	0.0303	0.0121	0.0380
25	0.0000	NaN	0.0853	0.1001	NaN	0.0848	0.0994	0.0000	0.0000	0.0375	0.1057	NaN
29	0.0000	NaN	0.0000	0.0000	0.0346	0.0000	0.0000	0.0216	0.0518	0.0600	NaN	0.0981
48	0.0008	0.0010	0.0033	0.0000	0.0002	0.0000	0.0009	0.0011	0.0000	0.0000	0.0912	0.0000

Compound Coumarin – Leaf disk toxicokinetic bioassay: total body

time	mg per larva											
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1	0.0883	0.0648	0.0665	NaN	0.0657	0.0491	0.0986	NaN	NaN	NaN	0.0527	0.0592
5	0.0198	NaN	0.0651	0.0055	0.0458	NaN	0.0025	0.0354	0.0332	NaN	0.0368	0.0500
24	0.0117	0.0047	0.0138	0.0031	0.0040	0.0044	0.0048	0.0047	0.0139	0.0089	0.0061	0.0142
25	0.0272	NaN	0.0186	NaN	0.0003	0.0003	0.0002	0.0003	0.0003	NaN	0.0002	0.0016
29	0.0061	0.0000	0.0000	NaN	0.0314	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
48	0.0060	0.0028	0.0077	0.0003	0.0005	0.0004	0.0001	0.0004	0.0071	0.0005	0.0006	0.0067

Data *Myzus persicae* honeydew– Compound quantities

Time is always in hours.

Compound A – Artificial diet toxicokinetic bioassay: honeydew

	mg per honeydew		
	time		
replicate	0-48 h	0-72 h	72-144 h
	0.004	0.013	0
	0.004	0.013	0
per well	0.0005	0.00163	0
	0.0005	0.00163	0

Compound B – Artificial diet toxicokinetic bioassay: honeydew

	mg per honeydew		
	time		
replicate	0-48 h	0-72 h	72-144 h
	0.004	0.001	
	0.003		
per well	0.0005	0.00013	0
	0.00038	0	0

Compound C – Artificial diet toxicokinetic bioassay: honeydew

	mg per honeydew		
	time		
replicate	0-48 h	0-72 h	72-144 h
	0.015		0.0003
	0.012		0.00028
per well	0.00188	0	3.7E-05
	0.0015	0	3.5E-05

Compound D – Artificial diet toxicokinetic bioassay: honeydew

	mg per honeydew	
	time	
replicate	0-72 h	72-144 h
	0.076	0.001
	0.074	0.001
	0.099	0.004
	0.099	0.004
per well	0.0095	0.00013
	0.00925	0.00013
	0.01238	0.0005

Compound E – Artificial diet toxicokinetic bioassay: honeydew

	mg per honeydew	
	time	
replicate	0-72 h	72-144 h
		0.025
		0.001
per well	0	0.00313
	0	0.00013

Data *Myzus persicae*– Compound quantities

Compound A – Artificial diet toxicokinetic bioassay: total body

time (h)	mg per aphids equivalent											
24	0.0002	NaN	NaN	0.0001	0.0001	0.0001	0.0000	0.0000	0.0000	0.0001	0.0001	0.0000
48	NaN	0.0003	0.0005	0.0003	NaN	0.0001	NaN	NaN	NaN	NaN	NaN	0.0003
72	0.0010	NaN	0.0006	0.0007	0.0011	0.0010	0.0010	0.0007	0.0014	0.0005	0.0006	0.0007
75	0.0001	0.0002	0.0003	0.0005	0.0001	0.0000	0.0000	0.0000	NaN	NaN	NaN	NaN
80	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	NaN	NaN	NaN	NaN	NaN	NaN
144	0.0000	0.0001	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	NaN	NaN	NaN

Compound B – Artificial diet toxicokinetic bioassay: total body

time (h)	mg per aphids equivalent								
24	0.0001	NaN	0.0000	0.0006	NaN	NaN	NaN	NaN	NaN
48	0.0000	0.0010	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
72	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN
75	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN
80	0.0000	0.0000	0.0000	0.0000	NaN	NaN	NaN	NaN	NaN
144	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	NaN

Compound C – Artificial diet toxicokinetic bioassay: total body

time (h)	mg per aphids equivalent								
24	0.0143	0	0	0	0.0167	0	0.0129	0.0406	0
48	0.0316	0.0037	0.0086	0.0098	0.0209	0.0238	0.0214	0.0181	NaN
72	0.0266	0.0094	0.0102	0.0245	0.0088	0.0036	0.0187	NaN	NaN
75	NaN	NaN	0.0103	0	0.0204	0.0538	0	0	NaN
80	0.0071	0.003	NaN	NaN	NaN	0.0032	NaN	NaN	NaN
144	0.0038	0.0024	0.0031	0	0.0049	0.0107	0	0	0.0074

Compound D – Artificial diet toxicokinetic bioassay: total body

time (h)	mg per aphids equivalent									
24	0.0090	0.0000	0.0080	0.0000	0.0000	0.0000	0.0030	0.0000	0.0200	0.0170
48	0.0160	0.0560	0.0140	0.0190	0.0070	NaN	NaN	NaN	NaN	NaN
72	0.0430	0.0130	0.0270	0.0210	0.0290	0.0100	0.0070	NaN	NaN	NaN
75	0.0010	0.0020	0.0020	0.0010	0.0000	0.0000	NaN	NaN	NaN	NaN
80	0.1300	0.0000	0.0000	0.0020	0.0000	0.0020	NaN	NaN	NaN	NaN
144	0.0010	0.0030	0.0000	0.0100	0.0040	0.0050	0.0040	NaN	NaN	NaN

Compound E – Artificial diet toxicokinetic bioassay: total body

time	mg per aphids equivalent									
24	0.0010	0.0020	0.0010	0.0000	0.0020	0.0010	0.0010	0.0000	0.0000	NaN
48	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	0.1310
72	0.0000	0.0010	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0300
75	0.0030	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	NaN	NaN
80	0.0020	0.0050	0.0000	0.0000	0.0020	0.0000	NaN	NaN	NaN	NaN
144	0.0030	0.0000	0.0000	0.0000	0.0020	0.0010	0.000	NaN	0.0010	0.0030

Compound F – Artificial diet toxicokinetic bioassay: total body

time	mg per aphids equivalent											
24	0.0010	0.0000	0.0002	0.0002	0.0011	0.0033	0.0000	0.0012	0.0000	0.0013	0.0000	0.0000
48	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0007	0.0000
72	0.0000	0.0000	0.0069	0.0057	0.0089	0.0046	0.0066	0.0068	0.0000	0.0014	0.0057	0.0000
75	0.0006	0.0000	0.0004	0.0003	0.0005	0.0004	0.0000	0.0003	NaN	NaN	NaN	0.0003
80	0.0038	0.0000	NaN	0.0003	0.0001	0.0003	0.0003	NaN	NaN	NaN	NaN	NaN
144	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0007	NaN	NaN	NaN	NaN

Compound G – Artificial diet toxicokinetic bioassay: total body

time (h)	mg per aphids equivalent											
24	0.0139	0.0000	0.0000	0.0194	0.0141	0.0121	0.0157	NaN	NaN	NaN	NaN	NaN
48	0.0230	0.0233	0.0235	0.0007	NaN	0.0103	0.1200	NaN	NaN	NaN	NaN	NaN
72	0.0204	0.0010	0.0000	NaN	NaN	0.0000	NaN	NaN	NaN	NaN	NaN	NaN
75	0.0026	0.0024	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
80	0.0029	0.0005	0.0012	0.0010	0.0011	0.0022	0.0009	0.0092	0.0019	0.0017	0.0011	0.0003
144	0.0005	0.0012	0.0010	0.0011	0.0022	0.0009	0.0092	0.0019	0.0017	0.0011	0.0003	NaN

Thesis publications

The following manuscripts and consent to use content of joint publications are included in the supporting information.

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Mathematisch-Naturwissenschaftliche Fakultät

Declaration of collaborative work in the case (b) of a monograph including manuscript.

The following joint publications and publication-drafts are included in the PhD Thesis of Clara Isis Römer (2024) with the title: **Insect model on toxicokinetics – foundation on species selectivity.**

Last Name, First Name:

List of Publications

1. RÖMER *et al.* (under review at Journal of Economic Entomology): Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model.
2. RÖMER *et al.* (draft): Comparison of absorption and excretion of test compounds in sucking versus chewing pests.
3. RÖMER *et al.* (draft): Comparative toxicokinetics of insecticide scaffolds and their putative biotransformation in target and non-target species.

Number	Accepted publication Yes/No	List of authors	Position of candidate in the list of authors	Scientific ideas by the candidate %	Data generation by the candidate %	Analysis & interpretation by the candidate %	Paper writing by the candidate %
1	under review	8	1	40	80	70	70
2	no	8	1	40	80	80	70
3	no	8	1	40	70	70	70

I confirm that the above-stated is correct.

18.March 2024
Date, Signature of the candidate

I/We certify that the above-stated is correct.

18 March 2024

Date, Signature of the doctoral committee or at least of one of the supervisors

Author	Author	Scientific	Data	Analysis &	Paper

	position	ideas	generation	interpretation	writing
Römer C. I.	1	x	x	x	x
Ashauer R.	2	x		x	x
Escher B. I.	3	x		x	x
Höfer K.	4		x		
Muehlebach M.	5	x		x	x
Sadeghi-Tehran P.	6		x		
Sherborne N.	7			x	x
Buchholz A.	8	x		x	x
Title of paper 1	Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model.				
Status in publication process	under review at Journal of Economic Entomology				

Author	Author position	Scientific ideas	Data generation	Analysis & interpretation	Paper writing
Römer C. I.	1	x	x	x	x
Ashauer R.	2	x		x	x
Escher B. I.	3	x		x	x
Hollender J.	4			x	x
Burkhard R.	5		x		
Höfer K.	6		x		
Muehlebach M.	7	x		x	
Buchholz A.	8	x		x	x
Title of paper 2	Comparison of absorption and excretion of test compounds in sucking versus chewing pests.				
Status in publication process	waiting for acceptance of manuscript 1				

Author	Author position	Scientific ideas	Data generation	Analysis & interpretation	Paper writing
Römer C. I.	1	x	x	x	x
Sherborne N.	2		x	x	x
Escher B. I.	3	x		x	x
Hollender J.	4			x	x
Höfer K.	5		x		
Muehlebach M.	6	x			
Buchholz A.	7	x		x	x
Ashauer R.	8	x		x	x
Title of paper 3	Comparative toxicokinetics of insecticide scaffolds and their putative biotransformation in target and non-target species.				
Status in publication process	waiting for acceptance of manuscript 1 & 2				

Manuscript 1 (submitted to Journal of Economic Entomology)

Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model.

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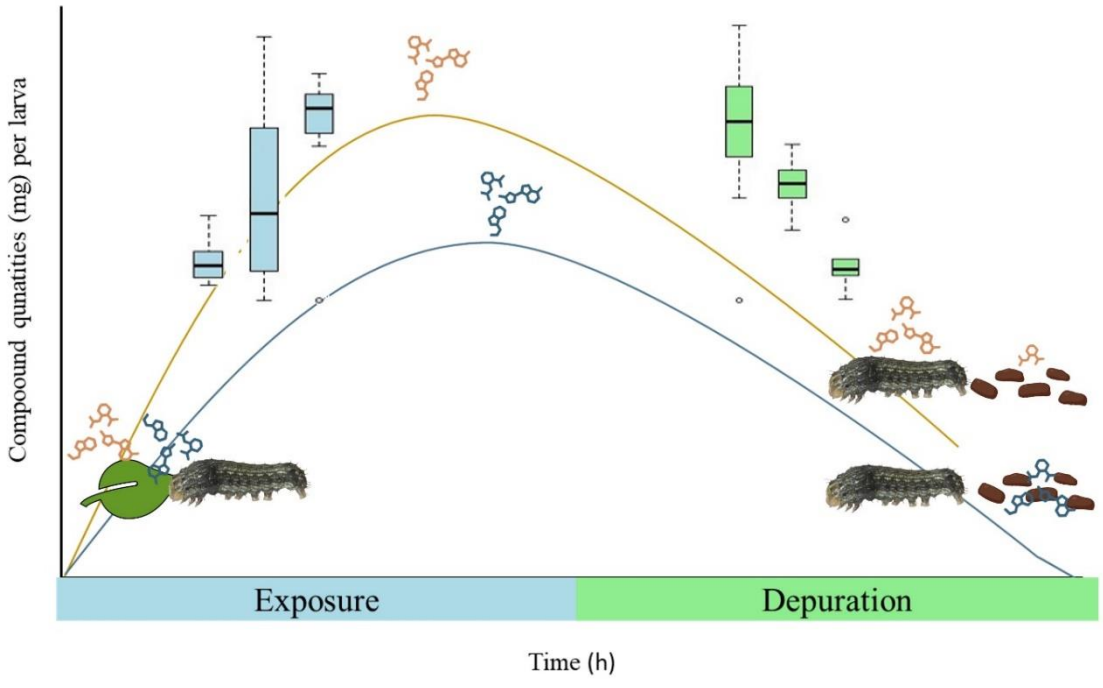
Fate of synthetic chemicals in the agronomic insect pest *Spodoptera littoralis:* experimental feeding-contact assay and toxicokinetic model



Abstract

Insecticides prevent or reduce insect crop damage, maintaining crop quality and quantity. Physiological traits, such as an insect's feeding behaviour, influence the way insecticides are absorbed and processed in the body (toxicokinetics), which can be exploited to improve species selectivity. To fully understand the uptake of insecticides, it is essential to study their total uptake and toxicokinetics independently of their toxic effects on insects. We studied the toxicokinetics (TK) of insecticidally inactive test compounds incorporating agro-like structural motifs in larvae of the Egyptian cotton leafworm (*Spodoptera littoralis*), and their distribution across all biological matrices, using laboratory experiments and modelling. We measured *Spodoptera* larval behaviour and temporal changes of whole-body concentrations of test compounds during feeding on treated soybean leaf disks and throughout a subsequent depuration period. Differences in the distribution of the total quantities of compounds were found between the biological matrices leaf, larva, and feces. Rate constants for uptake and elimination of test compounds were derived by calibrating a toxicokinetic model to the whole-body concentrations. Uptake and elimination rate constants depended on the physicochemical properties of the test compounds. Increasing hydrophobicity increased the bioaccumulation potential of test compounds. Incomplete quantities in larval matrices indicated that some compounds may undergo biotransformation. As fecal excretion was a major elimination pathway, the variable time of release and number of fecal pellets led to a high variability in the body burden. We provide quantitative models to predict the toxicokinetics and bioaccumulation potential of inactive insecticide analogues (parent compounds) in *Spodoptera*.

Keywords: agrochemistry, exposure, depuration, absorption, excretion.

Introduction

As both the global human population as well as global demand for agricultural products continue to increase, there is a continued need to develop effective methods and tools for regenerative agriculture (Roser et al. 2013). Insecticides have become an important part of this, but it is vital that their ecological impact is minimal and well understood. Combined effects of climate change and human population growth make even more important to ensure food availability and to avoid crop losses due to pests (Haile 2000, Meyers and Kalaitzandonakes 2015). Estimated global annual crop losses were around 52 % in 2015 (BVL, 2015), with pests and diseases accounting for a significant proportion of it. Insects, including beetles, aphids, and caterpillars, accounted for about 15 % of total losses. Effective pest management,

including insecticide use, is essential to maintain and improve crop yields by keeping pest populations below economic thresholds (Buntin 2000, Haile 2000, Tonnang et al. 2022). Insecticides also help to reduce post-harvest losses caused by pest damage during storage and transport. Understanding of the mechanisms contributing to these losses and of the efficacy of insecticides is therefore crucial to develop effective strategies for sustainable agriculture and ensure food security (Carvalho 2006, Seufert et al. 2012).

Insecticide exposure can have an impact on biological performance parameters of exposed individuals, from sub-lethal effects to mortality (Müller et al. 2017, Müller et al. 2019). Performance parameters such as feeding behaviour, body mass, and reproductive output are indicators of individual fitness of organisms (Gutsell and Russell 2013, Schuijt et al. 2021). When those insecticide effects are intended, we view them as aspects of efficacy. When similar effects occur in non-target organisms, we view them as unintended toxicity. Making insecticides more selective, i.e., optimising for efficacy, but with minimum environmental toxicity, is key for sustainable food production and biodiversity protection. Various methods in chemistry design are established to reduce the number of compounds to be synthesised and tested (Dudek 2006, Gichere 2021). This aims to improve efficacy against targets, while gaining selectivity on non-targets. Selectivity can be achieved through favourable environmental fate and bioavailability in target pests, as well as differentiating toxicokinetics and toxicodynamics.

The toxicokinetics (TK) of insecticides, which include processes such as uptake, distribution, biotransformation, and excretion, are important drivers of the biologically effective dose, which impacts on efficacy or toxicity. Species-specific differences in TK between target and non-target insects are particularly important as they may help to maximise efficacy against pests while minimising adverse environmental impact. Studies of TK across and within species (Ashauer et al. 2012, Nyman et al. 2014) could provide valuable insights for designing selectivity and therefore developing safer and more efficient insecticides.

Studies of TK in non-target organisms, and the corresponding experimental designs, are well established in environmental toxicology (Rubach et al. 2010, Nyman et al. 2014). However, studies on TK in target insect species are rare in the scientific literature. As a result, we up to date do not have available published standardised experimental design and data analysis workflow for toxicokinetics of chemicals in target

insects. Hence, this study aimed to develop such an assay combined with chemical analysis and toxicokinetic modelling. This was evaluated for potential use in chemistry optimisation.

The larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*, are severe agronomic chewing pests that can infest more than 80 different crops (De Groote, Kimenju et al. 2020) and are representative of foliar Lepidopteran pests. Our primary objective was to develop a *Spodoptera* bioassay combined with chemical quantification, to characterise the fate of chemicals in fed leaf disks and insects. The assay also measured behavioural responses to assess exposure-induced changes using image analysis. Our secondary objective was to build a species- specific toxicokinetic (TK) model capturing internal compound concentrations over time.

Materials & Methods

Test compounds

Four representative insecticidally inactive test compounds (log P range 1.43-3.57 and molecular mass 146-303 g/mol, Figure 1, Table 1) were synthesized in-house (≥ 95% purity). They incorporate structurally scaffolds or fragments with some degree of insecticide-likeness. Additionally, coumarin [CAS 91-64-5, (≥ 95% purity, Merck KGaA, Darmstadt, Deutschland), Figure 1, Table 1] was used as a reference for extraction and as a method standard. All compounds were dissolved at 2000 mg/L in water containing 15 % acetonitrile (ACN) (gradient grade for analytics 99.9 %) as solvent. A high dosing should also enhance the possibility of detecting putative metabolites. In addition, this rate did not show any adverse effects on larval performance parameters.

Plants

Soybean plants, *Glycine max* (L.) Merr cv. Toliman, were used in the *S. littoralis* feeding-contact assay. Four soybean seeds were germinated and grown per pot (Ø 6.5 cm) filled with white peat growth medium. Plants were grown in a greenhouse under controlled conditions (14 h light (27 Lux., 22 °C) and 8 h dark (18 °C) cycle, 65 ± 5 % relative humidity (RH)) and used in the assay after 14 days of germination. Two leaf disks (Ø 20 mm) were cut from two fully developed true leaves and stored on wet filter paper to minimise desiccation.

Insect

Egyptian cotton leafworm (*Spodoptera littoralis;* BOISDUVAL, 1833) were reared in the laboratory under standardised conditions (23 ± 1 °C, 55 ± 10 % RH), including an in-house artificial diet for both adults and larvae. This laboratory strain had not been exposed to insecticides.

Larvae stages were synchronised by transferring the derived second larval stage (L2) from artificial diet to an empty Petri dish (Ø 12 cm). The dish contained only dry filter paper and was covered with a cotton filter. Within two hours, the larvae moulted into the early third larval stage (L3).

***Spodoptera* bioassay**

The assay combined an exposure phase of 24 h followed by a depuration phase of 24 h under standardised conditions (25 ± 1 °C, 55 ± 10 % RH, 16-hour light/8-hour dark cycle, Figure 2). *S. littoralis* larvae were exposed to treated leaf disks, reflecting a preventative bioassay with oral and contact uptake of test compounds.

Before exposure, 50 µl of test solutions, containing 0.1 mg of test compounds, were evenly distributed on the leaf disks (Ø 20 mm) by shaking for 20 seconds at 300 rpm using a pipetting robot (Fluent® Automation Workstation, Tecan Group Ltd, Männedorf, Switzerland). The control treatment received a 15 % ACN-water solution. After 30 min evaporation of test solution, leaf disks were placed in a 12-well microtiter plate laid out with moist filter paper to maintain humidity (12-MTP, FalconTM, Northfield, Minnesota, USA).

One freshly moulted *S. littoralis* larva (L3) was placed on each leaf disk in a microtiter plate then covered with a transparent foil with evaporation holes. Larvae were exposed to treated leaf disks for 24 h, then transferred to a microtiter plate containing untreated leaf disks for a 24 h depuration period (Figure 2).

S. littoralis larvae samples were collected during the exposure period (T0-24 h) and the depuration period (T24-48 h). As a reference, 3 g leaf samples (n=133 pooled leaf disks) were collected from a parallel assay without larvae, and therefore no real mass balance can be established. Samples were taken after 0, 1, 5, 24, 25, 29, 48 hours (soybean samples: pooled leaf disks per time point, n=3; *S. littoralis* samples: one larva per time point, n=12). Feces samples (pooled feces pellets per larva, n=12) were collected at the end of the exposure (period T0-24 h) and depuration phase (period T24-48 h). All samples were transferred to 2.5 ml tubes (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop metabolism (Figure 2).

Larval performance parameters

Larval behaviour and performance parameters (larval size, food consumption, excretion, and movement) were observed by images acquisition in 1-hour-intervals during exposure period (Figure 2). Larval size (segmented pixels converted to mm^2), food consumption (proportion % leaf area) conversion of food to feces (count of feces pellets), and movement (comparison between frames) were recorded visually using methods described previously (Sadeghi-Tehran et al. 2017, Sadeghi-Tehran et al. 2019).

Residue measurements

Sample preparation

The same chemical analytical method was applied to all biological matrices to quantify compound concentrations, but extraction differed for larvae, feces, and leaf disks. As all samples were processed as a total mass of the given biological matrix (leaf disk, larva, feces), it is therefore not possible to distinguish whether the detected compound(s) were absorbed internally or adsorbed on the surface (Figure 2).

For each larva, the total body wet weight (wwt) was measured after thawing using a Sartorius-balance (BCE124I-1S Entris® II, Data Weighing Systems, Inc., Wood Dale, IL, USA). Larvae and feces samples were both homogenised using a macerator (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) with a ceramic ball (Ø 6.35 mm, MP Biomedicals™ zirconium oxide-coated beads, Lucerne Chem AG, Lucerne, Switzerland). Next, 500 µl ACN were added to each sample, before shaking for 3 h at 300 rpm and 20 °C using an Eppendorf ThermoMixer® (Merck & Cie, Schaffhausen, Switzerland). After shaking, samples were centrifuged at 9000 rpm for 2 min. Preparations of leaf samples involved addition of 30 ml ACN and a cleaning buffer step before centrifugation (buffer I 8g mixture: 450 g $MgSO_4$, 115 g sodium acetate). After centrifugation leaf and feces samples were filtered through a 0.20 µm pore size filter (CHROMAFIL®Xtra PET-20/13, Macherey-Nagel GmbH & Co.KG, Düren, Germany). Supernatants (150 µl) of each sample were transferred to analytical glass vials, with a 200 µl glass insert (Vials N11, with 0.2 ml insert, Macherey-Nagel GmBH & CO.KG, Düren, Germany).

Chemical analysis

Ultra High-Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) was performed using ACN as solvent. Spectra for parent compounds and their putative metabolites were recorded from all

samples on a Mass Spectrometer (Xevo TQ-XS Triple Quadrupole Mass Spectrometer) from Waters Corporation equipped with an Electrospray Ionization Source (ESI) (Figure 2).

Parent compound and metabolites in samples were chromatographically separated on an Acquity Iclass Plus system with an Acquity UPLC High Strength Silica (HSS) column (T3, 2.1 x 30 mm, particle size 1.8 µm) using a water solution (A) (90 % water, 10 % methanol, and 0.1 % formic acid) and (B) ACN (0.1 % formic acid). Samples were measured in gradient elution mode with fluctuating flow rates. The gradient flow consisted of following steps: initial flow rate of 1.0 ml/min of 80 % A/20 % B until 0.10 min, then from 0.10 min to 0.20 min to 25 % B, followed by a flow rate change to 0.750 ml/min till 1.20 min with 30 % B, from 1.20 min to 1.45 min to 100 % B, then until 1.45 min to 20 % eluent B, and finally, from end of the run at 2 min with a flow rate of 0.050 ml/min and 50 % B in an isocratic mode. Column temperature was maintained at 60 ± 5 °C, and sample injection volume was 2 µl. For MS detection conditions, the desolvation Gas Flow was set at 1000 L/h at temperature of 500 °C. The flow rate of the cone gas was set at 150 L/h, with capillary voltage of 3 kV, source temperature of 150 °C, and cone voltage ranging from 15 to 60 V. Detection of parent compound was performed by single ion recording (SIR) in a Mass Range of 120 to 1000 Da. The parent compound was quantified using a calibration series.

Toxicokinetic modelling

We determined uptake and elimination rates by calibrating a one-compartment first-order toxicokinetic (TK) model to the measured concentration in the larvae and leaf disks (internal concentration data).

The toxicokinetic model can be represented mathematically as:

(1)

$$\frac{d\,C_{\mathrm{i}}(\mathrm{t})}{dt} = k_{\mathrm{in}} * C(\mathrm{t})_{\mathrm{leaf}} - k_{\mathrm{out}} * C_{\mathrm{i}}(\mathrm{t})$$

where $C_i(t)$ represents the internal concentration of the parent compound [$mg_{compound}/mg_{wet\_weight}$] in and on the organism (whole body residue), t is time (h), $C(t)_{leaf}$ is the external concentration [$mg_{compound}/mg_{leaf}$] in and on the leaf disk, k_{in} [$mg_{leaf}/(mg_{wet\_weight}*h)$] the uptake rate constant and k_{out} [1/h] is the elimination rate constant.

Equation 1 was applied separately for each compound, yielding compound specific uptake and elimination rate constants. The ordinary differential equations (ODEs) were implemented using the

MATLAB (Version R2021a) Build Your Own Model (BYOM v60_beta5) platform (https://www.debtox.info/byom.html) and we used the maximum likelihood estimation with a normal likelihood function for model calibration by minimising the likelihood difference between measured and modelled internal body concentrations (Jager and Ashauer 2018b). Confidence intervals were calculated for the uptake and elimination rate constants with likelihood profiling, with an upper limit of 100 is set by the BYOM platform, which can be equated with infinity (Table 1, Figure S4).

Bioaccumulation factor

The bioaccumulation factor (BAF) is the ratio of the concentration of the test compound within the organism in comparison to the external source at steady state, specifically the concentration in *Spodoptera* larvae compared to the treated soybean leaf disk. The BAF can be calculated as the ratio between the uptake rate constant (k_{in}) and elimination rate constant (k_{out}).

(2)

$$\mathrm{BAF} = \frac{k_{\mathrm{in}}}{k_{\mathrm{out}}}$$

A BAF value can indicate whether the concentration of the test compound in the organism is higher than the concentration in the external source. To calculate the confidence intervals of the BAF, the model was run at a constant concentration (set to 1) until steady-state and the resulting confidence interval of the internal concentration equals the confidence interval of the BAF (Ashauer et al. 2010).

Results

Larval performance parameters

Larval performance parameters (food consumption, excretion, larval size, movement) did not deviate from untreated controls during the 24 h exposure (Figure 3, Figure 4). Larvae of all treated groups continued feeding without detectable influence of day and night shift. At the end of the 24 h exposure period all larvae had consumed almost the entire leaf disk (Figure 3, Figure S1). The average size of a larva after 24 hours of exposure was 55.9 mm^2 (Figure 4, Figure S2). On average, the larvae transformed one leaf disk (Ø 20 mm) into 33 feces pellets (Figure 3, Figure S3). Feces dropping usually started about 4 to 5 hours after infestation with larvae (Figure S3). The movement of the *S. littoralis* larvae between image frames was constant throughout the exposure (Figure 3). This means that exposure to test

compounds did not disrupt normal food consumption, defecation, growth, and movement pattern of L3-larvae.

Compound quantities in all compartments

Quantities of compound A, C and D on the leaf disks were consistent during 24 h of the exposure period, and therefore demonstrated stable exposure profiles (Figure 5 a). In contrast, quantities of compound B decreased by more than 50 %, as compared to the dosed amount, during the exposure period, from 0.1 mg to 0.05 mg of the parent compound per leaf disk sample within the first five hours of the experiment. Coumarin quantities started to decline after one hour down to 0.005 mg at 24 h (Figure 5 a).

The time-course of compound quantities in larvae differed between the compounds and during exposure and depuration periods (Figure 5 b). Quantities of compounds A, C and D increased during the 24 h exposure period and decreased during depuration period (Figure 5 b). Compounds C and D reached maximum levels (median 100 % of treated leaf disk) of the parent compound quantity in larval bodies after 24 h exposure, whereas compound A reached only 30 % (median) after 24 h and compound B reached 30 % already after 5 h. Coumarin showed maximum compound quantity levels of 60 % (median) within the larva after 1 h of exposure, which decreased to 15 % at the end of the period (Figure 5 b).

Feces samples represent the sum of all feces pellets of individual *S. littoralis* larvae sampled after exposure (T0-24 h) or depuration (T24-48 h) respectively. At the end of the exposure period, the fecal quantities of compounds A and coumarin were with about 60 % (median) of the dosed compound the highest detected fractions, whereas compound B, C, and D demonstrated quantities of parent compound in the range of 15-25 % (median) (Figure 5 c). Chemical quantities in feces pellets remained below 5 % (median) in the depuration period for all compounds, except compound D. Here the quantities in feces increased up to 30 % (median) of parent compound (Figure 5 c).

Toxicokinetic model

Overall, the compound treatments showed clear differences in the concentrations of parent compounds in the larvae and the resulting uptake and elimination rate constants and bioaccumulation factors (Table 1). The TK model fits the concentration at 24 h and the elimination period rather than the concentration in the first 5 h (Figure 6). All compounds and coumarin showed higher uptake rate constants than elimination rate constants, resulting in bioaccumulation to reach concentrations in larvae above the levels

in leaf disks (Table 1, Figure S4). The TK model curves (Figure 6) further highlights differences between the compounds, especially in their time-course of uptake and depuration. In the exposure phase, the concentrations at the first sampling point were already at a similar concentration level to those at the following sampling point (5 h). The model calibration of compound A, B, and C resulted in parameters hitting a boundary (k_{in} at upper limit) (Figure S4). Coumarin showed a rapid uptake, followed by a steep decline due to the declining exposure (Figure 6) and the model parameters converged with confidence intervals, which were well-identified (closed parameter likelihood plot Figure S4). The model of compound D also converged with closed confidence intervals (Figure S4).

Discussion

In a first investigative step performance parameters were analysed for potential effects of chemical exposure (Müller and Müller 2015). All larvae exhibited normal behavior during the exposure period of the experiments because no changes of behavioral patterns of larvae compared to control groups were detectable (Figure 4, Figure S1-3). We can therefore conclude that behavior is not predominantly responsible for detected differences in compound quantities in biological matrices (Kingsolver and Huey 2008, Ankley et al. 2010, Gergs et al. 2015).

During the exposure period, we found substantial variation of quantities in larval bodies over time (Figure 5 b, Figure 6). As measurable excretion of compounds begins with the first dropping of feces pellets after an average of four to five hours of feeding and the feces pellets contain a substantial amount of test chemicals, the highest variation in body tissue concentration variation was observed in this time frame (Figure 5, Figure 6). Interestingly, the variation in compound quantities in larval bodies is also reflected in the increased variation of quantities in feces (Figure 5 c). Whether this was caused by different exposure or elimination should be investigated in more detail in future studies. Compound uptake into larval bodies might lead to high biotransformation, resulting in low quantities in feces. This illustrates the interaction between bioavailability, uptake, and excretion. The substantial and quick elimination through fecal egestion is also an indication that only a limited quantity of compounds could be absorbed systemically into larval bodies. Some of the compounds might be passing the gut without being absorbed.

For stable chemicals one can expect 100 % recovery of the parent compound across all matrices and the apportionment between the different matrices describes the fate of chemicals over time in plant and larval

tissues in the given assay. Compound A, C and D demonstrated chemical stability on the leaf disk. Maximum levels of compound quantities were observed in *S. littoralis* bodies once the entire leaf disks were eaten up after 24 h, but with low quantities in feces. This suggests that the test compounds remained unchanged in these plant and insect matrices. In contrast, compound B reached the maximum quantities in larval bodies at 5 h. At 24 h quantities in larval bodies and feces were lower than 20 % (median) of the exposure dose. Abiotic degradation could be excluded by control experiments without larva, leaving only biotransformation as a likely cause of low recovery. The decline in compound quantities in leaf disks and larvae could potentially be explained with biotransformation (Figure 5).

Toxicokinetic (TK) modelling is a valuable tool for understanding species differences in uptake kinetics, bioaccumulation, and the role of metabolism. Standardised assays improve the reliability and reproducibility of data (Bonta 2002, Jager and Ashauer 2018a). TK modelling enables comprehensive assessments of non-target risks and informed environmental management decisions (Ashauer and Escher 2010, Hommen et al. 2015).The observed differences in compound quantities in larval bodies are explainable by different kinetic rate constants for uptake and elimination. Uptake rate constants varied by more than ten orders of magnitude between compounds, while excretion rate constants varied by only a factor of about two (Figure 6, Table 1). This was already demonstrated earlier with other organisms, such as annelids (Belfroid et al. 1993, Šmídová et al. 2021). TK models have been shown to be capable to predict the toxicokinetics of compounds in a range of organisms (Nyman et al. 2014) (Table 1). Here we show that the TK modelling approach that is well established in environmental toxicology and the risk assessment of non-target species risk assessments can be adapted to target organisms.

The current experimental design cannot distinguish between contact and oral uptake of test compounds. Therefore, we modelled the uptake over time without discriminating between both principal absorption routes. Nevertheless, varying absorption routes could be a major differentiator between target and non-target species due to different biology, e.g., feeding types. Beside food consumption, contact absorption by crawling on the leaf disk can contribute to substantial uptake (Chown and Nicolson 2004, Beran and Petschenka 2022). This uptake would then definitively lead to systemic exposure. The bioassay could be adapted to separate contact or oral exposure, with an assay design that uses only oral intake (forced feeding) or pure contact from treated surface (Hamby et al. 2013, Balabanidou et al. 2018, Denecke et al. 2018, Arlos et al. 2020).

After analyzing the experimental uptake curves and their variability, we recommend to sample with higher frequency during the beginning of exposure period (≤ 5 h), and at the beginning of the depuration period (Figure 6, Figure S4). This would help to better understand this critical part of the toxicokinetics, specifically the curvature of the modelled internal concentration. More data points would help to achieve also better and more robust model fits.

The simple first-order one-compartment TK model employed here is unable to explain all patterns in the body tissue concentration data because it cannot differentiate between exclusive gut passage and systemic uptake. At least those chemicals that show degradation in the insect should have had some systemic uptake unless the gut microbiome also contributes to biotransformation. Unfortunately, the TK model (Table 1, Figure, 6, Figure S4) does not capture well the observed uptake of compound A, B, and C, presumably due to the variability in the onset of the dropping of fecal pellets as this appears to be the most important elimination pathway. Nevertheless, under these assay conditions the TK model captures the basic patterns of TK in *S. littoralis* L3 larvae for five different test compounds (Table 1). Whilst the TK model generally reflected the compound concentrations within larval bodies, we also measured compound quantities in feces, but this information was not considered by the model. Additionally, the dilution of internal concentrations due to larval growth over time was not considered. *S. littoralis* larvae increased their body mass by a factor of 4 during the exposure, as they ate the entire leaf disk. Both, excretion via feces and growth dilution could be added to the toxicokinetic model. Here we wanted to apply the simplest model first to demonstrate the suitability of the method in general. More complex models could be considered in future studies if appropriate data can be generated. A combined understanding of the organisms' biology (performance parameter, total quantities of compound in insect body or excretion product) and more frequent measurements of internal concentrations could help to better understand the putative starting point of detoxification due to biotransformation.

Many phytophagous species, especially pest species such as Lepidoptera, possess a variety of enzymatic degradation pathways and detoxification mechanisms, such as excretion, to prevent bioaccumulation (Dow 1992, Schulz 1998, Roberts and Hutson 1999, Perić-Mataruga et al. 2019).

As molecules with higher Log P values tend to have a greater affinity for biological membranes (Hofstetter et al. 2018), their bioaccumulation potential is higher (Hawker and Connell 1985, Esser 1986).

In our study, the bioaccumulation factor (BAF) generally increased with increasing hydrophobicity, confirming this rule (Table 1), with the exception of coumarin, which is putatively metabolized. The residence time of a parent compound within a larval body not only can significantly influence the toxic effects on a Lepidopteran pest species, but has been shown to raise resistance potential due to enzymatic processes in pest species (Wing et al. 1998, Siegfried and Scharf 2001). In our experiments, all compounds were completely eliminated from the larval bodies within the depuration period (Figures 5, Figure 6).

In conclusion, we successfully developed and implemented a bioassay which characterised the fate of synthetic chemicals in plant, insect, and excretion in an agronomic relevant Lepidopteran pest. This study highlights the complexity of compound uptake, excretion, biotransformation, bioaccumulation, and biological response in *Spodoptera littoralis* larvae. These insights will support chemistry optimisation i.e., the identification of more selective insecticides which are more effective against target pests and which possess minimal environmental toxicity. the identification of more selective insecticides which are more effective against target pests, and which possess minimal environmental toxicity. Future studies could apply a similar experimental design and data analysis approach to other important Lepidopteran pest species. This experimental approach could contribute to a more comprehensive understanding of the uptake and excretion of agrochemicals.

TK models are a valuable approach to understand internal compound concentrations in target organisms and once parameterized, predict exposure under different conditions. Nevertheless, the total amounts in all compartments of the bioassay should be observed to obtain a realistic mass balance of the compound. Since we did not estimate the contribution of biotransformation within plant or insect, future research should include biotransformation measurements. Biotransformation could have a major impact on performance parameters, exposure, depuration, bioaccumulation, and finally toxicity (Ashauer et al. 2012, Rosch et al. 2016). It provides a better understanding of pest-specific patterns, which further supports the development of effective pesticides with the lowest possible environmental impact (Nyman et al. 2014).

Declarations' section

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors contribution

CIR: Formal analysis, Investigation, Methodology, Project administration, Software – Programming, Visualization, Writing – original draft, Writing – review & editing

RA: Conceptualization, Software – Programming, Supervision, Visualization, Writing – review & editing

BIE: Supervision, Writing – review & editing

KH: Formal analysis, Methodology, Software – Programming, Validation

MM: Conceptualization, Formal analysis, Supervision, Writing – review & editing

PST: Formal analysis, Methodology

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All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

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Table 1: Toxicokinetic model parameters (uptake and elimination rate constants, bioaccumulation factors) for *Spodoptera littoralis* (L3) larvae and chemical descriptors (log P, molecular weight) of tested compounds.

Compound	Log P	Molecular weight (g/mol)	Uptake rate constant (k_{in}) ($mg_{leaf}/(mg_{wet\_weight}*h)$)	Elimination rate constant (k_{out}) (1/h)	Bioaccumulation factor (BAF) ($mg_{leaf}/mg_{wetweight}$)
A	1.58	205.25	2.63 [0.61;>100]	2.24 [0.68;>100]	1.17 [0.78;1.58]
B	1.50	262.62	8.89 [3.31;>100]	5.43 [2.10;72.45]	1.64 [1.29;1.99]
C	2.24	302.25	4.86 [1.89;>100]	1.68 [0.63;45.21]	2.89 [2.26;3.85]
D	3.57	280.68	2.79 [1.26; 6.79]	0.73 [0.33;1.74]	3.82 [3.53;4.92]
Coumarin	1.43	146.14	18.71 [10.64;72.71]	4.52 [2.58;17.11]	4.16 [2.64;3.83]

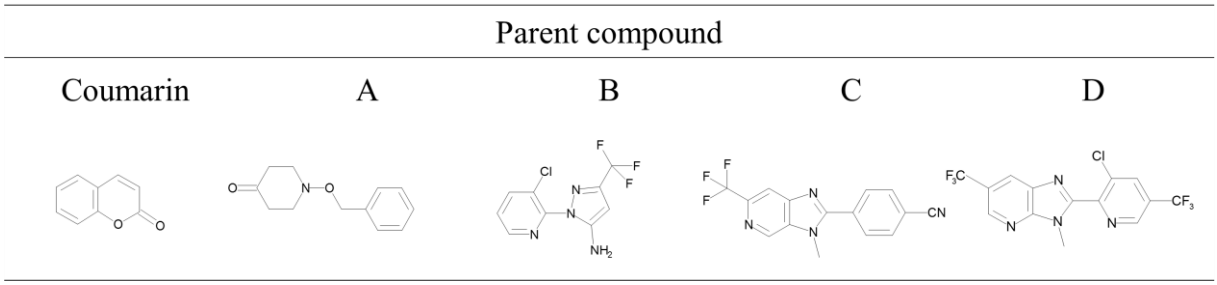

Figure 1: Structure of test compounds (A-D), and coumarin. (Measured log P and molecular mass shown in Table 1.)

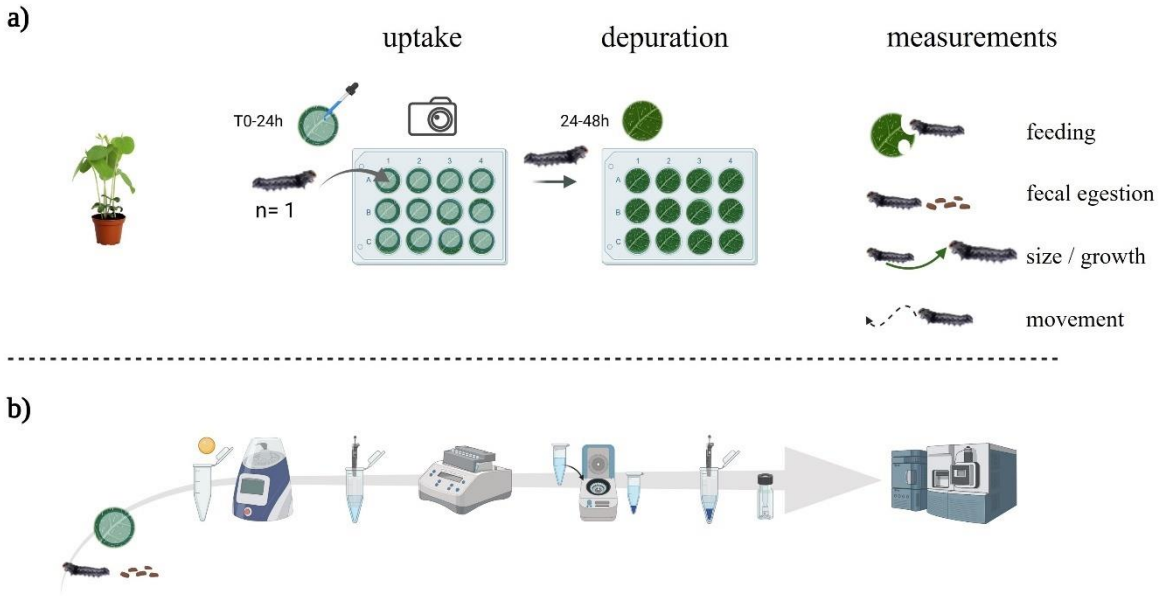

Figure 2: Toxicokinetic assay design: (a) Individual *Spodoptera littoralis* larvae were exposed to compound treated leaf disks. Feeding contact assay with a 24 h exposure period (including imaging) followed by a 24 h depuration period. (b) Schematic sample preparation. Biological samples were macerated. After extraction and centrifugation, the clear supernatant was used for residue measurements by Ultra-High Performance Liquid Chromatography-Mass Spectrometry. Figure created with BioRender.com.

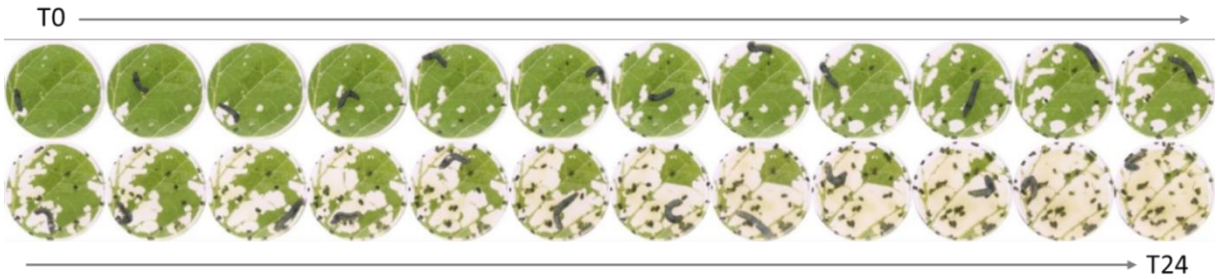

Figure 3: Image frames of the *Spodoptera littoralis* assay: Representative example of a single larva feeding pattern on a leaf disk observed in the toxicokinetic assay over 24 h of exposure (see also Fig. S1).

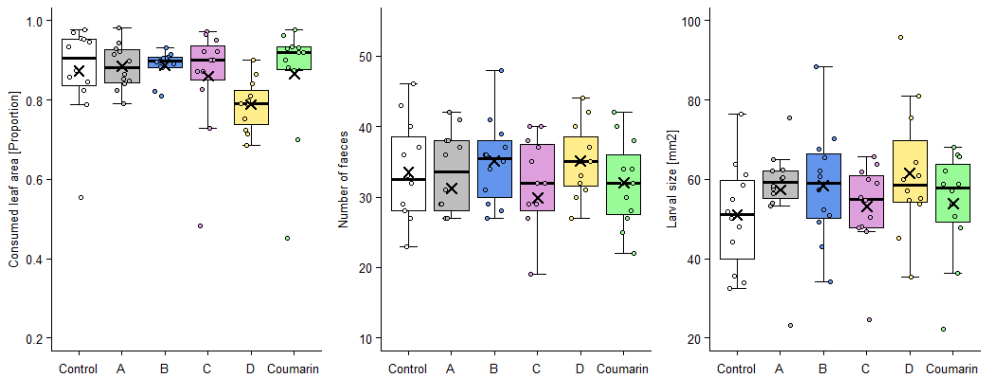

Figure 4: Physiological parameters of *Spodoptera littoralis* after 24 hours exposure period, created by image analysis. Consumed leaf area (Proportion = pixel per mm^2), number of feces pellets, and larval size, shown for all test groups (control, test compounds A-D, coumarin). Boxplots show interquartile ranges, medians (black lines), and means (×). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Individual data points (n = 12), including outliers, are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

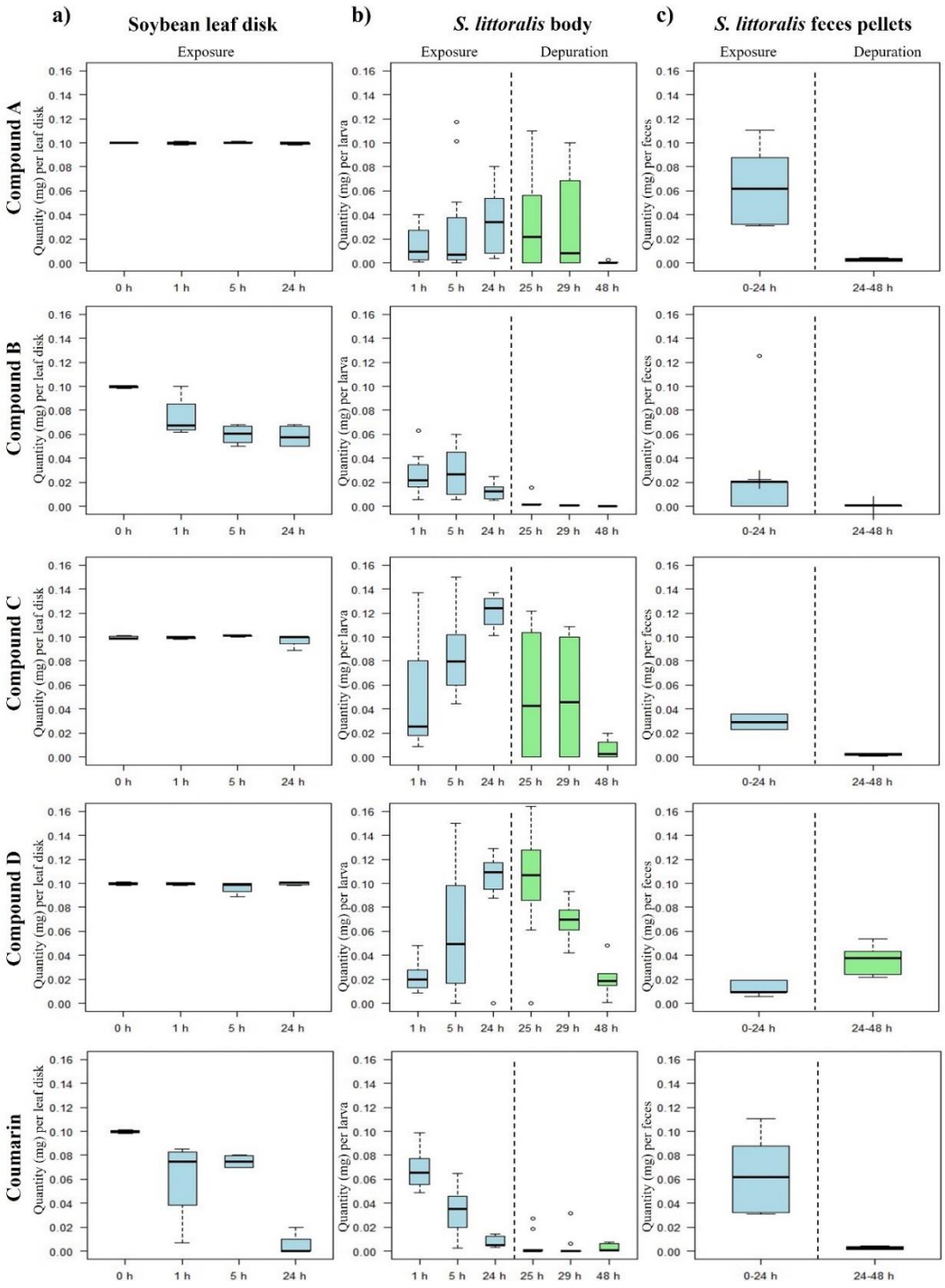

Figure 5: Compound quantities (mg) quantified per **a)** soybean leaf disk, **b)** *Spodoptera littoralis* larvae (body) and **c)** feces pellets. Exposed larvae fed on treated leaf disk for 24 hours, immediately after exposure interval larvae were transferred and fed on non-treated leaf disk for a follow-up depuration time of 24 hours. A separate bioassay without larvae feeding on the leaves was used for the leaf disk (a) measurements of the compound. Boxplots show interquartile ranges and medians (black lines). Whiskers

not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

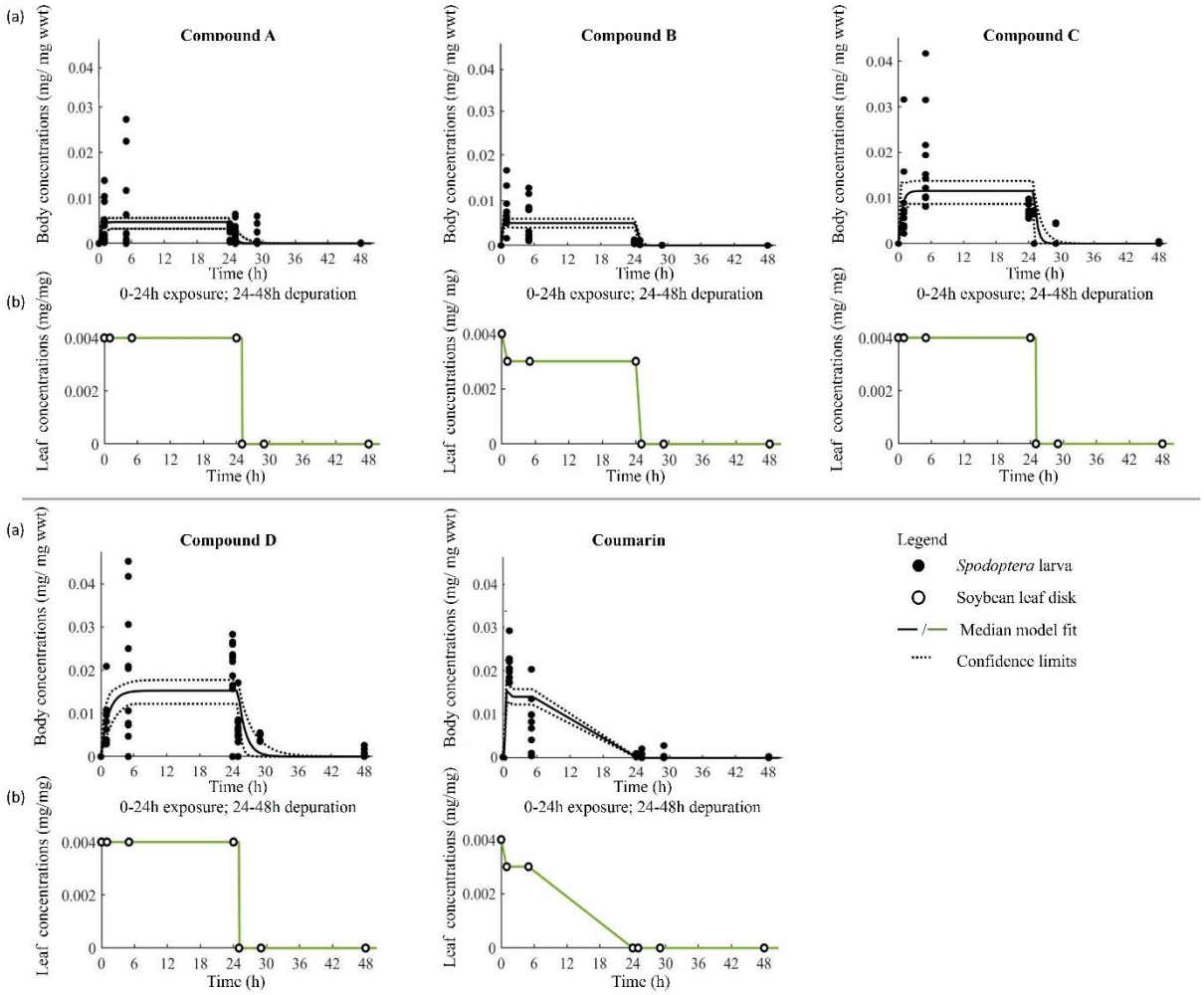

Figure 6: Leaf disk concentrations and body concentrations in *Spodoptera littoralis* larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Followed by transfer to untreated leaf disks for a depuration period of 24 hours. (a) TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 1) and 95 % confidence limits (dotted) of model fit represented by the lines. Dots indicate measured data. (b) Exposure scenarios in feeding contact assay: soybean leaf disk concentrations during exposure and depuration time (green line). Created using MATLAB (Version R2021a, Build Your Own Model).

Supporting information

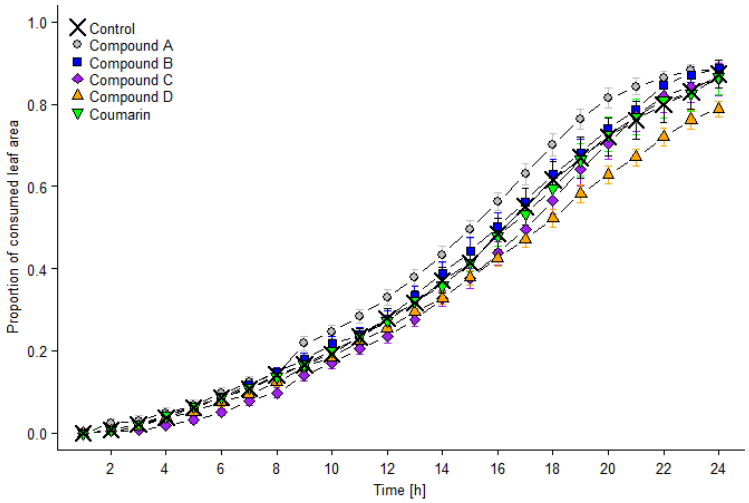

Figure S1: Performance parameters of *Spodoptera littoralis* larvae: consumed leaf area (n=12) during exposure period. Larvae exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Displayed data are the mean and standards error. Figures created using R (version 3.5.3; R Core Team, 2020).

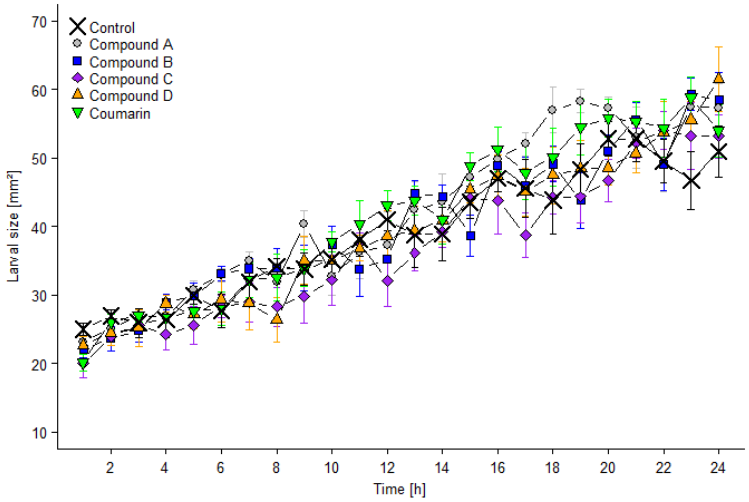

Figure S2: Performance parameters of *Spodoptera littoralis* larvae: larval size over time. Larvae (n=12) exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Displayed data are the mean and standards error. Figures created using R (version 3.5.3; R Core Team, 2020).

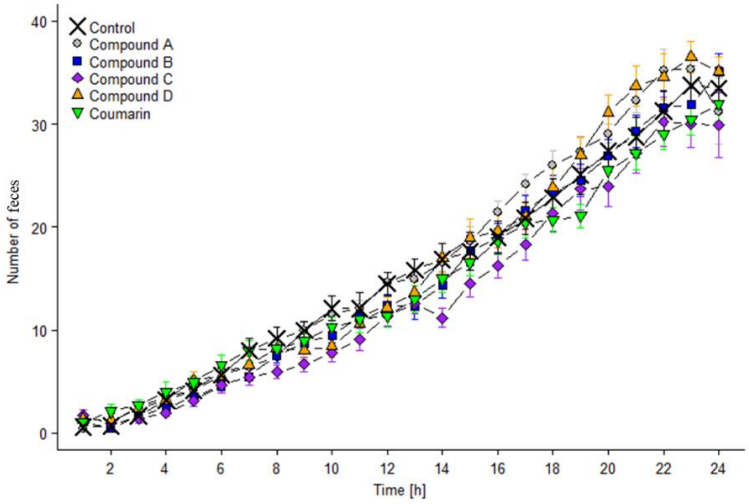

Figure S3: Performance parameters of *Spodoptera littoralis* larvae: number of feces pellets. Larvae (n=12) exposed to five test compounds. Exposed larvae fed on treated leaf disks for 24 hours. Displayed data are the mean and standards error. Figures created using R (version 3.5.3; R Core Team, 2020).

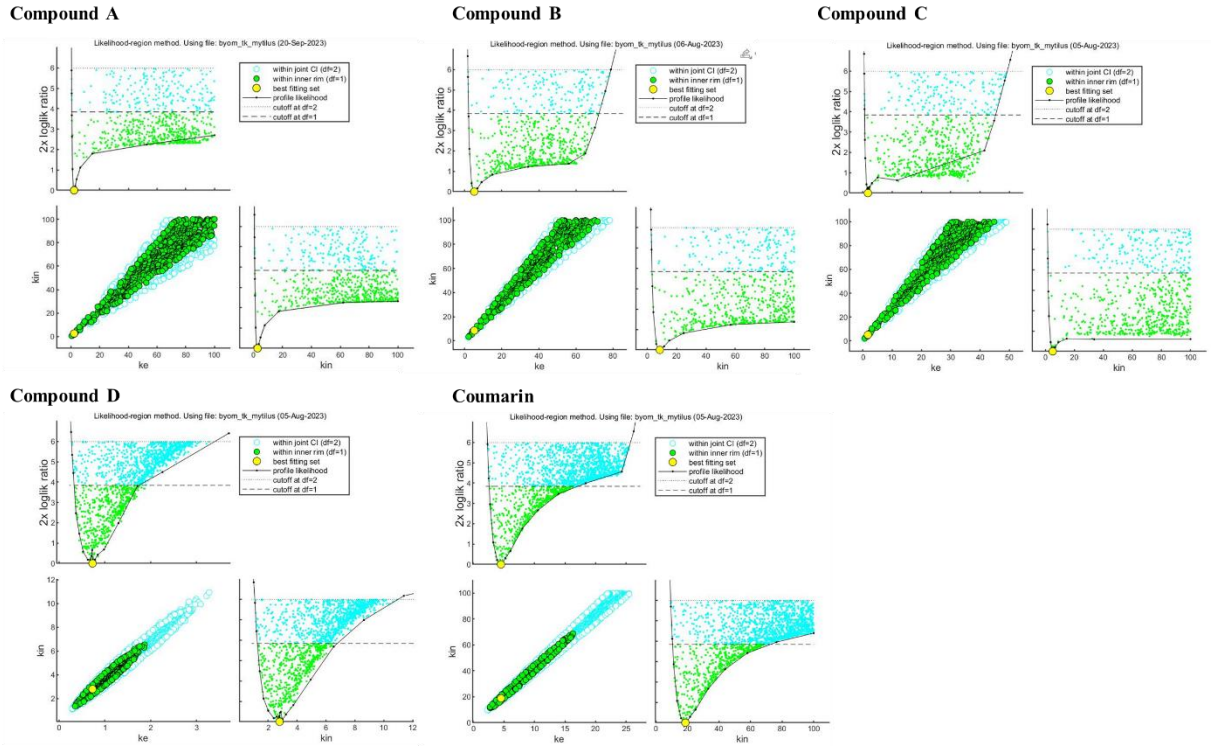

Figure S4: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* (n=12) feeding contact leaf disk assay for the five test compounds (A-D, coumarin). Likelihood profile for uptake (kin) and elimination (kout) rate constants during a 24 h exposure period and a 24 h. Created using MATLAB (Version R2021a, Build Your Own Model).

Table S1: Comparative toxicokinetic parameters, recovery and limit of quantification (LoQ) for the *Spodoptera littoralis* bioassay at the end of the 24h exposure period to different test compounds. This table presents the body mass of larvae (n=12), internal concentration (larval body, n=12), external concentration (leaf disk, n = 3), recovery percentage and limit of quantification adjusted for relative recovery.

Species	Test compounds	Ø Body mass per larva (mg)	Ø Internal concentration (mg /mg wet weight)*	Ø External concentration (mg/mg wet weight)	Ø Recovery (%)**	LoQ (mg /mg wet weight)***
S. litoralis	A	16.230	0.003	0.004	86	0.00003
	B	16.600	0.002	0.003	86	0.00003
	C	18.300	0.005	0.004	86	0.00002
	D	16.600	0.005	0.004	86	0.00003
	Coumarin	17.440	0.001	0.000	86	0.00002

* Average internal concentration at the end of the exposure period

** Relative recovery including matrix effects

*** Limit of Quantification (LoQ) with consideration of the relative recovery

Manuscript 2 (in submission process)

Prepared for the Journal of Pesticide Biochemistry and Physiology

Comparison of absorption and excretion of test compounds in sucking versus chewing pests.

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Abstract

Insecticides prevent or reduce insect damage on crops, thereby maintaining crop quality and quantity. Critical understanding on how pests interact with active ingredients is essential for developing new insect control solutions. Absorption into insect bodies of insecticides in pest species is an important process that confounds the efficacy of insecticides. This study investigated how the different feeding behaviour of two insect pests, *Spodoptera littoralis* and *Myzus persicae*, affects the absorption, metabolism, and excretion (AME) of insecticidally seven inactive test compounds with agrochemical-like structural motifs. We used a feeding contact assay for the Lepidopteran larvae (chewing pest) and an oral ingestion assay for the aphids (sucking pests) to investigate the AME of seven compounds The standardized assay comprised of a 24h or 72h exposure period with treated diet and a subsequent 24h or 72h depuration period with untreated diet. The results showed that *S. littoralis* larvae differed from *M. persicae* in absorbed compound quantities in insect bodies and excretion products at the end of the exposure and depuration periods. We suggest that this is caused by their unequal ingestion types and rates resulting in different uptake and excretion quantities. Further, we found differences in the metabolism (dynamics and biotransformation pathways) of compounds between both species. Notably, certain compounds remained detectable in both pests after the depuration period, suggesting compound and species-specific metabolism and excretion. Our results highlight the complex interplay between insect feeding biology and exposure to different compounds leading to species specific AME.

Keywords: ADME, feeding biology, absorption routes, metabolism, feces, honeydew

1. Introduction

Tailored insect control strategies are important to reduce damage from various pests and to maintain crop quality and quantity. The use of insecticides remains a key component in this regard and innovative solutions in modern agricultural chemistry are required (Akamatsu, 2011; Shelton and Lahm, 2015; Sparks and Lorsbach, 2017; Zhang, et al., 2018; Jeschke, 2021; Maienfisch and Mangelinckx, 2021).

However, their successful use is increasingly undermined by challenges such as the development of resistance to insecticides and pest shifts in crops (Corsi and Lamberth, 2015; Jeschke, 2016; Loso, et al., 2017; Sparks and Lorsbach, 2017). It is imperative that the use of insect control solutions is carefully selected considering pest biology and crop growth cycles to maximise crop protection (Stevens, et al., 1988; Bonaventure, 2012). Crop protection products get typically applied to crops by foliar spray. There are two principal application scenarios, i.e. curative or preventative treatments. In a curative spray, pests are directly exposed to the spray solution, whereas in a preventative treatment, pests get exposed to a dry spray deposit on the foliage (Wang and Liu, 2007).

The Absorption, Distribution, Metabolism, Excretion (ADME) framework supports the comprehensive understanding of the chemical fate of compounds within an organism. The effective dose on the leaf surface is determining the compound absorption by the insect as first step (Gerolt, 1983). There are two dominant uptake routes into the insect, oral and contact absorption (Ebeling, 1974; Clarke and Jewess, 1990; Missner and Pohl, 2009). Oral absorption by active feeding and contact absorption by passive diffusion across insect body surfaces are influenced by life stage and feeding behaviour of the insect. The internal distribution in insect tissues originates from several permeation steps across different biomembranes. This subsequently determines the effective dose at internal target sites such as the nervous system or endocrine glands. The metabolism (or biotransformation) of parent compounds by enzymes or sequestration in fat can detoxify the organism from these compounds (Rharrabe, et al., 2007; Roy, et al., 2016). The increase in hydrophilicity by oxidative transformations facilitates the excretion of compounds. Whereas lipophilic compounds are more likely to accumulate in fatty tissue.

Phytophagous insects create different excretion products, like feces or honeydew according to their diet. Excretion may include different metabolic processes of compounds.Further elimination (detoxification) strategies are enhanced excretion rates, regurgitation, or egg-laying (Broadbent, 1951; O'Donnell, 2008).

Overall detoxification is directly related to the kinetics of compound elimination, including metabolic processes and excretion mechanism (Smith, 1955; Febvay, et al., 1988).

Crop damaging insects exhibit different feeding behaviours on various plant organs and tissues; each resulting in different damage types, but also different uptake routes of crop protection products (Roy, et al., 2016; Talevi, 2022). Within this study we focus on two feeding types on leaves. Chewing and biting insects equipped with mandibles cut pieces of leaf material; notable examples include Lepidoptera larvae (caterpillars) (Krenn, 2019). This physical damage leads to reduced photosynthetic capacity, stunted growth and, in severe cases, to total crop loss. In an agronomic context, this feeding damage can lead to significant reductions in crop yield and quality, up to and including total production loss.

Another prominent feeding type is represented by sucking and piercing insects. They possess specialised mouthparts such as stylets, which allow the ingestion of cell liquids and plant saps (Buchholz, et al., 2015). Hemipteran species, such as aphids, are so called phloem feeders, i.e., their stylets take the sap from the plant vascular tissue (xylem and phloem) (Talevi, 2022). The resulting damage may be caused less by the direct ingestion of sap than by the transmission of viruses and other diseases (Ng and Perry, 2004).

Spodoptera littoralis and *Myzus persicae* are representative pests of great agronomic importance with wide distribution for both feeding types, often simplified as chewing and sucking pest (Mittler and Dadd, 1964; Alves, et al., 2021).

In order to develop effective insecticides against such diverse insect pests with the lowest possible environmental impact, it is important to understand the interaction of foliar applied compounds with the target pest. So far, there are rare, standardised assays for quantitative ADME comparisons between different pest species.

In this context, we developed a *Spodoptera littoralis* bioassay in which larvae were exposed by feeding and contact with treated leaves. The assay design followed the established approach in toxicokinetics which comprises an exposure period followed by a depuration period. The depuration period was initiated by transferring the larvae to untreated leaves. The excretion processes in both periods, exposure, and depuration, reflect larva's capability of compound elimination (detoxification) in addition to metabolism (Römer et al., submitted).

In a similar way, we present here an exposure / depuration bioassay for *Myzus persicae*. An artificial diet was prepared for the aphid (sachet) assay to provide different test compounds in an imbibable manner and at comparable exposure quantities. A leaf disk assay would carry the risk of compound specific foliar penetration rates and therefore unknown exposure quantities at the aphid feeding sites, the plant vascular tissues. This limits the exposure route to oral absorption, as contact absorption is anyhow negligible with this little moving pest.

Both bioassays allow the description and comparison of the chemical fate of test compounds in terms of absorption, metabolism (biotransformation) and excretion. Representative insecticidally inactive test compounds were selected to compare the unaffected A(D)ME between the chewing and sucking pest. In order to understand the fate of insecticide analogues, it is essential to study their A(D)ME independent from their toxic effects on insects.

2. Materials & Methods

The design of this study included an exposure and depuration period to measure total compound quantities in the insect body and their excretion (elimination) over time. During the exposure period, insects were exposed to test compounds through their food source (leaf disks or artificial diet). They were then transferred to an untreated food source for the depuration period. This was combined with frequent sampling of the insects and their excretion products, followed by solvent extraction and chemical analysis to determine parent compound quantities and their putative metabolites.

2.1. Test compounds

Seven insecticidally inactive test compounds with agrochemical related scaffolds were synthesised in house (≥ 95 % purity). They ranged in lipophilicity, Log P 1.43 - 4.7, and molecular mass, 146-433 g/mol (Table 1). The test compounds A to D were already described in a previous study building a toxicokinetic model based on this *S. littoralis* feeding-contact assay (Römer et al. submitted). All compounds were dissolved at 2000 mg/L in water containing 15 % acetonitrile (ACN) (gradient grade for analytics 99.9 %) as solvent and pipetted onto leaf disks for larval assay. A high rate of 0.1 mg compound per leaf disk or diet was chosen to ensure good analytical detection of compounds in all biological matrices (insects and excreta). In addition, this rate did not show any adverse effects on larval performance parameters. Aqueous diet solutions contained 100 mg/L for aphid assay.

2.2. Feeding contact assay – *Spodoptera littoralis*

The feeding-contact assay with the Egyptian cotton leafworm, *S. littoralis* larvae, was previously described (Römer et al. submitted). Briefly, synchronised third instar larvae were exposed to absorbed test compounds (0.1 mg pipetted in 50 µL onto soybean leaf disks, Ø 20 mm) under standardised conditions (25 ± 1 °C, 55 ± 10 % RH, 16-hour light/8-hour dark cycle). The assay included a 24-hour exposure period during which they were able to consume the entire leaf disk, followed by a transfer of larvae to untreated leaf disks and a 24-hour depuration period. Samples of larvae, leaf disks and feces were collected at various time-points for chemical analysis.

2.3. Oral ingestion assay– *Myzus persicae*

Many similar oral ingestion assays are described scince Mittler and Dadd, 1964. In this study, aphids were feeding on an aqueous artificial diet mimicking phloem sap as their natural food source . This assay was designed to record compound quantities in aphids and their excreted honeydew over time. Aphids consumed treated artificial diet for 72 hours whereby they were exposed to test compounds. They were subsequently transferred to untreated food for a 72-hour depuration period. Aphids and honeydew were sampled during both periods of the experiment.

2.3.1. Assay design

The aqueous artificial diet solution contained sucrose, minerals and amino acids according to (Febvay, et al., 1988). For the exposure period, 1 mL of the artificial diet containing 0.1 mg of the dissolved test compounds was added to 12 wells (replicates) of a 24-well microtiter plate (MT-plate, FalconTM, Northfield, Minnesota, USA). Control treatments contained the artificial diet with the corresponding solvent quantity. The MT-plates were then covered with a layer of stretched Parafilm™ and a perforated plate supporting aphid infestation on separate wells (Figure 2). For the depuration period, 24-well MT-plates were prepared in the same way, but the artificial diet did not contain test compounds.

2.3.2. Infestation of aphids

The Green peach aphids (*Myzus persicae*; Sulzer, 1776) originate from an asexual, wingless, laboratory strain that had not been exposed to insecticides before. Aphids were reared as mixed age population on pea seedlings under standardised conditions (20 ± 1 °C, 60 ± 10% RH, 16-hour light/8-hour dark cycle). Tips of infested pea seedlings were cut and placed into a Petri dish containing a dry filter paper and which

was covered with a cotton filter (Ø 14.5 cm). Aphids of all life stages were allowed to migrate from the drying pea seedling onto the filter paper within two hours. Only these vital aphids were used for experimentation. Aphid populations of 15-30 individuals were brushed onto the individual wells of the MT plates (Figure 1, Figure 5). Aphids readily started feeding through the Parafilm™ membrane. A cardboard was then placed to close the infested MT plate upside down. After 15 minutes incubation the cardboard and any non-feeding aphids were removed. An empty MT plate was placed under the infested MT plate to collect the honeydew. Since mixed age populations were used adult females may produce nymphs so that the number of individuals per well (replicate) could increase during experimentation.

2.3.3. Sampling

Since the number of infested aphids varied between wells and over time, the MT plates were photographed prior to insect sampling to capture the number of individuals by counting. Aphid and diet samples were taken at 0, 24, 48, 72 (end of exposure), 75, 80 and 144 h (end of depuration). Honeydew was collected at the end of the exposure (72 h) and depuration (144 h) period by dissolving it from the plate with 1000 µL ACN. Here, samples were pooled from all replicates (12 wells) per treatment. All sample types were transferred to 2.5 mL tubes (MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop any metabolism.

2.4. Compound quantification

The same chemical analysis method was applied to all biological matrices whereas the extraction methods differed for diet, insect, and excretion samples (Römer et al. submitted). All samples were processed as the total mass of the given biological matrix. Therefore, it is not possible to distinguish, for example, whether the compound(s) detected were absorbed internally or adsorbed on the insect surface. The wet weight of samples was measured using a Sartorius-balance (BCE124I-1S Entris® II, Data Weighing Systems, Inc., Wood Dale, IL, USA). All samples were homogenised using a macerator with a ceramic ball (Ø 6.35 mm) and dissolved in 500 µL ACN and extracted for 3 h (Römer et al. submitted). Afterwards samples were centrifuged at 9000 rpm for 2 min. Leaf, diet, feces and honeydew sample extracts were addtinoally filtered through a 0.20 µm pore size filter (CHROMAFIL®Xtra PET-20/13, Macherey-Nagel GmbH & Co.KG, Düren, Germany).

The quantity of test compounds in the biological matrices was determined by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) using ACN and water as solvents. Spectra of all

samples for parent compounds and their metabolite residues were recorded on a Waters Corporation Mass Spectrometer (Xevo TQ-XS Triple Quadrupole Mass Spectrometer) equipped with an electrospray ionization (ESI+) source. Putative metabolites were scanned using negative ion mode (ESI-) in a mass range of 120 to 1000 Da. The quantification of putative metabolites was semi-quantitative because reference standards were not available and had to be calculated by comparing its detected quantities with the detected amounts of the parent compound within the given calibration series.

2.5. Compound quantities in insect bodies over time

Total compound quantities were measured for individual replicates. They were expressed either as compound quantity per individual *S. littoralis* larva or per 'aphid equivalent'. The insect samples in the *M. persicae* aphid assay were standardised by dividing the measured compound quantity by the counted number of aphids at the sampling time-point. This was relevant since replicates (wells) contained a variable number of individuals. However, this approach does not consider differences in age and size of aphid individuals and the time-course of their reproduction.

2.6. Comparison of compound quantities in insects and excretion products

At the end of the exposure and depuration period, the quantities of the parent compound measured in insect bodies and in excretion products (feces or honeydew) were compared. Feces was sampled and measured for each replicate (*S. littoralis* larva) and time-point. While the honeydew samples represent the total quantity of each treatment group at the end of the exposure and depuration period. These compound quantities were correlated with the aphid number counted in all twelve replicates at the sampling time-point. This approach intends to provide a similar comparability to the compound quantities in aphid equivalents.

2.7. Calculation of diet uptake to body mass ratio

To further understand the relationship between compound quantities in insects and the ingestion rate of diet (diet uptake), we calculated the diet uptake (leaf disk or artificial diet) in relation to insect body masses. This relationship is defined as ‘diet uptake to body mass ratio’.

(1)

$$\text{diet uptake to body mass ratio}_{\text{end of exposure}} = \text{diet uptake} \,/\, \text{insect wet weight}$$

For *S. littoralis* it was determined at the end of the exposure period (24 h) when the larvae had consumed the entire leaf disk (average weight Ø 23 mg). The average weight of a *S. littoralis* larvae (Ø 17.14 mg) at 24 h was used in the calculation.

We could not estimate the ingestion rate of *Myzus persicae* in our assay. Therefore, we used the ingestion rate of 0.022 µL h^{-1} as reported by Rhodes *et al*. (1996) for pea aphids (*Acyrthosiphon pisum*) and an artificial diet as approximation. We calculated the diet uptake to body mass ratio by dividing the postulated ingestion volume after 72 h (1.58 µL) by the average wet weight of an aphid equivalent (Ø 0.42 mg).

3. Results

3.1. Measured compound quantities in both pest species

Notable differences in parent compound quantities were measured in entire larval (*S. littoralis*) and aphid (*M. persicae)* bodies in exposure and depuration periods of respective bioassays (Figure 2, Figure 3).

All compound quantities in *S. littoralis* larvae, except for compound B, increased during the exposure and decreased during the depuration period (Figure 2). Each compound reached its maximum quantity at different sampling time points during the exposure or depuration. Compound F reached its maximum quantity after 1 h of larval exposure on treated leaf disks, followed by compound B after 5 h. Compounds A, C, D, and E showed the time point of their maximum quantity after 24 h at the end of the exposure period. In contrast, compound G reached its maximum quantity after 25 h, which represents the first hour of the depuration period. Compound C had the highest (0.085 mg per larva) and compound F the lowest (0.029 mg per larva) overall quantity in larval bodies (Figure 2). Only compounds C and D remained above the limit of detection in larval bodies after 48 h at the end of the depuration period (Figure 2).

No uniform time courses of parent compound quantities were observed in *M. persicae* aphids (calculated equivalent based on aphid counts) (Figure 3). Compounds C and D showed an increase in aphid bodies during the exposure to treated diet followed by a decrease during the depuration. In contrast, the measured quantities of parent compound G decreased already during the exposure period with the maximum at 48 h. Compounds A, E, and F demonstrated comparable parent compound quantities in aphids during both periods. Compound D had the highest total quantity (0.021 mg per aphid equivalent) in *M. persicae* aphids (Figure 2-3). Conversely, compounds A, B, and E had the lowest total quantity in

aphids. At the end of the depuration period (144 h), compounds C, D, E, and G were still detectable in aphids (Figure 3).

3.2. Parent compound quantities in excretion relative to insect bodies

Compounds quantities in insect bodies of *S. littoralis* and *M. persicae* and their excretion products (feces and honeydew) differed between exposure and depuration periods (Figure 4).

c) *Spodoptera littoralis*

At the end of the exposure period (24 h), compounds C- G exhibited higher quantities in insects compared to their excreted feces. Conversely, compounds A and B showed higher quantities in feces than in insect bodies. Compound C reached the overall highest quantity in insects whereas compound A showed the highest quantity of parent compound in feces. At the end of the depuration period (48 h), compounds E and G still displayed higher quantities in insects compared to their excretion products; while compounds C and F showed relative higher quantities in feces (Figure 4a).

d) *Myzus persicae*

At the end of the exposure period (72 hours), compounds E, F, C, and D exhibited higher quantities in insects compared to their excretion product; whereas compounds B and A showed relative higher amounts in the honeydew (Figure 4b). Compound D displayed the highest quantity in the insect (aphid equivalent) at the end of the exposure period, followed by compounds G and C. In contrast, compound E exhibited the overall highest amount in honeydew followed by compound D at the end of the exposure period.

At the end of the depuration period (144 hours), relative higher quantities were found for compounds A, C, and D in insects, while relative higher quantities were measured for compound E in honeydew (Figure 4b).

3.3. Putative metabolism (biotransformation)

The putative metabolites observed in *Spodoptera littoralis* are described as mass changes (-14, +18, +16 Da) of test compounds over time (Table 1). All metabolites were found at shorter retention times in the reverse phase chromatography compared to the parent compounds indicating an increase of the polarity. A metabolite with a mass decrease of -14 Da, a potential demethylation,was detected for compounds E and F after 5 h larval exposure to treated leaf disks and for compound D after 24 h exposure. Another

metabolite with a mass increase of +18 Da, a potential water addition, was observed for compounds C and G after 5 h exposure (Table 1). The mass increase of +16 Da (oxidation) was detected for all compounds at 5 h (compounds C, D, E, G) and 24 h exposure (compound F). No metabolites were observed for compounds A and B (Table 1).

For *Myzus persicae* no mass decrease but mass increase by +18 and +16 Da were observed over time (Table 1). After 48 h of aphid exposure to treated diet, metabolites with a mass change of + 18 Da were observed for compounds B and G. A mass change of + 16 Da was observed for compounds A, B, D and E after 24 h, for compound G after 48 h, and for compound C after 72 h exposure (Table 1).

4. Discussion

This study investigated two agronomically relevant pest species, the Egyptian cotton leafworm, *S. littoralis* and the Green peach aphid, *M. persicae*, in dedicated bioassays consisting of an exposure period to insecticidally inactive test compounds followed by a depuration period. The results on measured parent compound quantities and putative metabolites suggest that the absorption (uptake), metabolism (biotransformation), and excretion (elimination) are influenced by the different feeding biology and/or physiology of both pest species.

Both bioassays differed regarding the absorption routes during the exposure period (Figure 1). The *S. littoralis* bioassay made of larvae feeding on treated leaf disks combined active oral absorption (larvae cutting leaf pieces with their mandibles) and passive contact (larvae crawling on spray deposits on leaf surfaces). This combination of two principal absorption routes is typical for field conditions where pests get preventatively exposed to foliar applied crop protection products. In the *M. persicae* assay oral absorption dominated because aphids were feeding on an artificial diet mimicking plant saps. The precise internal distribution of test compounds in different insect tissues remains unknown, as only entire insect bodies were analysed.

Absorption (uptake) and excretion of compounds

Both, absorption and excretion mechanisms, in species such as *S. littoralis* and *M. persicae* are influenced by insect physiology, feeding biology, and the chemical properties of ingested compounds. Differences in the excretion (elimination) of compounds may be due to variations in A(D)ME processes within the insect body. These processes have an impact on the residence time of compounds within the insect, which in turn affects the quantity of compounds found in excretion products. Typically, compounds with shorter

residence time are excreted more rapidly and thus may undergo no or less extensive metabolic transformation.

S. littoralis and *M. persicae* showed different patterns of compound quantities in their bodies and excretion products, feces or honeydew (Figure 4). Compounds with log P values lower than 1.58, explicitly A and B, were predominantly excreted already at the end of the exposure period for both species. In contrast, compounds with relative higher log P values (C: 2.24 and D: 3.57) were detected in feces of *S. littoralis* larvae in high quantities at the end of the depuration period (Figure 4a). These results are consistent with other *S. littoralis* studies, where consumed compounds remained unaltered at excretion (Ben-Aziz, et al., 1976).

In honeydew of *M. persicae* the measured quantities of compound D were significantly higher at the end of the exposure period than at the end of the depuration period; whereas the opposite was detected for compound E (Figure 4b). This suggests that compound D could already be excreted during its exposure whereas compound E may have had a longer residence time and could get gradually excreted during the subsequent depuration period.

Interestingly, compounds F and G were, regardless of the time of sampling, not detected in the excretion products of both pest species investigated (Figure 4). This observation strongly indicates that both compounds were completely metabolised and were therefore not excreted as parent compounds but as metabolites in feces or honeydew (Figure 4, Table 1). This suggests biotransformation as an effective elimination pathway (Smith, 1955). The predominance of parent compounds in excretion products may indicate either limited absorption potential in insect bodies and / or a fast excretion process.

Metabolism (biotransformation) of compounds

Metabolic processes have a crucial influence on the detectable quantities of parent compounds in given insect species, as they determine the concentration in the insect body, as well as in excretion products (feces or honeydew). In our study, we observed differences in the metabolism of test compounds, leading to different changes in their mass (Da). Specifically, we detected three prominent mass changes. The mass reduction of -14 Da was unique to *S. littoralis* larvae, suggesting a species-specific metabolic process, possibly demethylation, which could result in a more hydrophilic compound derivate which is easier to excrete (Table 1). (Rup, et al., 2006) also described demethylation as biotransformation pathway for mustard aphid (*Lipaphis erysimi*) feeding on radish plants. The fact that we did not detect this pathway may be due to the different diet and sampling dates in our assays or it may actually indicate a true species

difference. Metabolites of compounds A and B were not detected in *S. littoralis* larvae, but only in *M. persicae* (Table 1). Mass increases of +16 Da and +18 Da, indicative for oxidation processes such as hydroxylation and water addition, were observed in both species but not necessarily for each compound (Table 1). It is important to note that our results can only be taken as approximation due to possible limitations in our analytical method. Future research should aim for a comprehensive identification of metabolic pathways (Tolstikov, 2009).

Metabolism within insects influences the dynamics and quantities of measured compounds. *S. littoralis* indicated different biotransformation processes for compounds C, D, E, F and G (Figure 2, Table 1). Notably, metabolites of compound E emerged after 5 hours, coinciding with a temporary drop in the parent compound quantity before it increased again with prolonged exposure. This suggests continuous absorption in parallel to biotransformation.during the exposure period Compounds C, D and G demonstrated consistent increase in parent compounds in *S. littoralis* larvae irrespecitive to continuous metabolism. Conversely, the quantity of compound F did not increase once metabolic processes were detected; suggesting a higher biotransformation than uptake rate.

In aphids, the absorption of compound B seemed to decrease once a second metabolite appeared (Figure 3, Table 1). Compounds A and E were continuously metabolised with the start of the exposure and no increase in parent compounds in aphid bodies was detected. The onset of metabolism of compounds C and G appears to be well correlated with the decrease of parent compounds. This suggests a faster biotransformation than the oral ingestion of compounds. However, the absorption of compound D into aphid bodies continued to increase throughout the exposure period, regardless of an early onset of metabolism .

Our findings emphasize the complex interactions between the dynamics in compound absorption and metabolic processes across insect species, and indicate that biotransformation alone is not always an adequate indicator of effective compound elimination. A thorough analysis and identification of metabolites would be essential to confirm these observations and to better understand the dynamics between absorption and biotransformation rates defining the resulting compound quantities in insect bodies (Figure 2, Table 1).

Significance of feeding biology

To better understand the physiological differences between both pest species investigated, the total diet uptake over the exposure period (larvae: 24 h; aphids 72 h) was calculated in relation to the respective

body mass (see 2.7 in Material and Methods section). The resulting diet uptake to body mass ratio is 1.3 and 3.7 for *S. littoralis* larvae and *M. persicae* aphid equivalents, respectively. This reveals that aphids exhibit in general a 2.8 times higher absorption rate than *S. littoralis* larvae. In addition to the diet quantity, both insect species differ also significantly regarding their feeding style and therefore diet quality.

Feeding on foliar tissues provides a complex mixture of nutrients including carbohydrates, proteins and fats, as well as secondary plant metabolites such as alkaloids, terpenes, phenols, polyphenols or glycosides (Chown and Nicolson, 2004). These compounds could act as chemical defense mechanism by e.g. disrupting the digestion of herbivores (Jamieson, et al., 2017). Widespread polyphagous pests like *Spodoptera* have developed superfamilies of detoxification genes to arm themselves against plant toxins and xenobiotics (Amezian, et al., 2021). Their intestinal pH varies from neutral to slightly acidic in the foregut and to very alkaline in the midgut. In the hindgut, the pH might drop to the level of the foregut (Dow, 1992; Denecke, et al., 2018). The peritrophic matrix protects the intestinal mucosa in Lepidoptera; a barrier which is absent in aphids (Ortego, 2012).

The diet of aphids feeding on the vascular tissue is typically rich in carbohydrates, but often deficient in essential amino acids (unfavourable carbon-nitrogen ratio) which affects ingestion rates and population dynamics (Lescano, et al., 2022). To compensate the nutritional limitations of their diet, *M. persicae* have evolved symbiotic relationships with gut bacteria that aid in extraction or synthesis of essential nutrients, particularly amino acids (Skaljac, et al., 2018). Their gut system is also adapted to this unique diet, with a neutral or slightly acidic foregut, an acidic midgut optimised for nutrient absorption and enzymatic activity, and a neutral or slightly acidic hindgut (Auclair, 1963; Cristofoletti, et al., 2003).

Such fundamental differences in diet quantity, dietary composition, pH mileus and specialised symbiotic relationships define species specific differences in biotransformation pathways. Accordingly, the chemical fate and resulting uptake into internal insect tissues (bioavailability) will differ between both pest species.

Another aspect is the residence time of the diet in the respective gut system. The 2.8 times higher diet uptake to body mass ratio for aphids implies also a faster gut passage which is expected to affect both, uptake into internal insect tissues and exposure to metabolic processes. This might contribute to the overall low compound quantities measured in aphid bodies, the less diverse biotransformation compared to *S. littoralis* and the tendency for higher absorption rates with more lipohilic test compounds. However,

comprehensive metabolite identifications (qualitative and quantitative) are required to substantiate such correlations.

Critical insights on these complex interdepencies are of high value to pesticide research. Good knowledge on factors affecting ADME in target and non-target pests could support chemistry design of new insect control solutions providing effective pest control with minimal environmental impact.

Physicochemistry of test compounds

Poor bioavailability of pesticides in target organisms is one of the major bottlenecks in the discovery of pesticides. The molecular physicochemical characteristics can explain pesticide-likeness, including hydrophobicity, lipophilicity, electronic and structural properties, water solubility, and crystal packing (Chen, et al., 2022). Lipophilicity, most commonly referred to as the Log P, is a key property in transport processes, including intestinal absorption, membrane permeability, protein binding, and distribution to different tissues and organs (Van de Waterbeemd, 2007). An increase in the molecular mass or size is often observed to be associated with lower solubility and poor penetration through membranes (Tice, 2001). The McGowan volume is another molecular property known to influence absorption and distribution of agrochemicals (Clarke, 2009).

One could take measured compound quantities in insect bodies as a reference on biovailability for a given test compound (Figure 6). Our results appear to indicate that compounds between a Log P value of 2.2 (C) and 3.6 (D) demonstrated relative higher uptake into insect bodies of both species. The plot against molecular mass did not reveal an obvious correlation whereas the molecular volume suggests favourable absorption into *S. littoralis* larvae at a size of ca. 200 cm^3 mol^{-1}. Multi-variate data analyses on larger datasets will be required to better understand such complex interdepencies on bioavailbility.

Conclusions

This study highlighted the complex interactions involved in the uptake and elimination of different test compounds into two insect pests. Factors such as feeding behaviour, which determines diet quality and ingestion rates, the biotransformation capabilities of the insect and physicochemical properties of compounds, such as Log P and molar volume, are all important. Aphids have a relative higher diet uptake to body mass ratio than *S. littoralis* larvae, but overall absorption quantities of compounds in aphids bodies remained lower.

Measured compound quantities in insect bodies and excretion products at the end of the exposure and depuration periods in our bioassays could give an indication on the residence time of compounds within insect bodies. Less lipophilic compounds were detected at higher quantities in insect excretion products (feces and honeydew) than in respective insect bodies. Whereas increasing lipophilicity of compounds resulted in relative higher quantities in insect bodies.

This highlights that the description of the chemical fate of compounds in insects requires the consideration of the entire ADME processes and dynamics. A snapshot on e.g. concentrations in insects could cause misleading conclusions if excreted quantities get omitted. For example, unlike *S. littoralis*, we did not detect the demethylation pathway in *M. persicae* in our sampling setup. Furthermore, differences in species specific metabolism (dynamics and biotransformation pathways) emphasize the need for a comprehensive analysis (i.e. metabolite identification and quantification). Follow-up research is required to substantiate the possibility of a species selectivity. Better understanding of species specific differences in ADME, specifically in biotransformation, could pave the way for more selective pest control solutions by raising the effectiveness against target pests and minimising the impact on non-target organisms.

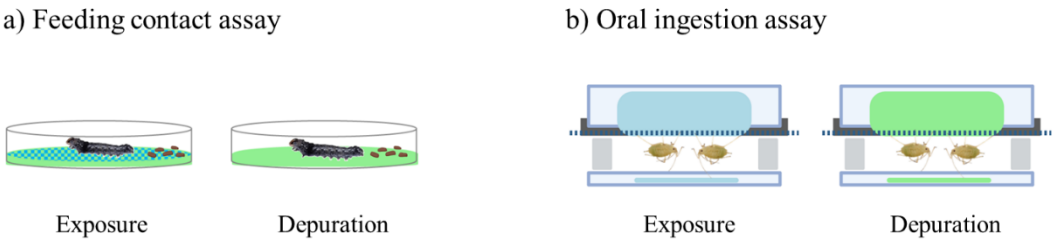


Figure 1: **a)** Schematic overview of the *Spodoptera littoralis* feeding contact assay in a 12-well microtiter plate with either compound-treated or untreated leaf disk. **b)** Schematic overview of the *Myzus persicae* oral ingestion assay in a 24-well microtiter plate, with either compound-treated or untreated diet. Blue = exposure preventative pipetted on leaf disk or in artificial diet, green = no treatment with compound. (Created with BioRender.com)

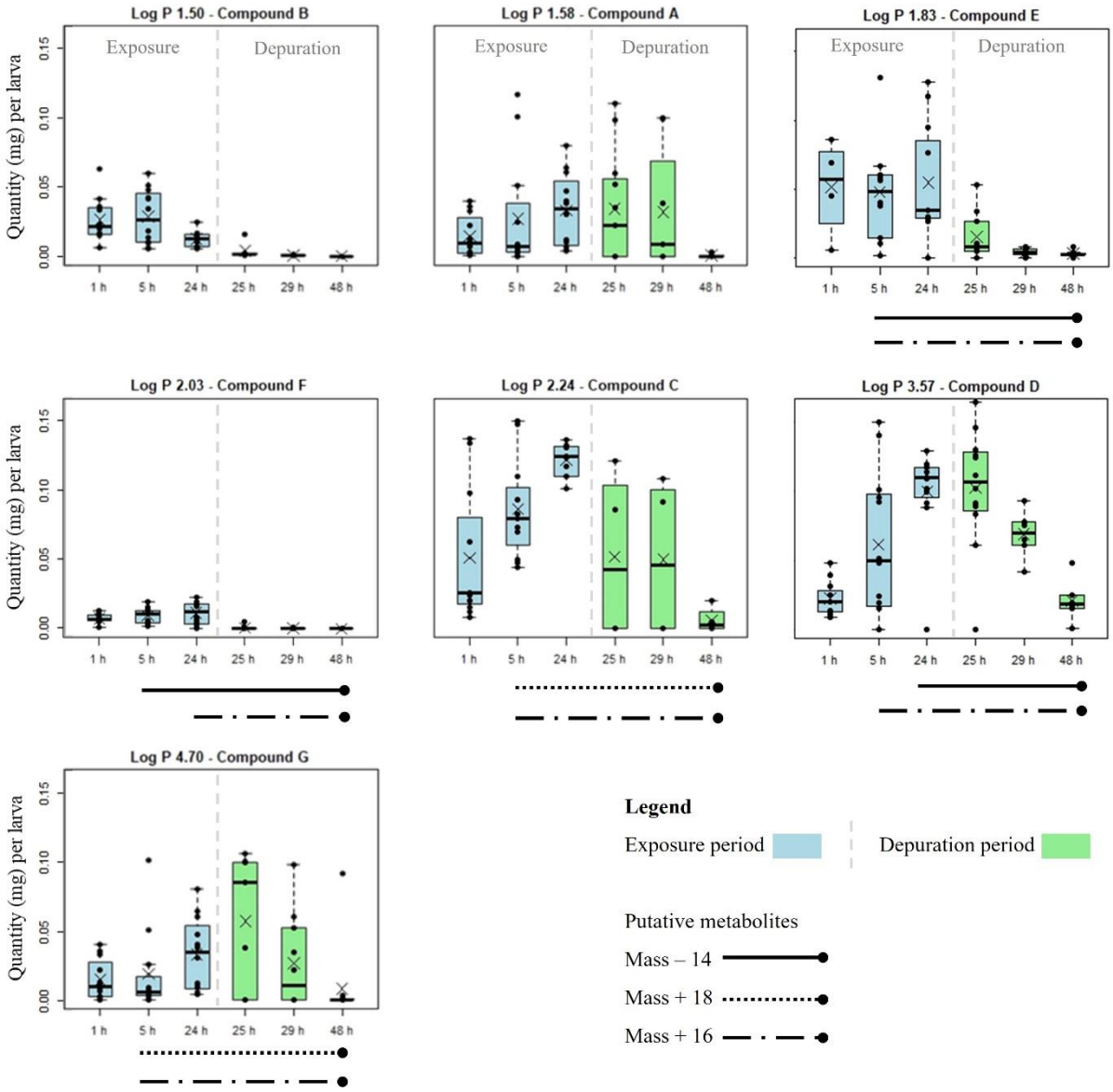


Figure 2: Compound quantities (mg) per *Spodoptera littoralis* larval bodies over time. The compounds are arranged in order of increasing log P (Table 1). Larvae consumed one treated leaf disk within 24 h (exposure), followed by one untreated leaf disks in the subsequent 24 h (depuration), (n=12 larva). Occurrence of putative metabolites (mass changes, Table 1) over time represented as different line types below the graphs, respectively . Boxplots show interquartile ranges, raw data points, mean (X), and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Parent compound quantities (A-D) in *S. littoralis* were already published in Römer et al (submitted). Figures created using R (version 3.5.3; R Core Team, 2020).

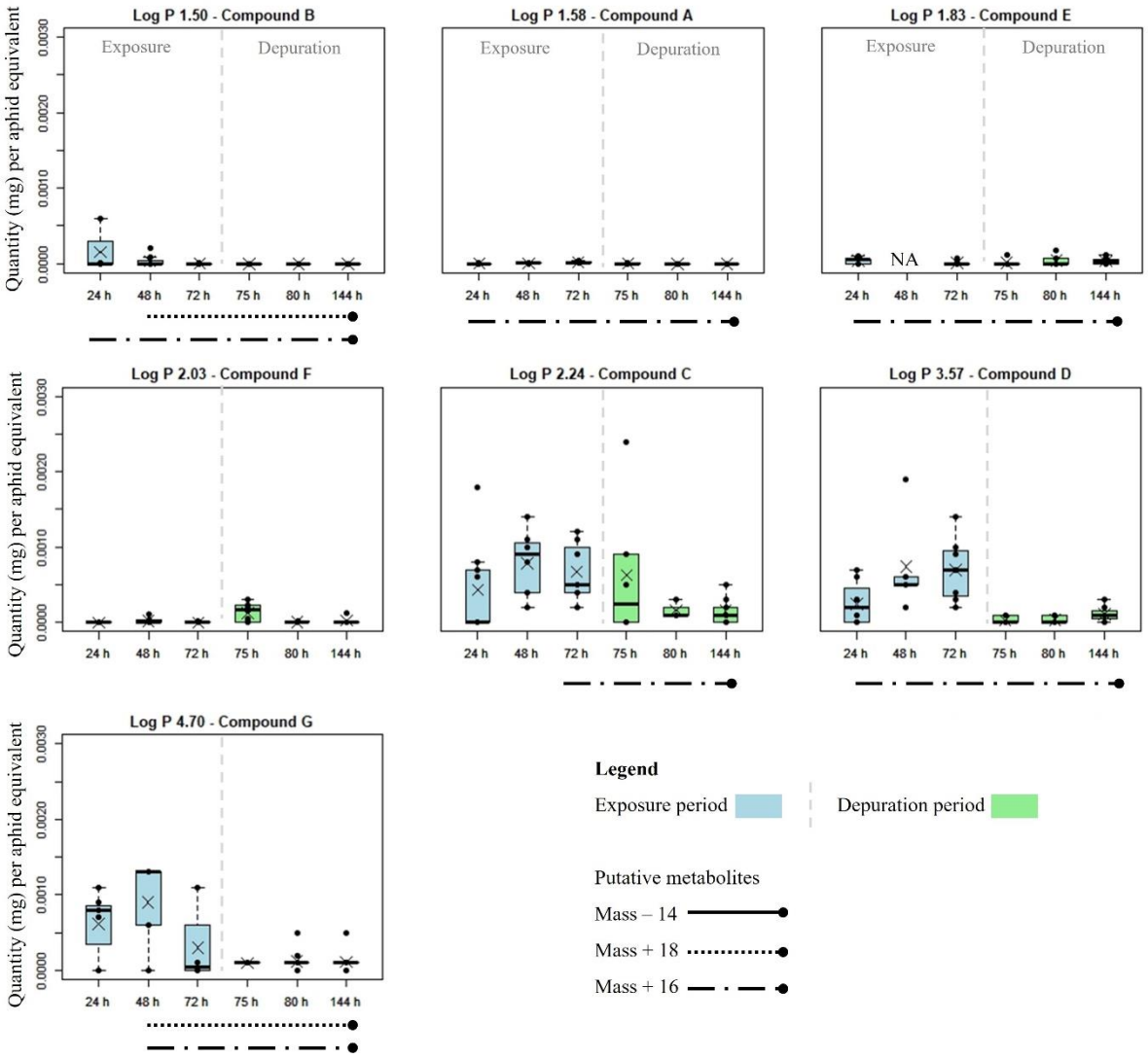


Figure 3: Compound quantities (mg) per *Myzus persicae* aphid (calculated equivalent based on aphid counts) bodies over time. The compounds are arranged in order of increasing log P (Table 1). Aphids fed on treated artificial diet for 72 h (exposure), followed by a 72-h feeding period on untreated diet (depuration) (n=8 replicates; aphid population per sampling time). Occurrence of putative metabolites (mass changes, Table 1) over time represented as different line types below the graphs, respectively (Table 1). Boxplots show interquartile ranges, raw data points, mean (X), and medians (black lines). Whiskers not exceeding 1.5 × of the interquartile range extend to the maximum and minimum. Outliers are shown as circles. Figures created using R (version 3.5.3; R Core Team, 2020).

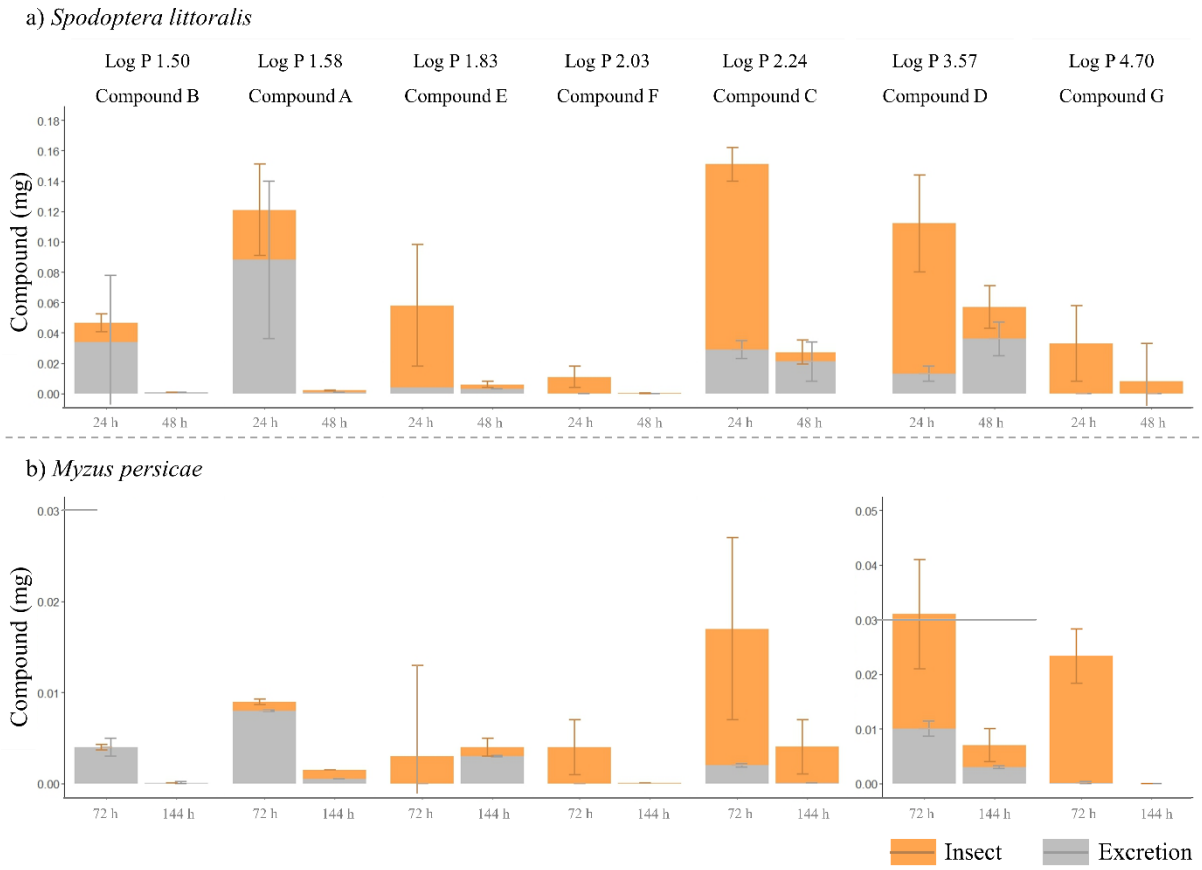
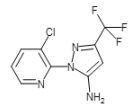
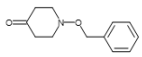
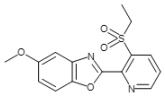
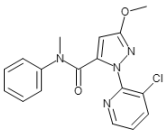
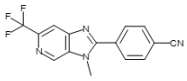
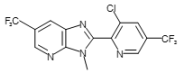
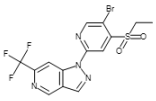


Figure 4: Distribution of measured parent compounds in insects (bodies) and excretion products (feces or honeydew). **a)** Compound quantity (mg) per *Spodoptera littoralis* larva (n=12)at the end of the exposure (0-24 h) and depuration (24-48 h) period, respectively. Larvae consumed one treated leaf disk within 24 h, followed by one untreated leaf disks in the subsequent 24 h. Parent compound quantities (A-D) *S. littoralis* were already published in Römer et al (submitted). **b)** Compound quantity (mg) per *Myzus persicae* aphid (calculated equivalent based on aphid counts, n=8) at the end of the exposure (0-72 h) and depuration (72-144 h) period, respectively. Aphids fed on treated artificial diet for 72 h, followed by a 72-h feeding period on untreated diet. (Horizonal grey line shows the different y-axis scale for compound D). Note the different y-axis for *S. littoralis* and *M. persicae.* Figures created using R (version 3.5.3; R Core Team, 2020).

Table 1: First time of detection of putative metabolites in insect samples of *Spodoptera littoralis* (larvae), and *Myzus persicae* (calculated equivalent based on aphid counts). Chemical structure, molecular mass, measured log P, and water solubility of test compounds (A-G). Putative metabolites detected by mass difference (in Dalton) to given parent compound. All metabolites detected in the exposure period were also detected at all subsequent sampling time points (i.e., entire depuration period).

Parent compound					Putative metabolites					
					Spodoptera littoralis (L3)			*Myzus persicae* (aphid equivalent)		
Chemical structure	Compound	Molecular mass (g/mol)	Log P	Water solibilty	Mass -14	Mass +18	Mass +16	Mass -14	Mass +18	Mass +16
	B	262.62	1.5	589	-	-	-	-	48 h	24 h
	A	205.25	1.58	1410	-	-	-	-	-	24 h
	E	318.35	1.84	120	5 h	-	5 h	-	-	24 h
	F	342.78	2.03	166	5 h	-	24 h	-	-	-
	C	302.25	2.24	10	-	5 h	5 h	-	-	72 h
	D	380.68	3.57	6.46	24 h	-	5 h	-	-	24 h
	G	435.22	4.7	0.26	-	5 h	5 h	-	48 h	48 h

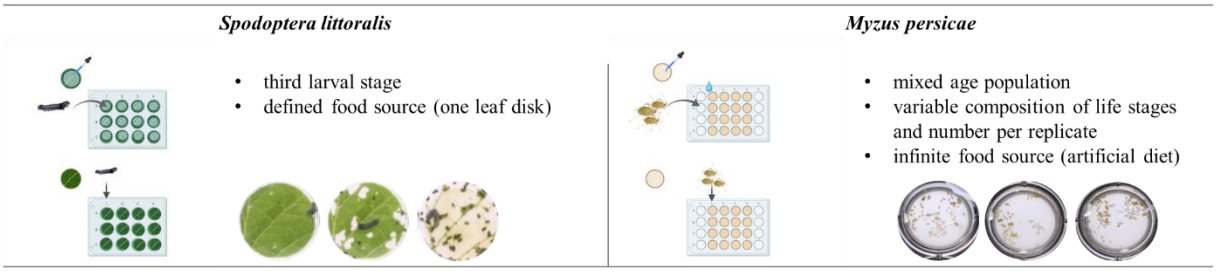


Figure 5: Bioassay conditions for *Spodoptera littoralis* and *Myzus persicae* highlighting the key differences. (Created with BioRender.com)

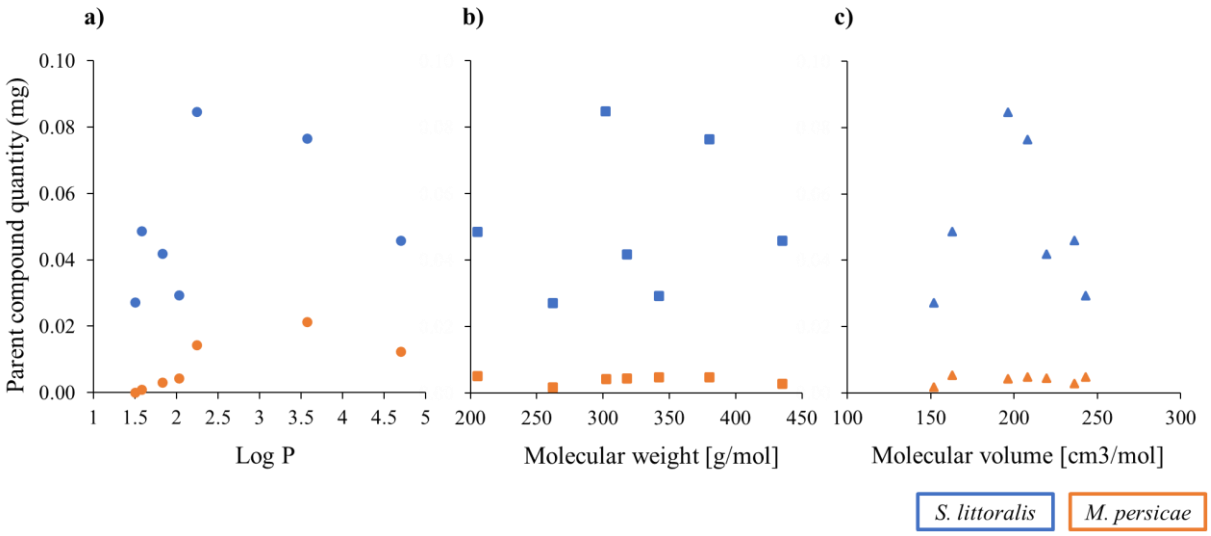


Figure 6: Parent compound quantities (mg) in *Spodoptera littoralis* (larvae, n=12) and *Myzus persicae* (calculated aphid equivalent, n=8) plotted against log P (a), molecular weight (b), and molecular volume (c) (Table 1). Dots represent the average of parent quantities (mg per insect) at the end of the exposure period.

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Supporting information

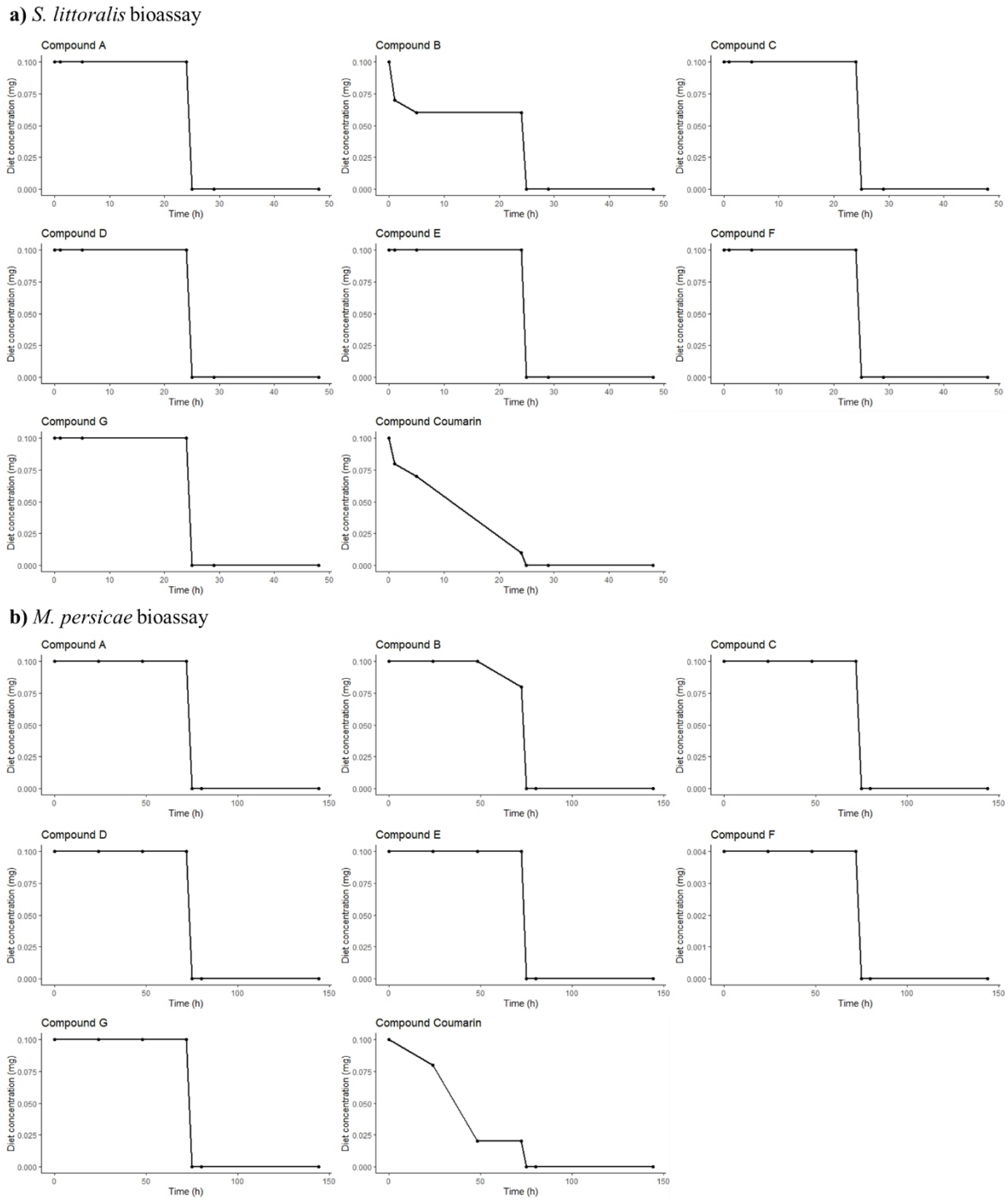

Figure SI 1: Exposure profiles: a) compound quantities (mg) in leaf disks (n=3) over time. b) Compound quantities (mg) in artificial diet (n=3) over time. Created using MATLAB (Version R2021a, Build Your Own Model).

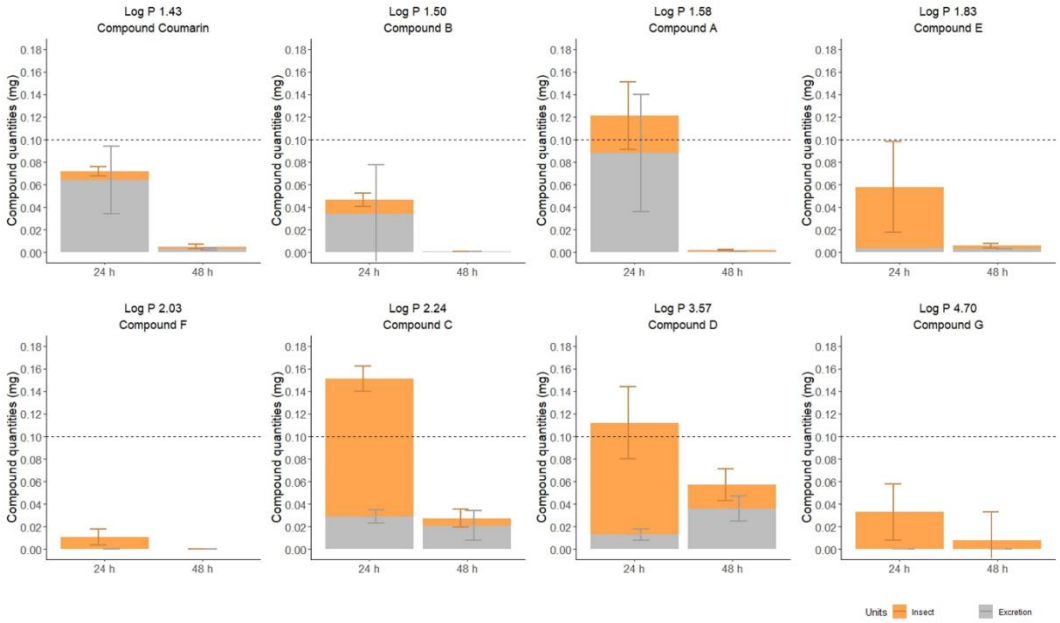

Figure SI 2: Parent compounds (sorted by log P) in insects and excretion. Quantified per *Spodoptera littoralis* (n=12) larvae (insect) and feces pellets (excretion). Exposed larvae fed on treated leaf disk for 24 hours, immediately after exposure interval larvae were transferred and fed on non-treated leaf disk for a follow-up depuration time of 24 hours. Parent compound quantities (A-D) *S. littoralis* were already published in Römer et al (submitted). Figures created using R (version 3.5.3; R Core Team, 2020).

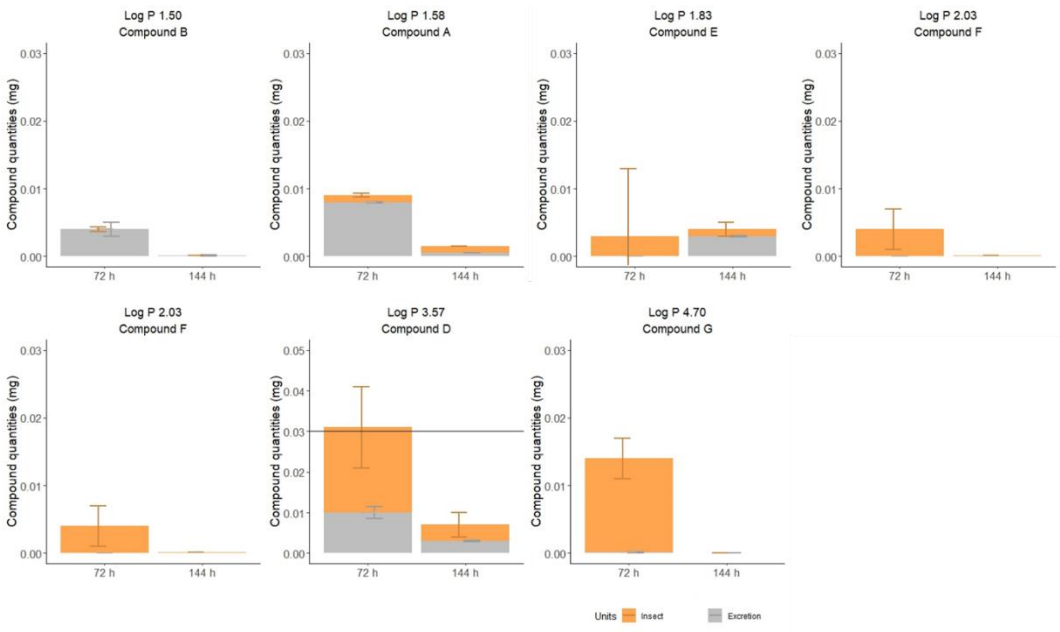

Figure SI 3: Parent compounds in insects and excretion. Quantified per *Myzus persicae* (n=8) aphids (body) and honeydew. Exposed aphids fed on treated diet for 72 hours, immediately after exposure interval aphids were transfers and fed on nontreated diet for a follow-up depuration time of 72 hours. Figures created using R (version 3.5.3; R Core Team, 2020).

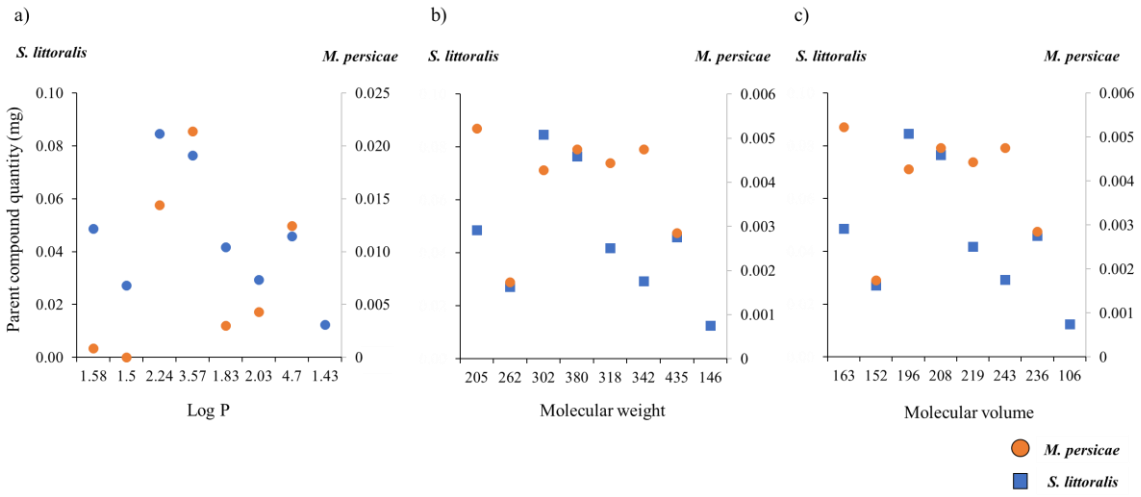

Figure SI 4: Parent compound quantities (mg) Spodoptera littoralis (larvae, n=12) and Myzus persicae (calculated aphid equivalent, n=8). Dots represent the average of parent quantities (mg per insect) at the end of the exposure period; plotted against Log P (a), molecular weight (b), and molecular volume (c).

Manuscript 3 (draft)

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Comparative toxicokinetics of insecticide scaffolds and their putative biotransformation in target and non-target species

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Keywords: ADME, Lepidoptera, Chironomidae, Metabolism, Detoxification

Synopsis: Differences in uptake, biotransformation and elimination between target and non-target insects suggest avenues for optimisation of pesticide selectivity.

Abstract:

Plant Protection Products (PPPs) are central to achieving high agricultural yields and to achieve the United Nations Sustainable Development Goal of 'Zero Hunger', whilst their environmental risks need to be minimised to achieve other goals such as the protection of 'Life on Land' and 'Life under Water'. Selective PPPs that maximise pest control efficacy, while minimising ecological impact, can be developed by harnessing differential toxicokinetic (TK) characteristics of target and non-target species. Here, we demonstrate how the complexity resulting from diversity in uptake, biotransformation and elimination observed in different species can be reduced through a combination of standardised assays and TK modelling. Using standardised bioassays that include an exposure and subsequent depuration period, we constructed quantitative TK models of seven inactive agrochemical-related test compounds and the reference compound Coumarin in target (*Spodoptera littoralis*) and non-target (*Chironomus riparius*) insect species. *S. littoralis* had a higher uptake and elimination potential, which could be beneficial for pest control, whereas *C. riparius* had slower metabolism and hence overall elimination kinetics, which poses a risk for bioaccumulation and potential food web impacts. Furthermore, the proposed comparative approach highlights differences in biotransformation capacities between species, suggesting avenues for chemical optimisation in PPP development. The integration of TK modelling is an important strategy to ensure PPP selectivity, with implications for both pests and non-target insects.

Introduction

Agriculture is at the nexus of several of the United Nations' Sustainable Development Goals[1]. High yields and productivity are key to achieve 'Zero Hunger'[2], whilst understanding and minimising the environmental impacts of agriculture is required to reach the goals to protect 'Life on Land' and 'Life below water'[3]. Plant Protection Products (PPPs), including insecticides, increase productivity and yield through their efficacy against target species, i.e., agricultural pests[4-6]. Environmental impacts of PPPs depend on their use, environmental fate, and toxicology profile. The toxicity of PPPs against non-target species and the efficacy against target species are determined by the same chemical and biological processes, however they are rarely investigated within the same framework, which hinders comparisons and understanding of the differences between target and non-target species that could be exploited to design more selective PPPs. Optimizing selectivity, i.e., maximising efficacy (against targets) and whilst minimising toxicity (against non-targets) is one important scientific lever to reduce environmental risks of PPPs and so make progress towards achieving the sustainable food production and the above-mentioned United Nations' Sustainable Development Goals.

Processes such as uptake, distribution, biotransformation, and elimination are important determinants of the biologically effective dose, which affects efficacy or toxicity, and can be described by the toxicokinetics (TK) of insecticides[7-9]. Species-specific differences in TK between target and non-target insects are particularly important as they may help to maximise efficacy against pests while minimising adverse environmental impact and because TK related properties could change through intentional chemical design. However, it is crucial to first determine and understand the toxicokinetic characteristics of research compounds in these organisms before using them in chemistry optimisation of new insecticides. Currently this understanding is hindered by the lack of comparative TK studies with target and non-target species. Such comparisons are further complicated by the very different biology and resulting uptake and elimination routes of different insect species. And to be most useful for intentional optimisation, the characterisation of TK differences between species needs to be quantitative and predictive.

To fill this knowledge gap, we combine specific bioassays with analytical approaches to measure internal concentrations of compounds and their putative biotransformation. We then use this information to construct quantitative, toxicokinetic models. Finally, we demonstrate how to use the TK models to shed light on interspecific patterns and explain inherent differences quantitatively.

Materials and methods

Study overview

We selected seven molecules that typify insecticide scaffolds plus one reference chemical and studied them in a comparative toxicokinetics approach using a dual-phase bioassay including both an exposure and a depuration phase. We investigated two taxa: *Spodoptera littoralis* larvae, a representative chewing pest due to its wide distribution and significant threat potential to agricultural crops, *Chironomus riparius* larvae, a representative non-target organism commonly found in freshwater systems and a standard toxicity test species[10]. The overall objective of our study, to elucidate and quantify species differences in toxicokinetics and biotransformation, was achieved through applying the same toxicokinetic modelling framework across all species-compound combinations.

Test compounds

Seven representative insecticidally inactive test compounds with agrochemical related scaffolds were synthesised in-house (Table S1). They ranged over three order of magnitude hydrophobicity (Log P 1.43 to 4.7) and a factor of three in molecular mass (146 to 433 g/mol, Table 1). Coumarin (CAS 91-64-5, Figure 1, Table 2) was used as a reference for extraction and as a method standard.

Toxicokinetic assays

The design of this study included a toxicokinetic bioassay combining an exposure and depuration phase for each combination of test compounds and species.

Spodoptera littoralis bioassay

The larval *S. littoralis* feeding-contact assay was previously described in Römer et al. (submitted). Synchronised third instar larvae were exposed to test compounds to test compounds (0.1 mg pipetted in 50 µL onto soybean leaf disks, Ø 20 mm) under standardised conditions (25 ± 1 °C, 55 ± 10 % RH, 16-hour light/8-hour dark cycle). The assay included a 24-hour exposure period followed by a 24-hour depuration period (with untreated leaf disks) under standardised conditions. Samples of larvae, leaf disks and feces were collected at various times for analysis.

Chironomus riparius bioassay

Harlequin fly larvae (*Chironomus riparius*; Meigen, 1804) were reared in the laboratory under standardised conditions (20 ± 1 °C, 60 ± 10 % relative humidity (RH), 16-hour light/8-hour dark cycle)

and fed with macerated fish powder (10 mg) for larval stages[10]. Adults were fed with honey-water solution. Larvae were derived from a laboratory strain (provided by IES Switzerland) that had not been exposed to insecticides. Larvae were selected by transferring egg clusters to water beakers (100 ml). The larvae hatched within four days. The first larval instar (3 days) was used for the bioassay. The toxicokinetic bioassay was adapted from the acute toxicity test recommended in the OECD guidelines[10]. All compounds were dissolved at 100 mg/L in water containing 15% acetonitrile (ACN) (gradient grade for analysis 99.9%) as solvent. The control treatment was given a water-ACN solution only. Assays were performed under standardised conditions (20 ± 1 °C, 55 ± 10 % RH, 16 h light/8 h dark cycle). *C. riparius* larvae were exposed to the test compounds for a period of 24 h in glass beakers (100 ml), each containing 0.1 mg of the test compound. The test solution was evenly distributed by manual stirring for 20 seconds. In addition, 10 mg of food was added to each beaker. During the exposure period (T 0-24 h), larvae were exposed to the treated water. Then the larvae were transferred to clean beakers with the same amount of food (10 mg) for the subsequent 48 h depuration period.

Larval samples (n=3, each containing 3 individuals pooled) and water samples (500 µl) were collected during the exposure (T 0-24 h) and depuration (T 24-72 h) periods. Larvae were collected with a single use pipette and rinsed in clean tab water for one minute. Samples were collected at 0, 2, 4, 6, 24, 26, 28, 30, 48, 50, 52, 54, 56 and 72 h. Each sample was separately transferred to 2.5 ml tubes (3 replicates per time point, MP Biomedicals™ FastPrep-24™ 5G, Lucerne Chem AG, Lucerne, Switzerland) and immediately frozen at -80 °C to stop metabolism.

Analytical chemistry

The same analytical approach was applied to all biological matrices to detect residues of the parent compounds and their putative metabolites. Samples were prepared as described in Römer et al. (submitted).

Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) using acetonitrile (ACN) as solvent was used to determine the amount of test compounds in the biological matrices. Spectra for parent compounds and metabolite residues were recorded from all samples on a Waters Corporation mass spectrometer (Xevo TQ-XS Triple Quadrupole Mass Spectrometer) equipped with an electrospray ionization (ESI) source. Chemical analysis of all biological matrices was performed as described in Römer et al. (submitted).

Toxicokinetic model

A toxicokinetic model using the Build Your Own Model (BYOM, version 64) platform was built in MATLAB (version R2021a). The TK model simulates the uptake, biotransformation, and elimination of test compounds within an organism as a series of ordinary differential equations (ODEs) as well as the organism growth over time to account for growth dilution. The model is calibrated separately for each species-compound pairing. Organism growth is modelled as a function of the current size of the organism[11]:

$$\frac{dW_w}{dt} = b(W_m^{\frac{1}{3}} W_w^{\frac{2}{3}} - W_w) \quad (1)$$

Where W_w is wet weight (mg $_{w.wt.}$), W_m is the maximum organism weight (mg $_{w.wt.}$) and b is the growth rate constant (1/h). The change in internal concentration (whole body residue), adjusted for growth dilution, is calculated as:

$$\frac{dC_{in}}{dt} = k_{in} C_e - k_e C_{in} - \sum_{i=1:n} k_{m_{f,i}} C_{in} - C_{in} W_w \frac{dW_w}{dt} \quad (2)$$

Where C_{in} is the internal concentration (mg/mg$_{w.wt.}$), k_{in} is the uptake rate constant (1/h), for *S. littoralis* and *C. riparius*, respectively), C_e is the external concentration (mg/mg$_{w.wt.}$ or mg/L, for *S. littoralis* and *C. riparius*, respectively), k_{out} is the elimination rate constant (1/h), $k_{m_{f,i}}$ is the formation rate constant of metabolite *i* (1/h). The metabolite concentration Met_i (mg/mg$_{w.wt.}$) of metabolite *i*, adjusted for growth dilution, is calculated as:

$$\frac{dMet_i}{dt} = k_{m_{f,i}} C_{in} - k_{m_{e,i}} Met_f - Met_f \frac{1}{W_w} \frac{dW_w}{dt} \quad (4)$$

Where $k_{m\_e,i}$ is the elimination rate constant of metabolite *i* (1/h). Equations (1-4) were integrated numerically.

Model calibration

The TK model was calibrated using the parameter space explorer algorithm developed by Tjalling Jager[12], which also generates parameter confidence intervals and stores a parameter sample. Resampling then facilitates creation of prediction intervals around simulated state-variables (e.g., internal concentrations) whilst accounting for parameter covariance. For each combination of species and test compound, all model parameters were jointly optimized by minimizing the minus log-likelihood. If the elimination rate

constant (k_{out}) was not well identified (i.e., if the confidence interval included parameter boundaries) it was fixed. To fix $k_{out,}$ the time at which 95% of steady state is reached ($t_{95\%}$) was used to calculate the parameter as follows:

$$k_{out} = -\frac{ln\ 0.05}{t_{95\%}} \quad (6)$$

We estimated $t_{95\%}$ directly from the data by assuming 95% of steady state was achieved at the first sampling time point (e.g., $t_{95\%}$ = 1 hour).

Bioaccumulation factor

The bioaccumulation factor (BAF) is the ratio of the concentration of the test compound within the organism in comparison to the concentration in the external source at steady state, specifically the concentration in *Spodoptera* larvae compared to the concentration in the treated soybean leaf disk or the concentration in *Chironomus* larvae compared to the concentration in water. The BAF can be calculated as the ratio between the uptake rate constant (k_{in}) and the sum of elimination rate constant (k_{out}) and all metabolite formation rate constants ($k_{m_{f,i}}$)

$$\mathrm{BAF} = \frac{k_{in}}{k_e + \sum k_{m_{f,i}}} \quad (7)$$

To calculate the confidence intervals of the BAF, whilst accounting for parameter covariation, the model was run at a constant concentration (set to 1) until steady state and the resulting confidence interval of the internal concentration equals the confidence interval of the BAF[13].

Results and Discussion

Standardised assays and TK modelling

Our study of the toxicokinetics and biotransformation of seven test compounds and Coumarin focussed on describing their differences in two insect species. We found that better TK descriptions are achieved in standardised assays and facilitate more detailed understanding of TK differences between target (*Spodoptera littoralis*) and non-target (*Chironomus riparius*) species using TK modelling (Figure 1, Table S1).

This comparative TK approach could fundamentally enhance research and development of more selective Plant Protection Products (PPPs). Specifically, toxicokinetic (TK) modelling has proven to be a valuable

tool to develop a deeper understanding of species differences in uptake kinetics, bioaccumulation, elimination, and the role of biotransformation. In this context the use of standardised assays, as implemented in this study, can significantly improve the reliability and reproducibility of data[14, 15]. When combined with toxicodynamic modelling, TK-TD modelling enables comprehensive assessments of non-target risks and informed environmental management decisions[7, 16-19]. A necessary further step of informed environmental management decisions is the comparison of the TK of compounds in a variety of target and non-target species, to figure out possible species selectivity, as for example resulting from differences in the uptake or elimination kinetics as well as their biotransformation potential (see also section below).

Toxicokinetics of test compounds

TK models could be successfully developed for all tested compounds in *S. littoralis* larvae (Figure 2). Such toxicokinetic bioassays were not successful with *C. riparius* for compounds C and G due to their effects (mortality) on that species (Figure 3). The elimination rate constants (k_{out}) of compound C, F and G for *S. littoralis* were not identifiable from the data, resulting in infinite confidence intervals, and were therefore fixed (Table 2). The *S. littoralis* model accurately captured both, the concentration dynamics at the end of the 24 h exposure, as well as during the elimination phase, but the concentrations observed at the initial sampling times (1 h, 5 h) were less well captured (Römer et al. submitted). The same was observed for *C. riparius* larvae exposed to compound D (Figure 3). For both species, internal concentrations were increasing during the exposure period, followed by decreases in the subsequent depuration phase (Figure 2, Figure 3). The only exception from this pattern was observed in *S. littoralis* larvae exposed to Coumarin (Figure 2), which was caused by an external decrease of concentration on the treated leaf disk (Figure S1).

TK analysis of *S. littoralis* larvae revealed clear differences between compounds in the concentrations of the parent compounds and their metabolites (Figure 2). Amount and concentration of the metabolites varied, indicating different biotransformation pathways and kinetics (Figure 2, Table 1, Table 2).

The size, growth, and body mass of an organism is a critical component in describing the uptake of a compound over time. The of growth (eq. 1) can be described by the term "growth dilution" in eq. 2, which means that the uptake of a compound would be diluted by the growth of the organism. In Römer et al. (submitted) it was shown that *S. littoralis* larvae can grow by a factor of 4 within the exposure period

(Figure S6). In contrast *C. riparius* hardly grew (Figure S7) . Application of growth dilution in the current model improved the model fit compared to the TK- model without considering growth dilution (Figure S6, Figure S8-9, Table S2, Römer et al.(submitted)), suggesting that accounting for growth dilution is a critical factor in accurate modelling of toxicokinetics in developing organisms.

The use of growth dilution reduced the variability of the uptake rate constants for compounds A-D and coumarin by more than half (comparison to Römer et al. submitted), which varied by 10 orders of magnitude without accounting for growth dilution. The rate constants of uptake (k_{in}) and elimination (k_{out}) varied over four orders of magnitude, demonstrating the benefit of including growth dilution in the model.

The uptake (k_{in}) and elimination (k_{out}) rate constants were generally higher in *S. littoralis* than in *C. riparius* with exception of compound D. The highest uptake rate constants were measured for compounds with a log P value between 2 and 2.5 (Table 2, Figure 4). This could suggest a more efficient uptake and distribution of the compounds within the target species, potentially indicating a bioavailability to PPPs desirable for pest control efficacy. In particular, the models for compounds A, B, C, F, and G revealed fast kinetics, indicating fast absorption into and processing within the organism. A flat, angular shape of the uptake curve indicates a rapid attainment of a steady state for compounds (Figure 2, Figure 3), however that pattern was imposed for compounds C, F and G by fixing the elimination rate constants.

In contrast to the fast kinetics of parent compounds some metabolites showed slow toxicokinetic patterns, resulting in more pronounced curvature of the model simulation (Figure 2, Figure 3). If a parent compound is biotransformed to a metabolite at a slow rate, this indicates slow biotransformation kinetics. This slow formation may be due to various factors such as low enzymatic activity, limited enzyme availability, or the inherent compound stability of the parent compound. As a result, the metabolite(s) appeared only gradually in the system. If the elimination of the metabolite is slow, perhaps due to inefficient elimination mechanisms and/or slow further biotransformation, this would lead to its concentration increasing slowly over time, even if the parent is already being eliminated..

Slow elimination of the metabolite can lead to its prolonged persistence in the system. In our studies with *S. littoralis*, we observed that “metabolite 3”, derived from parent compound E, exhibited slow elimination (Figure 2). This metabolite showed an increase in mass of 16 Da mass units, which is typically indicative of oxygen addition, such as hydroxylation. The slow kinetics observed for metabolite 3 suggest a prolonged residence time in the organism, suggesting that compound E is hydroxylated and

eliminated more slowly than other compounds tested. This may have implications for the bioaccumulation potential of compound E and its metabolites (Figure 2, Figure 5, Figure 6).

For the interpretation of the TK model in *C. riparius* larvae and comparison to *S. littoralis*, it is important to note that *C. riparius* bioassays were conducted with two fewer compounds than with *S. littoralis*. Compounds C and G were omitted as these experiments were impacted by toxicity due to the sensitivity of*C. riparius* larvae. The TK model of *C. riparius* provided insight into the toxicokinetic patterns of this species (Figure 3). No metabolites could be detected for compounds B and D, which may indicate either a lack of biotransformation or metabolite concentrations below the limit of detection (Figure 3, Table 1-2).

As in *S. littoralis*, differences in concentrations of parent compounds in the insect body were observed in *C. riparius*. The amount and concentration of metabolites varied which may indicate species-specific metabolic pathways or differences in uptake and distribution of the given compound. Coumarin was the only compound characterised by fast kinetics, while all others expressed slow kinetics patterns (Figure 3). The toxicokinetic parameters in *C. riparius* did show variability, with uptake rate constant (k_{in}) varying by three, and the elimination rate constant (k_{out}) by two orders of magnitude (Table 2).

Uptake (k_{in}) and elimination (k_{out}) were generally found to be higher in *S. littoralis* than in *C. riparius*, which was consistent for all compounds, except compound D (Figure 5).

Previous research has demonstrated the utility of TK models in predicting the dynamics of compound uptake and elimination in a wide range of organisms[20, 21]. Several studies have highlighted species-specific TK differences[9], e.g. in annelids[22, 23] or the insecticide chlorpyrifos in 15 freshwater arthropod species[24]. We observed that the uptake and elimination rate constants for the pest and non-pest species varied in different ranges. *S. littoralis* exhibited equal high variability in uptake and elimination rates (four orders of magnitude), whereas *C. riparius* displayed less and asymmetric variability, with uptake rates varying by three orders and elimination rates by only a factor of two (Table 2). *S. littoralis* may have a wider range of kinetic capabilities compared to *C. riparius*, which appears to have a more consistent and potentially lower overall elimination capacity.

Bioaccumulation of test compounds

Differences in TK between species are critical for bioaccumulation in organisms and potential biomagnification in food chains[25-28]. As *C. riparius* is an important food source for freshwater fish,

amphibians and various water birds and plays a central role in aquatic food webs[29, 30], any changes in its contaminant levels could have far-reaching consequences[25, 26, 31, 32]. Similarly, the pest species *S. littoralis* is an important food source for birds, small mammals, predatory insects and some reptiles, contributing to terrestrial food webs[33, 34].

The BAF was consistently higher in *C. riparius* (Figure 5c). However, the BAF in *C. riparius* does not appear to be the result of higher uptake per se, but rather of slower elimination kinetics (k_{out}). According to our results, the elimination seems to be the most important indicator of increased BAFs and species differences. The higher BAF in *C. riparius* may reflect a difference in compound elimination or biotransformation capacity between the species. Whether this can lead to biomagnification in aquatic food chains where *C. riparius* is prevalent depends also on diet-gut partitioning, compound uptake efficiency across the gut of predators, predator diet composition and feeding rates and food-web characteristics[35]. Further studies are needed to better understand the role of biotransformation in *C. riparius*, which has been shown to be important in other aquatic invertebrates (e.g., *Gammarus pulex*[36, 37].

Limitations of the applied toxicokinetic model in *S. littoralis* and *C. riparius*

The use of toxicokinetic (TK) models to predict the behaviour of compounds in biological systems is a powerful tool in environmental toxicology. However, the current model applied to two different species, *Spodoptera littoralis* and *Chironomus riparius*, has some limitations that must be considered when interpreting the results:

First of all, the two species have inherently different uptake routes due to their biology (behavioural ecology). *S. littoralis*, as a chewing-biting terrestrial insect, will encounter the compounds by both contact (tarsea or prolegs) and oral ingestion as it is crawling and feeds on preventatively applied leaf disks. In contrast, *C. riparius*, as an aquatic insect, is mainly exposed by passive uptake (contact). These differences in uptake routes may influence the distribution as well as the biotransformation of the compounds within each of the species and, as an inevitable consequence, the results of the TK model.

Secondly the model represents the total amount of compounds included in the body as an "insect homogenate", which also includes any compound adsorbed on the outer insect body. This approach may not accurately reflect true internal concentrations that are biologically relevant in toxicodynamics studies. Also, the model may not adequately cover early time points for parent compounds with fast kinetics leading to an underestimation of initial uptake. To solve this problem, and to accurately capture the

kinetics during the critical early uptake period, we recommend additional sampling times early during exposure and with higher frequency than later in the experiment. This sampling frequency should also be copied at the start of the depuration phase.

And finally, we have assumed, that mass changes infer the presence of metabolites without direct identification of such. This can lead to inaccuracies in the model if the assumed mass changes do not correspond to the actual metabolites formed in the organisms. This also includes the possibility that some metabolites may not be detected if their concentrations do not reach the limit of quantification. This is particularly relevant for *C. riparius*, which has a lower body mass than *S. littoralis* larvae (Ø17.15 mg). Whilst the concentration of metabolites (expressed in mg/mg wet weight) could theoretically be the same for both species, the absolute mass of compounds accumulated by *C. riparius* may be below the detection threshold required for analysis due to its smaller total biomass (Ø2.4 mg). In other words, despite potentially identical concentrations, the lower total amount of accumulated compounds in *C. riparius* compared to *S. littoralis* could pose a challenge for metabolite detection at the end of the exposure period (Table S4). In our study, we found that the limit of quantification of metabolites in *S. littoralis* is approximately one order of magnitude lower. Therefore, we have a higher probability of detecting metabolites in *S. littoralis* than in *C. riparius* (Table S4).

In summary, using a simple first-order, one-compartment TK model, we encountered limitations in fully explaining the patterns observed in the body tissue concentration data. Furthermore, the model does not distinguish between exclusive intestinal passage and systemic absorption. Despite these limitations, the TK model successfully captured the basic toxicokinetic patterns for both species for different test compounds within the assay conditions and provided information on slow and fast kinetic.

Biotransformation of test compounds

Biotransformation is an important physiological process, involving the enzymatic conversion of compounds into more hydrophilic, readily eliminable compounds. This process plays a key role in preventing bioaccumulation, facilitating detoxification, and ultimately influences the toxicity of compounds[37, 38]. Understanding the factors influencing biotransformation, such as insect life stage, sex, diet, and environmental conditions, is essential to predict the fate and effects of pesticides[39-42]. In addition, biotransformation is closely linked to the development of insecticide resistance, a growing concern in pest management strategies. Through in-depth analysis of biotransformation processes, researchers may be

able to identify critical specific metabolic patterns, including the appearance, quantity, and temporal dynamics of metabolites. This knowledge is crucial in monitoring species-specific metabolic responses to compounds, such as putative insecticides, and to detecting interspecies differences in biotransformation capabilities[9]. In a study investigating the biotransformation of contaminants in various freshwater invertebrates, including *C. riparius*, it was found that a lower proportion of compounds were recovered as parent compound in *C. riparius*, suggesting that different metabolic processes may occur in this species compared to *Lumbriculus variegatus*[43]. Indeed, this is consistent with our findings of differences in biotransformation capacity.

Differences in metabolism were detected between the species *S. littoralis* and *C. riparius*, each of it exhibiting unique biotransformation profiles detected and characterized by changes in the mass of the compounds tested (Table 2). Three prominent mass shifts were identified: a decrease of 14 Da and increases of 16 and 18 Da. These shifts are indicating specific biotransformation reactions occurring in the organisms: a demethylation reaction by the decrease in 14 Da, resulting in a more hydrophilic metabolite easier to eliminate, and oxidative transformations such as hydroxylation, indicated by the increases in 16 and 18 Da[44, 45] .

The biotransformation profiles showed that metabolites are present at different times and in different concentrations (Figure 2-3, Figure 5, Table 1-2). No metabolites were detected for compound B in either species, indicating no biotransformation or metabolites below the detection limit. Only three compounds produced the same four putative mass changes in both species across the range of compounds tested (Table 1-2, Figure 5). Specifically, two metabolites for compound F, one metabolite for compound E and Coumarin. *S. littoralis* showed a wide range of metabolites for compounds C, D, E and G, suggesting a more complex enzymatic system capable for detoxification. In contrast, *C. riparius* showed a more limited biotransformation capacity, indicating its comparatively lower ability to effectively detoxify and eliminate compounds. Nevertheless, the limited biotransformation capacity may have been caused by our limited quantification capabilities (lower detection limit in *S. littoralis* than in *C. riparius*) rather than by the species (Table S4). *S. littoralis* consistently produced two metabolites for all compounds, whereas *C. riparius* produced only two metabolites for compound F on one occasion.
In *S. littoralis* larvae, the metabolite formation rate constants (k_{m_f}) for compound E (+ 16 Da) and F (mass change of -14 and +16 Da) were faster compared to *C. riparius* (Figure 6 a). The formation rate constants of Coumarin metabolites were faster in *C. riparius* (Figure 6 a). The elimination rate constants

(k_{m_e}) of biotransformation products with a mass change of -14 (compound E and F) were faster in *S. littoralis*, suggesting a species-specific efficiency in these elimination processes (Figure 6 b). While the elimination rate constants of biotransformation products with a mass change of + 16 or + 18 (compound F, Coumarin) were faster in *C. riparius* (Figure 6 b). This suggests more efficient elimination mechanisms or greater detoxification capacity in *C. riparius* for these specific compounds.

An earlier study investigating the activity of detoxification enzymes in *Spodoptera frugiperda*, a species closely related to *S. littoralis*, found that various detoxification activities, including microsomal oxidases (such as hydroxylase, N-demethylase or O-demethylase) and hydrolases, were higher in an insecticide-resistant batch than in a susceptible strain[46]. This suggests a complex interaction with the compounds and potential biotransformation resulting in the detoxification of the compounds. Similarly, Novoselov et al.[47] and others have noted that *S. littoralis* can detoxify certain insecticides with neuronal mode of action through amino acid conjugation, suggesting an adaptive detoxification mechanism [48]. Thus, the biotransformation capabilities of this species, coupled with its feeding mechanism, digestive physiology, and the biochemical composition of its diet, may facilitate faster elimination and enable adaptation to plant toxins and insecticide resistance[49]. This results in challenges for the pest management of this species. In conclusion, it seems essential to better understand the metabolic pattern of *S. littoralis*, in order to potentially gain insights into the biotransformation capabilities associated with resistance. This could be done by running a comparative study on a resistant and a susceptible strain to compare biotransformation.

Insights into biotransformation can inform the design and use of pesticides to minimize environmental impact. By identifying compounds with low bioaccumulation potential in non-target organisms, it is possible to develop more environmentally benign pest control solutions. The observed differences in biotransformation capabilities of *S. littoralis* and *C. riparius* are good examples, as they could have a direct impact on the persistence of compounds in different ecological contexts. Different biotransformation rate constants for the same metabolite in the two species may indicate which of it has a higher biotransformation capacity. Biotransformation pathways more pronounced in non-target species may lead to either detoxification or unintended increased toxicity. Thus, it is important to further investigate potential detoxification mechanisms, not only in *C. riparius*, but also more generally across the diversity of non-target species.

Conclusion

Our study provides the blueprint for a species selective approach to the development of plant protection products (PPPs), that are both effective in pest control and have minimal ecological impact, through better understanding of TK. The complex interaction of uptake, biotransformation and elimination observed in different species lead to substantial species differences. By determining species-specific quantitative and qualitative differences in toxicokinetics and biotransformation through a combination of standardised assays and TK modelling, we can explain the cause of the differences in bioaccumulation.

Spodoptera littoralis larvae showed a higher uptake and elimination potential, which is beneficial for bioavailability in pest control. Conversely, *Chironomus riparius* had a lower elimination capacity, suggesting a greater propensity for bioaccumulation. This species also showed comparatively more limited biotransformation capacity, which can be of concern when non-target organisms are exposed to PPPs, whereas *S. littoralis* exhibited a wider range of biotransformation pathways. This difference provides an opportunity for compound optimisation, which could focus on improving the elimination rate in *C. riparius* or designing pro-pesticides that are only activated through the biotransformation pathways in *S. littoralis*. Although a much better understanding of biotransformation would be required than what we achieved here, such optimisation could include selecting compounds that are effective at lower concentrations, ensuring activation only with increased uptake in target pests, or significantly enhancing elimination in non-target species.

Thus, our results demonstrate that TK modelling approaches, which are well-established tools in environmental toxicology and non-target species risk assessment, can be effectively adapted to compare target and non-target organisms. This enables novel insights into species differences related to TK, which can be leveraged to design-in selectivity.

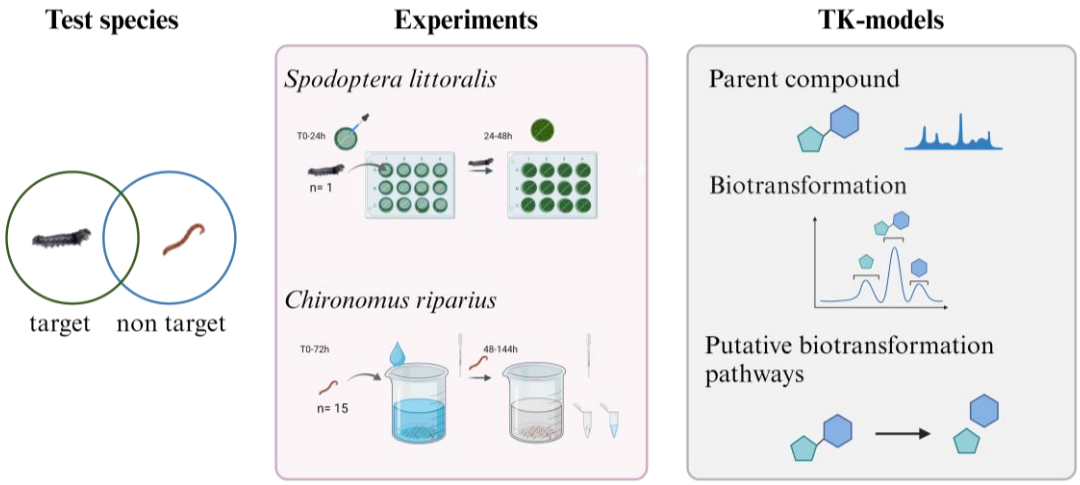


Figure 1: Toxicokinetic bioassay design: Individual *Spodoptera littoralis* larvae were exposed to compound treated leaf disks. Feeding contact assay with a 24h exposure period followed by a 24h depuration period. *Chironomus riparius* larvae (n=3 pooled) individuals were exposed to compound treated water with 10 mg food: 24h exposure period followed by a 48h depuration period. (Created using BioRender.com)

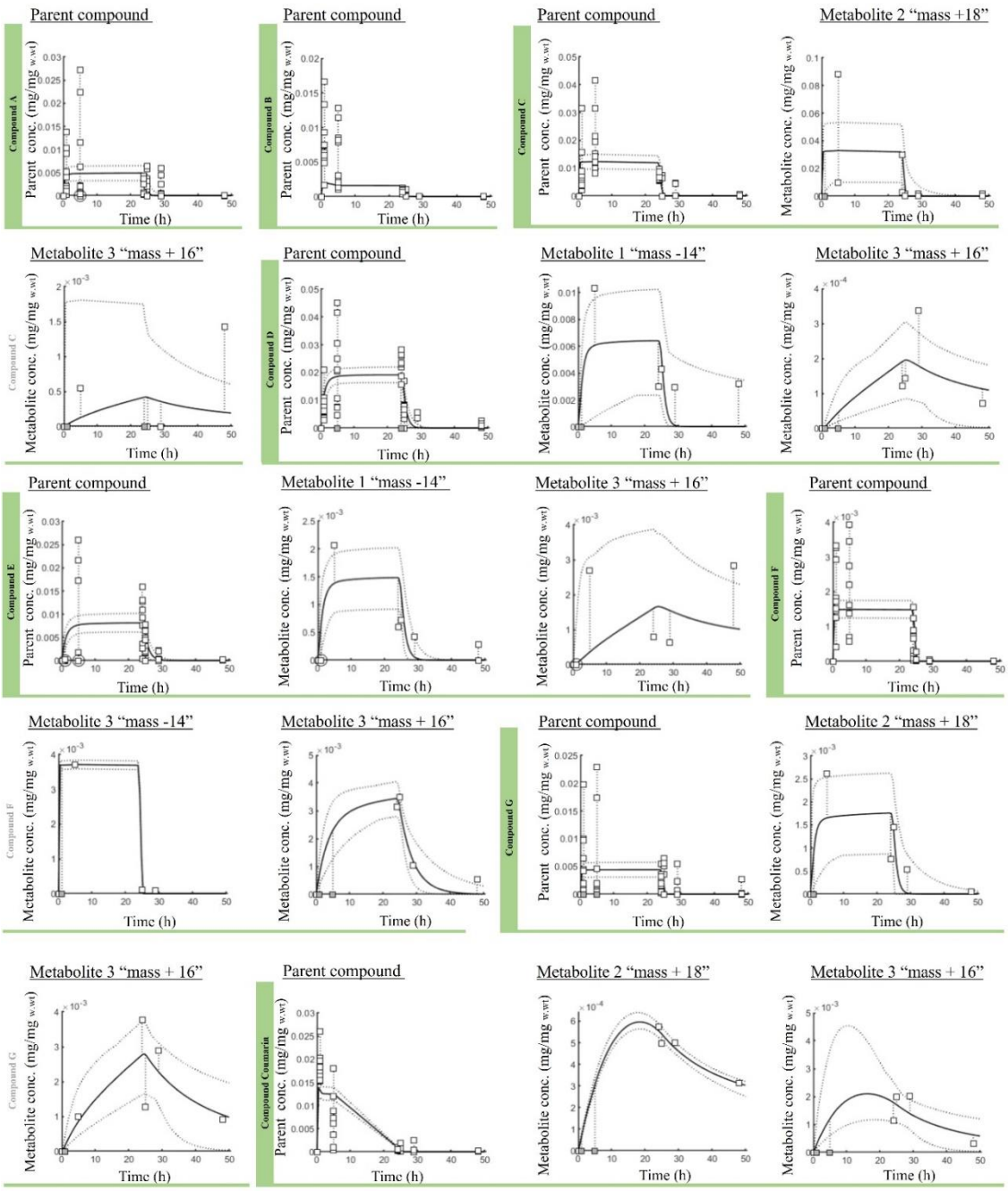


Figure 2: Body concentrations (mg/mg$_{w.wt.}$) in *Spodoptera littoralis* larvae (measured data: squares). Exposed larvae fed on treated leaf disks for 24 hours, followed by transfer to untreated leaf disks for a depuration period of 24 hours. TK-Model (best fit model: solid line, 95 % confidence limits: dotted lines) for *Spodoptera littoralis:* parent compound uptake and elimination, as well as formation and elimination of metabolites. See Table 2 for model parameter values. TK models of parent compound concentrations (A-D) *S. littoralis* were already published in Römer et al., (submitted).

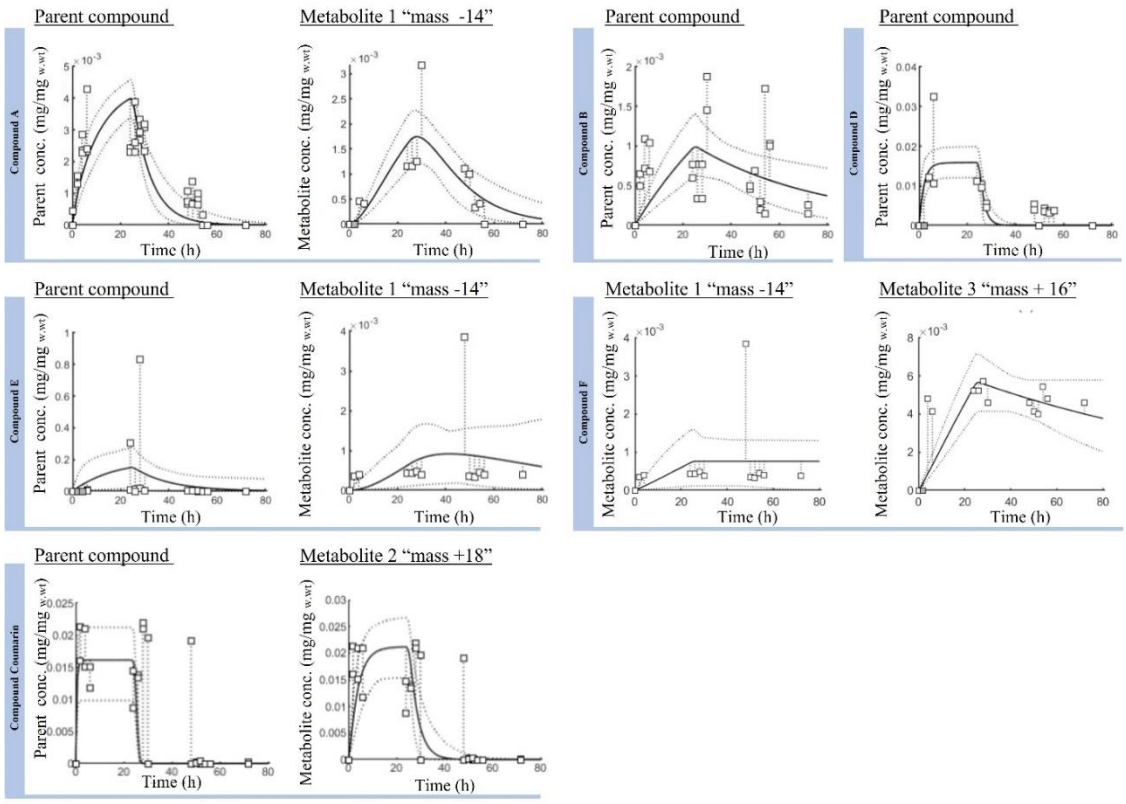


Figure 3: Body concentrations ($mg/mg_{w.wt.}$) in *Chironomus riparius* larvae (measured data: squares). Larvae were exposed in water for 24 hours, followed by transfer to clean water for a depuration period of 48 hours. TK-Model (best fit model: solid line, 95 % confidence limits: dotted lines) for *Chironomus riparius:* parent compound uptake and elimination, as well as formation and elimination of metabolites. See Table 2 for model parameter values.

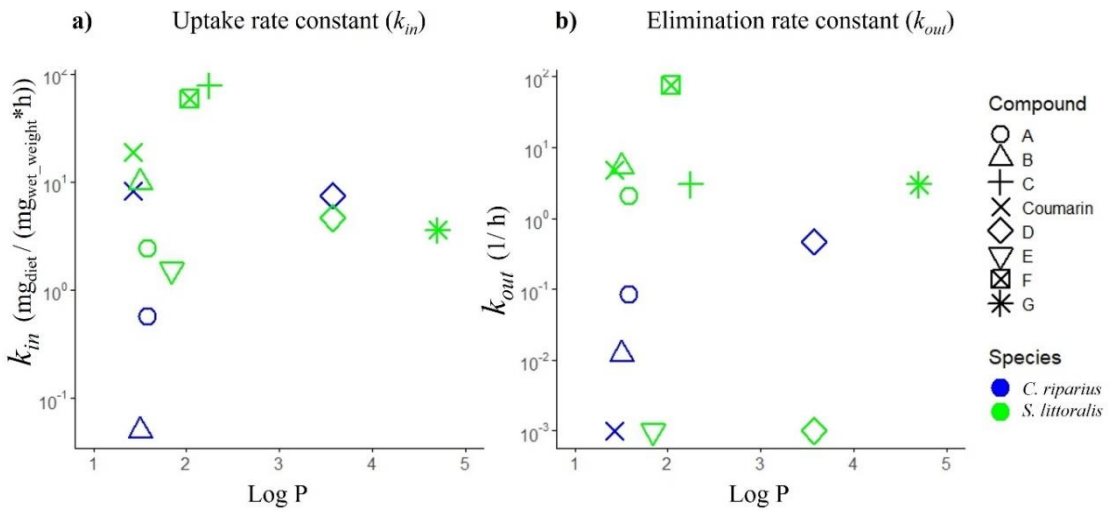


Figure 4: Toxicokinetic model parameters of parent compounds for *Spodoptera littoralis* and *Chironomus riparius* in relation to log P. (a) Uptake and (b) elimination rate constants of parent compounds. Note: compounds C, F and G were only detected and modeled in *S. littoralis*.

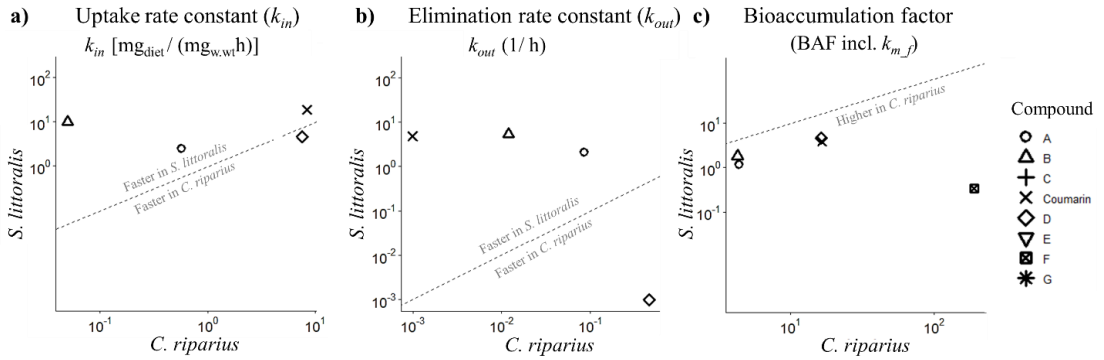


Figure 5: Toxicokinetic model parameters of parent compounds for *Spodoptera littoralis* and *Chironomus riparius* in correlation. (a) Uptake and (b) elimination rate constants of parent compounds, and (c) bioaccumulation factor. Note: compounds C, F and G were only detected and modeled in *S. littoralis*. Dashed 1:1 line.

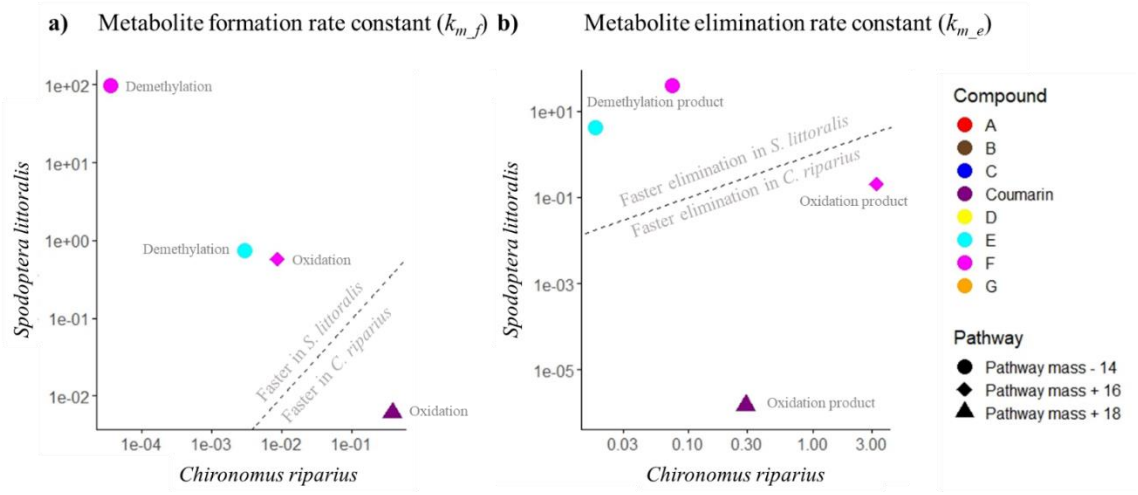


Figure 6: Biotransformation parameters of metabolites quantified in both species (Table 1, Table 2). (a) Metabolite formation rate constants and (b) metabolite elimination rate constants in *Spodoptera littoralis* and *Chironomus riparius*. Metabolites referred to by mass changes of decrease of 14 Da (demethylation) and increase of 16 or 18 Da (oxidation). Dashed 1:1 line.

Table 1: First time of detection of putative metabolites in samples of *Spodoptera littoralis* and *Chironomus riparius* larvae. Putative metabolites of compounds (A-G), and Coumarin detected by mass difference (in Dalton) to given parent compound. Metbaolite detecteion of S. *littoralis* were already published in Römer et al (paper 2).

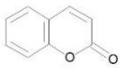
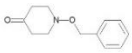
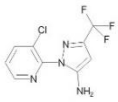
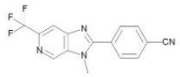
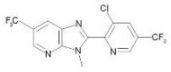
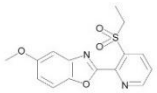
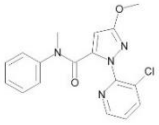
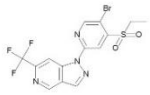
Parent compound		Putative metabolites					
		Spodoptera littoralis			*Chironomus riparius*		
Chemical structure	Compound	Mass -14	Mass +18	Mass +16	Mass -14	Mass +18	Mass +16
	Coumarin	-	24 h	24 h	-	2 h	-
	A	-	-	-	24	-	-
	B	-	-	-	-	-	-
	C	-	5 h	5 h	not observed		
	D	24 h	-	5 h	-	-	-
	E	5 h	-	5 h	6 h	-	-
	F	5 h	-	24 h	2 h	-	4 h
	G	-	5 h	5 h	not observed		

Table 2: Toxicokinetic model parameters (uptake and elimination rate constants, metabolite formation and elimination rate constants, and bioaccumulation factors BAF) for *Spodoptera littoralis* and *Chironomus riparius* larvae of tested compounds (A-G, Coumarin). **(**ND= not detected)

Species	Compound	k_{in} (mg_{diet} / ($mg_{w.wt}$h))	k_{out} (1/h)	$k_{m1\_f,i}$ (1/h)	$k_{m1\_e,i}$ (1/h)	$k_{m2\_f,i}$ (1/h)	$k_{m2\_e,i}$ (1/h)	BAF (mg_{diet} / ($mg_{w.wt}$)
S. littoralis	A	2.445 [0.7824;100.0]	2.117 [0.6774;100]	ND	ND	ND	ND	1.15 [0.71;1.43]
	B	9.869 [3.697;100.0]	5.417 [2.027;65.96]	ND	ND	ND	ND	1.82 [0.90;1.40]
	C	68.690 [3.337;100.0]	0.038 [0.001;38.19]	24.20 [0.3317;42.94]	9.031 [0.201;34.88]	0.002 [1.000e;06;15.85]	1.31E-06 [1.000e;06;100.0]	2.833 [2.30;3.58]
	C fixed	78.580 [9.594;100.0]	2.996	24.80 [0.3906;39.74]	9.195 [0.2412;32.12]	0.003 [1.000e;06;14.53]	1.56E-06 [1.000e;06;100.0]	2.816 [2.30;3.58]
	D	4.636 [2.692;8.254]	0.001 [0.001;1.507]	0.986 [0.01223;1.812]	2.913 [1.000e;06;8.457]	0.001 [0.0003;0.003]	0.004 [1.000e;06;0.21]	4.64 [1.37;2.30]
	E	1.531 [0.8016;3.198]	0.001 [0.001;1.094]	0.746 [0.0794;1.676]	4.041 [0.434;9.005]	0.015 [1.000e;06;1.07]	1.00E-06 [1.000e;06;100]	2.00 [1.36 ;2.28]
	F	58.850 [18.32;80.22]	75.870 [0.001;100]	96.00 [42.38;100]	38.350 [17.28;47.09]	0.586 [0.248;1.211]	0.203 [0.064;0.424]	0.341 [0.28;0.40]
	F fixed	35.45 [18.96;41.58]	2.996	99.84 [51.62;100]	40.07 [20.78;47.18]	0.575 [10.248;1.209]	0.200 [0.064;0.422]	0.342 [0.28;0.40]
	G	2.478 [0.8194;100]	1.651 [0.001;100]	0.624 [0.051;7.466]	1.567 [0.118;19.02]	0.052 [0.025;0.154]	0.019 [1.000e;06;0.186]	1.06 [0.64;1.30]
	G fixed	3.608 [2.452;9.217]	2.996	0.399 [0.047;6.044]	0.994 [0.104;14.90]	0.053 [0.025 ;0.153]	0.019 [1.000e;06;0.176]	1.04 [0.64;1.30]
	Coumarin	18.890 [10.20;100]	4.835 [2.577;26.80]	0.006 [0.005;0.007]	0.000 [1.000e;06; 0.008]	0.026 [0.012 ;0.091]	0.020 [1.000e;06;0.134]	3.90 [3.64; 4.69]
C. riparius	A	0.569 [0.341;0.886]	0.086 [0.001;0.17]	0.045 [0.023;0.100]	0.069 [0.031;0.184]	ND	ND	4.34 [3.694; 5.65]
	B	0.050 [0.0282;0.088]	0.012 [0.002;0.04]	ND	ND	ND	ND	4.25 [2.030 >500]
	D	7.542 [4.222;21.12]	0.462 [0.2686;1.331]	ND	ND	ND	ND	16.32 [10.86 ;19.75]
	E	12.610 [1.219;73.08]	0.066 [0.001;0.451]	0.0003 [4.829e;05;0.051]	0.018 [1.000e;06;9.722]	ND	ND	190.51 [41.76; >500]
	F	ND	ND	0.058 [0.011;0.208]	0.000001 [1.000e;06;0.077]	0.480 [0.3070;0.7599]	0.008 [1.000e;06;0.022]	ND
	Coumarin	8.325 [4.392 ; 17.00]	0.001 [0.001;0.557]	0.409 [0.171;0.789]	0.419 [0.194;0.749]	ND	ND	16.65 [14.61;26.85]

Acknowledgement

We would like to thank our Syngenta colleagues helping us to carry out this study, for interpreting the data and for insightful discussions. Special thanks go to Amund Løvik for technical assistance during the modelling. All contributions were essential to the success of this research project.

Supporting information

Compound properties

Table S1: Compound properties (log P, molecular weight, water solubility) of tested compounds.

Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Coumarin
Log P							
1.58	1.50	2.24	3.57	1.84	2.03	4.70	1.43
Molecular mass (g/mol)							
205.25	262.62	302.25	380.68	318.35	342.78	435.22	146.14
Water solibility (mg/L)							
1413	589	10	6.46	120	166	0.26	1050

Exposure scenarios

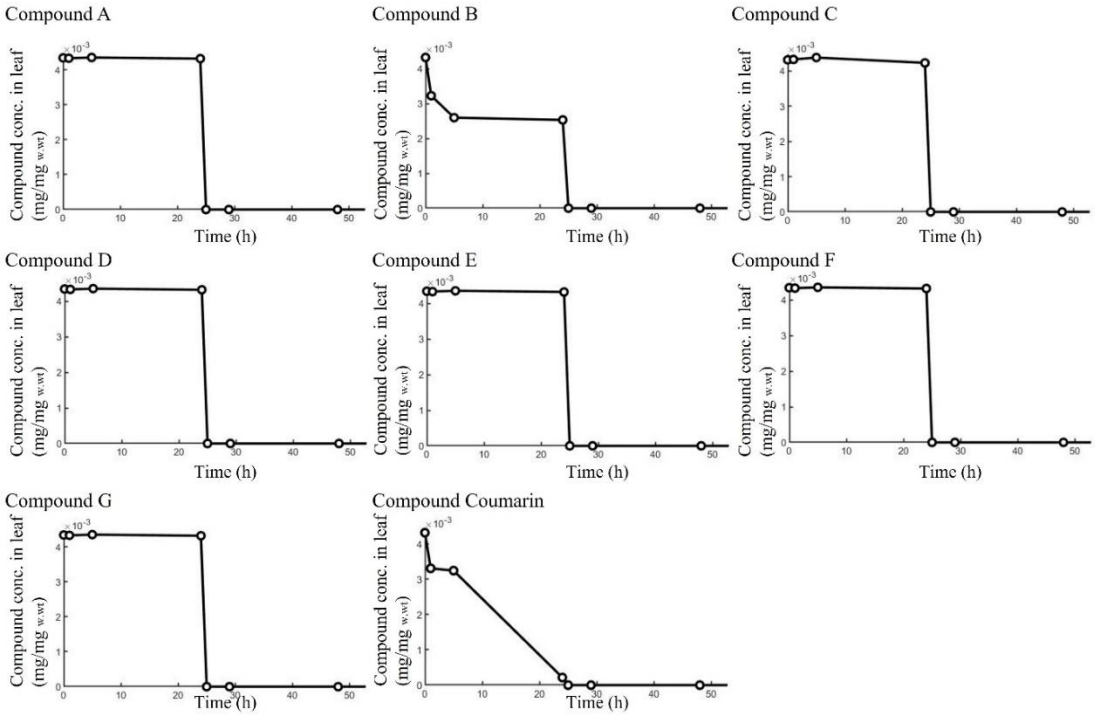


Figure S1: Exposure scenarios in the *Spodoptera littoralis* toxicokinetic study: soybean leaf disk concentrations during exposure (0- 24 h) and depuration time (24- 48 h) (mg/mg wet weight). Compound quantities (A-D) in *S. littoralis* were already investigated and published in Römer et al., (submitted).

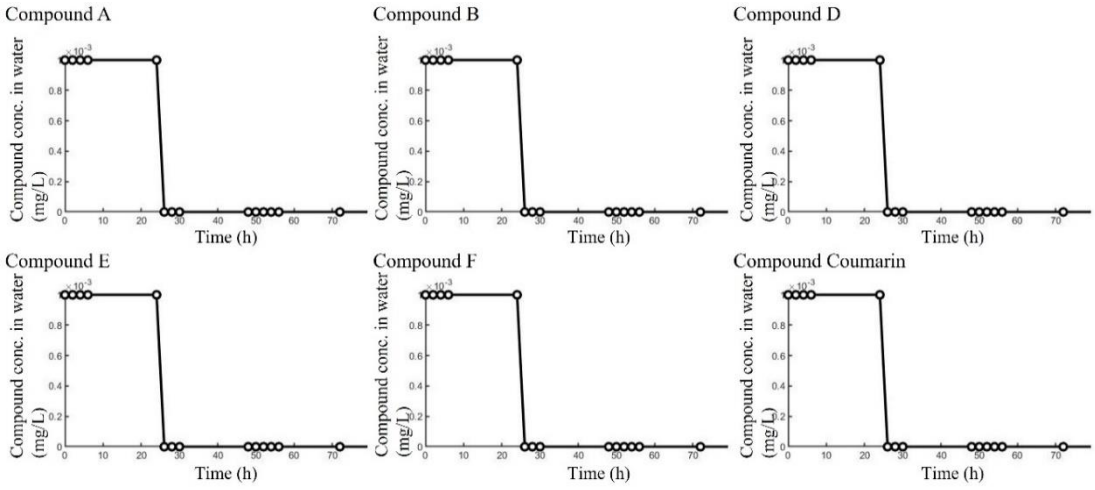


Figure S2: Exposure scenarios in the *Chironomus riparius* toxicokinetic study: water concentrations during exposure (0- 24 h) and depuration time (24- 72 h) (mg/L).

TK model likelihood profile

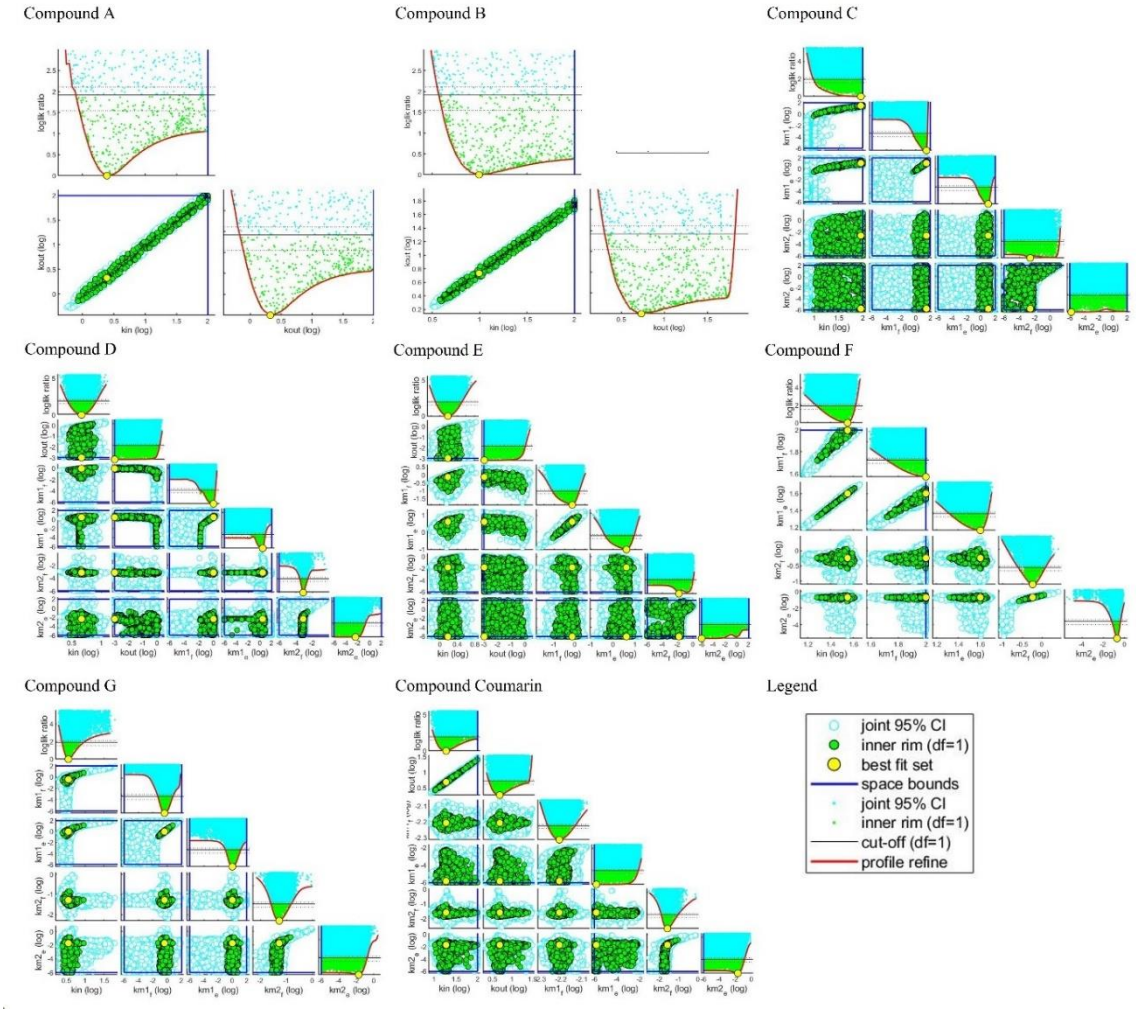


Figure S3: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* toxicokinetic assay for the five test compounds (A-G, Coumarin). Likelihood profile for uptake (k_{in}), elimination (k_{out}), metabolite formation (k_{m_f}), and metabolite elimination (k_{m_e}) rate constants during a 24 h exposure period and a 24 h depuration period.

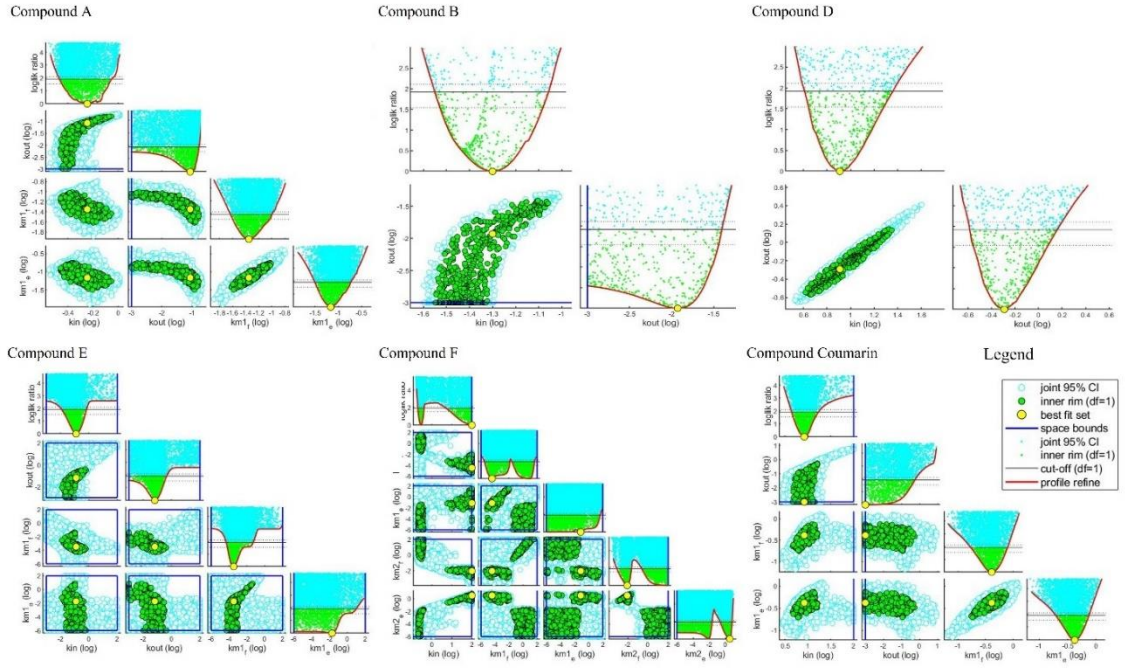


Figure S4: TK model likelihood profile for predicted parameters for the *Chironomus riparius* toxicokinetic assay for the five test compounds (A-F, Coumarin). Likelihood profile for uptake (k_{in}), elimination (k_{out}), metabolite formation (k_{m_f}), and metabolite elimination (k_{m_e}) rate constants during a 24 h exposure period and a 48 h depuration period.

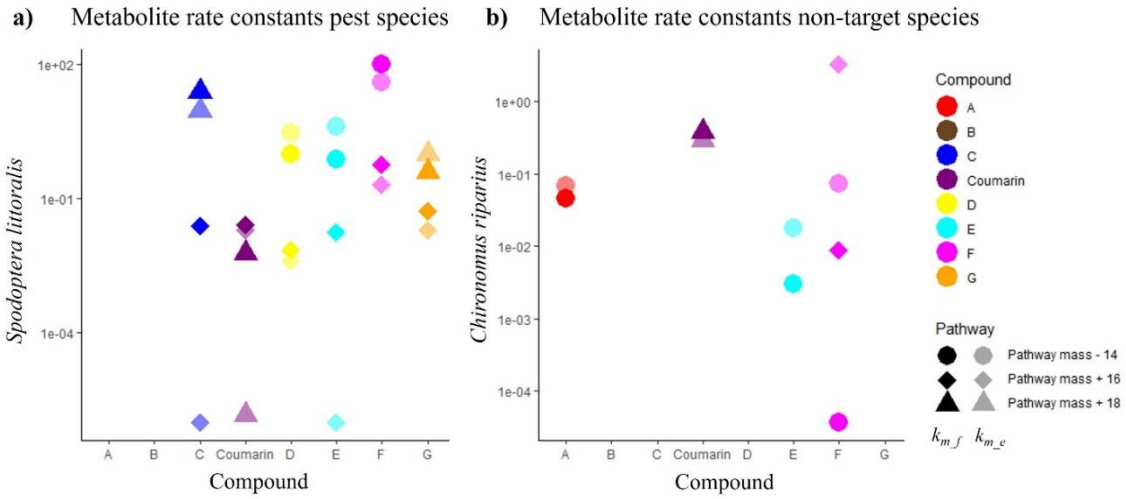


Figure S5: Biotransformation of all metabolites per species (Table 1, Table 2) in (a) *Spodoptera littoralis* and (b) *Chironomus riparius*. Metabolite fomation rate constants (k_{m_f}) shown as filled symbols, elimination rate constants (k_{m_e}) as empty symbols. Metabolites referred to by mass changes of decrease of 14 Da (demethylation) and increase of 16 or 18 (oxidation).

TK model parameters wet weight

Table S2: Toxicokinetic model parameters: Wet Weight growth rate (b, (1/h)) and Maximum Organism Size ($W_{m,}$ in $mg_{wet\ weight}$) for *Chironomus riparius* and *Spodoptera littoralis* larvae.

Species	Compound	b	[conficence interval]	Wm	[conficence interval]
Spodoptera littoralis	A	0.096	[0.067;0.130]	41.430	[32.41;60.03]
	B	0.094	[0.065;0.127]	43.410	[33.57;64.27]
	C	0.038	[0.020;0.059]	151.400	[79.37;500]
	C fixed	0.038	[0.020;0.060]	151.400	[79.37;500]
	D	0.094	[0.065;0.127]	43.410	[33.57;64.27]
	E	0.086	[0.057;0.120]	46.970	[35.12;75.04]
	F	0.037	[0.020;0.056]	159.600	[84.99;500]
	F fixed	0.037	[0.018;0.056]	159.600	[84.99;611.7]
	G	0.078	[0.051;0.108]	52.280	[38.42;86.99]
	G fixed	0.078	[0.051;0.108]	52.280	[38.42;86.99]
	Coumarin	0.081	[0.059;0.108]	54.440	[42.43;78.33]
Chironomus riparius	A	0.508	[0.055;1.000]	2.760	[2.577;4.367]
	B	0.001	[0.001;0.097]	500.000	[3.585;500]
	D	0.078	[0.001;0.227]	3.277	[3.048;500]
	E	1.000	[0.001;1.000]	2.484	[2.734;500]
	F	1.000	[0.001;1.000]	2.484	[2.734;500]
	Coumarin	0.000	[1.000e;06;500]	500.000	[1.000e;06;500]

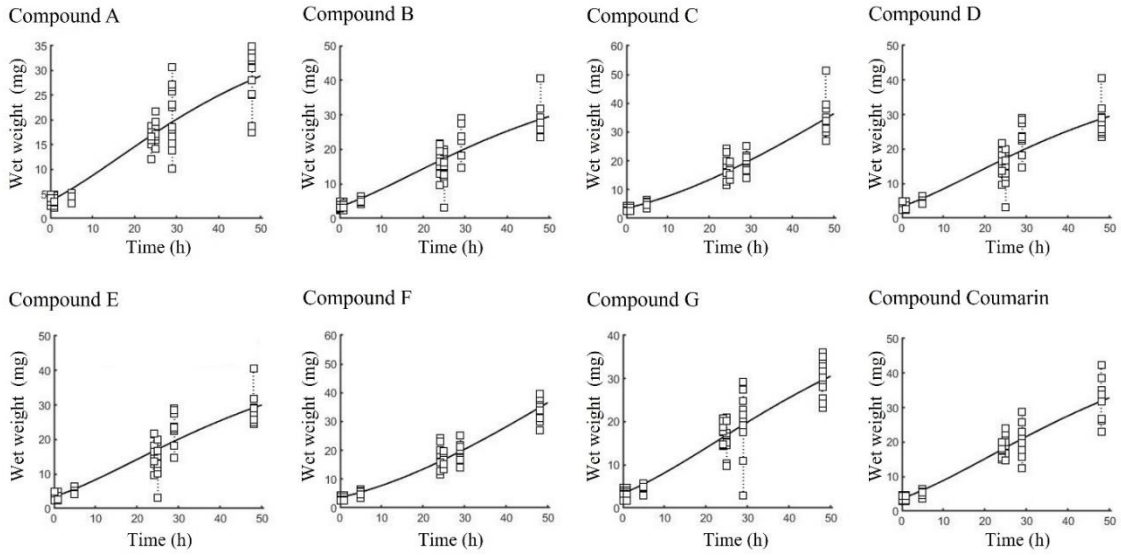


Figure S6: Wet weight (mg) of *Spodoptera littoralis* individuals during the toxicokinetic study.

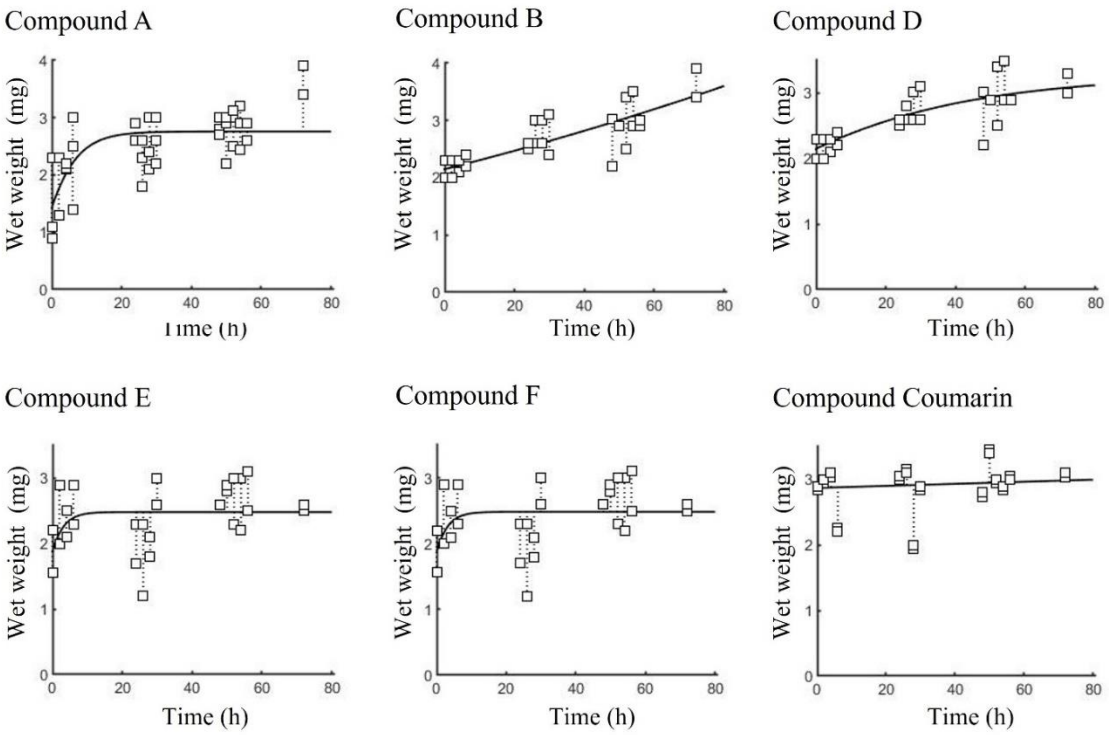


Figure S7: Wet weight (mg) of *Chironomus riparius* individuals during the toxicokinetic study.

TK model parameters wet weight: likelihood profile

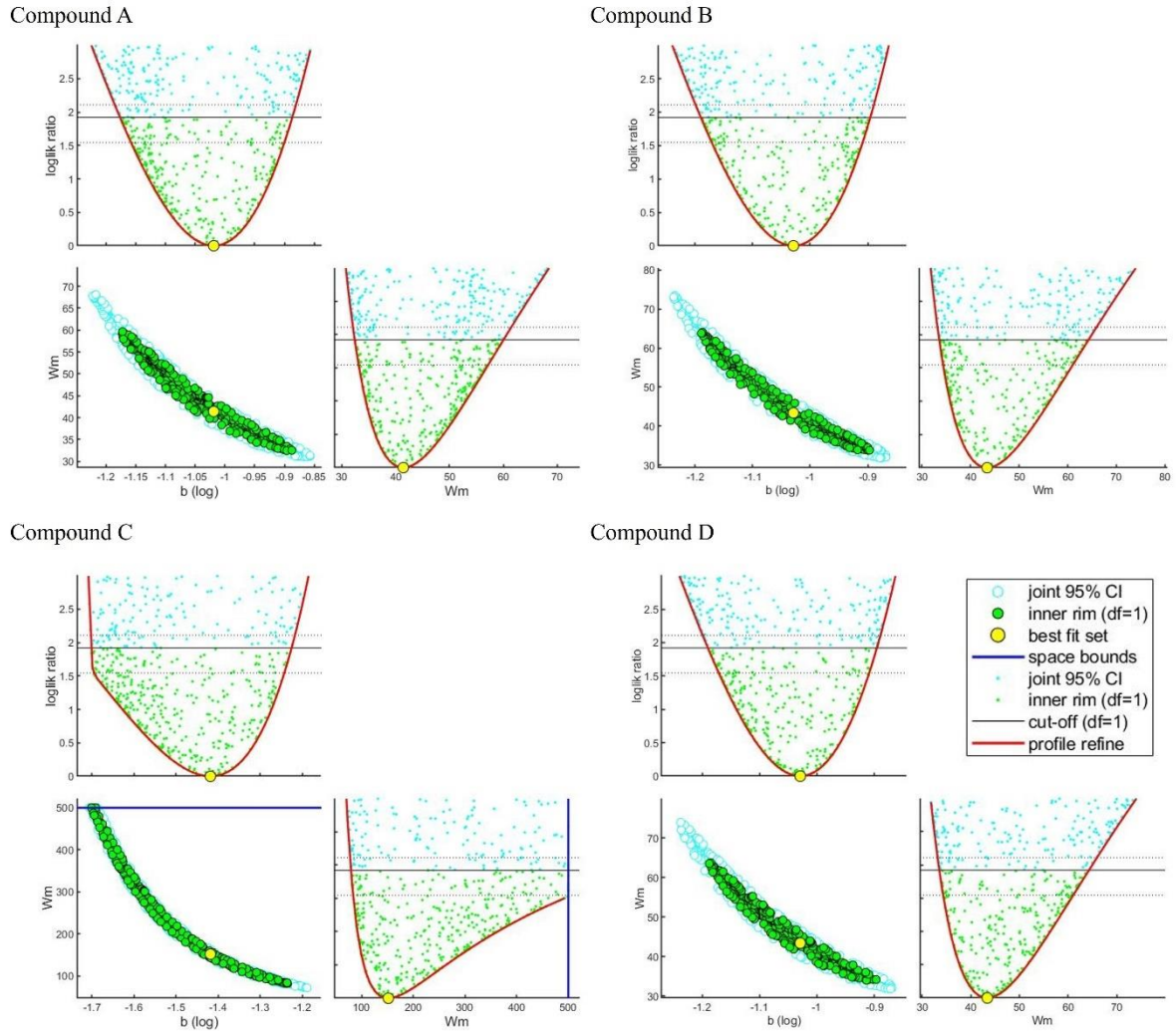


Figure S8: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (A-D). Likelihood profile Wet Weight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 48 h).

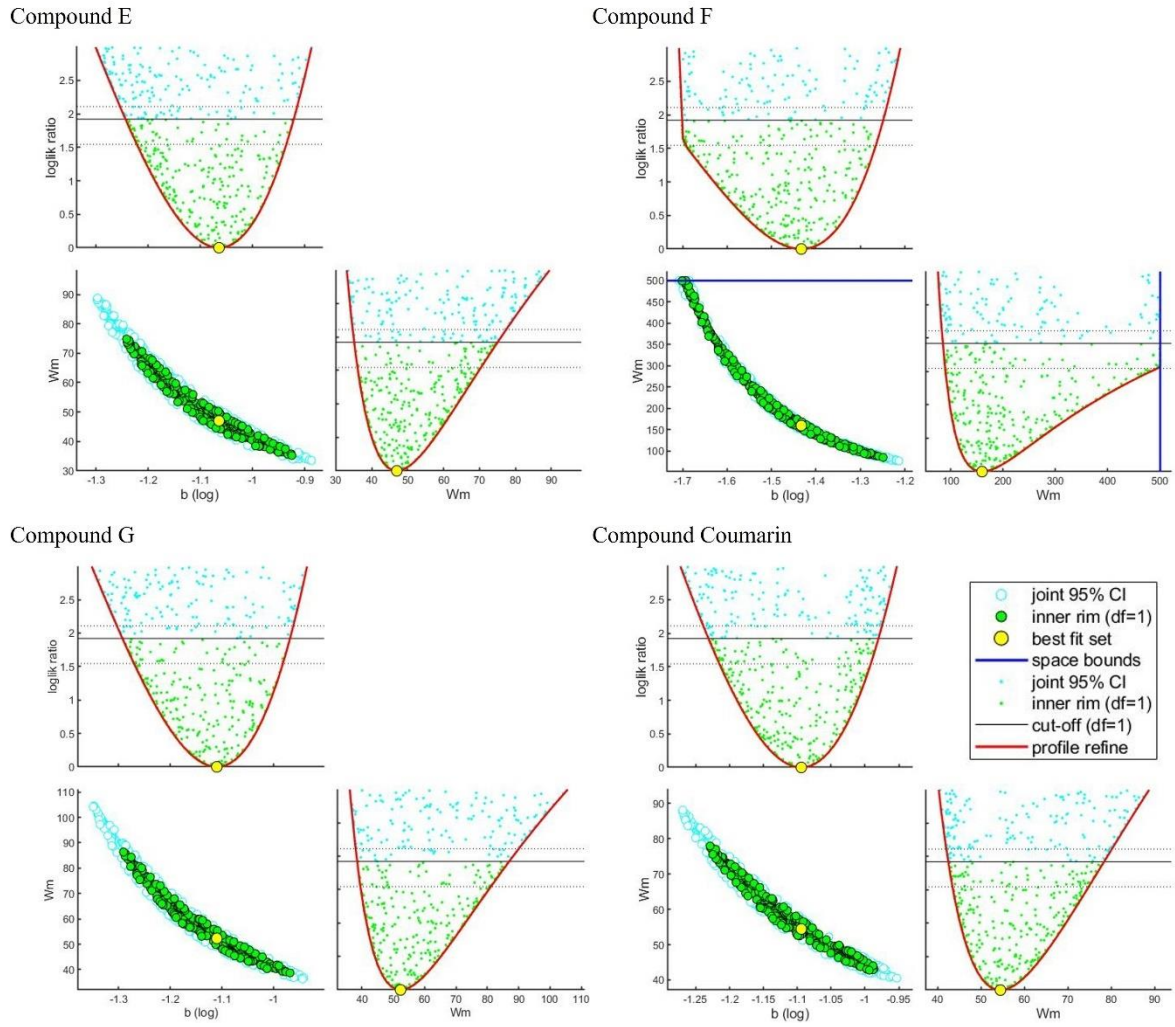


Figure S9: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds (E-G, Coumarin). Likelihood profile Wet Weight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 48 h).

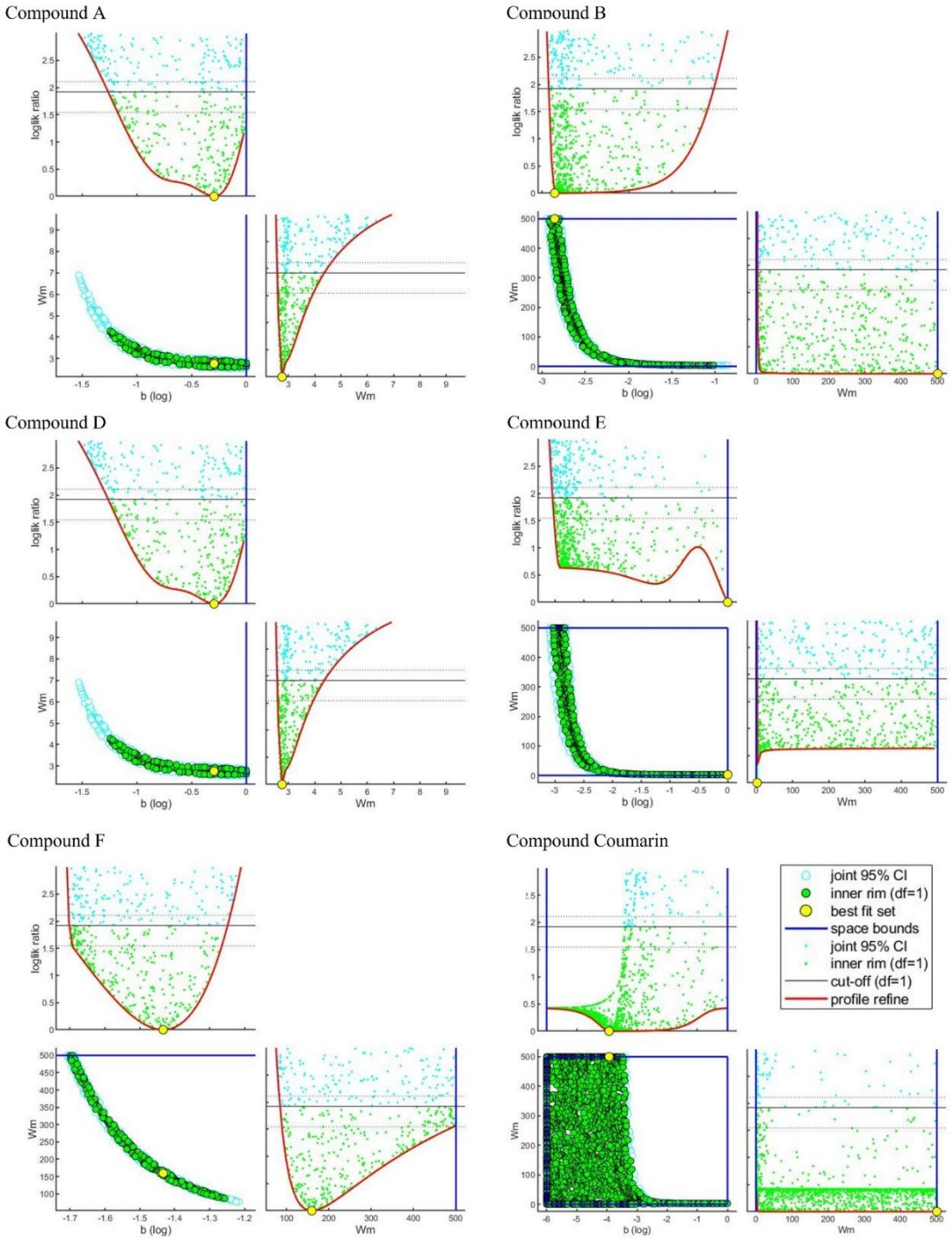


Figure S10: TK model likelihood profile for predicted parameters for the *Chironomus riparius* individuals during the toxicokinetic study for the test compounds (A-D). Likelihood profile Wet eight growth rate (b) and Maximum Organism Size (W_m) during exposure (0- 24 h) and depuration time (24- 72 h).

Table S3: Toxicokinetic model parameter symbols, names, and dimensions/ units.

Parameter symbol	Parameter Name	Dimensions/units
C_i	Internal parent concentration	Mass of parent / body mass
C_e	External parent concentration	Mass of parent / mass leaf / volume wat
W_w	Wet weight	Body mass
M_i	Metabolite concentration	Mass of metabolites / body mass
b	Growth rate	Time-1
k_{in}	Uptake rate	mass leaf/ body mass /time
k_{out}	Elimination rate	Time-1
k_{mf}	Metabolite formation rate	time-1
k_{me}	Metabolite elimination rate	time-1

Table S4: Comparative toxicokinetic parameters for *Spodoptera littoralis* and *Chironomus riparius.* This table presents the main toxicokinetic parameters: number of individuals per sample, body mass, internal concentration after exposure, external concentration, recovery percentage and limit of quantification adjusted for relative recovery.

Species	Test compounds	Number of individuals per sample	Ø Body mass per larva (mg)	Ø Internal concentration (mg /mg wet weight)*	Ø External concentration (mg/mg or mg/mL)	Ø Recovery (%)**	LoQ (mg /mg wet weight)**
C. riparius	A	2	2.630	0.002	0.001	87	0.00017
	B	2	2.550	0.001	0.001	87	0.00017
	C	ND	ND	ND	ND	ND	ND
	D	2	2.550	0.011	0.001	87	0.00017
	E	2	2.000	0.156	0.001	87	0.00022
	F	2	2.030	ND	0.001	87	0.00021
	G	ND	ND	ND	ND	ND	ND
	Coumarin	2	3.020	0.012	0.001	87	0.00014
S. litoralis	A	1	16.230	0.003	0.004	86	0.00003
	B	1	16.600	0.002	0.003	86	0.00003
	C	1	18.300	0.005	0.004	86	0.00002
	D	1	16.600	0.005	0.004	86	0.00003
	E	1	16.350	0.003	0.004	86	0.00003
	F	1	18.120	0.002	0.004	86	0.00002
	G	1	17.550	0.003	0.004	86	0.00002
	Coumarin	1	17.440	0.001	0.000	86	0.00002

* Average internal concentration at the end of the exposure period
** Relative recovery including matrix effects
*** Limit of Quantification (LoQ) with consideration of the relative recovery

TK model – not fixed compounds: likelihood profile

Compound C

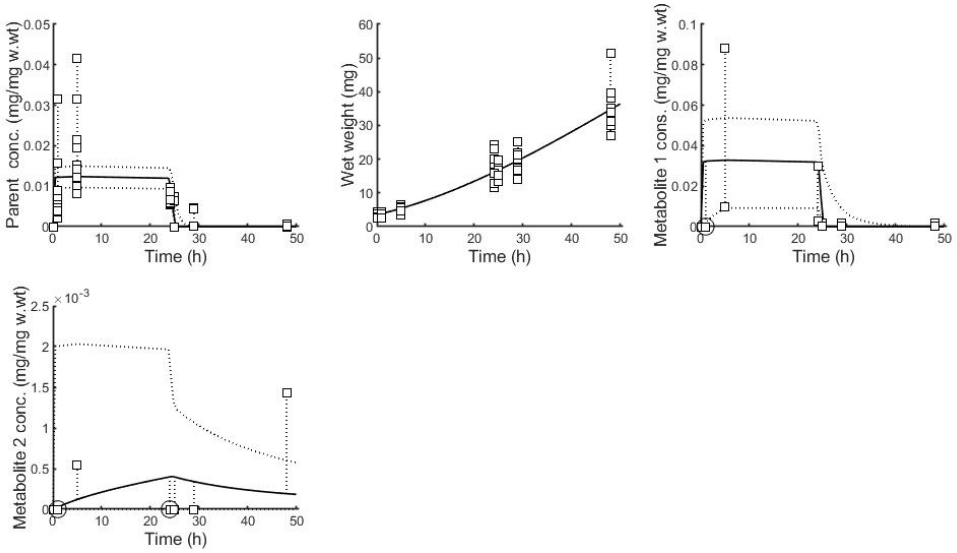


Figure S11: Body concentrations in *Spodoptera littoralis* larvae exposed to test compounds C. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 2) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data.

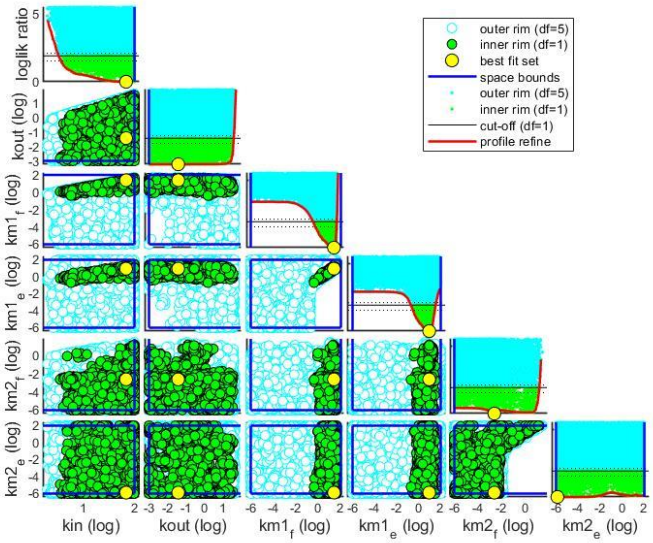


Figure S12: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds C.

Compound F

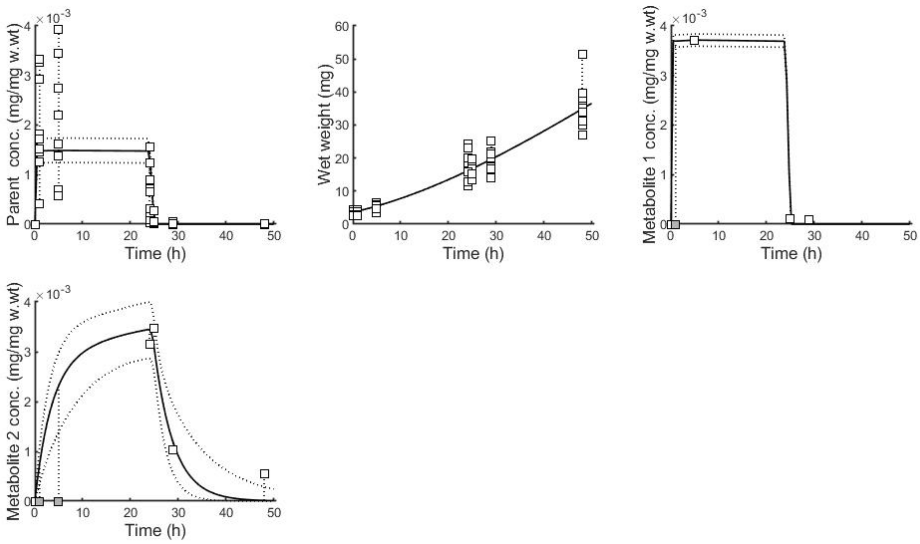


Figure S13: Body concentrations in *Spodoptera littoralis* larvae exposed to test compounds F. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 2) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data.

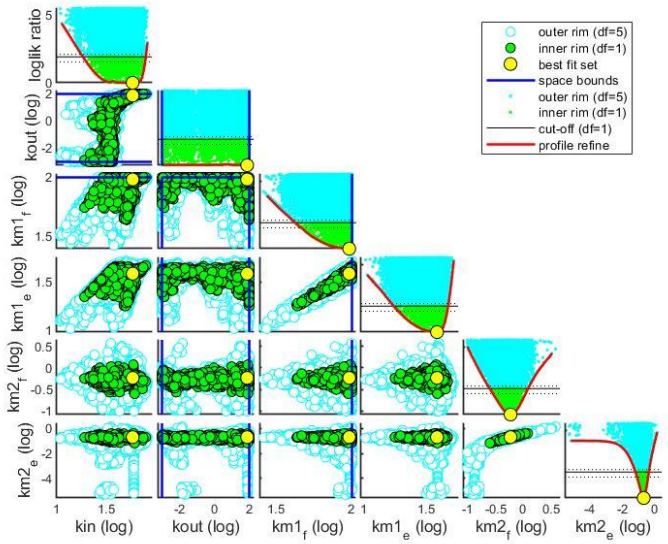


Figure S14: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds F.

Compound G

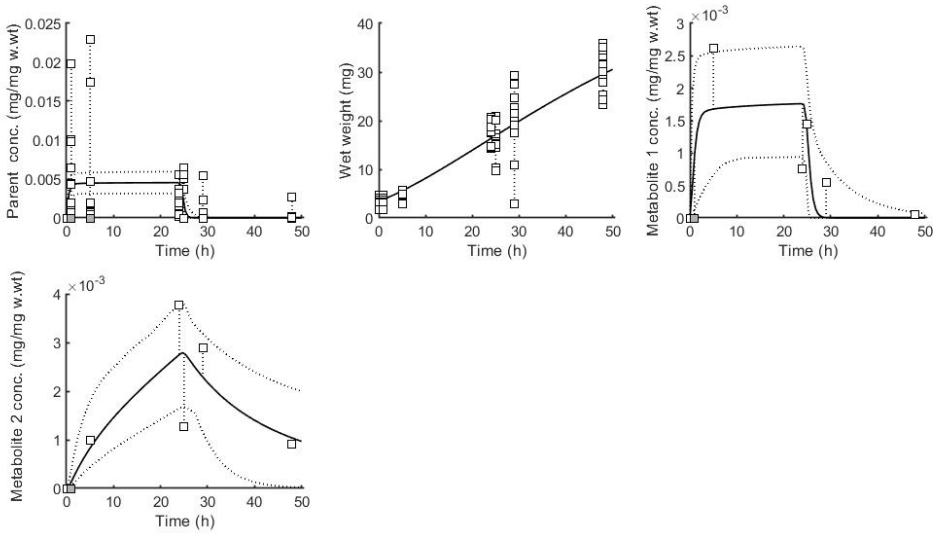


Figure S15: Body concentrations in *Spodoptera littoralis* larvae exposed to test compounds G. TK-Model for *Spodoptera littoralis:* parent compound uptake (mg/mg wet weight) and elimination (mg/mg wet weight), as well as metabolites (mg/mg wet weight). The model curve represents the best-fit parameter values (Table 2) and 95 % confidence limits (dotted) of model fit represented by the lines. Squares indicate measured data.

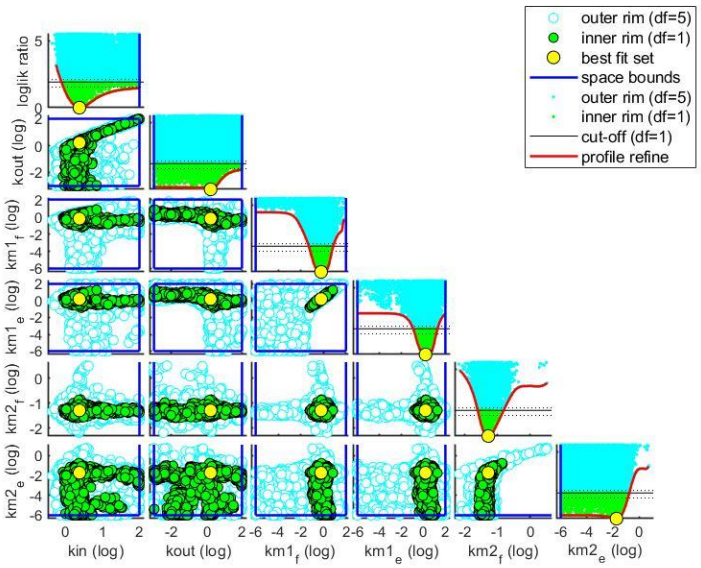


Figure S16: TK model likelihood profile for predicted parameters for the *Spodoptera littoralis* individuals during the toxicokinetic study for the test compounds G.

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