EFFEKTE MULTIPLER STRESSOREN AUF BODENORGANISMEN UND TERRESTRISCHE ÖKOSYSTEME

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Effekte multipler Stressoren auf Bodenorganismen und terrestrische Ökosysteme

INHALTSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

$\%_{vol}$	Prozentanteil, auf Volumen bezogen
$\%_{m}$	Prozentanteil, auf Masse bezogen
a.s.	Aktive Substanz, Wirkstoff in einer Formulierung
BBodSchG	Gesetz zum Schutz vor schädlichen Bodenveränderungen und zur Sanierung von Altlasten (Bundes-Bodenschutzgesetz)
BGBl	Bundesgesetzblatt
BiK-F	Senckenberg Biodiversität und Klimaforschung Zentrum, Frankfurt am Main
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
DBG	Deutsche bodenkundliche Gesellschaft
dw	dry weight, Trockengewicht
DWD	Deutscher Wetterdienst
EC	European Commission; Europäische Kommission
EC_X	X% effect concentration; X% Effektkonzentration
EFSA	European Food Safety Authority; Europäische Behörde für Lebensmittelsicherheit
EPA	Environmental Protection Agency; Umweltschutzbehörde der USA
FDR	Frequency domain reflectometry, Frequenzbereich-Reflektometrie
GmbH	Gesellschaft mit beschränkter Haftung
ha	Hektar = 10.000 Quadratmeter
IPCC	Intergovernmental Panel on Climate Change, Zwischenstaatlicher Ausschuss über Klimaveränderung, „Weltklimarat“
ISO	International Organization for Standardization; Internationale Organisation für Normung
KMU	Kleine und mittlere Unternehmen
log_{Kow}/log_{Pow}	logarithmierter Oktanol-Wasser-Verteilungskoeffizient
NOEC	No observed effect concentration; Höchste Konzentration, die zu keinem signifikanten Effekt führt
OECD	Organisation for Economic Co-operation and Development; Organisation für wirtschaftliche Zusammenarbeit und Entwicklung
PEC	Predicted environmental concentration, Prognostizierte Umweltkonzentration eines Stoffes
PIK	Potsdam-Institut für Klimafolgenforschung

ppm	Part per million; Anteile an einer Million
RP	Regierungspräsidium
SETAC	Society of Environmental Toxicology and Chemistry, Gesellschaft für Umwelttoxikologie und Chemie
SMS	Soil multi-species system, Terrestrischer Mikrokosmos
TER	Toxicity exposure ratio, Verhältnis von ermittelter Toxizität zur erwarteten Exposition
TME	Terrestrisches Modellökosystem
UBA	Umweltbundesamt
UCSD	University of California, San Diego; Universität von Kalifornien, San Diego
WHK_{max}	Maximale Wasserhaltekapazität

ABBILDUNGSVERZEICHNIS

TABELLENVERZEICHNIS

Zusammenfassung

Seit mehr als 200 Jahren gibt es durch die Wissenschaftler der Wetterstation auf dem Hohenpeißenberg systematische Wetterbeobachtungen, doch erst seit wenigen Jahren gibt es unter den Klimaforschern einen Konsens, dass sich das Klima durch anthropogene Einflüsse schon verändert hat – und weiter verändern wird. Die bisherigen Auswirkungen, wie zum Beispiel ein globaler Temperaturanstieg von 0,85°C seit Beginn der Industrialisierung, sind heute gut belegbar. Mögliche zukünftige Entwicklungen des Klimas werden heute ebenso erforscht wie die Auswirkungen des Klimawandels auf Mensch und Umwelt. Zu diesen Auswirkungen gehören unter anderem Folgen für die Landwirtschaft. Durch veränderte Niederschläge und den Temperaturanstieg werden sich die Lebensbedingungen von Bodenorganismen und Anbaubedingungen für Pflanzen ändern. Letztendlich ist aufgrund dieser Veränderungen auch ein verstärkter Einsatz von Pestiziden zu erwarten. Allerdings wurde bisher kaum untersucht, ob der Einsatz von Pestiziden in der Landwirtschaft unter den Bedingungen des Klimawandels (konkret durch die Interaktion von klimatischen und chemischen Faktoren) ein erhöhtes Umweltrisiko für Bodenorganismen darstellt. Bisher werden klimatische Faktoren bei den Tests für die Zulassung von Pestiziden nicht berücksichtigt.

Daher wurde diese Fragestellung in der hier vorliegenden Dissertation am Beispiel der Effekte von zwei zugelassenen Pestiziden auf Bodenorganismen unter verschiedenen klimatischen Bedingungen untersucht. Konkret wurden dazu mit Labor- und Halbfreilandversuchen die Wirkung eines Insektizids und eines Fungizids in Interaktion von Temperatur und Bodenfeuchte auf Vertreter zweier Invertebratengruppen (Collembola: zwei Arten; Enchytraeidae: eine Art) untersucht.

In einem modifizierten Standardtest mit Collembolen erhöhte sich die Toxizität des Insektizids Lambda-Cyhalothrin, wenn die Exposition der beiden Arten bei einer erhöhten Bodenfeuchte stattfindet. Die kühl adaptierte Art *Folsomia candida* reagierte bei erhöhter Testtemperatur am empfindlichsten auf diese Testsubstanz: Die EC_{50} aus diesem Experiment lag bei 2,84 mg (a.s.)/kg Boden Trockengewicht (dw). Unter Standardbedingungen, wie sie in Tests für die Zulassung von Pestiziden angewandt werden, lag die EC_{50} von *F. candida* dagegen bei 8,65 mg a.s./kg dw.

Unter den gleichen Versuchsbedingungen wurde auch das Fungizid Pyrimethanil an Collembolen getestet. Hier erwies sich die Testsubstanz für beide Arten bei gleichzeitigem Trockenstress und / oder erhöhter Temperatur als toxischer im Vergleich zu den Standard-Testbedingungen. Dabei zeigte *F. candida* mit einer EC_{50}

von 28,3 mg a.s./kg dw die höchste Empfindlichkeit. Ohne die veränderten klimatischen Faktoren, betrug die EC_{50} von *F. candida* 52,3 mg a.s./kg dw.
In Reproduktionstests mit der Enchytraeen-Art *Enchytraeus bigeminus* wurde die Bodenfeuchte als klimatischer Faktor in Kombination mit jeweils einer Testsubstanz untersucht. Bei beiden Chemikalien reagierte *E. bigeminus* in trockenem Boden empfindlicher. Die ermittelten EC_{50} betrugen 1,34 mg a.s./kg dw für Lambda-Cyhalothrin und 437 mg a.s./kg dw für Pyrimethanil. Getestet unter Standardbedingungen lagen die EC_{50}-Werte bei 3,79 bzw. 499 mg a.s./kg dw.
Neben den Laborexperimenten wurden Tests in „Terrestrischen Modellökosystemen“ (TME) mit den gleichen Chemikalien in Kombination mit variierender Bodenfeuchte als klimatischer Faktor vorgenommen. Diese Experimente wurden in Deutschland und in Portugal durchgeführt, um die Reaktion einer zentraleuropäischen und einer mediterranen Artengemeinschaft zu untersuchen. Aus der terrestrischen Lebensgemeinschaft wurden verschiedene Organismengruppen untersucht. Die Effekte auf Enchytraeen aus dem Experiment mit Pyrimethanil waren als Veröffentlichung Teil dieser Dissertation. In der portugiesischen Halbfreilandstudie wurden keine Effekte auf die Enchytraeen durch Pyrimethanil bei umweltrelevanten Konzentrationen festgestellt, jedoch beeinflusste die Bodenfeuchte die Zusammensetzung der Artengemeinschaft. Im deutschen TME-Experiment wurde eine verstärkte Wirkung des Fungizids in trockenem Boden festgestellt, d.h. die jeweiligen Effektkonzentrationen (niedrigste EC_{50} 3,48 mg a.s./kg dw für *Fridericia connata* in trockenem Boden) lagen deutlich unterhalb der aus den Labortests mit *Enchytraeus bigeminus* bekannten Werten (499 mg a.s./kg dw).
Zusammenfassend lässt sich feststellen, dass klimatische Faktoren die Effekte von Pflanzenschutzmittel auf Bodenorganismen beeinflussen können. Für Laborversuche ist eine generelle Berücksichtigung von klimatischen Faktoren im Zulassungsverfahren aus heutiger Sicht zu weit gegriffen. Die TME-Versuche zeigten sich als geeignetes Testverfahren, interaktive Effekte von Pestiziden und Klima bzw. multiplen Stressoren generell auf Artengemeinschaften zu untersuchen. Für TME-Experimente wäre unter Beachtung der Vielzahl möglicher Fragestellungen, Endpunkte und moderner statistischer Auswerteverfahren eine internationale Richtlinie wünschenswert.

1. EINLEITUNG

Seit einigen Jahrzehnten beobachten Forscher mit Besorgnis die Entwicklung unseres Klimas. Nicht unbedingt nur der globale Temperaturanstieg, sondern auch die Zunahme von extremen Wetterereignissen, wie langanhaltende Trockenheit oder die Hitzewelle im Sommer 2003, wird von der Bevölkerung und den Medien zunehmend im Zusammenhang mit dem Klimawandel diskutiert. Seine weitreichenden Konsequenzen sind nur teilweise abschätzbar, und Klimafolgenforscher versuchen Antworten auf die Frage zu finden, welche Bedingungen künftig in der Welt anzutreffen sind und wie Mensch und Umwelt damit umgehen werden. Da die klimatischen Einflüsse sämtliche Ökosysteme in ihren Grenzen, ihrer Zusammensetzung und Funktionalität beeinflussen können, ist es für deren Beurteilung wichtig, die Eigenschaften der Ökosysteme selbst zu kennen, um die schon vorhandenen Veränderungen wahrzunehmen und einzuschätzen.

Aufgrund des Klimawandels werden sich für die Landwirtschaft ebenfalls Änderungen ergeben, zum Beispiel durch eine verlängerte Vegetationsperiode oder das Auftreten neuer Agrarschädlinge. Bei der bisher durchgeführten Umweltrisikobeurteilung von Pflanzenschutzmitteln werden Aspekte des Klimawandels nicht untersucht, so dass es nur beschränkte Kenntnisse über die interaktive Wirkung von Chemikalien und klimatischen Einflüssen auf Organismen gibt.

Im Rahmen der Dissertation wurden veränderte klimatische Bedingungen in terrestrische ökotoxikologische Tests integriert und spezifische chemische und klimatische Auswirkungen auf Bodenorganismen untersucht. Diese Untersuchungen stellen einen Beitrag zur Klimafolgenforschung dar.

1.1 BÖDEN UND BODENORGANISMEN

Boden ist mehr als nur das, worauf wir stehen. Auch die Definitionen aus dem Duden („Erdreich, Erde“) und des BBodSchG („oberste Schicht der Erdkruste) lassen nicht abschätzen, welche vielfältigen Funktionen der Boden einnimmt. Er ist einerseits Lebensgrundlage und Lebensraum für Menschen, Tiere, Pflanzen und Bodenorganismen. Andererseits ist er Teil globaler Wasser- und Nährstoffkreisläufe und sorgt zugleich durch seine Filter-, Puffer- und Stoffumwandlungseigenschaften für einen Ausgleich der äußeren Einflüsse und trägt damit letztendlich auch zum Schutz des Grundwassers bei (BBodSchG 1998). So hängt die Ausgleichsfunktion eines Bodens maßgeblich von der Bodenorganismengemeinschaft ab. Zudem wird der Abbau bzw. die Metabolisierung von Pflanzenschutzmitteln in Böden deutlich stärker von biotischen als von abiotischen Prozessen gesteuert (Fenner et al. 2013). Die Artengemeinschaft und deren Wiedererholungspotential sind somit essentiell für den Erhalt sämtlicher Funktionen und ökosystemarer Leistungen des Bodens. Die Diversität, Masse und Abundanz der Artengemeinschaft ist wesentlich größer als die der Organismengemeinschaften in vielen anderen Ökosystemen, obgleich nach wie vor nicht sämtliche Arten beschrieben sind (Crawford et al. 2005, Wall et al. 2013). Ein Überblick über die wichtigsten Organismengruppen des Bodens, bzw. deren Einordnung innerhalb der Größenklassen, ist der Abbildung 1 zu entnehmen. Die Fotos zeigen die Organismengruppen, die auch im Rahmen des Forschungsprojektes untersucht wurden.

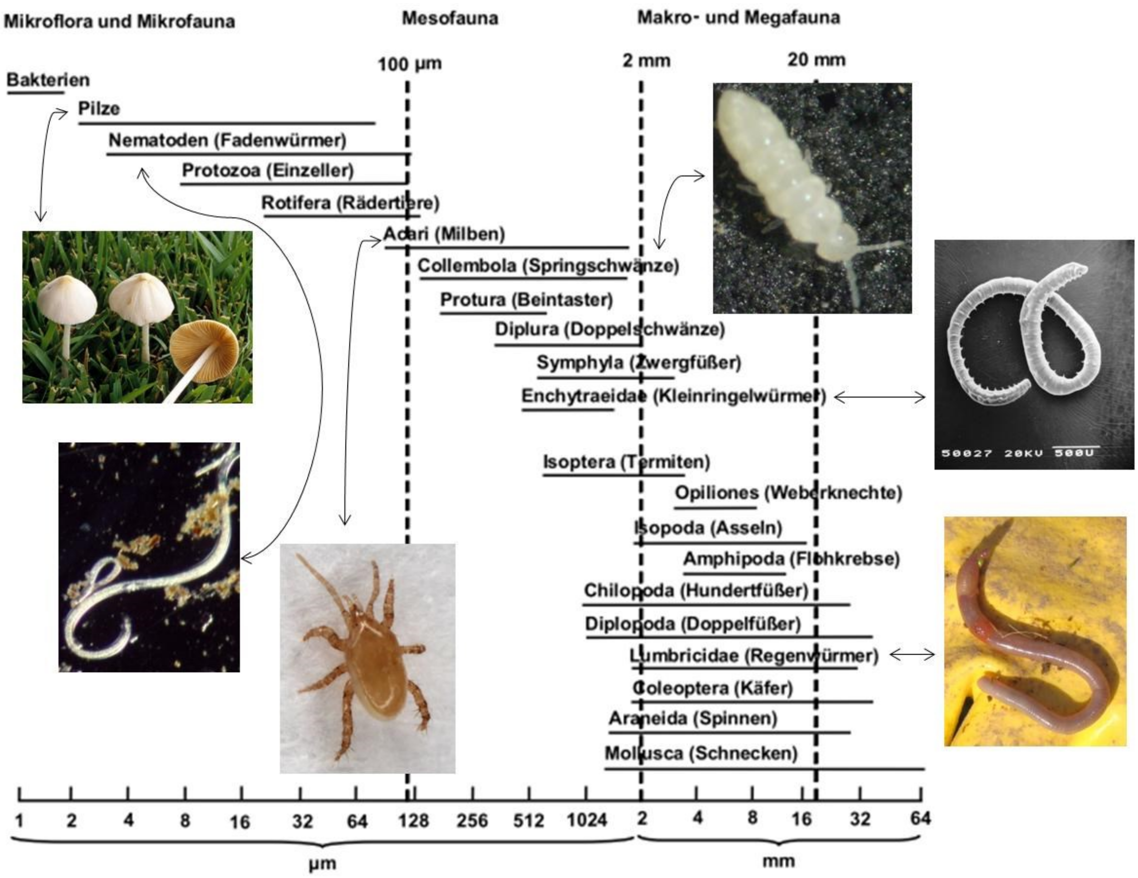

Abbildung 1: Größenklassen der Bodenorganismen, bezogen auf den Durchmesser der Organismen (nach Swift et al. 1979 aus UBA 2011). Urheber der Fotos: Pilze-Fred Stevens; Nematoden-Thomas Moser; Acari-Jarmo Holopainen; Enchytraeidae-Jörg Römbke; Lumbricidae-Pascale Lorenz.

Allen Gruppen (oder Teilen davon) ist gemein, dass sie als Destruenten am Um- bzw. Abbau von organischem Material beteiligt sind. Nematoden und Pilze waren Bestandteil der TME-Versuche. Für diese beiden Gruppen gibt es bisher keine standardisierten Richtlinien für ökotoxikologische Untersuchungen. Alle anderen abgebildeten Vertreter wurden sowohl in den TME-Versuchen als auch in Labortests untersucht, deren Ergebnisse teilweise veröffentlicht wurden (Annex I). Diese Gruppen (und wenige andere) werden in ökotoxikologischen Standardtests bei der Risikobeurteilung von Chemikalien untersucht (Kapitel 1.3.2 Risikobeurteilung in der terrestrischen Ökotoxikologie).

1.2 Klimawandel in Böden: Beobachtungen und Prognosen bezüglich klimatischer Faktoren

Anfang Mai 2015 wurde in der Presse ein neuer Rekordwert des atmosphärischen Kohlendioxidgehalts von über 400 ppm gemeldet, ein Messwert, der deutlich die Werte aus Untersuchungen von Eisbohrkernen der letzten 800.000 Jahre übersteigt (UCSD 2015). Der Anstieg des Kohlendioxidgehalts wird zusammen mit anderen klimarelevanten Gasen für den globalen Klimawandel verantwortlich gemacht. Seit Mitte des 19. Jahrhunderts wird das Klima der Erde unter wissenschaftlichen Aspekten beobachtet; zuerst vor allem anhand von Messungen der Lufttemperatur. Seitdem ist die globale Temperatur im Mittel um 0,85°C gestiegen, was durch eine Vielzahl weltweiter Messungen auf Land- und Meeresoberflächen belegt wird (IPCC 2013). Seit Mitte des 20. Jahrhunderts werden weitere Parameter, die mit dem Klimawandel verknüpft sind, regelmäßig erfasst. So konnte bis heute ein Anstieg der Meeresspiegelhöhe, eine Zunahme des atmosphärischen CO_2-Gehalts und auch eine Zunahme der Starkregenereignisse nachgewiesen werden (IPCC 2013). Für die Zukunft wird ein weiterer Temperaturanstieg prognostiziert, wobei dessen Höhe davon abhängt, wie ambitioniert Klimaschutzmaßnahmen umgesetzt werden bzw. ob durch eine Bevölkerungszunahme und den Einsatz fossiler Brennstoffe der Ausstoß von Treibhausgasen weiter zunehmen wird. Die möglichen Entwicklungen hat der Weltklimarat (IPCC) in vier verschiedene Modelle einfließen lassen, nach denen ein Temperaturanstieg zwischen 0,3 und 4,8°C bis Ende des 21. Jahrhunderts gegenüber der Klimaperiode 1986-2005 zu erwarten ist. Mit hoher Wahrscheinlichkeit werden Wetterextreme (z.B. Starkregenereignisse und Hitzewellen) häufiger und länger anhaltend auftreten (IPCC 2014). Dabei ist davon

auszugehen, dass die Niederschläge in Nordeuropa generell zunehmen, während sie in Südeuropa eher abnehmen werden (Kovats et al. 2014).
In Deutschland versuchen verschiedene Institute (z.B. das Max-Planck-Institut für Meteorologie oder das Potsdam-Institut für Klimafolgenforschung) mittels kleinräumigerer Modelle, präzisere Prognosen auch auf regionaler Ebene zu erstellen. Im Vergleich zum Referenzzeitraum 1971-2000 zeigen diese Modelle bis 2050 eine Erhöhung von etwa 1°C. Bis 2100 zeigen die Modelle eine Zunahme von mindestens 2,5°C, wobei zwei davon deutliche Gradienten von Nord nach Süd aufweisen. Bei der gesamten jährlichen Niederschlagsmenge wird sich in Deutschland bis ins Jahr 2100 bezogen auf den Zeitraum 1971-2000 keine große Änderung ergeben, doch werden sich die Niederschläge im Jahr anders verteilen: Im Winter werden sie um ca. 25% (maximal um 70%) zunehmen, während im Sommer je nach Modell und Region Rückgänge um etwa 25% (maximal 40%) angenommen werden (DWD 2015a).

Während es in Deutschland für die Luft (und teils auch für Oberflächengewässer) eine Vielzahl von Messstellen gibt, wird die Bodentemperatur nur an einem Standort (die Säkularstation in Potsdam), allerdings seit 1895 und in unterschiedlichen Tiefen bis zu 12 Meter, kontinuierlich erfasst. Dabei zeigt sich, dass in den für die meisten Bodenlebewesen und Abbauprozesse relevanten oberen Zentimetern des Bodens die Temperaturen seit Beginn der Aufzeichnungen ebenfalls ansteigen (Abbildung 2) (PIK 2015).

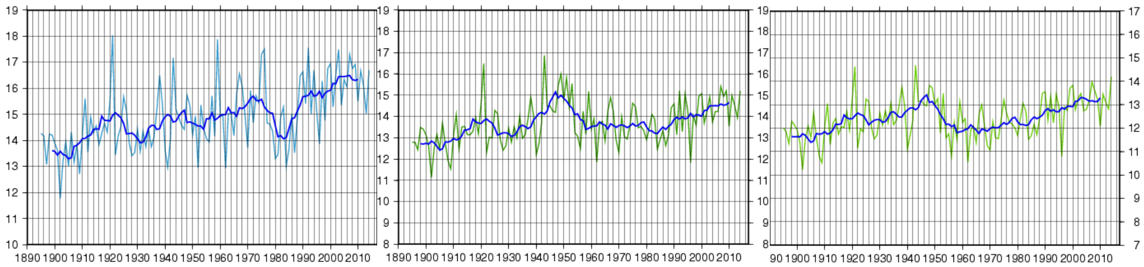

Abbildung 2: Temperatur (°C) in 2, 5 und 10 cm Bodentiefe in einem Sand-Braunpodsol (PIK 2015). Jeweils gezeigt sind die Einzelwerte sowie das 9jährige Mittel. Skalierungen der y-Achsen unterscheiden sich.

Bedingt durch die klimatischen Veränderungen, werden sich die Lebensbedingungen für die in den Böden vorkommenden Organismen erheblich ändern. So ist vermutlich innerhalb der oberen Bodenschicht (ca. 5 cm Tiefe), je nach Bodenart, mit einem ähnlichen Temperaturanstieg, wie von der IPCC und regionalen Modellen für die bodennahe Lufttemperatur vorausgesagt, zu rechnen. Aufgrund der veränderten Verteilung der Niederschläge im Jahresverlauf ist zudem anzunehmen, dass sich längere Trockenperioden und plötzliche Starkniederschläge in Zukunft häufen. Darüber hinaus werden sich dadurch nicht nur die Lebensbedingungen der Bodenorganismen (und damit auch die von ihnen erbrachten ökologischen Funktionen und Leistungen) verändern, sondern auch das Verhalten (speziell die Metabolisierung und der Abbau) von in den Boden gelangenden Chemikalien.

1.3 PESTIZIDE

Pflanzenschutzmittel sind in der modernen, konventionellen Landwirtschaft ein etablierter Garant für geringe Ernteausfälle und hohe Flächenerträge. Momentan sind 1430 Handelsprodukte mit 275 Wirkstoffen in ca. 750 Formulierungen in Deutschland zugelassen (BVL, 2015). Im Jahr 2013 wurden 110.000 t Formulierung mit entsprechend 44.000 t Wirkstoff in Deutschland abgesetzt. Den größten Anteil hatten, sowohl bei den Formulierungen als auch bei den Wirkstoffen, die Herbizide (ca. 40-50%), gefolgt von den Fungiziden (ca. 25%). Einen verhältnismäßig kleinen Anteil an Wirkstoffen und Mitteln haben Insektizide (ca. 3%) (BVL, 2013).

1.3.1 AUSGEWÄHLTE MODELLSUBSTANZEN

Für das mit dieser Dissertation verbundene Forschungsprojekt wurden zwei Modellsubstanzen ausgewählt, wobei zum einen deren Eigenschaften (z.B. die Wasserlöslichkeit), zum anderen ihre Verfügbarkeit (d.h. gesichert durch die Zulassung für den Projektzeitraum) als Entscheidungskriterien genutzt wurden. Des Weiteren war vor allem die Verfügbarkeit von Daten bezüglich Stoffverhalten, ökotoxikologischer Studien und chemischer Analytik wichtig. Ausführlichere Erläuterungen zur Wahl der Modellsubtanzen sind in den Publikationen im Annex enthalten.

Die erste Substanz war das Insektizid Lambda-Cyhalothrin. Es wird in vielerlei Kulturen, im Obst- und Gemüsebau, aber auch im Ackerbau und Forst, verwendet. In Deutschland wurden im Jahr 2013 zwischen 10 und 25 t Lambda-Cyhalothrin abgesetzt (BVL 2013). Die Substanz gehört zur Gruppe der Pyrethroide und wirkt nicht systemisch neurotoxisch bei Kontakt und Fraß (BVL 2011). Es wird in Deutschland derzeit in 14 Produkten gegen verschiedene saugende und beißende Insekten, z.B. Blattläuse, eingesetzt (Abbildung 3) (BVL 2015). In den terrestrischen Studien des Forschungsprojekts wurde Lambda-Cyhalothrin in der Formulierung

„Karate Zeon“ appliziert, das 100 g a.s./L enthält. „Karate Zeon“ darf pro Jahr zwei Mal in 10-14tägigem Abstand mit 75 mL/ha auf die Kulturen appliziert werden (BVL 2011). Eine Applikation entspricht etwa 10 µg a.s./kg Boden (Annahmen für Umrechnung in Annex I.I).

Abbildung 3: Wurzelhalsblattläuse (*Dysaphis crataegian*) an einer Möhre (RP Gießen, 2015)

Die zweite Modellsubstanz war das Fungizid Pyrimethanil, das derzeit in drei Formulierungen in Deutschland vertrieben und in vergleichbaren Mengen wie Lambda-Cyhalothrin eingesetzt wird (BVL 2013, 2015). Verwendet als Beizmittel wirkt es im Ackerbau gegen verschiedene Pilze; appliziert als Suspension in Tabak-, Wein- und Erdbeerkulturen wird es gegen Grauschimmel (*Botrytis cinerea*) (Abbildung 4) sowie beim Anbau von Kernobst gegen Schorf (*Venturia spp.*) eingesetzt (BVL 2015). Pyrimethanil stammt aus der Wirkstoffgruppe der Anilino-Pyrimidine und fungiert als Methionin-Biosynthesehemmer (Fritz 1997). Für die Studien dieser Dissertation wurde Pyrimethanil in Form des Mittels „Scala“ verwendet, das einen Wirkstoffanteil von 400 g a.s./L enthält. „Scala“ wird in einer maximalen einmaligen Aufwandmenge von 2,5 L/ha in Erdbeerkulturen angewandt (BVL 2009), was einer Konzentration von 1,33 mg a.s./kg entspricht (Annahmen für Umrechnung in Annex I.II, I.III).

Abbildung 4: Grauschimmelfäule *Botrytis cinerea* an Erdbeeren (RP Gießen, 2015)

1.3.2 Risikobeurteilung in der terrestrischen Ökotoxikologie

Pestizide müssen in der Europäischen Union vor ihrer Zulassung, je nach voraussichtlichem Anwendungsbereich und -häufigkeit, eine aufwändige Risikobeurteilung durchlaufen. Neben verschiedensten Anforderungen zu ihrer Humantoxizität und dem Verhalten in den unterschiedlichen Umweltkompartimenten (Stabilität, Mobilität, etc.), muss ein Risiko für Nichtziel-Organismen ausgeschlossen werden. Zu den Letzteren gehören unter anderem Vögel, Säugetiere und Bienen, aber auch wasser- und bodenlebende Organismen, deren Sensitivität gegenüber einem Wirkstoff durch standardisierte ökotoxikologische Tests überprüft wird. Dabei werden zuerst Laborexperimente eingesetzt, die bis heute generell bei 20°C und, im Fall von terrestrischen Tests, einer Bodenfeuchte von $50\%_m$ der maximalen Wasserhaltekapazität durchgeführt werden (OECD 2004a/b, 2008, 2009). Andere Umweltbedingungen, wie sie schon heute in verschiedenen Klimaregionen Europas vorzufinden sind (z.B. in mediterranen oder borealen Regionen) und bedingt durch klimatische Veränderungen eintreten können, werden in den Labortests nicht berücksichtigt.

Die Richtlinien beinhalten meist die experimentelle Beschreibung für eine oder wenige Stellvertreter-Arten, die nicht unbedingt wegen ihrer ökologischen Eigenschaften oder ihrer Sensitivität ausgewählt wurden, sondern vor allem aufgrund ihrer Eignung als Testorganismus (einfache Handhabung, reproduzierbare Ergebnisse, etc.). Sollte sich aufgrund der Ergebnisse dieser Tests ein Umweltrisiko nicht ausschließen lassen, sind weitere, komplexere Tests vorgeschrieben, die teilweise auch verschiedene Arten innerhalb eines Tests untersuchen. Jedoch fehlen für diese – mit Ausnahme des Regenwurm-Freilandtests (ISO 2014) – nähere Angaben zur Durchführung und Ergebnisbeurteilung.

In dem Forschungsprojekt wurden nun weitere klimatische Faktoren in die Laborexperimente integriert, um die Interaktion dieser Faktoren mit dem chemischen Stressor auf die Testorganismen zu untersuchen. Dabei sind, bedingt durch den Klimawandel, extreme Szenarien, wie sie in den Laborexperimenten getestet wurden, auch unter Freilandbedingungen vorstellbar. Daher wurden, zusätzlich zu den Einzelart-Tests im Labormaßstab, Experimente mit terrestrischen Modellökosystemen (TME) durchgeführt (siehe oben bzw. Annex I.IV.). Hier wurden natürliche Bodenkerne neben chemischem Stress auch verschiedenen Feuchtebedingungen ausgesetzt, um Auswirkungen der Interaktion auf die Artengemeinschaft zu erfassen.

1.4 INTEGRATION DER ARBEIT IN DEN AKTUELLEN FORSCHUNGSSTAND

1.4.1 HINTERGRUND DER ARBEIT

Die vorliegende Dissertation entstand im Rahmen des 2008 gegründeten Senckenberg Biodiversität und Klima Forschungszentrum (BiK-F) in Frankfurt am Main. Zu Beginn der ersten Förderphase des BiK-F wurden im Forschungsschwerpunkt „Adaptation und Klima" zwei Teilprojekte in der Ökotoxikologie initiiert (Müller et al. 2010). Die Fragestellungen aus dem aquatischen Bereich wurden an der Goethe-Universität in Frankfurt am Main bearbeitet, während die terrestrischen Experimente bei dem KMU-Partner von BiK-F, der ECT Oekotoxikologie GmbH in Flörsheim, durchgeführt wurden. Das Ziel beider Arbeitsgruppen war zu klären, ob bzw. wie sich die Effekte von Pestiziden unter dem Einfluss unterschiedlicher klimatischer Bedingungen ändern. Dabei sollten sowohl Auswirkungen auf einzelne Arten als auch Artengemeinschaften untersucht werden. Zu Beginn der Arbeit von BiK-F lag der Fokus der Publikationen zur Interaktion von klimatischen und chemischen Stressoren auf dem aquatischen Kompartiment (z.B. Sokolova und Lannig 2008; Schiedeck et al. 2007).

1.4.2 STAND DER FORSCHUNG BEZÜGLICH MULTIPLER STRESSOREN ZU BEGINN DER DISSERTATION

Zwei Übersichtsartikel, in denen sowohl aquatische als auch einige terrestrische Studien vorgestellt wurden, fassen diverse Labortests mit einzelnen Arten zur Interaktion klimatischer und chemischer Stressoren zusammen (Holmstrup et al. 2010; Laskowski et al. 2010). Diese Publikationen präsentieren in der Regel Studien, die mit den typischen „Standardorganismen" unter verschiedenen klimatischen Bedingungen durchgeführt wurden. Dazu gehörten beispielsweise *Folsomia candida* (Collembole), *Eisenia fetida* (Regenwurm) oder *Enchytraeus albidus* (Enchytraeidae). In den Tests wurden vor allem die Temperatur (z.B. Garcia et al. 2011; Lima et al. 2015) oder die Bodenfeuchte (z.B. Lima et al. 2011) verändert. Den bisherigen Publikationen ist zu entnehmen, dass die Effekte multipler Stressoren gegenüber einem Organismus nicht vorausgesagt werden können – selbst in den relativ einfachen Laborversuchen. Das Zusammenspiel mehrerer Faktoren kann sowohl in eine synergistische als auch antagonistische Interaktion münden und scheint nicht nur substanzabhängig, sondern auch artspezifisch zu sein. Daher erscheint es notwendig, entsprechende Interaktionen zusätzlich zu den Standardtests auch direkt in komplexeren Testsystemen zu untersuchen.

1.4.3 Stand der Forschung bezüglich komplexer terrestrischer Halbfreilandverfahren zu Beginn der Dissertation

Aufgrund der begrenzten Aussagekraft von Einzelart-Tests im Labor (Stichwort: Realitätsferne) werden seit etwa 30 Jahren auch in der terrestrischen Ökotoxikologie komplexe „Higher-Tier-Tests“ als Teil der Umweltrisikobeurteilung entwickelt. Im Folgenden werden dazu stellvertretend für verschiedenste Ansätze die beiden am weitesten entwickelten Verfahren kurz vorgestellt.

In dem „soil multi-species system“ (SMS) kann in kleinem Maßstab und mit geringem Aufwand die Reaktion einer künstlich zusammen gesetzten Artengemeinschaft (mindestens zwei trophische Ebenen abdeckend) auf Stressoren untersucht werden. In der Literatur finden sich sowohl Untersuchungen zu klimatischen Faktoren (Lang et al. 2014) als auch zu chemischen Einflüssen (Jensen und Scott-Fordsmand 2012; Santos et al. 2011; Sechi et al. 2014,). Des Weiteren wurde mit dieser Methode bereits eine Studie zur Interaktion klimatischer und chemischer Stressoren durchgeführt (Menezes-Oliveira et al., 2013). Auch wenn dieses Vorgehen aufgrund des recht einfachen Aufbaus einige Vorteile bei der praktischen Versuchsdurchführung oder der Auswertung bietet, wird damit keineswegs die Komplexität eines realen Ökosystems erreicht.

Eine andere, deutlich früher entwickelte, aber auch wesentlich komplexere Methode ist das „Terrestrische Modellökosystem“ (TME), für das inzwischen ein Richtlinienentwurf vorliegt (Schäffer et al. 2011). Hier werden ungestörte Bodenkerne mitsamt ihrer natürlichen Artengemeinschaft dem Freiland entnommen und diversen Stressoren ausgesetzt. Bereits seit den 1980er Jahren hat man auf diese Weise Chemikalien, Abwässer oder Flugasche auf verschiedenste Endpunkte wie mikrobielle Respiration bzw. Biomasse, Vegetation, oder die Diversität der Bodenfauna untersucht (Morgan und Knacker, 1994). Bis heute sind einige TME-Studien durchgeführt worden, häufig mit etwas variierender Herangehensweise und Endpunkten, aber stets mit einem einzelnen chemischen Stressor (Förster et al. 2011; Knacker et al. 2004; Scholz-Starke et al. 2013). Allerdings ist dieses System, nicht zuletzt aufgrund seiner Größe und damit auch der Stabilität der jeweiligen organismischen Beziehungen untereinander mit verschiedenen trophischen

Ebenen, durchaus für die Untersuchung von Kombinationen verschiedener Stressoren geeignet.

1.4.4 Ziele dieser Dissertation

In erster Linie sollte in dieser Forschungsarbeit untersucht werden, ob und wie klimatische Faktoren die Effekte der beiden Modellsubstanzen auf Bodenorganismen beeinflussen. Im Detail wurden dabei folgende Hypothesen analysiert:

- Lambda-Cyhalothrin und Pyrimethanil beeinflussen das Überleben und die Reproduktion von Bodenorganismen in umweltrelevanten Konzentrationen.
- Temperaturanstiege, wie sie durch den Klimawandel zu erwarten sind, wirken sich negativ auf kühl-adaptierte Boden-Organismen aus.
- Trockenheit beeinflusst Bodenorganismen auch ohne chemischen Stressor negativ.
- Höhere Temperaturen verstärken den toxischen Effekt chemischer Stressoren auf kühl-adaptierte Organsimen.
- Trockenheit intensiviert den chemischen Stress.
- Die Interaktion von klimatischen und chemischen Faktoren stellt bei zunehmendem Klima- und Landnutzungswandel ein potentielles Risiko für Bodenorganismen dar.

In einem ersten Schritt wurden zunächst mit drei Standardtestorganismen Labortests durchgeführt. Dazu zählten die Raubmilbe *Hypoaspis aculeifer*, die Enchytraee *Enchytraeus bigeminus*, sowie der Collembole *Folsomia candida*; alle drei gelten als eher kühl-adaptiert. Zusätzlich wurde der eher höhere Temperaturen präferierende Collembole *Sinella curviseta* untersucht. Alle Organismen wurden in multivariaten Reproduktionstests bei zwei Temperaturstufen (20°C und 26°C) mit jeweils drei Bodenfeuchtestufen (30, 50 und 70%$_m$ der maximalen Wasserhaltekapazität WHK_{max}) mit beiden Testsubstanzen unter Anwendung eines EC_x-Designs erforscht. Die Temperaturstufen wurden ausgewählt, da 20°C einerseits die Standard-Temperatur in den terrestrischen Labortest nach OECD darstellt (OECD 2004a/b, 2008, 2009). Andererseits basierte die erhöhte Temperatur auf der Prognose des vierten Sachstandsberichts der IPCC (Meehl et al. 2007), der im schlimmsten Fall von einer Temperaturerhöhung von bis zu 6,4°C ausgeht. Die Ausnahme stellt *E. bigeminus* dar, die nur in einem Temperaturbereich (23 ± 4°C) getestet wurde.

Die Bodenfeuchtestufen wurden ebenfalls auf Grundlage der OECD-Richtlinien ausgewählt. Standardisierte Labortests werden bei 50±10%$_m$ der WHK$_{max}$ durchgeführt. Daher mussten die beiden Extremstufen einerseits außerhalb dieses Bereiches liegen, sollten sich aber andererseits noch im Toleranzbereich der Testorganismen befinden (vgl. Jänsch et al. 2005, die für die typischen Labororganismen die ökologischen Präferenz- und Toleranzbereiche zusammengestellt hatten). Aufgrund dieser Information war davon auszugehen, dass unter den für diese Forschungsarbeit gewählten Bedingungen die Testorganismen zwar überleben werden, jedoch schon durch die beiden Extremfeuchtewerte oder die erhöhte Temperatur gestresst sein könnten.
Sowohl die Studien mit den Collembolen (Annex I.I. und I.II.) als auch die Experimente mit der Enchytraee (Annex I.III.) wurden als Bestandteil dieser Doktorarbeit veröffentlicht.

Um einen Eindruck über die Interaktion von Bodenfeuchte und chemischem Stress auf eine heimische und eine mediterrane Bodenartengemeinschaft zu erhalten, wurden bei der ECT Oekotoxikologie GmbH und an der Universität Coimbra in Portugal verschiedene TME-Experimente durchgeführt. Zu den untersuchten Endpunkten gehörte die Abundanz von Enchytraeen, Mikroarthropoden (v.a. Collembolen und Milben), Regenwürmern und Nematoden. In Portugal wurden zudem mehrere mikrobielle Endpunkte für beide Standorte ausgewertet. Des Weiteren wurde die Fraßaktivität von Bodenorganismen mittels Köderstreifen sowie verschiedene Vegetationsparameter in den Experimenten in Deutschland erfasst. An beiden Standorten wurden sowohl Pyrimethanil als auch Lambda-Cyhalothrin getestet. Zu der TME-Studie mit Pyrimethanil entstanden bisher eine Publikation mit Ergebnissen der mikrobiellen Endpunkte (Ng et al. 2014) sowie eine Veröffentlichung über die Effekte auf Enchytraeidae, die einen Teil dieser Thesis darstellt (Annex I.IV.).
Die TME-Experimente, die im Zusammenhang mit der vorliegenden Dissertation durchgeführt wurden, unterscheiden sich klar von bisherigen Veröffentlichungen, denn erstmals wurden in Halbfreiland-Tests mit ungestörtem Boden ein klimatischer Stressor in Kombination mit einem chemischen Stressor getestet und deren Effekte einzeln und in Interaktion auf eine Vielzahl von Endpunkten analysiert.

2. DISKUSSION

2.1 WICHTIGE ERGEBNISSE UND ERKENNTNISSE DER DISSERTATION

Im Folgenden werden die wichtigsten Ergebnisse, die im Zusammenhang mit der Dissertation entstanden und publiziert wurden, dargestellt. Die ausführlichen Beschreibungen der Experimente, deren Ergebnisse und eine detaillierte Diskussion können den Artikeln im Annex I entnommen werden.

Die Resultate der ersten Publikation (Annex I.I) über die Toxizität von Lambda-Cyhalothrin auf Collembolen unter verschiedenen klimatischen Faktoren im Labortest können wie folgt zusammengefasst werden:

- Um die Anforderungen der OECD-Richtlinie 232 (2009) bezüglich der Anzahl der Nachkommen in den Kontrollreplikaten zu erfüllen ($n \geq 100$), muss *Sinella curviseta* zu Testbeginn etwa 20-23 Tage alt sein.
- Bei niedriger Bodenfeuchte ist die Anzahl der Nachkommen in der Kontrolle bei beiden Arten signifikant reduziert.
- In den Kontrollen profitiert vor allem *F. candida* von hoher Bodenfeuchte.
- Die Toxizität von Lambda-Cyhalothrin wird von den Testfaktoren beeinflusst (Abbildung 5):

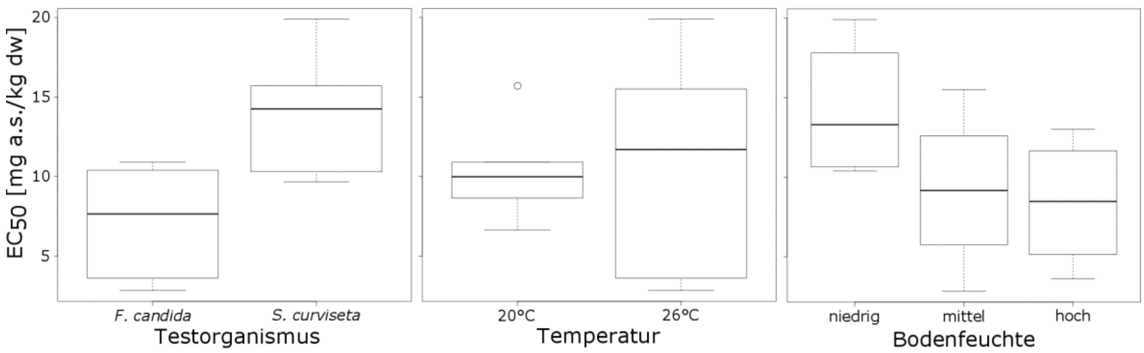

Abbildung 5: EC_{50}-Werte [mg Lambda-Cyhalothrin/kg Boden dw] aus den Collembolen-Reproduktionstests, dargestellt in Abhängigkeit von den Testfaktoren. Boxplots zeigen Mediane mit Quartilen. Antennen kennzeichnen den 1,5fachen Interquartilsabstand.

  - *F. candida* reagierte sensitiver auf Lambda-Cyhalothrin als *S. curviseta.*
  - Die Temperatur hatte keinen Einfluss auf die Toxizität von Lambda-Cyhalothrin auf Collembolen.
  - Lambda-Cyhalothrin ist bei hoher Bodenfeuchte toxischer für Collembolen.

- Die EC_{50}-Werte hingen von der Interaktion zwischen Temperatur und Art ab; d.h. *F. candida* reagierte bei 26°C sensitiver, während *S. curviseta* bei 20°C empfindlicher gegenüber Lambda-Cyhalothrin war.
- Der Schwund von Lambda-Cyhalothrin in der höchsten Testkonzentration (durch Abbau oder Adsorption) war unabhängig von den klimatischen Testfaktoren.

Eine ähnliche Versuchsreihe ging in eine weitere Publikation (Annex I.II.) ein. Erneut wurden Collembolen-Reproduktionstests unter verschiedenen Testbedingungen im Labor nach der OECD-Richtlinie 232 (2009) durchgeführt, jedoch wurde hier Pyrimethanil als Testsubstanz verwendet. Die wichtigsten Erkenntnisse sind nachfolgend aufgeführt:

- Erneut zeigte sich, dass bei niedriger Bodenfeuchte beide Arten weniger Nachkommen in den Kontrollen produzierten. *F. candida* offenbarte nochmals die Präferenz für hohe Bodenfeuchte, während sich bei *S. curviseta* kein Unterschied zwischen mittlerer und hoher Bodenfeuchte einstellte.
- Die EC_{50}-Werte für die Pyrimethanil-Toxizität variierten in Abhängigkeit der Testfaktoren (Abbildung 6):

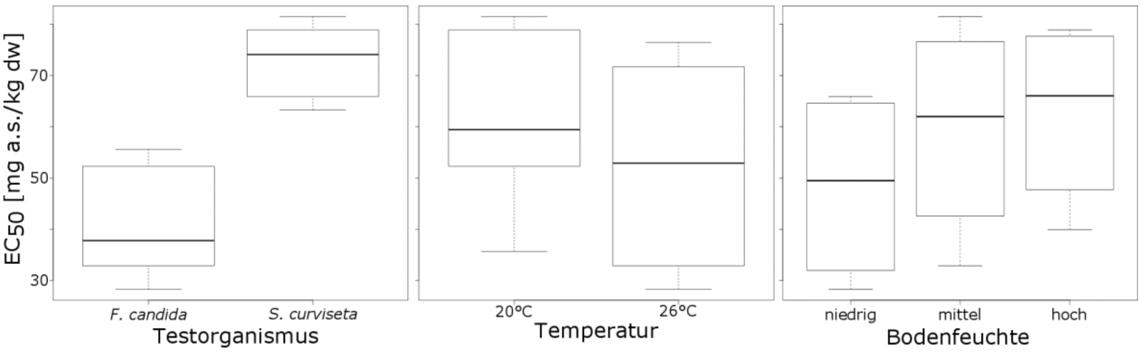

Abbildung 6: EC_{50}-Werte [mg Pyrimethanil/kg Boden dw] aus den Collembolen-Reproduktionstests, dargestellt in Abhängigkeit von den Testfaktoren. Boxplots zeigen Mediane mit Quartilen. Antennen kennzeichnen den 1,5fachen Interquartilsabstand.

- Erneut zeigte sich *F. candida* sensitiver als *S. curviseta* gegenüber der Testsubstanz.
- Beide Arten reagierten bei höherer Temperatur sensitiver gegenüber Pyrimethanil.
- Bei Trockenheit hatte Pyrimethanil eine höhere Toxizität gegenüber Collembolen.

- Bei Auswertung der EC_{50}-Werte zeigte sich eine signifikante Interaktion zwischen der jeweiligen Art und den klimatischen Faktoren. Das heißt, die Reaktion auf Pyrimethanil ist davon abhängig, wie eine Spezies auf Temperatur oder Bodenfeuchte reagiert.
- Die chemische Analytik der höchsten Testkonzentration zeigte unabhängig von klimatischen Faktoren keinen Abbau innerhalb der Versuchszeit von 28 Tagen.

Beide Laborstudien mit Collembolen-Reproduktionstests demonstrieren den Zusammenhang zwischen Toxizität und klimatischen Faktoren. Insbesondere die Bodenfeuchte spielte bei beiden Versuchen eine entscheidende Rolle. Die Collembolen reagierten unter trockenen Bedingungen in den Kontrollen mit hoher Adultmortalität und niedrigen Nachkommenzahlen. Das Experiment mit Pyrimethanil führte zu synergistischen Effekten unter Trockenstress. Jedoch zeigte sich im Versuch mit Lambda-Cyhalothrin, dass die offenbar schlechteren Ausgangsbedingungen nicht zwangsläufig zu einer höheren Toxizität der Modellsubstanz führten. In diesem Experiment war die Toxizität bei Trockenheit geringer als in feuchtem Boden. Das könnte an veränderten Stoffwechselprozessen der Organismen liegen, was zu unterschiedlicher interner oder externer Exposition führt (Hooper et al. 2013). Eine andere Möglichkeit wäre die Biomagnifikation: Durch den hohen $\log_{Kow}$ adsorbiert Lambda-Cyhalothrin an das Futter im Test, was von vitalen Tieren bei hoher Bodenfeuchte eher aufgenommen wird. Insgesamt zeigen die beiden Experimente, dass zwar ein deutlicher Einfluss klimatischer Faktoren auf die Toxizität einer Chemikalie stattfindet, die Ausmaße der Auswirkungen aber substanzspezifisch sind.

Die dritte Publikation, die im Rahmen des Forschungsprojekts entstand (Annex I.III.), beinhaltet zwei Reproduktionstests mit den beiden Modellsubstanzen und der fragmentierenden Enchytraeidae *Enchytraeus bigeminus* im Labor nach der OECD-Richtlinie 220 (2004a) bei verschiedenen Bodenfeuchten.

- In den Kontrollen mit trockenem Boden war die Reproduktion von *E. bigeminus* signifikant geringer im Vergleich zu den Behandlungen mit höherer Bodenfeuchte.

- Die EC_{50}-Werte für Lambda-Cyhalothrin erhöhten sich mit zunehmender Feuchte:
    - 1.34 mg a.s./kg dw in trockenem Boden
    - 3.79 mg a.s./kg dw in mäßig feuchtem Boden
    - 4.77 mg a.s./kg dw in feuchtem Boden
- Gleiches gilt für die EC_{50}-Werte, die im Pyrimethanil-Experiment generiert wurden:
    - 437 mg a.s./kg dw in trockenem Boden
    - 499 mg a.s./kg dw in mäßig feuchtem Boden
    - 829 mg a.s./kg dw in feuchtem Boden
- *E. bigeminus* ist bisher nicht namentlich als Testorganismus in der Richtlinie vorgeschlagen, eignet sich aber, aufgrund der einfachen Handhabbarkeit und der hohen Anzahl an Nachkommen, gut für die Durchführung ökotoxikologischer Test.

Auch die Laborstudie mit *E. bigeminus* weist auf die Interaktion zwischen der Sensitivität gegenüber einem chemischen Stressor und der Bodenfeuchte hin. Unabhängig von der Substanz zeigten sich unter augenscheinlichem Trockenstress synergistische Effekte.

Neben den Labortests wurden mehrere Halbfreilandstudien mit TME in Portugal und Deutschland durchgeführt, um realistischere Expositionsszenarien zu erhalten und ein größeres Artenspektrum abzudecken. Im Annex I.IV wird eine Veröffentlichung präsentiert, das zwei Halbfreilandstudien mit der Testsubstanz Pyrimethanil und dreierlei Bodenfeuchten beinhaltet. Dargestellt werden in dieser Publikation die Effekte auf die Abundanz der Enchytraeiden.

- Aus der Studie in Portugal wurden folgende Erkenntnisse gewonnen:
    - Eine neue Enchytraeiden-Spezies, *Achaeta coimbrensis*, konnte beschrieben werden.
    - Bei praxisüblicher Aufwandmenge von Pyrimethanil und deren 5facher Konzentration (2.24 -11.3 mg/kg dry soil) konnten keine Effekte auf die Abundanz der Enchytraeiden festgestellt werden.
    - Die Bodenfeuchte beeinflusste signifikant die Abundanz der Artengemeinschaft sowie einer einzelnen Art (*E. bulbosus*).

- Aus der Studie in Deutschland sind nachstehende Ergebnisse relevant:
    - Die niedrigste EC_{50} wurde für *Fridericia connata*, exponiert in trockenem Boden, ermittelt (3.48 mg a.s./kg dw).
    - Die höchste EC_{50} stammt ebenfalls von *Fridericia connata*, jedoch exponiert in feuchtem Boden (224 mg a.s./kg dw).
    - Der Umweltfaktor Bodenfeuchte wirkte sich signifikant auf die Sensitivität aus.
- Aus beiden Experimenten lassen sich folgende Erkenntnisse zusammenfassen:
    - Die Abundanz der Enchytraeiden eignet sich als Endpunkt für Halbfreilandversuche, jedoch sollten vier Replikate je Behandlung genutzt werden, um eine ausreichend hohe Abundanz der Schlüsselarten für die statistische Auswertung zu erzielen.
    - Ein breiter Konzentrationsbereich sollte abgedeckt sein ($n \geq 8$), um für möglichst viele Arten eine Effektkonzentration ermitteln zu können.

Die Ergebnisse der TME-Studien vertieften die Erkenntnisse über die Wirkung von Pyrimethanil auf eine komplexe Artengemeinschaft von Enchytraeiden. Pyrimethanil erwies sich im Halbfreiland als wesentlich toxischer gegenüber einzelnen Enchytraeiden-Arten, als es durch den Labortest mit der einzelnen Art *Enchytraeus bigeminus* zu erwarten war. Das kann unter anderem an der längeren Expositionsdauer liegen (8 im Gegensatz zu 3 Wochen). Es könnte aber auch darauf hindeuten, dass die für den Labortest ausgewählte Art oder das Testsystem insgesamt nicht sensitiv genug sind, um Auswirkungen unter Feldbedingungen abschätzen zu können. Außerdem wurde im Labor die Reproduktion als Endpunkt untersucht, während im Halbfreilandtest die Abundanz für die Berechnung der Effektkonzentrationen herangezogen wurde. Dabei spielen sich biotische Interaktionen (z.B. Prädation, Konkurrenz) im Halbfreiversuch ab, die die Abundanz einer Organismengruppe beeinflussen können und letztendlich die EC_{50}-Werte.

2.2 Empfehlungen für Praxis und Risikobeurteilung mit vorgestellten Testsystemen

2.2.1 LABOREXPERIMENTE

Die Ergebnisse der eigenen Arbeit bestätigen die publizierten Resultate (z.B. Laskowski et al. 2010, Holmstrup et al. 2010) insofern, dass klimatische Faktoren die Sensitivität von Organismen gegenüber einem chemischen Stressor verstärken oder abschwächen können. Der Einfluss von klimatischen Stressoren wird aber bisher nicht in der Risikobeurteilung von Pestiziden bedacht. Es wäre daher zu diskutieren, ob eine generelle Berücksichtigung weiterer Faktoren eine Rolle bei der Zulassung von Pestiziden (oder auch anderen Chemikalien, die potentiell in die Umwelt gelangen könnten,) spielen sollte. Neben den beiden untersuchten Faktoren Temperatur und Bodenfeuchte wären auch weitere Aspekte, wie z.B. pH-Wert bzw. der organische Anteil im Boden, Lichtdauer und -intensität als abiotische Faktoren denkbar. Auch zeigte sich in mehreren Studien, dass die Toxizität einer Chemikalie in natürlichen Böden, mit entsprechend verschiedenen abiotischen Faktoren, deutlich von derjenigen in OECD-Kunsterde abweichen kann (Shoults-Wilson et al. 2011, van Gestel et al. 2011).

Andererseits ist es die Idee des Labor-Experiments, unter vereinfachten Annahmen belastbare und reproduzierbare Ergebnisse zu generieren. In dem Collembolen-Reproduktionstest mit Lambda-Cyhalothrin (Annex I.I.) differierten die EC_{10}-Werte von *F. candida* bis zu einem Faktor von 6 beim Vergleich zwischen der Testung unter Standardbedingungen und der unter veränderten klimatischen Bedingungen. In den anderen Labor-Experimenten bzw. bei Betrachtung der EC_{50}-Werte war der Unterschied geringer. Durch die in der Risikobeurteilung für Pflanzenschutzmittel angewandten Sicherheitsfaktoren ist nicht von einer Gefährdung auf Grundlage der Laborergebnisse auszugehen.

In der Literatur finden sich kaum Studien, aus denen sich ein Faktor, basierend auf Schwellenwerten aus Standardtests (einer NOEC oder einer EC_x), wie er in eine Risikobeurteilung eingehen würde, und einem Schwellenwert, generiert unter simultanem Stress, errechnen ließe. Einige Publikationen betreffen entweder aquatische Organismen oder beschreiben nicht die Exposition gegenüber Pestiziden (Abdel-Lateif et al. 1998; Scherer et al. 2013). Die übrigen Veröffentlichungen, die sich mit der Interaktion von Umweltfaktoren und Pestiziden auf terrestrische

Organismen beschäftigen, folgen nicht den ISO- oder OECD-Richtlinien und sind damit generell nicht für eine formelle Risikobeurteilung ausgelegt. Holmstrup et al. (1998) beispielsweise setzten Kokons der Regenwurmarten *Aporrectodea caliginosa* und *Dendrobaena octaedra* erst einer Kupfer-Testlösung und anschließend niedriger Luftfeuchte aus. Sørensen und Holmstrup (2005) exponierten Collembolen zunächst gegenüber verschiedenen Chemikalien, u.a. Dimethoat und Cypermethrin und setzten sie anschließend Trockenstress in Form von reduzierter Luftfeuchte aus. Eine gleichzeitige Exposition gegenüber klimatischem Stress und einem Pestizid in Anlehnung an ISO- und OECD-Richtlinien sind in Studien von Lima et al. (2011, 2015) und De Silva et al. (2009) zu finden (Tabelle 1).

Tabelle 1: Gegenüberstellung von Toxizitätsdaten, die unter Standardtestbedingungen beziehungsweise bei simultanem klimatischem Stress generiert wurden. Quellen: a) Lima et al. 2011, b) Lima et al. 2015, c) De Silva et al. 2009.

Art	Endpunkt	Chemischer Stressor	Toxizität Standard	Klimatischer Stressor	Toxizität Interaktion	Faktor	Quelle
E. andrei	14d Mortalität	Carbaryl	LC_{50} = 26,2-54,7	Bodenfeuchte 10/20% WHK	LC50 < 20	1,3-2,7	a
F. candida	Reproduktion	Carbaryl	EC_{50} = 4,6	Temperatur 26°C	EC50 = 7,3	0,6	b
E. andrei	Biomasse	Carbaryl	EC_{50} > 100	Temperatur 26°C	EC50 = 50,8	> 2	b
E. andrei	Reproduktion	Chlorpyrifos	EC_{50} = 7,49	Temperatur 26°C	EC50 = 3,86	1,9	c
E. andrei	Reproduktion	Carbofuran	EC_{50} = 1,57	Temperatur 26°C	EC50 = 1,25	1,3	c
E. andrei	Reproduktion	Carbendazim	EC_{50} = 0,39	Temperatur 26°C	EC50 = 1,84	0,2	c

In der ersten Studie von Lima et al. (2011) wurde unter anderem der Regenwurm *Eisenia andrei* bei verschiedenen Bodenfeuchten gegenüber dem Insektizid Carbaryl exponiert. In den OECD-Richtlinien mit terrestrischen Invertebraten wird 40-60%$_m$ der WHK als Standard für die Bodenfeuchte vorgeschlagen. Bei diesen beiden Feuchtestufen stellte Lima et al. (2011) eine EC_{50} (Mortalität; 14 d) von 26,2 bzw. 54,7 mg/kg fest. Bei 10 und 20%$_m$ der WHK lagen die EC_{50}-Werte unter 20 mg/kg, so dass der Faktor dementsprechend 1,3 bzw. 2,7 entsprach. In der anderen Studie von Lima et al. (2015) wurden *F. candida* und *E. andrei* bei verschiedenen Temperaturen gegenüber Carbaryl exponiert. Den gleichen klimatischen Stressor wählte De Silva et al. (2009), die *E. andrei* in Reproduktionstests mit zwei Insektiziden und einem Fungizid untersuchten.

In den eigenen und den veröffentlichten Studien zeigte sich übereinstimmend, dass klimatische Faktoren die Sensitivität eines Organismus gegenüber einer Substanz verändern und synergistisch wirken können. Mit Ausnahme eines EC_{10}-Wertes (*F. candida*, Lambda-Cyhalothrin) deutet die Datenbasis bisher nicht auf zu starke

Effekte hin, die in umweltrelevanten Konzentrationen auftreten werden und nicht durch die Sicherheitsfaktoren in der Risikobeurteilung abgedeckt wären. Es ist somit zu diskutieren, ob der Mehrgewinn an Information den zusätzlichen Aufwand und damit eine vermehrte Durchführung von Tierversuchen rechtfertigt. Die Datenbasis ist bis zum heutigen Stand sehr überschaubar und beinhaltet lediglich vier Insektizide und zwei Fungizide (Tabelle 2), und dabei sind zusätzlich auch nur drei terrestrische Arten berücksichtigt.

Tabelle 2: Bisherige Veröffentlichungen zu Interaktionen von Pestiziden und klimatischen Faktoren im Vergleich zur Testung nach Richtlinien

Chemischer Stressor	**Verwendung**	**Gruppe**	**Autor**	**Jahr**
Carbaryl	Insektizid	Carbamat	Lima	2011/2015
Chlorpyrifos	Insektizid	Thiophosphorsäureester	De Silva	2009
Carbofuran	Insektizid	Carbamat	De Silva	2009
Carbendazim	Fungizid	Carbamat	De Silva	2009
Lambda-Cyhalothrin	Insektizid	Pyrethroid	Bandow	2013/2014
Pyrimethanil	Fungizid	Anilino-Pyrimidine	Bandow	2013/2014

Eine generelle Berücksichtigung von klimatischen Faktoren für alle in der Zulassung notwendigen ökotoxikologischen Verfahren ist aus heutiger Sicht zu weit gegriffen. Jedoch sind weitere Untersuchungen notwendig, denn nach derzeitigem Wissen scheint die veränderte Reaktion sowohl substanz- als auch artspezifisch zu sein. Bei einer größeren Datenbasis ließe sich vermutlich eher ein Zusammenhang für einzelne Pestizidgruppen erschließen, die zu besonders drastischen Effekten unter veränderten Bedingungen führen. Dieses Wissen könnte wiederum in die Zulassung oder Anwendungsempfehlung von Pflanzenschutzmitteln einfließen und wird von Wissenschaftlern und politischen Entscheidungsträgern gleichermaßen als notwendig erachtet (EC 2012; Stahl et al. 2013; Wenning et al. 2010).

2.2.2 Terrestrische Halbfreilandverfahren

In dem vorgestellten Forschungsprojekt wurde erstmals in einem komplexen TME-Experiment die Interaktion eines Pestizids und der Bodenfeuchte untersucht. Die Einstellung der Bodenfeuchte erwies sich als große Herausforderung. Während sich das portugiesische Experiment an realen Niederschlagswerten der Region orientierte, wurde in Deutschland versucht, den Feuchte-Konditionen aus den Laborversuchen möglichst nahe zu kommen. Die Herangehensweise aus Portugal bietet den Vorteil, dass die eigentliche Feuchte nicht betrachtet wird, sondern das

Bewässerungsregime den klimatischen Faktor darstellt. Letztendlich zeigte sich aber, dass die Bewässerungsregime zu relativ geringen Unterschieden zwischen den tatsächlichen Bodenfeuchte-Werten innerhalb des Versuchszeitraums führten. Zwar sind im deutschen Experiment die Unterschiede zwischen den Feuchte-Behandlungen deutlicher, aber durch Schwankungen innerhalb einer Behandlung dennoch weit von der Kontinuität von Laborbedingungen entfernt. Aus diesem Grund wurden die TME-Versuche, die vor Ort stattfanden, stets weiter entwickelt und optimiert.

Zunächst wurden Bodenfeuchte-Sensoren und Logger (ecoTech Umwelt-Meßsysteme GmbH, Bonn; HYDRA Sonden mit FDR-Messprinzip) beschafft, die die Bodenfeuchte in jedem einzelnen TME kontinuierlich alle sechs Stunden erfassten. Durch unterschiedlichen Bewuchs, der auch durch die Pestizide beeinflusst wurde, kam es zu individueller Evapotranspiration, was, verbunden mit der ungleichen Beschaffenheit der Wurzel- und Regenwurmgänge, eine spezifische Bewässerung erforderte. Durch die aktuellen Messwerte war die Bewässerung individuell an jeden TME angepasst. Einschränkend muss erwähnt werden, dass Bodenfeuchte sich sehr kleinräumig sowohl horizontal als auch vertikal ändert und die Sensoren nur einen beschränkten Ausschnitt des TME widerspiegeln. Nichtsdestotrotz wurde durch die individuelle Betreuung eine deutlich besser eingestellte Bodenfeuchte und Differenzierung zwischen den einzelnen TME-Gruppen erreicht.

Aufgrund des hohen Betreuungsaufwandes der TME-Experimente durch die tägliche manuelle Bewässerung war die Versuchsdauer auf acht bzw. 16 Wochen beschränkt. Um auch langfristige Effekte untersuchen zu können, wurde ein PC-Programm geschrieben, das die Messwerte der Logger verwendet und über die Differenz zum Feuchte-Sollwert die nötige Bewässerungsmenge errechnet. Diese wurde dann alle drei Stunden über Düsen automatisch auf die TME-Oberfläche gesprüht (Abbildung 7). Auf diese Weise konnten die Feuchtebedingungen exakt eingestellt und über einen Versuchszeitraum von zwölf Monaten aufrechterhalten werden.

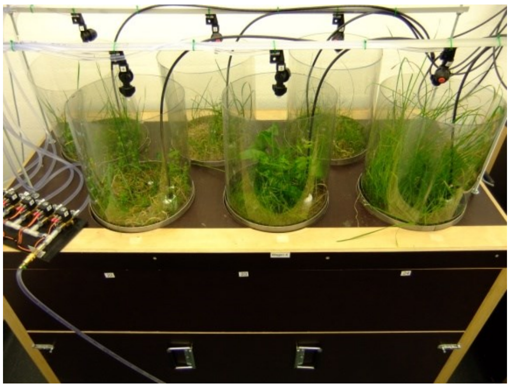

Abbildung 7: Aufbau eines TME-Versuchs in der Mesokosmenhalle von BiK-F, Frankfurt am Main. Sechs TME mit Feuchtesensoren und Sprühköpfen für eine automatisierte Bewässerung.

Für künftige TME-Experimente, in welchen die Bodenfeuchte als Faktor integriert werden soll, empfiehlt es sich somit, die Bodenfeuchte kontinuierlich zu überwachen, um etwaige Effekte auf Organismen besser zuordnen zu können.

In den bisherigen TME-Versuchen wurden entweder Rohre mit 18 oder mit 30 cm Durchmesser eingesetzt. Beide Typen bieten Vor- und Nachteile, die bedacht werden sollten: Die kleinen TME sind in experimenteller Hinsicht „Einwegsysteme“, das heißt, nach einer einmaligen Beprobung steht nicht mehr genug Material an der Oberfläche zu Verfügung, um eine weitere Probenentnahme durchzuführen (= destruktiver Ansatz). Die großen Rohre ermöglichen eine wiederholte Probenahme des gleichen Endpunkts innerhalb eines TME über die Versuchsdauer, allerdings nur für Proben mit kleinen Volumina (d.h. Mikroorganismen und Mesofauna). Bei letzterer Variante ist zu beachten, dass die entnommenen Proben eines einzelnen TME nicht als Replikate in die statistische Auswertung eingehen dürfen, da sie nicht unabhängig voneinander sind (Pseudoreplikation).

In der veröffentlichten TME-Studie (Annex I.IV) waren in dem deutschen Experiment alle Enchytraeen-Arten unter allen Feuchtebehandlungen wesentlich sensitiver als die EC_{50}-Werte aus dem Reproduktionstest im Labor (Annex I.III.) hätten erwarten lassen. Natürlich beruhen Vergleiche zwischen Labor- und Freilandtoxizitätsdaten immer auf Annahmen und Verallgemeinerungen, wie beispielsweise die Umrechnung von Aufwandvolumen/Fläche im TME-Versuch auf Konzentration/kg Boden im Laborversuch. Des Weiteren wird außer Acht gelassen, dass eine andere Bodenart zu einer veränderten Exposition führen kann und die Effekte des Pestizids auf Prädatoren oder Nahrung sich ebenfalls auf die untersuchten Testspezies auswirken können. Trotzdem steht die Frage im Raum, ob die Standard-Testsysteme mit Stellvertreter-Organismen, wie sie in der

Pflanzenschutzmittelzulassung angewandt werden, mit ihren Einzelart-Tests im Labor protektiv genug sind, um alle potentiell auftretende Nichtziel-Organismen zu schützen. Andererseits ist – provokant gefragt – auch darüber nachzudenken, ob der Schutz der sensitivsten Art im Freiland überhaupt das Ziel ist, solange sich die Population sensitiver Organismen bis zur erneuten Applikation erholen kann oder die jeweilige Bodenfunktion durch weniger empfindliche Arten erhalten bleibt. Letztendlich kann aber nur bei bekannter Sensitivität verschiedener Arten eingeschätzt werden, ob die Ausbringung einer Substanz ein Risiko für die Bodenorganismengemeinschaft darstellt oder nicht. Bis jetzt gibt es nur eine Richtlinie für Regenwurmfeldstudien (ISO 2014), die verschiedene Arten einer Organismengruppe untersucht, aber kein standardisiertes Testsystem, das die Effekte auf mehrere Arten oder gar auf eine ganze Artengemeinschaft berücksichtigt. Halbfreilandtests sind bisher nicht als Richtlinie verfügbar oder international standardisiert. Moe et al. (2013) sehen Mesokosmen-Experimente als ein wichtiges Instrument an, insbesondere für die Erforschung klimatischer und chemischer Interaktionen. Allerdings weisen sie auch auf die offenen Fragen bezüglich der statistischen Auswertung hin. Die Möglichkeiten und die in der Literatur beschriebenen statistischen Auswerteverfahren werden im Annex I.IV. beschrieben und diskutiert. Für aquatische Halbfreilandversuche existiert eine Anleitung der OECD (2006). Bis heute gibt es Bemühungen, auch für Versuche mit terrestrischen Modellökosystemen einen international gültigen Standard nach OECD oder ISO zu schaffen. Van Voris et al. (1985) entwarfen ein Testprotokoll, was später zu einer Richtlinie der US-Umweltschutzbehörde umgewandelt wurde (EPA 2012). Innerhalb Europas jedoch blieb es, trotz einer Vielzahl von Veröffentlichungen, bisher bei Seminaren und Veröffentlichungen mit Handlungsempfehlungen (UBA 1994, Schäffer et al. 2010). Darüber hinaus wurde ein umfangreicher Ringtest initiiert, der TME-Versuche an vier Standorten innerhalb Europas bei gleicher Methodik und Auswerteverfahren beinhaltete (Knacker et al. 2004, Sonderausgabe Ecotoxicology). Die Notwendigkeit einer Richtlinie für terrestrische Halbfreilandtests wurde in Fachkreisen insbesondere wegen des europäischen Zulassungsverfahrens für Pflanzenschutzmittel immer wieder betont (EC 2002; EC 2012; Schäffer et al. 2008; Toschki et al. *in prep*). Auch aus wissenschaftlicher Sicht ist eine Richtlinie wünschenswert, um mittels vergleichbarer Testsysteme die Sensitivität verschiedenster Organismengruppen und ihrer Arten zu erfassen (siehe oben) und künftige Studien anzuregen. Diese sollte einerseits neue statistische Methoden berücksichtigen, da zum einen in den letzten Jahrzehnten im Zuge immer besserer Rechenleistung auch mehrfaktorielle bzw. multivariate Methoden weiter entwickelt wurden (Beckerman 2014; Wang et al. 2012). Andererseits sollte eine Richtlinie die vielen Möglichkeiten an Endpunkten, mikrobielle, faunistische ebenso wie funktionelle, die ein TME-Experiment bietet, abdecken.

2.3 Einordnung der Ergebnisse in prognostizierte Szenarien

2.3.1 Klimatische Faktoren

Die Labortests wurden bei zwei Temperaturen durchgeführt, 20 und 26°C. Die erhöhte Temperatur orientierte sich an dem vierten Sachstandsbericht der IPCC, der im schlimmsten Fall von einem globalen Temperaturanstieg von 6,4°C ausging (Meehl et al. 2007). Der aktuelle, fünfte Sachstandsbericht prognostiziert „nur noch" einen maximalen Anstieg um 4,8°C bis 2100 (IPCC 2014). Auch wenn dieser Wert geringer ist, ist eine Bodentemperatur von 26°C in den belebten, oberen Bodenschichten keineswegs unrealistisch oder unwahrscheinlich. Zum einen werden Pflanzenschutzmittel für eine Anwendung in Südeuropa unter den gleichen Zulassungsbedingungen wie oben beschrieben getestet. Außerdem werden solche Temperaturen und noch wesentlich höhere auch heute schon in Mitteleuropa im Sommer in den belebten oberen Bodenschichten erreicht (Abbildung 8).

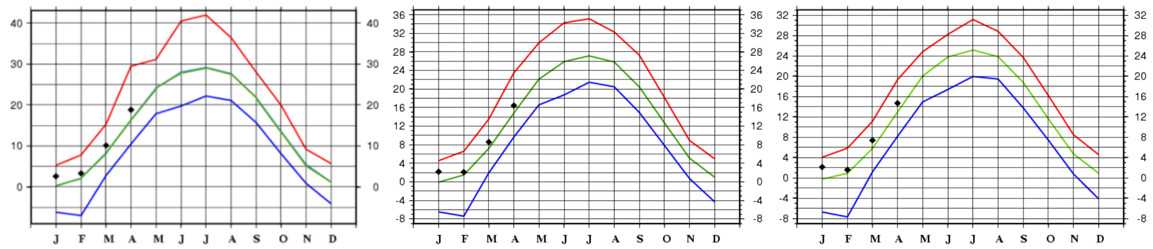

Abbildung 8: Temperatur (°C) in 2, 5 und 10 cm Bodentiefe in einem Sand-Braunpodsol im mittleren Jahresgang von 1895-2014 (PIK 2015). Grün: Monatliche mittlere Bodentemperatur; Rot: höchster Monatsmittelwert; Blau: tiefster Monatsmittelwert; zusätzliche Punkte: monatliche Mittelwerte von 2015. Skalierungen der y-Achsen unterscheiden sich.

Die Beurteilung der Bodenfeuchte in zukünftigen Szenarien ist wesentlich komplexer als die Abschätzung der Temperatur, nicht zuletzt, weil die Feuchte noch mehr als die Temperatur an die jeweiligen Bodeneigenschaften gekoppelt ist. Ob ein Boden dazu neigt, auszutrocknen oder ob Wasser nach einem Starkregenereignis versickert oder lange in den Bodenporen verbleibt, hängt in erster Linie mit dessen Korngrößenverteilung und damit der jeweiligen Bodenart zusammen. Bestimmt wird diese Bodeneigenschaft am ehesten anhand der Feldkapazität, also der Fähigkeit des Bodens, Wasser gegen die Schwerkraft in seinen Poren zu halten. Für gestörte (z.B. gesiebte) Bodenproben oder artifizielle Kunstböden, wie sie in ökotoxikologischen Laborversuchen eingesetzt werden, hat sich der Begriff der maximalen Wasserhaltekapazität (WHK_{max}) etabliert. Neben diesen beiden Kenngrößen gibt es noch weitere, die den Bodenwassergehalt angeben (nutzbare Feldkapazität, Saugspannung, pF-Wert, Wassergehalt [$\%_{vol}$], Wassergehalt [$\%_{m}$], relative Luftfeuchte [%]). Diese Einheiten sind teilweise nicht ineinander umzurechnen, was einen Vergleich zwischen verschiedenen Experimenten erschwert. Zudem ist die Bodenfeuchte kein Standard-Parameter, der im Rahmen von Monitoring-Programmen auf den Bodendauerbeobachtungsflächen erfasst wird. Jedoch wird in der Nähe Leipzigs seit 2009 bei einer Messstation des Deutschen Wetterdienstes (DWD) die Bodenfeuchte halbstündlich auf einem Wiesen-Standort aufgezeichnet. Der am Standort vorzufindende Bodentyp ist Parabraunerde, die Bodenart in den oberen 10 cm ist als sandig-toniger Lehm angegeben (Lehmann 2012). Die Bodenfeuchte wird mit der gleichen Methode erfasst, die auch in den späteren TME-Versuchen eingesetzt wurde (FDR-Sonden). Diese Werte sollen einen Anhaltspunkt geben, welchen Bodenfeuchteschwankungen die Mesofauna über das Jahr ausgesetzt ist (Abbildung 9). Bei Frost liefern die FDR-Sonden keine verlässlichen Werte, sodass bei der Datenaufbereitung die Messreihen der Tage, deren Durchschnittstemperatur kleiner 0,5°C war, aus der Grafik ausgeschlossen wurden.

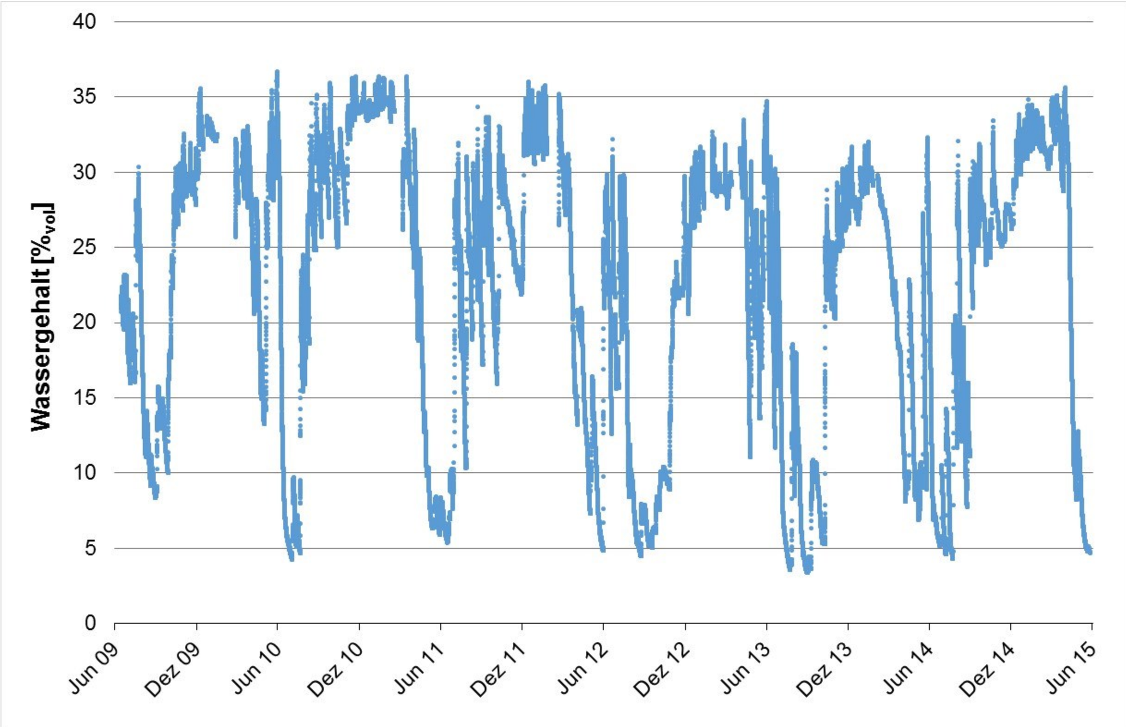

Abbildung 9: Volumetrischer Wassergehalt in 0-10 cm Tiefe in einem Wiesenstandort der DWD-Messstation Leipzig-Holzhausen. Messwerte von Tagen (*n* =176) mit einer Durchschnittstemperatur < 0,5°C wurden entfernt.

In den Laborexperimenten wurde Kunsterde nach OECD verwendet, und entsprechend zwischen 30 und 70%$_m$ der WHK_{max} befeuchtet. Um Masseprozent in Volumenprozent umzurechnen, wird von einer Dichte der Kunsterde von 1,1 g/cm³ ausgegangen (de Silva und van Gestel 2009; Garcia et al. 2008). Aufgrund des unterschiedlichen Torfanteils betrug die WHK_{max} in den Collembolentests etwa 50%$_m$ und in den Enchytraeentests etwa 60%$_m$. Dementsprechend lagen die Volumenprozente, auf die die Kunsterde eingestellt wurde, zwischen 13,6 und 38,2%$_{vol}$. Die vom DWD real gemessenen Bodenfeuchtewerte in der Wiese schwankten in den letzten sechs Jahren zwischen 3,4 und 36,7%$_{vol}$. Demnach stellten die Laborversuche ein realistisches Szenario dar, wobei der untere Extremwert der Feuchtewerte an Freilandstandorten sogar noch ausgeprägter ist.

2.3.2 Chemische Faktoren

Die niedrigste EC_{50} für Lambda-Cyhalothrin, die in dem Forschungsprojekt erfasst wurde, entstammt aus dem Enchytraeen-Labortest (Annex I.III). Unter simultanem Trockenstress (30%$_m$ WHK) war die Reproduktion von *Enchytraeus bigeminus* bei 1,34 mg a.s./kg dw Boden um 50% im Vergleich zur Negativ-Kontrolle reduziert. Die EC_{10} lag unter diesen Bedingungen bei 0,21 mg a.s./kg dw. Für die Risikobeurteilung

werden EC_{10}-Werte wie NOEC-Werte betrachtet und für die Berechnung des „Toxicity Exposure Ratio“ (TER, Verhältnis von ermittelter Toxizität zur erwarteten Exposition) herangezogen (EC 2003). Bei einer Datengrundlage resultierend aus chronischen Labortests gilt ein TER von >5 als akzeptabel. In den Publikationen (Annex I.I, I.II) wurden unter vereinfachenden Annahmen bezüglich Bodendichte und Verteilungstiefe eine zu erwartende Umweltkonzentration (PEC, predicted environmental concentration) von 0,010 mg a.s./kg dw nach Ausbringung der Formulierung Karate Zeon errechnet. Dabei wurde davon ausgegangen, dass das Insektizid einmalig in der höchsten zulässigen Aufwandmenge appliziert wird und die komplette Substanzmenge in die obere Bodenschicht gelangt. Dieser Annahme folgend, beträgt das Verhältnis von niedrigster EC_{10} und der genannten Umweltkonzentration 21. Ein Risiko für die getesteten Bodenorganismen (Enchytraeen und Collembolen) ist daher unwahrscheinlich.
Jedoch können in der behördlichen Risikobeurteilung eines Pestizids noch weitere Faktoren, wie z. B. Kultur und damit verbunden die Applikationshöhe und Abdrift, die Anzahl an Applikationen und deren zeitlicher Abstand sowie der pflanzliche Deckungsgrad, bei der Bestimmung der PEC berücksichtigt werden. In einer Risikobeurteilung von Lambda-Cyhalothrin wurde eine PEC von 0,078 mg a.s./kg dw kalkuliert (BVL 2011). Auf Basis dieser PEC beträgt das ermittelte Verhältnis für den TER 2,7. Ein Risiko für Enchytraeen kann somit nicht vollständig ausgeschlossen werden. In den Collembolenversuchen lag der niedrigste ermittelte EC_{10}-Wert bei 0,74 mg a.s./kg dw. Von einem Risiko für Collembolen ist auf Grundlage dieser Daten nicht auszugehen.
Der niedrigste für Pyrimethanil erfasste EC_{50}-Wert stammte aus dem TME-Versuch. Dort war die Abundanz von *Fridericia connata* nach achtwöchiger Exposition bei Trockenheit bei 3,48 mg a.s./kg dw halbiert. Die EC_{10}-Werte wurden in diesem Experiment nicht eigens ermittelt. Die vereinfachte PEC, wie sie in den Veröffentlichungen genannt wurde, beträgt 1,33 mg a.s./kg dw. In der offiziellen Risikobeurteilung wird von einer PEC im Boden von 1,1 mg a.s./kg dw ausgegangen (EFSA 2006). Das Verhältnis zwischen Toxizität und Exposition liegt in diesem Fall somit zwischen 2,6 und 3,2, was für die chronische Exposition, noch dazu für einen Halbfreilandtest, ein relativ geringer Wert ist. In einem Reproduktionstest mit einem anderen Oligochaeten, dem Kompostwurm *Eisenia fetida*, wurde ein Verhältnis von 3,75 ermittelt, worauf die europäische Behörde für Lebensmittelsicherheit (EFSA) einen Langzeit-Versuch unter Feldbedingungen mit Regenwürmern empfahl (EFSA

2006). Leider ist diese Studie nicht öffentlich zugänglich. Auf Grundlage eigener Ergebnisse und der Literaturdaten kann daher ein Risiko für Oligochaeten nicht ausgeschlossen werden.

2.4 Klimafolgen in Böden: Weitere Prognosen

Wie in den vorigen Kapiteln bereits erwähnt, können die Veränderungen, die mit dem Klimawandel einhergehen werden (1.2 Klimawandel in Böden: Beobachtungen), zu Veränderungen in Böden (einschließlich des Abbaus von Chemikalien) und zu klimatischem Stress für Bodenorganismen führen. Der Zusammenhang zwischen den Einflüssen des Klimawandels auf Böden und Bodenorganismen einerseits und die Pestizide andererseits wird in Abbildung 10 veranschaulicht.

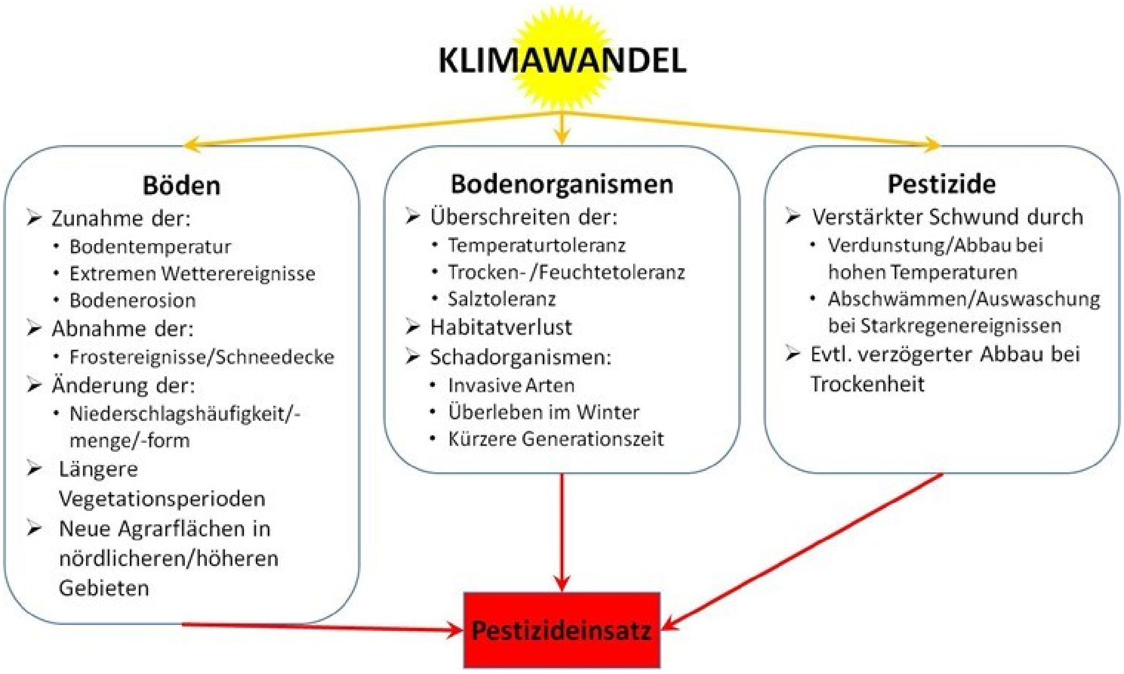

Abbildung 10: Die Einflüsse des Klimawandels auf Bodenorganismen und Böden in einer schematischen Darstellung unter Berücksichtigung der Auswirkungen auf den künftigen Einsatz von Pestiziden. Grafik modifiziert nach Schiedek et al. (2007).

Die genannten Faktoren von Temperatur und Niederschlag wirken sich zunächst direkt auf die Böden und die Lebensbedingungen darin aus. So wird sich die Vegetationsperiode verlängern, die im Frühjahr eher beginnt und im Herbst später endet (DWD 2015b). Zudem haben sich in der nördlichen Hemisphäre Flora und Fauna in den letzten 50 Jahren pro Dekade 6,1 km nordwärts beziehungsweise um 6,1 Höhenmeter nach oben bewegt (Thuiller 2007). Dadurch entstanden

Agrarflächen, die früher gar nicht bewirtschaftet werden konnten oder heute den Anbau neuer, thermophiler Kultursorten ermöglichen.

Neben dem bereits diskutierten Überschreiten der Temperatur- und Feuchtetoleranz kann auch durch starke Verdunstung an der Bodenoberfläche und verstärkten kapillaren Aufstieg eine Versalzung des Bodens eintreten, was zum Übersteigen der Salztoleranz von Bodenorganismen führen kann. Einzeln, aber vor allem in Kombination, können diese Stressoren zum Habitatverlust einzelner Arten führen (Schiedek et al. 2007). Im Gegensatz dazu kann eine Invasion neuer Arten erfolgen. In diesem Zusammenhang ist auch eine Ausbreitung invasiver Agrarschädlinge zu befürchten, wie z.B. die Tabakmottenschildlaus *Bemisia tabaci*, ein Wirtstier von pflanzenschädlichen Viren, das sich von den Küstenregionen des Mittelmeeres in mediterrane Länder ausbreitet (Gilioli et al. 2014). Aber auch die heimischen Schädlinge könnten sich bedingt durch den Klimawandel besser vermehren und/oder ausbreiten, was zu einer Verschärfung des Schaddrucks führen würde. Aufgrund milderer Winter und der damit verbunden geringeren Anzahl an Frosttagen (Kovats et al. 2014) überleben Schädlinge wie Insekten und Schnecken eher. Außerdem verkürzen höhere Temperaturen den Lebenszyklus von poikilothermen Organismen, sodass innerhalb einer Vegetationsperiode mehrere Generationen an Schädlingen heranwachsen würden.

Der Klimawandel wird sich dementsprechend auch auf den Pestizideinsatz auswirken (Abbildung 10). Einerseits werden sich Veränderungen bei Ziel- und Nichtzielorganismen ergeben. Andererseits beeinflussen klimatische Faktoren Pflanzenschutzmittel in ihren physikalisch-chemischen Eigenschaften. So ist zum Beispiel denkbar, dass bei höheren Temperaturen eine Substanz eher abgebaut wird oder verdunstet. Eine hohe Bodenfeuchte könnte ebenfalls den Abbauprozess beschleunigen oder Starkregenereignisse wasserlösliche Komponenten eines Pestizids auswaschen. Diese Einwirkungen auf Pestizide können deren Wirksamkeit verringern und damit zu verstärktem Chemikalien-Einsatz führen (Noyes et al. 2009). Andererseits sei erwähnt, dass eine niedrige Bodenfeuchte, bedingt durch die geringere Aktivität von Bodenorganismen, eher zum Verbleib eines Pestizids im Boden führen könnte.

Die genannten Veränderungen, einerseits bei den Pflanzenschutzmitteln selbst, andererseits die Erhöhung des Schaddrucks, können in Zukunft zu einem erhöhtem Pestizid-Einsatz führen. Die Pflanzenschutzmittel würden dabei länger und häufiger während einer Vegetationsperiode verwendet (Balbus et al. 2013; Bindi und Olesen

2011; Noyes et al. 2009). Durch Ausbreitung invasiver Arten ist außerdem denkbar, dass neue Wirkstoffe in Europa eingesetzt oder entwickelt werden müssten. Der bereits genannte invasive Agrar-Schädling, die Tabakmottenschildlaus *B. tabaci*, wird beispielsweise mit Pyriproxyfen bekämpft (Qureshi et al. 2007), einem Wachstumsregulator, der in Deutschland derzeit nicht zugelassen ist.

Außer einem erhöhten Pestizideinsatz ist mit weiteren Veränderungen in der Landwirtschaft zu rechnen. Dazu gehören unter anderem eine frühere Aussaat, verbunden mit einer früheren Reife oder eine verstärkte Bewässerung im Sommer. Durch den Einfluss des Klimawandels auf Böden und die Bodenartengemeinschaft werden sich weitreichende Konsequenzen für die Bodenfunktionen ergeben. In Hinblick auf die Ökosystemleistungen können sich, bedingt durch die klimatischen Faktoren und Starkregenereignisse, Wachstumsbedingungen für Pflanzen und landschaftliche Kulturen verschlechtern und damit die Lebensmittelversorgung verknappen. Durch Starkregenereignisse ist außerdem die Gefahr einer Bodenerosion gegeben und, bei geringem Flurabstand, eine verringerte Filterleistung des Bodens möglich, was sich negativ auf die Grundwasserqualität auswirken kann (UBA 2011).

Gezielte Maßnahmen in der Agrarwirtschaft können jedoch dazu beitragen, die Auswirkungen des Klimawandels abzumildern. Hierbei ist zum Beispiel der Anbau widerstandsfähiger und standortgerechter Sorten mit einer hohen Klimatoleranz sowie einer geringen Anfälligkeit gegenüber Schädlingsbefall zu nennen, ebenso wie der Einsatz erosionsmindernder und überschwemmungstoleranter Arten (UBA 2008). Letztendlich sollten allerdings nicht die Maßnahmen gegen die Folgen des Klimawandels bezüglich Landwirtschaft, Hochwasserschutz, Wasserversorgung, Artenschutz etc. in den Fokus von Wissenschaft, Politik und Gesellschaft rücken, sondern die Maßnahmen gegen den Klimawandel selbst.

3. SCHLUSSFOLGERUNGEN

Auf die untersuchten Hypothesen und die Ergebnisse der Experimente soll im Folgenden eingegangen werden:

- Lambda-Cyhalothrin und Pyrimethanil beeinflussen das Überleben und die Reproduktion von Bodenorganismen in umweltrelevanten Konzentrationen.
    - *Ohne zusätzlichen klimatischen Stressor wurden weder in den Laborexperimenten noch im TME-Versuch Effekte in umweltrelevanten Konzentrationen festgestellt.*
- Temperaturanstiege, wie sie durch den Klimawandel zu erwarten sind, wirken sich negativ auf kühl-adaptierte Boden-Organismen aus.
    - *In den Kontrollen der Reproduktionstests zeigte* F. candida *keine Reaktion auf eine höhere Temperatur, somit ist die Hypothese abzulehnen.*
- Trockenheit beeinflusst Bodenorganismen auch ohne chemischen Stressor negativ.
    - *Richtig, in den Kontrollen aller Labortests verringerte sich die Anzahl der Juvenilen unter Trockenheit.*
- Höhere Temperaturen verstärken den toxischen Effekt chemischer Stressoren auf kühl-adaptierte Organsimen.
    - *Diese Hypothese wurde verifiziert: Bei höherer Temperatur reagierte* F. candida *sensitiver auf beide Testsubstanzen.*
- Trockenheit intensiviert den chemischen Stress.
    - *In den Laborversuchen und dem TME-Versuch mit Pyrimethanil konnte diese Hypothese bestätigt werden. Gegenüber Lambda-Cyhalothrin reagierte nur* E. bigeminus *sensitiver bei simultanem Trockenstress.*
- Die Interaktion von klimatischen und chemischen Faktoren stellt bei zunehmendem Klima- und Landnutzungswandel ein potentielles Risiko für Bodenorganismen dar.
    - *In Laborversuchen waren die durch den klimatischen Stress verstärkten Effekte der Testchemikalien durch die in der Risikobeurteilung von Pestiziden üblichen Sicherheitsfaktoren abgedeckt. Im TME-Versuch konnte dagegen ein Risiko für Enchytraeen durch die Interaktion von Pyrimethanil und gleichzeitigem Trockenstress nicht ausgeschlossen werden, da bei diesem komplexen Testverfahren die Wirkung größer war als der Sicherheitsfaktor.*

Abschließend lässt sich feststellen, dass die im Rahmen der Dissertation durchgeführten Experimente geeignet waren, um die aufgestellten Hypothesen zu untersuchen. Der Nachweis für die Interaktion von klimatischen und chemischen Stressoren ist sowohl durch die Labor- als auch die Halbfreilandversuche erfolgt. In Laborversuchen waren die Effektkonzentrationen durch die Interaktion multipler Stressoren bis zu sechsmal kleiner als bei der Exposition gegenüber dem chemischen Stressor allein. Allerdings scheinen die Auswirkungen klimatischer Faktoren auf Chemikalieneffekte art- und substanzabhängig zu sein, sodass diesbezüglich weitere Forschung wünschenswert ist, um mögliche Zusammenhänge zu erkennen.

Die Ergebnisse der TME-Versuche belegen die prinzipielle Eignung dieses Testsystems, um eine Risikobeurteilung für Pestizide unter verschiedenen Bedingungen durchzuführen. Vier Replikate erscheinen sinnvoll, um ein notwendiges Maß an Stabilität und Vertrauenswürdigkeit der Freilanddaten zu erhalten. Acht Konzentrationen sollten mindestens abgedeckt sein, um belastbare Konzentrationswirkungsbeziehungen und damit verlässliche Effektkonzentrationen generieren zu können. Es wird empfohlen, dass, unter Einbeziehung von Vorschlägen aus der Literatur, die hier gemachten Erfahrungen für die Entwicklung einer internationalen TME-Richtlinie genutzt werden.

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Annex

I. Publikationen als Bestandteil der Thesis

I.I. INTERACTIVE EFFECTS OF LAMBDA-CYHALOTHRIN, SOIL MOISTURE, AND TEMPERATURE ON *FOLSOMIA CANDIDA* AND *SINELLA CURVISETA* (COLLEMBOLA)

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INTERACTIVE EFFECTS OF LAMBDA-CYHALOTHRIN, SOIL MOISTURE, AND TEMPERATURE ON *FOLSOMIA CANDIDA* AND *SINELLA CURVISETA* (COLLEMBOLA)

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Abstract: The authors investigated whether and how 2 environmental factors could influence the toxicity of a pyrethroid to 2 representatives of an important group of soil organisms. The impacts of different temperatures (20 °C and 26 °C) and soil moisture levels (30%, 50%, and 70% of water holding capacity) were investigated in combination with the insecticide lambda-cyhalothrin on the reproduction success of *Folsomia candida* and *Sinella curviseta* in a full factorial design. Testing was based on the standard collembolan reproduction test (Organisation for Economic Co-operation and Development, guideline 232) following an effect concentration design. The results showed an effect of environmental and chemical factors on the number of juveniles of these animals. Particularly in dry soil, the reproduction of both species was reduced, while higher soil moisture levels influenced the number of juveniles positively compared with the middle soil moisture level. In general, however, higher soil moisture led to increased sensitivity to lambda-cyhalothrin. In both organisms, temperature affected the toxicity of the pesticide but in different directions: high temperature led to higher toxicity in *F. candida* but to lower toxicity in *S. curviseta*. *Environ Toxicol Chem* 2014;33:654–661. © 2013 SETAC

Keywords: Toxic effect Insecticide Springtail Environmental factor Factorial design

INTRODUCTION

According to the Intergovernmental Panel on Climate Change, (IPCC) we will be confronted with rising global temperatures in future decades. The IPCC has modeled various scenarios depending on human population growth and consumption of fossil resources. The worst-case projection, A1FI, foresees a temperature increase of 6.4 °C by 2100 [1]. Furthermore, the IPCC predicts a substantial decrease of summer precipitation (up to 30–45%) and an increase in winter precipitation (up to 15–30%) in central Europe [2]. Changes in precipitation may lead to changes in soil moisture content, but a detailed forecast is difficult to make, because such measurements are usually not included in soil monitoring programs for technical reasons (e.g., rapid changes over time or within short distances, depending on soil characteristics). A proliferation of intense rain may, at least temporarily, lead to higher soil moisture, while an increase of periodicity of precipitation may cause drought stress to soil organisms.

When exposed to these climate-related factors, organisms may react differently to chemicals compared with reactions in standard laboratory or current field conditions. To date, however, additional factors have not been taken into consideration in the environmental risk assessment of pesticides.

As part of the activities of the research center BiK-F (Biodiversity and Climate Research Centre, Frankfurt, Germany), the effects of pesticides have been investigated under varying climatic scenarios in different experiments. The final objective of the present study was to evaluate a specific aspect of global climate change on soil ecosystems by comparing the combined effects of a pesticide and varying soil moisture levels and temperatures on soil organisms, such as Collembola.

Collembola (springtails) represent the most common, abundant, and widespread microarthropods in many soils [3,4]. Temperature and moisture reflect 2 key ecological factors affecting populations of soil-inhabiting Collembola [4]. They feed on a large assortment of food, mostly fungal hyphae. Collembolans are detritivorous and, thus, strongly involved in important functions of the soil organism community, in particular the decomposition of organic material [3].

Because collembolans are important representatives of soil mesofauna, several ecotoxicological guidelines have been developed with springtails, in particular with *Folsomia candida* (Isotomidae) [5–7]. This species has turned out to be an ideal candidate for standardized tests because it is easy to culture under laboratory conditions and reproduces parthenogenetically (such that gender ratio is not an issue in these reproduction tests). Therefore, comparatively large amounts of data for this organism are already available concerning the effects of chemicals such as metals [8], (veterinary) pharmaceuticals [9], or pesticides [10,11]. *Sinella curviseta* (Entomobryidae, Collembola) is listed among others as an alternative species in Organisation for Economic Co-operation and Development (OECD) guideline 232 [6]. *S. curviseta* and *F. candida* colonize similar habitats, and co-occurrence has been reported [12]. The epedaphic species *S. curviseta* prefers higher temperatures compared with the euedaphic *F. candida* [13–15]. Additional factors such as soil moisture or pesticides may influence the co-occurrence of these species. Literature studies [16,17] suggest that the complete range of interactions may occur between climatic and chemical influence, and they also present several examples of combined effects on ecotoxicological test organisms and underline the unpredictable implication of several stressors.

To evaluate possible interactions between environmental factors and a pesticide, a multifactorial laboratory study was conducted. As a model substance, the pyrethroid insecticide

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lambda-cyhalothrin (CAS number 91465-08-6) was investigated in reproduction tests with *F. candida* and *S. curviseta*. Lambda-cyhalothrin was chosen as a test substance because much data concerning mode of action, degradation, and so on are available [18], which helps to interpret the results of the present investigation. Because of its mode of action as a sodium-channel modulator, it disrupts signal transduction in the nervous system, leading to paralysis and death [19]. Furthermore, the half-life in soil of lambda-cyhalothrin extends across the applied test duration, and thus a continuous chemical exposure can be assumed. Finally, this insecticide is marketed and used globally in agriculture. We hypothesized that soil moisture and temperature could interact with the toxicity of lambda-cyhalothrin to *F. candida* and *S. curviseta*.

MATERIALS AND METHODS

Test organisms

Two species were chosen as test organisms: *Folsomia candida* (the "standard test species" and *Sinella curviseta. F. candida* is a parthenogenetic species occurring worldwide in caves, litter, and soils rich in humus. The other test species, *S. curviseta*, occurs in southeast Asia, especially in China; in the US northwest; and in parts of Europe in habitats similar to those of *F. candida*. The ecological optimum temperature for *S. curviseta* is considered to be 30 °C [13], whereas *F. candida* prefers 15 °C to 21 °C [14,15]. *S. curviseta* reproduces sexually, and parthenogenesis does not occur [20]. In the laboratory culture, the 2 species were kept separately in plastic vessels (approximately 200 mL) containing a layer of plaster of Paris (depth approximately 1 cm) and fed twice a week with dry baker yeast and a few drops of deionized water. There was no light in the climate chambers. To adapt the organisms to the 2 test temperatures, 2 cultures were established for each species at 20 ± 2 °C and 26 ± 2 °C, respectively, at least 16 mo prior to the test start.

Model substance lambda-cyhalothrin

Lambda-cyhalothrin contains 2 stereoisomers: (*S*)-α-cyano-3-phenoxybenzyl-(*Z*)-(1*R*.3*R*)-3-(2-chloro-3.3.3-trifluoroprop-1-enyl)-2.2-dimethylcyclo-propanecarboxylate and (*R*)-α-cyano-3-phenoxybenzyl-(Z)-(1*S*.3*S*)-3-(2-chloro-3.3.3-trifluoroprop-1-enyl)-2.2-dimethylcyclopropanecarboxylate. Under standardized laboratory conditions (20 °C and soil moisture of 40–60% water holding capacity), the mean half-life in soil is 56 d [18]. Lambda-cyhalothrin disrupts signal transduction in the nervous system, leading to paralysis and death [19]. Because of the high log octanol–water partition coefficient (K_{OW}) of 7.0, the substance tends to adsorb to organic matter in soil, which may lead to a reduced bioavailability in organic rich soils. Therefore, our reproduction tests followed the OECD 232 guideline [6], which instructs 5% peat usage as opposed to 10% peat as an indication by International Organization for Standardization (ISO) guideline 11267 [5]. Because cultivated soils rarely contain higher than 5% organic content, it recently became the scientific standard (also in environmental risk assessment) to use only 5% peat.

Water solubility of the compound is low (0.005 mg/L) [19]. In the present study, lambda-cyhalothrin was applied as Karate Zeon, a formulation distributed by Syngenta Crop Protection. The batch used in the present study contained 9.73% w/w corresponding to 103 g/L active substance, according to the certificate of analysis. Apart from lambda-cyhalothrin, propylene glycol (20–30% w/w), the solvent naphtha (5–10% w/w), and 1,2-benzisothiazol-3(2H)-one (0.05–0.1% w/w) are ingredients of Karate Zeon, pursuant to the safety data sheet [21]. All concentrations mentioned refer to the active substance (a.s.) of lambda-cyhalothrin and dry weight of soil. In Germany, the maximum single application rate for Karate Zeon is 75 mL/ha. This correlates to 7.73 g lambda-cyhalothrin/ha. Under the default assumptions used in European Union regulatory procedures (bulk soil density of 1.5 g/cm^3 and average incorporation depth of 5 cm [22]), this application rate would lead to a soil concentration of 10.3 μg a.s./kg dry weight soil. This conversion allows comparison of the expected environmental concentrations of lambda-cyhalothrin to the effect concentrations from the laboratory tests. The reproduction tests were performed with 1 negative control in untreated soil and 10 concentrations of lambda-cyhalothrin, starting from 0.125 mg a.s./kg dry weight soil up to 64 mg a.s./kg dry weight soil (nominal values) with a spacing factor of 2. The concentration range was selected based on previous range-finding tests (data not shown).

For chemical analysis of the highest concentration (64 mg a.s./kg dry wt soil), 1 additional vessel was prepared for each reproduction test. At the beginning and at the end of the reproduction test, 2 subsamples from this vessel were stored in a deep freezer until chemical analysis via gas chromatography with electron capture detection. The analysis was performed at Technologie Zentrum Wasser. The limit of quantification was determined as 5 μg a.s./kg dry weight soil. The loss in percentage of the initial concentration was calculated from the difference between the initial and the final measured concentrations (Supplemental Data, text and Figure S1).

Experimental testing

All tests were performed according to OECD 232 [6] under a 16 h/8 h light regime. The artificial soil consisted of 5% peat, 20% kaolin, 74% to 74.9% quartz sand, and 0.1% to 1% calcium carbonate to adjust the pH value to 6.5 ± 0.5. The maximum water holding capacity was determined as described in Annex 5 of OECD 232 [6]. In the tests with *F. candida* (20 °C and 26 °C) as well as in the test with *S. curviseta* (20 °C), the water holding capacity was 49.2%. In the test with *S. curviseta* (26 °C), the water holding capacity was 46.3%. To achieve the targeted moisture (in % w/w of water holding capacity), the soil was premoistened with the respective volume of deionized water minus the application volume of 10 mL, which was mixed into the soil at the test start. An additional vessel per treatment was prepared to assess the pH and the soil moisture at day 0 and day 28. The pH values were measured in calcium carbonate (0.01 mol/L), and soil moisture was determined gravimetrically. Once per week, moisture loss caused by evaporation was compensated with additional water until the initial weight was achieved again. At the beginning and in the middle of the experiment, collembolans were fed with a spatula tip of dry baker yeast (Dr. Oetker). All tests were performed in glass vessels containing 30 g of moist soil and covered with parafilm to reduce moisture loss. After 4 wk, the tests were terminated and the soil was floated with water. With the help of black ink, the water was colored to obtain a contrast to the animals, which were floating on the surface of the water. Via a stereomicroscope, individuals were counted to determine the mortality of the introduced springtails and the number of juveniles. The validity criteria of the reproduction test as defined in OECD guideline 232 [6] were as follows: adult mortality <20% and number of juveniles >100 per test vessel with a coefficient of variance <30%.

A pretest was conducted to identify the optimal age of *S. curviseta* individuals at the start of the test, which enabled us to meet the validity criteria required by OECD 232 [6]. Twenty individuals were put into each test vessel. The pretest was conducted with 5 groups (2 replicates each) of synchronized *S. curviseta* that differed in age at test start (10 d, 15 d, 20 d, 25 d, and 30 d). Individuals in a test group did not differ in age by more than 24 h.

The definitive reproduction tests were performed according to OECD guideline 232 [6] as well. To identify interactive effects between chemical and climatic factors, the soil was moistened to 30%, 50%, and 70% of the water holding capacity. These 3 moisture levels were combined with the above-mentioned 10 concentrations of lambda-cyhalothrin plus negative control. Two replicates were initiated per treatment, resulting in 66 vessels per definitive test. The tests with the 2 different species ran separately under 2 different temperatures, $20 \pm 2\,^{\circ}C$ and $26 \pm 2\,^{\circ}C$, which resulted in 4 reproduction tests in total. In accordance with OECD guideline 232 [6], 10 females of *F. candida* at age 9 d to 12 d taken from a synchronized culture were used per vessel in the reproduction tests. Because it is not possible to identify the gender of living *S. curviseta* morphologically, 20 individuals were put into each test vessel, assuming an even distribution of both genders (i.e., leading to 10 females per vessel). Based on the results of the pretest (Supplemental Data, Figure S2), *S. curviseta* individuals were used with an age of 20 d to 23 d. The experiments aimed to study the effects on the number of juveniles, but the mortality was assessed as well. The median lethal concentrations (LC50s) are presented in the Supplemental Data, Table S1.

Statistical analysis

All statistical analyses were conducted with R 2.13.1 [23] and the drc package [24]. A 3 parametric log-logistic function (LL2.3) was used to fit concentration–response curves referring to the number of juveniles, resulting from the 4 different reproduction tests

$$f_{(x)} = \frac{d}{1 + exp^{b(log[x]-e)}}$$

where x is the nominal concentration, b is the parameter relating to the slope at the inflection point, d is the upper limit (related to maximum reproduction), and e is the natural logarithm inflection point (equals the median effect concentration on the number of juveniles [EC50]).

The iterative model was fitted assuming a Poisson error distribution for the continuous reproduction data. Effect concentrations leading to 20% and 10% fewer juveniles (EC20 and EC10, respectively) and confidence intervals (95%) for all point estimates were also determined by the ED function using the fls method in drc.

The 3 fitted parameters were analyzed separately in linear models to identify their dependency on temperature, moisture, species, and interactions thereof. All parameters in these analyses were weighted by the reciprocal of their standard error. Starting with the full model (*parameter* ~ temperature × moisture × species), a stepwise backward-elimination procedure was applied [25]. In this procedure, the least significant factor combination was removed based on an analysis of variance until the minimum significant model was obtained (level of significance set to 0.05).

For the evaluation of measured test concentrations, it was assumed that the loss of substance was independent from the collembolan species. Hence, the tests with *F. candida* and *S. curviseta* were regarded as replicates to give more statistical power. To identify a possible influence of temperature and moisture on the fate of lambda-cyhalothrin, the loss of the pesticide was analyzed in a 2-way analysis of variance with the software Statistica (version 10). The results and their discussion can be found in Supplemental Data, *Chemical analysis*, and Figure S1.

The binomial data of survival were assessed in a 2 parametric log-logistic function (LL.2) with R 2.13.1 [23] and the drc package [24] to calculate the LC50 values (Supplemental Data, Table S1).

RESULTS AND DISCUSSION

Validity of definitive tests

At the beginning and at the end of a test, the pH value and soil moisture were measured as described in section *Experimental testing*. The pH ranged between 5.3 and 6.4. Even though the guideline demands a pH of 5.5 to 6.5, this difference can be regarded as negligible. The deviation of temperature during the tests did not exceed $2\,^{\circ}C$, as specified [6]. Because soil moisture was another test variable beside the insecticide, these values are of particular importance. Because of the parafilm lid and the weekly remoistening, it can be assumed that the actual soil moisture was independent of other test factors such as species, temperature, and concentration of the test item. Hence, the mean values of all additional vessels set aside for the measuring of pH and soil moisture from the same soil moisture level were calculated ($n = 44$, size of statistical sample). At day 0, the means of the actual moisture levels in these vessels were 28.8%, 50.2%, and 68.3% of water holding capacity, respectively, which is a deviation of $<2\%$ from the nominal values. At the end of the test, the means of gravimetrically determined soil moisture in the same vessels were 27.4%, 46.7%, and 66.6% of water holding capacity. Hence, the maximum deviation from the nominal moisture level was 3.4%. The criterion of the guideline is 40% to 60% of water holding capacity, meaning the acceptable deviation is of greater magnitude than in the presented investigation. However, it should be mentioned that the statements concerning pH and soil moisture cannot be transferred to the test vessels because they could not be measured directly.

The results of the pretest showed that *S. curviseta* at 20 d plus at the test start produced more than 100 juveniles within 28 d and thereby fulfilled the validity criterion as given in the OECD guideline for *F. candida* [6] (Supplemental Data, Figure S2). Individuals older than 30 d were not examined because at that age they would have reproduced at least once already. In an OECD standard reproduction test [6] by Xu et al. [26] individuals of *S. curviseta* with an age of 10 d to 12 d produced a mean number of juveniles of only 73 ($n = 8$) in the controls, which supports our findings.

Under standardized conditions in the definitive tests (i.e., $20\,^{\circ}C$ and 50% of water holding capacity), the validity test criterion concerning adult mortality was passed in the experiment with *S. curviseta*. In the respective control group of *F. candida*, 70% instead of at least 80% of the initially introduced collembolans survived. However, this deviation is considered to be acceptable because the main end point of this test is reproduction.

The control groups of both species met the validity criterion concerning number of juveniles as required for standard laboratory conditions (i.e., $20\,^{\circ}C$ and 50% of water holding capacity) by OECD guideline 232 [6]. *F. candida* had, for example, a mean reproductive output of 423 juveniles, which fits into the broad range of 29 to 1244 juveniles ($n = 35$) that were found in the control groups of the OECD ringtest [27]. In the tests with *S. curviseta*, 263.5 juveniles were counted in the control group of 20 °C and 50% of water holding capacity on average, which meets the validity criterion as defined for *F. candida*.

Interaction in definitive tests

The concentration–response curves referring to the number of juveniles are depicted in Figure 1, separately for species and temperature. The resulting values for EC10 and EC20 of each treatment and their referring confidence intervals are presented in Table 1.

Steepness at inflection point. The slope at the inflection point indicates how drastic the effect of an increase of the model substance might be. The estimates of parameter b are presented in Table 1. The single factors moisture and temperature were not significant, whereas the factor species significantly influenced the slope of the concentration–response curves (Table 2). This means that when exposed to the EC50, the species react differently to a further increase of chemical stress. According to Figure 1, the data from reproduction tests with *S. curviseta* led to a steeper concentration–response relationship in comparison with *F. candida*. Weltje [28] hypothesized the opposite—that populations of single genotypes (such as those produced by parthenogenetical reproduction) will exhibit steeper concentration–response curves than genetically more diverse populations because of the greater genetically determined reaction norms in the more diverse populations.

In addition, a significant interaction was detected between the parameters moisture and species: the steepness of the concentration–response curves differed between the species, with this difference being dependent on soil moisture. The significant interaction between species and soil moisture indicates that, compared with *F. candida*, *S. curviseta* might be more sensitive to small variations in lambda-cyhalothrin when soil water content differs.

Maximum reproduction. The upper limit of the concentration–response curve reflects the response at control level (e.g., the maximum reproduction without any chemical stressor). The estimates for parameter d are shown in Table 1. The statistical analysis for the upper limit verified the dependence of reproduction success on soil moisture (see Table 2). This parameter significantly influenced the number of juveniles. This influence is clearly visible in the concentration–response curves (Figure 1). Reproduction in dry soil was very low throughout all tests, in comparison with medium and very moist soil. In most of the tests, the upper limit reached its maximum at 70% of water holding capacity. Generally speaking, higher soil moisture led to higher reproductive output. Van Gestel and van Diepen [29] tested *F. candida* at different soil moisture levels (38–102% of water holding capacity, $n = 5$), using 7-d-old to

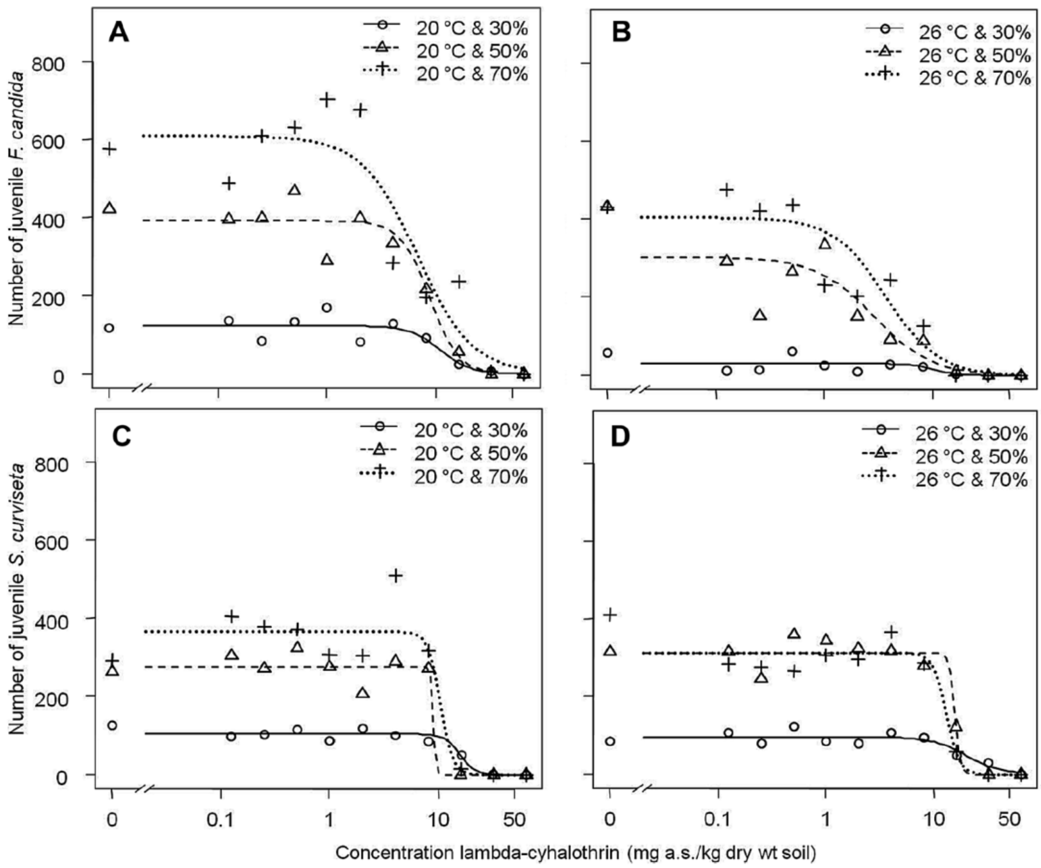

Figure 1. Number of juvenile *Folsomia candida* exposed to lambda-cyhalothrin (mg active substance [a.s.]/kg dry wt soil) under different soil moisture levels (percentage of water holding capacity) at (**A**) 20 °C ($n = 2$) and (**B**) 26 °C ($n = 2$). Number of juvenile *Sinella curviseta* exposed to lambda-cyhalothrin under different soil moisture levels at (**C**) 20 °C ($n = 2$) and (**D**) 26 °C ($n = 2$).

Table 1. Concentration leading to a 10% or 20% effect (EC10 and EC20, respectively; mg active substance/kg dry wt soil) with 95% confidence intervals of the reproduction test with *Folsomia candida* and *Sinella curviseta* exposed to lambda-cyhalothrin under different soil moisture levels (percentage of water holding capacity) at 20°C and 26°C ($n = 2$): Estimates of the parameters *b* (slope) and *d* (upper limit) and their standard errors calculated for each concentration–response curve

	EC10 (95% CI)	EC20 (95% CI)	*b* (SE) [a]	*d* (SE) [b]
F. candida 20 °C, 30% WHC	5.29 (4.21–6.37)	6.91 (5.77–8.04)	3.04 (0.27)	123.3 (3.04)
F. candida 20 °C, 50% WHC	4.39 (3.84–4.94)	5.64 (5.07–6.21)	3.24 (0.18)	391.8 (5.64)
F. candida 20 °C, 70% WHC	1.83 (1.57–2.09)	2.94 (2.60–3.28)	1.70 (0.05)	609.6 (8.05)
F. candida 26 °C, 30% WHC	6.19 (3.57–8.83)	7.49 (4.92–10.1)	4.23 (1.00)	29.39 (1.49)
F. candida 26 °C, 50% WHC	0.74 (0.55–0.92)	1.21 (0.97–1.45)	1.63 (0.08)	302.7 (6.90)
F. candida 26 °C, 70% WHC	1.06 (0.64–1.47)	1.66 (1.14–2.19)	1.79 (0.16)	405.2 (10.5)
S. curviseta 20 °C, 30% WHC	11.1 (9.27–12.9)	12.6 (11.1–14.1)	6.20 (1.09)	104.7 (2.59)
S. curviseta 20 °C, 50% WHC	8.72 (6.64–10.8)	9.06 (6.79–11.3)	38.3 (223)	277.2 (4.44)
S. curviseta 20 °C, 70% WHC	7.70 (7.03–8.37)	8.58 (7.93–9.23)	7.49 (0.52)	366.9 (5.12)
S. curviseta 26 °C, 30% WHC	9.02 (6.45–11.6)	12.1 (9.33–14.8)	2.77 (0.31)	95.63 (2.60)
S. curviseta 26 °C, 50% WHC	13.5 (8.66–18.2)	14.2 (10.7–17.7)	17.3 (31.8)	312.9 (4.42)
S. curviseta 26 °C, 70% WHC	9.50 (6.94–12.1)	10.7 (8.39–12.9)	7.08 (1.89)	312.9 (4.77)

[a]Estimates of the parameter slope and standard error.
[b]Estimates of the parameter upper limit and standard error.
CI = confidence interval; SE = standard error; WHC = water holding capacity.

10-d-old individuals in OECD soil with 10% peat. The highest reproduction was found in soils with a moisture level of 64% and 76% of water holding capacity, which corresponds with our results. Crouau et al. [30] tested 3 different moisture levels in a reproduction test (37%, 45%, and 53% of water holding capacity, $n =$ not specified), and they found this positive correlation as well; that is, the number of juveniles was highest in soil with the highest moisture content. Hence, the recommended soil moisture in the collembolan test guidelines [5–7] does not represent the ecological optimum of *F. candida*. The above-mentioned publications also indicate a reduced reproduction in dry soil, which was supported by all tests described in the present study. Unfortunately, no comparable data with reference to moisture preference or tolerance are available for *S. curviseta*. Because of the expected decrease of precipitation, both species might have difficulties in keeping a population constant in dry soil during a summer drought. Whether these fluctuations will fall within a normal seasonal cycle (i.e., ultimately ending up in the recovery of the populations) cannot be answered with the presented experiments.

The factor of temperature did not influence reproduction within its examined range (Table 2). Temperature effects on *F. candida* were examined by different authors (e.g., Hutson [31]), who found a reduction at 25 °C, referring to different end points (e.g., the period of oviposition or adult survival) in comparison with 20 °C. In the present study, however, negative effects were not observed at 26 °C. This might be a result of the long-term adaptation in the cultures at 20 °C and 26 °C.

The upper limits of *S. curviseta* were modeled as being in comparable magnitudes for 20 °C and 26 °C in medium moist and wet soil. However, there was a nonsignificant trend (slight increase at 26 °C) concerning reproductive output of *S. curviseta* in the control groups of 50% and 70% of water holding capacity. This corresponds with the few reference data found (e.g., Gist et al. [13]), which denoted the optimum temperature of *S. curviseta* at 30 °C.

Even though maximum reproduction did not differ between the species under the given test conditions (including different parental age at test start), a significant interaction of soil moisture and species was found (Table 2). This is linked to species-specific reactions to soil moisture: while *F. candida* profited

Table 2. Significant factors and factor combinations that influenced the shape of the concentration–response curves: Final versions of the backward-eliminated model for each parameter and the residuals of the model

Factor	*df*	Mean squares	*F*	Pr (>*F*)
Steepness at inflection point				
Moisture	1	6.19	1.84	0.217
Species	1	46.4	13.7	0.008
Temperature	1	8.98	2.66	0.147
Moisture × species	1	29.7	8.82	0.021
Residuals	7	3.37		
Maximum reproduction				
Moisture	1	59 715	76.9	<0.0001
Species	1	398	0.51	0.497
Temperature	1	3443	4.44	0.073
Moisture × species	1	5369	6.92	0.034
Residuals	7	776		
Toxicity of lambda-cyhalothrin				
Moisture	1	10.1	10.9	0.013
Species	1	28.1	30.1	<0.0001
Temperature	1	1.77	1.89	0.211
Temperature × species	1	10.8	11.6	0.011
Residuals	7	0.93		

df = degrees of freedom; Pr (>*F*) = *p* value.

enormously by increasing water content (especially at 20 °C), the response of *S. curviseta* was less strong by showing similar reproductive output at 50% and 70% water holding capacity. Furthermore, *F. candida* was more affected by drought stress in comparison with *S. curviseta*, especially at high temperatures. In this treatment (30% water holding capacity), the maximum reproduction of *F. candida* was calculated at 29 individuals, while that of *S. curviseta* was 96 (parameter d, Table 1). A study of Kærsgaard et al. [32] supports the enhanced sensitivity of *F. candida* to drought stress in contrast to *S. curviseta*. The authors exposed different collembolan species to various soil moisture levels (given in percentage of relative humidity) and classified *S. curviseta* as being considerably more resistant to desiccation than *F. candida*. If forecasts of long dry periods in summer materialize, *F. candida* may be less competitive than *S. curviseta*, especially when the temperature is high. On the other hand, this reflects only 1 possible scenario. In regions where precipitation events increase, *F. candida* may benefit in contrast to *S. curviseta*.

Toxicity of lambda-cyhalothrin. Average recovery of the highest nominal concentration of insecticide at the beginning of the tests was 77.4% (64.5%–87.2%). Taking into consideration that only data for this concentration are available, the presented results and statistical analysis are based on the nominal values.

The lowest EC10 resulted from the reproduction test with *F. candida*, conducted at 26 °C and soil moisture of 50% of water holding capacity. It was calculated with 0.74 mg a.s./kg dry weight soil, which is approximately 70-fold higher than the maximum application rate referring to 0.0103 mg a.s./kg dry weight soil. Hence, a risk for collembolans under field conditions is not expected under normal usage conditions.

The inflection point of the concentration–response curves is identical to the EC50—that is, the concentration that leads to a 50% effect and, hence, to 50% fewer juveniles compared with the untreated control. Similar to the other parameters, the estimates of the inflection points were investigated for their dependence on the fixed factors (Table 2). In Figure 2, the EC50 values and their confidence intervals derived from the 4 definitive reproduction tests are shown. Under standard conditions (20 °C and 50% of water holding capacity) *F. candida* and *S. curviseta* showed similar sensitivity to lambda-cyhalothrin, with the EC50 values differing less than 1.2-fold. Ecotoxicological data of lambda-cyhalothrin and collembolans exist in the literature only from no-observed-effect concentration (NOEC) or lowest-observed-effect concentration (LOEC) designed experiments. In accordance with the European Commission [33], we consider the EC10 as being comparable to NOEC values. The EC10 concerning number of juveniles of *F. candida* under standard conditions was calculated to be 4.39 mg a.s./kg dry weight soil (Table 1). In a reproduction test with *F. candida* according to ISO 11267 [5], a NOEC of 7.6 and a LOEC of 13 mg a.s./kg dry weight soil were determined by Heupel [34]. This is approximately twice as much as our EC10, but this value is difficult to compare with our results because it was determined in 10% peat, according to the ISO [5], in comparison with OECD 232 [6], which stipulates usage of 5% in artificial soil. An increased absorption of lipophilic substances to the organic material is probable, and thus bioavailability might have been reduced. Because cultivated soils rarely contain an organic content of more than 5%, a chemical testing soil with 10% peat may lead to underestimated toxicity.

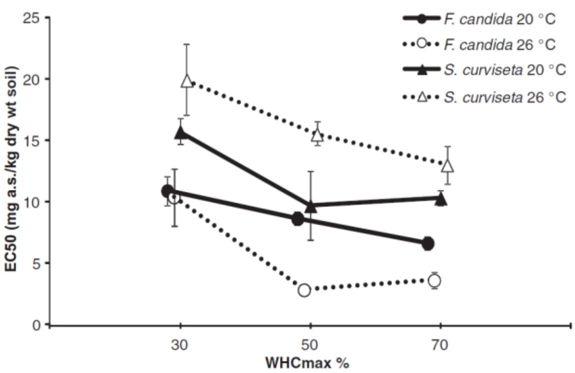

Figure 2. Interaction plot of median effect concentrations leading to 50% fewer *Folsomia candida* and *Sinella curviseta* juveniles (EC50; mg active substance [a.s.]/kg dry wt soil) and their confidence intervals against soil moisture (percentage of water holding capacity [WHC]); $n = 2$.

For both species, sensitivity to lambda-cyhalothrin was positively correlated with rising soil moisture. The EC50 values (as well as EC10 and EC20 values, see Table 1) were highest in dry soil. In the treatments with 50% and 70% of water holding capacity, the EC50 values were lower independent of species and temperature (no significant interaction of moisture with either species or temperature). The EC50 values calculated from tests with dry soil varied between 10.4 mg a.s./kg dry weight soil and 19.9 mg a.s./kg dry weight soil. For 50% water holding capacity, the EC50 ranged between 2.84 mg a.s./kg dry weight soil and 15.5 mg a.s./kg dry weight soil. In wet soil, they were calculated within 3.62 mg a.s./kg dry weight soil and 13.0 mg a.s./kg dry weight soil (Table 2). The reason for these results might be a reduced bioavailability of lambda-cyhalothrin in dry soil or a modified activity pattern of the collembolans. An investigation published by Sørensen and Holmstrup [35] examined the effects of drought stress on *F. candida* after previous exposure to another pyrethroid insecticide. The exposure to cypermethrin did not increase the sensitivity for drought stress. Other scientists found a strong relationship between chemicals and drought stress: Højer et al. [36] examined synergistic effects between 4-nonylphenol and different levels of relative humidity on *F. candida*. Exposure to 4-nonylphenol reduced the tolerance to drought stress, and reciprocally, the toxicity during drought stress was enhanced in comparison with the value obtained under optimal soil moisture conditions. Furthermore, Sjursen and Holmstrup [37] found an increased sensitivity of another collembolan species, *Protaphorura armata*, when it was exposed to pyrene at low relative humidity. All of the above-mentioned publications are based on investigations concerning air humidity. These values are only marginally comparable to our values, which refer to soil moisture. Because the latter has a more direct impact on the population dynamics of soil organisms, this parameter is more relevant when assessing impacts of climatic stress.

The reaction of the species clearly differed as well. Under identical treatment, the EC50 values of *F. candida* (calculated between 2.84 mg a.s./kg dry wt soil and 10.9 mg a.s./kg dry wt soil) were lower than those of *S. curviseta* (between 9.68 mg a.s./kg dry wt soil and 19.9 mg a.s./kg dry wt soil). To our knowledge, only 1 published article [12] has investigated the same species as in the present study. The authors of that study estimated various ecotoxicological data of the species after exposure to copper and found a higher sensitivity of *F. candida* in comparison with *S. curviseta* as well. However, a general conclusion on higher sensitivity of *F. candida* is premature because only 2 chemicals (1 metal and 1 organic pesticide) have been tested. Nevertheless, *F. candida* may be negatively impacted if exposed to chemical stressors under climatic stress (high temperature, low soil moisture) and, hence, is at a disadvantage under these circumstances compared with *S. curviseta*.

Temperature on its own did not significantly affect the sensitivity to lambda-cyhalothrin. Nevertheless, the increase of temperature from 20 °C to 26 °C led to an opposite response of the species: *F. candida* reacted more sensitively to the insecticide when exposed at a high temperature (up to a factor of 3 for EC50 and a factor of 6 for EC10). In comparison, *S. curviseta* was less sensitive to chemical stress at 26 °C (reduced by a factor of 1.6 for EC50). This aspect is responsible for the detected interaction between species and temperature. This means that the influence of temperature on the toxicity of the insecticide is species-specific. One reason is probably the different ecological temperature optimum. As mentioned above, *S. curviseta* is an epedaphic species with a preferred temperature of approximately 30 °C [13]. The euedaphic species *F. candida* has its optimal temperature between 15 °C and 21 °C [13,14]. Thus, it may be expected that chemical exposure leads to opposed effects under different temperatures. To date, there are very few available studies in the literature with Collembola concerning simultaneous heat and chemical stress and none on the effects of temperature and pyrethroids on soil micro-arthropods [16,17]. Nevertheless, it seems that *F. candida* might react more sensitively to chemical stress when exposed to increased temperature. In addition to the EC50 values, the LC50 values were calculated from the survival data for each factor combination. Most of them resemble the corresponding EC50 value or are even slightly lower. It could be argued that a mortality effect on adults obviously leads to a reduced number of juveniles. In a study of Jänsch et al. [38] the acute and chronic toxicity of lambda-cyhalothrin was investigated on the tropical isopod *Porcellionides pruinosus* in OECD soil using reproduction and mortality as end points, respectively. The EC50 and LC50 values were calculated to be 0.4 mg a.s./kg and 0.5 mg a.s./kg, respectively, which is similar to our findings. However, neither the test design of Jänsch et al. [38] nor the present study was intended to clarify whether the effects on number of juveniles are caused by adult mortality or juvenile mortality. Hence, the EC50 values should be regarded as an effect concentration on the population level.

Whether a population may be threatened by local extinction or recover after chemical or environmental stress depends on various factors, including the phenotypic plasticity, the frequency and duration of stress, and the reproduction strategy influence the potential of recovery. The examined species have different reproductive strategies. *F. candida* as a parthenogenetic species is a typical *r*-strategist with a shorter generation cycle in contrast to *S. curviseta*. If populations of both species in co-occurrence are affected by the same chemical or environmental stress, populations of *F. candida* may recover more quickly than those of *S. curviseta*.

Furthermore, *F. candida* is a euedaphic, while *S. curviseta* is an epedaphic, species. The latter could be expected to be more directly exposed to chemicals and varying environmental factors. The present study did not examine the potential of recovery of these species. However, a publication of Waagner et al. [39] demonstrated that *F. candida* has the potential to recover rapidly and completely after cessation of a 20-d drought. Coexposure to chemical stress may reduce this capacity of recovery, though such exposure schemes have not yet been investigated.

Further research is necessary, both on the level of individual species in the laboratory as well as semifield and field investigations. Based on these results and using population models, the effects of chemical and climatic change (especially soil moisture and temperature) interactions on soil organisms might become predictable.

CONCLUSION

The present study clearly demonstrated that the sensitivity of 2 collembolan species to a pyrethroid pesticide depends on environmental factors, most prominently on soil moisture. Our test results show that the toxicity (EC10) of such a pyrethroid can vary by up to a factor of 6 under differing climatic conditions. Such a change may alter the outcome of an environmental risk assessment. To date, however, it is not known how such alterations could be taken into account in the chemical registration procedure in Europe.

SUPPLEMENTAL DATA

Chemical analysis.
Table S1.
Figures S1 and S2. (973 KB DOC).

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I.II. INTERACTIVE EFFECTS OF PYRIMETHANIL, SOIL MOISTURE AND TEMPERATURE ON *FOLSOMIA CANDIDA* AND *SINELLA CURVISETA* (COLLEMBOLA)

Bandow, C., Karau, N. and J. Römbke (2014)

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Interactive effects of pyrimethanil, soil moisture and temperature on *Folsomia candida* and *Sinella curviseta* (Collembola)

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ABSTRACT

The aim of the presented investigation was to study effects resulting from specific aspects of climate change and chemical stress (individually or in interaction) on soil organisms. In detail, the interaction of different temperatures (20 °C and 26 °C) and soil moisture levels (30%, 50% and 70% of the water holding capacity) were examined in combination with the fungicide pyrimethanil on the reproduction of two Collembola species (*Folsomia candida* and *Sinella curviseta*). Testing was based on the standard collembolan reproduction test (OECD-Guideline 232), following an EC_X design. Low soil moisture led to a significant reduction of the juveniles in the control groups in contrast to medium or high soil moisture. Furthermore, the results showed a significant influence of both climatic factors on the toxicity of the fungicide. In general, both species reacted more sensitive when exposure was conducted in dry soil or at enhanced temperature.

1. Introduction

With reference to the fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC) we are faced with increasing global temperatures within the next decades. Depending on human population development and exploitation of fossil resources, IPCC modeled different scenarios. The worst case scenario, A1FI, forecasts a temperature increase of 6.4 °C by the year 2100 (Meehl et al., 2007). In connection with global climate change, IPCC also predict a shift of precipitation. A significant decline of summer precipitation (up to 30–45%) and an increase in winter precipitation (up to 15–30%) in central Europe is anticipated (Alcamo et al., 2007). Changes in precipitation may be linked to changes in soil moisture. However, a detailed prediction could not be given since such measurements are typically not integrated in soil monitoring programs due to technical reasons (e.g. its rapid changes within short distances or over time, depending on soil characteristics). A proliferation of intense rain events may cause higher soil moisture contents while a decrease of mean precipitation may lead to drought stress for soil organisms.

Soil organisms stressed by changes in soil moisture or temperature may respond differently to chemicals. This is particularly significant for plant protection products, since these chemicals are intentionally released to ecosystems targeting pest organisms, e.g. insects, pest plants or fungi. To exclude a potential risk for non-target organisms, e.g. earthworms, bees or collembolans, their environmental risk has to be assessed prior to market registration and laboratory tests, conducted in accordance to guidelines (e.g. OECD, ISO, etc.), are already requested. However, further potential stressors as they may occur due to climate change are not considered in the environmental risk assessment of pesticides to date.

As part of the research center BiK-F (Biodiversity and Climate Research Center, Frankfurt, Germany), one project aims to assess the effects of pesticides in combination with different climatic scenarios in several experiments. The main objective is to research one subset issue of global climate change on soil ecosystems by evaluating the combined effects of a pesticide and different soil moisture contents and temperatures on soil organisms, e.g. collembolans.

Collembolans (springtails) are one of the most prevalent and copious microarthropods of soil biocoenoses (Choi et al., 2006; Hopkin, 1997). Temperature and soil water content are ecological key factors that influence collembolan populations and their distribution (Choi et al., 2006). They live mainly on bacteria, dead organic material, fungal hyphae and fine roots. Hence,

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collembolans participate in essential soil functions, especially decomposition of organic material (Hopkin, 1997).

Since Collembola represent an important class of soil fauna, a couple of guidelines for chemical testing have been introduced using them. Firstly, *Folsomia candida* Willem, 1902 (Isotomidae) (ISO, 1999, 2011; OECD, 2009) is proposed as a "standard" test species based on its easy handling in the laboratory, cultivability, data availability and ecological relevance. The handling of this candidate is simple due to its' parthenogenetically reproduction strategy and hence, sex ratio is not relevant in a reproduction test. Many tests with different chemicals have already been performed with *F. candida*, for instance metals (e.g. Nakamori et al., 2008), (veterinary) pharmaceuticals (e.g. Noël et al., 2006) and pesticides (e.g. Campiche et al., 2007; Chernova et al., 1995).

As an alternative to *F. candida*, the species *Sinella curviseta* Brook, 1882(Entomobryidae) is proposed in OECD (2009) among further species. It is sexually reproducing and occurs in comparable habitats as *F. candida* (e.g. in the Zhejiang province (China); Xu et al., 2008). *S. curviseta* prefers higher temperatures in contrast to *F. candida* (Gist et al., 1974; Jänsch et al., 2005; Fountain and Hopkin, 2005). If temperature rises due to global climate change, it could be assumed that thermophilic collembolans (as e.g. *S. curviseta*) may prevail over collembolans that prefer cooler temperatures (as e.g. *F. candida*). Additional factors, both climatic and chemical, may affect this coexistence.

In order to examine a potential interaction of these factors, a multifactorial reproduction experiment with collembolan was performed. The fungicide pyrimethanil was selected as a model substance. Even though effects through pyrimethanil were investigated on arthropods, ecotoxicological data on Collembola are not available. However, data regarding for instance, degradation and fate in soil, mode of action, etc. are published (European Commission, 2005), which may be helpful to discuss the outcome of these experiments. Furthermore, with a half-life of 56 days, a near-constant exposure can be assumed during the 28 days lasting reproduction test. Finally, pyrimethanil is a broad spectrum fungicide with global use in agriculture.

To summarize, the main aim of this investigation was to answer, whether and how environmental factors impact the toxicity of the fungicide against the two selected collembolan species.

2. Material and methods

2.1. Test organisms

For the reproduction tests two species were selected as test organisms: *Folsomia candida* (the "standard test species") and *Sinella curviseta* (the "alternative collembolan species"). The parthenogenetic *F. candida* prefers an optimum temperature of 15–21 °C. Its clones are distributed worldwide in soils rich in humus and litter (Jänsch et al., 2005; Fountain and Hopkin, 2005). The second test species *S. curviseta*, is an obligate sexual reproducing organism (Waldorf, 1971). It colonizes similar habitats as *F. candida* and is found in Southeast Asia, North and Central America as well as parts of Europe (www.collembola.org). Its temperature optimum is 30 °C (Gist et al., 1974). The two species were cultured separately in plastic containers (ca. 200 mL) onto a 1 cm layer of plaster of Paris. The cultures were kept in the dark and fed twice a week with dry baker yeast and a few drops of deionized water. In order to acclimatize the collembolans to the two test temperatures, two cultures of each species were established at 20 ± 2 and 26 ± 2 °C, respectively.

2.2. Model substance pyrimethanil

The model substance pyrimethanil (CAS: 53112-28-0; IUPAC: N-(4,6-dimethylpyrimidin-2-yl) aniline) is a broad spectrum fungicide that belongs to the group of anilino-pyrimidines (mode-of-action: methionine biosynthesis inhibition). It restrains the secretion of hydrolytic enzymes of the fungi (FAO/WHO, 2007). Under aerobic laboratory conditions (20 °C) half life in soil was determined between 27 and 82 days with a mean of 56 days (European Commission, 2005). The water solubility (121 $mg/L_{25\,°C,\,pH\,6.1}$) is moderate as well as log K_{OW} ($2.84_{25\,°C,\,pH\,6.1}$) and hence, accumulation neither in biota nor in soil is expected (European Commission, 2005). In this study, pyrimethanil was applied as formulation "SCALA", distributed by BASF SE, Germany. This suspension concentrate contains 404.6 g/L active substance according to the certificate of analysis. Besides pyrimethanil, no further hazardous substances are ingredients of "SCALA" (BASF, 2005). All concentrations declared in this study refer to the active substance [a.s.] pyrimethanil and dry weight [dw] of soil. In Germany, the maximum single application rate for "Scala" is 2.5 L/ha in strawberry cultures (Federal Office of Consumer Protection and Food safety, 2009), i.e. 1011.5 g pyrimethanil per hectare. To allow a comparison of the effects from laboratory tests, this application rate has to be transformed into mg a.s./kg dw soil. To simplify the calculation, a soil density of 1.5 g/cm^3 and an average soil depth of 5 cm is assumed in EU regulatory procedures (European Economic Community, 2007). Thus, the maximum application rate complies with 1.35 mg a.s./kg dw soil. The reproduction tests were conducted with one negative control in untreated soil and 11 concentrations of pyrimethanil, starting from 0.98 up to 1000 mg a.s./kg dw soil (nominal values) with a spacing factor of two. The concentration range was chosen based on prior range finding tests (data not shown).

For chemical analysis, one extra vessel containing the highest concentration (1000 mg a.s./kg dw soil) was arranged for each experiment. At day 0 and day 28, two sub samples of this vessel were stored deep frozen for chemical analysis via HPLC/DAD. The chemical analyses were conducted at TZW (Technologie Zentrum Wasser) in Karlsruhe, Germany. The limit of quantification was assessed to be 0.5 mg a.s./kg dw soil.

2.3. Experimental testing

All tests were conducted as described in OECD (2009) with a 16/8 h light/dark regime. Ten female *F. candida* with an age between 9 and 12 days were used in the experiments. Due to the findings of Bandow et al. (2014) 20 individuals of *S. curviseta* were used with an age of 20–23 days. As it is not possible to recognize the sexes of the living individual of *S. curviseta* morphologically, 20 individuals were inserted into each test vessel, supposing an even distribution of both sexes due to randomization (i.e. achieving a comparable amount of females per test vessel).

The experiments with the two different species were performed independently at two different temperatures, 20 ± 2 and 26 ± 2 °C, resulting in four reproduction tests all in all.

The OECD soil contained 5% peat, 20% kaolin, 74–74.9% quartz sand and 0.1–1% calcium carbonate to regulate the pH value to 6.5 ± 0.5. The maximum water holding capacity (WHC) was determined according to Annex 5 of OECD (2009). In the tests with *S. curviseta* as well as in the test with *F. candida* conducted at 20 °C, the WHC was 49.2%. In the test with *F. candida* (26 °C), the WHC was 45.6%. To attain the desired soil moisture content (in % w/w of WHC), the soil was pre-moistened with the respective volume of deionized water less than the application volume of 10 mL, which was blended into the soil at the beginning of the test. In order to identify interactive effects between chemical and climatic factors, the soil was finally moistened to 30%, 50% and 70% of the water holding capacity, respectively. These three moisture levels were combined with the above mentioned 11 concentrations of pyrimethanil plus the negative control. Two replicates were used per treatment, resulting in 66 vessels per test. In the test with *S. curviseta* at 26 °C, only one vessel per treatment combination was initiated due to a lack of synchronized individuals. The experiments were conducted with glass vessels that were covered with parafilm. In general, one additional vessel per treatment combination was prepared to determine the soil moisture content and the pH at the test start and end of the test. The pH was assessed in calcium carbonate (0.01 mol/L), soil moisture content was measured gravimetrically. Once per week moisture loss due to evaporation was adjusted with deionized water until the initial weight was established again. At day 0 and day 14, a spatula tip of dry baker yeast (Dr. Oetker, Germany) was added as food to each test vessel. After 28 days the experiments were completed and the soil was floated with water. Black ink was used to color the water in order to achieve a high contrast to the pale collembolans that were on the surface of the water. With the help of a stereo microscope, individual animals were counted to assess mortality of the inserted collembolans and their reproduction, i.e. the number of juveniles. The effect concentrations regarding number of juveniles are presented in the following. The LC_{50} (median lethal concentration), which was calculated from adult mortality, is shown as Supplemental Data, Table 1. The validity criteria of the reproduction test as instructed by the OECD guideline 232 (2009) are an adult mortality less than 20% in the control. Furthermore, the mean number of juveniles has to exceed 100 per control vessel with a coefficient of variance below 30%.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2014.04.010.

2.4. Statistical analyses

The statistical analyses were performed with R 2.13.1 (R Development Core Team, 2011) and the drc package (Ritz and Streibig, 2005). A three parametric log-logistic function (LL2.3) was utilized to fit concentration–response curves of reproduction, resulting from the four different reproduction tests:

$$f(x) = \frac{d}{1 + \exp^{(b(\log(x)-e)}}$$

with x is the nominal concentration, b is the slope at inflection point; d is the upper limit (refer to maximum reproduction); and e is the ln inflection point (equals the median effect concentration, EC_{50}). The iterative model was fitted assuming a Poisson error distribution for the continuous reproduction data with many low number counts in replicates with high pyrimethanil concentrations. Additional effect concentrations EC_{20} and EC_{10} and confidence intervals (95%) for all point estimates were determined by the ED function applying the “fls” method in “drc” package (Ritz and Streibig, 2013).

Using the “drc” package again (Ritz and Streibig, 2013), the estimates of the three fitted parameters (b, d and e) were examined individually in linear models to discover their dependency on moisture, species, temperature and interactions thereof. All estimates of the parameters in these analyses were weighted by the reciprocal of their standard error. Starting with the full model (*parameter* ~ moisture*species*temperature), a stepwise backward-elimination procedure (Crawley, 2007) was used. In this procedure, the least significant factor combination was eliminated until the minimum significant model was achieved (level of significance $\alpha = 0.05$).

For the assessment of measured test concentrations in chemical analyses, we presumed that the fate of the test item was independent from the introduced collembolan species. Therefore, the test vessels of *F. candida* and *S. curviseta* were pooled as replicates to receive more statistical power. Again, a linear model with a stepwise backward-elimination was conducted. The model considered time, soil moisture content and temperature for a potential influence on the results of the chemical analysis (measured concentration ~ time*moisture*temperature). To achieve normal distribution, the data were transformed to their reciprocal values.

The binomial data of survival were assessed in a two parametric log-logistic function (LL.2) with fixed upper and lower limit (Ritz and Streibig, 2013) in order to calculate the LC_{50} values (Supplemental Data, Table 1).

3. Results and discussion

3.1. Chemical analyses

Mean recovery of the highest nominal concentration (1000 mg a.s./kg dw soil) at test start was calculated to be 101.8% (standard deviation = 24.4%, $n = 24$). The guideline does not request chemical analysis and therefore, no acceptable range is defined for terrestrial laboratory tests so far. It can be assumed however, that a deviation of less than 5% is acceptable for chemical analysis in soil. Hence, all results and statistical evaluations are based on the nominal values.

Within the test duration of 28 days no significant loss of pyrimethanil was observed. At the end of the tests a mean recovery of 100.7% (standard deviation = 12.6%, $n = 24$) of the nominal concentration was detected. This result is in line with the report of the European Commission (2005) that notified a significant retardation of degradation of high application rates.

Furthermore, the recovery of pyrimethanil was influenced neither by the climatic factors (temperature, soil moisture content) nor by a combination of the three factors (temperature: $F_{1,43} = 1.01$, $p = 0.320$; moisture: $F_{2,43} = 0.84$, $p = 0.440$; time: $F_{1,43} = 0.11$, $p = 0.738$). This could reveal the biological effects of the climatic impacts. Since there was no loss of pyrimethanil, enhanced effects are directly linked to the interaction of both chemical stress and climatic factors. However, it should be kept in mind that the chemical analysis was conducted only for the highest concentration. Lower concentrations could exhibit another environmental fate. But since the mean half life in soil is recorded to be 56 days (European Commission, 2005) and thence, twice as long as the test duration, a constant exposure may be assumed in all chemical treatments during the experiment.

3.2. Validity of the tests

At the start and at the end of a test, pH value and soil moisture content were determined as specified above. The pH values varied from 5.3 to 6.6. The variation of temperature during the experiments did not exceed 2 °C as required by OECD (2009). As soil moisture content was a further variable in addition to the chemical, the deviation to the nominal values should be minimized. Due to the Parafilm lid and remoistening once per week, the actual soil moisture content is considered to be independent from further test factors such as species, temperature and concentration of the test item. Consequently, the arithmetic mean of all gravimetrically determined values from one soil moisture level was computed ($n = 48$). At test start the means of the actual soil moisture contents were 29.3, 49.1 and 69.7% of WHC, respectively. The maximum divergence from the nominal moisture level was therefore less than 1%. At the end of the experiment, the means of soil moisture contents were 28.4, 48.5 and 68.5% of WHC and thus less than 2% from the nominal moisture level.

The validity criteria were only applied on the tests with 20 °C and 50% WHC soil moisture content, since they were defined for these test conditions. Regarding adult mortality, only *S. curviseta* fulfilled

Table 1
EC_{10} and EC_{20} values [mg a.s./kg dw soil] with their 95% confidence interval of the reproduction test with *Folsomia candida* and *Sinella curviseta* exposed to pyrimethanil under different soil moisture levels [% of water holding capacity (WHC)] at a temperature of 20 °C and 26 °C; $n = 2$; Estimates of the parameters *b* (slope), *d* (upper limit) and *e* (ln inflection point) and their standard errors calculated for each concentration–response curve.

Species	°C	% WHC	EC_{10} (CI)	EC_{20} (CI)	*b* (SE)	*d* (SE)	ln *e* (SE)
F. candida	20	30	31.7 (27–38)	33.1 (26–42)	18.5 (25.6)	50.9 (2.06)	3.58 (0.21)
		50	45.2 (22–92)	47.7 (26–87)	15.1 (16.7)	236 (4.11)	3.96 (0.21)
		70	41.4 (37–46)	46.2 (42–50)	7.44 (0.99)	490 (5.98)	4.02 (0.02)
	26	30	20.8 (17–25)	23.3 (20–27)	7.07 (1.50)	51.6 (2.09)	3.34 (0.04)
		50	16.1 (14–19)	20.9 (18–24)	3.07 (0.21)	300 (5.57)	3.49 (0.04)
		70	27.0 (25–30)	31.2 (29–34)	5.65 (0.34)	401 (5.82)	3.69 (0.03)
S. curviseta	20	30	43.5 (38–50)	49.9 (45–56)	5.85 (0.83)	107 (2.80)	4.15 (0.04)
		50	63.3 (58–70)	69.5 (64–75)	8.66 (0.71)	328 (4.80)	4.40 (0.03)
		70	59.6 (55–65)	66.1 (61–71)	7.86 (0.60)	290 (4.55)	4.37 (0.03)
	26	30	58.2 (43–79)	60.9 (53–69)	17.6 (33.9)	180 (5.07)	4.19 (0.09)
		50	60.3 (57–64)	64.3 (61–68)	12.7 (2.68)	353 (7.09)	4.27 (0.04)
		70	61.4 (56–68)	66.6 (61–73)	10.0 (1.18)	327 (6.81)	4.34 (0.04)

the requirements, while both species produced enough juveniles to reach the validity criterion of reproduction. In the test with *F. candida*, 185.5 juveniles were counted on average, which fits into the wide range of 29–1244 juveniles ($n = 35$) that were observed in the negative control groups of the OECD collembolan ringtest (Krogh, 2009). In the experiment with *S. curviseta*, an average of 308.5 juveniles were counted under the same conditions and thus more than necessary to meet the validity criterion as specified for *F. candida*. The coefficient of variation referring to the number of juveniles was less than 30% and hence, passed the validity criterion.

3.3. Interaction in the experiments

The concentration–response curves are illustrated in Fig. 1A–D individually for temperature and species. The respective parameters (slope, maximum reproduction and inflection point) of each curve as well as calculated effect concentrations (EC_{10} and EC_{20}) with their confidence intervals are obtainable in Table 1.

3.3.1. Steepness at inflection point

The steepness at the inflection point indicates how extreme the effect of an increase or decrease of the test substance might be. The estimates of this parameter (b) and their standard errors are presented in Table 1. The single factors moisture and temperature did not influence the steepness of the concentration–response curves significantly, whereas the factor species did significantly impact the slope (Table 2). Hence, when exposed to the EC_{50}, the two species react differently toward changes of the fungicide concentration. According to Table 1, the estimates show the tendency to be larger for *S. curviseta*. This is also visible in the Fig. 1A–D, where the data of reproduction tests with *S. curviseta* led to somewhat steeper concentration–response relationships in comparison to *F. candida*. Weltje (2003) stated that populations of single genotypes (like those of parthenogenetical reproduction) will show steeper concentration–response curves in contrast to genetically higher diverse populations, due to the greater genetically-determined reaction norms in the more diverse populations. In the presented investigation, the contrary occurred: the slopes of the concentration response curves from experiments with the parthenogenetic *F. candida* were less steep compared to the sexually reproducing *S. curviseta*. This finding was already observed in a prior study with these two species and lambda-cyhalothrin as a chemical stressor (Bandow et al., 2014). In that similar experiment, *S. curviseta* also showed a more drastic reaction in comparison to *F. candida* when exposed to somewhat lower or higher concentrations than the EC_{50}.

3.3.2. Maximum reproduction

The upper limit expresses the response at control level, e.g. the maximum reproduction without any chemical stressor. The estimates for this parameter (*d*) and their standard errors are shown in Table 1. The linear model for the upper limit confirmed the dependence of the number of juveniles on soil moisture content (see Table 2). This parameter highly significantly affected the reproduction success. That influence is well observable in the concentration–response curves (Fig. 1A–D). In general, the reproduction of both species was more successful in moist soil (50 and 70% of WHC), while the number of juveniles in dry soil (30% of WHC) was low throughout all tests. This effect may be directly linked to the soil moisture content itself but could also be caused by altering crumb structure or pore size distribution of the soil. However, it is impossible to distinguish this indirect influence from those of soil moisture. In a study of Van Gestel and van Diepen (1997) *F. candida* was examined at different soil moisture levels (38–102% of WHC, $n = 5$), using 7–10 day old collembolans in artificial OECD soil with 10% peat. The highest number of juveniles occurred in soils moistened to 64% and 76% WHC, which is in line with the findings of the present study. Crouau et al. (1999) investigated *F. candida* in a reproduction test according to ISO using artificial soil including 10% peat with a water content of 37, 45 and 53% of WHC (n = not specified). Again, the authors detected a positive correlation between mean population densities and soil moisture content. The forecited authors also found a low number of individuals in dry soil. Unfortunately, no data on ecological tolerance or preference regarding

Table 2
Significant factors and factor combinations that influenced the shape of the concentration-response curves; Results of reproduction tests with *Folsomia candida* and *Sinella curviseta* exposed to pyrimethanil in artificial soil (OECD guideline 232); Final versions of the backward eliminated model for each parameter and the residuals of the model; Df: Degrees of freedom, Mean Sq: Mean Squares; Pr(>*F*): *p*-value.

	Df	Mean Sq	*F* value	Pr(>*F*)
Steepness at inflection point				
Moisture	1	3.81	0.56	0.4741
Species	1	46.8	6.94	0.0300
Temperature	1	0.12	0.02	0.8979
Residuals	8	6.75		
Maximum reproduction				
Moisture	1	46,853	61.9	0.0001
Species	1	490	0.65	0.4475
Temperature	1	220	0.29	0.6063
Moisture*species	1	5569	7.36	0.0301
Residuals	7	757		
Toxicity of pyrimethanil				
Moisture	1	2.36	25.5	0.0023
Species	1	29.3	316	<0.0001
Temperature	1	2.61	28.2	0.0018
Temperature*species	1	1.39	15.0	0.0082
Moisture*species	1	0.56	6.00	0.0498
Residuals	6	0.09		

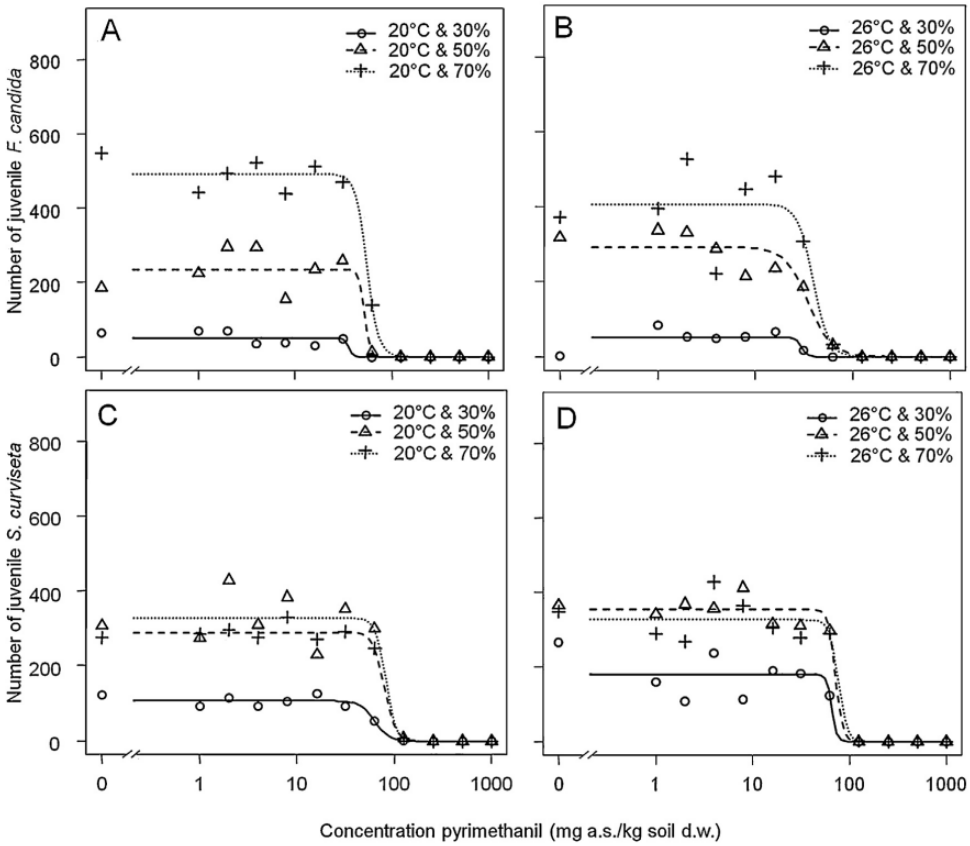

Fig. 1. (A) Number of juvenile *Folsomia candida* exposed to pyrimethanil [mg a.s./kg dw soil] under different soil moisture levels [% of water holding capacity (WHC)] at a temperature of 20 °C; $n = 2$. (B) Number of juvenile *Folsomia candida* exposed to pyrimethanil [mg a.s./kg dw soil] under different soil moisture levels [% of water holding capacity (WHC)] at a temperature of 26 °C; $n = 2$. (C) Number of juvenile *Sinella curviseta* exposed to pyrimethanil [mg a.s./kg dw soil] under different soil moisture levels [% of water holding capacity (WHC)] at a temperature of 20 °C; $n = 2$. (D) Number of juvenile *Sinella curviseta* exposed to pyrimethanil [mg a.s./kg dw soil] under different soil moisture levels [% of water holding capacity (WHC)] at a temperature of 26 °C; $n = 2$.

soil moisture exist in the literature for *S. curviseta*. However, even for *S. curviseta*, dry conditions do not seem to reflect the optimum. With regard to global climate change and the associated decrease of precipitation, both species, but especially *F. candida*, might have difficulties to produce enough offspring to keep a population stable in dry soils.

With regard to temperature, no significant influence on the maximum reproduction of collembolans was found. This might be due to the acclimatization to the respective temperatures during culturing. However, in two soil moisture treatments (30 and 70% of WHC) *F. candida* showed a tendency to prefer the cooler temperature of 20 °C. Effects due to enhanced temperature on *F. candida* were investigated by various scientists (Hutson, 1978; Snider and Butcher, 1973). They observed a decrease at 25 °C or 26 °C, with regard to different endpoints such as the period of oviposition, adult mortality, number of eggs and hatching success in comparison to 20 or 21 °C, respectively.

For *S. curviseta*, slightly higher upper limits of the concentration–response curves were modeled for a test temperature of 26 °C in contrast to 20 °C. This non-significant trend was observed in all soil moisture treatments. This fits to the few published data in literature. Gist et al. (1974) mentions a relatively high optimum temperature of 30 °C for *S. curviseta*, indicating a preference for warmer temperatures.

Even though the maximum reproduction did not differ between *F. candida* and *S. curviseta* under the given test conditions, a significant interaction between species and soil moisture content was detected. This is a result of the species-specific response toward soil moisture: in the experiments conducted with *F. candida* the upper limit reached its maximum in wet soil. In the tests with *S. curviseta*, such a clearly positive effect of high soil moisture was not noticed. Furthermore, *F. candida* was more impacted by drought stress in comparison to *S. curviseta*. These circumstances lead to the significant interaction of the factors soil moisture content and species.

If both species are subjected to global climate change within the same habitat, *S. curviseta* may not be affected as strongly as *F. candida*: *S. curviseta* tends to prefer warm temperature and furthermore, low soil moisture did not lead to extreme drought stress. On the other hand, the optimum temperature of *F. candida* might be exceeded and long, dry periods may lead to drought stress for this species and therefore, less resistance against environmental or anthropogenic factors. Nevertheless, it should be noted that this is only one supposable scenario among many others. If precipitation

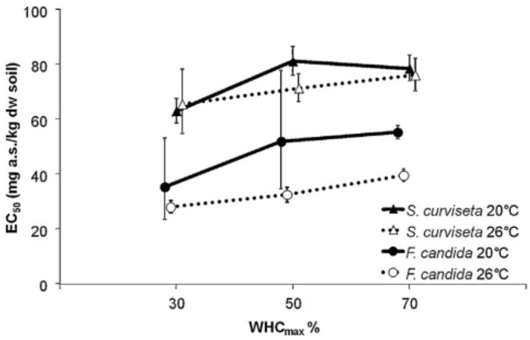

Fig. 2. Interaction plot: EC_{50} values [mg a.s./kg dw soil] and their confidence intervals against soil moisture [% of water holding capacity (WHC)]. Results of reproduction tests with *Folsomia candida* and *Sinella curviseta* exposed to pyrimethanil in artificial soil (OECD guideline 232). $n = 2$.

events increase, as is forecast for some regions, *F. candida* may benefit in comparison to *S. curviseta*.

3.3.3. Toxicity of pyrimethanil

The inflection point of a concentration–response curve is equal to the median effect concentration (EC_{50}), i.e. the concentration that causes a 50% effect and consequently 50% inhibition of maximum reproduction. In Fig. 2, the EC_{50} values and their referring confidence intervals resulting from the four reproduction tests are illustrated. As well as the other parameters, the estimates of the inflection points were examined for their dependence on the fixed factors (parameter *e*, Table 1).

The median effect concentration was significantly influenced by the soil moisture content (Table 2). For both species the highest EC_{50} values (as well as EC_{10} and EC_{20} values, see Table 1) were achieved in wet soil. The exception is the test with *S. curviseta* conducted at 20 °C: in this experiment, a slightly higher EC_{50} was recorded in medium moist soil. The EC_{50} values calculated in tests with dry soil ranged between 28.3 and 65.9 mg a.s./kg dw soil. For 50% WHC they were calculated within 32.9 and 81.5 mg a.s./kg dw soil and in wet soil between 39.9 and 78.9 mg a.s./kg dw soil. To simplify, the lower the soil moisture, the higher the toxicity. One cause might be due to a possibly higher concentration of the test item in the pore water in dry soil and accordingly a dilution of pyrimethanil in soil with high water content. Another reason might be an additive effect by the environmental factor inducing drought stress. Sørensen and Holmstrup (2005) studied the effects of drought stress on *F. candida* after preceding exposure to eight soil contaminants. Three of them affected the drought tolerance; one only weakly and the remaining four had no influence. None of their test substances has comparable characteristics to pyrimethanil, but that study shows the unpredictability of effects due to multiple stress.

The response of *F. candida* and *S. curviseta* were significantly different too (Fig. 2). When exposed to identical conditions the EC_{50} values of *F. candida* (computed between 28.3 and 55.6 mg a.s./kg dw soil) were conspicuously lower than those of *S. curviseta* (between 63.3 and 81.5 mg a.s./kg dw soil). This is confirmed by the linear model, which detected a significant influence by the factor species. So far, there have been only a few studies published that examined *F. candida* and *S. curviseta*. Xu et al. (2008) assessed a number of effects caused by copper on the two species. The authors also detected a higher sensitivity of *F. candida* in contrast to *S. curviseta*. In a study of Bandow et al. (2014) on interactive effects of lambda-cyhalothrin, *F. candida* turned out to be the more sensitive species as well. An overall statement on higher sensitivity of *F. candida* may not be drawn yet since only three chemicals have been tested so far (one metal and two organic pesticides). A reason might be the disparate characteristics of the two species: *F. candida* has a less hairy cuticle, *S. curviseta* has dense cover of setae and is generally an epigeic, possibly leading to different exposure. Under standardized test conditions (20 °C and 50% of WHC) there was a factor of about 1.6 between the EC_{50} values of each species. In literature only a few studies with pyrimethanil are available. Since they refer to aquatic insects, crustacean or earthworms, a comparison of ecotoxicological data is questionable.

The parameter temperature did influence the EC_{50} as well. The few published studies concerning temperature-dependent toxicity are rarely comparable to our investigation. Smit and van Gestel (1997) examined the influence of temperature (13–24 °C) on the toxicity of zinc in *F. candida*. The exposure time varied depending on exposure temperature: the higher the temperature, the shorter the test duration. Smit and van Gestel (1997) calculated increasing EC_{50} values with increasing temperature. That finding is contrary to the present study and might be due to the relatively low test temperatures among other obvious reasons like chemical properties and test design. Their highest exposure temperature (24 °C) was rather close to the published upper optimum of 21 °C (Fountain and Hopkin, 2005). Another study of Slotsbo et al. (2009) exposed *F. candida* to mercury for 24 h and afterwards to various temperature levels (20–35.5 °C). They observed a synergistic interaction between chemical effects and rising heat stress. Although that test design differed basically from the experiments in the present paper, it indicates that temperature may impact the toxicity of chemicals and/or sensitivity of the test organism. The test temperature of 26 °C led to higher sensitivity, especially in the case of *F. candida*: under the same soil moisture treatment all EC_{50} values are lower at high temperature (up to a factor of 2.8 for the EC_{10}). The EC_{50} values calculated from tests with *S. curviseta* did not show a definite trend. This species-dependent response toward temperature is responsible for the detected significant interaction of the factors temperature and species. This means that the influence of temperature for the toxicity of the fungicide is species-specific. The second significant factor combination was the interaction of species and soil moisture content. While *S. curviseta* did not show a clear preference on higher soil moisture (neither with nor without pesticide), *F. candida* profited enormously: in wet soil a higher maximum reproduction as well as a reduced sensitivity against pyrimethanil was observed. This inverse response of the two species toward soil moisture probably caused the examined interaction of these two factors.

In accordance with the European Commission the EC_{10} data could be considered as NOEC (No observed effect concentration) (European Commission, 2003). The lowest EC_{10} (16.1 mg a.s./kg dw soil) was detected in the experiment with *F. candida* conducted at 26 °C and 50% of WHC. When using pyrimethanil in agriculture, a maximum concentration of 1.35 mg a.s./kg dw soil may occur. Since this pyrimethanil concentration is more than 10 times below the EC_{10} a risk for collembolans under field conditions may not be expected.

In addition to the EC_{50}, the LC_{50} values were computed from the adult mortality of each single test (Supplemental Data, Table 1). All of them have similar size as the referring EC_{50} value, what might be due to the unspecific mode of action of the fungicide against Collembola. It could be argued that fewer adults due to mortality are apparently producing a lower number of juveniles. But a lower number of juveniles may also be caused by an acute effect on them. However, with this test design it is not possible to detect whether the effects on number of juveniles are caused by adult mortality or juvenile mortality. Hence, the EC_{50} values of the present study should be considered as effect concentrations on population level.

As mentioned above, the toxicity of pyrimethanil depended on all factors and factor combinations. Nevertheless it should be mentioned that the highest and lowest EC_{50} of each species differed by a factor less than 2. The greatest factor difference was observed for the EC_{10} values of *F. candida*. The EC_{10} under standardized laboratory conditions was about 2.8 times higher than the EC_{10} calculated at 26 °C and 50% WHC. One could argue that these factors are covered by uncertainty factors used in the environmental risk assessment of plant protection products (European Commission, 2002) and that the findings are negligible. Nevertheless in an experiment similar to the present but with another model substance, the effect concentrations differed up to a factor of 6 (Bandow et al., 2014). The different outcome of the studies may be caused by different modes of action, biosynthesis, bioavailability, degradation and thereby different toxicity through different internal and/or external exposure levels. In short, environmental factors can influence the toxicity of chemicals. This has been proved in several studies and reviews (Sørensen and Holmstrup, 2005; Holmstrup et al., 2010; Laskowski et al., 2010, etc.). How and to what extent this difference is, seems to be substance-dependent. A general conclusion may not be arrived yet.

4. Conclusion

When forced to low soil moisture, both collembolan species reacted with reduced reproduction. The impact through both environmental factors (soil moisture content and temperature) caused relatively slight shifts in the toxicity of pyrimethanil. Nevertheless they had a significant influence on the EC_{50}, a phenomenon already described in the literature for other chemicals. It is most likely that the dimension of impact is dependent on physical–chemical properties of the substance. So far, an interaction of environmental factors has not been examined within the chemical risk assessment, but might become even more relevant if global climate change accelerates.

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I.III. ENCHYTRAEUS BIGEMINUS (ENCHYTRAEIDAE, OLIGOCHAETA) AS A NEW CANDIDATE FOR ECOTOXICOLOGICAL LABORATORY TESTS

Bandow, C., Coors, A. and J. Römbke (2013)

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Enchytraeus bigeminus (Enchytraeidae, Oligochaeta) as a new candidate for ecotoxicological laboratory tests

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Abstract

In enchytraeid reproduction tests lambda-cyhalothrin and pyrimethanil were examined under three different soil moisture levels (30, 50 and 70% of the soil water holding capacity). The tests were performed with *Enchytraeus bigeminus* Nielsen & Christensen, 1963, a species that differs from other enchytraeid test species by its asexual mode of reproduction (fragmentation). The effect of chemical stressors depended on the soil moisture content. A higher toxicity was observed in soil with lower moisture levels. For lambda-cyhalothrin, the 21-day EC_{50} values for reproduction at the three levels of soil moisture were calculated to be 1.33, 3.79 and 4.75 mg active substance/kg dry weight soil, respectively. For pyrimethanil the values were 435, 499 and 829 mg active substance/kg dry weight soil. Apart from the evaluation of the combined effects of chemical stress and soil moisture, the appropriateness of the fragmenting test species *Enchytraeus bigeminus* was assessed. *E. bigeminus* tolerated temperature and pH variations, allowed obtaining reliable concentration-response relationships and was easy to handle and to culture in the laboratory. Hence this fragmenting species is considered to be suitable as an additional test species in ecotoxicological standard tests.

Keywords pyrimethanil | lambda-cyhalothrin | Clitellata | potworms | reproduction test | OECD

1. Introduction

Enchytraeids (Oligochaeta) are characteristic geobionts, involved in fertility and formation processes of many soils. In addition to their large relatives, the earthworms, they participate in the degradation of organic matter and in enhancing soil aeration and structure (Amorim 2005). Because of their importance in soil ecosystems, some representatives of this family have been chosen for standardized ecotoxicological laboratory test systems (ISO 16387 2003, OECD 220 2004). Test species are selected based on handling in the laboratory, cultivability, data availability and ecological relevance. The first choice and most common in chemical testing is *Enchytraeus albidus* Henle, 1837. Although it fulfils the first three criteria perfectly, its ecological relevance for ecotoxicological tests might be doubtful. It occurs mainly in decaying organic material, such as in the marine littoral or in compost (Schmelz & Collado 2010) and hence colonizes habitats that might not be representative for pesticide application. Both ISO and OECD guidelines recommend additional enchytraeid species, and the ones considered so far (*E. buchholzi* Vejdovský, 1879; *E. bulbosus* Nielsen & Christensen, 1963; *E. crypticus* Westheide & Graefe, 1992; *E. luxuriosus* Schmelz & Collado, 1999) have sexual reproduction in common. In this study, we explored the suitability of *Enchytraeus bigeminus* Nielsen & Christensen, 1963 as another possible species for ecotoxicological standard testing. *E. bigeminus* may reproduce sexually, but reproduces mostly by fragmentation. This might be advantageous because – due to its short generation cycle – test duration could be shortened and mass cultures can be initiated within a few days. Furthermore fragmenting species may offer a lower response variation due to genetic uniformity of the test population (Weltje 2003). However, effects of chemicals on sexual reproduction cannot be determined. In contrast to *E. albidus*, *E. bigeminus* colonizes habitats that are more representative for a possible pesticide usage. It belongs to the 'open landscape species' (Heck et al. 1999) and is found in lawns, grasslands and fields (Heck et al. 1999, Römbke et al. 2002).

To evaluate the advantages and limitations of *E. bigeminus* for chemical testing, two model substances were selected: lambda-cyhalothrin, an insecticide, and pyrimethanil, a fungicide. With these chemicals, reproduction tests according to OECD 220 (2004) were performed. The tests were conducted to examine the toxicity of the chemicals on the test species, but also to assess the effects of environmental factors. IPCC (Intergovernmental Panel on Climate Change) forecasts predict changes in temperature as well as in precipitation. The worst-case scenario forecasts a temperature rise up to 6.4°C by 2100 (Meehl et al. 2007). Furthermore a decline in summer precipitation (30–45 %) and an increase in winter precipitation (15–30 %) in central Europe is prognosed (Alcamo et al. 2007). Due to precipitation changes, a change in soil moisture may be expected. Therefore, we conducted the experimental tests at a wide temperature range of 19 to 27°C and at three defined levels of soil moisture: 30, 50 and 70 % of the maximum water holding capacity (WHC). Besides the effects of soil moisture on the toxicity of the pesticides, the tolerance of *E. bigeminus* against changing environmental factors is discussed.

2. Materials ans methods

2.1. Test species

The test species used in the present study was *E. bigeminus*. This species is not proposed as a possible candidate for ecotoxicological tests in the ISO and OECD guidelines and has thus far only rarely been used in laboratory studies (e.g. Christensen & Jensen 1995). It is one of six enchytraeid species known to reproduce mostly by fragmentation (Niva et al. 2012). Living individuals have a body length of between 0.5 and 15 mm (Schmelz et al. 2000). The species is probably common in Southern Europe and South America (i.e. Brazil), where it colonizes soils rich in organic matter (Schmelz & Collado 2010, Niva et al. 2012). It was also found in Iran (Dózsa-Farkas 1995). This might be an indication for a preference for warmer climatic zones. A list of records of *E. bigeminus* on a global scale is presented in Collado et al. (2012).

Christensen (1973) stated that its reproduction strategy depends on the density of individuals. At low densities it reproduces sexually, while at high densities only few mature individuals occur and reproduction by fragmenting strongly dominates. However, in the cultures used by us, clitellate individuals and cocoons were never observed. During fragmentation *E. bigeminus* divides into up to seven fragments. These need six days to regenerate a new anterior end and a further seven days for intermediary and posterior fragments at 20–22°C (Christensen 1964, Christensen 1973). The generation time of fragmenting *E. bigeminus* is comparatively short compared to the sexually reproducing test species proposed by OECD 220 (2004): 33 days (18°C) for *E. albidus* (Römbke & Moser 2002), 17.4 days (21°C) for *E. crypticus* (Westheide & Graefe 1992) and about 25 days (20°C) for *E. buchholzi* (Learner 1972). For *E. luxuriosus* and *E. bulbosus*, no data concerning life cycle are available. Therefore, the test duration was reduced to 3 weeks instead of the guideline's recommendation of 4 and 6 weeks, respectively.

The *E. bigeminus* culture was established without temperature control at 19–27°C. The animals were kept in plastic petri dishes containing a ca 5 mm layer of agar in constant darkness.

2.2. Model substances lambda-cyhalothrin and pyrimethanil

The reproduction tests were performed with two substances. They were selected according to the following criteria: for both of them ecotoxicological data are available, including Oligochaeta, but none for enchytraeids. Furthermore physical-chemical characteristics such as the mode of action and degradability are known and their half-life is longer than the applied test duration. Hence, a continuous chemical exposure can be assumed. Last but not least these are commercially available pesticides used globally in agriculture. The first was lambda-cyhalothrin, a pyrethroid insecticide with sodium-channel modulator as mode of action (CAS 91465-08-6). Its mean half life in soil is estimated to be 56 days under standard laboratory conditions (20°C and soil moisture of 40–60 % WHC) (European Commission 2001). Lambda-cyhalothrin has a high octanol water partition coefficient (Log $K_{OW} = 7.0$) (European Commission 2001) and, hence, tends to adsorb to organic matter, either to biota but certainly also to organic compounds in soils. Since water solubility is low ($0.005\ mg/l_{21°C,\ pH\ 6.5}$) (European Commission 2001) and to achieve a more direct link to agricultural reality, lambda-cyhalothrin was applied as the formulation 'Karate® Zeon™', distributed by Syngenta Crop Protection AG, Switzerland. For agricultural usage the maximum application rate for 'Karate® Zeon™' is 75 ml/ha. This rate corresponds to 7.73 g lambda-cyhalothrin per hectare. Applying the assumptions of EU regulatory procedures, namely a soil density of 1.5 g/cm³ and a mean incorporation depth of 5 cm (European Economic Community 2007), the maximum application rate would theoretically result in a soil concentration of 10.3 µg lambda-cyhalothrin/kg dw soil. This calculation provides the necessary comparison with data generated in laboratory experiments. The concentrations of the reproduction test were chosen based on previous range finding tests (data not shown) and included 10 concentrations with lambda-cyhalothrin plus one negative control in untreated soil. The concentrations were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 mg a.s./kg dw soil (nominal values).

The second model substance was pyrimethanil (CAS 53112-28-0), a broad spectrum fungicide, with methionin biosynthesis inhibition as mode of action. Under standardized laboratory conditions, its mean half life in soil has been determined to be 56 days. Its lipophilicity is moderate ($\log K_{OW} = 2.84$) as well as its water solubility (121 $mg/l_{25°C,\ pH\ 6.1}$) (European Commission 2005). Similar to lambda-cyhalothrin, pyrimethanil was applied as a commercial formulation, i.e. 'SCALA', distributed by BASF SE, Germany. The maximum application rate for Scala is 2.5 l/ha, equivalent to 1 kg pyrimethanil per hectare. Applying the same calculation as mentioned above, the maximum application rate would lead to 1.33 mg pyrimethanil/kg dw soil. The concentrations of the reproduction tests performed with pyrimethanil were 44.2, 62.5, 88.4, 125, 177, 250, 354, 500, 707 and 1000 mg a.s./kg dw soil (nominal values). Besides the chemical exposure, a negative control with untreated soil was also used. The concentration range was chosen based on prior range finding tests (data not shown).

All concentrations mentioned in this paper refer to the corresponding concentrations of the active substance (a.s.) in dry weight soil (kg dw soil), either lambda-cyhalothrin or pyrimethanil, as nominal values.

2.3. Experimental testing

All reproduction tests were conducted on the basis of OECD guideline 220 (2004), but with some deviations. The tests ran under a 16/8 h light/dark regime without climatic control at a temperature range of 19 to 27°C according to a minimum-maximum thermometer. The exposure was conducted in spiked OECD artificial soil, to which the chemical was added. OECD soil contains 10 % peat, 20 % kaolin, approximately 70 % quartz sand and circa 0.3 to 1 % calcium carbonate to regulate the pH-value. In order to evaluate a possible interaction of environmental and chemical stress, all chemical treatments (either with lambda-cyhalothrin or pyrimethanil) were combined with three different soil moisture levels (30, 50 and 70 % w/w of WHC).

The water holding capacity (WHC), measured as described in Annex 2 of OECD 220 (2004), of the soil used in the tests with lambda-cyhalothrin had a WHC of 60.4 %, while that of the soil in the tests with pyrimethanil was determined to be 58.1 %. Hence, water was added in a way that the actual water content in the three moisture levels corresponded to approximately 18, 30 and 41 % w/w. To achieve the desired soil moisture (in % w/w of WHC), the soil was pre-moistened before the start of the test with the respective volume of deionized water less the application volume of 10 ml. This was added at day 0 and mixed thoroughly in the soil. Each test vessel was filled with the respective amount of soil, corresponding to 20 g dry weight soil and covered with Parafilm afterwards. All vessels used in the experiments were made of glass with a volume of approximately 250 ml. Besides the test vessels, two extra vessels per treatment were established to assess the soil moisture and pH at the beginning and the end of the test. The soil moisture was measured gravimetrically (ca. 10 g fresh weight in tests with lambda-cyhalothrin, ca. 1 g fresh weight in case of pyrimethanil). The pH values were determined in calcium chloride for the tests with lambda-cyhalothrin and in 3 repetitions with a pH insertion electrode for the tests with pyrimethanil. The test design with 10 chemical concentrations plus negative controls combined with the three moisture levels led to 132 test vessels including four replicates per treatment plus 66 extra vessels for soil moisture and pH determination (two replicates per treatment) for each test substance.

At the beginning of an experiment, 10 full-grown enchytraeids of about 8–12 mm length were randomly inserted in the test vessels and fed with 50 mg autoclaved oat flakes. Thereafter, feeding (25 mg oat flakes) as well as remoistening to initial weights, was conducted once per week. Because of the short generation time of this fragmenting species, the test duration recommended in the ISO and OECD guidelines (4 weeks) was reduced to 3 weeks. After completion, the soil of the test vessels was saturated with 70 % (v/v) ethanol to kill the enchytraeids. After a couple minutes, each vessel was filled with deionized water and the suspension floated into a lab tray. Additionally 0.3 ml of diluted Rose Bengal (3 g Rose Bengal/l ethanol) were added as described in annex 6 of OECD 220 (2004). In order to receive a homogenous distribution of the stain and a flat layer of the soil, the lab tray was carefully slued. After at least 12 hours under the fume hood, the water was evaporated and the enchytraeids coloured deep violet. The individual enchytraeids laid above the soil particles and could be easily counted under a stereo microscope.

For *E. bigeminus* the validity criteria were established for small *Enchytraeus*-species applying OECD 220 (2004): at least 50 juveniles on average in the control. Furthermore the adult mortality should not exceed 20% at the end of the test. Since *E. bigeminus* may reproduce by fragmentation, the evaluation of adult mortality was omitted. Finally the coefficient of variation of the mean number of juveniles of the controls should not be higher than 50%. The endpoint statistically assessed in this study was the total number of individuals, referring to fragments and regenerated worms.

2.4. Statistical analysis

In order to assess an influence of soil moisture on reproduction in the absence of a chemical stressor, a two-way ANOVA was conducted with Statistica 10 (StatSoft, Inc. 2011) with number of individuals as the dependent variable and soil moisture and the two tests as fixed factors (n = 4). To achieve variance homogeneity of the dependent variable, a square root transformation was applied. The ANOVA was followed by a Bonferroni post hoc test with $\alpha = 5\%$.

The statistical evaluation of the concentration-response curves was conducted with R 2.13.1 (R Development Core Team 2011) and the drc package (Ritz & Streibig 2005). A three parametric log-logistic function (LL2.3) was used to fit the response (number of individuals at test end) to the concentration of the pesticide:

$f_{(x)} = d / (1 + \exp^{(b(\log(x)-e)})$ with x = nominal concentration, b = slope at inflection point, d = upper limit and e = ln inflection point. We assumed Poisson error distribution as the numbers of individuals are count data. From the fitted curves, EC_x values relating to 50%, 20% and 10% effect, respectively, were derived together with their respective 95% confidence intervals using the function 'fls' in the drc package.

3. Results

3.1. Validity of the tests

The pH in the tests with lambda-cyhalothrin was on average 6.21 (range: 6.11 – 6.34; n = 33) at the beginning of the experiment and 5.98 (range: 5.84–6.26; n = 66) at the end. Hence, the pH recommended by OECD 220 (2004) (= 6.0 ± 0.5) was achieved throughout the experiment. The pH values in the pyrimethanil test ranged from 5.25 to 7.20 with an average of 6.44 (n = 33) at the beginning of the experiment. After 21 days the average pH was 5.87 (range: 5.14–6.72; n = 66). The validity range for the pH according to OECD guideline 220 (2004), i.e. 6.0 ± 0.5, was not maintained for all additional vessels in this experiment. Potential consequences on the test outcome will be discussed below.

The means and standard deviations for each soil moisture treatment of the test with lambda-cyhalothrin were 33.9 ± 0.8, 53.7 ± 1.4 and 73.1 ± 1.7% (n = 33) at the beginning and 33.4 ± 1.7, 52.8 ± 1.6 and 73.4 ± 1.5% (n = 66) at end of the experiment. This clear variation allowed a separate analysis for further endpoints and discussion. According to OECD 220 (2004), the soil moisture should be adjusted to 50 ± 10% of WHC. The values in this experiment ranged between 29.2 and 36.6% WHC for the treatment with dry soil (n = 99) and between 48.2 and 55.9% WHC for the treatment aiming at 50% WHC (n = 99). In the soil with a WHC of 70%, the water content varied between 71.0 and 76.8% WHC (n = 99). Applying the same recommendations for maximum deviation, the actual soil moisture of this experiment fulfilled that criterion.

In the experiment with pyrimethanil, the following means and standard deviations for soil moisture were calculated for the respective treatment: 30.3 ± 1.3, 48.3 ± 1.3 and 68.7 ± 5.4% WHC (n = 33) at test start and 26.0 ± 3.1, 46.1 ± 3.1 and 61.2 ± 7.1% WHC (n = 66; for the treatment with 70% nominal soil moisture n = 65) at the end of the test. For technical reasons (amount of soil was too small), the gravimetric measurement of soil moisture in the test with pyrimethanil was performed with a smaller amount of soil. Hence, the deviations were larger in comparison to the study with lambda-cyhalothrin. For the nominal soil moisture of 30% of the WHC, the values ranged between 18.4 and 32.9% WHC (n = 99) during the experiment. For the treatment aiming 50% of the WHC, they ranged from 37.2 to 50.9% WHC (n = 99). Finally the treatment with a nominal value of 70% of the WHC, the values ranged between 47.0 and 76% WHC (n = 98). Even though the deviation of the nominal values were larger than recommended by the guideline (pH as well as soil moisture), it should be mentioned that these values result from additional vessels and need not necessarily reflect the conditions in the test vessels. Actually, the question whether an ecotoxicological test is valid or not depends on the fact whether specific validity criteria (most importantly the number of juveniles in the control vessels being larger than 50 individuals) are fulfilled or not.

In none of the vessels were cocoons observed, indicating that under test conditions fragmentation strongly dominates. Since 50% of WHC is the soil moisture required by OECD 220 (2004), only this moisture treatment was considered when determining the validity of the tests. In the experiment with lambda-cyhalothrin, between 144 and 425 enchytraeids (including fragments) were counted per control vessel (mean: 299; n = 4), while in the pyrimethanil study between 310 and 388 worms were found in the control vessels (mean: 355; n = 4). The coefficients of variation around the means were calculated as 40.5% in the study examining lambda-cyhalothrin and 9.71% in those of pyrimethanil. Hence, the validity criterion of both reproduction tests as defined for small species in the guideline (OECD 2004) was fulfilled.

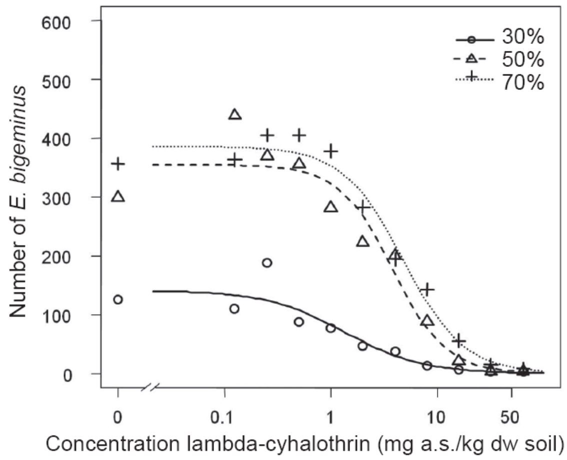

Figure 1. Effects of lambda-cyhalothrin [mg a.s./kg dw soil] on *E. bigeminus* in soils hydrated to different moisture levels expressed as percent of water holding capacity (WHC) (values are means; n = 4).

3.2. Impact of soil moisture on maximum reproduction

The concentration-response curves for the experiment lambda-cyhalothrin effects on enchytraeids are shown in Fig. 1. In the controls (as well as in the chemical treatments), a clear dependency of reproduction on soil moisture was visible in addition to the concentration-dependent reduction in reproduction (see below). Especially in vessels with soil moistened to 30% of WHC, the number of individuals was lower in comparison to the treatment with 50 and 70% WHC, respectively. In the latter two moisture regimes, comparable numbers of enchytraeids were counted, but with slightly greater values in 70% WHC.

In the reproduction tests with pyrimethanil (Fig. 2), the number of individuals in the control vessels was reduced by a simulated drought stress as well. In comparison to the first experiment with lambda-cyhalothrin, the enchytraeids seemed to be even more affected by drought, with 36 worms on average in contrast to an average of 126 in the first test. The treatment with a soil moisture of 70% WHC led to a marginally lower number of individuals in the control vessels compared to the 50% WHC treatment without a chemical stressor.

The two-way ANOVA of both experiments (Table 1) detected no difference between the numbers of worms in the control vessels of the two experiments. But as Figs 1 and 2 already indicate, a significant dependence between number of enchytraeids and the main factor 'soil moisture' was observed. Furthermore the Bonferroni post hoc test indicated a significant difference between the nominal groups of 30 and 50% as well as between 30 and 70% WHC, but none for the comparison of the groups 50 and 70% WHC. Hence, the simulated drought stress led to a considerable reduction of reproduction in comparison to the treatments with higher soil moisture.

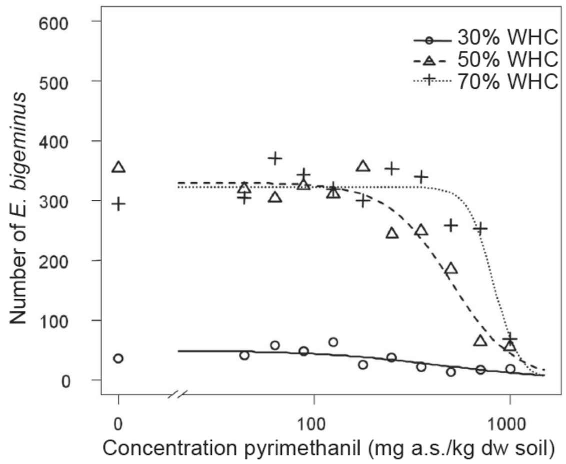

Figure 2. Effect of pyrimethanil [mg a.s./kg dw soil] on *E. bigeminus* in soils hydrated to different moisture levels expressed as percent of water holding capacity (WHC) (values are means; n = 4).

Table 1. Results of the two-way ANOVA of maximum reproduction in the controls.

	Df[a]	Mean Sq[b]	Fvalue	Pr (>F)[c]
experiment	1	24.7	2.41	0.138
moisture	2	232	22.6	< 0.001
experiment * moisture	2	27.5	2.67	0.096
residuals	18	10.3		

[a] – Degrees of freedom, [b] – Mean Squares, [c] – p-value

3.3. Toxicity of the model substances and their dependency on soil moisture

Besides the influence of soil moisture on the maximum reproduction in the controls, the concentration-response curves indicate a different reaction towards lambda-cyhalothrin for each moisture treatment (Fig. 1). The estimated EC_{50} values clearly increased with increasing moisture for both pesticides (Fig. 3). The EC_{50} values for each moisture treatment were calculated to be 1.34 (30% WHC), 3.79 (50% WHC) and 4.77 mg a.s./kg dw soil (70% WHC), respectively. These three EC_{50} values can be regarded as being clearly different since their confidence intervals do not overlap, indicating an increasing toxicity with decreasing soil moisture. EC_{10} and EC_{20} values as surrogate endpoints for NOECs (No Observed Effect Concentration) are presented with their confidence intervals in Table 2. Their confidence intervals of the upper two soil moisture levels overlap, indicating no influence at lower effect levels. A possible influence through dry soil was also observable at these effect levels.

The progressions of the three different concentration-response curves of the experiment with pyrimethanil are rather dissimilar as well (Fig. 2). That of the treatment with 30% WHC is very flat due to low numbers of enchytraeids throughout all chemical treatments. On the other hand, enchytraeids exposed to pyrimethanil at 70% WHC reacted less sensitively towards this substance and, hence, the decrease occurred in higher concentrations in comparison to the treatment at 50% WHC. The EC_{50} values (mg a.s./kg dw soil) were calculated to be 437, 499 and 829 in the treatments with 30, 50 and 70% of WHC, respectively. Due to the flat concentration-response curve of the treatment with 30%, the EC_{50} calculation was associated with uncertainties leading to relatively large confidence intervals. Therefore the EC_{50} determined in the two treatments at 30% and 50% WHC were not considered to be different since their confidence intervals overlapped. Only the EC_{50} resulting from the treatment at 70% WHC was clearly different from those determined at lower moisture levels. The EC_{10} and EC_{20} may be considered different among moisture levels, because their confidence intervals differed (Table 2). In both experiments a positive correlation between EC_{50} and soil moisture was observed: low soil moisture led to enhanced toxicity while higher soil moisture in general led to lower toxicity.

4. Discussion

4.1. Maximum reproduction

The validity criterion concerning the number of juveniles in the control vessels was fulfilled in both experiments in the treatments with medium soil moistures. In a ring test with *E. albidus*, a high coefficient of variation of the number of juveniles of the control vessels (= 43–48%) was already reported (Römbke & Moser 2002) and hence the range of variability that was found in our study can be regarded to be a normal deviation.

The statistical analysis confirmed an influence of moisture on the maximum reproduction, namely a decrease in offspring under simulated drought stress. Such an impairment of reproduction by low soil moistures has already been demonstrated for a number of enchytraeids. Beylich & Achazi (1999) found a decrease

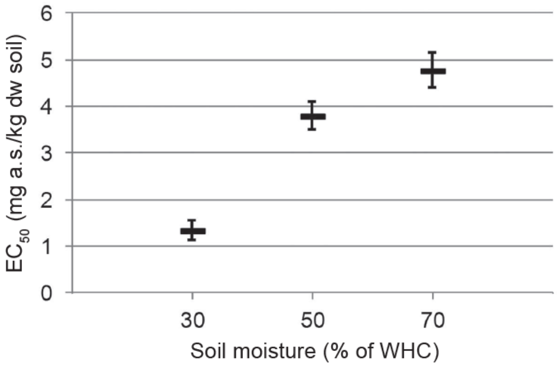

Figure 3. Relationship between the EC_{50} values for lambda-cyhalothrin [mg a.s./kg dw soil] with their 95% confidence intervals of the reproduction test with *E. bigeminus* and soil moisture [% of water holding capacity (WHC)]. n = 4.

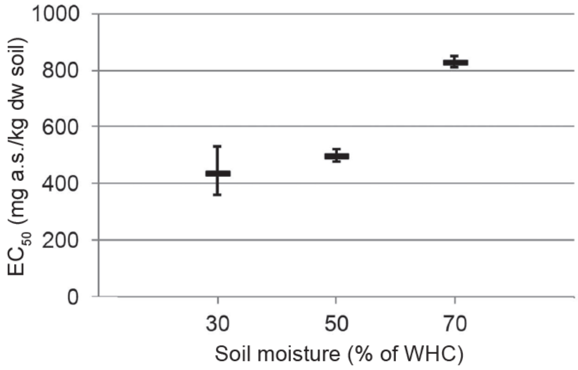

Figure 4. Relationship between the EC_{50} values for pyrimethanil [mg a.s./kg dw soil] with their 95% confidence intervals of the reproduction test with *E. bigeminus* and soil moisture [% of water holding capacity (WHC)]. n = 4.

Table 2. EC_{10} and EC_{20} values [mg a.s./kg d.w. soil] with their 95% confidence interval of the reproduction test with *E. bigeminus* exposed to lambda-cyhalothrin or pyrimethanil under different soil moisture levels [% of water holding capacity (WHC)]. n = 4.

	Moisture	Lambda-cyhalothrin	Pyrimethanil
EC_{10}	30	0.21 (0.16–0.28)	85.6 (56.4–130)
	50	1.06 (0.90–1.24)	220 (199–244)
	70	1.12 (0.97–1.30)	598 (560–639)
EC_{20}	30	0.41 (0.33–0.52)	156 (114–215)
	50	1.70 (1.49–1.93)	298 (276–322)
	70	1.92 (1.70–2.15)	675 (642–709)

in reproduction for *Enchytraeus buchholzi* in Lufa soil 2.2 containing 15% or less water content, comparable to 28.2% WHC in that soil. Dirven-van Breemen et al. (1994) examined the reactions of *E. albidus* and *Enchytraeus crypticus* to different soil moisture levels. They used OECD standard soil with 10% peat with a total water content of 15, 35, 55, 65 and 90% w/w. *E. albidus* reproduced best at 55%, *E. crypticus* at 35%, measured as the number of juveniles per adult per week. In treatments with the lowest soil moisture no juveniles were found, neither with *E. albidus* nor *E. crypticus*. The soil moisture of the study of Dirven-van Breemen et al. (1994) was reported in % w/w total water content. Our three soil moisture levels refer to approximately 18, 30 and 41% w/w total water content, respectively. For *E. bigeminus* no specific data are available in the literature, but our results are in agreement with published studies indicating that drought stress may reduce the reproduction success of enchytraeids.

4.2. Toxicity of model substances

The EC_x values followed a positive trend, i.e. higher toxicity (decreasing EC_x values), with decreasing soil moisture. The EC_{50} of lambda-cyhalothrin was reduced by a factor of 2.8 due to exposure at 30% WHC in contrast to the EC_{50} under standard conditions. An enhanced sensitivity due to drought stress may be of great relevance if global climate change worsens and aridity occurs in higher frequencies. At high soil moisture (70% WHC), the toxicity of lambda-cyhalothrin was slightly reduced in comparison to medium soil moisture by a factor of 1.3 for the EC_{50} value.

The toxicity of pyrimethanil showed a similar pattern regarding soil moisture. When exposed to pyrimethanil under simultaneous drought stress, the EC_x values were lower up to a factor of 2.6 for the EC_{10}, although the confidence intervals of the EC_{50} did not indicate a difference between the treatments at 30 and 50% of the WHC. The enchytraeids were less sensitive to the chemical stressor in the high soil moisture treatment (70% WHC), based on an increase of a factor of up to 2.7 in the EC_x values. Nevertheless it should be mentioned that there are several possible reasons for this dependency of toxicity on soil moisture: bioavailability and/or degradation may depend on soil moisture and thereby influence toxicity by influencing (internal and/or external) exposure levels. Toxicity may also be influenced by internal processes within the test organisms, i.e., lower metabolic activity or reduced fitness at lower soil moistures. The aspect concerning degradation might be of lower importance, since both substances have a mean half life in soil of 56 days under standard conditions, which is more than twice as long as the test duration.

Since no ecotoxicological data for enchytraeids are available for the test substances, a comparison with data of earthworm tests may be appropriate. Garcia et al. (2011) examined the toxicity of lambda-cyhalothrin in an earthworm reproduction test with *Eisenia fetida*. In OECD standard soil they detected an EC_{50} of 37.4 and a NOEC (No Observed Effect Concentration) of 10.0 mg a.s./kg dw soil. The earthworm EC_{50} value is about ten times greater in comparison to the EC_{50} generated under standard conditions with *E. bigeminus*. In accordance with the European Commission (2003), we compared our EC_{10} data that can be considered as NOECs. The EC_{10} of *E. bigeminus* (50% WHC) was calculated to be 1.06 mg a.s./kg dw soil (Table 2) and, hence, is also about a factor of 10 lower than the NOEC reported by Garcia et al. (2011). This difference is probably due to the use of different taxonomic families. It indicates a higher sensitivity of *E. bigeminus* towards lambda-cyhalothrin in comparison to the earthworm *E. fetida*. Nevertheless a risk for enchytraeids due to lambda-cyhalothrin even under additional drought stress appears unlikely, since the predicted environmental concentration in soil is about 20-fold lower than the lowest EC_{10} value.

For pyrimethanil a NOEC of 8.24 mg a.s./kg dw soil was reported for earthworm reproduction (European Commission 2005), which is considerably lower than the EC_{10} determined in the present study under standard conditions for *E. bigeminus* (220 mg a.s./kg dw soil). In the earthworm reproduction test, the chemical was sprayed onto the surface and, hence, the exposure scenario differed strongly from the one used in the present study (i.e. homogenous mixture in soil). A risk due to exposure to pyrimethanil is not expected for enchytraeids, as the lowest EC_{10} is more than 60-fold higher than the estimated concentration of the maximum application rate in the field.

4.3. *Enchytraeus bigeminus* as test organism

So far *E. bigeminus* has rarely been used in ecotoxicological tests. One of the aims of the present study was to examine whether and how a fragmenting species could be involved in standardized test systems. Neither the OECD (2004) guideline nor that of ISO (2003) proposes such a candidate. As stated above, the usage of a fragmenting species may be regarded as advantageous because test duration can be shortened and the variance of response may be reduced (Weltje 2003). In fact, the use of non-sexually reproducing species is often considered as positive since clonal laboratory cultures are more uniform genetically than cultures of sexually reproducing species, meaning that for the selection of test species this mode of reproduction is a positive or at least a neutral factor. However, *E. bigeminus* is polyploid, and different strains of the species sensu lato are highly divergent at the DNA level (Collado et al. 2012), suggesting different sensitivities to chemicals among strains. Therefore the use of the same strain in laboratory tests is recommendable. The molecular characterization of the strain of *E. bigeminus* (barcoding) used in these tests is under way (Schmelz, pers. com.).

Apart from *E. bigeminus*, another fragmenting enchytraeid species, *Cognettia sphagnetorum* (Vejdovský, 1879), has been proposed as a candidate species for ecotoxicological studies (e.g. Sjögren et al. 1995). However, due to its strong preference for acidic soils its use as a test species is limited and not applicable to agricultural soils that have a pH of usually > 5.5. Furthermore, maintaining stable laboratory cultures and obtaining reproducible test results is not straightforward (Römbke unpublished, Schmelz pers. com.).

In the reproduction tests a high tolerance against varying abiotic factors was observed for *E. bigeminus*, which might be due to the fact that this species is adapted to the environmental conditions in warmer climatic zones (Southern Europe, Brazil, Iran). Since the experiments were conducted without climatic control, the temperature varied between 19° and 27°C. The pH values deviated slightly from the range recommended by OECD (2004), but it is unlikely that these differences had a strong effect on the test results. In the validation of the enchytraeid reproduction test (OECD 2004), conducted in a ring test with 29 participants, it was recognized that small deviations do not have a great effect on enchytraeids in general, and hence only those tests with a pH lower than 4.5 and higher than 7.5 were excluded (Römbke & Moser 2002). It seems that *E. bigeminus* was not affected by different pH or temperature values and was also tolerant towards different moisture levels (even in the treatment at 30% WHC, the validity criterion was fulfilled in the study with lambda-cyhalothrin). This statement is backed by the fact that no outlier in abundance occurred in the experiments. In addition clear concentration-response relationships could be generated as well. Due to these results *E. bigeminus* might be a good choice for testing of chemicals and also for assessing different natural soils which possess a wide variation of environmental variables. Christensen & Jensen (1995) used *E. bigeminus* for assessing the toxicity of three pesticides (dimethoate, pirimicarb and fenpropimorph). Their test duration was even shorter (7 days). The formation of new segments in an active growth phase and mortality was evaluated. They detected clear concentration-response relationships for each chemical using *E. bigeminus* as a test organism. Although the test design differs considerably from the present study, it supports the finding that the fragmenting species *E. bigeminus* is a potential candidate for ecotoxicological tests that may offer robust results within a short time period.

5. Conclusions

With regard to the toxicity of the two model substances, clear effects on the number of descendants were detected that depended on soil moisture. Toxicity was enhanced in drier soils, while higher than standard soil moistures led to a reduced toxicity. Furthermore the fragmenting test species *E. bigeminus* can be recommended as a suitable candidate for chemical testing in standardized tests as well as in environmental monitoring, particularly for the examination of natural soils since *E. bigeminus* endures a rather broad range of abiotic factor variations.

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I.IV. A TME STUDY WITH THE FUNGICIDE PYRIMETHANIL COMBINED WITH DIFFERENT MOISTURE REGIMES – EFFECTS ON ENCHYTRAEIDS

Bandow, C., Ng, E-L, Schmelz, R.M., Sousa, J.P. and J. Römbke (2015)

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A TME study with the fungicide pyrimethanil combined with different moisture regimes: effects on enchytraeids

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Abstract Today's ecosystems are influenced by different factors that could evolve into stressors. Effects of pesticides, especially in agricultural areas, may interact with environmental factors, such as soil moisture fluctuation caused by global climate change. In this contribution, two semi-field studies conducted in Germany and Portugal with terrestrial model ecosystems are presented. Their aim was to assess the effects of the fungicide pyrimethanil under different soil moisture levels on Enchytraeidae. In Portugal a no observed effect concentration design was chosen, using two concentration levels: the maximum application rate (MAR) according to the safe use registration within the European Union and five times the MAR (1.82 and 9.09 mg/kg dry soil, respectively). Both concentrations did neither affect the total enchytraeid abundance nor single populations. In Germany an EC_x design (effect concentration) was conducted, using 11 concentrations. In general, 14 EC_{50} values for different combinations of single species, moisture level and sampling date were determined. The strongest effects were found in dry soil, particularly for *Fridericia connata* (EC_{50}: 3.48 mg/kg dry soil after 8 weeks of exposure). The advantages and challenges of these test designs are discussed with regard to the registration process of pesticides in the European Union. In any case, enchytraeids are suitable test organisms in such higher tier studies for the combined evaluation of chemical and climatic stressors due to their usually high diversity and abundances and their close contact with the soil solution.

Keywords Pesticide · Mesocosm · Semi-field · Oligochaeta · Multivariate analysis · Climate change

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Introduction

Plant protection products (PPPs) have to pass an environmental risk assessment with laboratory single species tests triggered by their inherent chemical properties (e.g., $\log_{POW}$) and their use patterns (e.g., target pest and crop type) prior to registration. In special cases, such as when a risk cannot be fully excluded by such lower tier tests, higher tier studies e.g., field studies, may be required. These field studies are regarded to be implementable for large geographical zones that differ considerably in terms of climate. Due to global climate change precipitation is projected to decrease in summer and to increase in winter in Europe (Schmidli et al. 2007). In addition, extreme precipitation events over most of the mid-latitude land masses will very likely become more intense and more frequent (IPCC 2013). In the standard test procedure of PPPs, including field studies, such climatic changes are not

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reflected in the test conditions. In fact, no interaction of various stressors is implemented in the guidelines so far. However, changes in environmental conditions such as soil moisture due to changes in rainfall may lead to different reactions of non-target organisms to PPPs. Therefore, there is a need for higher tier tests, which combine the reproducibility of laboratory tests with the complexity and diversity of soil organism communities of field studies. Furthermore, to be implemented, such tests should obtain robust results with moderate working efforts.

Here we report on two such higher tier tests; their overall aim was to assess the effect of irrigation events of differing intensity (mimicking extreme rain events) on the toxicity of the fungicide pyrimethanil towards soil organisms. Two semi-field studies using terrestrial model ecosystems (TMEs) were conducted. One study took place in Portugal applying a lowest observed effect concentration/no observed effect concentration (LOEC/NOEC) design, and the other study was conducted in Germany using an effect concentration (EC_x) design. The first approach roughly resembles the only soil higher tier study currently required in the European Union, i.e., the earthworm field study (ISO 2014). So far, the second approach is not fully implemented in the regulatory procedure for higher tier tests, but it is often used in laboratory studies and should be used preferably in ecotoxicological tests in general (EFSA 2009). While in both cases a different statistical evaluation is required, each offers specific advantages. The model substance, pyrimethanil, is a broad spectrum fungicide, globally marketed against *Venturia* spp. in pome cultures and *Botrytis cinerea* in strawberry and grape wine cultures. This PPP was chosen since much data is published regarding for example, its degradation in soil but also its effects on some soil organisms. For instance, in a long-term earthworm reproduction test in the laboratory a NOEC of 8.24 mg active substance (a.s.)/kg soil dw was derived (EC 2005). Furthermore, as pyrimethanil is listed in Annex I of the European Union, its future use and potentially global relevance can be assumed (EFSA 2006).

We investigated the possible interaction of the effects of the fungicide pyrimethanil and soil moisture on soil organisms. Three different levels of moisture were studied in the TMEs. Samples were taken from the top soil layer to examine the abundance and diversity of nematodes, microarthropods and enchytraeids. Furthermore, various soil microbial community structural and functional endpoints were assessed (Ng et al. 2014). Finally, earthworms were collected from the remaining soil. In this publication, only the results regarding effects on enchytraeids are presented. Enchytraeidae are small relatives of earthworms (Oligochaeta) and play an important role as decomposers in soil, mainly by regulating microbial growth and activity (Didden 1993; Mulder et al. 2011). For this reason, OECD published a standard guideline for the testing of chemicals describing a reproduction test in the laboratory with several enchytraeid species (OECD 2004). Alongside the TME studies, pyrimethanil was tested for its effects on enchytraeid reproduction using *Enchytraeus bigeminus* (Bandow et al. 2013). Using information gained from the single species test and this series of TME studies, the advantages and challenges of TMEs will be described. Specifically, the aims of these TME experiments were to

- study the effects of a fungicide on enchytraeids under semi-field conditions;
- find out, whether the effects of pyrimethanil on enchytraeids differ due to different soil moisture regimes;
- compare the results obtained at two different biogeographic regions (Mediterranean and Central European);
- assess enchytraeids as higher tier test organisms;
- determine the advantages and/or disadvantages of LOEC/NOEC and EC_x designs in higher tier test studies.

Materials and methods

Model substance pyrimethanil

The fungicide pyrimethanil [IUPAC: *N*-(4,6-dimethylpyrimidin-2-yl)aniline, CAS no 53112-28-0] was used as a model substance. Pyrimethanil belongs to the group of anilinopyrimidines, affecting the fungal methionine biosynthesis (US EPA 2007; FAO 2007). It has a moderate $\log_{POW}$ of 2.84 and a water solubility of 121 mg/L; thus, bioaccumulation may not be expected (PPDB 2009). The $DegT_{50}$ in soil was estimated as 27–82 days under aerobic laboratory conditions (20 °C) with a mean of 56 days (EC 2005). Under field conditions the $DegT_{50}$ was determined as 54 days (BVL 2009). In this study, the tests were performed with the formulation “Scala”, containing 404.6 g pyrimethanil as a.s./L according to the certificate of analysis. No further hazardous substances are known to be an ingredient of “Scala” (BASF 2005). In Portugal, the maximum single application rate (MAR) for “Scala” is 2.5 L/ha in grape cultures (BASF 2014). In Germany, the same MAR is used in strawberry cultures (BVL 2009). This rate corresponds to 1011.5 g pyrimethanil per hectare. To allow a comparison of the effects from laboratory tests, this application rate has to be transformed into mg a.s./kg soil. In the EU regulatory procedure for pesticides a standard soil density of 1.5 g/cm^3 and an average soil depth of 5 cm is assumed (EEC 2007). Thus, the MAR is supposed to be 1.33 mg a.s./kg soil. Using the measured soil density of the TME soil (1.1 g/cm^3), the environmental concentration would be 1.82 mg a.s./kg soil. All concentrations given in this manuscript refer to the a.s. pyrimethanil/kg dry weight soil as nominal concentrations.

Study performance

TME study in Portugal

In total, 72 intact TMEs (i.e., soil cores) were extracted from a field site in Coimbra, Portugal on February 19th, 2010 (for further description see Table 1; Ng et al. 2014). Since 2004, this site has been used without pesticide application for organic agriculture. Each core had a depth of 40 cm, and an inner diameter of 16.9 cm and thence, a soil surface of 224.2 cm^2. After extraction, the TMEs were placed into temperature-controlled carts in a greenhouse (see Knacker et al. 2004 for details of the technical setup). The soil cores received similar irrigation for 3 weeks for equilibration and acclimatization. Before the test substance was applied, the vegetation of each TME was trimmed down to assure a direct exposure to soil organisms. The concentrations were chosen in order to assess pyrimethanil under realistic field concentrations, which are expectable in agricultural practice. The study followed a NOEC/LOEC design, with three rates, three irrigation regimes and four replicates of each treatment combination. The rates were equal to the MAR and the fivefold MAR rate. Each TME received 50 mL test solution containing 5.54 µL Scala (referring to 2.24 mg pyrimethanil) and 27.7 µL Scala (11.3 mg pyrimethanil), respectively. The test solutions were prepared by weighing the respective amount of Scala individually and diluting them in 1 L of deionised water each. The application was done concentration-ascending with a 50 mL bulb pipette, meaning that the respective volume of test solution was applied spirally from inside outwards on each TME leading to fully moistened surface. For negative control, deionised water was applied. Assuming a 5 cm deep infiltration and applying the measured bulk density of 1.1 g/cm^3, these application rates correspond to 1.82 and 9.09 mg a.s./kg soil. After pesticide application, 50 mL artificial rain water (modified after Velthorst 1993) were poured onto the TME surface to seep the chemical into the soil.

After the application of pyrimethanil, three different irrigation regimes targeting low, medium and high soil moisture were implemented and combined with the chemical concentrations in a full factorial design. The first regime referred to the worst drought in Portugal in the last 150 years, which occurred in 2005 with an average rainfall of 0.3 mm/day between May and August. The second regime of 2.5 mm/day reflected the mean precipitation of the last 30 years for the region where TME were extracted from the field. For the third regime, an abnormally high rainfall of October till November 2006 was simulated with 8.25 mm/day. Site-specific rainfall was obtained from the Institute of Meteorology of Portugal (Ng et al. 2014).

Two weeks after pyrimethanil application, the first sampling of 36 TMEs took place. Enchytraeids were sampled using a cylindrical corer of 5.6 cm diameter and 8 cm depth. They were kept in plastic bags at 4 °C until extraction. Sampling and extraction were conducted

Table 1 Characteristics of the two field sites, where TME were extracted

Country	Germany	Portugal
Town	Floersheim	Coimbra
x coordinate longitude	5539593N	4451761N
y coordinate latitude	32U 456975E	29T 546618E
Mean precipitation (mm/a)	648.2	912.5
Mean annual (°C)	10.2	15.6
Altitude asl (m)	101	16
Slope angle	Light inclination	Flat area
Slope facing	South	
pH value	6.9 ($CaCl_2$)	7.5 (H_2O)
Water holding capacity	58.7	34.8
Soil type	Silty clay	Loamy sand
Organic matter (%)	2.93	1.63
Sand (%)	9.9	68.3
Silt (%)	48.2	20.2
Clay (%)	41.9	11.5
Contamination	No	No
Vegetation	Grass land	Cover crop of yellow lupin
Landuse	Protected landscape	Crop area

Soil properties are referring to the upper 10 cm according to ISO 22611-3 (2007). Species identification in vivo was based on the key and handling procedure described by Schmelz and Collado (2010). The second sampling was done 8 weeks after application, using the same procedure as described above. Additionally, soil samples were taken to determine pH and soil moisture gravimetrically from the first 10 cm depth.

TME study in Germany

One hundred and fourty-four TMEs were extracted at a meadow site with alluvial clay near Flörsheim, Germany on May 9th, 2010. Further site description is given in Table 1. The soil texture at the German and the Portuguese site clearly differs: silty clay versus loamy sand. For at least 10 years, neither pesticides nor fertilizers have been applied (the site belongs to a recreation area). This study was performed similar to the one in Portugal, but with different experimental design, an EC_x design with 11 concentrations of the test chemical plus one negative control. The design was also fully crossed (12 chemical levels × 3 moisture levels—see below), but with two replicates for each treatment combination. The nominal application rates per TME ranged from 0.447 to 1398 kg a.s./ha with a spacing factor of square root 5 in between. Thus, the application rates covered those used in the Portuguese study. Assuming an even distribution of the test substance in the upper 5 cm of the TME, these rates reflect a nominal concentration range of 0.81 and 2541 mg a.s./kg soil, respectively, based on the measured bulk density of 1.1 g/cm^3. The application was conducted according to the method described in the Portuguese description.

The irrigation regime targeted three different moisture levels (low, medium, high). For the TMEs with low moisture, mimicking a drought scenario, no water was applied in the first 4 weeks. After that, 50 mL were irrigated sporadically. The TMEs targeting medium soil moisture were irrigated daily with 100 mL in the first week, 50 mL between the second and fourth week and again 100 mL in the last four weeks. The last TME group (high soil moisture) was irrigated daily with 200 mL in the first week, 100 mL in the second to fourth week and finally 200 mL during weeks 5–8. The volume was dependent on plant growth, apparent soil moisture on the surface and occurrence of soakage.

The TME sampling was conducted 2 and 8 weeks after pyrimethanil application with a soil corer of 5.6 cm diameter, similarly to Portugal. However, sampling depth was 10 cm. Again, extraction method followed the ISO guideline (2007). The enchytraeids were identified alive following the key from Schmelz and Collado (2010). Samples were also collected from each TME for soil moisture and pH determinations.

Statistical analysis

The numbers of enchytraeids given in this paper and used in the statistical analysis refer directly to the total numbers extracted from the soil samples (i.e., without conversion to individuals per square meter). Both sets of data were first tested on interaction of the predictors irrigation regime/soil moisture level and time. Therefore, an interaction term was included into the referring statistical model. For the Portuguese experiment a generalized linear model (GLM) was used to identify which predictors mostly influenced the abundance of the enchytraeid community and individual species. For the German experiment the GLM approach could not be applied since no adequate model could be fitted to the data. Therefore, data analysis focused on the estimation of ecotoxicological endpoints (EC_{50} values). No indication of a significant dependence of the interaction (irrigation regime/soil moisture level × time) was found, neither for the community nor single species in the Portuguese experiment as well as for the EC_{50} values of the German study. Hence, this term was subsequently removed from the models to fulfill the demand of parsimony.

Portugal

The data were analyzed in R 3.1.0 (R Core Team 2014), applying the *mvabund* package for multivariate abundance data (Wang et al. 2012). Since the mean–variance relationship was quadratic, a negative binomial distribution was assumed for the count data (O'Hara and Kotze 2010; Warton et al. 2012). To strengthen the model, only those species with at least 10 individuals in any treatment were included in the analysis. In the GLM (function *manyglm*) the effects of the factors on the species community composition and on single selected species were estimated (abundance data of seven species ~ concentration + moisture level + time). The consequent analysis of deviance (function *anova.manyglm*) applied a resampling-based hypothesis testing to detect a possible significance of the factors in multivariate abundance data. To find out, which enchytraeid species caused significant effects, this anova function was completed by a univariate test. We used a likelihood-ratio-test and Monte Carlo resampling with 10,000 loops.

Germany

Since only two replicates were available the Portuguese GLM approach with its subsequent analysis of deviance was not applicable here. The evaluation of the EC_{50} values was conducted for selected enchytraeid species and their variation among the different moisture regimes and sampling time. To calculate EC_{50} values, concentration–

response curves were fitted to each data combination of species, soil moisture level and sampling time. As in the Portuguese study, only those species with ten or more individuals in any treatment were evaluated. The evaluation was conducted in R using the *drc* package (Ritz and Streibig 2005). For the fitting of the concentration–response curves either a Weibull or a log-logistic three parametric model was selected based on Akaike Information Criterion as well as expert judgment. From the concentration–response curves the EC_{50} values (plus their 95 % confidence intervals and standard error) were estimated using the ED-function of R. The subsequent three-way ANOVA included only those EC_{50} values, which had a standard error less than its respective values. The Shapiro–Wilk test indicated a normal distribution of the data and its residuals in the approached model. Furthermore, Levene's test proofed the homogeneity of variances. Thus, the data were left untransformed in the model. The linear model was performed to identify statistical significance resulting from all possible influencing factors (EC_{50} values ~ moisture level + time + species). The model was weighted by the reciprocal standard error of the EC_{50} values. Level of significance was set to $\alpha = 5$ %.

Results

Portugal

Two weeks after test start, the average soil moisture was 19.3, 20.0 and 20.8 % (w/w) in the three corresponding moisture levels. At the second sampling time (8 weeks after application of pyrimethanil), the average soil moisture values were 16.6, 18.9 and 19.7 % (w/w), targeting low, medium and high soil moisture, respectively. The values measured after 2 weeks did not vary significantly between irrigation regimes. However, those after 8 weeks were significantly different (Ng et al. 2014).

Overall, four enchytraeid genera were identified in the TMEs (Fig. 1): *Achaeta*, *Enchylea*, *Enchytraeus* and *Fridericia*. One *Achaeta*-species was identified as new to science: *Achaeta coimbrensis*, a species without pyriform glands, occurred in low numbers (Schmelz and Collado 2013). *Enchylea heteroducta*, the only species of that genus, was also very rare. This was the first find after its original description (Nielsen and Christensen 1963). The genus *Enchytraeus* included 3 species (*E. dichaetus*, *E. bulbosus* and *E. buchholzi*), each with comparable numbers (i.e., in total 200–400 individuals). Seven species belonged to the genus *Fridericia*, representing the largest group with 1910 individuals in total. The dominant species was *Fridericia bulboides* with 1133 individuals. Two *Fridericia* species could not be identified to species level and were called "*Fridericia* sp. *1*" and "*Fridericia* sp. *2*". These may be new species but no reference material of adequate quality is available (Schmelz and Collado 2013). The mean enchytraeid abundance in the controls, considering all irrigation regimes, was 34 individuals per sample ($n = 24$; Supplementary Data), which is equal to 13,800 individuals per square meter.

For the statistical evaluation, seven species, which fulfilled the above mentioned criteria, were selected: *E. buchholzi*, *E. bulbosus*, *E. dichaetus*, *F. bulboides*, *F. pretoriana*, *F. spec 1* and *F. tuberosa*. When assessing changes in community composition, the multivariate analysis did not detect a significant effect of pyrimethanil exposure (Table 2). The community composition was affected by soil moisture and differed between the sampling dates. Separate evaluation of species, using univariate analysis, showed a significant impact of soil moisture on *E. bulbosus*. The remaining enchytraeids were not significantly affected by any of the predictors. Since there was no effect of the pesticide in the tested concentrations, no ecotoxicological endpoint, e.g. NOEC/LOEC, could be estimated.

Germany

After 2 weeks, soil moisture was 27.7 % (w/w) in the low moisture treatment and 36.0 % (w/w) in the remaining two groups (medium and high moisture level). After 8 weeks the soil moisture in the three moisture levels was 18.6, 26.2 and 34.4 % (w/w), respectively.

In the German experiment, six genera were detected (Fig. 2). Again, the most abundant genus was *Fridericia* with 12 species and 2929 individuals in total. The genera *Marionina* and *Enchytraeus* contained four and 3 species, respectively. The remaining genera, *Achaeta*, *Buchholzia* and *Enchytronia*, were represented by just one species each. All in all, 22 enchytraeid species were identified. The most dominant species was *Fridericia semisetosa* with over 1000 individuals. Three of the *Marionina* species occurred in mean abundance numbers of about 200 individuals each. However, just 16 individuals of *M. hoffbaueri* were detected. The mean enchytraeid abundance recorded in the controls was 82 individuals per sample (Supplementary Data), which equals to 33,300 individuals per square meter.

After model fitting and screening EC_{50} values, only the endpoints for six species were analysed further as they complied with the acceptance criterion, i.e., their standard error being lower than the actual EC_{50} value. A total of 14 EC_{50} values encompassing the species *E. buchholzi*, *E. bulbosus*, *F. connata*, *F. paroniana*, *F. semisetosa* and *M. argentea* were considered (Table 3). These EC_{50} values ranged between 3.48 and 224 mg a.s./kg, depending on the

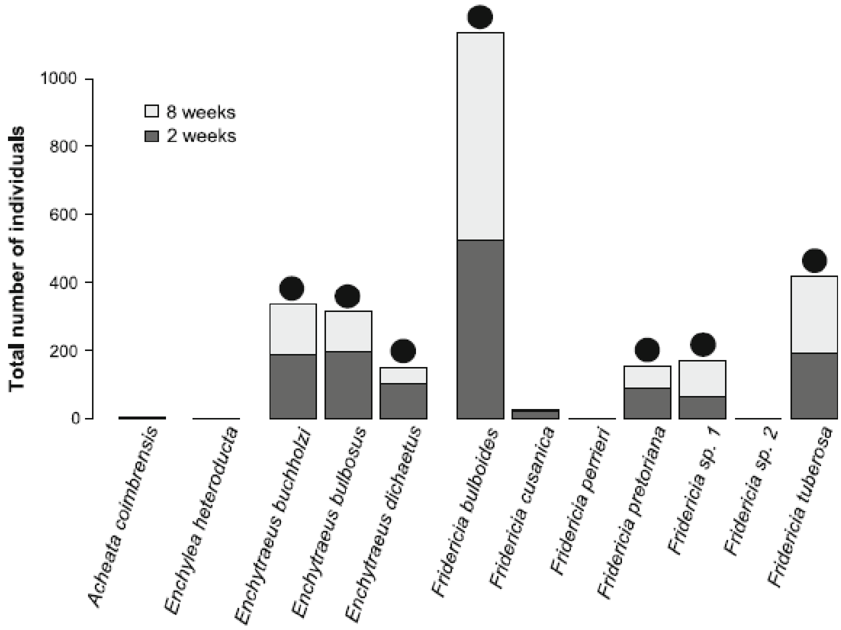

Fig. 1 Total abundance of each species found in the Portuguese TME experiment. Species marked with a *circle* were assessed in further evaluations

Table 2 Results of multivariate analyses adapting a generalized linear model to abundance data of seven species of the Portuguese TME experiment

	Concentration		Soil moisture		Sampling time	
	Deviation	Pr (>Dev)	Deviation	Pr (>Dev)	Deviation	Pr (>Dev)
Multivariate test	15.370	0.308	33.520	0.002	18.240	0.008
Univariate tests						
Enchytraeus buchholzi	2.331	0.749	5.948	0.249	2.497	0.370
Enchytraeus bulbosus	1.046	0.927	12.695	0.007	0.527	0.556
Enchytraeus dichaetus	0.496	0.949	0.252	0.884	5.174	0.140
Fridericia bulboides	3.304	0.688	4.024	0.419	1.382	0.556
Fridericia pretoriana	3.010	0.689	4.958	0.338	3.471	0.264
Fridericia sp. nov. *1*	0.062	0.970	2.818	0.554	4.215	0.204
Fridericia tuberosa	5.121	0.395	2.827	0.554	0.976	0.556

species, the soil moisture level and the sampling date. The results of the three-way ANOVA are shown in Table 4. The EC_{50} values of the six assessed species, with mean values ranging between 18.8 and 106 mg a.s./kg, did not significantly differ from each other (Fig. 3; Table 4). The EC_{50} values were significantly different at the two time points; i.e., the sensitivity was higher after 8 weeks of exposure to the treatments (Fig. 4). The long-term toxicity to the six species was thence higher than the short-term toxicity. The arithmetic mean of all EC_{50} values was determined to be 173 mg a.s./kg after 2 weeks and 47.6 mg a.s./kg after 8 weeks. Finally, the soil moisture significantly affected the sensitivity of the selected enchytraeid species (Fig. 5; Table 4), with mean EC_{50} values showing a positive relation with increasing moisture content. In the low moisture treatment, the EC_{50} values were the lowest with an arithmetic mean of 17.5 mg a.s./kg. In soil with the medium moisture content, mean EC_{50} was 77.7 mg a.s./kg, while in the high moisture level soil the EC_{50} was 105 mg a.s./kg. Therefore, the lower the soil moisture, the higher the sensitivity of enchytraeids to pyrimethanil.

Discussion

Effects of pyrimethanil on enchytraeids under different soil moisture regimes

The most important findings of the TME studies should be summarized first: In Portugal, the analysis of abundance data indicated a diffuse change in species composition in response to irrigation regime, where *E. bulbosus* was the

Fig. 2 Total abundance of each species found in the German TME experiment. Species marked with a *circle* were assessed in further evaluations

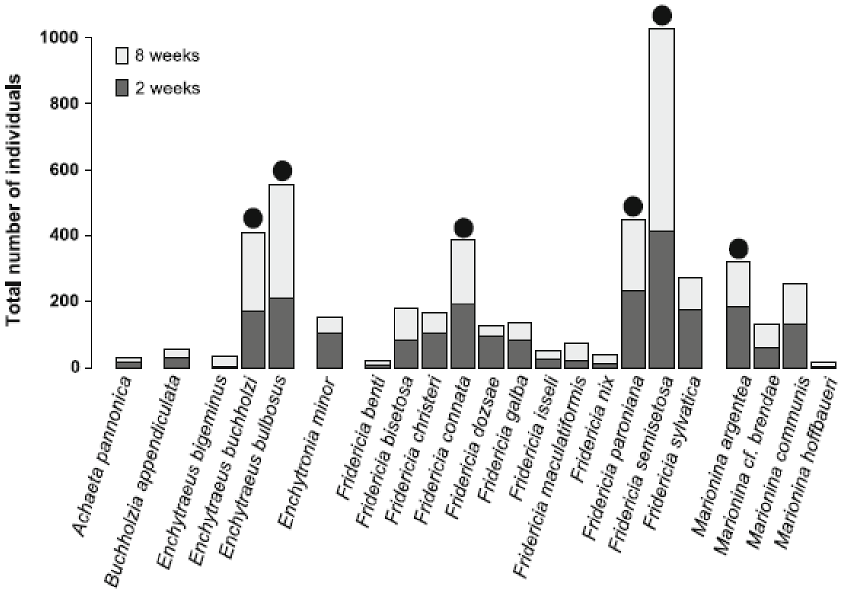

Table 3 Median effect concentration (EC_{50}) in (mg pyrimethanil/kg soil dry weight) of the assessed enchytraeid species with the respective confidence intervals in the German TME experiment

Species	Moisture	Time (weeks)	EC_{50}	CI lower	CI upper
Enchytraeus buchholzi	High	8	116	−69.0	300
Enchytraeus buchholzi	Medium	8	43.3	7.86	78.8
Enchytraeus bulbosus	Medium	8	18.8	5.43	32.2
Fridericia connata	Low	8	3.48	−0.39	7.34
Fridericia connata	High	2	224	88.0	568
Fridericia connata	Medium	8	47.3	6.39	88.2
Fridericia paroniana	Low	8	43.1	22.0	84.4
Fridericia paroniana	High	8	22.8	8.55	60.8
Fridericia paroniana	Medium	8	91.7	1.89	4456
Fridericia semisetosa	High	8	53.0	−9.42	116
Fridericia semisetosa	Medium	2	186	49.7	697
Fridericia semisetosa	Medium	8	79.1	−70.4	229
Marionina argentea	Low	8	5.78	−5.01	16.6
Marionina argentea	High	2	109	24.1	495

Table 4 Results of the three-way ANOVA of the EC_{50} values in the German TME experiment

	Df[a]	Mean Sq[b]	F value	Pr (>F)[c]
Soil moisture	2	4464	12.4	0.0115
Sampling time	1	13142	36.6	0.0018
Species	5	571	1.59	0.3110
Residuals	5	359		

[a] Degrees of freedom

[b] Mean squares

[c] *p* value

only species being significantly affected. The statistical evaluation did not show an effect of pyrimethanil. Additionally, the differences in irrigation regime did not alter the effect of pyrimethanil on the individual enchytraied species or community composition.

In Germany, the lowest EC_{50} value detected was that of *Fridericia connata* after 8 weeks exposure in dry soil (3.48 mg a.s./kg). The EC_{50} values cover a broad range and increase up to 224 mg a.s./kg. The EC_{50} values were significantly influenced by soil moisture, i.e., being lowest in dry soil.

In the Portuguese study, no effects of pyrimethanil were observed either on the species or on the community level. This result corresponds with the findings from the German experiment: 12 out of 14 EC_{50} values are considerably higher than the applied concentrations in the Portuguese

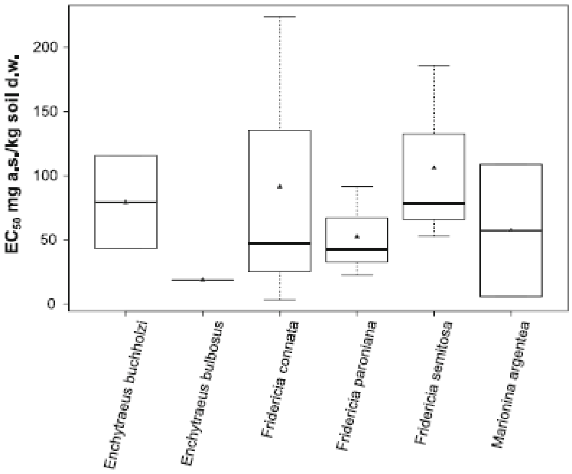

Fig. 3 Distribution of the EC_{50} values (mg pyrimethanil/kg soil dry weight) ($n = 14$) of the evaluated species in the German TME experiment. *Boxplots* show medians with quartiles. *Whiskers* indicate 1.5 interquartile ranges. *Triangles* symbolize arithmetic mean

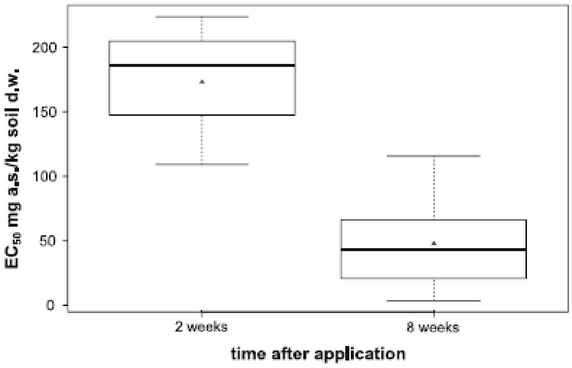

Fig. 4 Distribution of the EC_{50} values (mg pyrimethanil/kg soil dry weight) ($n = 14$) at 2 and 8 weeks after application in the German TME experiment. *Boxplots* show medians with quartiles. *Whiskers* indicate 1.5 interquartile ranges. *Triangles* symbolize arithmetic mean

experiment (1.82 and 9.09 mg a.s./kg). The lowest EC_{50} values were 3.48 mg a.s./kg for *F. connata* and 5.78 mg a.s./kg for *M. argentea*, both assessed using abundance data in dry soil after 8 weeks. Both species did not occur at the Portuguese site and they are perhaps particularly sensitive to pyrimethanil. Additionally, Bandow et al. (2013) reported the reproduction EC_{50} values for *Enchytraeus bigeminus* only at pyrimethanil concentration in the range of 50–100 times higher than the applied concentrations in the Portuguese experiment. In that publication a three-week enchytraeid reproduction test is presented. The EC_{50} derived under standard laboratory test conditions was 499 mg a.s./kg (Bandow et al. 2013) and thus, higher than all the EC_{50} values calculated in this German TME study. It

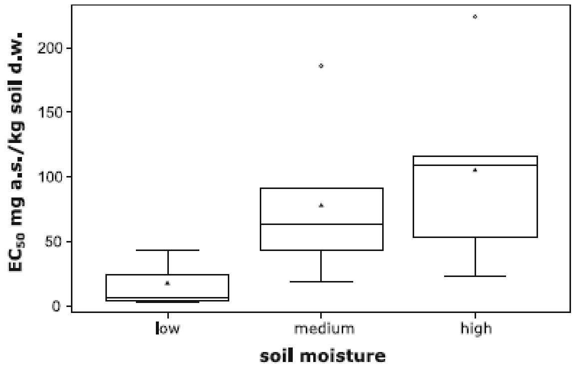

Fig. 5 Distribution of the EC_{50} values (mg pyrimethanil/kg soil dry weight) ($n = 14$) under different soil moisture regimen in the German TME experiment. *Boxplots* show medians with quartiles. *Whiskers* indicate 1.5 interquartile ranges. *Triangles* symbolize arithmetic mean

is possible that the chosen test organism *Enchytraeus bigeminus,* a small r-strategist with high reproduction rate, is not very sensitive to pyrimethanil or recovers within the test period. As such, pyrimethanil application based on the recommended rate does not seem to pose any observable risk to enchytraeids.

However, the lowest EC_{50} value (3.48 mg a.s./kg) found in the German TME study is only about two times higher than the theoretically predicted environmental concentration. To claim a risk for enchytraeids based on these results may go too far. Nevertheless, results from single species tests from laboratory studies might underestimate hazards. As further data for enchytraeids' response to pyrimethanil do not exist, this is a subject for further studies. Nevertheless, a NOEC of 8.24 mg a.s./kg (56 days) for the earthworm *Eisenia fetida* has been determined in a chronic (reproduction) laboratory test, which is the major standard test organism for terrestrial ecotoxicology (EC 2005). So, having the same test duration, this finding agrees with the German results of a relatively high sensitivity of Oligochaeta to pyrimethanil.

Results from the German TME study indicated that the EC_{50} values of the individual species can differ by as much as a factor of 75 (minimum 3.48 mg/kg, maximum 224 mg/kg) and no significant differences in EC_{50} between species could be identified. In the literature, there are no comparable TME studies that offer ecotoxicological data for different species, often because there were no strong effects on enchytraeids in the tested range. Apart from that, toxicity data of different species are hardly comparable when soil, exposure and test conditions differ. In the TME study of Scholz-Starke et al. (2013), lindane (2.4 kg a.s./ha) did not impact enchytraeids, while in the TME study of Moser et al. (2004b), only *Fridericia* species were negatively affected by carbendazim but not *Achaeta* sp. nor

Enchytraeus sp (up to 87.5 kg a.s./ha). In that study, NOEC and EC_{50} values were calculated for *Fridericia* (lowest NOEC and lowest EC_{50}: 9.72 and 0.67 kg a.s./ha, respectively), but not for single species. In an ongoing TME study, no consistent effects on enchytraeids were detected, either by lindane or by imidacloprid, both tested in relatively high concentrations (20 and 2 kg a.s./ha, respectively) (Toschki unpubl). Thus, any further discussion on interspecifically different sensitivity has to rely on some rare data from laboratory tests with different species of *Enchytraeus*. Amorim et al. (2005a, b) compared the sensitivity of *E. albidus* and *E. luxuriosus* to copper and phenmedipham, respectively. In both studies *E. luxuriosus* seemed to be the most sensitive species, but the differences were not large.

Two species were found at both of our TME study sites: *E. buchholzi* and *E. bulbosus*. While *E. buchholzi* occurred in similar abundances in both experiments, *E. bulbosus* was more abundant in the German experiment. In the Portuguese study no effects of pyrimethanil on *E. bulbosus* were observed, probably because of the low test concentrations. However, an effect of soil moisture was found. In the German experiment, only one EC_{50} could be identified (18.8 mg a.s./kg dw) in medium moist soil after 8 weeks of exposure. Thus, a particular sensitivity to low soil moisture could not be detected. However, the German abundance raw data showed a clear preference on *E. bulbosus* for higher soil moisture as well.

In the Portuguese study, the slight effects from the irrigation regime might be due to the rather small [just about 2 % (w/w)] but significant differences between the three soil moisture levels. However, other aspects not measured might influence the result as well.

A larger effect of soil moisture was found in the German study. The soil moisture decreased approximately by a factor of two during the duration of the study. As such, the enchytraeids in the German experiment experienced greater variations in soil moisture. The EC_{50} values were significantly influenced by soil moisture, i.e., being lowest in dry soil. So far, a comparable semi-field study with soil moisture as second test factor has not been published. However, enchytraeid laboratory studies detected a similar relationship between soil moisture and sensitivity to chemicals (Puurtinen and Martikainen 1997). González-Alcaraz et al. (2015) assessed the effects of metal contamination on *Enchytraeus crypticus*. When exposure was conducted in dry soil, the EC_{50} was reduced by a factor of 6.8 in comparison to the EC_{50} achieved from exposure in normal moist soil (González-Alcaraz et al. 2015). Bandow et al. (2013) also found a similar relationship between soil moisture and the intraspecific sensitivity of *Enchytraeus bigeminus* to pyrimethanil, with EC_{50} values of 437, 499 and 829 mg a.s./kg soil for moisture levels of approximately 18, 30 and 41 % (w/w), respectively. These publications could reflect a shift to preferred conditions and thus, a higher tolerance against chemical stress. On the other hand, different internal or external exposure due to higher metabolic activity or dilution of the compound may also impact the effect concentration.

In addition, the sampling time was found to influence the species community composition in Portugal and the EC_{50} values of the German experiment. As most of the small-bodied *Enchytraeus* have very short generation times of about 4 weeks, differences in the species' generation time may play a role here. Unfortunately, details about the generation time of the *Fridericia* species are not known, but at least in the case of the larger species, they are longer than those for *Enchytraeus*. The generation time of a species will have important implications on the ecosystem compositional respond to climate change. This area warrants further study.

Enchytraeid diversity in Portugal and German sites

In the Portuguese study, 12 different enchytraeid species were identified, seven of them not yet recorded for Portugal (Schmelz and Collado 2013), and one of them new to science (*Achaeta coimbrensis*). In other TME studies done in the vicinity of the study site, at least 21 species were identified, including five species that were also found in our study (*E. bulbosus*, *E. dichaetus*, *E. buchholzi*, *F. bulboides* and *F. perrieri*) (Moser 2004; Schmelz and Collado 2013). Since there is no other available information about the Portuguese enchytraeid fauna, it is not possible to assess these sites regarding their habitat function. However, the species number at the study site is comparable to that of crop sites in Central Europe (Römbke et al. 2013).

In the German TMEs, six enchytraeid genera with 22 species were found, which is higher than the average value for German grasslands = 12.2 ± 5.2 (Römbke et al. 2013). Three species—*B. appendiculata*, *F. bisetosa*, and *F. galba*–are commonly found in German grasslands (i.e., they occur at >50 % of all studied German grassland sites). Three other species—*E. buchholzi*, *F. christeri*, *F. paroniana*– which occur regularly but with <50 % frequency, were also found. It is clear that our TME study site is not a "typical" grassland. This assessment is supported by the richness and abundance of *Marionina* species and at the same time the complete lack of *Henlea* species, which is very uncommon for German grasslands. Soil texture, i.e., the high clay content, may play a role in this result. In a previous TME study, the site used was located about 30 km from the one in the present experiment. Up to 15 species were found in that grassland, a silty clay loam (Moser et al. 2004b). Another TME study, conducted by Scholz-Starke et al. (2013) in a meadow on sandy clayey loam in Western

Germany found 18 species, which is still less than in the present experiment. In summary, a highly diverse but not very common enchytraeid community was studied here.

In the uncontaminated control Portuguese samples, a mean abundance of 34 individuals was found. This corresponds to about 13,800 individuals per square meter, which is again comparable to the average enchytraeid density at crop sites in Central Europe (Römbke et al. 2013). In a previous TME experiment in the area, a mean value of 36,300 individuals per square meter was found in control TMEs (Moser et al. 2004b). This difference is probably related to site-specific differences in land use, although soil properties or small-scaled differences of microhabitat and food availability may influence the respective abundance.

In the controls of the German TME study, a mean abundance of 82 individuals was recorded, corresponding to circa 33,300 individuals per square meter. An average of 49,600 individuals per square meter was counted in the control samples in the study of Moser et al. (2004b). Scholz-Starke et al. (2013) found a maximum of 28,860 individuals per square meter in their TMEs. In a soil monitoring study involving 27 non-acid grasslands in North West Germany, the abundance per square meter was between 9000 and 75,000 (Beylich and Graefe 2009). Hence, although our study site had high species diversity, the mean abundance can be classified as being in a medium range among German grasslands.

Pros and cons of the statistical design adopted in TME studies

Common problems of field and semi-field studies are many stray findings and rare species. Most species found in natural habitats, including TMEs, have low abundances and an irregular spatial distribution, found mostly in clusters. Thus, the identification of ecotoxicological endpoints for single species is often difficult. This complicates statistical analysis and was the reason for the exclusion of 16 species in the German experiment, corresponding to 70 % of the species and 20 % of the individuals. Since the abundance data of one species had to be separated into three moisture and two sampling groups, respectively, absolute numbers per combination become smaller. Many combinations could not be evaluated because the number of individual data sets per combination was too small. In theory, 132 EC_{50} values could have been determined (22 species × 3 moisture levels × 2 samplings), but the statistical evaluation resulted in only 14 reliable EC_{50} values. In the Portuguese data analysis 5 out of 12 species were omitted, corresponding to 40 % of identified species and 1.5 % of the individuals. However, despite the field relevance of the tested concentrations and the robustness of the data due to a higher number of replicates, no ecotoxicological endpoints could be derived since no effects were found.

So, in order to get some meaningful results by enhancing statistical power at TME studies, either some factors (here: time or soil moisture) have to be excluded from the statistical evaluation or only those taxa with relatively high and even abundances could be evaluated. All these aspects and caveats must be kept in mind when considering the test design. Both versions (regression or ANOVA based) offer both challenges and advantages, which should be discussed based on the questions to be addressed when performing these semi-field studies.

Other TME studies described in literature use ordination methods like principal response curves (PRC). This method is also proposed in the OECD guidance document for aquatic mesocosms (OECD 2006). One weakness of this method is the necessity of log-transformation count data, which typically include zero counts (O'Hara and Kotze 2010). Another disadvantage could be seen in its relatively low statistical power for detecting between-treatment differences if differences in taxa between treatments are low. This results from violating the mean–variance relationship assumption (Warton et al. 2012). Last but not least, it cannot be used in studies addressing more than one stressor if the aim is to discriminate effects of different stressors,

Considering the pros and cons mentioned above, we recommend a mixed design. In order to avoid problems with stray findings, four replicates are favorable. A higher number of replicates offers a broader spectrum of multivariate statistical methods (e.g., PRC or Wang et al. 2012) to examine community effects or identify most impacted species. The use of NOEC as statistical endpoint should be avoided. As already and intensively discussed in the literature (e.g., Moser 2004; EFSA 2009), the NOEC approach has several disadvantages. Beside the aforesaid problems that occurred in this study, some other points should be emphasized here. Since the variability within the control group of field and semi-field studies is always high, the risk of a Type II error is high (i.e., differences between control and treatments are not identified). This could have been the case in our Portuguese study as well. Further problems are the dependence of the NOEC on the test concentrations and the uncertainty associated with the concept itself, i.e., to know the real concentration that did not cause any significant effect. Therefore, we recommend at least eight concentrations in order to create EC_{50} values. As long as abundances are high enough, the regression model could handle variability and non-monotonic trends. Thus, EC_{50} values with narrow confidence intervals or small standard errors are much more reliable than NOEC. They should be used for selected single species or the total community and finally taken for environmental risk assessment.

Enchytraeids and TMEs in higher tier testing of PPPs

In soil ecotoxicology, mesofauna such as enchytraeids, is generally covered by laboratory tests. However, as well as other groups, only a few representative species can be cultured. For field and semi-field tests the enchytraeids present themselves mostly as highly diverse and abundant taxa. Currently, earthworm field studies are commonly used in PPP registration (ISO 2014). In contrast to these large Oligochaeta, enchytraeids offer the advantage of having greater species diversity and occurrence in higher numbers. The morphological identification is very complex but may be supported or substituted by barcoding or metabarcoding methods in future (Kress et al. 2015; Orgiazzi et al. 2015). Furthermore, sampling is quick and simple, and less volume is necessary in comparison to earthworms. The same arguments are relevant for microorganism groups [e.g., fungi, nematodes (Moser et al. 2004a)] or other mesofauna groups [e.g., collembola, mites (Koolhaas et al. 2004)].

In its natural habitat single species are not solely stressed by the test substance but are potentially exposed to various stressors, biotic and abiotic. Therefore, it is necessary to find tools for realistic multistressor assessment (Segner et al. 2014). To use model ecosystems for chemical testing means combining the advantages of laboratory tests and those of field studies. An indoor TME could run under controlled environmental conditions (light regime and air temperature and moisture levels) in climatic chambers. Furthermore, by having a test system with undisturbed biotopes, natural biotic interactions remains and plenty of species and taxonomic groups can be examined in one test (Ng et al. 2014; Scholz-Starke et al. 2013). For these reasons, it should be considered to include TME testing, assessing different soil organism groups, in future PPP registration process. However, since soil moisture is one of the factors exerting a higher influence in many of the parameters assessed in TME experiments, it is of paramount importance to measure soil moisture levels in all TMEs during the test. This can be done either by small-sized in-between samplings for gravimetric moisture measurement or by electronic non-destructive approaches, using a soil moisture monitoring system. This allows not only controlling side effects of this abiotic parameter but also, as in this study, to examine them as a second stress factor.

Conclusion

Our studies provide a new aspect in terrestrial semi-field testing. Apart from a chemical factor differing soil moisture was involved, which influenced the results of both experiments. According to the Portuguese study, there is no risk to enchytraeids at field and fivefold field rates of the fungicide Pyrimethanil. However, in the German experiment the soil moisture significantly reduced the sensitivity and caused effect concentrations 2.6-fold higher than the lowest environmentally relevant concentration. With regard to global climate change and natural variations between different climate zones, climatic aspects will influence the toxicity of PPPs and should therefore be considered in environmental risk assessment. An idea to include these parameters might be a chemical testing under different climatic circumstances (e.g., higher temperature or different soil moisture). Field studies and semi-field studies could be conducted in different regions of Europe in order to evaluate influence of various climatic impacts on the toxicity of a pesticide.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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