Subletale Effekte des Insektizids Imidacloprid bei verschiedenen Regenwurmarten (*Eisenia fetida*, *Aporrectodea caliginosa* und *Lumbricus terrestris*) auf unterschiedlichen Ebenen biologischer Organisation (biochemisch, histologisch, organismisch)

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Zusammenfassung

1. Promotionsthema

Subletale Effekte des Insektizids Imidacloprid bei verschiedenen Regenwurmarten (*Eisenia fetida*, *Aporrectodea caliginosa* und *Lumbricus terrestris*) auf unterschiedlichen Ebenen biologischer Organisation (biochemisch, histologisch, organismisch).

2. Einleitung

Pflanzenschutzmittel werden weltweit in großen Mengen eingesetzt. Allein in Deutschland lag der Inlandsabsatz an Pflanzenschutzwirkstoffen im Jahre 2009 bei 38.757 Tonnen (BVL 2010). In der Europäischen Union (EU) unterliegen Zulassung und Anwendungsbestimmungen von Pflanzenschutzmitteln einem einheitlichen Regelwerk, welches auf einer EU-Richtlinie (91/414/EWG) aus dem Jahr 1991 basiert. Es schreibt eine gemeinschaftsweite Zulassungsprüfung neuer Wirkstoffe sowie die kontinuierliche Überprüfung aller in der EU im Einsatz befindlichen Pflanzenschutzmittelwirkstoffe vor (EEC 1991). Jeder einzelne EU-Mitgliedsstaat kann entscheiden, welche der von der Europäischen Kommission zugelassenen Wirkstoffe im eigenen Land tatsächlich zum Einsatz kommen dürfen. Das Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) ist in Deutschland die Zulassungsbehörde für Pflanzenschutzmittel. Es entscheidet basierend auf Empfehlungen eines unabhängigen Sachverständigenausschusses sowie auf Berichten von drei Bewertungsbehörden: dem Umweltbundesamt (UBA), dem Bundesinstitut für Risikobewertung (BfR) sowie dem Bundesinstitut für Kulturpflanzen (Julius Kühn-Institut (JKI)). Bei den Entscheidungen stehen laut BVL die Vermeidung von schädlichen Effekten auf die menschliche Gesundheit, der Schutz von Kulturpflanzen sowie die Vermeidung unvertretbarer Effekte auf den Naturhaushalt im Vordergrund (www.bvl.de). Obwohl Gesetze, Richtlinien und Verordnungen die Zulassung von und den Umgang mit Pflanzenschutzmitteln in Deutschland und in der EU regeln, konnten verschiedene ökotoxikologische Studien zeigen, dass Pestizide in umweltrelevanten Konzentrationen schädigend auf unterschiedliche Organismen (darunter auch Schlüsselarten) in aquatischen und terrestrischen Ökosystemen wirken können (Iwasa et al. 2004; Endlweber et al. 2006; Jemec et al. 2007; Capowiez et al. 2010).

Die Ökotoxikologie ist eine Umweltwissenschaft, welche aus den Disziplinen Umweltchemie, Toxikologie und Ökologie hervorgegangen ist und Auswirkungen von chemischen Stoffen auf die

belebte Natur (Organismen, Populationen und Ökosysteme) untersucht (Fent 2007). Ökotoxikologische Tests werden mit sogenannten Monitororganismen durchgeführt, welche sensitiv Umweltbelastungen anzeigen können. Regenwürmer eignen sich beispielsweise hervorragend als Monitororganismen zur Beurteilung von Schadeffekten in Böden. Aufgrund ihres Fressverhaltens, ihrer stark durchlässigen Epidermis und ihres großen Oberflächen-Volumen-Verhältnisses können sie leicht Schadstoffe assimilieren und sensitiv auf Belastungen reagieren (Bouché 1992). Zudem eignen sich Regenwürmer sehr gut als Monitororganismen, da sie von hoher ökologischer Relevanz für Böden sind. Durch ihre Lebensweisen leisten sie einen wichtigen Beitrag zum Abbau organischer Substanzen, zur Durchmischung, Auflockerung und besseren Belüftung von Böden und erhöhen damit die Bodenqualität (Scheu 1987; Edwards & Bohlen 1992, 1996; McCredie & Parker 1992; Lavelle 1997).

Schäden durch Chemikalien können auf unterschiedlichen biologischen Organisationsniveaus – von der molekularen bis hin zur ökosystemaren Ebene - auftreten. Die Ökotoxikologie bedient sich sogenannter "Biomarker", um Schadstoffbelastungen bei Organismen sichtbar zu machen (Triebskorn 2003; Köhler & Triebskorn 2004; Fent 2007). Biomarker kann man aufgrund unterschiedlicher Spezifität in zwei Gruppen einordnen. Die Antwort eines „*biomarker of exposure*“ kann eindeutig mit der Exposition und Wirkungsweise eines Stressors in Verbindung gebracht werden (Köhler & Triebskorn 2004). Dies ist beispielsweise der Fall bei der Hemmung der Aktivität des Enzyms Acetylcholinesterase, welche spezifisch durch Exposition gegenüber bestimmten Chemikaliengruppen (Carbamate, Organophosphate) hervorgerufen wird (Fukuto 1990). Ein „*biomarker of effect*“ hingegen zeigt keine spezifische Expositionssituation an, sondern gibt Aufschluss über Effekte, welche durch die Gesamtheit an wirkenden Einflüssen zustande kommen (Köhler & Triebskorn 2004). Zu letzterer Biomarkergruppe gehört beispielsweise die Induktion des Stressproteins Hsp70. Werden durch die Wirkung von Stressoren Proteine in Mitleidenschaft gezogen, so werden vermehrt Stressproteine der Hsp70-Gruppe gebildet, welche u.a. in der Lage sind, falsch gefaltete Proteine zu reparieren (Lindquist & Craig 1988).

Schadstoffbelastungen bei Organismen können auf molekularer, biochemischer, zellulärer und physiologischer Ebene sowie auf Verhaltensebene angezeigt werden (van Gestel & van Brummelen 1996; Fent 2007). Molekulare und biochemische Marker weisen eine hohe Sensitivität auf und eignen sich deshalb sehr gut, bereits geringe Belastungen anzuzeigen. Histologische Biomarker zeichnen sich dadurch aus, auch chronische sowie länger zurückliegende Schadstoffbelastungen anzeigen zu können. Zudem können sie Informationen über Wirkungsort und Wirkmechanismen liefern (Triebskorn 2003). Auf organismischer Ebene können beispielsweise Gewichts- oder Verhaltensänderungen von Versuchstieren gemessen werden. Da letztere häufig bereits bei geringen

Schadstoffkonzentrationen und kurze Zeit nach Schadstoffexposition beobachtet werden können, gelten Biomarker auf Verhaltensebene als sehr sensitiv. Diesen Markern wird zudem große Bedeutung beigemessen, da sich Änderungen im Verhalten von einzelnen Individuen auf die Integrität gesamter Populationen oder auf verschiedene Prozesse in Ökosystemen auswirken können (Little 1990; Doving 1991; Dell `Omo 2002). So kann beispielsweise eine verminderte Grabaktivität von Regenwürmern negativen Einfluss auf Gas- und Wassertransfereigenschaften von Böden haben (Capowiez et al. 2003a, 2006). In der Ökotoxikologie gelten Biomarker folglich als „biologische Frühwarnsysteme“, da sie bereits auf niedrigen biologischen Ebenen mögliche Effekte von Schadstoffexpositionen auf höheren Organisationsebenen (Populationen, Ökosysteme) vorhersagen können (Triebskorn 2003; Köhler & Triebskorn 2004; Fent 2007).

Durchgeführte Studien und Zielsetzung:

In der vorliegenden Arbeit wurden Auswirkungen einer Pestizidexposition an drei Regenwurmarten (*Eisenia fetida, Aporrectodea caliginosa und Lumbricus terrestris*) auf verschiedenen Ebenen biologischer Organisation untersucht. Bei allen Studien wurde das Insektizid Imidacloprid als Testsubstanz eingesetzt. Diese zur Gruppe der Neonicotinoide gehörende synthetische Substanz zählt derzeit zu den weltweit am meisten verwendeten Pflanzenschutzwirkstoffen (www.bayercropscience.com) und ist daher von hoher ökologischer Relevanz. Imidacloprid wirkt - wie alle Neonicotinoide - neurotoxisch auf Organismen durch irreversible Hemmung der postsynaptischen nicotinergen Acetylcholin-Rezeptoren, jedoch speziell schädigend auf Insekten im Vergleich zu Wirbeltieren (Matsuda et al. 2001). In vorangehenden ökotoxikologischen Studien konnten allerdings negative Effekte von Imidacloprid auf verschiedene Nicht-Zielorganismen, auch außerhalb der Klasse der Insekten, nachgewiesen werden (z.B. Zang et al. 2000; Iwasa et al. 2004; Kreutzweiser et al. 2009; Lukančič et al. 2010). Imidacloprid sowie weitere Neonicotinoide wurden zudem in jüngster Zeit häufig im Zusammenhang mit dem Bienenvölkerkollaps (colony collapse disorder (ccd)) diskutiert (Girolami et al. 2009; Maini et al. 2010).

Im ersten Teil der vorliegenden Dissertation wurden auf suborganismischer Ebene (durch die Messung der Induktion des Stressproteins (Hsp70) und durch histopathologische Untersuchungen) sowie auf organismischer Ebene (durch die Messung von Gewichtsänderungen und von Vermeidungsverhalten) in Laborversuchen Auswirkungen des Insektizids Imidacloprid auf drei verschiedene Regenwurmarten (*Eisenia fetida; Aporrectodea caliginosa und Lumbricus terrestris*) untersucht (Kapitel 1). Das Ziel dieser Studie war es, sowohl die Sensitivitäten der verschiedenen Biomarkerantworten als auch der unterschiedlichen Regenwurmarten anhand der Testsubstanz Imidacloprid zu vergleichen. Diese Studie wurde zum einen vor dem Hintergrund durchgeführt, dass

allein die für Regenwürmer von der EU für die Zulassung von Pflanzenschutzwirkstoffen vorgeschriebenen Standardtests - der „Akute Toxizitätstest bei *Eisenia fetida/ Eisenia andrei* (OECD Test 207, ISO 11268-1)“ und der „Regenwurm-Reproduktionstest bei *Eisenia fetida*/ *Eisenia andrei* (OECD Test 222, ISO 11268-2)“ (OECD 1984; 2004) – nicht ausreichend sind, um potentielle ökologisch relevante subletale Effekte zu erfassen. Zum anderen sollte mit diesem Forschungsansatz am Beispiel von Imidacloprid dazu beigetragen werden, zu überprüfen, wie groß die Aussagefähigkeit von ökotoxikologischen Regenwurmtests mit den Arten *E. fetida* und *E. andrei* ist. Diese aus Gründen der einfachen Beschaffung und Hälterung bei standardisierten Regenwurmtests häufig verwendeten Arten reagierten in einigen bislang bekannten Fällen weniger sensitiv gegenüber Umweltchemikalien als andere, häufig in Agrarböden vorkommende Regenwurmarten (Edwards & Coulson 1992; Fitzpatrick et al. 1992; Kula & Kokta 1992; Ma & Bodt 1993; Spurgeon & Weeks 1998; Muthukaruppan & Paramasamy 2010). Zudem muss der Einsatz von *E. fetida* und *E. andrei* auch vor dem Hintergrund ökologischer Relevanz kritisch gesehen werden: Beide Arten kommen sehr selten in landwirtschaftlich genutzten Böden vor.

Im zweiten Teil der vorliegenden Dissertation wurde ein neuer, ökologisch relevanter Biomarker für Regenwürmer auf Verhaltensebene entwickelt und etabliert (Kapitel 2). Dabei ging es um die Erstellung eines einfachen und standardisierbaren Protokolls zur akkuraten Bestimmung der Losungsproduktion (Kotproduktion) von zwei Regenwurmarten (*Lumbricus terrestris* und *Apporectodea caliginosa*). Wichtige Beiträge für die Funktion von Böden, welche Regenwürmer durch ihre Lebensweisen leisten, sind direkt an ihre Grab- und Fressaktivität gekoppelt (z.B. Bastardie et al. 2003; Capowiez et al. 2006). Da bei Regenwürmern die Menge an ausgeschiedener Losung in Relation zu der Menge an aufgenommener Erde gesetzt werden kann, kann die Losungsproduktion Anhaltspunkte zur Grab- bzw. Fressaktivität von Regenwürmern geben. Vorangehende Freilandstudien konnten zeigen, dass die Losungsproduktion von Regenwürmern an der Erdoberfläche von Golfrasen nach Einsatz von verschiedenen Pestiziden reduziert war (Baker et al. 1998; Lal et al. 2001). Mit solchen Studien kann allerdings nicht geklärt werden, ob die Regenwürmer generell weniger Losungen produzieren oder ob sie diese unter der Erde ausscheiden. Da die Quantität der Losungsproduktion von Regenwürmern abhängig von biotischen (z.B. untersuchte Regenwurmart und der Artenzusammensetzung) sowie abiotischen Faktoren (z.B. Anteil des organischen Materials im Boden, Bodendichte und Bodentemperatur) ist (Scheu 1987; Shipitalo et al. 1988; Scullion & Ramshaw 1998; Le Bayon & Binet 1999), ist es wichtig, für die Entwicklung eines Biotests auf Basis der Losungsproduktion standardisierte Bedingungen zu schaffen (Bodentyp; Bodenfeuchtigkeit; Temperatur; Regenwurmtestart). Ziel war es deshalb, einen ökotoxikologischen Labortest mit einer definierten und für Agrarflächen relevanten Regenwurmart (*L. terrestris*) unter standardisierten Bedingungen zu entwickeln. Dieser Biotest sollte es ermöglichen, die

Losungsproduktion von Kontrolltieren und unterschiedlich mit Pestiziden belasteten Tieren zu quantifizieren und zu vergleichen. Darüber hinaus sollte der Test zusätzlich auf eine weitere, häufig in Agrarböden vorkommende Regenwurmart (*A. caliginosa*) angepasst und beispielhaft zur Ermittlung von Effekten von Imidacloprid eingesetzt werden. Um die Sensitivität des neu entwickelten Biotests zu evaluieren, wurden die Ergebnisse zur Losungsproduktion jeweils mit gleichzeitig erarbeiteten Ergebnissen zum Biomarker Gewichtsänderung verglichen.

Im letzten Teil der vorliegenden Arbeit wurden Kurz- und Langzeiteffekte von Imidacloprid auf das Grabverhalten der Regenwurmarten *L. terrestris* und *A. caliginosa* ermittelt (Kapitel 3). Wie bereits erwähnt, können Änderungen im Grabverhalten von Regenwürmern negative Effekte auf verschiedene Prozesse in Böden haben (z.B. Bastardie et al. 2003; Capowiez et al. 2006). Daher gelten Grabverhaltensanalysen bei Regenwürmern in der Ökotoxikologie als ökologisch hochrelevante Werkzeuge. In der Vergangenheit wurden diese Biomarker bereits mehrfach erfolgreich angewendet (Capowiez et al. 2003b, 2006; Capowiez & Bérard 2006; Olvera-Velona et al. 2008). Häufig werden in der Ökotoxikologie akute Tests durchgeführt, wobei in den meisten Fällen wenig über die Übertragbarkeit der gemessenen Antworten auf längere Zeiträume bekannt ist. Um eine adäquate Risikoabschätzung der Auswirkungen von Umweltchemikalien auf die belebte Natur leisten zu können, ist die Messung von Langzeiteffekten in ökotoxikologischen Studien unerlässlich. Ziel der vorliegenden Studie war es deshalb, bei den ausgewählten Testorganismen nach Vorexposition gegenüber verschiedenen Konzentrationen von Imidacloprid das Grabverhalten zu messen und Kurzzeit- (24-96 h) sowie Langzeiteffekte (6 Wochen) dieses häufig eingesetzten Insektizids zu erfassen und zu vergleichen. Für die Kurzzeitexperimente wurde das Grabverhalten in 2D-Terrarien (Evans 1947) gemessen, da diese Methode sehr sensitiv Grabverhaltensänderungen in einer kurzen Zeitspanne anzeigen kann. Die 2D-Methode eignet sich allerdings nicht für die Messung von Langzeiteffekten, da die Terrarien zu wenig Raum für ein ausgedehntes System an Grabgängen bieten. Die Langzeitexperimente wurden daher in mit Erde gefüllten Kunststoffzylindern durchgeführt, und das Grabverhalten wurde mit Hilfe von 3D-Röntgenanalysen (Capowiez et al. 2003a) gemessen. Ein Vorteil der 3D-Methode besteht darin, dass die Lebensbedingungen in den Zylindern am ehesten denen eines natürlichen Habitats von Regenwürmern ähneln, wodurch die Übertragbarkeit der Laborergebnisse auf das Freiland verbessert wird.

3. Material und Methoden

3.1 Testorganismen, Erde und Imidacloprid

Adulte Tiere der beiden Arten *Lumbricus terrestris* und *Eisenia fetida* wurden von einem Angelsportgeschäft in Avignon (Frankreich) bezogen. Adulte *Aporrectodea caliginosa* wurden von Hand auf einer seit fünf Jahren unbehandelten INRA-Versuchsplantage in der Nähe von Avignon gesammelt.

Die Erde (23.4% Ton, 57% Schluff, 19.6% Sand, 28.3 g kg^{-1} organisches Material, pH = 8.3 (in Wasser), KAK (Kationenaustauschkapazität) = 8.2 cmol kg^{-1}, Wasserrückhaltekapazität = 0.247 g g^{-1}) für die gesamten Tests wurde von einer seit 10 Jahren stillgelegten und unbehandelten Apfelplantage in der Nähe von Avignon bezogen. Vor allen Tests wurden die Tiere in der Testerde jeweils für 7 Tage in abgedunkelten Klimakammern bei 12°C (*Lumbricus terrestris* und *Aporrectodea caliginosa*) bzw. 22°C (*Eisenia fetida*) akklimatisiert.

Das Insektizid Imidacloprid (255.66 g/ mol; Reinheitsgrad: 99.9%) wurde mit destilliertem Wasser zu den jeweils eingesetzten unterschiedlich konzentrierten Testlösungen angesetzt. Da die vorhergesagten Umweltkonzentrationen (PEC = predicted environmental concentration) von Imidacloprid zwischen 0.33 und 0.66 mg kg^{-1} Trockenerde liegen (abhängig von Land und Anbau) (Mostert et al. 2000; Oi 1999), wurde für die vorliegende Arbeit die höchste zu erwartende Umweltkonzentration (0.66 mg kg^{-1} Trockenerde) als einfache Applikation (1x) festgelegt. Diese entspricht einer Einsatzmenge von 244 g ha^{-1}.

3.2 Expositionsversuche

Die Kontamination der Testerde erfolgte nach dem Protokoll von Capowiez et al. (2005). Dafür wurde der Erde jeweils eine einheitliche Menge (40 ml pro 1 kg Erde) an den unterschiedlich konzentrierten Imidaclopridlösungen bzw. destilliertem Wasser (Kontrollerde) zugegeben und mit einem Spatel gut durchmischt. Die Kontaminationen wurden so durchgeführt, dass die Bodenfeuchte zu Beginn der Expositionsversuche jeweils 25% (der Trockenerde) betrug. Insgesamt wurden vier verschiedene Konzentrationen getestet: 0.2 (0.3x), 0.66 (1x), 2 (3x) und 4 mg kg^{-1} Trockenerde (6x).

Mit Ausnahme des Fluchttests wurden die Regenwürmer für alle anderen Tests einzeln in Petrischalen (Durchmesser = 10 cm) für verschiedene Zeiträume (zwischen 1 und 14 Tagen) gegenüber Imidacloprid exponiert. Die Petrischalen wurden mit jeweils 100 g gesiebter (3 mm Maschenweite), kontaminierter bzw. unkontaminierter (Kontrolle) Erde gefüllt und während der

Exposition in abgedunkelte Klimakammern bei 12°C (*Lumbricus terrestris* und *Aporrectodea caliginosa*) bzw. 22°C (*Eisenia fetida*) gestellt.

Vor und nach den Expositionsversuchen wurden die Regenwürmer mit Leitungswasser abgespült, vorsichtig mit Papiertüchern abgetrocknet und gewogen. Aus den gewonnenen Daten konnten Gewichtsänderungen berechnet werden. Das Wiegen der Tiere erfolgte ohne das „Entsanden" der Verdauungstrakte, so dass gemessene Gewichtsänderungen Aufschlüsse über Fress- bzw. Grabaktivität der einzelnen Tiere gaben.

3.3 Histologische Untersuchungen

Die Regenwürmer wurden direkt nach der Exposition für 6 h in Leitungswasser gehalten, um die Verdauungstrakte der Tiere zu entleeren. Anschließend wurden die Regenwürmer anästhesiert. Aus der Region direkt hinter dem Clitellum wurden jeweils zwei Querschnitte mit einer Dicke von 1-2 Segmenten angefertigt. Die Gewebeproben wurden in Bouin´schem Gemisch fixiert, in einem Einbettautomaten der Fa. *Leica* (TP 1020) über eine aufsteigende Alkoholreihe entwässert und anschließend in den Kunststoff Tecnovit eingebettet. Mit einem Rotationsmikrotom wurden daraufhin Dünnschnitte (5µm) angefertigt und diese mit Hämatoxylin-Eosin sowie mit Perjodsäure - Schiff´ sches Reagenz (PAS) - Alcianblau gefärbt. Lichtmikroskopisch wurden die Schnitte sowohl qualitativ beschrieben als auch semi-quantitativ über eine Einstufung der Zellzustände in verschiedene Kategorien (Kontroll-, Reaktions- und Destruktionszustand) bewertet.

3.4 Stressproteinanalysen (Hsp70)

Die Stressproteinanalysen wurden jeweils nur mit dem Hinterende (50-200 mg) eines Regenwurms durchgeführt. Nach Exposition gegenüber Imidacloprid wurden diese Hinterteile der Tiere in flüssigem Stickstoff schockgefroren. Zur weiteren Untersuchung wurden die Proben unter Zusatz eines Extraktionspuffers und mit Hilfe eines elektrischen Zerkleinerers homogenisiert und anschließend zentrifugiert. Aus dem Überstand konnte der Gesamtproteingehalt nach Bradford (1976) bestimmt werden. Daraufhin wurden jeweils aus dem Überstand gleiche Proteinmengen (20 µg) mit Hilfe einer Minigel-SDS-PAGE analysiert. Mittels Western-Blot wurden dann die Proteine auf Nitrozellulosemembranen übertragen und anschließend durch eine Peroxidasefärbereaktion immunochemisch sichtbar gemacht (1. Antikörper: mouse anti-human hsp70 IgG; 2. Antikorper: goat anti-mouse IgG, Peroxidase-Konjugat). Abschließend erfolgte eine densitometrische Quantifizierung der Proteinbanden.

3.5 Fluchttests

Die Fluchttests wurden in Plastikboxen verschiedener Größen (11 cm x 16 cm x 6 cm (für *A. caliginosa* und *E. fetida*) und 25 cm x 25 cm x 8 cm (für *L. terrestris*)) durchgeführt. Die Versuchsdurchführung wurde an das Standardprotokoll (ISO 2008) angelehnt. Für die Tests wurden die Boxen zur einen Hälfte mit kontaminierter, zur anderen Hälfte mit unkontaminierter Erde bis zu einer Höhe von 5 cm (*A. caliginosa* and *E. fetida*) bzw. 7 cm (*L. terrestris*) gefüllt. Dafür wurden je Hälfte 300 bzw. 1000g Erde benötigt, abhängig von der Größe der Box. Mit einem Plastikspatel wurde zwischen kontaminierten und unkontaminierten Teil eine klar sichtbare Grenze gezogen. Für die Fluchttests wurden drei verschiedene Konzentrationen verwendet: 0.2 (0.3x), 0.66 (1x) und 2 mg kg^{-1} Trockenerde (3x). Die Kontamination der Erde erfolgte genauso wie in den Expositionsversuchen (siehe 3.2).

Zu Beginn eines jeden Fluchttests wurden pro Box jeweils 10 Regenwürmer auf die Trennlinie gesetzt und anschließend jede Box mit einem perforierten Deckeln verschlossen. Daraufhin wurden die Boxen für 48h in abgedunkelte Klimakammern bei 12°C (*A. caliginosa* und *L. terrestris*) bzw. 22°C (*E. fetida*) gestellt. Nach Ablauf der Expositionszeit wurden kontaminierte und unkontaminierte Erde vorsichtig getrennt und die Anzahl der Regenwürmer in beiden Teilen bestimmt. Für jede Box konnte dann eine Nettoantwort (net response = NR) bestimmt werden, welche Aufschluss über den Grad der Vermeidung von bzw. der Attraktion zu der Testsubstanz gab:

$$NR = (C - T)/ N$$

[C ist die Anzahl der Regenwürmer in der Kontrollerde, T die Anzahl der Tiere in der kontaminierten Erde. N ist die Zahl der überlebenden Tiere.]

Folglich können Nettoantworten zwischen -1 und 1 liegen, wobei negative Antworten Attraktionsverhalten und positive Antworten Fluchtverhalten indizieren.

3.6 Bestimmung der Losungsproduktion

Für den Losungsproduktionstest wurden die Regenwürmer einzeln für 7 Tage in Petrischalen, gefüllt mit jeweils 100 g gesiebter (Maschenweite 3 mm), kontaminierter bzw. unkontaminierter (Kontrolle) Erde, exponiert. Zusätzlich wurden für die unterschiedlichen Ansätze jeweils 10 Petrischalen präpariert, in welche keine Regenwürmer gesetzt wurden und welche als Nullkontrollen dienten.
Nach den Expositionsversuchen wurde vorsichtig aus jeder Petrischale die Erde entnommen und diese gleichmäßig für 10 Sekunden gesiebt (Maschenweite 5.6 mm bzw. 4 mm für *L. terrestris* bzw. *A. caliginosa*). Die in den Sieben zurückgebliebenen Losungen wurden anschließend gewogen. Die Losungsproduktion konnte daraufhin durch Subtrahieren der Gewichte der aus den Leerkontrollen in

den Sieben verbliebenen Erde (Petrischalen ohne Regenwürmer) von den Gewichten aus den Expositionsversuchen in den Sieben verbliebenen Losungen plus restlicher Erde (Petrischalen mit Regenwürmern) bestimmt werden. Die Losungsproduktionsrate konnte dann als Losungsgewicht (frisch) pro Regenwurmgewicht (frisch) pro Expositionszeit (d) angegeben werden.

3.7 Grabverhaltensanalysen (2D und 3D)

Die Grabverhaltensanalysen auf zweidimensionaler Ebene wurden mit Hilfe von 2D-Terrarien (Evans 1947) durchgeführt. Die Terrarien bestanden aus zwei Glasplatten (30 cm x 42 cm), welche mit Hilfe von Kunststoffstegen und Klebeband parallel zueinander im Abstand von 3 mm (*A. caliginosa*) bzw. 5 mm (*L. terrestris*) fixiert wurden. Eine genaue Beschreibung der Terrarien kann der Studie von Capowiez (2000) entnommen werden. Nachdem die Regenwürmer jeweils einzeln für 1, 7 und 14 Tage gegenüber verschiedenen Konzentrationen von Imidacloprid in Petrischalen exponiert wurden (n=7), wurden die Tiere einzeln in die Terrarien eingesetzt. In der vorliegenden Arbeit waren die Terrarien mit gesiebter (2 mm) unkontaminierter Erde gefüllt und wurden während der Inkubationszeit von 4 Tagen in abgedunkelte Klimakammern (12°C) gestellt. Die Grabgänge wurden jeweils nach 24 h und 96 h auf Klarsichtfolien markiert und nach Versuchende digitalisiert. Anschließend wurden die folgenden Endpunkte berechnet: Gesamtgrablänge nach 24h und 96h und maximale Grabtiefe nach 24 h und 96 h.

Die Grabverhaltensanalysen im dreidimensionalen Raum (3D) wurden von Dr. Yvan Capowiez durchgeführt. Dafür wurden je zwei *L. terrestris* und vier *A. caliginosa* nach den Einzelexpositionsversuchen in Petrischalen in mit gesiebte (2 mm), unkontaminierte Testerde gefüllte Kunststoffzylinder (35 cm Höhe und 16 cm Durchmesser) gesetzt und in Klimakammern (12°C) für 6 Wochen inkubiert. Nach der Methode von Pierret et al. (2002) wurden anschließend die Grabgänge röntgentomographisch sichtbar gemacht und das Gesamtvolumen der Grabgänge berechnet.

4. Ergebnisse und Diskussion

4.1. Sensitivitäten verschiedener Biomarkerantworten beim Standardtestorganismus *Eisenia fetida* im Vergleich zu *Aporrectodea caliginosa* und *Lumbricus terrestris* nach Exposition gegenüber dem Insektizid Imidacloprid (Hsp70, Histopathologie, Vermeidungsverhalten, Gewichtsänderung)

a) *Dittbrenner N, Schmitt H, Capowiez Y, Triebskorn R (2011; in press): Sensitivity of Eisenia fetida in comparison to Aporrectodea caliginosa and Lumbricus terrestris after imidacloprid exposure. Body mass change and histopathology. Journal of Soils and Sediments*

b) *Dittbrenner N, Capowiez Y, Köhler H-R, Triebskorn R (2011; in press): Stress protein response (Hsp70) and avoidance behaviour in Eisenia fetida, Aporrectodea caliginosa and Lumbricus terrestris. Journal of Soils and Sediments*

Um die relativen Sensitivitäten des Standardtestorganismus *E. fetida* und der beiden für Agrarflächen relevanten Regenwurmarten *A. caliginosa* und *L. terrestris* gegenüber Imidacloprid zu untersuchen und zu vergleichen, wurden Biomarker auf verschiedenen Ebenen biologischer Organisation herangezogen. Im ersten Teil dieses Kapitels wurden die Regenwürmer auf Gewichtsänderungen hin getestet sowie histopathologischen Untersuchungen unterzogen. Dabei konnten für die beiden Arten *E. fetida* und *A. caliginosa* bereits bei Imidaclopridkonzentrationen von 0.2 (nach 7 d) und 0.66 mg kg^{1} Trockenerde (nach 14 d) signifikante Gewichtsabnahmen beobachtet werden, während für *L. terrestris* signifikante Effekte auf das Gewicht erst bei deutlich höheren Konzentrationen (ab 2 (nach 7 d) bzw. 4 mg kg^{-1} Trockenerde (nach 14 d)) nachgewiesen werden konnten. Die histologischen Untersuchungen zeigten, dass signifikante zelluläre Schädigungen bereits nach 24h Expositionszeit und bei den niedrigsten Testkonzentrationen (0.2 (*E. fetida* und *A. caliginosa*) und 0.66 mg kg^{-1} Trockenerde (*L. terrestris*)) auftraten. Insgesamt reagierten auch die einzelnen Monitororgane (Mitteldarm, Chloragog, Epidermis) der drei Testorganismen unterschiedlich sensitiv. Bei *A. caliginosa* reagierte das Mitteldarmgewebe (LOEC (1d): 0.2 mg kg^{-1} Trockenerde), bei den beiden anderen Arten die Epidermis am sensitivsten mit Schädigungen auf Imidacloprid (LOEC (nach 1 d): 0.2 (*E. fetida*) und 0.66 mg kg^{-1} Trockenerde (*L. terrestris*)). Zelluläre Veränderung nach Exposition gegenüber Imidacloprid waren bei *E. fetida* insgesamt am stärksten und *L. terrestris* am wenigsten stark ausgeprägt, jedoch war dieser Unterschied weniger eindeutig als für den Biomarker Gewichtsänderung.

Im zweiten Teil dieses Kapitels wurden bei den Testorganismen sowohl die Induktion des Stressproteins Hsp70 als auch das Vermeidungsverhalten gemessen. Während für *E. fetida* signifikante Änderungen des Hsp70-Levels bereits nach Exposition gegenüber der niedrigsten Imidaclopridkonzentration (0.2 mg kg^{-1} Trockenerde (14 d)) nachgewiesen werden konnten, wurden für die beiden anderen Regenwurmarten erst ab Konzentrationen von 2 (*A. caliginosa;* nach 1, 7 und 14 d) bzw. 4 mg kg^{-1} Trockenerde (*L. terrestris;* nach 14 d) signifikante Effekte beobachtet. Insgesamt konnte nur für eine Art (*A. caliginosa*) - und für diese nur in einem Expositionsansatz (0.2 mg kg^{-1} Trockenerde (14 d) - eine signifikante Erhöhung des Hsp70-Levels gemessen werden. Bei den anderen beobachteten Effekten handelte es sich jeweils um einen signifikanten Abfall des Stressproteinlevels. Bei den Fluchttests war *E. fetida* ebenfalls am sensitivsten und zeigte ein signifikantes Vermeidungsverhalten gegenüber allen mit Imidacloprid kontaminierten Testböden (0.2, 0.66 und 2 mg kg^{-1} Trockenerde). Für *L. terrestris* konnte kein signifikantes Vermeidungsverhalten nachgewiesen werden. *A. calignosa* zeigte sich sogar eine signifikante Attraktion bei der höchsten Testkonzentration (2 mg kg^{-1} Trockenerde).

Insgesamt konnten in dieser Studie verschiedene subletale Effekte bei allen drei Testorganismen bereits bei Exposition gegenüber umweltrelevanten Imidaclopridkonzentrationen (predicted environmental concentration (PEC) = 0.33-0.66 mg kg^{-1} Trockenerde (Oi 1999; Mostert et al. 2000)) nachgewiesen werden. Die vorliegenden Ergebnisse weisen darauf hin, dass der häufige Einsatz von Imidacloprid in der Landwirtschaft für Regenwürmer und damit auch für verschiedene Prozesse in Böden als kritisch angesehen werden sollte. Auch andere ökotoxikologische Studien, welche Auswirkungen von Imidacloprid (auf molekularer und physiologischer Ebene sowie auf Verhaltensebene) auf unterschiedliche Regenwurmarten untersuchten, konnten signifikante Effekte für ähnliche Konzentrationsbereiche (0.2–4 mg kg^{-1} Trockenerde) nachweisen (Luo et al. 1999; Zang et al. 2000; Lal et al. 2001; Mostert et al. 2002; Capowiez et al. 2003b).

Bei den eingesetzten Testorganismen zeigten die Biomarker Histopathologie und Gewichtsänderung insgesamt am sensitivsten und konstantesten die Schadstoffbelastung durch Imidacloprid an. Da angenommen wird, dass sich signifikante Gewichtsverluste bei Regenwürmern auf die Reproduktion und individuelle Überlebensrate auswirken können (Capowiez et al. 2005; Olvera-Velona et al. 2008), ist der Biomarker Gewichtsänderung von hoher ökologischer Relevanz. Auch vorangehende Studien konnten signifikante Gewichtsabnahmen bei unterschiedlichen Regenwurmarten (*E. fetida*, *L. terrestris, Aporrectodea nocturna* und *Allolobophora icterica*) nach Exposition gegenüber verschiedenen Konzentrationen von Imidacloprid beobachten (0.5 und 1.91 mg kg^{-1} Trockenerde) (Mostert et al. 2002; Capowiez et al. 2005; Goemz-Eyles et al. 2009).

Die ursprünglich für Mollusken entwickelte semi-quantitative histopathologische Bewertungsmethodik (Triebskorn & Köhler 2003) konnte auf die für die vorliegende Studie ausgewählten Regenwürmern angepasst werden. Sie hat sich in dieser Arbeit als geeignetes Werkzeug erwiesen, histologische Effekte einzustufen und die verschiedenen Regenwurmarten und Monitorgewebe in ihrer Sensitivität zu vergleichen. Hauptaugenmerke bisheriger histopathologischer Arbeiten mit Regenwürmern in der Ökotoxikologie waren sowohl die Epidermis und der Intestinaltrakt - da diese in direkten Kontakt mit Schadstoffen kommen - als auch das Chloragoggewebe, welchem man wichtige Funktionen bei Entgiftungsprozessen bei Regenwürmern zuschreibt (Prento 1987; Fischer & Molnár 1992; Vogel & Seifert 1992; Morowati 2000; Morgan & Turner 2005; Muthukaruppan et al. 2005; Chakra Reddy & Venkateswara Rao 2008; Muthukaruppan & Paramasamy 2010). Wie in der vorliegenden Studie mit Imidacloprid, konnten auch in verschiedenen vorangehenden histopathologischorientierten Arbeiten in den genannten Geweben negative Effekte nach Schadstoffexposition nachgewiesen werden (Gupta & Sundararaman 1988; Fischer & Molnár 1992; Vogel & Seifert 1992; Morowati 2000; Morgan & Turner 2005; Muthukaruppan et al. 2005; Chakra Reddy & Venkateswara Rao 2008; Muthukaruppan & Paramasamy 2010). Dass sich die Sensitivitäten der einzelnen Monitorgewebe in der vorliegenden Studie von Art zu Art unterschieden, könnte u.a. auf die verschiedenen Lebensweisen der Testorganismen zurückzuführen sein. So könnte beispielswiese die hohe Sensitivität des Mitteldarmgewebes von *A. caliginosa* mit der hohen Ingestions-Egestions-Rate dieser endogäischen Art zusammenhängen (Lavelle et al. 1989).

Die Sensitivität des standardisierten Fluchttests für Regenwürmern (ISO 2008) wird kontrovers diskutiert: Während einige Studien zu dem Ergebnis gelangten, dass der Test hochsensitiv Schadstoffbelastungen indizieren kann (Yeardley et al. 1996; Slimak 1997; Hund-Rinke et al. 2005; Garcia et al. 2008), konnten anderen Arbeiten zeigen, dass verschiedene Regenwurmarten unterschiedliche Schadstoffe in den Tests nicht gemieden haben (Hodge et al. 2000; Reinecke et al. 2002; Capowiez & Bérard 2006). Auch die vorliegende Studie erzielte widersprüchliche Ergebnisse und machte deutlich, dass eine der drei Arten (*E. fetida*) mit Imidacloprid kontaminierte Erde hochsensitiv mied, während die beiden anderen Arten selbst bei den höchsten Testkonzentrationen kein Vermeidungsverhalten zeigten. Pereira et al. (2010) gehen davon aus, dass der erfolgreiche Einsatz des Fluchttests stark von der Wirkungsweise der zu testenden Substanz abhängt, und raten dazu, den Test nicht zur Untersuchung neurotoxisch wirkender Schadstoffe heranzuziehen. Warum jedoch der Test in der vorliegenden Arbeit bei einer Art sehr gut und bei den anderen nicht funktionierte, konnte hier nicht geklärt werden. Gilman & Vardanis (1974) weisen darauf hin, dass sich Verhaltensantworten verschiedener Arten in ökotoxikologischen Studien stark unterscheiden können.

Die Stressproteinantwort (Hsp70) gilt in der Ökotoxikologie als sensitiver Effektbiomarker, welcher generelle Proteotoxizität anzeigen kann (Köhler et al. 1992; Ryan & Hightower 1996; Nadeau et al. 2001). In der vorliegenden Arbeit konnte insgesamt nur in einem Fall eine signifikante Induktion des Stressproteinlevels (Hsp70) nachgewiesen werden. Bei allen weiteren gemessenen Effekten handelte es sich jeweils um ein im Vergleich zur Kontrolle erniedrigtes Stressproteinlevel. Die Stressproteinantwort der 70kDa-Familie stellt für die in dieser Arbeit ausgewählten Testorganismen folglich nur einen schwachen Induktor dar. Die im Vergleich zur Kontrolle signifikant erniedrigten Stressproteinniveaus können auf eine allgemeine Stressüberlastung der Organismen hindeuten. Möglicherweise war in diesen Fällen bereits die Proteinbiosynthese gestört. *E. fetida* reagierte bei den in dieser Arbeit gemessenen Stressproteinantworten (Hsp70) am sensitivsten.

Insgesamt unterstreichen die vorliegenden Ergebnisse die Wichtigkeit des Einsatzes einer Biomarkerpalette, um die Toxizität einer Substanz genau einschätzen, und eine bessere Risikoabschätzung leisten zu können. Vergleicht man die Testorganismen in der vorliegenden Studie, so reagierte *E. fetida* insgesamt am sensitivsten. Die hohe Sensitivität des Standardtestorganismus *E. fetida* steht im Gegensatz zu einer Vielzahl anderer Forschungsergebnisse, welche zeigten, dass *E. feitda* relativ unsensitiv auf verschiedene Schadstoffe, wie beispielsweise Parathion, Propoxur und Chlorpyrifos reagierte (Edwards & Coulson 1992; Fitzpatrick et al. 1992; Kula & Kokta 1992; Ma & Bodt 1993; Spurgeon & Weeks 1998; Muthukaruppan & Paramasamy 2010). Schon Cairns (1986) behauptete, dass es so etwas wie die sensitivste Art *per se* nicht gäbe, und dass Multispeziestests für hochwertige ökotoxikologische Untersuchungen unerlässlich seien. Die Ergebnisse der vorliegenden Studie im Vergleich zu Ergebnissen vorangehender Arbeiten unterstützen diese Aussagen.

4.2. Entwicklung und Etablierung eines neuen Biomarkers auf Verhaltensebene für Regenwürmer

a) Capowiez Y, Dittbrenner N, Rault M, Triebskorn R, Hedde M, Mazzia C (2010): Earthworm cast production as a new behavioural biomarker for toxicity testing. Environmental Pollution 158, 388-393

b) Dittbrenner N, Triebskorn R, Moser I, Capowiez Y (2010): Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (Lumbricus terrestris and Aporrectodea caliginosa). Ecotoxicology 19, 1567-1573

Im ersten Teil dieses Kapitels ging es um die Entwicklung und Optimierung des Biotests „Losungsproduktion bei Regenwürmern“, welcher als Maß für eine generelle Aktivität der Tiere (Fress- und Grabaktivität) dienen sollte. Hierbei konnte für die Regenwurmart *L. terrestris* gezeigt werden, dass sich Siebe mit einer Maschenweite von 5,6 mm für die Trennung der Losungen von der Testerde am besten eignen. Der Wasseranteil in der Testerde wirkte sich sowohl auf die Menge der produzierten Losungen als auch auf die Eignung für das Trennen der Losungen von der Erde mittels Sieben aus. Es wurde nachgewiesen, dass sich ein Wassergehalt von 24% in der Testerde am besten für den Biotest eignet, da die getesteten Regenwürmer unter dieser Bedingung eine hohe Aktivität zeigten und zudem die Losungen nur selten mit Erde aggregierten. Des Weiteren wurde deutlich, dass das Sieben der Proben ohne ein vorheriges Trocknen direkt im Anschluss an die Expositionsversuche durchgeführt werden kann. Die Losungsproduktionsrate wurde in Losungsfrischgewicht [g] pro Regenwurmfrischgewicht [g] pro Anzahl der Expositionstage angegeben. Der auf der Basis dieser Erkenntnisse optimierte Losungsproduktionstest wurde anschließend auf seine Eignung zur Differenzierung der Toxizität von 6 verschiedenen Pestiziden (Methomyl, Chlorpyrifos-ethyl, Carbaryl, Ethyl-parathion, Azinphos-methyl und Imidacloprid) bei *L. terrestris* getestet. Als Referenz für die Sensitivität des Losungsproduktionstests wurden zudem Gewichtsänderungen gemessen. Bis auf die Substanz Azinphos-methyl konnte für alle getesteten Pestizide eine signifikant reduzierte Losungsproduktion von *L. terrestris* nach 7-tägiger Exposition gegenüber der jeweiligen Feldapplikationsmenge gemessen werden. Die Messungen der Gewichtsänderungen nach Exposition gegenüber der einzelnen Pestizide konnten mit Ausnahme von Imidacloprid jeweils bereits für die 0,1 fache Feldapplikationsrate signifikante negative Effekte für die getesteten Pestizide anzeigen.

Im zweiten Teil dieses Kapitels wurde der Losungsproduktionstest auf eine weitere, häufig in Agrarböden vorkommenden Regenwurmart, *A. caliginosa*, angepasst. Anschließend wurden mit dem Biotest Auswirkungen von Imidacloprid auf die Aktivität von *A. caliginosa* sowie auf die bereits im ersten Teil dieses Kapitels für den Losungsproduktionstest etablierte Regenwurmart *L. terrestris*

gemessen. Als Referenz wurden zudem für beide Arten Gewichtsänderungen nach Exposition gegenüber Imidacloprid ermittelt. Insgesamt konnte gezeigt werden, dass sich eine Siebmaschenweite von 4 mm am besten für den Losungsproduktionstest mit *A. caliginosa* eignete. Signifikant reduzierte Losungsproduktionsraten konnten für beide Testorganismen ab einer Imidaclopridkonzentration von 0.66 mg kg^{-1} Trockenerde beobachtet werden. Für *L. terrestris* konnte zudem eine signifikant erhöhte Losungsproduktionsrate bei der niedrigsten Testkonzentration (0.2 mg kg^{-1} Trockenerde) beobachtet werden. Signifikante Gewichtsabnahmen im Vergleich zur Kontrollgruppe wurden für beide Arten ab einer Imidaclopridkonzentration von 0.66 mg kg^{-1} Trockenerde gemessen.

Im vorliegenden Kapitel wurde ein Biotest für Regenwürmer auf Verhaltensebene entwickelt, der schnell und kostengünstig durchführbar ist. Er wurde auf die zwei Arten, *L. terrestris* und *A. caliginosa*, angepasst. Es konnte gezeigt werden, dass eine 7-tägige Exposition der Regenwürmer in Petrischalen, gefüllt mit gesiebter Testerde (3 mm; Bodenfeuchte 24%), sowie eine Trennung der Losungen von der Erde nach der Exposition mittels Sieben (Maschenweite 5,6 mm für *L. terrestris* und 4 mm für *A. caliginosa*) sich am besten für den Losungsproduktionstest eignete. Die mit dem Losungsproduktionstest gemessenen Effekte von Methomyl, Carbaryl, Azinphos-methyl, Imidacloprid, Chlorpyrifos-Ethyl und Ethyl-parathion auf die Aktivität von *L. terrestris* wurden jeweils für ähnliche Konzentrationsbereiche nachgewiesen, für welche auch vorangehende Forschungsarbeiten mit verschiedenen Regenwurmarten subletale Effekte beobachten konnten (Springett & Gray 1992; Booth & O´Halloran 2001; Gupta & Saxena 2003; Capowiez et al. 2005; Capowiez & Bérard 2006; Olvera-Velona et al. 2008; Gomez-Eyles et al. 2009; Pereira et al. 2010). Die im ersten Teil des vorliegenden Kapitels parallel zum Losungsproduktionstest durchgeführten Untersuchungen zu Gewichtsänderungen von *L. terrestris* nach Exposition gegenüber den verschiedenen Pestiziden waren jeweils sensitiver als die Aktivitätsmessungen mit dem Losungsproduktionstest (mit Ausnahme von Imidacloprid) und konnten bereits nach Exposition gegenüber der jeweils 0,1 fachen Applikationsmenge signifikante Effekte anzeigen. Bereits in Kapitel 1 der vorliegenden Dissertation wurde beobachtet, dass Gewichtsänderungen bei *L. terrestris*, *A. caliginosa* und *E. fetida* vergleichsweise sehr sensitiv Belastungen durch Imidacloprid anzeigen können. Auch in der Vergangenheit konnten verschiedene ökotoxikologische Studien zeigen, dass Gewichtsverluste bei Regenwürmern nach Pestizidexposition schon bei sehr geringen Konzentrationen auftreten können (Capowiez et al. 2005; Gomez-Eyles et al. 2009). Im zweiten Teil dieses Kapitels, in welchem Auswirkungen von Imidacloprid auf *A. caliginosa* und *L. terrestris* untersucht wurden, erwiesen sich die beiden eingesetzten Biomarker als vergleichbar sensitiv und konnten für beide Testorganismen jeweils signifikante negative Effekte auf Körpergewicht und Losungsproduktion ab einer Imidaclopridkonzentration von 0,66 mg kg^{-1} Trockenerde anzeigen. Die

signifikant erhöhte Losungsproduktion von *L. terrestris* bei Exposition gegenüber der niedrigsten Testkonzentration (0,2 mg kg^{-1} Trockenerde) könnte möglicherweise auf eine durch Fluchtverhalten erhöhte Aktivität und/ oder auf einen erhöhten Metabolismus aufgrund von Entgiftungsprozessen zurückgeführt werden.

Signifikante Gewichtsverluste bei Regenwürmern können von hoher ökologischer Relevanz sein, da diese die Tiere schwächen und sich damit auf Verhalten, Vermehrung und Überleben der Tiere auswirken können (Capowiez et al. 2005; Capowiez & Bérard 2006; Olvera-Velona et al. 2008). Da der Losungsproduktionstest indirekt die Aktivität von Regenwürmern misst, und da Regenwürmer durch ihre Aktivität wichtige Funktionen in Böden erfüllen, kann dieser neu entwickelte Test als ökologisch hoch relevant angesehen werden. Der Losungsproduktionstest hat sich in der vorliegenden Arbeit zudem als sehr sensitiv erwiesen und konnte für 5 von 6 getesteten Pestiziden eine signifikant verminderte Aktivität bei *L. terrestris* nach Exposition gegenüber normalen Feldapplikationsraten anzeigen. Der Test zeichnet sich neben den bereits genannten Eigenschaften auch durch eine schnelle und kostengünstige Handhabung aus und könnte Aktivitätsmessungen durch 2D-Terrarien (Capowiez et al. 2003b) ersetzen. Das Sieben könnte in Zukunft maschinell mit Schüttlern durchgeführt werden. Ein Nachteil des in dieser Arbeit entwickelten Tests liegt allerdings darin, dass nicht nachgewiesen werden kann, ob eine gemessene verminderte Aktivität auf eine physiologischen Schwächung der Tiere oder auf eine Vermeidung der Aufnahme kontaminierter Erde durch die Regenwürmer zurückzuführen ist. Letztlich sind aber beide Szenarien von ökologischer Bedeutung.

Da sowohl die im ersten als auch die im zweiten Teil dieses Kapitels subletale Effekte für jeweils umweltrelevante Konzentrationen von Pestiziden gemessen wurden, könnte ein vermehrter Einsatz dieser Substanzen als kritisch für Regenwürmer und damit auch für Böden sein.

4.3. Erfassung von Kurz- und Langzeiteffekten auf das Grabverhalten von Regenwürmern nach Exposition gegenüber dem Insektizid Imidacloprid

Dittbrenner N, Moser I, Triebskorn R, Capowiez Y (2011): Assessment of short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (Aporrectodea caliginosa and Lumbricus terrestris) by using 2D and 3D post-exposure techniques. Chemosphere 84: 1349-1355

Um Auswirkungen des Insektizids Imidacloprid auf das Grabverhalten von *Aporrectodea caliginosa* und *Lumbricus terrestris* zu analysieren, wurden im letzten Kapitel der vorliegenden Arbeit in Postexpositionsversuchen Kurz- (24-96 h) bzw. Langzeiteffekte (6 Wochen) mit Hilfe der 2D-Terrarienmethode (Evans 1947) bzw. 3D-Röntgenanalysen (Capowiez et al. 2003a) im Labor gemessen. In den Kurzzeitexperimenten (2D) konnten für *A. calignosa* bereits nach Exposition gegenüber der niedrigsten Testkonzentration (0.2 mg kg^{-1} Trockenerde) signifikante Auswirkungen auf das Grabverhalten nachgewiesen werden, während für *L. terrestris* erst nach Exposition gegenüber den höchsten Testkonzentrationen (2 und 4 mg kg^{-1} Trockenerde) negative Effekte gemessen wurden. Insgesamt erwiesen sich die Endpunkte „Gesamtgrablänge nach 24h“ und „maximale Grabtiefe nach 24h“ in den Kurzzeitversuchen als am sensitivsten. In den 3D-Langzeitversuchen, in welchen jeweils zwei *L. terrestris* und vier *A. caliginosa* in Testzylindern inkubiert wurden, konnte gezeigt werden, dass das Volumen der Grabgänge mit ansteigender Imidaclopridkonzentration (für den Bereich 0.2 – 4 mg kg^{-1} Trockenerde) signifikant linear abnahm ($p<0.01$; $R^2=0.326$). Bei den gegenüber der höchsten Imidaclopridkonzentration (4 mg kg^{-1} Trockenerde) exponierten Tieren war das von Grabgängen eingenommene Volumen im Vergleich zur Kontrolle um 40% reduziert. In der vorliegenden Studie konnte jedoch für die 3D-Experimente keine Methode entwickelt werden, die eine absolut fehlerfreie Zuordnung der Grabgänge zu den einzelnen Arten erlaubt hätte. Deshalb konnten an dieser Stelle nur Aussagen über Gesamtaktivität beider Arten gemacht und nicht die jeweilige Grabaktivität der einzelnen Arten gemessen werden.

Im vorliegenden Kapitel konnte in Laborversuchen gezeigt werden, dass das Insektizid Imidacloprid in den eingesetzten Konzentrationen sowohl Kurzzeit- als auch Langzeitauswirkungen auf das Grabverhalten der häufig in Agrarböden vorkommenden Regenwurmarten *Aporrectodea caliginosa* und *Lumbricus terrestris* haben kann. Die herangezogenen Endpunkte - Gesamtgrablänge und maximale Grabtiefe in den Kurzzeitversuchen (2D) sowie Gesamtvolumen der Grabgänge in den Langzeitversuchen (3D) – stellen ökologisch relevante Endpunkte dar, da sie Aufschluss über die Grabaktivität der Tiere geben und damit indirekt mit Bodenfunktionen, wie z.B. Wasser/ Gastransfereigenschaften in Verbindung gebracht werden können (Bastardie et al. 2003; Capowiez et al. 2006). Die in dieser Studie beobachteten Effekte wurden für ähnliche Konzentrationen (0.2-4 mg

kg^{-1} Trockenerde) gemessen, für welche frühere Arbeiten Auswirkungen von Imidacloprid auf verschiedene Regenwurmarten bereits diverse subletale Effekte nachweisen konnten (Luo et al. 1999; Zang et al. 2000; Lal et al. 2001; Mostert et al. 2002; Capowiez et al. 2003b). In den vorliegenden 2D-Experimenten reagierte *A. calignosa* deutlich sensitiver als *L. terrestris*. Mit den 3D-Versuchen konnten nur Aussagen über die Grabaktivität beider Arten in Kombination gemacht werden, da die einzelnen Antworten aufgrund methodischer Schwierigkeiten nicht getrennt werden konnten. Eine höhere Sensitivität gegenüber Imidacloprid wurde für *A. caliginosa* im Vergleich zu *L. terrestris* bereits in den unter Kapitel 1 beschriebenen Arbeiten gezeigt (Dittbrenner et al. 2011a,b). In dem vorliegenden Kapitel konnte nicht geklärt werden, ob die unterschiedlichen Sensitivitäten auf artspezifische Unterschiede oder ggf. auf für *L. terrestris* unzureichende Lebensbedingungen in den verwendeten 2D-Terrarien zurückzuführen waren. So können z.B. die Grabgänge von *L. terrestris* im Freiland eine maximale Grabtiefe von 40cm deutlich übersteigen (Shipitalo & Butt 1999).

Im vorliegenden Kapitel war die 2D-Methode sensitiver als die 3D-Analysen. Dies war zu erwarten, da die vorexponierten Regenwürmer für die Messungen in den 2D-Terrarien nur 96 h, jedoch für die 3D-Röntgenanalysen 6 Wochen in unkontaminierter Erde inkubiert wurden. Bei den 3D-Analysen hatten die Regenwürmer somit deutlich mehr Zeit, sich von der Schadstoffexposition zu erholen. Sowohl die 2D- als auch die 3D-Methodik wurden in dieser Arbeit als Postexpositionsanalysen eingesetzt. Der Vorteil dieser Herangehensweise ist, dass die tatsächliche Grabkapazität und damit physische Beeinträchtigungen der Regenwürmer gemessen werden. In vorangehenden ökotoxikologischen Studien wurde die Grabaktivität von Regenwürmern in kontaminierter Testerde bestimmt (Capowiez et al. 2003b; Capowiez et al. 2006; Olvera-Velona et al. 2008), weshalb in diesen Arbeiten nicht geklärt werden konnte, zu welchem Teil eine gemessene verminderte Grabaktivität auf Artefakte, wie z.B. Vermeidungsverhalten, zurückzuführen war.

Methodisch eigneten sich die Messungen in den 2D-Terrarien hervorragend, um ökologisch relevante Kurzzeitauswirkungen (24 bis 96 h Inkubation) auf das Grabverhalten der Testorganismen in einer relativ hohen Anzahl an Replikaten (n=7) zu messen. Langzeiteffekte auf das Grabverhalten von Regenwürmern können sehr gut und realitätsnahe mit der 3D-Röntgentechnik dargestellt werden. Der große Aufwand und die relativ hohen Kosten beim Einsatz dieser Methodik lassen jedoch nur einen limitierten Gebrauch zu, was häufig – wie auch in der vorliegenden Studie - die statistische Auswertung beeinträchtigt.

Da in der vorliegenden Studie Grabverhaltensänderungen auch für umweltrelevante Imidaclopridkonzentrationen (PEC (predicted environmental concentration) = 0.33-0.66 mg kg^{-1} Trockenerde) gemessen wurden, und da sich Grabverhaltensänderungen von Regenwürmern auf

verschiedene Bodenfunktionen auswirken können (Bastardie et al. 2003; Capowiez et al. 2006), muss der Einsatz von Imidacloprid in der Landwirtschaft als kritisch angesehen werden.

5. Synthese

In der vorliegenden Dissertation konnte in Laborversuchen gezeigt werden, dass das Insektizid Imidacloprid bei drei unterschiedlichen Regenwurmarten auch bereits in umweltrelevanten Konzentrationen auf verschiedenen Ebenen biologischer Organisation Schadeffekte hervorrufen kann. Unter anderem wurden bei den Testorganismen auch Effekte nachgewiesen, welche sich möglicherweise negativ auf Böden auswirken (z.B. reduzierte Grabaktivität). Daher muss der Einsatz von Imidacloprid in der Landwirtschaft als kritisch angesehen werden. Der in der vorliegenden Dissertation neu entwickelte und für zwei Regenwurmarten etablierte Losungsproduktionstest hat sich als sehr sensitiver, schneller und kostengünstiger Biotest erwiesen, welcher durch die indirekte Messung der Regenwurmaktivität von hoher ökologischer Relevanz ist. Der Grad der Sensitivität der eingesetzten Biomarker war in der Regel stark abhängig vom jeweiligen Testorganismus. Am konstantesten konnten die histologischen Untersuchungen sowie Gewichtsmessungen Schadeffekte bei allen Testorganismen anzeigen. Entgegen früher erhobener Forschungsergebnisse reagierte der Standardtestorganismus *Eisenia fetida* bei einigen Versuchen in der vorliegenden Arbeit am sensitivsten. Insgesamt machen die erzielten Ergebnisse deutlich, dass kein Testorganismus und kein Biotest *per se* am sensitivsten reagieren. Deshalb sollten für eine relevante Risikoabschätzung von Schadstoffen nach Möglichkeit immer verschiedene Biomarkertests kombiniert und mehrere Vertreter eines Taxons parallel geprüft werden.

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten

Kapitel 1

a) *Dittbrenner N, Schmitt H, Capowiez Y, Triebskorn R (2011; in press): Sensitivity of Eisenia fetida in comparison to Aporrectodea caliginosa and Lumbricus terrestris after imidacloprid exposure. Body mass change and histopathology. Journal of Soils and Sediments*

Eigenanteil gesamt 90%. Vollständiger Eigenanteil an der Versuchsplanung und an den Expositionsexperimenten. Mit Ausnahme der Bearbeitung und Auswertung der histologischen Proben von *Aporrectodea caliginosa*, welche von der Bachelor-Kandidatin Frau H. Schmitt durchgeführt wurden, wurde die übrige Bearbeitung und Auswertung für beide Biomarker von mir geleistet. Fachliche Betreuung erfolgte durch Frau Prof. Dr. R. Triebskorn (Universität Tübingen) und Herrn Dr. Y. Capowiez (INRA, Avignon, Frankreich). Das Verfassen und die Bearbeitung des Manuskripts wurden zu 100% von mir geleistet.

b) *Dittbrenner N, Capowiez Y, Köhler H-R, Triebskorn R (2011; in press): Stress protein response (Hsp70) and avoidance behaviour in Eisenia fetida, Aporrectodea caliginosa and Lumbricus terrestris. Journal of Soils and Sediments*

Eigenanteil gesamt 80%. Vollständiger Eigenanteil an der Versuchsplanung. Mit Ausnahme der Fluchttests mit *Aporrectodea caliginosa* und *Lumbricus terrestris*, welche von Herrn Dr. Y. Capowiez durchgeführt wurden, wurde die übrige Bearbeitung und Auswertung der Proben für die Fluchttests und Stressproteinanalysen von mir geleistet. Die fachliche Betreuung erfolgte durch Frau Prof. Dr. R. Triebskorn (Universität Tübingen), Herrn Dr. Y. Capowiez (INRA, Avignon, Frankreich) und Herrn Prof. Dr. H.-R. Köhler (Universität Tübingen). Das Verfassen und die Bearbeitung des Manuskripts wurden zu 100% von mir geleistet.

Kapitel 2

a) *Capowiez Y, Dittbrenner N, Rault M, Triebskorn R, Hedde M, Mazzia C (2010) Earthworm cast production as a new behavioural biomarker for toxicity testing. Environmental Pollution 158, 388-393*

Eigenanteil gesamt 20%. Vollständiger Eigenanteil an der Durchführung der Losungsproduktionstests mit dem Insektizid Imidacloprid. Alle übrigen Tests wurden von Herrn Dr. Y. Capowiez durchgeführt. Die Auswertung und Beurteilung der Ergebnisse sowie die Einstufung des Potenzials des Losungsproduktionstests als neuer Biomarker für Regenwürmer erfolgte mit Unterstützung von Frau Dr. M Rault, Frau Prof. Dr. R. Triebskorn, Herrn M. Hedde und Herrn Dr. C. Mazzia. Fachliche Betreuung erfolgte durch Herrn Dr. Y. Capowiez (INRA, Avignon, Frankreich). Das Verfassen und die Bearbeitung des Manuskripts erfolgten durch Herrn Dr. Y. Capowiez.

b) *Dittbrenner N, Triebskorn R, Moser I, Capowiez Y (2010) Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (Lumbricus terrestris and Aporrectodea caliginosa). Ecotoxicology 19, 1567-1573*

Eigenanteil gesamt 90%. Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die Expositionsexperimente sowie die Messungen der Gewichtsänderung wurden mit Unterstützung der Praktikantin Frau I. Moser durchgeführt. Die fachliche Betreuung erfolgte durch Herrn Dr. Y. Capowiez (INRA, Avignon, Frankreich) und Frau Prof. Dr. R. Triebskorn (Universität Tübingen). Das Verfassen und die Bearbeitung des Manuskripts wurden zu 100% von mir geleistet.

Kapitel 3

Dittbrenner N, Moser I, Triebskorn R, Capowiez Y (2011): Assessment of short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (Aporrectodea caliginosa and Lumbricus terrestris) by using 2D and 3D post-exposure techniques. Chemosphere 84: 1349-1355

Eigenanteil insgesamt: 60%. Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung der Grabverhaltensmessungen im zweidimensionalen Raum (2D). Die Grabverhaltensanalysen im dreidimensionalen Raum (3D) wurden von Herrn Dr. Y. Capowiez geplant, durchgeführt und ausgewertet. Die Durchführung der Grabverhaltensmessungen (2D) erfolgte mit Unterstützung der Praktikantin Frau I. Moser. Die fachliche Betreuung erfolgte durch Herrn Dr. Y. Capowiez (INRA, Avignon, Frankreich) und Frau Prof. Dr. R. Triebskorn (Universität Tübingen). Das Verfassen und die Bearbeitung des Manuskripts wurden zu 80% von mir geleistet.

Kapitel 1

a) Sensitivity of *Eisenia fetida* in comparison to *Aporrectodea caliginosa* and *Lumbricus terrestris* after imidacloprid exposure. Body mass change and histopathology. *Journal of Soils and Sediments, in press.*

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Abstract

Standard tests in ecotoxicology are often carried out with just one or a few species representing an entire taxon. This in consequence poses risk of underestimating the impact of toxicants on the environment. In earthworm ecotoxicity tests, the species *Eisenia fetida* or *Eisenia andrei* - both not present in most agricultural soils – are almost exclusively used. In the present study we have compared *E. fetida* and two other earthworm species - highly relevant in agricultural soils (*Aporrectodea caliginosa* and *Lumbricus terrestris*) – regarding their sensitivities towards soil contaminated with the widely used insecticide imidacloprid. In laboratory experiments, the specimens were individually exposed to various concentrations of the pesticide (0.2, 0.66, 2 and 4 mg kg $^{-1}$ dry soil) for 1, 7 and 14 days, and afterwards sub-lethal effects were assessed (body mass change and histopathology). Whereas significant changes in body mass in *E. fetida* and *A. caliginosa* occurred already after exposure to imidacloprid concentrations as low as 0.2 (7 days) and 0.66 mg kg $^{-1}$ dry soil (14 days), significant body mass changes in *L. terrestris* were only observed for much higher concentrations (starting at 2 (7 days) and 4 mg kg $^{-1}$ dry soil (14 days)). The histopathological examinations revealed that significant cellular changes occurred already after 24 h exposure to the lowest imidacloprid concentrations (0.2 (for E. fetida and A. caliginosa) and 0.66 mg kg -1 dry soil (for Lumbricus terrestris)), but the degree of detrimental effects as well as species-specific differences were dependent on the monitor tissue (midgut, chloragogenous tissue or skin). In general *E. fetida*

seemed to be more sensitive than *L. terrestris* concerning cellular alterations, but the hierarchy in species-specific differences was less obvious than for body mass change. The present study contributes to a better understanding of species-specific differences in sensitivity towards environmental toxicants. It also shows the necessity of including a range of species - being representatives of an animal taxon - in ecotoxicological tests in order to draw conclusions for risk assessment more precisely. Moreover, the study shows that the use of imidacloprid in agriculture may be of environmental concern.

Keywords: Earthworms – Imidacloprid - Standard test organism – Body mass change – Histopathology

1. Introduction

The intensive use of pesticides in agriculture bears a wide range of potential risks for the environment. The neonicotinoid imidacloprid is one of the most frequently used insecticides in agriculture worldwide, but is known to cause negative effects also on non-target and important organisms, including earthworms (Capowiez et al. 2003; 2005; 2006; 2010; Dittbrenner et al. 2010a; Kreutzweiser et al. 2009; Luo et al. 1999; Mostert et al. 2002). As earthworms contribute to a great extent to soil functioning, i.e. to soil fertility, soil formation and the breakdown of organic matter etc. (Edwards and Bohlen 1992; Edwards and Lofty 1972; 1996; McCredie and Parker 1992; Scheu 1987), detrimental effects of pesticides on these organisms may harm soils and soil quality indirectly. Due to their ecological importance, their high sensitivity towards many toxicants and their great abundance in different soils, the use of earthworms for pesticide toxicity testing is compulsory (EEC 2003). Since ecotoxicological standard tests with earthworms are primarily performed with the epigeic species *Eisenia fetida* and *Eisenia andrei* (OECD 1984; EEC 2003; OECD 2004; ISO 2008), it is indispensable (e.g. for an accurate setting of safety factors) to examine whether the sensitivity of *E. fetida* and *E. andrei* towards environmental toxicants differs significantly from the sensitivity of other earthworm species. In this context, it has already been claimed that possibly the Eisenia species as standard test organisms may be less sensitive than other earthworms and that comparative studies are therefore needed (Edwards and Coulson 1992; Fitzpatrick et al. 1992; Kula and Kokta 1992; Ma and Bodt 1993; Muthukaruppan and Paramasamy 2010; Spurgeon and Weeks 1998). When using earthworms in ecotoxicological studies one has to be aware of the fact that under natural conditions earthworms are exposed to environmental toxicants in different ways, depending on the “ecological type”

(anecic; endogeic; epigeic) the earthworm belongs to (Bouché 1992, Tomlin 1992). Since different ways of life can result in different frequencies and intensities of toxicant encounter under natural conditions, preferably one representative of each "ecological type" should be used for toxicity testing (Christensen and Mather 1994).

However, for a comprehensive ecotoxicological study in general and for a meaningful comparison of species-specific sensitivities in particular, not only lethal endpoints, but also sub-lethal responses should be regarded. For this purpose it is recommended to use a set of biomarkers including endpoints on different biological levels of biological organisation (Köhler and Triebskorn 2004). Based on this, we have chosen four different biomarkers to investigate sub-lethal effects of imidacloprid on three earthworm species in laboratory experiments in this study. We have focussed on histopathological changes and changes in body mass in the first part of this communication, as well as on molecular (changes in hsp70-level) and on behavioural responses (avoidance) in the second part of this study (Dittbrenner et al. 2010b).

Body mass change - as an integral part of the acute and chronic standard tests – is well established in toxicity testing and can sensitively indicate stress in earthworms (Capowiez and Bérard 2006; Dittbrenner et al. 2010a; Gomez-Eyles et al. 2009; Olvera-Velona et al. 2008; Zwahlen et al. 2003). Without clearing the earthworms intestines prior weighing, as it was done in the present study, body mass change can be interpreted as an indicator of earthworm activity (filling of the intestine) (Capowiez et al. 2010; Dittbrenner et al. 2010a). Since there is a link between earthworm activity and soil quality, this biomarker is of high ecological relevance. Cellular biomarkers have often been used successfully in ecotoxicological studies in different organisms (Cajaraville et al. 1995; Morowati 2000; Triebskorn et al. 1997; Triebskorn and Köhler 2003). In earthworms, former investigations in cellular toxicity testing have focussed on qualitative changes in the intestine, the chloragogenous tissue and the epidermis (Chakra Reddy and Venkateswara Rao 2008; Morowati 2000; Muthukaruppan 2005) or on quantitative changes in chloragogenous tissue (Fischer and Molnar 1992; Morgan and Turner 2005). According to our opinion, it is crucial to combine a qualitative description of histological conditions with a semi-quantitative analysis in order to precisely categorize effects. However, to the best of our knowledge, such an approach, has never been conducted in earthworm ecotoxicology before.

2. Material and methods

2.1 Test organisms, soil and imidacloprid

Adult earthworms of the species *Lumbricus terrestris* and *Eisenia fetida* were purchased from a fishery store in Avignon (France), while adult earthworms of the species *Aporrectodea caliginosa* were sampled manually from an INRA experimental orchard near Avignon (no pesticide application for 5 years). The test soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3 (in water), CEC = 8.2 cmol kg^{-1}, WHC = 0.247 g g^{-1}) was collected from an abandoned apple orchard (no pesticide application for 10 years) located in Montfavet near Avignon. Prior to the pesticide exposure and to the avoidance test, the earthworms were acclimatized for 7 days in the test soil in a dark climate chamber at 12°C (*A. caliginosa* and *L. terrestris*) and 22°C (*E. fetida*), respectively. General recommendations established by Fründ et al. (2010) were followed for handling of the test organisms.

The insecticide imidacloprid (255.66 g/mol; purity: 99.9%) was dissolved in distilled water to different concentrations used in this study. The predictive environmental concentration (PEC) of imidacloprid was found to be in the range of 0.33 - 0.66 mg kg^{-1} dry soil depending on the country and crop under consideration (Mostert et al. 2000; Oi 1999). We therefore have defined the normal application rate (1x) to be 0.66 mg kg^{-1} dry soil corresponding to a field application rate of 244 g ha^{-1}. Soil concentration values refer to a single application with a homogeneous distribution in the upper 5 cm of soil with a density of 1.5 kg l^{-1} and no crop interception.

2.2 Exposure experiments

Prior to the exposure experiments, the soil moisture was adjusted to 20% (80% of the WHC) and then soil spiking was conducted according to Capowiez et al. (2005), resulting in a final soil moisture of 25% of dry soil weight. For each tested earthworm species, three different imidacloprid concentrations (plus water control) were used: 0.2 (0.3x), 0.66 (1x) and 2 mg kg^{-1} dry soil (3x) for *A. caliginosa* and *E. fetida* as well as 0.66 (1x), 2 (3x) and 4 mg kg^{-1} dry soil (6x) for *L. terrestris*. We have chosen lower test concentrations for *E. fetida* and *A. caliginosa*, because the highest concentration used for *L. terrestris* was highly lethal for the former.

The earthworms were exposed individually for 7 and 14 days (for body mass change) and for 1, 7 and 14 days (for histology), respectively. The exposure experiments were conducted in Petri dishes (diameter = 10 cm) filled with 100 g contaminated or uncontaminated (control) soil in dark climate chambers at 12°C (*A. caliginosa* and *L. terrestris*) and 22°C (*E. fetida*), accordingly. Before and after

the exposure the earthworms were rinsed in tap water, gently dried and weighed. Weighing of the earthworms was done without clearing their intenstines, so body mass change was rather a measure of earthworm activity (filling of the gut) than a measure of growth.

2.3 Histology

Eight specimens of each species were used for each treatment (concentration; time period). After the exposure experiments, the earthworms for histological examination were kept in tap water for 6 h at 12°C or 22 °C (depending on the species) to clear the intestines from soil particles. This was necessary in order not to hinder microtome cutting by gut content . After these 6 h, the earthworms were anesthetized in carbonated water for 3 min and then cross cut twice directly behind the clitellum region in order to achieve tissue pieces with a length of two segments. After dissection of the earthworms, the samples were fixed in Bouin`s solution and stored at room temperature for several days. For further sample processing, samples were dehydrated in a graded series of ethanol and then embedded in Technovit 7100 resin (Heraeus Kulzer, Germany). Afterwards, they were cut into 5 µm sections on a microtome and spread on microscope-slides. Finally, the sections were stained by hematoxylin - eosin (HE) as well as by a combinded method using Periodic Acid-Schiff (PAS)-Alcianblue. A light microscope (Axioskop 2, Zeiss, Germany) was used to examine the samples.

Histology of the midgut and chloragogenous tissue and epidermis was qualitatively described and histopathological changes were semi-quantitatively assessed for each earthworm. According to the procedure described for mollluscs by Triebskorn and Köhler (2003) the state of cellular pathology was classified in 3 categories for each tissue investigated: control status (1); status of reaction (2); status of destruction (3). Table 1 shows the histopathological symptoms characterizing the 3 categories in the different monitor tissues. For each earthworm the different tissues were examined and a mean assessment value (MAV) was calculated for each monitor tissue of an exposure group.

2.4 Statistical analysis

Data for both semi-quantitative assessment of histopathology and for body mass change were tested for normality (Shapiro-Wilks-test) and were log-transformed when necessary. The data were then analysed by a one-way ANOVA and post-hoc comparisons were conducted using Tukey-HSD. Levels of significance were defined: $0.01 < P = 0.05$: $*$ (slightly significant); $0.001 < P = 0.01$: $**$ (significant); $P = 0.001$: $***$ (highly significant). Data for semi-quantitative assessment of histopathology and body mass change were correlated by means of linear regression.

Table 1. Classification of histopathological effects as a basis for the semi-quantitative assessment.

	Category 1: Control status	**Category 2: Status of reaction**	**Category 3: Status of destruction**
Cells of midgut tissue	• Columnar-shaped cells • Nucleus oval in shape • Clear cellular compart-mentation • Medium-dense cytoplasm	• Irregular cell shape • Irregular shape and altered size of nucleus • Irregular cellular compart-mentation • Cytoplasm of sparse density • Enhanced intercellular space	• Cells disintegrated
Chloragogenous cells	• Cone-shaped cells • Nucleus oval in shape • Moderate density of vacuoles	• Irregular cell shape and size (often swollen) • Irregular shape and altered size of nucleus • Enhanced vacuolization • Cells apically flattened	• Cells disintegrated
Epidermis	• Smooth surface of epidermis • Cells slightly filled with mucus • Clear cellular compart-mentation	• Irregular surface of epidermis • Cells strongly filled with mucus • Irregular cellular compart-mentation	• Cells disintegrated

3. Results

3.1 Mortality

Eight specimens of *E. fetida* died after 14 days exposure to imidacloprid concentrations of both 0.6 and 2 mg kg $^{-1}$ dry soil as well as six *E. fetida* after 7 days exposure to highest concentration (2 mg kg^{-1} dry soil). In addition, two *A. caliginosa* died after 14 days exposure to the highest imidacloprid concentration (2 mg kg^{-1} dry soil).

3.2 Body mass change after 7 days (n=36) and 14 days (n=18)

Eisenia fetida (Table 2): Significant losses in body mass were observed only after 7 days of exposure. All treatments differed significantly from the control group.

Aporrectodea caliginosa (Table 3): Significant differences between the treatments were observed both after 7 days and after 14 days of exposure. Compared to the control group, all exposure groups showed a significant decrease in body mass after 7 days, while after 14 days only the body mass of the 1x- and 3x- exposure groups were significantly decreased.

Lumbricus terrestris (Table 4): Body mass change differed significantly after 7 days and after 14 days of exposure. Significant losses were observed after 7 days in the 3x- and in the 6x- exposure groups compared to the control animals. After 14 days of exposure only the 6x- exposure group showed a significant loss in body mass compared to the control group.

Table 2 Mean initial body mass (+SD) and mean relative body mass (+SD) (expressed in percentage of the initial body mass) of *Eisenia fetida* after imidacloprid exposure for 7 days (n = 36) and 14 days (n = 18). Values in bold are significantly different from the control values ($p < 0.001$).

	0 mg kg^{-1} dry soil	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
Initial body mass [g]	0.38 (0.11)	0.42 (0.14)	0.36 (0.12)	0.36 (0.09)
Relative body mass after 7d [%]	126.1 (14.5)	**102 (13)**	**99.7 (31.2)**	**80.4 (10.3)** (n = 30)
Relative body mass after 14d [%]	115 (10.4)	107.4 (12.8)	**+** (n = 0)	**+** (n = 0)

Table 3 Mean initial body mass (+SD) and mean relative body mass (+SD) (expressed in percentage of the initial body mass) of *Aporrectodea caliginosa* after imidacloprid exposure for 7 days (n = 36) and 14 days (n = 18). Values in bold are significantly different from the control values ($p < 0.001$).

	0 mg kg^{-1} dry soil	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
Initial body mass [g]	0.56 (0.17)	0.59 (0.17)	0.62 (0.18)	0.62 (0.19)
Relative body mass after 7d [%]	119.6 (11.2)	**104.5 (11.9)**	**98 (10.3)**	**74.5 (9.1)**
Relative body mass after 14d [%]	112.4 (7.5)	106.7 (13.8)	**91 (8.7)**	**67.8 (10.2)** (n = 16)

Table 4 Mean initial body mass (+SD) and mean relative body mass (+SD) (expressed in percentage of the initial body mass) of *Lumbricus terrestris* after imidacloprid exposure for 7 days (n = 36) and 14 days (n = 18). Values in bold are significantly different from the control values ($p < 0.001$).

	0 mg kg^{-1} dry soil	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**	**4 mg kg^{-1} dry soil**
Initial body mass [g]	4.20 (1.17)	4.15 (0.89)	4.08 (1.55)	4.18 (1.09)
Relative body mass after 7d [%]	110 (11.4)	105.3 (14.2)	**90.7 (8.9)**	**81.6 (7.8)**
Relative body mass after 14d [%]	98.5 (19.1)	89.8 (15.6)	90 (9.7)	**79.9 (9.3)**

3.3 Histology

3.3.1 Qualitative assessment of histopathology

In general, the qualitative effects observed in the different monitor tissues were similar for all species and are summarized below:

Midgut tissue (Fig. 1): With increasing pesticide concentration and exposure time, the cells of the midgut tissue often showed a degradation of the cellular compartmentation as well as a reduced density of the cytoplasm and irregularly shaped nuclei. After exposure to the higher pesticide concentrations, increased occurrence of intercellular spaces as well as the disintegration of entire cells was observed.

Chloragogenous tissue (Fig. 2): In the chloragocytes, an increasing vacuolisation, an altered density of chloragosomes, a deformation of nuclei and a flattening of cellular apices were frequently observed with rising pesticide concentration and prolonged exposure time. Disintegration of entire chloragocytes sometimes occurred after exposure to higher pesticide concentrations.

Epidermis (Fig. 3): Due to pesticide exposure the cuticula was often corrugated, many mucocytes of the epidermis were hypertrophic and showed an increased mucus secretion. Moreover a degradation of cellular compartmentation occurred. After exposure to higher pesticide concentrations, disintegration of epidermal cells was sometimes observed.

In *A. caliginosa* many strongly filled mucocytes were already observed in the control group under laboratory conditions.

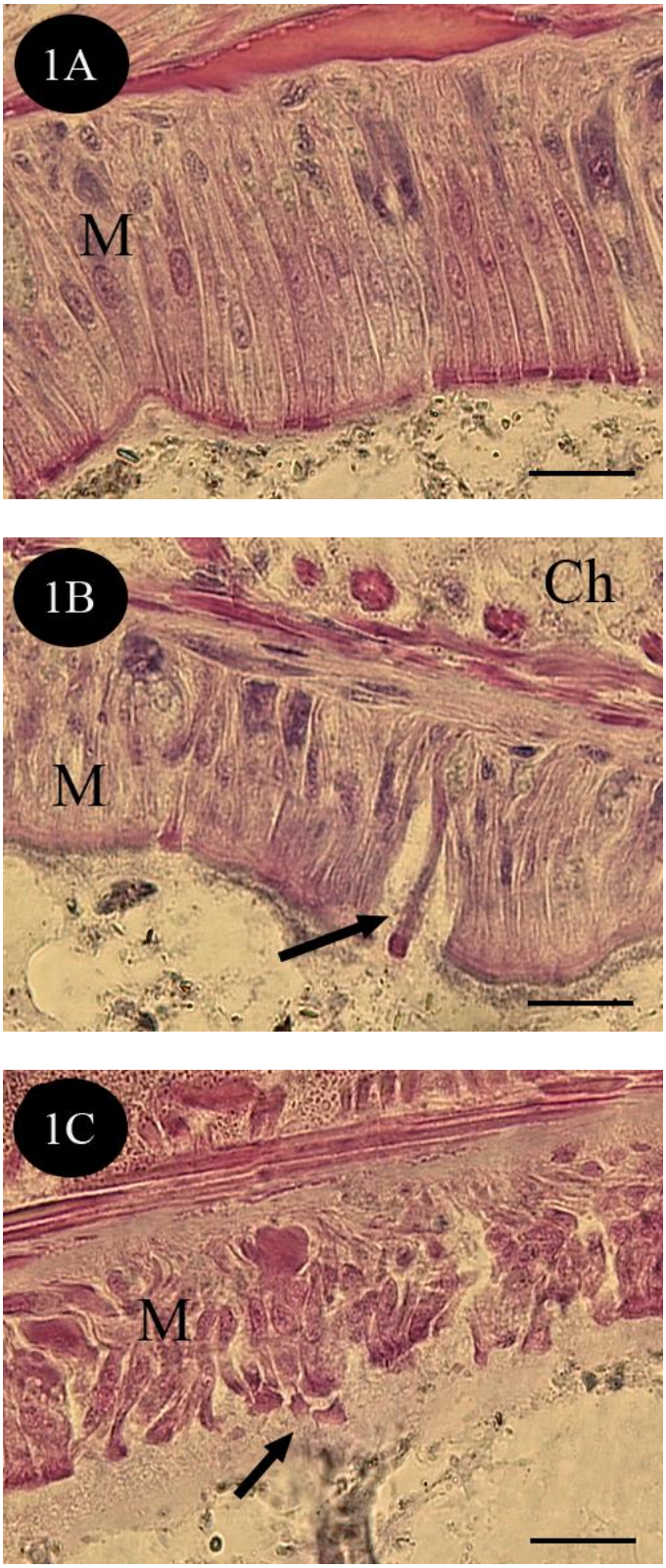

Figure 1: Midgut cells of *Lumbricus terrestris*. **A.** Control status: Midgut cells showing a clear compartmentation, a homogenous cytoplasma and ovally shaped nuclei (control group/ 1d exposure time). **B.** State of reaction: Irregularly shaped midgut cells and nuclei, disrupted cellular compartmentation and an increased occurrence of intercellular spaces (Arrow) (1x exposure group/ 14d exposure time). **C.** State of destruction: Many midgut cells are destroyed. Cell debris in the lumen (Arrow) (6x exposure group/ 7d exposure time). M, midgut cells; Ch, chloragocytes; Scale bars: 25 µm.

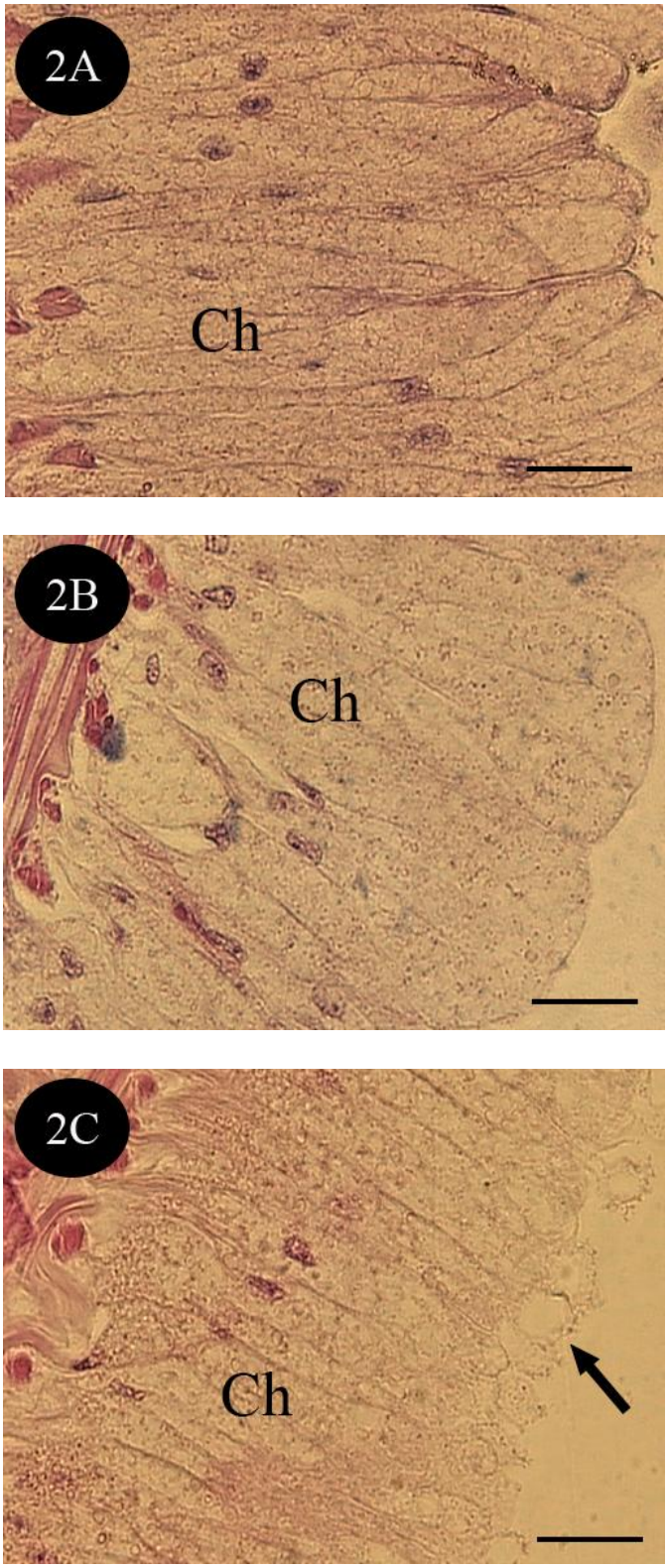

Figure 2: Chloragocytes of *Lumbricus terrestris*. **A.** Control status: Cone-shaped chloragocytes with oval or round nuclei and a moderate density of cytoplasma and vacuoles (control group/ 1d exposure time). **B.** State of reaction: Chloragocytes swollen and apically flattened. Cells showing irregularly shaped nuclei, a reduced density of cytoplasm and an enhanced vacuolisation (6x exposure group/ 7d exposure time). **C.** State of destruction: Many chloragocytes are disintegrated. Cell debris from the cellular apices in the coelomic cavity (Arrow) (6x exposure group/ 7d exposure time). Ch, chloragocytes; Scale bars: 25 µm.

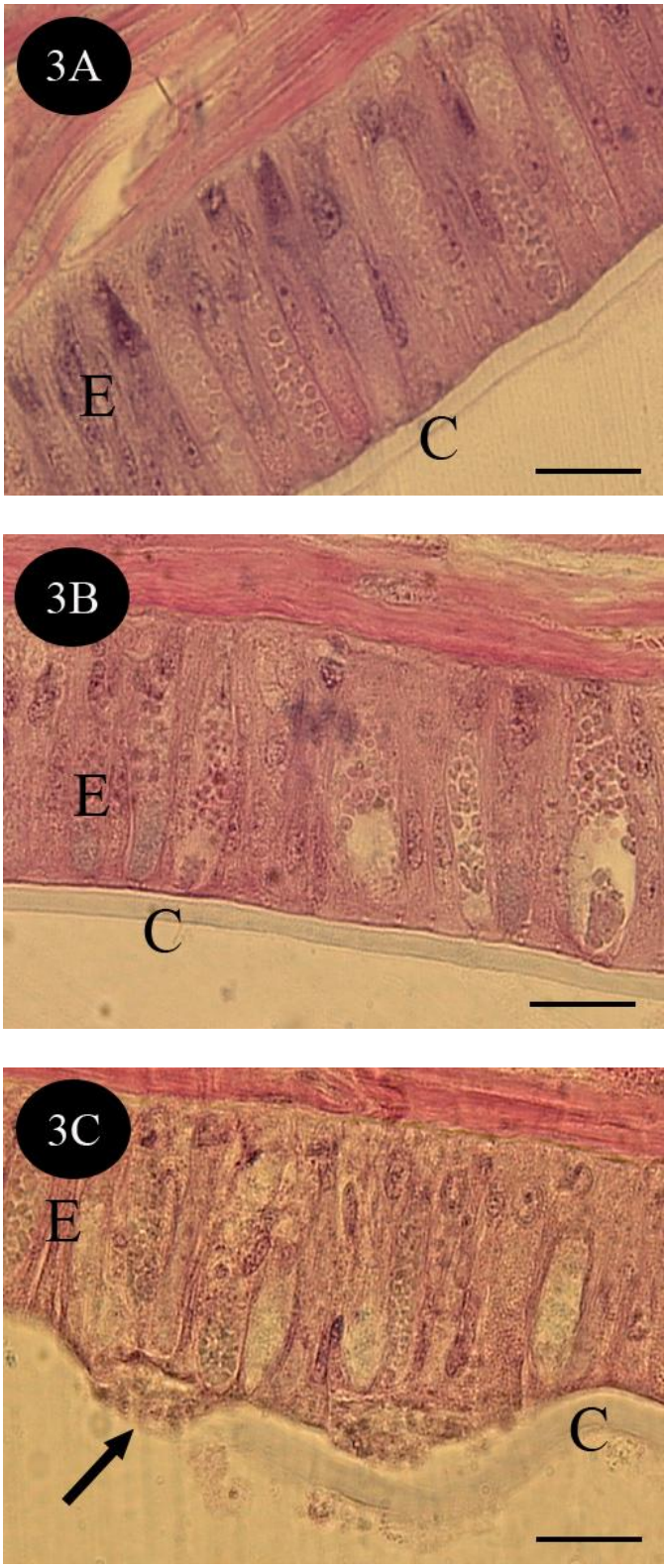

Figure 3: Epidermal cells of *Lumbricus terrestris*. **A.** Control status: Epidermal lining with a smooth cuticula and many slightly filled mucocytes with a clear cellular compartmentation (control group/ 1d exposure time). **B.** State of reaction: Epidermal cells with an irregular compartmentation. Increased occurrence of strongly filled mucocytes (1x exposure group/ 7d exposure time). **C.** State of destruction: Many epidermal cells are disintegrated. Corrugated epidermal surface and/ or destroyed cuticula. Cell debris (Arrow) (6x exposure group/ 14d exposure time). E, epidermis; C, cuticula; Scale bars: 25 µm.

3.3.2 Semi-quantitative assessment of histopathology

Eisenia fetida:

Midgut tissue (Fig. 4a): The condition of the midgut tissue deteriorated with increasing pesticide concentration and exposure time. After one day of exposure, the condition of the control group was significantly better compared to the 1x- and 3x- exposure groups. After 7 days, all exposure groups were significantly different from the control group.

Chloragogenous tissue (Fig. 4b): With increasing pesticide concentration and exposure time the condition of the chloragogenous tissue became worse. Except for the lowest pesticide concentration (0.2 mg kg^{-1} dry soil) after one day of exposure, significant effects were observed for all treatments compared to the respective control group.

Epidermis (Fig. 4c): The condition of the epidermis deteriorated with increasing pesticide concentration and exposure time. It was significantly impaired in all exposure treatments.

Aporrectodea caliginosa:

Midgut tissue: After 1 and 14 days of exposure time a significant impairment of the condition of the midgut tissue was observed in all treatments.

Chloragogenous tissue: Except for the highest imidacloprid concentration (2 mg kg^{-1} dry soil) and an exposure time of 14 days, no significant detrimental effects on the condition of the chloragogenous tissue were observed.

Epidermis: With increasing exposure time and pesticide concentration, the condition of the epidermis deteriorated. Significant impairment was observed for the highest imidacloprid concentration (2 mg kg^{-1} dry soil) after one day of exposure as well as for all treatments after 14 days of exposure.

Lumbricus terrestris:

Midgut tissue (Fig. 5a): With increasing imidacloprid concentration and exposure time, the condition of the midgut tissue deteriorated. After one day of exposure, this effect was significant for the 3x-exposure group, while after 7 days of exposure significant differences were observed for the 1x- and 3x- exposure groups compared to the control animals. All exposure groups were significantly impaired after 14 days of exposure.

Chloragogenous tissue (Fig. 5b): Except for the 1x- exposure group after one day, the condition of the chloragogenous tissue was impaired significantly in all other treatments.

Epidermis (Fig. 5c): The condition of the epidermis deteriorated significantly in all exposure groups compared to the control animals.

Significant positive correlations between body mass loss and semi-quantitative assessed impairment of the analysed tissues were found for all species: *E. fetida* ($p = 0.0007$ and $r^2 = 0.26$); *A. caliginosa* ($p < 0.0001$ and $r^2 = 0.23$)*; L. terrestris* ($P < 0.0001$ and $r^2 = 0.36$).

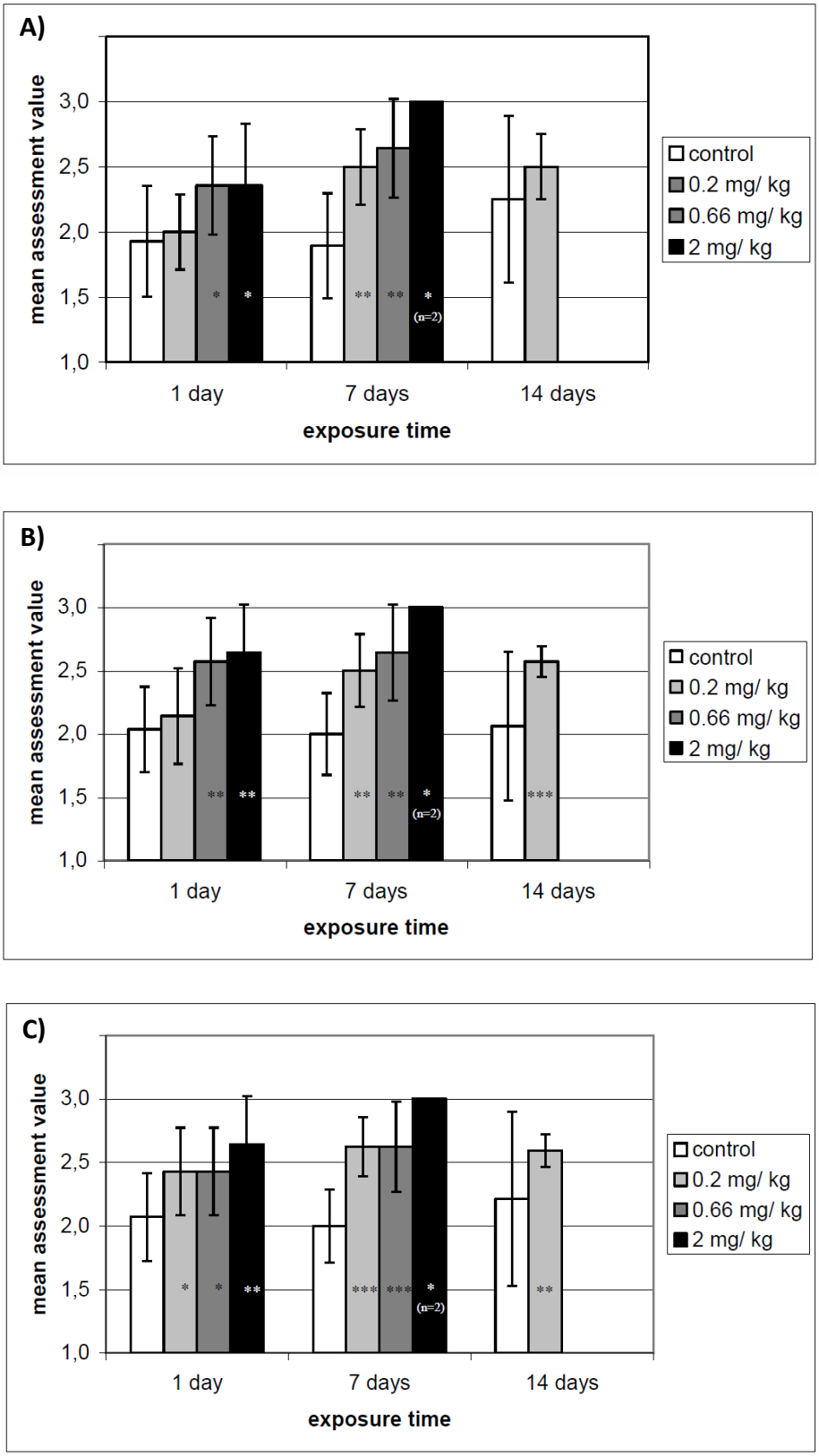

Figure 4: Mean assessment values (MAV) for the condition of different monitor tissues in *Eisenia fetida* after exposure to different concentrations of imidacloprid for 1, 7 and 14 days (means ± SD). Asterisks indicate significant differences from the control at 0.01 < *P* = 0.05 (*); 0.001< *P* = 0.01 (**) and *P* = 0.001 (***). **A.** Condition of midgut tissue. **B.** Condition of chloragogenous tissue. **C.** Condition of epidermis.

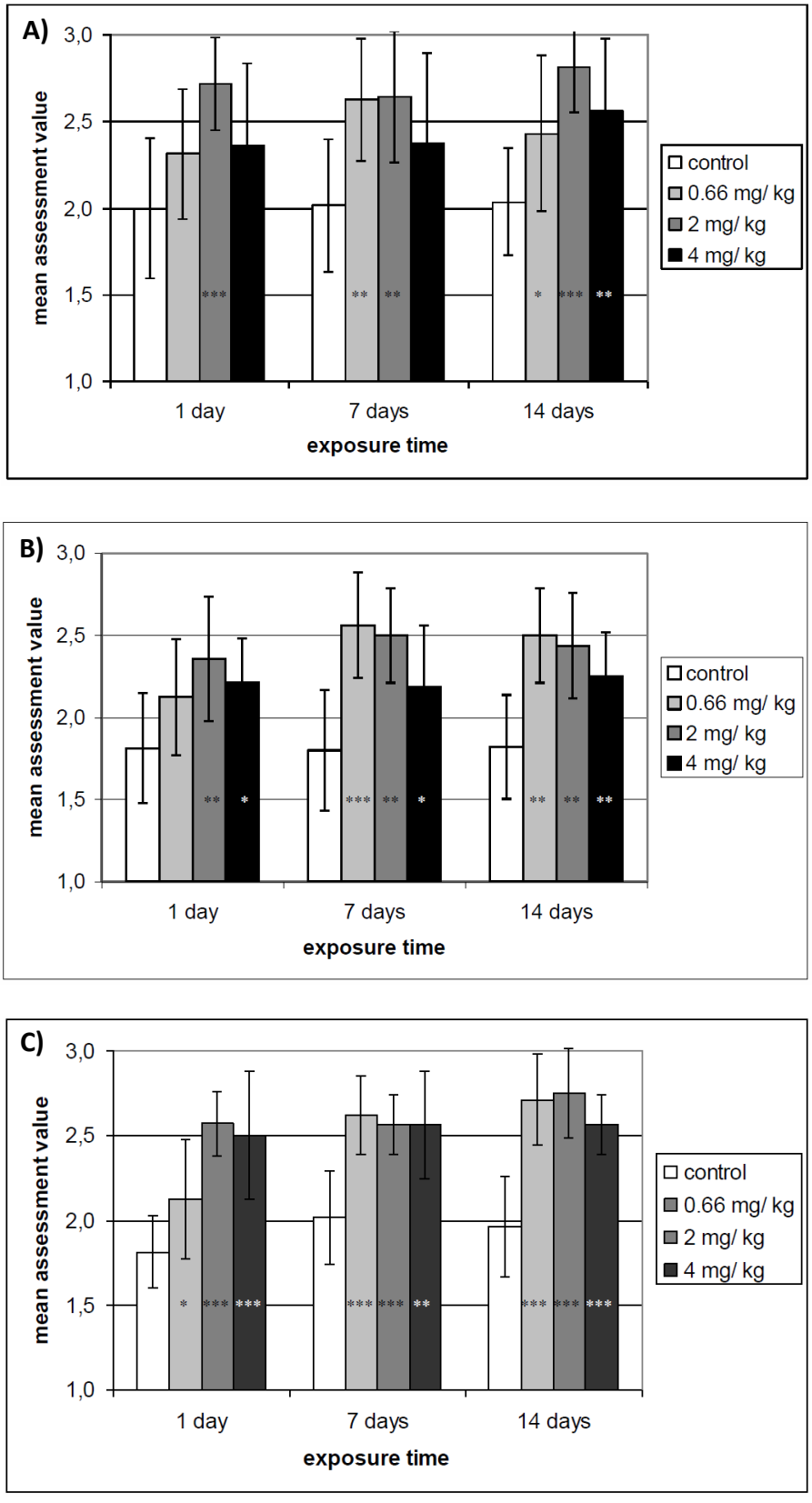

Figure 5: Mean assessment values (MAV) for the condition of different monitor tissues in *Lumbricus terrestris* after exposure to different concentrations of imidacloprid for 1, 7 and 14 days (means ± SD). Asterisks indicate significant differences from the control at 0.01 < *P* = 0.05 (*); 0.001< *P* = 0.01 (**) and *P* = 0.001 (***). **A.** Condition of midgut tissue. **B.** Condition of chloragogenous tissue. **C.** Condition of epidermis.

4. Discussion

In the present study sub-lethal effects in the tested earthworm species after imidacloprid exposure occurred in a similar range of concentrations (0.2–4 mg kg^{-1} dry soil) that were also found to have caused different sub-lethal effects on earthworms in previous studies (ranging from sperm deformities and changes in burrowing behaviour, in cast production as well as in body mass) (Capowiez et al. 2003; 2010; Dittbrenner et al. 2010a; Gomez-Eyles et al. 2009; Luo et al. 1999).

Body mass losses in earthworms (*E. fetida*, *Aporrectodea caliginosa*, *Ap. nocturna*, *Allolobophora icterica*, *L. terrestris*) due to pesticide exposure have often been observed in former studies (Capowiez et al. 2005; 2010; Dittbrenner et al. 2010a; Gomez-Eyles et al. 2009; Mostert et al. 2000) and have been discussed as to be of ecological relevance, since they may affect reproduction and survival (Capowiez et al. 2005; Luo et al. 1999; Olvera-Velona et al. 2008).

In our study, measurements in body mass change showed that after 7 days, significant detrimental effects occurred already at low imidacloprid concentrations (starting at 0.2 mg kg^{-1} dry soil) in *E. fetida* and *A. caliginosa*, while significant negative effects in *L. terrestris* were not observed until exposure towards the ten-fold concentration (2 mg kg^{-1} dry soil). After 14 days, all species showed a recovery at the respective 7 days LOEC-concentrations. However, enhanced adverse effects were observed at higher concentrations (at 0.66-2 mg kg^{-1} dry soil for *A. caliginosa* and at 4 mg kg^{-1} dry soil for *L. terrestris*). All *E. fetida* were even dead after 14 days exposure to 0.66 and 2 mg kg^{-1} dry soil. The results for body mass changes indicate that *E. fetida* and *A. caliginosa* were considerably more sensitive than *L. terrestris*. However, *A. caliginosa* did not seem to be much more sensitive than *L. terrestris* when looking at mortality data. Nevertheless, from our results one might presume that a recovery reaction in body mass can hardly occur after imidacloprid exposure at concentrations higher than 0.2 (in *E. fetida*), 0.66 (in *A. caliginosa*) or 4 mg kg^{-1} dry soil (in *L. terrestris*), respectively. This is due to the fact, that body mass continued to decrease at the mentioned concentrations after 14 days of exposure time. However, this hypothesis needs further investigations prolonging exposure times.

Earthworms are exposed to environmental toxicants either via skin (when moving) or intestine (when feeding). The chloragogenous tissue – often claimed to have some liver-like properties – is believed to play an important role in detoxification processes (Prento 1987; Vogel and Seifert 1992). Therefore many histopathological studies on earthworms have focussed on the examination of skin, intestine and chloragogenous tissue (Chakra Reddy and Venkateswara Rao 2008; Fischer and Molnár 1992; Morgan and Turner 2005; Morowati 2000; Muthukaruppan et al. 2005; Muthukaruppan and Paramasamy 2010).

In the present study, significant impairment of the midgut tissue was observed for all exposure times and in some cases even at the lowest imidacloprid concentration (e.g. at 0.2 mg kg^{-1} dry soil after 1 day in *A. caliginosa*). Overall, the observed histopathological effects in the midgut tissue ranged from altered shape and size of cells and nuclei, irregular cellular compartmentation to an increased occurrence of intercellular space and disintegrated cells at higher imidacloprid concentrations (starting at 0.66 mg kg^{-1} dry soil). In previous studies, similar damages were also found in the intestines of different earthworm species (*Pheretima elongata* and *E. fetida*) after exposure to different herbicides (glyphosate, butachlor) (Morowati 2000; Muthukaruppan and Paramasamy 2010).

The chloragogenous tissue of earthworms with its detoxification potential has been the object of intense ecotoxicological studies, wherein different effects due to toxicant exposure were described, ranging from cellular enlargement and nuclear swelling up to almost total depletion of chloragocytes (Chakra Reddy and Venkateswara Rao 2008; Fischer and Molnár 1992; Gupta and Sundararaman 1988; Morgan and Turner 2005; Morowati 2000; Muthukaruppan et al. 2005; Muthukaruppan and Paramasamy 2010; Vogel and Seifert 1992). The histopathological effects observed in this study were of the same quality and were found for all exposure periods and for the whole range of test concentrations (0.2-4 mg kg^{-1} dry soil).

Furthermore, significant cellular changes in the skin occurred after 1, 7 and 14 days of exposure time and in most cases already at low imidacloprid concentrations (e.g. at 0.2 mg kg^{-1} dry soil after 1 day in *E. fetida*). The observed detrimental effects often included a corrugated cuticula, hypertrophic mucocytes, increased mucus secretion and - at high imidacloprid concentrations - disintegrated epidermal cells. Similar effects were already described for earthworms (*E. fetida*) exposed to an organophosphorous pesticide or lead oxide, respectively (Chakra Reddy and Venkateswara Rao 2008; Venkateswara Rao et al. 2003). Increased mucus production in earthworms (following hypertrophy and hyperplasia of mucocytes) due to environmental stressors has often been described and interpreted as protective reaction (Günther and Greven 1990; Wielgus-Serafinska 1979).

Comparing the histopathological effects of imidacloprid on the different tissues, species-specific differences became evident, but were dependant on the monitor tissue. We have observed that *E. fetida* was slightly more sensitive than *L. terrestris* (e.g. when comparing condition of midgut tissue or of chloragogenous tissue after 1 day of exposure). Moreover, at first glance, *A. caliginosa* seemed to be the least sensitive species concerning cellular changes in general. Firstly, for none of the examined tissues significant differences between control and treated groups were detected after 7 days of exposure and, secondly, only one single significant difference between control and treated groups in the condition of chloragogenous tissue was observed for *A. caliginosa* in total. But when

looking at the condition of midgut tissue after 1 day of exposure, *A. caliginosa* even proved to be the most sensitive species (LOEC at 0.2 mg kg^{-1} dry soil). This fast and sensitive reaction could be due to comparatively high ingestion/ egestion rate of endogeic species (Lavelle et al. 1989), leading to an increased contact of intestine and contaminated soil. However, for the histopathological examination and analysis of *A. caliginosa* in this study, it is important to mention that even the control groups were often in a state of reaction (e.g. for all monitor tissues after 7 days exposure time). In this regard, one should not forget that, in comparison to the other two test species, *A. caliginosa* was collected in the field prior to the experiments and therefore might have suffered during acclimatisation to laboratory conditions. In addition, field collected specimens might be more heterogeneous in sensitivity due to fluctuating environmental conditions. However, the semi-quantitative assessment of histopathology applied in the present study proved to be a useful tool in order to describe and compare cellular changes sensitively. The necessity of increasing quantification in earthworm histology was already claimed in the past (Fischer and Molnár 1992).

In general, this study makes evident, that results from earthworm toxicity tests should be interpreted carefully and conclusions should not be drawn without considering earthworm origin (field or laboratory) and "ecological type. All in all, the significant positive correlation between degree of body mass loss and degree of cellular impairment might confirm the quality and coherence of the results obtained. Detoxification and regeneration processes might very likely have weakened the pesticide-exposed earthworms. This in consequence could have led to a reduced feeding and burrowing activity and finally to loss in body mass. Interestingly, during the 14 days of exposure, no clear regeneration was observed at the cellular level, while a recovery reaction was observed for body mass change. This might be explained by differences in biomarker kinetics, cellular regeneration being a relatively enduring process. Morowati (2000) found a cellular recovery of intestine and chloragogenous tissue in the earthworm *Pheretima elongata* not earlier than 21 days after glyphosate exposure.

In conclusion, we have found significant cellular impairment in the examined tissues as well as significant changes in body mass after exposure to a range of imidacloprid concentrations (0.2-4 mg kg^{-1} dry soil). Since the predicted environmental concentration (PEC) of the insecticide imidacloprid is between 0.33-0.66 mg kg^{-1} dry soil (depending on crop and country under consideration) (Mostert et al. 2000; Oi 1999) and since imidacloprid is frequently used in agriculture and can have a half-life in soil of greater than one year, (Sabbagh et al. 2002), the sub-lethal effects detected in this study may be of great environmental concern. Moreover the results obtained in our study indicate that there are species-specific differences in sensitivity after imidacloprid exposure, depending on the respective biomarker. Considerable species-specific differences in sensitivity towards *E. fetida* should

be noted and interpreted carefully, since most standard tests in earthworm ecotoxicology are carried out with the latter species. Even if *E. fetida* proved to be the most sensitive species in this study, always representative earthworm species for agricultural soil should additionally be used for toxicity testing.

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5. References

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b) Stress protein response (Hsp70) and avoidance behaviour in *Eisenia fetida*, *Aporrectodea caliginosa* and *Lumbricus terrestris*. *Journal of Soils and Sediments, in press.*

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Abstract

The earthworm species *Eisenia fetida* and *Eisenia andrei* have often been claimed to be comparatively insensitive when exposed to environmental toxicants. However, these species are almost exclusively used in ecotoxicological standard tests carried out with earthworms. In order to better evaluate potentially harmful environmental effects of toxicants, there is a need to compare responses of standard test organisms and closely related species after toxicant exposure. Therefore, in laboratory experiments, we have assessed sub-lethal effects of the insecticide imidacloprid on *E. fetida* and two other earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) using a biochemical (changes in stress protein level (hsp70)) and a behavioural biomarker (avoidance behaviour). In 48 hour dual choice experiments, *E. fetida* showed significant avoidance behaviour towards all test concentrations (0.2, 0.66 and 2 mg kg^{-1} dry soil), while *L. terrestris* and *A. caliginosa* did not avoid imidacloprid-contaminated soil. The latter species was even significantly attracted by the highest test concentration (2 mg kg^{-1} dry soil). The 1, 7 and 14 days exposure experiments for hsp70-analysis showed that significant changes in *E. fetida* already occurred at the lowest imidacloprid concentration (0.2 mg kg^{-1} dry soil and 14 days), while significant changes in hsp70 levels in the other species were not observed until exposure to 2 (*A. caliginosa;* after 1, 7 and 14 days) and 4 mg kg^{-1} dry soil (*L. terrestris;* after 14 days). The present study revealed species-specific differences in sensitivity after imidacloprid exposure, *E. fetida* being the most sensitive species and *L. terrestris* the least sensitive. Our study makes evident that sensitivities can vary greatly between closely related species and therefore highlights the importance of using multiple species in ecotoxicology - being representative for a whole taxon – in order to evaluate harmful environmental effects more

accurately. Moreover, some of the detected effects were found even at concentrations occurring under natural conditions. Since imidacloprid is frequently used in agriculture the present findings may be of substantial environmental concern.

Keywords: Earthworms – Imidacloprid - Standard test organism – Hsp70 – Avoidance behaviour

1. Introduction

Imidacloprid, a widely used neonicotinoid insecticide in agriculture, has been shown to cause a range of detrimental effects on different non-target organisms (e.g. Capowiez et al. 2005, 2010; Dittbrenner et al. 2010a; Duzguner and Erdogan 2010; Iwasa et al. 2004; Sawasdee and Köhler 2009). Earthworms as "soil engineers" play indispensable roles in many soils (Curry and Baker 1998; Edwards and Bohlen 1996; McCredie and Parker 1992; Scheu 1987; Scullion and Malik 2000) and the use of these animals as terrestrial model organisms in ecotoxicological tests is highly recommended (EEC 2003). In most tests the earthworm species *Eisenia fetida* and *Eisenia andrei* are used, but there is a need to perform more ecotoxicity tests using additional earthworm species – relevant for agricultural soils – in order to be able to evaluate risks for the natural environment more accurately (Bouché 1992; Christensen and Mather 1994; Dean-Ross 1983).

In eukaryotes, stress or heat shock proteins of the 70 kD class (hsp70) are induced in response to various biotic or abiotic stressors (Schlesinger 1990), due to their important role in protein repair (Hartl 1996; Lindquist and Craig 1988). The induction of hsp70s following toxicant exposure has often been observed in various organisms (e.g. Haap and Köhler 2009; Köhler et al. 1992; Nadeau et al. 2001; Scheil et al. 2010). In ecotoxicology, the stress protein induction is regarded a sensitive molecular biomarker of effect, indicating proteotoxicity (Köhler et al. 1992). However, due to its specific response kinetics, represented by an optimum curve (low-dose induction, high-dose down-regulation/ break-down), this marker should preferably be used in combination with histopathological or ultrastructural biomarkers (Köhler and Triebskorn 2004). However, only a few earthworm studies have adopted stress protein responses as biomarkers so far.

Behavioural studies are believed to be meaningful, because changes in behaviour might directly be linked to effects on higher ecological levels (Doving 1991; Little 1990; Scherrer 1992). The most frequently used test for earthworms on a behavioural level is the standardised avoidance test (ISO 2008). It is supposed to be a very sensitive as well as cost-effective tool in ecotoxicology (Schäfer 2003; Slimak 1997; Yeardley et al. 1996). However, some authors have claimed that the avoidance test might be useless when analysing the toxicity of non-irritant, narcotic and/or neurotoxic

compounds (Pereira et al. 2010; Scott-Fordsmand and Weeks 2000). Since behavioural responses in earthworms to environmental toxicants can differ to a great extent from species to species (Gilman and Vardanis 1974), the utilisation of several species can strongly improve the significance of a research study.

In the present communication, we have assessed sub-lethal effects of the insecticide imidacloprid on three different earthworm species using a molecular (induction of hsp70) as well as a behavioural biomarker (avoidance behaviour). We wanted to examine the sensitivity of the standard test organism *E. fetida* in comparison with two earthworm species highly relevant for many agricultural soils, in order to contribute to a better understanding of species-specific responses to environmental toxicants. The results obtained in this study will be discussed in connection to a parallel paper (Dittbrenner et al 2010b), which found *E. fetida* to be the most sensitive species using body mass change as well as histopathology as biomarkers.

2. Material and methods

2.1 Exposure experiments, test organisms, soil and imidacloprid

This has been described in detail earlier in this issue (Dittbrenner et al. 2010b).

2.2 Stress protein analysis (Hsp70)

For stress protein analysis, ten specimens of each species were used for each treatment (for each concentration and exposure time). After the exposure experiments (1, 7, 14 days), earthworms were individually shock frozen in liquid nitrogen and tissue pieces of the posterior ends (50 - 200 mg) were cut off and used for further processing. Tissue material was homogenised on ice in 200-800 µL extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor Sigma P4380, pH 7.5) – the volume depending on the sample mass. Homogenates were centrifuged (12min; 20000g at 4°C) and the total protein concentration in the supernatant was determined according to Bradford (1976). Subsequently, constant amounts of total protein (20 µg) were analysed by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide) for 20 min at 80 V and 120 min at 120 V. The protein was then transferred to nitrocellulose by semi-dry blotting and the filters subsequently blocked for 2 hours in 50% horse serum in Tris-buffered saline (TBS; 50 mM Tris; 150 mM NaCl; pH 7.5). After blocking, the filters were washed in TBS for 5 minutes and incubated in a monoclonal antibody solution (mouse anti-human Hsp70; Dianova, Hamburg, Germany, dilution

1:5000 in 10% horse serum/TBS) for 12 hours at room temperature. Then they were washed in TBS for 5 minutes and incubated in a second antibody solution (peroxidase-conjugated goat anti-mouse IgG Dianova, Germany, dilution 1:1000 in 10% horse serum/TBS) for 2 hours at room temperature. After repeated washing in TBS for 5 minutes, the antibody-complex was stained by 1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. Quantification of the grey scale values of the Western blot protein bands was performed by using a densitometric image analysis system (Herolab E.A.S.Y. Win 32, Germany). The samples were related to an hsp70 standard sample (prepared from total homogenate of an untreated *Lumbricus terrestris* individual), which was run in parallel on every gel.

2.3 Avoidance behaviour

The avoidance tests were carried out according to ISO recommendations (ISO 2008). Plastic containers of 11 cm x 16 cm x 6 cm (*A. caliginosa* and *E. fetida*) or 25 cm x 25 cm x 8 cm (*L. terrestris*) were filled with soil up to a height of 5 cm (*A. caliginosa* and *E. fetida*) or 7 cm (*L. terrestris*), respectively. One half of each container was first filled with uncontaminated soil, and then, the other half was filled with contaminated soil (300 g or 1000 g depending on the species). A clear boundary was created by using a plastic separator. Soil spiking was conducted in the same way as in the exposure experiments for histology and hsp70 analysis (see Dittbrenner et al. 2010b). Three different imidacloprid concentrations were tested for each species: 0.2 (0.3x), 0.66 (1x) and 2 mg kg^{-1} dry soil (3x). At the start of the experiment, ten earthworms were placed on the separation line between spiked and unspiked soil in each container. Then the containers were closed with transparent and perforated lids and placed in dark climate chambers at 12°C (*A. caliginosa* and *L. terrestris*) or 22°C (*E. fetida*) for 48 hours. Subsequently, the control soil was carefully separated from the contaminated soil and the number of earthworms in each section was counted. For each replicate, avoidance/ preference for the polluted soil were expressed as net response (NR), calculated as:

$$NR = (C - T)/ N$$

where C was the number of earthworms in the control soil, T the number of earthworms in the contaminated soil and N was the total number of surviving earthworms. This resulted in net response values ranging from -1 to 1. Negative net response values were indicating preference, while positive net response values were showing avoidance behaviour.

For all test species, each concentration was tested in eight replicates (n = 8).

2.4 Statistical analysis

The data analysis of the hsp70 responses was conducted in the same way as described earlier for histopathology and body mass change (Dittbrenner et al. 2010b). Response surfaces for hsp70 were computed using STATISTICA 5.0 (StatSoft, USA).

For the avoidance behaviour one-sample Wilcoxon tests were performed in order to determine if mean net responses were significantly different from zero ($\alpha = 0.05$).

3. Results

3.1 Mortality

After 14 days of exposure to the highest imidacloprid concentrations (0.66 and 2 mg kg^{-1} dry soil) ten *E. fetida* died each time. No mortality occurred in the other two test species.

3.2 Stress protein analysis (Hsp70)

In all three species, only few data on exposed individuals showed significance *vs*. the respective controls. However, the entirety of data subjected to surface plot analysis revealed distinct response patterns for the three different lumbricid species as displayed in Figs. 1 a-c.

In *Eisenia fetida* (Table 1/ Fig. 1a), the heat shock protein response significantly decreased after exposure to the highest imidacloprid concentrations (0.66 and 2 mg kg^{-1} dry soil) after 7 days. A significant increase of the hsp70 level was observed after exposure to 0.2 mg kg^{-1} dry soil imidacloprid and 14 days exposure time. In *Aporrectodea caliginosa* (Table 2/ Fig. 1b), the hsp70 level in *A. caliginosa* decreased significantly after 1, 7 and 14 days of exposure to the highest imidacloprid concentration (2 mg kg^{-1} dry soil). Except for a significant decrease of the hsp70 level after 14 days of exposure to the highest imidacloprid concentration (4 mg kg^{-1} dry soil), no other treatment caused a significant effect on the stress protein level in *Lumbricus terrestris* (Table 3/ Fig. 1c).

Table 1: Mean hsp70 levels (±SD) relative to the respective controls (expressed in percentages) of *Eisenia fetida* after imidacloprid exposure to three different concentrations for 1, 7 and 14 days (n = 10). Values in bold are significantly different from the according control values ($p < 0.05$). All specimens died after 14 days exposure to 0.66 and 2 mg kg^{-1} dry soil (+).

Concentration/ exposure time	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
1 d	92.4 (54.8)	84 (26.6)	95.7 (42.2)
7 d	113 (58)	**43 (32.4)**	**37.9 (24.9)**
14 d	**210.6 (54.5)**	**+**	**+**

Table 2: Mean hsp70 levels (±SD) relative to the respective controls (expressed in percentages) of *Aporrectodea caliginosa* after imidacloprid exposure to three different concentrations for 1, 7 and 14 days (n = 10). Values in bold are significantly different from the according control values ($p < 0.05$).

Concentration/ exposure time	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
1 d	116.5 (83.2)	82.6 (42.6)	**64.3 (35.3)**
7 d	115.3 (67.3)	161.7 (81.5)	**44.8 (38.7)**
14 d	107.8 (28.8)	128.5 (49)	**39.6 (32.2)**

Table 3: Mean hsp70 level (±SD) relative to the respective controls (expressed in percentages) of *Lumbricus terrestris* after imidacloprid exposure to three different concentrations for 1, 7 and 14 days (n = 10). Values in bold are significantly different from the according control values ($p < 0.05$).

Concentration/ exposure time	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**	**4 mg kg^{-1} dry soil**
1 d	139.9 (60.9)	114.2 (36.6)	89.2 (29)
7 d	103.8 (24.2)	126.2 (28.5)	110.4 (37)
14 d	98.2 (32.1)	89.1 (29.7)	**37.9 (16.6)**

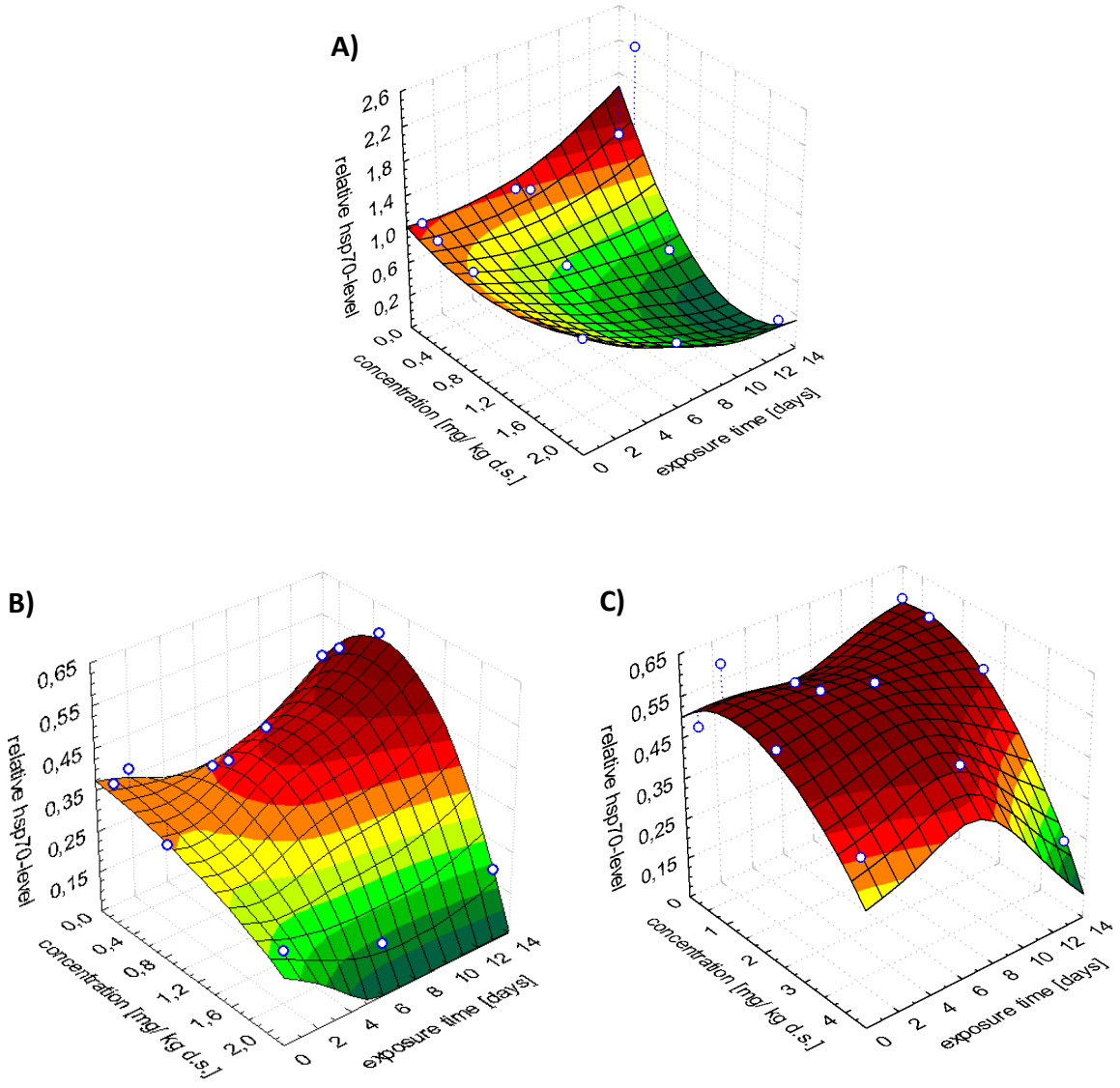

Figure 1: Hsp70 levels of three different earthworm species after 1, 7 and 14 days of exposure to different concentrations of imidacloprid (Surface plots were calculated on the basis of mean values (n = 10)). **A.** *Eisenia fetida*. **B.** *Aporrectodea caliginosa*. **C.** *Lumbricus terrestris*.

3.3 Avoidance behaviour (Fig. 2)

While a significant avoidance response occurred in *E. fetida* for all tested imidacloprid concentrations (0.2, 0.66 and 2 mg kg^{-1} dry soil), no significant effects were observed in *L. terrestris. A. caliginosa* showed a significant attraction in the treatment with the highest imidacloprid concentration (2 mg kg^{-1} dry soil).

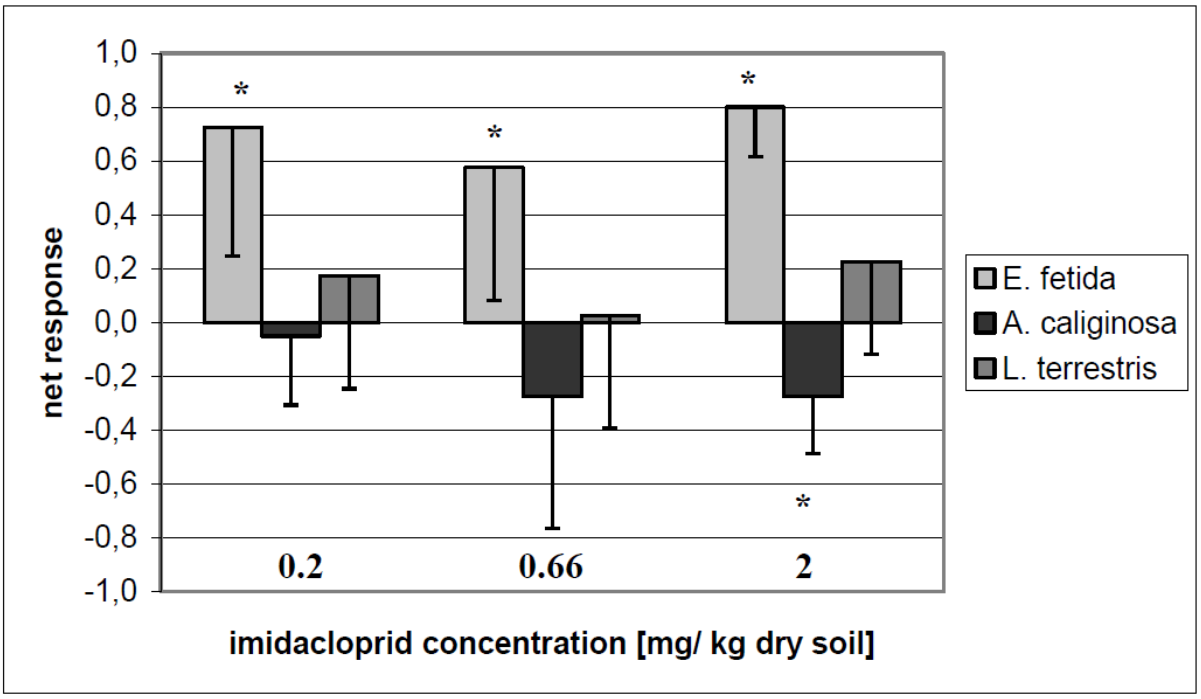

Figure 2: Mean net responses (-SD) of three different earthworm species (*Eisenia fetida*, *Aporrectodea caliginosa*, *Lumbricus terrestris*) after exposure to imidacloprid, determined in avoidance behaviour tests (n = 8). Positive responses show avoidance of the contaminated soil. Asterisks indicate net responses which were significantly different from zero at $p < 0.05$.

4. Discussion

In the present study we have detected sub-lethal effects of the insecticide imidacloprid in three different earthworm species in the laboratory. Effects were already observed at concentrations relevant for agricultural soils (predicted environmental concentration (PEC) = 0.33-0.66 mg kg^{-1} dry soil) (Mostert et al. 2000; Oi 1999). In the past, numerous studies have focussed on the impact of the insecticide imidacloprid on different earthworm species and a range of sub-lethal effects were found to occur in a comparable range of concentrations, which also caused sub-lethal effects in the present study (0.2–4 mg kg^{-1} dry soil) (e.g. Capowiez et al. 2003; 2010; Dittbrenner et al. 2010a; Lal et al. 2001; Luo et al. 1999; Mostert et al. 2002; Zang et al 2000).

On the biochemical level the response of heat shock proteins of the kD70 class (hsp70) represents a sensitive biomarker (of effect) in ecotoxicology, indicating general protein damage (Köhler et al. 1992; Nadeau et al. 2001; Ryan & Hightower 1996). Imidacloprid is a neurotoxin acting primarily on the nicotinic acetylcholine receptors (Matsuda et al. 2001). Therefore, after imidacloprid exposure mainly secondary effects due to increasing impairment of proteins can account for an induction of hsp70. A down-regulation (or break-down) of the hsp70 level usually results from stress overload causing severe constraints in overall body functions (e.g. cell damages affecting protein biosynthesis) (Köhler and Triebskorn 2004). As we have observed only minor up-regulations in hsp70 expression after imidacloprid exposure in the present study – only significant for *E. fetida* exposed to 0.2 mg kg^{-1} dry soil for 14 days – we can conclude that the investigated insecticide is a rather weak hsp70 inductor for the chosen test species. However, for all species investigated, significant down-regulations of the hsp70 level were found after different treatments. The results were revealing species-specific differences. While significant down-regulations after imidacloprid exposure in *E. fetida* were already observed for concentrations of 0.66 and 2 mg kg^{-1} dry soil, significant decreases of the hsp70 levels in the other species were not found until exposure to 2 (*A. caliginosa*) and 4 mg kg^{-1} dry soil (*L. terrestris*), respectively.

The surface plot analysis (Fig. 1a-c) showed that the response patterns of all species can be related to a theoretical hsp70 stress response scheme on the basis of Eckwert et al. (1997) (Fig 3a-c). While the exposure to the different concentrations of imidacloprid for 7 to 14 days in *A. caliginosa* covered the theoretical surface plot almost entirely (Fig 3b) the chosen ranges of imidacloprid concentrations only covered the “upper” (*L. terrestris*, with exception of the most severe exposure condition of 4 mg/kg imidacloprid for 14 d) (Fig 3c) or the “lower”, portion of the theoretical response surface (*E. fetida*) (Fig 3a). With respect to hsp70 responses, we can therefore conclude that *E. fetida* was the most sensitive, while *L. terrestris* was the least sensitive species tested. Species-dependent

differences in hsp70 levels after toxicant exposure – as we have observed them in the present study - were already reported in previous studies, e.g. an experiment comparing the hsp70 levels of two isopod species after heavy metal exposure (Arts et al. 2004).

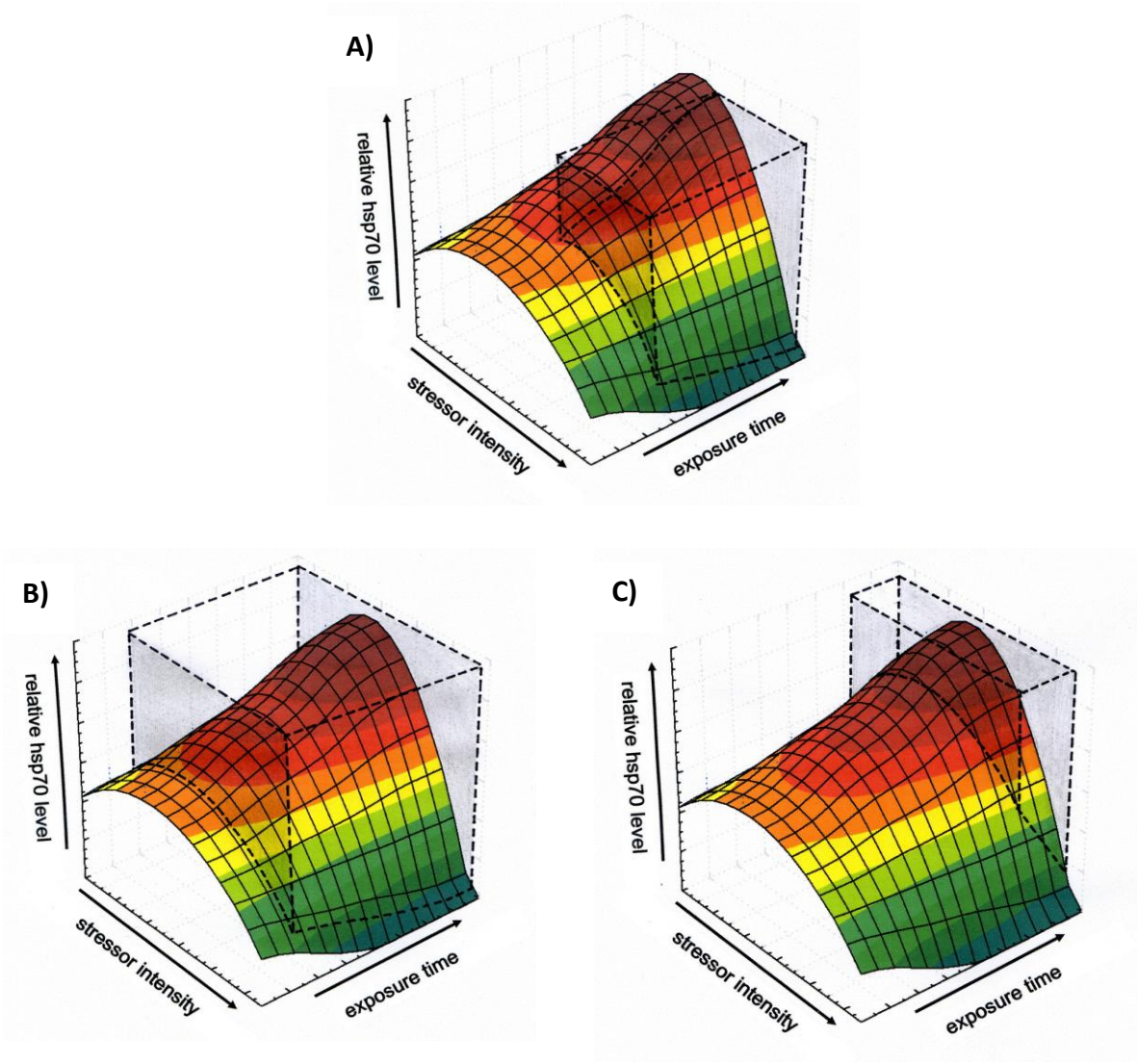

Figure 3: Theoretical surface plots for hsp70 levels as they can be deduced from the results of Eckwert et al. (1997) and approximate areas of coverage by the experimentally generated plots for the three lumbricid species tested. **A.** *Eisenia fetida*. **B.** *Aporrectodea caliginosa*. **C.** *Lumbricus terrestris*.

In earthworm ecotoxicology the avoidance test has a somewhat controversial reputation. Many authors have claimed the bioassay to be highly sensitive (e.g. Garcia et al. 2008; Hund-Rinke et al. 2005; Slimak 1997; Yeardley et al. 1996), while others have doubted its unrestrictive sensitivity after observing non-avoidance behaviour in earthworms exposed to different environmental toxicants (Capowiez and Bérard 2006; Hodge et al. 2000; Reinecke et al. 2002). Our results join the list of apparent inconsistencies in the sensitivity of the avoidance test. This is due to the fact that in our

study one species (*E. fetida*) significantly avoided imidacloprid, while the other two species (*L. terrestris* and *A. caliginosa*) did not. *A. caliginosa* even showed a significant attraction to the insecticide after exposure to the highest concentration (2 mg kg^{-1} dry soil). Pereira et al. (2010) claimed that the event of avoidance or non-avoidance is highly dependant on the mode of action of the toxicant under investigation. They have suggested that the avoidance test would not function well when neurotoxic compounds were tested. In our study, however, the avoidance test was a very sensitive tool for measuring effects of the neurotoxic insecticide imidacloprid in *E. fetida*, but not in *L. terrestris* and *A. caliginosa*. The non-avoidance of imidaclopriod in the latter two species is surprising and alarming, since severe cellular damage is very likely to occur in these species during 48 hours of exposure to (at least) the highest test concentration (2 mg kg^{-1} dry soil) (Dittbrenner et al. 2010b). These findings raise some questions: Were the species *L. terrestris* and *A. caliginosa* – in contrast to *E. fetida* – not capable of sensing imidacloprid by means of chemical receptors? Or did imidacloprid hamper the locomotory capacities of *L. terrestris* and *A. caliginosa,* but not of *E. fetida*? In this context, a previous study already showed that the species *A. caliginosa* did not avoid different pesticide-contaminated soils (diazinon; chlorpyrifos) (Hodge et al. 2000). Nevertheless, for *E. fetida* the capability of collective movement has been described and under natural conditions these earthworms are supposed to benefit from such behaviour by collectively escaping unfavourable conditions (Zirbes et al. 2010). Collective movement might explain the distinct avoidance behaviour observed for *E. fetida* at all test concentrations in our study.

In evaluating potential of the avoidance test for risk assessment, it was postulated that the assay should rather be regarded as a measure of repellence than of toxicity (Capowiez and Bérard 2006), which is reflected in e.g. earthworm avoidance of mustard (Gunn 1992) in comparison to non-avoidance of organophosphate insecticides (Hodge et al. 2000). However, if repellence leads to surface migration in earthworms, the avoidance test can still be considered as a tool measuring ecologically relevant changes in behaviour. Our study shows that results of avoidance tests have to be interpreted carefully and that the assay – even if very sensitive in many cases - should always be conducted in combination with different ecotoxicity tests (if possible using several earthworm species) in order to draw precise conclusions. Recommendations made by e.g. Hund-Rinke et al. (2005) - stating that reproduction tests in earthworms might only be carried out if a substance has been indicated as to be toxic in the avoidance test – may lead to severe underestimations of effects.

When comparing our results for the avoidance test and the hsp70 response, good accordance was conspicuous. While *E. fetida* reacted very sensitively in both bioassays, *L. terrestris* did not show significant avoidance behaviour and, in a single case only, a significant down-regulation in hsp70 level was observed (4 mg kg^{-1} dry soil / 14 days). Furthermore, *A. caliginosa* did not respond very

sensitively in either bioassay. For the latter species however, the question arose whether the significantly down-regulated hsp70 level after 24 hours exposure to 2 mg kg^{-1} dry soil imidacloprid and the significant attraction during 48 hours towards the same insecticide concentration could be linked. Possibly the severe constraints in overall body functions (indicated by the low hsp70 level) due to exposure to the highest concentration (2 mg kg^{-1} dry soil), might have handicapped the avoidance behaviour of *A. caliginosa*. All in all, the results obtained in the present study confirmed the hierarchy in species-specific sensitivity regarding imidacloprid-exposure, which was described earlier in this issue for body mass changes and histopathology (Dittbrenner et al. 2010b).

The well-acknowledged principle of hierarchical stress responses across different organisational levels highlights the importance of linking effects occurring at different biological levels in order to improve risk assessment. In the past, molecular (cholinesterase (ChE) activity) and behavioural biomarkers (burrowing behaviour; avoidance) in earthworms have already been used in combination (Capowiez et al. 2003; Gupta and Sundararaman 1991; Olvera-Velona et al. 2008; Pereira et al. 2010). In comparing these studies no clear tendency, in terms of preferential suitability of biomarkers for measuring environmental stress was observed. Biomarker sensitivities were strongly dependant on the respective toxicant mode of action, therefore combining the responses on different biological levels enhanced the accuracy of every single study. The same was true when considering the results of the present study and the results of a parallel communication in this issue (Dittbrenner et al. 2010b). In theory, one expects the molecular changes (hsp70 level) to occur earlier than changes on cellular and individual levels (body mass, behaviour). Nonetheless, consolidating the results of the present study and its parallel communication (Dittbrenner et al 2010b), body mass change and histopathology proved to be the most sensitive and reliable biomarkers overall. Avoidance behaviour, however, was a very sensitive toxicity marker in the case of *E. fetida*. These circumstances emphasise the importance of using a set of biomarkers and of linking responses on different biological levels.

Concerning species-specific differences in sensitivity, *E. fetida* proved to be most sensitive, whereas *L. terrestris* seemed to be the least sensitive species for all biomarkers used. It is striking that these differences in sensitivity do exactly reflect differences in average body mass, *L. terrestris* being the heaviest and *E. fetida* being the lightest species. Species-specific differences may partly be explained by relatively higher toxicant contact (via skin) of lighter species in comparison to heavier species, due to differences in surface/ volume-ratio. However, the real extent of toxicant exposure in different species is very hard to predict, since it also depends on the ecology of the respective earthworm species and therefore on factors such as soil ingestion/egestion-rates or frequency of soil surface movement (Lavelle et al 1989). Nevertheless, the results are in contrast to several previous studies in

which *E. fetida* proved to be comparatively insensitive when exposed to different environmental toxicants (polychlorinated biphenyls; parathion; propoxur; chlorpyrifos) (Fitzpatrick et 1992; Kula and Kokta 1992; Ma and Bodt 1993). Cairns (1986) claimed that there is no such thing as the most sensitive species and that multispecies testing is of higher value for protecting the environment. The latter approach, however, is not always easy to carry out, since much higher costs would be incurred. But in the case of testing only one species on a trophic level, a further increase of safety factors would lead to a better protection of the environment.

In conclusion, we have observed sub-lethal effects in three different earthworm species already after imidacloprid exposure to environmentally relevant concentrations. The biomarkers used (avoidance behaviour and changes in hsp70 level) were in good accordance and revealed species-specific differences. *E. fetida* proved to be the most sensitive species, while *L. terrestris* was the least sensitive species. The present results, linked to results of a parallel communication and compared to previous studies, made it clearly evident that it is preferable to have a set of biomarkers (linking different biological levels) as well as to use multiple species in order to improve risk assessment.

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Kapitel 2

a) Earthworm cast production as a new behavioural biomarker for toxicity testing. ***Environmental Pollution 158, 388-393.***

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Abstract

There is currently a lack of ecotoxicity tests adapted to earthworm species of higher ecological relevance and whose endpoints could be directly related to their ecological role in the soil. We propose a new and relatively simple ecotoxicity test based on the estimation of cast production (CP) by *Lumbricus terrestris* under laboratory conditions. CP was found to be linearly correlated to earthworm biomass and to be greatly influenced by soil water content. Azinphos-methyl had no effect on CP at all the concentrations tested. Significant decreases were observed at the normal application rate for other pesticides with (imidacloprid, carbaryl, methomyl) or without (ethyl-parathion and chlorpyrifos-ethyl) a clear concentration-effect response. For the highest concentration tested, reduction in CP varied between 35 and 67%. CP is straightforward and rapidly measured and ecologically meaningful. We thus believe it to be of great use as an endpoint in ecotoxicity testing.

Keywords: Egestion rate – Imidacloprid – Carbamates – Organophosphorous insecticides- *Lumbricus terrestris*

1. Introduction

Earthworms, as ecosystem engineers (Jones et al., 1994) have a great influence on many physical (transfer properties), chemical (biogeochemical cycles) and biological (interactions with other components of the soil ecosystem) processes that occur in the soil (Mc Coy et al., 1994; Görres et al., 2001). They are therefore important terrestrial model organisms requiring toxicity testing. To date, a number of normalized tests using *Eisenia fetida* and focusing on mortality, reproduction and behaviour (avoidance) are available. Changes in behaviour are promising targets for ecotoxicological studies because the results can be linked to effects at the ecosystem level (Little, 1990; Doving, 1991; Scherrer, 1992). In the case of earthworms, changes in behaviour such as modified or reduced burrowing activity are crucial factors because these could have drastic effects on soil functioning (Capowiez et al., 2006). Although significant modifications to earthworm burrow systems due to the presence of pollutants have been sometimes observed (Eijsackers et al., 2001; Capowiez et al., 2003), it is difficult to study earthworm behaviour because these animals are concealed by the soil in which they live. Previous attempts to study the effects of pollutants on earthworm burrowing behaviour were based on the use of X-ray tomography (Capowiez et al., 2006) or 2D terraria (Capowiez and Bérard, 2006) which are expensive and have very specific methods resulting in a limited number of observations. Furthermore both methods require either image analysis or complex mathematical analysis to translate the observations into measurements (endpoints). Overall, even if of scientific value, these techniques may prove difficult to justify.

Earthworms burrow in the soil either by ingesting soil particles or by pushing them aside (Lee and Foster, 1991). In addition, soil ingestion is necessary for alimentary reasons. After gut transfer, the soil is egested in a specific feature: the cast. It is deposited either on the soil surface or in the soil itself (Whalen et al., 2004). Cast production (CP) therefore contributes to soil bioturbation, i.e. the disruption and mixing of soils or sediments by organisms that live and/or feed in them and/or simply pass through them. Casts per se play an important ecological role in the soil (Lee and Foster, 1991; Blanchart, 1992; Le Bayon and Binet, 1999) and, equally, they can also be used as a proxy for earthworm activity. This latter approach was recently adopted by Loranger-Mercidis et al. (2008) to study potential interactions between earthworms and woodlice. Subsurface and/or surface cast production was examined in a great number of studies under controlled or natural conditions. Casting was shown to be influenced by biotic factors, such as the earthworm species under consideration (Scheu, 1987; Hindell et al., 1994), species association (Scullion and Ramshaw, 1988), but also by abiotic conditions, such as soil bulk density (Le Bayon and Binet, 1999), organic matter type and quantity (Shipitalo et al., 1988; Flegel et al., 1998; Buck et al., 1999), temperature, or water potential (Scheu, 1987; Hindell et al., 1994; Daniel et al., 1996). Overall, the results are difficult to

compare directly since the methodologies used to sample earthworm casts were often very different (hand collecting, wet or dry sieving) and the results have been expressed in a variety of ways (number, area covered or weight of casts related to earthworm fresh or dry weight).

There is some limited evidence that pesticides can affect CP. It is well known that the application of some pesticides to golf courses (turf grasses) results in lower surface CP (Baker et al., 1998; Lal et al., 2001). However, we do not know in these cases if earthworms were casting more below ground (to avoid pesticides at the soil surface) or if earthworm abundance simply decreased. In studies of aquatic systems, measurements of the egestion rate of sediments by benthic invertebrates such as oligochaetes (*Lumbriculus variegatus*) or bivalves (*Hydrobia ulvae*) have been successfully used in sediment toxicity testing (Leppänen and Kukkonen, 1998; Shipp and Grant, 2006; Penttinen et al., 2008). To our knowledge, the effect of pollutants on earthworm CP has not been studied to date for ecotoxicology purposes. To set the foundations for using CP measurements in ecotoxicity testing, we initially conducted a series of experiments to investigate the influence of earthworm weight and soil moisture content on CP. We then studied the effectiveness of using CP as a biomarker for exposure of *L. terrestris* earthworms to 6 different pesticides. The aim of the study is to propose a new ecotoxicity test which produces reproducible and relevant results, is relatively easy to conduct and could be considered as a possible candidate for a standardized test in soil risk assessment protocols.

2. Materials and Methods

2.1. Soil, earthworms and the sieving protocol

Soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3, CEC = 8.2 cmol kg^{-1}) was collected from an apple orchard abandoned in 1995 and located in Montfavet near Avignon, in south-eastern France. The water holding capacity (WHC) of the soil was 0.247 g g^{-1}. Total heavy metal concentrations were measured in the soil (Cu = 30.0, Zn = 76.8, Pb = 30.0 and Cd = 0.290 mg kg^{-1}). Adult and subadult earthworms (*Lumbricus terrestris*) were purchased from a local supplier (fisheries store). They were raised in Canadian farms and are therefore available all year round. The worms were acclimated in the orchard soil for four days prior to experiments.

In all experiments, the soil was primarily sieved at 3 mm. Soil moisture was measured and adjusted to the desired value by adding distilled water, mixing the soil and then setting it apart for 2 days in a dark chamber at 12°C to reach equilibrium. Before each experiment, the soil was re-sieved and the moisture content was verified. At the beginning of each experiment, earthworms were washed in tap water, blotted dried on filter paper, weighed (without gut voiding) and individually placed for 7 days

in 100 g moist soil in crystal transparent polystyrene round boxes (diameter = 10 cm; height = 3 cm; purchased at Caubère, Yebles, France) and called hereafter Petri dishes. Controls were set up in which no earthworms were added to the soil in the Petri dishes. Earthworms were weighed again (without gut voiding) at the end of the experiment.

In many previous studies (Scheu, 1987; Shipitalo et al., 1988; Buck et al., 1999), casts were collected manually after visual assessment (according to shape and size). For the sake of reproducibility and accuracy, in this study we separated out casts using a set of 4 sieves (diameter = 15 cm and mesh sizes = 5.6, 4, 3.15, 2.5 mm) since earthworm activity may modify soil granulometry leading to an increase in the amount of soil retained in some sieves (casting) and a decrease in others (soil consumption). All soil from each Petri dish, including the soil that adhered to the walls of the dishes which was removed with a knife, was sieved taking care not to break up the casts. The set of sieves was manually shaken for 10 s. The soil retained in each sieve was weighed. The effect of earthworm bioturbation was then examined by determining the changes in the particle size distribution (PSD), i.e. weight of fresh soil in each sieve minus the corresponding weight of soil for the control soil (without earthworm bioturbation).

2.2. Optimisation of the sieving protocol

To optimise the protocol, we independently assessed CP dependence on (i) earthworm weight, (ii) soil water content and (iii) drying the soil at the end of the experiment. Most authors (Scheu, 1987; Shipitalo et al., 1988; Curry and Baker, 1998) expressed CP on a weight basis (i.e. in g of soil per g of earthworm body mass) so we investigated the relationship between earthworm weight and CP using 60 Petri dishes. Adults or subadults of *Lumbricus terrestris* with a large range of body masses (from 1.13 to 6.38 g with a mean of 3.69 and a standard deviation of 1.26 g) were placed in fifty of them. Ten Petri dishes without earthworms were set up as a control.

Earthworm activity (burrowing and casting) is influenced by water potential and hence soil water content (Kretzschmar, 1991; Daniel et al., 1996). However, soils with high water contents are difficult to sieve because wet soil aggregates tend to combine. We therefore studied the relationship between CP and water content using 100 Petri dishes and 5 different soil water contents (15, 18, 21, 24 and 27% expressed on a weight/weight basis) ranging from 60 to 110% of the WHC. For each soil water content, 20 Petri dishes were fill with moist soil, 10 without earthworms (control) and 10 containing one *L. terrestris*. Because earthworm weights were variable and ranged from 2.47 to 5.62 g, a blocking procedure was used so that mean earthworm weight was similar in each treatment (Mc Indoe et al., 1998). This blocking procedure was used for the following experiments.

We hypothesised that waiting for the soil to dry at the end of the experiment may facilitate soil sieving. This in turn could influence the results of CP as a possible ecotoxicity test. To investigate this idea, a test was carried out using 160 Petri dishes and a carbamate insecticide (Lannate©, DuPont) the active ingredient of which is methomyl. Moist soil was prepared at initially 19.2% water content. Soil was contaminated by manually spraying each kg of soil with 40 ml of a solution containing increasing concentrations of methomyl. Control soil was sprayed with the same quantity of water. The spiking procedure, which generated good levels of pollution homogeneity, was described in detail and tested by Capowiez et al. (2005). The methomyl concentrations were chosen based on the usual application rate and calculation of the PEC (Predicted Environmental Concentration i.e. a single application of 750 g a.i. ha^{-1}, with a homogeneous distribution in the first 5 cm of soil, no crop interception and a soil density of 1.5 kg l^{-1}). This normal application rate (2.025 mg a.i. kg^{-1} of wet soil) is termed '1X' and we then used the following concentrations: '10X', '0.1X' accordingly. For each pesticide concentration, 40 Petri dishes were filled with moist soil and one *L. terrestris* (range weight was 1.69-5.97) was added in half of them. The final soil water content was 24%. After 7 days of exposure at 12°C in a dark chamber, earthworms were weighed. The soil in half of the Petri dishes was sieved immediately. The other half was sieved after the Petri dishes were dried for 4 days at laboratory temperature (about 25°C). The soil water content of 3 Petri dishes was measured in each treatment. Because casts were dried, this time CP was expressed in % of weight of the available soil g^{-1} fresh body mass day^{-1} to enable direct comparisons between fresh and dried soil (each worm was in 100 g of moist soil).

2.3. Effect of 6 pesticides on cast production

The sensitivity of CP as a behavioural biomarker was tested using 6 pesticides (Table 1). Four of these were long-standing and broad-spectrum insecticides belonging to the carbamate and organophosphorous families, which are currently still used in apple orchards in Provence (South-East of France) due to the increasing resistance of codling moths to other pesticides (Sauphanor et al., 2000). The fifth (ethyl-parathion) was banned by the EEC in 2001 but was used in the present study as a model pesticide so that comparisons with previously published data (Olvera-Velona et al., 2008) were possible. The last (imidacloprid) is a relatively new insecticide belonging to the family of neonicotinoids, which is currently used in peach orchards against aphids. This pesticide was previously shown to cause behavioural effects on earthworms even at low concentrations close to the PEC (Capowiez and Bérard, 2006). It was then used in the present study at lower concentrations than these other pesticides (Table 1). Four of these pesticides were classified as very toxic or extremely toxic to earthworms by Edwards and Bohlen (1996) whereas they found insufficient

evidence to categorize azinphos-methyl. Imidacloprid was initially categorized as moderately toxic to earthworms by Elbert et al. (1990). The soil spiking procedure was as explained previously except that only 20 Petri dishes were filled with most soil for each pesticide concentration, 10 contained an earthworm and 10 did not. The final soil water content was 24%. The adults or subadults of *L. terrestris* used had an average weight of 4.01 g (the range of weight was 1.57-6.09 g). After 7 days of exposure in a dark chamber at 12°C, the earthworms were re-weighed and the soil was sieved immediately by the same operator (wearing a mask when handling polluted).

Table 1: Pesticides used in the study on earthworm cast production, their trade names, main target organisms, Predictive Environmental Concentration (PEC) and median lethal concentrations (LC_{50}) for the effect on earthworm survival taken from the literature.

Active ingredient	**Commercial name**	**Targets**	**Application Rate ($g\ ha^{-1}$)**	**PEC = Soil concentration ($mg\ a.i.\ kg^{-1}$ dry soil)***	**LD_{50} ($mg\ kg^{-1}$ dry soil)**	**Duration of the toxicity test**	**Species used**
Methomyl	Lannate	Codling moth Aphids	750	2.025	90[(a)]	14	*E. fetida*
Chlorpyrifos-ethyl	Pyrinex ME	Codling moth	500	1.35	129[(b)]	14	*E. fetida*
Carbaryl	Sevin L85	Codling moth	850	2.295	9[(c)]	7	*Pheretima*
Ethyl-parathion[(1)]	Oleobladan	Codling moth	250	0.675	32[(d)]	14	*A. caliginosa*
Azinphos-methyl	Gusathion XL	Codling moth Aphids	437.5	1.18	158[(e)]	14	*E. fetida*
Imidacloprid	Confidor	Aphids	70	0.189	10.7[(b)]	14	*E. fetida* *E. andrei*

* single application with an homogeneous distribution in the first 5 cm of soil with a density of 1.5 $kg\ l^{-1}$ and no crop interception.

[(1)] forbidden since 2001 in EEC

[(a)] Armstrong et al (1991) ; [(b)] Agritox database (www.dive.afssa.fr/agritox/index.php); [(c)] Mostert et al 2002 ; [(d)] Olvera-Velona et al (2008) ; [(e)] Heimbach (1986).

2.4. Statistical analyses

Data were tested for normal distribution and homogeneity of variance and then were log-transformed before the ANOVA or regression analysis as necessary. The relationships between CP (in g of fresh soil day^{-1}) and earthworm fresh weight were assessed using linear regression. To study the effects of the soil water content on CP (in g g^{-1} day^{-1}) we performed a one-way ANOVA. The effect of drying the soil at the end of the experiment was assessed with a two-way ANOVA with soil drying and pesticide dose as factors. To study the effects of the 6 pesticides on CP or weight loss, we performed 6 independent one-way ANOVA. All tests were carried out using R-software (R 2005). In every case the significance threshold was set to 5%.

3. Results

3.1. Optimisation of the sieving protocol

In all experiments, positive values of PSD were consistently found for only the largest sieve size (5.6 mm). Because the values of PSD obtained for the second sieve (4 mm) were either very low or negative it was then decided to compute the CP using only the values obtained with the largest sieve size.

A positive and linear correlation was observed between the raw CP (expressed in g of soil day^{-1}) and earthworm weight ($R^2 = 0.623$; $p < 0.01$; CP = 0.674 EW + 0.068 with CP, (cast production in g day^{-1} of soil) and EW, (earthworm fresh weight in g)). Thus, in the following experiments, the CP was expressed as cast fresh weight per earthworm fresh body mass per day (g g^{-1} day^{-1}).

The soil water content (up to 24%) had a significant effect on CP which increased from 0.034 at 15% to 0.752 g g^{-1} day^{-1} at 24% soil water content (Table 2). For the highest soil water content (27%), we did not observe a significantly lower CP value than for 24% but the variability was much higher. Furthermore, at 27% soil water content, very large amounts of soil were found in the 5.6 mm sieve from the control without earthworms (Table 2) even if the soil was initially sieved at 3 mm.

After 4 days at ambient temperature, the mean soil water content decreased from 20.7 to 1.7 % with no significant difference between the treatments, i.e. methomyl concentration (data not shown). This time, CP was expressed as the % of soil weight available g^{-1} of body mass day^{-1} so that the amount of dry and fresh casts can be compared. CP was significantly influenced by the pesticide concentration ($p < 0.01$) but not by the drying procedure ($p = 0.192$). The interaction between these

two factors was not significant. The presence of methomyl led to significant decreases in CP of 45 % and 65% at 1X and 10X, respectively, compared to the control (Fig. 1).

Table 2: Effect of the soil water content on *Lumbricus terrestris* cast production (means and standard deviations) and the amount of soil retained in the sieve with the largest mesh size (5.6 mm) from control Petri dishes without earthworms (n=10). Values labeled with the same letter are not significantly different at the 5% level.

Soil water content ($g\ g^{-1}$)	**15%**	**18%**	**21%**	**24%**	**27%**
Cast production (fresh g g^{-1} fresh body mass day^{-1})	0.034^{c} (0.037)	0.125^{c} (0.075)	0.357^{b} (0.101)	0.752^{a} (0.148)	0.712^{a} (0.275)
Soil in the sieve with the largest mesh size from controls without earthworms (g)	0.00 (0.00)	0.03 (0.01)	0.06 (0.01)	0.10 (0.01)	14.22 (0.34)

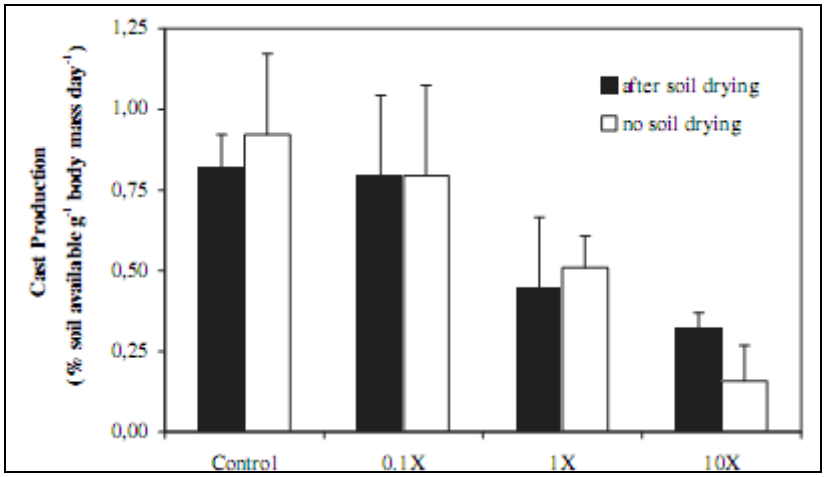

Figure 1: Effect of different concentrations in methomyl (1X = normal application rate) on *Lumbricus terrestris* cast production (means and standard deviations) expressed in % of soil available (each earthworm was given 100 g of moist soil) g^{-1} of fresh body mass day^{-1}. Cast production was estimated either immediately at the end of the 7 days exposure period (no soil drying) or 4 days later (after soil had been allowed to dry).

3.2. Effects of the 6 pesticides on CP and body mass

Following finding from the optimisation protocol, soil water content was set at 24% and sieving was carried out immediately after the 7th day of exposure. Azinphos-methyl was the only pesticide which did not have a significant effect on CP (Fig. 2). Chlorpyrifos-ethyl and ethyl-parathion had a significant

effect on CP but without a clear concentration-effect relationship (10X was not significantly different from 0.1X). In addition, the decreases in CP for 10X were limited to 38 and 35% for chlorpyrifos-ethyl and ethyl-parathion, respectively (Fig. 2). The other three pesticides showed significant effects on CP with a concentration-effect response. The decreases in CP for the highest pesticide concentration were 67, 54 and 61% for methomyl, carbaryl and imidacloprid, respectively. Due to the limited number of concentrations tested it was not possible to compute an EC_{50}. For chlorpyrifos-ethyl and carbaryl, significant decreases in CP were observed for the 0.1X treatment.

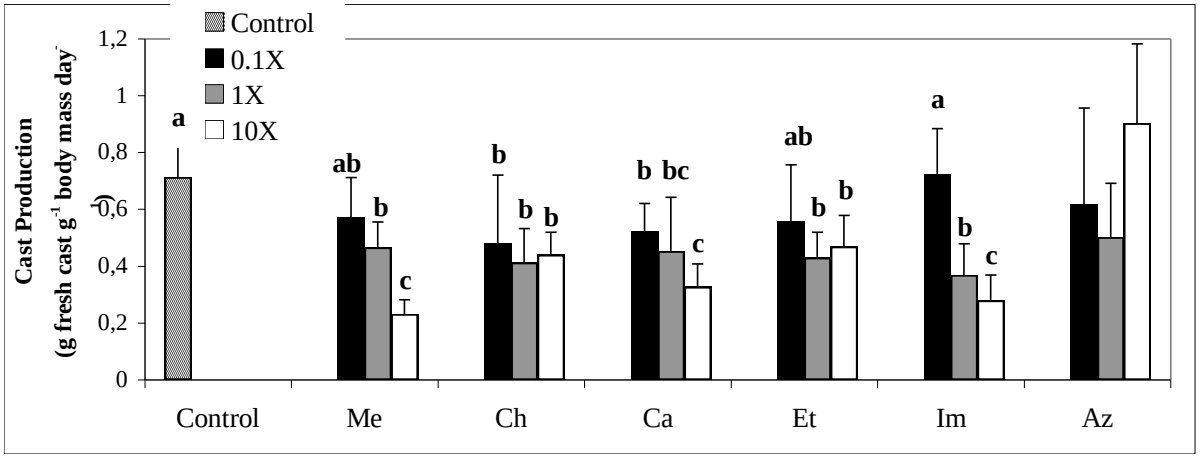

Figure 2: Cast production (means and standard deviations) of *Lumbricus terrestris* after exposure to 6 different pesticides for 7 days expressed in g fresh casts g^{-1} fresh body mass day^{-1} (n=10). Bars labeled with the same letters are not significantly different at the 5% level (each pesticide was tested separately). Me = methomyl ; Ch = chlorpyrifos-ethyl ; Ca = carbaryl ; Et = ethyl-parathion ; Im = imidacloprid ; Az = azinphos-methyl.

Body mass changes of *L. terrestris* after the 7 days of exposure showed significant differences even for the lowest concentrations (0.1X) tested except for imidacloprid (Table 3). Whereas earthworms in the control soil gained weight (11%), a decrease in body mass was observed at 1X for methomyl, carbaryl and ethyl-parathion and for all pesticides at 10X. Overall, the body mass decreases were limited and smaller than 20%(except for methomyl).

Table 3: Body mass changes (means and standard deviations) of *Lumbricus terrestris* due to exposure to 6 different pesticides for 7 days expressed as a percentage of initial body mass set at 100%. Values in bold are significantly different from the control value (n=10). Values labeled with the same letters are not significantly different at the 5% level (each pesticide was tested separately).

	Control	0.1X	1X	10X
Methomyl	111.0^{a} (8.2)	**100.5^{b} (15.6)**	**93.4^{b} (7.4)**	**76.6^{c} (9.4)**
Chlorpyrifos-ethyl		**101.1^{b} (7.0)**	**100^{b} (5.5)**	**97.6^{b} (12.2)**
Carbaryl		**99.7^{b} (9.2)**	**89.0^{c} (10.0)**	**81.9^{c} (12.2)**
Ethyl-parathion		**103.3^{b} (9.0)**	**95.4^{c} (5.0)**	**85.7^{d} (4.3)**
Imidacloprid		111.8^{a} (9.4)	**103.6^{b} (12.1)**	**93.9^{c} (8.3)**
Azynphos-methyl		**95.7^{b} (7.6)**	**101.1^{b} (8.2)**	**81.4^{c} (11.1)**

4. Discussion

4.1. Optimisation of the sieving test

In the present study we focused on some aspects of the sieving protocol in order to determine if (i) earthworms of various body mass can be used, and, whether (ii) the soil water content during the experiment or during the sieving has an influence on CP itself and on our ability to accurately measure CP.

As expected, we observed a significant and linear relationship between initial earthworm body mass and CP (expressed in g of soil). The same result was found by Scheu (1987) for *A. caliginosa* and most authors express CP in g of dry or wet soil g^{-1} of body mass day^{-1}. Occasionally, very small earthworms (juveniles) were reported to produce a greater weight of casts than adults or subadults: Bolton and Phillipson (1976) observed this for *Aporrectodea rosea* but only at 14.8°C the highest of the 3 temperatures tested and Daniel et al. (1996) reported a similar finding for *Aporrectodea nocturna*. In both cases, only surface casts were included in the study. Furthermore, in the latter study, CP was expressed in g dry cast g^{-1} of earthworm dry weight. Indeed, the lack of standardized units for expressing CP often makes direct comparisons between results from different studies difficult or impossible. In this study, because we did not dry the casts or only focus on surface casts, the mean values obtained for *L. terrestris* CP (0.714 g of fresh soil g^{-1} body mass day^{-1}) were higher than the range of values reported by previous authors: 0.10-0.18 for Shipitalo et al (1988), 0.25 for Hartenstein and Amico (1983) and 0.25-0.43 g g^{-1} day^{-1} for Buck et al. (1999).

As observed by many authors (Kretzschmar, 1991; Hindell et al., 1994; Daniel et al., 1996), earthworm activity and therefore CP increased with the soil water content. However, for the highest

soil water content we tested, non-biogenic soil aggregates tended to combine and sieving became difficult leading to higher values than expected for the controls (without earthworms) and a high variability in the CP measurements. It therefore appeared that, for the soil used in this study, the optimal soil water content to accurately estimate CP is 24%. In addition, drying the soil at the end of the experiment had no influence on CP estimations, meaning that soil can be sieved immediately at the end of the experiment.

The protocol we used to estimate CP is relatively simple and not time-consuming (if only one sieve adapted to the earthworm species is used). Hand shaking can be a source of variability so in this study, all tests were carried out by the same person. Diaz-Zorita et al. (2007) showed that sieving duration has no effect when using sieves with a mesh size larger than 4 mm. In future experiments, however, CP estimations could be standardized by using automatic sieve shakers.

4.2. Cast production is a promising biomarker

A decrease in CP in the presence of pesticides appears to be a sensitive marker of toxic effects. Different kinds of responses, from no effect to a clear concentration effect, were observed. These different responses are in relative agreement with previously determined characteristics of the toxicity of the tested pesticides to earthworms (Table 1). Although the LD_{50} for each of the pesticides used were computed on different earthworm species and under different time frames, it is still relevant to divide the LD_{50} by the PEC, to give a toxicity ranking as is frequently done in ecological risk assessments. Three different groups of values were obtained: (i) below 10 for carbaryl; (ii) between 40 and 60 for methomyl, methyl-parathion and imidacloprid; (iii) close to 100 for chlorpyrifos-methyl and azinphos-methyl (the higher this value is, the lower is predicted toxicity). Interestingly, the effects on CP were approximately the same as this ranking: azinphos showed no toxic effects, ethyl-parathion and chlorpyrifos-ethyl led to a significant decrease in CP without a clear concentration-effect response and finally there was a clear concentration-effect response with methomyl, imidacloprid and carbaryl. CP appears to be generally highly sensitive to pesticides with the 1X concentration of 5 of the pesticides leading to a significant decrease in CP and with 2 of the pesticides showing effects at even the 0.1X concentration. Importantly, this indicates that field (or even 10 times lower) concentrations of currently used pesticides may have a negative influence on earthworm behaviour, and thus, also on soil functioning.

Earthworms exposed to the pesticides tested showed significant weight losses even at low concentrations (except for imidacloprid at 0.1X). This was unexpected because a previous study using endogeic earthworms exposed to ethyl-parathion, reported that this marker was less sensitive and

more variable than behavioural endpoints related to burrowing behaviour (Olvera-Velona et al., 2008). Weight loss was significant but the range of variation was rather limited (rarely more than 20%) in this experiment. Furthermore, if the pesticides are ranked using this marker it does not correlate with the toxicity given by the LD_{50} and PEC data (for example azinphos-methyl appeared to be as toxic as methomyl and imidacloprid at the 10X concentration).

Regarding published sublethal effects on earthworms, no data were found for methomyl. Springett and Gray (1992) reported that azinphos methyl had negative effects on *A. caliginiosa* growth but when applied at a normal application rate every week for 100 days. For chlorpyrifos-ethyl, studies reported no sublethal effects (Mostert et al., 2000; Booth and O'Halloran, 2001). For carbaryl, imidacloprid and ethyl-parathion, significant influences on sublethal, including behavioural endpoints, were observed for concentrations close to or below the PEC reported in Table 1 (Gupta and Saxena, 2003; Capowiez et al., 2005; Capowiez and Bérard, 2006; Olvera-Velona et al., 2008; Gomez-Eyles et al., 2009). Our findings on CP are in agreement with these previous studies. Interestingly, in their recent study, Gomez-Eyles et al. (2009) measured the feeding rate of *E. fetida* after exposure to imidacloprid (estimated by the quantity of ingested manure). They showed that this endpoint was very sensitive and observed significant reductions in manure ingestion at imidacloprid concentrations as low as 1.91 mg kg^{-1}.

In the present study, most of the casts were sampled using the sieve with a mesh size of 5.6 mm. For all the other sieves, values of PSD were generally negative or very low. This 5.65 mm value is rather high compared to the 2 mm threshold size generally used to isolate earthworm casts (Blanchart, 1992). However it should be noted that only adults or subadults, hence large *L. terrestris* (with a mean weight of 4.01 g), were used in this study. It is known that other soil organisms such as fungi and other micro-organisms can also influence soil aggregation (Tisdall and Oades, 1982; Elmholt et al., 2008). In our experimental set up these organisms are also exposed to the pesticides and their role in soil aggregation may or may not have been modified (Helfrich et al., 2008). Theoretically, this could have influenced CP. However, because we measured CP using only the larger mesh size and an earthworm-free control consisting of soil alone was used for each pesticide concentration, we suggest that the influence of these organisms in our estimation of CP was negligible.

Due to their important roles in soil ecosystems, earthworms are terrestrial model organisms requiring testing. Currently several standardised tests using earthworms are available. The first tests examined earthworm mortality (on paper filter or in artificial or natural soils) in order to establish the LD_{50} (ISO, 1993). Then other tests were developed based on sublethal endpoints such as reproduction (ISO, 1998) or behaviour (avoidance; ISO, 2008). *E. fetida* is the model organism in all of these tests and, for example, the reproduction test is only possible with epigeic worms due to their

short reproduction cycle. Another drawback is that this reproduction test takes 28 days. The avoidance test is a behavioural test with several advantages (simple, quick and cheap) but one drawback: this is not a measure of toxicity but rather a measure of repellence (Capowiez and Bérard, 2006), and thus is termed 'measure of habitat modification'. As there is not always a direct relationship between avoidance and toxicity, an improvement of this test was recently proposed by Sanchez-Hernandez (2006). Our behavioural test, based on estimations of CP, is straightforward, quick, does not require specific equipment and mitigates most of the drawbacks listed above for other behavioural standardised tests. Of special importance, it is adapted to earthworm species with higher ecological relevance (Lowe and Butt 2005) and takes only 7 days. Furthermore, the results are easy to interpret: CP simply represents the quantity of soil the earthworms egested and may be correlated to the quantity ingested (feeding behaviour). However, a disadvantage of this test worth mentioning is that when a decrease in CP was observed, our results do not clearly determine whether earthworms ingested less soil because their health was affected or because the polluted soil acted as a repellent. Another drawback of CP as a biomarker is that it appears to be a difficult approach for use with *E. fetida* since our first attempts to estimate CP for epigeic earthworms (*E. fetida* or *Dendrobaena veneta*) failed even using sieves with smaller mesh sizes.

In conclusion, CP appears to be a relevant (i.e. ecologically meaningful) and promising biomarker for ecotoxicity tests. Some aspects should be carefully considered for this marker to be widely used and accepted. First of all, preliminary tests need to be carried out to adapt (i) mesh size and test duration to the earthworm species under investigation and (ii) soil water content to soil texture. The key parameters are the soil moisture content and the initial soil particle size distribution. These parameters are not easy to control for large quantities of soil, so we recommend that in order to minimize variability, the soil to be incubated with and without earthworms should be prepared at the same time for each pesticide or pollutant concentration.

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b) Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*). *Ecotoxicology 19, 1567-1573.*

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Abstract

Earthworms play key roles in soils and sub-lethal effects of environmental toxicants on these organisms should be taken seriously, since they might have detrimental effects on higher ecological levels. In laboratory experiments we have assessed sub-lethal effects (body mass change and cast production) of imidacloprid on two earthworm species commonly found in different agricultural soils (*Lumbricus terrestris* and *Aporrectodea caliginosa*). After 7 days of exposure in contaminated soil, a significant loss of body mass was found in both species exposed to imidacloprid concentrations as low as 0.66 mg kg^{-1} dry soil. These losses ranged from 18.3% to 39% for *A. caliginosa* and from 7.4% to 32.4% for *L. terrestris*, respectively. Changes in cast production, a new biomarker previously validated using *L. terrestris*, was assessed by soil sieving using the recommended mesh size (5.6 mm) for *L. terrestris* and three different mesh sizes for *A. caliginosa* (5.6, 4 and 3.15 mm). The 4 mm mesh size proved to be the most suitable sieve size for *A. caliginosa*. Cast production increased by 26.2% in *A. caliginosa* and by 28.1% in *L. terrestris* at the lowest imidacloprid concentration tested (0.2 mg kg^{-1} dry soil), but significantly decreased at higher concentrations (equal to and above 0.66 mg kg^{-1}dry soil) in both earthworm species after the 7 days exposure experiment. These decreases in cast production ranged from 44.5% to 96.9% in *A. caliginosa* and from 42.4% to 95.7% in *L. terrestris.* The EC_{50} for cast production were 0.84 (*L. terrestris*) and 0.76 mg kg^{-1}dry soil (*A. caliginosa*), respectively. The detected sub-lethal effects were found close to the Predicted Environmental Concentration (PEC) of imidacloprid, which is in the range of 0.33 - 0.66 mg kg^{-1} dry soil. The biomarkers used in the

present study, body mass change and changes in cast production, may be of ecological relevance and have shown high sensitivity for imidacloprid exposure of earthworms. The measurement of changes in cast production should be considered for inclusion in current standard tests.

Keywords: Earthworms – Imidacloprid – Cast production – Body mass change

1. Introduction

Imidacloprid is a relatively new neonicotinoid insecticide which is commonly used worldwide in agriculture against sucking insects. It shows selective toxicity for insects (Matsuda et al. 2001), but there is evidence of effects on non-target and ecologically important organisms (Mostert et al. 2002; Iwasa et al. 2004; Kreutzweiser et al. 2009).

Earthworms are of crucial importance for soil functioning, they make important contributions to the breakdown of organic matter, soil fertility, and to the formation of soils (Edwards and Bohlen 1992; 1996). Because of their ecological importance, their high biomass in soil and their frequently observed sensitivity to relatively low concentrations of environmental toxicants, they are ideal test organisms for soil risk assessment (Bouché 1992; EEC 2003).

The few existing standard tests for earthworms are carried out mainly with *Eisenia fetida* and *Eisenia andrei* and focus on mortality, reproduction and avoidance behaviour (OECD 1984; EEC 2003; OECD 2004; ISO 2008). However, *E. fetida* and *E. andrei* are epigeic species ecologically not relevant for pesticide testing since these earthworms are absent from most agricultural soil and often claimed to be less sensitive to environmental toxicants than other earthworm species (Edwards and Coulson 1992; Spurgeon and Weeks 1998). According to our opinion, the relevance of a toxicity test is based on the possibility to link the response to indispensable soil functions (EEC 2003). In this context, some behavioural endpoints are important and efforts should be made to develop, optimise and increase their use in earthworm toxicity testing. However, care must be taken, since behavioural responses might vary greatly from species to species (Gilman and Vardanis 1974).

Earthworm behavioural biomarkers range from avoidance tests (Slimak et al. 1997; Hodge et al. 2000; Schaefer 2003) to studies on burrowing behaviour using 2D and 3D techniques (Hans and Beg 1992; Capowiez et al. 2003; Capowiez et al. 2006). Avoidance behaviour can be measured easily, but

results must be interpreted carefully, since some toxicants might attract organisms, whereas others might repel them (Yeardley et al. 1996). Studying changes in earthworm behaviour via 2D and 3D techniques is very sensitive and promising, but time consuming and therefore not worth taking into account as potential standard tests (Eijsackers et al. 2001; Capowiez et al. 2006). Edwards and Lofty (1972) claimed that cast production is an important indication of earthworm activity and some studies have shown reduced cast production of earthworms (Cook et al. 1980; Lal et al. 2001) or reduced ingestion rates of earthworms after pesticide treatment (Gomez-Eyles et al. 2009). Capowiez et al. (2010) have developed a new and standardisable protocol for toxicity testing, based on changes in earthworm cast production as a proxy of changes in activity. It was successfully applied to an anecic species (*Lumbricus terrestris*) using six pesticides, but the suitability of this test for endogeic species need to be tested. In their study, these authors have found a significant decrease of cast production after exposure to imidacloprid at concentrations as low as the normal application rate (70 g ha^{-1}), but no effect for a concentration ten times lower.

On a physiological level, body mass change has already been successfully used as a biomarker in earthworms and is well established since it is an integral part of the acute and chronic standard tests. It can be interpreted as indication of general health (Leland et al. 2001; Zwahlen et al. 2003; Olvera-Velona et al. 2008).

The aim of the present study was then to assess sub-lethal effects of imidacloprid on two species ecologically relevant for agricultural soil (*Lumbricus terrestris* and *Aporrectodea caliginosa*) by using the earthworm cast production test and the measurement of body mass changes. Therefore the suitability of cast production test for *A. caliginosa* had to be evaluated and the test had to be adapted to this endogeic species. With the present study we also wanted to contribute to the development of the cast production test as a potential standard test. Moreover we wanted to determine if imidacloprid concentrations between the normal application rate and one third of it would still have significant effects in both earthworm species.

2. Material and methods

2.1 Test organisms, soil and pesticide

Adult earthworms of the species *Aporrectodea caliginosa* were collected from an INRA experimental orchard near Avignon (France), where no pesticide was applied for 5 years. Adult and subadult earthworms of the species *Lumbricus terrestris* (raised in Canadian farms) were purchased from a fishery store in Avignon. The soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3 (in water), CEC = 8.2 cmol kg^{-1}, WHC = 0.247 g g^{-1}) was collected from another orchard (abandoned for about 10 years,) close to Avignon (France). Before the experiments, the earthworms were acclimatized for 7 days in the orchard soil in a climate chamber (12°C) under conditions of complete darkness. Handling of our test organisms prior to the experiments was based on the general recommendations established by Fründ et al. (2010).

The insecticide imidacloprid (255.66 g/mol; purity: 99.9%) was purchased from FLUKA (No.37894) and dissolved in distilled water to different concentrations.

2.2 Experimental design

Prior to the exposure experiments, the soil was sieved to 3 mm. Soil water content was measured and adjusted to 20% (80% of the WHC) by adding distilled water thoroughly mixed and sieved again to 3 mm. Then soil spiking (for both, pesticide exposure groups and control groups) was conducted according to the protocol of Capowiez et al. (2005) by adding 40 ml of water or solution containing the adapted pesticide concentration, reaching a final soil water content of 25% (of dry soil weight). The predictive environmental concentration (PEC) of imidacloprid was found to be in the range of 0.33 - 0.66 mg kg^{-1} dry soil depending on the country and crop under consideration (Oi 1999; Mostert et al. 2000). We have defined the normal application rate (1x) as to be 0.66 mg kg^{-1} corresponding to a field application rate of 244 g ha^{-1}. The soil concentration value refers to a single application with a homogeneous distribution in the upper 5 cm of soil with a density of 1.5 kg l^{-1} and no crop interception. For both the cast production test and body mass change measurements, 25 individuals of each species were exposed to each of the following concentrations: 0 (control), 0.2 (0.3x), 0.66 (1x), 2 (3x) and 4 (6x) mg kg^{-1} dry soil (the latter concentration was only used for experiments conducted with *L. terrestris,* since it was lethal for *A. caliginosa*). Additionally the same concentrations were used to pollute the soil without earthworms as controls for the cast-production-test (n = 10 for each concentration tested).

Petri dishes (diameter = 10 cm) were filled with 100 g of uncontaminated (control group with distilled water) or contaminated moist soil. For the exposure experiments each earthworm was rinsed in tap water, gently dried on filter paper, weighed and randomly put in individual Petri dishes. Afterwards the dishes were placed in a dark chamber at 12°C. After 7 days of exposure, the earthworms were taken out of the dishes with soil, rinsed, gently dried on a filter paper and weighed again and the soil was kept apart for the cast production test. The weighing procedures before and after the experiment were done without voiding the guts of the earthworms, so body mass change was rather an indication for feeding activity than for growth (the latter is limited for adult earthworms during an exposure time of 7 days).

The cast production test was conducted according to the protocol of Capowiez et al. (2010). Therefore, the soil in each dish was taken out carefully and sieved (diameter = 15 cm) manually by consistently shaking the sieve for 10 s. To determine the cast production of *L. terrestris*, we used a sieve with a mesh size of 5.6 mm since Capowiez et al. (2010) showed that values found for smaller mesh sizes (4 and 3.15 mm) were either very low or zero. For *A. caliginosa* the soil was sieved using a set of sieves (mesh sizes = 5.6, 4 and 3.15 mm). The additional 3.15 and 4 mm sieves were chosen in order to determine the most suitable mesh size for this species since cast produced by *A. caliginosa* were relatively smaller.

The remaining soil in each sieve was weighed and cast production was computed by subtracting the mass of the remaining soil in the control sieves (control without earthworms) from the corresponding mass in the sieves of the test samples. Since a linear correlation was found for *L. terrestris* between weight of produced casts and earthworm biomass (Capowiez et al*.,* 2010), every single value for cast weight in each Petri dish was divided by the body mass of the corresponding earthworm. This resulted in cast production values expressed as cast fresh weight per earthworm fresh body mass per exposure day (g g^{-1} day^{-1}). Because we could not find a significant correlation between cast production and earthworm body mass in *A. caliginosa*, expressing cast production rate dependant on earthworm body mass became dispensable. But for the sake of a better comparison, we have used cast production rate (in g g^{-1} fresh body mass day^{-1}) in both species as a unit for expressing the amount of produced casts.

2.3 Statistical analysis

Data (body mass change or cast production) were tested for normality and homoscedasticity and were log-transformed when necessary. For both species, the effect of imidacloprid concentration either on body mass change or cast production was analysed by a one-way ANOVA and post-hoc

comparisons were done using Tukey-HSD. The relationship between cast production and earthworm body mass was investigated using linear regression. Median effective concentrations (EC_{50}) for cast production were computed using the linear interpolation (ICp) method.

3. Results

No mortality was observed except that one individual died at one time in the 0.3x and 1x exposure group of *L. terrestris* and in the 3x exposure group of *A. caliginosa*.

3.1 Body mass change

For *A. caliginosa* significant losses in body mass were observed ($p < 0.0001$; $F=115.2$; $df=3$). The 1x and 3x exposure groups were significantly different from the control group at $p < 0.05$ (Table 1). For *L. terrestris* body mass change differed significantly as well ($p < 0.0001$ $F=50.8$; $df=4$). The 1x, 3x and 6x exposure groups were significantly different (compared to the control group) at $p < 0.05$ (Table 2).

Table 1: Mean initial body mass (+SD), mean body mass after 7d (+SD) and mean body mass after 7d relative to initial body mass (+SD) (expressed in percentage) of *Aporrectodea caliginosa* after imidacloprid exposure for 7 days (n = 25). Values in bold are significantly different from the control values ($p < 0.05$).

	0 mg kg^{-1} dry soil	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
Initial body mass [g]	0.61 (0.15)	0.66 (0.19)	0.60 (0.16)	0.65 (0.20)
Body mass after 7d [g]	0.67 (0.18)	0.68 (0.18)	0.54 (0.14)	0.46 (0.13)
Relative body mass after 7d [%]	109.3 (10.6)	104.5 (7.5)	**91 (9.3)**	**70.3 (5.9)**

Table 2: Mean initial body mass (+SD), mean body mass after 7d (+SD) and mean body mass after 7d relative to initial body mass (+SD) (expressed in percentage) of *Lumbricus terrestris* after imidacloprid exposure for 7 days (n = 25). Values in bold are significantly different from the control values ($p < 0.05$).

	0 mg kg^{-1} dry soil	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**	**4 mg kg^{-1} dry soil**
Initial body mass [g]	4.10 (1.09)	3.91 (1.03)	3.95 (0.68)	4 (1.58)	4.12 (1)
Body mass after 7d [g]	4.74 (1.35)	4.56 (1.29)	4.28 (0.8)	3.82 (1.69)	3.43 (1.26)
Relative body mass after 7d [%]	115.6 (7.9)	116.6 (9.4)	**108.4 (12.1)**	**95.5 (8.3)**	**83.3 (4.7)**

3.2 Cast production

First of all the results obtained for *A. caliginosa* using three different sieve sizes (3.15, 4 and 5.6 mm) were compared. Four and 5.6 mm mesh size resulted in the same significant differences, but higher cast weights were obtained when using the 4 mm mesh size (Table 3). For 3.15 mm mesh size no significant difference between control group and 1x-exposure group was observed (Table 3).

While a significant but low positive relationship between cast production and earthworm body mass was found for the control group of *L. terrestris* ($P = 0.001$ and $r^2 = 0.2$), cast production (for all mesh sizes) of the control group of *A. caliginosa* did not correlate with earthworm body mass.

Significant differences in cast production were observed in the earthworm species *A. caliginosa* ($p < 0.0001$; $F=35.8$; $df=3$). In comparison to the control group, mean cast production significantly decreased for the 1x and 3x exposure groups (-44.5% and -96.9% respectively) at $p < 0.05$ (Table 3).

Table 3: Effect of mesh size on the estimation of mean cast production (+SD) (in g cast weight g^{-1} earthworm body mass day^{-1}) of *Aporrectodea caliginosa* after exposure to different concentrations of imidacloprid for 7 days (n = 25). Values in bold are significantly different from the control values ($p < 0.05$).

Mesh size [mm]	**0 mg kg^{-1} dry soil**	**0.2 mg kg^{-1} dry soil**	**0.66 mg kg^{-1} dry soil**	**2 mg kg^{-1} dry soil**
3.15	2.63 (1.27)	3.38 (1.75)	1.8 (1.7)	**-0.07 (1.32)**
4	2.56 (1.27)	3.23 (1.57)	**1.42 (1.34)**	**0.08 (1.23)**
5.6	1.89 (1.06)	2.44 (1.18)	**0.93 (0.9)**	**-0.01 (0.83)**

Compared to the control group, mean cast production in the species *L. terrestris* was found to be significantly different in all exposure groups ($p < 0.0001$; $F=139.1$; $df=4$). For the 0.3x group cast production increased (+28.1%) and considerably decreased in the 1x, 3x and 6x exposure groups (-42.4%; -57.2% and -95.7%, respectively) at $p < 0.05$ (Fig. 1). The EC_{50} for changes in cast production were 0.84 (*L.terrestris*) and 0.76 mg kg^{-1} dry soil (*A. caliginosa*), respectively. They were calculated by also including the induction of cast production in the 0.3x exposure groups in both species.

In general, mean cast production rate was about 4 times higher in *A. caliginosa* (2.56 g g^{-1} day^{-1}) than in *L. terrestris* (0.65 g g^{-1} day^{-1}) when comparing the control groups (mesh size = 4 and 5.6 mm respectively).

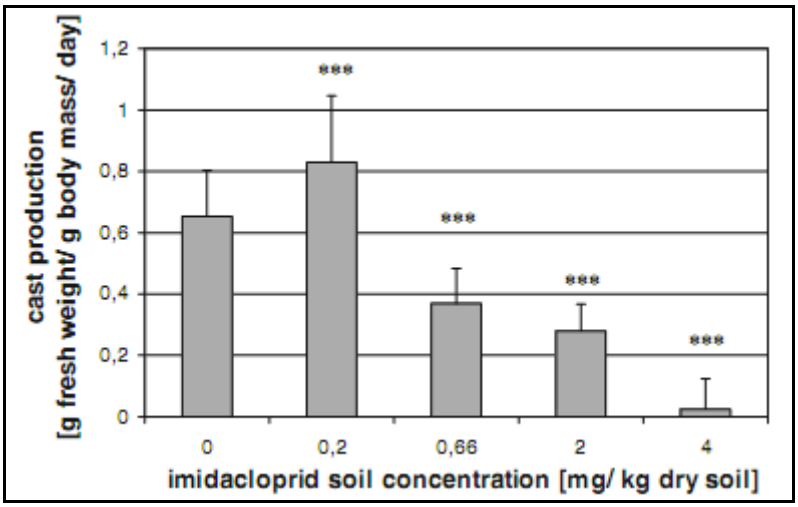

Figure 1: Mean cast production (+SD) (in g casts g^{-1} earthworm body mass day^{-1}) of *Lumbricus terrestris* after exposure to different concentrations of imidacloprid for 7 days (n = 25). A sieve with a mesh size of 5.6mm was used. Asterisks indicate significant differences from the control ($p < 0.001$).

4. Discussion

Imidacloprid is known to cause different sub-lethal effects in earthworms as e.g. at behavioural, physiological as well as on molecular levels (Luo et al. 1999; Schaefer 2003; Capowiez et al. 2006; Olvera et al. 2008; Gomez-Eyles et al. 2009). The LC_{50} value of imidacloprid in different earthworm species (*Eisenia fetida*; *Lumbricus terrestris*; *Aporrectodea nocturna*; *Allolobophora icterica; Pheretima* group) is between 2.3 and 4 mg kg^{-1} dry soil (Luo et al. 1999; Mostert et al. 2002; Capowiez et al. 2006; Gomez-Eyles et al. 2009).

Changes in earthworm body mass (as a biomarker of effect) are thought to be of ecological relevance. It is often assumed that high losses in body mass may lead to negative effects on

reproduction and survival (Capowiez et al. 2005; Olvera-Velona, et al. 2008). We found body mass change to be significantly different from the control groups starting for concentrations of imidacloprid as low as 0.66 mg kg^{-1} dry soil for both species. Our results proved to be in the range of those found in previous studies, in which significant effects of imidacloprid on body mass change in different earthworm species (*Eisenia fetida*, *Aporrectodea nocturna*, *Allolobophora icterica*, *Lumbricus terrestris*) - exposed in soils with different properties - were found at concentrations between 0.5 and 1.91 mg kg^{-1} dry soil (Mostert et al. 2002; Capowiez et al. 2005; Gomez-Eyles et al. 2009). Nevertheless, one has to bear in mind that in our study body mass change was rather a measure of earthworm activity (filling of the gut) than of growth, since the exposure time was relatively short and since *L. terrestris* and *A. caliginosa* are slower growing species than the common test species *E. fetida* or *E. andrei.* However, the entire biological implications (for the ecosystems functions) of these losses in body mass due to reduced earthworm activity are hard to predict from such short-term exposures.

The measurement of changes in cast production is a new and promising behavioural biomarker in ecotoxicology. It is of ecological relevance, since reduced cast production demonstrates a reduced (feeding) activity and in consequence might have indirect impacts on soils (Capowiez et al. 2010). Indeed cast production is an important function of earthworm activities i.e. organic matter mixing with soil aggregates and thus organic matter protection and hot spots for micro-organisms (Binet and Le Bayon 1999).

In this study, cast production increased in both species after exposure to the lowest concentration of imidacloprid (0.2 mg kg^{-1} dry soil), but only significantly for *L. terrestris*, while the exposures to higher concentrations (0.66 – 4 mg kg^{-1} dry soil) caused significant decreases for both species. Other studies have observed reduced total or surface cast production after imidacloprid exposure for *L. terrestris* at concentrations starting from 0.5 mg kg^{-1} dry soil using the same test soil as was used in this study (Capowiez et al. 2006; 2010). In former studies reduced total or surface cast production was also described after exposure to a range of other different pesticides and species in laboratory as well as in field experiments (Lal et al. 2001; Capowiez et al. 2010). In our study, the increase of cast production after exposure to the lowest concentration might be explained by higher earthworm activity due to escaping or avoidance behaviour and/ or an increased metabolic rate possibly caused by detoxification processes. Avoidance behaviour may not harm earthworms directly, but could still have negative impacts on soils, like consequences related to reduced leaf-litter breakdown (Kreutzweiser et al. 2009). The drastic decrease in cast production at higher concentrations found in this study might be due to the neurotoxic effect of imidacloprid inhibiting earthworms in their normal feeding behaviour. In general, such biphasic dose responses (low-dose stimulation and high-

dose inhibitory effect) are referred to as the phenomenon of hormesis and can often be observed in earthworms after exposure to environmental agents (Spurgeon et al. 2004; Hackenberger et al. 2008; Zhang et al. 2009).

Cast production rate in the control groups was about four times higher in *A. caliginosa* compared to *L. terrestris*. This could possibly be explained by higher ingestion/ egestion rates of endogeic species (Lavelle et al. 1989), but also by allometric differences of metabolic rates due to body mass differences. The cast production rate found for the control group of *L. terrestris* (0.65 g g^{-1} fresh body body mass day^{-1}) is close to the one described by Capowiez et al. (2010) for the same species (between 0.8 and 0.9 g g^{-1} fresh body mass day^{-1}), indicating a good reproducibility. In previous studies on cast production of *A. caliginosa* (Scheu 1987; Curry and Baker 1998), casts were dried before weighing and therefore direct comparisons with our results are difficult.

However, the measurement of changes in cast production proved to be a sensitive biomarker for sub-lethal effects in both tested earthworm species. This was unclear prior to our study, since the mean body mass of *A. caliginosa* was about one sixth/ seventh of the one of *L. terrestris* (which was used as a model organism in the protocol of Capowiez et al. (2010)) and therefore their casts could have been too small to separate from the soil using the sieving method. Compared to the results of two biggest mesh sizes (5.6 mm and 4 mm), the use of the smallest mesh size (3.15 mm) did not result in significant differences between control and 1x-exposure group and therefore 3.15 mm seemed to be the least suitable mesh size for the sieving test with *A. caliginosa* under our experimental conditions. The two biggest mesh sizes used to assess the cast production of *A. caliginosa* led to the same significant differences, but the sieve with the 4 mm mesh size proved to be more suitable, because it led to higher cast weights (Table 3). In general, using the sieving method always has to be adapted to the chosen earthworm species.

Even if this biomarker seems not to be suited for epigeic earthworm species (Capowiez et al. 2010), it seems to be very likely that the method functions well in anecic and endogeic earthworm species.

In conclusion, sub-lethal effects of imidacloprid on two earthworm species were found in the range of the predicted environmental concentrations (PEC) (0.33 - 0.66 mg kg^{-1}) of this substance. Considering the extensive use of imidacloprid in agriculture, the effects could be of high importance, since they might be related to constraints of earthworms in their role as “soil engineers” and consequently may have crucial impacts on soils.

Both parameters tested, body mass change and cast production, proved to be sensitive biomarkers, easy and rapid to handle. The recently developed cast production test seems promising for future

earthworm ecotoxicity testing and might be considered for inclusion in current standard tests or even as potential standard test itself.

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Kapitel 3

Assessment of short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) by using 2D and 3D post-exposure techniques. *Chemosphere 84, 1349-1355.*

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Abstract

Adverse effects of agrochemicals on earthworms' burrowing behaviour can have crucial impacts on the entire ecosystem. In the present study, we have therefore assessed short- and long-term effects on burrowing behaviour in the earthworm species *Aporrectodea caliginosa* and *Lumbricus terrestris* after exposure to a range of imidacloprid concentrations (0.2 – 4 mg kg^{-1} dry soil) for different exposure times (1, 7, 14 days). 2D-terraria were used for the examination of post-exposure short-term effects (24-96h), while post-exposure long-term effects were assessed by means of X-ray burrow reconstruction in three dimensional soil cores (6 weeks). For the latter each core was incubated with two specimens of *L. terrestris* and four of *A. calignosa*. Short-term effects on the burrowing behaviour (2D) of *A. caliginosa* were already detected at the lowest test concentration (0.2 mg kg^{-1} dry soil), whereas such effects in *L. terrestris* were not observed until exposure to concentrations 10 times higher (2 mg kg^{-1} dry soil). For both species tested in the 2D-terraria, "total burrow length after 24h" and "maximal burrow depth after 24h" were the most sensitive endpoints. 3D reconstructions of the burrow systems made by both earthworm species in the repacked soil cores revealed a significant linear decrease in burrow volume with increasing imidacloprid concentration. Since many of the observed effects occurred at imidacloprid concentrations relevant to natural conditions and since reduced activities of earthworms in soils can have crucial impacts on the ecosystem level, our results are of environmental concern.

Keywords: earthworms - imidacloprid - burrowing behaviour - post-exposure effects

1. Introduction

The neonicotinoid insecticide imidacloprid is extensively used in agriculture worldwide. Currently even in Germany around 1000 tons of imidacloprid are produced per year. (www.bvl.de) Soil concentrations after field application can be as high as 0.66 mg kg^{-1} dry soil (Oi, 1999), which is in the range of effect concentrations for soil organisms (e.g. Capowiez et al., 2010; Dittbrenner et al., 2010; Lal et al., 2001; Luo et al., 1999; Mostert et al., 2002; Zang et al., 2000). The beneficial role of earthworms in soils, influencing a range of chemical, physical and biological processes, is beyond dispute (Curry and Baker, 1998; Edwards and Bohlen, 1992; McCredie and Parker, 1992; Scheu, 1987). Since they are, in addition, easy to culture and to handle for experiments, earthworms have become standard test organisms in ecotoxicology (EEC, 2003; OECD, 1984; OECD, 2004). Over the last decades ecotoxicologists have more and more realised the great importance of conducting behavioural tests in order to better evaluate adverse effects of toxicants on the environment (Doving, 1991; Little, 1990; Scherrer, 1992). Changes in behaviour of earthworms can be of crucial importance for soils, and thus can result in adverse effects on soil functions (Capowiez et al., 2006). The most frequently used behavioural test for earthworms is the standardised avoidance test (ISO, 2008), which is based on a 48h exposure. It has proven to be very sensitive in many studies (Hund-Rinke et al., 2005; Natal Da Luz et al., 2004; Pereira et al., 2010; Slimak, 1997; Yeardley et al., 1996). However, in some cases non-avoidance of different toxicants (diazinon; chlorpyrifos; imidacloprid; ivermectin) or even significant attraction has been observed (Capowiez and Bérard, 2006; Dittbrenner et al., 2011, submitted; Hodge et al., 2000; Torkhani et al., 2011). Thus, the avoidance test is rather considered to be a measure of repellence than of toxicity (Capowiez and Bérard, 2006). Since the beneficial role of earthworms is mainly dependent on their burrowing activity, examining whether and how toxicants hamper the earthworms burrowing behaviour is highly important. Burrowing behaviour can be assessed by using 2D-terraria (Evans, 1947) or X-ray tomography in repacked soil cores (3D) (Capowiez et al., 2003a). Adverse effects on burrowing behaviour were shown several times in previous studies by means of the 2D-terraria (Capowiez et al., 2003b; Capowiez and Bérard, 2006; Hans and Beg 1992; Olvera-Velona et al., 2008). This method has the advantage of being easy, quick and inexpensive. However, it can only be used for short-term exposure periods, since space for burrow systems is very limited. In contrast, the 3D-approach opens the possibility to study long-term effects and is adapted to the study of modifications of soil functions (Capowiez et al., 2006), and thus, represents a more realistic exposure scenario. However, it is more expensive and time-consuming to carry out. Measuring chronic effects of toxicants on the environment is highly important for risk assessment, but such effects are difficult to predict from short-term experiments. With respect to imidacloprid, the majority of behavioural studies on earthworm behaviour have solely focussed on acute effects (Capowiez et al., 2003b; Capowiez and

Bérard, 2006; Capowiez et al., 2009; Dittbrenner et al., 2011b, submitted) and little is known about the predictability of these results in the long run and thus about their ecological relevance.

In the present study, we have compared short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) by using 2D-terraria and repacked soil cores (3D). Both methods have been conducted by firstly carrying out pre-exposure experiments of earthworms individually in Petri dishes and subsequently measuring burrowing behaviour in uncontaminated 2D-terraria or 3D-soil cores. By doing so, bias like e.g. avoidance behaviour due to the presence of a toxicant is excluded. In our experiments the measured burrowing behaviour therefore indicates effective burrowing capacities of the earthworms after toxicant exposure.

2. Material and methods

2.1. Earthworms, soil and imidacloprid

Specimens of the species *Lumbricus terrestris* were purchased from a fishing store in Avignon (France), while specimens of the species *Aporrectodea caliginosa* were sampled from an untreated INRA experimental orchard (no pesticide application for 5 years). For all experiments only adult earthworms were used. From another untreated orchard located close to Avignon (France) and abandoned for more than 10 years, the test soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3 (in water), CEC = 8.2 cmol kg^{-1}) was collected. The earthworms were acclimatised in the test soil for 7 days in a dark climate chamber (12°C) prior the exposure experiments. For handling of the test organisms the general recommendations of Fründ et al. (2010) were followed. The insecticide imidacloprid was purchased from FLUKA (No.37894) and was dissolved in distilled water to different test concentrations.

2.2. Exposure experiments

Prior to the exposure experiments, the soil was sieved to 3 mm and the soil-water content was adjusted to 20% by adding distilled water. Subsequently, soil spiking (for both exposure and control groups) was conducted according to the protocol of Capowiez et al. (2005). Therefore 40 ml of water or adapted imidacloprid solution were added to the test soil, reaching a final soil-water content of 25% (i.e. about 80% of the WHC). Since the highest value for the predicted environmental concentration (PEC) of imidacloprid was found to be 0.66 mg kg^{-1} dry soil (Oi, 1999), we have defined this concentration to be the normal application rate (termed 1x). For both biomarkers the earthworms were exposed to the following imidacloprid concentrations: 0 mg kg^{-1} (control); 0.2 mg kg^{-1} (0.3X); 0.66 mg kg^{-1} (1X); 2 mg kg^{-1} (3X) and 4 mg kg^{-1} dry soil (6X). For the measurements in the

2D-terraria the lowest concentration (0.2 mg kg^{-1} dry soil) was not tested in *L. terrestris* and the highest concentration (4 mg kg^{-1} dry soil) was not tested in *A. caliginosa*. For the latter species an imidacloprid concentration of 4 mg kg^{-1} dry soil was already lethal.

The exposure experiments were carried out individually in Petri dishes (diameter = 10 cm) filled with 100 g of uncontaminated (control group with distilled water) or contaminated soil, respectively. Before and after the exposure experiments each specimen was rinsed in tap water, gently dried on filter paper and weighed. The Petri dishes were placed in dark climate chambers (12°C) during earthworm exposure.

2.3. Burrowing behaviour (2D)

Burrowing behaviour was assessed by means of 2D-terraria (Evans, 1947) after 1, 7 and 14 days of exposure time using 7 replicates for each treatment (n = 7). The 2D terraria consisted of two glass sheets (30 cm x 42 cm) and were adapted to the according species by fixing the sheets 3 mm (*A. caliginosa*) or 5 mm (*L. terrestris*) apart. The terraria were filled with sieved soil (2 mm). A detailed description of the 2D-terraria can be found in Capowiez (2000). After the exposure experiments in Petri dishes, the earthworms were put individually in the 2D terraria for four days and were kept in dark climate chambers (at 12°C). The burrows were marked after 24 and 96h on transparent sheets and were then digitised. Then the following four endpoints were computed for each individual: total length of burrows after 24 and 96h as well as maximal burrow depth after 24 and 96h in the terraria.

2.4. Burrowing behaviour in repacked soil cores (3D)

Since several detrimental effects were observed in both species only after 7 days of exposure time, we have chosen the latter period as the pre-exposure duration for the 3D long-term experiments. Repacked soil cores were prepared using PVC cylinders (35 cm in length and 16 cm in diameter) lined with a mixture of sealing varnish and sharp fine sand to prevent the earthworms from crawling along the PVC walls. A hydraulic press was used to compact five cores simultaneously. Cores were compacted by applying a pressure of 270 kPa for 5 minutes on sieved soil at 23 % moisture content (gravimetric). This treatment resulted in a soil dry bulk density of 1.1 $g.cm^{-3}$. To minimise variations in soil bulk density between the top and bottom of the cores, the soil was compacted stepwise in 12 layers. Each layer comprised 600 g of soil. The final thickness of each layer was approximately 2.5 cm. Before adding a new soil layer, the surface of the previous layer was gently scratched using a small rake to increase cohesion between layers. The bottom of each core was sealed and the top was closed using a perforated lid to prevent significant water losses. A total of 25 cores were created and 6 earthworms (2 *L. terrestris* and 4 *A. caliginosa*) were put in each core according to the following pre-exposure treatments: control,

0.3X, 1X, 3X and 6X with 4 replicates (cores). Two cores were incubated with either 2 *L. terrestris* or 4 *A. caliginosa* coming from the control group (unexposed). Another three cores were left without earthworms (control cores).

The cores were incubated in a dark climate chamber at 12°C. Water (5 ml per core) was supplied weekly. Food (5 g of dried grass) was added at the top of each core. After 6 weeks, chloroform (10 ml) was applied to each core to kill the earthworms and prevent them from further burrowing. At the end of the experiment, cores were imaged using a medical X-ray tomograph (General Electrics; brightspeed exel) at the INRA Nancy centre to obtain a set of images 1.25 mm thick every 1.25 mm. The settings at which the X-ray beam was operated were 50 mA and 120 kV.

The burrow system in each core was reconstructed following the method proposed by Pierret et al. (2002). In brief, macropores were traced starting from the darkest voxels by studying local variation in mean grey level when the current voxel was included in the current macropore. Macropores that were too small (less than 100 voxels, i.e. about 0.5 cm^3) were discarded. At this stage, the volume of macroporosity, and the number of burrows (a burrow is a set of connected voxels) were computed.

Since the tested earthworm species differed greatly in size (thus in body diameter), we studied the distribution of macropore area for each species (results not shown) in order to finally be able to define which burrows were made by which species. This was done by analysing the 2D images of cores incubated with only *L. terrestris* or *A. caliginosa*. We found that the best approximation for the allocation of macropores was that macropores larger than 25 mm^2 were most likely made by *L. terrestris*, while smaller ones were most likely created by *A. caliginosa* (assuming that the interspecific interactions between earthworms would not result in variations in mean burrow diameter). In addition to the assessment of overall effects on both species in combination, the latter approach enabled us to isolate and study behavioural effects on every single species.

2.5. Statistical analysis

Data were tested for normality and not normally distributed data sets were log-transformed. The effects of imidacloprid contamination on the burrow length of *L. terrestris* and A*. caliginosa* in the 2D-terraria were examined by using one-way ANOVA and by conducting post-hoc comparisons using Tukey-Kramer HSD. For the analysis of effects on maximal burrow depth in the 2D-terraria, Kruskal-Wallis tests followed by Holm-Bonferroni corrections were carried out.

For the 3D results, the number of replicates (n=4) was too low and the variability of the data, as often observed for behavioural studies, was too high to use classical inference tests. The overall effect of imidacloprid concentrations on the 3D burrowing behaviour of both earthworm species was then analysed using a log-linear regression. The data for the estimation of macropores made by each

species were not statistically tested since we did not find a method to accurately allocate all burrows to a definite species.

3. Results

3.1. Post-exposure modifications of burrowing behaviour in 2D

a) *Aporrectodea caliginosa:*

A. caliginosa maximal burrow depth (measured after 24h and 96h in the terraria) decreased with increasing imidacloprid concentration and exposure time (Table 1). The following treatments differed significantly from the according control groups ($p < 0.05$): 1d/ 3X, 7d/ 0.3X, 7d/ 1X, 7d/ 3X, 14d/ 0.3X, 14d/ 1X and 14d/ 3X (after 24h in the terraria) as well as for 7d/ 3X, 14d/ 0.3X, 14d/ 1X and 14d/ 3X (after 96h in the terraria).

The total length of burrows made by *A. caliginosa* (measured after 24h and 96h in the terraria) also decreased with increasing imidacloprid concentration and exposure time (Table 1). Significant differences to the control groups were observed for the following treatments ($p < 0.05$): 1d/ 3X, 7d/ 0.3X, 7d/ 1X, 7d/ 3X, 14d/ 1X and 14d/ 3X (after 24h in the terraria) as well as for 1d/ 1X, 7d/ 3X and 14d/ 3X (after 96h in the terraria).

b) *Lumbricus terrestris:*

In *L. terrestris* maximal burrow depth (measured after 24h and 96h in the terrarium) decreased with increasing imidacloprid concentration and exposure time (Table 2). However, this was only significant when maximal burrow depth was measured after 24h in the terrarium and when the earthworms were exposed to 2 (3X) and 4 mg kg^{-1} dry soil (6X) for 7 days as well as to 2 (3X) mg kg^{-1} dry soil ($p < 0.05$).

The total length of burrows made by *L. terrestris* (measured after 24h and 96h in the terrarium) also decreased with rising exposure concentration and time (Table 2). However, significant differences in comparison to the control groups were only found after 7 days exposure to imidacloprid concentrations of 2 (3X) and 4 mg kg^{-1} dry soil (6X) ($p < 0.05$).

Table 1: Characteristics of the burrows systems made by *Aporrectodea caliginosa* (Mean ± SD) in the 2D-terraria dependent on exposure time (1, 7, 14 days) and imidacloprid concentration (1X; 3X; 6X). The concentrations are indicated in relation to the predicted environmental concentration 1X (0.66 mg kg^{-1} of dry soil). Values for the contaminated groups are expressed in percentage of the respective controls. Values in bold are significantly different from the according control values ($p < 0.05$).

	1d				7d				14d			
	control	**0.3X [%]**	**1X [%]**	**3X [%]**	**control**	**0.3X [%]**	**1X [%]**	**3X [%]**	**control**	**0.3X [%]**	**1X [%]**	**3X [%]**
Maximal burrow depth after 24h [cm]	23.37 (10.38)	92.38 (43.84)	69.55 (43.13)	**25.14 (10.93)**	20.47 (11.51)	**42.74 (26.62)**	**40.54 (20.98)**	**11.92 (9.9)**	21.07 (9.76)	**43.29 (23.04)**	**22.24 (13.31)**	**13.08 (14.59)**
Maximal burrow depth after 96h [cm]	32.84 (11.03)	96.01 (31.83)	74.25 (37.84)	63.19 (43.59)	29.43 (12.26)	81.90 (40.9)	70.62 (48.83)	**39.53 (34.79)**	35.2 (8.54)	**55.75 (33.34)**	**48.30 (32.20)**	**26.72 (35.69)**
Total burrow length after 24h [cm]	35.05 (10.51)	100.3 (10.53)	77.43 (20.32)	**63.47 (24.7)**	29.64 (9.65)	**65.18 (22)**	**58.62 (18.03)**	**15.70 (12.21)**	26.83 (8.35)	74.1 (32.46)	**42.22 (9.45)**	**15.7 (15.65)**
Total burrow length after 96h [cm]	99.41 (23.07)	95.65 (20.78)	**75.97 (21.64)**	85.5 (31)	68.91 (20.35)	93.85 (47.37)	90.32 (17.17)	**38.55 (27.24)**	69.59 (21.41)	71.74 (31.42)	73.26 (18.82)	**32.86 (30.34)**

Table 2: Characteristics of the burrows systems made by *Lumbricus terrestris* (Mean ± SD) in the 2D-terraria dependent on exposure time (1, 7, 14 days) and imidacloprid concentration (1X; 3X; 6X). The concentrations are indicated in relation to the predicted environmental concentration 1X (0.66 mg kg^{-1} of dry soil). Values for the contaminated groups are expressed in percentage of the respective controls. Values in bold are significantly different from the according control values ($p < 0.05$).

	1d				7d				14d			
	control	**1X [%]**	**3X [%]**	**6X [%]**	**control**	**1X [%]**	**3X [%]**	**6X [%]**	**control**	**1X [%]**	**3X [%]**	**6X [%]**
Maximal burrow depth after 24h [cm]	16.91 (10.42)	68.81 (54.54)	41.66 (50.59)	39.9 (34.04)	12.74 (7.16)	84.4 (41.05)	**35.01 (37.63)**	**30.64 (47.79)**	20.72 (7.76)	69.35 (53.53)	**51.93 (55.95)**	61.97 (53.99)
Maximal burrow depth after 96h [cm]	33.02 (11.25)	92.84 (42.88)	76.6 (55.62)	105.84 (36.68)	32.82 (12.32)	77.03 (47.26)	55.41 (45.36)	90.66 (37.85)	36.69 (5.82)	93.74 (41.36)	83.13 (43.59)	97.21 (17.18)
Total burrow length after 24h [cm]	19.48 (12)	75.88 (56.5)	51.18 (43.26)	74.76 (64.71)	20.64 (12.35)	90.16 (38.03)	**31.54 (38.9)**	**18.93 (30.18)**	25.49 (8.8)	85.92 (39.3)	86.28 (45.43)	57.83 (48.16)
Total burrow length after 96h [cm]	58.93 (24.35)	74.17 (34.68)	69.7 (50.36)	108.52 (37.49)	72.42 (33.52)	91.72 (51.26)	45.43 (39.43)	62.34 (40.01)	78.5 (30.04)	90.53 (43.6)	107.68 (53.06)	66.01 (19.55)

3.3 Post-exposure modifications of burrowing behaviour in 3D

The 3D burrow systems made by the two uncontaminated earthworm species clearly differed visually: Burrows made by *L. terrestris* had a greater diameter and were more continuous than the ones made by *A. caliginosa* (Figure 1). For both species, we observed a higher density of burrows in the upper part of the cores. For the combined controls ("both species"), we could visually distinguish between the burrows made by each species due to the diameter differences and no modification in burrowing patterns due to inter-specific disturbances were observed. In addition, no visual difference for the burrow systems of the pre-exposed earthworms (1X, 3X, 6X) compared to the burrow systems of control earthworms ("both species") became obvious.

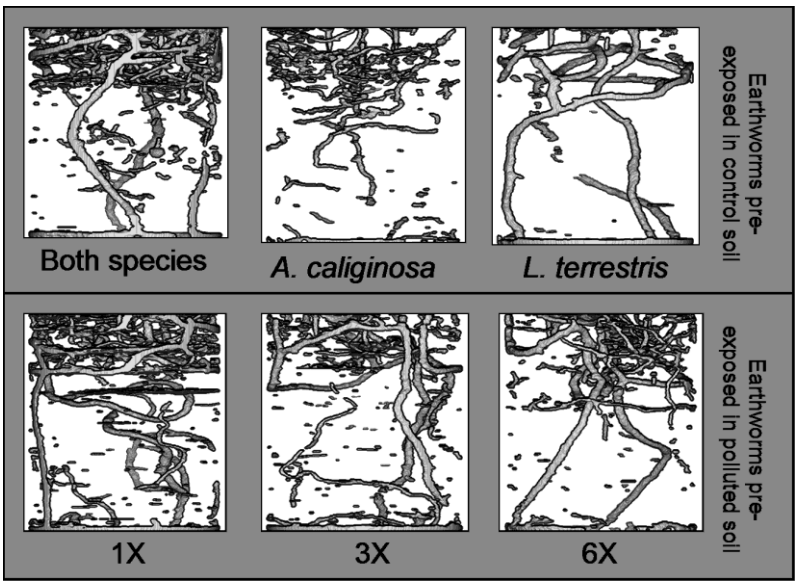

Figure 1: Examples for 3D reconstructions of the burrow systems made by *L. terrestris* and *A. caliginosa* in the repacked soil cores during 6 weeks of incubation. Colours range from soft to dark grey according to the distance from the point of observation. Burrow systems shown in Fig. 1 on top were observed after exposure to control soil of either both species (4 *A. caliginosa* and 2 *L. terrestris*), or each species separately (4 *A. caliginosa* or 2 *L. terrestris*). The 3 burrow systems shown at the bottom - made by both species (4 *A. caliginosa* and 2 *L. terrestris*) - were observed after exposure to different imidacloprid concentrations (1, 3 or 6X with 1X meaning 0.66 mg active ingredient per kg of dry soil).

However, quantifications of the burrow volumes enabled us to detect a significant decrease with increasing imidacloprid concentration (Figure 2). The log-linear regression was significant ($p<0.01$), but R^2 was low ($R^2=0.326$) due to the high variability. The approximated macropore area threshold used to separate burrows made by each species in 2D images (25 mm^2) was not ideal, since about 15% of the macropores were misclassified in the two cores incubated with only one species (Figure 3). This prevented us from applying a statistical analysis to the results. Moreover, we were not able to show significant differences in the amount of macropores created by each species, even if a trend

of fewer macropores for *A. caliginosa* at the highest imidacloprid concentration was observed in comparison to the control.

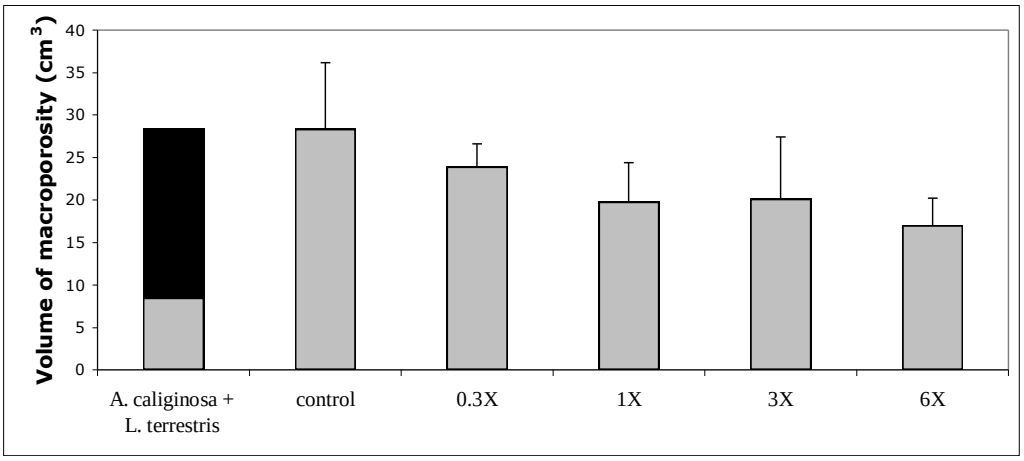

Figure 2: Mean volume of macroporosity (+ SD) measured in the cores incubated with 2 *L. terrestris* and 4 *A. caliginosa*. The first bar is the sum of the macroporosity found in the mono-specific cores with either 2 *L. terrestris* (grey) or 4. *A. caliginosa* (black). The imidacloprid concentrations are indicated in relation to the predicted environmental concentration 1X (0.66 mg kg^{-1} of dry soil).

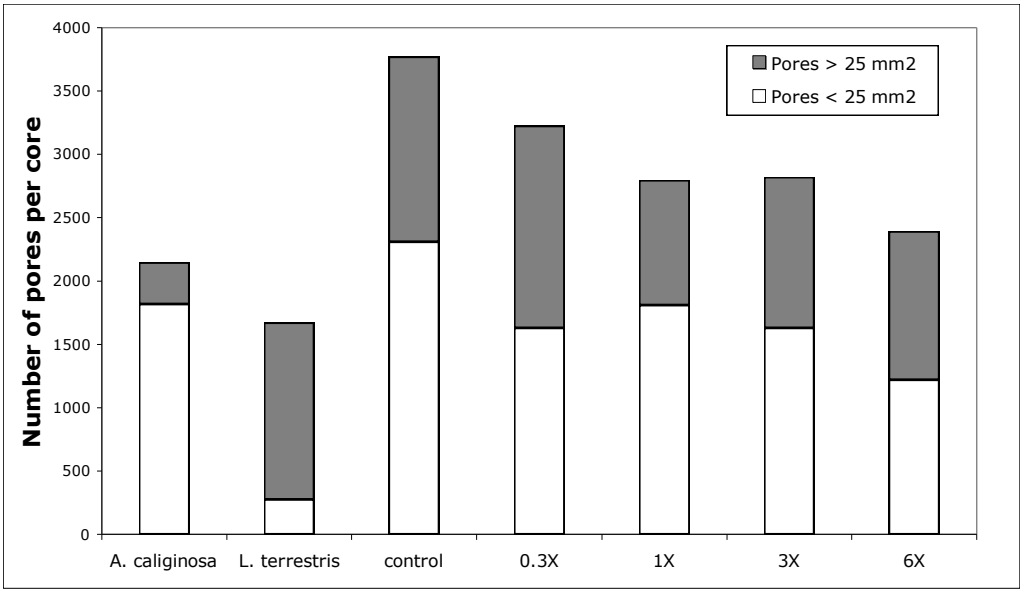

Figure 3: Mean total number of macropores in the successive 2D images resulting from X-ray tomography analysis of the repacked soil cores. The 2 first bars represent the mono-specific cores of either 4 *A. caliginosa* or 2 *L. terrestris* after pre-exposure in control soil. The following bars represent the soil cores of both species (4 *A. caliginosa* or 2 *L. terrestris)* for 6 weeks after a pre-exposure of 7 days in either control soil or polluted soil using different imidacloprid concentrations. The imidacloprid concentrations are indicated in relation to the predicted environmental concentration 1X (0.66 mg kg^{-1} of dry soil). The grey part of each bar represents the number of pores with a pore area greater than 25 mm^2 and thus assumed to have been made by *L. terrestris*. The white part of each bar represents the number of pores with a surface area smaller than 25 mm^2 and therefore assumed to have been made by *A. caliginosa*.

4. Discussion

In the present study both acute and chronic behavioural effects were detected for a similar range of concentrations (0.2-4 mg kg^{-1} dry soil), for which previous ecotoxicological studies have observed different sub-lethal effects on earthworms after imidacloprid exposure (e.g. Capowiez et al., 2003b; 2010; Dittbrenner et al., 2010a; Lal et al., 2001; Luo et al., 1999; Mostert et al., 2002; Zang et al., 2000). Since behavioural changes can have detrimental effects on higher levels of biological organisation (Doving, 1991; Little, 1990; Scherrer, 1992) and since imidacloprid is frequently used in agriculture and was shown in the present study to have adverse effects on the earthworms' burrowing behaviour, already at concentrations relevant to natural conditions, (predicted environmental concentration (PEC) = 0.33-0.66 mg kg^{-1} dry soil) (Mostert et al., 2000; Oi, 1999), our results might be of great environmental concern.

The results obtained for burrowing behaviour by means of 2D-terraria showed that significant adverse effects in *A. caliginosa* occurred already at very low imidacloprid concentrations (at 0.2 mg kg^{-1} dry soil after 7 and 14 d as well as at 0.66 mg kg^{-1} dry soil after 1d), while significant negative effects for *L. terrestris* were only detected after exposure to ten times higher concentrations (2 and 4 mg kg^{-1} dry soil after 7 days of exposure). In a previous study on effects of imidacloprid on the burrowing behaviour of two other earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*) also using 2D-terraria, significantly reduced burrow lengths, burrow reuse rates and covered distances were observed for both species starting at soil concentrations of 0.5 mg kg^{-1} dry soil (Capowiez et al., 2003b). However, in the latter approach – unlike the experimental design of the present study - the earthworms were not pre-exposed in Petri dishes, but were directly put into 2D-terraria filled with contaminated soil. Therefore changes on the effective burrow capacities of the earthworms could not be measured and thus the study could not reveal whether the detected effects were only due to physiological damage caused by the toxicant or whether the effects were influenced by bias (e.g. avoidance behaviour). In our study, however, we were able to exclude such bias and to demonstrate that the effects on burrowing behaviour were due to physiological damage in the earthworms.

Nevertheless, a lower sensitivity of *L. terrestris* compared with *A. caliginosa* towards imidacloprid has already been observed in a range of previous studies (Dittbrenner et al., 2010; Dittbrenner et al., 2011a, submitted; 2011b, submitted) and might generally be due to species-specific differences in sensitivity and / or to the relatively low surface-volume ratio of *L. terrestris*. With respect to behavioural measurements by means of 2D-terraria, this method was used with smaller species in the past (Capowiez et al., 2003b; Olvera-Velona et al., 2008). Even if the 2D-terraria were adapted to *L. terrestris* in the present study, they still might have been too small in order to offer sufficient space to detect effects more precisely. For example the maximal burrow depth of *L. terrestris* could easily

exceed 40cm (Shipitalo and Butt, 1999). In addition the burrow systems of *L. terrestris* are less complex than the ones of endogeic species (*A. caliginosa*) (Bastardie et al., 2003a; Jégou et al., 1998) meaning that behavioural effects in *A. caliginosa* might become more obvious at an earlier stage. Moreover anecic species like *L. terrestris* are known to reuse their burrows more often and to show less overall burrowing activity than endogeic species such as *A. caliginosa* (Capowiez et al., 2003a; Bastardie et al., 2003a). All of these facts might add up and lead to a hindered detection of detrimental effects on burrowing behaviour in *L. terrestris* compared with *A. caliginosa*, when using the present approach. However, one of our previous studies on sub-lethal effects of imidacloprid on *L. terrestris* showed significant cellular alterations as well as significant body mass losses occurring in this species already at lower imidacloprid concentrations (e.g. 0.66 mg kg^{-1} dry soil after 7d) (Dittbrenner et al., 2011a, submitted). This might support that the present 2D measurements were rather limited to sensitively detect sub-lethal effects in *L. terrestris*.

In general, total burrow length and maximal burrow depth after 24h incubation in the 2D-terraria proved to be the most sensitive endpoints in the present study. Total burrow length represents a very important behavioural aspect, since it indicates general burrowing activity. Changes in maximal burrow depth should also be of high ecological importance, since reduced depths of earthworm burrow systems under natural conditions might have detrimental effects on gas / water transfer properties of soils and as a consequence might affect the whole ecosystem (Bastardie et al., 2003b, Capowiez et al., 2006).

While for *L. terrestris* significant effects on burrowing behaviour (2D) were found only after 7 days exposure time, significant detrimental effects for *A. calignosa* were found for all exposure periods (1, 7 and 14d), and the number of significant endpoints grew with increasing exposure time. It seems very likely that *A. caliginosa* strongly suffered from the imidacloprid exposure towards the highest concentrations (0.66 and 2 mg kg^{-1} dry soil) and - unlike *L. terrestris* – was not able to adapt to the presence of the insecticide in our experiments. However, when looking at the effects measured during incubation in the 2D-terraria, one can clearly recognise that for both total burrow length as well as maximal burrow depth, the number of significant effects diminished with increasing incubation time (24h vs 96h). This makes evident, that without the presence of the insecticide after the exposure the earthworms were capable of recovering from adverse effects.

With respect to imidacloprid exposure of *L. terrestris* and *A. caliginosa*, measuring behavioural effects by means of 2D-terraria was more sensitive than by means of the avoidance test (ISO 17512-1 (2008)) - the most frequently used test to investigate impacts on behaviour in earthworms (Dittbrenner et al., 2011b, submitted). However, a recently developed behavioural bioassay – the earthworm cast production test (Capowiez et al., 2010) - proved to be of a similar sensitivity (in the case of *L. terrestris* even of higher sensitivity) to the post-exposure 2D-burrowing behaviour

assessment, when effects of imidacloprid on the earthworm species *L. terrestris* and *A. caliginosa* were examined (Capowiez et al., 2010; Dittbrenner et al., 2010).

The 3D burrow reconstructions of the control treatments are in agreement with the typical burrowing patterns known for each species (Jégou et al., 1998; Langmaack et al., 1999), indicating favourable conditions for both species in the repacked soil cores. The overall burrowing activity - measured by means of macropore volume in the soil cores - decreased significantly and log-linearly with increasing imidacloprid concentration (from 0.2 to 4 mg kg^{-1} dry soil). The mean burrow volume in the cores with earthworms pre-exposed to the highest imidacloprid concentration (4 mg kg^{-1} dry soil) showed a decrease of about 40% relative to the earthworms pre-exposed in control soil. Although we did not find a perfect way to classify macropores according to the earthworm species in the present study, our approximation by means of macropore area gave satisfying results. By using this approximation and then separating the burrow systems of both species, a trend for greater sensitivity of *A. caliginosa* compared to *L. terrestris* could be elucidated at the highest imidacloprid concentration, which, however, could not be confirmed statistically. In comparison to the results obtained by the 2D measurements, the 3D measurements were less sensitive, but proved to be a mere confirmation of the effects observed for burrowing activity in the 2D-terraria. Significant long-term effects of imidacloprid on the burrowing behaviour of *L. terrestris* and *A. caliginosa* were measured in the soil cores, but due to a relatively low replicate number only a linear regression could be used to analyse the present data.

However, it was not surprising to discover that the detected long-term effects (3D) were less obvious than the measured short-term effects (2D), given that the earthworms spent six weeks in uncontaminated soil cores compared to 96h in uncontaminated 2D-terraria. Nevertheless, the results obtained by means of 3D burrow reconstruction should raise great concern about the frequent use of imidacloprid in agriculture, since a reduced burrowing activity of earthworms over a longer period of time might have crucial effects on soils. Moreover, under natural conditions effects are likely to be more severe than the ones measured in the soil cores, since (1) the half-life of imidacloprid in soils can be greater than one year (Sabbagh et al., 2002) resulting in a long-term exposure of earthworms, and (2) also because a number of different pesticides are often used in one plot favouring mixture toxicity.

In conclusion, we have found significant adverse effects of imidacloprid on the burrowing behaviour of two earthworm species (*A. caliginosa* and *L. terrestris*) in environmentally relevant concentrations in both short- and long-term experiments in the laboratory. With respect to the measurements of burrowing activity, the results obtained from the 3D reconstruction of burrows proved to be a mere confirmation of the results observed by means of 2D-terraria, but were less sensitive. Due to the frequent use of imidacloprid in agriculture and its relatively long half-life in soils, our results are of

environmental concern. From a methodological point of view, 2D terraria appeared to be a convenient tool (high number of replicates, responses after only 24 or 96h) to analyse ecologically meaningful modifications of earthworm behaviour but for short term effects. 3D reconstructions provided a relevant tool to determine modifications of earthworm behaviour and some of their effects on the soil functioning (e.g. soil transfer properties). However the cost and the complexity of this method generally results in a low number of replicates and thus prevents a powerful statistical analysis.

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Tagungsbeiträge

SETAC Europe 20th Annual Meeting, Sevilla, Spanien (05.2010)

Poster: Dittbrenner N, Triebskorn R, Moser I, Capowiez Y. Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*)

Meeting StEvE, Annual Meeting of PhD-Students in Evolution and Ecology at research facilities in Tübingen (11.2010)

Vortrag: Dittbrenner N. Sub-lethal effects of the insecticide imidacloprid on three different earthworm species (*Eisenia fetida*, *Aporrectodea caliginosa* and *Lumbricus terrestris*)